CPS 2022 RFP – FINAL PROJECT REPORT

Project Title:
Occurrence and transfer of pathogens from the production environment to leafy greens grown in controlled environment agriculture

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Objectives:

1. Risk-assessment of *Salmonella* and *Listeria monocytogenes* contamination in controlled environment agriculture (CEA) facilities: detection of potential sources and routes of contamination.
2. Establishment of genetic correlations of isolates to identify the distribution patterns of *Salmonella* and *L. monocytogenes* across different sources and routes of contamination.
3. Evaluation of foodborne associated traffic patterns using abiotic surrogates from the indoor production environment to leafy greens.
4. Evaluation of the efficacy of practical and feasible sanitation strategies implemented for indoor systems against *Salmonella* and *L. monocytogenes* contamination.

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FINAL REPORT

Summary of Findings and Recommendations

Main findings:

1. Water Sources as Primary Contamination Vectors

- Irrigation water and nutrient solutions: *Salmonella* was detected in irrigation water and nutrient solutions in substrate-based systems, confirming water as a major transmission route.
- Drainage systems: *Listeria monocytogenes* was found in hydroponic system drainage water, highlighting the risk of persistent contamination in water recirculation systems.

2. Soil and Growing Media as Pathogen Reservoirs

- *L. monocytogenes* was identified in soil-based and hydroponic CEA systems, particularly on worker booties, confirming soil and floors as a key contamination source.

3. Worker-Related Cross-Contamination

- *L. monocytogenes* was detected on worker booties, indicating that contaminated footwear can spread pathogens across different areas within the facility.
- Experimental tracking with *L. innocua* confirmed that boots and trolley wheels can transfer contamination, highlighting the role of equipment movement in pathogen spread.

4. Harvest Equipment and Maintenance

- Harvest bins were identified as high-risk contamination points, particularly when improperly sanitized.
- Inadequate cleaning protocols allow organic matter to accumulate on surfaces (e.g., on damaged and undamaged plastic lids and bins), which might protect bacteria from removal during sanitation.

Recommendations: Mitigation Strategies

- Enhanced water monitoring and treatment to minimize contamination risks (e.g., filtration, disinfection).
- Strict sanitation protocols for high-risk surfaces, ensuring effective cleaning and sanitation of equipment and facilities.
- Implementation of environmental monitoring programs (EMPs) to detect contamination hotspots and prevent pathogen persistence and spread.

These findings highlight the importance of an integrated approach that includes environmental monitoring (EM) sampling, trend analysis, and proactive response strategies. In the case of contamination, timely and targeted control measures should be implemented to prevent pathogen persistence and spread in CEA systems.

Abstract

Controlled environment agriculture (CEA) has emerged as a promising solution for year-round leafy green production. However, contamination risks from foodborne pathogens such as *Salmonella* and *Listeria monocytogenes* remain a concern. This study aimed to assess contamination risks in different CEA systems, establish genetic correlations of isolates, evaluate pathogen transmission routes, and determine the effectiveness of sanitation measures. Three distinct CEA systems were included: **soil-based with re-growth, hydroponic, and substrate-based with re-growth**. Environmental monitoring (EM) was conducted at multiple sampling points, including water, substrate, nutrient solution (growing media), and equipment. *L. monocytogenes* was confirmed in soil-based and hydroponic systems, with hotspots identified in booties, harvest bins, and nutrient drainage systems. *Salmonella* isolates were detected only in the substrate-based system, particularly in irrigation water and produce. Whole-genome sequencing (WGS) of presumptive isolates revealed genetic diversity among strains and help to identify potential contamination routes, highlighting: i) **water sources as a primary vector of contamination, ii) the role of soil and growing media as potential reservoirs, and iii) the risk of contaminated footwear spreading pathogens across different facility areas**.

To examine pathogen spread, *L. innocua* was used as a surrogate to track worker movement and equipment use. Results showed that boots and trolleys could transfer contaminants, though high temperatures and low humidity significantly reduced pathogen survival. The study also evaluated sanitation protocols for harvest bins, a major contamination point. While no *Listeria* spp. were detected after washing and disinfection, trials with *L. innocua* demonstrated that organic matter can protect bacteria from removal, reinforcing the need for improved sanitation.

Findings from this project contribute to science-based risk assessment for CEA operations, highlighting the need for enhanced environmental monitoring programs, sanitation protocols, and risk mitigation strategies. The study provides practical recommendations for industry stakeholders, aligning with regulatory guidelines to improve food safety in indoor farming.

Background

Primary production of leafy greens has traditionally depended on conventional outdoor farming for agricultural production, benefiting from ample land resources and favorable climatic conditions. However, indoor farming has been experiencing steady growth, with an annual increase of 5% in total acreage for leafy greens and approximately 11% for fruiting vegetables across the U.S. and Canada. This expansion plays a key role in ensuring the year-round availability of fresh produce (Walter et al., 2020). The field of **Controlled Environment Agriculture** (CEA) has advanced significantly, evolving from basic protective structures like shade houses and hoop tunnels to highly sophisticated, fully enclosed vertical farming systems. These modern facilities incorporate precise control of lighting, irrigation, and ventilation, optimizing conditions for high-yield crop production while minimizing environmental impact. However, despite their controlled nature, **indoor farming systems are not inherently safer than traditional open-field agriculture**, as contamination risks persist due to potential hazards introduced through production practices and operational procedures.

Specific guidelines outlining **Good Agricultural Practices (GAPs)** and **Good Hygiene Practices (GHPs)** for CEA operations already exist (FAO, 2017). These guidelines include general recommendations, such as: i) **Protected facility structures** should be strategically located, designed, and constructed to prevent contamination and avoid pest harborage; ii) **Worker training and sanitation practices** must be implemented **across all operations**; iii) **Proper water management** and appropriate use of **soil amendment** are critical for **risk control and reduction**.

In January 2022, the **FDA** published the final report of the first domestic investigation into a foodborne illness outbreak associated with leafy greens grown in a CEA operation (illness onset: June – August 2021). The specific CEA facility involved used high-density hydroponic growing techniques, including deep-water culture and floating raft production methods. The **FDA report** released on January 14, 2022, identified multiple factors that may have contributed to ***Salmonella*** contamination in packaged leafy greens, including: i) the presence of a different *Salmonella* serotype in the pond water used for cultivation; ii) Improper storage of growth media; iii) Suboptimal water management practices; and iv) Inadequate sanitation measures, which failed to prevent the introduction and spread of pathogens of public health significance (FDA, 2022). On the other hand, ***Listeria monocytogenes*** is a human pathogen widely present in the environment (soil, water, and organic material). Unlike most bacteria, *L. monocytogenes* can survive and multiply at low temperatures, making it a potential concern in fresh fruit and vegetables. The bacterium can persist in the environment for over a decade, and temporary hygiene failures, poor sanitation practices, and unhygienic equipment design can contribute to contamination (EFSA, 2018). Environmental contamination by *L. monocytogenes* has been shown to play a significant role in fresh produce contamination (Norton et al., 2001; Simmons & Wiedmann, 2018). However, little is known about the likelihood of *L. monocytogenes* persistence in CEA systems. Although **leafy greens grown in CEA systems have been linked to one confirmed multistate foodborne disease outbreak**, **several recalls** of **hydroponically grown leafy greens** in **Canada and the USA** suggest that **contamination can still occur in indoor environments** (Topalcengiz et al., 2024). Most prevalence studies on foodborne pathogens have focused on open-field production environments (Belias et al., 2020; Castro-Ibañez et al., 2015; Harrand et al., 2020), as well as packing houses and fresh-cut processing facilities (Belias et al., 2022; Castro-Ibañez et al., 2016; Sullivan & Wiedmann, 2020). However, further research is needed to develop science-based guidance for systematically assessing food safety risks in leafy greens grown under CEA systems.

In 2022, the FDA published a list of requirements and recommendations applicable to growers engaged in CEA (FDA, 2022), including: 1) to develop a keen understanding of potential sources and routes of contamination including the raw materials and inputs used, as well as possible sources of contamination throughout the operation; 2) to implement effective sanitation procedures and sampling plans, ensuring that cleaning procedures do not contribute to the dispersion of microbial contaminants that may be present; 3) to assess growing operations to ensure implementation of appropriate science- and risk-based preventive measures, including applicable required provisions of the FDA Food Safety Modernization Act (FSMA) Produce Safety Rule and good agricultural practices (GAPs); 4) to verify the effectiveness of routine monitoring of processing and storage environments to prevent pathogen growth in harvested leafy greens; 5) to ensure that all growing pond water is safe and of adequate sanitary quality for its intended use, which includes implementing measures (such as water treatment) necessary to reduce the potential for contamination by known or reasonably foreseeable hazards; 6) to perform a root cause analysis when a pathogen is identified in the growing environment, in raw agricultural inputs such as water, or in the

agricultural commodity to determine how the contamination likely occurred and implement appropriate prevention and verification measures; 7) to assess and mitigate risks associated with adjacent and nearby land uses, in both rural and more urbanized settings. However, more research was still needed such as:

1. Implementation of standardized Environmental Monitoring Programs (EMPs) for CEA operations.
2. Conducting field-based risk assessment studies to better understand risk sources and contamination routes.
3. Acquisition of data-driven development of corrective actions and mitigation strategies.
4. Comprehensive research on the behavior of persisting bacterial forms in CEA.
5. Identifying and prioritizing food safety risks within each CEA operation, pinpointing potential contamination of hotspots.

Answers to these questions will allow growers to accomplish the FDA requirements and recommendations to avoid specific sources and routes of contamination of leafy greens with *Salmonella* and *L. monocytogenes* when produced in CEA facilities.

Research Methods

Objective 1. Risk-assessment of *Salmonella* and *L. monocytogenes* contamination in CEA facilities: detection of potential sources and routes of contamination.

In this study, we conducted environmental monitoring of three different CEA systems, each representing a distinct cultivation approach (**Figure 1**):

CEA System 1: Soil-Based System with Re-Growth (**Soil + Re-Growth**): Plants are grown directly in soil within a controlled environment. After harvesting, re-growth occurs, meaning plants regenerate new leaves from the same plant. This growing system is common in leafy greens and herbs, where multiple harvests are possible from a single planting. Example crop: Batavia lettuce.

CEA System 2: **Hydroponic** System: A soilless cultivation method where plant roots are immersed in a nutrient-rich solution in a nutrient film technique (NFT). This CEA system provides precise nutrient delivery and optimized water usage, often leading to higher yields. It is widely used for lettuce, leafy greens, and fruiting vegetables such as tomatoes and peppers. Example crop: Pak Choi.

CEA System 3: Substrate-Based System with Re-Growth (**Substrate + Re-Growth**): Plants are grown in an inert substrate instead of soil. The system allows for multiple harvests from the same plant, similar to the soil-based re-growth approach. It offers better root aeration and disease control compared to traditional soil cultivation. Example crop: Lamb's lettuce.

Each CEA system was assessed over three visits at intervals of 3 to 4 months. **Table 1** presents the dates of environmental monitoring (EM) sampling cycles conducted in the different CEA systems. Before sampling, the research team together with the facility staff conducted an initial site visit at each facility. The primary objective was to map and identify key risk areas to determine the most critical sampling points. Sampling locations within the CEA systems were risk-rated based on factors such as facility layout, environmental conditions, and operational practices. This risk assessment helped define the appropriate number of test points, taking into account the physical size of the facility and the complexity of cultivation

processes. In each CEA system, a specific set of sampling points was selected and consistently monitored during each EM sampling. **Tables 2–4** summarize the sampling points included in the different EMs.

About 15 different sampling points were screened in each EM for the detection of *Salmonella* and *L. monocytogenes*. Depending on the sampling site, 3 or 5 replicates were taken, with a total of 483 samples (**Table 5**). The sites selected were sampled using hydrasponge sterile swabs (3M), pre-moistened with BPW or ½ Fraser broth (Scharlab, Barcelona, Spain) for *Salmonella* and *L. monocytogenes*, respectively. In large surface areas (e.g., conveyor belts and boxes), approx. > 900 cm^2 (approx. 30 x 30 cm) was swabbed. In small areas, approx. < 81 cm^2 was swabbed. For other surface areas, individual units (e.g., gloves or knives) were swabbed. For produce, 50 g was taken per sample while for soil, and substrate samples 25 g. For water sampling, a modified Moore swab (MMS) (Sbodio et al., 2013) was used as an effective method to capture the filtration of 10 L and concentrate in the swab the expected low concentration of pathogens present. In the laboratory, filtration was performed on 50 or 10 mL using sterile 0.45 µm cellulose nitrate filters coupled with a vacuum system (Sartorius, Madrid, Spain). For the isolation and identification of *Salmonella*, the UNE-EN ISO 19250:2010 standard was followed. Briefly, samples of soil, crop, SMM and sponges were placed into a sterile bag containing a sufficient amount of Buffered Peptone Water (BPW) to ensure submersion, and were homogenized by hand massaging. The homogenate was then incubated at 36 ± 2 °C for 18 ± 2 h. After pre-enrichment, the samples were transferred for enrichment in selective Rappaport-Vassiliades Soja Peptone Broth (RVS broth) incubated at 41.5 ± 1 °C for 24 ± 3 h and in Muller-Kauffmann Tetrathionate Novobiocin Broth, incubated at 37 ± 1 °C for 24 ± 3 h. Following enrichment, the samples were streaked onto two selective mediums (Xylose-Lysine-Desoxycholate Agar, XLD and *Salmonella* Chromogenic Agar) and incubated at 37 ± 2 °C for 24 ± 3 h for colony isolation. For water samples, a 0.45 µm membrane filter was used with a filter holder manifold. The filter was then pre-enriched in BPW at 36 ± 2 °C for 18 ± 2 h, following the same protocol described for sponges. Presumptive *Salmonella* colonies were further isolated and the *invA* gene was identified by PCR (Malorny et al., 2003). In the case of *L. monocytogenes*, samples were analyzed according to the ISO 11290-1:2017 and ISO 11290-2:2017 standard methods for detection. Briefly, primary enrichment in ½ Fraser broth (incubated for 18-24 h at 37 °C) followed by secondary enrichment Fraser broth (incubated for 18-24 h at 37 °C) and plating in two selective media, Brilliance™ Listeria Agar (formerly Oxoid Chromogenic Listeria Agar (OCLA), and modified oxford agar, MOX (Oxoid, UK). Presumptive colonies were analyzed by conventional PCR (Bio rad® thermal cycler, CA) with specific primers to confirm the presence of *iap* and *hly* genes. For each PCR, positive *L. monocytogenes* CECT 5672 from the Spanish Culture Collection (CECT) and negative (sterile distilled water), controls were included. Template DNA for PCR was prepared by the boiling method. The PCR products were analyzed by agarose gel electrophoresis at 80 V70 min and Red-dye staining (Biotium Inc., CA). UV fluorescence emission was recorded and quantified by using ImageQuant™ LAS 500 (GE Healthcare Bio-Sciences AB, Björkgatan, Sweeden). Presumptive *Listeria* spp., *L. monocytogenes* and *Salmonella* isolates obtained from the different EM sampling cycles were further confirmed using the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF), before performing genetic characterization through next-generation sequencing (NGS).

Objective 2. *Establishment of genetic correlations of isolates to identify the distribution patterns of* Salmonella *and* L. monocytogenes *across different sources and routes of contamination.*

Presumptive *Salmonella* and *L. monocytogenes* isolates were streaked on Brain Heart Infusion agar plates and incubated at 37 °C for 24 h. Genomic DNA from all isolates was purified using the Gentra Puregene Yeast/Bacterial Kit (Qiagen). DNA quantification was performed by Agilent 4150 TapeStation System (Agilent Technologies, Inc.). A total of 12 *L. monocytogenes* and 13 *Salmonella* DNA extracts were sent to Macrogen for sequencing. Library construction was carried out using Nextera DNA XT, following the manufacturer's protocols, and sequencing was performed on the NovaSeq X platform (150 bp paired-end sequencing). Raw read data (fastq) were quality checked using FastQC v0.11.7 (Andrews, 2010), and l adapters sequences were removed using Trimmomatic (v0.38) (Bolger et al., 2014). Then, the filtered reads were mapped to reference genomes of *L. monocytogenes* (GCF_000196035.1; 2,944,528 bp) and S. enterica (GCF_000006945.2; 4,951,383 bp) *using BWA v0.7.17* with the BWA-MEM algorithm (Li, 2013). Picard was used for duplicate read removal (https://broadinstitute.github.io/picard/). Variant detection was performed using GATK 4.2.0.0, with HaplotypeCaller identifying SNPs and indels (McKenna et al., 2010). SnpEff 4.3t was used for variant annotation (Cingolani et al., 2012). Manta 1.6.0 and Control-FREEC 11.6 were employed for structural variant detection (Chen et al., 2016) and copy number variation analysis (Boeva et al., 2011), respectively. Mapping efficiency was evaluated based on the percentage of mapped reads, which indicates the proportion of sequences correctly aligned to the reference genome. Proper mapping ensures accurate sequencing results.

Objective 3*. Evaluation of foodborne associated traffic patterns using abiotic surrogates from the indoor production environment to leafy greens.*

A CEA facility located in the CEBAS-CSIC experimental farm was prepared to evaluate the traffic patterns of *L. innocua* (**Picture 1**). In this study, worker's boots and trolley wheels were identified as potential vectors of contamination. The simulated transmission of microbial contamination was conducted as follows: ***1.*** *Contamination source for boots/wheels*: The boots and wheels were inoculated by immersion in a contaminated solution designed to simulate a puddle of water from greenhouse irrigation, which could harbor pathogens, and adhere to the surfaces. The inoculum of *L. innocua* was prepared by reviving a stock culture from frozen storage at -80 °C in BHI broth. The culture was incubated at 37 °C for 18–24 h followed by centrifugation (4000 rpm 10 min). A final dilution was made with sterile PBS 1X to achieve a concentration of 4-5 log CFU/100 mL. Boot and wheel surfaces were immersed in the inoculum solution to ensure complete coverage (**Picture 2**). ***2.*** *Bacteria adhesion*: *L. innocua* adhered to the surfaces of the boots and wheels as contained ridges and grooves that facilitated bacteria attachment. After inoculation, the boots and wheels were used for stepping on the greenhouse floor (T0). ***3.*** *Movement and spread*: As workers and trolleys moved throughout the CEA facility, they served as vehicles for pathogen transfer through direct contact with the floor (**Picture 3**). The experiment was designed to identify traffic patterns at different floor points (FP). A total of 13 floor points (FPs) were distributed along four corridors to evaluate the transfer of contamination across the CEA facility. A diagram of the floor points from FP1 to FP13 and the movement in the CEA corridors is shown in **Figure 2**. The process was repeated twice, with two independent trials conducted for the boots and two for the wheels. Samples were collected at time 0 (T0) and again after 24 h (T24) at each point to assess bacterial survival (**Picture 4**). ***4.*** *Environmental factors influencing the survival*: The CEA environment, depending on its climatic conditions, could either facilitate bacteria survival or contribute to bacteria die-off. Factors such as temperature (°C), relative humidity (%), and radiation (W/m^2), which could influence the survival of the surrogate *L. innocua*, were

monitored and recorded at T0 and T24 over the two trials for both boots and wheels (**Table 12**). ***5.*** *Sample collection*: At both time points (T0 and T24), samples were taken from the floor, boots and wheels using sponge-swabs humidified in distilled water. After swabbing the floor surfaces, the sponges were submerged in Listeria Broth (LB) and refrigerated during transport to the lab. Once in the lab, the sponges were gently massaged to release any attached bacteria. Samples were tested using the FDA BAM method, with some modifications. The protocol was the same previously used for the environmental monitoring of CEA facilities which included the enumeration of *L. innocua* through the pour plate method. Samples were plated on selective Brilliance™ Listeria Agar and incubated for 24-48 h at 37 °C. The limit of quantification for the enumeration was 100 CFU/swab.

Objective 4. *Evaluation of the efficacy of practical and feasible sanitation strategies implemented for indoor systems against Salmonella and L. monocytogenes contamination.*

Task 1: Sampling in selected CEA facilities before and after implementation of control measures. To address this task, the harvest bins used after harvest for placing the leafy greens and subsequently for vacuum cooling before transporting them to clients were selected, as they were identified as hotspots for pathogen detection. A commercial tunnel washing system for harvest bins was used to evaluate the efficacy of the cleaning and disinfection. The washing system consisted of two stages: The first stage involved a water spray shower at medium pressure and the second stage included an alkaline disinfection solution from Ecolab (**Figure 3**). To evaluate the effectiveness of this cleaning process, 20 bins were randomly sampled from different pallets both before and after cleaning (**Picture 5**). Two trials were carried out: one in mid-May and another at the beginning of July 2024 for the detection of *L. monocytogenes*/*Listeria* spp. on dirty and clean harvest bins. For that, 20 individual bins were sampled. Sponges were used to swab the entire surface of each bin, inside and outside (**Picture 6**). For the detection of *Listeria* spp. and *L. monocytogenes*, the ISO 11290-1:2017 standard was followed, with minor modifications. These modifications included primary and secondary enrichment steps using Half-Fraser and Fraser broths, followed by plating on the selective media OCLA. The enumeration of total bacterial counts (TBC) was carried out using surface plating of serial dilutions prepared with buffered peptone water (ISO 4833-1:2013 for the enumeration of microorganisms in food products). Plate Count Agar (PCA) plates were incubated at 37 °C for 44 hours. The results were expressed as log colony-forming units per swabbed surface area (log CFU/bin).

Task 2: Efficacy of cleaning and sanitizing practices to eliminate pathogen contamination on surfaces with different levels of damage. The ability of *L. innocua* to survive on plastic lids, simulating harvest bins with damage, and the efficacy of a washing step using a water rinse were evaluated. Damaged and undamaged polypropylene lids (9x9 cm) were inoculated with *L. innocua*. The inoculum was prepared at a concentration of 9.69 log CFU/mL and lids were sprayed with 3 mL (4.82 log CFU/cm^2) (**Picture 7)**. After inoculation, half of the lids were washed with water and analyzed at time 0 (T0, 1 h after inoculation) and half were stored for 24 h (T24) at 25 °C and 60-70% RH. Thus, four conditions were compared: **T0**: inoculated, **T0 + washing**: inoculated and washed, **T24**: inoculated after 24 h storage, and **T24+ washing**: inoculated after 24 h of storage and then washed. To ensure effective surface sampling and recovery of cells from scratches (which may be protected from swabbing), two sampling procedures were compared: 1. *Swabbing*: Sterile sponges moistened with sterile water were used to collect the inoculum. The sponges were placed in sterile bags containing 100 mL of peptone water (2 g/L). 2. *Massage method*. The entire lid was placed in sterile bags with 100 mL of peptone water (2 g/L) and the bags were shaken/stomached.

Enumeration *of L. innocua* was performed at both T0 and T24 by spreading 1 mL of sample on OCLA plates. Additionally, dirty bins were inoculated with *L. innocua* to assess whether if dirty surfaces promote better survival of the bacteria. In addition to enumeration, the viability of *L. innocua* was also determined. Viability was assessed using the propidium monoazide and ethidium monoazide (PMA-EMA) viability qPCR (v-qPCR) protocol (Truchado et al., 2020).

Results and Discussion

Objective 1

Based on the selected culture-based methods, a total of 31 presumptive *Listeria* isolates were identified in the different EM samplings conducted across the three CEA systems (**Table 6**). The majority of these presumptive isolates (23) were found in CEA System 1 (Soil + Re-Growth), followed by 8 isolates in CEA System 2 (Hydroponics), while no isolates were confirmed as potential *Listeria* in CEA System 3 (Substrate + Re-Growth). Among the presumptive *Listeria* positive isolates, MALDI-TOF analysis confirmed only one *L. monocytogenes* positive sample, which was found on booties in CEA System 1 (Soil + Re-Growth). Additionally, 12 positive isolates, detected in harvest bins and columns, were identified as *L. innocua* (**Table 6**). The presumptive *L. monocytogenes* isolate found in CEA System 1 (Soil + Re-Growth) aligns with the well-documented persistence of *L. monocytogenes* in soil (Ivanek et al., 2006; Strawn et al., 2013). *L. monocytogenes* is known to survive in soil for long periods, facilitated by its ability to withstand harsh environmental conditions, such as nutrient deprivation and desiccation (Vivant et al., 2013).

In CEA System 2 (Hydroponics), 8 presumptive *Listeria* isolates were found in the booties and nutrient solution and drainage solutions. MALDI-TOF analysis confirmed two *L. monocytogenes* positive sample points: one isolate in the booties and three isolates in the drainage nutritive solution (**Table 6**). This finding is consistent with previous research, which highlights water systems in hydroponic setups as favorable environments for the survival and dissemination of *L. monocytogenes* (Danyluk & Schaffner, 2011). The ability of *L. monocytogenes* to form biofilms could favor the long-term persistence in these wet environments, potentially leading to recurrent contamination events. The presence of *L. monocytogenes* in CEA systems raises significant food safety concerns, as this pathogen is known for its persistence in agricultural and food processing environments, as well as its ability to form biofilms, which protect it from sanitation measures (Carpentier & Cerf, 2011; Ferreira et al., 2014). The presumptive positive detection in the booties confirms that floor surfaces can serve as a key reservoir for this microorganism. These findings further support the use of booties as a representative sampling method for aggregating potential contamination from large surface areas (Wu et al., 2023). These results suggest that this pathogen can persist in production environments and pose a risk of cross-contamination. Booties testing positive in two of the CEA Systems further supports the role of soil and floors as primary contamination sources, as workers can unknowingly spread the pathogen through foot traffic (Hoelzer et al., 2014). The detection of *L. innocua* in harvest bins suggests potential risks of *L. monocytogenes* presence and possible contamination of produce after harvest, which could be attributed to inadequate cleaning and sanitizing practices, as reported in prior studies (Colagiorgi et al., 2017). In CEA System 3 (Substrate + Re-growth), the presumptive *Listeria* isolate was confirmed as *Enterococcus faecalis* by MALDI-TOF analysis (**Table 6**).

For *Salmonella*, the only CEA system with positive sampling points for this pathogen was CEA System 3 (Substrate + Re-growth). Among all the samples analyzed, 26 presumptive *Salmonella* isolates were selected using culture-based methods that combined enrichment broth and selective agar media (**Table 7**). In EM1, MALDI-TOF analyses confirmed *Salmonella* positive samples in the pre-harvest product, in the nutrient solution, and the irrigation community water. In EM2 however, MALDI-TOF analysis did not confirm *Salmonella* positive samples: instead, it identified *Proteus mirabilis* in some presumptive isolates, as also observed in EM1 from the nutrient solution and irrigation community water (**Table 7**). Most of the presumptive positive isolates were linked to water sources, particularly irrigation water and the nutrient solution. However, it is important to highlight that some presumptive positive samples were also found in the pre-harvest product, corresponding to EM1. The detection of positive *Salmonella* isolates in only one CEA system (Substrate + Re-growth) suggests that specific environmental conditions in this system may have favored the pathogen persistence or introduction. The association of *Salmonella* with water sources is expected, as water is a well-documented vector for bacterial contamination in agricultural environments (Strawn et al., 2013; Truitt et al., 2018). However, the presence of *Salmonella* in the pre-harvest product raises additional food safety concerns regarding the effectiveness of irrigation water disinfection treatments (Banach & Van Der Fels-Klerx, 2020). Water sources, particularly irrigation water and nutrient solutions, are critical risk factors for *Salmonella* contamination in fresh produce production systems (Allende & Monaghan, 2015). The detection of *Salmonella* in irrigation water suggests potential contamination from contaminated water sources or cross-contamination from the nutrient-recirculating system (Pachepsky et al., 2011). The primary route of *Salmonella* contamination in growing spinach leaves within a hydroponic system originates from the plant's roots rather than from direct leaf contamination (Koseki et al., 2011). These findings emphasize the need for regular water quality monitoring and effective disinfection strategies to minimize microbial risks in CEA systems (Weller et al., 2015).

Objective 2

The presumptive isolates identified by MALDI-TOF were genetically characterized using NGS. The obtained sequences were evaluated based on key sequencing quality parameters, including the total number of sequenced bases, read count, GC content (%), and Q20/Q30 quality metrics, which reflect sequencing accuracy (**Table 8**). Overall, the data were of high quality, suitable for genomic analyses, with good sequencing accuracy and overall coverage. However, for variant analysis, samples with Q30 values above 90% provide more reliable results. In all the cases, filtered reads were aligned to reference genomes of different databases. The results obtained after analyzing the assembled sequences against the reference genomes are shown in **Tables 9** and **10**. The isolates 9B2, 1C2, 2C3, 2C4, D4, and B2 exhibited a low percentage of mapped reads, which may be attributed to issues in library preparation or specific genomic characteristics. Some of the analyzed isolates were identified as another species or remained unidentified, corresponding to samples with a low percentage of mapped reads (**Table 8**).

In the case of presumptive *L. monocytogenes* isolates, the assembled sequence data of five *L. monocytogenes* isolates were genotyped using core genome MLST (cgMLST) based on a 1,748-gene scheme via the BIGSdb-Pasteur v1.36.7 web server (Moura et al., 2016). The results indicated that the isolates belonged to Lineage II, Sequence Type (ST) 155, and Clonal Complex (CC 155) (**Table 9**). For *Salmonella*, sequencing data were uploaded and analyzed on the EnteroBase web-based platform (http://enterobase.warwick.ac.uk/species/index/senterica). EnteroBase analysis included serovar

prediction using the Achtman 7-gene MLST scheme, sequence type determination, cgMLST, and wgMLST. The PubMLST database for *Salmonella* (https://pubmlst.org/organisms/salmonella-spp) was also used to obtain MLST profiles, indexing each unique combination of alleles with an ST designation (**Table 10**). Regardless of the database used, all isolates identified as *Salmonella* corresponded to the species *Salmonella enterica*. Five of them were classified as ST 3614 and five as ST 1815. Additionally, EnteroBase indicated that these STs corresponded to the serotypes II 41:z10:1,2 and Mikawasima. *Salmonella enterica* serovar Mikawasima is an uncommon serovar in the EU (EFSA, 2013). In Europe, since 2004, five Mikawasima strains have been isolated from vegetables, including tomatoes (EFSA, 2013). This serotype and ST 1815 were also isolated from iceberg lettuce analyzed in Turkey (Gunel et al., 2015).

Objective 3

L. innocua was enumerated in the boots and the wheels at T0 in both trials (**Table 11**). The initial inoculum (4.6 log CFU/swab, mean value in Trials 1 and 2) was transferred by the boots to FP1 (4.5 log CFU/swab, mean value in Trials 1 and 2). In the case of the wheels, the initial inoculum (5.5 log CFU/swab mean value) was transferred to FP1 (3.8 log CFU/swab mean value) and FP2 (3.1 log CFU/swab). Enumeration was only possible at the first floor points (0 meters). In the wheel trials, bacteria were also enumerated at floor points spaced at 30 and 60 meters, as the inoculum size was higher than in the boot trials (**Table 11**). However, enumeration was not possible either at any other floor points at T0 or at any one after 24 h (T24). In cases where *L. innocua* enumeration was not observed using culture method, viability was confirmed by the positive Fraser results in OCLA. As shown in **Figure 4**, *L. innocua* was detected at T0 after being transferred by either boots or wheels to multiple floor points at varying distances from the inoculation site. However, *L. innocua* only survived after 24 h (T24) when transferred by boots. One possible explanation for this difference could be the higher temperature recorded during the wheel trials than in the boots (32.1 vs 28.8 °C maximum and 20.9 vs 18.5 °C minimum temperature in the wheel and the boot trials, respectively) (**Table 12**). Relative humidity was high but comparable between boots and wheels (82.6% and 85.9% maximum and 56.2% and 56.5% minimum RH for the boot and wheel trials, respectively). The radiation intensity was also similar between the boot and the wheel trials (1036 vs 1010 W/m^2) (**Table 12**).

Results obtained from Objective 1 showed a low occurrence of positive *L. monocytogenes*/*Listeria* spp. samples when compared with previous results on fresh-cut processing plants (Gil et al., 2024). This low prevalence can be attributed to the high temperatures and low humidity conditions under which only competitive microbiota were able to survive in the CEA indoor environment. The results obtained in the present objective indicate that the potential risk of microbial contamination being transmitted via workers or equipment, such as trolleys, is very low under similar climatic conditions of high temperatures and low humidity. This study highlights that, while pathogens can be transferred by workers' boots and trolley wheels, they are unable to survive under the prevailing environmental conditions. Our findings suggest that high temperatures significantly impacted pathogen survival. Previous studies have shown that pathogen survival in soil and soil amendments is influenced by moisture content and temperature. In contrast, temperature and other parameters such as pH and turbidity affect survival in irrigation water. Pathogens like *L. monocytogenes* can survive for extended periods, but this largely depends on the moisture levels and the temperature of the soil (Black et al., 2021). Higher soil moisture generally enhances pathogen survival, while elevated temperatures can reduce their persistence. Because CEA

systems lack the benefit of UV light exposure due to indoor settings, they may require enhanced vigilance during the cleaning and sanitation of harvesting equipment to prevent contamination (Leaman et al., 2023). In the climatic conditions tested in our study, contamination from the floor played a minor role in food safety.

Objective 4

When the valuation of the efficacy of cleaning and sanitizing operation for harvest bins was examined, it was observed that no *Listeria* spp. or *L. monocytogenes* were detected in either dirty or clean bins. One possible explanation could be that *Listeria* spp./*L. monocytogenes* may have gone undetected due to the presence of competing microorganisms, which could suppress their growth during the enrichment incubation process (Dailey et al., 2014). It should be considered that several bacterial species can outcompete *Listeria* spp. and interfere with detection. This is particularly problematic when the competing microorganisms are present at levels 5-7 logs higher than *L. monocytogenes* at the start of enrichment. In our study, the total bacterial levels were very high (> 7 log CFU/swab), which may have further hindered the detection of *Listeria* spp. **Figure 5** shows that there was no significant difference in the total aerobic count between dirty and clean bins.

Our findings showed that even when a surface is damaged, the potential for the survival of human pathogens is very low and the only significant effect is the water rinse in removing the contamination (**Figure 6**). Several factors have previously been shown to affect *L. monocytogenes* biofilm formation, including temperature, time, surface type, and nutrient availability (Dygico et al., 2020). These authors highlighted specific surfaces of concern, with a positive correlation between surface roughness and biofilm formation. In our study, the low nutrient availability might have played a crucial role in reducing inoculum survival and enhancing the wash-off effect of the water rinse. When the trial was repeated on dirty surfaces rich in organic matter, similar to the surfaces of the harvest bins, *L. innocua* survived and remained viable 24 h after inoculation. The results indicated that a water rinse was not effective in removing the inoculated *L. innocua* as the presence of organic matter helped to trap the bacteria (**Table 13**).

Outcomes and Accomplishments

Findings from this project contribute to science-based risk assessment for CEA, emphasizing the need for enhanced monitoring, improved sanitation protocols, and targeted risk mitigation strategies. Based on the results obtained, the main outputs and accomplishments of the project include:

- **Water was identified as a primary contamination vector**: *Salmonella* was detected in irrigation water and nutrient solutions in substrate-based systems, confirming water as a key contamination source. *L. monocytogenes* was found in hydroponic drainage systems, highlighting persistent contamination risks in recirculating water.
- **Soil/floors contamination vectors:** *L. monocytogenes* was confirmed in both soil-based and hydroponic systems, demonstrating that soil and floors can be primary reservoirs of this pathogen in the produce cultivation environment. This finding reinforces the importance of using booties for testing large surface areas to effectively identify contamination points across the facility and evaluate the efficacy of cleaning and sanitizing practices.

- **Transfer and survival of contamination:** Experimental tracking with *L. innocua* demonstrated that worker boots and trolley wheels can spread bacteria, which can persist until the environmental conditions favor their growth. In our study, high temperatures and low humidity reduced pathogen survival, suggesting that environmental factors play a critical role in contamination survival and persistence.
- **Harvesting cleaning and sanitizing practices**: Harvest bins were identified as potential contamination points for the harvested product. Our results showed that organic matter can protect bacteria from removal, allowing them to survive after contamination. These findings reinforce the need for efficient cleaning protocols as well as monitoring the effectiveness of sanitation practices.

Recommendations for Risk Mitigation Strategies in CEA

Environmental monitoring programs (EMPs) are essential to identify contamination hotspots and prevent pathogen persistence and spread. Enhanced water monitoring is recommended, including the use of a device such as the Moore swab to collect large volume samples. If contamination is detected, water disinfection treatments should be recommended (e.g., filtration, disinfection). Cleaning and sanitizing procedures should be implemented for harvesting equipment, ensuring that these processes are consistently followed and validated for effectiveness. These outputs provide science-based guidance for CEA growers to reduce food safety risks and enhance microbial quality control in indoor farming systems.

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APPENDICES

Presentations

Ana Allende. *Listeria monocytogenes* in the production and processing environment of fruits and vegetables: The known knowns and the known unknowns of environment contamination. Invited Speaker. Teagasc Food Research Centre, Ashtown, Dublin. 24-25 May, 2023.

Ana Allende. Assessing the efficiency and practicality of the decision-making processes and microbiological criteria developed within the Codex framework for fresh fruits and vegetables. Invited speaker. Workshop on advancing controlled environment agriculture on land and in space in the next 20 years. USDA-ARS, University of Toledo, OH, United States, 27th-29th of June,2023.

Ana Allende. Produce safety systems of the future: What we know and what we should know. Invited Speaker. Fresh Produce Safety Conference 2023: food safety in a changing world. Fresh Produce Safety Centre Australia and New Zealand, Sydney, Australia, 10th of August, 2023.

Ana Allende. Sustainable and Safe Production of Fresh Produce: Does Controlled Environmental Agriculture answer all challenges? Invited Speaker. Singapore International Food Forum 2024 (SIFF2024), National University of Singapore, Singapore, 1-2 August, 2024.

Ayala-San Nicolás, M., Allende, A., Illán-Ortega, G., Truchado, P., Gil, M.I. 2024. Risk assessment of *Listeria monocytogenes* contamination in controlled agriculture systems. XXIII National Congress of Food Microbiology. Oral presentation. Murcia, Spain, 9-12 September.

Maria I. Gil. Contamination risks of *Listeria monocytogenes* in plant-based foods from production to processing. 2024. AESAN (Spanish Agency for Food Safety and Nutrition). Invited presentation at the seminar: '*Listeria monocytogenes*: A Challenge for Food Safety.' Madrid, Spain, June 6.

Plant-based foods and new consumption trends. 2024. AESAN (Spanish Agency for Food Safety and Nutrition). Invited presentation at the seminar: 'Objective: *Listeria* Under Control.' Madrid, Spain, December 12.

Publications *(in preparation)*

Environmental Monitoring for *Listeria monocytogenes*/*Listeria* spp. in a Soil Growth Controlled Environment Agriculture System. Implementation of Harvesting Cleaning. *Publication in preparation*.

Prevalence and Persistence of *Listeria monocytogenes* in Hydroponic Controlled Environment Agriculture (CEA) of Pak Choi: Transfer route in Indoor Production. *Publication in preparation.*

Environmental Monitoring of Controlled Environment Agriculture Facilities for *Salmonella. Publication in preparation.*

Applying Multiple Logistic Regression to Identify *Listeria monocytogenes* Contamination Vectors and Transmission Pathway. *Publication in preparation.*

Budget Summary

This project was awarded $225,864 in research funds. The majority of the grant funds were spent on salaries and wages, which accounted for a significant portion of the total budget. Almost all of the allocated funds in this category were utilized, demonstrating that personnel costs were a key priority in the project. Materials and supplies also represented a substantial share of the expenses, and in fact, spending in this category slightly exceeded the original allocation. This suggests that operational needs may have required additional resources beyond what was initially budgeted. Travel expenses also represent an important component; a large amount of that budget will be used in the next trip to the CPS conference. Remaining budget in "Other direct expenses" will be expended in the registration for the conference. Finally, indirect costs, which were calculated as a percentage of personnel costs, were mostly utilized but not fully exhausted. This suggests that adjustments in personnel expenditures had a minor impact on overhead allocations.

Overall, the project managed its budget effectively, spending almost all of the granted funds, with some flexibility in categories such as materials and supplies, while keeping other costs, like miscellaneous direct expenses, lower than expected.

Tables, Figures and Pictures (see below)

Table 1. Dates of the EM sampling cycles performed in the three different CEA systems.

CEA SYSTEM	SAMPLING DATES
Soil + Re-growth	May 2023
	October 2023
	April 2024
Hydroponic	July 2023
	September 2023
	March 2024
Substrate + Re-growth	October 2023
	December 2023
	February 2024

Table 2. Sampling points included in the EM of the soil + re-growth CEA system (CEA System 1).

#	Sampling point	Description	Sample Type	Replicate
1	Soil Amendments		Soil	1
2	Soil Amendments		Soil	2
3	Soil Amendments		Soil	3
4	Soil		Soil	1
5	Soil		Soil	2
6	Soil		Soil	3
7	Irrigation water from reservoir		Water	1
8	Irrigation water from reservoir		Water	2
9	Irrigation water from reservoir		Water	3
10	Irrigation water from sprinklers		Water	1
11	Irrigation water from sprinklers		Water	2
12	Irrigation water from sprinklers		Water	3
13	Harvested Batavia lettuce		Product	1
14	Harvested Batavia lettuce		Product	2
15	Harvested Batavia lettuce		Product	3
16	Harvested Batavia lettuce		Product	4
17	Harvested Batavia lettuce		Product	5
18	Precooled Batavia lettuce		Product	1
19	Precooled Batavia lettuce		Product	2
20	Precooled Batavia lettuce		Product	3
21	Precooled Batavia lettuce		Product	4
22	Precooled Batavia lettuce		Product	5
23	Harvest bins		Surface	1
24	Harvest bins		Surface	2
25	Harvest bins		Surface	3
26	Cooling bins		Surface	1
27	Cooling bins		Surface	2
28	Cooling bins		Surface	3

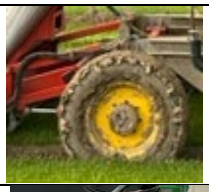
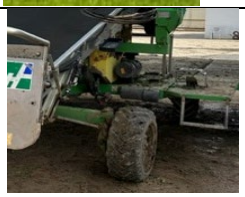
29	Leaf vacuum cleaner machine		Surface	1
30	Leaf vacuum cleaner machine		Surface	2
31	Leaf vacuum cleaner machine		Surface	3
32	Harvesting machine		Surface	1
3	Harvesting machine		Surface	2
34	Harvesting machine		Surface	3
35	Wheels vacuum cleaner machine		Surface	1
36	Wheels vacuum cleaner machine		Surface	2
37	Wheels vacuum cleaner machine		Surface	3
38	Wheels harvesting machine		Surface	1
39	Wheels harvesting machine		Surface	2
40	Wheels harvesting machine		Surface	3
41	Columns		Surface	1
42	Columns		Surface	2
43	Columns		Surface	3
44	Booties		Surface	1
45	Booties		Surface	2
46	Booties		Surface	3

Table 3. Sampling points included in the EM of hydroponic CEA system (CEA System 2).

#	Sampling point	Description	Sample Type	Replicate
1	Hydroponic system (NFT)		Surface	1
2	Hydroponic system (NFT)		Surface	2
3	Hydroponic system (NFT)		Surface	3
4	Knife		Surface	1
5	Knife		Surface	2
6	Knife		Surface	3
7	Harvest bins		Surface	1
8	Harvest bins		Surface	2
9	Harvest bins		Surface	3
10	Conveyor belt		Surface	1
11	Conveyor belt		Surface	2
12	Conveyor belt		Surface	3
13	Booties (Floor)		Surface	1
14	Booties (Floor)		Surface	2
15	Booties (Floor)		Surface	3
23	Pre-harvest pak choi		Product	1
24	Pre-harvest pak choi		Product	2
25	Pre-harvest pak choi		Product	3
26	Pre-harvest pak choi		Product	4

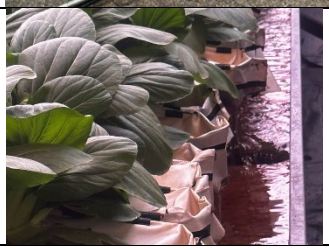
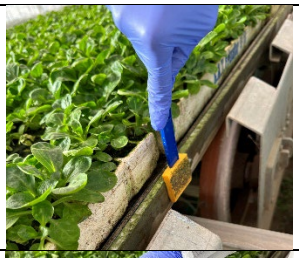
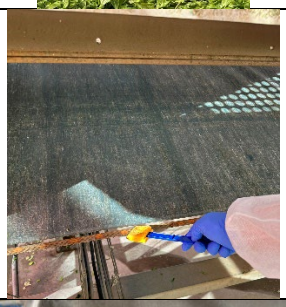
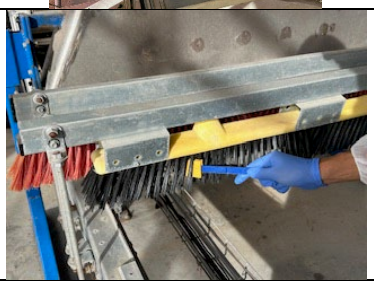
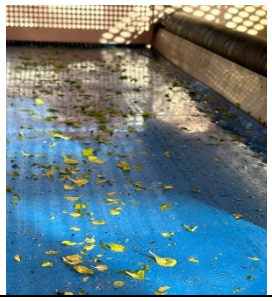
27	Pre-harvest pak choi		Product	5
29	Harvested pak choi		Product	1
30	Harvested pak choi		Product	2
31	Harvested pak choi		Product	3
32	Harvested pak choi		Product	
33	Harvested pak choi		Product	
32	Nutrient solution		Water	1
33	Nutrient solution		Water	2
34	Nutrient solution		Water	3
35	Irrigation community water		Water	1
36	Irrigation community water		Water	2
37	Irrigation community water		Water	3
38	Drainage of nutrient solution		Water	1
39	Drainage of nutrient solution		Water	2
40	Drainage of nutrient solution		Water	3

Table 4. Sampling points included in the EM of substrate + re-growth CEA system (CEA System 3).

#	Sampling point	Description	Sample Type	Replicate
1	Moving platforms		Surface	1
2	Moving platforms		Surface	2
3	Moving platforms		Surface	3
4	Trays		Surface	1
5	Trays		Surface	2
6	Trays		Surface	3
7	Columns		Surface	1
8	Columns		Surface	2
9	Columns		Surface	3
10	Cutting blades		Surface	1
11	Cutting blades		Surface	2
12	Cutting blades		Surface	3
13	Brushes		Surface	1
14	Brushes		Surface	2
15	Brushes		Surface	3
16	Vacuum machine		Surface	1
17	Vacuum machine		Surface	2
18	Vacuum machine		Surface	3

19	Conveyor belt of harvested product		Surface	1
20	Conveyor belt of harvested product		Surface	2
21	Conveyor belt of harvested product		Surface	3
22	Bins for harvested product		Surface	1
23	Bins for harvested product		Surface	2
24	Bins for harvested product		Surface	3
25	Booties (floor)		Surface	1
26	Booties (floor)		Surface	2
27	Booties (floor)		Surface	3
28	Pre-harvest lamb's lettuce		Product	1
29	Pre-harvest lamb's lettuce		Product	2
30	Pre-harvest lamb's lettuce		Product	3
31	Pre-harvest lamb's lettuce		Product	4
32	Pre-harvest lamb's lettuce		Product	5
33	Harvested lamb's lettuce		Product	1
34	Harvested lamb's lettuce		Product	2
35	Harvested lamb's lettuce		Product	3
36	Harvested lamb's lettuce		Product	4
37	Harvested lamb's lettuce		Product	5
38	Substrate		Soil	1
39	Substrate		Soil	2

40	Substrate		Soil	3
41	Nutrient solution		Water	1
42	Nutrient solution		Water	2
43	Nutrient solution		Water	3
44	Irrigation water from the community		Water	1
45	Irrigation water from the community		Water	2
46	Irrigation water from the community		Water	3

Table 5. Total number of samples taken at each CEA system.

CEA System	Sample type	EM	Replicates	Total samples
Soil+Re-growth	Product (2)	1	5	10
		2	5	10
		3	5	10
	Water (2)	1	3	6
		2	3	6
		3	3	6
	Soil (2)	1	3	6
		2	3	6
		3	5	10
	Surfaces (9)	1	3	27
		2	3	27
		3	5	45
TOTAL				**169**
Hydroponic	Product (2)	1	5	10
		2	5	10
		3	5	10
	Water, nutrient and drainage solution (3)	1	3	9
		2	3	9
		3	3	9
	Surfaces (9)	1	3	27
		2	3	27
		3	5	45
TOTAL				**156**
Substrate+Re-growth	Product (2)	1	5	10
		2	5	10
		3	5	10
	Water and nutrient solution (2)	1	3	6
		2	3	6
		3	3	6
	Substrate (1)	1	3	3
		2	3	3
		3	5	5
	Surfaces (9)	1	3	27
		2	3	27
		3	5	45
TOTAL				**158**

Table 6. Presumptive *L. monocytogenes*/*Listeria* spp. isolates found in the EM sampling of the three CEA systems.

CEA SYSTEM	EM	SAMPLING POINT	Isolate ID	*hly*	*iap*	*SigB*	MALDITOF
Soil + Re-growth	1	Harvest bins	1C1		+	+	*L. innocua*
			1C2		+	+	*L. innocua*
			1C3		+	+	*L. innocua*
		Harvest bins after vacuum cooling	2B2		+	+	*L. innocua*
			2B3		+	+	*L. innocua*
			2B4		+	+	*L. innocua*
		Columns	7A1		+	+	*L. innocua*
			7A3		+	+	*L. innocua*
			7A5		+	+	*L. innocua*
			7C1		+	+	*L. innocua*
			7C3		+	+	*L. innocua*
			7C4		+	+	*L. innocua*
		Booties	9B2	-	+	-	*L. aquatica*
	2	Harvest bins	1C2	-	+	-	*L. fleischmannii*
		Harvest bins after vacuum cooling	2C1		+	-	*No Identification*
			2C3		+	-	*L. grayi*
			2C4		+	-	*L. aquatica*
		Vacuum machine	3B2		+	-	*Enterococcus faecalis*
			3B3		+	-	*Enterococcus faecalis*
			3B4		+	-	*Enterococcus faecalis*
	3	Booties	9D2	+	-	+	*Enterococcus faecalis*
			9D4	+	-	+	*Enterococcus faecalis*
			9D5	+	-	+	*L. monocytogenes*
Hydroponic	1	Booties	20A1	+	+	+	*L. monocytogenes*
			20A3	+	+	+	*Enterococcus faecalis*
			20A4		+		*Enterococcus casseliflavus*
		Drainage nutrient solution	26A1	+	+	+	*L. monocytogenes*
			26A2	+	+	-	*L. monocytogenes*

			26A3	+	+	+	*L. monocytogenes*
	2	Nutrient solution	23A2		+	-	*No identification*
Substrate + Re-growth	3	Booties	39A4		+	-	*Enterococcus faecalis*

Table 7. Presumptive *Salmonella* isolates found in the EM sampling of the three CEA systems identified by MALDI-TOF.

CEA SYSTEM	EM	SAMPLING POINT	Isolate ID	Müller S. Plus	Müller XLD	Rappaport S. Plus	Rappaport XLD	MALDI-TOF MS
Substrate + Re-growth	1	Product before harvest	D1	Positive				*Salmonella spp.*
			D2	Positive				*Salmonella spp.*
			D3		Positive			*Salmonella spp.*
			D4		Positive			*Salmonella spp.*
			D5			Positive		*Salmonella spp.*
			D6			Positive		*Salmonella spp.*
		Nutrient solution	B1			Positive		*Salmonella spp.*
			B2			Positive		*Salmonella spp.*
			B3				Positive	*Salmonella spp.*
			B4				Positive	*Salmonella spp.*
			C1			Positive		*Proteus mirabilis*
			C2			Positive		*Proteus mirabilis*
		Irrigation community water	A1			Positive		*Salmonella spp.*
			A2			Positive		*Proteus mirabilis*
			B1			Positive		*Proteus mirabilis*
			B2			Positive		*Proteus mirabilis*
			B3				Positive	*Proteus mirabilis*
			B4				Positive	*Proteus mirabilis*
			B5		Positive			*Proteus mirabilis*
			B6		Positive			*Salmonella spp.*
			C1		Positive			*Salmonella spp.*
			C2		Positive			*Proteus mirabilis*
	2	Nutrient solution	A1				Positive	*Proteus mirabilis*
			C1				Positive	*Proteus mirabilis*
			E1				Positive	*Proteus mirabilis*
		Irrigation community water	B1	Positive				*Proteus mirabilis*

Table 8. Sample ID and sequencing quality parameters for *Salmonella* and *Listeria* isolates, including the total number of sequenced bases, read count, GC content (%), Q20 and Q30 quality metrics, mapped reads (%), and variants, in comparison to the reference genomes of *Listeria monocytogenes* (GCF_000196035.1; 2,944,528 bp) and *Salmonella enterica* (GCF_000006945.2; 4,951,383 bp).

Sample ID	Total bases (bp)	Total reads	GC (%)	AT (%)	Q20 (%)	Q30 (%)	Mapped Reads (%)	Variants
1C1	3,004,916,397	20,451,442	38.6	61.4	98.8	95.3	79.85	**155,581**
2B3	1,775,389,981	12,274,798	38.6	61.4	98.5	94.2	77.15	**153,405**
7C1	2,727,746,915	18,794,632	38.7	61.3	98.5	94.2	77.30	**153,035**
9B2	2,869,348,710	19,838,970	37.0	63.0	98.5	94.3	3.07	**2,675**
1C2	2,911,829,622	19,847,948	39.5	60.5	99.0	95.9	11.70	**17,592**
2C3	2,677,643,558	18,540,102	41.4	58.6	98.5	94.0	10.26	**12,576**
2C4	2,181,508,922	15,034,500	41.1	58.9	98.6	94.6	10.38	**12,469**
9D5	2,905,465,478	20,063,094	39.1	60.9	98.5	94.0	94.59	**27,322**
20A1	2,142,833,486	14,796,230	39.2	60.8	98.5	94.0	94.51	**27,419**
26A1	1,663,730,391	11,497,430	39.1	60.9	98.6	94.4	94.58	**27,782**
26A2	2,837,723,406	19,535,566	39.1	60.9	98.6	94.6	94.43	**27,701**
26A3	2,896,814,863	20,012,858	39.1	60.9	98.5	94.3	94.52	**27,649**
D1	2,801,219,120	18,551,120	53.5	46.5	95.2	87.2	78.86	**126,077**
D2	3,197,890,080	21,178,080	53.3	46.7	95.5	87.6	79.49	**126,019**
D3	2,687,278,748	17,796,548	53.3	46.7	95.5	87.6	79.70	**126,142**
D4	2,517,998,084	16,675,484	61.8	38.2	94.4	85.3	27.59	**126,935**
D5	2,636,657,508	17,461,308	53.4	46.6	95.3	87.2	79.63	**126,219**
D6	2,226,794,584	14,746,984	53.4	46.6	95.2	86.9	79.63	**125,924**
B1	2,363,543,808	15,652,608	53.4	46.6	95.7	88.2	93.19	**38,447**
B2	2,289,553,204	15,162,604	52.5	47.5	95.1	86.5	39.28	**136,062**
B3	2,946,784,932	19,515,132	53.4	46.6	95.6	88.0	91.15	**38,540**
B4	3,524,938,262	23,343,962	53.3	46.7	95.7	88.0	90.80	**38,466**
A1	2,746,420,314	18,188,214	53.9	46.1	95.0	86.5	92.48	**38,502**
B6	2,637,026,250	17,463,750	53.4	46.6	95.4	87.4	92.99	**38,461**
C1	**3,140,583,768**	**20,798,568**	**53.6**	**46.4**	**95.2**	**86.8**	**93.02**	**38,409**

- Sample ID: Sample name.
- Total Bases (bp): Total number of bases sequenced.
- Total Reads: Total number of reads. For illumina paired-end sequencing, this value refers to the sum of read1 and read2.
- GC (%): Ratio of GC content.
- AT (%): Ratio of AT content.
- Q20 (%): Ratio of bases that have phred quality score of over 20.
- Q30 (%): Ratio of bases that have phred quality score of over 30.
- Mapped Reads (%): % of reads mapped to the reference genome.
- Variants: Number of variants (insertions, deletions, SNPs) compared to reference genome.

Table 9. Sample ID, CEA system, EM, sampling point, and species and serotype prediction results for *Listeria* isolates.

SAMPLE ID	CEA System	EM	SAMPLE POINT	MALDI-TOF MS	SoluGenomics		BIGSdb-Pasteur		
					Species	ST	ST	CC	Lineage
1C1	Soil + Re-growth	1	Harvest bins	*L. innocua*	*L. innocua*				
2B3	Soil + Re-growth	1	Harvest bins after vacuum cooling	*L. innocua*	*L. innocua*				
7C1	Soil + Re-growth	1	Columns	*L. innocua*	*L. innocua*				
9B2	Soil + Re-growth	1	Booties	*L. aquatica*	*Bacillus circulans*				
1C2	Soil + Re-growth	2	Harvest bins	*L. fleischmannii*	*No identification*				
2C3	Soil + Re-growth	2	Harvest bins after vacuum cooling	*L. grayi*	*No identification*				
2C4	Soil + Re-growth	2	Harvest bins after vacuum cooling	*L. aquatica*	*No identification*				
9D5	Soil + Re-growth	3	Booties	*L. monocytogenes*	*L. monocytogenes*	155	155	155	II
20A1	Hydroponics	1	Booties	*L. monocytogenes*	*L. monocytogenes*	155	155	155	II
26A1	Hydroponics	1	Drainage nutrient solution	*L. monocytogenes*	*L. monocytogenes*	155	155	155	II
26A2	Hydroponics	1	Drainage nutrient solution	*L. monocytogenes*	*L. monocytogenes*	155	155	155	II
26A3	Hydroponics	1	Drainage nutrient solution	*L. monocytogenes*	*L. monocytogenes*	155	155	155	II

Table 10. Sample ID, CEA system, EM, sampling point, species and serotype prediction results for *Salmonella* isolates.

SAMPLE ID	CEA System	SAMPLE POINT	EM	MALDI-TOF	PubMLST[1]		EnteroBase[2]					
					ST	CC	Species	rMLST	Serotype	ST	cgMLST	wgMLST
D1	Substrate + Re-growth	Product before harvest	1	*Salmonella* spp.	3614	-	*Salmonella enterica*	New	II 41:z10:1,2	3614	498354	597780
D2	Substrate + Re-growth	Product before harvest	1	*Salmonella* spp.	3614	-	*Salmonella enterica*	New	II 41:z10:1,2	3614	498342	597765
D3	Substrate + Re-growth	Product before harvest	1	*Salmonella* spp.	3614	-	*Salmonella enterica*	New	II 41:z10:1,2	3614	498342	597762
D4	Substrate + Re-growth	Product before harvest	1	*Salmonella* spp.			*Pseudomonas aeruginosa*					
D5	Substrate + Re-growth	Product before harvest	1	*Salmonella* spp.	3614	-	*Salmonella enterica*	New	II 41:z10:1,2	3614	498346	597770
D6	Substrate + Re-growth	Product before harvest	1	*Salmonella* spp.	3614	-	*Salmonella enterica*	New	II 41:z10:1,2	3614	498342	597808
B1	Substrate + Re-growth	Nutrient solution	1	*Salmonella* spp.	1815	247	*Salmonella enterica*	8615	Mikawasima	1815	498339	597775
B2	Substrate + Re-growth	Nutrient solution	1	*Salmonella* spp.			*Escherichia coli / Shigella*					
B3	Substrate + Re-growth	Nutrient solution	1	*Salmonella* spp.	1815	247	*Salmonella enterica*	8615	Mikawasima	1815	498339	597771

[1] Available at: https://pubmlst.org/

[2] Available at: https://enterobase.warwick.ac.uk/ EnteroBase is updated daily by scanning the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) to look for new sequences. For more information about *Salmonella enterica* subsp. *enterica* serovar Mikawasima: https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?lvl=0&id=149388 Serotipe II 41:z10:1,2, is similar to II Negev (https://www.pasteur.fr/sites/default/files/veng_0.pdf)

B4	Substrate + Re-growth	Nutrient solution	1	*Salmonella* spp.	1815	247	*Salmonella enterica*	8615	Mikawasima	1815	498343	597766
A1	Substrate + Re-growth	Irrigation community water	1	*Salmonella* spp.	1815	247	*Salmonella enterica*	8615	Mikawasima	1815	498355	597782
B6	Substrate + Re-growth	Irrigation community water	1	*Salmonella* spp.	1815	247	*Salmonella enterica*	8615	Mikawasima	1815	498339	597778
C1	Substrate + Re-growth	Irrigation community water	1	*Salmonella* spp.	1815	247	*Salmonella enterica*	8615	Mikawasima	1815	498339	597781

Table 11. *Listeria innocua* enumeration at the corridor floor points in the CEA facility from artificially contaminated boots and wheels, with floor points spaced at intervals starting from 0 meters and extending every 6 meters. Measurements were conducted in two trials (Trial 1 and Trial 2) at time 0 (T0, post inoculation) as quantification by culturable bacteria was not possible at T24 (after 24 h).

	Trial	Time	Floor distance (m)	Enumeration (Log CFU/floor point)
Boots				
	1	**T0**	Boots	4.76
			0	4.59
	2	**T0**	Boots	4.49
			0	4.45
Wheel				
	1	**T0**	Wheel	5.40
			0	4.00
			6	2.90
			30	3.10
	2	**T0**	Wheel	5.60
			0	3.60
			6	3.10

LoQ = 100 CFU/swab per floor point.

Table 12. Maximum and minimum temperature, radiation and relative humidity inside the CEA facility from 6:00 to 20:00 at time 0 (after inoculation T0) and after 24 hours (T24) in trials 1 and 2.

Surface	Trial	Time	Date	Temperature (ºC)		Radiation (W/m^2)	Relative humidity (%)	
				Max	Min	Max	Max	Min
Boots	1	0	28/05/2024	29.6	20.2	1038.4	84.6	59.4
		24	29/05/2024	27.9	17.6	1050.4	81.9	55.6
	2	0	05/06/2024	29.2	18.0	1073.1	82.8	55.1
		24	06/06/2024	28.3	18.3	982.0	81.1	54.9
Wheel	1	0	11/06/2024	28.2	20.1	965.5	84.9	62.2
		24	12/06/2024	34.2	18.8	985.3	84.8	48.6
	2	0	09/07/2024	32.1	22.1	1045.0	85.3	61.3
		24	10/07/2024	33.8	22.4	1046.0	88.4	53.8

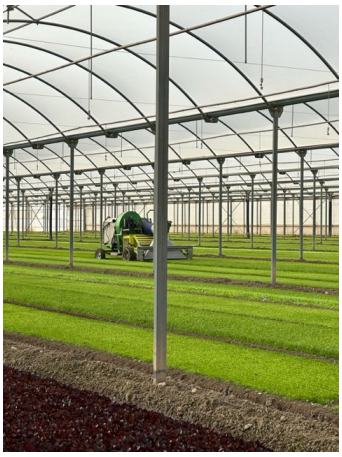
Table 13. Counts of *Listeria innocua* on dirty harvest bins with and without water rinse, 1 hour after inoculation (T0) and after 24 hours (T24) of storage at 25 °C and 60-70% RH (T24).

Time	Water rinse	Log CFU/crate	Viable cells (crate)
T0	No	5.74±0.17	5.56±0.14
	Yes	4.14±0.06	4.73±0.51
T24	No	4.14±0.04	4.68±0.52
	Yes	4.21±0.00	5.02±0.07

Values are means (n = 3) with standard deviations. Levels of culturable bacteria were obtained by plate count, levels of viable bacteria by v-qPCR + EMA + PMAxx.

Figure 1. Controlled environment agriculture (CEA) production systems selected for the three Environmental Monitoring sampling cycles.

Soil + re-growth



Hydroponic

Substrate + re-growth

Figure 2. Representation of *Listeria innocua* traffic patterns in the CEA indoor production environment at various floor points, each spaced 60 meters apart.

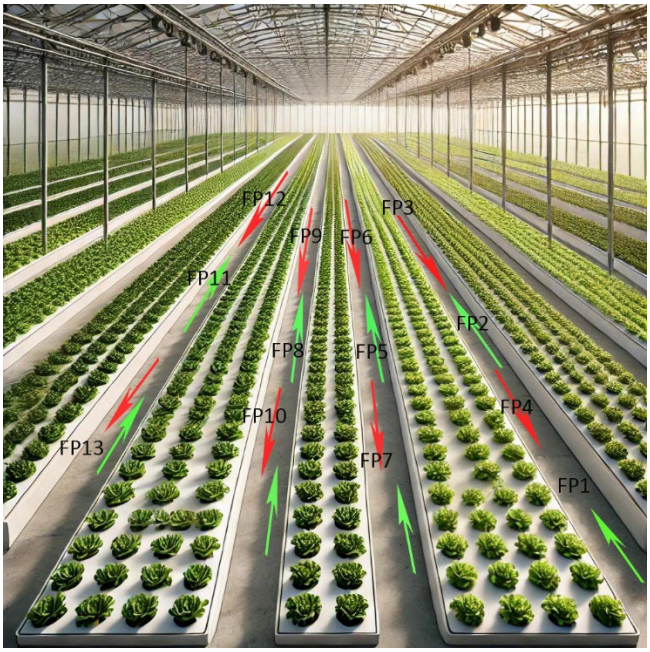
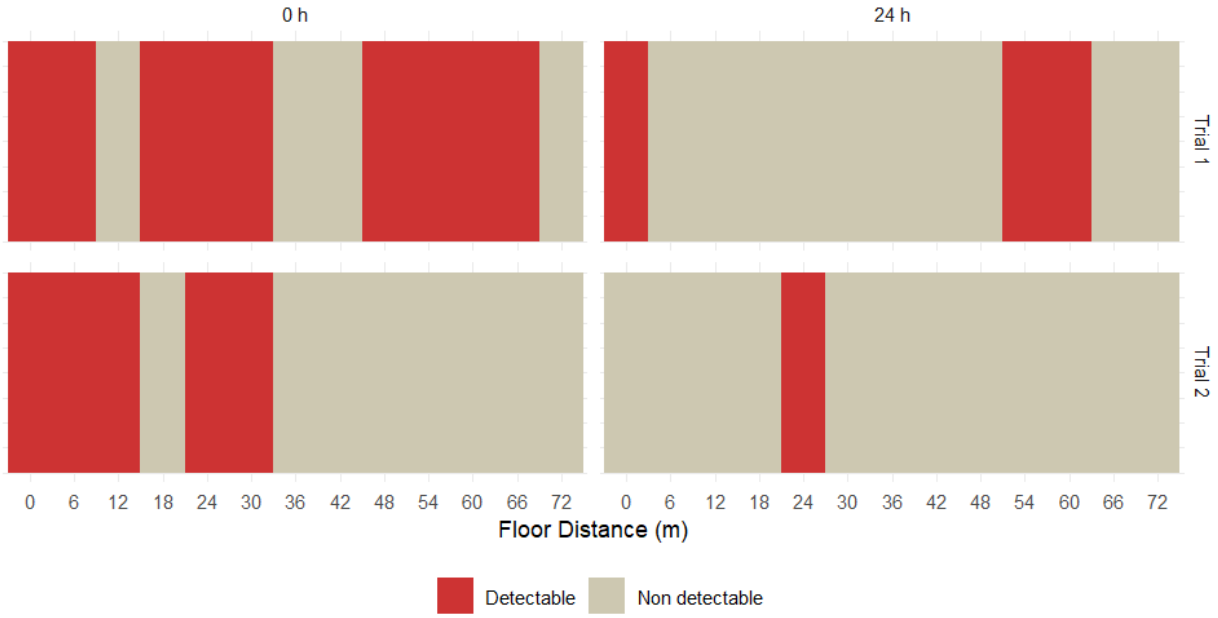


Figure 3. Wash tunnel for cleaning the harvest bins.

Figure 4. *Listeria innocua* detection after enrichment (+ positive Fraser enrichment confirmation in OCLA) in the CEA facility from artificially contaminated boots and wheels in trials 1 and 2 at time 0 (after inoculation T0) and after 24 hours (T24) with floor points spaced at intervals starting from 0 meters (FP1) and extending every 6 meters to 72 meters (FP13).

BOOTS



WHEELS

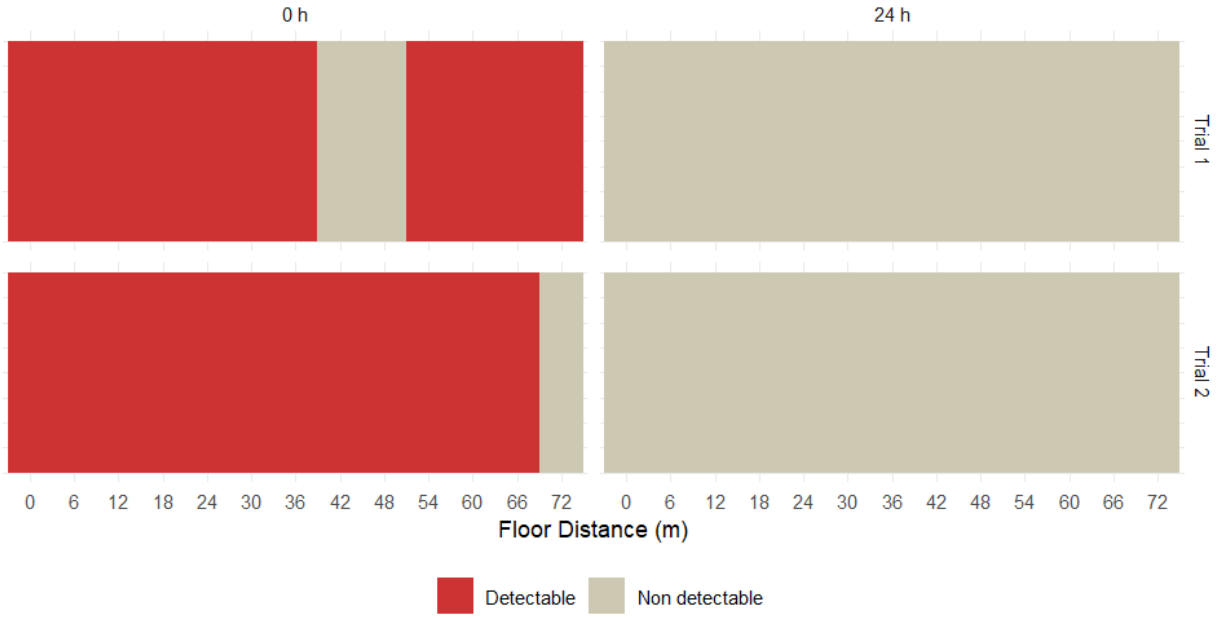


Figure 5. Total bacteria count in the harvest bins before cleaning (dirty) and after cleaning (clean). The values represent the mean bacterial count from 39 dirty bins and 38 clean bins.

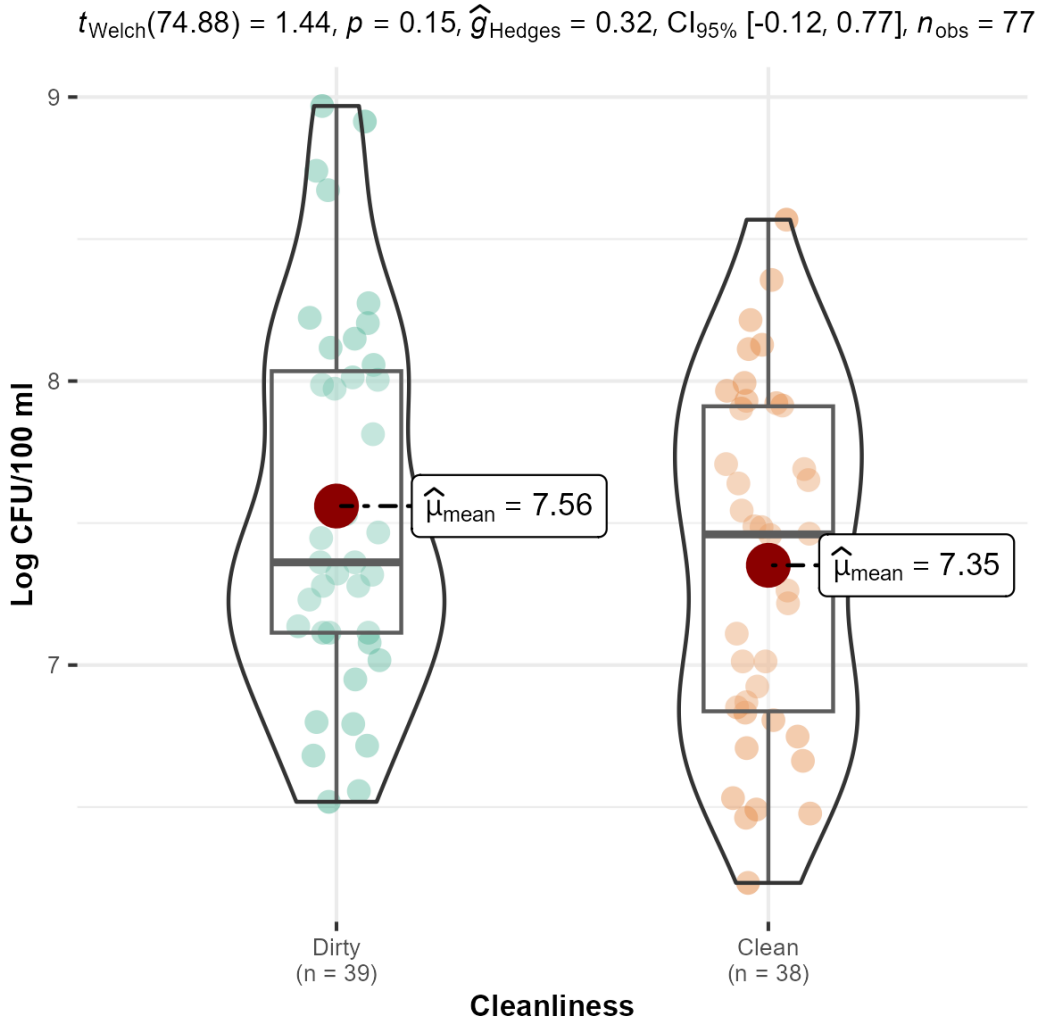
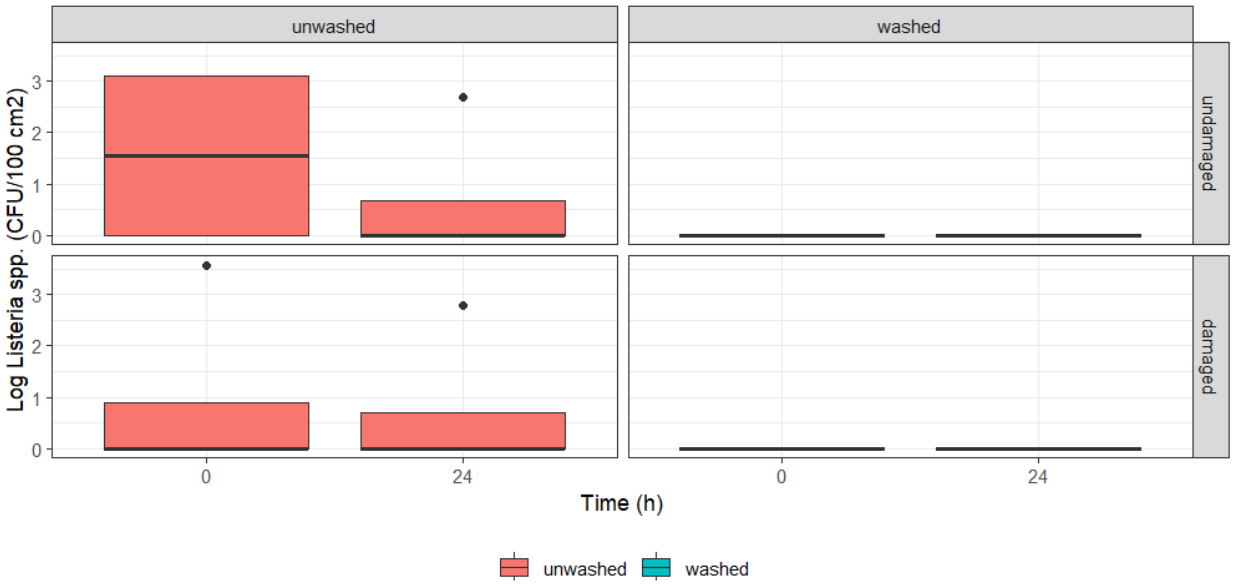


Figure 6. Enumeration of *Listeria innocua* on undamaged and damaged polypropylene lids unwashed and washed with water at T0 (post-inoculation) and after 24 hours (T24) of storage at 25 °C and 60–70% RH.



Picture 1. Exterior and interior views of the CEA facility, which was set up to evaluate the traffic patterns of *L. innocua.*

Picture 2. Boot and wheel surfaces submerged in the inoculum solution.

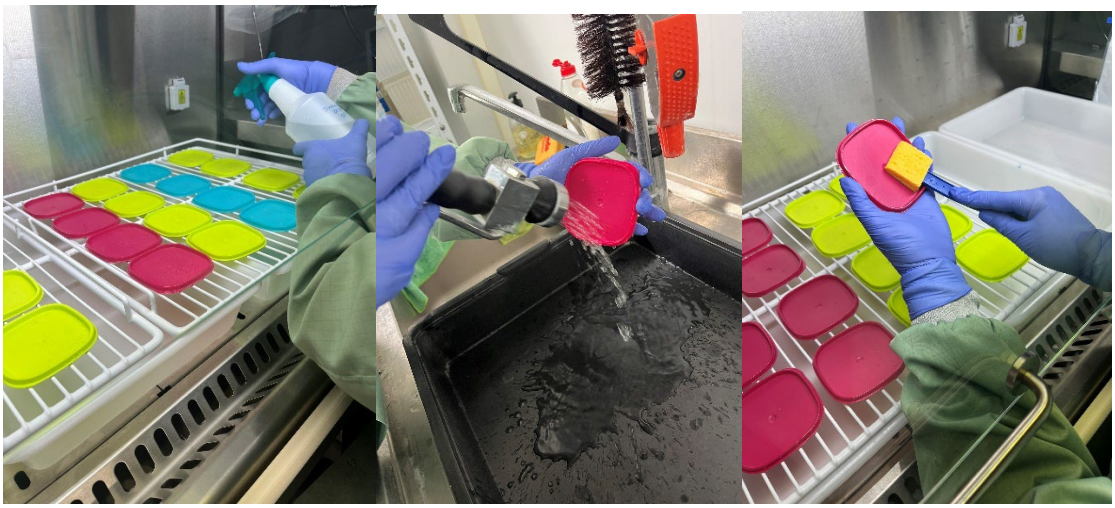
Picture 3. Boot and wheel surfaces transferring the inoculum throughout the CEA corridors.

Picture 4. Samples collected at time 0 (T0, 1h after inoculation) and after 24 h (T24) at each sampling point to assess bacterial survival.

Picture 5. Harvest prepared for cleaning, tunnel set up for sanitation, and cleaned bins ready for distribution to CEA facilities.

Picture 6. Swabbing of bins.

Picture 7. Inoculation of lids with Listeria innocua (A), rinsing with water (B) and swabbing with a sponge-swab (C).



Picture 8. Storage of inoculated harvest bins at 25 °C and 62% RH.