VALORIZACIÓN DE SUBPRODUCTOS DE LA INDUSTRIA AGROALIMENTARIA COMO ANTIMICROBIANOS NATURALES FRENTE A MICROORGANISMOS PATÓGENOS MEDIANTE TECNOLOGÍAS NO TÉRMICAS DE CONSERVACIÓN

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TESIS DOCTORAL

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CERTIFICAN:

Que el trabajo que presenta Maria Sanz Puig para optar al grado de Doctor por la Universitat Politècnica de València, con el título Valorización de subproductos de la industria agroalimentaria como antimicrobianos naturales frente a microorganismos patógenos mediante tecnologías no térmicas de conservación, ha sido realizado bajo nuestra dirección, en el Instituto de Agroquímica y Tecnología de Alimentos del Consejo Superior de Investigaciones Científicas.

Y para que así conste a los efectos oportunos, firman este certificado en Paterna, a 28 de junio de 2017.

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La presente tesis doctoral se enmarca en el Programa de Doctorado en Ciencia, Tecnología y Gestión Alimentaria de la Universitat Politècnica de València.

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A mi hermana, Lourdes

A Carlos

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RESUMEN

La industria agroalimentaria genera, como resultado de sus procesos de producción, grandes cantidades de subproductos que suponen un impacto negativo a nivel económico y medioambiental. Es por ello que, en la actualidad, su revalorización es uno de los objetivos principales de la Unión Europea en apoyo al desarrollo sostenible.

La presente tesis doctoral se centra en la revalorización de subproductos de la industria hortofructícola como antimicrobianos naturales en sí mismos o combinados con tecnologías no-térmicas de conservación de alimentos como los Pulsos Eléctricos de Alta Intensidad (PEF) o las Altas Presiones Hidrostáticas (HHP) frente a los microorganismos patógenos transmitidos por alimentos más importantes. Además, trata de evaluar el desarrollo de resistencias en los microorganismos a los tratamientos antimicrobianos subletales estudiados y sus posibles cambios de virulencia usando *C. elegans* como organismo modelo.

Los subproductos hortofructícolas estudiados han demostrado un importante efecto antimicrobiano frente a los principales patógenos alimentarios, así como los extractos ASE y las infusiones obtenidas a partir de los mismos, siendo el microorganismo más sensible *S.* Typhimurium. Además, la aplicación de forma combinada de tratamientos subletales de PEF y HHP con infusiones de subproductos ha dado lugar a la aparición de sinergias que permiten alcanzar los niveles deseados de inactivación microbiana (5 ciclos logarítmicos) en un menor periodo de tiempo.

La aplicación de los tratamientos antimicrobianos subletales estudiados de forma consecutiva se ha demostrado que da lugar a la generación de resistencia microbiana en *S.* Typhimurium. Sin embargo, los estudios con *C. elegans* ponen de manifiesto que el desarrollo de esta resistencia antimicrobiana

no lleva consigo el aumento de su virulencia al infectar a un organismo hospedador.

En base a todo lo anterior, podemos concluir que la revalorización de los subproductos de la industria hortofructícola como antimicrobianos naturales es una alternativa viable para su utilización como medida de control adicional de la seguridad microbiológica de productos alimenticios por sí mismos o en combinación con tratamientos de PEF o HHP, dado su efecto sinérgico.

RESUM

La industria agroalimentària genera, com a resultat dels seus processos de producció, grans quantitats de subproductes que suposen un impacte negatiu a nivell econòmic y mediambiental. És per això que, en la actualitat, la seua revalorització és un dels objectius principals de la Unió Europea en recolzament al desenvolupament sostenible.

La present tesi doctoral es centra en la revalorització de subproductes de la industria hortofructícola com a antimicrobians naturals per sí mateixa o combinats amb tecnologies no-tèrmiques de conservació d'aliments com els Polsos Elèctrics d'Alta Intensitat (PEF) o les Altes Pressions Hidrostàtiques (HHP) front als microorganismes patògens transmesos per aliments més importants. A més, tracta d'avaluar el desenvolupament de resistències en els microorganismes als tractaments antimicrobians subletals estudiats i els seus possibles canvis de virulència utilitzant *C. elegans* com a organisme model.

Els subproductes hortofructícoles estudiats han demostrat un important efecte antimicrobià front als principals patògens alimentaris, així com els extractes ASE i les infusions obtingudes a partir dels mateixos, sent el microorganisme més sensible *S.* Typhimurium. A més, l'aplicació de forma combinada de tractaments subletals de PEF i HHP amb infusions de subproductes ha donat lloc a l'aparició de sinèrgies que permeten aplegar als nivells desitjats d'inactivació microbiana (5 cicles logarítmics) en un menor període de temps.

L'aplicació dels tractaments antimicrobians subletals estudiats de forma consecutiva s'ha demostrat que dona lloc a la generació de resistència microbiana en *S.* Typhimurium. No obstant això, els estudis amb *C. elegans* posen de manifest que el desenvolupament d'esta resistència antimicrobiana no du implícit l'augment de la seua virulència al infectar a un organisme hospedador.

En base a tot lo anterior, podem concloure que la revalorització dels subproductes de la industria hortofructícola com a antimicrobians naturals és una alternativa viable per a la seua utilització com a mesura de control addicional de la seguretat microbiològica de productes alimentaris per sí mateixa o en combinació amb tractaments de PEF o HHP, donat el seu efecte sinèrgic.

SUMMARY

Agri-food industry generates, because of its production processes, high amount of by-products, which cause a negative impact both economically and environmentally. For this reason, nowadays, their revalorization is one of the main aims of European Union in support of sustainable development.

This doctoral thesis is focused on the revalorization of by-products from the horticultural industry as natural antimicrobials, by themselves or combined with no-thermal technologies for food preservation, like Pulsed Electric Fields (PEF) or High Hydrostatic Pressure (HHP) against the main foodborne pathogens. Also, it pretends to evaluate the microbial resistance development against the subletal antimicrobial treatments under study and the possible virulence changes using *C. elegans* as a model organism.

The agri-food by-products studied have shown an important antimicrobial effect against the main foodborne pathogens, also the ASE extracts and the infusions obtained therefrom, being *S.* Typhimurium the most sensitive microorganism. In addition, the combination of subletal treatments of PEF and HHP with by-product infusions has resulted in the emergence of synergies, which permit us to achieve the desirable levels of microbial inactivation (5 log cycles) in a minor period of time.

The application of subletal antimicrobial treatments under study consecutively, has shown that it causes microbial resistance development in *S.* Typhimurium. However, *C. elegans* studies show that the development of microbial resistance not imply the increase of its virulence against a host organism.

For all these reasons, we can conclude that the revalorization of agri-food by-products as natural antimicrobials is a viable alternative as an additional

control measure to ensure the microbial food safety by themselves or combined with PEF or HHP treatments, due to their synergistic effect.

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JUSTIFICACIÓN DEL TEMA

1. JUSTIFICACIÓN DEL TEMA

La industria agroalimentaria constituye uno de los sectores industriales más importantes a nivel mundial. Fruto de su intensa actividad de procesado, se producen grandes cantidades de residuos agroindustriales en todo el mundo, que, a priori, no tienen ningún valor para la empresa que los produce. Sin embargo, su eliminación conlleva un impacto altamente negativo, tanto económico como medioambiental.

Es por ello que la revalorización de los residuos de la industria agroalimentaria, convirtiéndolos en subproductos de interés para su utilización en los mismos u otros procesos industriales se ha convertido, a día de hoy, en uno de los objetivos principales de la Unión Europea en apoyo al desarrollo sostenible (EUROSTAT, 2010).

Una de las posibles vías de revalorización de estos subproductos agroindustriales es su utilización como aditivos naturales o ingredientes en la formulación de nuevos productos, aprovechando su riqueza en compuestos bioactivos, que les confiere capacidad antioxidante, anticancerígena o antimicrobiana.

Además, su revalorización como ingredientes naturales con propiedades beneficiosas para la salud en la formulación de nuevos productos, o la reformulación de otros ya existentes, da respuesta a la creciente demanda de los consumidores actuales, cada día más exigentes en la búsqueda y elección de aquellos productos del mercado funcionales, libres de aditivos sintéticos, frescos o mínimamente procesados, que conserven sus características organolépticas y nutricionales.

El desarrollo de productos de alto valor añadido, que conserven dichas propiedades nutricionales mejoradas con respecto a los tratados mediante tecnologías térmicas convencionales, es uno de los ejes directores de la I+D de

las empresas, que apuestan por la competitividad mediante el lanzamiento de productos innovadores, procesados mediante tecnologías no-térmicas de conservación de alimentos, entre ellas las Altas Presiones Hidrostáticas (HHP) y los Pulsos Eléctricos de Alta Intensidad (PEF).

Sin embargo, la aplicación de tratamientos antimicrobianos sub-letales que mejoran y/ó conservan en gran medida el atractivo nutricional y organoléptico del producto, puede representar un riesgo que debe ser convenientemente evaluado. Dicho riesgo implica la posible supervivencia microbiana bajo el concepto de células subletalmente dañadas, que generalmente acaban muriendo, pero en el peor de los casos, podrían recuperarse en condiciones óptimas, pudiendo desarrollar resistencias al tratamiento antimicrobiano y/o cambios en la virulencia del microorganismo. El estudio del riesgo asociado a dichas poblaciones minoritarias resulta fundamental en la evaluación de la efectividad asociada a procesos mínimos de conservación de alimentos, especialmente bajo la aplicación de la tecnología de barreras.

Por todo ello, la presente tesis doctoral se ha centrado en la revalorización de subproductos de la industria agroalimentaria como antimicrobianos naturales, evaluando su potencial antimicrobiano individualmente, y en combinación con tecnologías no-térmicas de conservación, con el objetivo de conseguir sinergias en su capacidad antimicrobiana, reduciendo la intensidad del tratamiento requerido, y mejorando así las características nutricionales y organolépticas del producto final. Asimismo, se ha evaluado la posible generación de resistencia o cambios de virulencia microbiana mediante el uso del organismo modelo *C. elegans*.

ANTECEDENTES BIBLIOGRÁFICOS

2. ANTECEDENTES BIBLIOGRÁFICOS

2.1 SUBPRODUCTOS DE LA INDUSTRIA AGROALIMENTARIA

En la actualidad, y como consecuencia de la intensa actividad de la industria agroalimentaria, se producen grandes cantidades de residuos en todo el mundo. Restos de hojas, tallos, frutos que no cumplen el estándar de calidad comercial, restos de pieles y pepitas, todos ellos generados como resultado de sus procesos de producción, con escaso o nulo valor económico para la empresa que los produce. Sin embargo, la eliminación de los subproductos agroalimentarios supone un sobrecoste para la empresa productora, así como un impacto negativo en el medio ambiente (O'Shea *et al*., 2012).

En la Unión Europea se producen, aproximadamente, un millón de toneladas de subproductos vegetales al año provenientes de la industria hortofructícola (Stojceska *et al*., 2008). Estos subproductos, obtenidos en grandes cantidades del procesado de vegetales y frutas, pueden resultar, sin embargo, interesantes por su composición. En este sentido, la revalorización de los subproductos de la agricultura y la industria alimentaria, se ha convertido en un eje prioritario de la Unión Europea (EUROSTAT, 2010) en apoyo al desarrollo sostenible.

Trabajos de investigación diversos y recientes, profundizan en los objetivos de recuperar, revalorizar y/o reciclar estos subproductos. Así, se han desarrollado diferentes aplicaciones que permiten la revalorización de los subproductos de la industria agroalimentaria para alimentación animal, fertilizantes, industria papelera, extracción de aceites esenciales y fragancias, compostaje, bioconversión, o su utilización como nuevos ingredientes en la formulación de nuevos productos (Kelbert *et al.*, 2015; Cañete-Rodríguez *et al.*, 2016; Marín *et al.*, 2015). En el caso de la formulación de nuevos productos con aplicación en industria alimentaria, los subproductos vegetales pueden ser

revalorizados como fuente tanto de componentes de alto valor nutricional como de compuestos bioactivos que les confieren capacidad antioxidante, anticancerígena o antimicrobiana (Martin-Luengo *et al*., 2011).

Desde el punto de vista del interés de esta tesis doctoral, las familias más importantes de vegetales cuyos subproductos son ricos en compuestos bioactivos son las de *Citrus*, *Brassicaceae* y *Fabaceae.*

2.1.1 Citrus

Los cítricos son los frutales más cultivados en todo el mundo, con una producción de, aproximadamente, 100 millones de toneladas anuales (Djilas *et al.,* 2009). Especialmente, los países del Mediterráneo presentan una importante producción y procesado de cítricos. En conjunto, los países de la Unión Europea producen 10 millones de toneladas al año de cítricos, aproximadamente.

Sin embargo, el procesado de cítricos genera grandes cantidades de subproductos (15 millones de toneladas al año en todo el mundo), que pueden ser revalorizados. Su utilización en la industria alimentaria se inició alrededor de 1920 y ha ido aumentando significativamente desde la década de los 80.

Los subproductos de cítricos se caracterizan por poseer compuestos bioactivos como aceites esenciales, vitaminas o flavonoides expresamente en su piel, semillas o pulpa (Sawalha *et al*., 2009; Callaway *et al*., 2008; Ghafar *et al*., 2010).

2.1.2 Brassicaceae

Esta familia vegetal se encuentra liderada en producción por el broccoli (*Brassica oleracea* var. Italica) y la coliflor (*Brassica oleracea* var. Botrytis) con valores de hasta 24.175.040 toneladas en 2014. El 75% de esta producción pertenece a China e India (FAOSTAT, 2017a). Estos vegetales son una fuente

importante de vitaminas antioxidantes, concretamente, vitamina A, C, E y ácido fítico, destacando también en cuanto a la presencia de metabolitos secundarios como carotenoides, cumarinas, glucosinolatos, compuestos fenólicos y terpenos (Cartea *et al*., 2010).

Las brassicas contienen también el enzima mirosinasa, responsable de la hidrólisis de los glucosinolatos en gases. Esta reacción ocurre cuando se produce una ruptura de tejido. Así, cuando la mirosinasa y los glucosinolatos entran en contacto, se produce la formación y liberación de los gases, entre los que se encuentran isotiocianatos, nitrilos y tiocianatos (Mori y Borek, 2010). Estas sustancias volátiles pueden ser utilizadas como tratamientos en el proceso de conservación de frutas y vegetales durante el almacenamiento o envasado en atmósfera modificada (Mari *et al.,* 2008).

2.1.3 Fabaceae

La familia *Fabaceae* destaca por ser una de las fuentes vegetales más ricas en proteína (20-25 %) de alto valor biológico, alcanzando valores de hasta el 38 % en legumbres como la soja y el cacahuete. La soja (*Glycine max*) es la legumbre más consumida en el mundo y su producción alcanza los 200 millones de toneladas al año (FAOSTAT, 2017b). En la actualidad, el principal productor de soja son los EEUU (32%), seguidos por Brasil (28%) y Argentina (21%) (Nahashon y Kilonzo-Nthenge, 2011).

La soja normalmente se procesa industrialmente para la obtención de bebida de soja y tofu. Este procesado incluye la extracción de agua y la producción de un subproducto llamado okara (Zhong y Zhao, 2015).

El okara deshidratado está compuesto por proteínas (24%), fibra (12-14.5%) y lípidos (8-15%), aunque también contiene potasio, calcio o niacina. Actualmente se utiliza como pienso para el ganado, concretamente cerdos y vacas, como fertilizante natural o para la elaboración de compost rico en

nitrógeno. Una pequeña parte se utiliza también en alimentación humana (Li *et al*., 2013; Vong y Liu, 2016).

2.2 ANTIMICROBIANOS NATURALES

Los antimicrobianos naturales procedentes de plantas y animales están alcanzando gran popularidad como alternativa a los productos sintéticos. En el contexto de la presente tesis, los antimicrobianos naturales se consideran también como una estrategia más en conservación no térmica de alimentos.

Los consumidores se encuentran cada vez más sensibilizados con la necesidad de cuidar su alimentación y seguir una dieta saludable, relacionada con la prevención del desarrollo de ciertas enfermedades, por lo que optan cada vez más por productos con ingredientes naturales seguros, en detrimento de los aditivos químicos (Carocho *et al.*, 2014). Todo esto propicia que el consumidor se preocupe más por la elección de los productos alimentarios que consume y se vuelve cada vez más crítico y exigente rechazando el consumo de aditivos sintéticos y seleccionando los aditivos naturales.

Los subproductos hortofructícolas ocupan una categoría privilegiada en este ámbito debido, sobre todo, a las propiedades beneficiosas para la salud asociadas a sus fitoquímicos (Naziri *et al.*, 2014; Teixeira *et al.*, 2014). Por ello, resulta especialmente interesante su revalorización y aprovechamiento como ingredientes bioactivos.

Como se ha comentado anteriormente, los subproductos de la industria hortofructícola constituyen una fuente rica de compuestos como azúcares, minerales, ácidos orgánicos, fibra alimentaria y compuestos bioactivos, que presentan características versátiles y una amplia gama de acción, que puede incluir actividad antioxidante, antitumoral, antiviral, o antibacteriana, entre

otras (Djilas *et al.*, 2009). La actividad bioactiva la confiere, fundamentalmente, un grupo diverso de metabolitos secundarios de plantas que pueden variar en su estructura (variaciones en su anillo heterocíclico) y en su ruta biosintética (Azmir *et al.*, 2013).

Uno de los grupos de compuestos responsables de la capacidad bioactiva de los residuos agroindustriales son los compuestos **polifenólicos.** Estos se pueden clasificar según el número de anillos aromáticos y su estructura. Así, los podemos agrupar en ácidos fenólicos (ácidos benzoicos y ácido cinámico), flavonoides, ligninas y estilbenos (Manch *et al.*, 2004). Entre todos ellos, los flavonoides son el tipo más abundante e incluye diferentes grupos como los flavanoles, las flavanonas, las antocianidinas, las flavonas, los flavonoles o las isoflavonas, entre otros (Andersen, 2006). Además, los polifenoles pueden estar asociados con diversos hidratos de carbono y ácidos orgánicos. Sus funciones en la planta se encuentran relacionadas con la polinización y la defensa frente a agentes patógenos y radiación ultravioleta.

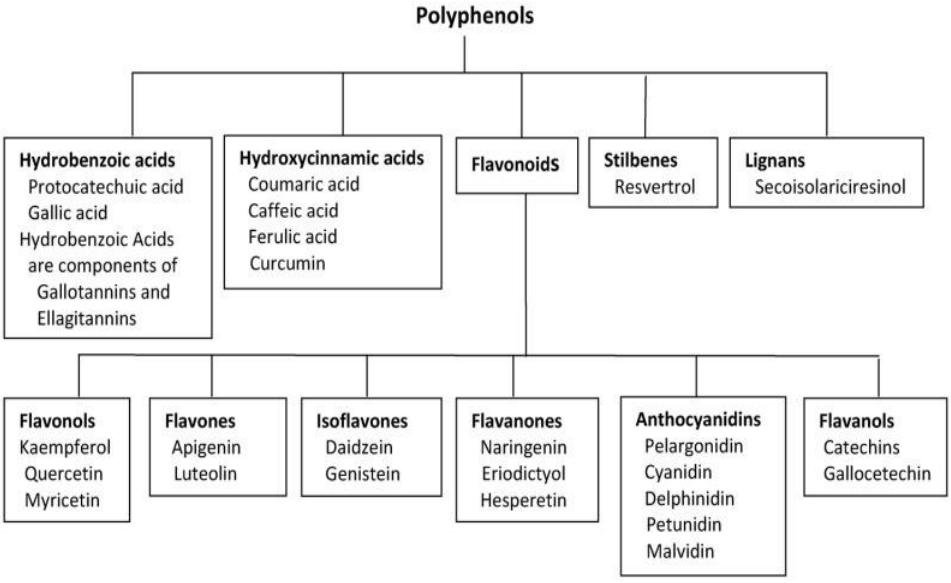

Figura 2.1. Clasificación de los polifenoles (Hardman, 2014).

Estudios previos han demostrado que estos compuestos poseen una importante capacidad antioxidante, así como de descomponer peróxidos, quelante de metales e inhibidora de radicales libres. Pero, además, a los compuestos fenólicos se les atribuyen otros mecanismos de acción que incluyen la actividad antitumoral, antiviral, antibacteriana, cardioprotectora y antimutagénica (Balasundram *et al*., 2006).

Los polifenoles se encuentran principalmente en bebidas como el té o el vino tinto, así como en productos hortofructícolas como legumbres, cereales, frutas, bulbos, bayas, y semillas. Algunos de ellos se encuentran más específicamente en ciertos alimentos como las flavanonas en cítricos o las isoflavonas en la soja. Aun así, la mayoría de alimentos contienen mezclas de polifenoles, lo que dificulta su caracterización pormenorizada. La concentración de polifenoles en los alimentos varía, además, según numerosos factores ambientales, tecnológicos y genéticos (Manch *et al*., 2004).

En los cítricos, concretamente en la piel, se pueden encontrar gran cantidad de flavonoides en comparación con otras partes del fruto. Estos flavonoides se pueden clasificar según su estructura, siendo principalmente, flavonas, flavanonas, flavonoles, isoflavonas, antocianidinas y flavanoles. Se les atribuye una gran capacidad antioxidante, antimicrobiana, anticancerígena, antiviral, antiinflamatoria y son efectivos frente a la fragilidad capilar y en la agregación óptima de las plaquetas humanas (Senevirathne *et al.*, 2009).

Por su parte, los compuestos fenólicos presentes en las brassicas ejercen efectos protectores (Dekker *et al*., 2000). Los flavonoides confieren a estos vegetales una importante capacidad antioxidante y antimicrobiana (Volden, 2009). Estudios previos han evidenciado que las brassicas, particularmente la *Brassica oleracea*, L., subsp. Botrytis (coliflor), posee un contenido elevado de compuestos fenólicos, siendo aproximadamente 270 mg de ácido gálico por

100 gramos de porción comestible seca el total de polifenoles (Pincchi *et al*., 2012).

Las propiedades antioxidantes y antimicrobianas de estos vegetales pueden tener un impacto importante en el campo de los nutracéuticos y en la industria de procesado de alimentos, principalmente por su contenido en polifenoles y en glucosinolatos (Cabello-Hurtado *et al*., 2012; O'Shea *et al*., 2012). Además, cultivos como la soja son ricos en isoflavonas, polisacáridos, fitoesteroles, saponinas y fitatos, que le confieren una actividad antioxidante y antimicrobiana relevante (Guan *et al.*, 2016).

Por lo tanto, todas las propiedades que se les atribuyen a estos compuestos presentes en los subproductos de la industria hortofructícola les confieren un creciente interés para su aprovechamiento como aditivos alimentarios o suplementos con alto valor nutricional, convirtiendo su revalorización en económicamente viable y atractiva (Djilas *et al*., 2009).

La formulación de nuevos productos ricos en compuestos bioactivos funcionales que, además de ejercer sus destacadas propiedades para la salud, proporcionen un potencial antimicrobiano demostrado, y que bien solos o combinados con tratamientos mínimos de conservación (térmicos / no térmicos) permitan prolongar la vida útil de los nuevos productos diseñados, es uno de los campos de investigación más relevantes en el ámbito de la conservación de alimentos. En este sentido destaca la aplicabilidad de los subproductos de la industria agroalimentaria como ingredientes en sí mismos, con potencial tecnológico o funcional, fuentes ricas en compuestos con propiedades antimicrobianas y valor nutricional destacado (p.e. contenido en fibra), que añadidos a los alimentos permiten mejorar el perfil nutricional, bioactivo y/o organoléptico del producto, al tiempo que ejercen un efecto

antimicrobiano aditivo o sinérgico a otros procesos tradicionales y emergentes de conservación (Pina-Pérez *et al.* 2012).

2.3 NUEVAS TECNOLOGÍAS DE CONSERVACIÓN DE ALIMENTOS. TECNOLOGÍAS NO TÉRMICAS.

Tradicionalmente, la conservación de alimentos se ha fundamentado en el control del efecto de la temperatura sobre el producto (tratamientos a elevada temperatura; conservación en frío), el control de la actividad de agua (deshidratación, salazón, concentración, baja $a_w < 0.75$) o el pH en el alimento (acidez de los productos como medida de conservación). En la actualidad, estas tecnologías siguen siendo las más utilizadas en la conservación de alimentos.

Entre los procesos de conservación tradicionales más utilizados destaca el tratamiento térmico, que consiste en la aplicación de una temperatura entre 60 y 140 ^{o}C sobre el alimento durante un tiempo determinado (desde segundos a minutos). Esto provoca la trasferencia de una gran cantidad de energía sobre el alimento, pudiendo provocar reacciones no deseables (desnaturalización de las proteínas, caramelización de los azúcares, oxidación de los lípidos), cambios en sus propiedades (color, textura) o la formación de subproductos no deseables (Wang *et al.*, 2016). Sin embargo, el tratamiento térmico tiene un bajo coste en relación con su efectividad, asegurando la estabilidad microbiológica del producto. Por lo tanto, el principal inconveniente del tratamiento térmico es la pérdida de calidad del alimento debido a modificaciones en las propiedades fisicoquímicas y/o nutricionales del mismo, que comprometen la aceptabilidad y preferencia del producto por parte del consumidor (Deliza *et al*., 2005).

Esta situación genera que la innovación tecnológica en la industria alimentaria se haya centrado en la investigación y desarrollo de procesos

alternativos de conservación que sean capaces de satisfacer las exigencias del consumidor, es decir, en la obtención de alimentos seguros y que, además, mantengan en mayor proporción sus propiedades organolépticas y nutricionales incrementando la vida útil del producto.

La investigación en tecnologías de conservación de alimentos se realiza bajo los siguientes objetivos: optimización de los tratamientos térmicos tradicionales, aplicación de procesos mínimos de conservación consistentes en la combinación de diferentes tratamientos de baja intensidad y el desarrollo y validación de tecnologías alternativas de conservación de alimentos en las que la temperatura no ejerce un papel principal (Rivas, 2012).

De esta manera, en los últimos 20 años nacen las tecnologías no térmicas de conservación de alimentos. Se trata de tecnologías en las que el uso de temperaturas elevadas no es el principal factor responsable de la estabilización del alimento y se reduce, por tanto, el impacto negativo en la calidad nutricional y organoléptica del producto. En la actualidad existen diferentes tecnologías no térmicas de conservación de alimentos, y algunas son empleadas exitosamente en la industria, aunque su desarrollo continúa para mejorar sus prestaciones tanto a nivel de inactivación de microorganismos y conservación de las características naturales de los alimentos como de su eficiencia energética. Estas nuevas tecnologías permiten la pasteurización de alimentos sin modificar significativamente las propiedades fisicoquímicas y nutricionales del producto. Algunas de ellas son la radiación por ultrasonidos, los campos magnéticos oscilantes, la radiación con rayos ultravioleta, las altas presiones hidrostáticas (HHP) o los pulsos eléctricos de alta intensidad (PEF) (Herrero, 2006).

Las tecnologías no térmicas de conservación de alimentos se adaptan a las exigencias de los diferentes productos presentes en el mercado, siendo

versátiles y efectivas en su aplicación tanto a alimentos sólidos como líquidos. Así, la tecnología de PEF es más apropiada para su aplicación en alimentos líquidos y semi-líquidos (p.e. purés) y las HHP permiten tratar tanto alimentos líquidos como sólidos, ya envasados. Cada tecnología de procesado de alimentos presenta sus ventajas e inconvenientes, de manera que es necesario un estudio previo de las características del producto alimentario que se desea procesar para poder seleccionar la tecnología más adecuada a sus características.

2.3.1 Altas Presiones Hidrostáticas

La primera vez que las HHP se aplicaron a un alimento fue en un trabajo realizado por Hite (1899), en el que se intentó esterilizar leche mediante presurización, demostrando la reducción de la población microbiana alcanzada tras la aplicación de esta tecnología. Posteriormente, se estudió el efecto de las HHP en frutas y hortalizas (Hite *et al*., 1914). Pero no fue hasta la década de los 80 cuando realmente se empezó a investigar exhaustivamente el tratamiento de alimentos por HHP. Los primeros estudios realizados sobre matrices alimentarias se llevaron a cabo en EEUU en 1982 en la Universidad de Delaware (Hoover *et al.,* 1989; Hoover, 1993). Seguidamente, en 1986, la Universidad de Kyoto, en Japón, inició nuevas líneas de investigación en este ámbito (Hayashi, 1989ab).

Japón fue el país pionero en la producción y comercialización de alimentos tratados por HHP. Los primeros en comercializarse fueron zumos y derivados de frutas, concretamente, mermeladas de fresa, frambuesa, kiwi y manzana (Ledward *et al*., 1995), comercializados por la empresa japonesa Meidi-Ya Food Co.

En la actualidad las HHP es la tecnología no térmica más utilizada industrialmente en el tratamiento de productos alimentarios,

comercializándose gran cantidad de productos tratados por HHP en Japón, EEUU y Europa. Se pueden encontrar mermeladas, zumos, jaleas, concentrados, purés de frutas, postres, patés, productos lácteos o productos cárnicos curados y cocidos loncheados y preparados listos para su consumo, entre otros, tratados por HHP.

En la industria alimentaria se utilizan, principalmente, sistemas de pasteurización en frío (T^a< 40ºC), con tiempos de tratamiento entre 4 y 10 minutos y presiones normalmente superiores a 400 MPa e inferiores a 700 MPa (Téllez-Luis *et al*., 2009).

La utilización de HHP se rige por dos principios fundamentales:

- Principio de Le Chatelier: cualquier fenómeno que va acompañado de disminución de volumen se favorece al aumentar la presión y viceversa.
- Ley de Pascal: una presión externa aplicada a un fluido confinado se transmite de forma uniforme e instantánea en todas las direcciones.

Por ello, las HHP pueden aplicarse a alimentos líquidos o a productos convenientemente envasados, sumergidos en un fluido de presurización. De esta manera, la presión aplicada permite un tratamiento isostático y uniforme independientemente de cual sea el tamaño, forma y volumen del alimento (Herrero y Romero, 2006).

El equipo de HHP está formado por una cámara de presurización, una bomba generadora de presión y un fluido transmisor de la presión. La presión en el interior de la cámara se alcanza mediante una compresión indirecta (San Martín *et al*., 2002; Patterson, 2005). La bomba de presión transmite el fluido presurizado desde un depósito hasta la cámara de presurización correctamente cerrada (Figura 2).

Los alimentos, una vez envasados, se introducen en la cámara de presurización, ésta se cierra correctamente y se llena con fluido trasmisor. A continuación, la bomba de presión comienza a presurizar el fluido y, una vez se alcanza la presión deseada, se detiene el bombeo de fluido, las válvulas se cierran y la presión se mantiene constante (Farr, 1990).

El envase del alimento debe ser parcialmente flexible y deformable para el tratamiento por HHP (ha de tolerar reducciones de volumen de hasta un 15%). Normalmente se utilizan envases a base de copolímeros de etilino-alcohol vinílico (EVOH) o alcohol polivinílico (PVOH) (Barbosa-Cánovas *et al*., 2005).

Figura 2.2. a) Sistema de tratamiento por HHP en alimentos pre-envasados por compresión indirecta (Moreau, 1995). b) Equipo de HHP a escala de planta piloto en el Instituto de Agroquímica y Tecnología de los Alimentos (IATA), Valencia.

El volumen de la cámara de presurización puede variar desde menos de un litro, para aplicaciones a escala de laboratorio utilizando presiones hasta 1000 MPa, hasta alrededor de 400 litros, para aplicaciones de procesado industrial de alimentos utilizando como máximo 600 MPa. El volumen permitido de producto debe de ocupar entre el 50 y el 75% del espacio interno de la cámara (Téllez-Luis*et al*., 2009).

En un tratamiento por HHP, las principales variables que intervienen son el nivel de presión y el tiempo de tratamiento. Sin embargo, debido a que el tratamiento tiene lugar en condiciones adiabáticas, el aumento de presión produce un aumento de la temperatura, de diferente magnitud según las características del líquido presurizante (Toepfl *et al*., 2007). Por esta razón, la temperatura es la tercera variable importante a tener en cuenta en un tratamiento por HHP (Balasubramaniam *et al*., 2004). El incremento de la temperatura adiabática puede variar entre 3 y 9 ^{o}C por cada 100 MPa, según la composición del producto, su temperatura inicial y la presión aplicada sobre el mismo (Heij *et al*., 2003; Toepfl *et al*., 2007).

En cuanto a los fluidos transmisores de presión, el etanol y el etilenglicol generan gran cantidad de calor durante la compresión. El agua es el más utilizado en procesos de conservación de alimentos (Buzrul *et al.*, 2008).

Los mecanismos de inactivación de microorganismos por HHP han sido descritos por diversos grupos de investigadores, siendo los efectos a nivel celular que afectan a la viabilidad de los microorganismos como la disminución en la síntesis de ADN, el aumento de la permeabilidad en las membranas celulares, la desnaturalización de proteínas y enzimas o cambios en la morfología celular, de los más destacados. Algunos de estos efectos, son reversibles a bajas presiones (<200 MPa) pero irreversibles a presiones más altas (>300 MPa). El efecto de las HHP sobre la inactivación microbiana depende de las variables de tratamiento (presión, tiempo y temperatura), de la composición del alimento y del tipo de microorganismo. La inactivación microbiana por HHP se produce en mayor medida si el microorganismo se encuentra en la etapa logarítmica de crecimiento. Generalmente, los microorganismos más sensibles al tratamiento por HHP son los Gram negativos, seguidos por las levaduras, los hongos, los Gram positivos y las esporas. Las

células vegetativas se pueden inactivar a presiones entre 400 y 600 MPa mantenidas durante pocos minutos (3-10 min) (Daryaei *et al.*, 2016). Las esporas bacterianas son las más resistentes a la presión requiriendo de combinaciones de presión (600-1200 MPa) y temperatura moderada para su inactivación (p.e. 600MPa – 90 °C, frente a esporas de *Bacillus cereus*) (Evelyn y Silva, 2016).

En las condiciones en las que se trabaja habitualmente en el procesado de alimentos por HHP no se ven afectados los enlaces covalentes y no se alteran los aromas ni el valor nutricional del producto, pero sí que se pueden producir cambios de color y apariencia y modificaciones en la textura, que varían según el alimento presurizado.

Aunque inicialmente las HHP se empezaron a utilizar con el objetivo de la conservación de alimentos, los cambios que ocasionan en diversos productos han demostrado su potencial en la elaboración de algunos productos alimenticios. Así, la aplicación de HHP se puede emplear para obtener geles de pescado, carne, huevo o leche, ablandar la textura en carnes y pescados, inactivar toxinas, retardar o acelerar procesos de maduración o fermentación enzimática, congelar a temperaturas superiores a cero evitando la formación de cristales de hielo, disminuir el punto de fusión de lípidos, conseguir la agregación de alimentos sólidos o en polvo o impedir el pardeamiento y la oxidación lipídica. Además, la aplicación de HHP favorece la difusión de solutos en los alimentos, la solubilización de gases y la extracción de compuestos (Toledo-del-Arbol, 2016).

2.3.2 Pulsos Eléctricos de Alta Intensidad

Los primeros estudios relacionados con la aplicación de PEF para la inactivación de microorganismos datan de los años 60 del siglo XX. Sin embargo, su paso del laboratorio a la industria alimentaria se ha prolongado en

el tiempo. Esto se debe a que el escalado de la tecnología a nivel industrial requería de la posibilidad de realizar el tratamiento en flujo continuo, y esto no fue posible hasta los años 80 (Puértolas *et al*., 2013).

Los primeros equipos de PEF para el tratamiento de alimentos líquidos fueron comercializados por la compañía Krupp Maschinentechnik (Alemania) en 1985, pero no tuvieron mucho éxito industrial debido al elevado coste de los equipos y al aumento de temperatura que generaban en los productos, asociada a la elevada intensidad de los pulsos aplicados. En los años 90, la empresa PurePulse Technologies (EEUU) decidió desarrollar un equipo de PEF que alcanzara velocidades de flujo de hasta 2000 L/h, pero no se llegó a comercializar debido a la complejidad del proceso y a que los resultados a escala de planta piloto no fueron los deseados (Puértolas *et al.,* 2013).

En los últimos años, el Instituto Alemán de Tecnología Alimentaria (DIL, Alemania) y las empresas Diversified Technologies Inc. (EEUU) y ScandiNova Systems (Suecia) han comercializado equipos industriales de PEF que alcanzan hasta 50 kW de potencia y 2000 L/h de capacidad. Así, la empresa Genesis Juice Corp. (EEUU) empezó a comercializar hace unos años zumos de frutas tratados por PEF (OSU-5), que preservaban en mayor medida sus características nutricionales y sensoriales y presentaban una mayor vida útil que aquellos tratados por pasteurización térmica.

La tecnología de PEF destaca entre las tecnologías no térmicas de conservación de alimentos por su corto tiempo de tratamiento y su baja aplicación de calor. En la actualidad, es una de las tecnologías más prometedoras para la pasteurización de alimentos líquidos. Se considera una de las tecnologías no térmicas más eficaces para el control de microorganismos en alimentos líquidos, permitiendo la inactivación de células vegetativas de

bacterias patógenas y levaduras en diversos alimentos, sobretodo en alimentos ácidos (Saldaña, 2012).

Su efecto sobre los microorganismos se basa en la destrucción o alteración de su membrana celular como consecuencia de la aplicación de una intensidad de campo eléctrico que genera una diferencia de potencial entre ambas partes de dicha membrana, dando lugar a la formación de poros. Este fenómeno se conoce como electroporación y produce la pérdida de la integridad de membrana celular y aumenta su permeabilidad de forma transitoria o permanente, causando la destrucción de la célula afectada (Herrero y Romero, 2006). La electroporación irreversible puede inactivar las células vegetativas de bacterias, levaduras o mohos a temperaturas inferiores a las del tratamiento térmico convencional.

Esta tecnología puede aumentar su eficacia si se combina con otros factores como la temperatura, pH, fuerza iónica o agentes antimicrobianos, o si se aplica sobre células estresadas, especialmente si el factor estresante afecta a la integridad de la membrana (Saldaña *et al*., 2012).

El tratamiento por PEF consiste en la aplicación de pulsos eléctricos de corta duración (1 – 10 µs) a intensidades de campo eléctricas altas (15-40 kV/cm) en alimentos situados entre dos electrodos, uno de ellos conectado a tierra y el otro al generador de PEF, produciéndose un campo eléctrico en el espacio comprendido entre ambos electrodos.

El equipo de PEF está formado por un generador de pulsos, una cámara de tratamiento, un mecanismo que impulsa el alimento a través del sistema y dispositivos que controlan la temperatura. El generador de pulsos está formado por un generador de corriente de voltaje continuo a partir de la corriente alterna de la red eléctrica, un generador de energía, un sistema de almacenamiento de energía eléctrica mediante condensadores y componentes

que liberan energía en forma de pulsos con las características deseadas mediante la combinación de condensadores, resistencias e interruptores (Rivas, 2012).

Los tipos de pulsos eléctricos más utilizados son de caída exponencial (incremento rápido del voltaje y disminución exponencial a lo largo del tiempo) o de onda cuadrada (incremento rápido de voltaje, que se mantiene constante durante un tiempo determinado y disminuye rápidamente).

El efecto del tratamiento por PEF en la inactivación de microorganismos y enzimas depende de una serie de parámetros propios de la tecnología, pero también de la naturaleza del alimento o del microorganismo a tratar. Cabe destacar los siguientes factores (Amiali *et al*., 2004; Rodrigo *et al*., 2003):

- Factores técnicos: intensidad de campo eléctrico, forma del pulso, amplitud del pulso, tiempo de tratamiento, energía del pulso, temperatura.
- Factores biológicos: resistencia del microorganismo, fase de crecimiento, concentración celular.
- Factores relacionados con el producto: pH, actividad de agua, fuerza dieléctrica, grasas y proteínas.

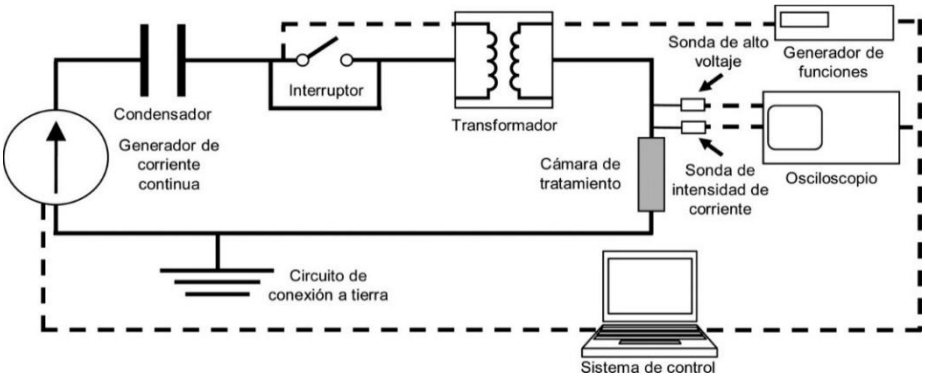

Figura 2.3. Esquema de un equipo de PEF de flujo continuo (Puértolas *et al.*, 2013).

Esta tecnología presenta diversas ventajas, pero también algunas limitaciones. Éstas están asociadas principalmente a su aplicación limitada sólo a alimentos líquidos homogéneos o que posean pequeñas partículas o burbujas de gas, con una conductividad eléctrica y viscosidad comprendida en un determinado intervalo. Además, se trata de una tecnología que se puede aplicar para la pasteurización de alimentos, no siendo eficaz en la inactivación de esporas de microorganismos y, en el caso de alimentos con baja acidez, es necesario su posterior almacenamiento en refrigeración. Estos inconvenientes pueden mejorarse adaptando la formulación de los productos a procesar o ajustando las condiciones de tratamiento, de manera que aumente la eficacia del mismo.

El tratamiento por PEF es eficaz en la inactivación de microorganismos patógenos y su combinación con temperaturas moderadas permite inactivar enzimas y microorganismos de forma similar a la pasteurización térmica, preservando además la calidad del alimento (características organolépticas y nutricionales) en comparación con los tratamientos convencionales. Así, su aplicación en la industria alimentaria, focalizada en la pasteurización de alimentos y el aumento de su vida útil respecto a los productos tratados térmicamente ha crecido significativamente (Knorr, 2011).

Figura 2.4. Equipo de PEF OSU-4D existente en las instalaciones del IATA-CSIC.

2.4 TECNOLOGÍA DE BARRERAS

Tradicionalmente se ha tratado de alcanzar los niveles deseados de inactivación microbiana en los alimentos a través de la aplicación de un solo tratamiento, generalmente térmico, o del control de parámetros como el pH, la sal, los conservantes, el envase, la temperatura de almacenamiento o la a_w del alimento, lo cual requería la aplicación de tratamientos de elevada intensidad que afectaban a las características organolépticas y nutricionales de los productos tratados. Sin embargo, la estabilidad microbiológica y seguridad del alimento está afectada por una combinación de varios factores sobre los cuales se puede actuar de forma simultánea para alcanzar los objetivos deseados de seguridad alimentaria del producto. Esto es lo que se conoce como "Tecnología de Barreras", término que fue propuesto por primera vez por Leistner en 1978.

La tecnología de barreras consiste en la aplicación de forma combinada de diferentes tratamientos de conservación de alimentos de baja intensidad (sub-letales) con el objetivo de obtener un efecto sinérgico entre ellos que permita la inactivación de los microorganismos patógenos presentes en el producto (Leinster, 2000). La tecnología de barreras es ventajosa porque nos permite evitar la aplicación de un solo tratamiento de elevada intensidad para la conservación del alimento, y propicia la aparición de sinergias entre los diferentes tratamientos sub-letales aplicados (Rahman, 2015).

Cuando una célula es sometida a un tratamiento sub-letal (tratamiento térmico, pH, HHP, PEF, incubación en un medio estresante) puede sobrevivir o morir, pero, de las que sobreviven, un porcentaje de células estarán dañadas y otro porcentaje serán células intactas (células que no han sufrido ningún daño). Las células dañadas, en condiciones óptimas, se pueden recuperar y volver de nuevo a su condición de células intactas. En cambio, si no están en condiciones óptimas para reparar el daño celular, no se recuperan y mueren. Esto provoca

la obtención de una población de células dañadas no controlada. En este punto, cobra verdadera importancia la tecnología de barreras porque mediante la combinación de diferentes tratamientos sub-letales se puede obtener un efecto antimicrobiano sinérgico que nos permita inactivar toda la carga microbiana presente en el producto, y ofrecer así alimentos seguros y de calidad al consumidor.

Concretamente, el efecto de los tratamientos de HHP o PEF en la inactivación de microorganismos patógenos (*Listeria monocytogenes, Salmonella spp., Bacillus cereus, Escherichia coli O157:H7, Cronobacter sakazakii,* entre otros) presentes en alimentos, así como su capacidad de preservar la calidad del alimento y aumentar su vida útil ha sido ampliamente demostrado (Pina-Pérez *et al.*, 2009; Sanz-Puig *et al.*, 2016; Mukhopadhyay*et al.*, 2016). Sin embargo, en ocasiones es necesaria la aplicación de tratamientos de elevada intensidad para conseguir los niveles deseados de inactivación microbiana. Por ello, resulta una opción interesante la combinación de estas tecnologías con la adición de antimicrobianos naturales con el objetivo de reducir la intensidad del tratamiento requerido y aprovechar el efecto de las sinergias generadas (Pina-Pérez *et al*., 2012; Oliveira *et al*., 2015; Montiel *et al*., 2015).

2.5 GENERACIÓN DE RESISTENCIAS EN MICROORGANISMOS

La aplicación de tratamientos de conservación sub-letales puede generar resistencias en los principales patógenos transmitidos por alimentos.

Los antimicrobianos son una amplia gama de productos de síntesis o producidos por animales o plantas que tienen como finalidad combatir infecciones o protegerse de la acción de distintos microorganismos. Muchos de ellos se utilizan en terapias humanas como son los antibióticos pero cada vez

están alcanzando más popularidad los antimicrobianos naturales procedentes de plantas y animales para conservar alimentos, bien usándolos como único método de conservación o bien en combinación con otras tecnologías no térmicas y térmicas suaves, de tal forma que en su conjunto consiguen el efecto conservador deseado como se indicó anteriormente.

Aunque el uso de pequeñas dosis o intensidades de antimicrobianos naturales o procedimientos físicos de conservación tiene sus ventajas bajo el punto de vista de su pequeño impacto sobre la calidad nutricional o sensorial, también tienen algunos inconvenientes, ya observados en el uso de antibióticos utilizados en terapia para humanos o para animales de granja.

Inicialmente los antimicrobianos (fundamentalmente antibióticos) se empezaron a utilizar para tratar infecciones bacterianas tanto en humanos como en animales, pero en la década de los años 50 se observó en la industria agropecuaria que la administración de pequeñas dosis de antimicrobianos aceleraba el crecimiento de animales sanos. Uno de los efectos del uso de antibióticos a dosis subletales es la creación de resistencias a dichos antibióticos e incluso resistencias cruzadas. Ya en la década de 1970 se asociaron las dosis subletales con el desarrollo de resistencias microbianas y, en consecuencia, se inició la regulación en el uso de antibióticos en terapia de animales o como promotores del crecimiento (Espino, 2007; Andersson y Hughes, 2014).

Los antimicrobianos naturales obtenidos de animales o plantas, no antibióticos, usados como conservantes a dosis subletales podrían producir también resistencias a dichos antimicrobianos e incluso afectar a la eficacia de los antibióticos usados en terapia humana o animal debido a cambios en la membrana de los microorganismos (Zanini *et al.*, 2014). De la misma manera

puede ocurrir un efecto similar con el uso de dosis subletales de los tratamientos no térmicos de conservación.

En la actualidad, el desarrollo de resistencias microbianas es un grave problema de salud pública que afecta a todas las especies bacterianas y se pueden transferir al hombre desde el ambiente a través de la cadena alimentaria. La utilización de aguas contaminadas para el regadío de los cultivos y de heces para el abono de los mismos propicia la diseminación de resistencias. Además, la intensa actividad del metabolismo bacteriano en el tracto gastrointestinal de animales y humanos lo convierte en un ecosistema extremadamente favorable para el intercambio de genes de resistencia entre bacterias (FAOSTAT, 2017a). Las resistencias adquiridas por los microorganismos se pueden diseminar rápidamente entre ellos debido a su facilidad para intercambiar material genético. Así, los elementos móviles del ADN bacteriano son los principales responsables de la diseminación de una amplia gama de factores genéticos que confieren resistencia a antimicrobianos.

Los microorganismos pueden adquirir resistencia a las sustancias antimicrobianas de diferentes maneras como, por ejemplo, la modificación de la permeabilidad de su membrana celular, la alteración de las glicoproteínas presentes en su pared celular, la inhibición de determinados enzimas, la eliminación del antimicrobiano mediante sistemas de bombeo o la modificación de la diana sobre la que actúan (Kottwitz *et al*., 2013).

Esta fuerte capacidad de expansión de genes de resistencia puede dar lugar a la aparición de multiresistencias, es decir, microorganismos que adquieran la capacidad de ser resistentes a varios tipos de antimicrobianos simultáneamente, suponiendo un problema mucho mayor de salud pública (Medeiros *et al*., 2011).

Los dos géneros con mayor riesgo de transferencia zoonótica de resistencias microbianas son *Salmonella* spp. y *E. coli*. En la década de los 90 empezaron a emerger cepas tanto de *E. coli* como de *Salmonella* spp*.,* principalmente de los serovares *S.* Typhimurium y *S.* Enteritidis, resistentes a diferentes grupos de antimicrobianos utilizados en el tratamiento clínico de infecciones, que fueron aisladas tanto de humanos como de animales o alimentos (Puig *et al.*, 2011; Quesada *et al.*, 2016). Además, se han encontrado casos de multiresistencia en *E. coli* y en *Salmonella* spp., principalmente,en *S.* Typhimurium (Arthur *et al*., 2008). Por ello, este serovar requiere ser tratado con especial atención debido a su elevada virulencia en humanos y en animales y su creciente resistencia a antimicrobianos (Zanini *et al*., 2015).

Así mismo, las modificaciones a nivel celular que les confieren a los microorganismos la capacidad de adquirir resistencias a diferentes antimicrobianos, pueden implicar también un cambio en su virulencia frente a un organismo hospedador.

2.6 INDUCCIÓN DE CAMBIOS DE VIRULENCIA EN MICROORGANISMOS

Tal como se ha comentado anteriormente, los tratamientos subletales del tipo que sea podrían inducir cambios en la virulencia de los microorganismos patógenos. Cambios que podrían derivar en microorganismos más virulentos o menos virulentos (Silva et al 2015). Para los estudios de virulencia, así como de los cambios de virulencia de los microorganismos se pueden usar, entre otros, modelos *in vitro* como las células CACO-2, que nos permiten realizar estudios relacionados con la función y diferenciación de las células intestinales *in vitro* (Monente *et al.*, 2015; Maestre *et al.*, 2013) o modelos *in vivo* basados en el efecto sobre el nematodo *C. elegans,* el cual

resulta especialmente interesante ya que posee un sistema digestivo e inmunológico similar al humano (Altun *et al.*, 2009; Balla *et al.*, 2013).

C. elegans pertenece al filum *Nematoda*, al género *Caenorhabditis* y a la familia *Rhabditidae* (Strange, 2006;Sommer, 2005). Se trata de un organismo multicelular, que ha sido ampliamente estudiado a lo largo de cuatro décadas (Aitlhadj *et al*., 2014; Corsi *et al*., 2015). Vive en el suelo, especialmente en zonas con vegetación en descomposición y se alimenta de todo tipo de bacterias (Balla *et al*., 2013; Edgley *et al*., 2015).

C. elegans no representa ningún peligro para el ser humano ya que no es un organismo infeccioso ni patogénico. Tampoco es parasitario y no tiene importancia a nivel económico (Edgley *et al*., 2015). Además, presenta muchas ventajas para su estudio en el laboratorio ya que tiene un ciclo de vida corto (entre 18 y 21 días) y se puede usar una lupa binocular para su visualización, ya que tieneun tamaño de 1 mm aproximadamente.

Figura 2.5.*C. elegans* visto al microscopio Nikon Eclipse 9i del IATA-CSIC.

Su manejo en el laboratorio es sencillo, ya que su tasa de reproducción es elevada, se pueden crioconservar durante largos periodos de tiempo y crecen con facilidad en medios de cultivo. Todas estas características convierten a *C. elegans* en una de las mejores opciones a la hora de elegir una herramienta en el laboratorio para detectar los posibles cambios de virulencia, o el efecto que esta tiene sobre el nematodo en distintas fases de su ciclo de vida.

Su principal ventaja es su transparencia permitiendo visualizar cambios a nivel celular, por ejemplo, la colonización del intestino por microorganismos patógenos utilizando el marcaje por fluorescencia (Irazoqui *et al*., 2010). Además, las células epiteliales del intestino de *C. elegans* son morfológicamente muy similares a las de los mamíferos, por lo que se trata de un buen modelo para estudiar las infecciones intestinales en humanos (Kawli *et al*., 2010).

2.6.1. *C. elegans:* Características fisiológicas y ciclo vital

C. elegans tiene un cuerpo cilíndrico no segmentado, que se estrecha en las extremidades. Internamente presenta dos tubos concéntricos, separados por el pseudoceloma. En el tubo interior se encuentran la faringe, el intestino y la gónada y en el tubo exterior se encuentra la cutícula, la hipodermis, el sistema excretor, las neuronas y los músculos. Las estructuras neuronales de la cabeza incluyen los órganos sensoriales que permiten que los nematodos puedan reaccionar al gusto o tacto, así como a la temperatura o a la luz (Edgley *et al.*, 2015).

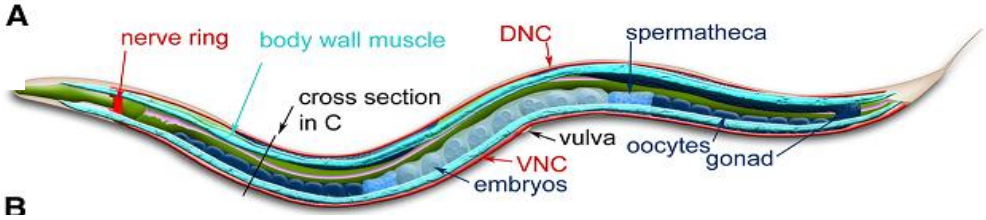

Figura 2.6. Anatomía de *C. elegans* hermafrodita(Corsi*et al.*, 2015).

C. elegans cuenta con un sistema muscular que está formado por dos tipos de músculo: sarcómero múltiple o estriado y sarcómero simple o no estriado. El sarcómero múltiple o estriado es mucho más abundante en *C. elegans*, ya que es el que constituye la pared del cuerpo y es el responsable de su movilidad (Krause *et al.,* 2012). La mayor parte de sus músculos se forman durante la embriogénesis y cumplen funciones como el paso del alimento a través de la faringe, la contracción intestinal o la puesta de huevos (Corsi*et al.,* 2015).

Además presenta un sistema nervioso sencillo, formado por neuronios, conectados entre ellos formando vias neuronales. Los neuronios mecanico-sensoriales regulan su comportamiento a nivel de movilidad, puesta de huevos o bombeo de la faringe. Concretamente, su movilidad se rige por un patrón sinusoidal de los músculos de la region ventral y dorsal, los cuales responden a estimulos mecanico-sensoriales a través de neuronios motores (Aballat *et al.*, 2013).

El sistema digestivo en los individuos hermafroditas consta de tres partes diferenciadas: la cavidad bucal y faringe, el intestino y el recto o ano. Su alimento principal son microorganismos del suelo y materia orgánica en descomposición, que atraviesan la boca y la faringe, donde son trituradas por dientes entrelazados. La faringe actúa como un filtro, ya que tiene la capacidad de separar las partículas del fluido y expulsar el fluido. A continuación, el alimento llega al intestino a través de la válvula faringe-intestinal, donde se produce la digestión. El tiempo que permanece el alimento en el intestino es un factor importante en la nutrición del nematodo. Finalmente, se produce la defecación a través del recto y el ano (Altun *et al*., 2009).

El sistema reproductor está formado por una gónada formada por dos brazos, cada uno de los cuales presenta ovario, oviducto, espermateca, útero y

vulva. Cada uno de los espermatozoides fecunda un ovocito y, tras la fertilización, los huevos comienzan su maduración. Una vez se agota el número de espermatozoides, se finaliza la puesta de huevos.

Cada ovocito es fecundado por un espermatozoide y se convierte en un embrión en desarrollo. El desarrollo embrionario se produce en el útero y posteriormente los huevos salen al exterior a través de la vulva (Robertson *et al.*, 2015). En algunos casos el nematodo es infértil debido a mutaciones que afectan a alguno de los gametos. En este caso los ovocitos no fertilizados se acumulan en el útero y se produce la muerte del nematodo (Marcello y Singson *et al.*, 2010).

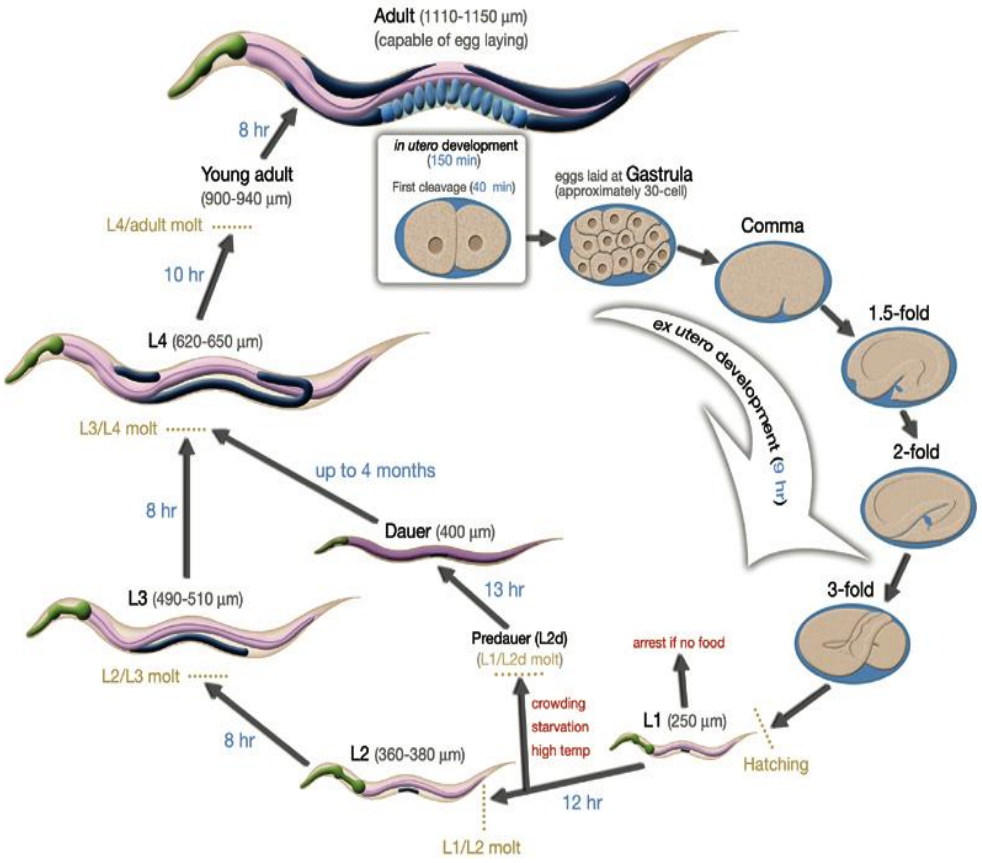

Figura 2.7. Ciclo de vida de *C. elegans* (Wormatlas).

En condiciones óptimas el nematodo adulto fecunda los huevos y estos empiezan a desarrollarse pasando por 3 fases: fase embrionaria o embriogénesis, fase larvaria (comprende los estadios larvarios L1, L2, L3 y L4) y fase adulta.

El proceso de embriogénesis consta de dos fases:

- Fase de proliferación: se forma el embrión, se generan las células embrionarias y, a continuación, se producen múltiples divisiones celulares.
- Fase de morfogénesis u organogénesis: el embrión se alarga y se produce el desarrollo y diferenciación de todos sus tejidos y órganos hasta alcanzar la estructura de un nematodo adulto (Altun *et al.*, 2009).

A continuación, tiene lugar la eclosión del huevo y el inicio del desarrollo post-embrionario o fase larvaria, en la que se produce el desarrollo de la larva hasta alcanzar el estadio adulto. En presencia de alimento y en condiciones óptimas de crecimiento, se inicia la división celular a un ritmo muy acelerado, de forma sincronizada entre individuos (Rougvie *et al.*, 2013). El desarrollo de la fase larvaria tiene lugar en 4 etapas diferenciadas (L1, L2, L3 y L4). Seguidamente, el nematodo entra en la Fase Adulta, en la cual los individuos hermafroditas ponen sus primeros huevos, llegando a poner alrededor de 300 huevos.

2.6.2.Modelo in vivo en el estudio de patogénesis bacteriana

Aunque inicialmente Sydney Brenner propuso *C. elegans* como un modelo genético en 1963 para estudios de biología del desarrollo y neurobiología, su simplicidad y fácil manejo en el laboratorio ha propiciado que, posteriormente, su uso se haya extendido a otras áreas como la biología de la evolución, la interacción entre parásito y hospedador, o determinadas

enfermedades humanas (Corsi *et al.,* 2015). De hecho,entre el 60 y el 80% de los genes humanos han sido identificados también en *C. elegans*.

Williams *et al*., 1988, propusieron a *C. elegans* por primera vez para estudiar la toxicidad en humanos, concretamente, de metales pesados y pesticidas. Pero pronto emergió como modelo para estudiar las enfermedades infecciosas en humanos producidas por microorganismos patógenos, (Jain *et al.*, 2013), entre ellos, *S.* Typhimurium, *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, *Serratia marcescens* o *Yersinia pestis (*Zou *et al.,* 2014; Lee *et al.*, 2013*).* Para ello, los microorganismos patógenos se crecen en medios de cultivo en condiciones óptimas y, posteriormente, infectan al nematodo de forma natural introduciéndose normalmente por la boca y recorriendo el tracto digestivo (faringe, intestino y recto) (Schulenburg *et al*., 2004).

La interacción que existe entre las células del hospedador y el patógeno es un aspecto crucial en el proceso de infección bacteriana. Sin embargo, hasta la fecha son pocos los patógenos alimentarios estudiados utilizando *C. elegans* como modelo de patogenecidad. Bacterias como *Listeria monocytogenes (*Thomsen *et al.,* 2006*)*, *S.* Typhimurium (Ibarra et al., 2009), *Staphyclococcus aureus* (Sifri *et al.*, 2003), y *Vibrio cholerae* son aquellos patógenos sobre los que más se ha profundizado. Aspectos como el grado de colonización intestinal, la expresión génica, o la activación de una respuesta inmune específica frente a dichos patógenos, son algunas de las líneas en las que se centran las investigaciones actuales (Balla *et al.*, 2013; Portal-Celhay *et al.*, 2012; Jain *et al.*, 2013.

En condiciones de laboratorio, *C. elegans* se alimenta de *E. coli* OP50, una cepa de *E. coli* no patógena auxótrofa, que satisface sus requerimientos nutricionales. Estudios recientes realizados con este organismo modelo aportan relevante información relativa a la influencia que la microbiota intestinal (p.e.

Bacillus subtilis) ejerce sobre aspectos como la longevidad, la estimulación del sistema inmune y la prevención de cierto tipo de enfermedades (Sánchez-Blanco et al., 2016). En los estudios de exposición a microorganismos patógenos, la evaluación de cambios en las características fenotípicas del nematodo, como esperanza de vida, movilidad, fertilidad, y concentración bacteriana intestinal con respecto al control (alimentado con *E.coli* OP50) son algunas de las técnicas más sencillas y no invasivas utilizadas en los estudios de patógeno-hospedador (Marsh y May, 2012).

En el citado contexto, y a la vanguardia en los estudios realizados sobre patógenos alimentarios, resulta de especial interés la utilización del modelo *C. elegans* como organismo modelo-hospedador para el estudio de cambios de virulencia en microorganismos patógenos sometidos a tratamientos subletales de conservación, o expuestos a sustancias antimicrobianas naturales incorporadas a nuevos alimentos.

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OBJETIVOS

3. OBJETIVOS

El objetivo general de la presente tesis doctoral es la revalorización de subproductos de la industria agroalimentaria como antimicrobianos naturales, por sí mismos, y en combinación con tecnologías no-térmicas de conservación de alimentos frente a los patógenos más relevantes en seguridad alimentaria.

Con esta finalidad se han planteado los objetivos específicos que se detallan a continuación:

1. Evaluar el potencial antimicrobiano de subproductos de la industria agroalimentaria frente a los principales patógenos transmitidos por alimentos.
2. Evaluar el potencial antimicrobiano de infusiones de subproductos de la industria agroalimentaria en combinación con un tratamiento subletal de PEF y HHP frente a microorganismos patógenos.
3. Evaluar el desarrollo de resistencias microbianas a los tratamientos subletales estudiados aplicados de forma consecutiva y sus posibles cambios de virulencia utilizando *C. elegans* como organismo modelo *in vivo*.

PLAN DE TRABAJO

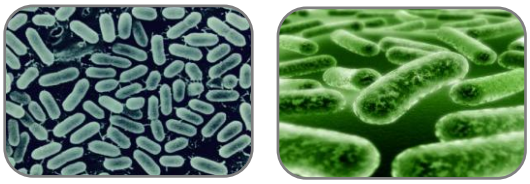
4. PLAN DE TRABAJO

El plan de trabajo llevado a cabo para alcanzar los objetivos de la tesis es el que se detalla a continuación:

1. Revisión bibliográfica.
2. Evaluar el potencial antimicrobiano de subproductos de la industria agroalimentaria brutos deshidratados frente a los principales patógenos transmitidos por alimentos a diferentes concentraciones y temperaturas de incubación.

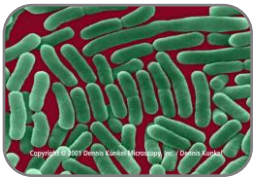
0.5, 1, 2, 5, 10, 15 %

5, 10, 22, 37 ºC



L. monocytogenes

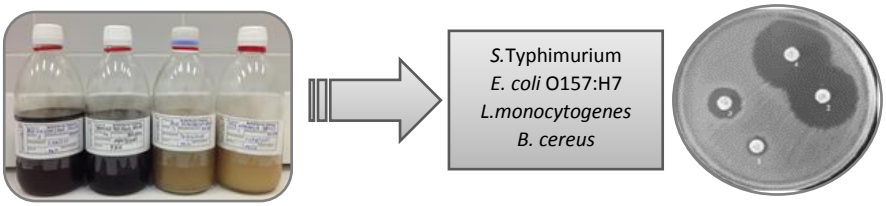
B. cereus



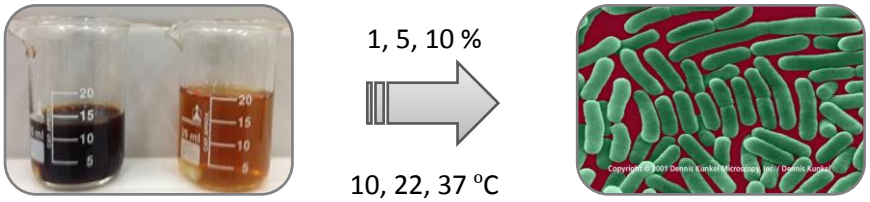
S. Typhimurium

E. coli O157:H7

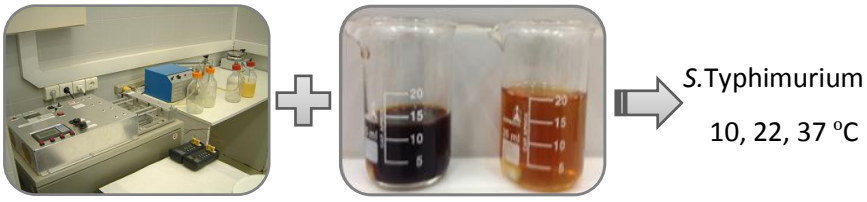
3. Evaluar la capacidad antimicrobiana de extractos ASE obtenidos a partir de subproductos brutos deshidratados frente a los microorganismos patógenos alimentarios más importantes.



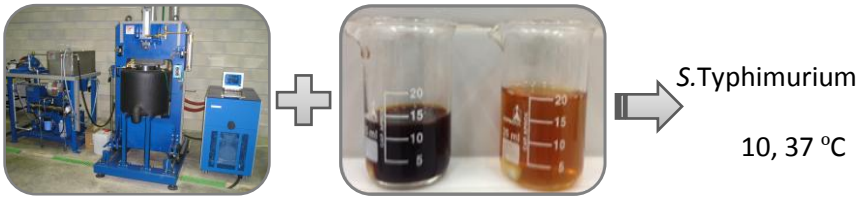
4. Evaluar el efecto antimicrobiano de infusiones obtenidas a partir de subproductos brutos deshidratados de mandarina y coliflor frente a *S.* Typhimurium a diferentes concentraciones y temperaturas de incubación.



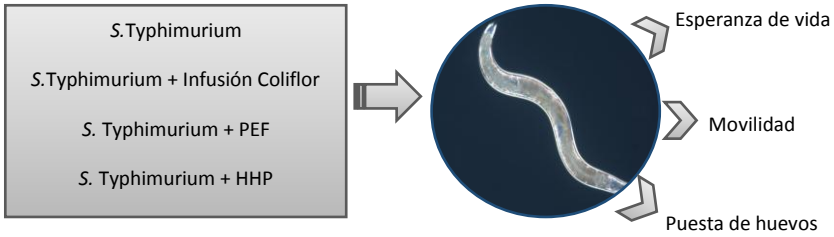
5. Estudiar la actividad antimicrobiana de infusiones de los subproductos de coliflor y mandarina al 10% en combinación con un tratamiento subletal de PEF frente a *S.* Typhimurium a diferentes temperaturas de incubación.



6. Estudiar la capacidad antimicrobiana de infusiones de los subproductos de coliflor y mandarina al 10 % en combinación con un tratamiento subletal de HHP frente a *S*. Typhimurium a diferentes temperaturas de incubación.



7. Evaluar el desarrollo de resistencias microbianas a los tratamientos subletales estudiados aplicados de forma consecutiva y sus posibles cambios de virulencia utilizando *C. elegans* como organismo modelo *in vivo*.



RESULTADOS

CAPÍTULO 5.1 EVALUACIÓN DEL POTENCIAL ANTIMICROBIANO DE LOS RESIDUOS DE LA AGROINDUSTRIA: COLIFLOR, BRÓCOLI, SOJA, MANDARINA, NARANJA Y LIMÓN BRUTOS DESHIDRATADOS

CAPÍTULO 5.1.1.

Sanz-Puig, M., Pina-Pérez, M.C., Criado, M.N., Rodrigo, D., Martínez-López, A.

Antimicrobial potential of Cauliflower, Broccoli and Okara By-products Against Foodborne Bacteria

Foodborne Pathogens and Disease, 12, 1. (2015).

Abstract

The antimicrobial potential of cauliflower, broccoli, and okara by-products was assessed against gram-positive and gram-negative bacteria. *Salmonella* enterica serovar Typhimurium, *Escherichia coli* O157:H7, *Bacillus cereus*, and *Listeria monocytogenes* serovar 4b growth behavior was assessed under exposure to 5% vegetable by-products studied, to reference medium (buffered peptone water (1‰ (w/v))), at 37°C. Although the by-products were not effective against *L. monocytogenes* they were bactericidal against *S.* Typhimurium, *E. coli* O157:H7, and *B. cereus*. The most promising results were achieved with the cauliflower – *S.* Typhimurium combination, because the bacterial population was reduced by 3.11 $\log_{10}$ cycles after 10 h of incubation at 37°C as a result of 5% cauliflower addition. Further studies were carried out for this combination, at different cauliflower concentrations (0, 0.5, 1, 5, 10, 15%) and at temperatures in the range [5–37]°C. The greatest inactivation level (6.11 $\log_{10}$ cycles) was achieved at refrigeration temperature (5°C) as a result of 15% cauliflower addition. Both temperature and cauliflower concentration

significantly ($p \leq 0.05$) influenced the *S.* Typhimurium inactivation level. The kinetic parameters were adjusted to mathematical models. The modified Gompertz mathematical model provided an accurate fit (RMSE [0.00009–0.21] and adjusted-R2 [0.81–0.99]) to experimental *S.* Typhimurium survival curves describing inactivation kinetics of the pathogen to the antimicrobial effect of cauliflower by-product.

5.1.1.1 INTRODUCTION

Every year, the food processing industries generate large amounts of food waste worldwide. The elimination of these residues usually involves a cost to the producer, due to landfill or incineration, which generates negative effects on the environment (O'Shea *et al*., 2012). Therefore nowadays many studies focus on recovery, recycling, and upgrading of food waste, turning it into by-products for use as operating supplies or as ingredients in new product formulations. Recognition of the value of by-products that can be incorporated into new production process would reduce demand for raw materials and restrain exploitation of natural resources, with consequent benefits to our society.

The valorization of agriculture and food by-products is a requirement of the European Union (EUROSTAT, 2010) supporting sustainable development. Vegetable residues are cheap, available in large amounts, and characterized by high dietary fiber content (Stojceska *et al*., 2008). So far, some valuable applications of these agri-food wastes involve animal feedstocks, fertilizers, paper industry application, extraction of essential oils and fragrances, composting, bioconversion, and new ingredients in product formulations (Henningsson *et al*., 2004).

For new product formulations, vegetable by-products could be a valuable source of nutritional and antimicrobial compounds. Among them, there are two

very important plant families, *Brassicaceae* and *Fabaceae*. Broccoli and cauliflower, the main crops of the *Brassicaceae* family, and soybean, the main crop of *Fabaceae*, contain phytochemical components with reported antioxidant and anticarcinogenic properties (Tyug *et al*., 2010). Worldwide production of broccoli and cauliflower was 22,226,957 tons in 2009. About 75% of this production belongs to China and India (USDA, 2009). The antioxidant properties of these vegetables could have a significant impact in the field of nutraceuticals and in food processing industry applications (Cabello-Hurtado *et al*., 2012), mainly because of the polyphenol and glucosinolate contents (O'Shea *et al*., 2012).

With regard to *Fabaceae*, soybean is one of the most commonly consumed legumes in the world, with 200 million tons produced per year (FAOSTAT, 2010). Nowadays, the main producer is the United States (32%), followed by Brazil (28%) and Argentina (21%) (Nahashon and Kilonzo-Nthenge, 2011). After extraction of water from soybeans to produce soy milk and tofu, a by-product called okara is obtained. Consequently, scientific and industrial research is required to find potential applications of okara from environmental and food technology viewpoints (O'Toole, 1999).

In this context, and for valorization purposes, the antimicrobial effect of vegetable by-products from the raw material of broccoli (*Brassica oleracea* italica), cauliflower (*Brassica oleracea* botrytis), and soybean (*Glycine max*) was evaluated against Gram-positive and Gram-negative foodborne pathogens.

5.1.1.2 MATERIAL AND METHODS

5.1.1.2.1 Bacterial cultures and growth conditions

Pure cultures of *Listeria monocytogenes* serovar 4b (CECT 4032), *Bacillus cereus* (CECT 131), *Salmonella enterica* serovar Typhimurium (CECT 443) and

Escherichia coli O157:H7 (CECT 5947) were provided freeze-dried by the Spanish Type Culture Collection. The *B. cereus* culture was rehydrated with 10 mL of brain heart infusion (BHI) broth (Scharlab Chemie, Barcelona, Spain), whereas tryptic soy broth (TSB) (Scharlab Chemie, Barcelona, Spain) was used for *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* rehydration. After 20 minutes, the rehydrated culture was transferred to 500 mL of BHI broth or TSB, respectively, and incubated at 32°C for *B. cereus* and at 37°C for the other microorganisms, with continuous shaking (Selecta Unitronic) at 200 rpm for 14 hours to obtain cells in a stationary growth stage. Growth curves were obtained by plate count (colony forming units per mL (CFU/mL)). The cells were centrifuged (Beckman Avanti J-25) twice at 4000 x g at 4°C for 15 minutes and then resuspended in BHI broth or TSB, respectively. After the second centrifugation, the cells were resuspended in 20 mL of BHI broth or TSB with 20% glycerol, and then dispensed in 2 mL vials to a final concentration of 10^8 obtained by plate count. The 2 mL samples were immediately frozen and stored at –80 °C until needed for the kinetic inactivation studies.

5.1.1.2.2 Antimicrobial substances

Cauliflower, broccoli, and okara by-products from vegetable raw materials were provided as leaf residues from primary production. Each raw by-product was tested to screen its bacteriological quality. The bacteriological analysis determined the presence/absence of microbial contamination and was carried out according to the procedures described by Aycicek *et al*. (2006). The samples studied presented positive contamination with *L. monocytogenes* and *B. cereus* (Gram-positives), chiefly in broccoli and cauliflower samples, below 5 CFU/g. In contrast, no samples were contaminated with *E. coli* O157:H7 or *S.* Typhimurium (Gram-negatives).

The raw by-product was washed in sterile water to eliminate contaminating substances, dried, triturated, and homogenized using a laboratory grinder (JANKE & KUNKEL IKA-Labortechnik) to obtain a powder with a particle size of 40 μ_m, which was used to perform the experiments (Brandi *et al*., 2006).

5.1.1.2.3 Total Phenolic Compounds of vegetable by-products

The total phenol contents of the vegetable by-products were determined spectrophotometrically according to the Folin–Ciocalteu colorimetric method (Singleton and Rossi, 1965). Gallic acid calibration standards with concentrations of 0, 100, 200, 300, 400, 500, 600, 700, 800 and 1000 ppm were prepared. Three mL of sodium carbonate solution (2% (w/v)) (Sigma-Aldrich Co. LLC, USA) and 100 μ_L of Folin-Ciocalteu reagent (1:1 (v/v)) (Sigma-Aldrich Co. LLC, USA) were added to an aliquot of 100 μ_L from each gallic acid standard (Sigma-Aldrich Co. LLC, USA) or sample tube. The mixture was vortexed (Heidolph) and allowed to stand at room temperature in the dark for 1 h. Absorbance was measured at 750 nm using a Lan Optics Model PG1800 spectrophotometer (Labolan, Spain), and the results were expressed as milligrams of gallic acid equivalents per liter.

5.1.1.2.4 Substrate and inoculation

Buffered peptone water (Scharlab Chemie, Barcelona, Spain) (1‰ (w/v)) was used as reference substrate in the present study (Pina-Pérez *et al*., 2007; Lin *et al*., 2010). The reference medium was supplemented with natural vegetable by-product and later, was inoculated of stock culture to a final concentration of 10^7 CFU/mL. In an initial research step, the antimicrobial potential of each by-product: cauliflower, broccoli, and okara, was tested against the microorganisms studied under specific conditions: (i) 5% (w/v) of

vegetable by-product addition to medium, and (ii) at the optimal incubation temperature for each microorganism, for 10 h. The plates of *B. cereus* were incubated at 30°C during 48 hours on BHI agar (Scharlab Chemie, Barcelona, Spain), the plates of *E. coli* O157:H7 and *S.* Typhimurium were incubated at 37°C during 24 hours on TSA; and the plates of *L. monocytogenes* were incubated at 37°C during 48 hours on TSA.

A second experimental step was conducted, based on the results obtained in the first one. The vegetable by-product with the greatest bactericidal effect was tested over a wide concentration range [0–15]% (w/v) against the most sensitive microorganism. Moreover, to test the influence of temperature on the antimicrobial potential of the vegetable by-product studied, incubation was carried out at four temperatures (5, 10, 22 and 37 ^{o}C).

5.1.1.2.5 Viable microorganism count

At regular time intervals (hours), the cell suspension for each sample was evaluated by plate count after serial dilution with 1‰ (w/v) buffered peptone water. Each dilution was plated in duplicate. Experiments were carried out in triplicate and the plate counts used for enumeration (CFU/mL).

5.1.1.2.6 Modeling of microorganism inactivation

Microbial behavior was fitted to a modified Gompertz equation to mathematically describe the bacterial inactivation kinetics under the intervention of the most effective vegetable by-product at different concentrations and temperatures (Linton *et al*., 1995):

$$Log_{10}\left(\frac{N}{N_0}\right) = Ce^{-e^{-BM}} - Ce^{-e^{-B(t-M)}} \quad (1)$$

where N is the cell concentration at time t (CFU/mL), N_0 is the initial cell concentration (CFU/mL); C is the difference between upper and lower value of asymptote; B is the relative death rate at time M, and M being the time at which the absolute death rate is maximal. Minus sign before C means the microbial inactivation.

Subsequently, with B, C and M obtained values, the maximum death rate (μ_{max}) can be calculated as follows:

$$\mu_{max} = \frac{BC}{e} \quad (2)$$

5.1.1.2.7 Data analysis and model evaluation

The statistical analysis was performed with STATGRAPHICS Centurion XV (version 15.1.03; STATGRAPHICS, Warrenton, VA). This analysis included average and standard deviation calculations for the three replications and an ANOVA analysis to test significant differences depending on incubation conditions. The goodness of fit of the model was assessed by using adjusted regression coefficient (adjusted-R^2) and root mean square error (RMSE) (López *et al*., 2004).

5.1.1.3 RESULTS AND DISCUSSION

5.1.1.3.1 Antimicrobial effect of vegetable by-products against Gram-positive and Gram-negative bacteria

The antimicrobial effect of broccoli, cauliflower, and okara by-products was evaluated. Figure 1 shows the survival curves obtained for (a) Gram + and (b) Gram – bacteria in 1‰ buffered peptone water supplemented/not supplemented with vegetable by-product at 5% concentration. As can be seen in the figure, the vegetable by-products studied exerted a bactericidal effect

with reduction of the bacterial population over time when incubation was carried out at optimal growth temperature for each microorganism, with the exception of *L. monocytogenes*. Among the Gram-positive bacteria, *L. monocytogenes* was not affected by exposure to the vegetable by-products (5%, 37°C, 10 h), with no significant reduction of the initial load ($p > 0.05$); in contrast, the *B. cereus* population was reduced in the range [0.61–2.32] log cycles by vegetable by-product intervention under the same conditions. In the case of the Gram-negative bacteria, *S.* Typhimurium was highly sensitive compared with *E. coli* O157:H7 under exposure to Brassicas, but was more resistant to the antimicrobial effect of okara than *E. coli* O157:H7.

Many studies have supported that Gram-positive were more susceptible to antimicrobial effect of plant essential oils and extracts than Gram-negative bacteria (Jayaprakasha *et al*., 2003; Smith-Palmer *et al*., 1998). In contrast, other studies have found more sensitivity against other natural extracts or essential oils in Gram-negative than Gram-positive bacteria (Di Pasqua *et al*., 2005; Hu *et al*., 2004).

Figure 2 shows the inactivation levels achieved for each by-product–microorganism combination after the complete incubation period. Under the conditions studied, the bactericidal effect against *E. coli* O157:H7, *S.* Typhimurium and *B. cereus* achieved a minimum value of 0.48±0.05 $\log_{10}$ cycles reduction under broccoli intervention against *E. coli* O157:H7, and a maximum value of 3.11±0.50 $\log_{10}$ cycles reduction by the effect of cauliflower against *S.* Typhimurium. From the results obtained, it is possible to establish a ranking based on the sensitivity of each microorganism to the antimicrobial effect of the by-products studied. With regard to the microorganisms' susceptibility to the antimicrobial effect of cauliflower, the ranking can be established as follows: *S.* Typhimurium (3.11±0.15 $\log_{10}$ cycles reduction) > *B. cereus* (2.31±0.025 $\log_{10}$ cycles reduction) > *E. coli* O157:H7 (0.53±0.09 $\log_{10}$ cycles

reduction). For broccoli, the sensitivity of the microorganisms studied can be ordered as follows: *B. cereus* (2.25±0.05 $\log_{10}$ cycles reduction) > *S.* Typhimurium (0.49±0.225 $\log_{10}$ cycles reduction) > *E. coli* O157:H7 (0.48±0.05 $\log_{10}$ cycles reduction). With regard to the sensitivity of the microorganisms studied to the antimicrobial effect of okara, *E. coli* O157:H7 and *S.* Typhimurium seemed to produce similar results (1.15±0.05 and 1.07±0.025 $\log_{10}$ cycles reduction, respectively), followed by *B. cereus* (0.61±0.03 $\log_{10}$ cycles reduction).

According to the results obtained, under the conditions studied (i) okara was the most bactericidal by-product against *E. coli* O157:H7 (1.15 $\log_{10}$ cycles); (ii) cauliflower and broccoli showed the highest antimicrobial effect against *B. cereus* (2.25 and 2.31 $\log_{10}$ cycles respectively), and (iii) cauliflower was the most effective vegetable by-product against *S.* Typhimurium (3.11 $\log_{10}$ cycles). So it is possible to conclude that addition of cauliflower at 5% achieved the greatest reduction in bacterial levels, showing the most bactericidal capability among the vegetable by-products studied.

Although the antioxidant capacity of *Brassicaceae* and *Fabaceae* is widely known and has been attributed mainly to their polyphenol contents (O'Shea *et al*., 2012; Tyug *et al*., 2010), so far the antimicrobial effect of these plants has scarcely been studied (Hu *et al*., 2004). With respect to the soybean by-product, okara, there are previous studies indicating antimicrobial effect of soybean derivatives (Roubos-Van den Hil *et al*., 2010; O'Toole, 1999).

As far as we know the antimicrobial capability of these raw agri-food by-products from primary production: cauliflower, broccoli, and okara, has not previously been reported. To our knowledge, only qualitative studies of the antimicrobial potential of other vegetable by-products have been carried out, establishing a correlation between functional properties attributed to these

vegetables and their polyphenol contents (Roubos-Van den Hil *et al*., 2010; Fattouch *et al*., 2007). The observed bactericidal capability of cauliflower, broccoli, and okara by-products against the foodborne pathogens studied could be due to the effect of their high polyphenol content.

As can be seen in Table 1, cauliflower extract had the highest polyphenol content (mg galic acid/L), followed by extract of broccoli, finally, okara extract. The same order of by-products appears regarding antimicrobial capacity. Therefore, it is possible to establish a relationship between the polyphenol content of tested by-products and their antimicrobial activity.

Table 5.1.1.**1**. Total polyphenol content in by-product extracts.

Extract	Polyphenol content (mg galic acid/L)
Cauliflower 15%	11359,8135 ± 747,9627
Broccoli 15%	9091,6660 ± 605,2390
Okara 15%	873,7500 ± 64,9519

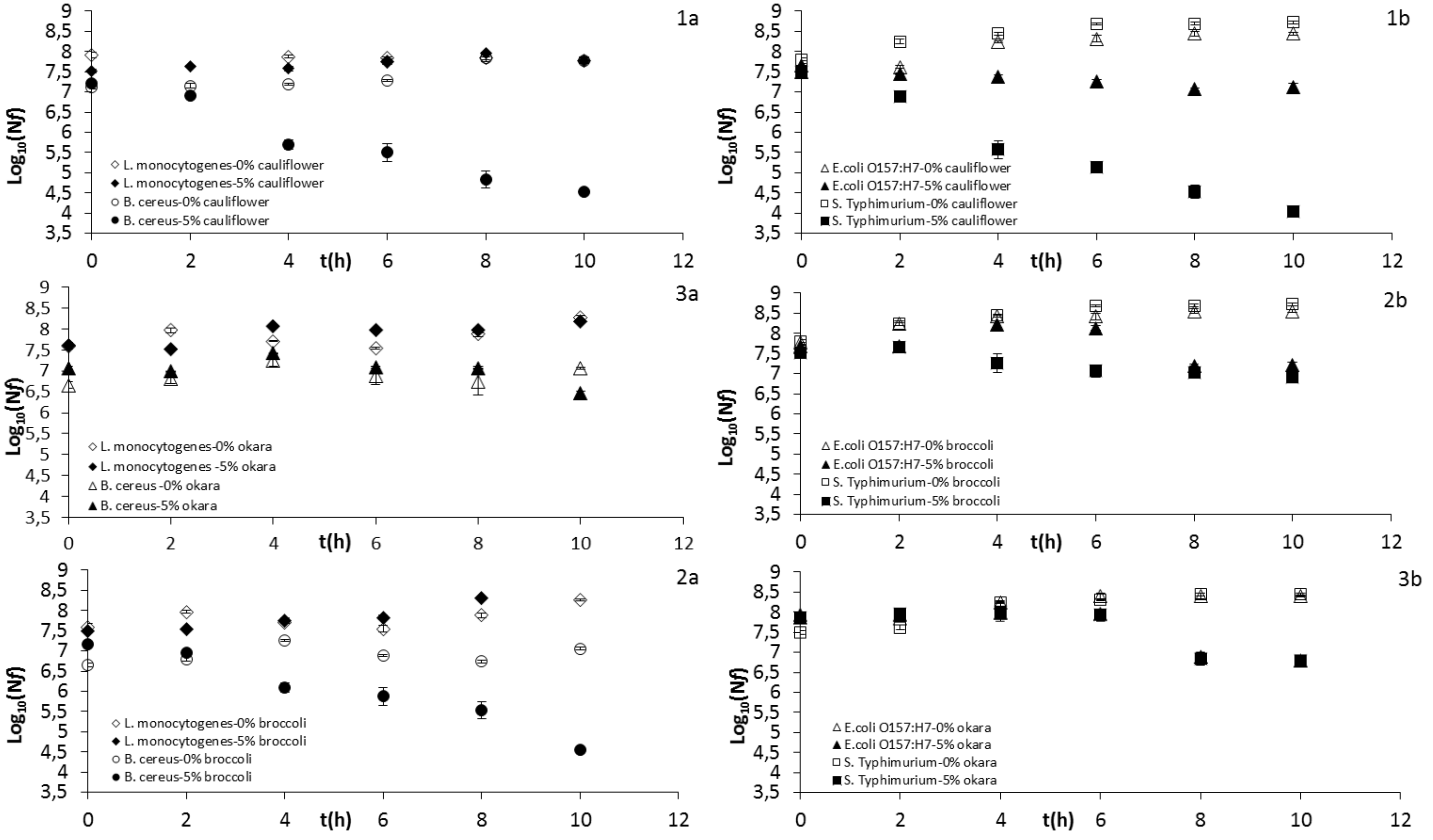


Figure 5.1.1.**1**. Survival curves of Gram-positive bacteria (*Listeria monocytogenes* and *Bacillus cereus*) (a); and Gram-negative bacteria (*E. coli* O157:H7 and *Salmonella* Typhimurium) (b), obtained at optimal growth incubation temperature, when cauliflower (1), broccoli (2), or okara (3) are added at 5% (w/v) in reference medium (1‰ (w/v) buffered peptone water).

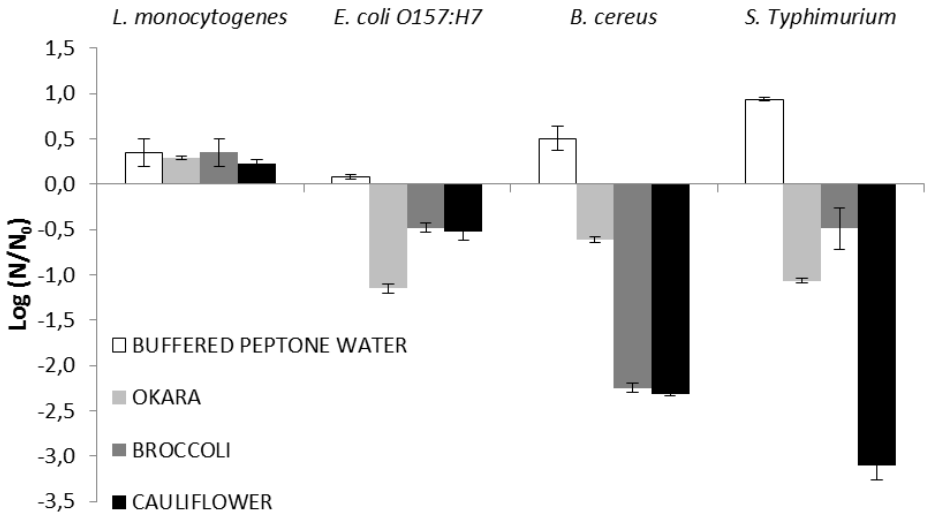


Figure 5.1.1.**2**. Inactivation levels of *L. monocytogenes*, *E. coli* O157:H7, *S.* Typhimurium and *B. cereus* cells after 10 hours at 37 °C, under the effect of 5% (w/v) cauliflower, broccoli, and okara.

5.1.1.3.2 Effect of temperature and concentration of cauliflower against *S.* Typhimurium

In view of the results obtained, intensive study was conducted on the antimicrobial effect of cauliflower (the most effective vegetable by-product), based on its effect on the most sensitive microorganism, *S.* Typhimurium. Various concentrations of cauliflower (0, 0.5, 1, 2, 5, 10, and 15%) were assessed against *S.* Typhimurium at different temperatures: 5°C (refrigeration), 10°C (abuse in refrigeration), 22°C (room temperature), and 37°C (optimal temperature).

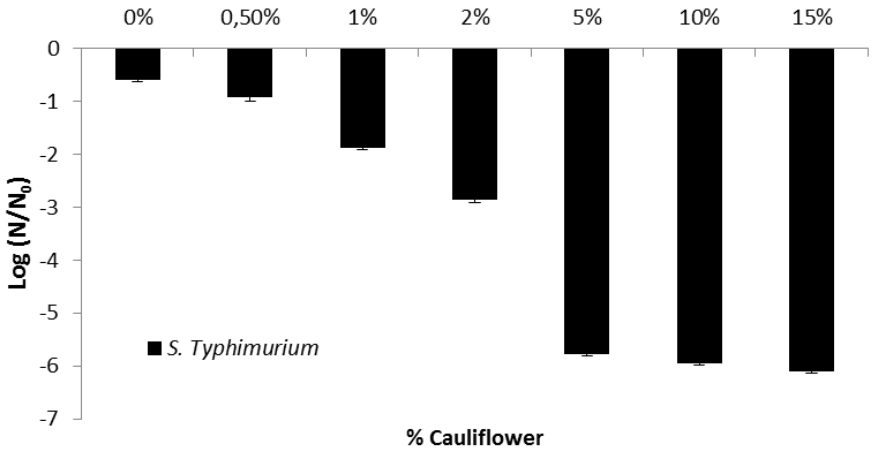


Figure 5.1.1.**3**. Inactivation levels of *S.* Typhimurium in reference medium supplemented/not supplemented with 0.5, 1, 2, 5, 10, and 15% (w/v) of cauliflower after 432 hours at 5 °C.

For all the temperatures, the higher the concentration of cauliflower, the greater the reduction of cell population ($p \leq 0.05$). Figure 3 shows the concentration effect of cauliflower at 5°C. As can be seen graphically, the *S.* Typhimurium cell population was reduced 0.6, 0.93, 1.88, and 2.86 $\log_{10}$ cycles at concentrations of 0%, 0.5%, 1%, and 2%, respectively, and about 6 $\log_{10}$

cycles at concentrations of 5%, 10%, and 15%, reaching a maximum reduction level (6.11 $\log_{10}$ cycles) at the highest cauliflower concentration (15%). These results are in agreement with studies conducted by Brandi *et al*. (2006) on the antimicrobial potential of Brassica leaf juice in reference media. The influence of concentration level on the antimicrobial capability of *Brassicaceae* species was reported previously with disc diffusion method (Blazevic *et al*., 2010; Sousa *et al*., 2008).

With regard to temperature effect, Figure 4 shows the reduction in the growth of the cell population due to temperature with respect to the growth behavior at 37°C, when cauliflower was added to the medium at 5%. When the temperature was reduced from 37 to 22°C, a reduction level of 0.20 $\log_{10}$ cycles was observed; reducing the temperature from 37 to 10°C achieved a reduction of 0.51 $\log_{10}$ cycles; and when the temperature was reduced from 37°C to refrigeration level (5°C), the reduction of bacterial counts was 1.19 $\log_{10}$ cycles. Therefore, under exposure to the same concentration of cauliflower by-product, the lower the incubation temperature, the higher the bacterial reduction. Figure 4 also shows the concentration effect at different temperatures. It can be seen that cauliflower exerted a higher concentration effect at 5°C and 37°C than at 10°C and 22°C, with slightly more bactericidal effect of 5% cauliflower against *S.* Typhimurium at 5°C than at 37°C. The results are in agreement with the studies carried by Cava *et al*. (2007) against *L. monocytogenes*.

Cauliflower extract added to reference medium at a concentration of 5% and incubated at 37°C for 10 hours not only inhibits *S.* Typhimurium growth, but also reduces the microbial load levels by 4 $\log_{10}$ cycles. These results are in agreement with the results obtained by Brandi *et al*. (2006) against *Salmonella* spp. and *E. coli* spp. by adding 20% cauliflower extract (leaf juice) at the same temperature. Meanwhile, the enhanced antimicrobial capability of natural

ingredients against several foodborne pathogens has been observed previously at refrigeration temperature in other studies (Ferrer *et al*., 2009; Iturriaga *et al*., 2012), which are in agreement with the present results.

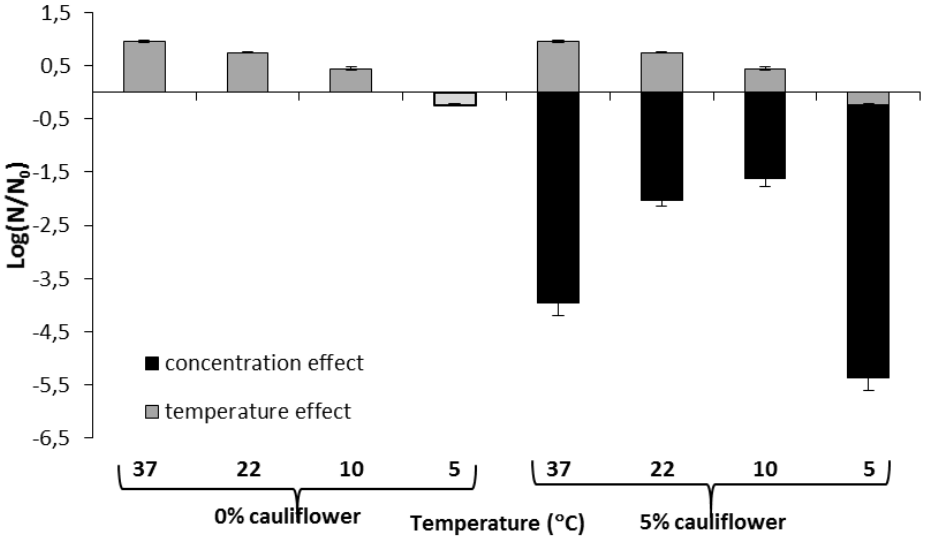


Figure 5.1.1.4. Temperature effect on reduction in growth of initial cell population with respect to growth behavior at 37 °C and concentration effect at various temperatures studied.

The results obtained were adjusted to the modified Gompertz distribution function, which has been used by other authors to provide an accurate fits to microbial behavior under exposure to natural antimicrobials (Belda-Galbis, 2013; Gammariello, 2008). The Gompertz kinetic parameters are presented in Table 2, in which is showed a negative μ values due to belonging to inactivation kinetics, with the exception of the 0% cauliflower – 22^{o}C combination, which has a positive value because the microorganism grows under these conditions. The μ_{max} values, calculated with C, B and M parameters, give us information about the maximum growth/dead rate. As can be seen in Table 2, μ values, are generally higher at higher temperature and cauliflower extract concentration. Therefore, both temperature and concentration lead to an increase of the inactivation rate, contributing to antimicrobial effect. To our

knowledge, no previous studies have reported the mathematical modelling of microbial inactivation/survival using raw cauliflower extract. There are few studies on the antimicrobial effect of *Brassicaceae* vegetables, and most of them evaluated the antimicrobial activity qualitatively, using inhibition zones (Hu *et al*., 2004; Blazevic *et al*., 2010; Sousa *et al*., 2008).

Table 5.1.1.2. Values of C, B and M parameters of modified Gompertz equation and the growth/dead rate (μ) for *S.* Typhimurium inactivation with 0%, 5%, 10% and 15% of cauliflower at 5, 10 and 22 °C. R2 and MSE values are indicators of goodness of fit.

		Kinetic parameters				Accuracy of the fit	
Temperature	**Concentration**	**C**	**B**	**M**	**μ**	**R^2**	**MSE**
5 °C	0%	0,973858	-0,00827136	0,232782	-0,00296332	0,998644	0,000095
	5%	385,051	-0,00010065	16,9334	-0,01425688	0,960919	0,273448
	10%	213,648	-0,00018538	7,22053	-0,01457057	0,972636	0,150502
	15%	11,8737	-0,00510458	5,16327	-0,02229726	0,975164	0,156017
10 °C	0%	3,9796	0,0122761	0,306152	0,01797237	0,856983	0,029367
	5%	2,11021	-0,05126	0,13689	-0,03979328	0,998721	0,000452
	10%	2,6055	-0,0574663	0,361006	-0,05508202	0,983660	0,009024
	15%	4,78427	-0,0538037	0,551275	-0,09469637	0,960864	0,211304
22 °C	0%	3,92077	0,0831159	0,574861	0,11988394	0,940552	0,020777
	5%	3,41821	-0,0354045	0,979768	-0,04452078	0,900027	0,026786
	10%	1,40628	-0,0911854	0,821861	-0,04717399	0,931711	0,010978
	15%	1,69713	-0,0731222	0,604788	-0,04565306	0,886458	0,018668

5.1.1.4 CONCLUSION

According to the results presented in this research work, cauliflower extract can be considered as potential material with antimicrobial properties with important economic and food safety applications. Animal feed supplementation, or development of new additives based on the bactericidal effect of this extract for vegetable creams and ready-to-eat plates, which are pasteurized and stored at refrigeration temperature (5 °C), present important challenges to food processors.

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CAPÍTULO 5.1.2

Sanz-Puig, M., Pina-Pérez, M.C., Rodrigo, D., Martínez.López, A.

Antimicrobial activity of cauliflower (*Brassica oleracea* var. Botrytis) by-product against *Listeria monocytogenes*

Food Control, 50, 435-440. (2015).

Abstract

The antimicrobial potential of cauliflower by-product was assessed against *Listeria monocytogenes* at different concentrations [0–15]% (w/v) and incubation temperatures [5–22] ^{o}C, in reference medium. Survival curves under cauliflower by-product exposure versus time were obtained. The bactericidal effect of the cauliflower by-product was shown at concentration levels ≥ 5% (w/v) at all temperatures studied. Both temperature and cauliflower by-product concentration significantly ($p \leq 0.05$) influenced the reduction levels achieved in the initial *L. monocytogenes* contamination. Growth/inactivation kinetics of *L. monocytogenes* under cauliflower by-product exposure were fitted to a modified Gompertz equation for each of the conditions studied (concentration–temperature combinations), and maximum inactivation rate (μ_{max}) and lag phase duration (t_{lag}) parameters were obtained. It was observed that the higher the incubation temperature and the cauliflower by-product concentration added to the reference medium, the higher the μ_{max} and the lower t_{lag}. In spite of this, the maximum inactivation level achieved at stationary phase was 2.25 $\log_{10}$ cycles after 20 days of exposure to a 15% (w/v) concentration of

cauliflower added to reference medium. Both conclusions indicate the effective control that cauliflower by-product could provide as an additional preservation measure during shelf-life of refrigerated RTE products, specifically when there is an accidental rise in storage temperature, e.g. in cold chain breakdown situations.

5.1.2.1 INTRODUCTION

Listeria monocytogenes is an opportunistic psychrotrophic microorganism with a reported capability of multiplying itself at temperatures down to a few degrees below 0°C, persisting in refrigerated industrial settings. The incidence of listeriosis mainly affects young and elderly people (over 65 years), pregnant women and immune-compromised people (Gambarin *et al*., 2012; Adzitey *et al*., 2010), with high morbidity and mortality rates associated with *L. monocytogenes* (about 30%). Nowadays, *L. monocytogenes* is one of the most worrying foodborne pathogens, with one of the highest hospitalization rates (91%) and long-term sequels in affected patients (Denny & McLauchlin, 2008). Despite the fact that a wide variety of foods may be contaminated with *L. monocytogenes*, outbreaks and sporadic cases of listeriosis are predominately associated with ready-to-eat (RTE) foods – a large, heterogeneous category of foodstuffs that can be subdivided in many different ways and vary from country to country according to local eating habits, availability and integrity of the chill chain, and regulations specifying, for example, the maximum temperature at retail level.

Recent sporadic cases of listeriosis have been described in Europe (from 2006 to 2010) (Cairns & Payne, 2009; Goulet *et al*., 2009; Kvistholm *et al*., 2010). A large outbreak was recorded in Canada in 2008 (PHAC, 2008), and there has been an increasing number of *L. monocytogenes* food isolates in the USA and Canada in recent years. RTE products are likely to act as vehicles for

transmission of *L. monocytogenes*, mainly because they do not require additional preparation or cooking before consumption. RTE products (e.g. pasteurized milk, ice cream, fermented meat and cold smoked fish) can be contaminated by *L. monocytogenes* during post-processing steps, and then it can proliferate during storage at refrigeration temperature because of the psychrotrophic nature of the microorganism (Cobo *et al*., 2009; Zhu *et al*., 2005). In order to prevent *L. monocytogenes* contamination in RTE products, some natural bioactive substances with antimicrobial capability are added to control pathogenic bacteria in food systems (Lianou *et al*., 2007). Among possible added natural antimicrobials, increasing interest is focusing on vegetable by-products, as rich natural sources of fibre, vitamins, minerals, secondary plant metabolites and antioxidants.

These vegetable residues from the food industry that are mainly destined to landfill or incineration, causing important economic and environmental problems, can be re-evaluated as supplements for animal feed or, in novel approaches, as food additives with bioactive properties, specifically antioxidant and antimicrobial, to be added in the formulation of new food products for human consumption (Fernández-López *et al*., 2005; Viuda-Martos *et al*., 2007). Compounds such as polyphenols, flavonoids and glucosinolates have been reported as being responsible for the bioactive properties attributed to vegetables. These bioactive compounds are mainly retained in cellular tissues, leaves and roots of vegetables (Hu *et al*., 2004; Ayaz *et al*., 2008). Consequently, assessment of the potential antimicrobial capability of vegetable by-products is a novel approach for food technologists and scientists to work on.

Among these by-products, one of most the important groups consists of members of the *Brassicaceae* family, which are among the most extended food crops in many countries (Cabello-Hurtado *et al*., 2012). Cauliflower (*Brassica*

oleracea var. Botrytis) is one of the main *Brassicaceae* crops, with edible parts, such as leaves and stems, which have been widely described as sources of fibre and antioxidant substances. These bioactive properties give them the healthy, nutritious quality so extensively documented currently (Stojceska *et al*., 2008; Volden *et al*., 2009; Köksal *et al*., 2007; Brandi *et al*., 2006).

In this context, the main objective of the present study is to evaluate the antimicrobial activity of cauliflower by-product against *L. monocytogenes* at several temperatures and cauliflower by-product concentrations.

5.1.2.2 MATERIAL AND METHODS

5.1.2.2.1 Microbiology

A pure culture of *L. monocytogenes* (CECT 4032), which has a food origin and has been associated with meningitis after eating soft cheese, was provided freeze-dried by the Spanish Type Culture Collection and was rehydrated with 10 mL of tryptic soy broth (TSB) (Scharlab Chemie, Barcelona, Spain). After 20 min, the rehydrated culture was transferred to 500 mL of TSB and incubated at 37 °C, with continuous shaking at 200 rpm for 14 h to obtain cells in a stationary growth stage. Growth curves were obtained by plate count (colony forming units per mL (CFU/mL)). The cells were centrifuged twice at 4000 g at 4 C for 15 min and then resus-pended in TSB. After the second centrifugation, the cells were resuspended in 50 mL of TSB with 20% glycerol, and then dispensed in 2 mL vials to a final concentration of 108 colony forming units per millilitre (CFU/mL). The 2 mL samples were immediately frozen and stored at 80 C until needed for the kinetic inactivation studies.

5.1.2.2.2 Antimicrobial substances

Cauliflower by-product was provided as leaf residue from pri-mary production and was tested to screen its bacteriological quality. The

bacteriological analysis determined the presence/ absence of microbial contamination and was carried out according to Aycicek, Oguz, and Karci (2006) procedures. Cauliflower by-product samples presented positive contamination with *L. monocytogenes* and *Bacillus cereus* (Gram-positives), mostly below 5 CFU/g. No samples were contaminated by *Escherichia coli* O157:H7 or *S.* Typhimurium (Gram-negatives).

The raw by-product was washed in sterile water to eliminate contaminating substances, and then dried, triturated and homogenized using a laboratory grinder to obtain a powder with a particle size of 40 mm, which was used to perform the experiments.

5.1.2.2.3 Total phenolic compounds

The total phenol content of the cauliflower by-product was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965). Gallic acid calibration standards with concentrations of 0, 100, 200, 300, 400, 500, 600, 700, 800 and 1000 ppm were prepared. Three mL of sodium carbonate solution (2% (w/v)) (Sigma-Aldrich Co. LLC, USA) and 100 mL of Folin-Ciocalteu reagent (1:1 (v/v)) (Sigma-Aldrich Co. LLC, USA) were added to an aliquot of 100 mL from each gallic acid standard (Sigma-Aldrich Co. LLC, USA) or sample tube. The mixture was vortexed and allowed to stand at room temperature in the dark for 1 h. Absorbance was measured at 750 nm using a Lan Optics Model PG1800 spectrophotometer (Labolan, Spain), and the results were expressed as mg of gallic acid equivalents (GAE)/L.

5.1.2.2.4 Substrate and inoculation

Buffered peptone water (Scharlab Chemie, Barcelona, Spain) (1‰ (w/v)) was used as the reference substrate, in accordance with previous antimicrobial

capability determination studies (Lin, Sheu, Hsu, & Tsai, 2010; O'Bryan at al., 2008). Then, 1 mL from a vial of stock culture was added to reference medium to a final concentration of 10^7 CFU/mL. The inoculated medium was supplemented with natural cauliflower by-product. The antimicrobial potential of the cauliflower by-product was tested against *L. monocytogenes* over a wide concentration range, [0-15]% (w/v), and the influence of incubation temperature on the antimicrobial potential of the vegetable by-product was assessed at 5 °C, 10 °C and 22 °C. Inactivation curves were prolonged to achieve a stationary point. The plates were incubated at 37 °C for 48 h in TSA (Scharlab Chemie, Barcelona, Spain).

5.1.2.2.5 Viable microorganisms count

At regular time intervals (hours), the cell suspension was evaluated for each sample by plate count after serial dilution with 1‰ (w/v) buffered peptone water. Each dilution was plated and the plates were incubated. The plate counts were used for (CFU)/mL enumeration.

5.1.2.2.6 Mathematical modelling of microbial inactivation

Microbial behaviour was fitted to a modified Gompertz equation to mathematically describe the bacterial inactivation kinetics under the intervention of cauliflower by-product at different by-product concentrations and temperatures (Linton, Carter, Pierson, & Hackney, 1995):

$$Log_{10}\left(\frac{N}{N_0}\right) = Ce^{-e^{-BM}} - Ce^{-e^{-B(t-M)}} \quad [1]$$

where N is the cell concentration at time t (CFU/mL); N_0 is the initial cell concentration (CFU/mL); C is the difference between the upper and lower values of the asymptote; B is the relative death rate at time M, M being the

time at which the absolute death rate is maximal. A minus sign before C means microbial inactivation.

Subsequently, with the B, C and M values obtained, the maximum death rate (μ_{max}) and the lag phase duration (t_{lag}) were calculated as follows.

$$\mu_{max} = \frac{BC}{e} \quad [2]$$

$$t_{lag} = M - \left(\frac{1}{B}\right) + \frac{Log_{10}N_0 - A}{\frac{BC}{e}};\ A = Log_{10}N_0 + Ce^{-e^{BM}} \quad [3]$$

5.1.2.2.7 Data analysis and model evaluation

The statistical analysis was performed with STATGRAPHICS Centurion XV (version 15.1.03; STATGRAPHICS, Warrenton, VA). The analysis included average and standard deviation calculations for the three repetitions and an ANOVA analysis to test significant differences depending on incubation conditions. The goodness of fit of the model was assessed by using adjusted regression coefficient (adjusted-R^2) and root mean square error (RMSE).

5.1.2.3 RESULTS AND DISCUSSION

5.1.2.3.1 Effect of cauliflower by-product concentration and incubation temperature

The antioxidant capacity of *Brassicaceae* is widely known and has been attributed mainly to their polyphenol contents (O'Shea, Arendt, & Gallagher, 2012). However, the antimicrobial effect of these plants has scarcely been studied (Hu *et al*., 2004).

As far as we know, only qualitative studies of the antimicrobial potential of other vegetable by-products have been carried out, establishing a correlation between functional properties attributed to these vegetables and their polyphenol contents (Fattouch *et al*., 2007; Roubos-Van den Hil, Schols, Rob Nout, Zwietering, & Grup-pen, 2010).

Among the most abundant phenolic compounds present in cauliflower, various authors have reported the presence of high levels of ferulic acid, chlorogenic acid, gallic acid and catechin (Cartea, Francisco, Soengas, & Velasco, 2011; Mahroop-Raja, Raja, Mohamed-Imran, & Habeeb-Rahman, 2011). In our case, the cauliflower by-product has a polyphenol content of 11359,8135 ± 747,96277 (mg galic acid/L). Therefore, the observed bactericidal capability of cauliflower by-product against *L. monocytogenes* could be due to the effect of its high polyphenol content. The antimicrobial potential of the cauliflower by-product against *L. monocytogenes* was assessed at several incubation tem-peratures of 5, 10 and 22 °C and by-product concentrations (0, 0.5, 1, 2, 5, 10, 15% (w/v)).

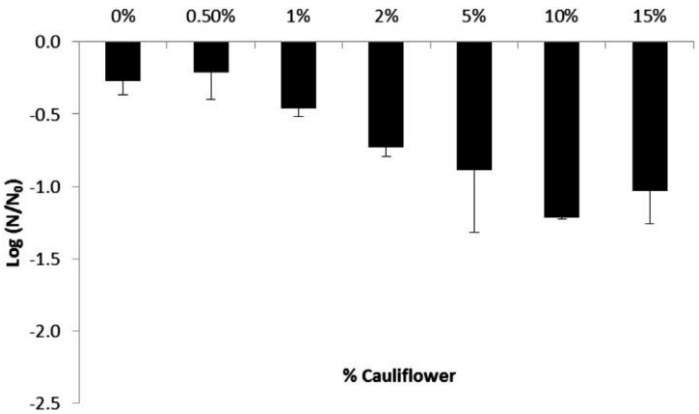

Figure 5.1.2.**1**. *L. monocytogenes* inactivation levels under exposure to 0%, 0.5%, 1%, 2%, 5%, 10% and 15% cauliflower by-product at 22 °C.

The observed bactericidal effect of cauliflower against *L. monocytogenes* was significantly affected (p≤0.05) by both the incubation

temperature and the concentration of cauliflower by-product added. Regarding concentration effect, generally it was seen that the higher the cauliflower by-product concentration, the higher the $\log_{10}$ cycles listerial reduction level, as is shown in Fig. 1 after 24 h of incubation at 22 °C. Very few researchers have carried out studies on the antimicrobial capacity of cauliflower, such as the study of Hu *et al*., (2004) with the disk diffusion method; or the results obtained by Brandi *et al*., (2006) in which a similar inactivation pattern was observed, depending on the cauliflower leaf juice concentration added to reference media against *L. monocytogenes* at 37 °C. Concentration-dependent microbial inactivation levels have also been observed by other authors (Kim, Cho, & Han, 2013; Pina-Perez, Martínez-Lopez, & Rodrigo, 2013), with other vegetables, such as olive, chamnamul, fatsia or cocoa, mainly attributed to their rich polyphenol content.

Regarding the temperature-time incubation effect, according to the experimental results obtained the lowest incubation temperature was associated with the highest antimicrobial effect of the cauliflower by-product once the stationary point had been reached, after 480 h at 5 °C, 75 h at 10 °C, and 24 h at 22 °C. However, at the highest temperature studied (22 °C) the growth inhibition kinetics advanced considerably faster than at low temperatures (5-10 °C), in spite of the final low inactivation level achieved once the stationary point had been reached. For explanatory purposes, an incubation period of 24 h was considered in order to compare the influence of temperature on the by-product bactericidal effectiveness. Microbial $\log_{10}$ cycle reduction levels after 24 h of incubation are shown in Fig. 2 at the temperatures studied (5, 10 and 22 °C) when *L. monocytogenes* was exposed to different cauliflower by-product concentrations. As can be seen in Fig. 2, at 5 °C and 10 °C, no bactericidal effect (<0.5 $\log_{10}$ cycles reduction) (FAO/ WHO, 2009) of the cauliflower by-product concentrations studied was detected after a

storage period of 24 h. However, at 22 °C all cauliflower by-product concentrations showed an inhibitory effect, being the minimum bactericidal concentration 2% (w/v). Under these conditions, a maximum reduction level of 1.21 $\log_{10}$ cycles was achieved after 24 h of exposure to a cauliflower by-product concentration of 10% (w/v). In spite of the longer time required by the listerial population to start to die at 5 and 10 °C under cauliflower by-product exposure, a lower minimum inhibitory concentration was observed in reference medium at 5 °C with 0.5% (w/v) of cauliflower by-product; 10% (w/v) was the minimum bactericidal concentration at the same temperature.

In the present study, the maximum reduction level of 2.25 $\log_{10}$ cycles of the *L. monocytogenes* bacterial population in reference medium was achieved when the bacteria were exposed to a 15% (w/v) concentration of cauliflower by-product at refrigeration temperature (5 °C) after an incubation period of 480 h (20 days) (Fig. 3). The most effective antimicrobial capability of natural ingredients/substances has previously been obtained by various authors at refrigeration temperatures by means of significantly higher microbial reduction levels than the ones obtained at refrigeration abuse or room temperatures (Iturriaga, Olabarrieta, & Martínez de Marañon, 2012; Kong, Chena, Xing, & Park, 2010).

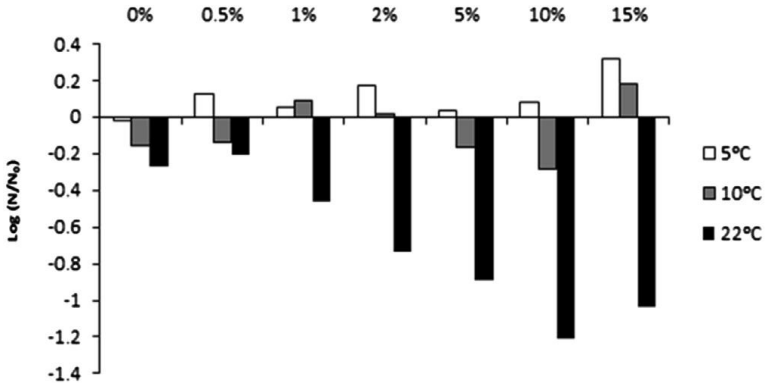

Figura 5.1.2.**2.** *L. monocytogenes* inactivation levels after 24 h incubation under exposure to 5%, 10% and 15% cauliflower by-product at 5 °C, 10 °C and 22 °C.

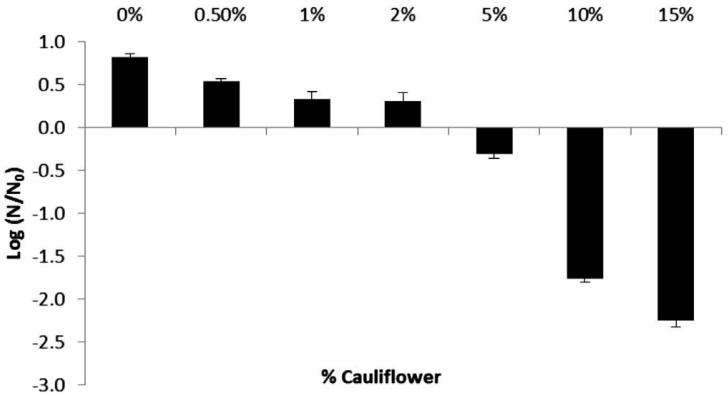

Figura 5.1.2.**3**. *L. monocytogenes* inactivation levels under exposure to 0%, 0.5%, 1%, 2%, 5%, 10% and 15% cauliflower by-product at 5 ^{o}C.

5.1.2.3.2 Kinetics of *L. monocytogenes* growth inhibition by cauliflower by-product

In order to complete the evaluation of the proposed future applicability of cauliflower by-product as a natural antimicrobial additive in new formulated products for human consumption the experimental results were fitted to the modified Gompertz equation. This model has previously been used to provide accurate fits to microbial behaviour of *Listeria* innocua under exposure to natural antimicrobials (Char, Guerrero, & Almazora, 2009, 2010). The Gompertz kinetic parameters and model goodness of fit coefficients are presented in Table 1. An ANOVA analysis confirmed the influence of both factors studied (concentration and temperature) ($p \leq 0.05$) on *L. monocytogenes* inactivation kinetic parameters (B, C, M).

Maximum death rate (μ_{max}) and lag phase duration (t_{lag}) values were calculated from B, C and M parameters, according to Equations (3) and (4), for each concentration-temperature combination (Table 1). As can be seen in the table, increasing by-product concentrations and temperatures are associated with the highest maximum death rate (μ_{max}) and lowest lag phase duration (t_{lag})

values. These results are in agreement with the studies of Char *et al*., (2009, 2010) in which the kinetic parameters followed the same behaviour pattern when the temperature and concentration of other natural products were modified, specifically for Gram-positive bacteria. According to Ferrer, Ramon, Muguerza, Marco, and Martínez (2009) *B. cereus*, also showed similar kinetic parameter patterns, at higher temperatures and with higher concentrations of other natural substances.

A deeper study was carried out, based on secondary response surface models (Fig. 4a and b), used to define the μ_{max} and t_{lag} dependence on temperature and by-product concentration by means of the following polynomials:

$$\mu_{max} = 0.097 - 0.004[\text{Conc}] - 0.021[T] + 0.0002[\text{Conc}]^2 + 0.0006[T]^2 \quad \left(R^2 0.929\right)$$

$$t_{lag} = 6974.97 - 246.992[\text{Conc}] - 821.531[T] + 8.522[\text{Conc}]*[T] + 4.839[\text{Conc}]^2 + 23.388[T]^2 \quad \left(R^2 = 0.912\right)$$

As can be seen in Fig. 4a and b, at refrigeration temperatures (≤5 °C), μ_{max} values are close to zero, and t_{lag} values are about 2500 h. However, in the abuse refrigeration temperature range (5-10 °C), there is an increase in μ_{max} and a decrease in t_{lag}, achieving the maximum values of μ_{max} and the minimum values of t_{lag} at room temperature (range 15-22 °C). Therefore, the antimicrobial potential of cauliflower by-product is specifically effective as an additional control measure during the shelf-life of pasteurized RTE products, exerting an effective bacteriostatic effect over a short period of time, at abuse refrigeration temperatures, or in broken cold chain situations.

To date, few studies have been published about the antimicrobial effect of *Brassicaceae* vegetables, and most of them evaluate the antimicrobial

activity qualitatively, using inhibition zones (Blazevic *et al*., 2010; Char at al., 2010). To our knowledge, no previous studies deal with the mathematical modelling of microbial inactivation/survival kinetics due to vegetable by-products. Therefore, mathematical modelling of raw cauliflower by-product intervention opens up the possibility of using vegetable by-products as food ingredients in the near future.

Although the promising antimicrobial properties of cauliflower by-product, it shows a characteristic taste and odour that could not be accepted in any RTE products at effective concentrations, such as occurs in other studies (Klein *et al*., 2013). Therefore, is important to carry on a sensorial study with the aim to find the concentration of cauliflower by-product with an antimicrobial capability and sensorial acceptance (Valero & Giner, 2006) and the RTE products where it can be added, such as salads, meat dishes and garnishes.

Tabla 5.1.2.**1**. Modified Gompertz equation kinetic parameters (μ_{max} and t_{lag}) and accuracy of model fit (adjusted-R2 and MSE) for *L. monocytogenes* inactivation under exposure to 5%, 10% and 15% (w/v) cauliflower by-product concentrations at 5 ºC, 10 ºC and 22 ºC.

		Gompertz kinetic parameters					Accuracy of fit	
Temperatures	**Concentration**	**C**	**B**	**M**	μ_{max}	t_{lag}	R^2	**MSE**
5 °C	0%	10.451±0.956	-0.0011±0.000	1213.25±212.17	-0.004±0.001	4106.11±735.01	0.963	0.003
	5%	8.604±0.296	-0.002±0.001	965.81±157.87	-0.006±0.001	2485.04±409.11	0.933	0.008
	10%	9.831±0.802	-0.003±0.001	582.99±35.14	-0.009±0.001	1792.28±182.83	0.960	0.035
	15%	8.970±1.164	-0.004±0.002	498.83±62.981	-0.013±0.003	1447.90±380.93	0.896	1310
10 °C	0%	12.185±4.925	-0.006±0.007	178.01±109.38	-0.041±0.012	584.41±159.29	0.882	0.042
	5%	11.333±5.561	-0.003±0.004	334.63±244.23	-0.071±0.018	243.17±33.57	0.952	0.008
	10%	9.324±0.968	-0.009±0.008	178.15±91.58	-0.073±0.012	221.65±24.55	0.974	0.011
	15%	9.232±0.929	-0.012±0.009	144.89±60.40	-0.083±0.014	189.10±23.47	0.808	0.391
22 °C	0%	9.240±0.809	-0.019±0.012	82.85±34.83	-0.081±0.021	257.71±71.77	0.953	0.023
	5%	12.230±5.646	-0.012±0.011	78.97±29.27	-0.095±0.022	235.85±76.65	0.956	0.004
	10%	10.708±1.629	-0.021±0.008	51.28±6.48	-0.097±0.021	199.53±37.98	0.986	0.012
	15%	9.848±2.692	-0.024±0.017	49.17±14.11	-0.105±0.032	151.09±30.07	0.935	0.083

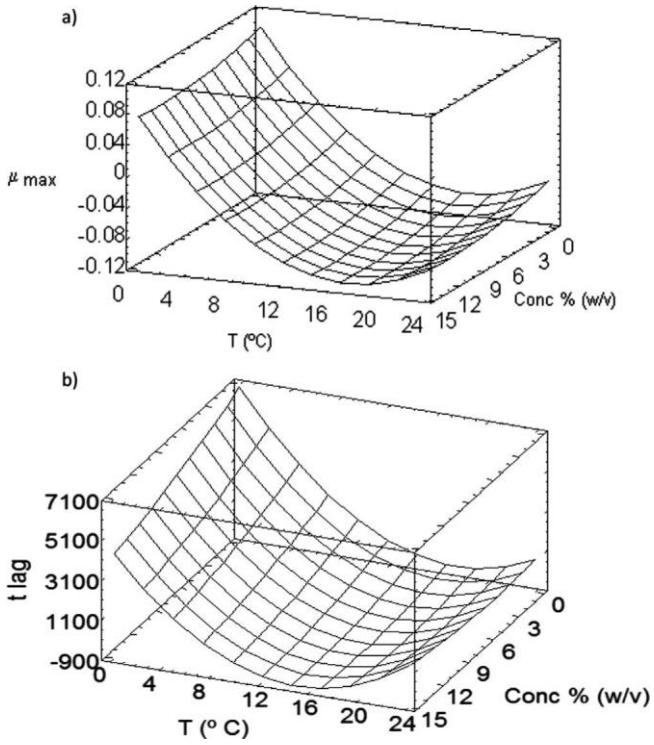

Figure 5.1.2.**4**. Three-dimensional relationship between the influence of cauliflower by-product concentration (% (w/v)) and incubation temperature (^{o}C) on the maximum death rate (μ_{max}) and lag phase duration (t_{lag}) values, defining *L. monocytogenes* growth inhibition in reference medium due to cauliflower by-product antimicrobial capability.

5.1.2.4 CONCLUSIONS

The present study proposes a quantitative approach to assess the antimicrobial potential of a vegetable by-product, cauliflower, against one of the foodborne pathogens of greatest concern to the scientific community and public health authorities. As RTE products are the main vehicle of *L. monocytogenes* transmission, they seem to be possible food matrices for supplementation with various concentrations of cauliflower by-product as an antimicrobial, to act effectively during the chilling period. According to the results obtained, maximum inactivation levels can be achieved with high

concentrations (>5% (w/v)) and low temperature (5 °C), and this would be specifically useful for pasteurized products with a limited shelf-life under refrigeration, controlling and reducing the *L. monocytogenes* load by up to 2.25 log cycles. Moreover, this natural product has demonstrated its value as a way of controlling bacterial load under possible cold chain breakdown (T ≥ 10 °C).

In spite of the promising possibilities of this vegetable from a functional and antimicrobial point of view, the intense odour and taste of cauliflower might not be suitable for addition in some RTE products. However, it would be particularly appropriate in vegetable salad, prepared meat dishes, or ready-to-eat garnishes, which might be possible food matrices for supplementation with these novel natural preservatives.

5.1.2.5 ACKNOWLEDGEMENTS

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CAPÍTULO 5.1.3.

Sanz-Puig, M., Pina-Pérez, M.C., Martínez-López, A., Rodrigo, D.

***Escherichia coli* O157:H7 and *Salmonella* Typhimurium inactivation by the effect of mandarin, lemon, and orange by-products in reference medium and in oat-fruit juice mixed beverage**

LWT- Food Science and Technology, 66, 7-14. (2016).

Abstract

The antimicrobial capability of three water extracts of citrus peels was evaluated against *S.* Typhimurium and *E. coli* O157:H7 at various concentrations (0.5, 1, 5, 10%) and temperatures (5, 10, 22°C) in a reference medium. The best of them was mandarin by-product, achieving a maximum inactivation level against *S.* Typhimurium (8 $\log_{10}$ cycles) with 5% at 5°C. Also, this by-product had the highest total polyphenol content. Mandarin by-product showed a bactericidal effect in a food matrix also at 5°C (≈2 $\log_{10}$ cycles). All results were adjusted to the Weibull model and the *b* values indicated that the higher concentration of mandarin, the greater the inactivation rate in reference medium, without significant differences between 5 and 10%. Similarly, in the food matrix, the inactivation rate of *S.* Typhimurium was higher when the mandarin by-product was added. Therefore, the mandarin by-product could be used as a control measure of *S.* Typhimurium in pasteurized products, which are stored under refrigeration.

5.1.3.1 INTRODUCTION

Citrus is the largest fruit crop worldwide, with an annual production of approximately 100 million tons. The main world producers are Brazil, the USA

and Mediterranean countries (Djilas, 2009; Ghafar, Prasad, Weng, & Ismail, 2010). The industrial production of juices and other citrus derivatives generates approximately 15 million tons of citrus waste a year worldwide, which mainly consists of peel, seeds, and the fruit pulp. *Citrus* waste is usually consigned to landfill or incineration, which generates negative effects on the environment and a cost to the producers (O'Shea, Arendt, & Gallagher, 2012).

This valueless citrus waste can be considered as a renewable source of raw material whose use in various industrial fields could have a double benefit, economic and technological, as a result of its valorization (Martín-Luengo, Yates, Diaz, Saez Rojo, & Gonzalez Gil, 2011; Schieber, Stintzing, & Carle, 2001). Since 2010 generalized agri-food by-product valorization has been a European Union requirement (EUROSTAT, 2010) and many research studies nowadays are focused on recovering, revaluing, and recycling these by-products. One way of valorizing these by-products is the formulation of new products with added nutritional value. *Citrus* by-products are rich in functional compounds such as carotenoids and flavonoids, among others (O'Shea *et al*., 2012), whose antioxidant, anticarcinogenic, antiviral, and anti-inflammatory properties are well known. *Citrus* derivative compounds have an important nutritional and flavoring value, and an antimicrobial capability has also been attributed to some of them, mainly due to ferulic acid, hydrocinnamic acid, yaniding glucoside, hisperidin, vitamin C, carotenoid, and naringin (Ghafar *et al*., 2010). In this sense, they could be used like natural antimicrobials to control the growth of foodborne pathogens, replacing the chemical compounds which are used currently. Also, they could be used as an additional control measure of the microbial growth in situations of cold chain breakdown in pasteurized food that is stored in refrigeration (Sanz-Puig *et al*., 2015).

In this context, the aim of this study was to evaluate the anti-microbial effect of water extracts of by-products of citrus fruits e mandarin, orange, and

lemon e against two of the foodborne pathogens of most concern that are found in low-acid beverages: *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7.

5.1.3.2 MATERIAL AND METHODS

5.1.3.2.1 Microbiology

Pure cultures of *S.* Typhimurium (CECT 443) and *E. coli* O157:H7 (CECT 5947) were provided freeze-dried by the Spanish Type Cul-ture Collection. Both cultures were rehydrated with 10 mL of Tryptic Soy Broth (TSB) (Scharlab Chemie, Barcelona, Spain). After 20 min, the rehydrated culture was transferred to 500 mL of TSB and incubated at 37 °C with continuous shaking at 200 rpm for 14 h to obtain cells in a stationary growth stage. The cells were centrifuged twice at 4000 g at 4 °C for 15 min and then resuspended in TSB. After the second centrifugation, the cells were resuspended in 20 mL of TSB with 20% glycerol, and then dispensed in 2 mL vials with a final concentration of 10^8 colony forming units per milliliter (CFU/mL). The 2 mL samples were immediately frozen and stored at 80 °C until needed for the kinetic inactivation studies.

5.1.3.2.2 Citrus by-products

Dehydrated peel residues from mandarin (*Citrus* reticulata), orange (*Citrus* sinensis) and lemon (*Citrus lemon*) were provided from primary production (Indulleida, S.A.). Each raw by-product was tested to screen its bacteriological quality. The bacteriological analysis determined the presence/absence of microbial contamination with *Listeria monocytogenes* and *Bacillus cereus* (Gram-positives), or *E. coli* O157:H7 and *S.* Typhimurium (Gram-negatives), and was carried out according to the procedures described by

Aycicek, Oguz, and Karci (2006). No samples studied presented contamination with any of the microorganisms tested.

5.1.3.2.3 Total phenolic compounds

The total phenol content of the citrus by-products was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965). Gallic acid calibration standards with concentrations of 0, 100, 200, 300, 400, 500, 600, 700, 800, and 1000 ppm were prepared. Three mL of sodium carbonate solution (2% (w/v)) (Sigma-Aldrich Co. LLC, USA) and 100 mL of Folin-Ciocalteu reagent (1:1 (v/v)) (Sigma-Aldrich Co. LLC, USA) were added to an aliquot of 100 mL from each gallic acid standard (Sigma-Aldrich Co. LLC, USA) or sample tube. The mixture was shaken and allowed to stand at room temperature in the dark for 1 h. Absorbance was measured at 750 nm using a Lan Optics Model PG1800 spectrophotometer (Labolan, Spain), and the results were expressed as mg of gallic acid equivalents (GAE)/L.

5.1.3.2.4 Antimicrobial assay

Buffered peptone water (Scharlab Chemie, Barcelona, Spain) (0.1% (w/v)) was used as a reference substrate in the present study. For the assessment of citrus by-product antimicrobial capability, 1 mL of each vial of stock culture was added to reference substrate at a final concentration of 10^7 CFU/mL. The inoculated medium (buffered peptone water) was supplemented with dehydrated peel residues at different concentrations (0.5, 1, 5, and 10% (w/v)). All the samples were then incubated at different temperatures (5, 10, and 22 °C). At regular time intervals (hours), the cell suspension for each sample was evaluated by plate count in Tryptic Soy Agar (TSA) (Scharlab Chemie, Barcelona, Spain) after serial dilution with 0.1% (w/v) buffered peptone water. The plates were incubated at 37 °C for 24 h. Each dilution was plated in duplicate. The

experiments were carried out in triplicate and the plate counts were used for CFU/mL enumeration.

A second set of experiments was conducted. The most effective antimicrobial of the three tested in the reference medium was evaluated against *S.* Typhimurium in various formulated beverages.

Finally, in order to compare the results, the behavior of both microorganisms under exposure to citrus by-product was characterized by estimating the minimal inhibitory concentration (MIC), being the lowest concentration of antimicrobial substance that is able to inhibit microbial growth (Guillier *et al*., 2007).

Also, the minimal bactericidal concentration (MBC) was estimated, being the lowest concentration of antimicrobial substance that is able to exert a bactericidal effect against the microorganism under study (Bär *et al*., 2009).

5.1.3.2.5 Food matrix

The antimicrobial potential of the most bactericidal citrus by-product was tested against both pathogens in complex food matrices. Firstly, an oat beverage (OB) was used in this set of experiments. The beverage used was supplemented with the most effective citrus by-product and compared with the non-supplemented beverage. The concentration of the by-product was the minimum bactericidal concentration (MBC), and the incubation temperature was 5 °C, a typical temperature for storage of beverages of this kind. Secondly, an oat beverage containing 32.5% papaya, 10% mango, and 7.5% orange (OB-FM) was used. As in the case of the oat beverage, this beverage was supplemented with the most effective antimicrobial by-product using the minimum bactericidal concentration (MBC). The results were compared with those obtained in the non-supplemented OB-FM beverage.

The food matrices considered, OB (supplemented/not supplemented) and OB-FM (supplemented/not supplemented with the most bactericidal by-product), were inoculated with an initial microbial population of 10^8 CFU/mL. The bacterial growth/death during refrigerated storage was monitored by means of viable cell counts.

5.1.3.2.6 Modeling of microorganism inactivation

The microbial behavior was fitted to a Weibull equation (Peleg & Cole, 1998) to obtain a mathematical description of the kinetics of bacterial inactivation by the citrus by-product:

$$\log_{10}\big(S(t)\big) = -b \times t^n \quad (1)$$

where t is the time (hours), S is the survival fraction, i.e., the quotient between the cell concentration at time t (Nt) (CFU/mL) and the initial cell concentration (N_0) (CFU/mL); *b* is the scale factor and *n* is the form factor.

5.1.3.2.7 Data analysis and model evaluation

The statistical analysis was performed with STATGRAPHICS Centurion XV (version 15.1.03; STATGRAPHICS, Warrenton, VA).

This analysis included average and standard deviation calculations for the three repetitions and an ANOVA analysis to test significant differences depending on incubation conditions. The goodness of fit of the model was assessed by using the adjusted regression coefficient (adjusted-R2) (Lopez, 2004).

5.1.3.3 RESULTS AND DISCUSSION

5.1.3.3.1 Antimicrobial capacity of citrus by-products against *S.* Typhimurium

The antimicrobial effect of the mandarin, orange, and lemon by-products was evaluated against *S.* Typhimurium cells during 96 h of incubation at 5 and 10 ^{o}C and 24 h of incubation at 22 °C. Fig. 1 shows the log cycle reduction achieved for each combination.

With regard to the effect of temperature, *S.* Typhimurium growth was inhibited in non-supplemented reference medium (0%) with refrigerated incubation of 5 ^{o}C, while at 10 ^{o}C detectable growth was observed after 96 h, and it was higher at 22 ^{o}C. Therefore it can be concluded that low temperature acts as an effective bacterial proliferation barrier against *S.* Typhimurium, which is in agreement with the findings of other authors (Okada *et al*., 2013).

In general, all by-products tested reduced the microbial load of *S.* Typhimurium regardless of the incubation temperature, with a maximum reduction very close to 8 $\log_{10}$ cycles at 5% and 10% mandarin by-product and 5 and 10 °C incubation temperature. We note that mandarin was the most effective by-product, followed by orange and lemon.

With regard to the by-product concentration, only 5 and 10% of orange and lemon by-products could be considered as an additional control measure for *S.* Typhimurium in the case of a cold chain break (22 °C), at least for 24 h. In contrast, for mandarin by-product, all concentrations tested could be used. In the case of temperature abuse (10 ^{o}C), 5 and 10% of by-product could also be considered as an additional control measure for this microorganism, at least for 96 h, although in orange by-product no significant differences ($p \geq 0.05$) were observed among the concentrations studied.

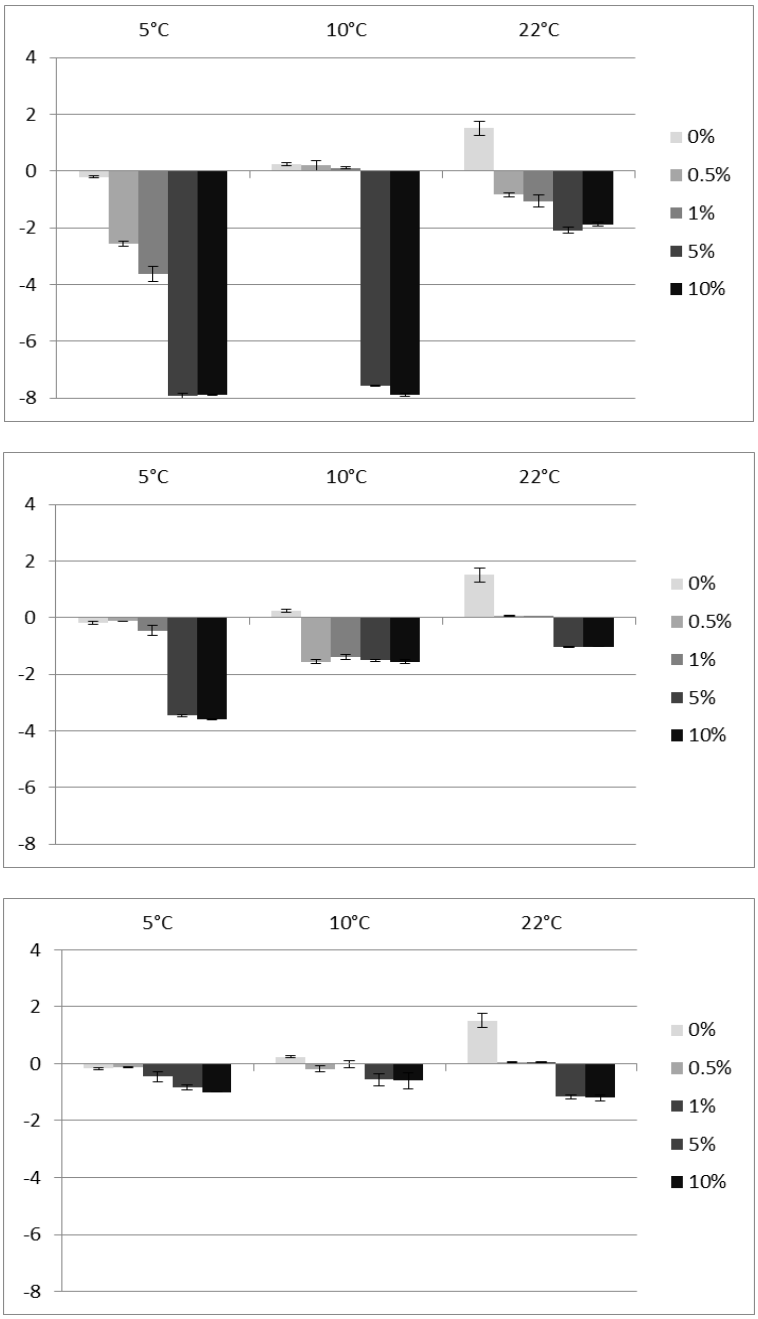


Figure 5.1.3.**1**. Inactivation levels (Log_{10} (N_f/N_0)) of *S.* Typhimurium in contact with various (0, 0.5,1,5,10%) citric by-products concentrations: mandarin (a), orange (b), and lemon (c) in buffered peptone water, incubated at different temperatures (5, 10, and 22 ^{o}C).

An ANOVA analysis concluded that both incubation temperature and by-product concentration had a significant impact ($p \leq 0.05$) on *S.* Typhimurium cell survival. As can be seen in Figure 1, at all temperatures an increase in citrus by-product concentration was accompanied by greater microorganism growth inhibition or inactivation. However, no significant differences were observed between inactivation levels achieved when citrus by-product was added to the medium at 5-10 %, with inactivation levels very close to 8 $\log_{10}$ cycles at incubation temperatures of 5 and 10 ^{o}C in samples with mandarin by-product.

The antimicrobial potential of the by-products studied could be particularly relevant under the concept of hurdle barriers, acting as an additional measure to control bacterial proliferation in situations of abuse temperature (10 ^{o}C) or in the case of cold chain breakdown (22 ^{o}C) in pasteurized food products which must be storage at refrigeration temperatures. They can be added to this kind of products (fruit or vegetable creams or beverages) like an ingredient and control the microbial growth during their storage period. However, these by-products have a low but characteristic taste and odor that could not be accepted by the consumers at high concentrations. Therefore, is important to carry on a sensorial study with the aim to find the concentration of by-product with an antimicrobial capability and sensorial acceptance (Valero & Giner, 2006) and the food products where it could be added.

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for each citrus by-product in relation to incubation temperature were calculated (Table 1). *S.* Typhimurium is highly sensitive to contact with citrus by-products, with very low MIC and MBC values (0.5%). The microbial sensitivity of *S.* Typhimurium depends on both the temperature and the citrus by-product type ($p \leq 0.05$). The lowest MBC was obtained for mandarin at 5 and 22 ^{o}C; while lemon and orange required a

smaller MBC than mandarin to be effective against *S.* Typhimurium when the incubation temperature was 10 ºC.

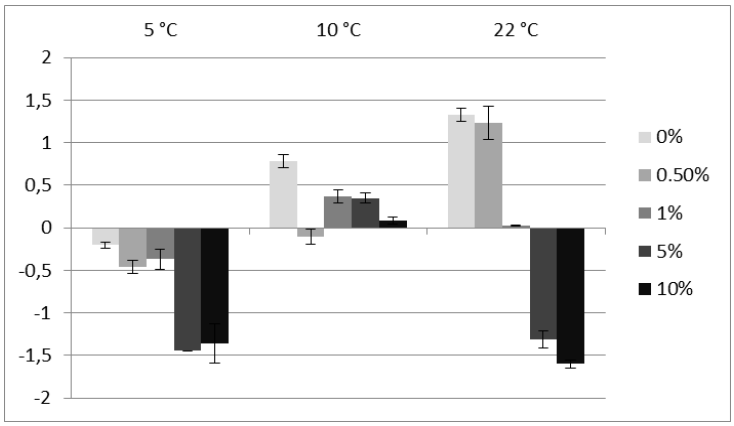
Table 5.1.3.**1**. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) for *S.* Typhimurium in the conditions tested. No significant effects (-).

	***S.* Typhimurium**		
Temperature (C)	**By-product**	**MIC (%)**	**MBC (%)**
5	**Mandarin**	-	0.5
	Orange	-	1
	Lemon	-	1
10	**Mandarin**	0.5	5
	Orange	-	0.5
	Lemon	-	0.5
22	**Mandarin**	-	0.5
	Orange	0.5	5
	Lemon	0.5	5

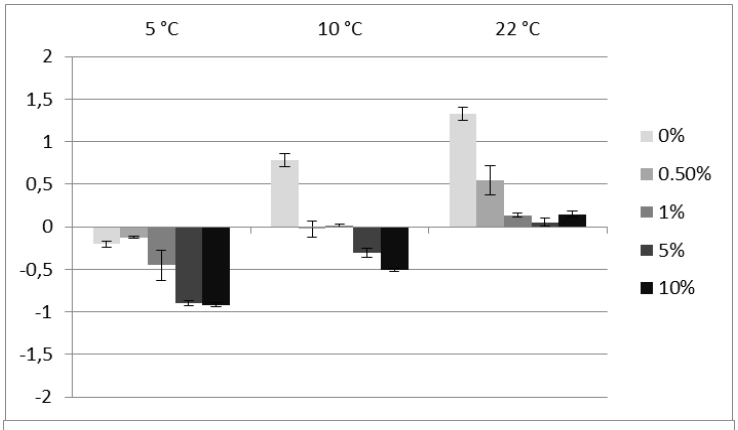
Generally, the MBC at refrigeration temperatures was lower than at room temperature (22 ºC). This may be because refrigeration temperatures have a bacteriostatic capacity and exert a synergistic or additive effect with the by-product concentration. Other authors have shown the bacteriostatic capacity of refrigeration temperatures and have attributed it to a stress response mechanism that is activated in microorganisms at low temperatures (Shapiro & Cowen, 2012).

5.1.3.3.2 Antimicrobial capacity of citrus by-products against *E. coli* O157:H7

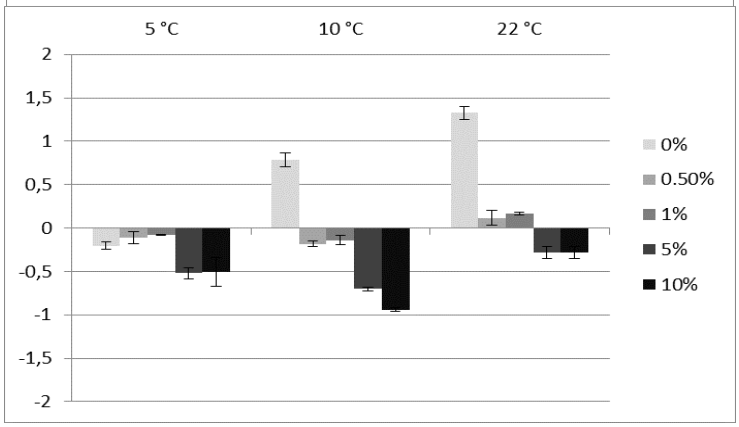
The results for the effect of the citrus by-products on *E. coli* O157:H7 are shown in Figure 2.



a)



b)



c)

Figure 5.1.3.**2**. Inactivation levels (Log_{10} (Nf/N_0)) of *E. coli* O157:H7 in contact with various (0, 0.5,1, 5,10%) citric by-product concentrations: mandarin (a), orange (b), and lemon (c) in buffered peptone water, incubated at different temperatures (5, 10, and 22 °C).

As can be seen, low temperature (5 ^{o}C) inhibited *E. coli* O157:H7 cell growth in reference medium (0% by-product), while at 10 (abuse of temperature) and 22 ^{o}C (cold chain break) the microorganism was able to grow. Focusing on the effect of by-product concentration, 5 and 10% mandarin and orange by-product had a bactericidal effect (0.5 $\log_{10}$ cycles), reducing *E. coli* O157:H7 counts by a maximum of 1.5 $\log_{10}$ cycles. The effect of 5 and 10% concentrations on the bacteriostatic or bactericidal effect at temperatures other than 5 ^{o}C depended on the citrus by-product used. Concentrations lower than 5% appear to have a bacteriostatic effect, slowing down growth of the microorganisms. Note that at 10 ^{o}C *E. coli* O157:H7 started to grow and addition of the mandarin by-product showed a bacteriostatic capacity. In contrast, addition of the orange and lemon by-products did not have any antimicrobial (bacteriostatic or bactericidal) effect at this temperature. At 22 °C, the by-products studied had a bacteriostatic effect against *E. coli* O157:H7 when they were added at 5% (w/v), and addition of mandarin by-product at 10% (w/v) had a bactericidal effect, achieving a maximum reduction of 1.6 $\log_{10}$ cycles.

The mandarin by-product also showed the highest antimicrobial potential against *E. coli* O157:H7, with reductions of 1.3 and 1.6 $\log_{10}$ cycles at 5 and 22 ^{o}C, respectively. The orange and lemon by-products achieved a bactericidal effect, with reductions ranging from 0.5 to 1 $\log_{10}$ cycles at refrigeration temperatures, and both exerted a bacteriostatic effect at 22 ^{o}C.

It is important to note that the effect of the by-products depended on the microorganism tested and the polyphenol structure (Taguri *et al*., 2011). In our case, *S.* Typhimurium was more sensitive than *E. coli* O157:H7 to the various by-products used. This might indicate that each antimicrobial could be specific against a particular microorganism or group of microorganisms.

An ANOVA analysis of data for *E. coli* O157:H7 revealed that for all the by-products studied both incubation temperature and by-product concentration had a significant influence on the antimicrobial activity against *E. coli* O157:H7 ($p < 0.05$), achieving the highest antimicrobial effect by 5 and 10% by-product addition, without significant differences between them.

Table 2 shows the MIC and MBC of the citrus by-products against *E. coli* O157:H7 for each combination of the factors (temperature - concentration) tested. The MIC values are 0.5% at all the temperatures studied, and the MBC values are between 1 and 5%, both being influenced by the incubation temperature and the type of citrus by-product added.

The mandarin by-product had a bactericidal effect at 5 ^{o}C, a bacteriostatic effect at 10 ^{o}C, and both at 22 ^{o}C. However, although the orange and lemon by-products have the same MIC and MBC values as the mandarin by-product at 10 and 22 ^{o}C, they showed a lower antimicrobial capacity expressed as $\log_{10}$ cycle reduction. Therefore, under the conditions studied, it is possible to conclude that *E. coli* O157:H7 has less sensitivity to the citrus by-products studied than *S.* Typhimurium.

Table 5.1.3.**2**. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) for *E. coli* O157:H7 in the conditions tested. No significant effects (-).

E. coli O157:H7			
Temperature (°C)	***By-product***	***MIC (%)***	***MBC (%)***
5	***Mandarin***	-	5
	Orange	-	1
	Lemon	-	5
10	***Mandarin***	0.5	-
	Orange	0.5	5
	Lemon	-	5
22	***Mandarin***	0.5	5
	Orange	0.5	-
	Lemon	-	5

It is well known that the antimicrobial effect of many natural products in a real or buffered medium is influenced by environmental factors (e.g., pH and

temperature conditions), the concentration of the natural ingredient, and the sensitiveness of the microbe (e.g., strain, virulence) (Bajpai *et al*., 2012).

Table 3 shows the pH values for the citrus by-products tested at concentrations of 5 and 10%. Although it has traditionally been accepted that pH plays an important part in inhibiting cellular activity, the table shows that the citrus by-product with the lowest pH value is lemon, while the by-product with the best antimicrobial effect against the microorganisms under study is mandarin. This result appears to indicate that pH is not the most important factor that influences citrus by-product antimicrobial activity.

Table 5.1.3.**3**. pH values measured for mandarin, orange, and lemon by-products at concentrations of 5 and 10%.

	Mandarin		Orange		Lemon	
	5%	10%	5%	10%	5%	10%
pH	4.39 ± 0.02	4.24 ± 0.01	4.85 ± 0.04	4.54 ± 0.02	3.92 ± 0.06	3.77± 0.06

5.1.3.3.3 Polyphenol concentration of citrus by-products

The bacteriostatic and bactericidal capacities of citrus by-products could be significantly influenced by their composition, mainly because of their polyphenol content. Numerous studies show that they have many bioactive compounds such as polyphenols, including ferulic acid, hydrocinnamic acid, cyaniding glucoside, hisperidin, carotenoid, and naringin, in their peel and seeds, which have antioxidant and antimicrobial properties (Ghafar *et al*., 2010). Table 4 shows the polyphenol content measured for each citrus by-product under study. As can be seen in the table, the mandarin by-product has the highest total polyphenol content, followed by orange and then lemon. In this case, the total polyphenol content coincides with the antimicrobial capacity of the by-products: the citrus by-product with the highest polyphenol content, mandarin, is the one with the greatest antimicrobial capacity, followed by the orange and lemon by-products. Therefore we can conclude that polyphenol

content may be directly related to antimicrobial activity, in accordance with other studies (Devi *et al*.,2008).

Table 5.1.3.**4**. Total polyphenol content in by-product extracts.

Citrus by-product	Polyphenol content (mg gallic acid/L)
Mandarin 10%	5111.50± 201.93
Orange 10%	4809.72± 287.47
Lemon 10%	4600.00± 20.00

5.1.3.3.4 Mathematical modeling of *S.* Typhimurium and *E. coli* O157:H7 inactivation

The experimental curves obtained for *S.* Typhimurium and *E. coli* O157:H7 were fitted to a Weibull distribution function, owing to its simplicity and robustness for describing inactivation kinetics (De Oliveira *et al*., 2011).

The results of the fitting are shown in Tables 5 and 6. The *b* value is related to inactivation rate: the higher the *b* value, the faster the microorganism dies. The Weibull kinetic *b* values for *S.* Typhimurium (Table 5) increase with higher by-product concentrations, achieving the maximum inactivation rate at 5% by-product concentration, without significant differences (>0.05) between the *b* values at 5 and 10% by-product concentration.

The same pattern occurs in the *E. coli* O157:H7 inactivation kinetics. As can be seen in Table 6, at lower by-product concentrations the *b* values are close to 0 or negative, owing to microorganism growth. However, at higher citrus by-product concentrations the *b* value increases, without significant differences between 5 and 10% (w/v) addition.

Tabla 5.1.3.**5**. Weibull kinetic values for *S.* Typhimurium inactivation under the citrus by-product effect at various concentrations (% (w/v)) and temperatures (ºC).

% By-product			0%	0.5%	0,01	0,05	0,1
MANDARIN	5 ºC	b	0.017±0.008	0.027±0.013	0.033±0.011	0.107±0.016	0.090±0.010
		n	0.561±0.067	0.548±0.230	0.855±0.074	0.613±0.044	0.652±0.018
		R^2	0.925	0.921	0.928	0.938	0.931
	10 ºC	b	-0.001±0.001	-0.009±0.016	-0.027±0.037	-0.001±0.001	-0.018±0.006
		n	1.460±0.284	0.663±0.497	0.859±0.561	3.796±0.968	0.344±0.230
		R^2	0.960	0.949	0.955	0.930	0.953
	22 ºC	b	-0.036±0.002	-0.015±0.021	-0.116±0.037	0.358±0.178	0.291±0.036
		n	1.138±0.026	1.798±0.912	0.180±0.253	0.450±0.188	0.544±0.041
		R^2	0.973	0.921	0.953	0.942	0.912
ORANGE	*5 ºC*	b	0.005±0.006	0.001±0.001	0.014±0.019	0.001±0.001	0.001±0.002
		n	1.013±0.547	1.385±0.501	1.849±0.470	1.843±0.051	1.532±0.569
		R^2	0.941	0.945	0.965	0.957	0.921
	10 ºC	b	-0.001±0.001	0.029±0.028	0.033±0.007	0.023±0.009	0.079±0.013
		n	1.457±0.280	0.403±0.239	0.437±0.035	0.559±0.130	0.438±0.060
		R^2	0.960	0.966	0.939	0.963	0.921
	22 ºC	b	-0.036±0.002	-0.038±0.010	-0.051±0.057	0.020±0.028	0.029±0.069
		n	1.138±0.026	1.012±0.071	0.647±0.154	1.078±1.265	0.347±0.490
		R^2	0.973	0.922	0.970	0.939	0.925
LEMON	5 ºC	b	0.017±0.006	0.008±0.005	0.017±0.011	0.003±0.003	0.001±0.001
		n	0.526±0.049	0.785±0.091	0.349±0.212	1.137±0.257	1.485±0.007
		R^2	0.935	0.913	0.939	0.927	0.915
	10 ºC	b	-0.001±0.001	0.038±0.029	0.014±0.019	0.032±0.018	0.839±0.121
		n	1.378±0.168	0.460±0.132	0.808±0.337	0.669±0.093	0.037±0.038
		R^2	0.952	0.933	0.925	0.935	0.985
	22 ºC	b	-0.036±0.002	-0.018±0.018	-0.019±0.017	0.035±0.001	0.001±0.001
		n	1.138±0.026	0.961±0.645	0.769±0.344	0.672±0.094	2.905±1.919
		R^2	0.973	0.961	0.954	0.923	0.965

* The *b* value is negative when the microorganism grows and positive when the microorganism dies.

Table 5.1.3.**6**. Weibull kinetic values for *E. coli* O157:H7 inactivation under the citrus by-product effect at various concentrations (% (w/v)) and temperatures (°C).

% By-product			0%	0.5%	0,01	0,05	0,1
MANDARIN	5 °C	b	0.017±0.008	0.027±0.013	0.033±0.011	0.107±0.016	0.090±0.010
		n	0.561±0.067	0.548±0.230	0.855±0.074	0.613±0.044	0.652±0.018
		R^2	0.925	0.921	0.928	0.938	0.931
	10 °C	b	-0.001±0.001	-0.009±0.016	-0.027±0.037	-0.001±0.001	-0.018±0.006
		n	1.460±0.284	0.663±0.497	0.859±0.561	3.796±0.968	0.344±0.230
		R^2	0.960	0.949	0.955	0.930	0.953
	22 °C	b	-0.036±0.002	-0.015±0.021	-0.116±0.037	0.358±0.178	0.291±0.036
		n	1.138±0.026	1.798±0.912	0.180±0.253	0.450±0.188	0.544±0.041
		R^2	0.973	0.921	0.953	0.942	0.912
ORANGE	*5 °C*	b	0.005±0.006	0.001±0.001	0.014±0.019	0.001±0.001	0.001±0.002
		n	1.013±0.547	1.385±0.501	1.849±0.470	1.843±0.051	1.532±0.569
		R^2	0.941	0.945	0.965	0.957	0.921
	10 °C	b	-0.001±0.001	0.029±0.028	0.033±0.007	0.023±0.009	0.079±0.013
		n	1.457±0.280	0.403±0.239	0.437±0.035	0.559±0.130	0.438±0.060
		R^2	0.960	0.966	0.939	0.963	0.921
	22 °C	b	-0.036±0.002	-0.038±0.010	-0.051±0.057	0.020±0.028	0.029±0.069
		n	1.138±0.026	1.012±0.071	0.647±0.154	1.078±1.265	0.347±0.490
		R^2	0.973	0.922	0.970	0.939	0.925
LEMON	5 °C	b	0.017±0.006	0.008±0.005	0.017±0.011	0.003±0.003	0.001±0.001
		n	0.526±0.049	0.785±0.091	0.349±0.212	1.137±0.257	1.485±0.007
		R^2	0.935	0.913	0.939	0.927	0.915
	10 °C	b	-0.001±0.001	0.038±0.029	0.014±0.019	0.032±0.018	0.839±0.121
		n	1.378±0.168	0.460±0.132	0.808±0.337	0.669±0.093	0.037±0.038
		R^2	0.952	0.933	0.925	0.935	0.985
	22 °C	b	-0.036±0.002	-0.018±0.018	-0.019±0.017	0.035±0.001	0.001±0.001
		n	1.138±0.026	0.961±0.645	0.769±0.344	0.672±0.094	2.905±1.919
		R^2	0.973	0.961	0.954	0.923	0.965

* The *b* value is negative when the microorganism grows and positive when the microorganism dies.

Therefore the concentration of citrus by-product added affects the inactivation rate of the two Gram-negative microorganisms studied. In contrast, there does not appear to be a relationship between incubation temperature and *b* value, and, therefore, with the rate of microorganism inactivation.

5.1.3.3.5 Antimicrobial potential of mandarin by-product incorporated in an oat-based beverage

According to the results in the previous sections, mandarin (MND) had the highest antimicrobial potential among the citrus by-products studied in reference medium.

Table 7 shows the inactivation levels reached in *S.* Typhimurium and *E. coli* O157:H7 in oat beverage (OB) supplemented or not supplemented with mandarin during the refrigerated storage period of 144 h at 5 ^{o}C. Although temperature produces some log reductions in the microbial load, an additive effect can be attributed to the mandarin by-product added to the real beverages, producing an additional reduction for *S.* Typhimurium of 0.47 $\log_{10}$ cycles when MND was incorporated in OB and 0.68 $\log_{10}$ cycles when MND was added to oat-based beverage with fruit juice mixture (OB + FM); and for *E. coli* O157:H7 additional reductions close to 1.18 $\log_{10}$ cycles were achieved when MND was incorporated in OB, and 0.65 $\log_{10}$ cycles when MND was added to OB + FM. Although MND had higher effectiveness against *S.* Typhimurium in reference medium, *E. coli* O157:H7 was more sensitive when MND was added to the food matrices studied. It can be observed that the inactivation levels achieved for both microorganisms in OB + FM were significantly ($p \leq 0.05$) higher than those achieved in OB. Some research studies have shown that many fruits are rich in bioactive compounds with antioxidant properties, such as polyphenols, which could also have additional antimicrobial properties against foodborne pathogens (Ghasemi *et al*, 2009; Mandalari *et al*., 2007).

Table 5.1.3.**7**. Inactivation levels (log_{10} cycles) achieved in the food matrices studied for both *S.* Typhimurium and *E. coli* O157:H7 by the intervention of mandarin (MND) by-product added at MBC 5% during a refrigerated storage period of 144 h at 5 °C.

Microorganism	Storage time (h)	OB	OB+MND	OB+FM	OB+FM+MND
E. coli O157:H7	0	0	0	0	0
	24	-0.10±0.00	-0.92±0.05	-0.91±0.05	-1.75±0.12
	48	-0.15±0.04	-0.96±0.04	-0.96±0.07	-1.92±0.06
	96	-0.72±0.06	-1.12±0.08	-1.06±0.05	-1.73±0.06
	144	-0.83±0.06	-2.01±0.13	-1.57±0.07	-2.22±0.23
S. Typhimurium	0	0	0	0	0
	24	-0.10±0.00	-0.77±0.03	-0.59±0.02	-1.20±0.11
	48	-0.15±0.02	-0.94±0.02	-0.64±0.05	-1.32±0.07
	96	-0.48±0.01	-0.98±0.05	-0.85±0.05	-1.54±0.06
	144	-0.65±0.06	-1.12±0.08	-1.17±0.06	-1.85±0.10

OB: Oat beverage; OB+MND: Oat beverage supplemented with 5% (w/v) mandarin; OB+FM: Oat beverage and fruit juice (papaya, mango, and orange) mixture; OB+FM+MND: Oat beverage and fruit juice mixture supplemented with 5% (w/v) mandarin.

According to the results obtained, the bactericidal effect of mandarin on both microorganisms was higher in reference medium than in food matrix. When the mandarin by-product was added to a real matrix, its antimicrobial effectiveness against *S.* Typhimurium was 75% less than when it was added to the reference medium. The interference of the real substrate was remarkable

in the case of the *S.* Typhimurium growth/death pattern under refrigeration using OB as the food matrix. The addition of MND (5% (w/v)) in reference medium resulted in a reduction of 8 $\log_{10}$ cycles for *S.* Typhimurium, while incorporation of this by-product in OB only produced a reduction close to 1 $\log_{10}$ cycle under the same time and temperature storage conditions (96 h, 5 °C). Several authors attribute to food matrix complexity a protective effect that reduces the effectiveness of many control treatments (Gutierrez, Barry-Ryan, & Bourke, 2008). The protective effect of a lipid-rich substrate such as oat milk could affect the antimicrobial potential of mandarin against *S.* Typhimurium (Di Pascua, Hoskins, Betts, & Mauriello, 2006).

The addition of a papaya, mango, and orange juice mixture to the beverage studied significantly increased the inactivation values at each storage point recorded for both microbial populations. After the complete storage period, *S.* Typhimurium inactivation was almost doubled (increasing from 0.74 $\log_{10}$ cycles in OB to 1.25 in OB + FM) by the additional effect of the fruit juices. This may be because mango, orange, and papaya are fruits rich in bioactive substances such as polyphenol compounds (Tomas-Barberan & Espín, 2001), which might produce an antimicrobial effect against the microorganisms studied. Also, the acid pH of the beverage (pH 4.6) might contribute to the antimicrobial effect shown when the fruit juice mixture was added. The supplementation of OB + FM with 5% (w/v) MND increased the final *S.* Typhimurium inactivation level to a maximum of 1.85 $\log_{10}$ cycles compared with the 1.12 $\log_{10}$ cycles achieved in OB + MND, and it increased the maximum *E. coli* O157:H7 inactivation level to 2.22 $\log_{10}$ cycles compared with the 2.01 $\log_{10}$ cycles achieved in OB + MND.

Table 5.1.3.**8.** Weibull kinetic parameters of *E. coli* O157:H7 and *S.* Typhimurium inactivation in Oat beverage and Oat beverage e fruit juice mixture when supplemented/not supplemented with 5% (w/v) mandarin by-product under refrigerated storage (144 h, 5 °C).

Beverage		OB	OB + MND	OB + FM	OB + FM + MND
S. Typhimurium	B	0.014 ± 0.003	0.461 ± 0.015	0.137 ± 0.002	0.571 ± 0.006
	N	0.746 ± 0.012	0.179 ± 0.022	0.419 ± 0.025	0.219 ± 0.011
	Adj-R^2	0.903	0.968	0.962	0.983
	RMSE	0.071	0.051	0.055	0.002
E. coli O157:H7	B	0.018 ± 0.003	0.121 ± 0.011	0.261 ± 0.001	0.802 ± 0.026
	N	0.767 ± 0.025	0.541 ± 0.023	0.325 ± 0.031	0.221 ± 0.021
	Adj-R^2	0.946	0.887	0.915	0.993
	RMSE	0.091	0.062	0.022	0.001

OB: Oat beverage; OB + MND: Oat beverage supplemented with 5% (w/v) mandarin; OB + FM: Oat beverage and fruit juice (papaya, mango, and orange) mixture; OB + FM + MND: Oat beverage and fruit juice mixture supplemented with 5% (w/v) mandarin.

5.1.3.3.6 Mathematical modeling of antimicrobial effect of mandarin by-product addition in an oat-based beverage

The results obtained for microbial inactivation in the oat-based beverage and oat-based beverage with fruit juice mixture, both supplemented/not supplemented with mandarin by-product addition, were fitted to a Weibull distribution function and their kinetic parameters were obtained. The *b* and *n* values obtained are shown in Table 8. In all cases the *n* values are below 0, indicating a concave survival pattern for the microorganisms studied in the beverage. With regard to the scale factor, the *b* values in the fruit juice mixture were higher than those obtained in the oat beverage, indicating the influence of the juice mixture on the microbial inactivation response. The addition of mandarin increased inactivation rates in both OB and OB + FM, with a maximum of 0.571 ± 0.006 for *S.* Typhimurium inactivation and 0.802 ± 0.026 for *E. coli* O157:H7 inactivation in OB + FM supplemented with mandarin by-product.

5.1.3.4 CONCLUSIONS

In conclusion, the three citrus by-products under study showed an antimicrobial effect against *S.* Typhimurium. The maximum reduction level was achieved by the mandarin by-product, followed by the orange and lemon by-products.

The same order can be observed in their polyphenol content, so there may be a relationship between the polyphenol content of the citrus by-products and their antimicrobial activity.

Also, the mandarin by-product was able to exert an antimicrobial effect both on a reference medium (8 $\log_{10}$ cycles for *S.* Typhimurium and 1.6 $\log_{10}$

cycles for *E. coli* O157:H7) and on a real food matrix, an oat-based beverage supplemented/not supplemented with a fruit juice mixture (≈2 $\log_{10}$ cycle reductions for *S.* Typhimurium and *E. coli* O157:H7). Therefore this by-product could be used as an ingredient for technological purposes owing to its potential to act as an additional control measure inhibiting bacterial proliferation, e.g., in pasteurized foods, which have limited refrigerated storage.

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CAPÍTULO 5.1.4

Sanz-Puig, M., Pina-Pérez, M.C., Martínez, A., Rodrigo, D.

Use of natural antimicrobials as a treatment option to control *Salmonella* Typhymurium.

Salmonella: Prevalence, Risk factors and Treatment Options. ISBN: 978-1-63463-680-3. (2015).

Abstract

Salmonella is a foodborne pathogen that causes a huge amount of cases of typhoid fever, gastroenteritis, and deaths every year throughout the world. Although salmonellosis cases in humans have decreased in the last five years, *Salmonella* remains the second most common zoonosis in humans. Foodborne outbreaks caused by *Salmonella* have also reduced in recent years, but they have been linked with contamination of eggs and egg products, cheese, mixed foods, and fresh fruits and vegetables. Therefore control measures for this microorganism are very important to prevent and control *Salmonella* at relevant stages of production, processing, and distribution, especially in primary production, thus reducing its prevalence and the risk it poses to public health. In this context, research carried out to find antimicrobial compounds from natural sources is important because they could be used as additives in new product formulations, where they could exercise an additional measure to control *Salmonella* growth and have an important impact from economic and food safety points of view.

By-products from the food industry are a potential source of inexpensive raw materials, and are rich in bioactive components whose technological and antimicrobial properties are still scarcely studied. With the aim of covering this gap, the objective of the present study was focused on evaluating the antimicrobial properties of three citrus by-products – mandarin, orange, and lemon – against *Salmonella enterica* serovar Typhimurium, in reference medium, under various incubation conditions with differences in temperature and by-product concentration. According to the results obtained, it can be concluded that all the citrus by-products showed a bacteriostatic and/or bactericidal effect under the conditions studied, the mandarin by-product being the most effective one. Maximum reduction levels in the microbial population attained values of ≈8 $\log_{10}$ cycles at refrigeration temperature (5 °C). Consequently, it can be concluded that citrus by-products have effective antimicrobial activity, and could act as an additional barrier to microbial growth when added to pasteurized beverages that are stored under refrigeration, contributing additionally to meeting the zero waste targets set by the European Union.

5.1.4.1 Salmonella: A foodborne pathogen

Salmonella is one of the most important foodborne pathogens worldwide, producing an illness called Salmonellosis that causes over 90,000 human cases per year in the European Union. Salmonellosis is a zoonotic disease that can be transmitted between animals and humans directly or indirectly, and it usually produces diarrhea, nausea, fever, and abdominal cramps, although if it infects the bloodstream it can be life-threatening (EFSA, 2014).

Salmonella is usually present in the intestines of birds and mammals and can be transferred to humans through contaminated foods such as eggs and

raw meat from pigs, turkeys, and chickens. The incubation period ranges from five hours to seven days, but the clinical signs usually appear 12 to 16 hours after ingestion of contaminated food and the syndrome lasts between two and seven days. Usually infections occur in people at risk, young, elderly, or immunocompromised people (Forshell and Wierup, 2006).

In the same way as with humans, *Salmonella* infects animals too. There are serovars of *Salmonella* that are adapted to specific animal species, such as *S.* Abortus ovis (sheep), *S.* Cholerae suis (pigs), *S.* Gallinarum (poultry), *S.* Abortus equi (horses), and *S.* Dublin (cattle). These serovars are not pathogenic to humans, but if humans are infected these serovars cause septicemia. In contrast, these host-adapted serovars cause abortions and severe gastroenteritis in their animal hosts (EU, 2003).

Among *Salmonella* species, there is a group consisting of *S.* Typhimurium, *S.* Enteritidis, *S.* Hadar, *S.* Infantis, and others, which infect both humans and animals. They can establish an animal infection without clinical signs during a variable time period, which can produce a potential zoonosis. Also, serovars that are usually non-pathogenic can cause disease in animal species used for food products under stress conditions (Forshell and Wierup, 2006).

Salmonella zoonosis can be transmitted from various animal sources. The food categories with the highest hazard in relation to zoonosis are raw meat, raw and undercooked poultry meat, eggs and their derivative products, unpasteurized milk and its derivative products, sprouted seeds, unpasteurized fruit juices, and home-made mayonnaise.

Therefore, *Salmonella* control measures are very important to guarantee human health. In this connection, since 1980, when the WHO formulated a three-point defense strategy against *Salmonella* (WHO, 1980), the following measures have been carried out:

- Pre-harvest control: Control of *Salmonella* in food-producing animals. Establishment of monitoring programs to find and control sources of *Salmonella* infection and prevent further outbreaks, with the aim of producing *Salmonella*-free animals.

- Harvest control: Guarantee hygiene during slaughter and processing of meat and meat products.

- Post-harvest control: Educate both the food industry and consumers about good hygiene practices.

Pursuing the same goal, in 2003 the European Union set up control measures to combat zoonosis, considering *Salmonella* as a priority because of the high number of cases of salmonellosis every year and their economic cost. In this connection, several programs to control *Salmonella* have been implemented in all Member States of the European Union.

In these programs, EFSA provides recommendations for control and reduction measures, with the aim of supporting the reduction of *Salmonella* in the food chain (reduction targets in poultry flocks and poultry meat, use of vaccines and antimicrobials to control *Salmonella*). Also, EFSA conducted studies on the prevalence of *Salmonella* in food and food-producing animals and evaluation of the risk factors that affect its prevalence in animals and food.

The application of these programs and the coordinated efforts made by all EU members have resulted in a significant reduction of human cases of *Salmonella* amounting to almost 50% in 5 years (2004–2009). The prevalence of *Salmonella* in flocks of laying hens has also been reduced to 2% or less in all EU Member States, from original values of 20% in some of them. The main reason for the decrease in *Salmonella* cases in humans is probably the reduction of these bacteria in laying hen flocks, because eggs are the most important source of human infections in the EU (EFSA, 2012).

However, although salmonellosis cases in humans have decreased in the last five years, *Salmonella* remains the second most common zoonosis in humans, with almost 200,000 reported human cases in 2012. Therefore measures for the control and inactivation of this microorganism are very important to prevent, detect, and control *Salmonella* at relevant stages of production, processing, and distribution, especially in primary production, to reduce its prevalence and the risk it poses to public health.

5.1.4.2 Salmonella control measures

Salmonella has an important effect on foodborne illnesses; therefore control measures against this microorganism are necessary.

Traditionally, antimicrobial drugs such as antibiotics or chemical substances have been used to control *Salmonella* spp., among other foodborne pathogens. However, the development of resistance to these antimicrobial agents by the microorganisms and public concern about the health damage caused by synthetic additives have led consumers to reject chemical preservatives in food products. Therefore processors and scientists are working together to find antimicrobial compounds of natural origin. In this connection, many extracts of plant origin have proved to have a large range of bioactive compounds with antioxidant, anti-carcinogenic, anti-inflammatory, or antimicrobial properties, including polyphenol compounds and essential oils from various plants (Dai and Mumper, 2010), such as clove and cinnamon (Cava *et al*., 2007), olives (Ferrer *et al*., 2009), brassicas (Brandi *et al*., 2006), and *Stevia rebaudiana* (Belda-Galbis *et al*., 2014), among others.

The bioactive properties of these natural compounds can be considered as natural additives in the development of new products with the goal of eliminating chemical additives in our food (Khan *et al*., 2014).

Essential oils are secondary plant metabolites that exert a potential antimicrobial effect to control foodborne pathogenic bacteria, such as *Salmonella* species, owing to the presence of bioactive volatile compounds. Although their antimicrobial capacity depends on several factors, such as temperature, pH, microbial population load, and favorable environment, they are potent antimicrobials with a low toxic effect, and this makes them green preservatives for microbial control in the food industry. Consequently, the use of essential oils has become an important area of research for future applications as pathogen control measures both in human food systems and in animal nutrition (Bajpai *et al*., 2012; Palaniappan and Holley, 2010; Losa, 2001; Borsoi *et al*., 2011). Many research studies have demonstrated their antimicrobial effect against *Salmonella* in different food models, such as the effect shown by oregano in salads, meat products, and tomatoes (Koutsoumanis *et al*., 1999; Govaris *et al*., 2010; Gunduz *et al*., 2010b), the effect of cinnamon in liquid whole egg (Valverde *et al*., 2010), or the effect of citral in fishery products (Kim *et al*., 1995b) against *Salmonella*.

The bioactive capacity of essential oils is generally attributed to their chemical compounds, such as polyphenolic or terpene groups. Many studies have shown the antimicrobial effect of various polyphenol plant extracts against *Salmonella* (Bajpai *et al*., 2012). For example, the polyphenol content of raspberry, cloudberry, and strawberry (species of the *Rubus* and *Fragaria* genera) showed an antibacterial effect against several strains of *Salmonella* (Puupponen-Pimia *et al*., 2001); Karapinar *et al*. (2007) showed that koruk (unripe grape from *Vitis vinifera*) juice is effective to inhibit *S.* Typhimurium; and Fattouch *et al*. (2007) showed that Tunisian quince (*Cydonia oblonga* Miller) pulp and peel polyphenolic extracts exerted antimicrobial activity against *Salmonella* spp. isolated from food.

Moreover, these natural bioactive compounds from plants can also be extracted from food industry waste, which generally consists of peel, seeds, and outer leaves of fruits and vegetables that remain after food processing (Marín *et al*., 2007; O'Shea *et al*., 2012), and that are usually consigned to landfill or incineration and represent a cost and an environmental problem for the companies involved.

However, these apparently valueless wastes can be considered as a renewable source of raw material, and their use can have a double benefit: reducing pollution and turning them into substances with added value (Martín-Luengo *et al*., 2011; Schieber *et al*., 2001).

Moreover, agri-food by-product valorization is a requirement of the European Union (EUROSTAT, 2010), and many research studies nowadays are focused on recovering, revaluing, and recycling these by-products.

Various ways of using these by-products have been developed. They can be used in agriculture as phytochemical compounds, in waste water treatment as biosorbents, in feed production, in the paper industry, as fuel, or as additives in the development of new products (Gracia, 2004).

An important percentage of total by-product production worldwide consists of citrus by-products. *Citrus* is the largest fruit crop worldwide, with 100 million tons of annual production, mainly from Brazil, the US, and Mediterranean countries (Djilas, 2009). The industrial production of marmalade and citrus segments and the extraction of flavonoids and essential oils result in 15 million tons of citrus waste a year throughout the world.

Many research studies have shown the health benefits of bioactive compounds that have been found in citrus by-products, mainly phenolic compounds such as carotenoids and flavonols (Sawalha *et al*., 2009; Ghafar *et al*., 2009; Igual *et al*., 2013). Consequently, the use of citrus by-products as

functional ingredients in the development of new food products is a promising possibility.

Therefore the aim of this research study is to evaluate the antimicrobial effect of mandarin, orange, and lemon by-products against *Salmonella enterica* serovar Typhimurium under various incubation conditions.

5.1.4.3 Use of natural antimicrobials against *Salmonella*

In this work, the antimicrobial effect of three citrus by-products (mandarin, orange, and lemon) against *S.* Typhimurium was evaluated at different conditions, with incubation temperatures in the range [5–22] °C and various citrus by-product concentrations (0, 0.5, 1, 5, 10%), in reference medium (buffered peptone water (1‰ (w/v))).

The decimal log cycles of *S.* Typhimurium inactivation under the conditions studied, after 96 hours of incubation at 5 and 10 °C and 24 hours at 22 °C, are shown in Tables 1 (mandarin), 2 (orange), and 3 (lemon). As can be seen in these tables, the three citrus by-products that were tested showed an antimicrobial effect against *S.* Typhimurium. Mandarin was the by-product with the best antimicrobial effect, achieving a maximum inactivation level of approximately 8 log cycles at refrigeration temperature (5 °C) with 5% mandarin by-product addition. Moreover, at 10 °C and 22 °C mandarin by-product also had a bacteriostatic effect, with maximum values of 8 and 2 log cycles of microbial inactivation, respectively.

Mandarin by-product effectiveness was followed by orange by-product, which achieved a maximum of 3.59 log cycles of microbial inactivation, also at refrigeration temperature (5 °C), with 10% orange addition. At 10 °C, orange by-product also had a bactericidal effect, with a maximum of 1.5 log cycles of microbial inactivation, and at 22 °C it was bacteriostatic.

Finally, although lemon by-product was able to inhibit *S.* Typhimurium growth at all the concentrations and temperatures studied, it was the natural extract that showed the smallest antimicrobial effect, with a maximum inactivation level of 1.22 log cycles when 10% of lemon by-product was added at 22 °C.

If we compare the data in the three tables, it can be seen that the higher the citrus by-product concentration, the greater the *S.* Typhimurium inactivation level achieved by the three by-products under study. An ANOVA analysis confirmed that the citrus by-product concentration had a significant influence ($p \leq 0.05$) on the antimicrobial effect observed against *S.* Typhimurium, although there were no significant differences between 5 and 10% by-product addition.

Regarding the effect of temperature, the tables show that microbial inactivation levels were generally higher at lower temperatures. An ANOVA analysis confirmed that temperature had a significant influence ($p \leq 0.05$) on the antimicrobial effect observed against *S.* Typhimurium. Therefore, refrigeration temperature also showed an antimicrobial effect against *S.* Typhimurium.

Figure 1 shows the evolution of the microbial load during the incubation period, under exposure to 5% (w/v) of each of the citrus by-products, at 5, 10, and 22 °C.

Table 5.1.4.**1**. Inactivation levels (log cycles) of *S.* Typhimurium under exposure to mandarin by-product at different conditions of temperature and by-product concentration.

Mandarin by-product concentration	*S.* Typhimurium inactivation levels		
	5 °C	10 °C	22 °C
0%	-0.1761	0.2404	1.5117
0.5%	-2.5539	0.2032	-0.8391
1%	-3.6193	0.1123	-1.0591
5%	-7.9243	-7.5798	-2.0838
10%	-7.8865	-7.8921	-1.8792

Table 5.1.4.2. Inactivation levels (log cycles) of *S.* Typhimurium under exposure to orange by-product at different conditions of temperature and by-product concentration.

Orange by-product concentration	*S.* Typhimurium inactivation levels		
	5 °C	10 °C	22 °C
0%	-0.1761	0.2404	1.5117
0.5%	-0.1231	-1.5607	0.0570
1%	-0.4506	-1.3912	0.0536
5%	-3.4610	-1.4993	-1.0269
10%	-3.5911	-1.5586	-1.0280

Tabla 5.1.4.**3**. Inactivation levels (log cycles) of *S.* Typhimurium under exposure to lemon by-product at different conditions of temperature and by-product concentration.

Lemon by-product concentration	*S.* Typhimurium inactivation levels		
	5 °C	10 °C	22 °C
0%	-0.1761	0.2404	1.5117
0.5%	-0.1231	-0.1845	0.0570
1%	-0.4506	-0.0209	0.0536
5%	-0.8329	-0.5616	-1.1622
10%	-0.9945	-0.5991	-1.2013

If we compare these results with the control sample (buffered peptone water without citrus by-product addition), we can see that at refrigeration temperature (5 °C) *S.* Typhimurium growth was inhibited even in the control sample, as also occurred in other research studies (Yang *et al*., 2001; Mañas *et al*., 2003).

However, at the same temperature, the addition of 5% of mandarin or orange by-product had a bactericidal effect, achieving a maximum of 8 $\log_{10}$ cycles of *S.* Typhimurium inactivation in the case of mandarin.

In contrast, at 10 °C *S.* Typhimurium started to grow in control samples, but the addition of 5% of mandarin and orange by-product showed a bactericidal effect, again achieving a maximum of 8 log cycles of microbial inactivation by mandarin by-product. The addition of lemon by-product had a bacteriostatic effect.

At 22 °C, the initial population of *S.* Typhimurium started to grow, but addition of 5% (w/v) of the three citrus by-products under study had an inhibitory effect on microbial growth.

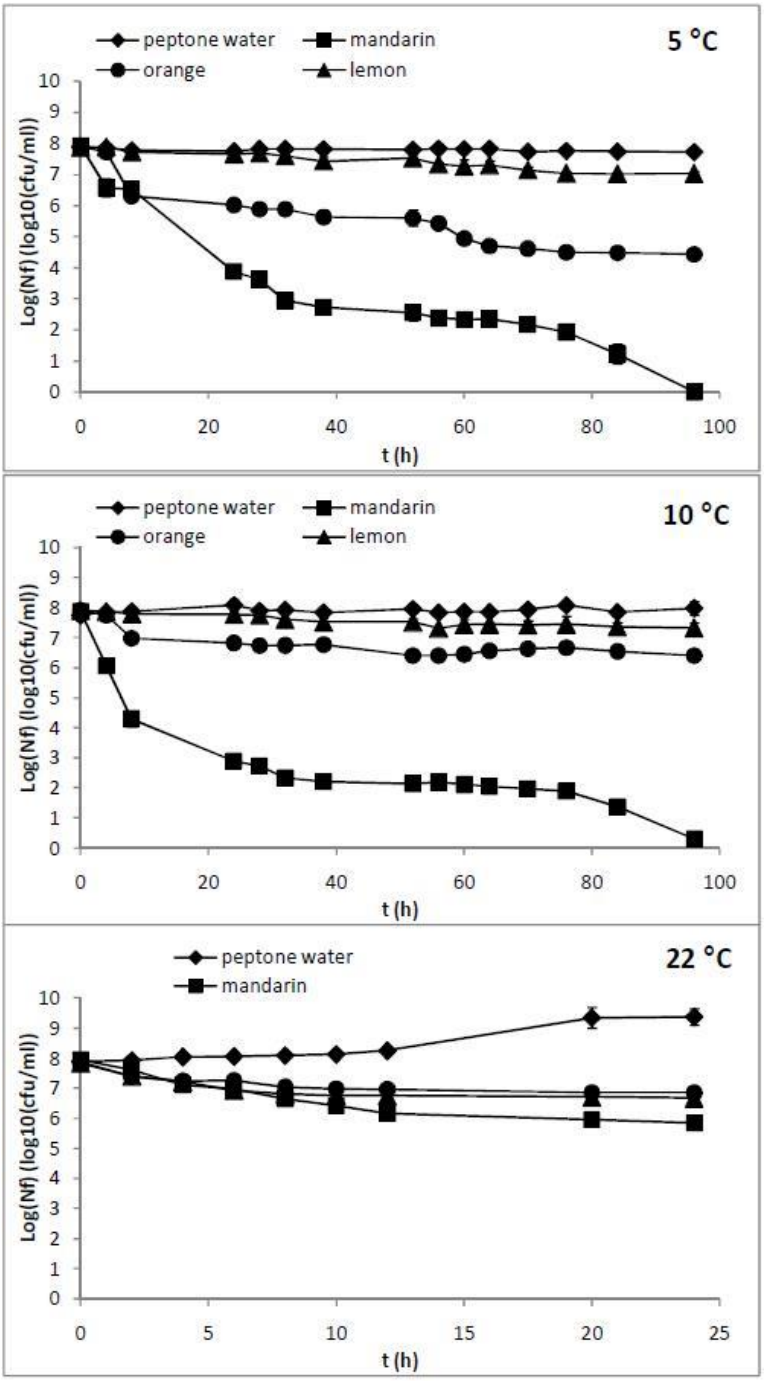

Figura 5.1.4.**1**. Evolution of initial *S.* Typhimurium cell population under the effect of mandarin, orange, and lemon by-product at 5% at 5, 10, and 22 °C.

The bacteriostatic effect of refrigeration temperature has also been extensively recognized by various authors, who attributed this effect to a stress response mechanism of microorganisms, due to molecular changes and metabolic defense mechanisms (Shapiro and Cowen, 2012). Similarly, this control measure can help to enhance the effectiveness of other thermal and non-thermal technologies, especially during the shelf life of the product, achieving additive or synergistic effects in the reduction of the bacterial load until consumption. In the present study, the combination of the two factors, refrigeration temperature and citrus by-product concentration in the medium, showed a synergistic effect regarding microbial inactivation at 5 and 10 °C. The same results were observed by De Oliveira *et al*. (2013), who showed the antimicrobial effect of oregano and lemongrass essential oils against *Salmonella* Enteritidis in ground beef at refrigeration temperature.

In addition to temperature and by-product concentration, there are other factors that can influence *S.* Typhimurium growth, such as pH and citrus by-product composition.

In acid products, pH has an important control effect on microbial growth (Alali *et al*., 2012). However, the pH values of the three citrus by-products are in the range of 3.77 to 4.54, with the lemon by-product having the lowest pH value, followed by the orange and mandarin by-products, respectively. On the other hand, the citrus by-product that showed the highest antimicrobial capacity was mandarin. Thus there was no concordance between the pH value and the antimicrobial effect of the by-products under study against *S.* Typhimurium. Consequently, in this case pH was not the most important factor that contributed to the capacity of these citrus by-products to inhibit microbial growth.

Regarding the citrus by-product composition, many studies show the antimicrobial properties of bioactive compounds of citrus peels and seeds,

which mainly belong to the polyphenol group (Espina *et al*., 2011; Viuda-Martos *et al*., 2008). They are lipidic compounds with aromatic properties, and they also have an antimicrobial effect that is of interest for the pharmaceutical and food industries (Sobrino-López *et al*., 2006). The total polyphenol content of the three citrus by-products under study was in the range of 4600 to 5111 mg gallic acid/L, with the mandarin by-product having the highest value, followed by orange and lemon, respectively. Therefore in this case it is possible to establish a concordance between the antimicrobial effect of the citrus by-products and their polyphenol content. The mechanism of action of these compounds is still not well understood, but the most accepted hypothesis is that their hydrophobic components can break down the lipid components of the bacterial membrane and then the cell content is released to the exterior (Trípoli *et al*., 2007).

In conclusion, all the citrus by-products under study showed a bactericidal and bacteriostatic capacity against *S.* Typhimurium, with the mandarin by -product having the best antimicrobial capacity, especially at refrigeration temperature. Their demonstrated beneficial properties, both nutritional and bioactive, make them possible candidates to be added to food products for both animals and humans as a microbial control measure.

The bactericidal and bacteriostatic capacity that they demonstrated suggests the possibility of using them as natural bacteriostatic compounds on crops as a measure to control the growth of foodborne pathogens, in liquid form on vegetables and cereals and as wax on fruit peel.

Furthermore, in view of their capacity to inactivate zoonotic microorganisms such as *S.* Typhimurium, they might play an important role in control of zoonotic cases if they are added to the feed of animals for human consumption.

On the other hand, their antimicrobial effect against *S.* Typhimurium, especially at refrigeration temperature, opens the door to the possibility of using them as ingredients in food products for humans that are subjected to pasteurization treatment and subsequently stored at refrigeration temperature, as a control measure against foodborne pathogens such as *S.* Typhimurium. Thus they might be a possible solution in the increasing search by food producers for new products with added value, in response to increasing consumer demand for natural products with health benefits (O'Shea *et al*., 2012).

Consequently, agri-food by-products could be re-valorized as antimicrobial additives and would no longer be an economic problem; on the contrary, the valorization of these natural compounds could represent an economic benefit for food companies, adding nutritional and antimicrobial potential to newly developed products.

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CAPÍTULO 5.2. EVALUACIÓN DEL POTENCIAL ANTIMICROBIANO DE EXTRACTOS DE RESIDUOS DE LA AGROINDUSTRIA OBTENIDOS MEDIANTE ASE

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Effect of polyphenol content on the antimicrobial activity of natural extracts from agro-industrial by-products

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Abstract

The main objective of the present study was to investigate the effect of the conditions of extraction by Accelerated Solvent Extraction (ASE) technology on the bioactive antimicrobial activity of extracts from by-products of cauliflower, broccoli, orange, and mandarin. The antimicrobial activity of extracts, with concentrated phenol content, was evaluated against four of the most important foodborne pathogens: *Salmonella enterica* serovar Typhimurium, *Escherichia coli* O157:H7, *Bacillus cereus*, and *Listeria monocytogenes*. The largest phenol content (1252.12 ± 38.29 μg gallic acid/mL) was recovered from cauliflower extract. Cauliflower and mandarin extracts were effective against both Gram-positive and Gram-negative bacteria, showing the highest inhibition zones, 16 ± 0.1 mm and 17 ± 0.1 mm respectively, against 10^5 cfu/mL *S.* Typhimurium. The antimicrobial effectiveness of extracts was influenced by the ASE extraction conditions, initial contamination level, and microbial strain.

5.2.1 INTRODUCTION

Owing to consumer concerns about synthetic additives, there is a growing interest toward the use of natural substances obtained from plants as functional food ingredients (Viuda-Martos *et al*., 2007). At the same time, agro-industrial activities like fruit and vegetable processing result in a huge quantity of wastes representing an important economic problem for producers and an environmental challenge (O'Shea *et al*., 2012). However, many of them have bioactive compounds that can be recovered and used in other industrial processes. Many studies show that fruit and vegetable by-products and their extracts are significant sources of dietary fiber and bioactive compounds with high nutritional value (Fattouch *et al*., 2007). These bioactive compounds can be phenolic compounds, essential oils, flavonoids, carotenoids, and vitamin C, whose antioxidant, anticarcinogenic, anti-inflammatory, antiviral, and antimicrobial properties have been reported (Ghafar *et al*., 2010). Their recovering permit to increasing the added value of these residues and to some extent mitigating the environmental problem, according with the requirement of zero wastes of European Union (EUROSTAT, 2010). Therefore approaches involving the use of agri-food wastes as by-products to obtain food additives or supplements are now being encouraged. This antimicrobial effects are of huge interest for the food industry.

New extraction procedures have been proposed with the aim of extracting bioactive compounds from plants, reducing extraction time and solvent consumption and improving analyte recovery (Ballard *et al*, 2009). Among them, accelerated solvent extraction (ASE) maximizes sample throughput and minimizes phytochemical degradation, and it is a suitable method that is particularly useful for comparing the phenolic content of fruit, food materials, and by-products (Wibisono *et al*., 2009).

The aim of this study was to test the antimicrobial effectiveness of cauliflower, broccoli, mandarin, and orange by-product extracts against four foodborne pathogens – *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Bacillus cereus*– and how their effectiveness is affected by ASE extraction conditions applied to the type and initial quantity of microbial contamination.

5.2.2 MATERIALS AND METHODS

5.2.2.1 Bacterial cultures

Glycerinated cryovials of *L. monocytogenes* (CECT 4032), *B. cereus* (CECT 131), *S.* Typhimurium (CECT 443), and *E. coli* O157:H7 (CECT 5947) were obtained from lyophilized cultures provided by the Spanish Type Culture Collection, using the methods described by Belda-Galbis *et al*., (2013) for *L. monocytogenes* and *E. coli* O157:H7, by Pina-Pérez *et al*., (2013) for *B. cereus* and by Pina-Pérez *et al*., (2012) for *S.* Typhimurium.

5.2.2.2 Obtainment of natural extracts from vegetable by-products

Dehydrated natural by-products of broccoli, cauliflower, mandarin, and orange were provided dehydrated directly from primary production.

Brassicaceae and *Citrus* extracts were obtained by the ASE technology. Accelerated solvent extraction (ASE) is a sample preparation technique that greatly reduces the amount of time and solvent required to achieve analyte extraction. The rate of extraction was greatly enhanced and the % recovery of analytes consistently increased over traditional techniques such as Soxlhet by using elevated temperature and pressure to achieve extraction from solid and semi-solid matrices in very short periods (Fig. 1). To perform an extraction, the solid sample is loaded into a sample cell (11 or 22 mL) which is loaded onto a cell tray and collection vessels are loaded onto a collection tray. A robotic arm

transfers each cell separately into the oven for extraction. The oven is maintained at the selected operating temperature throughout the extractions. The extraction cell design allows operation of the extractions at elevated pressures (1600 psi) to maintain the solvents as liquids at temperatures above their boiling points. Once the cell is placed in the oven, the pump immediately begins to deliver the solvent of choice to the sample cell. Single solvents or premixed solvents can be used from a single collection vessel, or any combination of up to three different solvents can be programmed. ASE is attracting interest as it features short extraction times, low solvent use, high extraction yields, and provides a high level of automation (Hofler, 2002). The carotenes have been extracted by using ASE system (Breithaupt, 2004), as well as aflatoxins (Sheibani & Ghaziaskar, 2009), glucosinolates (Mohn *et al*., 2007) or polyphenols (Talcott *et al*., 2003).

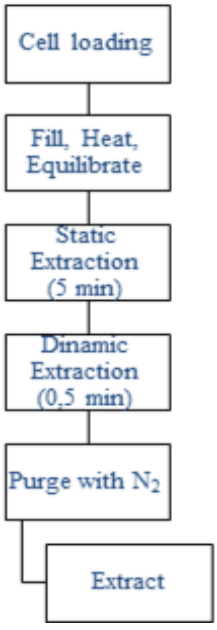

Figure 5.2.**1**. ASE procedure used to obtain the extracts from the by-products of cauliflower, broccoli, mandarin and orange.

Accelerated Solvent Extraction (ASE 350 by Dionex, Vertex Technics) was employed for obtain the cauliflower, broccoli, mandarin and orange extracts, such as shows the Figure 1. The extraction was assumed to be affected by two independent variables: the extraction temperature and the number of extraction cycles.

In this case, a mixture of ethanol and water (20:80) was used as a solvent, the extraction temperature was fixed in the range of 20–120 °C, and the extraction cycles were in the range of one to four. Static extraction time was fixed at 5 minutes and pressure level at 1600 psi (approximately 11 MPa). Each sample was obtained in quadruplicate.

5.2.2.3 Determination of phenol content

A modified version of the Glories' method (Glories, 1979) was used to determine the total phenol content and phenolic composition of ASE extracts. Samples were diluted 1:10 with 10% ethanol and, later, 0.25 mL of sample or standard were placed in a test tube and added 0.25 mL of 0.1% HCl in 95% ethanol and 4.55 mL of 2% HCl. The solution was mixed with a vortex (Heidolph) and allowed to incubated for approximately 15 min before reading the absorbance at 272, 323, 368, and 522 nm with a spectrophotometer. To estimate total phenolic content, the absorbance (A) at 272 nm was used, and the absorbance of, A323 nm, A368 nm and A522 nm were used to estimate tartaric esters, flavonols and anthocyanins, respectively. Standards used were gallic acid (Sigma Aldrich Co., Madrid, Spain) in 10% ethanol for total phenolics, caffeic acid (Sigma Aldrich Co., Madrid Spain) in 10% ethanol for tartaric esters, quercetin (Sigma Aldrich Co., Madrid, Spain) in 95% ethanol for flavonols, and malvidin-3-glucoside (Extrasynthese) in 10% ethanol for anthocyanins.

The statistical analysis was performed with STATGRAPHICS Centurion XV (version 15.1.03; STATGRAPHICS, Warrenton, VA). The analysis included

average and standard deviation calculations for the three repetitions and an ANOVA analysis to test significant differences depending on extraction conditions.

5.2.2.4 Determination of antimicrobial activity

Antimicrobial activity of the natural extracts was measured using the agar diffusion method. One mililiter of stock vials of each microorganism at different concentrations (10^5 cfu/mL and 10^7 cfu/mL) was spread on the surface of Mueller-Hinton agar plates (Scharlau, S.A., Barcelona, Spain). Sterile filter paper discs (7 mm in diameter) were impregnated with 50 µL of the vegetable extracts. The extract was replaced with buffered peptone water (Scharlab, S.A., Barcelona, Spain) as a control sample.

The plates were then kept at ambient temperature for 30 min to allow diffusion of the extracts prior to incubation at 30 °C for 48 hours for *B. cereus*, at 37 °C for 48 hours for *L. monocytogenes*, and at 37 °C for 24 hours for *S.* Typhimurium and *E. coli* O157:H7.

Finally, the inhibition diameter of each disc was measured with a slide gauge. Studies were carried out in triplicate and the average and standard deviation of three values were calculated using STATGRAPHICS Centurion XV (version 15.1.03; STATGRAPHICS, Warrenton, VA).

5.2.3 RESULTS AND DISCUSSION

5.2.3.1 Phenol content in broccoli, cauliflower, mandarin, and orange ASE extracts

Vegetable ASE extracts were characterized by means of their total phenolic content expressed in mg gallic acid/L, and then some phenolic families, which are present in a wide variety of fruits and vegetables, such as flavonols, tartaric esters and anthocianins, were characterized from the total

phenolic content. Table 1 presents the total phenol content of extracts for the various extraction conditions (temperature and extraction cycles) and their polyphenolic characterization, expressed in percentages.

Independently of extraction conditions, maximum phenol content (1252.12 ± 38.29 mg gallic acid/L) was obtained from cauliflower leaf by-product, followed by mandarin, broccoli, and orange with 893.67 ± 105.84, 748.15 ± 86.70, and 570.48 ± 26.55 mg gallic acid/L, respectively.

Regarding the effect of temperature on the amount of total phenolic compounds extracted, Wibisono *et al*. (2009) reported that the total phenol content of turnip leaf, Red Delicious apple puree, and elderberry extracts was slightly greater when the samples were extracted at a higher temperature (100 °C) than when they were extracted at a lower temperature (40 °C). However, no significant effect ($p \leq 0.05$) on the phenol content of the cauliflower extract was found when the extraction was carried out at 80 °C as compared with the extracts obtained at 100 °C (as can be seen in Table 1). Similar results could be observed with the mandarin and broccoli extracts, where no significant differences ($p \leq 0.05$) were found between the extraction temperatures, 100 and 120 °C (mandarin) or 20 and 120 °C (broccoli), regardless the number of cycles. In the case of orange extract the total phenol content at an extraction temperature of 120 °C with 4 cycles was higher than an extraction temperature of 80 °C with 2 cycles.

Regarding the number of cycles for the same by-product and extraction temperature, it appears that they did not affect the total phenol content of the extracts. The total polyphenol contents of the mandarin extracts obtained with 2 and 4 cycles at 120 °C were not significantly different ($p \leq 0.05$).

A comparison of the total polyphenol content in the mandarin and orange extracts, extracted under the same conditions, 4 cycles at 120 °C, shows

that mandarin extract had more total phenol content than orange extract ($p \leq 0.05$). A comparison of the total polyphenol content of the orange and cauliflower extracts, extracted with 2 cycles at 80 °C, indicates that the total phenol content expressed as gallic acid/L was higher in cauliflower than in orange ($p \leq 0.05$). According to these results it appears that orange by-products have the lowest total phenol content, and accordingly this by-product is the least attractive for the valorization industry.

Also, the polyphenol characterization of each extract, permit us to know the main polyphenol families which are present in each ASE extract. Both cauliflower and broccoli extracts have a similar polyphenol pattern, with tartaric esters as the main polyphenol family, followed by flavonols and anthocyanins. Cartea *et al*., (2011), also found a similar polyphenolic composition of *Brassicaceae* vegetables. In the case of *Citrus* by-products, tartaric esters are also the main polyphenol group, with percentage values higher than in *Brassicaeae* extracts, followed by flavonols and anthocyanins. The percentages of the three polyphenol families analysed are higher in mandarin than in orange extracts, being, in contrast, the percentage of "other polyphenols" higher in orange than in mandarin extracts (table 1). These differences could be due to hydroxycinnamic acids and flavanones such as naringin, which can be found in higher amounts in orange than in mandarin extracts (Abad-García *et al*., 2014; Khan *et al*., 2014).

Table 5.2.**1**: Total phenol content in by-product extracts.

Extract	Temperature (°C)	Cycles	Total Phenol content (mg gallic acid/L)
Broccoli	20	3	734.60 ± 82.90[a]
	120	1	748.15 ± 86.70[a]
Cauliflower	80	2	1252.12 ± 38.29[b]
	100	2	1071.87 ± 108.04[b]
Orange	80	2	294.34 ± 19.20[c]
	120	4	570.48 ± 26.55[d]
Mandarin	100	4	836.24 ± 107.62[e]
	120	2	893.67 ± 105.84[e]
	120	4	810.40 ± 68.36[e]

[a-e]: superscript letters are indicating significant differences between rows, according to an ANOVA analysis.

Table 5.2.**2:** Total phenol content and antimicrobial effect of vegetable extracts (50 µl), tested by disk diffusion method, against *L. monocytogenes*, *B. cereus*, *S.* Typhymurium and *E.coli* O157:H7 (10^5 CFU/mL)*.

Extract	Extraction conditions		Inhibition halo (mm)			
	Polyphenol Content (mg gallic acid/L)	T°C/Cycles	*L. monocytogenes*	*B. cereus*	*S.* Typhimurium	*E. coli* O157:H7
Broccoli	734.60 ± 82.90[a]	20/3	NI	NI	NI	11 ± 1.4
Broccoli	748.15 ± 86.70[a]	120/1	NI	NI	NI	NI
Cauliflower	1252.12 ± 38.29[b]	80/2	13 ± 1.4	12 ± 0.5	16 ± 1	9 ± 0.2
Cauliflower	1071.87 ± 108.04[b]	100/2	14 ± 1.4	15 ± 2	15 ± 1	NI
Mandarin	893.67 ± 105.84[e]	120/2	NI	NI	14 ± 0.6	8 ± 0.1
Mandarin	836.24 ± 107.62[e]	100/4	8 ± 0.1	NI	17 ± 0.4	10 ± 0.1
Mandararin	810.40 ± 68.36e	120/4	11 ± 0.6	13 ± 0.1	15 ± 0.1	8 ± 0.8
Orange	294.34 ± 19.20[c]	80/2	NI	NI	11 ± 0.1	NI
Orange	**570.48 ± 26.55d**	**120/4**	**NI**	**NI**	**10±0.1**	**NI**

*Diameter (mean and SD) of inhibition zone (mm) including disc diameter of 7 mm. NI: No inhibition.

Table 5.2.**3**: Total phenol content and antimicrobial effect of vegetable extracts (50 µl), tested by disk diffusion method, against *L. monocytogenes*, *B. cereus*, *S.* Typhymurium and *E.coli* O157:H7 (10^7 CFU/mL)*.

Extract	Extraction conditions		Inhibition halo (mm)			
	Polyphenol Content (mg gallic acid/L)	T°C/Cycles	*L.monocytogenes*	*B. cereus*	*S.* Typhimurium	*E. coli* O157:H7
Broccoli	734.60 ± 82.90[a]	20/3	NI	NI	NI	10.5 ± 2.38 mm
Broccoli	748.15 ± 86.70[a]	120/1	NI	NI	NI	NI
Cauliflower	1252.12 ± 38.29[b]	80/2	11.5 ± 0.7 mm	15 ± 0.5 mm	NI	9 ± 0.2 mm
Cauliflower	1071.87 ± 108.04[b]	100/2	11 ± 0.0 mm	11 ± 1 mm	NI	NI
Mandarin	893.67 ± 105.84[e]	120/2	NI	NI	12 ± 0.2 mm	10 ± 0.1 mm
Mandarin	836.24 ± 107.62[e]	100/4	NI	NI	12 ± 0.5 mm	9 ± 1 mm
Mandararin	810.40 ± 68.36[e]	120/4	NI	9 ± 1 mm	NI	9 ± 0.5 mm
Orange	294.34 ± 19.20[c]	80/2	NI	NI	11 ± 1 mm	NI
Orange	570.48 ± 26.55[d]	120/4	NI	NI	NI	8±0.5 mm

*Diameter (mean and SD) of inhibition zone (mm) including disc diameter of 7 mm.
NI: No inhibition.

Regarding the effect of extraction conditions in the content of different families of polyphenols (Table 1), the higher number of extraction cycles, the lower flavonols and anthocyanins content for mandarin extracts, as occurs with the flavanols of grape skin in the study carried out by Mané *et al*., (2007). Those authors indicated two possible explanations: the exposure to organic solvents leads to reduction of extractability of cellular components in next extraction procedures and the enzymatic oxidation could produce the degradation of polyphenolic compounds. Also, it can be seen in mandarin extract that, when the temperature was higher, the flavonol content was lower, probably due to the fact that high temperature increased the hidrolyzation and oxidation of phenolic compounds, such as was indicated by Dai *et al*., (2010).

5.2.3.2 Antimicrobial activity of broccoli, cauliflower, mandarin, and orange extracts

The ASE extracts, were then tested for antimicrobial activity against four foodborne pathogens: *S.* Typhimurium, *B. cereus*, *E. coli* O157:H7, and *L. monocytogenes*.

Tables 2 and 3 show the inhibition halo for each plant by-product and the total phenol content of each extract. Comparing the results, it could be said that, in general, the larger the inoculum concentration, the smaller the inhibition halo, without inhibition halo in many cases. This was especially relevant for *S.* Typhimurium. All the extracts except broccoli exerted some inhibition against this microorganism when the initial microbial concentration was 10^5, but a non-inhibition halo or a decrease in the halo diameter was observed for almost all the extracts when the initial microbial concentration was 10^7. The effect of inoculum concentration on the efficiency of antimicrobial substances was also observed by Silva-Angulo *et al*., (2014). Those authors indicated that inoculum size affected the antibacterial effect of carvacrol on

Listeria innocua and *L. monocytogenes* and this effect should be taken into account in growth kinetic studies. In our study, similar results have been obtained in practically all the studied combinations.

As shown on Table 2, cauliflower extract followed by mandarin extract presented the highest antimicrobial activity against the microorganisms tested. Both extracts were effective against Gram (+) and Gram (–) bacteria, with *S.* Typhimurium being the most sensitive microorganism of the tested microorganism. Cauliflower showed its highest antimicrobial effect with a maximum inhibition zone of 16 ± 1 mm against the 10^5 cfu/mL inoculum concentration of *S.* Typhimurium for the 80 °C, 2 cycle extract (polyphenol content of 1252.12 ± 38.29 mg gallic acid/L), while the mandarin extract obtained with 4 cycles at 100 °C (polyphenol content of 836.24 ± 107.62 mg gallic acid/L) achieved the greatest inhibition zone, 17 ± 0.4 mm, against *S.* Typhimurium at an inoculum concentration of 10^5 cfu/mL.

Regardless of the extraction conditions, the orange extracts were only effective against *S.* Typhimurium. According to these results, orange extracts seem to have some specificity against *S.* Typhimurium, with a similar inhibition halo both at 120 °C with 4 cycles and at 80 °C with 2 cycles against 10^5 of initial cell population, although the first of them has a greater total phenol content. The cauliflower and mandarin extracts were also effective against *L. monocytogenes*. Similar inhibition halo for different cauliflower extracts were obtained, which could be expected, considering that there are no significant differences ($p > 0.05$) in the phenol contents of the cauliflower extracts obtained at different temperatures with the same number of cycles. Mandarin extracts also produced an inhibitory halo in *L. monocytogenes*, although the diameter depended on the extraction conditions because no significant differences ($p > 0.05$) were found among the phenol contents of the mandarin extracts.

B. cereus vegetative cells were also inhibited by the cauliflower and mandarin extracts. The biggest inhibition halo (15 ± 2 mm) was achieved with cauliflower extracts obtained with 2 cycles at 100 °C.

Regarding *E. coli* O157:H7, the broccoli extract was the only one that was effective against this microorganism (11 ± 1.4 mm halo), but only with extracts obtained with 3 cycles at 20 °C. There was no significant ($p \leq 0.05$) difference between the phenol contents of the two extracts (3 cycles at 20 °C and 1 cycle at 120 °C).

Generally, considering the effect of the extracts on the various microorganisms, it appears that the extraction conditions are the parameters that have most influence on the activity of the extracts, there were no significant ($p \leq 0.05$) differences in total phenol content among extracts of the same plant genus, these extraction conditions can influence in the concentration of the different polyphenol families in the same plant genus (Table 1). Previous studies by Wibisono *et al*. (2009) confirmed that for the ASE technique 3 cycles (10 min at 40 °C; 2 min at 100 °C) provided optimal conditions for maximizing phenol extraction from apple pomace. Temperature and extraction cycles were also determined as influential when applied to *Cynara* spp. biomass and bioactive compound extraction by ASE (Ciancolini, 2012).

Total phenol content values, obtained for each of the extracts under study, appears to exert an influence in their antimicrobial potential. As can be seen in the results obtained, the extracts whose phenol content was higher, were the extracts with greater antimicrobial activity. In fact, both cauliflower, among *Brassicaceae*, and mandarin, among *Citrus*, were the ASE extracts with the highest antimicrobial effect against all the microorganisms studied, corresponding with the extracts with the highest phenol contents.

For the effect of the extracts the type of microorganism is also important. In general, the Gram-negative bacteria showed higher sensitivity to exposure to *Brassicaceae* and *Citrus* extracts than the Gram-positive bacteria. According to Martin-Luengo *et al*. (2007), bergamot minimum inhibitory concentrations (MIC, μg/mL) range between 400 and 800 μg/mL against *S.* Typhimurium and *E. coli* K-12, whereas 1000 μg/mL was necessary to inhibit *B. cereus*, and no antimicrobial effect was exerted against *L. monocytogenes*. Similarly, the results of Hu *et al*. (2004) also demonstrated a higher antimicrobial effect of cabbage extracts against Gram-negative bacteria than against Gram-positive bacteria.

With regard to the extraction conditions of the various ASE extracts (temperature and cycles), for broccoli and orange there were no significant ($p \leq 0.05$) differences between the antimicrobial activity of the same extract obtained at different temperatures or with a different number of cycles. However, for cauliflower and mandarin a rise of temperature (from 80 to 100 °C and from 100 to 120 °C) caused the disappearance of antimicrobial activity against *E. coli* O157:H7 and *S.* Typhimurium, respectively. This behaviour could be attributable to the phenolic profile, which might be dependent on the combination of temperature and extraction process cycles, such as occurs with the lower flavonol content at higher temperatures in mandarin extracts.

5.2.4 CONCLUSIONS

The results showed above clearly suggest that vegetable by-products are a potential, economical, and promising source of phenolic compounds with a high antimicrobial effect. However, it is important to achieve optimization of the extraction process because of the effect of those conditions on the antimicrobial activity. The present study provides valuable information about the value of vegetable by-products for food safety improvement and potential new alternatives for food functional supplementation, and extraction

conditions for the ASE technique. Some microbial specificity was found for orange and broccoli extracts. Probably the extraction conditions affect the phenol profile. Studying the phenol profile of each extract could help in understanding the differences observed in the inhibitory capability of extracts from the same plant genus despite the fact that there were no significant differences in the polyphenol contents of the extracts of each genus.

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CAPÍTULO 5.3 EVALUACIÓN DEL POTENCIAL ANTIMICROBIANO DEL TRATAMIENTO POR PEF COMBINADO CON LAS INFUSIONES EN CALIENTE DE LOS SUBPRODUCTOS DE COLIFLOR Y MANDARINA FRENTE A *S.* Typhimurium

Sanz-Puig, M., Santos-Carvalho, L., Cunha, L.M., Pina-Pérez, M.C., Martinez, A., Rodrigo, D.

Effect of Pulsed Electric Fields (PEF) combined with natural antimicrobial by- products against *S.* Typhimurium

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Abstract

The effect against *Salmonella enterica* serovar Typhimurium of PEF treatment combined with cauliflower and mandarin by-product infusions at several concentrations (0, 1, 5, and 10% (w/v)) was evaluated at various incubation temperatures (10, 22 and 37 °C). The possible synergistic antimicrobial action of the combined process of Pulsed Electric Field (PEF) technology followed by exposure to the by-product infusions and the occurrence of sublethal cellular damage were also studied. Antimicrobial kinetics of by-product infusions alone or following PEF treatment were fitted to a Weibull model. Both mandarin and cauliflower by-product infusions showed a maximum antimicrobial effect against *S.* Typhimurium after 10 hours at 37 °C when the microorganism was exposed to 10% of by-product infusion, achieving reductions of initial bacterial load up to undetectable levels. The effect of the PEF treatment (20 kV - 900 μs) caused a reduction of 4 log cycles of the initial

cell population (10^8 cfu/mL) of *S.* Typhimurium and 1 log cycle (90%) of cellular damage. Moreover, when the PEF pre-treated *S.* Typhimurium population was subjected to subsequent incubation in the presence of both by-product [10%] infusions, the microbial inactivation was faster, achieving a reduction of the initial bacterial load (4 $\log_{10}$ cycles) up to undetectable levels in 2 hours. The kinetic values of the Weibull model were obtained. The higher the concentration of by-product infusion, temperature, and PEF treatment applied, the greater the kinetic parameter "*b*" values, which are related to the microbial inactivation rate. Therefore, the addition of cauliflower and mandarin by-product infusions could be a good additional control measure contributing to ensure bacterial counts below recommended limits in pasteurized PEF products during their storage at refrigeration temperatures.

5.3.1 INTRODUCTION

In the last few years, international organizations such as the World Health Organization (WHO) and Food Agricultural Organization (FAO) have shown their concern about microbiological contamination in the food chain, because population mobility and food globalization have led to an increase in food outbreaks (WHO, 2008; EFSA, 2010).

One of the most important foodborne pathogens is *Salmonella*, which causes approximately 93.8 million foodborne illness outbreaks and 155,000 deaths per year (Majowicz *et al*., 2010). *Salmonella enterica* serovar Typhimurium is especially related to meat, eggs, and fresh fruits and vegetables (EFSA, 2011). In the last few years, the incidence of these foodborne outbreaks has been greater, and has increased people's concern about them (Pui *et al*., 2011). Therefore, one of the aims of current food research is to avoid outbreaks caused by foodborne pathogens such as *Salmonella*.

Traditionally, thermal treatment was the most used mechanism to guarantee the microbial safety of food products. Now, however, new non-thermal treatments have been developed to preserve food products, maintaining their organoleptic and nutritional properties (Knorr *et al*., 2011; Barret &Lloyd, 2011). Among the most validated non-thermal treatments applied to food preservation, a notable tendency is the addition of natural antimicrobial compounds from plants (Cava *et al*., 2007; Ferrer *et al*., 2009) or the application of new non-thermal technologies such as High Hydrostatic Pressure or Pulsed Electric Fields (PEF) (Aymerich *et al*., 2005; Mosqueda-Melgar *et al*., 2012).

The development of non-thermal technologies such as PEF for food preservation has increased in recent years, mainly because of the demand for potential methods to ensure not only the microbiological harmlessness of products but also the preservation of their organoleptic and nutritional properties. In this respect, PEF technology appears to be a good alternative to thermal pasteurization processes, only applied to liquid products but with good prospects for being used in the dairy and juice industries (Pina-Pérez *et al*., 2012). In fact, there are several studies that show that the antimicrobial reduction achieved by PEF treatments both in reference media and in food products with various bacteria (Saldaña *et al*., 2011; Pina-Pérez *et al*., 2012; Monfort *et al*., 2012) could be up to 6 $\log_{10}$ cycles. Moreover, recently many studies have tested a wide variety of hurdle combination technologies that reduce the intensity of treatments through the synergistic effect of combinations (Iu *et al*., 2001; Pina-Pérez *et al*., 2009).

Many research studies have also demonstrated the antimicrobial properties of compounds such as polyphenols, carotenoids, and flavonoids, which we can find in some fruits and vegetables (Djilas *et al*., 2009; O'Shea *et al*., 2012). Among them, both *Citrus* and *Brassicaceae* families have been shown

to contain bioactive compounds with antioxidant, anti-inflammatory, anticarcinogenic, and antimicrobial capacity (Ghafar *et al*., 2010; Igual *et al*., 2013). These bioactive compounds are usually in the peel, pulp, or leaves of these fruits and vegetables. Consequently, we can also find them in agro-industrial by-products, large amounts of which are generated in the food industry. Moreover, the revalorization of food by-products can avoid the economic and environmental costs that they create for producers (Martin-Luengo *et al*., 2011) and help to meet the requirements of the European Union (EUROSTAT, 2010).

In this study, the antimicrobial capacity of two infusions of agro-industrial by-products, cauliflower and mandarin, alone or combined with several PEF treatments, against *Salmonella enterica* serovar Typhimurium was evaluated.

5.3.2 MATERIALS AND METHODS

5.3.2.1 Microorganism

A freeze-dried pure culture of *Salmonella enterica* serovar Typhimurium (CECT 443) was provided by the Spanish Type Culture Collection. It was rehydrated with 10 mL of tryptic soy broth (TSB) (Scharlab Chemie). After 20 min, the rehydrated culture was transferred to 500 mL of TSB and incubated at 37 °C, with continuous shaking (Selecta Unitronic) at 200 rpm for 14 h. The cells were centrifuged (Beckman Avanti J-25) twice at 4000 rpm at 4 °C for 15 min and then resuspended in TSB. After the second centrifugation, the cells were resuspended in 20 mL of TSB with 20% glycerol and then dispensed in 2 mL vials to a final concentration of 7.6×10^9 cfu/mL obtained by plate count. The 2 mL samples were immediately frozen and stored at -80 °C until needed for the kinetic inactivation studies.

5.3.2.2 Antimicrobial substances

Cauliflower and mandarin by-products from agro-industrial raw materials were provided as dehydrated residues from primary production of TRASA S.L. and INDULLEIDA S.A., respectively. The raw by-products were washed in sterile water to eliminate contaminating substances, dried, triturated, and homogenized using a laboratory grinder (Janke & Kunkel Ika-Labortechnik) to obtain a powder with a particle size of 40 μm, which was used to perform the experiments (Brandi *et al*., 2006).

5.3.2.3 Preparation of by-product infusions

Infusions at 10% (w/v) from dried cauliflower and mandarin by-products were obtained by boiling in buffered peptone water (0.1% (w/v)) for 30 min. After this, the infusions were centrifuged at 4000 rpm – 15 min at 4 °C for cauliflower and at 3000 rpm – 5 min in the case of mandarin. Then the infusions were filtered three times, using filters with a pore size of 11 and 2.5 (Whatman), and 0.45 μm (PVDF syringe filter) to sterilize the infusions before use.

Finally, from the 10% infusions of cauliflower and mandarin by-products it was obtained 1 and 5% infusions by diluting them with buffered peptone water (0.1% (w/v)). For the control sample, buffered peptone water (0.1% (w/v)) without addition of infusion was used.

5.3.2.4 Pulsed Electric Field treatment (PEF)

Initially, one sample of pure culture prepared and stored frozen (2 mL), was diluted in 18 mL of buffered peptone water (Scharlab Chemie, Barcelona, Spain) 0.1% (w/v). Later, 1 mL of this dilution with approximately 10^8 cfu/mL initial concentration of *S.* Typhimurium was inoculated in buffered peptone water (Scharlab Chemie, Barcelona, Spain)(0.3% (w/v)) and was then treated by

PEF. The PEF equipment (OSU-4D, designed by Ohio State University) consists in eight chambers connected in series with a diameter of 0.23 cm. Between chambers there was connected cooling coils and submerged in a refrigerated bath (20± 0.5 °C). The intensity, voltage and pulse of treatment were recorded by an oscilloscope (Tektronic TDS 210, Tektronic, OR). The pulses are square-wave bipolar, with a duration of 2.5 μs, the flow was 30 mL/min (set using a gear pump (Cole-Parmer 75210-25, Cole-Instruments Parmer, IL)) and the medium was buffered peptone water 0.3% (w/v) because its conductivity (2,57 mS/cm at 25 °C) was optimal to applied PEF treatment. The pulse frequency was in the range 164 – 904 Hz and the temperature increased from 13 to 45 °C during the treatment.

First we applied a screening of 20 PEF treatments ([10–40] kV/cm; [40–220 μs]) to the sample and from all of them we chose a treatment of 20 kV/cm – 900 μs, an intermediate treatment that is able to produce 4 log cycles of microbial inactivation and 1 log cycle of cellular damage.

5.3.2.5 Evaluation of antimicrobial capacity

Both treated and untreated *S.* Typhimurium samples were inoculated in tubes with cauliflower and mandarin infusions (1, 5, and 10% (w/v)) and incubated at 10 and 37 °C. During the incubations, the *S*. Typhimurium population was determined by plate count in Tryptic Soy Agar (TSA) (Scharlab Chemie, Barcelona, Spain) at regular time intervals after serial dilution with 0.1% (w/v) buffered peptone water. The initial counts in the samples without PEF treatment were 10^7 cfu/mL and in the samples with previous PEF treatment were 10^3 cfu/mL. The plates were incubated at 37 °C for 24 hours. All analysis was done in triplicate.

5.3.2.6 Evaluation of cellular damage

In the same way as with the antimicrobial capacity evaluation, cellular damage was evaluated by plate count after several decimal dilutions of 1 mL of sample in buffered peptone water at regular time intervals, in TSA and in TSA with 3% of NaCl. The addition of 3% of salt converts TSA (general medium) into a selective mediumin which only intact cells will grow, while in the TSA medium all viable cells (damaged and intact) will grow. The damaged and dead cell counts were obtained by using the following equations:

$$Damaged\ cells = log\left(\frac{CFU/mL\ nonselective}{CFU/mL\ selective}\right) \quad (1)$$

$$Dead\ cells = log\left(\frac{CFU/mL\ \ nonselective\ t2}{CFU/mL\ nonselective\ t1}\right) \quad (2)$$

where CFU/mL selective is the count in selective medium (TSA with 3% NaCl); CFU/mL nonselective is the count in non-selective medium (TSA) and *t* is the time. Differences in damaged cell counts lower than 0,5 log cycles were not considered.

5.3.2.7 Total polyphenol content

The total phenol content of the cauliflower and mandarin by-product infusions was determined spectrophotometrically according to the Folin–Ciocalteu colorimetric method (Singleton & Rossi, 1965). We prepared gallic acid calibration standards with concentrations of 0, 100, 200, 300, 400, 500, 600, 700, 800, and 1000 ppm. Three mL of sodium carbonate solution (2% (w/v)) (Sigma-Aldrich Co. LLC, USA) and 100 µL of Folin–Ciocalteu reagent (1:1 (v/v)) (Sigma-Aldrich Co. LLC, USA) were added to an aliquot of 100 µL from

each gallic acid standard (Sigma-Aldrich Co. LLC, USA) or sample tube. The mixture was shaken and allowed to stand at room temperature in the dark for 1 h. Absorbance was measured at 750 nm using a Lan Optics Model PG1800 spectrophotometer (Labolan, Spain), and the results were expressed as mg of gallic acid equivalents (GAE)/L.

5.3.2.8 Mathematical modelling of *S.* Typhimurium inactivation

The microbial behavior of *S.* Typhimurium was fitted to a Weibull equation (Peleg & Cole, 1998):

$$\log_{10}\big(S(t)\big) = -b \times t^{n} \qquad (3)$$

where t is the time (hours), S is the survival fraction, i.e., the quotient between the cell concentration at time t (N_t) (CFU/mL) and the initial cell concentration (N_0) (CFU/mL); *b* is the scale factor, and *n* is the form factor.

5.3.2.9 Statistical analysis

The statistical analysis was performed with STATGRAPHICS Centurion XV (version 15.1.03; STATGRAPHICS, Warrenton, VA).

Also, average and standard deviation calculations for the three repetitions and an ANOVA analysis to test significant differences between samples were carried out. The goodness of fit of the model was assessed by using the adjusted regression coefficient (adjusted-R^2) (López *et al*., 2004). Assumptions regarding the application of the Weibull model to fit the data were performed in accordance with Cunha *et al*. (2006).

5.3.3 RESULTS AND DISCUSSION

5.3.3.1 Antimicrobial effect of cauliflower and mandarin by-product infusions against *S.* Typhimurium

The antimicrobial effect of mandarin and cauliflower by-product infusions against *S.* Typhimurium was evaluated under different incubation conditions combining concentrations of infusion in the range [0–10]% and temperatures of 10, 22, and 37 °C. The samples were incubated until the population of *S.* Typhimurium became stable, in case of growth, or until it reached the method detection limit in the cases in which it was inactivated.

When *S.* Typhimurium was exposed to cauliflower infusion (Figure 1), the 1% concentration did not produce an antimicrobial effect and the microorganism grew, showing a behavior similar to that of the control sample without by-products (0%). However, the 5 and 10% cauliflower concentrations had an antimicrobial effect against *S.* Typhimurium, achieving complete bacterial reduction at 10% of cauliflower infusion at all the temperatures tested (10, 22 and 37 °C). Obviously, at lower incubation temperatures the time necessary for microbial inactivation was longer, probably owing to the reduction of its metabolic activity and also the low permeability of the cell membranes at cold temperatures, which would slow down the effect of antimicrobials. These results are in agreement with other studies, such as McDonald *et al*., 1999, or Swinnen *et al*., 2004.

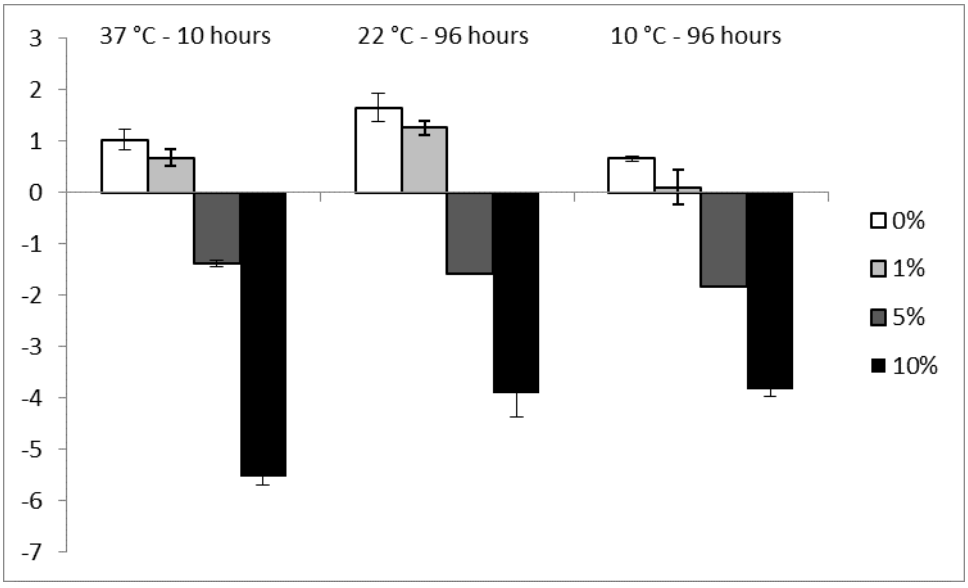

Figure 5.3.**1**. *S.* Typhimurium inactivation levels achieved with different concentrations of cauliflower by-product infusion (0, 1, 5, 10%) and various incubation temperatures (10, 22, 37 °C).

In previous studies (Sanz-Puig *et al*., 2015), the antimicrobial potential of cauliflower by-product infusion obtained with buffered peptone water at ambient temperature was tested against *S.* Typhimurium and other bacteria, and now, if we compare the antimicrobial effect of cauliflower by-product infusion obtained at ambient temperature and 100 °C, we can conclude that the infusion obtained at 100 °C exerts a higher antimicrobial effect than the infusion obtained at ambient temperature, achieving total inactivation in a shorter period of time. Jaiswall *et al*. (2012) also reported the antimicrobial properties of different extracts from several brassicas against Gram – and Gram + bacteria. Also, Burris *et al*. (2012) tested the antimicrobial activity of aqueous extracts of yerba mate against *E. coli*, achieving approximately 4–5 log cycle reductions in apple juice with 40 mg/mL extract.

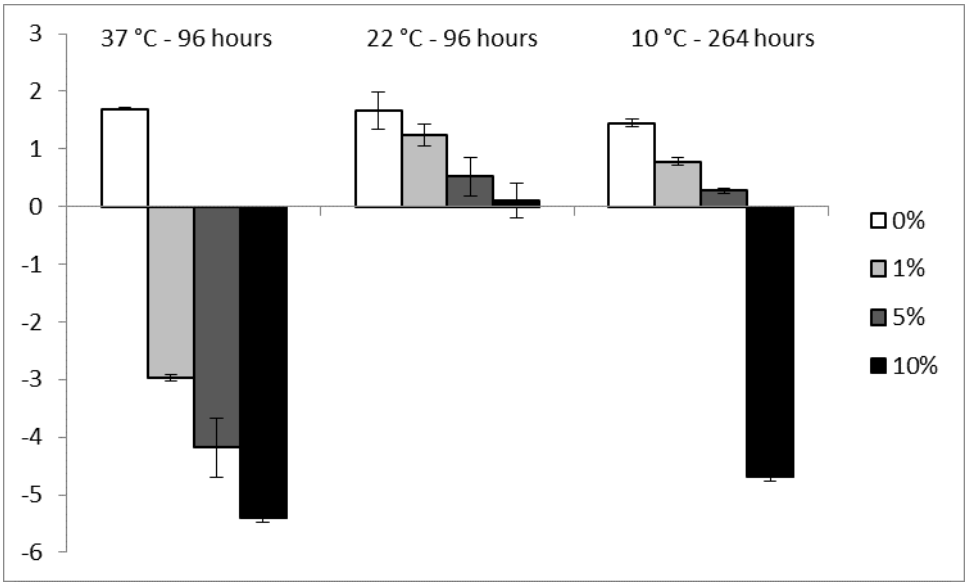

Figure 5.3.**2**: *S.* Typhimurium inactivation levels achieved with different concentrations of mandarin by-product infusion (0, 1, 5, 10%) and various incubation temperatures (10, 22, 37 °C).

The bactericidal effect of the mandarin by-product infusion (Figure 2) was only exerted at 37 and 10 °C. At 22 °C, the infusion only showed a bacteriostatic effect, slowing down the *S.* Typhimurium growth, at all the concentrations studied. In contrast, at 37 °C, the bactericidal effect of the mandarin infusion was effective at all concentrations, and the higher the concentration, the higher the antimicrobial effect, achieving total inactivation of the initial bacterial load with 10% of mandarin by-product infusion after 96 hours of exposure. Finally, at 10 °C, the concentrations of 1 and 5% exerted a bacteriostatic effect (slowing down the growth) against *S.* Typhimurium, while 10% was a bactericidal concentration, achieving complete inactivation of the bacterial inoculum after 264 hours of incubation. Our results are in agreement with Espina *et al*. (2011), who showed the antimicrobial effect of mandarin and other citrus fruits using the agar disc diffusion technique.

Previous studies have indicated the antimicrobial properties of species of both families studied, *Brassicaceae* and *Citrus*, mainly due to the fact that they are rich in various phytochemicals with antimicrobial properties.

Polyphenols are among the most important phytochemicals with antimicrobial potential (Daglia, 2012). The high antimicrobial effect produced by both cauliflower and mandarin by-product infusions might be related to their total polyphenol contents, which are shown in Table 1. Although there are no statistical differences between the total polyphenol contents of the cauliflower and mandarin by-product infusions, the fact that the cauliflower infusion has a higher value than the mandarin infusion or their different polyphenolic profile could be the main causes of the greater antimicrobial effect of cauliflower infusion against *S.* Typhimurium. Our results are in agreement with those obtained by Adámez *et al*. (2012), who showed that aqueous extracts from grapeseeds (*Vitis vinifera* L.) had a total polyphenol content of 6000 mg/L gallic acid, approximately, and exerted an antimicrobial effect against Gram-positive and Gram-negative bacteria, achieving a maximum microbial reduction of 10^5 cfu/mL with the highest extract concentration tested (100 µL/mL).

Table 5.3.**1**. Total polyphenol content of cauliflower and mandarin by-product infusions at 10%.

By-product Infusion	Total Polyphenol Content (mg gallic acid/L)
Mandarin 10%	3958.75 ± 185.62
Cauliflower 10%	4560.0 433.90

5.3.3.2 Antimicrobial effect of pulsed electric fields (PEF) followed by exposure to cauliflower and mandarin by-product infusions against *S.* Typhimurium

Results of the effect of the exposure of bacterial cells to PEF and the cauliflower and mandarin infusions can be seen in Figures 3 and 4. When the samples inoculated with 10^8 cfu/mL of *S.* Typhimurium were treated by PEF, reductions of 4 log cycles were achieved in the *S.* Typhimurium bacterial load.

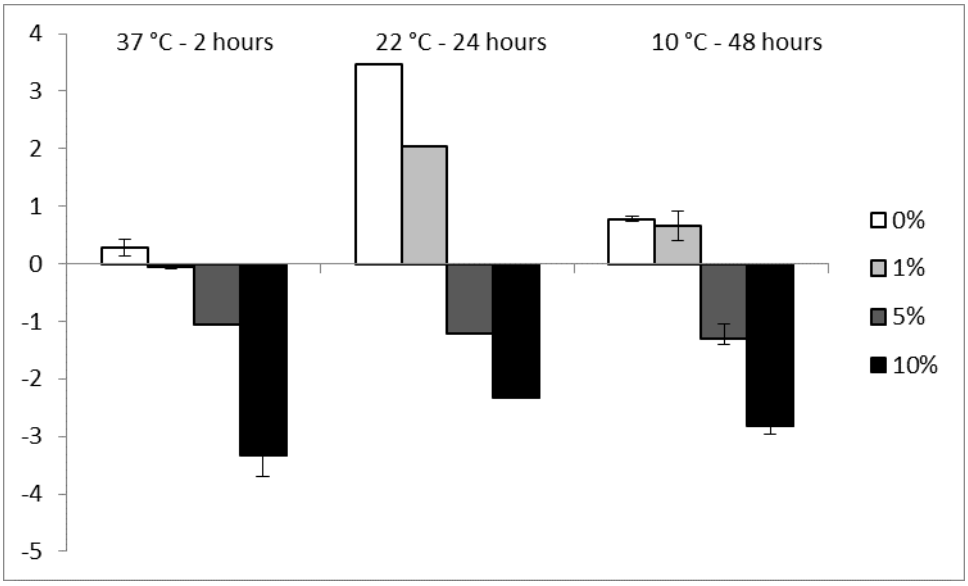

Figure 5.3.3: Inactivation levels of *S.* Typhimurium cells treated by PEF and incubated with different concentrations of cauliflower by-product infusion (0, 1, 5, 10%) and various incubation temperatures (10, 22, 37 °C).

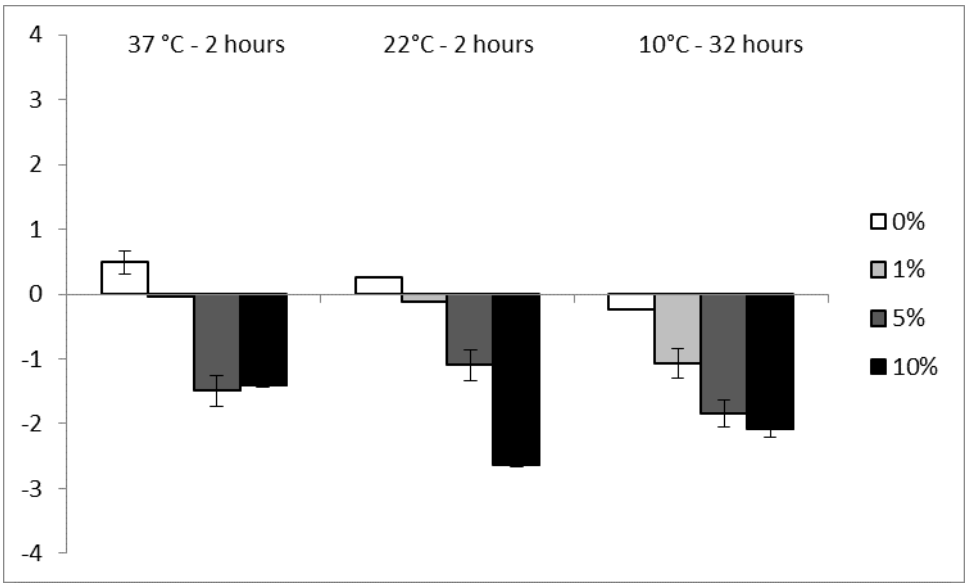

Figure 5.3.4: Inactivation levels of *S.* Typhimurium cells treated by PEF and incubated with different concentrations of mandarin by-product infusion (0, 1, 5, 10%) and various incubation temperatures (10, 22, 37 °C).

PEF-treated samples were incubated in the presence of cauliflower and mandarin by-product infusions at concentrations of 0, 1, 5, and 10% and at different temperatures (10, 22 and 37 °C).

When PEF-treated samples of *S.* Typhimurium were incubated with cauliflower infusion (Figure 3), both 5 and 10% concentrations exerted a bactericidal effect, while the concentration of 1% had a bacteriostatic effect, slowing down the microbial growth at all the temperatures tested. The treated bacterial population was reduced completely after exposure to 5 and 10% of cauliflower for (i) 2 hours at 37 °C, (ii) 24 hours at 22 °C and (iii) 48 hours at 10 °C.

Also, when the treated samples were incubated with mandarin by-product infusion (Figure 4), at 22 and 37 °C the 1% concentration had a bacteriostatic effect against *S.* Typhimurium and concentrations of 5 and 10% exerted a bactericidal effect. However, all concentrations of mandarin by-

product infusion had a bactericidal effect at the temperature of 10°C. The time necessary for inactivation by mandarin by-product infusion of bacteria surviving the PEF treatment was (i) 2 hours at 22 and 37 °C, and (ii) 32 hours at 10 °C.

The results obtained are in agreement with other research studies with PEF and other natural compounds (Pina- Pérez *et al*., 2012; Mosqueda-Melgar *et al*., 2012).

5.3.3.3 Evolution of *S.* Typhimurium different cell populations (intact, damaged and dead) under exposure to by-product infusions combined or not with PEF pre-treatment

The *S.* Typhimurium cellular damage caused by the addition of cauliflower and mandarin by-product infusions to the media, alone or following the application of PEF treatment was evaluated during incubations 10, 22 and 37 ^{o}C and at different infusion concentrations (0, 1, 5, 10 %).

As an example, Figure 5 shows the intact, damaged, and dead cells of *S.* Typhimurium during their incubation at 37 °C with/without 5% of cauliflower infusion and with/without PEF pre-treatment (20kV/cm – 900μs). The control sample (a), 0% cauliflower infusion without PEF treatment, grew during incubation and the damaged cells were maintained at low levels. However, for the PEF-treated sample (b), in addition to the 4 log cycles of inactivation due to the treatment, an additional percentage of damage was observed due to the effect of PEF treatment, which was higher than in the control sample and decreased after 2h incubation time due to these damaged cells were recovered. In contrast, when the initial population of *S.* Typhimurium was incubated with 5% of cauliflower infusion (c) the number of intact cells decreased and the death of bacterial cells increased during the incubation period. The amount of damaged cells increased during incubation owing to the effect of the cauliflower infusion addition.

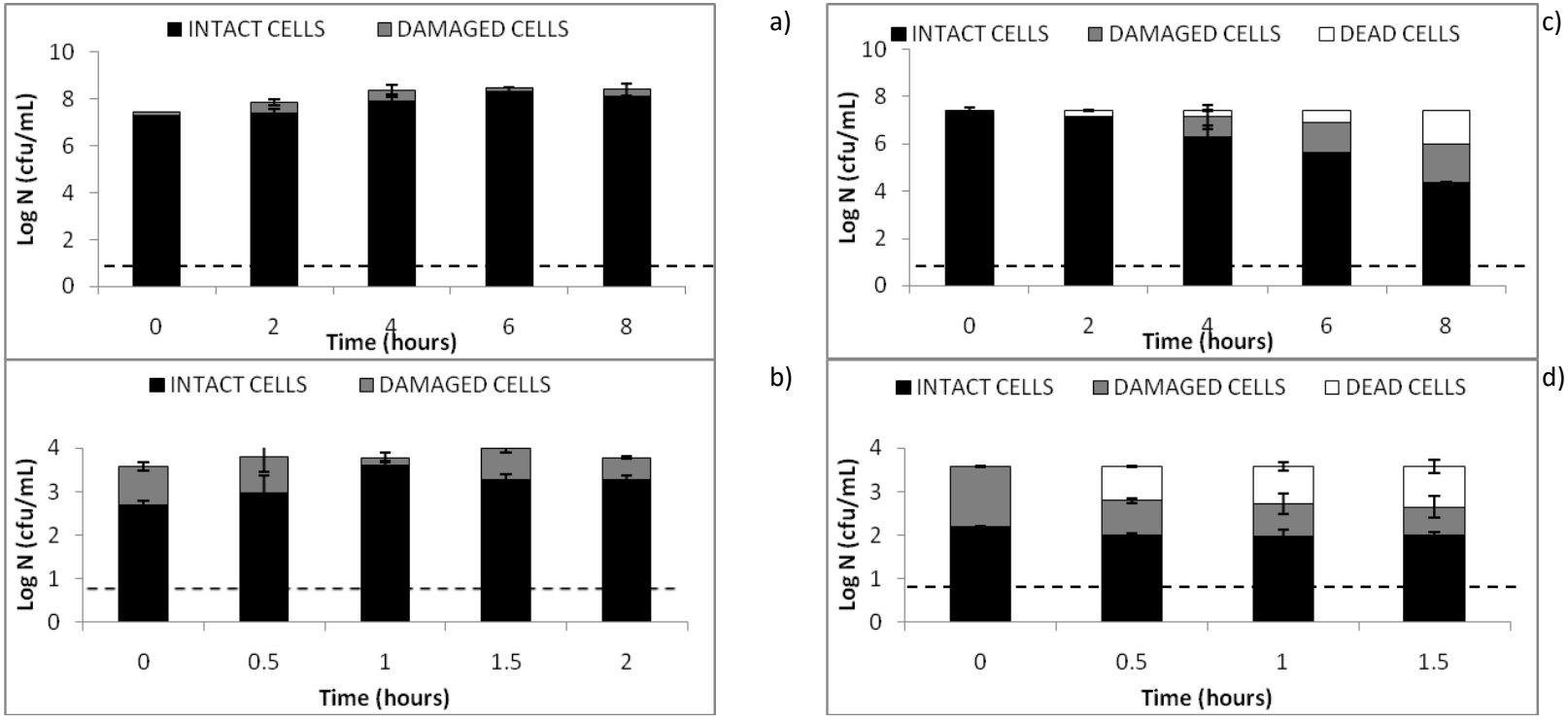

Figure 5.3.**5**: Cellular damage of *S.* Typhimurium caused by Pulsed Electric Field treatment (20 kV/cm – 900 µs) combined/not combined with the addition of 5% cauliflower by-product infusion at 37 °C. a) 0% cauliflower by-product infusion – without PEF treatment, b) 0% cauliflower by-product infusion – with PEF treatment, c) 5% cauliflower by-product infusion – without PEF treatment, d) 5% cauliflower by-product infusion – with PEF treatment. Detection limit.

Finally, in the sample that was treated by PEF and then exposed to 5% of cauliflower infusion (d), the cellular damage was the highest, approximately 1.5 log cycles of the PEF survival population (4 $\log_{10}$ cycles). During incubation of this sample, intact cells (selective medium) seem to be constant with the incubation time, but the counts in non-selective medium were reduced, therefore, dead cells increased, and damaged cells decreased progressively up to undetectable limits. This situation was reached in a shorter period of time than in the other samples (1.5 hours), owing to the combined effect of PEF treatment and addition of the cauliflower infusion. In fact, if we focused in hour 1.5-2, we can see that when the microorganism was incubated with cauliflower infusion there was 7.13 log cycles of intact cells, in contrast, when PEF treatment was applied produced a reduction of intact cells until 3.27 log cycles and 0.5 log cycles of cellular damage and, finally, the combination of PEF treatment and cauliflower infusion caused a reduction until 1.99 log cycles of intact cells and 0.65 log cycles of cellular damage.

Figure 6 shows the results obtained for *S.* Typhimurium treated/not treated by PEF and incubated at 10 °C with/without 10% of mandarin by-product infusion. With regard to the mandarin by-product infusion, for example in a concentration of 10% incubated at 10 °C, the control sample (a) showed growth behavior again. When the initial *S.* Typhimurium population was treated by PEF (b), it was reduced by 4 log cycles and some of the survival cells were damaged. During the incubation period the damaged population remained static, because 10 °C is a refrigeration temperature that slows down microbial metabolic activity (Belda-Galbis *et al*., 2014; Okada *et al*., 2013). When *S.* Typhimurium was incubated with 10% of mandarin by-product infusion (c), the intact cells decreased, the dead cells increased, and the sublethal damage increased with the incubation time, achieving complete bacterial inactivation at 240 hours.

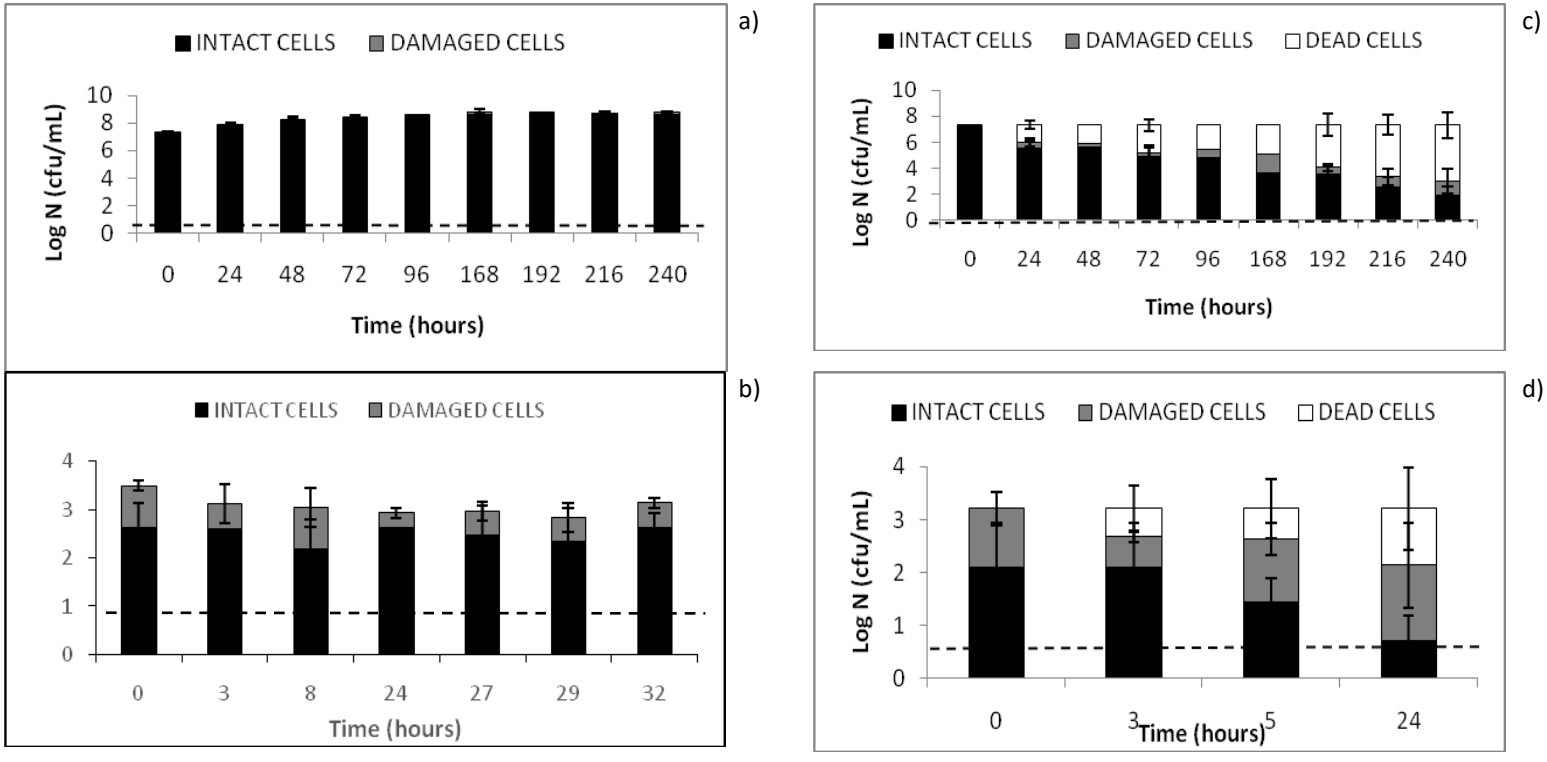

Figure 5.3.6: Cellular damage of *S.* Typhimurium caused by Pulsed Electric Field treatment (20 kV/cm – 900 µs) combined/not combined with the addition of 10% mandarin by-product infusion at 10 °C. a) 0% mandarin by-product infusion – without PEF treatment, b) 0% mandarin by-product infusion – with PEF treatment, c) 10% mandarin by-product infusion – without PEF treatment, d) 10% mandarin by-product infusion – with PEF treatment. Detection limit.

However, the effect of PEF treatment and subsequent exposure to addition of the 10% mandarin infusion (d) caused (i) a reduction of 4 log cycles in the initial cell population, and approximately 1.5 log cycles of damaged cells owing to the PEF treatment, (ii) a reduction of survival cells during the incubation period, achieving total inactivation (undetectable limits) in a shorter time (24 hours) than the sample incubated only with mandarin infusion, owing to the combined hurdle effect and (iii) the proportion of sublethal damage cells increased. Also, we can compare the microbial population at 24 hours: when the microbial cells were incubated with mandarin infusion there was 5.52 log cycles of intact cells at 24 hours, when PEF treatment was applied there was 2.62 log cycles of intact cells and 0.29 log cycles of damaged cells at the same time and when PEF treatment was combined with cauliflower infusion only there was 1.43 log cycles of damaged cells and the population of intact cells was below the detection limit of the skill.

The cellular damage produced by PEF treatment has already been tested in other research studies with other *Enterobacteriaceae* such as *E. coli* (Rivas *et al*., 2012), but the present study also demonstrates its synergistic effect with the antimicrobial effect of infusions from agro-industrial by-products.

5.3.3.4 Mathematical modelling of *S.* Typhimurium inactivation

The effect of the treatments producing inactivation was also evaluated by fitting the experimental results to the Weibull distribution function. The values of *b* (scale factor), also considered as the kinetic parameter (Cunha *et al*., 1998), and *n* (form factor) obtained for the various conditions are shown in Tables 2, 3, 4 and 5. There are *n* values higher and lower than 1, indicating that the survival pattern for *S.* Typhimurium has a concave or convex form, depending on the conditions. Tables 2 and 4 show the *b* values obtained for *S.* Typhimurium inactivation with different concentrations (0, 1, 5, and 10%) of

mandarin and cauliflower by-products, respectively. It can be observed that, at all temperatures, when the mandarin and cauliflower by-product concentration was increased, the microbial inactivation rate was greater. Tables 3 and 5 show the*b* values obtained for *S.* Typhimurium inactivation, previously treated by PEF and incubated in the presence of different concentrations of the infusions. It can be seen that the higher the by-product concentration, the greater the microbial inactivation rate. Finally, if we compare Tables 2 and 3 or Tables 4 and 5 (with and without PEF treatment), we can see that the rate of *S.* Typhimurium inactivation was higher in the samples that had been treated by PEF before exposure to mandarin and cauliflower than in the samples without PEF treatment. Higher *b* values mean less resistance of the cells to the treatments given.

Table 5.3.**2**. Weibull kinetic parameters (scale factor "*b*" and form factor "*n*") for *S.* Typhimurium inactivation with different concentrations of mandarin by-product (0, 1, 5, and 10%) at different incubation temperatures (10, 22, and 37 °C). R2 and MSE values are indicators of goodness of fit. --- Microbial cells grew.

T °C	% Mandarin	*b* (t^{-1})	*n*	R^2 *adjusted*	*MSE*
10 °C	0	---	0.520±0.034	0.959	0.063
	1	---	0.492±0.145	0.957	0.094
	5	---	0.978±0.862	0.965	0.163
	10	0.190±0.098	0.564±0.131	0.986	0.100
T °C	**% Mandarin**	***b* (t^{-1})**	***n***	***R^2 adjusted***	***MSE***
22 °C	0	---	0.149±0.018	0.979	0.062
	1	---	0.462±0.191	0.984	0.130
	5	---	0.458±0.070	0.987	0.681
	10	0.007±0.060	0.602±0.187	0.976	0.659
T °C	**% Mandarin**	***b* (t^{-1})**	***n***	***R^2 adjusted***	***MSE***
37 °C	0	---	0.110±0.042	0.977	0.0134
	1	0.003±0.004	1.768±0.443	0.984	12.778
	5	0.065±0.037	0.913±0.091	0.958	9.434
	10	0.780±0.398	0.369±0.209	0.998	7.662

Table 5.3.**3**. Weibull kinetic parameters (scale factor "*b*" and form factor "*n*") for *S.* Typhimurium inactivation with different concentrations of mandarin by-product (0, 1, 5, and 10%) at different incubation temperatures (10, 22, and 37 °C) after PEF treatment. R2 and MSE values are indicators of goodness of fit. --- Microbial cells grow.

T °C	% Mandarin	*b* (t^{-1})	n	R2 adjusted	MSE
10 °C	0	0.005±0.005	1.388±0.371	0.953	0.083
	1	0.038±0.042	1.187±0.655	0.972	0.377
	5	0.857±0.197	0.212±0.066	0.977	0.204
	10	1.348±0.531	0.159±0.093	0.974	1.604
T °C	**% Mandarin**	**b (t-1)**	**n**	**R2 adjusted**	**MSE**
22 °C	0	---	1.625±0.102	0.986	0.043
	1	0.234±0.076	0.490±0.097	0.992	0.007
	5	0.777±0.148	0.447±0.136	0.986	0.007
	10	1.734±0.355	0.338±0.103	0.998	4.693
T °C	**% Mandarin**	**b (t-1)**	**n**	**R2 adjusted**	**MSE**
37 °C	0	---	1.233±0.067	0.990	0.025
	1	---	2.542±0.396	0.989	0.047
	5	0.992±0.019	0.321±0.067	0.993	0.009
	10	1.391±0.252	0.277±0.050	0.977	0.018

Table 5.3.**4**. Weibull kinetic parameters (scale factor "*b*" and form factor "*n*") for *S.* Typhimurium inactivation with different concentrations of cauliflower by-product (0, 1, 5, and 10%) at different incubation temperatures (10, 22, and 37 °C). R2 and MSE values are indicators of goodness of fit. --- Microbial cells grow.

T °C	% Cauliflower	*b* (t^{-1})	n	R2 adjusted	MSE
10 °C	0	---	0.510±0.153	0.977	0.361
	1	---	1.119±0.406	0.989	0.851
	5	0.060±0.007	0.802±0.062	0.960	1.647
	10	0.095±0.018	0.811±0.029	0.981	0.084
T °C	% Cauliflower	b (t-1)	n	R2 adjusted	MSE
22 °C	0	---	0.433±0.010	0.992	0.339
	1	---	0.439±0.015	0.972	0.845
	5	0.034±0.003	0.831±0.019	0.953	0.345
	10	0.170±0.032	0.695±0.027	0.978	0.510
T °C	% Cauliflower	b (t-1)	n	R2 adjusted	MSE
37 °C	0	---	0.341±0.002	0.958	0.058
	1	---	0.263±0.137	0.999	0.225
	5	0.022±0.002	1.985±0.016	0.960	0.094
	10	0.382±0.037	0.970±0.158	0.991	0.429

Table 5.3.**5**. Weibull kinetic parameters (scale factor "*b*" and form factor "*n*") for *S.* Typhimurium inactivation with different concentrations of cauliflower by-product (0, 1, 5, and 10%) at different incubation temperatures (10, 22, and 37 °C) after PEF treatment. R2 and MSE values are indicators of goodness of fit. --- Microbial cells grow.

T °C	% Cauliflower	b (t^{-1})	n	R2 adjusted	MSE
10 °C	0	---	0.362±0.107	0.992	0.287
	1	---	0.349±0.001	0.968	0.023
	5	0.047±0.009	0.859±0.047	0.965	0.079
	10	0.350±0.033	0.321±0.150	0.983	0.225
T °C	**% Cauliflower**	**b (t-1)**	**n**	**R2 adjusted**	**MSE**
22 °C	0	---	1.637±0.072	0.968	0.133
	1	---	1.480±0.016	0.951	0.332
	5	0.124±0.013	0.875±0.030	0.986	0.033
	10	0.272±0.052	0.706±0.062	0.970	0.053
T °C	**% Cauliflower**	**b (t-1)**	**n**	**R2 adjusted**	**MSE**
37 °C	0	---	0.538±0.219	0.980	0.007
	1	---	0.259±0.225	0.963	0.013
	5	0.875±0.022	0.156±0.081	0.972	0.005
	10	1.459±0.068	0.254±0.050	0.995	0.003

5.3.4 CONCLUSIONS

Both mandarin and cauliflower by-product infusions showed a substantial antimicrobial capacity against *S.* Typhimurium directly related to the concentration, and probably due to the polyphenol content.

The results of the present study reveal that the addition of infusions from by-products could be a good option to ensure food safety in PEF-treated products, exerting a higher antimicrobial effect against *S.* Typhimurium than when they are applied separately. It could also be an additional control measure when problems with the refrigeration chain arise. Accordingly, mandarin and cauliflower by-product infusions appear to be tasty alternative antimicrobial ingredients that could contribute to the food safety of PEF-treated products by application of the hurdle technology concept.

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CAPÍTULO 5.4 EVALUACIÓN DEL POTENCIAL ANTIMICROBIANO DEL TRATAMIENTO POR HHP COMBINADO CON LAS INFUSIONES DE LOS SUBPRODUCTOS DE COLIFLOR Y MANDARINA FRENTE A *S.* Typhimurium

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Combined effect of High Hydrostatic Pressure (HHP) and antimicrobial from agro-industrial by-products against *S.* Typhimurium

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Abstract

The inactivation potential of HHP treatment (200 MPa - 2 min) was evaluated against *Salmonella enterica* serovar Typhimurium in cauliflower and mandarin by-product infusions at 37 and 10 °C. By-product infusions exerted a strong antimicrobial effect used alone, achieving 5 log cycles of bacterial reduction for cauliflower by-product infusion after 10 hours and for mandarin by-product infusion after 80 hours, at 37 °C.

The HHP treatment caused only one log cycle of cellular damage, but when inoculated cauliflower or mandarin by-product infusions were subjected to HHP treatment the antimicrobial effect against *S.* Typhimurium was enhanced, achieving 5 log cycles of inactivation in 6 hours at 37 °C in both cases. Inactivation curves were adjusted to the Weibull equation and the kinetic parameters (*b* and *n*) were obtained. When HHP treatment was combined with by-product infusions, the inactivation rates were greater than when either of the by-product infusions was added separately. In conclusion, a

synergistic antimicrobial effect against *S.* Typhimurium appeared to take place when HHP treatment was combined with cauliflower or mandarin by-product infusion. These infusions could be considered as an additional microbial control measure to guarantee the food safety and food quality of pasteurized food products that are stored under refrigeration.

5.4.1 INTRODUCTION

Salmonella spp. is a foodborne pathogen, which cause approximately 93.8 million of foodborne disease outbreaks worldwide and 155000 deaths per year (Majowicz *et al*., 2010). In 2010, 99020 cases of salmonellosis were reported in the EU and *Salmonella* spp. were mainly detected in chicken and turkey (EFSA, 2011). In the USA, more than 40000 cases of salmonellosis are detected every year and products of animal origin are the main source of *Salmonella* spp. (Finstad, O'Bryan, Marcy, Crandall, & Ricke, 2012). Although more than 2500 serotypes of *Salmonella enterica* have been identified, *Salmonella enterica* serovars Typhimurium and Enteritidis are the most common causes of human salmonellosis worldwide (Kramarenko, Nurmoja, Karssin, & Meremae, 2014).

Therefore, the food industry needs to guarantee food safety in relation to *S. enterica*. Many products potentially contaminated with *S. enterica* are now processed by using new non-thermal technologies, such as oscillatory magnetic fields, radiation, ultrasounds, pulsed electric fields (PEF) and high hydrostatic pressure (HHP), and studies are needed to identify the different control measures alone or combined to fight against *S. enterica*. Among them, HHP technology has shown that it can achieve suitable levels of microbial inactivation, preserving sensory and nutritive properties (Polydera, Stoforos, & Taoukis, 2003).

Moreover, several research studies have shown that the antimicrobial effect of HHP treatment is greater when it is combined with various natural antimicrobials, achieving a synergistic effect between them (Oliveira, Ramos, Eamos, Piccoli, & Cristianini, 2015; Montiel, Martín-Cabrejas, & Media, 2015; Feyaerts, Rogiers, Corthouts, & Michiels, 2015). This synergistic effect allows the use of lower intensities in HHP treatments and lower concentrations of natural antimicrobials, achieving the same microbial reduction with less impact on sensory and nutritional properties (Pina-Pérez, Rodrigo, & Martínez, 2015).

Several studies have shown that some vegetable by-products are good sources of bioactive compounds with health benefits such as antioxidant, anti-inflammatory and antimicrobial properties (Balasundram, Sundram, & Samman, 2006; Peschel, Sanchez, Diekmann, Plescher, Gartzia, & Jimenez, 2006). Specifically, by-products of *Citrus* species (mandarin, orange, lemon) have phenolic compounds and essential oils with antimicrobial properties (Khan, Abert-Vian, Fabiano-Tixier, Dangles, & Chemat, 2010; Ramful, Bahorun, Bourdon, Tarnus, &Aruoma, 2010; He, Shan, Wu, Liu, Chen,&Yao, 2011; Dembitsky*et al*., 2012). Similarly, some by-products from *Brassicaceae* species have also shown an antimicrobial effect against bacterial pathogens owing to compounds such as glucosinolates, flavonoids and polyphenols (Ahmet *et al*., 2008; Stojceska *et al*., 2008; Volden, Bengtsson, & Wicklund, 2009; Köksal *et al*., 2007; Brandi, Amagliani, Schiavano, De Santi, & Sisti, 2006). The presence of bioactive compounds with antimicrobial properties in by-products from *Citrus* and *Brassicaceae* species opens the door for their revalorization as natural antimicrobials in the biocontrol of foodborne pathogens.

The food industry generates high amounts of waste worldwide. In the European Union, one million tons of vegetable residues from the food industry are produced every year (Stojceska, Ainsworth, Plunkett, Ibanoglu, & Ibanoglu, 2008). Generally, vegetable waste consists of peel, seeds and leaves and other

inedible fractions, which are used to feed animals or disposed of by landfill or incineration (Marín, Soler-Rivas, Benavente-García, Castillo, & Peréz-Alvarez, 2007; Peréz-Jiménez & Viuda-Martos, 2015).

This residual waste is an economic and environmental problem for the agri-food industry. Therefore, many research studies are seeking new strategies for revalorization of this waste, focusing on ways of providing it with added value by obtaining bioactive products for use in animal feeding, in biocontrol or as fertilizers (Llorach, Espín, Tomás-Barberán, & Ferreres, 2003;Marín *et al*., 2007). In this way, revalorization of residual waste or by-products as bioactive compounds could produce economic benefits for the agri-food industry (Wijngaard, Roble, & Brunton, 2009).

For all these reasons, the aim of this study is to evaluate the antimicrobial effect of infusions of mandarin (*Citrus reticulata*) and cauliflower (*Brassica oleracea* L. var.*botrytis*) by-products, alone or combined with HHP treatment, against *S.* Typhimurium stored at different temperatures.

5.4.2 MATERIALS AND METHODS

5.4.2.1 Microorganism

Glycerinated cryovials of *S.* Typhimurium (CECT 443) were obtained from freeze-dried cultures provided by the Spanish Type Culture Collection, using the method described by Sanz-Puig *et al*., 2015.

5.4.2.2 Preparation of cauliflower and mandarin by-product infusions

Cauliflower and mandarin by-products were provided from agro-industrial primary production of TRASA S.L. and INDULLEIDA S.A., respectively. Both by-products were processed according to Brandi, Amagliani, Schiavano,

De Santi and Sisti (2006). In brief, they were washed in sterile water, dried, triturated and homogenized with a laboratory grinder (Janke & Kunkel,IKA-Labortechnik) to obtain a powder with a particle size of 40 μ_m.

A 10% (w/v) infusion of cauliflower or mandarin by-product was obtained byboiling the powder in 0.1% (w/v) buffered peptone water (Scharlab, S.A., Barcelona, Spain) for 30 min. Then the infusions were centrifuged at 4 °C, at 2450g for 15 min for the cauliflower by-product infusion and at 1378g for 5 min in the case of the mandarin by-product infusion. Finally, both infusions were filtered through filters (Whatman) with a pore size of 11 and 2.5 µm and then sterilized by filtering through a PVDF syringe filter with a pore size of 0.45 μ_m.

5.4.2.3 Antimicrobial effect of by-product infusions

Both by-product infusions were inoculated with 10^8 cfu/mL of *S.* Typhimurium and incubated at 10 and 37 °C until its inactivation. The microbial inactivation curves were obtained by removal of aliquots at regular time intervals and plate count in Tryptic Soy Agar (TSA, Scharlab Chemie, Barcelona, Spain) after serial dilution with 0.1% (w/v) buffered peptone water. The plates were incubated at 37 °C for 24 hours. All analysis was done in triplicate.

5.4.2.4 Selection of High Hydrostatic Pressure treatment

Firstly, the inactivation of *S.* Typhimurium (10^8 cfu/mL) by HHP treatment was evaluated at several levels of pressure and time (Table 1). Initial load and surviving microorganisms after each HHP treatment were obtained by plate count. From these treatments, 200 MPa – 2 min was chosen because it did not cause death and produced only one log cycle of cellular damage in the initial *S.* Typhimurium population.

Table 5.4.1. HHP treatments tested against *S.* Typhimurium.

Pressure (MPa)	Time (min)	Pressure (MPa)	Time (min)
500	5	350	5
450	5	200	2 and 5
400	5	100	2 and 5

5.4.2.5 Combined antimicrobial effect of HHP treatment and cauliflower or mandarin by-product infusion against *S.* Typhimurium

To evaluate the antimicrobial effect of cauliflower and mandarin by-product infusions combined with HHP treatment, samples of both infusions were inoculated with *S.* Typhimurium (10^8 cfu/mL) and then they were treated by HHP (200 MPa – 2 min) and incubated at 10 and 37 °C. Control samples were stored at the same temperatures in 0.1% buffered peptone water. In all cases, the inactivation curves of *S.* Typhimurium were obtained by removal of aliquots at regular time intervals during the incubation period and plate count.

5.4.2.6 Cellular damage evaluation

S. Typhimurium cell damage was evaluated for all combinations of HHP treatment – by-product infusion addition. For this purpose, microbial plate counts were carried out in TSA (general culture) and TSA with 3% of NaCl (Wuytack *et al.*, 2003;Arroyo, Somolinos, Cebrian, Condon, & Pagan, 2010) as selective medium.

Cell damage was obtained according to the following equation:

$$Damaged\ cells = log\left(\frac{CFU/mL\ nonselective}{CFU/mL\ selective}\right) \quad (1)$$

where CFU/mL selective is the count in selective medium (TSA with 3% NaCl); and CFU/mL nonselective is the count in non-selective medium (TSA).

5.4.2.7 Mathematical modelling

The kinetic inactivation curves of *S.* Typhimurium were adjusted by using the Weibull model (Peleg & Cole, 1998, Fernandez, Salmerón, Fernandez, & Martinez, 1999):

$$\log_{10}\left(S(t)\right) = -b \times t^{n}$$

where *t* is the time (hours), S is the survival fraction, i.e., the quotient between the cell concentration at time t (Nt) (cfu/mL) and the initial cell concentration (N_0) (cfu/mL), *b* is the scale factor and *n* is the form factor.

5.4.2.8 Statistical analysis

Average and standard deviation calculations for the three repetitions and an ANOVA analysis to test significant differences between samples were carried out. The goodness of fit of the model was assessed by using the adjusted regression coefficient (adjusted-R^2) (López *et al*., 2004). The statistical analysis was performed with STATGRAPHICS Centurion XV (version 15.1.03; STATGRAPHICS, Warrenton, VA).

5.4.3 RESULTS AND DISCUSSION

5.4.3.1 Antimicrobial effect of cauliflower and mandarin by-product infusions, HHP treatment and the combination of them against *S.* Typhimurium

Figures 1 and 2 show the inactivation curves for *S.* Typhimurium during its incubation at 37 °C (a) and 10 °C (b) with or without cauliflower or mandarin (10% (w/v)) by-product infusion, respectively, and with or without HHP treatment (200 MPa – 2 min).

As can be seen, *S.* Typhimurium grew at both temperatures, 37 and 10 °C, in the control samples (buffered peptone water). When the microorganism was treated by HHP (200 MPa – 2 min), almost no inactivation effect was achieved and cells finally grew as in the control sample. However, when the microorganism was incubated with cauliflower by-product infusion, 5 log cycle reductions were achieved in the microbial load after 10 hours of storage at 37°C, and after approximately 110 hours of storage at 10 °C. Similarly, when *S.* Typhimurium was incubated with mandarin by-product infusion, 5 log cycle reductions were obtained in 80 and 240 hours during incubation at 37 and 10 °C, respectively. Therefore, although both cauliflower and mandarin by-product infusions exert a strong antimicrobial effect against *S.* Typhimurium, cauliflower by-product infusion appears to be more effective against the microorganism, achieving 5 log cycles of microbial reduction in a shorter period of time. Both agroindustrial by-products exert a relevant antimicrobial effect against *S.* Typhimurium, which could be explained due to their polyphenolic profile. On the one hand, mandarin and other *Citrus* by-products are rich in polyphenols as eriodyctiol, naringenin or hesperetin, which have an intense antimicrobial effect against gram-negative bacteria as *S.* Typhimurium (Mandalari *et al*., 2007; Sanz-Puig *et al*., 2016). On the other hand, cauliflower and other *Brassicaceae* by-products have been characterized and possess several

flavonols, anthocyanins and phenolics with antimicrobial activity against *S.*Typhimurium (Olsen *et al*., 2010, Sanz-Puig *et al*., 2015).

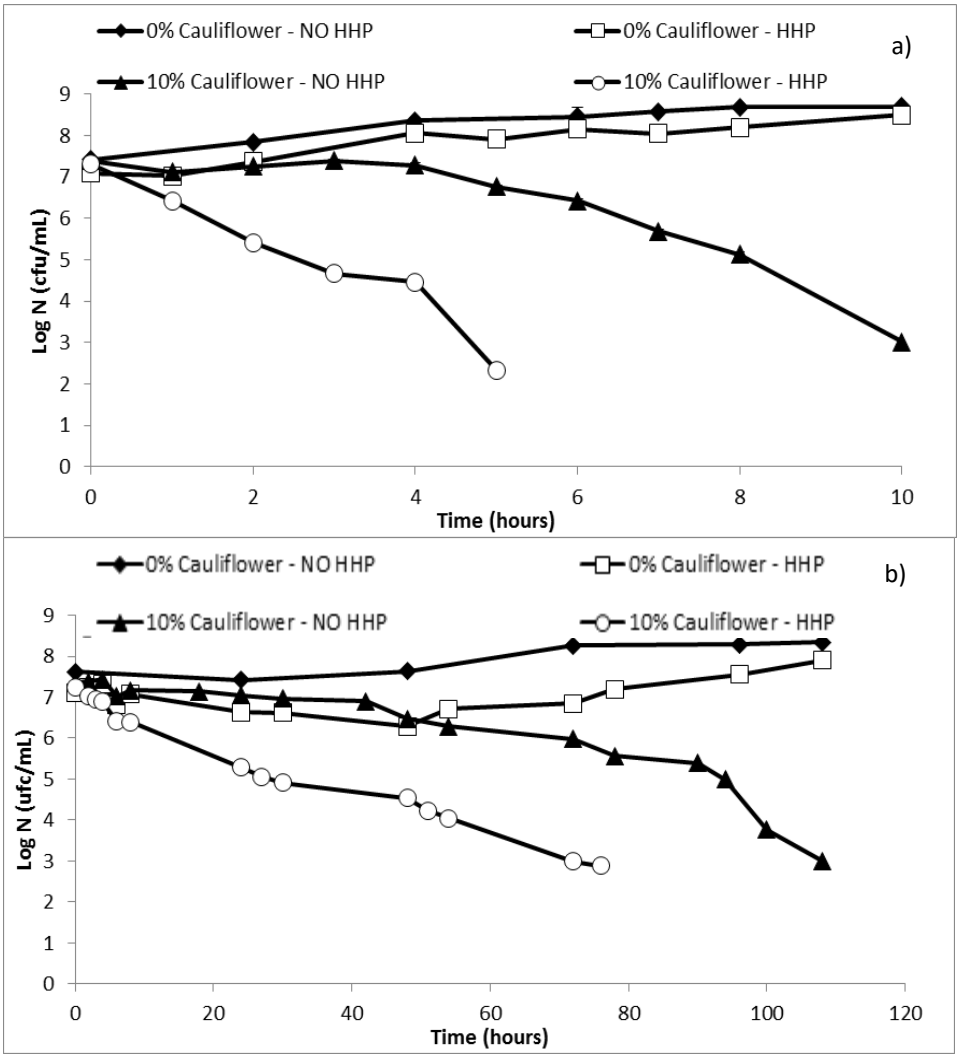

Figure 5.4.1: Inactivation levels of *S.* Typhimurium exposed to 10% cauliflower infusion, HHP treatment (200 MPa – 2 min) and a combination of both treatments during incubation at 37 °C (a) and 10 °C (b).

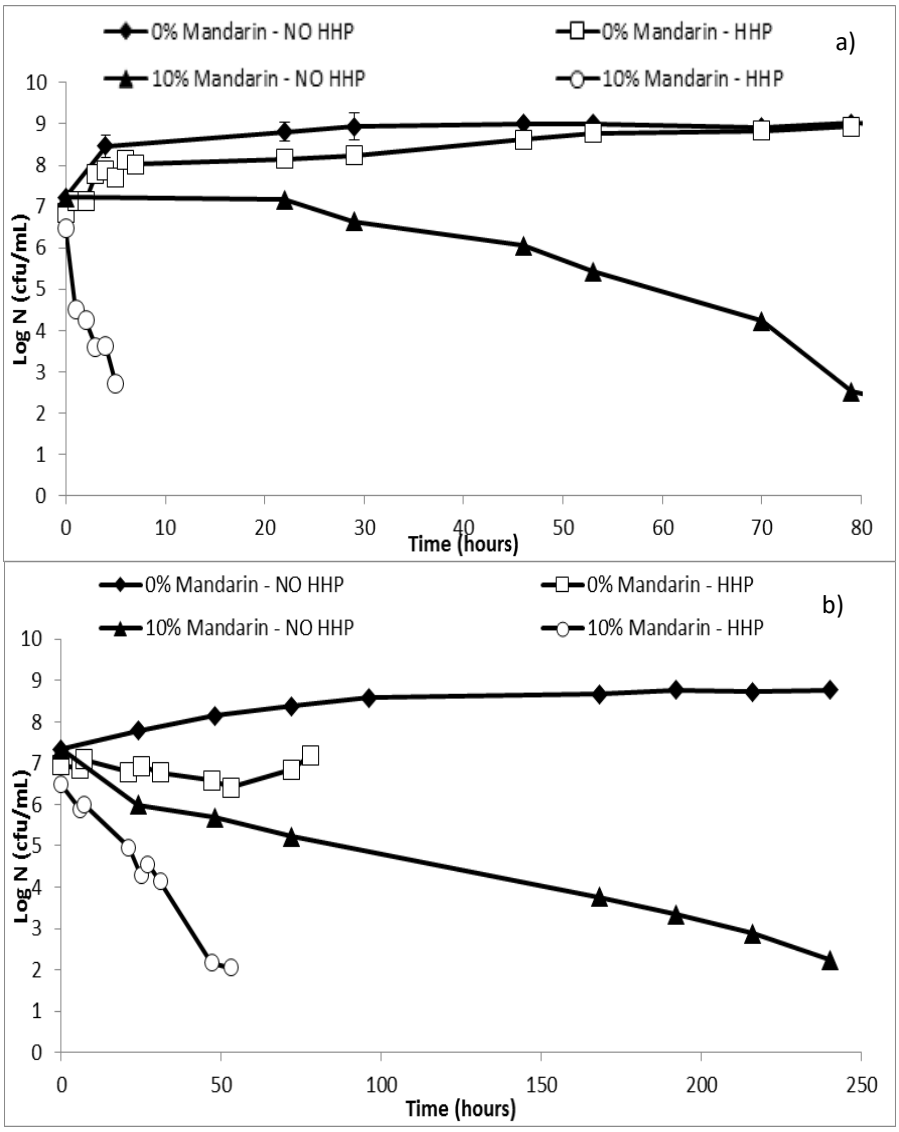

Figure 5.4.2: Inactivation levels of *S.* Typhimurium exposed to 10% mandarin infusion, HHP treatment (200 MPa – 2 min) and a combination of both treatments during incubation at 37 °C (a) and 10 °C (b).

When the HHP treatment was applied in combination with 10% of cauliflower or mandarin by-product infusion, the antimicrobial effect against *S.* Typhimurium was greater, achieving a reduction of 5 log cycles after 5 and 80 hours of incubation at 37 and 10 °C, respectively, in cauliflower by-product infusion, and after 5 and 54 hours of storage at 37 and 10 °C, respectively, for mandarin by-product infusion. These results appear to indicate that there are synergistic effects between HHP treatment and the addition of cauliflower and mandarin by-product infusions, because the combination of them reduces the time required to achieve 5 log cycles of inactivation at different incubation temperatures. These results are in agreement with other research studies in which HHP treatments were combined with other natural antimicrobials (Montiel *et al*., 2015; Oliveira *et al*., 2015). Obviously, at low temperature (10 °C) the microbial inactivation was slower than at optimal temperature (37 °C), probably because of a reduction in its metabolic activity, leading to an increase in the time for cell recovery, as previously indicated by McDonald and Sun (1999) and Swinnen, Bernaerts,Dens, Geeraerd and Van Impe (2004).

5.4.3.2 Study of cellular damage in *S.* Typhimurium population exposed to cauliflower or mandarin by-product infusion combined/not combined with HHP pre-treatment

As can be seen in Figure 3, the control sample grew (a and c), and the HHP treatment (b and d) generated a small percentage of damaged cells while the intact cell population was maintained throughout the incubation. In contrast, when *S.* Typhimurium was exposed to 10% cauliflower by-product infusion (Figure 4), both at 10 and 37°C (a and c), the number of intact cells decreased, the number of dead cells increased and there was a subpopulation of damaged cells that were not able to repair the injury and were dead after 100 hours (4 days) at 10 °C and 10 hours at 37 °C. The combination of HHP treatment and exposure to cauliflower by-product infusion during storage of

treated samples at 10 and 37°C (b and d) resulted in a number of damaged *S.* Typhimurium cells that progressively died during the storage period, achieving complete inactivation in shorter periods of time: 76 and 6 hours at 10 and 37 °C, respectively.

Incubation of *S.* Typhimurium in 10% mandarin by-product infusion alone (Figure 5) exerted an antimicrobial effect against the microorganism, causing a decrease in intact cells, an increase in dead cells and a slowly decreasing concentration of damaged cells, achieving complete microbial inactivation at 240 hours (10 days) and 94 hours (4 days) at 10 and 37 °C (a and c, respectively). When HHP treatment was applied in combination with mandarin by-product infusion the intact cells decreased, the dead cells increased and the damaged cells died during the storage period, achieving total inactivation in a shorter period of time than the result obtained with the infusion alone (54 hours at 10 °C and 6 hours at 37 °C) (b and d).

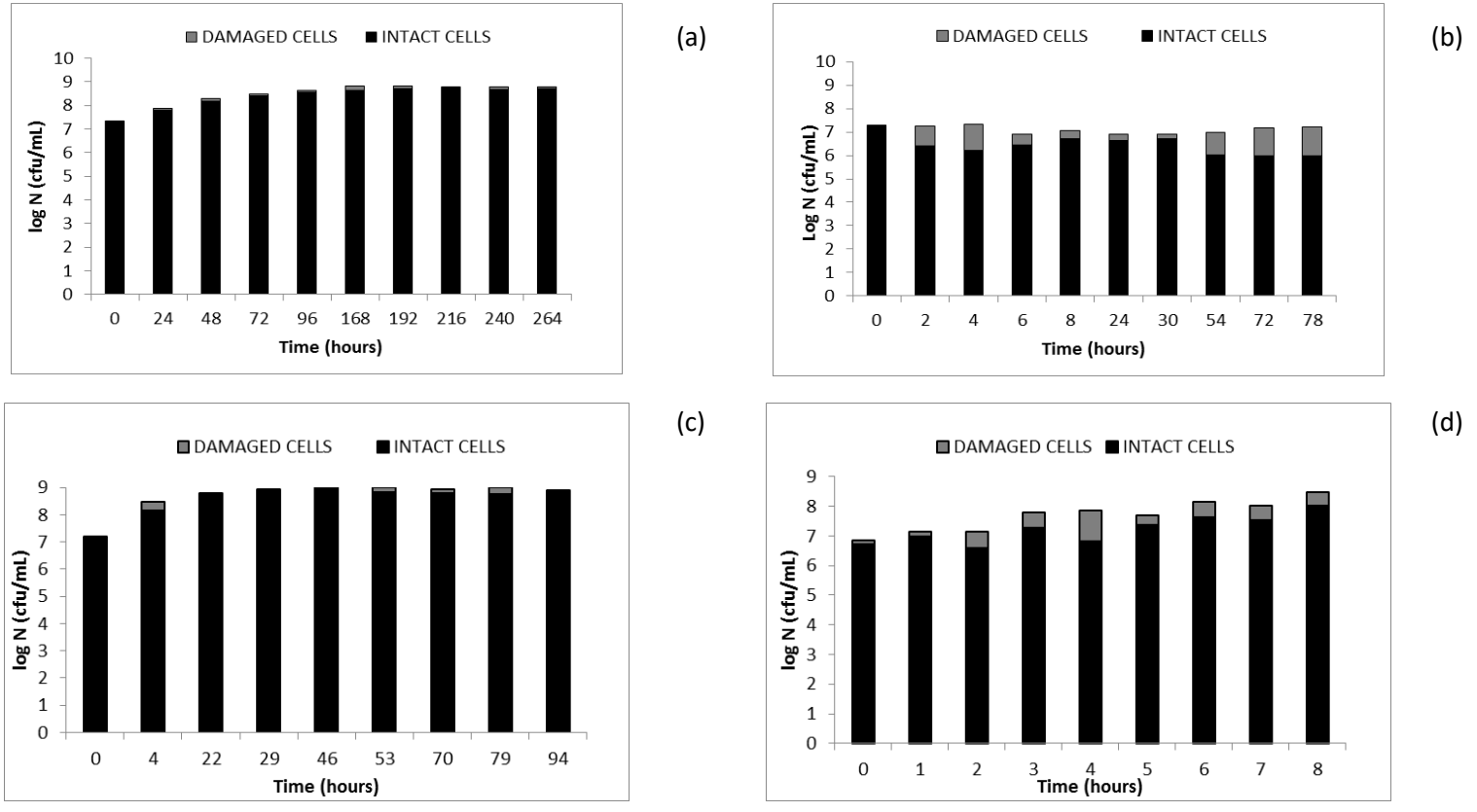

Figure 5.4.3: *S.* Typhimurium population analysis in control sample (buffered peptone water) (a) and samples treated by HHP (b) at 10 °C and *S.* Typhimurium population analysis in control sample (buffered peptone water) (c) and samples treated by HHP (d) at 37 °C.

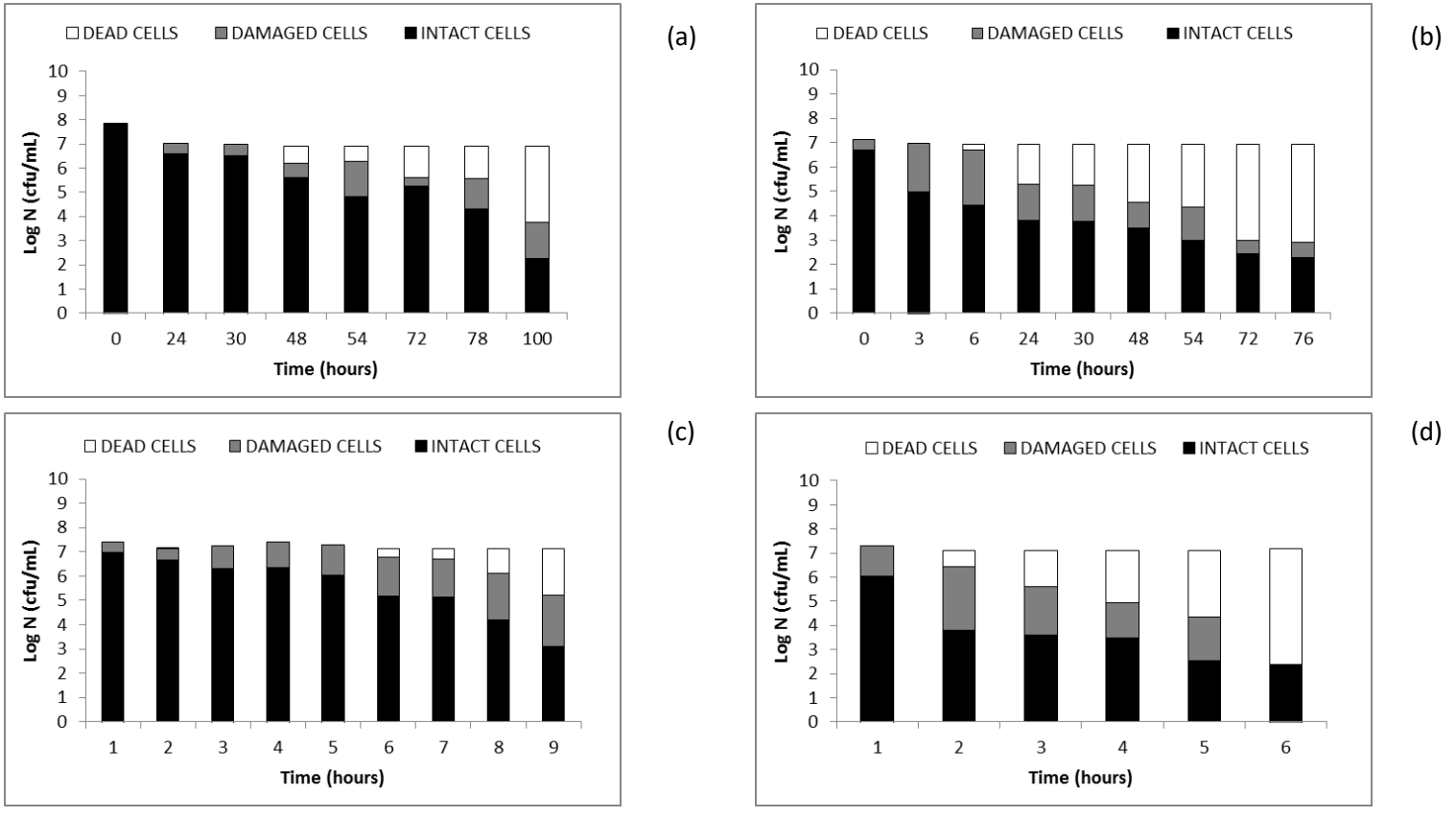

Figure 5.4.4: *S.* Typhimurium population analysis with exposure to 10% cauliflower by-product infusion at 10 °C (a), with a combination of both treatments at 10 °C (b), with cauliflower by-product infusion at 37 °C (c) and with a combination of both treatments at 37 °C (d).

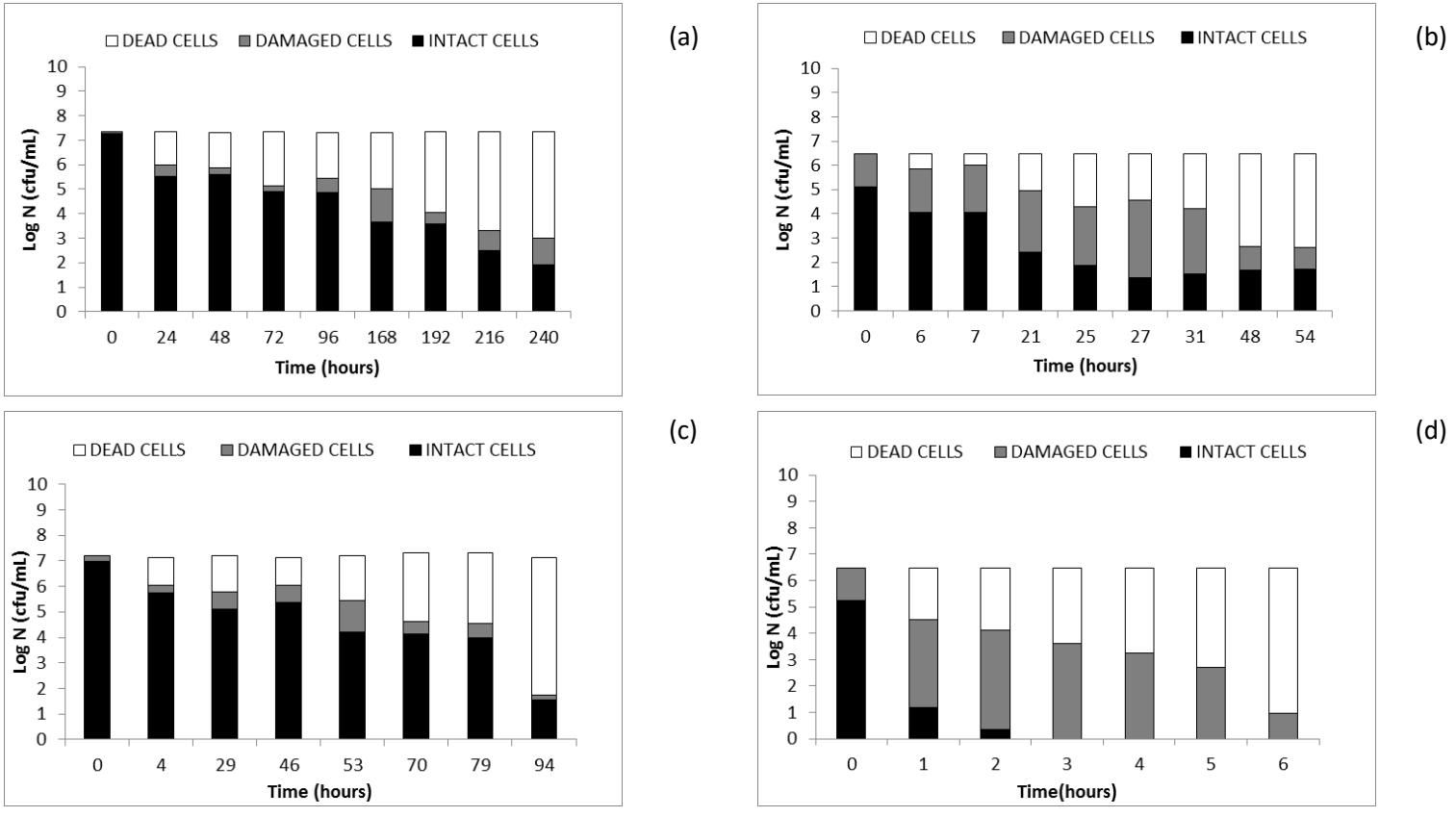

Figure 5.4.5: *S.* Typhimurium population analysis with exposure to 10% mandarin by-product infusion at 10 °C (a), with a combination of both treatments at 10 °C (b), with mandarin by-product infusion at 37 °C (c) and with a combination of both treatments at 37 °C (d).

The synergistic antimicrobial potential between by-product infusions and HHP could be mainly due to the existence of sublethally damaged cells after HHP treatment (Prieto-Calvo, Prieto, López, & Alvarez-Ordoñez, 2014) which, in the presence of antimicrobial natural compounds in by-product infusions, cannot recover and finally die (Somolinos, García, Pagán, & Mackey, 2008; Espina, García-Gonzalo, Laglaoui, Mackey, & Pagán, 2013). Thus the damaged cells – which in normal conditions would be a risk population because, if they recovered, they might acquire different characteristics from those of the initial population (greater virulence or resistance to various antimicrobials or antibiotics) – are eliminated by the combination of treatments (HHP + infusion). Therefore, sublethal HHP treatments could be used in combination with these antimicrobial by-product infusions to reduce the food safety risk.

5.4.3.3 Mathematical modelling of *S.* Typhimurium inactivation

The *S.* Typhimurium inactivation results obtained with different combinations of HHP treatment and cauliflower and mandarin by-product infusions, incubated at 10 and 37 °C, were fitted to the Weibull model to obtain their kinetic values (*b* and *n*), which are shown in Table 2 with its standard deviations.

The values of b (scale factor, which is directly related to the inactivation rate) were significantly (p-value<0.05) higher when the samples of *S.* Typhimurium were exposed to HHP treatment combined with incubation with 10% of by-product infusion than when they were exposed to cauliflower or mandarin by-product infusion alone. In fact, both at 37 °C and 10 °C, the inactivation rate was doubled when the two treatments were applied together. Therefore, it can be concluded that the HHP pre-treatment improves significantly the antimicrobial effect of the by-product infusions and the

combination of the two treatments exerts a synergistic effect, which increases the microorganism inactivation rate.

Table 5.4.2. Weibull kinetic parameters (*b* and *n*) of *S.* Typhimurium inactivation with/without cauliflower or mandarin by-product infusion, with or without HHP treatment, and the combination of them. R2 and MSE values are indicators of goodness of fit.

Infusion	Temperature	HHP	*b*	*n*	R^2adjusted	MSE
Cauliflower	37 °C	--	0.38±0.03	1.27±0.16	0.986	0.170
		200 MPa 2 min	0.55±0.04	1.49±0.04	0.965	0.340
	10 °C	--	0.09±0.01	0.65±0.05	0.969	0.013
		200 MPa 2 min	0.26±0.01	0.64±0.006	0.966	0.198
Mandarin	37 °C	--	0.77±0.39	0.15±0.07	0.986	0.170
		200 MPa 2 min	1.38±0.08	0.65±0.18	0.965	0.340
	10 °C	--	0.13±0.03	0.65±0.04	0.986	0.041
		200 MPa 2 min	0.23±0.05	0.70±0.06	0.992	0.104

5.4.4 CONCLUSIONS

Cauliflower and mandarin by-product infusions have shown a strong antimicrobial effect against *S.* Typhimurium. The antimicrobial effect of these infusions could be due to their polyphenolic profile, as indicated by Sanz-Puig, Pina-Pérez, Martínez-López and Rodrigo (2016). This effect improves significantly when bacterial exposure to these infusions is combined with a sublethal HHP treatment (200 MPa – 2min), achieving reductions of 5 log cycles in short periods of time (5–6 hours).

Therefore, the inclusion of cauliflower and mandarin by-product infusions in vegetable products pasteurized by HHP technology could be used as an additional control measure to guarantee the food safety and food quality of food stored at refrigeration temperatures, preventing negative consequences of possible cold chain break situations.

5.4.5 AKNOWLEDGEMENTS

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CAPÍTULO 5.5 EVALUACIÓN DE LOS CAMBIOS QUE SE PRODUCEN EN *S.* Typhimurium COMO CONSECUENCIA DE LA APLICACIÓN DE TRATAMIENTOS SUBLETALES: POSIBLES CAMBIOS DE RESISTENCIA Y DE VIRULENCIA

CAPÍTULO 5.5.1

Sanz-Puig, M., Arana-Lozano, A., Pina-Pérez, M.C., Martinez, A., Rodrigo, D.

Study of *Salmonella enterica* serovar Typhimurium resistance to natural antimicrobial substances and changes in its virulence, using *Caenorhabditis elegans* as a model organism

Foodborne Pathogens and Disease. (2017 - Submitted).

Abstract

The emergence of microbial resistance is a serious food safety problem. Therefore, the aim of this study was to evaluate the resistance of *Salmonella enterica* serovar Typhimurium against an antimicrobial extract from cauliflower by-product applied repeatedly and to investigate its changes in virulence, using *Caenorhabditis elegans* as a model organism. To evaluate the microbial resistance, *S.* Typhimurium was exposed repeatedly to the antimicrobial extract, and the microbial population before and after each treatment was evaluated by plate count. The results obtained show that *S.* Typhimurium developed microbial resistance against cauliflower by-product extract in the course of three consecutive exposures. The virulence changes study indicated that the lifespan of *C. elegans* populations fed with *S.* Typhimurium treated once and three times was longer than that of the control or of nematodes fed

with untreated *Salmonella*. The egg-laying rate was higher and they had higher mobility than *C. elegans* fed with untreated *S.* Typhimurium. However, there were no significant differences between *C. elegans* fed with *S*. Typhimurium treated once and three times. Therefore, *S.* Typhimurium treated with this antimicrobial extract becomes resistant and appears to be less virulent than untreated *S.* Typhimurium.

5.5.1.1 INTRODUCTION

Salmonella spp. is the main cause of foodborne diseases in many countries. EFSA reports indicate that there are around 100,000 outbreaks per year in the European Union (EU) (EFSA, 2014). Human infection is caused by consumption of contaminated foods from infected animals or animal products (eggs, chicken, turkey and pork) or of cross-contaminated foods, such as fruits and vegetables (FDA, 2012). Generally, *Salmonella enterica* serovars Typhimurium and Enteritidis are the most frequent serotypes involved in salmonellosis outbreaks (20.2 and 39.5% of cases, respectively) (EFSA, 2015).

Consequently, with the aim of protecting consumers from foodborne pathogens, the EU adopted the "from farm to fork" approach in order to guarantee the highest level of food safety (European Commission, 2004). With this approach, risks are assessed throughout all stages of the food chain, and there is a focus on effective communication of risks. The result of its application is that the number of salmonellosis cases has decreased in recent years (a 21.7% reduction between 2013 and 2014) (EFSA, 2014). Despite the decrease in cases, EFSA and European Centre for Disease Prevention and Control (ECDC) reported in 2015 that *Salmonella* and *Campylobacter* genera have significant levels of antibiotic resistance in animals and humans (EFSA, 2015). To understand the situation, many studies have been carried out to evaluate the

effect of new antimicrobial strategies (non-thermal technologies and natural antimicrobials) on their possible adaptations, development of resistance, and changes in virulence (Kisluk *et al*., 2013).

Consumers nowadays are worried about chemical additives because they are related to allergies and other health problems, and they prefer natural and minimally processed products with natural additives from plants. Some of these natural compounds are essential oils or bioactive compounds with antimicrobial activity (polyphenols, flavonoids and glucosinolates) (O'Shea *et al*., 2012; Wilson *et al*., 2011; Hu *et al*., 2004). These bioactive compounds can be obtained from agro-industrial by-products (Fernández-López *et al*., 2005;Viuda-Martos *et al*., 2007), contributing to the EU requirement to recover, revalue and recycle food by-products in order to support sustainable development (EUROSTAT, 2010).

Natural by-products from *Brassicaceae* plants such as cauliflower (*Brassica oleracea* var. Botrytis) are rich in antimicrobial compounds such as glucosinolates, isothiocyanates, thiocyanates, indoles, and polyphenols, which have been shown to exert a lethal effect against several microorganisms, both Gram-positive (*Listeria monocytogenes*) and Gram-negative (*S.* Typhimurium) (Sanz-Puig *et al*, 2015a; Sanz-Puig *et al*., 2015b). However, these natural products can produce a similar response in microorganisms to antibiotics, and some cross-resistance may even arise (Kisluk *et al*., 2013). In view of the possible implication of natural products in generating resistance in treated microorganisms, the aim of this study was firstly to evaluate the development of *S.* Typhimurium resistance against an antimicrobial extract from cauliflower by-product after repeated exposure and then to evaluate the possible changes in its virulence, using *C. elegans* as a model organism.

5.5.1.2 MATERIALS AND METHODS

5.5.1.2.1 Cauliflower by-product extract

Cauliflower by-product (mainly, external leaves of cauliflower plants) was provided as dehydrated residues from primary production of TRASA S.L. and was washed in sterile water to eliminate contaminants, dried, and homogenized using a laboratory grinder (Janke & Kunkel Ika-Labortechnik) (Brandi *et al*., 2006).

Infusions at 5% (w/v) from dried cauliflower by-product were obtained as follows: Buffered peptone water (0.1% (w/v)) was boiled and then, the dry by-product was added and let infuse for 30 min (Sanz-Puig *et al*., 2016). The infusions were then centrifuged at 4000 rpm for 15 min at 4 °C and filtered three times, using filters with a pore size of 11 and 2.5 µm to eliminate smaller particles (Whatman), and 0.45 µm (PVDF syringe filter) to sterilize.

5.5.1.2.2 Bacterial strain

A pure culture of *S.* Typhimurium (CECT 443) was provided freeze-dried by the Spanish Type Culture Collection. It was rehydrated with 10 mL of tryptic soy broth (TSB) (ScharlabChemie), and after 20 min it was transferred to 500 mL of TSB and incubated at 37 °C, with continuous shaking (Selecta Unitronic) at 200 rpm for 14 h to obtain cells in a stationary growth stage. The cells were centrifuged (Beckman Avanti J-25) twice at 4000 rpm at 4 °C for 15 min and then resuspended in TSB. Cells were finally resuspended in 20 mL of TSB, then dispensed in 2 mL vials with glycerol at 20% to a final concentration of 10^8cfu/mL obtained by plate count, and finally frozen and stored at -80 °C.

5.5.1.2.3 Evaluation of microbial resistance

The initial population of *S.* Typhimurium (10^7 cfu/mL) was exposed to three consecutive antimicrobial treatments. Each treatment consisted in

exposure to cauliflower by-product infusion at 5% for 4 h at 37 °C in continuous shaking (200 rpm). Later, the sample was centrifuged to recover the microbial cells. Recovered cells were grown in TSB culture overnight to achieve stationary phase (10^9 cfu/mL), inoculated in cauliflower by-product infusion (10^7cfu/mL) and treated again using the same conditions as described above. Before and after each treatment, the antimicrobial effect against the *S.* Typhimurium population was evaluated by plate count in tryptic soy agar (TSA) (ScharlabChemie). The entire experiment was carried out in triplicate with three different infusion batches and stocks of the various *S.* Typhimurium populations obtained from each treatment step were stored at –80 °C. From the *S.* Typhimurium populations obtained, *S.* Typhimurium treated once and *S.* Typhimurium treated three times with cauliflower by-product infusion were chosen to evaluate the changes in their virulence against *C. elegans*, because they were the most different populations with regard to their resistance to the antimicrobial treatment.

5.5.1.2.4 *C. elegans* studies

C. elegans strain N2, obtained from the College of Biological Sciences, Minnesota University, USA, was used to evaluate the possible virulence changes in *S.* Typhimurium population as a consequence of repeated exposure to antimicrobial from cauliflower by-product infusion. *C. elegans* was maintained in plates with Nematode Growth Medium (NGM) agar and a bacterial lawn of *E. coli* OP50 (Stiernagle, 2006).

In order to evaluate the effect of different *S.* Typhimurium population on the lifespan of *C. elegans*, 5 repetitions of 50 synchronized young adult nematodes (250 total), distributed in 5 plates of 10 worms each, were transferred to NGM agar with a lawn of *S.* Typhimurium. This was repeated for each of the *S.* Typhimurium population studied (untreated *S.* Typhimurium, *S.*

Typhimurium treated once, and *S.* Typhimurium treated three times with cauliflower by-product infusion). The worms were maintained at 20 °C during their life cycle (approximately three weeks) and were examined with a binocular microscope (COMECTA S.A.) at 48 h intervals. Worms were considered dead when they did not move and did not respond to stimulation (contact with a platinum worm picker).

We also studied the effect of selected *S.* Typhimurium populations on the mobility and egg laying of *C. elegans*. For this purpose, 50 young adult nematodes, distributed in 5 plates with 10 worms each, were transferred to NGM plates with a lawn of untreated *S.* Typhimurium or *S.* Typhimurium treated with antimicrobial by-product infusion, and they were examined at 48 h intervals, focusing on the number of movements in a short period of time (10 seconds) to evaluate their mobility, and on the number of eggs to evaluate their egg laying.

All the experiments carried out had a negative control with *C. elegans* in NGM plates with an *E. coli* OP50 lawn.

5.5.1.2.5 Statistical analysis of data

Mean and standard deviation were calculated in all cases. Also, ANOVA and Kruskal-Wallis tests were performed to detect significant differences between microbial cell populations and in behavior of nematodes fed with different *S.* Typhimurium populations ($p\text{-value} \leq 0.05$).

In addition, the lifespan studies with *C. elegans* were analyzed with the Kaplan-Meier method, with which it is possible to obtain life tables subdivided into intervals, the survival function and the hazard function, and percentiles. All statistical analyses were performed using Statgraphics Centurion XII software (StatPoint Technologies, Inc., Warrenton, VA, USA).

5.5.1.3 RESULTS AND DISCUSSION

5.5.1.3.1 Evaluation of *S.* Typhimurium evolution to antimicrobial treatment

The microbial resistance developed by *S.* Typhimurium against 5% cauliflower by-product infusion applied consecutively (three times) was studied. Figure 1 shows the microbial inactivation (log cycles) of the initial cell population (10^5 cfu/mL) caused by each treatment. As can be seen, an inactivation of 1.04 log cycles of the *S.* Typhimurium population was achieved after the first treatment. However, when the recovered cells were exposed to the next treatment a growth of 0.282 log cycles was observed. When recovered cells from the second treatment were exposed again to the infusion, growth of 0.831 log cycles was seen in the *S.* Typhimurium population. These results appear to indicate that the *S.* Typhimurium population developed microbial resistance in the course of exposure to 5% cauliflower by-product infusion three times.

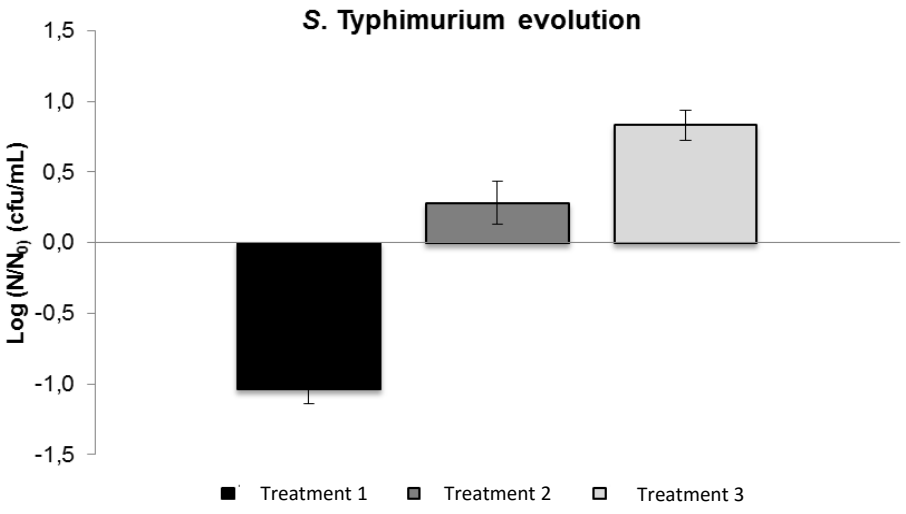

Figure 5.5.1.1. *S.* Typhimurium evolution by repeated antimicrobial treatments with 5% cauliflower by-product infusion.

The application of sublethal treatments that inactive only part of the initial microbial population help to create bacterial subpopulations that during the repair process can acquire or develop resistance to the antimicrobial used. This development of resistance could involve structural and functional changes in microbial cells, specifically in their cell membranes (McMahon *et al*., 2007). Moreover, the injured subpopulations can also modify their virulence behavior due to the environmental conditions causes genetically mutations that can modify the microbial virulence, as indicated by Rajkovic *et al*. (2009).

Many scientific studies have demonstrated the microbial resistance of *S.* Typhimurium to several antibiotics such as cephalosporins or fluoroquinolones (Kariuki *et al*., 2015; Crump *et al*., 2015), but there are not many studies about the resistance developed when this microorganism is treated with natural antimicrobials (essential oils or polyphenols). Several authors, such as Kisluk *et al*. (2013), Ultee *et al*. (2000), or Di Pasqua *et al*. (2006), have shown specific adaptation of *E. coli*, *S.* Senftenberg, *S.* Typhimurium, and *Bacillus cereus* to sublethal concentrations of essential oil components. This indicates that *Salmonella* and other enteric bacteria are able to develop various strategies to adapt to the stress conditions produced by some plant components, such as various antimicrobial compounds. Further research studies are needed to investigate the occurrence of adaptations and microbial resistance to repeated exposure to natural antimicrobials because they possess a potential risk that may affect food safety. This means that it is necessary to design optimal application conditions for sublethal treatments in order to avoid the development of adaptation and resistance (Boxstael *et al*., 2012; Louden *et al*., 2012; Torpdahl *et al*., 2013).

5.5.1.3.2 Determination of virulence changes in *S.* Typhimurium with *C. elegans*: lifespan studies

The lifespan of nematodes fed with *S.* Typhimurium treated once or three times (populations that showed the highest differences in microbial resistance) was compared with the lifespan of those fed with untreated *S.* Typhimurium. Figure 2 shows the percentage of surviving nematodes during their life cycle, when they were fed with untreated *S.* Typhimurium and *S.* Typhimurium treated once or three times with cauliflower by-product infusion. The survival rate during their life cycle was always higher (p-value < 0.05) for *C. elegans* fed with treated *S.* Typhimurium than for *C. elegans* fed with untreated *S.* Typhimurium. Specifically, the life span was up to 21 days for untreated *S.* Typhimurium, and 23 and 25 days for nematodes fed with *S.* Typhimurium treated once and three times, respectively. However, non-significant differences were found between nematode populations fed with *S.* Typhimurium treated once and three times (p-value > 0.05). When the results were compared with those obtained for *C. elegans* fed with *E. coli* OP50, the lifespan of nematodes fed with treated *S.* Typhimurium was higher than that of samples fed with *E. coli* OP50.

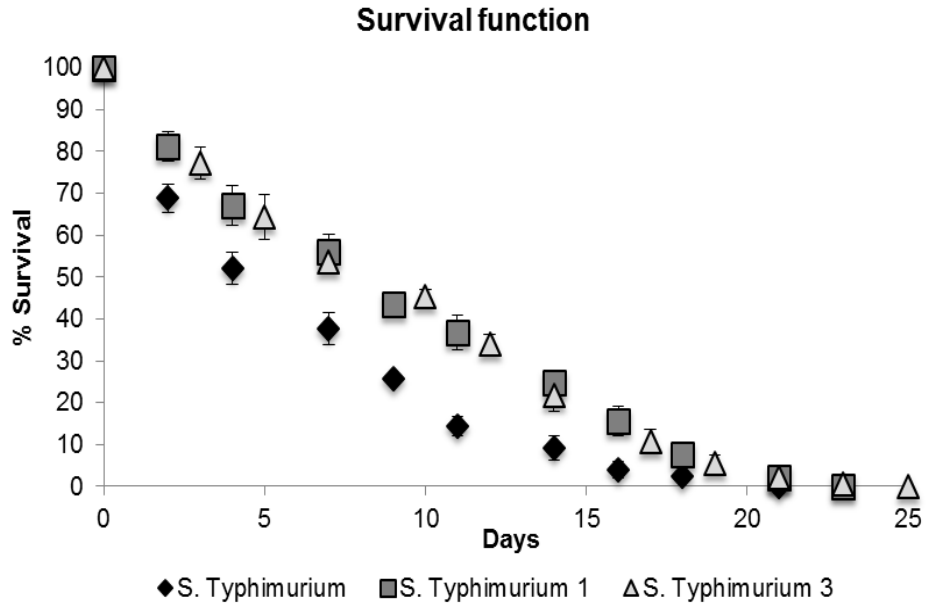

Figure 5.5.1.2. *C. elegans* survival function when fed with untreated *S.* Typhimurium and *S.* Typhimurium treated one and three times with cauliflower by-product infusion.

According to studies carried out by Sifri *et al*. (2005), at a constant temperature of 20 °C (optimal temperature for their growth) the lifespan cycle is up to 21 days. However, the lifespan of *C. elegans* is determined by various environmental factors, such as temperature and availability of bacteria on which to feed (Allen *et al*., 2015). The normal laboratory feeding conditions for *C. elegans* are with *E. coli* OP50, but when this bacterium was replaced with *S.* Typhimurium the lifespan decreased significantly (Labrousse *et al*., 2000; Aballay *et al*., 2000).

There are studies that show the virulence of *S.* Typhimurium against a nematode population. The worms have a significantly shorter life span when are infected by *S.* Typhimurium. Also, the motility of the worms and the rate of pharyngeal pumping gradually declined when are fed with *S.* Typhimurium SL1344 until the nematodes became immobile and died. The lumen of the worms became distended. Moreover, *S.* Typhimurium may affect the egg-laying

process (Aballay *et al*., 2000; Labrousse *et al*, 2000). However, when this bacterium is treated with cauliflower by-product infusion it appears to be less virulent against *C. elegans*.

The experimental data were subjected to a Kaplan-Meier analysis to obtain the survival function and the hazard function for different *C. elegans* samples fed with different *S.* Typhimurium populations. Table 1 shows percentiles of estimated survival distribution for each of the *C. elegans* populations. If we focus on the 5%, we can see that there are significant differences (p-value < 0.05) between *C. elegans* fed with treated *S.* Typhimurium (treated once: 19.3 days, and three times: 19.9 days) and untreated *S.* Typhimurium (16.5 days), the survival distribution being higher for nematodes fed with treated bacteria.

TABLE 5.5.1.1. Percentiles for *C. elegans* lifespan when fed with the different *S.* Typhimurium populations.

	***S.* Typhimurium**		***S*. Typhimurium 1**		***S*. Typhimurium 3**	
Percentile	**Time (days)**	**Standard Error**	**Time (days)**	**Standard Error**	**Time (days)**	**Standard Error**
75.0	2.4	0,204	3.9	0,502	3.3	0,562
50.0	5.3	0,607	8.5	1,019	8.2	1,542
25.0	10.2	1,095	13.9	0,866	13.4	0,874
10.0	13.8	3,084	18.4	3,993	17.3	7,401
5.0	16.5	5,789	19.9	4,086	19.3	6,681

Figure 3 shows the hazard function for each of the *C. elegans* populations studied. As we can see in the figure, the hazard rate is always higher in the control sample (fed with *S.* Typhimurium) than in samples fed with treated *S.* Typhimurium. Besides, between day 14th and 17th hazard rate for *C. elegans* fed by *S.* Typhimurium treated once and three times is constant or decreases while for *C.elegans* fed by untreated *S.*Typhimurium their hazard rate begins to increase on the 14th day. However, in optimal conditions, when nematodes are fed with *E. coli* OP50, their hazard rate also increases rapidly

around the 17th day, probably because by then the nematodes are already considered old.

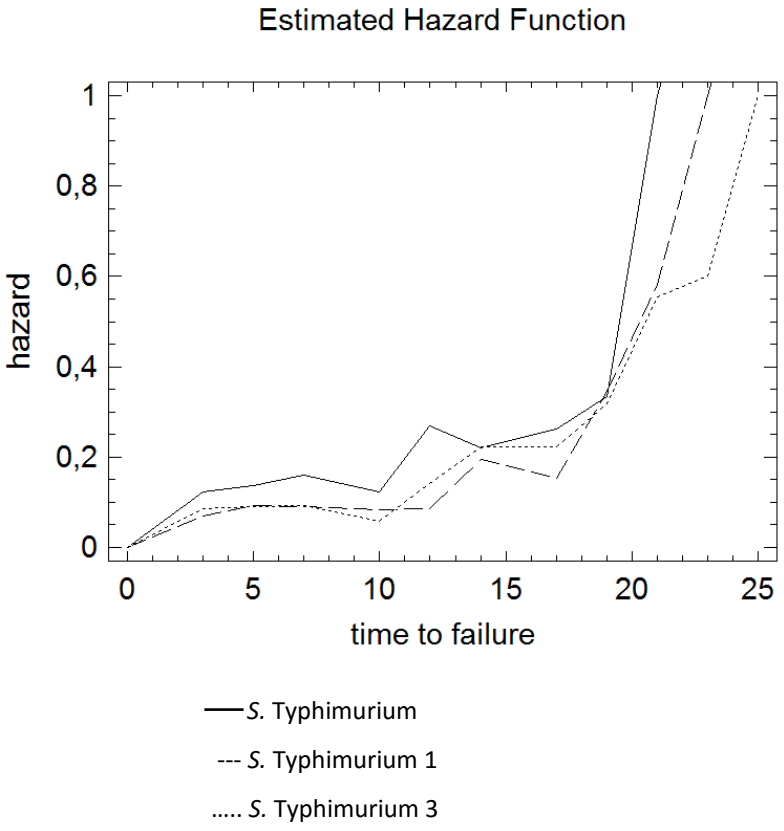

Figure 5.5.1.3. *C. elegans* hazard function when fed with untreated *S.* Typhimurium and *S.* Typhimurium treated one and three times with cauliflower by-product infusion.

5.5.1.3.3 Determination of virulence changes in *S.* Typhimurium with *C. elegans*: egg-laying studies

The next study on the effect of the three *S.* Typhimurium populations on *C. elegans* was related to the amount and frequency of egg laying during their lifespan. In optimal conditions, *C. elegans* lays about 300–350 eggs during its life cycle (Lavigne *et al*., 2006). However, when the nematodes were fed and infected with *S.* Typhimurium they only laid eggs until the 5th day. The same occurred when they were infected with *S.* Typhimurium treated once and three

times with the antimicrobial by-product infusion. Figure 4 shows a comparison of the number of eggs laid by *C. elegans* fed with the three different *S.* Typhimurium populations in the first two time intervals. In the first interval (0 – 2 days) we found significant differences (p-value < 0.05) between the three populations: nematodes fed with untreated *S.* Typhimurium laid fewer eggs than nematodes fed with *S.* Typhimurium treated once. Also, when the nematodes were fed with *S.* Typhimurium treated three times they laid a higher number of eggs than when they were fed with *S.* Typhimurium treated once.

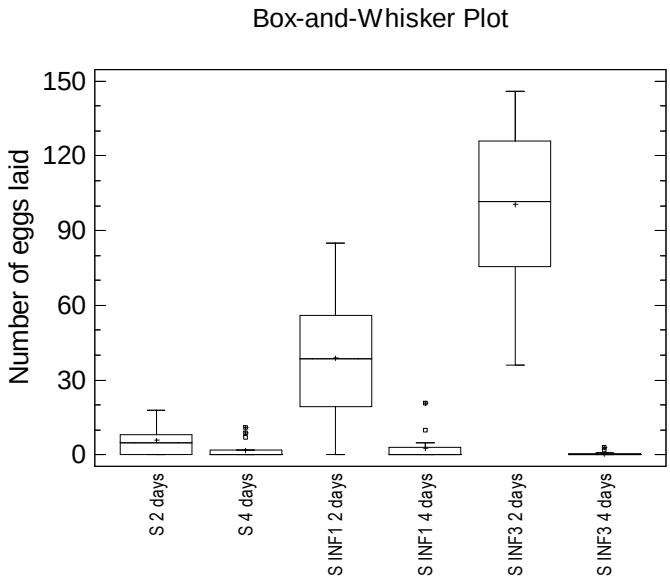

Figure 5.5.1.4. Eggs laid during two first time intervals by *C. elegans* fed with different *S.* Typhimurium populations.

These results are in agreement with other research studies that have shown that live cells of *S.* Typhimurium accumulate in the lumen of the intestine of *C. elegans* and it appears to be completely infected by the 5th day of bacterial infection, coinciding with the day when egg laying stopped in our study. Also, other studies have demonstrated that *S.* Typhimurium infection can affect *C. elegans* egg laying and the eggs hatch internally, which contribute

significantly to killing the worms in the first days of their lifespan (Labrousse *et al*., 2000; Aballay *et al*., 2000).

Furthermore, scientific studies (Gardner *et al*., 2013) have demonstrated that when *C. elegans* is exposed to harmful substances in the environment, such as pathogenic bacteria like *Enterococcus faecalis*, they retain eggs in their uterus to protect their progeny. This could explain why *C. elegans* infected with *S.* Typhimurium only lays eggs until the 5th day as also exposed by Gardner *et al*., 2013. Moreover, when the worms were infected with *S.* Typhimurium treated once and three times with cauliflower by-product infusion they laid more eggs than when they were infected with untreated *S.* Typhimurium.

5.5.1.3.4 Determination of virulence changes in *S.* Typhimurium with *C. elegans*: mobility studies

Finally, we studied the mobility of *C. elegans* fed with untreated *S.* Typhimurium and *S.* Typhimurium treated once and three times with cauliflower by-product infusion. As Figure 5 shows, mobility decreased in all the populations of *C. elegans* during their life cycle. However, during the first 5 days the worms infected with treated *S.* Typhimurium had significantly (p-value < 0.05) better mobility than the worms infected with untreated *S.* Typhimurium, probably corresponding to the progressive bacterial infection in the intestinal lumen of *C. elegans* (Labrousse *et al*., 2000). Therefore, treated *S.* Typhimurium was less virulent against *C. elegans* than untreated *S.* Typhimurium.

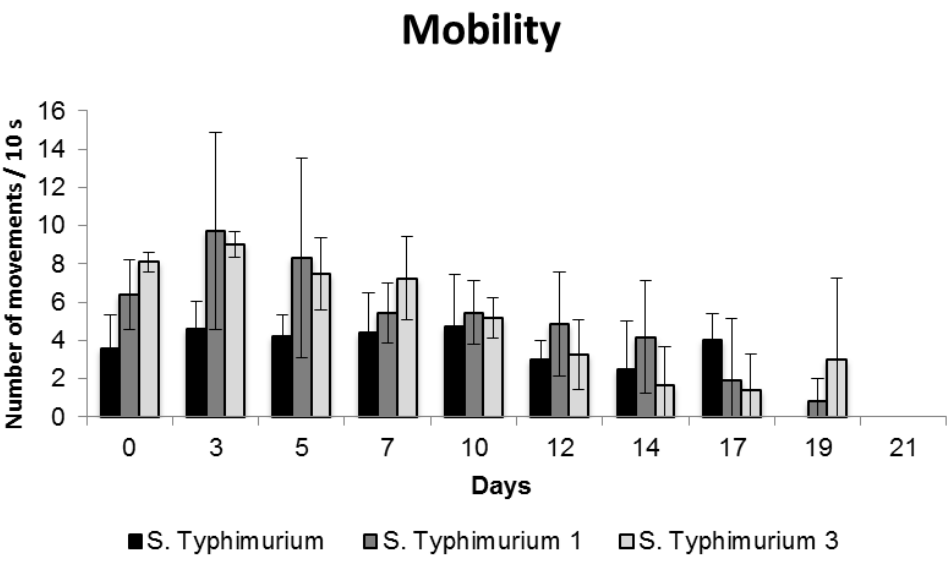

Figure 5.5.1.5. Mobility of *C. elegans* fed with different *S.* Typhimurium populations during their lifespan.

5.5.1.4 CONCLUSIONS

From the results presented in this research work it can be concluded that *S.* Typhimurium develops microbial resistance to natural antimicrobial extract after consecutive exposures. Also, treated *S.* Typhimurium populations show less virulence against *C. elegans* than untreated ones. Therefore, in this study, the microbial changes occurred in *S.* Typhimurium to become resistant against natural antimicrobial causes the reduction of virulence against a model organism. Nevertheless, more studies with different *Salmonella* strains and with different natural antimicrobials are necessary to find more information about the resistance and virulence changes in this microorganism.

5.5.1.5 ACKNOWLEDGMENTS

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CAPÍTULO 5.5.2

Sanz-Puig, M., Torres, C., Cunha, L.M., Martinez, A., Rodrigo, D.

Evaluation of *S.* Typhimurium resistance to Pulsed Electric Fields treatment and their virulence changes against *C. elegans*

Food Microbiology. (2017 - Submitted).

Abstract

The goal of this study was, firstly, to evaluate the development of *Salmonella enterica* serovar Typhimurium microbial resistance against PEF treatment and to study the possible virulence changes in *S.* Typhimurium using *C. elegans* as a model organism. For this purpose, *S.* Typhimurium underwent repeated treatments with PEF until it became resistant and, then, *C. elegans* was fed with three different *S.* Tyhimurium subpopulations untreated and treated by PEF once and four times. Their lifespan, mobility and eggs laying were analysed. The results shown that *S.* Typhimurium became resistant to PEF treatment after the fourth consecutive PEF treatments. Moreover, the results obtained with *C. elegans* as a host organism shown that there were not significant differences in lifespan between untreated *S.* Typhimurium and *S.* Typhimurium treated once, but there was significant differences between them and *S.* Typhimurium treated four times, showing in this last case a largest lifespan. Also, the *C. elegans* eggs laying pattern was modified when they were fed with the three *S.* Typhimurium subpopulations. All of them stop their eggs laying before the 5^{th} day but there was significant differences in the number of eggs laid in the first two days of their lifespan among the three subpopulations

studied. In conclusion, when *S.* Typhimurium was treated repeatedly by PEF develops microbial resistance against it but decrease its virulence against a host organism as *C. elegans*.

5.5.2.1 INTRODUCTION

Nowadays, consumers demand healthier food products, guaranteeing their food safety and quality. Therefore, food industries and research groups have been developing new technologies for food preservation which permit us to maintain the original organoleptic properties of food products and to increase their shelf life (Otunola *et al*., 2008).

Many non-thermal technologies have been developed like ionizing radiation, ultraviolet light, ozone, High Hydrostatic Pressure and Pulsed Electric Fields (PEF) (Lado *et al*., 2002; Devlieghere *et al*., 2004; Ramos *et al*., 2006). Among them, PEF treatment has been seen as a promising alternative cool treatment to the conventional thermal pasteurization for liquid products (Saldaña *et al*., 2014).

PEF technology consists on the application of short pulses (1-10 s) of high intensity of the electrical field (15-80 kV/cm) in pumpable food products that pass between two electrodes (Devlieghere *et al*., 2004; Mosqueda-Melgar *et al*., 2012). This technology produces the microbial inactivation breaking the cellular membranesof microorganisms, without significant reductions in colour, flavour and nutrients of food products (Mosqueda-Melgar *et al*., 2008). This technology can be included in those producing sublethal treatments.

Up to now, this technology has been tested mainly with fruit juices (Timmermans *et al*., 2014) like carrot (Xiang *et al*., 2014), blueberries (Lamanauskas *et al*., 2015), pomegranate (Guo *et al*., 2014), pineapple

(Dastgheib *et al*., 2014), orange (Buckow *et al*., 2013) or apple (Bi *et al*., 2013) and with other products like milk (Smith *et al*., 2002; Riener *et al*., 2009; Valizadeh *et al*., 2009; Bermudez-Aguirre *et al*., 2011; Sharma *et al*., 2014).

However, as occurs with other sublethal treatments (Kostyanev *et al*., 2015; Laxminarayan *et al*., 2013), microorganisms could develop a microbial resistance against PEF treatment when they are submitted to sublethal treatments consecutively. Resistant microbial population might suppose a risk for the consumers because it is a new population whose virulence is unknown (Capita *et al*., 2013).

Therefore, the aim of this research study was, firstly, to evaluate the microbial resistance against PEF treatment developed by *Salmonella enterica* serovar Typhimurium, which is one of the most relevant foodborne pathogens and is of concern to public health (Coburn *et al*., 2007), and, secondly, to study the possible virulence changes in *S.* Typhimurium using *C. elegans* as a model organism because it is simple and easy to use in the laboratory (Ewbank *et al*., 2011).

5.5.2.2 MATERIAL AND METHODS

5.5.2.2.1 Microbial strain

The freeze-dried *S.* Typhimurium was provided from the Spanish Type Culture Collection (CECT 443). The pure culture was rehydrated with tryptic soy broth (TSB) (Scharlab Chemie) and incubated in continuous shaking (Selecta Unitronic) for 14 h at 37 °C to obtain cells stock. Then, the cells were centrifuged (Beckman Avanti J-25) twice at 2450 g at 4 °C for 15 min and resuspended in TSB. At the end, the cells were resuspended in TSB with 20%

glycerol and dispensed in vials of 2 mL, to a final concentration of 10^8 cfu/mL. The cryovials were frozen and stored until we need at -80 °C.

5.5.2.2.2 PEF treatment against *S.* Typhimurium

First of all, based in previous studies (Sanz-Puig *et al*., 2016) in which *S.* Typhimurium initial population (10^8 cfu/mL) was treated with different PEF conditions (10 - 40 kV/cm and 40-1900 µs), we chose the treatment of 30 kV/cm - 300 µs because it was an intermediate sublethal treatment that only caused 2,5 log cycles of cellular reductions in *S.* Typhimurium.

5.5.2.2.3 Evaluation of microbial resistance development

To evaluate the development of *S.* Typhimurium microbial resistance against PEF treatment, an initial population of this microorganism (10^8 cfu/mL) was treated by PEF at 30 kV/cm for 300 µs repeatedly (4 times). Between PEF treatments, *S.* Typhimurium population was grown, incubating it in TSB overnight with continuous shaking and 37 °C. Later, the microbial cells were recovered by centrifugation (2450 g – 15 min). Before and after each PEF treatment, the concentration of *S.* Typhimurium was calculated by plate count in TSA (Scharlau, Scharlab).

All of *S.* Typhimurium populations obtained after the application of consecutive PEF treatments were frozen stored at -80 °C. Among them, we decided to use *S.* Typhimurium treated once and four times with PEF to study their virulence changes against *C. elegans*, because they were the *S.* Typhimurium populations that showed the greatest differences related to their resistance to PEF treatment.

5.5.2.2.4 *C. elegans* studies

C. elegans is a nematode that has been used as a model organism to evaluate the virulence of different microorgamisms. In this study it is used to investigate virulence changes in *S.* Typhimurium populations treated once and four times by PEF. *C. elegans* was provided by "College of Biological Sciences, Minnesota University", USA, and its optimal conditions to grow in the laboratory are plates of Nematode Growth Medium (NGM) Agar, with a bacterial lawn of *E. coli* OP50 at 20 °C (Stiernagle, 2006).

To evaluate the virulence changes of the chosen *S.* Typhimurium populations, the microbial lawn of *E. coli* OP50 was replaced by *S.* Typhimurium as a control, and *S.* Typhimurium treated once and four times by PEF monitoring the *C. elegans* behavior focusing on their lifespan, their mobility and their eggs laying.

5.5.2.2.5 Lifespan studies

Lifespan studies were carried out with 250 nematodes, distributed in 25 plates (5 repetitions of 5 plates) of 10 synchronized nematodes, which were fed during their life span with *S.* Typhimurium and *S.* Typhimurium treated once and four times by PEF. At regular intervals of 48 hours, all plates were examined with a binocular microscope (COMECTA S.A.), counting the number of live worms. We considered dead worms when they did not move and did not respond to stimulate.

5.5.2.2.6 Mobility

The mobility of 25 nematodes, distributed in five repetitions of five worms (25 plates, with one worm in each one), was examined along their life span every 48 hours, focusing in the number of waves that each worm

produced in 10 seconds. These studies were carried out with nematodes fed with the three selected subpopulations of *S.* Typhimurium.

5.5.2.2.7 Eggs laying

The eggs laying of 25 nematodes, distributed in five repetitions of five worms (25 plates, with one worm in each one), fed with a lawn of three selected *S.* Typhimurium subpopulations, were analyzed at regular intervals of 48 hours, counting the number of eggs that they laid.

5.5.2.2.8 Statistical analysis

Both the results obtained by plate count for the development of *S.* Typhimurium resistance against PEF treatment and the results obtained by *C. elegans* were analyzed calculating the average and standard deviation. In addition, ANOVA, Friedman and Kruskal-Wallis analysis were necessary to evaluate the significant differences (p-value < 0.05) between different populations of *S.* Typhimurium and *C. elegans* populations fed with different *S.* Typhimurium.

Besides, Kaplan-Meier analysis was carried out to obtain de survival and hazard function from lifespan studies with *C. elegans*, using Statgraphics Centurion XII software (StatPoint Technologies, Inc., Warrenton, VA, USA).

5.5.2.3 RESULTS AND DISCUSSION

5.5.2.3.1 Development of *S.* Typhimurium resistance against PEF treatment

An initial *S.* Typhimurium concentration (10^8 cfu/mL) was consecutively treated by PEF (30 kV/cm - 300 μs) until it becomes resistant (four times). The resistance was evaluated focusing on the number of survival microorganisms in each consecutive treatment. The results obtained are shown in Figure 1. The

first treatment produced 2,91 log cycles of inactivation; later, the second and the third treatment produced lower inactivation levels 1,23 and 0,57 log cycles, respectively, that can be attributed to an increase of its microbial resistance against PEF treatment. Finally, the fourth treatment caused 0,73 log cycles of inactivation, slightly greater than the third treatment but without significant differences (p-value < 0,05) between them. The results showed that a resistance to treatment could be occurred until the third consecutive treatments, stabilizing in the fourth treatment.

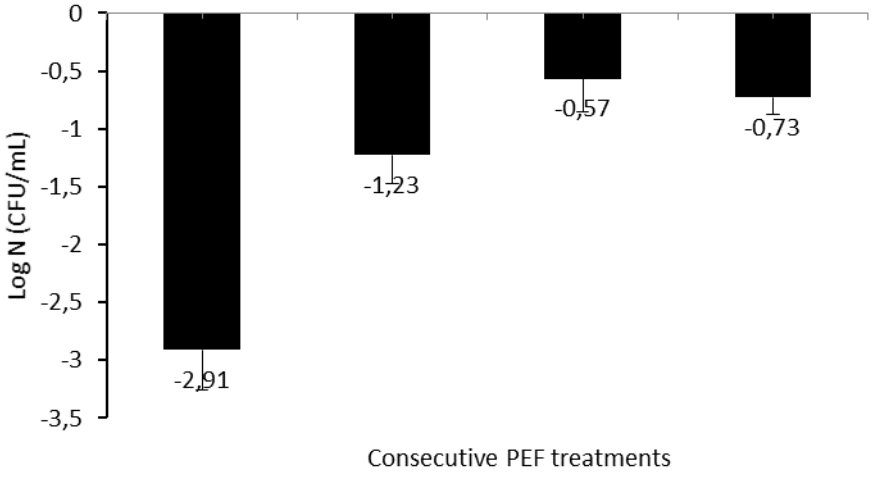

Figure 5.5.2.1: Inactivation of *S.* Typhimurium (log cycles) after the application of consecutive PEF treatments.

The development of *S.* Typhimurium microbial resistance against PEF treatment has not been studied before, there are only a few studies like Sagarzazu *et al*., (2013), who test it but with a lower intensity of PEF treatment. In addition, there are other studies on microbial resistance of *Enterobacter sakazakii* (Arroyo *et al*., 2010) or *Campylobacter jejuni* (Sagarzazu *et al*., 2010) in which were tested different conditions or PEF parameters combined with other compounds like citral, but they had not tested the effect of consecutive PEF treatments.

Microbial resistance against PEF treatment could be an important problem to food industry due to PEF treatment generates pores in microbial cell membrane to inactivate them. However, there are a subpopulation of microbial cells, which are not inactivated and remains damaged and, finally, recover their damage and grow, and could becoming mutant cells with unknown virulence (Zimmermann *et al*., 1974; Garcia *et al*., 2005b; Soliva-Fortuny *et al*., 2009; Puértolas *et al*., 2012).

5.5.2.3.2 Evaluation of changes in *S.* Typhimurium virulence using *C. elegans*

The evaluation whether the resistance development of *S.* Typhimurium against PEF treatment included microbial virulence changes was carried out using *C. elegans*. The nematode was fed with untreated *S.* Typhimurium and *S.* Typhimurium treated once and four times by PEF and its lifespan, mobility and eggs laying were analysed at regular time intervals of 48 hours.

5.5.2.3.3 Lifespan studies

The effects of virulence level of different *S.* Typhimurium populations against defence mechanisms of *C. elegans* was assessed by life span studies. Lifespan of nematodes in optimal conditions, 20 °C and fed with *E. coli* OP50, is around three weeks.

Table 5.5.2.1: Percentiles for *C. elegans* lifespan when fed with the different S. Typhimurium populations.

Microorganism	Percentil at 20% (days)
***S.* Typhimurium untreated**	10.13 ±1.12
***S.* Typhimurium treated once**	11.36 ±1.97
***S.* Typhimurium treated four times**	13.31 ±0.84

Results obtained were analysed using Kaplan-Meier test, which provides the survival and hazard function (Figures 2 and 3, respectively) and the percentiles (Table 1), indicating the percentage of worms surviving some amount of times. As can be seen at Figure 2, it seems that *C. elegans* fed with *S.* Typhimurium treated four times by PEF has a greater survival probability than the populations fed with the other *S.* Typhimurium subpopulations. Also, nematodes fed with untreated and treated once *S.* Typhimurium had very close survival probabilities.

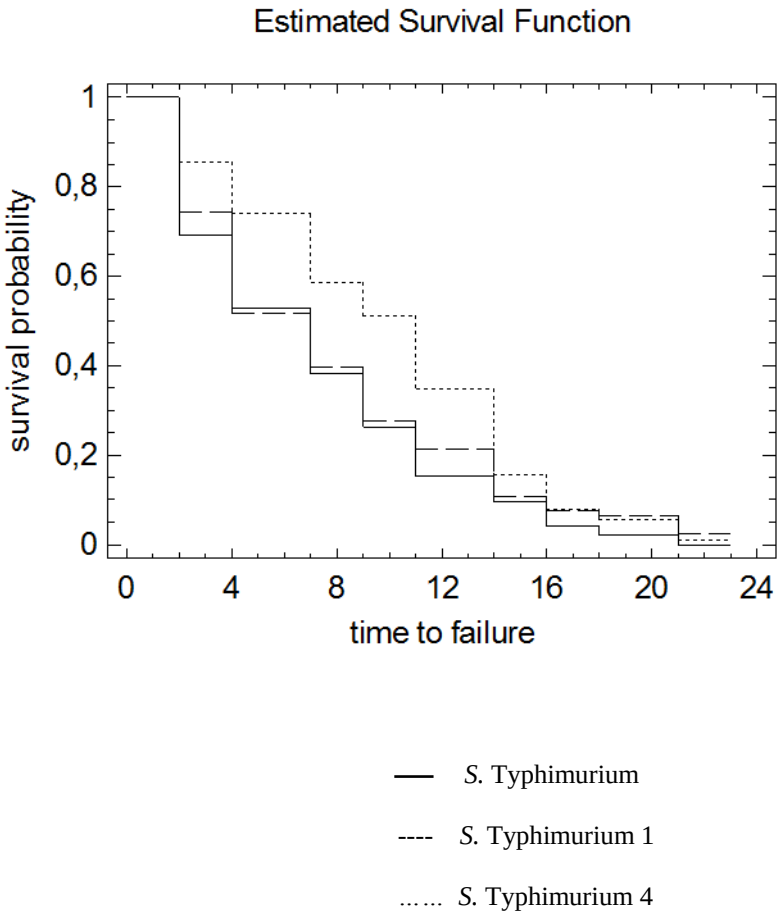

Figure 5.5.2.2. Survival probability of worms fed with untreated *S.* Typhimurium and *S.* Typhimurium treated once and four times by PEF.

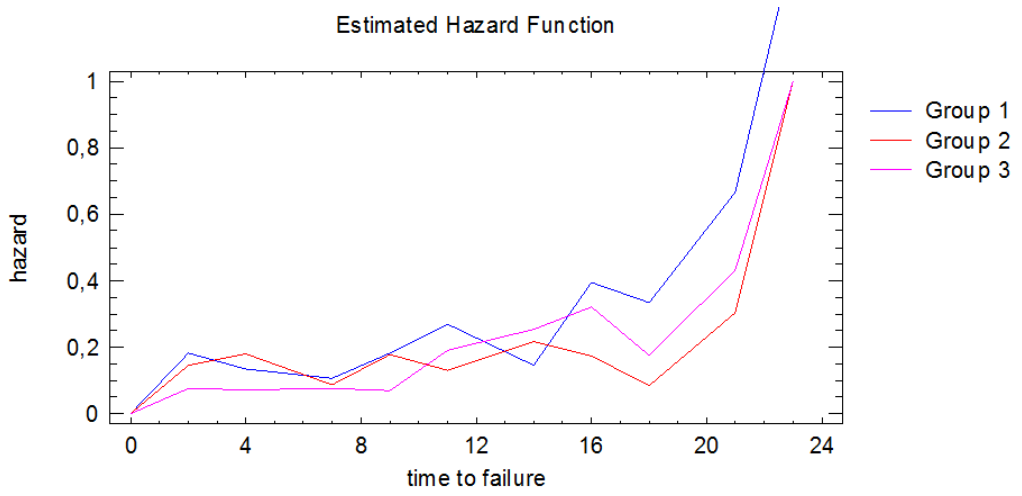

Figure 5.5.2.3. *C. elegans* hazard function when fed with untreated *S.* Typhimurium and *S.* Typhimurium treated one and four times with PEF.

Additionally the Friedman test was used to evaluate the differences between the three populations. This test, with a 90% significant level, confirmed that there were not significant differences (p-value < 0.05) between nematodes fed with untreated *S.* Typhimurium and *S.* Typhimurium treated once with PEF. However, there were significant differences (p-value < 0.05) between *C. elegans* fed with *S.* Typhimurium treated four times by PEF and the other populations.

In addittion, percentiles presented in Table 1 shows the estimated days for 20% percentile of nematodes were alive. These data confirms that there were not significant differences between nematodes fed with untreated and treated once *S.* Typhimurium and, in contrast, there were significant differences between nematodes fed with *S.* Typhimurium treated four times with PEF and the other subpopulations. As a matter of fact, in the percentile 20%, nematodes fed with untreated and treated once *S.* Typhimurium subpopulations achieved this percentile at day 10 and 11, respectively, whereas the nematodes fed with *S.* Typhimurium treated four times achieved this percentile at 13,3 days. From that moment, all nematodes populations

stabilized their survival probability, which is lower after the 14th day. This fact could be related with several factors such as the effects of the persistent infection of *S.* Typhimurium in the intestinal lumen or the aging of *C. elegans*.

Kaplan-Meier analysis provides also the hazard function for *C. elegans*, which gives complementary information to survival function indicating the death risk during the lifespan. In the first time intervals of lifespan there were not significant differences (p-value > 0,05) between different populations of *C. elegans*. But, after the 18th day, the hazard function increased in the three populations, probably due to the few survival population (lower than 20%) and the age of the nematodes (the higher age, the greater probability to death). Anyway, the hazard function of nematodes fed with untreated *S.* Typhimurium increased early than the others, whose hazard functions were increased at the same time.

Some authors have described that *C. elegans* dead early when is infected by *S.* Typhimurium than in optimal conditions because *S.* Typhimurium persistent infection colonizes the intestinal lumen and the bacterial cells increase whereas the intestinal cells decrease (Aballay *et al*., 2000; Labrousse *et al*., 2000; Aballay *et al*., 2002).

Aballay *et al*., (2000) show that in the first four days of *S.* Typhimurium infection, the 50% of nematodes dead. These results are in agreement with those obtained in the present work for untreated *S.* Typhimurium. When the nematodes become older, they start losing their intestinal immunity and pathogen cells are accumulated, causing a reduction in their lifespan (Portal-Celhay *et al*., 2012). Also, the nematodes' pharynx is a neuromuscular bomb that control de amount of bacteria that arrives to intestine and when the nematodes aging, the pharynx lost its capacity and the number of microbial cells that achieve the intestinal lumen is higher, contributing to increase the death risk (Avery, 1993).

Therefore, the higher survival probability of the nematodes fed with bacteria treated four times permit us to conclude that *S.* Typhimurium, when suffered repeated PEF treatments develops a microbial resistance but, in contrast, its virulence could decrease. This is very relevant conclusion for food preservation industry because is an important factor to consider bearing in mind the safety of PEF technology.

5.5.2.3.4 Mobility studies

C. elegans' mobility is induced by contraction of the body wall muscles in the ventral-dorsal plane (Ghosh and Hope, 2010). Figure 4 shows the results obtained on number of movements of *C. elegans* during 10 seconds, reported every 48 hours.

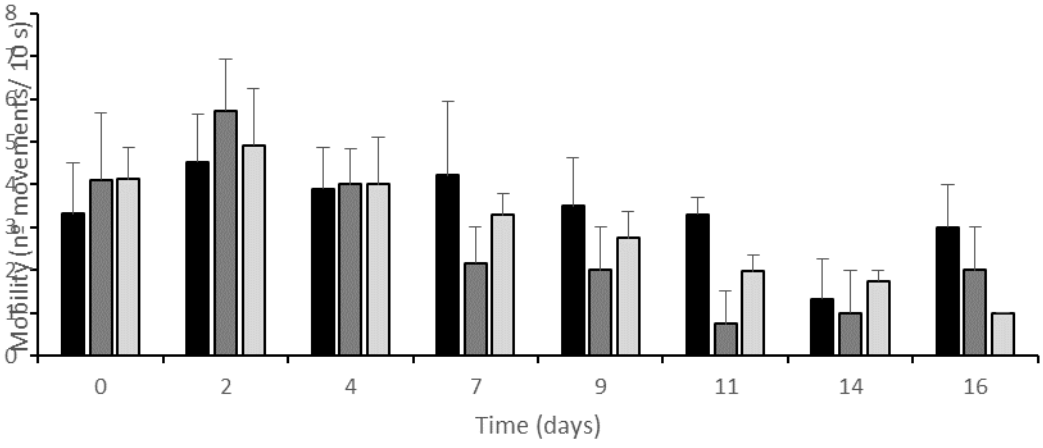

Figure 5.5.2.4: Mobility of *C. elegans* (number of movements in 10 seconds) during their life cycle when they were fed with untreated *S.* Typhimurium and *S.* Typhimurium treated once and three times by PEF.

A Kruskal-Wallis test confirmed that, with 90% of confidence interval, there were not significant differences between nematodes fed with three subpopulations of *S.* Typhimurium. However, the results shown in the figure indicated that the mobility was greater at first days of their life cycle, presumably when their metabolic activity was higher. It could be due to the fact

that when *C. elegans* contacts with pathogen bacteria it is recognised through nervous system, which is connected with the muscles by motor nerves (Altun *et al*., 2009 and Kawli *et al*., 2010). Then, the nematodes become stressed, and they try to avoid the bacteria causing faster movements. However, they cannot avoid the pathogenic bacteria because it is extended over the whole surface of the plate and, finally, *S.* Typhimurium will infect them. Moreover, the mobility could also decrease along their lifespan due to their age.

5.5.2.3.5 Eggs lying studies

The number of eggs laid by *C. elegans* fed with *S.* Typhimurium untreated and treated once and four times by PEF was reported every 48 hours. *C. elegans* lays eggs along its lifespan when it growth in optimal conditions, although the amount of eggs laid is higher at the first steps and decrease during its lifespan. However, when nematodes are infected by pathogenic bacteria, the eggs laying pattern is altered and they lay a greater number of eggs during the firsts days of the lifespan and they stop the eggs laying after the 5^{th} day. The results obtained in the first two control intervals (96 hours) are presented in Figure 5. As can be seen in the figure, nematodes laid a higher amount of eggs in the first time interval (0-2 days) than in the second (2-4 days). Moreover, there was significant differences between three populations of *C. elegans* in the first control interval (p-valor < 0.05) but there was not significant differences between three populations in the second control interval (Kruskal-Wallis test with a confident interval of 90%).

These results could be due to the PEF technology modify the pathogenic mechanisms of *S.* Typhimurium, increasing the stress mechanism of *C. elegans* when it was exposed to treated bacteria because nematodes could feel threatened by the unknown bacterial population generated by PEF. The stress mechanisms correspond with the r-strategy, in which the nematodes

increase its reproductive tax in a short period of time (Hodgkin *et al*., 1991; Schulenburg *et al*., 2004). This strategy permits them to protect themselves against pathogenic bacteria and to ensure the continuity of his offspring before they died.

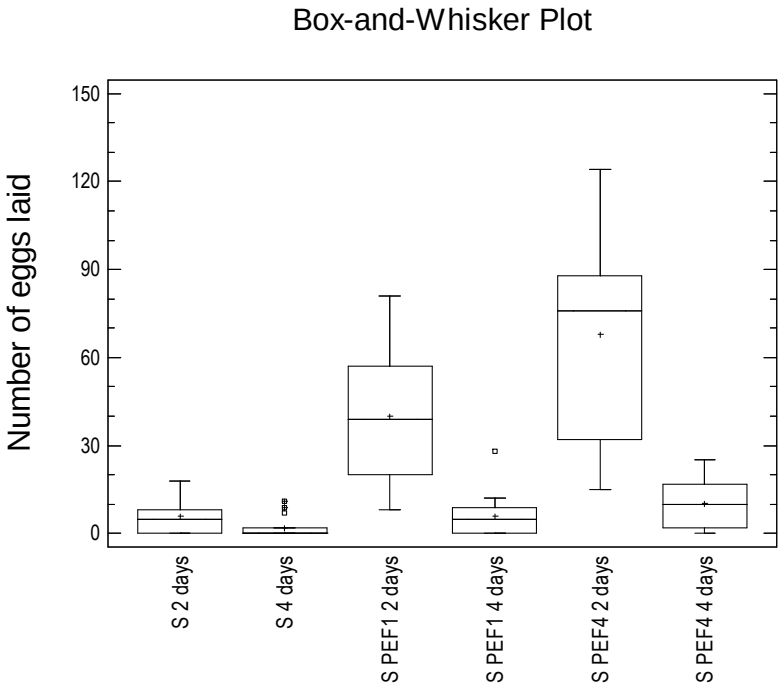

Figure 5.5.2.5. Eggs laid by worms fed by untreated *S.* Typhimurium and *S.* Typhimurium treated once and four times by PEF in first two time intervals.

In addittion, there was a relationship between the eggs laying and the hazard function of nematodes. At first time intervals, the number of eggs laid was in-depth whereas the risk was low. In a research study carried out by Aballay *et al*., 2000 and 2002, they suggested that *S.* Typhimurium could affect the eggs laying pattern and the nematodes exposed to this bacteria laid high amount of eggs and, once they had left offspring, they died by the intestinal infection.

After the first four days *C. elegans* stop its eggs laying and its survival probability decreases quickly when they were fed with *S.* Typhimurium untreated or treated once by PEF. In contrast, *C. elegans* fed with *S.* Typhimurium treated four times by PEF maintained their survival probability after the fourth day. This fact confirms that PEF treatment affects the bacterial virulence, decreasing its pathogenicity.

5.5.2.4 CONCLUSIONS

Results obtained in this study could conclude that *S*. Typhimurium develops microbial resistance against PEF treatment when it was applied repeatedly, but, in contrast, its virulence decreases against a host organism like *C. elegans*. This behaviour could vary among different bacteria so individualized studies are needed depending on the tangent pathogen.

Therefore, it can be concluded that sub-lethal treatments with non-thermal technologies to food preservation are able to inactivate *S.* Typhimurium population on food products but also generate damaged microbial subpopulations that should be controlled to avoid the development of microbial resistance and future risks emerging.

5.5.2.5 ACKNOWLEDGEMENTS

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CAPÍTULO 5.5.3

Sanz-Puig, M., Velázquez-Moreira, A., Guerrero-Beltrán, J.A., Martínez, A., Rodrigo, D.

Validation of High Hydrostatic Pressure treatment against *Salmonella enterica* serovar Typhimurium using *Caenorhabditis elegans*

Food Research International. (2017 - Submitted).

Abstract

HHP treatment is one of the most successful non-thermal technologies to food preservation due to its versatility and capability to achieve enough microbial inactivation maintaining better than traditional preservation methods the organoleptical and nutritional properties of food products. Nevertheless, those non-thermal treatments can lead to sublethally damaged cells that, in case of receiving consecutive treatments, they could develop microbial resistances to the HHP treatment and eventually produce changes in the virulence of microorganism. In the present work, an initial population of *Salmonella enterica* serovar Typhimurium underwent four consecutive HHP treatments and the inactivation achieved with each one was analyzed. Then three microbial populations: untreated *S.* Typhimurium and *S.* Typhimurium treated once and four times by HHP were selected, and the possible virulence changes using *C. elegans* as a model organism were studied. The results obtained showed that *S.* Typhimurium developed a microbial resistance against HHP treatment. Regarding if this resistance increase implied changes on virulence, results indicated that the survival probability of worms feed with

HHP treated *S.* Typhimurium populations was greater than whose were fed with untreated *S.* Typhimurium in the first time intervals of lifespan. In contrast, the hazard function was higher in nematodes fed with HHP treated *S.* Typhimurium beyond 16th day than nematodes fed with untreated *S.* Typhimurium. Additionally, it was observed increased eggs laying and mobility at first days of their lifespan. Those results appear to indicate that some decrease on virulence was achieved on *S.* Typhimurium despite of the increment of resistance to the HHP treatment.

5.5.3.1 INTRODUCTION

Food preservation is necessary to guarantee the food safety and to avoid foodborne outbreaks, but it is also necessary the highest level of nutritional and sensorial quality of preserved foods. Consequently, in last years, new technologies have been developed to preserve food products maintaining better than traditional preservation methods their organoleptic and nutritional properties. Among them, High Hydrostatic Pressure (HHP) is one of the most successful technologies. This technology allows reducing the microbial population of food, using lower temperatures than thermal pasteurization (Rendueles *et al*., 2011). Therefore, sensorial and nutritional properties of food products are preserved being a technology to choice for minimally processed products. It can also be applied both in low water and in liquid foods. For these reasons, HHP treatment has turned in a good option for food products whose properties may be affected by thermal pasteurization (Barbosa-Canovas and Juliano, 2008).

However, almost all preservation treatments produce microbial damaged populations that could be able to develop a resistance against them, like has occurred with some antibiotic treatments (Kostyanev *et al*., 2015; Laxminarayan *et al*., 2013). Although there are very few research studies about

this, foodborne pathogens could also develop resistance against natural antimicrobials from plants or new pasteurization treatments such as HHP (Vanlint, D., 2013; Kisluk *et al*., 2013). In consequence, it appears interesting to study the development of microbial adaptations and resistances against antimicrobial treatments and the possible changes in pathogen virulence.

One of the most important foodborne pathogens contaminating raw food is *Salmonella spp.* It is the most frequent cause of foodborne outbreaks (22,5%), being eggs and egg-products the main contributors (44,9%), because it can be found them in sweets, chocolate or pork meet (EFSA, 2015). Also, salmonellosis is the second most frequent zoonotic disease in the European Union, with 82,694 cases in 2013, being the most frequent serotips *Salmonella enterica* serovar Enteritidis and Typhimurium, with 39,5% and 20.2% of confirmed cases, respectively (EFSA, 2015).

Therefore, it is important to evaluate the possible resistance developed by *S.* Typhimurium against HHP treatments due to many products contaminated by this microorganism are used as raw material for food preparation. It is also important to know whether those sublethal treatments could induce virulence changes. A good option is using the nematode *Caenorhabditis elegans* as a model organism, due to its manipulation is easy in the laboratory and it has been used as a model organism in many studies (Chai-Hoon *et al.,* 2010; Silva *et al*., 2015).

For all these reasons, the goal of this research study was to evaluate the possible development of a microbial resistance of *S.* Typhimurium against a subletal HHP treatment applied repeatedly and study the response of the *C. elegans* fed with the different *S.* Typhimurium HHP treated subpopulations.

5.5.3.2 MATERIAL AND METHODS

5.5.3.2.1 Bacterial strain

The Spanish Type Culture Collection provided us a pure culture freeze-dried of *S.* Typhimurium (CECT 443). It was rehydrated with tryptic soy broth (TSB) (Scharlab Chemie) and, was transferred to 500 mL of TSB and incubated at 37 °C, with continuous shaking (Selecta Unitronic) at 200 rpm for 14 h to obtain cells stock. Later, the cells were centrifuged (Beckman Avanti J-25) twice at 2450 g at 4 °C for 15 min and resuspended in TSB. Finally, the cells were resuspended in 20 mL of TSB with 20% glycerol and then dispensed in vials of 2 mL, to a final concentration of 10^8 cfu/mL, and finally frozen and stored at -80 °C.

5.5.3.2.2 HHP treatment against *S.* Typhimurium

Firstly, based on previous results (Sanz-Puig *et al*., 2016), *S.* Typhimurium with an initial concentration of 10^8 cfu/mL, was treated by HHP at 250 MPa for 5 minutes, because it was atreatment which produced few inactivaction (0,5 log cycles) and a high percentage of damaged cells. These studies were carried on by triplicate.

HHP treatments were done using the EPSI NV equipment (Temse, Belgium) (Pina-Pérez *et al*., 2007).

5.5.3.2.3 Evaluation of microbial resistance

HHP treatments of 250 MPa for 5 minutes were applied to *S.* Typhimurium repeatedly. Before and after each treatment, the microbial population was evaluated by plate count in triptic soy agar (TSA) (Scharlab Chemie) and, between HHP treatments, *S.* Typhimurium cells were grown in TSB overnight with continuous shaking at 37 ^{o}C to achieve the stationary phase and centrifuged at 2450 g for 15 min to recovery them.

Stocks of different *S.* Typhimurium populations obtained were stored at -80 ^{o}C. Among them, *S.* Typhimurium HHP-treated populations that shown the most different pattern in resistance were chosen to evaluate the possible changes in their virulence by using *C. elegans*. All treatments have been done by triplicate.

5.5.3.2.4 *C. elegans* studies

C. elegans strain N2, was provided from "College of Biological Sciences, Minnesota University" USA. This nematode was used as a model organism to evaluate the possible virulence changes in *S.* Typhimurium populations caused by consecutive HHP-treatments. In optimal laboratory conditions, *C. elegans* was remained in plates at 20 ^{o}C with Nematode Growth Medium (NGM) agar and a bacterial lawn of *E. coli* OP50 (Stiernagle, 2006). For virulence studies the lawn of *E. coli* OP50 was changed by a lawn of each one of selected populations of *S.* Typhimurium.

Therefore, the worms were fed with untreated *S.* Typhimurium and with *S.* Typhimurium HHP-treated once and four times by HHP and, in all cases, the studies were focused in their lifespan, their eggs laying and their mobility.

To study their lifespan, 50 synchronized nematodes were fed with each one of selected populations of *S.* Typhimurium during their lifespan, examining them at regular intervals of 48 hours with a binocular microscope (COMECTA S.A.). Worms were considered dead when they did not move and did not respond to stimulate. Each experiment was done in five repetitions, with 250 nematodes.

To study the mobility, five synchronized nematodes were fed with different *S.* Typhimurium populations during their lifespan and were examined at each 48 hours intervals counting the number of movements in 10 seconds.

Each experiment was carried out in five repetitions, with a total of 25 *C. elegans*.

Also, it was studied the effect of selected *S*. Typhimurium populations in the eggs laying of *C.* elegans. 25 synchronized nematodes, distributed in five repetitions of five worms, were fed with a lawn of different *S.* Typhimurium populations and were examined at 48 hours' intervals, focusing in the number of eggs that they laid.

All the experiments carried out had a negative control with *C. elegans* in NGM plates with an *E. coli* OP50 lawn.

5.5.3.2.5 Statistical analysis of data

The results obtained of the evaluation of *S.* Typhimurium resistance against HHP-treatment were analyzed calculating the mean and standard deviation. The same procedure was used with results obtained with *C. elegans*. In addition, ANOVA and Kruskal-Wallis analyses were performed to detect significant differences between microbial cell populations and among nematodes feeding with different *S.* Typhimurium populations ($p\text{-value}<0.05$).

Moreover, the lifespan results were analyzed with the Kaplan-Meier method, allowing to obtain the survival and hazard function for each condition. All statistical analyses were done using Statgraphics Centurion XII software (StatPoint Technologies, Inc., Warrenton, VA, USA).

5.5.3.3 RESULTS AND DISCUSSION

5.5.3.3.1 Study of *S.* Typhimurium resistance after consecutive HHP treatments

The possible microbial resistance developed by *S.* Typhimurium against HHP treatment was evaluated by submitting an initial microbial population of 10^8 cfu/mL to consecutive HHP treatments at 250 MPa for 5 minutes.

Figure 1 shows the inactivation (log cycles) caused in the different *S.* Typhimurium populations after the 250 MPa for 5 minutes pressure treatment. After the first HHP treatment a reduction of 2,62 log cycles was achieved, the second HHP treatment caused 1,8 log cycles of microbial reductions, the third treatment produced 0,76 log cycles on bacterial inactivation and, finally, the fourth treatment only 0,67 log cycles of *S.* Typhimurium inactivation was achieved. According to the results obtained, it appears that *S.* Typhimurium developed a microbial resistance to HHP treatment when it was applied consecutively (four times). These results are in agreement with results obtained by Fioretto *et al*., (2005) with *S.* Enteritidis. Also, other authors such as Buzrul, (2004), suggest that HHP treatments applied consecutively in food products could increase the microbial resistance to pressure and temperature.

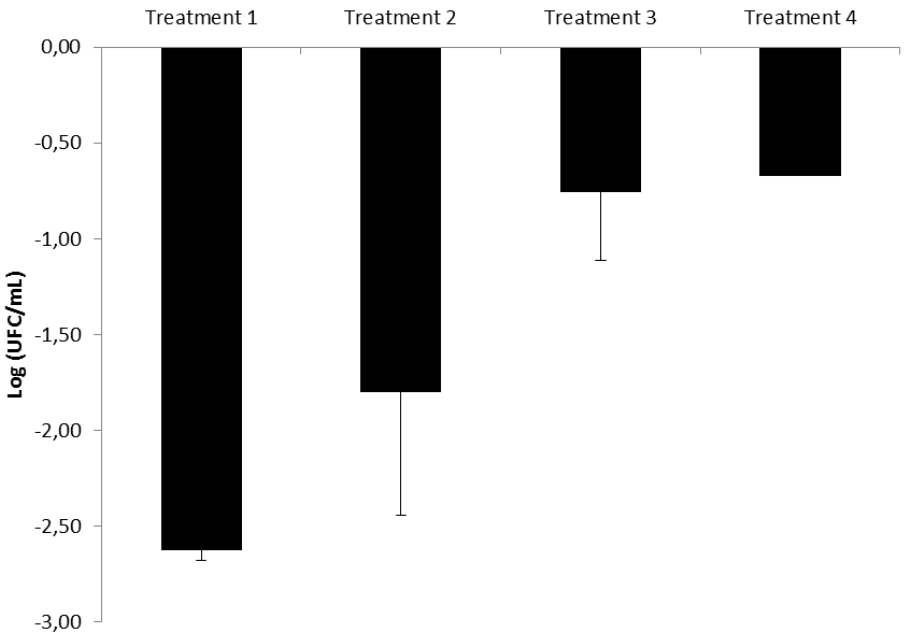

Figure 5.5.3.1. Inactivation of *S.* Typhimurium after consecutive HHP treatment (250 MPa – 5 min).

5.5.3.3.2 Evaluation of virulence changes in *S.* Typhimurium using *C. elegans*

Possible virulence changes caused in *S.* Typhimurium by four consecutive HHP-treatments were evaluated by feeding *C. elegans* with *S.* Typhimurium treated once and four times and comparing with *C. elegans* fed with untreated *S.* Typhimurium. Studies carried out with *C. elegans* were lifespan, eggs lying and mobility.

5.5.3.3.3 Lifespan studies

The estimated survival function for nematodes during their lifespan, while they were fed with different populations of *S.* Typhimurium were analysed applying the Kaplan-Meyer method and the results are presented in Figure 2. As can be seen in this figure, the survival probability was decreasing during their lifespan, being practically all nematodes dead at 16-20th days. Although the nematodes fed with *S.* Typhimurium treated by HHP showed a greater survival probability in the first time intervals of their lifespan than the nematodes fed with untreated *S.* Typhimurium, there was not apparent differences between nematodes fed with different *S.* Typhimurium populations in the last time intervals. Even so, the nematodes population fed with untreated *S.* Typhimurium reached greater survival probability in the last step of lifespan than nematodes fed with HHP treated *S.* Typhimurium.

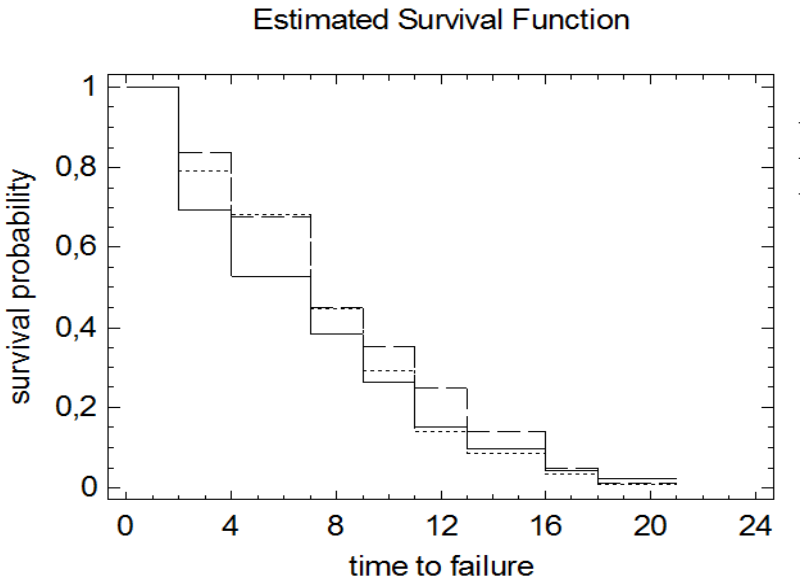

Figure 5.5.3.2. Survival probability of worms fed with untreated *S.* Typhimurium and *S.* Typhimurium treated once and four times.

On the same way, Table 1 shows the percentiles for lifespan of *C. elegans* fed with *S.* Typhimurium untreated and treated by HHP once and four times. As can be seen, the 50% of nematodes was died at day 4^{th} or 6^{th} depending on if they were fed with untreated or treated *S.* Typhimurium, respectively. This confirms that the survival probability of nematodes was greater in the first time intervals when they were fed treated *S.* Typhimurium. Moreover, only 5% of nematodes survived beyond 15^{th} day, again without apparent differences between nematodes fed with different *S.* Typhimurium sub-populations. Finally, focusing in the last time intervals of lifespan, the 1% of nematodes survived beyond 19 days when they were fed with untreated *S.* Typhimurium, 18 days when they were fed with *S.* Typhimurium treated once by HHP and 17 days when they were fed with *S.* Typhimurium treated 4 times with HHP. It is in according with that at the end of lifespan the nematodes fed with untreated *S.*

Typhimurium shown slightly greater survival probability than those that were fed with treated *S.* Typhimurium.

Table 5.5.3.1. Percentiles for lifespan (days) of *C. elegans* fed with untreated *S.* Typhimurium and *S.* Typhimurium treated once and four times.

Percentil %	*S*. Typhimurium	*S*. Typhimurium 1	*S*. Typhimurium 4
75,0	1,6 ± 0.2	3.1 ± 0.4	2,8 ± 0.6
50,0	4,6 ± 0.9	6.3 ± 0.5	6,3 ± 0.4
25,0	9,2 ± 1.1	11,0 ± 1.0	9,6 ± 0.7
10,0	12,9 ± 4.8	14,3 ± 1.8	12,4 ± 4.5
5,0	15,6 ± 4.0	15,9 ±1.8	15,0 ± 4.2
1,0	19,6 ± 31,9	18,5 ± 72,2	17,8 ± 14,6

The Kaplan-Meyer analysis also gives the hazard function, showed in Figure 3. It can be seen that there are not apparent differences until 15th day, but after this moment the hazard function of *C. elegans* fed with *S.* Typhimurium treated once and four times by HHP increases faster than the control sample (untreated *S.* Typhimurium). It indicated that in the last days of their lifespan, nematodes fed with these microbial sub-populations had a higher hazard to die than nematodes fed with control *S.* Typhimurium sub-population.

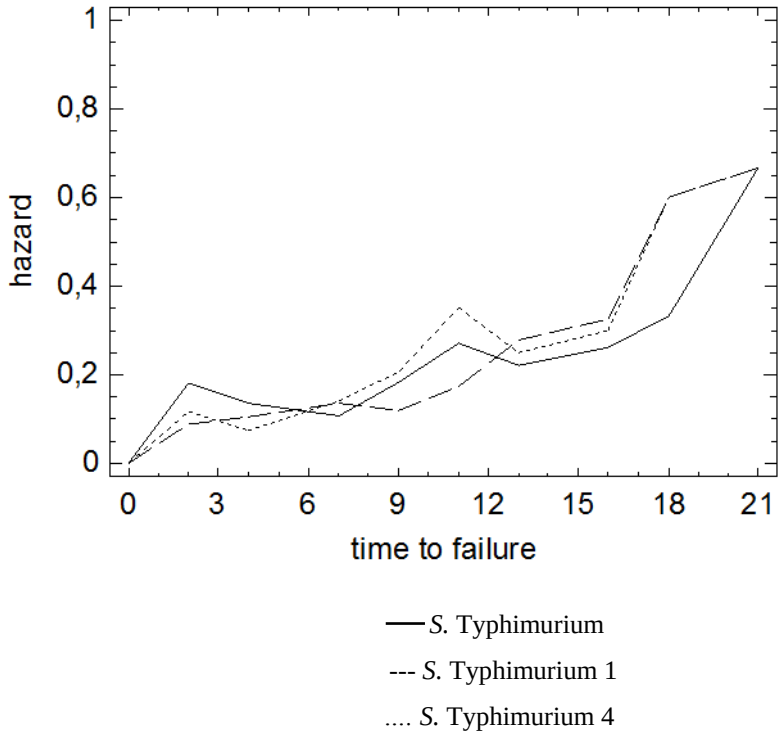

Figure 5.5.3.3. Hazard function of nematodes fed with untreated *S.* Typhimurium and *S.* Typhimurium treated once and four times.

Other research studies with *C. elegans* fed with other microorganisms achieved different results. For example, in the study carried on by Silva *et al*., 2015, only 5% of *C. elegans* fed with *Listeria monocytogenes* untreated and treated with citral survive beyond 14th day, without significant differences between them (p-value > 0.05). However, the 5% of nematodes fed with *L. monocytogenes* treated with carvacrol survive beyond 13 days, with significant differences (p-value < 0.05) with control sample (untreated *L. monocytogenes*).

5.5.3.3.4 Eggs laying studies

Eggs laying studies were carried out with *C. elegans* fed with untreated *S.* Typhimurium and *S.* Typhimurium treated once and four times, counting the

number of eggs laid by each one of 25 worms during their lifespan, in time intervals of 48 hours.

C. elegans fed with its natural feed (*E. coli* OP50) lays eggs during their life cycle (aprox. 3 weeks) in optimal conditions (Lavigne *et al*., 2006); results are in agreement with those found in the present study (data not shown). Nevertheless, when *S.* Typhimurium infected them, they only lays eggs until 5th day. Figure 4 shows the number of eggs laid by the worms fed with three selected populations of *S.* Typhimurium during the first two time intervals (0-2 and 2-4 days). As can be seen in the figure, there are significant differences (p-value < 0.05) in the egg laying pattern of nematodes fed with *S.*Typhimurium HHP treated and untreated. Both fed with HHP treated microbial populations once and four times laid a greater amount of eggs (around 80 eggs of average) than those fed with not treated microorganism (around 20 eggs of average). Focusing in the egg laying pattern by intervals, worms fed with *S.* Typhimurium HHP treated laid higher number of eggs during first time interval (0 – 2 days) than during the second (2 – 4 days). In contrast, there was no significant differences (p-value > 0,05) between the number of eggs laid by nematodes fed with *S.* Typhimurium treated once or four times with HHP.

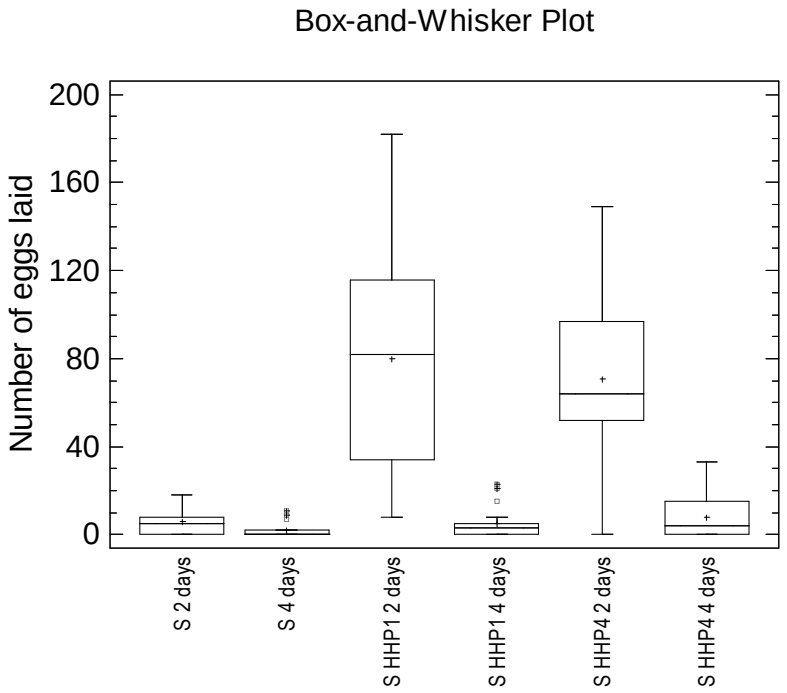

Figure 5.5.3.4. Eggs laid by worms fed by untreated *S.* Typhimurium and *S.* Typhimurium treated once and four times by HHP in first two time intervals.

The results indicate that *S.* Typhimurium affects the reproductive system of *C. elegans* and changes their eggs laying pattern, increasing the amount and frequency of eggs laying during the first five days. These results are in agreement of Aballay *et al*.,2000, who suggested that eggs laid process of *C. elegans* can be affected when they are fed with *S.* Typhimurium. There are research studies that demonstrate that the infection with *S.* Typhimurium reach the whole lumen intestine at 5th day (Labrousse *et al*., 2000), and match with the day in which nematodes stop their eggs laying. Moreover, in this study it has been proven by first time that the infection with *S.* Typhimurium treated by HHP caused a greater number of eggs laid by nematodes although they stopped their eggs laying at the same time than with the control sample. It can be

explained with some research studies that show that *C. elegans* retains eggs in their uterus when environmental conditions are harmful until the conditions become optimal again (Gradner *et al*., 2013).

Therefore, we can conclude that *S.* Typhimurium treated by HHP increase its resistance to treatment but in some aspects decrease its virulence against *C. elegans*.

5.5.3.3.5 Mobility studies

Finally, it was studied the mobility of *C. elegans* fed with untreated *S.* Typhimurium and *S.* Typhimurium treated once and four times with HHP. The results obtained are showed in Figure 5. As can be seen in this figure, all populations of *C. elegans* reduced their mobility during their life cycle. Although they not shown differences between nematodes fed with different *S.* Typhimurium populations, at first time intervals the nematodes fed with *S.* Typhimurium treated by HHP had higher mobility than nematodes that were fed with untreated *S.* Typhimurium, probably due to pathogenic bacteria causes stress in *C. elegans*, producing faster movements initially (Altun *et al*., 2009). This effect was not observed at the end of the process likely due to treated *S.* Typhimurium populations were less virulent than untreated.

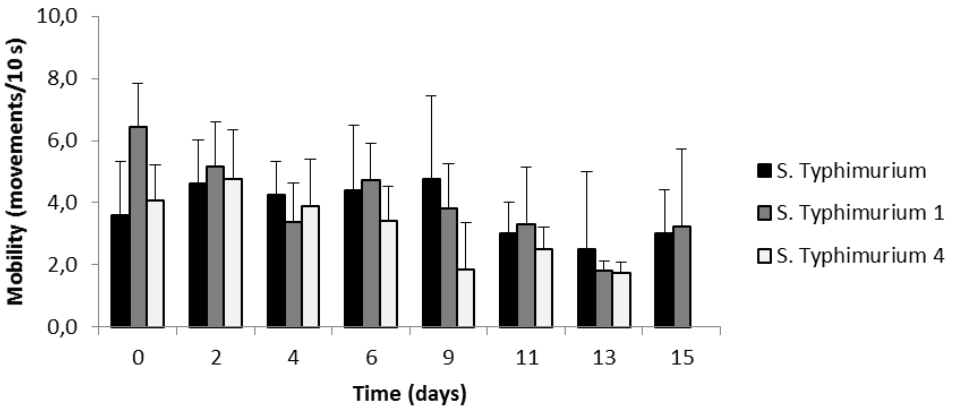

Figure 5.5.3.5. Mobility of worms fed by untreated *S.* Typhimurium and *S.* Typhimurium treated once and four times by HHP.

5.5.3.4 CONCLUSIONS

In view of the results, it can be concluded that a sublethal HHP treatment (250 MPa, 5 min) applied repeatedly caused the development of microbial resistance in *S.* Typhimurium. Also, when *C. elegans* was infected with these microbial sub-populations, their survival function was greater in the first time intervals for the nematodes fed with treated *S.* Typhimurium than untreated one. Moreover,their hazard function increased earlier for nematodes fed with *S.* Typhimurium treated once and four times by HHP than in nematodes fed with untreated *S.* Typhimurium. Also, the infection of *C. elegans* with treated and untreated *S.* Typhimurium caused changes in their eggs laying, increasing the number of eggs laid when *C. elegans* was fed with treated *S.* Typhimurium, probably due to the fact that they appear to be less virulent against *C. elegans* than untreated cells.

Therefore, in this study it has been shown that a repeated sublethal HHP treatment increases *S.* Typhimurium resistance but decreases its virulence and the hazard function and the egg laying ratio increases. Therefore, the HHP treatment applied in food industries should be chosen trying to avoid the development of microbial resistance and possible changes in their virulence.

5.5.3.5 ACKNOWLEGDEMENTS

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CAPÍTULO 5.6 ESCALADO INDUSTRIAL DE LA INFUSIÓN DE SUBPRODUCTO DE COLIFLOR

Sanz-Puig, M., Pina-Pérez, M.C., Martínez, A., Rodrigo, D.

Antimicrobial cauliflower by-product infusion: from lab to pilot scale

Journal of Food Engineering. (2017 - Submitted).

Abstract

Revalorization of plant by-products from food industry is a good option to recover their bioactive compounds. Cauliflower by-product infusion had demonstrated in previous studies to exert an important antimicrobial effect against *S.* Typhimurium. For these reason, the aim of this research study was to scale-up the laboratory cauliflower by-product infusion to pilot scale and evaluate its antimicrobial capacity against *S.* Typhimurium. Firstly, cauliflower by-product infusion was obtained under different conditions of time and temperature and its antimicrobial activity was determined by disk diffusion method, selecting the conditions 100 °C – 30 min to be used in the next step of this research study. Selected cauliflower by-product infusion was scaled up to pilot scale and its antimicrobial activity was evaluated obtaining the microbial inactivation curve. Results obtained showed that scaled infusion maintained the antimicrobial effect exerted by lab scale infusion, achieving a reduction of 5 log cycles in 8 hours at 37 °C. Therefore, it can be concluded that cauliflower by-product infusion maintain its antimicrobial activity in an industrial scale and

could be used as an additional antimicrobial measure to control the food safety in food industries.

5.6.1 INTRODUCTION

Nowadays, food industry is increasingly interested in new food preservatives with natural origin due to their antimicrobial and antioxidant benefits. Also, the revalorization of plant waste from agroindustrial production, which are usually destined to incineration or landfill, present itself a great opportunity to recover bioactive compounds remaining in their by-products (Zibetti *et al*., 2013).

Cauliflower by-product has demonstrated in previous studies to be rich in many bioactive compounds with antimicrobial activity, mainly polyphenolic compounds (Sanz-Puig *et al*., 2015a, Sanz-Puig *et al*., 2015b). Also, cauliflower by-product infusion has showed to be able to exert an important antimicrobial effect against *Salmonella enterica* serovar Typhimurium in lab studies (Sanz-Puig *et al*., 2016; Sanz-Puig *et al*., 2017).

All previous results permit us to propose cauliflower by-product infusion as a good option to control the food safety and food quality of pasteurized food products during their storage in refrigeration after their industrial production. Nevertheless, in order to use the cauliflower by-product infusion at food industry, it is necessary to confirm its antimicrobial activity when it is produced in an industrial scale.

For all these reasons, the main goal of this research study is to scale-up the cauliflower by-product infusion at different conditions and evaluate its antimicrobial activity against *S.* Typhimurium using both a qualitative and quantitative method and, finally, propose the best conditions to obtain a

cauliflower by-product infusion ready to use as a natural preservative in industrial food products.

5.6.2 MATERIALS AND METHODS

5.6.2.1 Microorganism

Glycerinated cryovials of *S.* Typhimurium (CECT 443) were obtained from freeze-dried cultures provided by the Spanish Type Culture Collection, using the method described by Sanz-Puig *et al*. 2015.

5.6.2.2 Preparation of cauliflower by-product infusion

Cauliflower by-product was provided from agro-industrial primary production. It was washed in sterile water, dried, triturated, and homogenized with a laboratory grinder (Janke & Kunkel, IKA-Labortechnik) to obtain a powder with a particle size of 40 μm.

A 10% (w/v) infusion of cauliflower by-product was obtained by immersing the powder in 0.1% (w/v) buffered peptone water (Scharlab, S.A., Barcelona, Spain) under several conditions, which are showed in Table 1.

Then the infusion was centrifuged at 4 °C, at 2450 g for 15 min and finally, it was filtered through filters (Whatman) with a pore size of 11 and 2.5 μm and then sterilized by filtering through a PVDF syringe filter with a pore size of 0.45 μm.

Initially the infusion was scaled up from a laboratory scale (100 mL) to 1000 mL and finally to 50 L, evaluating its antimicrobial activity in each step.

Table 5.6.1. Conditions of cauliflower by-product infusion (1000 mL).

Temperature (ºC)	Time (min)
Room temperature	15 min
Room temperature	30 min
100 ºC	15 min
100 ºC	30 min

5.6.2.3 Evaluation of antimicrobial activity

Firstly, the antimicrobial effect against *S.* Typhimurium was evaluated qualitatively, using the agar diffusion method. One millilitre of microorganism (10^7 cfu/mL) was spread on the surface of Mueller-Hinton agar plates (Scharlau, S.A., Barcelona, Spain). Sterile filter paper discs (7 mm in diameter) were impregnated with 50 µL of the vegetable extracts. The extract was replaced with buffered peptone water (Scharlab, S.A., Barcelona, Spain) as a control sample. The plates were then kept at ambient temperature for 30 min to allow diffusion of the extracts prior to incubation at 37 °C during 24 hours. Finally, the inhibition halo of each disc was measured with a slide gauge. Studies were carried out in triplicate and the average and standard deviation of three values were calculated using STATGRAPHICS Centurion XV (version 15.1.03; STATGRAPHICS, Warrenton, VA).

Later, the antimicrobial activity against *S.* Typhimurium was evaluated quantitatively. Infusion was inoculated with 10^8 cfu/mL of *S.* Typhimurium and incubated at 37 °C. The microbial inactivation curves were obtained by removal of aliquots at regular time intervals and plate count in Tryptic Soy Agar (TSA, Scharlab Chemie, Barcelona, Spain) after serial dilution with 0.1% (w/v) buffered peptone water. The plates were incubated at 37 °C for 24 hours. All analysis was done in triplicate.

5.6.3 RESULTS AND DISCUSSION

The antimicrobial activity of cauliflower by-product infusions (1000 mL), obtained under different conditions, was evaluated by agar diffusion method. The results obtained are shown in Table 2.

Table 5.6.2.. Inhibition halo (mm) of *S.* Typhimurium under different cauliflower by-product infusions (1000 mL).

Sample	Inhibition Halo (mm)
Control (H_2O Peptone 0.1%)	7
Room temperature, 15 min	13,133 ± 2.031
Room temperature, 30 min	17,181 ± 2.136
100 °C, 15 min	15,500 ± 0.816
100 °C, 30 min	18,937 ± 3.214

Generally, cauliflower by-product infusions obtained during 30 min showed higher inhibition halo than which obtained during 15 min, both at room temperature and 100 °C. It indicates that the higher contact time with cauliflower by-product, the greater antimicrobial activity of the infusion against *S.* Typhimurium. In addition, inhibition halos were greater when the infusion was obtained at 100 °C than when it was obtained at room temperature in both time conditions. Thus, the higher the infusion temperature, the greater antimicrobial capacity against *S.* Typhimurium. These results are in agreement with previous studies (Castiglioni *et al*. 2015) in which the temperature and time conditions were able to modify the bioactive compounds extracted during an infusion process. This could explain the differences in their antimicrobial activity against *S.* Typhimurium.

Nevertheless, the best antimicrobial effect against *S.* Typhimurium was obtained by 100 °C – 30 min cauliflower by-product infusion (18.937 ± 3.214

mm). It permits us to conclude that these were the best conditions for the infusion process and it was selected to scale up in the next steps of this research study.

In a secondary step, cauliflower by-product infusion (100 °C – 30 min) was scaled to 50 L and its antimicrobial capacity against *S.* Typhimurium was evaluated quantitatively, obtaining the microbial inactivation curve. Figure 1 shows the *S.* Typhimurium inactivation curves when it was incubated at 37 °C during 10 hours with cauliflower by-product infusion obtained at lab scale (100 mL) and pilot scale (50 L). As it can be seen in this figure, the antimicrobial capacity of cauliflower by-product infusion was maintained when it was scaled up, achieving 5 log cycles of microbial inactivation in 8 hours at 37 °C, both when it was incubated with lab and pilot scale cauliflower by-product infusion. Similar results have been obtained when other vegetable extracts like grape or lemon, have been scaled up (Prado *et al*., 2012; Zibetti *et al*., 2013; Prado *et al*., 2014; Pérez-López *et al*., 2014).

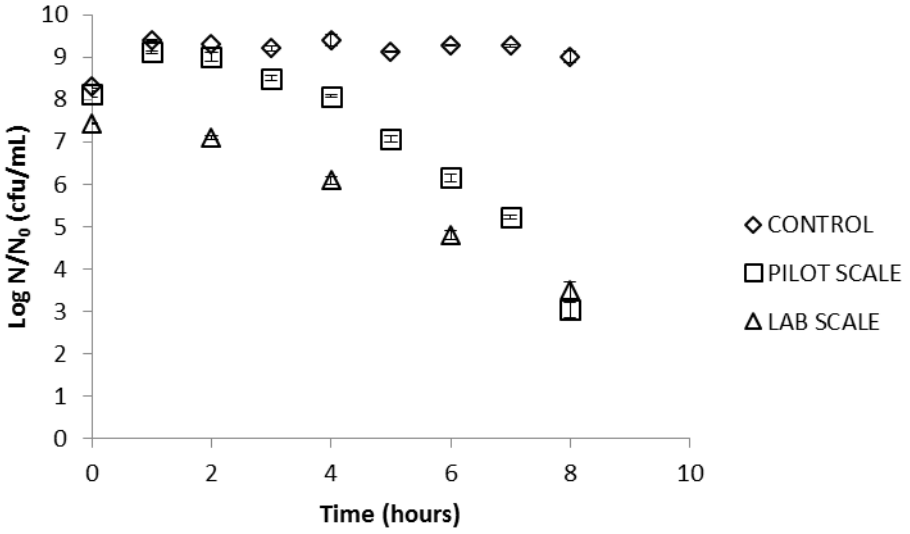

Figure 5.6.1. *S.* Typhimurium inactivation curves under the incubation with cauliflower by-product infusion obtained in a lab scale (100 mL) and pilot scale (50 L).

5.6.4 CONCLUSIONS

According to the results presented in this research work, it can be concluded that cauliflower by-product infusion exert an important antimicrobial effect against *S.* Typhimurium and this effect is maintained when the infusion is scaled up. Therefore, this cauliflower by-product infusion is ready to be used as an antimicrobial control measure in food products in an industrial scale.

5.6.5 AKNOWLEDGEMENTS

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DISCUSIÓN

6. DISCUSIÓN GENERAL

De acuerdo a los resultados obtenidos, los antimicrobianos naturales procedentes de residuos de la agroindustria pueden ser una alternativa efectiva al uso de conservantes sintéticos, respondiendo así a las exigencias de los consumidores cada vez más informados y preocupados por su salud. Este nuevo consumidor demanda alimentos exentos de aditivos químicos (etiquetado limpio o "clean label"), que contengan ingredientes naturales, sencillos, de uso tradicional y, en la medida de lo posible, productos lo más parecido al fresco o sometidos a tratamientos mínimos. En este sentido, en el año 2013 el 27% de los nuevos productos lanzados en Europa fueron comercializados bajo el concepto "*clean label*". Como se ha podido apreciar en diferentes partes de esta tesis, los resultados están alineados con las demandas en la I+D de la industria alimentaria, y con los esfuerzos en la investigación y desarrollo a nivel internacional sobre el uso de ingredientes con función tecnológica mejorada y que garanticen dicho etiquetado limpio.

Los productos vegetales, frutas, hortalizas, semillas y granos son fuente de compuestos bioactivos, entre los que destacan grupos moleculares de elevada capacidad antimicrobiana, fundamentalmente compuestos polifenólicos. En la presente tesis doctoral se ha trabajado en el aprovechamiento de los antimicrobianos de origen vegetal como estrategia de conservación de alimentos, evaluando al tiempo la efectividad y posibles riesgos asociados como son la adaptación/resistencia microbiana en bacterias tratadas/expuestas a los mismos, aplicándolos solos, o combinados con tecnologías de conservación no térmicas (tecnología de barreras). La presente propuesta de revalorización de subproductos agroalimentarios contribuye a generar conocimiento en uno de los Retos de la Sociedad, siendo eje prioritario contemplado en el H2020 el uso eficiente de recursos y materias primas,

mediante el aprovechamiento de un elevado volumen de residuos vegetales generados por la industria agraria. Evaluar la efectividad de dichos subproductos frente a los patógenos alimentarios de mayor relevancia en seguridad alimentaria es el objetivo fundamental de la presente tesis doctoral.

6.1 CAPACIDAD ANTIMICROBIANA DE SUBPRODUCTOS VEGETALES: SUBPRODUCTOS EN BRUTO, SUBPRODUCTOS OBTENIDOS MEDIANTE ACCELERATED SOLVENT EXTRACTION (ASE) Y SUBPRODUCTOS INFUSIONADOS EN CALIENTE

Los resultados de la presente tesis han mostrado, en primer lugar, la capacidad antimicrobiana de 6 subproductos deshidratados procedentes de la industria agroalimentaria: coliflor, brócoli, okara, mandarina, naranja y limón, frente a 4 patógenos alimentarios: *S.* Typhimurium, *E. coli* O157:H7, *L. monocytogenes* y *B. cereus,* a diferentes concentraciones de subproducto (0.5, 1, 2, 5, 10, 15 %) (p/v) y temperaturas de incubación (5, 10, 22, 37 ^{o}C).

Estos resultados correspondientes al Capítulo I indican que todos los subproductos brutos deshidratados presentaron actividad antimicrobiana frente a los patógenos estudiados, sin embargo no todos tuvieron la misma capacidad, destacándose el subproducto de coliflor, entre las *brassicas*, y el subproducto de mandarina, entre los cítricos, como los de mayor capacidad antimicrobiana tanto frente a bacterias Gram-positivas (*L. monocytogenes* y *B. cereus*) como Gram-negativas (*S*. Typhimurium y *E. coli* O157:H7). De todos los microorganismos estudiados, *S.* Typhimurium resultó ser el más sensible al efecto antimicrobiano de estos subproductos. Así, se alcanzó un efecto bactericida máximo próximo a 6 ciclos logarítmicos de inactivación tras la exposición de *S.* Typhimurium al subproducto de coliflor durante 432 horas (18 días), y un efecto bactericida de 8 ciclos logarítmicos al incubar dicho microorganismo con el subproducto de

mandarina durante 96 horas (4 días), ambos efectos por exposición a una concentración del 10 % (p/v) de subproducto, en medio de referencia, a una temperatura de incubación de 5 ^{o}C.

Pensando en los posibles responsables de dicha actividad antimicrobiana, bien bacteriostática o bactericida, el perfil polifenólico de ambos subproductos (coliflor y mandarina) se postula como responsable de la misma frente a los diferentes patógenos estudiados, coincidiendo con lo publicado previamente por otros autores (O'Shea et al 2012, Roubos-Van den Hil et al, 2010 o Ghafar et al., 2010). Ambos subproductos, coliflor y mandarina poseen un elevado contenido en compuestos fenólicos (ver Tabla 6.1).

Tabla 6.1. Contenido total de polifenoles en los subproductos de coliflor y mandarina brutos deshidratados y de los extractos ASE y las infusiones en caliente obtenidas a partir de los mismos.

Contenido Total Polifenoles (mg ácido gálico/L)		
	Coliflor 10% (p/v)	**Mandarina 10 % (p/v)**
Subproductos Brutos Deshidratados	7573.21 ± 747.96	5111.50 ± 201.93
Extractos ASE	1252.12 ± 38.29	836.24 ± 107.62
Infusiones en Caliente	4560.00 ± 433.90	3958.75 ± 185.62

De acuerdo a la bibliografía, los principales polifenoles presentes en la coliflor y que podrían ser los responsables de su potente actividad antimicrobiana son el ácido ferúlico, el ácido clorogénico, el ácido gálico y la catequina (Cartea et al, 2011; Mahroop-Raja et al., 2011). Por su parte, los principales compuestos fenólicos presentes en el subproducto de mandarina descritos en bibliografía son el eriodictiol, el ácido ferúlico, el ácido hidroxicinámico, el glicósido de cianuro, la hesperidina, la vitamina C, los

carotenoides y la naringina (Mandalari et al., 2007; Ghafar et al., 2010), a los que previamente se les han atribuido propiedades antioxidantes y antimicrobianas (Espina et al, 2011; Viuda-Martos et al, 2008).

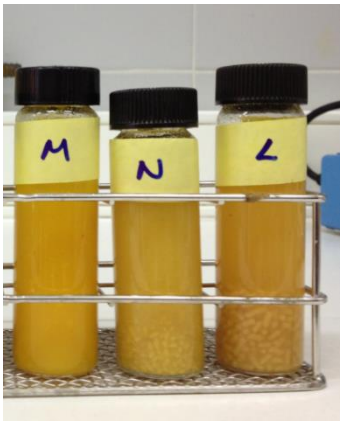

Figura 6.1. Subproductos cítricos (mandarina (M), naranja (N) y limón (L)) al 10 % (p/v) en agua de peptona (0.1 %).

Aunque el efecto antimicrobiano de los subproductos brutos deshidratados fue evidente, la falta de homogeneidad al ser disueltos en medio de referencia a diferentes concentraciones fue el principal problema encontrado en su utilización (ver Figura 6.1). Como solución para mejorar su homogeneidad y facilitar su aplicabilidad, se procedió a la obtención de extractos a partir de los subproductos brutos deshidratados mediante el uso de diferentes metodologías. Para ello, se procedió a evaluar si la tecnología de extracción ASE (Accelerated Solvent Extraction) permitía obtener extractos con capacidad antimicrobiana a partir de los subproductos de coliflor, brócoli, mandarina y naranja. Los subproductos de okara y limón se desestimaron ya en este punto por ser los subproductos que habían presentado un menor efecto antimicrobiano en el apartado anterior.

De igual manera que lo obtenido en el Capítulo I, los extractos obtenidos mediante la tecnología ASE (ver Capítulo II) fueron efectivos tanto frente a las bacterias Gram-positivas como Gram-negativas en estudio, siendo los extractos obtenidos a partir de los subproductos de coliflor y mandarina los que presentaron una mayor capacidad antimicrobiana (16 ± 1 mm de inhibición de *S.* Typhimurium por parte del extracto del subproducto de coliflor y 17 ± 0.4 mm de inhibición por parte del extracto del subproducto de mandarina), coincidiendo además con los extractos que contenían un valor más elevado en polifenoles de acuerdo a esta tecnología de extracción (Tabla 6.1). Aun así, se observó una reducción muy acusada del contenido polifenólico en los extractos ASE frente a los subproductos brutos. *S.* Typhimurium continuó siendo el microorganismo más sensible a los extractos estudiados.

Si bien la tecnología ASE puede ser un procedimiento prometedor en la extracción y preparación de concentrados polifenólicos procedentes de tejidos vegetales (p.e. *Vitis vinífera*), de acuerdo a los estudios previos de Rajha et al. (2014), el potencial antioxidante de dichos concentrados puede verse reducido sobre todo cuando dicha tecnología se aplica a temperaturas superiores a 100 °C ya que la diversidad de compuestos flavonoides y otros antioxidantes presentes en el tejido bruto pueden verse seriamente reducidos por dicho proceso. Además, el uso de solventes de extracción de síntesis resta interés a este método de extracción en el contexto de la presente tesis doctoral en la que se pretende estudiar la aplicación como antimicrobianos de sustancias naturales.

Ya que la obtención de extractos a partir de los subproductos deshidratados mediante la técnica de extracción ASE, además de generar residuos, afecta considerablemente al contenido en polifenoles de los extractos, se optó por la alternativa de la infusión en caliente tal como se describe en el Capítulo III y IV a partir de los subproductos deshidratados con el objetivo de

obtener extractos homogéneos, de fácil aplicabilidad, y que mantuvieran, en mayor medida que por la técnica ASE, tanto el contenido en compuestos polifenólicos como su funcionalidad antimicrobiana (Adwan y Mhanna, 2008). De igual manera que para la técnica ASE, se seleccionaron únicamente los subproductos de coliflor y mandarina debido a que fueron los que mejores resultados habían presentado en los dos capítulos anteriores.

En este caso (infusión en caliente), los resultados obtenidos con el subproducto de coliflor mostraron un extraordinario poder antimicrobiano frente a *S.* Typhimurium a todas las concentraciones y temperaturas estudiadas, siendo capaz de inactivar hasta 5 ciclos logaritmos de *S.* Typhimurium en 10 h a 37 ^{o}C y en 110 h (4.5 días) a 10 ^{o}C. Por otra parte, la infusión en caliente del subproducto de mandarina resultó tener un efecto bacteriostático a 22 ^{o}C y bactericida a 10 y 37 ^{o}C, alcanzando los 5 ciclos logarítmicos de inactivación para *S.* Typhimurium al 10 % en 80 y 240 horas (10 días) a 37 y 10 ^{o}C, respectivamente.

Además, con la infusión en caliente de los subproductos brutos se mejora la homogeneidad y aplicabilidad de los mismos a las distintas concentraciones y temperaturas (ver Figura 6.2).

Figura 6.2. Infusiones en caliente de subproducto de coliflor y mandarina al 10 %.

Se procedió también a comparar el contenido polifenólico de las infusiones obtenidas en caliente *versus* el correspondiente a los subproductos deshidratados brutos (ver Tabla 6.1). Se puede observar un descenso en el contenido polifenólico de ambos subproductos al ser infusionados en caliente, debido, posiblemente, a que la infusión a 100 ºC, durante 30 min degradó algún compuesto de carácter polifenólico. Sin embargo, y de acuerdo a los estudios de Rajha et al. (2014), no sólo el contenido en compuestos polifenólicos, sino la diversidad de los mismos presentes finalmente en el extracto, actuarían de modo determinante en su potencial antimicrobiano.

Si comparamos el efecto bactericida obtenido para las infusiones en caliente y los subproductos brutos frente a *S.* Typhimurium (Figura 6.3) se observa cómo, bajo las mismas condiciones de incubación, las infusiones en caliente de coliflor y mandarina mantuvieron o mejoraron el efecto antimicrobiano obtenido con los subproductos brutos, y su aplicabilidad mejoró notablemente. Por estos motivos se decidió seleccionar las infusiones en caliente de los subproductos de coliflor y mandarina al 10 % para los siguientes estudios realizados en la presente tesis doctoral.

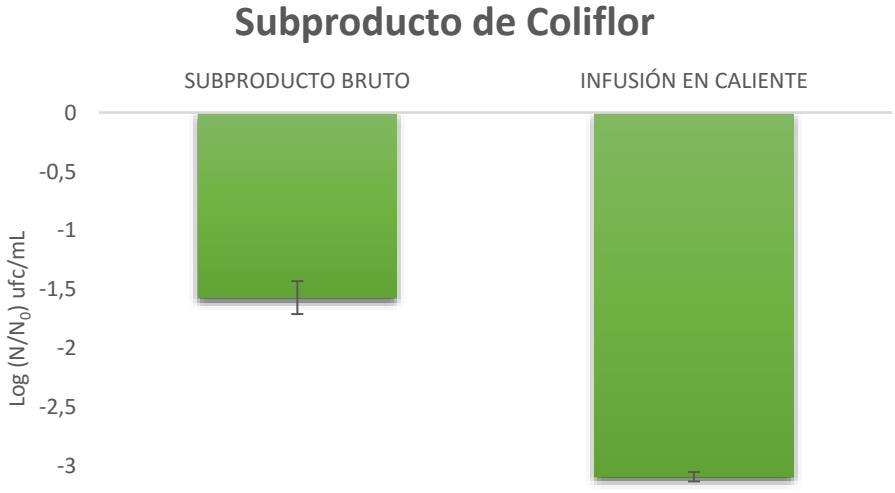

Figura 6.3. Ciclos logarítmicos de inactivación de *S.* Typhimurium tras la incubación durante 75 horas con el subproducto bruto y la infusión en caliente de coliflor al 10 % a 10 ºC.

6.2 SUBPRODUCTOS VEGETALES BAJO EL CONCEPTO DE TECNOLOGÍA DE BARRERAS

En la actualidad, se está haciendo hincapié en el uso de distintas barreras para controlar la proliferación microbiana durante el almacenamiento de los alimentos. Este concepto permite usar menos intensidad en los procedimientos de conservación individuales para conseguir un mayor efecto conjunto sobre la inactivación y control de los microorganismos. En consecuencia, el potente efecto antimicrobiano de las infusiones de los subproductos de coliflor y mandarina obtenido en los estudios llevados a cabo en el Capítulo III y IV, les confiere un gran potencial para su incorporación en un sistema de tecnología de barreras. En este sentido, serían medidas de control frente a la proliferación bacteriana durante el almacenamiento refrigerado de alimentos vegetales o zumos de frutas que hayan sido pasteurizados, contribuyendo a garantizar la seguridad microbiológica y la calidad del producto a lo largo de su vida útil. Combinando el efecto de un subproducto natural infusionado, y el uso de temperaturas de refrigeración, bajo el concepto de "Tecnología de Barreras", es posible alcanzar valores de inactivación microbiana elevadas (> 5 ciclos $\log_{10}$) mediante el efecto sinérgico de ambos tratamientos.

Considerando la posible integración de los subproductos vegetales infusionados en la formulación de nuevos alimentos, surge la necesidad de evaluar el efecto antimicrobiano de los mismos en combinación con otras tecnologías o procesos mínimos de conservación, con el objetivo de obtener productos seguros desde un punto de vista microbiológico, en los que se pueda mantener en mejor medida el valor nutricional del alimento sin procesar, siendo respetuosos con el medio ambiente y realizando una eficiente revaloración de recursos. En consecuencia, se planteó estudiar el efecto antimicrobiano frente a *S.* Typhimurium de las infusiones de subproductos de coliflor y mandarina en

combinación con tratamientos de conservación subletales aplicados por tecnologías no térmicas de conservación que se encuentran en pleno desarrollo e implantación a nivel industrial como alternativa a los tradicionales tratamientos térmicos de pasteurización: Pulsos Eléctricos de Alta Intensidad (PEF) y Altas Presiones Hidrostáticas (HHP).

En el Capítulo III se muestran los resultados obtenidos frente a *S.* Typhimurium, inoculada a una concentración de 10^8 ufc/mL sometida al efecto de un tratamiento subletal por PEF (20 kV/cm – 900 μs), seguido de una incubación en presencia de infusión de subproducto de coliflor y mandarina a diferentes concentraciones (1, 5, 10 %) y temperaturas (10, 22, 37 ^{o}C). Tras el tratamiento de PEF se consiguieron 4 ciclos logarítmicos de inactivación microbiana. Durante la incubación posterior, ambas infusiones mostraron un efecto bacteriostático a la concentración de 1 %, mientras que las concentraciones de 5 y 10 % ejercieron un efecto bactericida durante el almacenamiento. En el caso de la infusión de coliflor al 10 %, se inactivaron 4 ciclos logarítmicos adicionales, alcanzándose la inactivación microbiana completa en un periodo más corto de tiempo que cuando se aplicaba únicamente la infusión (en 2 horas a 37 ^{o}C, en 24 horas a 22 ^{o}C y en 48 horas a 10 ^{o}C) (Tabla 6.2). Del mismo modo, la incubación con infusión de mandarina al 10 % permitió una inactivación microbiana completa en un tiempo menor que cuando no se aplicaba el tratamiento previo de PEF (2 horas a 22 y 37 ^{o}C y en 32 horas a 10 ^{o}C) (Tabla 6.3).

Tabla 6.2 Tiempo necesario para la inactivación completa de una población inicial de 10^8 ufc/mL de *S.* Typhimurium durante su incubación con infusión de coliflor al 10 % (p/v), en combinación o no con un tratamiento de PEF.

Temperatura	Coliflor 10% (p/v)	Coliflor + PEF
37 °C	10 h	2 h
22 °C	140 h	24 h
10 °C	168 h	48 h

Tabla 6.3 Tiempo necesario para la inactivación completa de una población inicial de 10^8 ufc/mL de *S.* Typhimurium durante su incubación con infusión de mandarina al 10 % (p/v), en combinación o no con un tratamiento de PEF.

Temperatura	Mandarina 10% (p/v)	Mandarina + PEF
37 °C	110 h	2 h
22 °C	---	2 h
10 °C	300 h	32 h

La aplicación de tecnologías de conservación en condiciones subletales, como se propone en este trabajo, parece una buena estrategia bajo el punto de vista de la inocuidad microbiana, además de que puede contribuir en el mantenimiento de la calidad de los alimentos ya que están sometidos a condiciones menos estresantes. Sin embargo, bajo estas condiciones pueden aparecer células dañadas subletalmente (Criado et al., 2015; Lim *et al.*, 2013). Las células dañadas son células viables no cultivables en medios selectivos (Wu, 2008; Wesche *et al.*, 2009) y cuyos mecanismos de resistencia pueden ser diferentes a aquellos de las células intactas, por lo tanto, es interesante hacer un estudio de la evolución de esta población. Tal como se puede observar en la

Figura 6.4., el porcentaje de células dañadas y muertas tras 24 horas en incubación con infusión de mandarina al 10 % - 10 ºC fue mucho mayor (aproximadamente 45 % y 33 %, respectivamente) cuando la población microbiana había sido sometida previamente a un tratamiento de PEF que cuando la infusión de mandarina fue aplicada como único tratamiento antimicrobiano (aproximadamente 6 % y 18 %, respectivamente). Por su parte, el porcentaje de células que permanecieron intactas fue mucho menor cuando se aplicaron los dos tratamientos antimicrobianos de forma consecutiva (aproximadamente 22 %) respecto a cuándo se aplicó solamente la infusión de mandarina (aproximadamente 75 %).

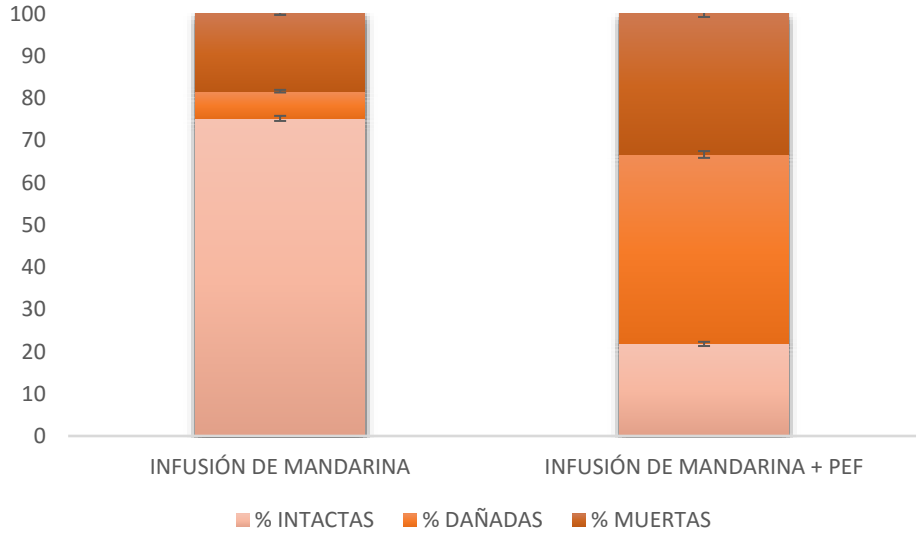

Figura 6.4. Porcentaje de células intactas, dañadas y muertas después de 24 horas en incubación con infusión de mandarina al 10 % tras haber recibido o no un tratamiento previo de PEF.

Por lo tanto, es posible afirmar que existe un efecto sinérgico entre el tratamiento subletal de PEF y la incubación posterior con infusión de subproducto de coliflor o mandarina al 10 %, que permitió la inactivación completa de una población inicial de 10^8 ufc/mL de *S.* Typhimurium en un periodo de tiempo mucho más corto (ver Tabla 6.2 y 6.3). Combinaciones similares entre tratamientos subletales de PEF y otros extractos naturales con

propiedades antimicrobianas han dado lugar a efectos sinérgicos en la inactivación microbiana de otros patógenos alimentarios (Pina-Pérez et al., 2012; Mosqueda-Melgar et al., 2012).

En el Capítulo IV se exponen los resultados obtenidos para *S.* Typhimurium (10^8 ufc/mL) al combinar un tratamiento subletal de HHP (200 MPa – 2 min) con el efecto de la incubación en presencia de infusión de subproducto de coliflor y mandarina al 10 % a 10 y 37 ^{o}C hasta un máximo de 5 días. Con el tratamiento por HHP, no se alcanzaron niveles significativos de inactivación microbiana, únicamente se produjo 1 ciclo logarítmico de daño celular. La posterior incubación de las células tratadas en presencia de las infusiones de subproducto de coliflor y mandarina al 10 % produjo un efecto sinérgico que permitió reducir considerablemente el tiempo necesario para alcanzar la inactivación de *S.* Typhimurium con respecto a los niveles de reducción obtenidos mediante la aplicación de las infusiones en solitario, tal y como se puede observar en las Tablas 6.4 y 6.5.

Tabla 6.4 Tiempo necesario para la inactivación completa de una población inicial de 10^8 ufc/mL de *S.* Typhimurium durante su incubación con infusión de coliflor al 10 % (p/v), en combinación o no con un tratamiento de HHP.

Temperatura	Coliflor 10% (p/v)	Coliflor + HHP
37 °C	10 h	5 h
10 °C	110 h	75 h

Tabla 6.5 Tiempo necesario para la inactivación completa de una población inicial de 10^8 ufc/mL de *S.* Typhimurium durante su incubación con infusión de mandarina al 10 % (p/v), en combinación o no con un tratamiento de HHP.

Temperatura	Mandarina 10% (p/v)	Mandarina + HHP
37 °C	96 h	6 h
10 °C	250 h	54 h

Los datos mostrados en las Tablas 6.2, 6.3, 6.4 y 6.5 evidencian el efecto sinérgico de tratamientos subletales de ambas tecnologías de conservación no térmicas (PEF y HHP) con la capacidad antimicrobiana de las infusiones de subproducto de coliflor y mandarina, reduciendo en gran medida el tiempo necesario para alcanzar la inactivación microbiana completa de *S.* Typhimurium. Efectos sinérgicos similares han sido demostrados en estudios previos como el llevado a cabo por Belda-Galbis (2017), en el que se observó un efecto antimicrobiano sinérgico entre un tratamiento subletal de HHP y un antimicrobiano natural (infusión de *Stevia rebaudiana* Bertoni) frente a *L. monocytogenes*.

El porcentaje de daño celular generado fue mayor cuando se combinaron ambos tratamientos antimicrobianos, HHP y exposición a las infusiones de coliflor y mandarina (Prieto-Calvo et al., 2014; Espina et al., 2013).

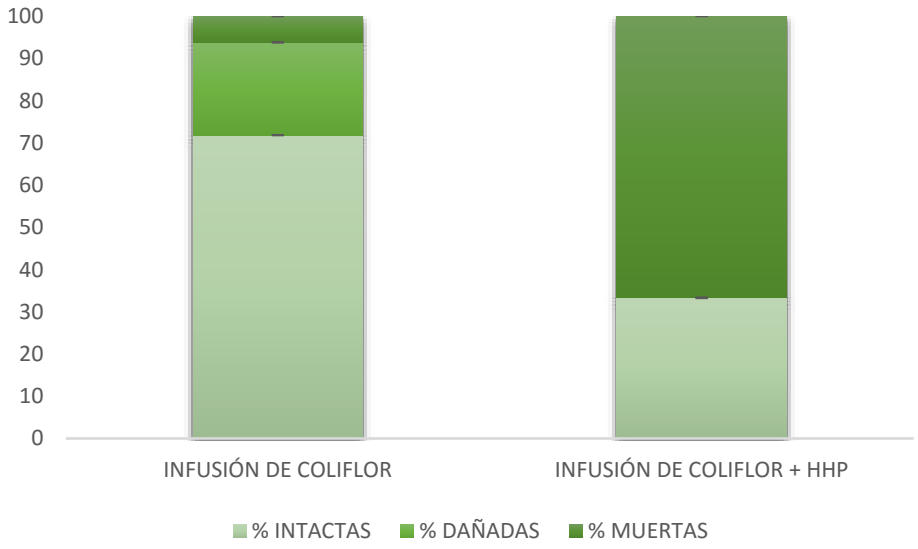

Figura 6.5. Porcentaje de células intactas, dañadas y muertas después de 6 horas en incubación con infusión de coliflor al 10 % tras haber recibido o no un tratamiento previo de HHP.

Como puede observarse en la Figura 6.5., el porcentaje de células que permanecieron intactas después de la incubación durante 6 horas a 37 ^{o}C en infusión de coliflor al 10 % fue mucho menor cuando habían recibido un tratamiento previo de HHP (aproximadamente 33 %) respecto a cuándo únicamente fueron incubadas con la infusión de coliflor como tratamiento antimicrobiano (aproximadamente 72 %). Además, cuando se aplicaron los dos tratamientos antimicrobianos combinados, el porcentaje de células dañadas fue inexistente, evolucionando todas ellas a células muertas (aproximadamente 67 %), respecto al porcentaje de, aproximadamente, 6 % de células muertas alcanzado cuando no se aplicó el tratamiento previo de HHP.

De esta manera, se evidencia la efectividad de las infusiones en caliente de coliflor y mandarina como estrategia de control antimicrobiano, en el marco de la tecnología de barreras, en combinación con tratamientos subletales de PEF y HHP. Sin embargo, como hemos visto (Capítulos III y IV), estos tratamientos generan poblaciones de células dañadas, que en condiciones normales podrían recuperarse, adaptándose a las nuevas condiciones de incubación, adquiriendo nuevas características o capacidades, y que sin embargo, al combinarse con la posterior exposición a subproductos de coliflor y mandarina infusionados, evolucionan a células muertas, eliminando así su riesgo potencial para la seguridad alimentaria.

6.3 MODELIZACIÓN MATEMÁTICA DE LOS RESULTADOS OBTENIDOS PARA LAS DISTINTAS ESTRATEGIAS DE CONSERVACIÓN EN ESTUDIO

Todos los resultados de actividad antimicrobiana de los diferentes subproductos y sus extractos, así como sus combinaciones con tratamientos subletales de PEF o HHP, obtenidos mediante curvas de inactivación en los Capítulos I, III y IV, fueron ajustados a modelos matemáticos, concretamente a la función modificada de Gompertz y a la distribución de frecuencias de Weibull, ampliamente utilizados en estudios similares por su simplicidad y robustez (Belda-Galbis, 2013; Gammariello, 2008; Char et al., 2010). Estos modelos nos permiten calcular los parámetros cinéticos que definen el patrón de inactivación microbiana bajo las diferentes condiciones de estudio, corroborando que la concentración de subproducto y la temperatura de incubación, influyen significativamente en el efecto antimicrobiano observado (Tabla 6.6). En presencia de los subproductos estudiados se observa inactivación microbiana caracterizada por la velocidad de inactivación mostrada en la tabla 6.6.

Tabla 6.6. Valores del parámetro cinético *b* de Weibull obtenidos para la inactivación microbiana de *S.* Typhimurium en presencia de infusión de coliflor y mandarina al 10 %, combinadas o no con tratamientos previos de HHP o PEF durante su incubación a 10 y a 37 ºC.

		INFUSIONES 10%	INFUSIONES + HHP	INFUSIONES + PEF
COLIFLOR	37 ºC	0.38 ± 0.03	0.55 ± 0.04	1.46 ± 0.07
	10 ºC	0.09 ± 0.01	0.26 ± 0.01	0.35 ± 0.033
MANDARINA	37 ºC	0.77 ± 0.39	1.38 ± 0.08	1.39 ± 0.25
	10 ºC	0.13 ± 0.03	0.23 ± 0.05	1.35 ± 0.53

Teniendo en cuenta que tras la aplicación de un tratamiento subletal de PEF o HHP la población de *S.* Typhimurium incubada en medio de referencia no moría sino que entraba en fase de crecimiento, las variables cinéticas nos permitieron comprobar que existe un aumento significativo de la velocidad de inactivación microbiana cuando la población inicial de *S.* Typhimurium es sometida a un tratamiento subletal de PEF o HHP previo a su incubación en presencia de las infusiones de subproductos de coliflor y mandarina, en comparación con su velocidad de inactivación cuando eran incubadas únicamente con las infusiones, sin tratamiento previo, constatando así el efecto sinérgico de la combinación de ambos tratamientos antimicrobianos frente a *S.* Typhimurium (ver Tabla 6.6).

Los estudios realizados hasta la fecha en la evaluación de la capacidad antimicrobiana de las brassicas y los cítricos, se han realizado desde un enfoque cualitativo (Blazevic et al, 2010; Sousa et al., 2008). Los datos mostrados en el Capítulo I, III y IV contribuyen por primera vez a evaluar de forma cuantitativa, y mediante modelización matemática, el importante efecto antimicrobiano

presente en los extractos de estos subproductos hortofructícolas por sí mismos, o en combinación con tecnologías no-térmicas de conservación de alimentos mediante tratamientos subletales de PEF o HHP.

6.4 CAMBIOS DE VIRULENCIA EN CÉLULAS DE *SALMONELLA* TRATADAS MEDIANTE PEF, HHP, Y ANTIMICROBIANOS NATURALES UTILIZANDO *C. ELEGANS* COMO MODELO IN VIVO

A pesar de los beneficios que supone la aplicación combinada de tratamientos subletales desde el punto de vista, tanto de la inocuidad como de su menor impacto potencial sobre la calidad del alimento, existen riesgos derivados de su aplicación que conviene evaluar. Entre ellos, la posibilidad de que la aplicación de tratamientos subletales de forma continuada pueda dar lugar a una población de células dañadas que, en condiciones óptimas, se recuperaría y podría adquirir nuevas características o capacidades que le permitan desarrollar resistencia al tratamiento antimicrobiano aplicado y/o cambios en su virulencia al infectar al organismo hospedador.

Como se describió en el Capítulo V se observó que bajo la aplicación de todos los tratamientos subletales estudiados, *S.* Typhimurium fue capaz de desarrollar mecanismos de resistencia. De este modo, *S.* Typhimurium se hizo resistente tras ser expuesta a 3 tratamientos subletales consecutivos con infusión de coliflor y a 4 tratamientos consecutivos con PEF y HHP. Aunque la aparición de resistencia microbiana a antimicrobianos naturales procedentes de plantas (residuos de agroindustria o plantas aromáticas) no ha sido muy estudiada hasta el momento, algunos autores como Kisluk et al (2013) ó Di Pasqua et al., (2006) han demostrado la capacidad de generar adaptaciones microbianas por exposición a concentraciones subletales de aceites esenciales, en patógenos alimentarios como *E. coli, S.* Typhimurium o *B. cereus*. Sin

embargo, aunque las poblaciones microbianas que desarrollan resistencia a un tratamiento antimicrobiano pueden modificar también su virulencia (Rajkovic et al., 2009), en el estudio presentado en esta tesis, un aumento de la resistencia antimicrobiana no pareció llevar implícito un aumento de la virulencia del microorganismo. De hecho, los resultados del Capítulo V han demostrado que la infección de *C. elegans* con el microorganismo *S.* Typhimurium resistente/adaptado a cada uno de los 3 tratamientos estudiados (PEF, HHP e infusión de subproducto de coliflor) disminuye su efecto virulento sobre *C. elegans*, acortando en menor medida su esperanza de vida (23-25 días) respecto a los valores obtenidos cuando *C. elegans* es expuesto al microorganismo no-tratado (21 días). De la misma forma, los percentiles estimados para la distribución de supervivencia de *C. elegans* alimentado con *S.* Typhimurium tratada o no con cada uno de los tratamientos subletales estudiados muestran cómo el tiempo de supervivencia es mayor cuando *C. elegans* es infectado con *S.* Typhimurium tratada con respecto a la no tratada (5% de nematodos supervivientes en 19 días cuando son infectados con *S.* Typhimurium tratada con infusión de subproducto de coliflor respecto a 16 días cuando son infectados con *S.* Typhimurium no tratada).

No obstante hay que destacar, que la esperanza de vida de *C. elegans* en condiciones óptimas (alimentado con *E. coli* OP50) es de aproximadamente 3 semanas, pero la infección por *S.* Typhimurium provoca una disminución de la misma, debido a que las células bacterianas van colonizando el lumen de su intestino y a medida que los nematodos van envejeciendo, su sistema inmune intestinal va perdiendo efectividad, permitiendo en mayor medida la acumulación de células patógenas y ocasionando su muerte en un periodo de tiempo menor (Portal- Celhay et al., 2012; Labrousse et al., 2000; Aballay et al., 2000) (ver Figura 6.6).

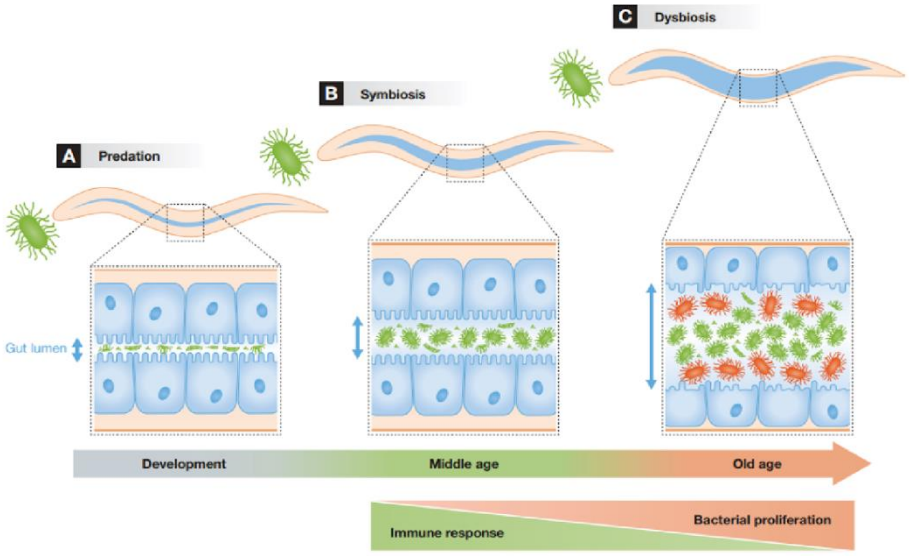

Figura 6.6. Evolución de la microbiota intestinal, propia: (verde) - patógena (rojo), en *C. elegans* a medida que avanza el ciclo de vida del nematodo (Cabreiro y Gems, 2013).

En este sentido los estudios de Portal - Celhay et al. (2012) definen una clara relación inversa entre la carga microbiana acumulada en el intestino del nemátodo, y la longevidad del mismo. Bajo condiciones de alimentación óptimas para *C. elegans* (*E. coli* OP50 10^2 ufc/mL a tiempo 0) la carga microbiana intestinal aumenta de 2 a 4 ciclos $\log_{10}$ durante los primeros 4 días de estudio,

permaneciendo en dichos valores hasta el 14º día del ciclo, momento en el que ha muerto el 50 % de la población. Sin embargo, en los estudios de exposición realizados con *Salmonella* SL 1344 (10^2 ufc/mL) se alcanzan valores de carga microbiana acumulada a nivel intestinal próximos a 5 ciclos $\log_{10}$ tras 4 días de estudio, valores que siguen en ligero aumento hasta el día 8º del ciclo, reduciendo la esperanza de vida del nematodo en más de 5 días respecto del control alimentado con *E. coli* OP50.

El patrón de reproducción del nematodo *C. elegans* también se ve afectado por la infección con *S.* Typhimurium, deteniendo su puesta de huevos a partir del día 5, tiempo que coincide, según estudios previos, con el requerido para alcanzar la infección completa del lumen del nematodo por parte de *S.* Typhimurium (Gardner et al., 2013; Labrousse et al., 2000; Aballay et al., 2000). Además, otros estudios han demostrado que la infección por *S.* Typhimurium provoca que *C. elegans* cese en su puesta de huevos y los retenga en su útero como mecanismo de defensa para proteger su progenie en situaciones desfavorables. Esto provoca que en ocasiones los huevos eclosionen en su interior, provocando la muerte anticipada del nematodo. Sin embargo, cuando *C. elegans* fue infectado con *S.* Typhimurium resistente a cada uno de los 3 tratamientos evaluados se observó, además, que, aunque *C. elegans* detenía su puesta de huevos el día 5 del ciclo, el número de huevos que ponía durante esos primeros días era significativamente mayor. Esto puede deberse a que las poblaciones de *S.* Typhimurium resistentes a los 3 tratamientos antimicrobianos estudiados pueden haber modificado su mecanismo de patogenicidad aumentando el estrés generado en *C. elegans*, el cual activa un mecanismo de defensa a modo de respuesta que consiste en aumentar su tasa de reproducción en un periodo corto de tiempo con el objetivo de protegerse de las bacterias patógenas y asegurar la continuidad de su progenie antes morir a causa de la infección intestinal (Schulenburg et al., 2004).

La movilidad de *C. elegans* cuando fue infectado por *S.* Typhimurium resistente a cada uno de los 3 tratamientos antimicrobianos en estudio fue disminuyendo a lo largo de su vida, tal y como ocurre cuando es infectado con *S.* Typhimurium no-tratada, no apreciándose diferencias significativas. Esto puede deberse a que cuando los nematodos entran en contacto con una bacteria patógena, su sistema nervioso activa los nervios motores que comunican con los músculos del eje ventral-dorsal. Los nematodos tratan de evitar al patógeno aumentando su actividad metabólica y realizando movimientos rápidos. Sin embargo, no pueden evitar a los microorganismos patógenos, ya que se encuentran extendidos por toda la superficie de la placa Petri en la que se encuentran y acaban siendo infectados. Además, *C. elegans* en condiciones óptimas también disminuye su movilidad a lo largo de su vida debido a su envejecimiento (Altun et al., 2009; Kawli et al., 2010).

Todo esto nos permite concluir que, aunque *S.* Typhimurium es capaz de desarrollar resistencia frente a tratamientos antimicrobianos subletales, las modificaciones fenotípicas que han conllevado en estos estudios la adquisición de esa resistencia no van asociadas a una mayor virulencia durante su infección a un organismo hospedador, sino que la virulencia de su patogenicidad se ve disminuida.

6.5 VALIDACIÓN DEL EFECTO ANTIMICROBIANO DE LA INFUSIÓN DE COLIFLOR MEDIANTE EL ESCALADO EN PLANTA PILOTO

Teniendo en cuenta los buenos resultados obtenidos tras evaluar la capacidad antimicrobiana de las infusiones, su efecto combinado con las tecnologías de PEF y HHP y sus posibles efectos sobre la virulencia de *S*. Typhimurium, en el capítulo VI, se llevó a cabo la evaluación de la infusión obtenida en condiciones semi-industriales (50 litros).

Los resultados mostraron que la capacidad antimicrobiana de la infusión de coliflor se mantiene al escalar su producción a nivel industrial (6 ciclos logarítmicos de inactivación en 8 horas a 37 ^{o}C). Estos resultados nos confirman, definitivamente, el potencial que presenta la revalorización de los subproductos de la industria agroalimentaria en nuestro caso como antimicrobianos naturales para mantener la calidad y la seguridad alimentaria en sus productos durante la vida útil en refrigeración de los mismos.

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CONCLUSIONES

7. CONCLUSIONES

A continuación se detallan las principales conclusiones obtenidas en la presente tesis doctoral:

I. Los subproductos de coliflor, brócoli, okara, mandarina, naranja y limón brutos deshidratados poseen un elevado potencial antimicrobiano frente a los patógenos alimentarios más relevantes, tanto Gram positivos como Gram negativos, siendo *S.* Typhimurium el más sensible, alcanzándose hasta 8 reducciones logarítmicas.

II. Los extractos obtenidos mediante la tecnología ASE también presentan capacidad antimicrobiana frente a los principales patógenos alimentarios.

III. Las infusiones obtenidas a partir de los subproductos de coliflor y mandarina poseen un marcado potencial antimicrobiano frente a *S.* Typhimurium, llegando a producir una reducción de 5 ciclos logarítmicos en 10 h y en 80 h a 37 ^{o}C respectivamente; mejorando notablemente su homogeneidad y aplicabilidad en relación a los subproductos brutos.

IV. La capacidad antimicrobiana de los subproductos podría estar relacionada tanto con el contenido como con el perfil de compuestos polifenólicos. De acuerdo a los resultados, los subproductos en bruto son los que tienen mayor contenido en polifenoles, seguido de las infusiones y de los extractos ASE.

V. La combinación de infusiones de los subproductos de coliflor y mandarina con tratamientos subletales de PEF y HHP tienen un efecto sinérgico sobre la inactivación de *S.* Typhimurium. Esta sinergia se debe, probablemente, al mayor porcentaje de células dañadas que se generan por la combinación de infusiones y tecnologías de conservación no térmicas.

VI. Los datos de inactivación en presencia de las infusiones solas o combinadas con PEF o HHP se ajustaron bien a la distribución de frecuencias de Weibull obteniéndose los parámetros cinéticos para las diferentes condiciones estudiadas. El parámetro cinético de velocidad se incrementa hasta en 10 veces con la combinación de tratamientos, reflejando de esta manera el efecto sinérgico observado.

VII. La aplicación de tratamientos subletales consecutivos con infusión de subproducto de coliflor, PEF o HHP da lugar a la aparición de resistencia microbiana en *S.* Typhimurium, reflejadas bien en una menor inactivación o incluso en el crecimiento del microorganismo tras el tratamiento.

VIII. Los estudios realizados con *C. elegans* como organismo modelo indican que los incrementos en la resistencia observados en *S.* Typhimurium no conllevan aumento en su virulencia ya que se observa un aumento en la esperanza de vida y la puesta de huevos del nematodo alimentado con la *S.* Typhimurium resistente.

IX. Por último y como conclusión general, podemos decir que es posible la revalorización de los subproductos de la industria agroalimentaria estudiados como antimicrobianos naturales, pudiendo ser una medida de control adicional del crecimiento microbiano en caso de que se produzca rotura de la cadena de frio, ya que se alcanzan elevados niveles de inactivación de patógenos alimentarios, y dado su efecto sinérgico en combinación con tratamientos subletales de PEF o HHP.

ANEXOS

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Antimicrobial Potential of Cauliflower, Broccoli, and Okara Byproducts Against Foodborne Bacteria

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Abstract

The antimicrobial potential of cauliflower, broccoli, and okara byproducts was assessed against Gram-positive and Gram-negative bacteria. *Salmonella enterica* serovar Typhimurium, *Escherichia coli* O157:H7, *Bacillus cereus*, and *Listeria monocytogenes* serovar 4b growth behavior was assessed under exposure to 5% vegetable byproducts added to the reference medium, buffered peptone water (0.1% [wt/vol]), at 37°C. Although the byproducts were not effective against *L. monocytogenes*, they were bactericidal against *Salmonella* Typhimurium, *E. coli* O157:H7, and *B. cereus*. The most promising results were achieved with the cauliflower–*Salmonella* Typhimurium combination, because the bacterial population was reduced by 3.11 $\log_{10}$ cycles after 10 h of incubation at 37°C as a result of 5% cauliflower addition. Further studies were carried out for this combination, at different cauliflower concentrations (0, 0.5, 1, 5, 10, and 15%) and at temperatures in the range of 5–37°C. The greatest inactivation level (6.11 $\log_{10}$ cycles) was achieved at refrigeration temperature (5°C) using 15% cauliflower addition. Both temperature and cauliflower concentration significantly ($p \leq 0.05$) influenced the *Salmonella* Typhimurium inactivation level. The kinetic parameters were adjusted to mathematical models. The modified Gompertz mathematical model provided an accurate fit (root-mean-square error (RMSE) [0.00009–0.21] and *adjusted*-R^2 [0.81–0.99]) to experimental *Salmonella* Typhimurium survival curves describing inactivation kinetics of the pathogen to the antimicrobial effect of cauliflower byproduct.

Introduction

Every year, the food-processing industries generate large amounts of food waste worldwide. The elimination of these residues usually involves a cost to the producer, due to landfill or incineration, which generates negative effects on the environment (O'Shea *et al.*, 2012). Thus, several studies focus on recovery, recycling, and upgrading of food waste, turning it into byproducts for use as operating supplies or as ingredients in new product formulations. Recognition of the value of byproducts that can be incorporated into new production processes would reduce demand for raw materials and restrain exploitation of natural resources, with consequent benefits to society.

The valorization of agriculture and food byproducts is a requirement of the European Union (EUROSTAT, 2010) supporting sustainable development. Vegetable residues are inexpensive, available in large amounts, and characterized by high dietary fiber content (Stojceska *et al.*, 2008). So far, some valuable applications of these agrifood wastes involve animal feedstocks, fertilizers, paper industry application, extraction of essential oils and fragrances, composting, bioconversion, and new ingredients in product formulations (Henningsson *et al.*, 2004).

For new product formulations, vegetable byproducts could be a valuable source of nutritional and antimicrobial compounds. Among them, there are two very important plant families, Brassicaceae and Fabaceae. Broccoli and cauliflower, the main crops of the Brassicaceae family, and soybean, the main crop of Fabaceae, contain phytochemical components with reported antioxidant and anticarcinogenic properties (Tyug *et al.*, 2010). Worldwide production of broccoli and cauliflower was 22,226,957 tons in 2009. About 75% of this production belongs to China and India (USDA, 2009). The antioxidant properties of these vegetables could have a significant impact in the field of nutraceuticals and in food-processing industry applications (Cabello-Hurtado *et al.*, 2012), mainly because of the polyphenol and glucosinolate contents (O'Shea *et al.*, 2012).

With regard to Fabaceae, soybean is one of the most commonly consumed legumes in the world, with 200 million tons produced per year (FAOSTAT, 2010). Currently, the main producer is the United States (32%), followed by Brazil (28%) and Argentina (21%) (Nahashon and Kilonzo-Nthenge, 2011). After extraction of water from soybeans to produce soymilk and tofu, a byproduct called okara is obtained. Consequently, scientific and industrial research is required to find potential applications

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Short communication

Antimicrobial activity of cauliflower (*Brassica oleracea* var. Botrytis) by-product against *Listeria monocytogenes*

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ABSTRACT

The antimicrobial potential of cauliflower by-product was assessed against *Listeria monocytogenes* at different concentrations [0–15]% (w/v) and incubation temperatures [5–22] °C, in reference medium. Survival curves under cauliflower by-product exposure versus time were obtained. The bactericidal effect of the cauliflower by-product was shown at concentration levels ≥5% (w/v) at all temperatures studied. Both temperature and cauliflower by-product concentration significantly ($p \leq 0.05$) influenced the reduction levels achieved in the initial *L. monocytogenes* contamination. Growth/inactivation kinetics of *L. monocytogenes* under cauliflower by-product exposure were fitted to a modified Gompertz equation for each of the conditions studied (concentration–temperature combinations), and maximum inactivation rate (μ_{max}) and lag phase duration (t_{lag}) parameters were obtained. It was observed that the higher the incubation temperature and the cauliflower by-product concentration added to the reference medium, the higher the μ_{max} and the lower t_{lag}. In spite of this, the maximum inactivation level achieved at stationary phase was 2.25 $\log_{10}$ cycles after 20 days of exposure to a 15% (w/v) concentration of cauliflower added to reference medium. Both conclusions indicate the effective control that cauliflower by-product could provide as an additional preservation measure during shelf-life of refrigerated RTE products, specifically when there is an accidental rise in storage temperature, e.g. in cold chain breakdown situations.

1. Introduction

Listeria monocytogenes is an opportunistic psychrotrophic microorganism with a reported capability of multiplying itself at temperatures down to a few degrees below 0 °C, persisting in refrigerated industrial settings. The incidence of listeriosis mainly affects young and elderly people (over 65 years), pregnant women and immune-compromised people (Adzitey et al., 2010; Gambarin et al., 2012), with high morbidity and mortality rates associated with *L. monocytogenes* (about 30%). Nowadays, *L. monocytogenes* is one of the most worrying foodborne pathogens, with one of the highest hospitalization rates (91%) and long-term sequels in affected patients (Denny & McLauchlin, 2008). Despite the fact that a wide variety of foods may be contaminated with *L. monocytogenes*, outbreaks and sporadic cases of listeriosis are predominately associated with ready-to-eat (RTE) foods – a large, heterogeneous category of foodstuffs that can be subdivided in many different ways and vary from country to country according to local eating habits, availability and integrity of the chill chain, and regulations specifying, for example, the maximum temperature at retail level.

Recent sporadic cases of listeriosis have been described in Europe (from 2006 to 2010) (Cairns & Payne, 2009; Goulet, Hedberg, Le Monnier, & de Valk, 2008; Kvistholm et al., 2010). A large outbreak was recorded in Canada in 2008 (PHAC, 2008), and there has been an increasing number of *L. monocytogenes* food isolates in the USA and Canada in recent years. RTE products are likely to act as vehicles for transmission of *L. monocytogenes*, mainly because they do not require additional preparation or cooking before consumption. RTE products (e.g. pasteurized milk, ice cream, fermented meat and cold smoked fish) can be contaminated by *L. monocytogenes* during post-processing steps, and then it can proliferate during storage at refrigeration temperature because of the psychrotrophic nature of the microorganism (Cobo et al., 2009; Zhu, Du, Cordray, & Uk Ahn, 2005). In order to prevent *L. monocytogenes* contamination in RTE products, some natural bioactive substances with antimicrobial capability are added to control pathogenic bacteria in food systems (Lianou & Sofos, 2007). Among possible added natural antimicrobials, increasing interest is focussing on vegetable by-products, as rich natural sources of fibre, vitamins, minerals, secondary plant metabolites and antioxidants.

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Escherichia coli O157:H7 and *Salmonella* Typhimurium inactivation by the effect of mandarin, lemon, and orange by-products in reference medium and in oat-fruit juice mixed beverage

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ABSTRACT

The antimicrobial capability of three water extracts of citrus peels was evaluated against *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7 at various concentrations (0.5, 1, 5, 10%) and temperatures (5, 10, 22 °C) in a reference medium. The best of them was mandarin by-product, achieving a maximum inactivation level against *S.* Typhimurium (8 $\log_{10}$ cycles) with 5% at 5 °C. Also, this by-product had the highest total polyphenol content. Mandarin by-product showed a bactericidal effect in a food matrix also at 5 °C (≈2 $\log_{10}$ cycles). All results were adjusted to the Weibull model and the *b* values indicated that the higher concentration of mandarin, the greater the inactivation rate in reference medium, without significant differences between 5 and 10%. Similarly, in the food matrix, the inactivation rate of *S.* Typhimurium was higher when the mandarin by-product was added. Therefore, the mandarin by-product could be used as a control measure of *S.* Typhimurium in pasteurized products, which are stored under refrigeration.

1. Introduction

Citrus is the largest fruit crop worldwide, with an annual production of approximately 100 million tons. The main world producers are Brazil, the USA and Mediterranean countries (Djilas, 2009; Ghafar, Prasad, Weng, & Ismail, 2010). The industrial production of juices and other citrus derivatives generates approximately 15 million tons of citrus waste a year worldwide, which mainly consists of peel, seeds, and the fruit pulp. Citrus waste is usually consigned to landfill or incineration, which generates negative effects on the environment and a cost to the producers (O'Shea, Arendt, & Gallagher, 2012).

This valueless citrus waste can be considered as a renewable source of raw material whose use in various industrial fields could have a double benefit, economic and technological, as a result of its valorization (Martín-Luengo, Yates, Diaz, Saez Rojo, & Gonzalez Gil, 2011; Schieber, Stintzing, & Carle, 2001;). Since 2010 generalized agri-food by-product valorization has been a European Union requirement (EUROSTAT, 2010) and many research studies nowadays are focused on recovering, revaluing, and recycling these by-products. One way of valorizing these by-products is the formulation of new products with added nutritional value. Citrus by-products are rich in functional compounds such as carotenoids and flavonoids, among others (O'Shea et al., 2012), whose antioxidant, anticarcinogenic, antiviral, and anti-inflammatory properties are well known. Citrus derivative compounds have an important nutritional and flavoring value, and an antimicrobial capability has also been attributed to some of them, mainly due to ferulic acid, hydrocinnamic acid, yaniding glucoside, hisperidin, vitamin C, carotenoid, and naringin (Ghafar et al., 2010). In this sense, they could be used like natural antimicrobials to control the growth of foodborne pathogens, replacing the chemical compounds which are used currently. Also, they could be used as an additional control measure of the microbial growth in situations of cold chain breakdown in pasteurized food that is stored in refrigeration (Sanz-Puig, Pina-Pérez, Rodrigo, & Martínez-López, 2015).

In this context, the aim of this study was to evaluate the antimicrobial effect of water extracts of by-products of citrus fruits – mandarin, orange, and lemon – against two of the foodborne pathogens of most concern that are found in low-acid beverages: *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7.

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Chapter

USE OF NATURAL ANTIMICROBIALS AS A TREATMENT OPTION TO CONTROL *SALMONELLA* TYPHYMURIUM

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ABSTRACT

Salmonella is a foodborne pathogen that causes a huge amount of cases of typhoid fever, gastroenteritis, and deaths every year throughout the world. Although salmonellosis cases in humans have decreased in the last five years, *Salmonella* remains the second most common zoonosis in humans. Foodborne outbreaks caused by *Salmonella* have also reduced in recent years, but they have been linked with contamination of eggs and egg products, cheese, mixed foods, and fresh fruits and vegetables. Therefore control measures for this microorganism are very important to prevent and control *Salmonella* at relevant stages of production, processing, and distribution, especially in primary production, thus reducing its prevalence and the risk it poses to public health. In this context, research carried out to find antimicrobial compounds from natural sources is important because they could be used as additives in new product formulations, where they could exercise an additional measure to control *Salmonella* growth and have an important impact from economic and food safety points of view.

By-products from the food industry are a potential source of inexpensive raw materials, and are rich in bioactive components whose technological and antimicrobial properties are still scarcely studied. With the aim of covering this gap, the objective of the present study was focused on evaluating the antimicrobial properties of three citrus by-products – mandarin, orange, and lemon – against *Salmonella enterica* serovar

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Effect of polyphenol content on the antimicrobial activity of natural extracts from agro-industrial by-products

Einfluss des Polyphenolgehaltes auf die antimikrobielle Wirkung natürlicher Extrakte aus agarindustriellen Nebenprodukten

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Summary

The main objective of the present study was to investigate the effect of the conditions of extraction by Accelerated Solvent Extraction (ASE) technology on the bioactive antimicrobial activity of extracts from by-products of cauliflower, broccoli, orange, and mandarin. The antimicrobial activity of extracts, with concentrated phenol content, was evaluated against four of the most important foodborne pathogens: *Salmonella enterica* serovar Typhimurium, *Escherichia coli* O157:H7, *Bacillus cereus,* and *Listeria monocytogenes*. The largest phenol content (1252.12 ± 38.29 µg gallic acid/mL) was recovered from cauliflower extract. Cauliflower and mandarin extracts were effective against both Gram-positive and Gram-negative bacteria, showing the highest inhibition zones, 16 ± 1 mm and 17 ± 0.4 mm respectively, against 10^5 cfu/mL *S.* Typhimurium. The antimicrobial effectiveness of the extracts was influenced by the ASE extraction conditions, initial contamination level, and microbial strain.

Keywords: vegetable by-products, Accelerated Solvent Extraction, foodborne pathogens, phenols, natural antimicrobials

Zusammenfassung

Das Hauptziel der vorliegenden Studie war es, die Auswirkungen der Extraktionsbedingungen der Accelerated Solvent Extraction (ASE) Technologie auf die bioaktive antimikrobielle Wirkung von Extrakten aus Nebenprodukten von Blumenkohl, Brokkoli, Orange und Mandarine zu untersuchen. Die antimikrobielle Wirkung der Extrakte mit konzentriertem Phenolgehalt wurde auf vier der wichtigsten lebensmittelbedingten Krankheitserreger untersucht: *Salmonella enterica* Serovar Typhimurium, *Escherichia coli* O157: H7, *Bacillus cereus* und *Listeria monocytogenes*. Der höhste Phenolgehalt (1252,12 ± 38,29 µg Gallussäure/ml) wurde aus dem Blumenkohl-Extrakt gewonnen. Blumenkohl- und Mandarinen-Extrakte waren sowohl gegen Gram-positive als auch Gram-negative Bakterien wirksam. Die größten Hemmzonen (16 ± 1 mm und 17 ± 1 mm) waren gegen *S.* Typhimurium zu beobachten. Die antimikrobielle Wirksamkeit der Extrakte wurde von den ASE-Extraktionsbedingungen, des anfänglichen Kontaminationsgrades und der Bakterienstämme beeinflusst.

Schlüsselwörter: Beschleunigte Lösemittelextraktion, lebensmittelbedingte Krankheitserreger, Phenol, natürliche antimikrobielle Stoffe

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Effect of pulsed electric fields (PEF) combined with natural antimicrobial by-products against *S.* Typhimurium

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ABSTRACT

The effect against *Salmonella enterica* serovar Typhimurium of PEF treatment combined with cauliflower and mandarin by-product infusions at several concentrations (0, 1, 5, and 10% (w/v)) was evaluated at various incubation temperatures (10, 22, and 37 °C). The possible synergistic antimicrobial action of the combined process of Pulsed Electric Field (PEF) technology followed by exposure to the by-product infusions and the occurrence of sublethal cellular damage were also studied. Antimicrobial kinetics of by-product infusions alone or following PEF treatment were fitted to a Weibull model. Both mandarin and cauliflower by-product infusions showed a maximum antimicrobial effect against *S.* Typhimurium after 10 h at 37 °C when the microorganism was exposed to 10% of by-product infusion, achieving reductions of initial bacterial load up to undetectable levels. The effect of the PEF treatment (20 kV–900 μs) caused a reduction of 4 log cycles of the initial cell population (10^8 cfu/mL) of *S.* Typhimurium and 1 log cycle (90%) of cellular damage. Moreover, when the PEF pre-treated *S.* Typhimurium population was subjected to subsequent incubation in the presence of both by-product [10%] infusions, the microbial inactivation was faster, achieving a reduction of the initial bacterial load (4 $\log_{10}$ cycles) up to undetectable levels in 2 h. The kinetic values of the Weibull model were obtained. The higher the concentration of by-product infusion, temperature, and PEF treatment applied, the greater the kinetic parameter "b" values, which are related to the microbial inactivation rate. Therefore, the addition of cauliflower and mandarin by-product infusions could be a good additional control measure contributing to ensure bacterial counts below recommended limits in pasteurized PEF products during their storage at refrigeration temperatures.

1. Introduction

In the last few years, international organizations such as the World Health Organization (WHO) and Food Agricultural Organization (FAO) have shown their concern about microbiological contamination in the food chain, because population mobility and food globalization have led to an increase in food outbreaks (WHO, 2008; EFSA, 2010).

One of the most important foodborne pathogens is *Salmonella*, which causes approximately 93.8 million foodborne illness outbreaks and 155,000 deaths per year (Majowicz et al., 2010). *Salmonella enterica* serovar Typhimurium is especially related to meat, eggs, and fresh fruits and vegetables (EFSA, 2011). In the last few years, the incidence of these foodborne outbreaks has been greater, and has increased people's concern about them (Pui et al., 2011). Therefore, one of the aims of current food research is to avoid outbreaks caused by foodborne pathogens such as *Salmonella*.

Traditionally, thermal treatment was the most used mechanism to guarantee the microbial safety of food products. Now, however, new non-thermal treatments have been developed to preserve food products, maintaining their organoleptic and nutritional properties (Knorr et al., 2011; Barret & Lloyd, 2011). Among the most validated non-thermal treatments applied to food preservation, a notable tendency is the addition of natural antimicrobial compounds from plants (Cava, Nowak, Taboada, & Marin-Iniesta, 2007; Ferrer, Ramón, Muguerza, Marco, & Martinez, 2009) or the application of new non-thermal technologies such as High Hydrostatic Pressure or Pulsed Electric Fields (PEF) (Aymerich, Jofré, Garriga, & Hugas, 2005; Mosqueda-Melgar, Raybaudi-Massilia, & Martín-Belloso, 2012).

The development of non-thermal technologies such as PEF for food preservation has increased in recent years, mainly because of the demand for potential methods to ensure not only the microbiological harmlessness of products but also the preservation of their organoleptic and nutritional properties. In this respect, PEF technology appears to be a good alternative to thermal pasteurization processes, only applied to liquid products but with good prospects for being used in the dairy and juice industries (Pina-Pérez, Martínez-López, & Rodrigo, 2012). In

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Combined effect of high hydrostatic pressure (HHP) and antimicrobial from agro-industrial by-products against *S.* Typhimurium

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ABSTRACT

The inactivation potential of HHP treatment (200 MPa-2 min) was evaluated against *Salmonella enterica* serovar Typhimurium in cauliflower and mandarin by-product infusions at 37 and 10 °C. By-product infusions exerted a strong antimicrobial effect used alone, achieving 5 log cycles of bacterial reduction for cauliflower by-product infusion after 10 h and for mandarin by-product infusion after 80 h, at 37 °C. The HHP treatment caused only one log cycle of cellular damage, but when inoculated cauliflower or mandarin by-product infusions were subjected to HHP treatment the antimicrobial effect against *S.* Typhimurium was enhanced, achieving 5 log cycles of inactivation in 6 h at 37 °C in both cases. Inactivation curves were adjusted to the Weibull equation and the kinetic parameters (*b* and *n*) were obtained. When HHP treatment was combined with by-product infusions, the inactivation rates were greater than when either of the by-product infusions was added separately. In conclusion, a synergistic antimicrobial effect against *S.* Typhimurium appeared to take place when HHP treatment was combined with cauliflower or mandarin by-product infusion. These infusions could be considered as an additional microbial control measure to guarantee the food safety and food quality of pasteurized food products that are stored under refrigeration.

1. Introduction

Salmonella spp. is a foodborne pathogen, which cause approximately 93.8 million of foodborne disease outbreaks worldwide and 155,000 deaths per year (Majowicz et al., 2010). In 2010, 99,020 cases of salmonellosis were reported in the EU and *Salmonella* spp. were mainly detected in chicken and turkey (EFSA, 2011). In the USA, more than 40,000 cases of salmonellosis are detected every year and products of animal origin are the main source of *Salmonella* spp. (Finstad, O'Bryan, Marcy, Crandall, & Ricke, 2012). Although more than 2500 serotypes of *Salmonella enterica* have been identified, *Salmonella enterica* serovars Typhimurium and Enteritidis are the most common causes of human salmonellosis worldwide (Kramarenko, Nurmoja, Karssin, & Meremae, 2014).

Therefore, the food industry needs to guarantee food safety in relation to *S. enterica*. Many products potentially contaminated with *S. enterica* are now processed by using new non-thermal technologies, such as oscillatory magnetic fields, radiation, ultrasounds, pulsed electric fields and high hydrostatic pressure (HHP), and studies are needed to identify the different control measures alone or combined to fight against *S. enterica*. Among them, HHP technology has shown that it can achieve suitable levels of microbial inactivation, preserving sensory and nutritive properties (Polydera, Stoforos, & Taoukis, 2003).

Moreover, several research studies have shown that the antimicrobial effect of HHP treatment is greater when it is combined with various natural antimicrobials, achieving a synergistic effect between them (Feyaerts, Rogiers, Corthouts, & Michiels, 2015; Montiel, Martín-Cabrejas, & Media, 2015; Oliveira, Ramos, Eamos, Piccoli, & Cristianini, 2015). This synergistic effect allows the use of lower intensities in HHP treatments and lower concentrations of natural antimicrobials, achieving the same microbial reduction with less impact on sensory and nutritional properties (Pina-Pérez, Rodrigo, & Martínez, 2015).

Several studies have shown that some vegetable by-products are good sources of bioactive compounds with health benefits such as antioxidant, anti-inflammatory and antimicrobial properties (Balasundram, Sundram, & Samman, 2006; Peschel et al., 2006).

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