UNIVERSIDAD DE CHILE - FACULTAD DE CIENCIAS - ESCUELA DE PREGRADO

Laboratory analysis of industrial food safety protocols for fruit export

Seminario de Título entregado a la Universidad de Chile en cumplimiento parcial de los requisitos para optar al Título de Ingeniero en Biotecnología Molecular.

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INFORME DE APROBACIÓN SEMINARIO DE TITULO

Se informa a la Escuela de Pregrado de la Facultad de Ciencias, de la Universidad de Chile que el Seminario de Título, presentado por el Sr.

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Ha sido aprobado por la Comisión de Evaluación, en cumplimiento parcial de los requisitos para optar al Título de Ingeniero en Biotecnología Molecular.

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Santiago de Chile, 1 de Agosto de 2018

INFORMACIÓN PRELIMINAR

ii. Autobiografía del autor

Francisco José Iglesis Honorato

Nací un martes 29 de Julio de 1986 en Santiago de Chile, hijo de Haydee Honorato, tecnólogo medico de profesión, y Rodrigo Iglesis, un medico cirujano recién graduado. Ambos provenientes de familias numerosas, trabajadores entusiastas y apasionados por la salud humana, desde pequeño guiaron mi interés por la ciencia, particularmente matemáticas y biología.

Mis años formativos de enseñanza básica y media los cursé en el otrora, Colegio Las Américas, destacando en el área de ciencias naturales. En 2010 fui aceptado en la Facultad de Ciencias de la Universidad de Chile para estudiar Ingeniería en Biotecnología Molecular. Con el pasar de los años me especialicé en electrónica e informática, enfocando mi aprendizaje a la instrumentación y el desarrollo de sensores.

Durante 2016, el actual Director de Investigación de Ciencia Pura, Juan Pablo Matte (PhD), me ofreció trabajo como investigador asociado y diseñador de prototipos para varios proyectos. Actualmente, me encuentro empleado como Jefe de Proyectos, desarrollando un sistema de iluminación de precisión con tecnología *Internet of Things*.

iii. Dedicatoria

Este trabajo este dedicado a mis padres, familia y amigos, por apoyar mis decisiones, las buenas y las malas, las que me condujeron a este momento. Sin su apoyo y cariño, nada de esto hubiese sido posible. Con su ayuda y consejo, es más fácil abrirse camino. Un abrazo afectuoso para todos ellos.

iv. Agradecimientos

A mis profesores y compañeros de trabajo, por su paciencia, dedicación y apoyo. Su empuje constante por alcanzar metas altas me mantuvo enfocado en tiempos difíciles. Agradecimientos especiales a Ciencia Pura SpA y al Dr. Patricio Arce-Johnson y a su equipo.

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BLR%: Bacterial Load Reduction Percentage

BLRF: Bacterial Load Reduction Factor

CFU: Colony Forming Units

SH: Sodium Hypochlorite

SD: Standard deviation

RESUMEN

La industria chilena hortofrutícola enfrenta grandes desafíos en el área de inocuidad alimentaria y bioseguridad. Garantizar el cumplimiento de los más altos estándares internacionales, le permite a empresas exportadoras, mantener su liderazgo en los más exigentes y demandantes mercados extranjeros.

Para validar la inocuidad de sus productos, una empresa chilena líder en la exportación de fruta congelada, solicitó a Ciencia Pura SpA, una compañía enfocada en I+D+i (investigación, desarrollo e innovación), evaluar algunos de sus protocolos de bioseguridad, los cuales deben garantizar la ausencia de patógenos posiblemente letales y ubicuos, como las bacterias *Listeria monocytogenes* y ciertas cepas de *Escherichia coli*. Este seminario de título se desarrolla en el marco de la solución propuesta por Ciencia Pura SpA para satisfacer la solicitud de la empresa solicitante.

La investigación desarrollada consta de dos series de experimentos con objetivos distintos: la primera, determinar la efectividad del proceso de lavado de la fruta con una solución de hipoclorito de sodio a diferentes concentraciones, ensayando distintos tiempos de exposición al agente desinfectante, sobre la superficie de fruta previamente inoculada; y la segunda serie, analizar la supervivencia bacteriana en la superficie de fruta inoculada, en condiciones de almacenamiento previas y posteriores a su congelación. Los estudios se realizaron sobre cuatro especies de *berries* cultivadas con gran éxito económico en territorio nacional: frutillas, frambuesas, moras y arándanos. La disminución de la carga bacteriana, y por ende el éxito del tratamiento o almacenaje, fue evaluada comparando el número de Unidades Formadoras de Colonias (*CFU*) sobre una

placa de agar, al ensayar muestras de la superficie de los frutos, luego de inocularlos, y de someterlos a ensayo de lavado o almacenaje.

Las características estudiadas de los protocolos utilizados por la empresa cumplen con las regulaciones demandadas por la industria, sin embargo, la relación entre concentración de cloro y tiempo de exposición al desinfectante podrían no ser las óptimas en todos los casos ensayados. Respecto a los ensayos de supervivencia bacteriana bajo diferentes condiciones de almacenamiento y transporte, un efecto bactericida fue observado en las cuatro especies estudiadas, siendo especialmente destacable en frambuesas.

ABSTRACT

Food exporting companies are always struggling to ensure their biosafety protocols are up to satisfy the most demanding international markets, focusing on high reliability to guarantee operations overseas.

To validate innocuity of their exported goods, an important Chilean food export company, asked Ciencia Pura SpA, a company focused on R&D in the agricultural sector, to evaluate that in their frozen berries production chain the risk of contamination from potentially dangerous microbiological risks, such as the health-threatening bacteria *Listeria monocytogenes* and *Escherichia coli*, is nil. This seminar is the result of the research proposed and conducted by Ciencia Pura SpA.

The upcoming investigation is divided as two sets of experiments with different objectives: the first one, to determine the disinfection effectiveness of household bleach (sodium hypochlorite dissolution), at different concentrations and exposure times, in the surface of bacteria-inoculated berries; the second set, to analyze bacterial growth in fruit surface at different storage conditions (pre-freezing and freezing temperatures). The research was carried out on four commercially successful berry fruits grown in Chilean territory: straw-, rasp-, black- and blue-berries. Bacterial load of inoculated berries was assessed to determine disinfection efficiency and bacterial survival, comparing Colony Forming Unit (CFU) occurrence in an agar plate culture before and after the treatments.

La investigación desarrollada consta de dos series de experimentos con objetivos distintos: la primera, determinar la efectividad del proceso de lavado de la fruta con una solución de hipoclorito de sodio a diferentes concentraciones, ensayando distintos tiempos de exposición al agente desinfectante, sobre la superficie de fruta previamente inoculada; y la segunda serie, analizar la supervivencia bacteriana en la superficie de fruta inoculada, en condiciones de almacenamiento previas y posteriores a su congelación. Los estudios se realizaron sobre cuatro especies de *berries* cultivadas con gran éxito económico en territorio nacional: frutillas, frambuesas, moras y arándanos. La disminución de la carga bacteriana, y por ende el éxito del tratamiento o almacenaje, fue evaluada comparando el número de Unidades Formadoras de Colonias (*CFU*) sobre una placa de agar, al ensayar muestras de la superficie de los frutos, luego de inocularlos, y de someterlos a ensayo de lavado o almacenaje.

Although actual sanitizing methods inside the company fully comply with industry's safety regulations, we found that current SH (sodium hypochlorite) concentration and exposure times for disinfection could not be optimal in all cases. Regarding bacterial survival at different storage temperature conditions (*Transportation* and *Exporting*), a bactericide effect was observed in all four species tested, being especially noteworthy in raspberries. Let it be noted that after time frames commonly observed in overseas exports (up to 90 days), total bacteria eradication was achieved at some point, in all berries assayed.

INTRODUCTION

Several outbreaks of harmful bacteria or viruses over the whole world have been reported in the past decades for contaminated berry fruits, such as strawberries, blueberries and raspberries (Tavoschi *et al.*, 2015). The outbreaks frequently occurred in locations geographically distant from the contamination origin, as certain pathogens can survive in freezing conditions over long transportation periods. These outbreaks mean important expenses in public-health, and the measures to contain them are often implemented too late to impede human casualties, with some iconic cases such as the shigatoxigenic *E. coli* contaminated fenugreek sprouts in Germany during 2011 (europa.eu, 2012) and the ongoing listeriosis outbreak in South Africa since last year, the worst in history (timeslive.co.za, 2018). These two bacteria species, *Listeria monocytogenes* and *Escherichia coli* are ubiquitous; of high fitness and survival rates during disinfection; grow in several types of different food, from raw meat to green vegetables; and although most strains of *E. coli* are non-pathogenic, both bacteria can cause a severe illness when ingested even in relatively small loads, as there are also *E. coli* pathogenic strains that can cause severe gastrointestinal compromise in healthy humans (Radoshevich & Cossart, 2017; Lim *et al.*, 2010).

Safety regulations and protocols inside the food industry must be maintained at the highest level to comply with domestic and international safety regulations. For this purpose, constant monitoring and development of new strategies for handling food is a must. Fruit is more often than not consumed raw, making it an even more relevant target for strict food safety regulations. At the same time these protocols must guarantee the eradication of harmful pathogens, they must do so with special care as to not damage the fruit or leave toxic residues in the process. Although there is no international

consensus for a unique regulation, policies aim to achieve maximum consumption safety, completely rejecting export cargo shipments from potentially contaminated countries, generating substantial economic losses (fortune.com, 2016). United States Food and Drug Administration (FDA) established admissible *Listeria* loads at the limit of detection (zero tolerance) (Archer, 2018). *Escherichia coli* is generally permitted to be within a range; while it's an indicator associated to fecal contamination, it's only potentially dangerous, as only a couple of serotypes are health-threating at small loads and should be completely absent.

To achieve disinfection in various industries, household bleach, a diluted solution of sodium hypochlorite, is used. This substance is a ubiquitous and general-purpose disinfectant. Its action mechanism is due to the powerful oxidizing potential of chlorine (Fair *et al.*, 1948) and is therefore an unspecific and wide-spectrum bactericide agent (Bloomfield & Uso, 1985). SH works as an oxidizer and its action is thus limited by the initial number of oxidizing agents (molecules of chlorine ion) in the solution. Because of this, the antibacterial properties of the disinfectant solution may decrease over time, as chlorine ions are spent while oxidizing bacterial components. It has been previously reported (Brackett, 1987) that chlorine concentrations less than about 50 ppm show no antimicrobial effect, but exposure to 50 ppm or greater, results in total eradication of *L. monocytogenes* from nutrient tryptic soy broth.

Harmful bacteria colonization and development could occur in all of the production stages; in the field, animal presence, particularly their feces; at harvest, bad habits from workers; and at collection centers, packaging and transportation, cross-contamination and bad hygiene at the production line. In every step of the complex industrial process there are norms and strict demands to minimize this occurrence to a minimum. Training personnel and developing improved protocols are typical means to achieve the

aforementioned goal. Common household disinfectants are used for sanitization all along the production line in several industries over a wide variety of industrial equipment and products. In the export of frozen berry fruits, the regulations allow the use of diluted sodium hypochlorite

Chile is an important producer and exporter of fruit, and as such, the industry is subject to constant revision. Ciencia Pura's client, a fruit freezing and exporting company, committed to food safety, have decided to validate their sanitization fruit export protocols, testing them in an external specialized laboratory, evaluating them to fulfill the most demanding international standards. Considering they sell ready-to-eat food that must travel long distances to first world markets, their processes are under constant scrutiny and revision.

In particular, the company was worried about two scenarios: the first, an unsuccessful washing process prior to freezing, where the bacterial load of the fruit's surface is significantly reduced; and the second, the growth of any remaining hazardous bacteria in the time it takes for overseas transportation. The company had determined a set of parameters for sanitizing the fruit that typically reduces bacterial load two orders of magnitude. This reduction is reported by the company to be enough to reduce bacterial load to non-hazard levels.

Ciencia Pura was appointed to conduct the experimental design and the research to conclude if the washing and storage protocols were over the standard required to fulfill international safety regulations. The study was carried out in four commercial berry fruits inoculated with two well-known, health-threatening, naturally-occurring bacteria: *Listeria monocytogenes* (Ramaswamy *et al.*, 2007) and *Escherichia coli* (Kaper *et al.*, 2004). According to the company's goals, the experiments performed in this project can be understood as two independent studies: to review their fruit washing processes efficacy,

and to determine no hazardous bacteria grow over frozen fruit in the period under storage conditions. Briefly, the experiments consisted in inoculating sterile fruit with known concentrations of the aforesaid bacteria under sterile conditions, expose the fruit to the washing treatments or storage time, and then, measure bacterial concentration remnant at the fruit surface.

To assess and measure the bacterial concentration and the effectiveness of the washing and storage treatment, samples taken from the surface of the inoculated fruits are plated in solid agar medium using a modified drop method (Jett *et al.*, 1997; Fawcett *et al.*, 2013; Thomas *et al.*, 2015). After incubation time, colony forming units (CFUs) are counted and results analyzed. To validate the method, an impartial third party, Universidad Católica's research division, DICTUC, determined, by different methods, the bacterial load on dedicated replicate samples.

In addition to analyzing the efficacy of their sanitization processes, the projection of this work is to propose modifications to their protocols for improving their production line throughput.

In summary, the objectives of this project can be summarized as below.

Objectives

General objective:

- Validate the effectiveness of current fruit sanitization protocols used inside the company.

Specific objectives:

- Quantitatively compare disinfection levels achieved with current and modified sanitization parameters (SH concentration and exposure times), in *Listeria monocytogenes* and *Escherichia coli*-inoculated berry fruits.
- Analyze *Listeria monocytogenes* and *Escherichia coli* bacterial growth in stored fruit: prior to freezing, at transportation temperatures (14ºC) over 48 hours, and at exporting temperatures (–20°C), over 90 days of storage.

MATERIALS & METHODS

Fruit acquisition

Strawberries, blueberries, blackberries and raspberries were received from the company to be used as samples. The fruit was harvested and immediately transported, for 5 hours, to the laboratory under refrigerated conditions (10-15ºC). Once at the lab, the fruit was kept at 4°C and used 0-24 hours later.

Fruit sterilization

The chlorine gas sterilization method was used. In preliminary tests, an optimal protocol was established for completely eradicating the natural occurrence of bacteria without compromising the organoleptic properties of the fruit. The method was carried out as follows. The fruits were put into a desiccator together with a beaker containing a 150 mL solution of bleach (4.9% active chlorine). To produce the gas, 3.5 ml of 37% HCl where carefully added to the beaker and the desiccator was closed. The fruit was maintained in contact with the gas for 3 hours and then placed, under sterile conditions, into a sterile container and kept at 4°C until the start of the experiments, 0-4 hours later.

Bacteria inoculation

The strains used in this study were *Listeria monocytogenes* ATCC 19115 and *Escherichia coli* ATCC 25922. *E. coli* was grown in LB medium for 24 hours and *Listeria monocytogenes*, for 48 hours. Once the bacteria culture reached $OD_{600} = 2$, a dilution of the culture was performed to adjust to 10^9 CFU/mL. The fruit was immersed into the bacterial suspension for 1 second and then dried for 5 minutes. This method was established by a preliminary assay, finding the best sampling time after inoculation to capture a representative colony number on the surface of the berries. These samples were used to quantify the initial bacterial content and to evaluate the bacterial load

reduction for the different treatments. Sterilized non-inoculated berries were left as control samples.

Samples collection

Washing assays: Immediately after inoculation protocol, a sterile, calibrated loop with $MgCl_2$ 10 mM was rubbed 3 times over the fruit surfaces, by the same researcher to minimize the effect of systematic errors, and put into a sterile 96-well-plate containing 90 µL of a $MgCl_2$ 10mM solution. Two extra serial dilutions were performed in different wells, diluting 10µl of the first well and mixing in a second well with 90 µL of a $MgCl_2$ 10mM solution to get 1/100 of the initial concentration, repeating a second time to obtain 1/10000 of the initial concentration. Once these pre-wash samples were acquired, the inoculated berries underwent a SH-wash with different settings. The post-wash samples were taken in the same way as described for pre-wash samples.

Three different SH concentrations (C1, C2 and C3) and three different exposure times/sanitizing flows (T1, T2 and T3) were studied for each type of berry. Two washing methods were used: submersion for strawberries and blueberries, and spray-wash for raspberries and blackberries. In the former, exposure time to SH (immersion duration) was the controlled variable, while in the latter, it was sanitizing flow (milliliters of SH solution applied per kilogram of fruit). Exposure time/sanitizing flow is displayed in seconds for fruit washed by submersion, and in mL/Kg for spray-washed fruit. A summary of this experimental design is shown in Table 1.

Table 1: Summary of the SH concentration and flow or exposure time for each washing treatment.

	Strawberry	Blueberry	Raspberry	Blackberry
C1	10 ppm	10 ppm	10 ppm	10 ppm
C2	30 ppm	25 ppm	20 ppm	20 ppm
C3	80 ppm	80 ppm	40 ppm	40 ppm
T1	10 s	10 s	20 mL/Kg	20 mL/Kg
T2	20 s	20 s	40 mL/Kg	40 mL/Kg
T3	*	*	*	100 mL/Kg

SH concentrations and flow / exposure times used. SH concentration is given in parts per million (ppm). Depending on application method, exposure time is given in seconds (s) for strawberry and blueberry (submersion), and in milliliters per kilogram (mL/Kg) for blackberry and raspberry (spray). (*) This assay was not deemed necessary.

Three fruit samples washed with C2T2 settings were also sent to another laboratory (DICTUC) for analysis, providing a third-party outcome as validation control for results found in this project.

For each wash treatment a set of three fruits were inoculated as a replicate, sampled and assessed; a standard average of the three was used. Serially-diluted samples were plated for each treatment and CFU were assessed wherever possible, calculating a weighted average and its corresponding weighted standard deviation (SD).

Samples of each berry type were washed with a mock treatment with sterile water (chlorine-free) and set as control group. These results are labeled H2O instead of C1, C2 or C3.

Storage assays: For rasp-, black- and blue-berries, a sample of three inoculated fruits was obtained. The strawberries were studied in two formats: as a whole, sampling in the surface (skin) of the fruit, and with a clean cut, sampling in the flesh of the fruit: because strawberry harvesting results in these two fruit conditions. For the first set of storage-oriented experiments, inoculated samples were taken immediately for

quantification while other samples were stored at 14ºC for 24 or 48 hours before sampling, by rubbing a sterile loop containing $MgCl_2$ 10mM three times on different spot over the fruit surface. The samples were then placed in a sterile 96-well-plate containing 90 µL $MgCl_2$ 10mM. For the second experiment, a sample was taken immediately after inoculation and then the fruit was stored at -20ºC for 15, 30, 45, 60, 75 or 90 days. A second sample was taken after thaw for each period of time.

Bacterial quantification from fruit

The inoculated fruit samples were 1/100 serially diluted 3 times to cover a wide range of concentrations, to achieve a suitable resolution for *the drop method* (Jett *et al.*, 1997; Fawcett *et al.*, 2013; Thomas *et al.*, 2015). This method consisted in plating, for each dilution, an 8µL drop of the sample in square plates (12cm X 12cm) with agar-LB solid medium, and then incubating for 24 hours at 40°C for *E. coli* and 48 hours for *Listeria monocytogenes*. (Fang *et al.*, 2014, Gonthier *et al.*, 2001). After the incubation period, pictures of the plates were taken individually and the number of bacteria colonies were counted by hand. Adjusting according to dilution factor and plating volume, bacterial load was calculated and is displayed as Colony Forming Units per milliliter (CFU/mL).

DICTUC's samples

An independent assay was sought after for data validation and analysis reliability. Sterilized, inoculated and washed samples were obtained as replicates for some treatments, using the same methods as above. Samples were then stored at 4°C until used by a different staff at DICTUC's laboratories, without breaking the cold chain. DICTUC emitted a report after samples were evaluated, measuring bacterial concentration of *Escherichia coli* using AOAC method 991.14 (Petrifilm) and presence or absence of *Listeria monocytogenes* using a method from FDA's Bacteriological Analytical Manual (chapter 10, 2003 edition).

Statistical analysis

Data were analyzed using a single factor ANOVA to recognize significant difference from at least a pair of samples, and individual comparison of two samples was achieved by Student's t-Test.

To select the right t-test, an F-test for two samples was conducted to test for equal variance, and if the test was accepted, a t-test for two samples assuming equal variance was used. Otherwise a t-test for two samples assuming unequal variance was employed. All statistical analyses were carried out with Microsoft Excel ®.

Data summarize

For the results of a determined assay, a weighted average ($\bar{X}$) of CFU counted in the different dilutions was used; i.e. CFUs calculated for a sample diluted to 1/10th of the original have more weight than the number of CFU determined for a sample diluted to 1/100th, according to equation (1).

$$\bar{X} = \frac{\sum_{i=1}^{n} w_i x_i}{\sum_{i=1}^{n} w_i} \qquad (1)$$

The weighted arithmetic mean ($\bar{X}$) formula is displayed in equation (1), where x_i represents the non-empty data set (CFUs assessed) with non-negative weights, w_i (dilution factor).

The standard deviation (SD) is also weighted to give more influence to higher concentrations than less. It's calculated according to equation (2).

$$SD = \sqrt{\frac{\sum_{i=1}^{n} w_i (x_i - \bar{X})^2}{\sum_{i=1}^{n} w_i}} \qquad (2)$$

RESULTS

Sodium Hypochlorite bactericide effects

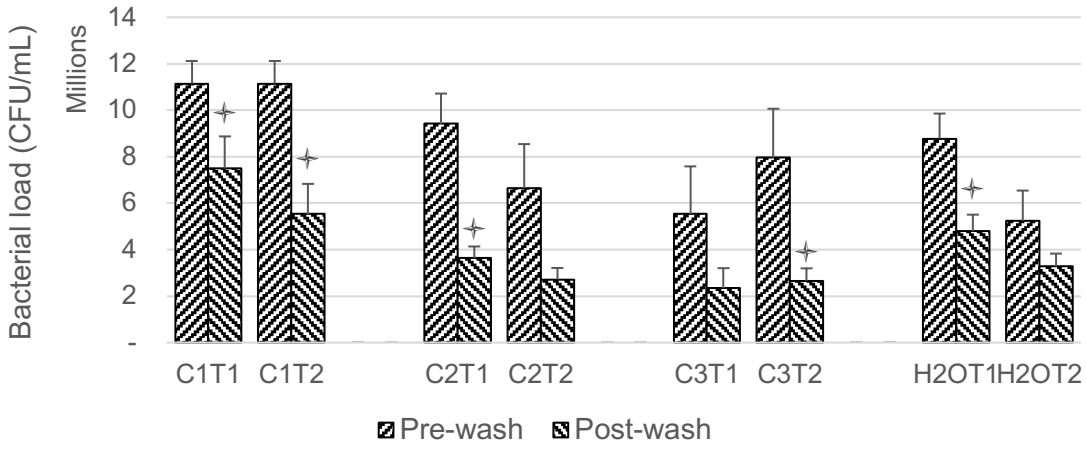
A study on the bactericide potential of SH was previously conducted by Ciencia Pura. These results show that higher drops in bacterial load are achieved at higher SH concentrations, however, disinfection capacity was limited, as the oxidizing reagent (chlorine) is consumed as bacteria die. Once the maximum bacterial drop was achieved, an increase in bacterial load was observed, indicating that disinfection was no longer active and bacteria could restart growth.

Disinfection efficiency of washing treatments

To assess the efficiency of the disinfection method, *Colony Forming Units* (CFU) developed in an agar plate from samples acquired from *Listeria monocytogenes* and *Escherichia coli*-inoculated berries, before and after washing, were counted and compared. This data was then normalized as '*bacterial load reduction percentage*' (data can be found in SUPPLEMENTARY MATERIAL). To further analyze the data, the base-10 logarithm arithmetic difference between the initial number of CFU/mL (pre-wash) and the number of CFU/mL after SH treatment (post-wash), was calculated. The value of this arithmetic difference was named Bacterial Load Reduction Factor (BLRF).

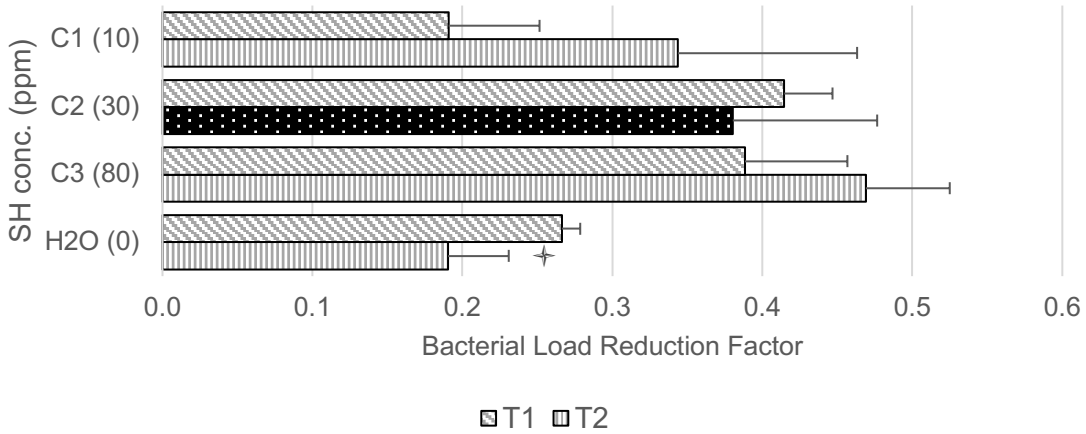
The following figures show the results found for inoculated and then sanitized/*washed* berries. For each type of berry (straw-, rasp-, black- and blue-), and bacteria (*Listeria monocytogenes* and *Escherichia coli*), two figures are presented: the first shows bacterial load (CFU/mL) before and after being washed, and the second, bacterial load reduction normalized as BLRF, for each treatment. Data tables can be found in SUPPLEMENTARY MATERIAL.

Figure 1: Bacterial load (CFU/mL) assessed in *Listeria*-inoculated strawberries, before and after each SH treatment.



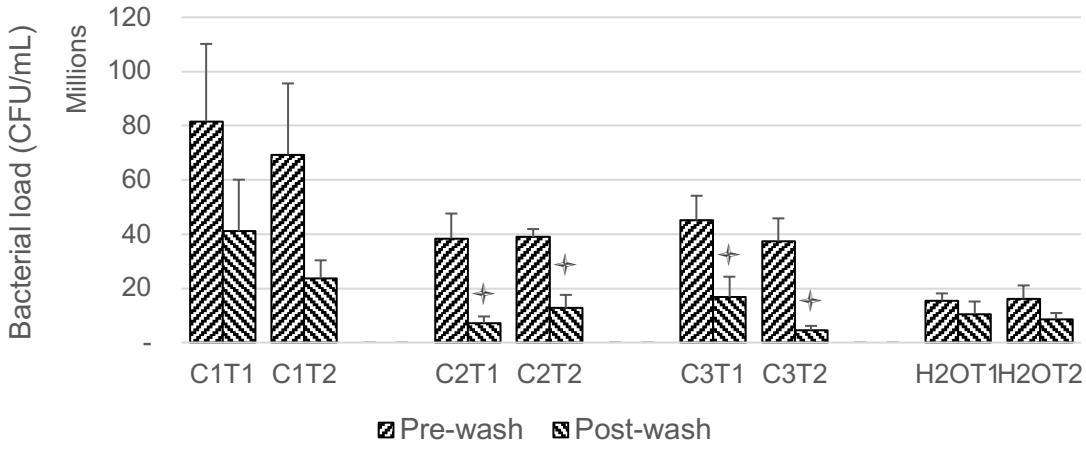
Bacterial load was assessed from samples before and after being sanitized. The bacterial count in Colony Forming Units (CFU) per mL is shown for every performed treatment (CxTx). Standard deviation bars are shown. Post-wash bacterial loads marked with "✦" have statistically significant differences (p-value < 0.05) when compared to the same treatment's pre-wash bacterial load. Assayed samples n=3.

Figure 2: Logarithmic bacterial load reduction in *Listeria*-inoculated strawberries after SH treatments.



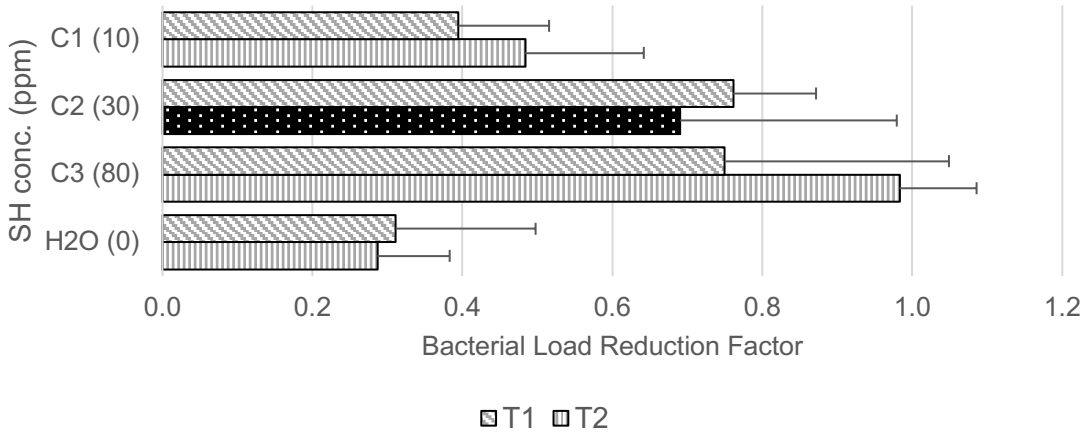
Bacterial Load Reduction Factor (BLRF) for each SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1, T2 and T3). BLRF is a normalized value for indicating logarithmic bacterial load drop after treatment. Standard deviation bars are shown. Treatments marked with "✦" have statistically significant differences (p-value < 0.05) with C2T2 (black bar), the current settings used at the company. Assayed samples n=3.

Figure 3: Bacterial load (CFU/mL) assessed in *Listeria*-inoculated raspberries, before and after each SH treatment.



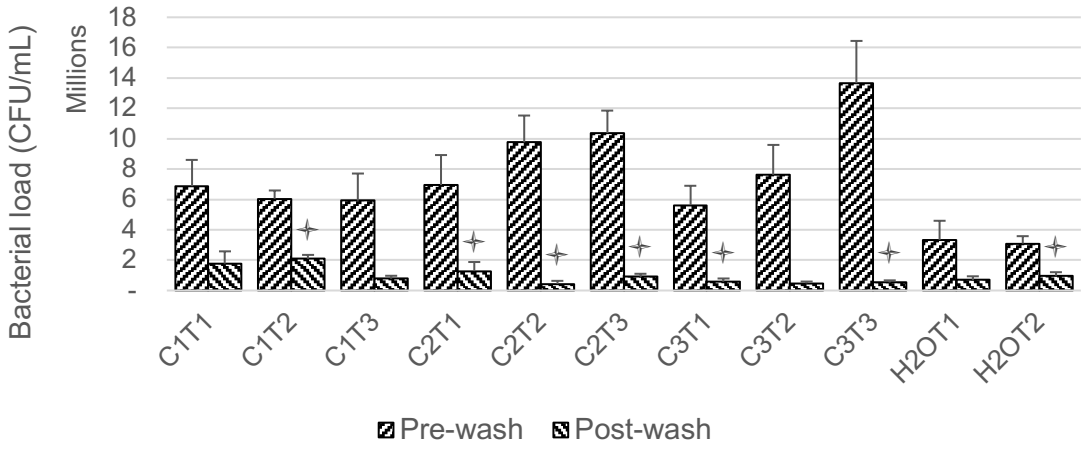
Bacterial load was assessed from samples before and after being sanitized. The bacterial count in Colony Forming Units (CFU) per mL is shown for every performed treatment (CxTx). Standard deviation bars are shown. Post-wash bacterial loads marked with "✦" have statistically significant differences (p-value < 0.05) when compared to the same treatment's pre-wash bacterial load. Assayed samples n=3.

Figure 4: Logarithmic bacterial load reduction in *Listeria*-inoculated raspberries after SH treatments.



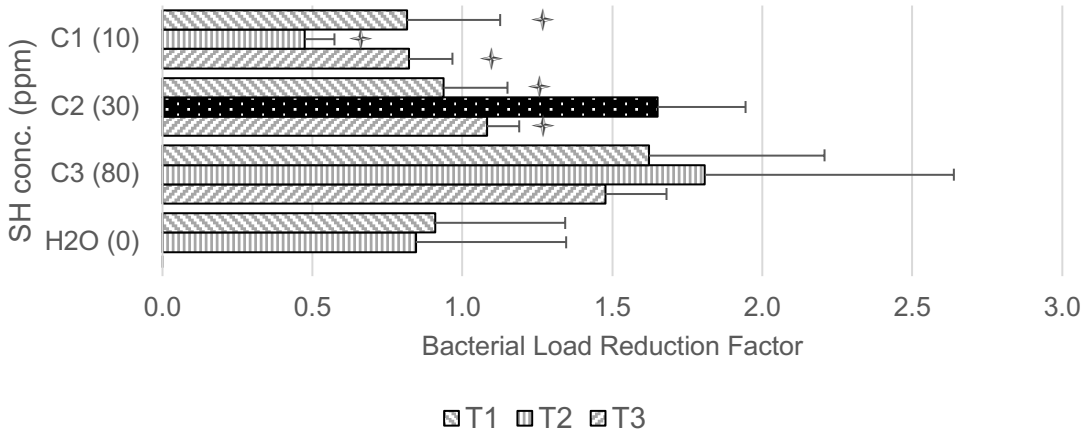
Bacterial Load Reduction Factor (BLRF) for each SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1, T2 and T3). BLRF is a normalized value for indicating logarithmic bacterial load drop after treatment. Standard deviation bars are shown. Treatments marked with "✦" have statistically significant differences (p-value < 0.05) with C2T2 (black bar), the current settings used at the company. Assayed samples n=3.

Figure 5: Bacterial load (CFU/mL) assessed in *Listeria*-inoculated blackberries, before and after each SH treatment.



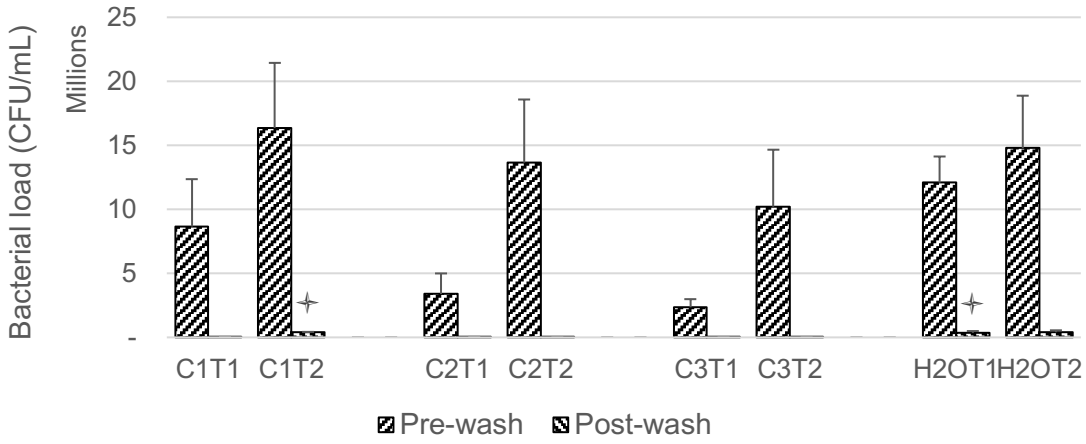
Bacterial load was assessed from samples before and after being sanitized. The bacterial count in Colony Forming Units (CFU) per mL is shown for every performed treatment (CxTx). Standard deviation bars are shown. Post-wash bacterial loads marked with "✦" have statistically significant differences (p-value < 0.05) when compared to the same treatment's pre-wash bacterial load. Assayed samples n=3.

Figure 6: Logarithmic bacterial load reduction in *Listeria*-inoculated blackberries after SH treatments.



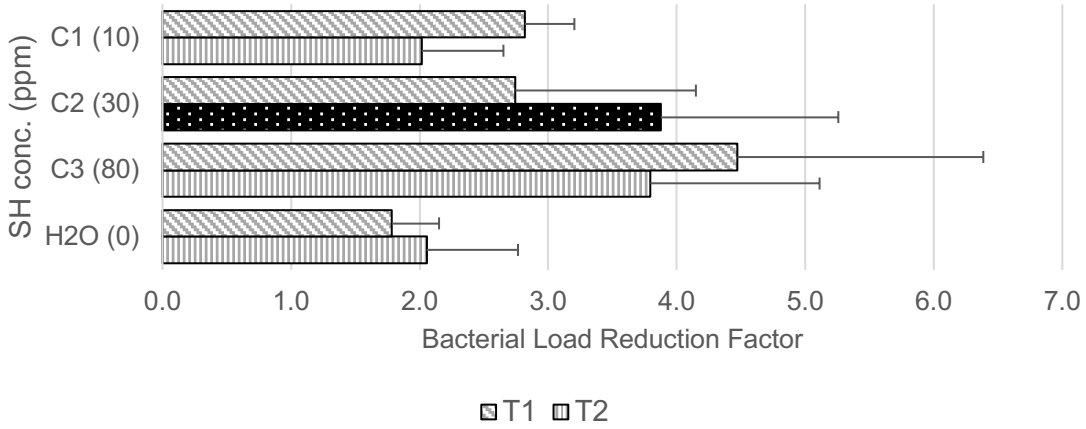
Bacterial Load Reduction Factor (BLRF) for each SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1, T2 and T3). BLRF is a normalized value for indicating logarithmic bacterial load drop after treatment. Standard deviation bars are shown. Treatments marked with "✦" have statistically significant differences (p-value < 0.05) with C2T2 (black bar), the current settings used at the company. Assayed samples n=3.

Figure 7: Bacterial load (CFU/mL) assessed in *Listeria*-inoculated blueberries, before and after each SH treatment.



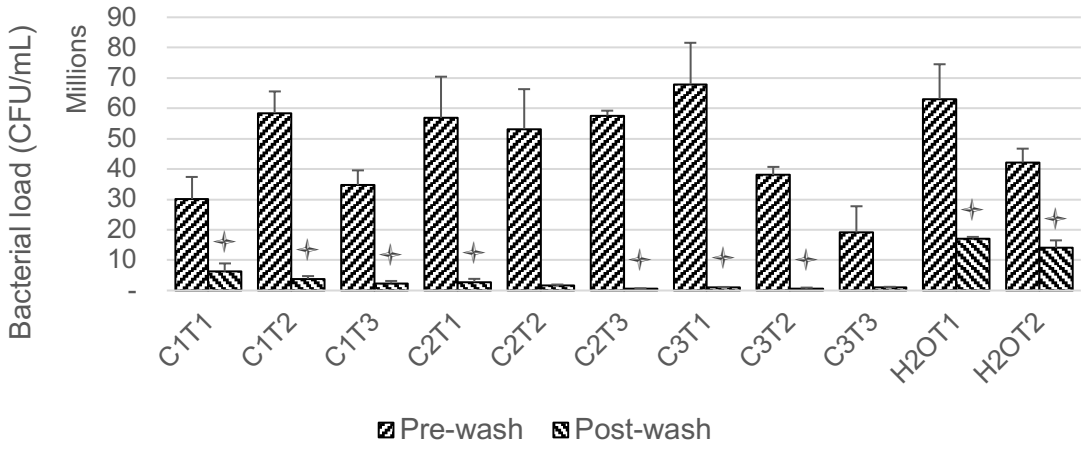
Bacterial load was assessed from samples before and after being sanitized. The bacterial count in Colony Forming Units (CFU) per mL is shown for every performed treatment (CxTx). Standard deviation bars are shown. Post-wash bacterial loads marked with "✦" have statistically significant differences (p-value < 0.05) when compared to the same treatment's pre-wash bacterial load. Assayed samples n=3.

Figure 8: Logarithmic bacterial load reduction in *Listeria*-inoculated blueberries after SH treatments.



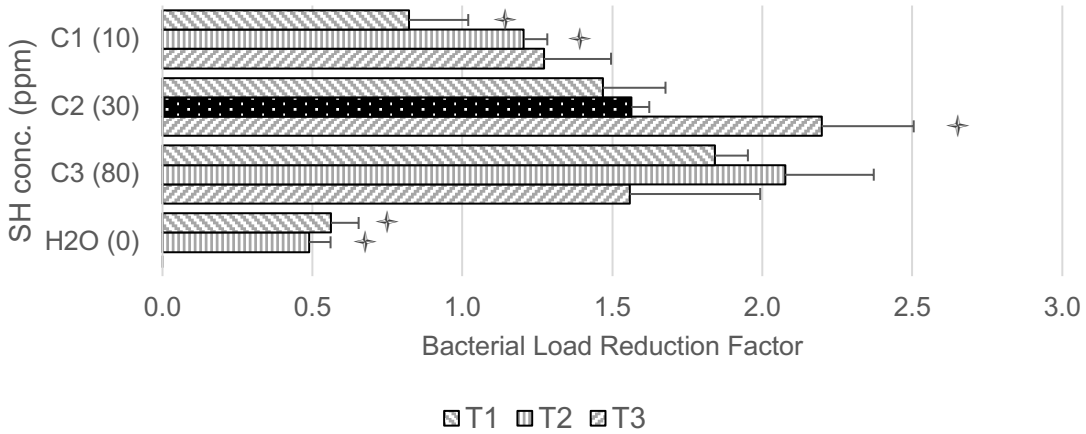
Bacterial Load Reduction Factor (BLRF) for each SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1, T2 and T3). BLRF is a normalized value for indicating logarithmic bacterial load drop after treatment. Standard deviation bars are shown. Treatments marked with "✦" have statistically significant differences (p-value < 0.05) with C2T2 (black bar), the current settings used at the company. Assayed samples n=3.

Figure 9: Bacterial load (CFU/mL) assessed in *E. coli*-inoculated strawberries, before and after each SH treatment.



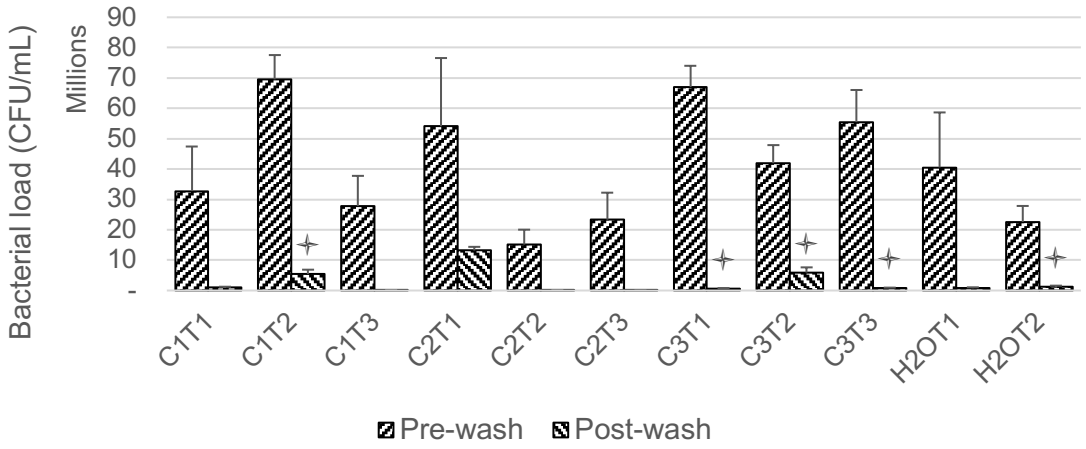
Bacterial load was assessed from samples before and after being sanitized. The bacterial count in Colony Forming Units (CFU) per mL is shown for every performed treatment (CxTx). Standard deviation bars are shown. Post-wash bacterial loads marked with "✦" have statistically significant differences (p-value < 0.05) when compared to the same treatment's pre-wash bacterial load. Assayed samples n=3.

Figure 10: Logarithmic bacterial load reduction in *E. coli*-inoculated strawberries after SH treatments.



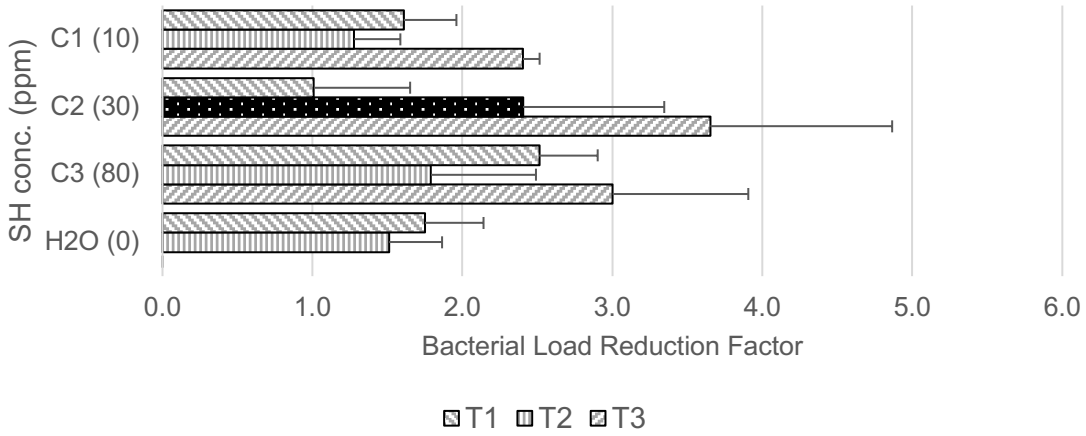
Bacterial Load Reduction Factor (BLRF) for each SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1, T2 and T3). BLRF is a normalized value for indicating logarithmic bacterial load drop after treatment. Standard deviation bars are shown. Treatments marked with "✦" have statistically significant differences (p-value < 0.05) with C2T2 (black bar), the current settings used at the company. Assayed samples n=3.

Figure 11: Bacterial load (CFU/mL) assessed in *E. coli*-inoculated raspberries, before and after each SH treatment.



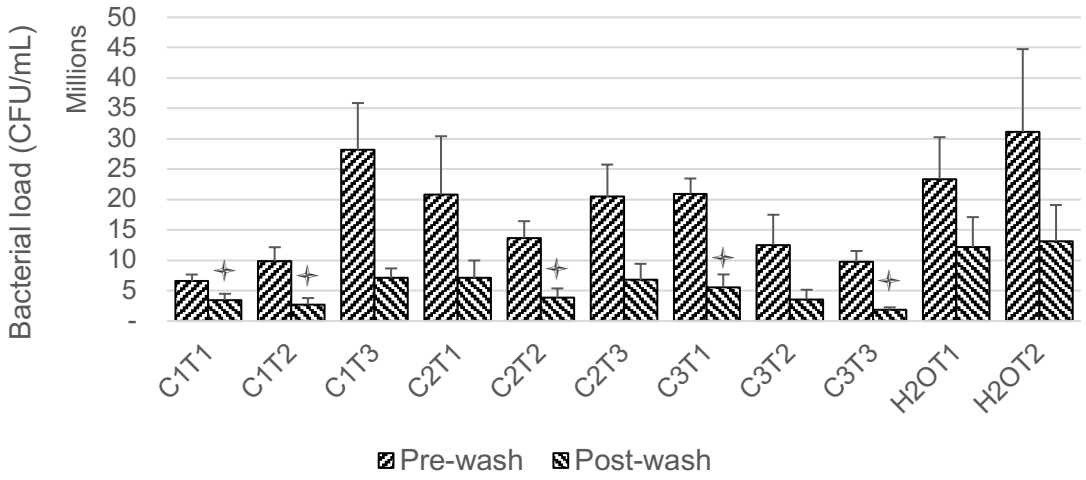
Bacterial load was assessed from samples before and after being sanitized. The bacterial count in Colony Forming Units (CFU) per mL is shown for every performed treatment (CxTx). Standard deviation bars are shown. Post-wash bacterial loads marked with "✦" have statistically significant differences (p-value < 0.05) when compared to the same treatment's pre-wash bacterial load. Assayed samples n=3.

Figure 12: Logarithmic bacterial load reduction in *E. coli*-inoculated raspberries after SH treatments.



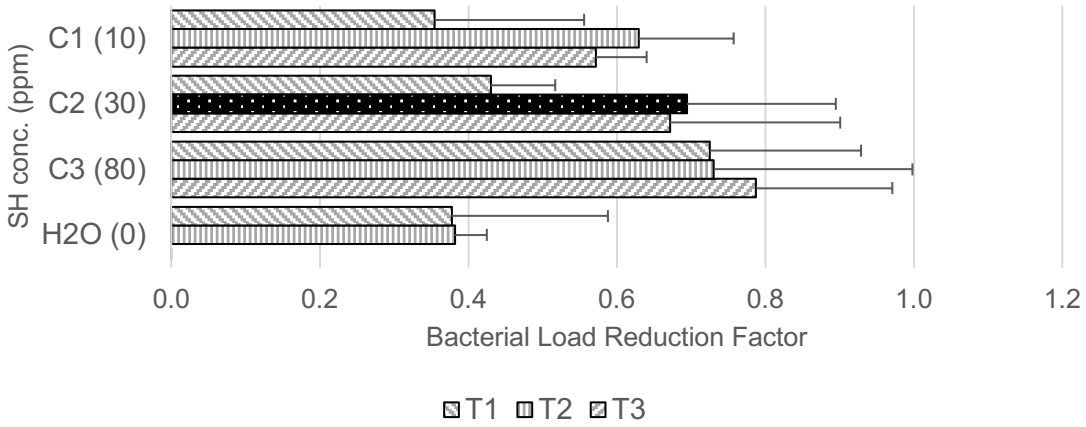
Bacterial Load Reduction Factor (BLRF) for each SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1, T2 and T3). BLRF is a normalized value for indicating logarithmic bacterial load drop after treatment. Standard deviation bars are shown. Treatments marked with "✦" have statistically significant differences (p-value < 0.05) with C2T2 (black bar), the current settings used at the company. Assayed samples n=3.

Figure 13: Bacterial load (CFU/mL) assessed in *E. coli*-inoculated blackberries, before and after each SH treatment.



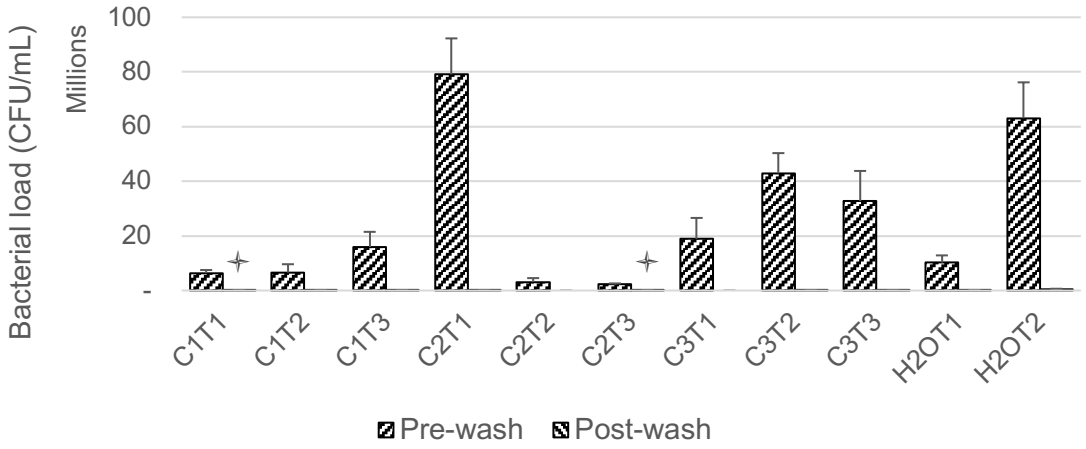
Bacterial load was assessed from samples before and after being sanitized. The bacterial count in Colony Forming Units (CFU) per mL is shown for every performed treatment (CxTx). Standard deviation bars are shown. Post-wash bacterial loads marked with "✦" have statistically significant differences (p-value < 0.05) when compared to the same treatment's pre-wash bacterial load. Assayed samples n=3.

Figure 14: Logarithmic bacterial load reduction in *E. coli*-inoculated blackberries after SH treatments.



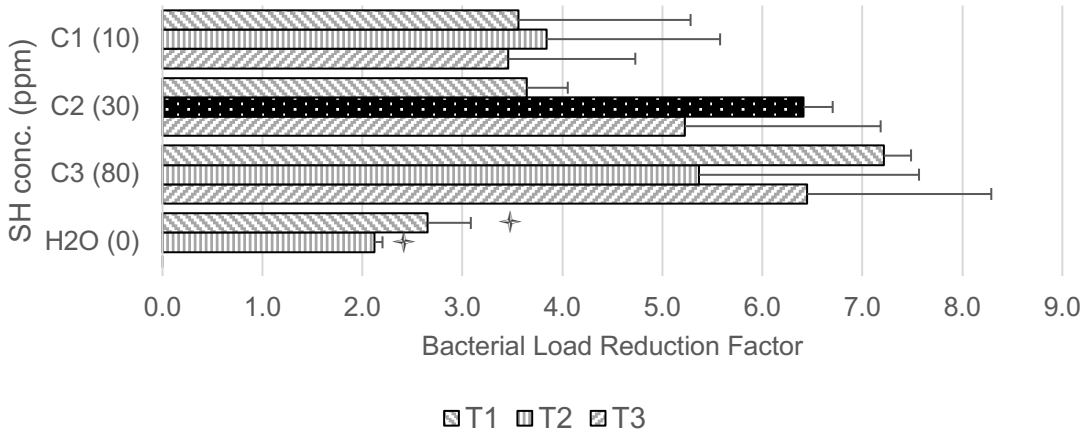
Bacterial Load Reduction Factor (BLRF) for each SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1, T2 and T3). BLRF is a normalized value for indicating logarithmic bacterial load drop after treatment. Standard deviation bars are shown. Treatments marked with "✦" have statistically significant differences (p-value < 0.05) with C2T2 (black bar), the current settings used at the company. Assayed samples n=3.

Figure 15: Bacterial load (CFU/mL) assessed in *E. coli*-inoculated blueberries, before and after each SH treatment.



Bacterial load was assessed from samples before and after being sanitized. The bacterial count in Colony Forming Units (CFU) per mL is shown for every performed treatment (CxTx). Standard deviation bars are shown. Post-wash bacterial loads marked with "✦" have statistically significant differences (p-value < 0.05) when compared to the same treatment's pre-wash bacterial load. Assayed samples n=3.

Figure 16: Logarithmic bacterial load reduction in *E. coli*-inoculated blueberries after SH treatments.



Bacterial Load Reduction Factor (BLRF) for each SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1, T2 and T3). BLRF is a normalized value for indicating logarithmic bacterial load drop after treatment. Standard deviation bars are shown. Treatments marked with "✦" have statistically significant differences (p-value < 0.05) with C2T2 (black bar), the current settings used at the company. Assayed samples n=3.

DICTUC's analysis on disinfection

To validate the results of this study, an independent assessment using a different method was held at DICTUC's laboratories. Samples obtained and treated under the same protocols as C2T2 groups, both pre-washed and post-washed, were analyzed.

Presence or absence of *Listeria monocytogenes* in the samples was reported back, however DICTUC's *L. monocytogenes* assays only reveal presence or absence and doesn't quantify bacterial load. Sterile samples weren't contaminated and presence or absence was in accordance with the results of this study.

For *E. coli*-inoculated fruit, total bacterial load was reported back as *Colony Forming Units* (CFU) *per gram* of fruit. Because of differences in the quantification method, bacterial load was informed in a different unit than the one used in this study (CFU/mL), and direct comparison couldn't be done. To contrast and validate results, Bacterial Load Reduction Factor for DICTUC's data was calculated accordingly, and is displayed alongside C2T2 reduction factors from this study in Table 2.

Table 2: Assessed *Bacterial Load Reduction Factor* for DICTUC's samples in *E. coli*-inoculated berries.

	Bacterial Load Reduction Factor			
	Strawberry	Raspberry	Blackberry	Blueberry
DICTUC	1.00	0.78	0.15	2.57
This study†	1.56	2.41	0.69	6.41
Relative % difference*	156%	309%	460%	249%

† Bacterial Load Reduction Factors from C2T2 treatments.
* Relative percentage difference of this study's BLRF and DICTUC's

Surface bacteria development under transportation and storage conditions

To assess bacterial growth over the surface of berry fruits, strawberries, blueberries, blackberries and raspberries were inoculated and the bacterial load was determined after scheduled time frames.

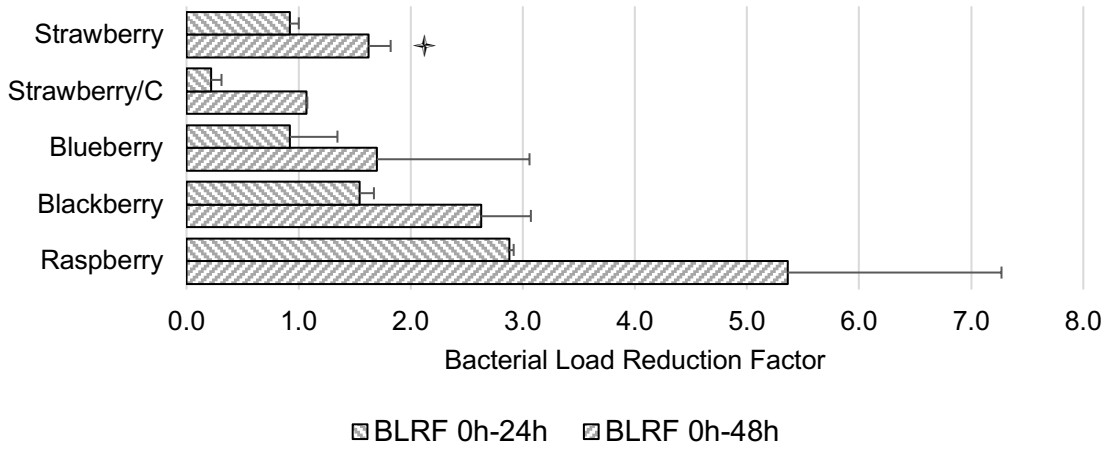
Two storage conditions were studied: short-term storage (24 and 48 hours) at non-freezing temperatures (14ºC) and long-term storage (every 15 days for 90 days) at freezing temperatures (-20º).

Samples in the upcoming assays were not washed by any means.

Transportation conditions: short-term storage at non-freezing temperatures

These next results show how the bacteria studied develop under transportation conditions and time frames. The next two figures show the Bacterial Load Reduction Factor calculated for each type of inoculated berry, sampling immediately after inoculation (reference) and after 24 or 48 hours. For this assay only, strawberries were inoculated and sampled in the skin (*Strawberry*) and in the exposed flesh (*Strawberry/C*).

Figure 17: Bacterial Load Reduction Factor of Listeria-inoculated berries under transportation conditions.



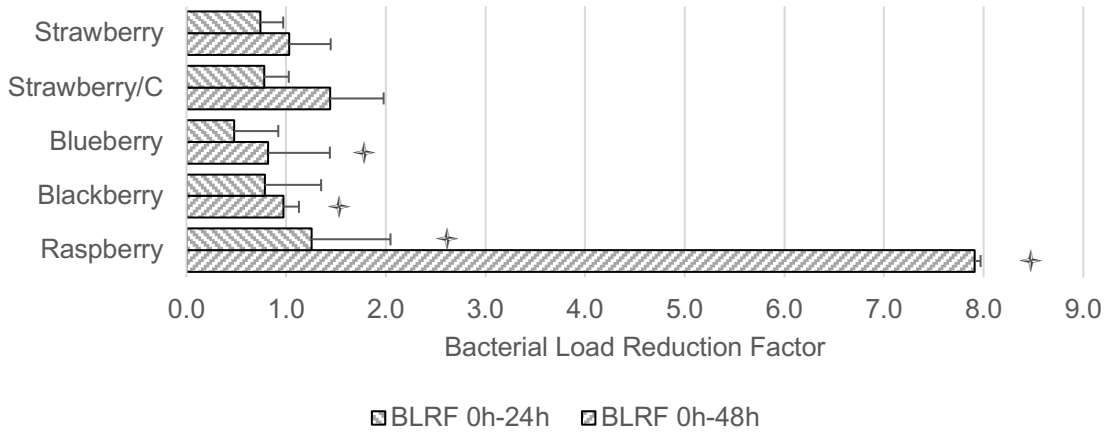
Bacterial Load Reduction Factor (BLRF) for each berry and short-term storage condition. BLRF is a normalized value for indicating logarithmic bacterial load drop. *Strawberry/C* corresponds to a second type of sampling method for strawberries, where inoculation and sampling occurred in the fruit's exposed flesh. Bar marking "✦" shows that statistically significant differences (p-value < 0.05) were found between 0hr and 24hr/48hr samples. Assayed samples n=3.

Table 3: Bacterial load reduction percentage for Listeria-inoculated berries under transportation conditions.

	Strawberry		Strawberry/C		Blueberry		Blackberry		Raspberry	
	BLR%	SD	**BLR%**	SD	**BLR%**	SD	**BLR%**	SD	**BLR%**	SD
0h-24h	88%	2%	38%	14%	70%	68%	97%	1%	100%	0%
0h-48h	97%	2%	91%	0%	95%	2%	99%	3%	100%	0%

Bacterial Load Reduction Percentage (BLR%) for each berry and short-term storage condition. Raw data was normalized as BLR%, where 100% reduction indicates an absolute eradication of bacteria. *Strawberry/C* corresponds to a second type of sampling method for strawberries, where inoculation and sampling occurred in the fruit's exposed flesh. Assayed samples n=3. SD: Standard deviation.

Figure 18: Bacterial Load Reduction Factor of *E. coli*-inoculated berries under transportation conditions.



Bacterial Load Reduction Factor (BLRF) for each berry and short-term storage condition. BLRF is a normalized value for indicating logarithmic bacterial load drop. *Strawberry/C* corresponds to a second type of sampling method for strawberries, where inoculation and sampling occurred in the fruit's exposed flesh. Bar marking "✦" shows that statistically significant differences (p-value < 0.05) were found between 0hr and 24hr/48hr samples. Assayed samples n=3.

Table 4: Bacterial load reduction percentage for *E. coli*-inoculated berries under transportation conditions.

	Strawberry		Strawberry/C		Blueberry		Blackberry		Raspberry	
	BLR%	SD	**BLR%**	SD	**BLR%**	SD	**BLR%**	SD	**BLR%**	SD
0h-24h	79%	12%	80%	13%	49%	40%	68%	26%	79%	26%
0h-48h	88%	6%	93%	7%	70%	22%	89%	4%	100%	0%

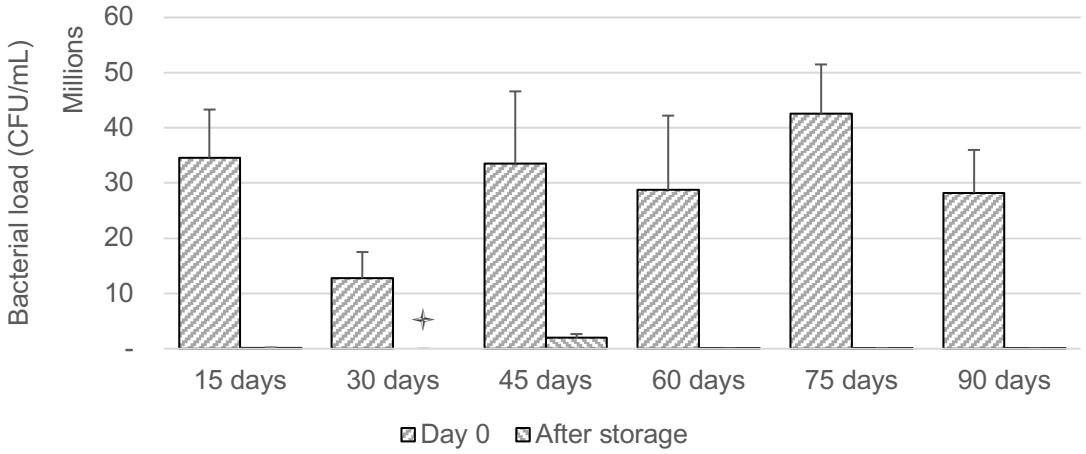
Bacterial Load Reduction Percentage (BLR%) for each berry and short-term storage condition. Raw data was normalized as BLR%, where 100% reduction indicates an absolute eradication of bacteria. *Strawberry/C* corresponds to a second type of sampling method for strawberries, where inoculation and sampling occurred in the fruit's exposed flesh. Assayed samples n=3. SD: Standard deviation.

Exporting conditions: Long-term storage under freezing temperatures

The last set of experiments shows bacterial growth over long periods of time once the fruit has been, and kept, frozen, a condition that mimics long overseas exports.

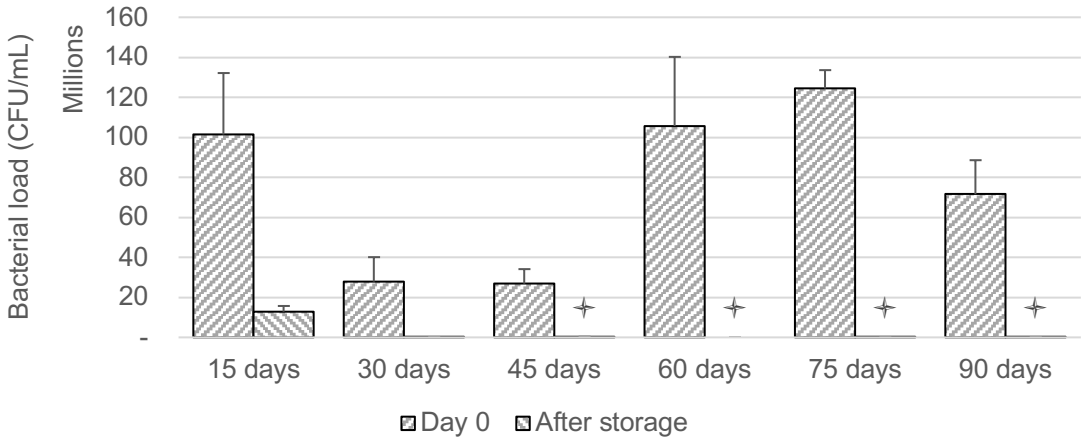
The forthcoming figures shows average bacterial load as *Colony Forming Units per mL* of incubated samples obtained from inoculated berries. A set of samples were inoculated while a subset was immediately assessed (*Day 0*) and another (*After storage*) kept frozen at -20ºC for the specified number of days.

Figure 19: Average bacterial load of Listeria-inoculated strawberries under exporting conditions.



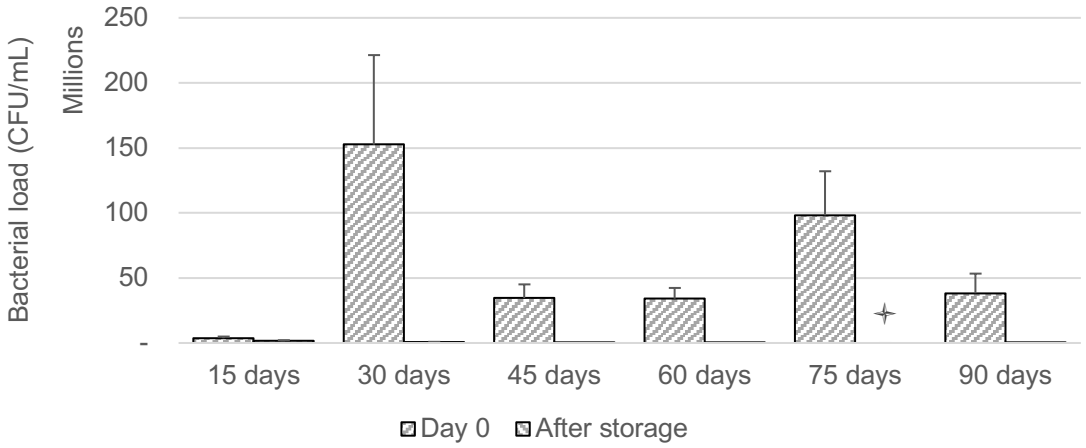
Bacterial load averaged over all fruit samples, assessed immediately after inoculation (*Day 0*) and after being stored frozen for the specified number of days (*After storage)*. Bars marked with "✦" shows that statistically significant differences (p-value < 0.05) were found between them. Assayed samples n=3.

Figure 20: Average bacterial load of Listeria-inoculated raspberries under exporting conditions.



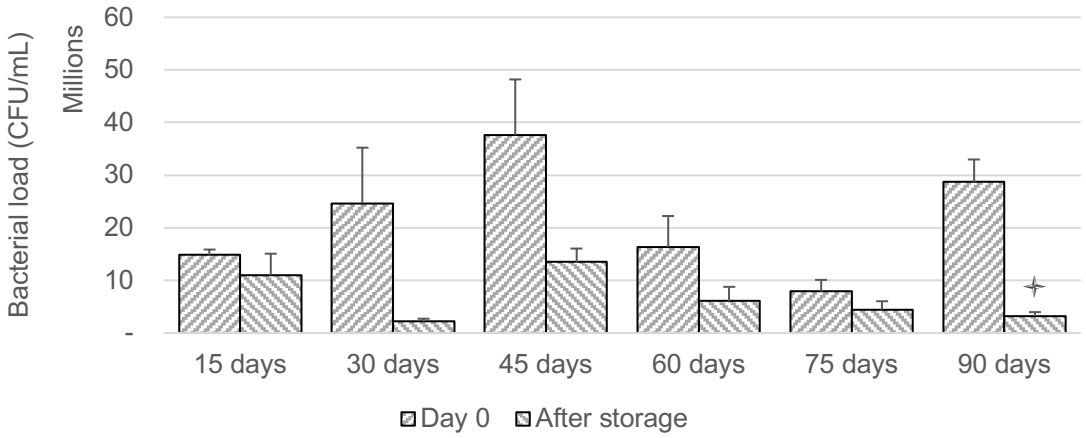
Bacterial load averaged over all fruit samples, assessed immediately after inoculation (*Day 0*) and after being stored frozen for the specified number of days (*After storage)*. Bars marked with "✦" shows that statistically significant differences (p-value < 0.05) were found between them. Assayed samples n=3.

Figure 21: Average bacterial load of Listeria-inoculated blackberries under exporting conditions.



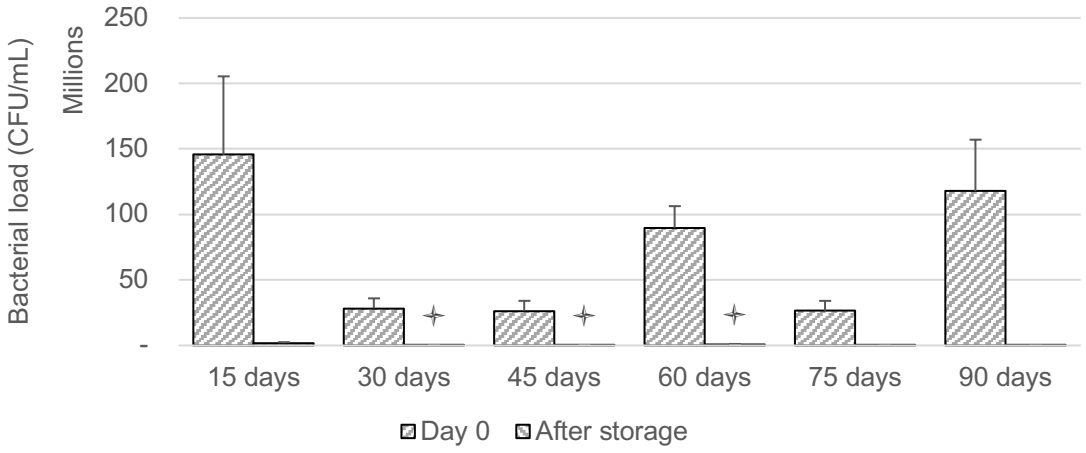
Bacterial load averaged over all fruit samples, assessed immediately after inoculation (*Day 0*) and after being stored frozen for the specified number of days (*After storage)*. Bars marked with "✦" shows that statistically significant differences (p-value < 0.05) were found between them. Assayed samples n=3.

Figure 22: Average bacterial load of Listeria-inoculated blueberries under exporting conditions.



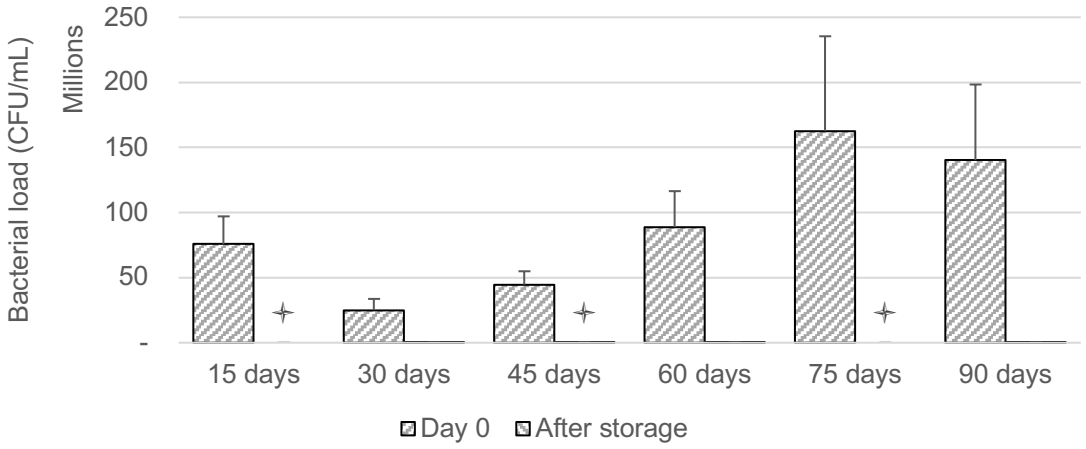
Bacterial load averaged over all fruit samples, assessed immediately after inoculation (*Day 0*) and after being stored frozen for the specified number of days (*After storage)*. Bars marked with "✦" shows that statistically significant differences (p-value < 0.05) were found between them. Assayed samples n=3.

Figure 23: Average bacterial load of *E. coli*-inoculated strawberries under exporting conditions.



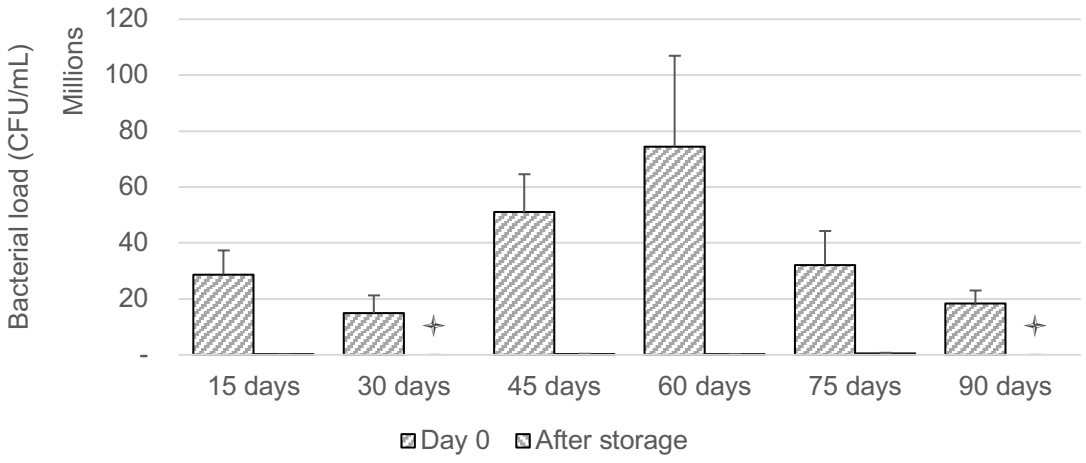
Bacterial load averaged over all fruit samples, assessed immediately after inoculation (*Day 0*) and after being stored frozen for the specified number of days (*After storage)*. Bars marked with "✦" shows that statistically significant differences (p-value < 0.05) were found between them. Assayed samples n=3.

Figure 24: Average bacterial load of *E. coli*-inoculated raspberries under exporting conditions.



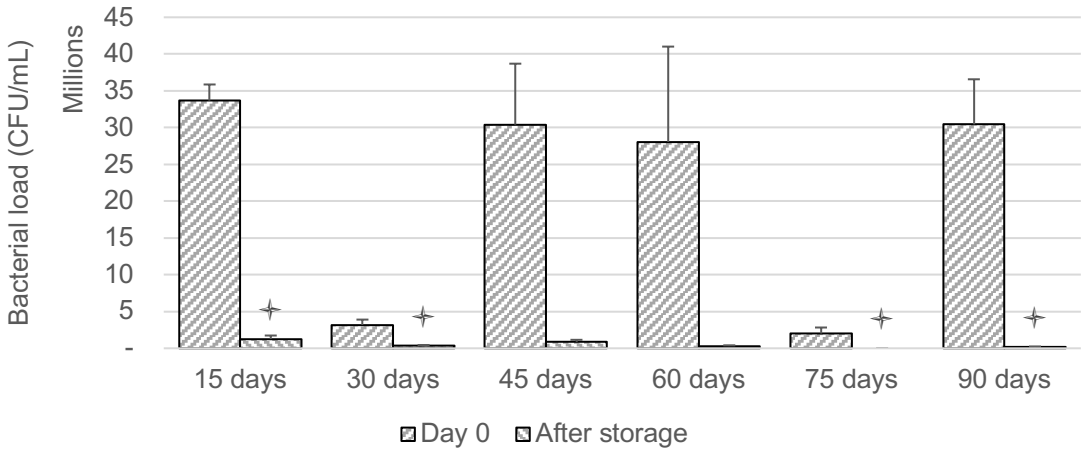
Bacterial load averaged over all fruit samples, assessed immediately after inoculation (*Day 0*) and after being stored frozen for the specified number of days (*After storage)*. Bars marked with "✦" shows that statistically significant differences (p-value < 0.05) were found between them. Assayed samples n=3.

Figure 25: Average bacterial load of *E. coli*-inoculated blackberries under exporting conditions.



Bacterial load averaged over all fruit samples, assessed immediately after inoculation (*Day 0*) and after being stored frozen for the specified number of days (*After storage)*. Bars marked with "✦" shows that statistically significant differences (p-value < 0.05) were found between them. Assayed samples n=3.

Figure 26: Average bacterial load of *E. coli*-inoculated blueberries under exporting conditions.



Bacterial load averaged over all fruit samples, assessed immediately after inoculation (*Day 0*) and after being stored frozen for the specified number of days (*After storage)*. Bars marked with "✦" shows that statistically significant differences (p-value < 0.05) were found between them. Assayed samples n=3.

DISCUSSION

Disinfection properties of bleach

The results show a general tendency of greater disinfection potential at higher SH concentrations. This behavior is expected, as the number of oxidizing agents in the solution goes up, unspecific cell damage reduces bacterial load by hindering general cellular processes. However, this unspecific cell damage also downgrades fruit quality as a product, harming organoleptic properties, and is potentially harmful for general human consumption.

Washing treatments

The control groups, consisting of fruit washed with sterile water as a mock treatment, showed significant disinfectant action in some fruit, however, this sub-standard, chlorine-free method proved unreliable, with low disinfection levels in some berries and high in others, exhibiting low reliability. It was privately stated by the company, that a common industry's practice is exporting raspberries without washing them. The company prefers to wash them anyway, for added safety. The decrease in bacterial load seen in sterile water-only treatments might be due to dragging forces impeding bacteria growth and colonization. Also, an extensively reported antimicrobial effect has been described for members of the *Rosaceae* family (strawberries, blackberries and raspberries), over a wide variety of human pathogenic bacteria (Heinonen, 2007; Nohynek *et al.*, 2006; Puupponen-Pimiä *et al.*, 2001, 2005a, 2005b; Ryan *et al.*, 2001). Blueberries also display high antimicrobial activity, related to their high anthocyanin content (Burdulis *et al*., 2009). This berry also displayed high tolerance to inoculation, probably related to his "conspicuous wax layer" (Chu *et al*., 2018). The aforementioned

properties can account for the high bacterial load decrease observed in the control groups H2OT1 and H2OT2.

Different post-inoculation, pre-wash bacterial load was observed between berry species (data not shown); i.e. Listeria-inoculated raspberries showed up to 10 times higher CFU count than other berries. This phenomenon could be the result of natural differences intrinsic to the fruit, i.e. differences in shape or the presence of protective wax layers. Low variance between samples of the same group supports this idea and rejects experimental bias.

Strawberries were washed by submerging the fruit in a diluted SH solution imitating the wash protocol used in production. Listeria-inoculated samples of this berry showed very weak correlation between disinfection level and both parameters measured, with no statistically significant differences found between current company's parameters (C2T2) and other tested treatments (Figure 2). In *E. coli*-inoculated strawberries, C1 concentrations proved unreliable, while increasing to C3 didn't improve sanitization power significantly.

Blueberries, also washed by submersion, show very weak correlation between concentration and disinfection level, and almost no correlation with exposure time, exhibiting no statistically significant differences in any treatment.

Blackberries were washed by spraying them with a SH solution, simulating the company's method. Current parameters showed exceptional performance in both bacteria assayed, with no treatment displaying statistically significant improvement.

As discussed previously, it is admissible for this berry to be exported without being washed. Also, it was observed that no statistically significant differences were found for BLRFs in raspberries, for both bacteria tested. This latter observation supports the former idea, with the most important bactericide/bacteriostatic role being played by the fruit's

own properties, reducing SH-related disinfection to anecdotic extents; raspberries and blackberries share several similarities, even at the production line level, making it easier for the company to wash them "*just in case*".

It was also noted in the *E. coli*-inoculated berries, that an increase in flow/exposure time in C3 treatments did not show significant increase over sanitizing efficiency, suggesting the possibility of better throughput in the disinfection process by increasing concentration to C3 and using lower flows/exposure times. On the other hand, Listeria-inoculated berries treated at low, medium and high SH concentrations, showed to be flow/exposure time-dependent in relation to disinfection efficiency. However, C3T1 showed in most cases disinfection potential equivalent to C2T2, suggesting the possibility of better throughput in the disinfection process by increasing concentration to C3 and using lower flows/exposure times. In addition to these results, at C3 concentrations for all berries tested, the increasing in flow/exposure time did not show a significant increase in sanitizing efficiency.

DICTUC's analyses got lower BLRFs, and although the values show significative differences in some berries, the rates are consistently lower, in the same order of magnitude and in general accordance with the ones found in this study. This apparent lower BLRF is probably related to the reported bactericide effect. The samples had to wait extra time in DICTUC's laboratory before analysis, this reduced both pre-washed and post-washed bacterial loads in the samples, and so the results show an apparent lower than expected difference for the treatment.

Storage assays

In both storage experiments (short-and long-term), a powerful bactericide effect is observed across all berry types.

Berries that underwent short-term storage assays (transportation conditions), displayed a considerable bacterial load drop after 24 hours, with an even more potent germicide effect observed after 48 hours. Raspberries show the most remarkable bactericide effect, a total eradication of Listeria after 24 hours and *E. coli* after 48 hours (Tables 3 and 4).

No major significant differences were found between BLRFs for 24 and 48 hours of storage (Figures 17 and 18), and in general, the latter shows slightly higher bacterial drops. These results prove that longer transportation times, prior to freezing, does not deleteriously impact food safety.

A noteworthy result was found for strawberries, where its exposed flesh exhibited no noticeable differences with assays performed over its epicarp (skin), showing that bactericide properties are also present in the mesocarp (flesh).

All berries assayed for long-term storage (exporting conditions), showed a significant bactericide effect since day 15, in accordance with the findings of preceding storage analyses. Raspberries displayed the most notorious bactericide effect, showing high bacterial load drops and BLRFs with consistent statistically significant differences, while exhibiting very effective inoculations. Comparisons between BLRFs found in short-term and long-term storage assays, suggests that the bactericide effects reported are due to intrinsic properties of the fruit, and that freezing conditions didn't play an important role on bacterial survival.

Blueberries showed significantly lower BLRFs compared to other berries, particularly in long-term storage assays (Tables S19 and S20). These berries proved

difficult to inoculate because of its low-energy surface (Skurtys *et al*., 2011), displaying very low wettability.

Different methods were used for washing the fruits (submersion method for strawberries and blueberries, and spray-wash method for raspberries and blackberries) in accordance with current protocols inside the company. It was disclosed to this investigation that the purpose of using different methods is due to fruit resistance to externally applied physical forces. Raspberries and blackberries are prone to fragmentation when exposed to intense flows, such as those used in submersion method. Although the action of the disinfection agent (SH) is presumed to be reduced in spray-washed berries due to a lesser amount of oxidizer molecules in direct contact with the surface of the fruit, the method proved to be reliable and consistent.

As it should be with any business producing ready-to-eat food, biosafety is the most critical item in the company's agenda; prior to implementing any change in the production chain that could affect innocuity, revalidation of the results presented in this study must be done, considering a major increase in the number of samples being assayed.

PROJECTIONS

Disinfection methods are designed to provide reliable ways of assuring safe human consumption, in accordance with industry's and health organisms' regulations. Technology advancements can provide new means to achieve this, integrating reliable, cost-efficient and environmentally friendly solutions that would prevent economic losses by minimizing the likelihood of microbiological contamination.

Although ozone treatment has been in use for more than a century in industrial sanitization, application as a powerful oxidizing agent for biocontrol in food production chains, replacing chlorine, is being studied since only a couple decades, and formally approved by the FDA in 2001 (foodsafetymagazine.com, 2002). This interest in conceiving new reliable methods for disinfection, often based in trivial knowledge, such as the properties of ozone, are probably boosted by the imperative social need to improve environmental impact of industrial processes. Even though rudimentary methods may prove to be empirically efficient and reliable, research needs to be done to confirm these claims and implement them as standard practices.

While efforts may be put in improving disinfection methods, a good approach to enhance biosafety in the production chain would be to also focus on reducing the chance of contamination in the first place. Simple actions, like enforcing better hand sanitization in the workers area, or forbidding animal access to the harvesting or growing areas, could play a major role in reducing contamination risk. Innocuity could be achieved not only by disinfection of the final product, but also by implementing effective disinfection protocols all the way through the production chain. While it makes sense to focus on the final stages of production at the beginning, when adequate methods have been designed and implemented, resources could be destined to control possible contamination points of

origin, further reducing the possibility of an outbreak. All possible sources of contamination should be considered, such as inadequate harvesters' hygienic habits, deficient machinery cleaning practices, cross-contamination in trucks previously used for transporting livestock, etc. Also, innocuity in the packing process must be considered.

It was disclosed by the company that in shared production lines, where different berries are processed, all-purpose practices are performed to every type of fruit. Without specific knowledge about the berry's particular characteristics, it's not an unwise practice to assume similar characteristics and apply the same procedures, however, an in-depth analysis of every particular berry type helps to better allocate resources and improve overall productivity. Studies like the one conducted in this seminar aids in this task, showing that resources appointed for R&D projects can guide decisions to improve the production chain, materializing in real profit for the company. By including detailed industrial costs for each protocol tested, a cost-benefit analysis can be performed and changes introduced for improved efficiency.

Although the bactericide effect observed has been previously reported in the literature, its powerful effect observed in this study is undeniably remarkable. This investigation also adds value to the fruit evaluated by suggesting new research ventures in developing or extracting naturally-occurring effective bactericides. Also, the physicochemical properties of the fruit's epicarp probably play a major role in the bactericide effect, and are a possible source of insight in the lookout for new and novel supramolecular structures with bactericide effect.

The spray/mist method currently used by the company to wash the fruit may be improved by sprinkling with a customized droplet size for each berry type. Disinfection efficacy is modified by the oxidizing agent's penetration capacity and wettability, both potentially modulated by droplet's size, which in turn can be controlled by the pump's

pressure and nozzle properties of the mist generator. Having notoriously different epicarp characteristics, that alter penetration and wettability, the disinfected fruit can benefit from customized settings.

The findings of this study could be disclosed, fully or partially, to strategic partners and clients around the globe, as rigorous proof of the company's commitment to reliability and biosafety.

CONCLUSIONS

While there are industry's regulations to follow to comply with exporting regulations, constant monitoring and fine-tuning of ordinary processes allow for a better allocation of resources.

Bacterial Load Reduction Factor (BLRF) proved to be a useful instrument for studying bacterial load drops.

Current company sanitizing parameters (C2T2) proved to be effective at removing bacteria from the surface of berries assayed in this study.

Bacteria tended to die naturally on the surface of berries; with the bactericide/bacteriostatic effect being fast-acting and especially effective in raspberries. Bacteria couldn't colonize and survive successfully over the fruit's surface.

Supported by the cited references and by the results of this study, an advantageous practice for the company could be the use of SH washing protocols at higher SH concentrations. At these higher settings, equal or better disinfection potentials are expected. A detailed study, with the costs associated to the implementation of each new treatment and an in-depth evaluation of non-detrimental effects in fruit quality, is necessary to determine whether a different setting would be a better combination in the production line.

Berry fruit is a broad classification where fruit with differing characteristics and origins is grouped together by rough similarities in organoleptic and physical properties. Although being classified together, the berries assayed in this study proved to have diverse properties regarding their bactericide effect and ease of bacterial colonization. This in-depth individual knowledge about the fruit processed can increases productivity by improving resource allocation.

It is a common practice to perform certain industry routines *´based on averages`*, leaving margin for fine-tuning of the processes that can help reduce costs, increase throughput and improve productivity.

The plating method used in this study for bacteria quantification, termed *modified drop method*, proved to be a reliable, resource-efficient technique, as results comparison with DICTUC's show.

In overall, the objectives of this work have been successfully accomplished. With this deeper understanding of bacterial survival and proliferation, the company could potentially be able to optimize their processes by increasing production line throughput and/or reducing costs.

BIBLIOGRAPHY

Archer, D. (2018). The evolution of FDA's policy on *Listeria monocytogenes* in ready-to-eat foods in the United States. Current Opinion in Food Science **20:** 64-68.

Bloomeld, S. and Uso, E. (1985). The antibacterial properties of sodium hypochlorite and sodium dichloroisocyanurate as hospital disinfectants. Journal of Hospital Infection **6(1):** 20-30.

Brackett, R. (1987). Antimicrobial Effect of Chlorine on *Listeria monocytogenes*. Journal of Food Protection **50(12):** 999-1008.

Burdulis, D., Sarkinas, A., Jasutiené, I., Stackevicené, E., Nikolajevas, L. and Janulis, V. (2009). Comparative study of anthocyanin composition, antimicrobial and antioxidant activity in bilberry (*Vaccinium myrtillus L.*) and blueberry (*Vaccinium corymbosum L.*) fruits. Acta poloniae pharmaceutica **66(4):** 399-408.

Chu, W., Gao, H., Chen, H., Fang, X. and Zheng, Y. (2018). Effects of cuticular wax on the postharvest quality of blueberry fruit. Food Chemistry **239:** 68-74.

European Food Safety Authority (EFSA), *E. coli*: Rapid response in a crisis., http://www.efsa.europa.eu/en/press/news/120711 (accessed Mar. 22, 2018).

Fair, G., Morris, J., Chang, S., Weil, I. and Burden, R. (1948). The Behavior of Chlorine as a Water Disinfectant. Source Journal (American Water Works Association) **40(10):** 1051-1061.

Fang, T. and Huang, L. (2014). Growth and survival kinetics of *Listeria monocytogenes* in cooked egg whites. Food Control **36(1):** 191-198.

Fawcett, N., Mathers, A., Cheruvanky, A. and Sifri, C. (2013). Modified Track-plating: A Rapid, Accurate and Economical Quantitative Culture Method to Measure the Composition and Resistance Profile of the Gut Microbiome (P 1333).

Food Safety Magazine, 'Recent Ozone Applications in Food Processing and Sanitation' by Rice, R., Graham, D. and Lowe, M. http://www.foodsafetymagazine.com/magazine-archive1/octobernovember-2002/recent-ozone-applications-in-food-processing-and-sanitation/ (accessed Mar 10, 2019).

Fortune, 'Why Our Food Keeps Making Us Sick' by Kowitt, B. http://fortune.com/food-contamination/ (accessed May 22, 2018).

Gonthier, A., Guerin-Faublee, V., Tilly, B. and Delignette-Muller, M. (2001). Optimal growth temperature of O157 and non-O157 *Escherichia coli* strains. Letters in Applied Microbiology **33(5):** 352-356.

Heinonen, M. (2007). Antioxidant activity and antimicrobial effect of berry phenolics - A Finnish perspective. Molecular Nutrition and Food Research **51(6):** 684-691.

Jett, B., Hatter, K., Huycke, M. and Gilmore, M. (1997). Simplified agar plate method for quantifying viable bacteria. BioTechniques **23(4):** 648-650.

Kaper, J., Nataro, J. and Mobley, H. (2004). Pathogenic *Escherichia coli*. Nature Reviews Microbiology **2(2):** 123-140.

Lim, J. Y., Yoon, J. W. and Hovde, C. J. (2010). A Brief Overview of *Escherichia coli* O157:H7 and Its Plasmid O157. Journal of Microbiology and Biotechnology **20(1):** 5-14.

Nohynek, L., Alakomi, H., Kähkönen, M., Heinonen, M., Helander, I., Oksman-Caldentey, K. and Puupponen-Pimiä, R. (2006). Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens. Nutrition and Cancer **54(1):** 18-32.

Puupponen-Pimiä, R., Nohynek, L., Hartmann-Schmidlin, S., Kähkönen, M., Heinonen, M., Määttä-Riihinen, K. and Oksman-Caldentey, K. (2005a). Berry phenolics selectively inhibit the growth of intestinal pathogens. Journal of Applied Microbiology **98(4):** 991-1000.

Puupponen-Pimiä, R., Nohynek, L., Alakomi, H. L. and Oksman-Caldentey, K. (2005b). Bioactive berry compounds - Novel tools against human pathogens. Applied Microbiology and Biotechnology **67(1):** 8-18.

Puupponen-Pimiä, R., Nohynek, L., Meier, C., Kähkönen, M., Heinonen, M., Hopia, A. and Oksman-Caldentey, K. (2001). Antimicrobial properties of phenolic compounds from berries. Journal of Applied Microbiology **90(4):** 494-507.

Radoshevich, L. and Cossart, P. (2017). *Listeria monocytogenes*: to-wards a complete picture of its physiology and pathogenesis. Nature Reviews Microbiology **16:** 32-46.

Ramaswamy, V., Cresence, V., Rejitha, J., Lekshmi, M., Dharsana, K., Prasad, S. and Vijila, H. (2007). Listeria: review of epidemiology and pathogenesis. Journal of Microbiology, Immunology and Infection **40(1):** 4-13.

Ryan, T., Wilkinson, J. and Cavanagh, H. (2001). Antibacterial activity of raspberry cordial *in vitro*. Research in Veterinary Science **71(3):** 155-159.

Skurtys, O., Velásquez, P., Henriquez, O., Matiacevich, S., Enrione, J. and Osorio, F. (2011). Wetting behavior of chitosan solutions on blueberry epicarp with or without epicuticular waxes. LWT – Food Science and Technology **44(6):** 1449-1457.

Thomas, P., Sekhar, A., Upreti, R., Mujawar, M. and Pasha, S. (2015). Optimization of single plate-serial dilution spotting (SP-SDS) with sample anchoring as an assured method for bacterial and yeast CFU enumeration and single colony isolation from diverse samples. Biotechnology Reports **8:** 45-55.

Tavoschi, L., Severi, E., Niskanen, T., Boelaert, F., Rizzi, V., Liebana,E., Gomes Dias, J., Nichols, G., Takkinen, J. and Coulombier, D. (2015). Food-borne diseases associated with frozen berries consumption: a historical perspective, European Union, 1983 to 2013. Eurosurveillance **20(29):** 20-30.

TimesLIVE, 'Death toll from listeria outbreak rises to more than 200' By Tanisha Heiberg., https://www.timeslive.co.za/news/south-africa/2018-05-18-death-toll-from-listeria-outbreak-rises-to-more-than-200/ (accessed June 1, 2018).

Vattem, D., Lin, Y., Labbe, R. and Shetty, K. (2004). Phenolic antioxidant mobilization in cranberry pomace by solid-state bioprocessing using food grade fungus *Lentinus edodes* and effect on antimicrobial activity against select food borne pathogens. Innovative Food Science and Emerging Technologies **5(1):** 81-91.

SUPPLEMENTARY MATERIAL

Table S1: Data values for "Logarithmic bacterial load reduction in Listeria-inoculated strawberries after SH treatments" (Figure 2).

	T1		T2	
SH conc. (ppm)	**BLRF**	**SD**	**BLRF**	**SD**
H2O (0)	0.27	0.01	0.19	0.04
C3 (80)	0.39	0.07	0.47	0.06
C2 (30)	0.41	0.03	0.38	0.10
C1 (10)	0.19	0.06	0.34	0.12

Bacterial Load Reduction Factor (BLRF) for each treatment: SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1 and T2). BLRF is a normalized value for indicating logarithmic bacterial load drop achieved after sanitization treatment. SD: Standard deviation.

Table S2: Data values for "Logarithmic bacterial load reduction in Listeria-inoculated raspberries after SH treatments" (Figure 4).

	T1		T2	
SH conc. (ppm)	**BLRF**	**SD**	**BLRF**	**SD**
H2O (0)	0.31	0.19	0.29	0.10
C3 (80)	0.75	0.30	0.98	0.10
C2 (30)	0.76	0.11	0.69	0.29
C1 (10)	0.39	0.12	0.48	0.16

Bacterial Load Reduction Factor (BLRF) for each treatment: SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1 and T2). BLRF is a normalized value for indicating logarithmic bacterial load drop achieved after sanitization treatment. SD: Standard deviation.

Table S3: Data values for "Logarithmic bacterial load reduction in Listeria-inoculated blackberries after SH treatments" (Figure 6).

	T1		T2		T3	
SH conc. (ppm)	**BLRF**	**SD**	**BLRF**	**SD**	**BLRF**	**SD**
H2O (0)	0.91	0.43	0.84	0.50		
C3 (80)	1.62	0.59	1.81	0.83	1.48	0.20
C2 (30)	0.94	0.21	1.65	0.29	1.08	0.11
C1 (10)	0.81	0.31	0.47	0.10	0.82	0.15

Bacterial Load Reduction Factor (BLRF) for each treatment: SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1, T2 and T3). BLRF is a normalized value for indicating logarithmic bacterial load drop achieved after sanitization treatment. SD: Standard deviation.

Table S4: Data values for "Logarithmic bacterial load reduction in Listeria-inoculated blueberries after SH treatments" (Figure 8).

	T1		T2	
SH conc. (ppm)	**BLRF**	**SD**	**BLRF**	**SD**
H2O (0)	1.78	0.37	2.06	0.71
C3 (80)	4.47	1.91	3.80	1.32
C2 (30)	2.75	1.40	3.88	1.38
C1 (10)	2.82	0.39	2.02	0.63

Bacterial Load Reduction Factor (BLRF) for each treatment: SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1 and T2). BLRF is a normalized value for indicating logarithmic bacterial load drop achieved after sanitization treatment. SD: Standard deviation.

Table S5: Data values for "Logarithmic bacterial load reduction in *E. coli*-inoculated strawberries after SH treatments" (Figure 10).

	T1		T2		T3	
SH conc. (ppm)	**BLRF**	**SD**	**BLRF**	**SD**	**BLRF**	**SD**
H2O (0)	0.56	0.09	0.49	0.07		
C3 (80)	1.84	0.11	2.08	0.30	1.56	0.43
C2 (30)	1.47	0.21	1.56	0.06	2.20	0.31
C1 (10)	0.82	0.20	1.20	0.08	1.27	0.22

Bacterial Load Reduction Factor (BLRF) for each treatment: SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1, T2 and T3). BLRF is a normalized value for indicating logarithmic bacterial load drop achieved after sanitization treatment. SD: Standard deviation.

Table S6: Data values for "Logarithmic bacterial load reduction in *E. coli*-inoculated raspberries after SH treatments" (Figure 12).

	T1		T2		T3	
SH conc. (ppm)	**BLRF**	**SD**	**BLRF**	**SD**	**BLRF**	**SD**
H2O (0)	1.75	0.39	1.51	0.35		
C3 (80)	2.51	0.39	1.79	0.70	3.00	0.90
C2 (30)	1.01	0.64	2.41	0.94	3.65	1.21
C1 (10)	1.61	0.35	1.28	0.31	2.40	0.11

Bacterial Load Reduction Factor (BLRF) for each treatment: SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1, T2 and T3). BLRF is a normalized value for indicating logarithmic bacterial load drop achieved after sanitization treatment. SD: Standard deviation.

Table S7: Data values for "Logarithmic bacterial load reduction in *E. coli*-inoculated blackberries after SH treatments" (Figure 14).

	T1		T2		T3	
SH conc. (ppm)	**BLRF**	**SD**	**BLRF**	**SD**	**BLRF**	**SD**
H2O (0)	0.38	0.21	0.38	0.04		
C3 (80)	0.73	0.20	0.73	0.27	0.79	0.18
C2 (30)	0.43	0.09	0.69	0.20	0.67	0.23
C1 (10)	0.36	0.20	0.63	0.13	0.57	0.07

Bacterial Load Reduction Factor (BLRF) for each treatment: SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1, T2 and T3). BLRF is a normalized value for indicating logarithmic bacterial load drop achieved after sanitization treatment. SD: Standard deviation.

Table S8: Data values for "Logarithmic bacterial load reduction in *E. coli*-inoculated blueberries after SH treatments" (Figure 16).

	T1		T2		T3	
SH conc. (ppm)	**BLRF**	**SD**	**BLRF**	**SD**	**BLRF**	**SD**
H2O (0)	2.65	0.43	2.12	0.08		
C3 (80)	7.21	0.27	5.37	2.20	6.45	1.84
C2 (30)	3.64	0.41	6.41	0.29	5.22	1.96
C1 (10)	3.56	1.72	3.84	1.74	3.46	1.27

Bacterial Load Reduction Factor (BLRF) for each treatment: SH concentration (C1, C2, C3 and H2O) and exposure/flow time (T1, T2 and T3). BLRF is a normalized value for indicating logarithmic bacterial load drop achieved after sanitization treatment. SD:

Standard deviation.

Table S9: Bacterial load reduction percentage for Listeria-inoculated strawberries.

	T1		T2	
SH conc. (ppm)	**BLR%**	**SD**	**BLR%**	**SD**
H2O (0)	46%	2%	35%	6%
C3 (80)	59%	6%	66%	5%
C2 (30)	61%	3%	57%	10%
C1 (10)	35%	9%	53%	14%

Bacterial Load Reduction Percentage (BLR%) for each treatment. Raw data was normalized as BLR%, where 100% reduction indicates an absolute eradication of bacteria after sanitization. SD: Standard deviation.

Table S10: Bacterial load reduction percentage for Listeria-inoculated raspberries.

	T1		T2	
SH conc. (ppm)	**BLR%**	**SD**	**BLR%**	**SD**
H2O (0)	46%	24%	47%	12%
C3 (80)	78%	14%	89%	2%
C2 (30)	82%	4%	75%	15%
C1 (10)	58%	11%	65%	14%

Bacterial Load Reduction Percentage (BLR%) for each treatment. Raw data was normalized as BLR%, where 100% reduction indicates an absolute eradication of bacteria after sanitization. SD: Standard deviation.

Table S11: Bacterial load reduction percentage for Listeria-inoculated blackberries.

	T1		T2		T3	
SH conc. (ppm)	**BLR%**	**SD**	**BLR%**	**SD**	**BLR%**	**SD**
H2O (0)	80%	19%	85%	17%		
C3 (80)	95%	5%	96%	4%	96%	2%
C2 (30)	87%	6%	97%	2%	91%	2%
C1 (10)	81%	11%	66%	7%	84%	9%

Bacterial Load Reduction Percentage (BLR%) for each treatment. Raw data was normalized as BLR%, where 100% reduction indicates an absolute eradication of bacteria after sanitization. SD: Standard deviation.

Table S12: Bacterial load reduction percentage for Listeria-inoculated blueberries.

	T1		T2	
SH conc. (ppm)	**BLR%**	**SD**	**BLR%**	**SD**
H2O (0)	98%	2%	96%	10%
C3 (80)	100%	0%	100%	0%
C2 (30)	99%	0%	100%	1%
C1 (10)	100%	0%	98%	1%

Bacterial Load Reduction Percentage (BLR%) for each treatment. Raw data was normalized as BLR%, where 100% reduction indicates an absolute eradication of bacteria after sanitization. SD: Standard deviation.

Table S13: Bacterial load reduction percentage for *E. coli*-inoculated strawberries.

	T1		T2		T3	
SH conc. (ppm)	**BLR%**	**SD**	**BLR%**	**SD**	**BLR%**	**SD**
H2O (0)	72%	6%	68%	5%		
C3 (80)	99%	0%	99%	1%	95%	6%
C2 (30)	96%	2%	97%	0%	99%	0%
C1 (10)	83%	8%	94%	1%	94%	3%

Bacterial Load Reduction Percentage (BLR%) for each treatment. Raw data was normalized as BLR%, where 100% reduction indicates an absolute eradication of bacteria after sanitization. SD: Standard deviation.

Table S14: Bacterial load reduction percentage for *E. coli*-inoculated raspberries.

	T1		T2		T3	
SH conc. (ppm)	**BLR%**	**SD**	**BLR%**	**SD**	**BLR%**	**SD**
H2O (0)	97%	3%	96%	3%		
C3 (80)	100%	0%	94%	8%	99%	1%
C2 (30)	84%	8%	99%	0%	100%	0%
C1 (10)	97%	3%	94%	3%	100%	0%

Bacterial Load Reduction Percentage (BLR%) for each treatment. Raw data was normalized as BLR%, where 100% reduction indicates an absolute eradication of bacteria after sanitization. SD: Standard deviation.

Table S15: Bacterial load reduction percentage for *E. coli*-inoculated blackberries.

	T1		T2		T3	
SH conc. (ppm)	**BLR%**	**SD**	**BLR%**	**SD**	**BLR%**	**SD**
H2O (0)	53%	19%	58%	6%		
C3 (80)	79%	9%	78%	9%	82%	9%
C2 (30)	62%	9%	78%	8%	76%	12%
C1 (10)	51%	22%	76%	7%	73%	5%

Bacterial Load Reduction Percentage (BLR%) for each treatment. Raw data was normalized as BLR%, where 100% reduction indicates an absolute eradication of bacteria after sanitization. SD: Standard deviation.

Table S16: Bacterial load reduction percentage for *E. coli*-inoculated blueberries.

	T1		T2		T3	
SH conc. (ppm)	**BLR%**	**SD**	**BLR%**	**SD**	**BLR%**	**SD**
H2O (0)	99%	2%	99%	0%		
C3 (80)	100%	0%	100%	0%	100%	0%
C2 (30)	100%	0%	100%	0%	100%	1%
C1 (10)	100%	0%	100%	0%	100%	1%

Bacterial Load Reduction Percentage (BLR%) for each treatment. Raw data was normalized as BLR%, where 100% reduction indicates an absolute eradication of bacteria after sanitization. SD: Standard deviation.

Table S17: Data values for "Bacterial Load Reduction Factor of Listeria-inoculated berries under transportation conditions" (Figure 17).

	BLRF 24h	**SD**	**BLRF 48h**	**SD**
Raspberry	2.88	0.04	5.36	1.91
Blackberry	1.54	0.13	2.63	0.44
Blueberry	0.92	0.42	1.70	1.36
Strawberry/C	0.22	0.09	1.07	0.01
Strawberry	0.92	0.08	1.62	0.20

Bacterial Load Reduction Factor (BLRF) for berry fruits stored at 14ºC for 24 or 48 hours. BLRF is a normalized value for indicating logarithmic bacterial load drop observed after storage. SD: Standard deviation.

Table S18: Data values for "Bacterial Load Reduction Factor of *E coli*-inoculated berries under transportation conditions" (Figure 18).

	BLRF 24h	SD	**BLRF 48h**	SD
Raspberry	1.25	0.79	7.91	0.06
Blackberry	0.78	0.57	0.97	0.16
Blueberry	0.48	0.44	0.82	0.62
Strawberry/C	0.78	0.25	1.44	0.54
Strawberry	0.74	0.23	1.03	0.42

Bacterial Load Reduction Factor (BLRF) for berry fruits stored at 14ºC for 24 or 48 hours. BLRF is a normalized value for indicating logarithmic bacterial load drop observed after storage. SD: Standard deviation.

Table S19: Data values for "Average bacterial load of Listeria-inoculated strawberries (Figure 19), raspberries (Figure 20), blackberries (Figure 21) and blueberries (Figure 22) under exporting conditions".

	Strawberry		**Blueberry**		**Blackberry**		**Raspberry**	
	BLRF	SD	**BLRF**	SD	**BLRF**	SD	**BLRF**	SD
15 days	2.66	0.51	0.51	0.48	1.55	0.81	1.67	0.78
30 days	7.38	0.19	0.90	0.07	1.11	0.41	4.06	2.38
45 days	4.89	2.76	0.02	0.06	1.07	0.21	3.12	0.86
60 days	3.86	1.51	0.47	0.27	1.68	0.27	7.69	0.30
75 days	4.56	2.50	0.59	0.20	6.54	0.16	6.36	1.73
90 days	5.18	1.81	0.75	0.20	5.59	1.79	2.83	0.30

Bacterial Load Reduction Factor (BLRF) for berry fruits stored at -20ºC for 90 days and sampled every 15 days. BLRF is a normalized value for indicating logarithmic bacterial load drop observed after storage. SD: Standard deviation.

Table S20: Data values for "Average bacterial load of *E. coli*-inoculated strawberries (Figure 23), raspberries (Figure 24), blackberries (Figure 25) and blueberries (Figure 26) under exporting conditions".

	Strawberry		**Blueberry**		**Blackberry**		**Raspberry**	
	BLRF	SD	**BLRF**	SD	**BLRF**	SD	**BLRF**	SD
15 days	2.46	0.54	1.59	0.24	2.91	0.39	7.86	0.15
30 days	6.21	2.33	2.41	0.63	7.20	0.23	7.03	1.48
45 days	5.01	2.28	1.71	0.28	4.55	2.39	5.78	2.22
60 days	2.46	0.65	2.10	0.09	2.38	0.35	5.74	2.08
75 days	7.22	1.16	7.53	0.02	5.40	2.55	7.80	0.13
90 days	7.30	1.29	2.76	0.33	7.26	0.22	7.09	1.45

Bacterial Load Reduction Factor (BLRF) for berry fruits stored at -20ºC for 90 days and sampled every 15 days. BLRF is a normalized value for indicating logarithmic bacterial load drop observed after storage. SD: Standard deviation.