CENTER for PRODUCE SAFETY

CPS 2022 RFP
FINAL PROJECT REPORT

Project Title
Identification of study routes and mechanisms for distribution and establishment of *Listeria monocytogenes* and *Listeria* spp. in avocado packing environments

Project Period
April 1, 2022 – December 31, 2023 (extended to February 29, 2024)

Principal Investigator
Alejandro Castillo, PhD
Texas A&M AgriLife Research
Department of Food Science and Technology
College Station, TX 77843-2256
T: 979-845-3565
E: a-castillo@tamu.edu

Co-Principal Investigators
Rosana Moreira, PhD
Texas A&M AgriLife Research
Department of Biological and Agricultural Engineering
College Station, TX 77843-2117
T: 979-847-8794
E: rmoreira@tamu.edu

Sapna Chitlapilly Dass, PhD
Texas A&M AgriLife Research
Department of Animal Science
College Station, TX 77843-2471
T: 979-458-8177
E: sapnacd@tamu.edu

M. Ofelia Rodriguez-García, PhD
Centro Universitario de Ciencias Exactas e Ingenierías
Universidad de Guadalajara
Guadalajara, Jalisco, Mexico 44430
T: +52-333-134-2222 ext. 27521
E: ofelia.rodriguez@academicos.udg.mx

Objectives

1. *Apply molecular and observational approaches to determine harborage sites and spreading mechanisms for Listeria spp. in the environment inside avocado dry packing facilities in Mexico and sanitation practices potentially leading to this colonization and spreading.*
2. *Develop a map of areas of potential Listeria spp. cross contamination to avocado fruits during packing based on whole genome sequencing (WGS) and multilocus sequence typing (MLST).*
3. *Conduct a mechanistic evaluation of the effect of plant layout, avocado contact surface materials, and environmental factors (temperature and humidity) on attachment and biofilm formation on avocado-contact surfaces during avocado packing.*
4. *Develop a simulation model with discrete time event simulation to predict the levels of cross contamination in a fresh avocado processing facility with visualization of the whole processing line.*

Funding for this project was provided through the CPS Campaign for Research

FINAL REPORT

Abstract

This project was divided in 4 objectives that aimed at tracking the distribution of *Listeria* spp. in the avocado packing environment of Mexican packing plants that export to the United States. During the first objective, 1,003 samples of non-food contact surfaces were collected and tested for *Listeria* spp.; 4% of these samples tested positive for *Listeria* spp., indicating that, although infrequent, the avocado packing environment may be vulnerable to the establishment of these microorganisms in the plant. The analysis of environmental samples also indicated that during the wet season the prevalence of *Listeria* spp. increases. There were no differences in isolation of *Listeria* spp. between the 3 packing plants tested. Isolates that were confirmed by biochemical and serological tests as *Listeria* spp. were further subjected to DNA extraction and sequencing. The whole genome sequencing indicated DNA similarities between isolates, which would be sufficient to develop a map of sites in the plants where the same clonal complex (CC) is found. (Unfortunately, the third-party laboratories have not yet sent the phylogenetic tree needed to confirm that these CCs belong to *Listeria* spp. Therefore, these data are still preliminary.) Various surfaces of equipment and utensils were sampled before and after applying cleaning procedures. The number of samples testing positive for *Listeria* spp. did not decrease after cleaning and sanitizing, indicating a need for re-evaluating sanitation programs. In inoculation trials conducted in a laboratory setting, wet cleaning of stainless-steel surfaces resulted in a greater reduction of *Listeria monocytogenes* (*LM*) than when dry cleaning was applied. In laboratory trials, *LM* showed ability to produce biofilm on various materials used in equipment and utensils. It was also estimated that between 10 and 30% of the *LM* present on the surface of contaminated materials used in equipment and utensils can be transferred to the avocados within 35 s to 15 minutes of contact.

Background

Listeria monocytogenes continues to be a major concern for the avocado industry, while research on reducing the risk of foodborne illness linked to fresh avocados is scarce. A Google Scholar search on Avocado Bioactive Compounds returned 4,970 results between 2023-2024. In contrast, a search on Avocado Listeria returned 734 results. In both cases, at least one half of the hits on the first five pages were not related to the search. Of the new research articles published since 2023, only a few address the issue of *LM* in avocados, and these publications are more focused on remediation measures as opposed to preventive measures, or research focused on quality aspects such as extending shelf-life of fresh avocados. Garcia-Frutos et al. (2023) developed a steam pasteurization process that reduced *Salmonella* and *LM* by ~4 log cycles. Dong et al. (2023) inoculated fresh avocados with *Salmonella* and *LM* and found that while LM survived on the avocado surface for at least 14 days at 7 and 25 °C, *Salmonella* grew on the avocados when stored at 25 °C. These authors then treated the avocados with various antimicrobials, with varying magnitudes of reduction. From this article, the finding of bacterial growth in non-refrigerated avocados seemed more relevant. Many other papers have been published but deal with quality. A more recent review (Lieu et al., 2024) presents an array of methods for avocado preservation, including known alternatives such as irradiation, essential oils, antagonistic organisms etc. We can conclude that very little information can be added to the background written in the original proposal for this project. Therefore, a summary of the points made will be shown:

1. Although no cases of listeriosis have been identified, the FDA avocado sampling (FDA, 2018) indicated the presence of this pathogen on the rind of avocados, and to some extent, in the pulp as well. Although there have been recalls of avocados and avocado products

(FDA, 2019, 2019A, 2019B, 2020), a search in the FDA recall webpages returned no new recalls.
2. There are suspicions that some listeriosis cases may have been linked to avocados but remained non-detected (Pomeroy et al., 2021)
3. Most of the avocados consumed in the US are imported (ERS 2022). Of those, most come from Mexico.
4. Little research has been conducted on the safety of fresh avocado, and most of the studies are from the University of Guadalajara (Enriquez-Hernandez, 2007; Garcia-Frutos et al., 2020; Rodriguez-Garcia et al., 2011; Rodriguez-Herrera, 2018).
5. Most data available in publications relate to the ability of *LM* to grow or survive in processed avocado products (Iturriaga et al., 2002). Other studies have tested disinfection strategies during packing (Rodriguez-Garcia et al., 2011) or on pathogen internalization during postharvest washing of avocados (Chen et al., 2016).
6. This project focused on the effect of sanitation practices on the vulnerability of the avocado packing plants to support potential harborage of *Listeria* spp.

This project will focus on evaluating the impact of current sanitation practices at dry avocado packing plants on the ability of *LM* to colonize the packing plant environment and the areas of the plant that potentially facilitate harborage. To do this, a well-designed sampling plan based on the concept of zones was developed and WGS methods were used to track the transfer and relocation of specific *LM* strains. Intensive environmental sampling/testing and fingerprinting has been conducted for avocado processing plants (Styrdom et al., 2013), using the results to develop strategies to prevent product cross-contamination (Strydom et al., 2016). We believe this can be achieved in whole avocado packing plants.

Research Methods (by objective)

OBJECTIVE 1: As described above, Objective 1 consisted of determining harborage of *Listeria* spp. along the avocado packing process. The activities to achieve this objective included the following 3 tasks:

Task 1. Conduct survey for risk assessment data collection

A survey questionnaire will be prepared following a deductive scaling process assuming that all respondents will have knowledge of the process of avocado packing, following the method of Hinkin (1998), using scales to facilitate statistical analysis. The method of Ganassali (2008) will also be applied to reduce the risk of web survey failure. The questions will be separated in 3 major sections: General information about plant management and organization, food safety management, and sanitation practices, developing the instrument so that the 3 sections are separated and do not interact (Hinkin, 1998). Content validity assessment will consist of a preliminary application of the survey using a small cohort of naïve respondents such as UDG students to obtain evidence of content adequacy. After the instrument has been improved based on the validity assessment, it will be uploaded to a UDG server. A letter of invitation to participate will be sent (e-mail) to APEAM and APEAJAL to share with their members who run a packing operation (65 in Michoacán and 19 in Jalisco, N=84). Consultation will be started with these associations to inquire their preferences about sending the invitations, sending them directly to the packers if these associations or their members so prefer. The letters will include the link to a UDG server where the questionnaire may be answered online. The questionnaire will be designed to take an expected time of 30 min to fill each section and may be saved and accessed again at different times. Questions will be designed to produce a straight, concise answer. The page where the questionnaire will be posted will be open for 6 months after

emailing the invitations, to allow data processing during Year 1. Data will be analyzed by Chi-Square test to compare differences between variables and generate consensus about how packers view specific points of concern in the avocado process and sanitation practices. Both the numeric and qualitative evaluation of responses to key questions will be used for conducting of a risk assessment for the cross-contamination of LM on various zones of the plant.

Task 2. Conduct environmental sampling and molecular testing

The activities for Task 2 were executed by Co-PI Rodriguez and were conducted at the Food Microbiology and Safety Laboratory (LMIA) of University of Guadalajara (UDG). From January-October 2023, a total of 1,003 environmental samples were collected from three avocado packing facilities (684 samples during the dry season and 319 in the wet season). Samples at each facility were collected during packing operation. All three facilities followed the same procedure for packing (**Figure 1**), but at different scale (average 80 tons/day to 220 t/day). Sampling staff followed the hygiene and safety practices of each plant. All samples collected were tested for detection of *Listeria* spp. and counts of *Enterobacteriaceae*. The latter group was included as a precautionary step in case *Listeria* spp. was not detected, to at least have data on plant sanitation.

Floors. A floor sampling swab device (FSSD) was developed for this study. This swab device was composed of a polypropylene oval (39 x 13.5 cm) framed in an aluminum handle, which was covered with a sterile bag and then a cotton gauze swab constructed with cheesecloth (grade 100, 90 x 60 cm) and superimposed elastic. The 90 x 60 cm fabric was folded to get a 45 x 60 cm rectangle, and then folded three times to get a 45 x 15 cm rectangle shape. Short sides were sewed and joined with elastic. The FSSD was moistened with 200 ml of Dey Engley broth (D/E broth) and swabbed on floor surfaces (**Figure 2**). Floor samples included the areas of receiving avocados, packing and cold storage, and included regular concrete floors and stainless-steel floors from platforms at sorting step. After sampling, the sample was placed in a sterile plastic bag containing 200 ml D/E broth.

Air. Air samples (1,000 L) were collected from avocado receiving and packing rooms, as well as from cold rooms, using a MAS-100VF® Merck Millipore air sampler adjusted to a flow rate of 100 L/min. Each sample was collected by placing a plate of tryptic soy agar supplemented with yeast extract 0.6% (TSE-YE) in a 100 x 15 mm petri dish in the air sampler. The sampler then was turned on and maintained activated for 10 min. After 10 min sampling, the TSE-YE plate was collected and placed in a sterile plastic bag.

Surface samples. Surface samples were all from non-food contact areas related to zones 2, 3 and 4 (**Table 1**), These samples included outsides of equipment holding conveyor belts, rollers, sizer clips, nylon brushes, soft belt conveyors, PVC and stainless conveyor rods, singulation line, as well as metal/plastic equipment-holding frames, fans, walls, drains and cleaning utensils. Drain and other similar surfaces were samples with sterile swabs moistened with D/E broth and placed in tubes with 5 ml of D/E broth. All other surfaces were collected with a sterile sponge (3.5 x 7.5 cm, Whirl-Pak) moistened with D/E broth and placed in sterile bags with 25 ml D/E broth.

Sample transportation, processing and analysis. All samples were maintained and transported in coolers at 4 °C, and sample analysis occurred within 24 h after collection. In the laboratory, the TSA-YE agar where the air samples were collected, was extracted from the petri dish using sterile membrane filter flat tweezers and transferred to a stomacher bag with 100 ml buffered peptone water (BPW), and homogenized by sonication. This mixture then was

incubated at 35 °C for 18-24 h to resuscitate any *Listeria* cells potentially injured after impinging on the agar surface during sampling. After this enrichment, where any *Listeria* spp. cells were expected to propagate, 25 ml were transferred to 225 ml UVM broth for primary enrichment. Floor samples were homogenized in their original sampling bag by massaging in the 300 ml D/E broth, whereas swab samples were homogenized in their tube with 5 ml D/E broth by agitation in a vortex mixer for 1 min, and all other samples were homogenized by pummeling in their original D/E broth in a stomacher for 1 min. The contents of the sampling bags were then added to 225 ml UVM broth for continuing *Listeria* analysis following the USDA-FSIS Microbiology Laboratory Guidebook (GLM), Method Number 8.13 (FSIS, 2021). Briefly, all samples in Modified University of Vermont broth (UVM, primary enrichment) were incubated at 30 °C for 20-26 h. After this primary enrichment, 0.1 ml was transferred from each sample to a modified Oxford agar (MOX) plate and incubated at 35 °C for 24–28 h. Separately, 0.1 ml was transferred from UVM to morpholinepropanesulfonic acid–buffered *Listeria* enrichment broth (MOPS-BLEB, secondary enrichment), incubating at 35 °C for 18-24. After secondary enrichment, automated test in the 3M™ Molecular Detection System (MDS) was conducted, following the User's Guide for conducting every step using this molecular method. For this study, the *Listeria* spp. kit was used. All samples that tested positive for *Listeria* spp. were then confirmed by cultural methods including plating, Gram stain, biochemical and serological tests to identify to the species level.

Counts of *Enterobacteriaceae*. For each sample collected and transported as described above, 2 mL of the sample after homogenizing prior to transfer to UVM (or BPW in the case of air samples), were separated for enumeration of *Enterobacteriaceae*. This test was conducted by preparing decimal dilutions and pour-plating on violet red bile agar supplemented with glucose (VRBA+G). After agar solidification, all plates were overlayed with the same VRBA+G to homogenize the glucose-fermentative colony morphology.

Temperature and relative humidity (RH). Immediately before surface sample collection, the temperature of the actual surfaces from which samples were collected for microbiological analysis, was measured with an infrared thermometer with adjustment for surface measurement. Temperature as well as RH of the air during collection of air samples were measured with an Easy Vision Model EA20 hygro-thermometer (Extech, Punjab, India).

Statistical analysis. Data for percent detection of *Listeria* spp. were compared between type of surface, packing plant and their interactions, as well as between seasons (dry vs. wet) using the Chi-square analysis in Statgraphics Centurion XV. For counts of *Enterobacteriaceae*, the data were compared for differences between plants, type of surface and their interactions using LS Means test in JMP Pro 17 (SAS Institute). Data for temperature and RH were analyzed with JMP Pro 17, conducting ANOVA with Tukey's mean separation.

Task 3. Characterize and evaluate cleaning and sanitizing procedures

Evaluation of cleaning and sanitizing procedures. This task included in-plant as well as laboratory trials. The in-plant activities were executed under the supervision of O. Rodriguez and were conducted at the Food Microbiology and Safety Laboratory (LMIA) of University of Guadalajara (UDG). The laboratory trials were executed under the supervision of A. Castillo and were conducted at the Texas A&M AgriLife Research Food Microbiology Laboratories.

In-plant trials. Between July and October 2023, 360 surface samples were collected during 9 visits at one avocado packing facility. Samples from packing equipment/processing line, receiving crates, floor and drains were included (**Table 2**). Samples were collected at the end of the processing day, before the cleaning and sanitizing procedures, and after the cleaning and

sanitation procedures early morning on the next day. All samples were collected before and after execution of cleaning and sanitizing procedures. Presence of *Listeria* spp., total aerobic plate count, *Enterobacteriaceae*, yeast, molds, ATP levels and visual evaluation were determined. A visual scale (1-4) was developed to evaluate the cleaning of surfaces (**Table 3**).

Sample transportation and analysis. All samples were maintained and transported in coolers at 4 °C, and sample analysis occurred within 24 h after collection. From each sample, aliquots of 1 ml were serially diluted and plated on Petrifilm for Aerobic Counts, *Enterobacteriaceae*, Yeast and Molds. Plates for total counts were incubated at 35 °C/24 h and *Enterobacteriaceae* plates incubated at 35 °C/24 h. Yeast and mold Petrifilm plates were incubated at 30 °C/5 days. All results were expressed as CFU/100 cm^2. For *Listeria* spp. all surface samples were pre-enriched with 225 ml of UVM broth and incubated at 30 °C for 24 h. After pre-enrichment, aliquots of 200 μl were transferred into MOPS-BLEB selective enrichment and incubated at 35 °C/24 h. Screening test for *Listeria* spp. was performed using MDS. Presumptive positive samples were confirmed by isolating and biochemical identification of isolated colonies as described in USDA-FSIS MLG 8.13.

Laboratory trials. These experiments included the use of pilot equipment inside a biosafety level 2 facility, to inoculate with *LM* to evaluate effectiveness of wet vs. dry cleaning, which cannot happen in-plant. Preliminary experiments showed a consistent lack of detectable counts by sanitizing. Therefore, these results are focused on before and after cleaning only to determine effect of the cleaning approach (wet vs. dry) on reduction of *LM.*

Pilot packing equipment. Motor-operated conveyor belt units were used in pilot equipment to simulate the cleaning and sanitizing procedure reported to be used at avocado packing plants. This pilot equipment was made of stainless steel and has a polyvinyl chloride (PVC) belt attached, which is activated by a motor. The PVC belt was intended to be the food-contact surface whereas the stainless-steel parts were not to be in contact with avocados when packing operations were to be conducted, which represents real-life procedures. Two pilot equipment units were used: one for wet cleaning and the other for dry cleaning. The wet process unit was sprayed with sterile distilled water on the stainless-steel and PVC components, every 24 h for 3 days before the experiment was started, whereas the equipment for dry cleaning was not exposed to any added moisture.

Inoculum preparation and inoculation. Three strains of *LM* (0072, 0104 and 0089) were used to prepare a strain cocktail for inoculating the pilot equipment. Each strain was grown in 240 ml tryptic soy broth supplemented with yeast extract (TSE-YE) at 37 °C for 24 h. Four 240-ml bottles were incubated for each strain. After incubation, 50 ml from each culture was transferred to a 50 ml centrifuge tube and washed 3 times by centrifugation at 3500 rpm for 15 min, then discarding the supernatant, and resuspending the pellet in 50 ml phosphate buffered saline (PBS). After 3-time cell wash, the suspensions for each strain were mixed in a vortex mixer to form a 3-strain *LM* cocktail. The volume of the cocktail suspension was adjusted to have enough volume to complete inoculation of all areas in the experiment; the bacterial concentration in this suspension was confirmed by plate counting to determine the initial concentration of *LM* per cm^2 of the inoculated area. The 3-strain cocktail was transferred to a sterile spray bottle to apply inoculum onto the surfaces of the pilot packing equipment. Prior to inoculating, Hass avocados were placed on the conveyor and left to run on the active conveyor for 1 h to simulate equipment to be cleaned and sanitized after process in commercial packing plants. To inoculate the pilot packing equipment, 100 cm^2 areas were delimitated using colored tape. An adequate number of 100-cm^2 areas was prepared to prevent sampling the same surface more than one time. Each 100-cm^2 area was spray-inoculated by squeezing the spray bottle 15 times and then

spreading with a sterile roller to evenly distribute the inoculum over the entire area. The volume applied by this method on each surface was previously determined to be 5 ml.

Experimental design. The effect of the time at which the equipment was exposed to *LM* before cleaning was studied by allowing dwell times after inoculation, letting the inoculum dry for 1 or 8 h at room temperature. After each of these dwell times, the surfaces were sampled and then the equipment was cleaned. As described below, the equipment surfaces which were sampled before cleaning, were again sampled after cleaning. A second experiment was conducted to confirm the effect of wet vs dry cleaning on the log reduction after cleaning and after sanitizing, using a single dwell time of 4 h prior to cleaning.

Cleaning. After the first sampling, cleaning was conducted by both wet and dry methods. For the wet cleaning the surfaces were cleaned with a mix of water and a non-acidic, alkaline-based cleaner soap (foam-brite), applied with brushes and microfiber towels and wipes and rinsed (waited 15 min before sampling). For the dry cleaning, new brushes were used to clean the surface, a second step was cleaning with new and clean microfiber towel. Repeat procedure of sample, stomach, serial dilutions & plating, mentioned before.

Cleaning. After the first sampling, cleaning procedures are applied on the equipment. For the wet cleaning procedure, materials were cleaned with a mix of water and a non-acidic, alkaline-based cleaner soap (foam-brite), applied with brushes and microfiber towels and wipes (waited 15 min before sampling). For the dry cleaning, new brushes were used to clean the surface, a second step was cleaning with new and clean microfiber towel. Repeat procedure of sample, stomach, serial dilutions & plating, mentioned before.

Sampling and microbiological testing. Sample collection was achieved by using a 3M sponge stick moistened with 10 ml of D/E neutralizing broth to rub over the entire 100-cm^2 with vertical, horizontal and angled movements. The sponge was then separated from the stick inside the bag containing 10 ml D/E broth and pummeled in a stomacher for 1 min. Serial dilutions were made of each sample bag and surface-spread onto modified Oxford agar with the *Listeria* supplement (MOX). The plated MOX was incubated at 37 °C for 48 h. This sampling and plating approach resulted in a detection limit of -1.0 log CFU/cm^2.

Statistical analysis. For the in-plant trials, the percent detection of *Listeria* before and after cleaning and sanitizing was compared by Chi-square test. Counts of indicator organisms and other cleanliness measurements (ATP) were compared by ANOVA, using Statgraphic Centurion. For laboratory trials, all data was converted to log value and compared by LS Means procedure to determine the effects of dwell time (1 vs. 8 h), sampling time (before vs. after cleaning), cleaning approach (wet vs. dry) and type of surface (stainless steel vs. PVC conveyor). The log reduction by cleaning was also determined by subtracting the log value after cleaning from that value before cleaning, where before vs. after cleaning was no longer a possible effect.

OBJECTIVE 2. *Develop a map of areas of potential Listeria spp. cross contamination to avocado fruits during packing based on whole genome sequencing (WGS) and multilocus sequence typing (MLST).*

Various surfaces and floors in the avocado processing plants were sampled by swabbing with a sterile sponge pre-moistened with 25 ml D/E broth. The collected samples were transferred back to the laboratory in a refrigerated cooler. The samples were then enriched with UVM medium for 26 h at 30 °C. Further, 0.1 ml of the enriched cultures were incubated with MOPS-

BLEB medium for 24 h at 35 °C, selective for *Listeria*. The presence of *Listeria* in the obtained samples were confirmed thorough the 3M Molecular Detection System. After the confirmation of the presence of *Listeria*, positive enriched samples were randomly selected for the whole genome sequencing (WGS) and 16S rRNA amplicon sequencing experiments.

Whole genome sequencing (WGS) of the isolates. 100 µl of the enriched cultures were plated on the TSA plates for the isolation of individual isolates. About 19 pure isolates were selected for the DNA extraction using Wizard Genomic DNA purification kit (Promega, USA) according to manufacturer's instructions. Further, the obtained DNA was quantified, quality checked and subjected to library preparation. During library preparation, DNA was fragmented, end-polished, A-tailed, and ligated with full-length adapters for Illumina sequencing. The prepared library was then purified, assessed on fragment analyzer system and quantified with Qubit before proceeding for sequencing on Illumina NovaSeq platform (150 bp PE).

The obtained raw FASTP sequences were quality checked with FastQC, trimmed with Trimmomatic and paired-ends merged with PEAR (Bolger et al., 2014; Zhang et al., 2014; Chen et al., 2018). The quality check was carried out with the following parameters: (1) Discard a paired reads if either one read contains adapter contamination (>10 nucleotides aligned to the adapter, a lowing ≤10% mismatches); (2) Discard a paired reads if more than 10% of bases are uncertain in either one read; (3) Discard a paired reads if the proportion of low quality (Phred quality <5) bases is over 50% in either one read. Quality-filtered reads were then subjected to the reference-based assembly process against a reference genome with Burrows-Wheeler Aligner (BWA) tool (parameters: mem-t 4-k 32-M) (Li & Durbin, 2009). The obtained Sequence Alignment/Map (SAM) data was sorted with SAM tools (parameters: sort -@ 6 -m 2G) and the duplications checked with Picard (parameters: VALIDATION_STRINGENCY=SILENT) (Li et al., 2009). SNPs/InDels were detected through GATK tools with the following parameters: SNP: QD<2.0, FS> 60.0, MQ < 30.0, HaplotypeScore > 13.0, MappingQualityRankSum <-12.5, ReadPosRankSum <-8.0 INDEL: QD < 2.0, FS > 200.0, ReadPosRankSum <-20.0 (McKenna et al., 2007). Sequence variants (SVs) were detected with Breakdancer (parameter: -q 20) and variation annotation with ANNOVAR (Wang et al., 2010).

16S rRNA amplicon analysis. 1 ml of the enriched broth was subjected to the total DNA extraction through Zymo Quick-DNA Faecal/Soil kit. The obtained DNA was quantified, quality checked and subjected to PCR amplification of 16S rRNA genes. Barcoded bacterial universal primers (515F & 806R) were used to amplify the V4-V5 region of 16S rRNA gene (Walters et al., 2016). Each 15 µl reaction mixture consisted of 15 µL of Phusion® High- Fidelity PCR Master Mix (New England Biolabs); 0.2 µM of forward and reverse primers, and about 10 ng template DNA. PCR conditions were as follows: initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s and 72 °C for 5 min. The obtained PCR products were purified and subjected to library preparation. The prepared library was then purified, assessed on fragment analyzer system and quantified with Qubit before sequencing on Illumina NovaSeq platform (250 bp PE).

The obtained raw fastq reads were demultiplexed with cutadapt tool and the paired-end sequences were merged with FLASH (Magoc and Salzberg, 2011). The resulting data was quality-filtered with Fastp and the chimera was removed with the vsearch tool comparing against the silva database (16S rRNA database). The sequences were clustered with ≥97% similarity using Uparse software and the representative OTUs were subjected to taxonomy analysis using mothur algorithm against SILVA database (Edgar, 2013; Quast et al., 2012). The phylogenetic relationship among the OTUs were established using the multiple sequence alignment MUSCLE software (Edgar 2004). The OTUs were normalized before proceeding for the relative abundance estimation, alpha diversity and beta diversity calculations. Alpha

diversity metrics for community richness & community diversity and the rarefaction curves were calculated with R. Similarly, beta diversity analysis like non-metric multidimensional scaling (NMDS) was performed in R to visualize the difference among the groups.

OBJECTIVE 3. *Conduct a mechanistic evaluation of the effect of plant layout, avocado contact surface materials, and environmental factors (temperature and humidity) on attachment and biofilm formation on avocado-contact surfaces during avocado packing.*

Task 1. Surface dynamics and LM attachment

Physical characterizations of the surfaces. Scanning electron microscopy (SEM) was employed to investigate various aspects of different surfaces, including understanding the surface topography of uncontaminated avocados and packing plant materials, analyzing preferential adhesion trends of bacteria with dissimilar surface morphology and roughness, and characterizing biofilm formation over time intervals. To assess adhesion capacity, all surfaces were spot-inoculated with 100 µL from a 9-log bacterial suspension and stored for 3 h, 4 days, and 10 days. Additionally, biofilm formation was examined. For SEM observations, the samples of inoculated surfaces, including avocado rinds, and various pieces of surplus materials used in avocado packing process, were fixed by immersion in 2.5% glutaraldehyde solution (VWR Chemicals, OH; pH 7.0) for 4 h and washed with 1% peptone water. Subsequently, the specimens underwent dehydration through sequential immersion in 20%, 40%, 60%, and 80% ethanol solutions for 10 min each, followed by immersion in 100% ethanol for 1 h at 37 °C. Prior to SEM characterization, all samples were sputter-coated with a thin layer (10 nm) of Pt/Pd to enhance electrical conductivity. Surface roughness was quantified using an atomic force microscope (AFM) with a silicon tip in tapping mode, scanning at a size of 20 µm and a rate of 0.5 Hz (Dimension Icon, Bruker, Billerica, MA). Data analysis of the AFM micrographs was conducted using Nanoscope Analysis 1.8 (Bruker, Billerica, MA).

Wetting behavior and contact angle. The surface wetting characteristics of avocados and packing materials were measured using DI water and Diiodomethane (Sigma-Aldrich, St. Louis, MO) to determine static contact angles. The produce was washed under running DI water to remove residual dirt and grit, followed by wiping with lint-free wipes and drying for 1-2 hours at 25 °C. Separate syringes were dedicated to each liquid to prevent contamination, and approximately 3-5 µL of the solution was dropped onto the targeted surface, with immediate image capture using a KSV Camera 200 Goniometer (Espoo, Finland). Contact angle values were reported after 9 repeats of the procedure. Surface energy of the substrates was calculated using Fowkes theory (Owens and Wendt, 1969), following methods similar to (Kozbial et al., 2014) for surface free energy calculation. Images captured with the KSV camera were analyzed using ImageJ (National Institutes of Health (NIH), Bethesda, MD), employing the “drop-analysis-LB-ADSA plugin”. For avocado, the wax was extracted by submerging avocados in hexane, followed by hexane extraction using a rotary evaporator under 40 °C and 290 Pa vacuum pressure. The resulting wax was then placed on a glass slide for measurement of its contact angle with water and diiodomethane.

Sample preparation. Two varieties of avocados, including Hass and Green-Skin (Fuerte), were purchased from a local store. These varieties were selected to compare the rough vs. smooth skin surfaces. Additionally, various packing materials were provided by avocado plants in Mexico. These materials included black rollers, white and blue conveyor belts, strip curtain separators made of polyvinyl chloride (PVC), pieces of plastic crates used for collecting avocados and stainless steel 304. All these materials were obtained from real equipment, and some of them were already surplus after the plant management decided that it was time for

replacement. Used materials were chosen as opposed to new and non-used materials to provide a realistic scenario to simulate the intensive use of equipment and utensils made from these materials for use in commercial avocado packing.

Preparation of bacterial cultures. A strain of *LM* (LIS 0104) isolated from listeriosis cases during the cantaloupe outbreak of 2011 was used to surface-inoculate avocado and packing materials. This isolate was reactivated from storage at -80 °C by repeated transfers in tryptic soy broth (TSB) and then streaking on tryptic soy agar (TSA) slants to generate working cultures. This working culture was stored at 5 °C. Prior to use, the culture was reactivated by 2 consecutive transfers in TSB, incubating for 24 hours at 37 °C. The cells in the TSB culture then were harvested from the second transfer suspensions by centrifugation for 15 min at 15,000 × g in a Rotor S40 centrifuge (Jouan B4i, Scimetrics, Inc), followed by elimination of the supernatant. The pellet was resuspended into the same volume of phosphate-buffered saline (PBS) solution and then centrifuged again for 15 min. This procedure was repeated at least 3 times. A suspension of the bacterium was prepared by resuspending the washed bacterial pellet into the same volume of PBS. The concentration of bacteria in the suspension was calculated by decimally diluting 1 mL of the suspension, followed by MOX agar plating. The concentration of bacteria in the suspension was observed to be around 8.9 ± 0.1 $\log_{10}$ *LM* CFU/mL.

Avocado inoculation. Prior to inoculation, the avocados were carefully inspected, and those with visible damage were discarded. Selected avocados, characterized by firm texture and green color, were then washed using distilled water and placed on sanitized stainless-steel stands to dry at room temperature. The *LM* suspensions, harvested as described above, were activated from a prepared slant and diluted in PBS by mixing 30 mL of bacterial suspension in 270 mL of PBS. Various avocados were inoculated by submerging in this suspension. Immediately after immersion (zero time) and after 1, 3, and 6 h of contact with the suspension, avocados were removed and sampled for loosely attached bacteria by placing in a sterile Whirl-Pak bag with PBS, gently shaking to release cells that were not strongly attached (loosely attached). The same avocado was then placed in another bag with PBS and was sonicated to dislodge cells that were not released by the previous shake. These cells were considered to be strongly attached. The suspensions with the loosely and with the strongly attached *LM* cells were used to prepare decimal dilutions and plate on MOX agar to determine bacterial concentration. Non-inoculated control samples were included at each time for comparison.

Packing plant materials inoculation. The procedure for inoculating and quantifying loosely and strongly attached cells on the surfaces of the different packing materials involved preparing the 5 different materials described above. The inoculation procedure was similar to the process described for avocado, except that each piece of the test materials was cut to a 6.25 ± 0.5 cm^2 area. These materials were washed with distilled water and sanitized with 70% ethanol to eliminate any potential contamination. Subsequently, the materials were placed on a sanitized stainless-steel stand and allowed to dry for 2 h at 25 °C. The *LM* suspension was activated and enumerated as described above, and a volume of 2 ml of this suspension was transferred to Whirl-Pak bags containing 18 mL of PBS, gently stirred, and used to submerge the samples for different time intervals of 0, 1, 3, 6 hours at 25 °C. The bacterial concentration in the inoculum was verified by plate counting, to determine whether any changes occurred over the 6 h contact time. At each time, samples were removed from the bacterial suspension and drained in a sanitized rack. Loosely attached cells were counted by transferring the samples to sterile Whirl-Pak bags containing 20 mL of PBS, followed by gentle shaking, then enumerating by plating serial dilutions. The samples then were placed in another sterile Whirl-Pak bag with PBS, sonicated, and the bacterial concentration in the suspension determined by plating serial

dilutions. All plating was conducted on MOX agar as strongly attached bacteria. Additionally, a control set was included for each time interval.

Bacterial enumeration through SEM. The *LM* was streaked onto TSA plates, and an isolated bacterial colony was grown in TSB by incubating overnight at 35-37 °C. The mean counts in this culture were determined to be 8.9 ± 0.2 log CFU/mL. Hass and Green-Skin avocados, as well as the 5 types of packing plant materials, were inoculated. Avocado samples were carefully washed, drained on a sanitized stainless-steel rack for two hour and then used to aseptically excise 10-cm^2 core pieces with a sanitized borer of ~10 cm^2. In addition, pre-cut (6.5 cm^2) pieces of packing materials were washed and sanitized and drained as described above. Three pieces of each avocado (Hass and Green-Skin) and 6 different pieces of packing plants materials were then spot-inoculated with 100 µL of the *LM* suspension, then let to stand for 3 h to allow sufficient time for bacteria to attach to the target samples. Subsequently, all surfaces were washed in PBS to remove excess bacteria and fixed in 2.5% glutaraldehyde solution for 4 h and washed using PBS. Stepwise dehydrations were performed with 20%, 40%, 60%, and 80% ethanol for 10 min each to remove water content, followed by storage for 1 h at 37 °C in 100% ethanol. Before SEM characterization, all the produce were sputter coated with a thin layer (10 nm) of Pt/Pd to improve its electrical conductivity. The number of bacteria in each image was counted using ImageJ software, with at least five different locations of each sample.

Task 2. Biofilm formation and transfer from equipment to avocado

Observing biofilm formation using SEM. Before initiation, the growth media were inoculated with an isolated bacterial colony and allowed to grow overnight at 35-37 °C, with the initial concentration measured (8.9 ± 0.2 log CFU/mL). Then, the materials were spot-inoculated with about 9 log CFU per sample by distributing 100 µl of cell suspension in 10 drops (10 µl each) on the material surface within a 2- to 3-cm diameter circle. Next, the environment was set up by placing the inoculated samples inside a fish-tank like chamber with wet filter paper to provide high humidity, storing at room temperature for up to 10 days. During storage, the relative humidity was measured every 3 h using a hygrometer. After 3, 144, and 240 h of storage, samples were removed from the chamber and prepared for SEM observation. After retrieval, the samples were washed by dipping them in PBS buffer to remove excess bacteria and then fixed, washed, and dehydrating as described above. Adequate dehydration is paramount to minimize off-gassing during subsequent steps. Before SEM characterization, all the samples were sputter-coated with a thin layer (10 nm) of Pt/Pd to improve their electrical conductivity. A direct microscopic count of cells by SEM was conducted. The inoculum level used permitted a reasonable assumption that most cells counted were the *LM.*

Transfer of LM from inoculated materials to fresh avocado. The growth media were inoculated with an isolated bacterial colony and allowed to grow overnight at 35-37 °C, with the initial concentration measured (8.9 ± 0.2 log CFU/mL). Six different large pieces of packing plant materials were selected and submerged in activated inoculum for 4 h to allow sufficient time for bacteria to attach to the target samples. The samples were then removed and placed on a sanitized rack for sufficient drainage. Avocado rinds, which were scissored and cut by a cylinder into 9.3 ± 0.1 cm^2 pieces, were then placed on the contaminated materials for 36, 180, and 900 seconds, respectively. Subsequently, the avocado samples were removed from contact with the materials, and loosely attached bacteria were counted by transferring the samples to Whirl-Pak bags containing 18 mL of PBS, followed by gentle shaking and enumeration through serial dilution. The samples were rinsed, placed in another sterile Whirl-Pak bag with PBS, sonicated, and the bacterial concentration in the suspension was determined through serial dilutions and plating on MOX agar as strongly attached bacteria.

OBJECTIVE 4. *Develop a simulation model with discrete time event simulation to predict the levels of cross contamination in a fresh avocado processing facility with visualization of the whole processing line.*

This objective was executed by Co-PI Moreira. The model was to be designed to:

1. Track *Listeria* and avocado movements in time,
2. Evaluate microbial contamination in different equipment/surfaces and calculate the probability events of cross-contamination between avocados and equipment,
3. Determine the number of avocados and their level of contamination at the end of the processing line.

The number of contaminated avocados and avocado contamination levels will be computed and visualized on plots and diagrams. Diagrams representing equipment variables will also be produced to track changes in these variables.

Model development. Data from a packing house facility was utilized to develop the agent-based model. The model was constructed in NetLogo 6.2.2, following the general structure of the model developed by Madamba et al. (2022). The simulation is conducted with one-second time steps over an 8-h period. The first hour simulates the potential introduction and spread of *Listeria* within the facility, while the final 7 hours involve simulated environmental monitoring for model validation. The simulator provides food safety insight to farms and processors by integrating *Listeria* cross-contamination data since it determines how many avocados are contaminated, and how to reduce the number of contamination cases in the facility. The core of the system is a discrete event simulation, which models the processing facility with uncertainty present in both input (fresh harvested avocado microorganism load) and in-the-facility cross-contamination including people, equipment, and crates. **Figure 35** shows a schematic of avocados arriving at the processing facility evaluated in this study.

Assumptions. The FS-ABS (Food Safety Agent Based Simulation) is initialized with a batch of avocado arriving at the processing facility from the field. The avocados are then transported to the processing line, which consists of crate turning system, a brush conveyor, a ramp-up conveyor with fans, a roller conveyor, a sorting machine, a separator curtain, a wheel conveyor, a blue conveyor, and a packaging area where workers randomly selected avocados and place them in 25 kg crates. It is assumed that the avocados and the equipment are independently transported and operated, respectively, during the process. Each piece of equipment as well as avocados and workers have specified microorganism loads. **Figure 36** presents an overview of the whole system. The system models the flow of a product from receiving to packaging, i.e., the product is either cross-contaminated or non- contaminated.

The processing line and experimental setup. The whole avocado process evaluated in this study consists of a commercial Hass avocado processing line capable of processing approximately 110,831 kg of avocado per hour. Avocados with a random contamination or no contamination levels are fed into the processing line and processed and packed. It takes 180 seconds to process 1250 kg of avocados (4,167 avocados). The daily production (8 hours) is about 881,845 kg (448,000 lb) of avocados in that facility. Whole avocados (0.3 kg each) are processed at a rate of about 23 kg/s (1,388 kg/min).

Modeling of cross-contamination. It is assumed that there is no net loss of microorganisms during cross-contamination, that is, the total bacterial load is conserved during the transfer process between the two contacting surfaces. The population size is a discrete integer value and there are only *i* positive observations. The binomial probability distribution function is used to approximate the number of microbial loads to be transferred. The transfer coefficient ($0 < kx < 1$) is the number of microbes that will be moved from one contacting surface to the other, 'surface-A' and 'surface-B' (k_{AB}, k_{BA}).

$$\chi \sim Binomial\,(N_{oA}, k_{AB}) \quad [1]$$

$$\psi \sim Binomial\,(N_{oB}, k_{BA}) \quad [2]$$

$$N_A = N_{oA} - \chi + \psi \quad [3]$$

$$N_B = N_{oB} - \chi + \psi \quad [4]$$

where N_{oA} and N_{oB} are the initial *Listeria* population on surface A and B (in CFU), respectively; N_A and N_B are the *Listeria* population on surface A and B after cross-contamination (in CFU), respectively; χ is the *Listeria* population transferred from surface A to surface B (in CFU), and ψ is the *Listeria* population transferred from surface B to surface A (in CFU).

Cross-contamination transfer input probabilities. Table 23 shows the data used for the distribution for each equipment material surfaces (stainless steel, separator curtain, blue and white conveyors, wheels, crates) obtained from Dr. Castillo group (2024). Because of the lack of data, the transfer rate from avocados to the surfaces and from gloves to avocados and avocados to gloves were taken from Madamba et al. (2022). The transfer rate from the brusher conveyor was assumed to be higher than the other surfaces. The roller brushes are used to remove the dust/soil, bacteria, and insects from the product's skin in addition to polish the products. These brushes are made of polyethylene and camelhair. These rollers are source of cross contamination as they can attach microorganisms in their surface (Calle et al., 2018). The data provided by Dr. Castillo included *Listeria* transfer rates from contaminated equipment to avocados, but the model would not be complete without data on transfer from avocados to equipment. These data are yet to be provided by Dr. Castillo.

Description of the FS-AB simulator and assumptions:

(i) State variables and scale. In the FS-AB simulator (**Figure 36**), the components responsible for the dynamics of the cross-contamination are avocados, space, and time. Agents are either mobile or patch agents. The avocados are agents that are transported from patch to patch in each equipment. Patch agents are presented by a grid where each one contains traits that are modified at each model run. Avocados are transported through the processing line moving from patch-to-patch agents that have their own attributes, including levels of contamination and probability of microbial transfers. Space is represented by grid-based equipment with multiple attributes. Space provides the environmental basis for movements and interactions of lettuces. Each piece of equipment has a different surface area. **Table 24** describes each grid element of the facility and provides the location ID. Other attributes of the patches are fixed parameters of the equipment, represented as raster data: contamination levels and transfer probabilities. Time was represented by discrete time steps corresponding to one-fourth of a 180 s (3 min) processing time (720-time steps).

(ii) Computer simulation. At each time step, the time counter is updated. The variables for the avocado are updated as they move, get cross-contaminated, are decontaminated, and

then packaged. The patch agent variables are also updated accordingly as they become cross-contaminated. The variables are updated immediately during model runs.

(iii) Design concepts. A user-friendly interface was created to follow spatial and temporal variations in model outputs. Global level outputs, such as the number of contaminated avocados, the avocado contamination levels, were computed, and visualized on plots and diagrams. Diagrams representing equipment variables were also produced to track changes in these variables.

(iv) Agent interactions. A main feature of the modelled processing facility is the cross-contamination between different agents. Spatial-temporal dynamics of contacts are influenced by changes in the global state of the system, which affect interactions between lettuces and the equipment. Random events were introduced in the model to represent the equipment's heterogeneity. A realistic pattern of variability was introduced into deterministic rules by defining probability distribution functions. For example, the stochasticity of mobiles agents was represented by varying their locations in the equipment during initialization of each model run by introducing a random element in agent movements. The lettuce dynamics include a random selection of lettuces that are cross contaminated at each time step.

Statistical analysis. The Mean Squared Error (MSE), Root Means Squared Error (RMSE), $\chi 2$, and t-test were used to compare the models to the experimental data. The two-sample t-test was used to evaluate the level of significance changes on the outputs from different experiments using R version 4.0.2 (2020-06-22).

Model verification, and validation. To be able to validate the model, data from the facility must be provided so we may recreate analogous sampling scenarios that targeted the same equipment surfaces within the model. The model will run on the TAMU Supercomputer to assess the contamination status of agents representing historical sampling data. One thousand replications will be used per condition (3 total) to validate the model, which corresponded to 389 hours CPU time, taking about 20 hours to complete the job. The model will also be verified to be functioning correctly using NetLogo's own debugging tool for code integrity. Model mechanics will be tested using extreme scenarios and simplified models that only ran isolated parts of the original systems.

Sensitivity analysis. Sensitivity analysis will be used to confirm that changes in the input parameters correspond with logical changes in the simulation results. A deterministic 1-way sensitivity analysis will be performed to examine the output and look for a significant change ($p < 0.05$) in the number of contaminated avocados and contamination levels (log CFU/bag). In this study, deciding the minimum level of contamination that would not lead to an infection is a complex process, requiring not just scientific judgment but also social values from consumers and manufactures (Madamba et al., 2022). Therefore, a tolerance levels (TL) of $TL < 0.01$CFU/g (1 CFU/100 g) was considered since more than 1% of probability infection is considered unsafe for food processors (Puerta-Gomez et al., 2013). Therefore, avocados were assumed safe if the average contamination levels where below that tolerance limit. During a single simulation run, avocado movements and various statistical results, e.g., current avocado microbial load, will be visualized. After each simulation run, the average cross-contamination at each equipment/avocado, number of avocados decontaminated, and the average CFU/g in each bag will be reported. Multiple runs with varying input parameters and stochastic factors will be done. Subsequently, the aggregated results will be analyzed to identify the impact of those parameters on the processing performance.

Research Results (by objective)

OBJECTIVE 1:

Task 1. Conduct survey for risk assessment data collection

Unfortunately, the response by avocado packers to answer the questionnaire online was minimal and, in some of the few responses, the questionnaires were left incomplete. The plan presented by O. Rodriguez of having a prepared questionnaire to have avocado packers answer it physically during a meeting was also not viable due to the low number of individuals attending the meeting that was held in November of 2023. The questionnaire is still posted at https://tamu.qualtrics.com/jfe/form/SV_ehT1ATzTpCrCbDo. Only 16 people accessed the survey. Of these, 10 (63%) completed the survey. All answered that they used dry cleaning in their facility, they measure temperature and relative humidity routinely, conducted cleaning at the end of every day, and used alcohol-based cleaning products.

Task 2. ***Conduct environmental sampling and molecular testing***

Prevalence of *Listeria* spp. according to the environmental monitoring. The overall prevalence of *Listeria* spp. in environmental samples was 4.5% (**Figure 3**). Of these, none was isolated from air, whereas all other samples tested positive for *Listeria* spp. in at least 1 iteration. The percentage of positive samples ranged between 1.8 to 20. The highest percent isolation of *Listeria* spp. occurred in floors with 20% prevalence, followed by receiving crates (8.0%), transport vehicles such as carts and forklifts (6.0%), cleaning utensils (5.0%), drains (3.0%), non-food contact surfaces of the packing line's machinery (1.8%), and surfaces of the building such as walls and ceilings (2.5%). These data are summarized in **Table 4**. **Figure 4** shows the number of samples that tested positive to *Listeria* spp. at each plant. There were no differences in the percent detection of *Listeria* spp. between plants ($P \geq 0.05$). In contrast, the weather showed a significant effect ($P < 0.05$) in the percent detection of *Listeria* spp., from 3.3% during the months with dry weather (January-April), to 9.4% during the wet weather months (June-September). These data are shown in **Figure 5**.

Of the samples testing positive, a representative number of isolates was subjected to biochemical and serological tests for confirmation of genus *Listeria* and species identification. For 58 of these isolates, species identification was completed. Of the 58 *Listeria* spp. identified, 30 (51.7%) corresponded to *L. innocua,* 5 (8.6%) to *L. welshimerii*, 2 (3.4%) to *L. grayi*, 2 (3.4%) to *L. ivanovii.* 2 (3.4%) to *L. seeligeri*, and 12 (20.7%) to *L. monocytogenes*. In 3 isolates the species could not be identified (**Table 5**).

***Enterobacteriaceae* counts. Table 6** shows descriptive statistics for the finding of *Enterobacteriaceae* on various surfaces. Overall, in 40% of the samples the levels of these indicator organisms were below the limit of detection, which depending on the sample type, varied between 0.01–0.7 CFU/cm^2 for surfaces, and 10 CFU/sample for drains, and 100 CFU/m^3 for air samples. Of those samples with countable plates, the median of the counts ranged between 2–18 CFU/cm^2 for surfaces and was 3,200 CFU/m^3 for air samples and 3,200 CFU/sample in drains (**Table 7**). **Table 8** shows the effects of the packing plant and type of surface, as well as the interactions between these factors, in the counts of *Enterobacteriaceae.* There was no effect of the packing plant, indicating that the level of sanitation as indicated by the count of *Enterobacteriaceae*, is similar in all these plants, whereas the type of sample showed an effect on the counts of these organisms. In this case, floors, cleaning utensils and transport vehicles such as forklifts showed higher counts ($P < 0.05$). There were also interactions between the type of surfaces and the packing plant. Regarding air and drain samples, there was only an effect of the packing plant in air samples but not in the sampling

season (dry vs. wet), whereas none of these factors affected the counts of *Enterobacteriaceae* in drains (**Table 9**). The effect of the sampling iteration also was tested. As seen in **Figure 6**, there were significant ($P < 0.05$) differences between the sampling date. However, these did not seem to be related to the weather, as the counts shown during the cold months (January-February) were as high as those during the wet months (June-September).

The counts of *Enterobacteriaceae*, an indicator of plant sanitation, were included in this project as a safety measure in case the prevalence of *Listeria* spp. in the avocado packing environment was too low to draw conclusions. However, the number of *Listeria* spp. positive samples permitted an evaluation of the distribution of these environmental microorganisms despite its low prevalence. The counts of *Enterobacteriaceae* seemed to be relatively low although with great variations within each sample type (see **Table 7**). There seemed to be a constant presence of background microbiota, which is typical of agricultural environments, but these data were difficult to assess due to the lack of a reference standard.

To determine a possible correlation between the temperature at the plant and the isolation of *Listeria* spp., the temperature at the actual surface from which a surface sample was collected, was measured. In this case, due to the low prevalence of *Listeria* a correlation could not be established with reliability. The data on temperature measurements by type of surface are shown in **Table 10** for each packing plant. Also, **Table 11** shows the general statistics for temperature of various surfaces in the avocado packing environment. These data are influenced by the samplings at cold rooms, leading to minimum temperatures below 5 °C for samples of air, floors, building surfaces (walls, frames etc.) and forklifts, which are often moving product inside the cold rooms. Still, the medians show a relatively constant temperature inside the avocado packing facilities.

The relative humidity (RH) of the air was also measured for the areas from which air samples were collected for microbiological testing. There was a significant (but expected) increase in RH during the wet season. Attempts to establish a correlation between the isolation of *Listeria* spp. and RH yielded non-reliable results. However, it is interesting to show that there was also an increase in the percent positive samples during the wet season (see Figure 5).

Task 3. Characterize and evaluate cleaning and sanitizing procedures

In-plant trials:

Humidity and temperature before and after applying cleaning procedures. The average temperature (± one standard deviation) prior to the application of cleaning and sanitization procedures was 24.2 °C (± 2.7 °C), while the temperature following the cleaning and disinfection procedure was 21.4 °C (± 2.0 °C). This difference is statistically significant ($P < 0.05$). There were no significant differences observed among most surfaces, except for the receiving crates, which showed the lowest temperatures. On the other hand, the relative humidity prior to the application of the cleaning and disinfection procedure was 73.3% (± 10.8%), whereas after application it was 78.4% (± 6.7%). This difference is statistically significant ($P < 0.05$).

Isolation of *Listeria* spp. on surfaces before and after cleaning and sanitizing procedures. The prevalence of *Listeria* spp. on surfaces before and after the cleaning and sanitizing procedure is summarized in **Table 12**. Overall, *Listeria* spp. were detected in 30 (16.7%) samples before the cleaning and sanitizing procedures and in 29 (16.1%) samples after the application of cleaning and sanitizing procedures. Prior to cleaning and sanitizing procedures, the most frequent positive samples were found on receiving crates (16/180), floor (8/180), exit ramp 2 (3/180), transporting bands (2/180), and brushes (1/180); whereas, after the cleaning and sanitizing procedure, the most frequent positive samples were found on receiving crates (17/180), drains (5/180), floor (4/180), and brushes (3/180). There was no statistically significant

difference (P > 0.05) in the frequency of *Listeria* spp. between contact surfaces before and after the application of the cleaning and sanitizing procedure.

Levels of indicator organisms before and after cleaning and sanitizing procedures. The levels of microbiological indicators are summarized in **Table 13**. A significant difference (95% confidence) was observed in the levels of APC before and after the cleaning and disinfection procedure for the samples of transporting bands, exit ramps 1, and exit ramps 2, with an average reduction of 1.3, 2.0, and 0.8 Log CFU/100 cm^2, respectively. The levels of *Enterobacteriaceae* showed statistically significant differences (95% confidence) for the samples of transporting bands, floor, exit ramp 1, and exit ramp 2, with reductions of 1.6, 0.8, 1.7, and 2.1 Log CFU/100 cm^2, respectively. The levels of yeast showed statistically significant differences (95% confidence) for the samples of drains and exit ramp 2, with reductions of 1.5 and 0.7 Log CFU/100 cm^2, respectively. The levels of molds showed statistically significant differences (95% confidence) for the samples of transporting bands, strip curtains, exit ramp 1, rollers, and exit ramp 2, with an average reduction of 1.4, 0.6, 0.8, 0.5, and 1.1 Log CFU/100 cm^2, respectively.

Using the visual scale to evaluate the cleaning of the surface. The levels of visible soiling on surfaces before and after the application of the cleaning and disinfection procedure are summarized in **Table 14**. Prior to the application of the cleaning and sanitization procedure, samples from brushes, sizers, receiving crates, exit ramp 1, rollers, drains, and floor were frequently found with visible dirt (levels 3 and 4); whereas, after the application of the cleaning and disinfection procedures, visible dirt was primarily reported in samples from brushes, receiving crates, and drains. A statistically significant difference (P < 0.05) was observed in the level of visible soiling on transporting bands, sizers, strip curtains, exit ramp 1, rollers, exit ramp 2, and floor. In these cases, the level of visible soiling was lower after the application of the cleaning and sanitization procedure.

ATP levels on surfaces before and after cleaning and sanitizing procedures. The ATP levels (Log RLU/100 cm^2) are summarized in **Table 14**. Prior to the application of the cleaning and disinfection procedure, average levels between 2.62 and 3.79 Log RLU/100 cm^2 were observed. Following the application of the cleaning and disinfection procedure, levels were reported between 2.83 and 3.36 Log RLU/100 cm^2. There was no statistically significant difference (95% confidence) observed for the ATP level on any of the surfaces in the avocado packing plant.

Laboratory trials:

Table 15 show the effects of dwell time (sampling 1 vs. 8 h after having inoculated), sampling time (before or after cleaning), cleaning approach (wet vs. dry cleaning) and the type of equipment surface tested (stainless-steel surfaces vs PVC conveyor belt), where the conveyor would represent the food contact surface in an avocado packing plant and the stainless steel the adjacent structures that do not have contact with the product. All independent variables showed a significant effect. The *LM* before and after cleaning were 3.5 and 1.4 log CFU/cm^2, respectively, and the counts on stainless-steel structures vs. PVC conveyor were 2.0 and 2.9 log CFU/cm^2. The differences between these counts were significant, and numerically there is an obvious difference, to conclude that the cleaning procedure resulted in a significant reduction of this pathogen and that stainless steel consistently presented lower counts in comparison to the PVC conveyor. However, the main effects of the cleaning approach and wet vs. dry cleaning were significant while showing counts that seem to be not too different from one another. An observation of the data for interactions, particularly dwell time and cleaning approach, it is obvious that 1 h dwell time resulted in a lower count prior to cleaning, whereas

for 8 h dwell time, these differences are not very different. This may be useful for the avocado packing industry to develop adequate cleaning strategies. As indicated, these comparisons were not possible for sanitizing procedures after cleaning due to a complete reduction, but the effect of adequate cleaning highlights. In general, it seems to be that stainless steel tends to be more refractory to holding *LM* compared to PVC, and this effect is more evident for equipment that was wet vs. dry. Within dry and wet equipment, the differences in counts after 1 or 8 h dwell times were significant before cleaning, numerically these differences are too low, raising the question whether these statistically significant differences would have any biological significance. After cleaning, the counts were generally not different. While observations such as dwell time and type of surface seemed to apply better to the behavior of *LM* before cleaning, the effect of the cleaning procedure was difficult to determine. In the second experiments, a single dwell time was established, and the *LM* count data were processed to determine the magnitude of the reduction obtained by wet vs. dry cleaning. The results are shown in **Figure 8**, where the greatest reduction (1.8 log cycles) was obtained by wet-cleaning stainless-steel structures. This reduction was significantly different from the reduction on stainless-steel surfaces obtained by dry cleaning. For the PVC conveyor, there was no significant difference between wet vs. dry cleaning.

OBJECTIVE 2:

Whole genome analysis. Nineteen isolates obtained from the plates were selected for the WGS sequencing study and were sequenced on the Illumina NovaSeq platform generating 8-9 million reads on average with highest being 20 million reads and the lowest being 6.5 million reads. The quality analysis resulted in retaining over 97% of the reads and the Q30 values were above 90% for all the isolates. The GC content of the isolates varied between 36.5 to 51% (**Table 16**).

Following the quality analysis the sequences were assembled using a reference-based assembly approach using the BWA tool. The genome sequence of *LM* EGD-e (ATCC BAA-679) with a genome size of 2,944,528 bp and a GC content of 37.98% was used as the reference genome. Mapping statistics (< 30%) indicated that 4 isolates namely A1, A2, A4, A13 and A22 were not related to the reference strain. This was further confirmed by the GC% values of the above-mentioned isolates differing widely from the reference genome. Further, the average depths were between 101.05X and 517.83X, and 1X coverages ranged from 1.35% to 90.93% (**Table 17**). Since the reads contained a good coverage threshold, further analysis on mutation detection were carried out.

Single nucleotide polymorphism (SNP) refers to a variation in a single nucleotide which may occur at some specific position in the genome, including transition and transversion of a single nucleotide. SNPs in the assembled genomes were calculated with GATK tools. The total number of SNPs varied widely for the isolated organisms ranging from 276 to 52205. SNPs of the isolates closely related to the reference organism ranged from 37552 to 52205. Among the variations in the exonic region, synonymous mutations outnumbered non-synonymous mutations while stop gain mutations outnumbered stop loss mutations. Similarly, mutations in the downstream region outnumbered upstream region. The average ratio of transitions to transversions was around 400 among the genomes. SNP quality distribution plots by plotting the distribution of SNP support reads number, distances between adjacent SNPs, and the cumulative distribution of SNP quality confirmed the above-mentioned results (**Figure 9**). Further the categories of the SNP mutations were analyzed. For any whole-genome sequencing project, the SNPs could be placed in any of the six categories. For example, when T>C mutation appears on either of the double strand,1 the A>G mutation will be found in the same

position of the other chain. Therefore, the T>C and A>G mutations are classified into one category. Results indicated that C: G>T: A were highest class followed by T: A>C: G category of SNPs (**Figure 10**).

InDel refers to the small insertion or deletion in the DNA and were detected in the genomes using GATK. InDel analysis indicated that the total number varied widely for the isolates ranging from 49 to 3148. InDels of the isolates closely related to the reference organism ranged from 1356 to 3148. Among the variations in the exonic region, synonymous mutations outnumbered non-synonymous mutations while stop gain mutations outnumbered stop loss mutations. Similarly, mutations in the downstream region outnumbered upstream region (**Figure 11**).

Structural variants (SVs) are genomic variation with mutations of relatively larger size (>50 bp), including deletions, duplications, insertions, inversions and translocations. BreakDancer software were used to detect insertion (INS), deletion (DEL), inversion (INV), intra-chromosomal translocation (ITX) and inter-chromosomal translocation (CTX) mutations, based on the reference genome mapping results and the detected insert size. The detected SVs were filtered by removing those with less than 2 supporting PE reads, the INS, DEL and INV were further annotated by ANNOVAR. The total SVs varied between 6 to 809, while the value for the closely related organisms ranged between 118 to 809. SVs in the exonic regions were higher compared to the other regions and the ITX outnumbered other translocations.

Using this information, a risk map was attempted. Of all isolates that tested positive for *Listeria* spp., 19 were subjected to whole genome sequencing and referenced against *LM* EGD-e (ATCC BAA-679) strain. One isolate failed the quality test and could not complete the sequence, and 4 isolates had mapping rates of 0.87%, 0.71%, 0.96% and 0.83% compared to the reference genome, which was too low and were discarded as *Listeria* strains. Fourteen isolates yielded percent mapping rates between 28.92 and 54.71. The higher the rate the more likely to share DNA. The sources of these isolates are shown in **Table 18**.

Figure 12 shows the points during the process flow where similarities were found at each packing plant. Unfortunately, at the time of report submission, the phylogenetic tree, needed for completing the tracking map was still not received from the third party used for sequencing, which prevented from tracking individual isolates with repeated sequences obtained from more than one source. As soon as we receive such information, we will add it to this report.

16S rRNA amplicon analysis. 35 randomly selected enrichment broth were analyzed for the 16S rRNA amplicon analysis. On an average, about 0.2 million reads were generated for the samples analyzed and after the QA step, about 95% reads were retained. Further, the Q30 values were above 90% for all the samples analyzed. The reads were further demultiplexed and trimmed.

Following the QA step, in order to study the microbial community composition in each sample, OTUs were obtained by clustering with 97% identity against 16S rRNA database of Silva version 138.1. Based on the analysis Firmicutes were found to be dominant phyla observed among the samples followed by Actinobacteria. At genus level, *Listeria* was found to be the dominant genus followed by *Enterococcus* and *Microbacterium* (**Figure 13**). This was expected and does not indicate that *Listeria* spp. are, in fact, predominant in environmental samples. The predominance of the genus *Listeria* in these samples occurred because the samples were from enriched medium, used with the purpose of propagating *Listeria* to compare16S gene sequencing to the 3M Molecular Detection System (MDS) as a potential tool for identification without the need for further confirmation.

Diversity analysis was performed after normalizing the reads at 155951. Alpha diversity is applied to the analysis of microbial community diversity within the sample by analyzing the diversity of a single sample, reflecting the richness and diversity of microbial communities in each sample, including a series of statistical analysis. Alpha diversity analysis indicated that the observed species varied between 2 to 14. Shannon index varied between 0.001 to 0.168 while the Simpson index varied between 0.045 to 0.001. Chao1 values varied between 2 and 14.

A rarefaction curve is created by selecting randomly certain amount of sequencing data from the samples, then counting the number of the species they represent (i.e., the number of OTUs). The Rarefaction curves can directly reflect the rationality of the sequencing data volume and indirectly reflect the richness of microbial community in the samples. Rarefaction analysis indicated that the sufficient sampling has been carried out. Statistical tests indicated that there are no significant differences between the samples (**Figure 14**).

Before proceeding for beta diversity analysis, the samples grouped into two, namely surface and floors. NMDS is a non-linear model designed for a better representation of non-linear biological data structure, aiming at overcoming the flaws in methods based on a linear model. NMDS of the analyzed samples indicated that there is no difference between the groups (**Figure 15**).

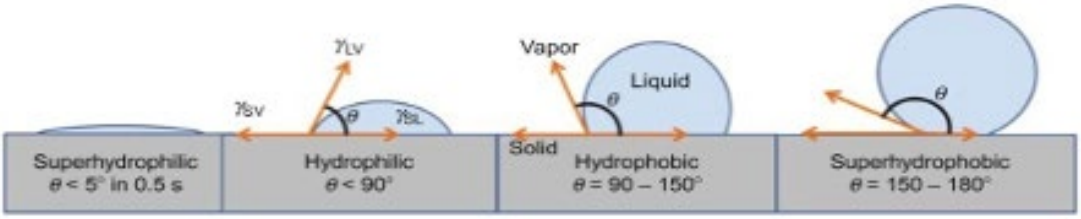
OBJECTIVE 3:

Task 1. Surface dynamics and potential for attachment

Topographical study. To gain a deeper insight into the physical morphology, surface characterization techniques such as SEM and AFM were employed to analyze the surfaces of all samples. **Figure 16** illustrates the diverse topographical profiles observed across the samples. Stainless-steel surfaces exhibited prominent peaks and valleys, indicative of its typical rough surface texture. Hass avocados displayed a distinctive network-like structure, with notable chunks of wax distributed across the surface. Conversely, the black roller surfaces exhibited significant signs of wear and erosion, likely due to extensive usage. In contrast, other surfaces appeared comparatively smoother, suggesting varying degrees of surface integrity and wear.

Roughness. To understand surface roughness, AFM was utilized. **Figure 17** illustrates the 3D atomic force microscopic images of each sample. In measuring surface roughness, we use two important numbers: Rq and Ra. Rq, or root mean square roughness, offers insight into the root mean square of surface height deviations from the mean line within a defined sampling length, thereby depicting the variation in height across the surface. On the other hand, Ra, or arithmetic average roughness, presents the arithmetic average of absolute values of surface height deviations from the mean line within the same sampling length, providing an indication of the surface's average roughness. While both parameters contribute to understanding surface roughness, Ra tends to be more sensitive to extreme values, whereas Rq offers a more comprehensive overview of the overall variation in surface texture. According to **Figure 18**, when comparing the surface roughness of different samples, the highest Rq is observed for Black Roller, followed by Stainless Steel and Hass Avocado. A higher Rq indicates greater roughness. Conversely, the lowest Rq is associated with the Blue Conveyor Belt. Columns with the same color do not exhibit statistically significant differences in roughness. **Table 19** displays the results of Rq and Ra, with letters (A, B, C, D) indicating the level of significance determined through statistical analysis performed on the Rq values. Samples with identical letters adjacent to their Rq values do not display statistically significant differences in roughness.

Wetting behavior of samples. The water contact angle is a measure of how much a water droplet spreads out or beads up on a solid surface. It tells us if a surface repels water (hydrophobic) or attracts it (hydrophilic). The impact of surface chemistry and wetting behavior on bacterial adhesion has been firmly established (Oh et al., 2018), emphasizing the necessity of comprehensively understanding these properties before proceeding with further evaluation studies.



To investigate the wetting behavior, water contact angles (WCAs) were measured for all samples. It was noted that the Blue Conveyor Belt exhibited the highest hydrophobicity, with a WCA of 106±5.5° (as depicted in **Figure 19**). Conversely, the White Conveyor Belt demonstrated the highest hydrophilicity, with a WCA of 83±3.9°. WCA of Hass Avocado was 86.7±1° (illustrated in **Figure 20**), which means compared to other material has higher hydrophilicity except white conveyor belt.

Bacterial attachment on avocado surface. The aim of this section is to monitor the adhesion of *LM* to the avocado surface over time. **Table 20** illustrates a clear trend: as time progresses, the bacterial count gradually increases. Notably, the highest bacterial population is observed after 6 hours of inoculation. Throughout all stages of inoculation, the population of loosely attached bacteria consistently exceeds that of strongly attached bacteria, with a difference ranging from 0.9 to 1.3 log cfu/cm^2 at each time point. For Hass avocados, after 6 hours of inoculation, the counts were 6.6 ± 0.1 and 5.1 ± 0.1 log cfu/cm^2 for loose and strong attachment, respectively, based on agar plate counting (displayed in **Figure 21**).

Bacterial attachment on packing plant material surfaces. According to **Figure 22**, there is a gradual increase in the amount of loosely attached bacteria over time. For instance, black roller shows 6.2 log cfu/cm^2 of loosely attached bacteria at 6 hours compared to 5.7 log cfu/cm^2 at time 0. **Figure 23** illustrates a clearer trend of increasing bacteria over time. In all samples, bacterial attachment is notably stronger after 6 hours of inoculation. For example, black roller exhibits 7.02 log cfu/cm^2 at 6 hours compared to 5.4 log cfu/cm^2 at 0 hours. **Table 21** presents detailed data regarding this phenomenon.

Strength of attachment (S_R). The attachment strength (S_R), defined by Dickson and Koohmaraie (1989), is a ratio of strongly attached (S_R) cells to the total bacterial population and does not directly reflect the attractive forces between bacteria and sample surfaces. It is calculated using the formula: (S_R) = [(SA) / (SA + LA)]. **Figure 24** compares different materials in terms of attachment strength (S_R), with the highest S_R observed at 0.5, while Hass avocado exhibits the lowest attachment strength. This suggests that prioritizing surface cleaning in packing plants is more important than cleaning individual avocados. **Figure 25** demonstrates that over time, the strength of attachment increases, indicating that bacteria adhere more strongly to surfaces as time progresses.

Bacterial enumeration through SEM. In terms of average levels of microbial contamination, the samples are ranked as follows (**Table 22**): The highest average contamination level is exhibited by Stainless steel at 7.2 log CFU/cm^2, closely followed by White conveyor belt at 7.0 log CFU/cm^2, Separator Curtain at 6.8 log CFU/cm^2, Blue conveyor belt at 6.7 log CFU/cm^2,

Avocado Hass at 6.6 log CFU/cm^2, and Black roller also at 6.6 log CFU/cm^2. This ranking suggests that the highest average contamination levels are observed in Stainless steel and White conveyor belt, while slightly lower levels are found in Separator Curtain and Blue conveyor belt, and the lowest levels are detected in Avocado Hass and Black roller.

Task 2. Biofilm formation and transfer from equipment to avocado

Biofilm formation using SEM. **Figure 26** illustrates the Hass avocado following a 3-hour spot-inoculation with the distribution of a 100-µl *LM* suspension with a concentration of 9 logs. The SEM image captures bacterial behavior, revealing their tendency to hide and spread across the avocado. Bacteria exhibit adhesion to various parts of the avocado skin, particularly within valleys. During the process, relative humidity (RH) and temperature were monitored every 3 hours, measuring 92% ± 2 for RH and 72± 2 °F for temperature. **Figures 27 to 31** depict scanning electron micrographs of all utilized packing plant materials after storage periods of 3 hours, 4 days, and 10 days at 72± 2 °F and 92% ± 2 RH. These images reveal the development of biofilms. At the 3-hour mark of storage, no observable biofilm formation was evident. Instead, single or double bacteria were observed adhering across the surface. However, after 4 days, a well-defined biofilm containing numerous bacteria was present, indicating clustering and the formation of biofilm linkages, a thin fibers, generally associated with the production of extracellular polymers (Donlan, 2002). After 10 days, material surfaces were covered with slime, although only a few bacteria were detected in some samples. This pattern corresponds to findings from another study on tomatoes (Iturriaga et al., 2007).

Transfer of LM from inoculated materials to fresh avocado. The transfer rate of LM from different contaminated surfaces to fresh avocado was assessed. Initially, all materials were contaminated under the same conditions with LM suspension. Subsequently, fresh avocado was brought into contact with the contaminated surfaces. Some bacteria were loosely attached to the avocado surface, while others adhered strongly.

In the results depicted in **Figure 32**, varying degrees of bacterial transfer were observed among the contaminated materials at the 15-minute time interval. For example, higher transfer rates were noted for the Blue conveyor Belt and Stainless steel surfaces, with values of 0.4 and 0.36, respectively. Conversely, relatively lower transfer rates were observed for the Separator curtain and Red box surfaces, with values of 0.20 and 0.16, respectively. At the 3-minute time interval, fluctuations in bacterial transfer rates were observed among the materials. The Black roller surface exhibited a transfer rate of 0.32, while slightly lower rates were observed for the Blue conveyor Belt and Stainless steel surfaces (0.31 and 0.28, respectively). Notably, significantly lower transfer rates were observed for the S White conveyor Belt and Red box surfaces compared to the 15-minute interval. During the shortest duration of 36 seconds, lower transfer rates were generally observed across all materials. The Black Roller, Stainless steel and Blue conveyor Belt surfaces exhibited the highest transfer rates at this interval, followed by the Separator curtain and White conveyor Belt surfaces. Interestingly, the Red Box surface showed an extremely low transfer rate, indicating minimal bacterial transfer.

In **Figure 33**, which deals with strongly attached *LM* to the avocado surface transferred from contaminated materials, the similar trends were observed. At 15 minutes, Blue conveyor Belt had the highest transfer rate to avocado, followed closely by Black roller and Stainless steel. During 3 minutes, Blue conveyor Belt maintained a relatively high transfer rate, while Stainless steel showed a decrease, suggesting shorter durations may reduce contamination risks. At 36 seconds, Blue conveyor Belt showed a significant decrease in transfer rate, while Black roller maintained a relatively high rate. Stainless steel showed lower transfer rates, and Separator curtain had minimal bacterial transfer, indicating potential risk reduction with shorter contact durations.

The graph (**Figure 34**) illustrates the transfer rates of total bacteria (LA+SA) from various contaminated surfaces to fresh avocado regardless the time. Among the surfaces analyzed, the Black roller and Blue conveyor belt exhibit the highest transfer rates, positioned at the upper end of the graph. They are followed by Stainless steel, which demonstrates a moderate transfer rate. In contrast, the White Conveyor Belt, Separator Curtain, and Red Box surfaces display lower transfer rates, positioned towards the lower end of the graph. This visual representation highlights the differing degrees of bacterial transfer among the surfaces, with some showing notably higher rates than others.

OBJECTIVE 4:

Preliminary results:

Scenario 1 – About 4% incoming avocados are contaminated with 1 log CFU/avocado
The simulation of scenario 1 involved running the program on a laptop computer, which processed 20,000 avocados over approximately 1.5 hours (equivalent to 90 minutes or 30 batches of 3 minutes each). It was assumed that 4.2% (833 avocados) of the total avocados introduced to the packinghouse were contaminated with 1 log CFU/avocado. Prior to processing, the facility was considered clean with no existing pathogen contamination. The detection limit for pathogens was assumed to be 0.04 CFU.

Figure 37 illustrates the progression of avocado contamination throughout the process. By the end of the processing line, 2,001 avocados (10% of the total) were contaminated with *Listeria*. Most contamination stemmed from cross-contamination originating from equipment that had been exposed to contaminated incoming avocados. However, by the end of the process, equipment within the facility did not exhibit any contamination above the detection limit.

Scenario 2 – About 4% incoming avocados are contaminated with 3 log CFU/avocado
In this scenario, we processed a total of 7,000 avocados, taking approximately 36.5 seconds (equivalent to 12 batches of 3 minutes each). It was assumed that approximately 4% (256 avocados) of the avocados introduced to the packinghouse were contaminated with a microbial load of 3 log CFU/avocado. Prior to processing, the facility was presumed to be free of any pathogen contamination. The detection limit for pathogens was set at 0.04 CFU. By the end of the processing line, 5,623 avocados (80% of the total) were found to be contaminated with *Listeria*. The primary source of contamination stemmed from cross-contamination originating from equipment that had come into contact with the incoming contaminated avocados, with 526 avocados contaminated with 3 log CFU of *LM.* In comparison with scenario 1, considering only the first 7,000 processed avocados, scenario 2 yielded 8 times more contaminated avocados. This highlights the danger associated with highly contaminated avocados entering the packinghouse.

Figure 38 illustrates the average and standard deviation (represented by error bars) of cross-contaminations within each component of the packinghouse. Additionally, **Figure 39** depicts the changes in percentage and level of contamination throughout the processing. The results reveal that the separator curtain (L6) emerged as the most heavily cross-contaminated element in the packinghouse, with approximately 46% ± 16% of its surface contaminated during the process. The average level of cross-contamination for this component was approximately 1.092 ± 0.546 log CFU. Furthermore, the roller conveyor, blue conveyor belt, and white conveyor belt were identified as significant sources of cross-contamination within the facility.

Scenario 3 – About 4% of the first 1,000 avocados were contaminated with 3 log CFU *Listeria*

In this scenario, out of the 7,000 avocados processed, it was assumed that 4% of the initial 1,000 avocados introduced were contaminated with 3 log CFU of Listeria, while the remaining 6,000 avocados were assumed to be uncontaminated. The results indicated that 20% (1,429) of the total processed avocados were contaminated.

Figure 40 illustrates the significant variability in the percentage of area cross-contaminated within each component. These changes occurred during the process as cross-contamination from contaminated surfaces affected the processed avocados. **Figure 41** depicts that the maximum level of cross-contamination was observed around 300-400 seconds into the process. This pattern contrasts with the results obtained from scenario 2, where cross-contamination affected all components throughout the entire process. The average level of cross-contamination across the facility was calculated at 0.804 ± 0.252 log CFU. This scenario underscores that not only the level of contamination but also the number of initially contaminated avocados entering the processing stream significantly impacts the safety of the processed avocados.

Conclusions:

The initial findings suggest that the developed FS-AB simulation effectively captures the inherent complexity of an avocado packinghouse with precision. However, to fully visualize and simulate the process, additional data are needed, including details on areas of contamination within the facility and transfer rates from component surfaces to avocados. This would enable modeling of production, cleaning, and sanitation shifts based on facility-provided information. To conduct a comprehensive analysis of the efficacy of the developed model, we will execute the program using the TAMU supercomputer. This high-performance computing resource will facilitate more extensive simulations and enable a deeper understanding of the system's behavior and performance.

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Outcomes and Accomplishments

This project aimed at tracking *Listeria* spp. in avocado packing plants where fruit washing is eliminated but they apply wet cleaning and sanitation practices. In-plant and laboratory studies comparing dry cleaning vs. wet cleaning were conducted, and the data should permit evaluation of current sanitation practices and possible room for improvement in preventing *Listeria* spp. in the avocado packing environment. After completion of this project, we are better equipped to understand the potential for *Listeria* to colonize the packing plant environment and the areas of the plant that potentially facilitate harborage at avocado dry packing plants in Mexico. Results will help packers to implement practices that improves food safety during avocado dry packing.

Specific accomplishments include the collection of data previously nonexistent, which will be useful for continuing work with the avocado industry and will provide light on the distribution of *Listeria* spp. in the avocado packing environment. This can allow identification of routes of transmission of these organisms over the plant environment, allowing in turn the development of science-based strategies for preventing cross-contamination.

A significant accomplishment is the contribution with data related to cleaning and sanitation procedures, both in-plant and in laboratory settings. These data have permitted diagnosis of key factors in cleaning and sanitizing equipment in avocado packing plants. Another important accomplishment is the finding that *L. monocytogenes* can form biofilms under simulated avocado packing conditions. Most studies on biofilm formation force the conditions to promote the biofilm, which results in an artificial setting which does not always represent the real conditions of post-harvest handling of produce. Finally, the data will permit the development of a small tool for predicting the transfer of *Listeria* spp. from contaminated equipment to avocados and should help in development of control measures to prevent the presence and distribution of *LM* in the plant. From these data, the following plans are under development, which will in turn become an outcome when implemented:

1. The development of best practices for sanitation in avocado packing plants.
2. The development of training programs that target the understanding the effect of current practices in promoting equipment contamination.
3. A plan for broadcasting the efforts of the avocado industry in Mexico, which should improve the acceptance of foreign avocados and possibly increase shipments at competitive prices (mutual benefit).
4. Publication of articles on food safety of fresh avocados. Most of the publications are focused on potential remediation measures, but publications on preventive food safety measures are scarce.

Regarding Objective 4 outcomes and accomplishments, the model showed that *LM* is notoriously challenging to control once established in a packinghouse. Decision support tools like the agent-based model can play a crucial role in understanding cross-contamination dynamics and devising effective mitigation strategies. Our study with the avocado facility can demonstrate the adaptability of agent-based models in addressing food safety concerns by replicating unique packinghouse components. In summary, agent-based modeling (ABM) has proven invaluable in understanding and simulating the complexities of avocado processing in packinghouses. By representing interactions between avocados and various equipment within the facility, ABM offers a framework for comprehending the intricate dynamics involved. Moreover, ABM facilitates predictive modeling, allowing for the simulation of avocado contamination levels over time. This capability is particularly useful for assessing cross-contamination during processing and forecasting pathogen spread based on avocado interactions and environmental factors such as pathogen transfer rates. Furthermore, ABM provides the flexibility to explore alternative scenarios and hypotheses by adjusting parameters

and initial conditions. This allows for experimentation and hypothesis testing in a virtual environment, eliminating the need for costly real-world trials.

Summary of Findings and Recommendations

Findings:

1. The presence of *Listeria* spp. is low but constant in the avocado packing environment.
2. Current in-plant cleaning and sanitizing procedures did not seem to be effective in eradicating *Listeria* from packing equipment. Visual inspections showed steps that may have negative effects in the effectiveness of the cleaning and sanitizing procedure.
3. Whole genome sequencing may be a useful tool to track *Listeria* and identify possible sites of establishment inside avocado packing plants.
4. *LM* can attach to the surfaces of equipment and utensils and, if the atmosphere is of high RH, can follow an efficient biofilm formation path.
5. Dry cleaning did not show advantages over wet cleaning in pilot-plant studies.
6. The developed model, while highly intricate, demands substantial computational resources to simulate the process accurately. Once fully validated and verified, employing machine learning techniques could streamline simulation time, allowing wider accessibility for avocado producers and packinghouse managers.
7. Based on the preliminary results outlined in this report, meticulous attention is warranted regarding both the quantity and level of contamination, particularly with pathogens like *Listeria*, which serve as significant sources of cross-contamination within the facility and vice versa.

Recommendations include:

1. Intensify cleaning and sanitizing, following an effective procedure with adequate verification of effectiveness.
2. Although reducing humidity in the plant is highly desirable, there is room for improvement of the procedures for maintaining dry equipment during pre-operative cleaning and sanitizing.
3. If switching to a fully dry packing process is being considered, keep in mind that pilot studies showed that dry cleaning may not necessarily result in a greater prevention or eradication of *Listeria* spp.
4. To achieve the development of a model for predicting the risk of contamination that helps address risk reduction strategies during sanitation, comprehensive data from the facility are essential, encompassing all areas susceptible to contamination, cleaning protocols, and experimental data pertaining to surfaces in contact with avocados. However, the efficacy of the model is contingent upon the availability of scarce contamination data. Targeting *Listeria* originating from primary contamination sources, such as contaminated incoming avocados, appears to be the most effective approach in reducing prevalence, offering a potentially applicable strategy facility wide. Therefore, focusing on internal corrective measures, such as enhancing surface sanitation frequency and decreasing contamination spread between equipment, designed to the specific conditions of each facility, may prove to be more logical.

APPENDICES

Publications and Presentations

- ✓ CPS 2023 Annual Symposium, Atlanta, Georgia, June 2023 Poster presentation. Alex Castillo
- ✓ Presentation of Results: "Seminar: Sanitation in avocado packing facilities. Challenges and Solutions" aimed at the safety personnel of the three packing houses that participated in the research, as well as members of other avocado packing plants. (The invitation and organization were through the University of Guadalajara and Association of Avocado Producers and Exporters. Site: REDI Zapotlan Auditorium. Ciudad Guzman, Jalisco) February 16, 2024

- ✓ Two manuscripts are in preparation.

Budget Summary

This project was awarded a total of $344,220 in research funds, and all funds were spent.

Appendix A. Figures and Tables for Objectives 1–4.

Appendix B. Methodology for Objective 1. *Task 3. Characterize and validate cleaning and sanitizing procedures*

(see below)

Appendix A. Figures and Tables for Objectives 1–4.

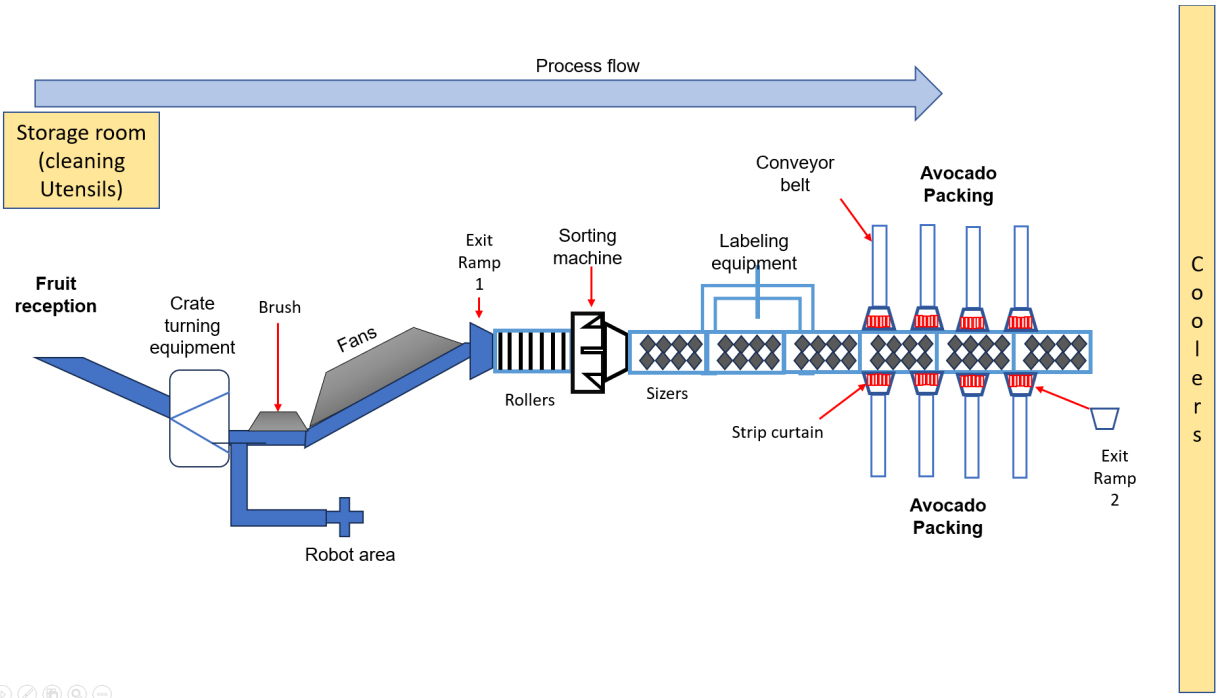


Figure 1. Avocado dry packing flow process

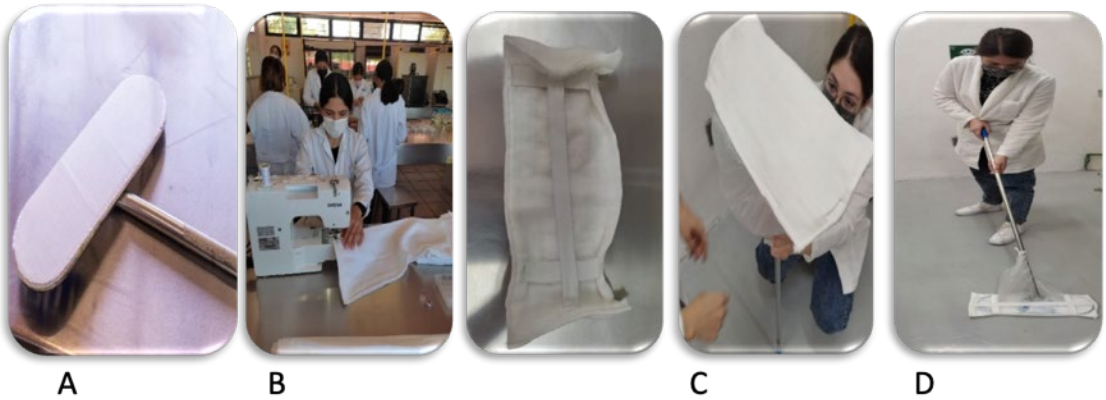


Figure 2. Floor sampling swab device (FSSD). Floor swabs designed for floor sampling at avocado packing plants. A) Aluminum frame and handle covered with polypropylene. B) Confectioning swab. C) Assembled MFSS. D) Floor sampling

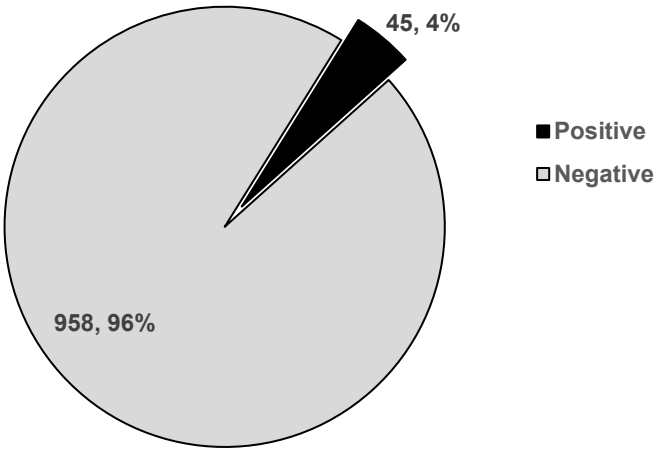


Figure 3. Overall prevalence of* Listeria *spp. in environmental samples collected at 3 avocado plants in Mexico

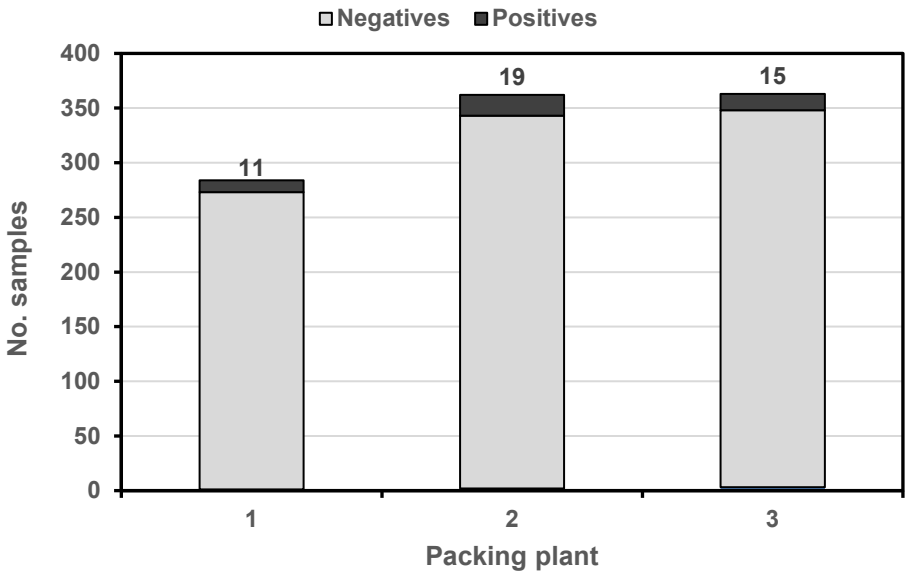


Figure 4. Prevalence of* Listeria *spp. in environmental samples collected at 3 avocado packing plants in Mexico. Pearson's Chi-Square test indicated no difference between proportions of positive samples between plants.

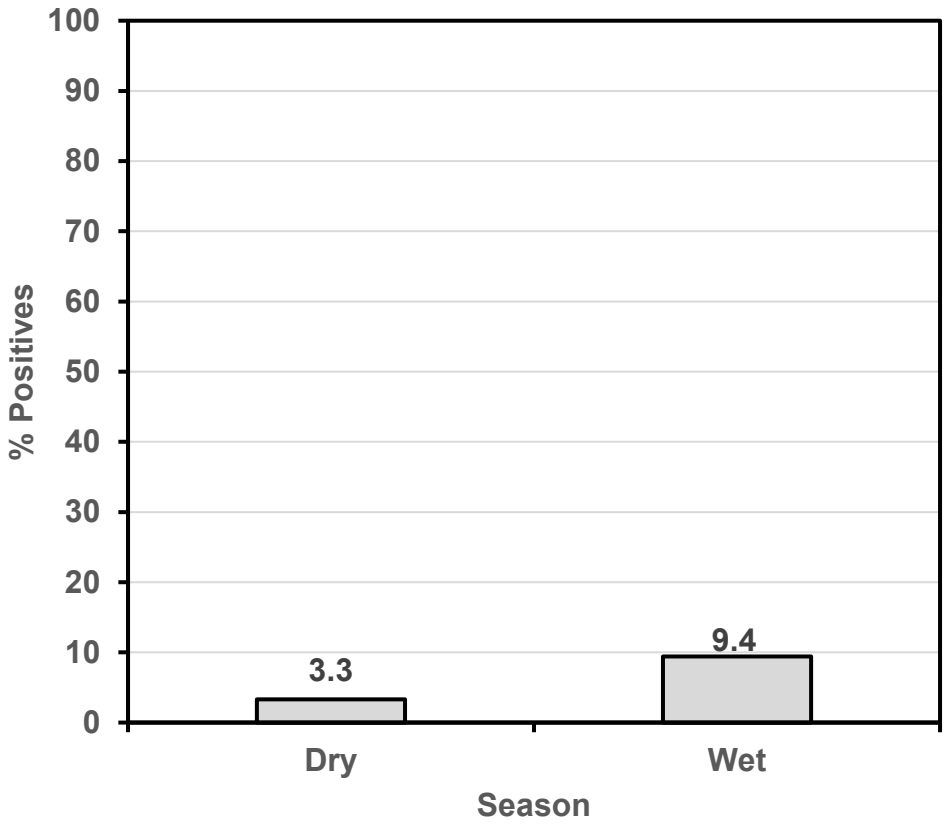


Figure 5. Effect of the wet season on the isolation of* Listeria *spp. in the environment at 3 avocado packing plants in Mexico. The difference between proportions of positive samples for each season was significant (P < 0.05).

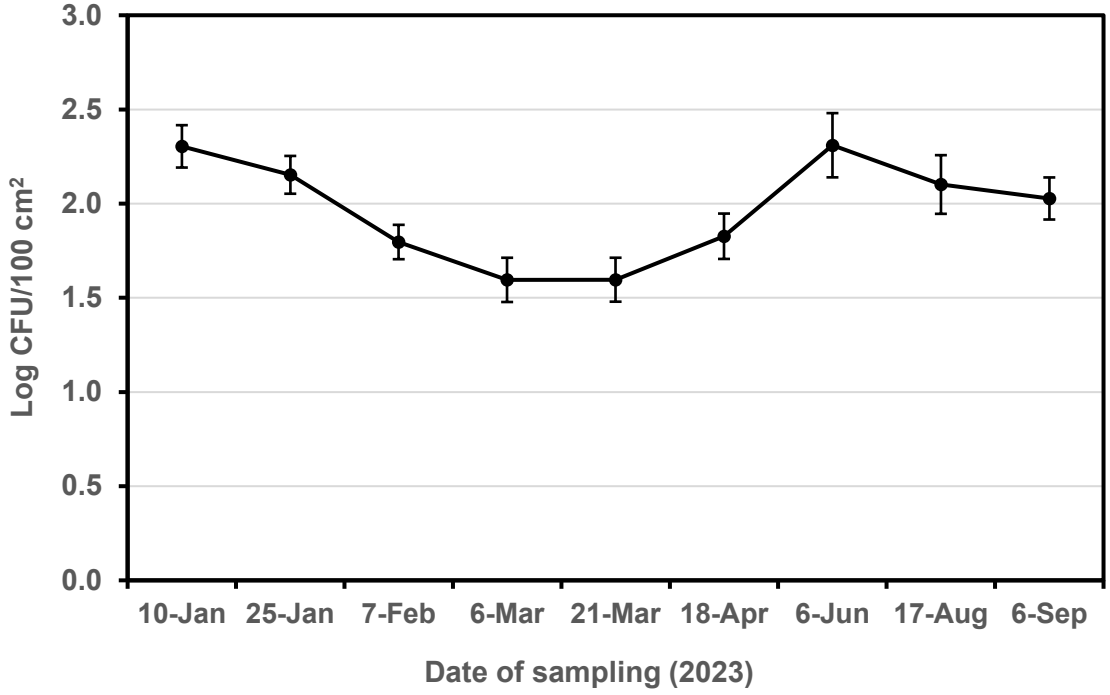


Figure 6. Counts of* Enterobacteriaceae *on diverse surfaces in the environment at 3 avocado packing plants in Mexico over a 9-month sampling period

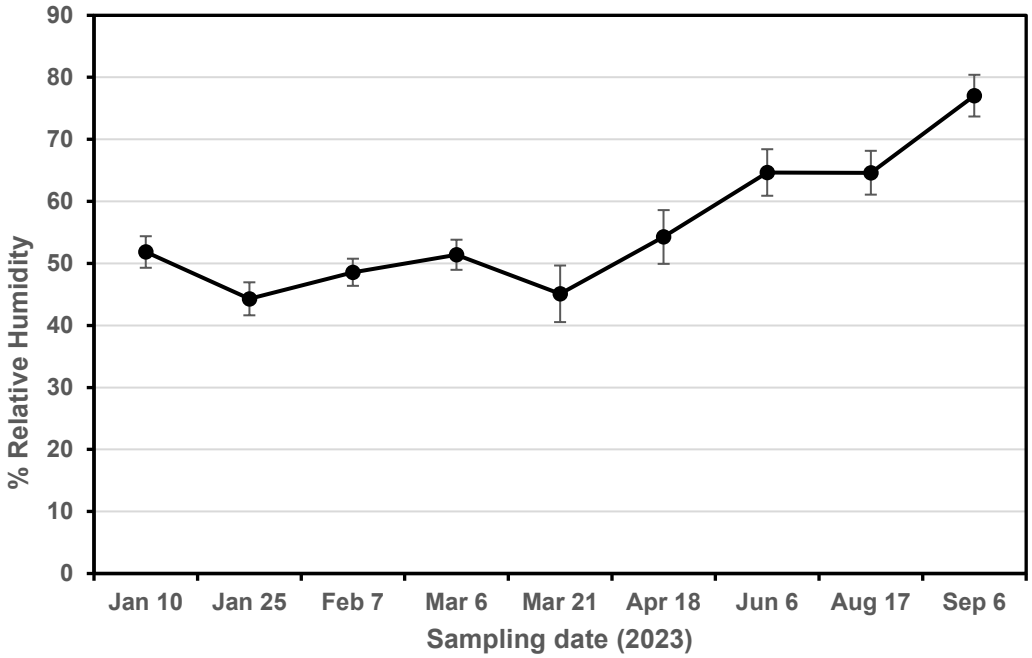


Figure 7. Relative humidity of the air at areas where environmental samples were collected in 3 avocado packing plants in Mexico.

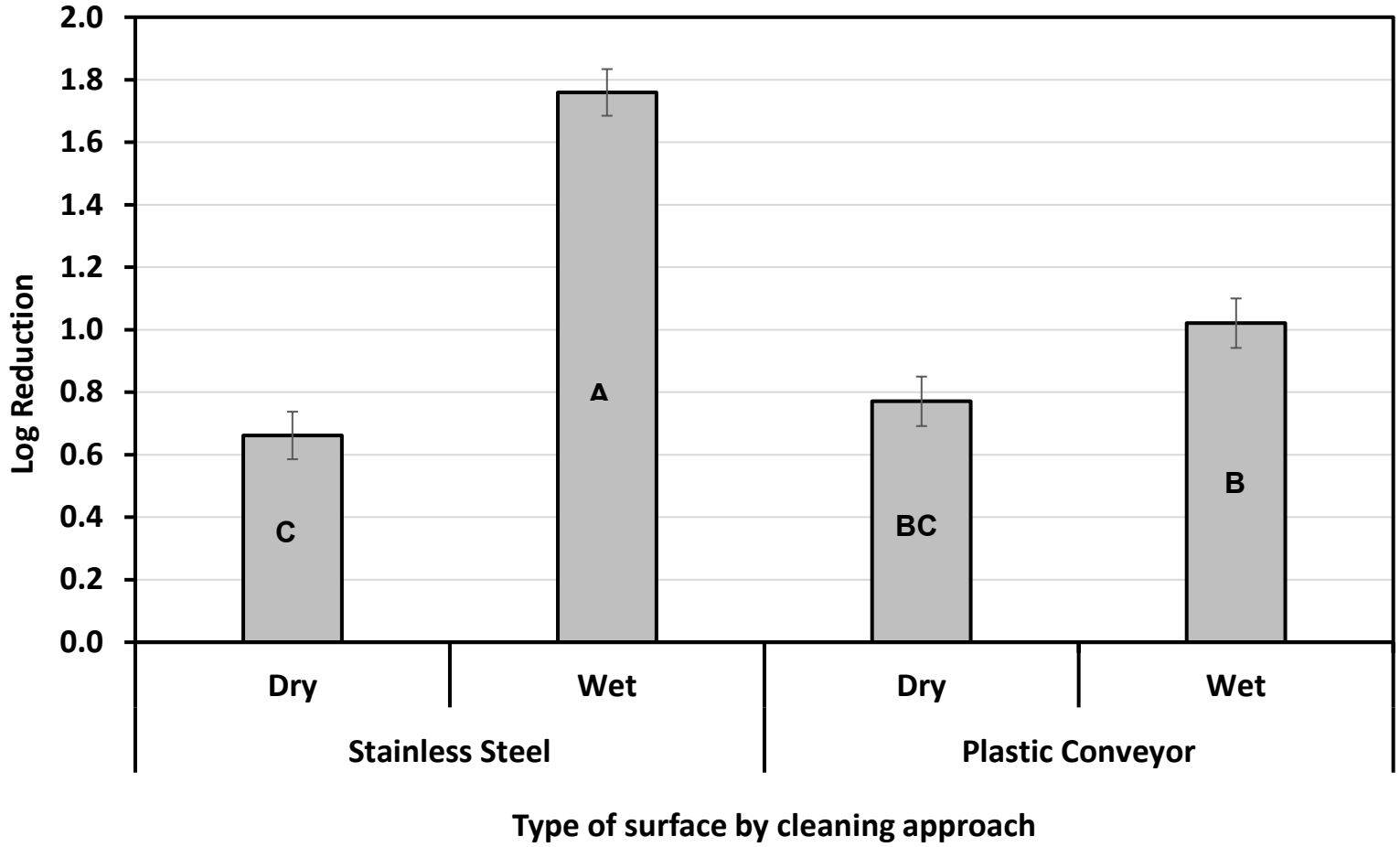


Figure 8. Log reductions of* LM *after wet or dry cleaning on inoculated pilot packing plant equipment. Log reductions calculated by subtracting the log CFU/cm^2 after cleaning from those before cleaning. Taller bars indicate greater reduction, and columns not showing the same letter are significantly different ($P < 0.05$).

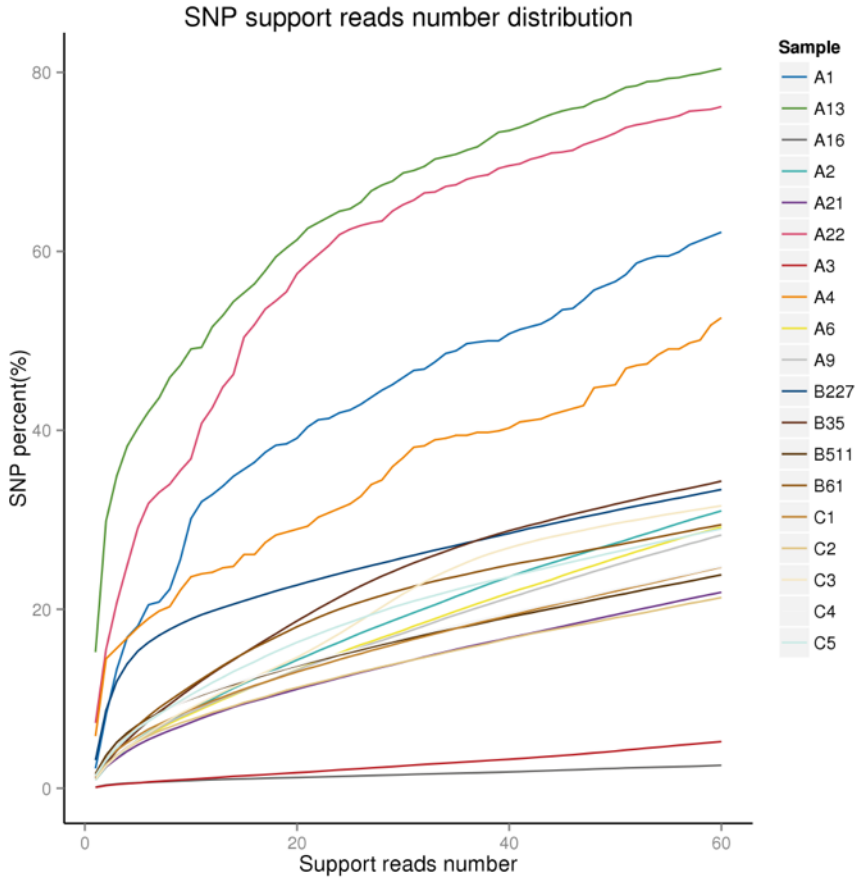


Figure 9. Cumulative distribution of SNP quality

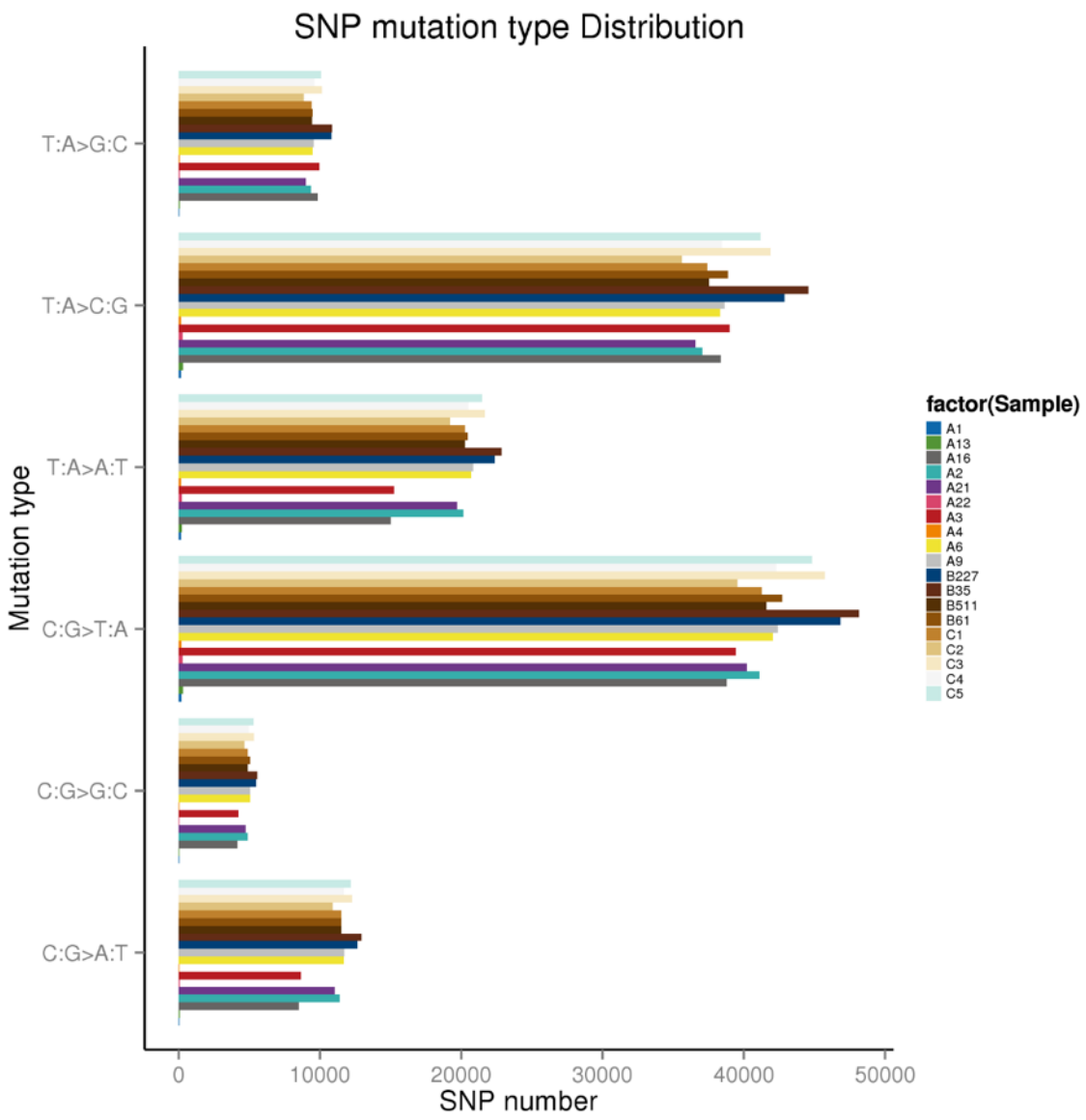


Figure 10. Frequency of SNP mutations. The x-axis represents the number of the SNPs, and y-axis indicates the mutation types.

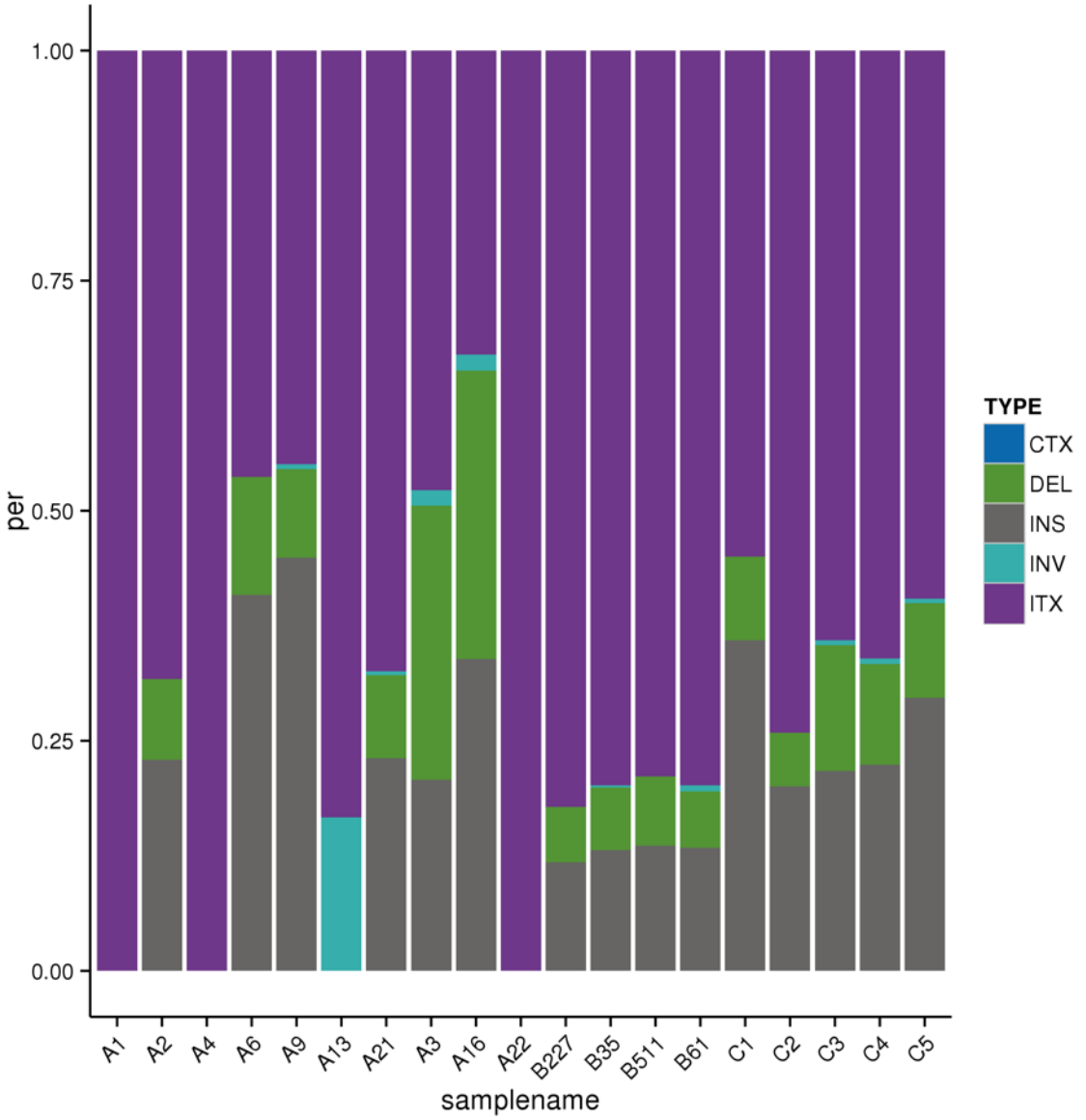


Figure 11. Variation type statistics distribution of SVs. The x-axis represents samples, and the y-axis indicates the proportion of each type of SVs.

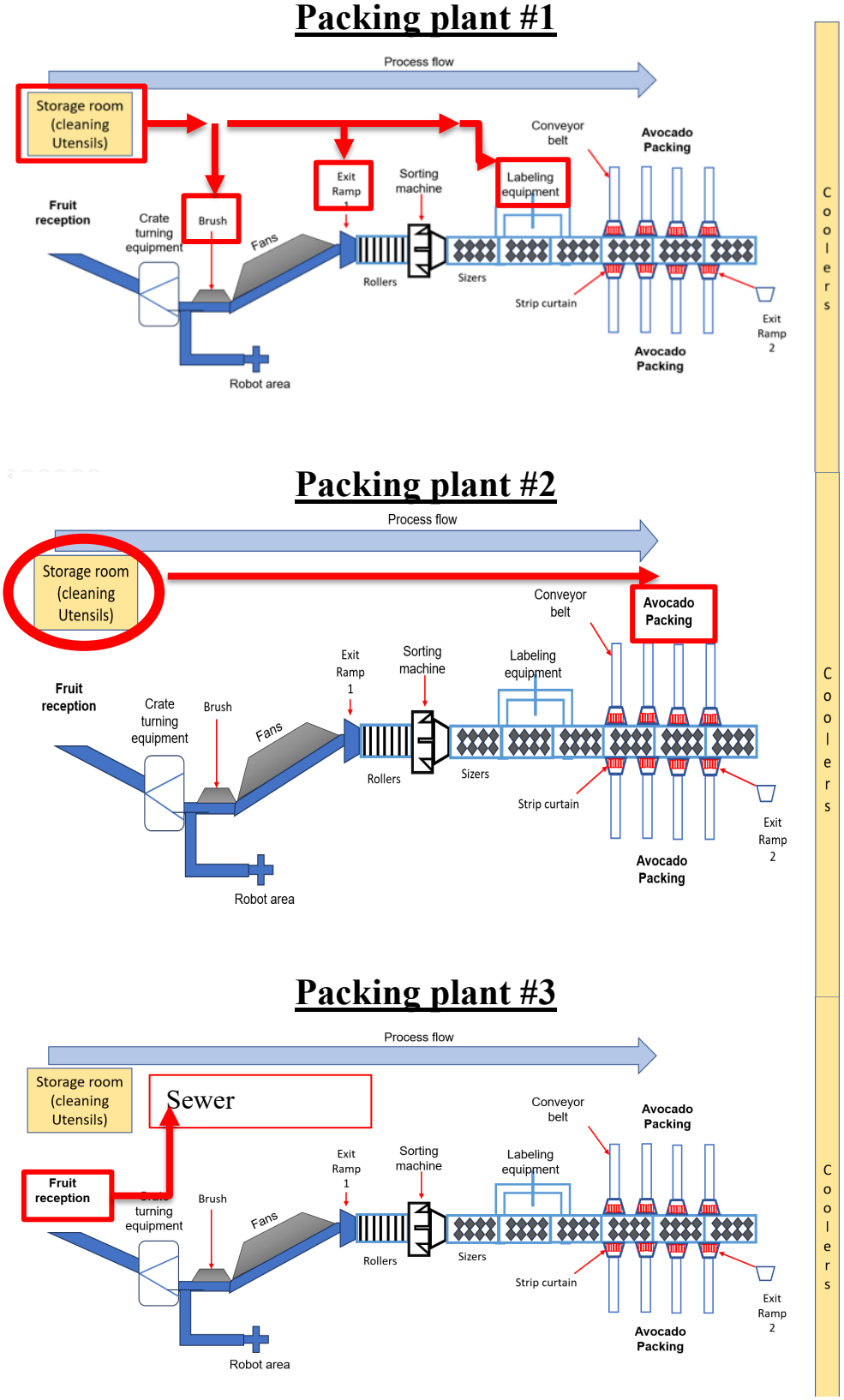


Figure 12. Distribution of* Listeria *isolates with mapping rates indicating DNA similarities

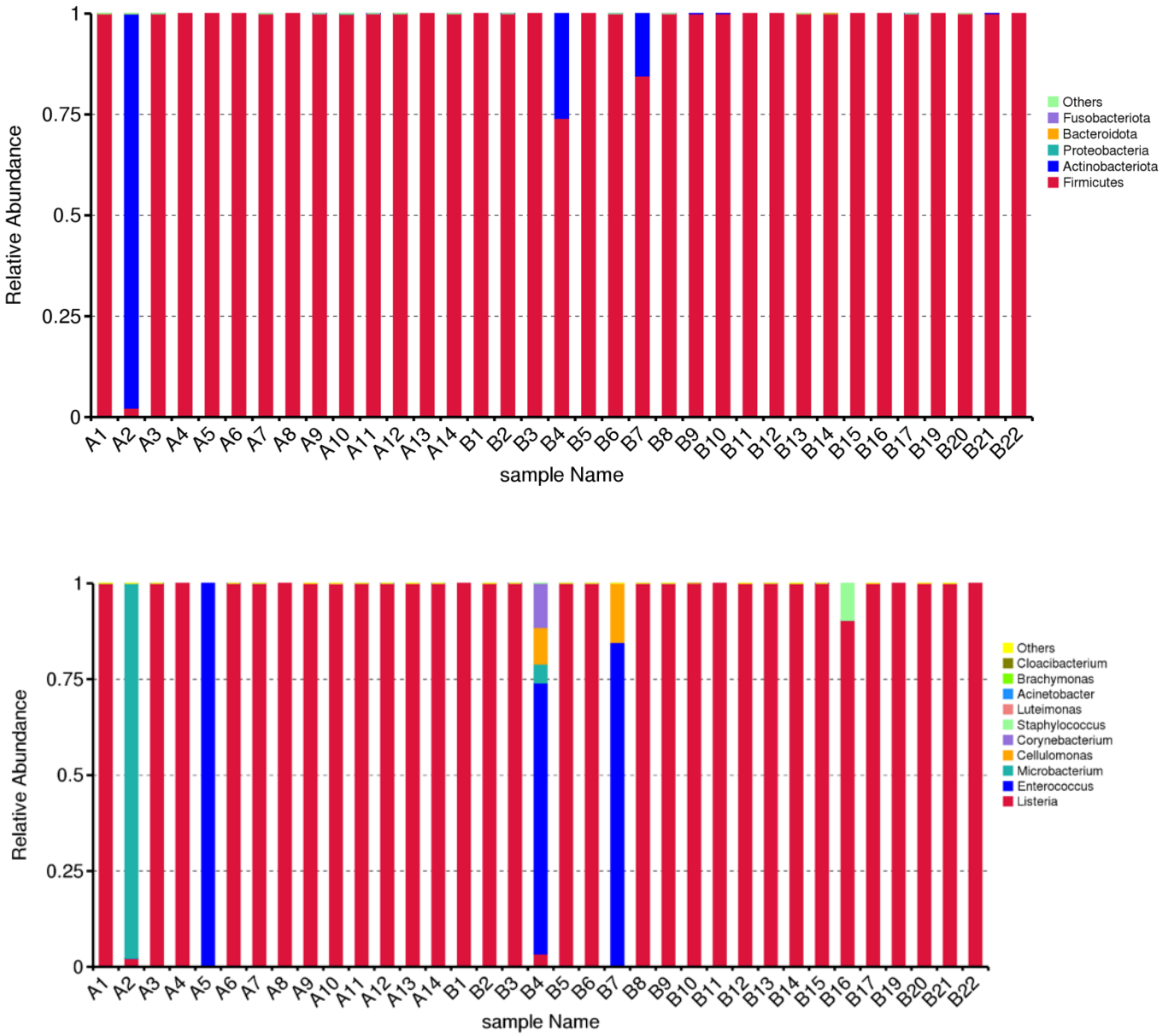


Figure 13. Relative abundance of A. Phylum and B. Genus

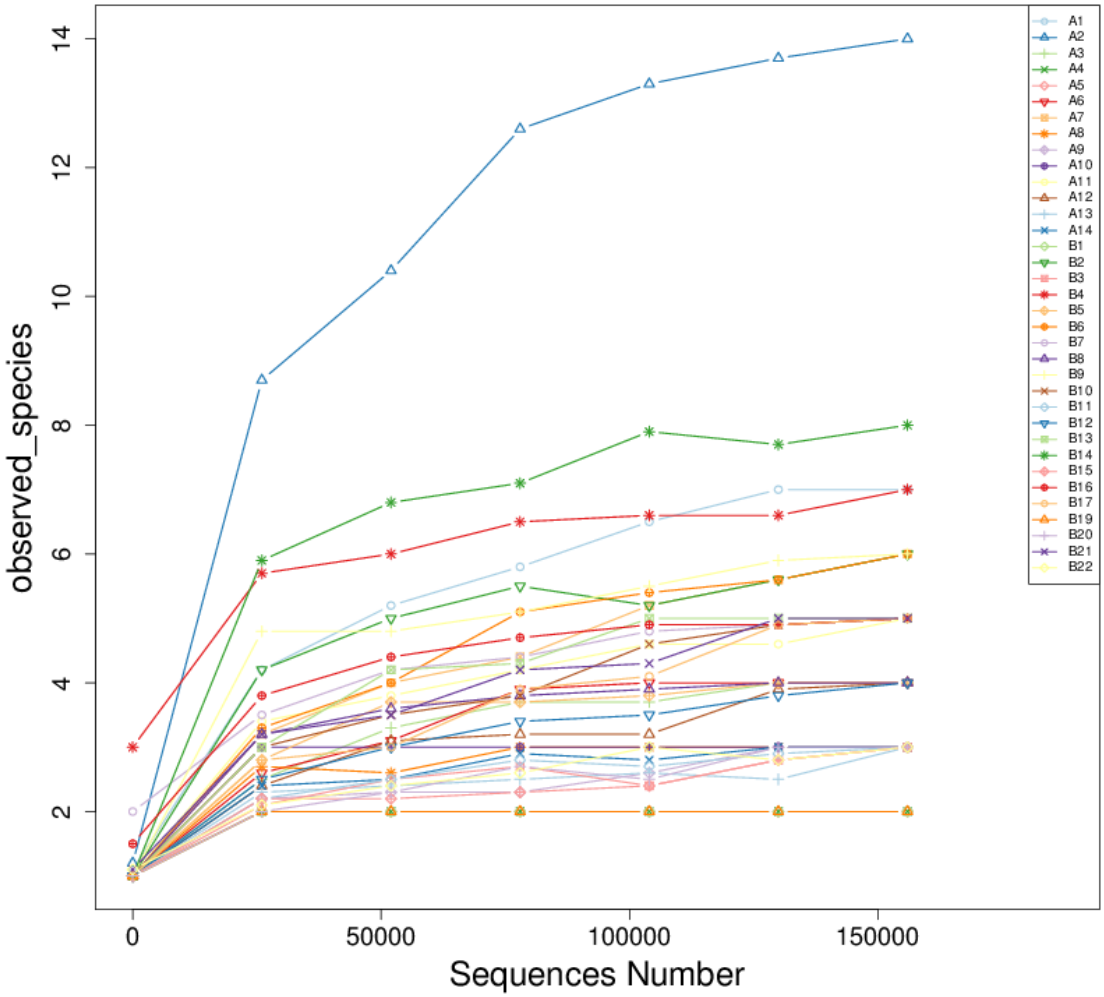


Figure 14. Rarefaction curves indicating the number of observed species against the sequence number

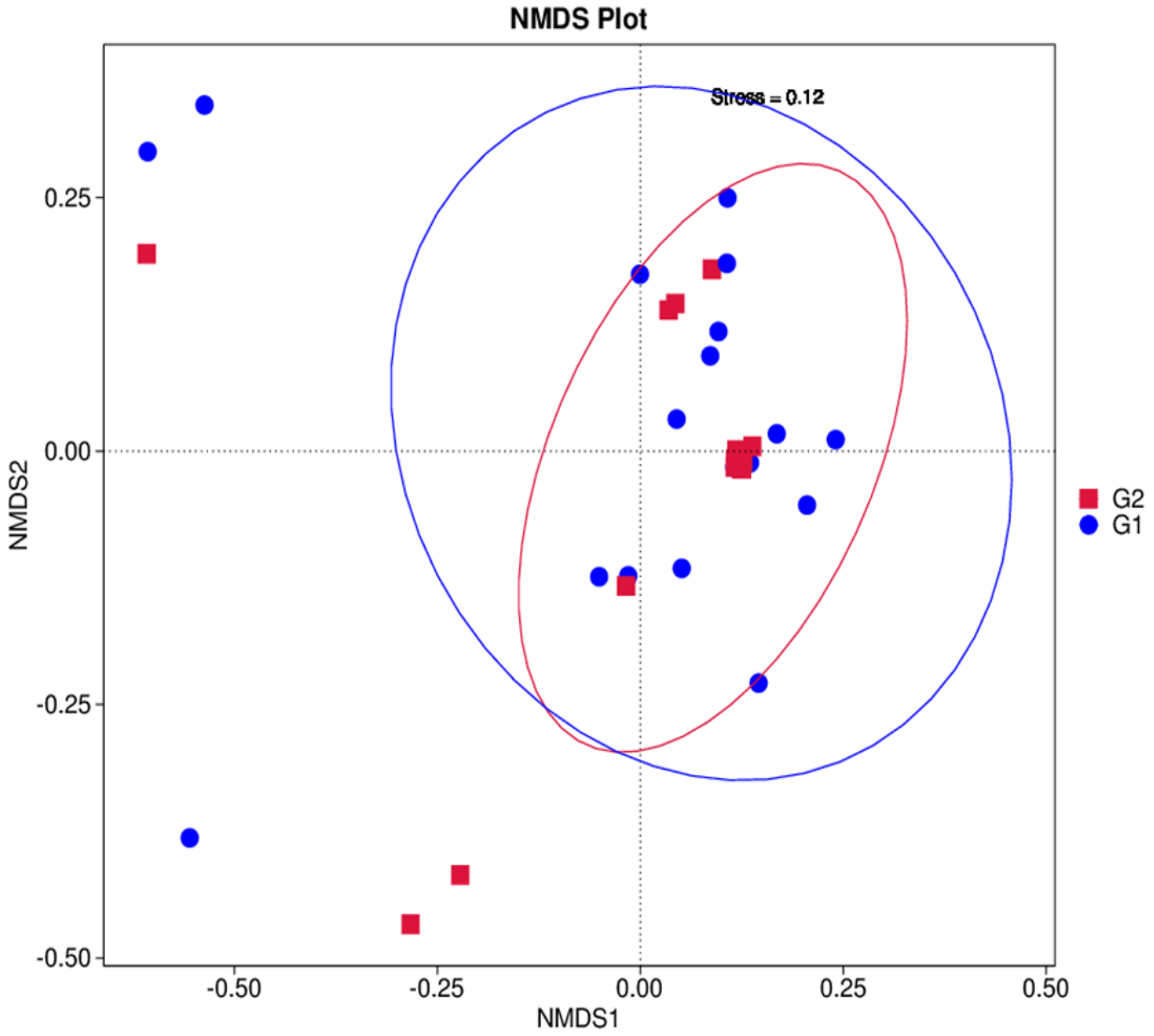


Figure 15. NMDS based on the Unweighted Unifrac distance

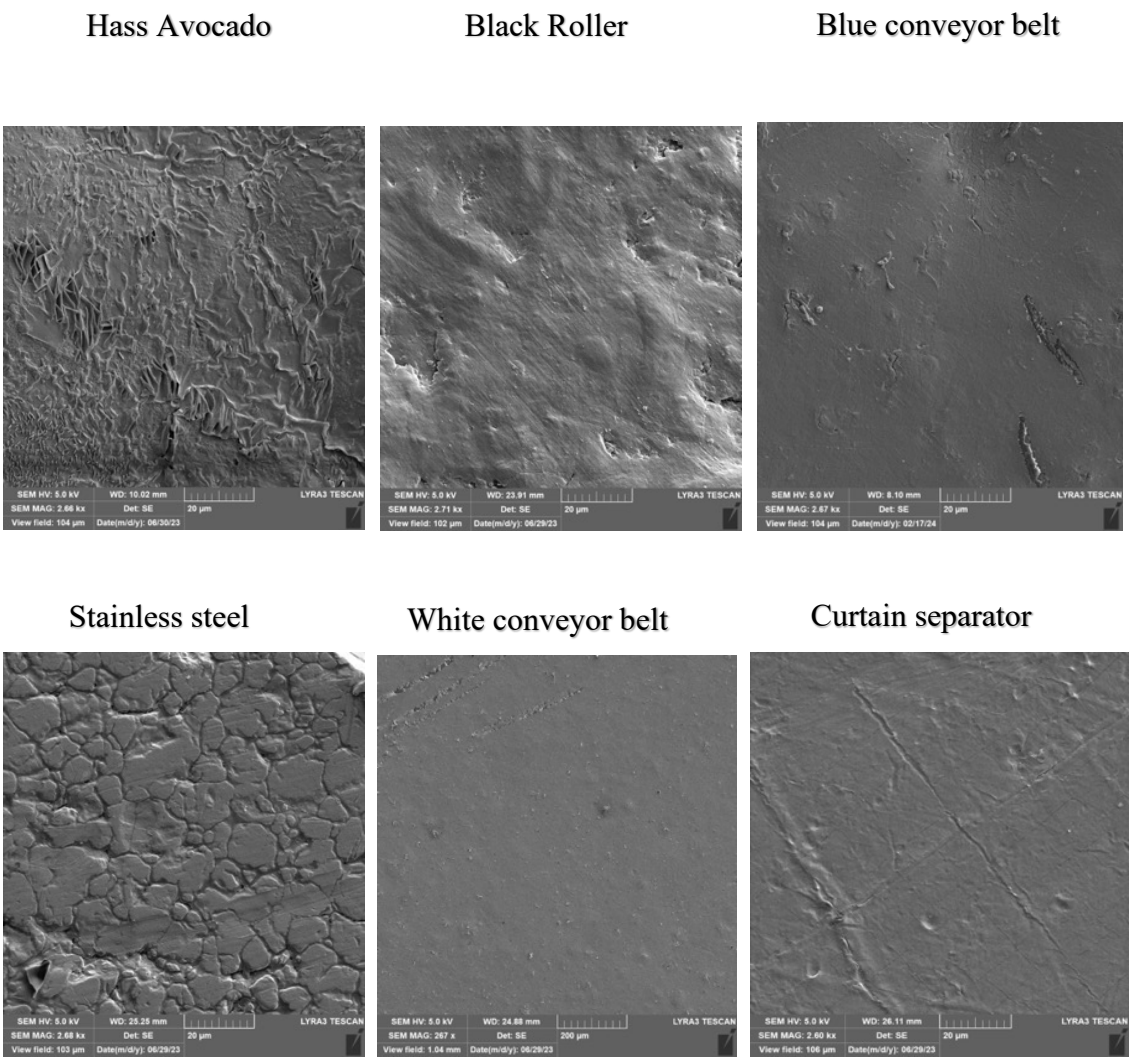


Figure 16. Scanning electron microscopy (SEM) images of surfaces, obtained at the same magnification (magnification, 2.6kx), with a scale bar of 20 µm (embedded in the image).

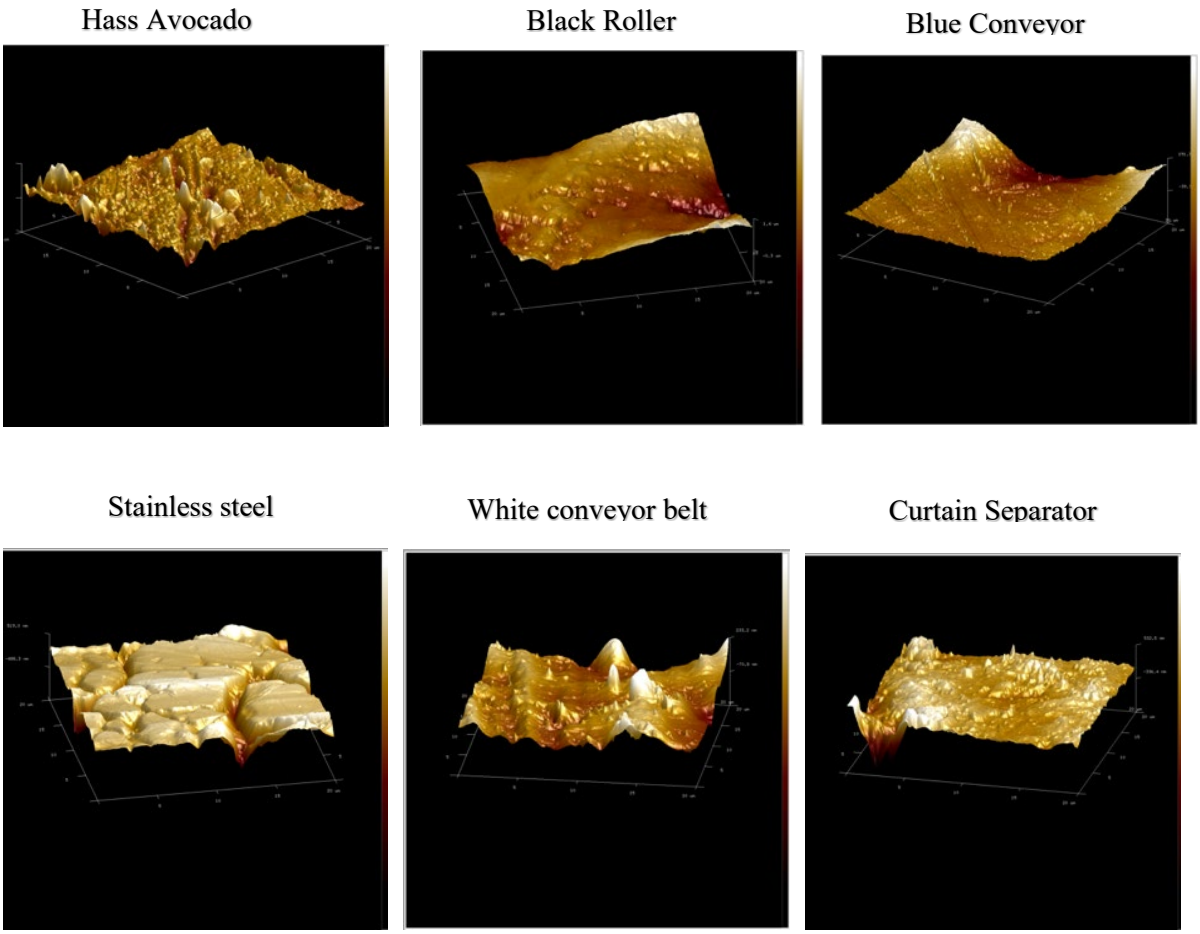


Figure 17. Atomic force microscopic 3D images of surfaces of avocado and of surplus avocado packing materials

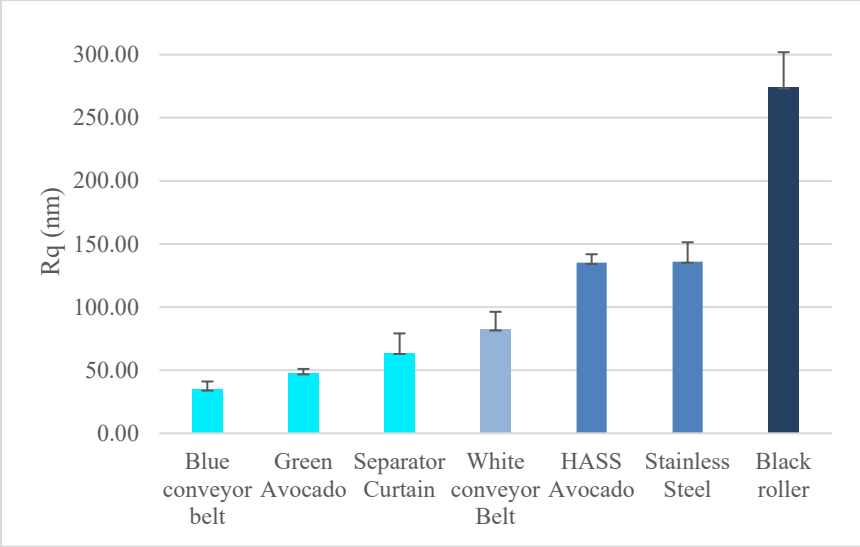


Figure 18. Comparing various surfaces in terms of roughness, different color presents statistical significance; p<0.05

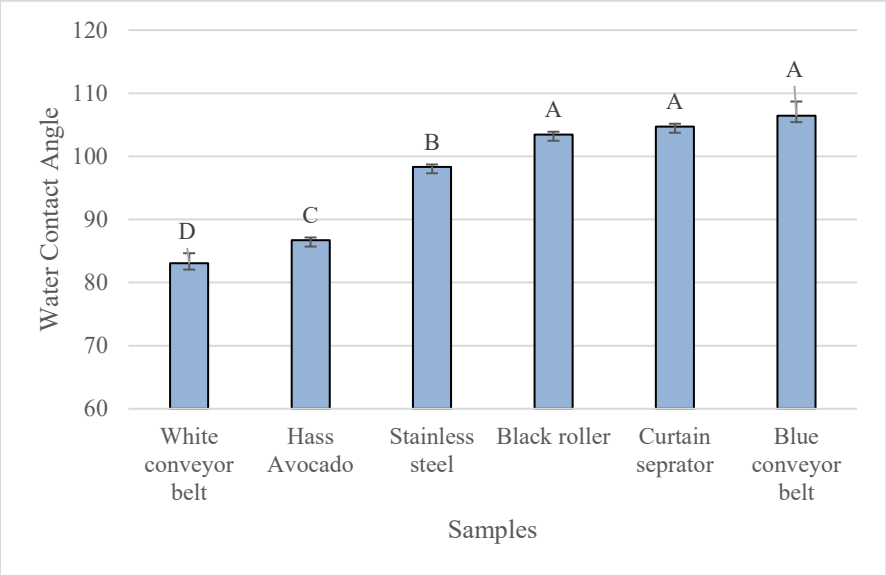


Figure 19. Comparison of the static water contact angle on the surface of various samples (With the alphabetical representation of statistical significance; p<0.05)

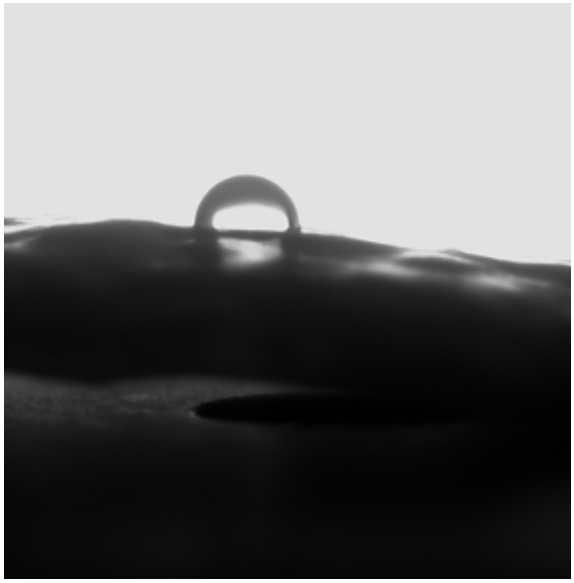


Figure 20. Water Contact Angle of Hass Avocado: average :86.7±1° C

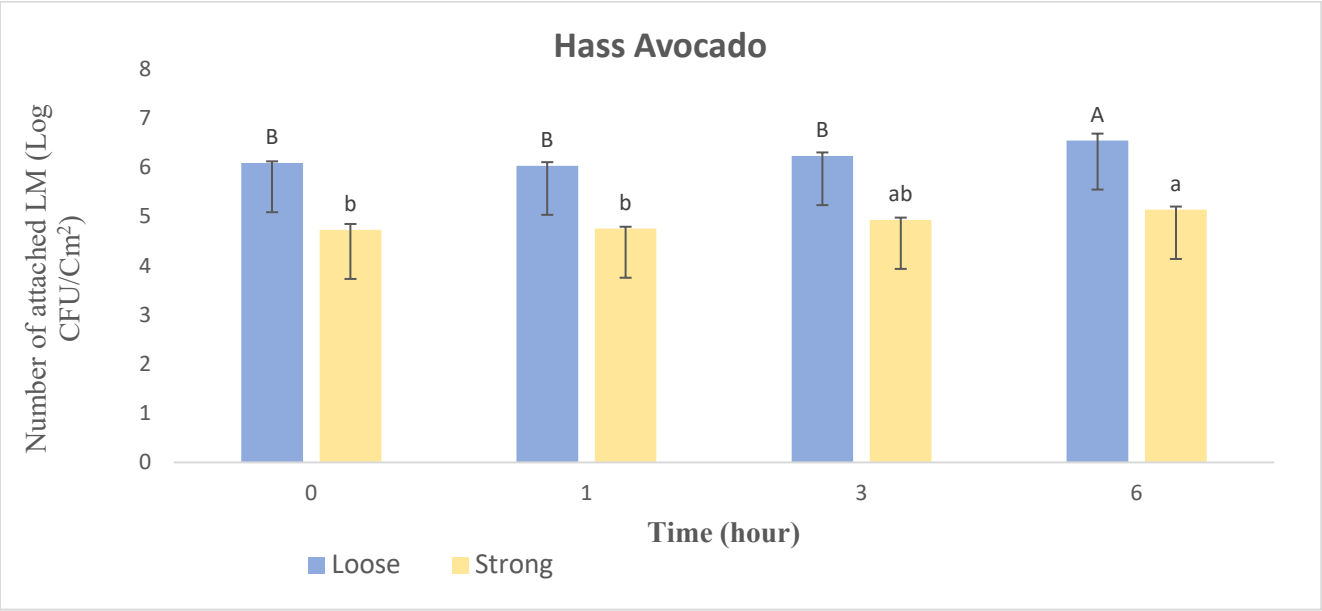


Figure 21. Comparing Loose and Strong attachment of* LM *on Hass avocados, Log-scale enumeration of the surface attached bacteria calculated via agar plating

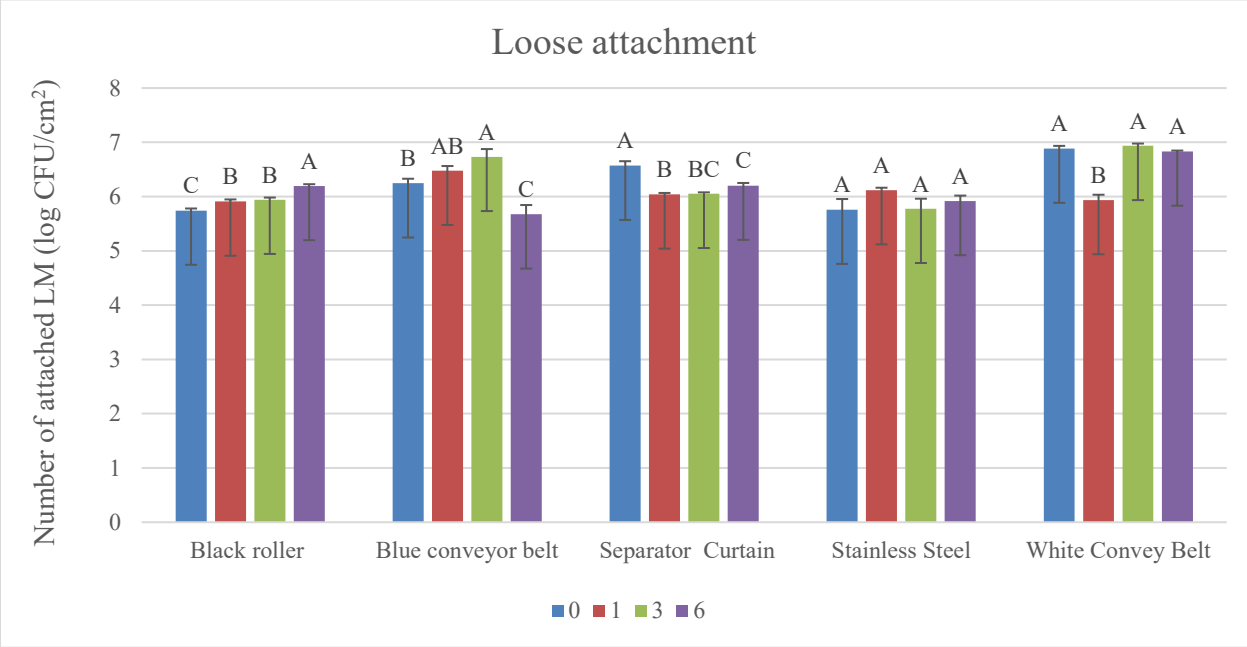


Figure 22. Comparing Loose attachment of* LM *on packing plant materials. The statistical analysis has been performed on the results of Loose attachment for each material separately, where letters denote the level of significance.

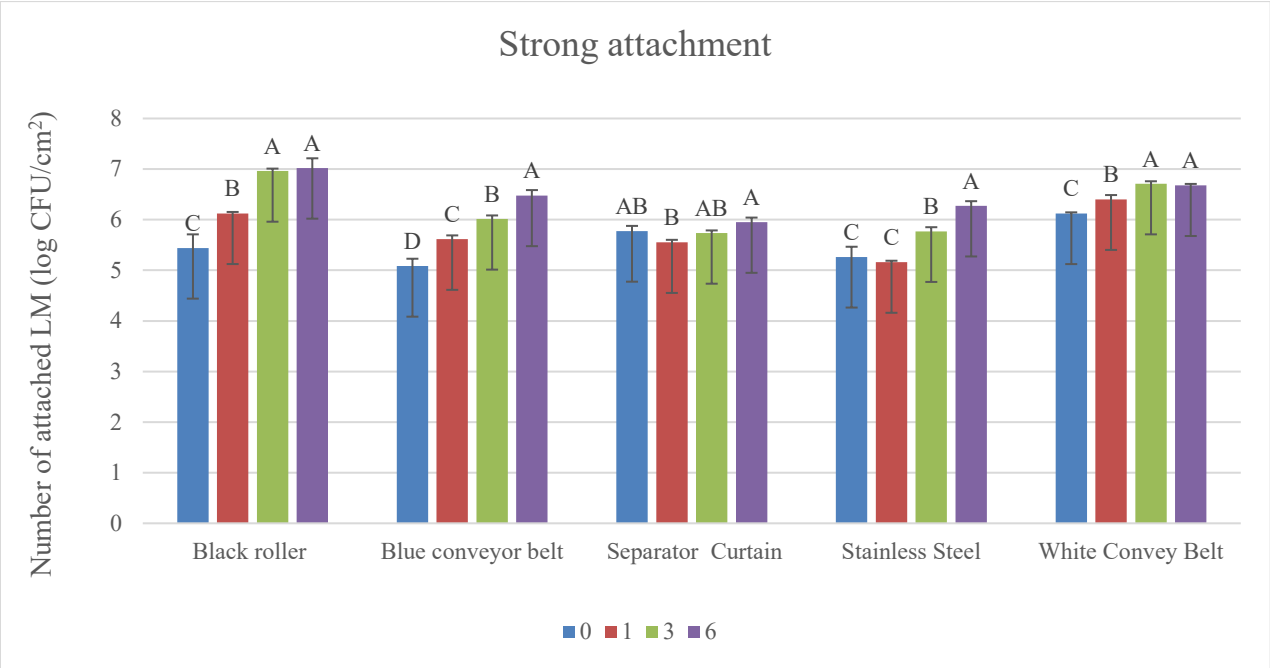


Figure 23. Comparing Strong attachment of* LM *on packing plant materials. The statistical analysis has been performed on the results of Strong attachment for each material separately, where letters denote the level of significance.

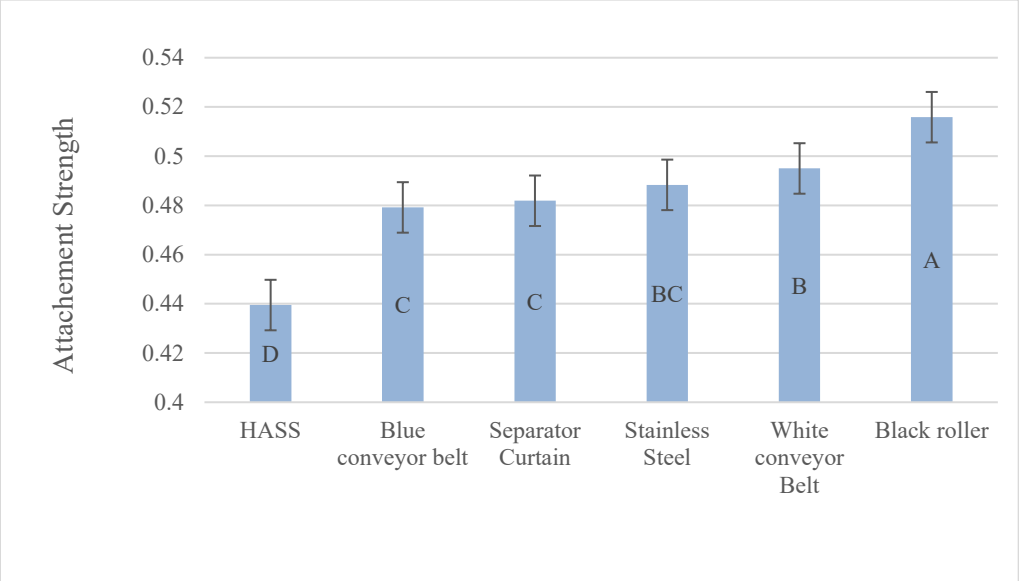


Figure 24. Strength of attachment (SR) of* LM *on all samples during all inoculation times at 25 (ºC)

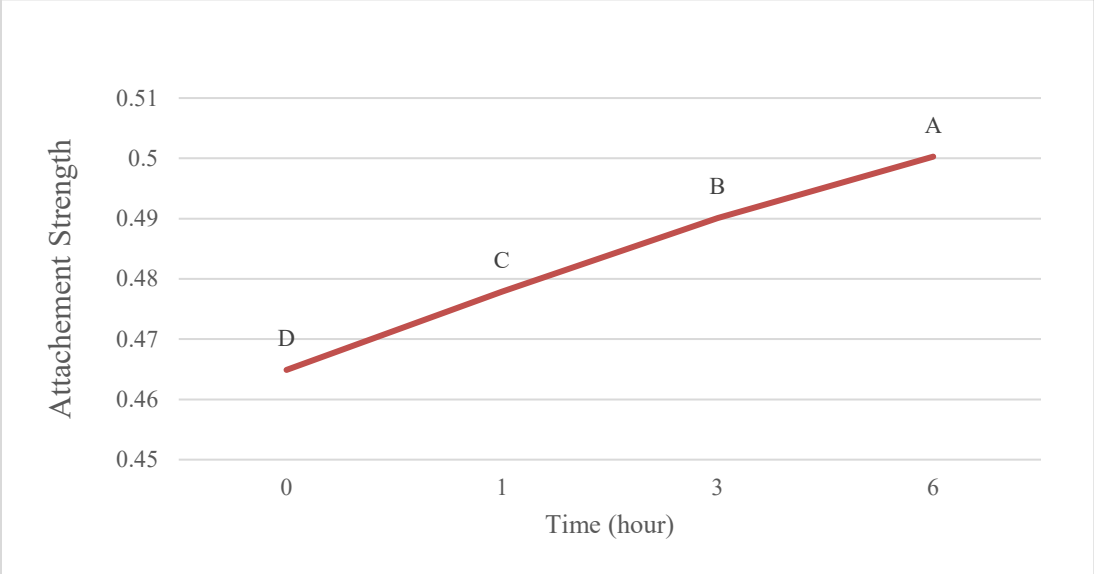


Figure 25. Strength of attachment of* LM *over the inoculation time

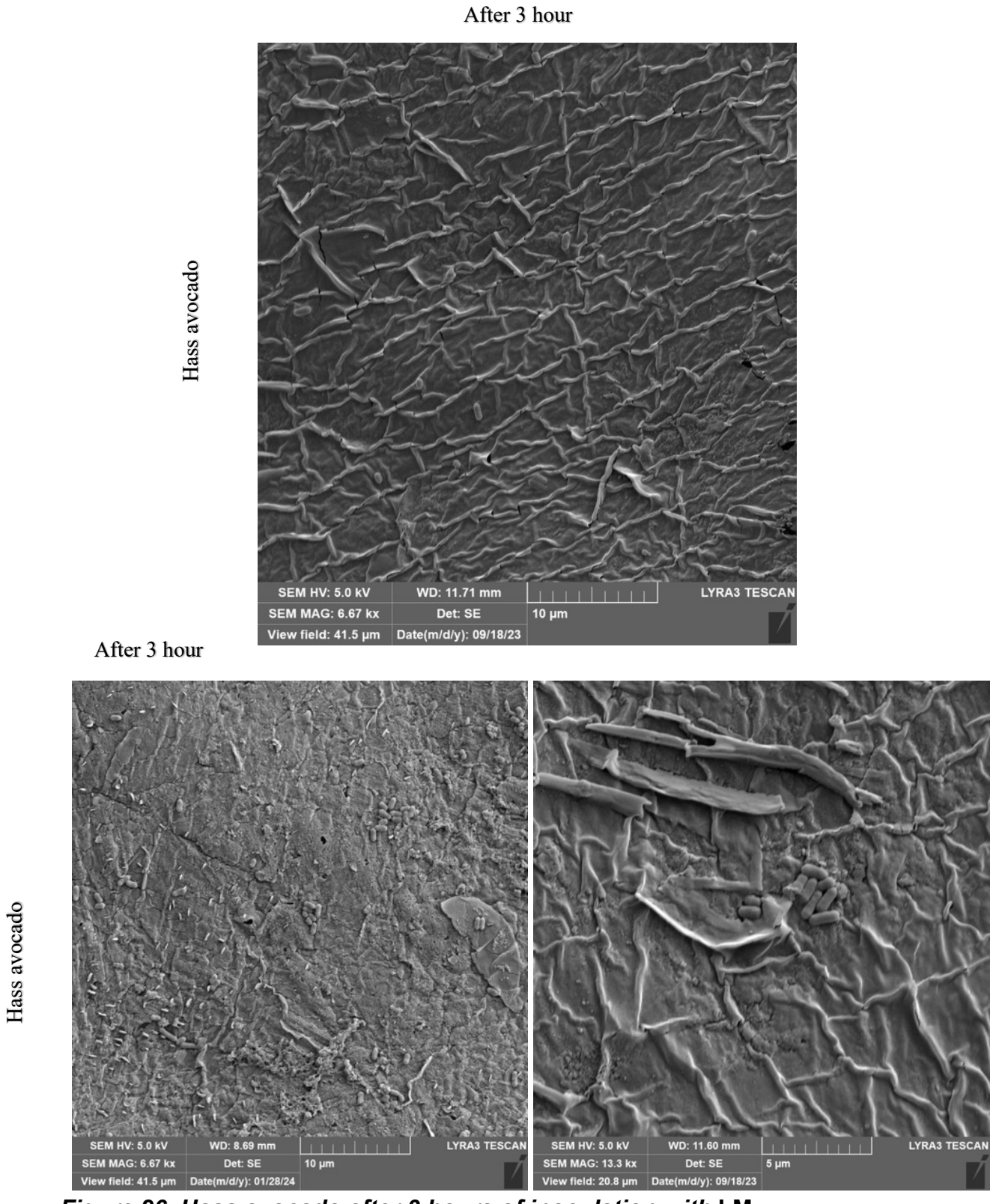


***Figure 26. Hass avocado after 3 hours of inoculation with* LM.**

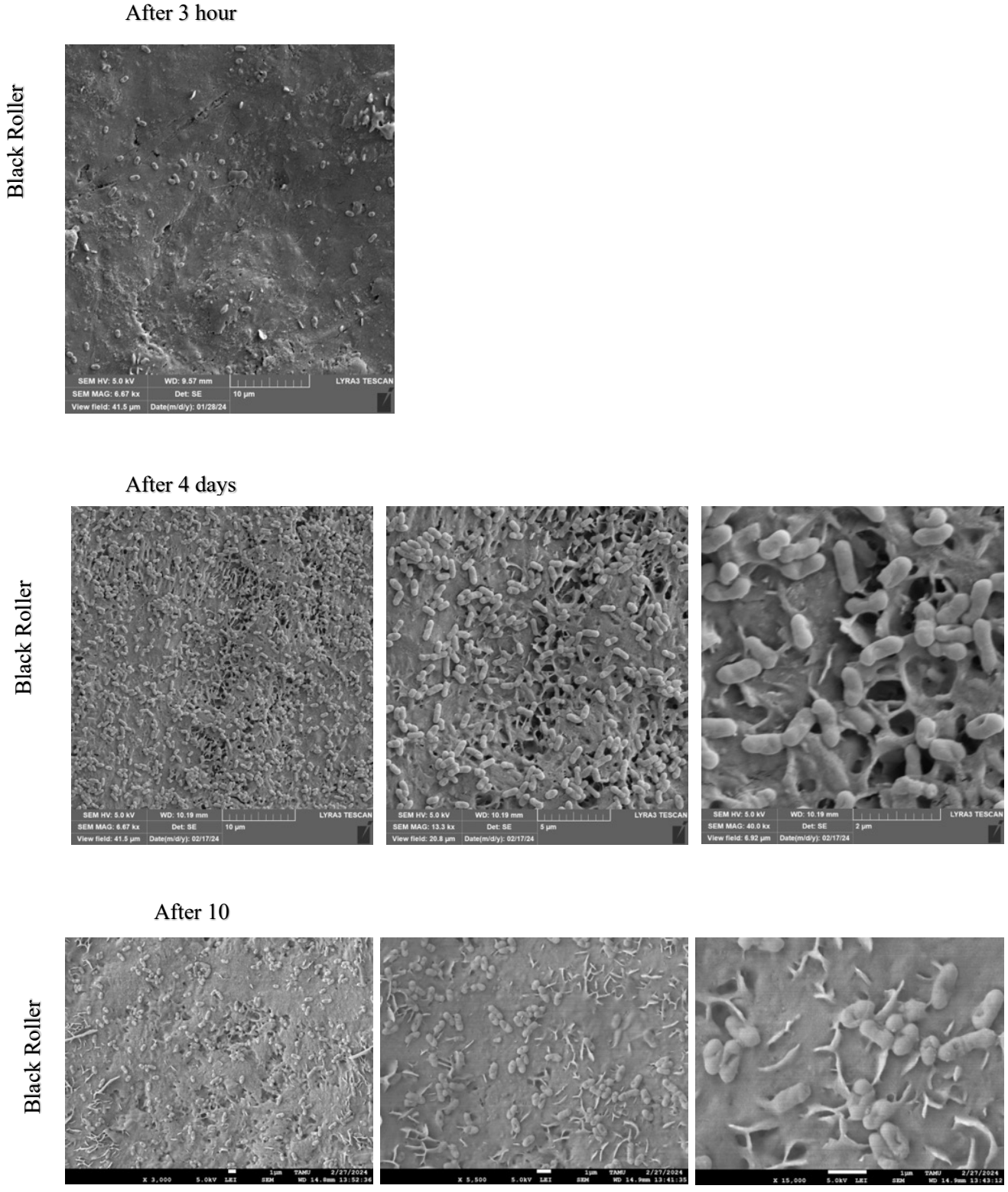
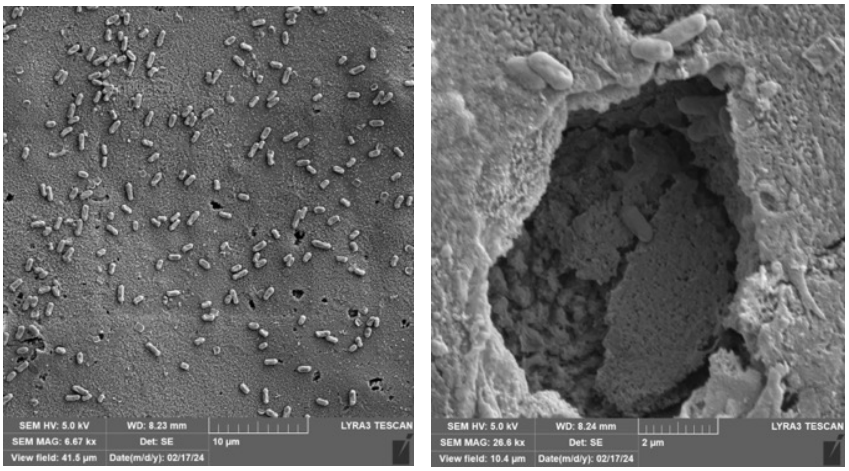


Figure 27. Biofilm formation of* LM *on Black roller after 3 hours, 4 days, and 10 days of inoculation with different scale bars, respectively.

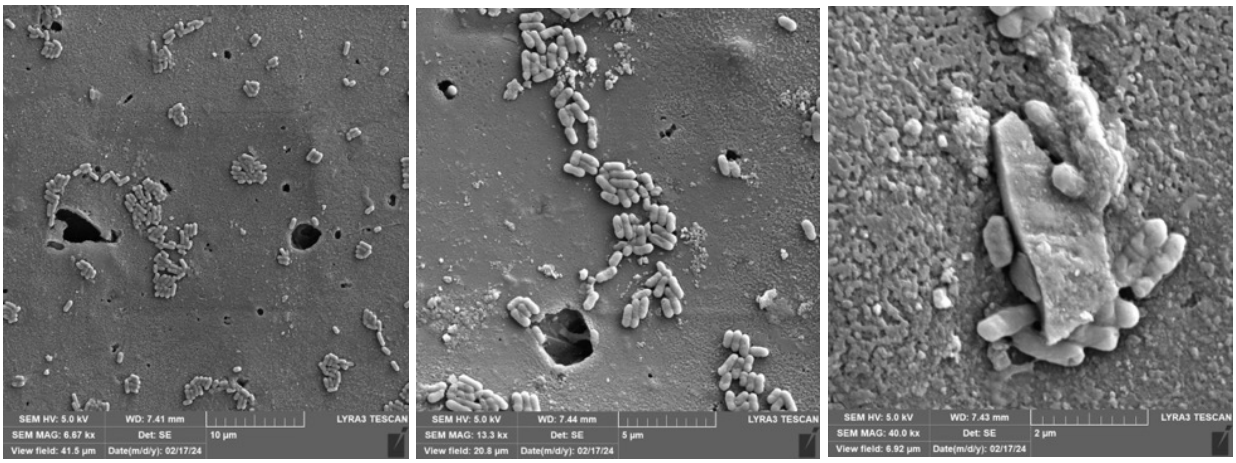
After 3 hours

Blue conveyor belt



After 4 days

Blue conveyor belt



After 10

Blue conveyor belt

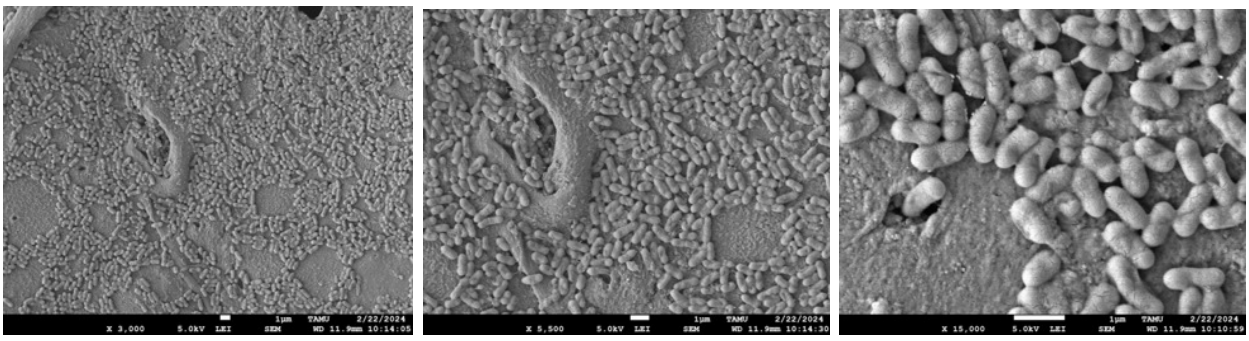


Figure 28. Biofilm formation of* LM *on Blue conveyor belt after 3 hours, 4 days, and 10 days of inoculation with different scale bars, respectively

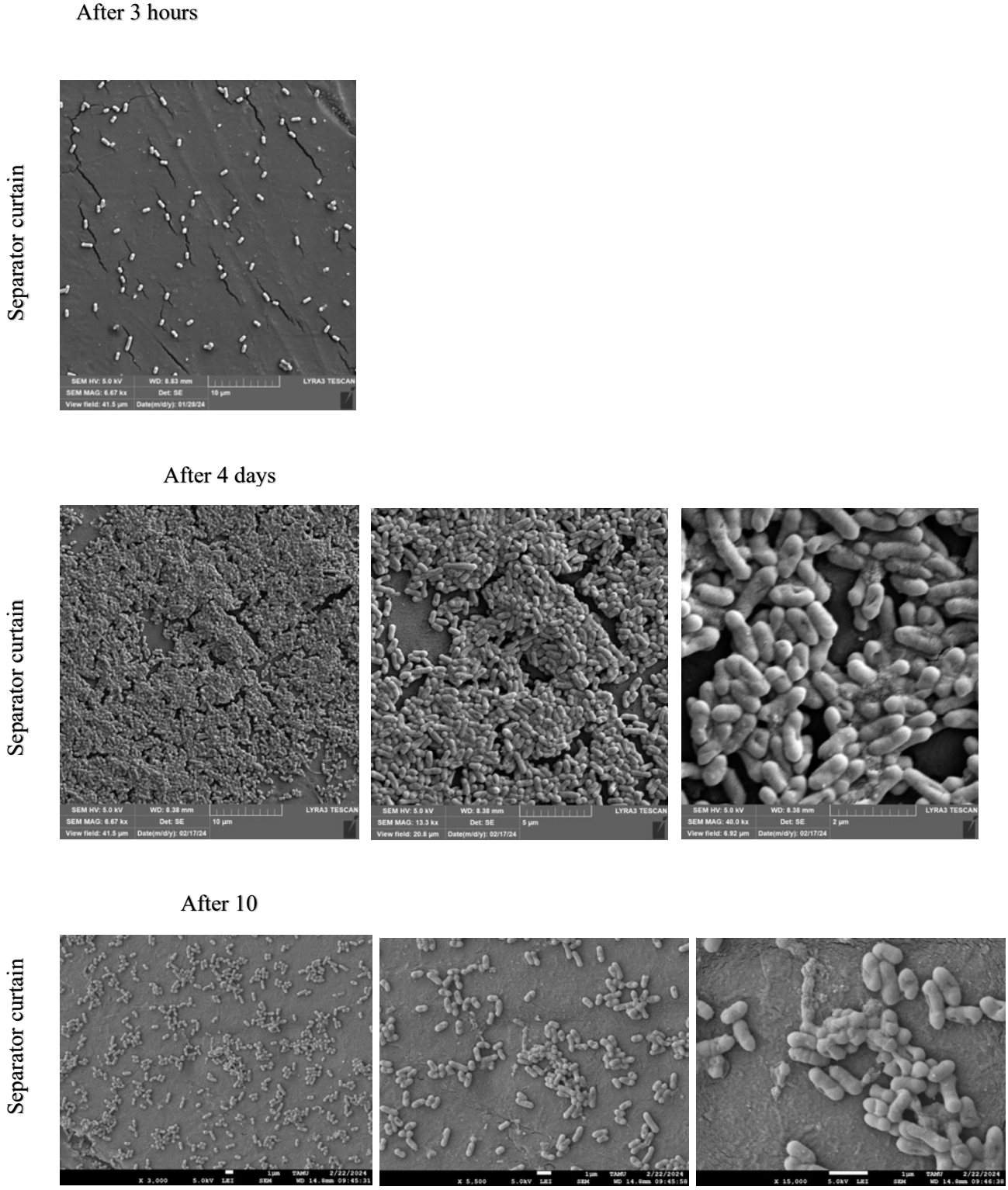


Figure 29. Biofilm formation of* LM *on Separator Curtain after 3 hours, 4 days, and 10 days of inoculation with different scale bars, respectively

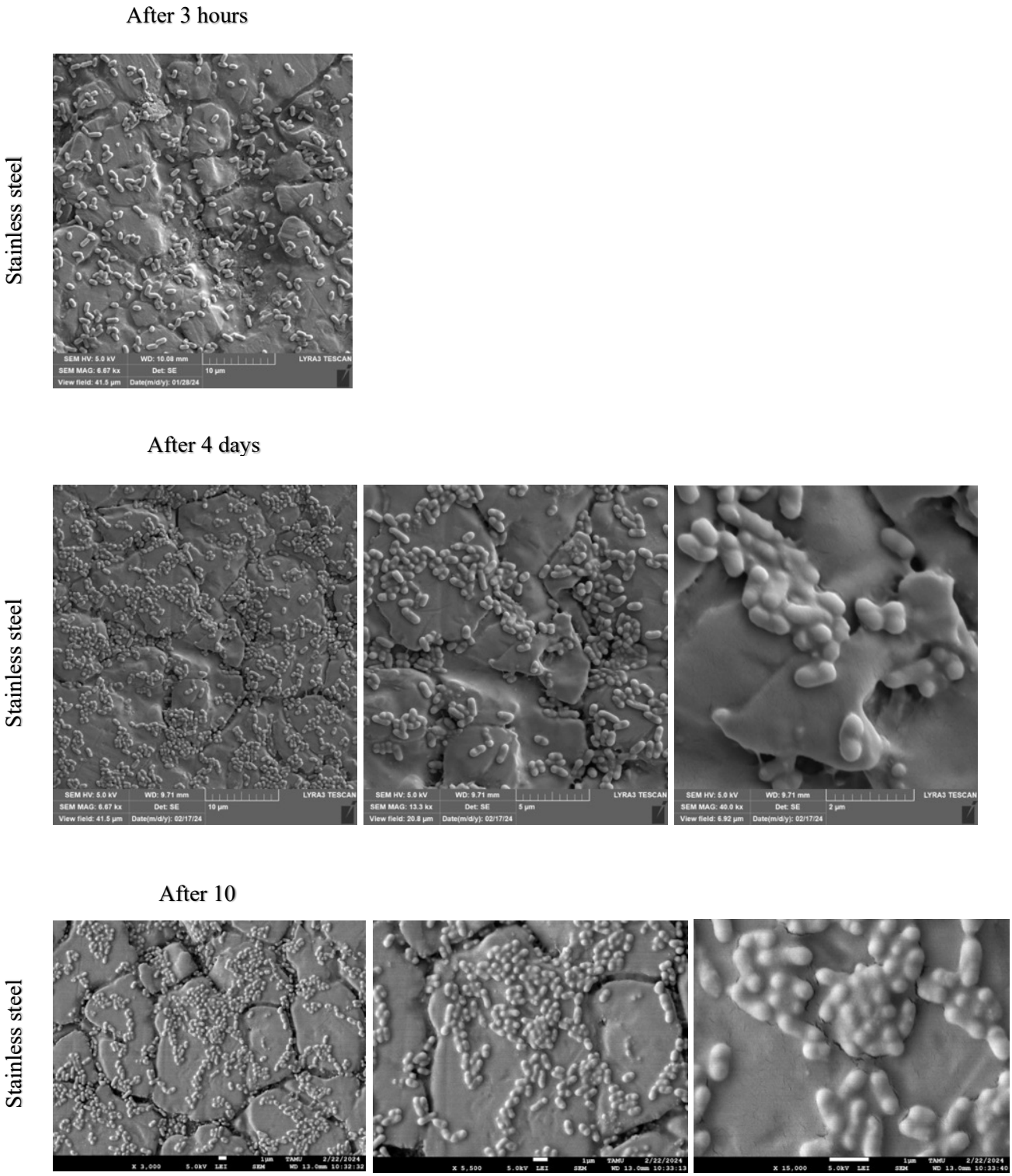


Figure 30. Biofilm formation of* LM *on Stainless steel after 3 hours, 4 days, and 10 days of inoculation with different scale bars, respectively

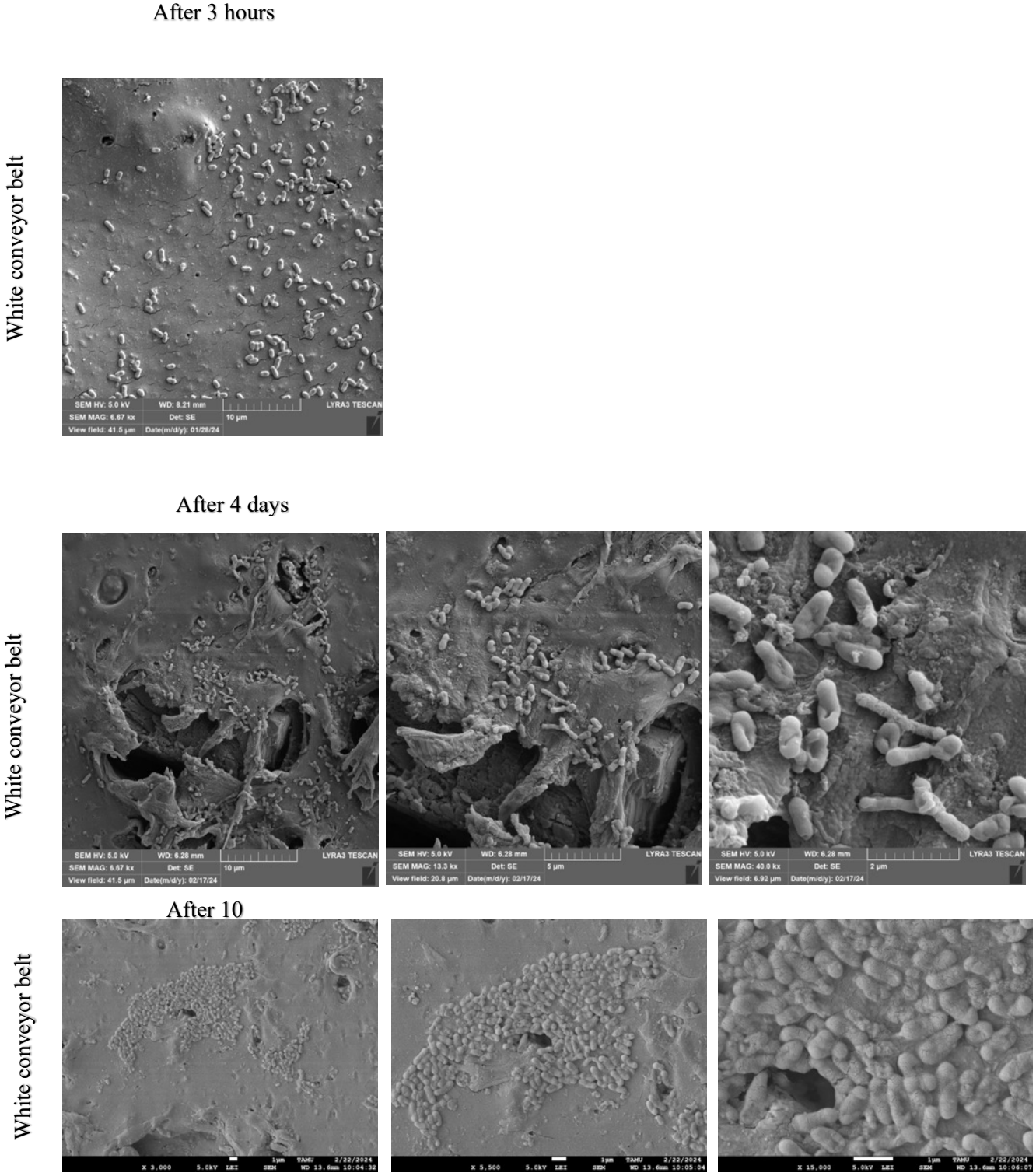


Figure 31: Biofilm formation of* LM *on White conveyor belt after 3 hours, 4 days, and 10 days of inoculation with different scale bars, respectively

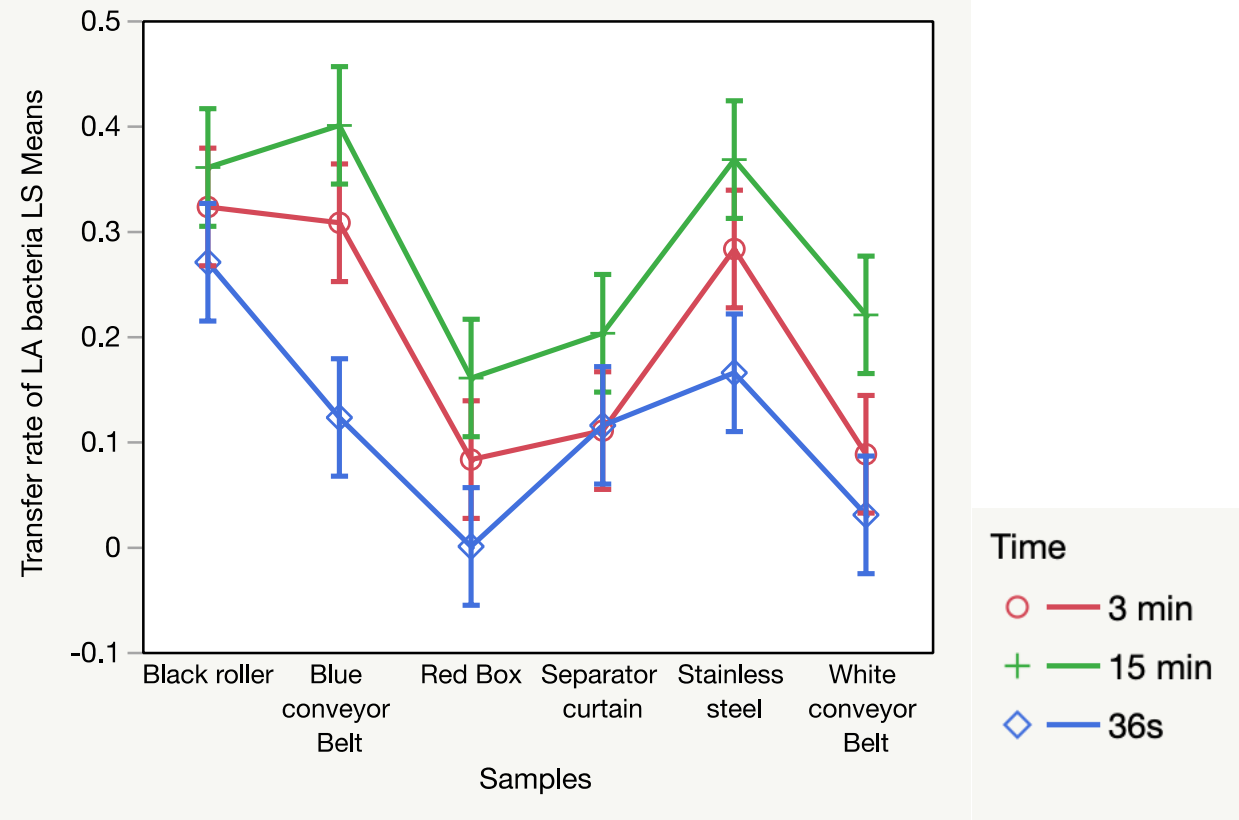


Figure 32. Transfer Rate of Loosely Attached* LM *from Various Contaminated Materials to Avocado

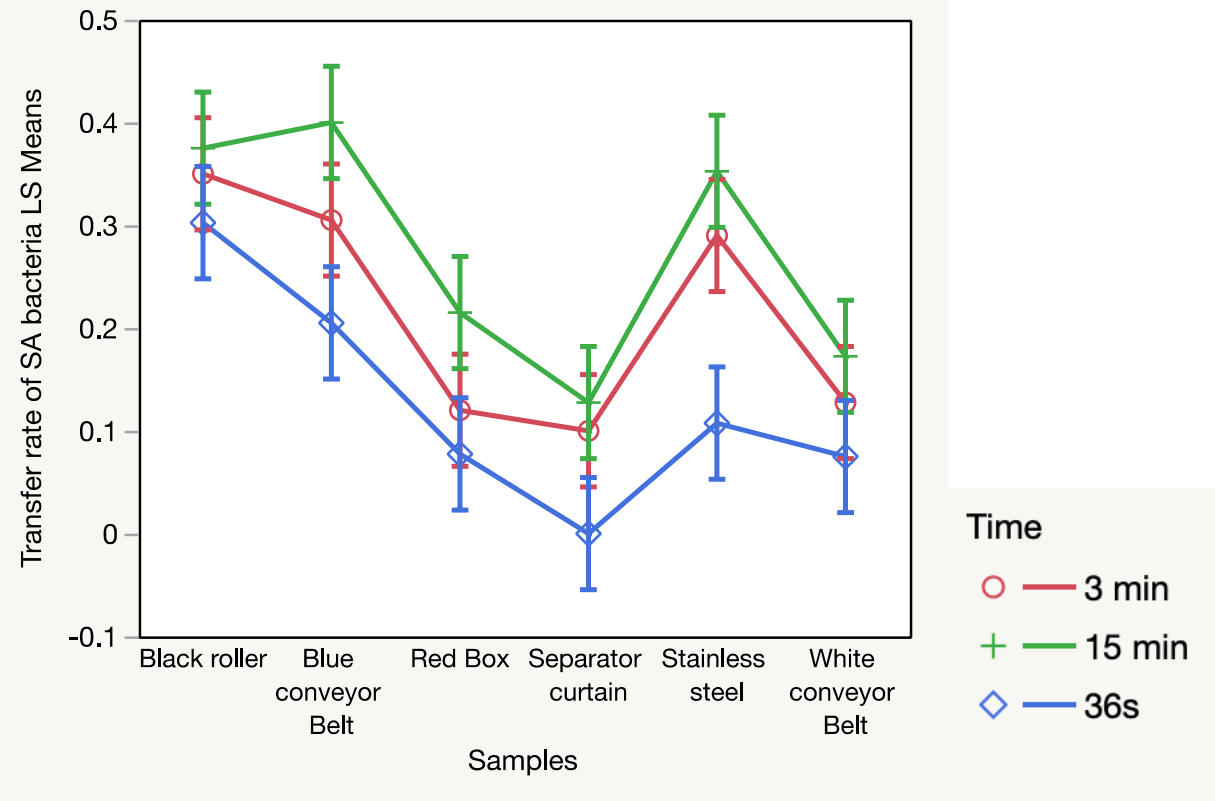


Figure 33. Transfer Rate of strongly Attached* LM *from Various Contaminated Materials to Avocado

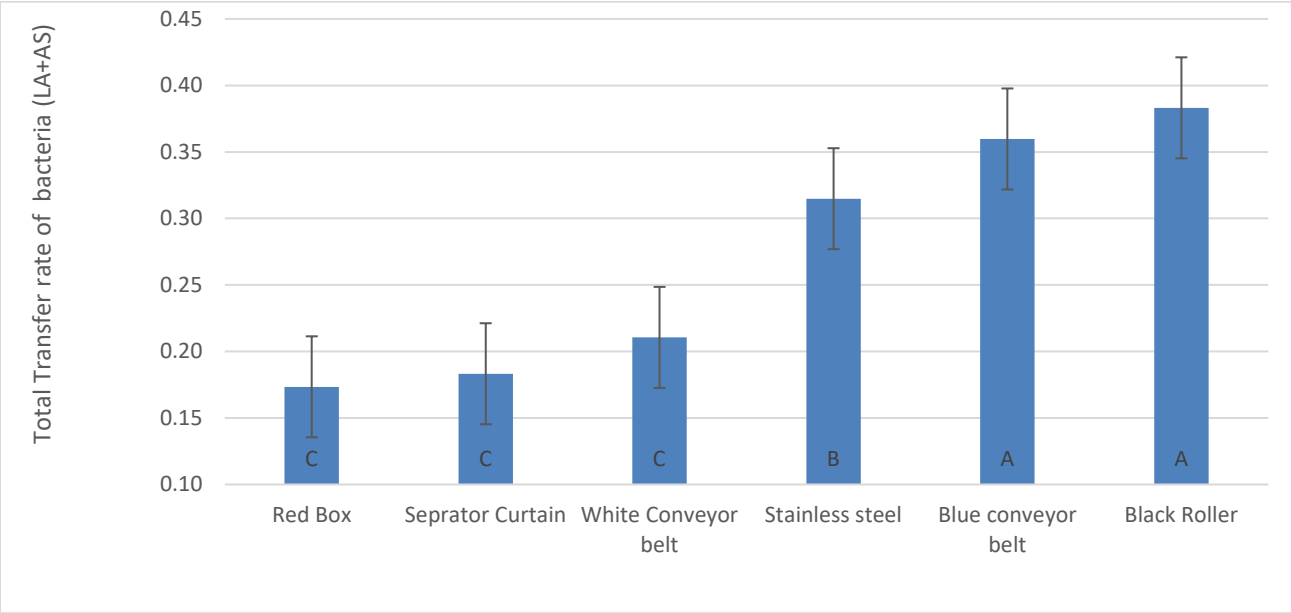


Figure 34. Total transfer Rate of Attached* LM *(LA+SA) from Various Contaminated Materials to Avocado.

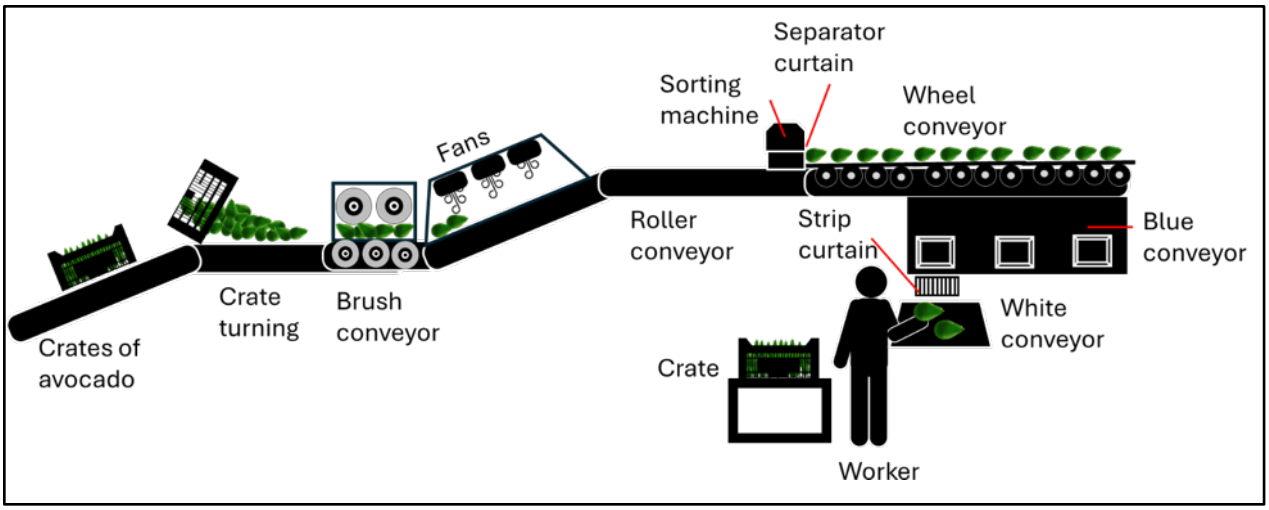


Figure 35. Modeled avocado processing facility schematic.

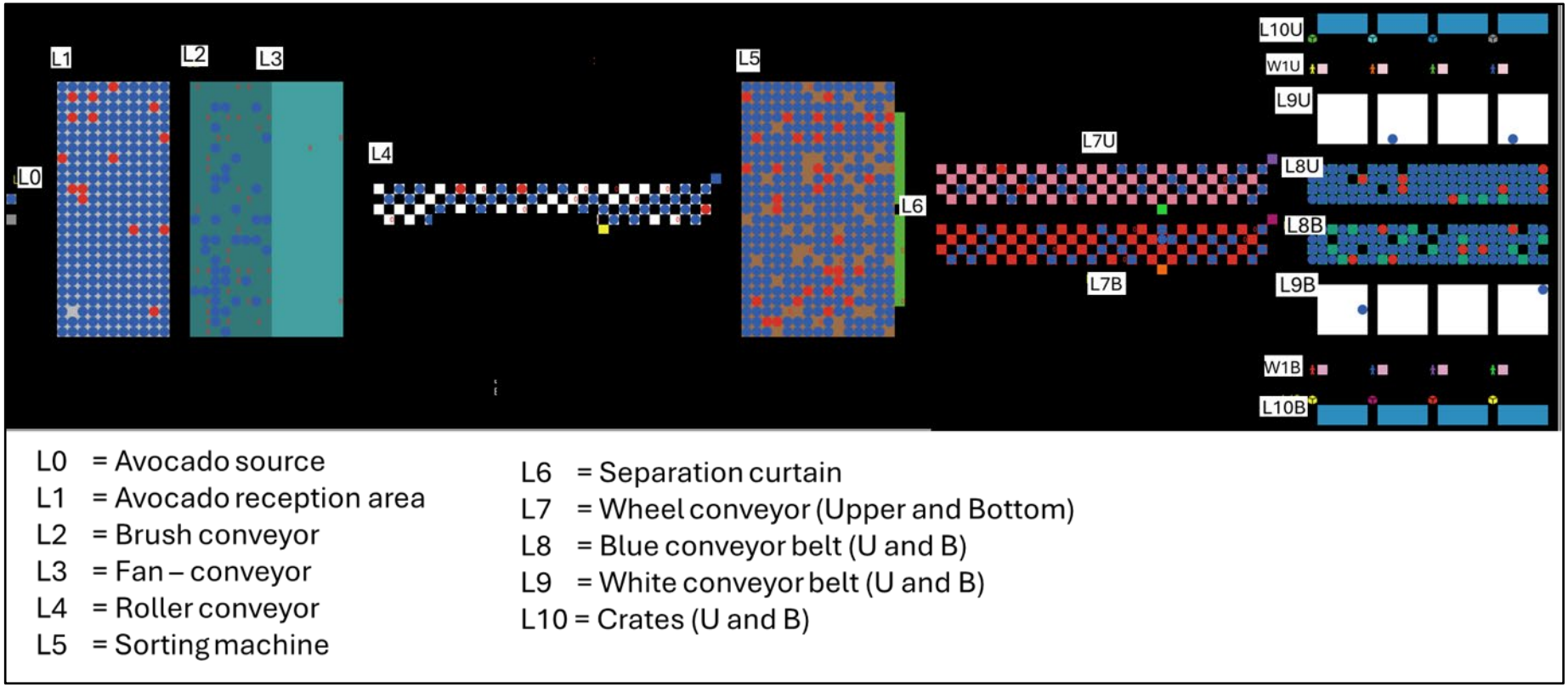


Figure 36. The 2-D grid for the different fixed agent locations in the whole avocado processing facility FS-AM simulator (the red dots are contaminated avocados). With this model we can track Listeria and avocado movements in time.

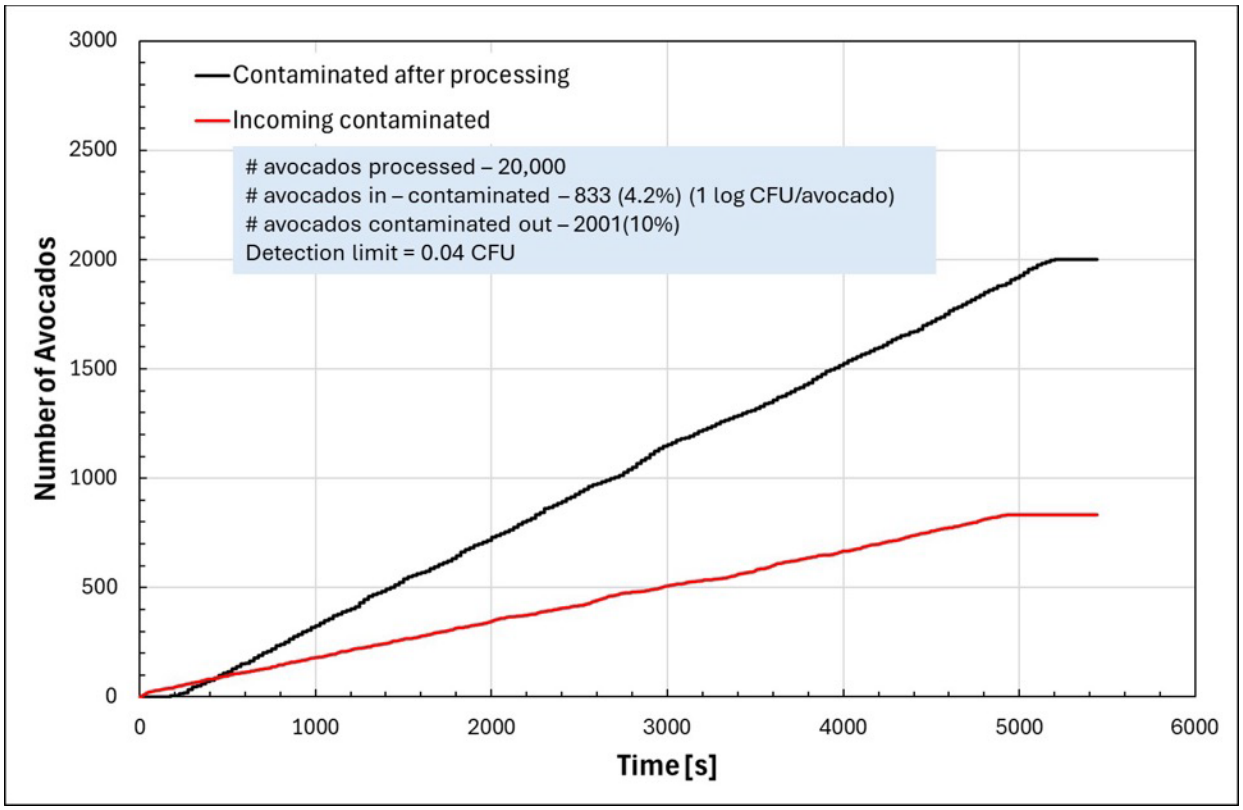


Figure 37. Simulation of a packinghouse processing 20,000 avocados for 1.5 hours (Scenario 1).

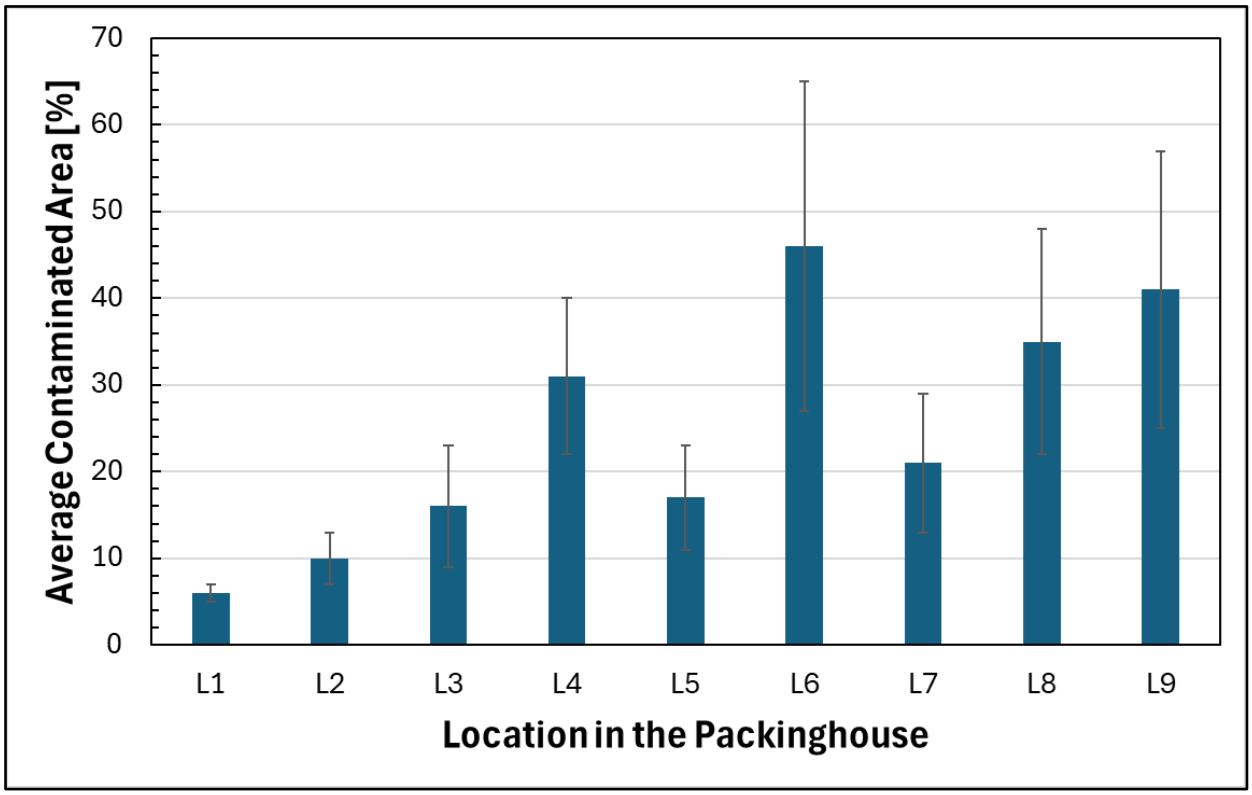


Figure 38. Average and standard deviation cross-contamination within each component of the packinghouse (Scenario 2).
L1 = fruit reception; L2 = brush conveyor; L3 = Fan/conveyor; L4 =roller conveyor; L5 = sorting machine; L6 = separator curtain; L7 = wheel conveyor; L8 = blue conveyor; L9 = white conveyor.

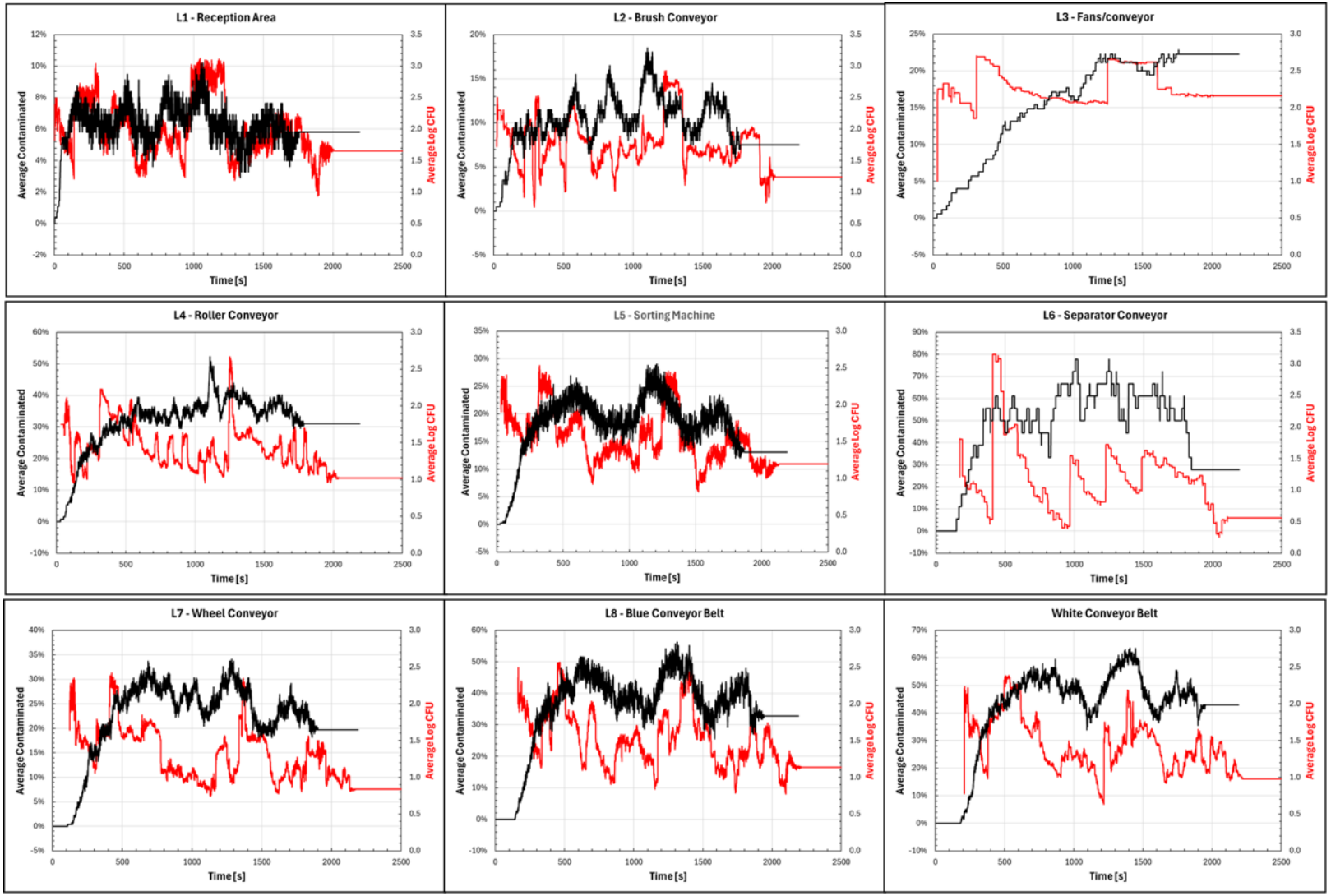


Figure 39. Simulation of packing house processing 7,000 avocados for 36.5 minutes (Scenario 2).

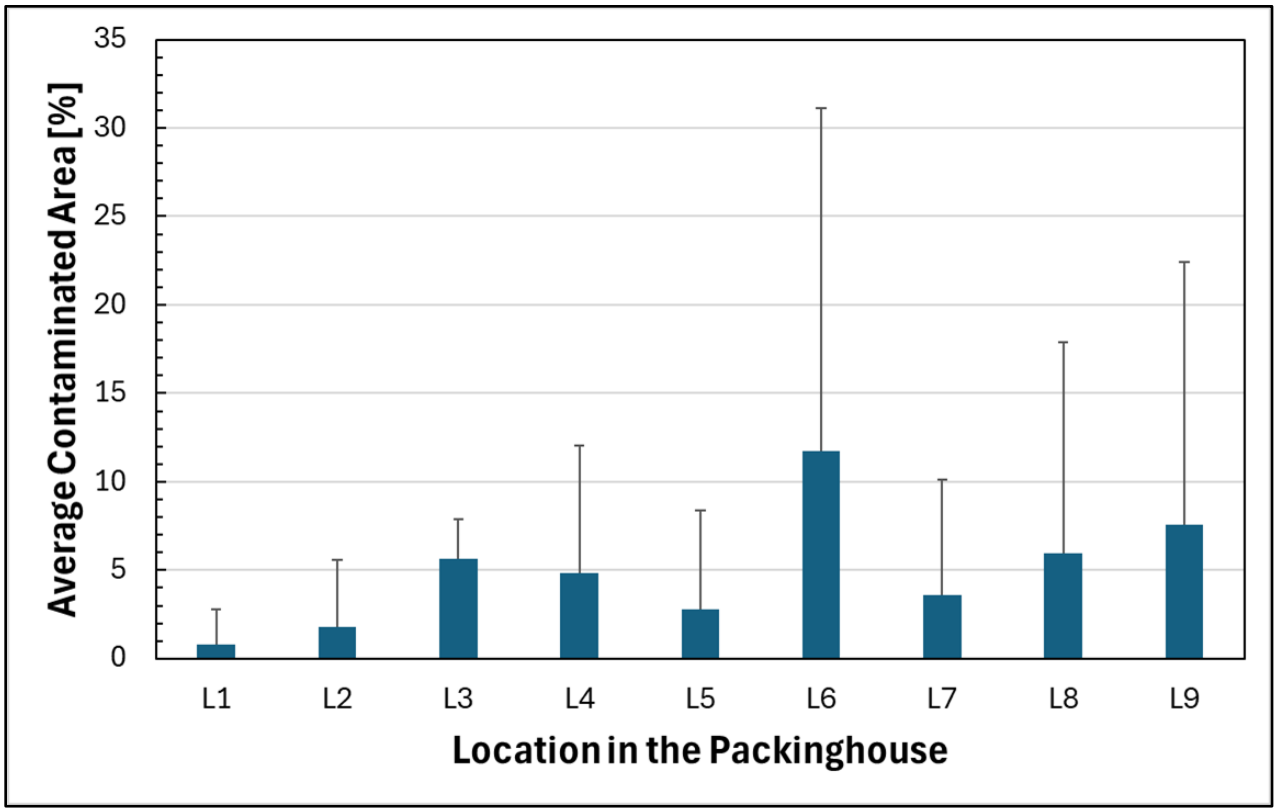


Figure 40. Average and standard deviation cross-contamination within each component of the packinghouse (Scenario 3).
L1 = fruit reception; L2 = brush conveyor; L3 = Fan/conveyor; L4 =roller conveyor; L5 = sorting machine; L6 = separator curtain; L7 = wheel conveyor; L8 = blue conveyor; L9 = white conveyor.

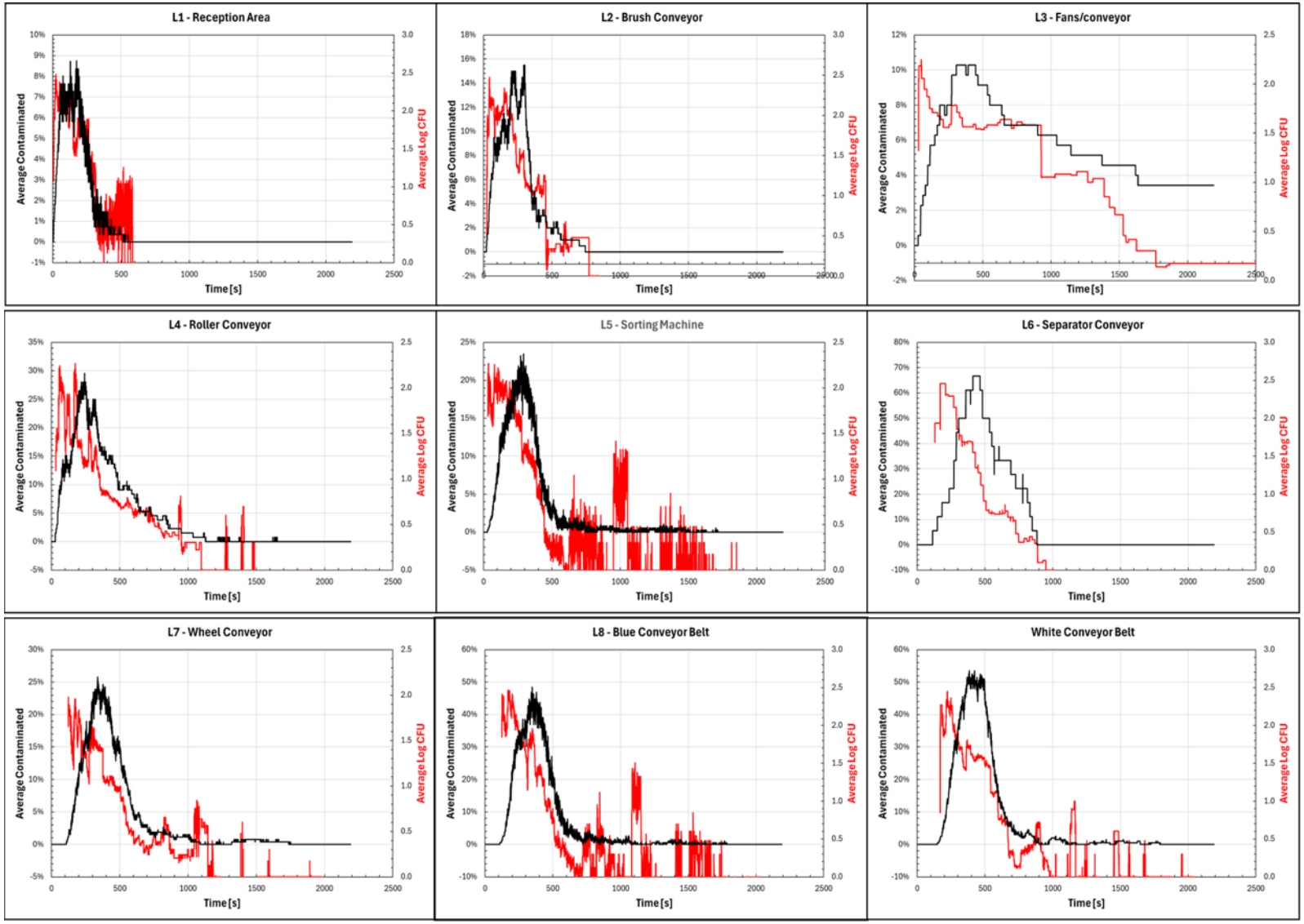


Figure 41. Simulation of packing house processing 7,000 avocados for 36.5 minutes (Scenario 3).

Table 1. Description of sampling sites and locations, and sample preparation for analysis (All samples were not food contact surfaces)

Site name	Example of Surface type and location	Surface area sampled	Type and volume of diluent/pre-enrichment broth added	Limit of Detection	
					UNITs
Air		1000 L (1 m^3)	BPW (100 ml)	100	CFU/m^3
Drain		Swab the entire drain cover	DEB (10 mL)	10	CFU /sample
Floor	Concrete Stainless steel	10,000 cm^2	DEB (200 mL)	0.2	CFU/cm^2
BLD	Door handle Curtains, Fan Racks, Railing, Wooden pallets, Walls	50 cm^2 (5 x 10 cm) 100 cm^2 (10 x 10 cm) 250 cm^2 (5 x 50 cm) 375 cm^2 (7.5 x 50 cm) 750 cm^2 (15 x 50 cm) 2500 cm^2 (50 x 50 cm)	DEB (25 mL)	0.1 0.25 0.1 0.06 0.03 0.01	CFU/cm^2
CU	Mops, brooms, squeegees etc.	Swab aprox 250 cm2 (5 x 50 cm)	DEB (25 mL)	0.1	CFU/cm^2
PE/PL	Clip sizers, rollers, conveyors, Keyboards Curtains, Fan, control panel, scales	25 cm^2 (5 x 5 cm) 50 cm^2 (5 x 10 cm) 100 cm^2 (10 x 10 cm) 250 cm^2 (5 x 50 cm) 375 cm^2 (7.5 x 50 cm) 750 cm^2 (15 x 50 cm) 2500 cm^2 (50 x 50 cm)	DEB (25 mL)	1 0.5 0.25 1 0.66 0.03 0.01	CFU/cm^2
RC	Receiving crate	2500 cm^2	DEB (25 mL)	0.1	CFU/cm^2
TV	Forklifts,	375 cm^2	DEB (25 mL)	0.06	CFU/cm^2

BLD: Buildings; PE/PL: CU: Cleaning utensils; PE/PL: Packing equipment/packing line; RC: Receiving crates; TV: Transport vehicles
BPW Buffered Peptone Water; DEB Dey Engley Broth

Table 2. Description of sampling sites and locations, and sample preparation for analysis

Site name	Example of Surface type	Surface area sampled	Type and volume of diluent/pre-enrichment broth added	Limit of Detection	
				CFU/ cm^2	**CFU/ 100 cm^2**
Brushes	PVC	Swab 96 cm^2 (8 × 12 cm)	DEB (25 mL)	0.26	26
Conveyor belts	PVC	Sponge 2025 cm^2 (45 × 45 cm)	DEB (25 mL)	0.01	1
Exit ramps	PVC	Sponge 1075 cm^2	DEB (25 mL)	0.02	2
Sizing clips	PVC	Sponge 270 cm^2	DEB (25 mL)	0.09	9
Conveyor Rollers	Stainless steel	Sponge 900 cm^2	DEB (25 mL)	0.03	3
Strip Curtain	PVC	Sponge 1890 cm^2	DEB (25 mL)	0.01	1
Receiving crates	PP	Sponge 2500 cm^2 (50 × 50 cm)	DEB (25 mL)	0.01	1
Floor	Stainless steel and concrete	Modified Floor Moore Swab 10,000 cm^2 (100 × 100 cm)	DEB (25 mL)	0.0025	0.25
Drains		Swab	DEB (25 mL)	0.25	25

Table 3 Visual scale for cleaning evaluation

Value	Description
1	No visible dirt. Wiping with a white tissue there is no dirt/dust
2	No visible dirt. Wiping with a white tissue there is dirt/dust
3	Spots of dirt are slightly visible
4	Spots of dirt/soil/organic material are visible

Table 4. Frequency of *Listeria* spp. in environmental samples from three avocado dry packaging plants in Mexico

Sample	Material	Collected samples	Positives	Frequency (%)
Floor	concrete, stainless steel	126	25	20
Air	1000 L	118	0	0
Drains	Smooth concrete	34	1	3
BLD: Buildings	(cooler area, storage area, walls, ceiling, doors, windows, pipes, curtains, fans, pallets, pallet racks)	237	6	2.5
CU: Cleaning utensils	(Trash containers, brooms, dustpan, mops)	41	2	5
PE/PL: Packing equipment, packing line	(side of conveyor belt, side of: rollers, brushes, tables, and ramps) (polypropylene bins)	389	7	1.8
RC: Receiving crates	(Forklift, manual pallet jacks)	26	2¥	8
TV: Transport vehicles		32	2	6
Total		**1,003**[1]	**45**	**4.5**

[1] Total of samples collected
¥RC samples were collected only during the first three sampling events.

Table 5. *Listeria* species isolated from positive environmental areas during the year 2023 at three avocado facilities

	Number of positive samples						
Listeria spp.	Floor (N=126)	Drain (N=34)	BLD (N=237)	CU (N=41)	PE/PL (N=389)	RC (N=26)	TV (N=32)
L. innocua	19		3	1	4	2	1
L. welshimeri	2		1	1	1		
L. grayi	1		1				
L. ivannovi	1		1				
L. seeligeri			1	1			
LM	7		2	1	2		1
Other	1	1	1		1		
Totals	31	1	10	4	8	2	2

BLD: Buildings (wood pallets, wall); CU: Cleaning utensils (brooms); PE/PL: Packing equipment/packing line (sides of: conveyor rollers, plastic curtain, exit ramps, scales); RC: receiving crates; TV: Transport vehicles (manual pallet jack).

Table 6. Counts of *Enterobacteriaceae* (CFU/cm^2) in environmental samples from 3 avocado dry packaging plants in Mexico.

Sample type	Description	N	Number positives	Geometric mean of positives	Min	Max	Med
Floor	concrete, stainless steel	126	104	8.36	0.02	794	2.00
BLD: Buildings	(cooler area, storage area, walls, ceiling, doors, windows, pipes, curtains, fans, pallets, pallet racks)	236	104	2.37	0.01	31,622,776	2.0
CU: Cleaning utensils	(Trash containers, brooms, dustpan, mops)	41	28	22.39	0.20	794	17.78
PE/PL: Packing equipment, packing line.	(sides of conveyor belts, rollers, brushes, tables, mats)	389	235	2.48	0.01	2 511	2.51
RC: Receiving crates	(Cardboard, polypropylene bins)	26	16	1.58	0.1	25	1.78
TV: Transport vehicles	(Forklifts, manual pallet jacks)	32	26	9.23	0.25	199	9.23

Table 7. *Enterobacteriaceae* levels (CFU/sample) in environmental samples from three avocado dry packaging plants in Mexico.

Sample type	Description	N	Number positives	Geometric mean of positives	Min	Max	Med
Air	1000 L	80	13	4,800	10	1,000,000	3,200
Drains	Smooth concrete	34	27	5,600	31	10,000,000	3,200

Table 8. Effects of packing plant and type of surface on the counts of *Enterobacteriaceae* on environmental surface samples collected at 3 avocado packing plants in Mexico

Primary effect: Packing Plant	
Plant	*Enterobacteriaceae* count[a]
1	2.2 ± 0.13 A[b]
2	2.3 ± 0.10 A
3	2.1 ± 0.12 A
P = 0.22	

Primary effect: Type of surface	
Surface	*Enterobacteriaceae* count
BLD	1.6 ± 0.08 B
CU	2.9 ± 0.20 A
Floor	2.6 ± 0.10 A
PE/PL	1.9 ± 0.06 B
RC	1.4 ± 0.25 B
TV	2.7 ± 0.21 A
P = < 0.0001	

Mixed effect: Interactions Packing Plant * Type of Surface		
Packing plant	Type of surface	*Enterobacteriaceae* count
1	BLD	1.6 ± 0.13 DF
	CU	3.3 ± 0.44 ABC
	Floor	2.4 ± 0.17 ABCE
	PE/PL	1.9 ± 0.10 CDEF
	RC	1.0 ± 0.47 DEF
	TV	2.9 ± 0.38 ABCDE
2	BLD	1.4 ± 0.12 F
	CU	2.9 ± 0.30 AB
	Floor	2.7 ± 0.17 A
	PE/PL	1.6 ± 0.10 DF
	RC	2.1 ± 0.30 ABCDEF
	TV	3.2 ± 0.38 ABC
3	BLD	1.9 ± 0.15 BCDEF
	CU	2.5 ± 0.26 ABCDE
	Floor	2.6 ± 0.20 ABCE
	PE/PL	2.1 ± 0.11 ABCDE
	RC	1.1 ± 0.52 ABCDEF
	TV	2.2 ± 0.31 ABCDEF
	P = 0.0042	

[a] Values indicate LS Means of the log CFU/100 cm^2 ± standard error
[b] Within each effect, LS Means followed by same letter are not significantly different $P < 0.05$.

Table 9. Counts of *Enterobacteriaceae* in samples of air and drains collected from 3 avocado packing plants in Mexico

Type of sample[a]	Main effects	Identifier	Mean counts[b]	Significance of Differences[c]
Air	Packing plant	Plant 1	2.7 ± 0.137 A[a]	S
		Plant 2	2.1 ± 0.143 B	
		Plant 3	2.8 ± 0.189 A	
	Sampling season	Dry	2.6 ± 0.111	NS
		Wet	2.6 ± 0.192	
	Sampling iteration	25-Jan	1.7 ± 0.000	NS
		6-Mar	2.7 ± 0.000	
		21-Mar	2.4 ± 0.166	
		18-Apr	2.4 ± 0.294	
		6-Jun	3.0 ± 0.282	
		17-Aug	2.4 ± 0.319	
		6-Sep	2.8 ± 0.080	
Drains	Packing plant	Plant 1	3.2 ±0.395	NS
		Plant 2	3.0 ±0.492	
		Plant 3	4.0 ±0.596	
	Sampling season	Dry	3.5 ±0.321	NS
		Wet	2.5 ±0.357	
	Sampling iteration	10-Jan	3.9 ± 1.252	NS
		25-Jan	3.1 ± 0.240	
		7-Feb	3.6 ± 0.758	
		6-Mar	1.9 ± 0.323	
		21-Mar	3.9 ± 1.406	
		18-Apr	3.5 ± 0.636	
		6-Jun	4.5 ± 0.636	
		17-Aug	2.3 ± 0.567	
		6-Sep	2.7 ± 0.524	

[a] Air samples consisted of 1,000 L of air impinged onto a TSA plate at a speed of 100 L/minute. Drain samples included the entire structure of the drain, swabbing the surface and reaching as deep as possible in the internal surfaces.
[b] Counts expressed as mean log CFU/m^3 for air or per drain unit.
[c] S, significant ($P < 0.05$); NS, not significant ($P \geq 0.05$)

Table 10. Temperature of surfaces[a] sampled at 3 avocado packing plants in Mexico

Plant	Type of surface sampled	Temperature[b]
1	Air[c]	18.4 ± 0.87 B
	BLD	14.9 ± 0.70 C
	CU	21.3 ± 2.14 ABC
	Drain	21.9 ± 1.35 AB
	Floor	18.7 ± 1.24 BC
	PE/PL	23.4 ± 0.53 A
	RC	21.2 ± 2.63 ABC
	TV	18.2 ± 2.14 ABC
2	Air	19.4 ± 0.75 BC
	BLD	17.8 ± 0.53 C
	CU	22.7 ± 1.28 AB
	Drain	22.6 ± 1.88 ABC
	Floor	18.8 ± 0.76 BC
	PE/PL	24.2 ± 0.43 A
	RC	21.3 ± 1.28 ABC
	TV	22.6 ± 1.65 ABC
3	Air	20.3 ± 0.98 B
	BLD	20.2 ± 0.79 B
	CU	24.7 ± 1.41 AB
	Drain	23.2 ± 2.60 AB
	Floor	19.9 ± 1.08 B
	PE/PL	27.6 ± 0.57 A
	RC	24.3 ± 2.60 AB
	TV	25.6 ± 1.75 AB

[a] In the case of air samples, the temperature was measured from the air surrounding the air sampler. All measurement

[b] Values indicate LS mean temperatures in °C ± standard error.

[c] See Table 1 for definition of acronyms and description of samples.

Table 11. Temperatures on environment surfaces prior to sample collection at 3 avocado packing plants in Mexico

Type of surface[a]	Temperature[b]	Minimum	Maximum	Median
Air	19.4 ± 0.64 BC[c]	2.5	28.5	21.6
BLD	17.6 ± 0.54 C	2.3	34.6	19.4
CU	23.4 ± 0.45 A	14.0	28.0	23.8
Drain	22.3 ± 0.38 AB	18.5	25.6	22.7
Floor	19.1 ± 0.69 BC	3.6	26.9	21.4
PE/PL	25.0 ± 0.18 A	5.5	36.5	25.1
RC	21.9 ± 0.55 AB	17.6	28.1	21.0
TV	22.8 ± 0.88 AB	5.5	27.8	23.4

[a] See Table 1 for definition of acronyms and description of samples.
[b] Values show the means ± standard deviation of temperature in °C.
[b] Within means, values followed by same letter are not significantly different (P ≥ 0.05)

Table 12. Evaluation of cleaning procedures. Summary of positive samples to *Listeria* spp. on surfaces of avocado packing plant before and after application of cleaning procedures.

Surface	N	Before	After
Transporting bands	18	2	-
Brushes	9	1	3
Sizers	18	-	-
Receiving Crates	44	16	17
Drains*	9	-	5
Strip curtains	17	-	-
Floor*	18	8	4
Exit ramp 1	9	-	-
Rollers	18	-	-
Exit ramp 2	18	3	-
Total	180	30	29

Table 13. Evaluation of cleaning procedures. Summary of indicator counts before and after applying cleaning procedures on surfaces of avocado packing plant.

Indicator group	Surface	N	Mean Log CFU/100 cm^2 Before	Mean Log CFU/100 cm^2 After	Std. Error	Sig.
APC	Transporting bands	18	4.22	2.91	0.278	**
	Brushes	9	7.16	6.67	0.393	
	Sizers	18	5.25	4.56	0.278	
	Receiving Crates	44	6.23	5.97	0.178	
	Drains	9	7.04	6.36	0.393	
	Strip curtains	17	3.75	3.11	0.286	
	Floor	18	5.13	4.49	0.278	
	Exit ramp 1	9	6.08	4.04	0.393	**
	Rollers	18	4.41	4.03	0.278	
	Exit ramp 2	18	4.79	2.95	0.278	**
ENT	Transporting bands	18	2.43	0.84	0.285	**
	Brushes	9	5.35	5.04	0.403	
	Sizers	18	3.24	2.99	0.285	
	Receiving Crates	44	3.62	3.52	0.182	
	Drains	9	3.95	4.11	0.403	
	Strip curtains	18	1.72	1.10	0.285	
	Floor	18	3.43	2.60	0.285	**
	Exit ramp 1	9	4.05	2.33	0.403	**
	Rollers	18	3.18	2.76	0.285	
	Exit ramp 2	18	3.08	0.98	0.285	**
YEASTS	Transporting bands	16	1.17	0.82	0.261	
	Brushes	8	4.37	3.91	0.369	
	Sizers	16	2.48	1.96	0.261	
	Receiving Crates	39	4.58	4.36	0.167	
	Drains	8	4.55	3.01	0.369	**
	Strip curtains	16	0.86	0.35	0.261	
	Floor	15	2.56	2.32	0.269	
	Exit ramp 1	8	2.19	1.76	0.369	
	Rollers	16	1.95	1.64	0.261	
	Exit ramp 2	16	1.50	0.79	0.261	**
MOLDS	Transporting bands	16	1.98	0.60	0.155	**
	Brushes	8	3.66	3.80	0.219	
	Sizers	16	2.16	2.29	0.155	
	Receiving Crates	39	3.19	2.97	0.099	
	Drains	8	2.70	2.43	0.219	
	Strip curtains	16	0.96	0.36	0.155	**
	Floor	16	2.09	2.13	0.155	
	Exit ramp 1	8	2.35	1.52	0.219	**
	Rollers	16	1.82	1.35	0.155	**
	Exit ramp 2	16	2.08	1.00	0.155	**

** Significant difference at 95% confidence.

Table 14. Evaluation of cleaning procedures. Summary of soil levels (1-4 scale) and ATP (Log RLU/100 cm^2) on surfaces of avocado packing plant before and after application of cleaning procedures.

Variable	Surface	N	Before	After	Std. Error	Sig.
Soil level	Transporting bands	16	2.88	1.31	0.163	**
	Brushes	8	3.88	3.63	0.230	
	Sizers	16	3.44	2.25	0.163	**
	Receiving Crates	34	3.68	3.33	0.112	
	Drains	7	3.43	3.17	0.246	
	Strip curtains	16	2.63	1.63	0.163	**
	Floor	16	3.38	2.50	0.163	**
	Exit ramp 1	8	3.25	1.50	0.230	**
	Rollers	16	3.50	1.25	0.163	**
	Exit ramp 2	16	2.88	1.50	0.163	**
ATP	Transporting bands	16	3.07	2.76	0.200	
	Brushes	7	2.83	2.92	0.303	
	Sizers	16	3.03	3.14	0.200	
	Receiving Crates	34	2.79	3.07	0.137	
	Drains	6	3.03	2.80	0.327	
	Strip curtains	16	3.17	3.01	0.200	
	Floor	14	2.89	2.83	0.214	
	Exit ramp 1	8	2.62	2.85	0.283	
	Rollers	15	3.79	3.31	0.207	
	Exit ramp 2	16	3.17	3.36	0.200	

Visual scale: 1. No visible dirt. Wiping with a white tissue there is no dirt/dust. 2. No visible dirt. Wiping with a white tissue there is dirt/dust. 3. Spots of dirt are slightly visible. 4. Spots of dirt/soil/organic material are visible.
** Significant difference at 95% confidence.

Table 15. Main effects on counts of *LM* on equipment surfaces

Main effects	Identifier		*LM* count[a]	P[b]
Dwell time	1 h		2.4A	<0.0001
	8 h		2.5B	
Sampling time	Before cleaning		3.5A	<0.0001
	After cleaning		1.4B	
Cleaning approach	Wet		2.6A	<0.0001
	Dry		2.3B	
Type of surface	Stainless steel		2.0A	<0.0001
	Conveyor belt (PVC)		2.9B	
				STERR=0.024
Interactions				
	Type of surface* Dwell time			
Factors			Log count	
Stainless steel	1 h		2.1A	<0.0001
	8 h		1.9B	STERR=0.333
Conveyor belt (PVC)	1 h		2.7C	
	8 h		3.1D	
	Dwell time*sampling time			
1 h	Before		3.3B	<0.0001
	After		1.4C	STERR=0.333
8 h	Before		3.7A	
	After		1.3D	
	Type of surface*cleaning approach*time of sampling			
Stainless steel	Wet	Before	3.2C	= 0.0020
		After	1.2F	STERR=0.047
	Dry	Before	2.9D	
		After	0.8G	
Conveyor belt	Wet	Before	4.1A	
		After	1.8E	
	Dry	Before	3.8B	
		After	1.8E	
	Cleaning approach*dwell time*sampling time			
Wet	1 h	Before	3.5B	=0.0271
		After	1.5D	STERR=0.047
	8 h	Before	3.8A	
		After	1.4D	
Dry	1 h	Before	3.1C	
		After	1.4DE	
	8 h	Before	3.6AB	
		After	1.2E	

[a] Counts expressed in Log CFU/cm^2 of equipment. Within each effect, LS Means followed by same letter are not significantly different (P ≥ 0.05).
[b] Significance level (P) was set at <0.05. STERR means standard error.

Table 16. Statistics of Sequencing Data

Sample name	Raw reads	Raw data (G)	Clean data (G)	Effective (%)	Error (%)	Q20 (%)	Q30 (%)	GC (%)
A1	9952812	1.5	1.5	99.67	0.03	97.57	93.03	47.69
A2	7536474	1.1	1.1	97.93	0.03	96.47	91.72	43.91
A4	10356154	1.6	1.5	98.44	0.03	97.37	92.62	40.81
A6	6531518	1.0	1.0	99.57	0.03	97.55	92.66	37.91
A9	7142002	1.1	1.1	99.49	0.03	97.69	92.98	37.77
A13	6662162	1.0	1.0	99.39	0.03	97.60	92.98	42.52
A21	11064160	1.7	1.7	99.44	0.03	97.63	92.87	37.98
A3	9646604	1.4	1.4	99.52	0.03	97.38	92.27	36.55
A16	20444318	3.1	3.1	99.51	0.03	97.46	92.47	37.71
A22	7334114	1.1	1.1	99.36	0.03	96.53	90.86	51.02
B227	6993378	1.0	1.0	98.38	0.03	97.44	92.60	37.44
B35	7381598	1.1	1.1	98.99	0.03	97.62	92.92	37.58
B511	8010048	1.2	1.2	98.68	0.03	97.52	92.81	37.69
B61	7719076	1.2	1.1	99.1	0.03	97.68	93.01	37.46
C1	6514644	1.0	1.0	99.5	0.03	97.52	92.55	37.52
C2	7824682	1.2	1.2	99.22	0.03	97.34	92.25	37.49
C3	8792228	1.3	1.3	99.35	0.03	97.59	92.81	37.36
C4	7327402	1.1	1.1	99.46	0.03	97.49	92.54	37.39
C5	7604194	1.1	1.1	99.48	0.03	97.54	92.63	37.47

Table 17. Statistics of mapping rate, depth and coverage

Sample	Mapped reads	Total reads	Mapping rate (%)	Average depth(X)	Coverage at least 1X (%)	Coverage at least 4X (%)
A1	70,725	9,919,552	0.71	186.54	1.35	1.14
A2	2,134,874	7,380,840	28.92	136.85	64.79	61.16
A4	88,423	10,195,058	0.87	215.07	1.38	1.10
A6	2,263,297	6,503,180	34.80	139.08	66.04	62.39
A9	2,397,153	7,105,240	33.74	148.10	65.90	62.21
A13	55,175	6,621,480	0.83	101.05	2.00	1.20
A21	3,692,167	11,001,856	33.56	224.16	67.18	63.60
A3	4,319,698	9,600,016	45.00	204.34	90.72	90.31
A16	11,129,413	20,343,670	54.71	517.83	90.93	90.58
A22	69,772	7,287,416	0.96	169.82	1.47	1.17
B227	3,298,826	6,879,758	47.95	163.84	80.59	65.46
B35	3,466,735	7,307,352	47.44	192.97	71.25	66.91
B511	3,894,890	7,904,364	49.28	230.14	67.86	62.99
B61	3,758,397	7,649,744	49.13	215.77	69.60	65.38
C1	3,142,403	6,481,998	48.48	189.55	67.17	62.38
C2	3,806,743	7,763,514	49.03	234.06	66.26	62.13
C3	4,394,821	8,734,942	50.31	242.58	71.26	67.41
C4	3,592,738	7,287,580	49.30	204.46	69.96	64.97
C5	3,673,861	7,564,938	48.56	196.78	70.82	66.11

Table 18. Sources of isolates yielding sequencing with mapping rate ≥ 28.92%.

Novogene Tag	Type	Material	Description	Mapping rate %	Packing plant
B35	Surface	Stainless Steel	Surface of Equipment	47.44	1
B61	Surface	PVC	Sewer	49.13	3
C3	Surface	Stainless Steel	Surface of Equipment	50.31	1
C1	Floor	Concrete	Floor below equipment (end of process)	48.48	2
A9	Surface	Steel	Hydraulic skate	33.74	2
B227	Floor	Concrete	Wearhouse floor	47.95	3
C2	Surface	Plastic	Broom	49.03	1
A16	Surface	Stainless Steel	Surface of Equipment	54.71	1
A3	Surface	Steel	Hydraulic skate	45	1
A2	Surface	Plastic	Broom	28.92	1
A6	Surface	Galvanized roof metal panel	maintanance area	34.8	1
C4	Floor	Concrete	Wearhouse floor	49.3	3
C5	Floor	Steel with antislip	Floor below equipment (middle of process)	48.56	1
B511	Surface	Plastic	broom	49.28	1

Table 19. Atomic force microscopy

Samples	Rq	Ra
HASS Avocado	135.2 ± 23.3^{A}	76.2 ± 29.1
Blue conveyor Belt	34.9 ± 18.7^{D}	24.4 ± 12.5
Old Curtain separator	63.9 ± 45.9^{CD}	43.1 ± 28.1
Black Roller	274.3 ± 78.4^{A}	215.4 ± 57.5
Stainless Steel	136.1 ± 45.9^{B}	99.7 ± 38.9
White conveyor belt	82.5 ± 41.5^{C}	60.5 ± 28.9
Green Avocado	47.7 ± 9.9^{B}	36.7 ± 7.9

Values are expressed as means ± standard deviation
The statistical analysis has been performed on the Rq results, where letters denote the level of significance

Table 20. Comparison of populations of loosely and strongly attached *LM* cells on Hass avocados

Variety	Time (hour)	Loose (log CFU/cm^2)	Strong (log CFU/cm^2)
Hass Avocado	0	6.1 ± 0.0^{B}	4.7 ± 0.1^{b}
Hass Avocado	1	6.0 ± 0.1^{B}	4.8 ± 0.0^{b}
Hass Avocado	3	6.2 ± 0.1^{B}	4.9 ± 0.0^{ab}
Hass Avocado	6	6.6 ± 0.1^{A}	5.1 ± 0.1^{a}

Values are expressed as means ± standard error
The statistical analysis has been performed on the results of Loose and Strong separately, where letters denote the level of significance.

Table 21. Populations of loosely and strongly attached *LM* on diverse materials used in avocado packing equipment and utensils.

Type	Time (hour)	Loose (log CFU/cm^2)	Strong (log CFU/cm^2)
Black roller	0	5.7 ± 0.0	5.4 ± 0.3
Black roller	1	5.9 ± 0.0	6.1 ± 0.0
Black roller	3	5.9 ± 0.0	7.0 ± 0.0
Black roller	6	6.2 ± 0.0	7.0 ± 0.2
Separator Curtain	0	6.6 ± 0.1	5.8 ± 0.1
Separator Curtain	1	6.0 ± 0.0	5.6 ± 0.0
Separator Curtain	3	6.1 ± 0.0	5.7 ± 0.1
Separator Curtain	6	6.2 ± 0.0	6.0 ± 0.1
Stainless steel	0	5.8 ± 0.2	5.3 ± 0.2
Stainless steel	1	6.1 ± 0.0	5.2 ± 0.0
Stainless steel	3	5.8 ± 0.2	5.8 ± 0.1
Stainless steel	6	5.9 ± 0.1	6.3 ± 0.1
White Convey Belt	0	6.9 ± 0.0	6.1 ± 0.0
White Convey Belt	1	5.9 ± 0.1	6.4 ± 0.1
White Convey Belt	3	6.9 ± 0.0	6.7 ± 0.0
White Convey Belt	6	6.8 ± 0.0	6.7 ± 0.0
Blue conveyor belt	0	6.2 ± 0.1	5.1 ± 0.1
Blue conveyor belt	1	6.5 ± 0.1	5.6 ± 0.1
Blue conveyor belt	3	6.7 ± 0.1	6.0 ± 0.1
Blue conveyor belt	6	5.7 ± 0.2	6.5 ± 0.1

Table 22. Direct SEM count of attached *LM* cells on different avocado packing materials.

Samples	Number of attached LM
Black roller	6.6 ± 0.0
Blue conveyor belt	6.7 ± 0.1
Separator Curtain	6.8 ± 0.0
Stainless steel	7.2 ± 0.1
White conveyor belt	7.0 ± 0.2
Avocado Hass	6.6 ± 0.1

Values are expressed in mean log cells/cm^2 ± standard deviation

Table 23. Cross-contamination rates*, XC, between processing equipment and the produce (lettuce).

XC [%]	Direction	Location	ID	Distribution [%]
$XC_{B,Av}$	Contaminated brush conveyor to avocado	Brush conveyor	L2	$T[35, 45, 48]$
$XC_{Av,B}$	Contaminated avocado to brush conveyor	Brush conveyor	L2	$T[2,10, 28]$
$XC_{F,Av}$	Contaminated fan/conveyor to avocado	Fan/ conveyor	L3	$T[4.7, 36, 45.8]$
$XC_{Av,F}$	Contaminated avocado to fan/conveyor	Fan/ conveyor	L3	$T[0, 0.62, 1.39]$
$XC_{Rc,Av}$	Contaminated roller conveyor to avocado/SS	Roller conveyor	L4	$T[4.7, 36, 45.8]$
$XC_{Av,Rc}$	Contaminated avocado to roller conveyor/SS	Roller conveyor	L4	$T[0, 0.62, 1.39]$
$XC_{Sc,Av}$	Contaminated sorting machine to avocado	Separator curtain	L5	$T[4.7, 36, 45.8]$
$XC_{Av,Sc}$	Contaminated avocado to sorting machine	Separator curtain	L5	$T[0, 0.62, 1.39]$
$XC_{Sc,Av}$	Contaminated separator/curtain to avocado	Separator curtain	L6	$T[5, 20, 30.8]$
$XC_{Av,Sc}$	Contaminated avocado to separator/curtain	Separator curtain	L6	$T[0, 0.48,1.2]$
$XC_{W,Av}$	Contaminated wheel conveyor to avocado	Wheel conveyor	L7	$T[32.4, 41,43]$
$XC_{Av,W}$	Contaminated avocado to wheel conveyor	Wheel conveyor	L7	$T[0, 0.06, 0.38]$
$XC_{Bc,Av}$	Contaminated blue conveyor to avocado	Blue conveyor/fan	L8	$T[19.5,26,48.2]$
$XC_{Av,Bc}$	Contaminated avocado to blue conveyor	Blue conveyor/fan	L8	$T[0, 0.25, 0.53]$
$XC_{Sc,Sl}$	Contaminated white conveyor to avocado	White conveyor	L9	$T[6.6, 22, 37.3]$
$XC_{Sl,Dc}$	Contaminated avocado to white conveyor	White conveyor	L9	$T[0, 0.35, 1.59]$
$XC_{Sc,Sl}$	Contaminated red box to avocado	Crate	L10	$T[5.7, 15, 27.9]$
$XC_{Sl,Dc}$	Contaminated avocado to red box	Crate	L10	$T[0, 0.35, 1.59]$
$XC_{H,Av}$	Contaminated hands/gloves to avocado	Worker	W1	$T[3, 10, 30]$
$XC_{Av,H}$	Contaminated avocado to hands/gloves	Worker	W1	$T[0, 1, 3]$

Table 24. Description of each location ID in the processing facility

Location ID	Equipment	Number of patches (area)
L0	Avocado Source	2
L1	Reception area	275 (11x25)
L2	Brush conveyor	200 (8x25)
L3	Fan/conveyor	175 (7x25)
L4	Roller conveyor	132 (33x4)
L5	Sorting machine	375 (25x15)
L6	Separator curtain	18 (9x1)
L7	Wheel conveyor (2)	132 (33x4)
L8	Blue conveyor (2)	96 (4x24)
L9	White conveyor (8)	25(5x5)
L10	Crates	10(2x5)
W	Workers	8

Appendix B. Methodology for Objective 1. ***Task 3. Characterize and validate cleaning and sanitizing procedures***

Gallery of surfaces sampled at avocado packing plant. a) Curtain strip; b) transporting band; c) plastic slope; d) rollers; e) brush exit; f) brush; g) drain; h) sizers; i) floor; j) reception container