CENTER for PRODUCE SAFETY

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FINAL PROJECT REPORT

Project Title
Verification and validation of environmental monitoring programs for biofilm control in the packinghouse

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Principal Investigator
Paul Dawson
Clemson University
Department of Food, Nutrition and Packaging Sciences
226B Life Science Facility
Clemson, SC 29634-0316
T: 864-656-1138
E: pdawson@clemson.edu

Objectives

1. *Determine physiological and structural parameters (growth rate, microbial composition, morphology, and erosion or detachment) of microbial biofilms of resident microbiota and Listeria monocytogenes on food-contact surfaces in zone 1 in a simulated packinghouse environment.*
2. *Determine factors (microbial, physical, environmental) important in L. monocytogenes biofilm persistence and colonization of the packinghouse. Determine L. monocytogenes growth parameters in refrigeration conditions.*
3. *Validate the biofilm development parameters in a pilot plant study; build a mathematical model of biofilm growth and detachment and develop an Excel add-in to predict optimal sampling time and sanitation schedule.*

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FINAL REPORT

Abstract
Fresh produce processing facility and fruit packinghouses play a crucial role for the national supply of fresh fruits and vegetables. Since these products are consumed without major preparation, food safety of these products is of major concern. In this project we aimed to investigate the surface associated microflora in the stone fruit packinghouses for their biofilm forming capabilities, microbial composition, and their interactions with *Listeria monocytogenes* in multiple-species biofilms. The growth rate of the biofilms was aimed to create a tool to predict the biofilm growth rate in the packinghouse for optimal environmental monitoring practices. More than 800 environmental samples were collected from nine stone fruit packing facilities in California and South Carolina. The samples were collected before and after packing operations, and they were evaluated by total plate count and for biofilm formation capability, and, for select samples, the multi-species present were identified at the genus level by 16S rRNA metagenomics. Microbial plate counts and metagenomics indicated three different scenarios: (i) plate counts and microbial diversity were lower for the samples collected before packing than those collected after packing operations, (ii) plate counts and microbial composition were higher in the samples collected before packing than after, and (iii) for one facility there were no significant differences in the before and after samples. Metagenomics revealed that the surface associated microbiota is more diverse in the wax/wash area of the plant (wet surfaces) than in the sorting area (dry surfaces). The environmental strains are robust biofilm-formers and have almost a double growth rate than that of *L. monocytogenes*. The pathogen can form complex monoculture biofilms, but in all performed experiments *Listeria* integrated in the environmental biofilm matrix.

Background
From an ecological standpoint, the packinghouse is an open, dynamic environment where microorganisms can be introduced by fruits, packing materials and workers. To survive in the packinghouse, bacteria will adhere to surfaces and resist adverse environmental conditions such as shear forces associated with packaging and cleaning operations, sanitation and disinfection procedures, absence of nutrients, osmotic stress and/or desiccation. Adhered cells can then form biofilms in favorable environmental conditions (e.g., favorable temperature and humidity) and in the presence of suitable sources of nutrients available. Favorable conditions for biofilm formation can vary greatly depending on the type of microorganism. Biofilms, which usually consist of four main components, namely (i) water, (ii) the living microbial population, (iii) the surface-substrate to which cells are adhered, and (iv) the associated extracellular matrix, are networks of microcolonies and often have patchy distributions. Biofilms can be single or multi species and can also detach, thereby becoming a source of contamination for other sites.

L. monocytogenes is known to form biofilms, but there are significant differences between phylogenetic divisions. In monocultures, *L. monocytogenes* biofilms have been described by different representations ranging from flat films to honeycomb structures. In addition, the pathogen can form biofilms in mixed cultures with the resident packinghouse microflora. Over time, biofilms can grow with a rate depending on the microbial species and presence of nutrients, reach a steady state with no growth or decay over time, and then decay due to cell loss or biofilm detachment. The detachment of microbial cells is important because it can contaminate other surfaces. Spontaneous detachment can occur as erosion (continual detachment of small portions of the biofilm) and/or sloughing as a massive loss of the biomass. The particle size and detachment frequency depend on the shear forces and the presence of nutrients. Monitoring procedures for *L. monocytogenes* in packinghouses as part of their sanitation protocols are not harmonized in terms of frequency of sampling and sampling sites

(one size does not fit all). Moreover, some of the food-contact materials are less than optimal for cleaning and sanitation, and the packing process does not have a 'kill step' for harmful bacteria which imposes strict sanitation and EMP rules.

The goal of this research was to provide data for the development of sampling tools and plans that can be used to assist in the validation of the metrics of a packinghouse sanitation plan. Improving sampling strategies based on biofilm growth rate and detachment *L. monocytogenes* and/or background microflora would not only improve efficacy of treatments and cleaning procedures but also reduce recontamination events.

Research Methods and Results

Bacterial strains and growth condition: *L. monocytogenes* Petite Scott A (ATCC 49594) and a derivative expressing green fluorescent protein (GFP) from plasmid pNF8 were used for biofilm growth. All strains were maintained as freezer stocks (-80°C) in 25% glycerol and propagated on Tryptic Soy Agar (TSA) plates, and Tryptic Soy Broth (TSB) unless stated otherwise. *L. monocytogenes* Petite Scott A pNF8 was grown on media supplemented with 10 μg ml^{-1} for plasmid maintenance. Biofilms of *Listeria* and packinghouse strains were grown either in TSB, Listeria Synthetic Media (LSM - formulation in Supplemental Table 4) and residual water from peach wash. LSM is a chemically defined medium, in which microorganisms utilize glucose as the main carbon source and glutamate as the nitrogen source. Inoculums for biofilm growth were prepared in 2X LSM, but after inoculation and attachment of cells the biofilms were grown in 0.5X LSM, except biofilms grown in refrigeration temperature, which required 2X LSM. Water from peach wash (PE) was obtained from a California packinghouse. Briefly, around 1000 lbs. of fruit were washed through a miniature brush test bed simulating the wash conditions in the packing season. The peaches had been previously de-fuzzed, washed, waxed and placed in cold storage. The resulting PE had 8.30 pH, 0.19 ppm total chlorine, and 0.03 ppm free chlorine. The PE was stored at -20°C, and prior to use in experiments was thawed and filter-sterilized through a 0.22 μm pore size filtration unit. Statistical analysis and box plots were performed in IBM SPSS Statistics 25.

Objective 1. *Determine physiological and structural parameters (growth rate, microbial composition, morphology, and erosion or detachment) of microbial biofilms of resident microbiota and L. monocytogenes on food-contact surfaces in Zone 1 in the packinghouse. Determine the optimal conditions for sampling for L. monocytogenes detection in mixed biofilms.*

Sampling of the stone fruit packinghouses: To characterize the background microflora present in the packinghouses we sampled a facility in South Carolina and 8 packing facilities and cold storage in California (6 packinghouses, one packing shed, and one cold storage facility). Depending on the type of the surface, sampling was performed with sterile Q-Swabs (Hygiena™ Q-Swab) or 3M Sponge-Stick (Nelson Jameson) containing 1 ml and 10 ml buffered peptone water, respectively. The use of hydrated Q-Swab or 3M Sponge was dictated by the topography of the specific sampling location (e.g., swabbing of tufted brush filaments, irregular surface of the sizer cups versus extended flat surfaces such as collection table). For each packinghouse, sampling was performed (i) in the select area of the pack line before the start of packing operations, and (ii) in approximately the same area after the packing operations. The South Carolina packing facility was also sampled immediately after the cleaning operations because the specific packing schedule allowed for multiple daily samplings. The California facilities were sampled once per each session (session defined as before and after packing), and the South Carolina facility was visited and sampled on two different packing days. Collected environmental samples were stored at 4°C until processed in the lab.

For bacterial enumeration, individual sponges and swabs were vortexed (Q-Swab) or homogenized (3M Sponge) and serial dilutions (10^{-4} to 10^{-14}) were made from the homogenate in sterile phosphate saline buffer (PBS) and plated on Plate Count Agar (PCA). Plates were incubated at 20°C for 48 hours and then colonies were enumerated. Subsequently, the microbial mixtures from the homogenate were mixed with 50% sterile glycerol (1:1 vol/vol) and stored at -80°C for further experiments.

Biofilm screening of collected environmental samples: To compare the biofilm-forming abilities of the collected environmental samples, bacterial suspensions were first standardized by optical density measurements, and then inoculated in growth medium in a ratio of 1:100 in the individual wells of 48-well plates (400 microliters total volume), with duplicate replicate wells created for each species. Plates were incubated for 24 hours at room temperature with moderate shaking. After incubation, the suspended cells in each well were removed, and the wells were rinsed three times with sterile water. Then, 400 µL of 0.1% crystal violet solution was added to the emptied wells to bind to the biofilm-forming species present on the well walls. The crystal violet solution was then removed from the wells after 30 min, and wells were rinsed with distilled water twice to remove the unbound dye. The bound crystal violet was solubilized with 400 µL of 33% acetic acid solution, and the absorbance of solution in each well was measured at 590 nm. Control wells containing sterile media were treated by the same staining and rinsing procedure and used as blanks for background subtraction. The biofilm-forming experiments were conducted in triple independent experiments.

Microbiota profiling by Next Generation Sequencing of the V3-V4 regions of the 16S rRNA gene was performed for selected environmental samples (Supplemental Tables 1–3). Samples were selected for genetic identification based on the (i) overall facility plate counts, (ii) location within facility to allow analysis of samples collected from wet and dry surfaces, and (iii) their biofilm formation profile based on the crystal violet assay data. The homogenates from *individual* Q-Swabs or 3M Sponges were allowed to recover in Tryptic Soy Broth (TSB) overnight, to allow recovery of all microbial species (including those injured during cold storage) and then genomic DNA was extracted using ZymoBIOMICS DNA Miniprep kit according to manufacturer's instructions. Genomic DNA stocks were verified for their concentration with Qubit™ fluorometer (Qubit dsDNA HS Assay) and for purity with a BioTeK Epoch 2 microplate spectrophotometer. A 16S rRNA library was constructed with *Quick*-16S NGS Library Prep Kit. For each of the 92 environmental samples selected for microbiome analysis, approximately 15-20 ng of purified DNA was used as template to amplify the hypervariable region V3-V4 of the bacterial 16S rRNA gene. The quantitative PCR (qPCR) first amplification step (primers V3-V4) resulted in an amplicon of about 460 base pair (bp). In a short, second qPCR amplification step, index primers were ligated to the amplicons for multiplexing. The DNA fragments resulted after the addition of barcodes were 596 bp. Both qPCR steps were performed with a Roche 96 Lightcycler Real-Time PCR system. Quality and quantity of each sequencing library were assessed using Agilent's 4150 Tapestation using gDNA Screen Tape and gDNA Reagents both from Agilent and picogreen measurements, respectively. The libraries were then pooled in equal concentrations according to picogreen and qPCR measurements for an average concentration of 80 ng per sample. The pool was then quantified using the KAPA Biosystems, and then according to the qPCR measurements, 10 pM of pooled libraries was loaded onto a MiSeqDX flow cell and sequenced using MiSeq Reagent Kit v3 600 Cycles PE (Paired end 300 bp). The paired-end MiSeq Illumina reads (2 × 300 bp) were assembled according to the overlap and fastq files were processed to generate individual files, which could then be analyzed. All identical sequences were merged into one. The fastq files were processed using Mothur. The pipeline was run on the Palmetto cluster. Sequences were merged using "make.contig" command, quality filtered by "screen.seqs" command, dereplicated, and aligned to the 16S

rRNA reference database (SILVA). The unique sequences were clustered into OTUs at 97% identity. Data was used to determine the operational taxonomic unit (OTU) clusters.

Biofilm formation in flow cells of single and multi-species of *L monocytogenes* packinghouse microflora: A biofilm apparatus was developed for individual flow-through enclosures where biofilms were grown under constant laminar flow. During biofilm growth, the medium flown from the reservoir was maintained constant by the calibrated peristaltic pumps (8 individual channels/ 1 fluid cell per channel. Late exponential growth phase cultures of *L monocytogenes* Petite Scott A (optical density 0.25) grown in 2X LSM were inoculated in the flow cells (10 ml each, through a separate inlet tubing with a sterile syringe) and the cells were allowed to attach for one hour. Experiments involved (i) biofilms of *L monocytogenes*, (ii) biofilms of mixed microbiota from select sampling points, and (iii) mixed *L monocytogenes* biofilms with mixed microbiota. After the attachment phase the media was pumped at a flow rate of 0.5 ml min -1. For the biofilms involving environmental microbiota and mixed *Listeria* biofilms, microbial strains were inoculated at 1:1 ratio (vol: vol of plant microbiota and *L monocytogenes*).

Confocal microscopy was performed for select samples at Clemson University Light Imaging Facility. After removing from the flow cells enclosures, biofilms were fixed by incubating in 4% paraformaldehyde at room temperature for 30 minutes. Biofilms were rinsed twice in phosphate buffer and imaged with a Leica SP8 MCS multiphoton microscope.

Objective 1 Results

The microbial populations of the packinghouse surfaces were quantified by estimating the colony-forming units per surface area sampled. Since food-contact surfaces are not flat and are constructed with a variety of materials (stainless steel, nylon filament brushes, PVC etc.), sampling was performed with either a swab or a sponge. The surface of the sampled area could not be determined directly, therefore a statistical comparison across the sampling points would result in errors. However, comparison was possible for the plate counts obtained on samples before and after packing, collected approximately at the same location. For each facility, sampling involved the wash/wax area (fruit pads, brushes, drains), dry area-sorting equipment (sizer cups, conveyor belts, and rods) and sorting tables and dry drains. Most of the collected samples were from zone 1, but occasionally non-food contact surfaces were sampled (conveyor for the transport of packaging material, walls, metal frame supporting the equipment, or condensation collection trays).

Comparison of means of before and after packing for the approximately same sampling point (paired sample *t*-test, alpha 0.05) indicated that there were significant differences in the means. The mean comparison reveals three distinct situations (**Table 1**).

First, plants 1, 5, 6 and 7 (statistical summary in Table 1 and data in Supplemental Figure 1) had significantly higher means in the samples collected right after packing operations. In general, the cleaning operations most likely reduced significantly the microbial surface contamination that is inherently associated with fruit packing. There were sampled spots where the means before and after were not significantly different such as cracks in the walls, modular belts, sizer cups, polyurethane foam that coats the sorting tables, wax brushes. That would be a possible indicator of surfaces overlooked by the cleaning crew. The overall lower microbial count at plant 7 could be due to a number of factors (i) the facility packed only one type of stone fruit, (ii) there was an aggressive wash of the incoming fruits with higher concentrations of chlorine, and (iii) there were certain district modifications of the packing line. For example, the soft belts and PVC conveyor rods for the incoming fruit (not washed) leading to the brush bed and from the brush bed (fruit washed and waxed) had been replaced by stainless steel rod conveyors. The sampling data indicated, in general, that stainless steel is a more hygienic material and has a lower microbial load than the rest of the soft materials.

Second, plants 2 and 4 also presented significant differences in the means of before and after packing. However, in these 2 facilities the significantly higher means were from the samples collected *before* packing. The sampling was performed in the morning before packing operations, not right after cleaning, where surfaces had been cleaned and sanitized the night before and they were dry at the time of the sampling. These results suggest that the residual microorganisms left after cleaning had favorable conditions for growth and could multiply overnight in the interval before packing. The lower means in the after packing samples indicate that some of the surface associated microbes dissociate, and they are disrupted by the packing operations.

The third scenario is plant 3. In this facility, cleaning and sanitation operations are performed right *before the start of packing operations for the day*. The means of the before and after packing were not significantly different, suggesting that bacteria could replicate on surfaces overnight, but the cleaning and sanitation removed some of the microorganisms.

The ability of the isolated bacterial strains to form biofilms was also evaluated by the crystal violet method (example Supplemental Figure 2). Data is shown for the ninety-two isolates (the same selected for identification through genomic sequencing from plants 1, 2 and 3 (see Supplemental Tables 1, 2 and 3). Environmental samples (groups that were collected on the same swab or sponge) were grown in 48-well plates, and the resulting biofilms were analyzed spectrophotometrically. The assay can quantify the total biofilm biomass (cells and extracellular polymers). The strains that were isolated from the food-contact surfaces showed in general a high biofilm-forming capability, i.e., the average biofilm biomass values ranged from 0.2 to 3.8. To test the influence of the surface type (material- e.g., brush, stainless steel, belts) on the biofilm formation by the environmental community, the results were analyzed by one way ANOVA. Samples were grouped as (i) non-contact surfaces, (ii) contact stainless steel, (iii) contact brushes, and (iv) contact PVC (belts, siders, rods). Means of biofilms obtained from the Facility 1 environmental samples that were collected before packing were not statistically different among the different groups (Supplemental Figure 3). These biofilms, however, were concentrated at the air-water interface of the well (pellicle) and sometimes biofilms were washed away during staining-distaining procedures (Box plots, Supplemental Figure 3). The after-pack samples collected from Facility 1 indicated that the brush samples formed significantly more biofilms (p 0.05) than the other groups. Similarly, Facility 2, brush group samples (wash, defuzz, sponge and wax) had significantly higher biofilm-forming capability then the rest of the surfaces in before and after packing environmental samples (Supplemental Figure 4). In facility 3, the stainless-steel group had the lowest biomass and was significantly lower than the brush and the PVC group (p at 0.05 level). The stainless-steel group was not significantly different than the non-contact surfaces in the before pack collected samples. The after-pack group had the non-contact surfaces (floor, drain, collection table) with the highest biofilm-forming capability than the rest of the sample groups. The biofilm forming data seems to suggest that differences in the biofilm formation capability are probably microbial adaptations due to plant sanitation schedule and management.

Composition of the microbial community: The 16S metagenomic analysis showed that the microbial population of the surfaces consists mainly of microorganisms from the phyla Bacteria, Bacterioides, Acidobacteria, Actinobacteria (especially for the after packing samples), Proteobacteria, Gammaproteobacteria, Firmicutes, and Bacilli. The composition of the microbial population varied for the before and after packing samples (Supplemental Figures 6-7, Facility 2; Supplemental Figures 8-9, Facility 3; and Supplemental Figures 10-11, Facility 1). In general, the before packing samples seem to be more diverse than the after packing samples for Facility 2 (it correlates with the plate count data). Also, samples collected in the wash/wax area of the packinghouse were more diverse than the samples collected in the dry (sorting area) of the

facilities. Microbial composition was different between the dry-wet area: Facility 2 indicated Firmicutes, Proteobacteria, Bacterioidetes isolated in the dry area and Proteobacteria, Lactobacillales and Clostridia isolated in the wash/wax area.

A diversity analysis was performed to compare the unique and shared OTUs between the microbial species detected before and after packing. The sanitation techniques do not remove the microorganisms entirely, or the residual microorganisms have favorable conditions to recover of surfaces, since there are 893 before-after shared OTUs for Facility 1, 117 shared OTUs for Facility 2, and 897 shared OTUs for Facility 3 (**Table 2**). The metagenomic analysis did not include samples collected right after cleaning/sanitation procedures.

Biofilm formation of *L. monocytogenes* single and mixed cultures with packinghouse strains: The pathogen can form biofilms in single cultures. A mature biofilm of *L. monocytogenes* Petite Scott A grown in LSM, under laminar flow conditions (no turbulent flow) can form in 72 hours. In the pure *Listeria* biofilms, cells orient themselves in groups (palisades) and grow vertically around microchannels that presumably transport oxygen and nutrients to the cells located in the lower levels of the biofilm (**Figure 1A** and **1B**). The orientation around the microchannels gives the overall impression of honeycomb appearance (1B) of the biofilm. The biofilm at 24 hours of growth is patchy with cells building the biofilm structure vertically. The 48-h grown biofilm is uniform, less patchy, with cells forming a uniform layer of biofilm. The honeycomb structure is still visible, with cells orienting themselves around biofilm's microchannels for nutrients and oxygen (**Figure 1D**). After 72 hours of growth, the biofilm is mature with the structure collapsing and with cells and clumps of cells ready to detach and colonize other surfaces (**Figure 1E** and **1F**). In contrast, environmental strains have a rapid growth rate, and they outcompete *L. monocytogenes*. The multiple species biofilms seem to incorporate Listeria and no particular pattern was observed for the studied mixed biofilm. The pathogen growth was not inhibited in any of the combinations, suggesting that there was no competitive exclusion. There were two types of environmental biofilms formed in the cells: constant cell number with increased extracellular matrix (Supplemental Figure 16C, D and E), and biofilms with less extracellular polymer production and increased cell number.

Objective 2. *Determine factors important in L. monocytogenes persistence and colonization of the packinghouse. Determine L. monocytogenes growth parameters in refrigeration conditions.*

Microscopy observations indicated that *L. monocytogenes* biofilms have a metabolism with reduced cellular redox potential. Staining the *L. monocytogenes* biofilms with 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC) resulted in no dye incorporated by the biofilm cells. CTC is a tetrazolium salt that is used for staining and quantification of live, actively respiring microorganisms. CTC is soluble in water and forms a nearly colorless nonfluorescent solution outside the microbial cell. Inside the cell, the dye interferes with the electron transport system, as a redox participant instead of oxygen, the actual final electron acceptor. Actively respiring bacteria placed in CTC solution reduce it to insoluble formazan (CTC formazan), which accumulates in the cells, and stains them red. Formazan emits a red fluorescence emission peak (630 nm) when excited at 480 nm. On the other hand, inactive cells show no accumulation of CTC formazan. In our observations, *L monocytogenes* grown as biofilm did not accumulate formazan (metabolically inactive) (**Figure 2A**), however, the environmental packinghouse strains did utilize the dye as a final electron receptor (**Figure 2B**).

We then investigated the cells' metabolic state in single and multiple species biofilms by the real-time quantification of significant genes involved in aerobic respiration, and the level of the electron carriers in the cells (NAD+/NADH and NADP+/NADPH). Since biofilms have a complex

structure, composed of multiple species of microorganisms, we studied the architecture and metabolic activity of cells within the biofilm layers by laser capture microdissection microscopy (LMD). The method allows viewing of the vertical biofilm sections (using any of the microscope lenses) and the laser dissection or cut of the area of interest. Cut samples can be obtained from as little as one cell up to small groups from particular regions in the biofilms (Supplemental Figure 12A and B).

L. monocytogenes biofilms were grown on coupons with the flow-through system. Listeria minimal media (LSM) flowed at a constant rate of 0.5 ml/min and biofilms were allowed to develop for 72 hours. The flow cells were than dismantled and the biofilm coupons were covered with Tissue Tek OCT (optimal cutting temperature compound) and frozen immediately on dry ice. The biofilms were treated with the gel using the same gasket from the flow cell, carefully not to shift any biofilm cells during treatment. Embedded biofilms were then mounted on the cryotome chuck with cold OCT gel and then biofilms were sliced starting with 5 microns thick slices. Experiments were performed to optimize the slice thickness. Slices were then mounted on Leica PEN (polyethylene naphthalate) membrane slides and used for laser microdissection (Leica with DFC7000T camera).

Microdissected areas of the biofilms were cut from different biofilm layers and then captured using TriReagent in caps of microcentrifuge tubes (Supplemental Figure 12A and B). Samples obtained by LCM were then purified for RNA isolation following established protocols. The obtained nucleic acid from different layers were used to obtain test the hypothesis that *L. monocytogenes* biofilms have heterogenous metabolic activity. Target gene for measuring expression was the global regulator *rexA* (transcription factor that responds to intracellular redox potential in Gram-positive bacteria). Probes were synthesized with by PrimerQuest (IDT DNA) with a 5' 6-carboxyfluorescein (6-FAM) reporter dye and a 3' ZEN™dark quencher dye (Supplemental Table 5). Transcript levels were normalized against housekeeping genes *rpoS* and *bglA*, and qRT-PCR was performed on a Roche 96 Lightcycler. Real-time PCR was performed with LightCycler® EvoScript RNA Probes Master in a one-step RT qPCR. The differences in the transcript levels of *rexA* (Supplemental Figure 12C) using RNA template from cells cut from different thickness of the biofilms indicated that the biofilm is heterogenous and that *L. monocytogenes* cells have altered metabolic activity with the biofilm layers.

To investigate the interference in the overall reducing power of *L. monocytogenes* cells in biofilms, we measured the level of the main enzymes involved in microbial respiration: oxidized nicotinamide adenine dinucleotide (NAD+) and reduced nicotinamide adenine dinucleotide (NADH) and then a second set consisting of oxidized nicotinamide adenine dinucleotide phosphate (NADP+) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). These enzymes are part of the oxidative phosphorylation system in the cell and help maintain a proton concentration gradient across the microbial membrane by transferring electrons to molecular oxygen.

The concentration of redox enzymes in the cells was measured with a luminescence assay (Promega Corporation). Typically, the redox potential of NADP+/NADPH at standard physiological conditions is similar to NAD+/NADH although the function of the cofactors is different, and their presence (ratios) indicates different metabolic state of the microbial cell. NADP+/NADPH is used for anabolic redox reactions, whereas NAD+/NADH is used for oxidation reactions (degradation of compounds in the microbial cell). The results indicated that the ranges of the electron carriers varied significantly, but the important result was their ratio in the cells. In the background microflora cells (*Pseudomonas* and *Burkholderia* isolated in the previous project), the primary electron carriers were represented by high level of NAD+/NADH, and significantly lower NADP+/NADPH. In contrast, *L. monocytogenes* biofilms presented a flipped ratio of electron carriers: high NADP+/NADPH and low NAD+/NADH ratio (Supplemental Figure 12D). In other firmicutes, RexA has an important role in central metabolism, oxidative stress, and biofilm formation. It is possible that in *L. monocytogenes* high levels of RexA are

associated with biofilm formation and that the NAD/NADP ratio change is a physiological adaptation to the sessile fife.

The low level of reducing power in *L. monocytogenes* has practical implications. Subliminal concentrations of strong oxidizing agents such as chlorine can initiate the rapid oxidation in the cells of highly reactive molecules, such as NADH. In general, environmental isolates indicated a high redox balance (high NAD/NADH ratio) in mixed species biofilms (Figure 2B and Supplemental Figure 13A). In these conditions, if low concentrations of chlorine reach the mixed species biofilm, the sanitizer would react preferentially with the environmental isolates' biomass and not *L. monocytogenes*. Inactivation studies were not performed for this objective, but our results suggest that *L. monocytogenes* may be present in a reduced metabolic state in single culture or complex biofilms, which can help protect the pathogen against oxidative stress and sanitizers that are applied in suboptimal concentrations.

Growth kinetics of individual Listeria monocytogenes monoculture biofilms on simulated packinghouse surfaces and in refrigeration conditions:
L. monocytogenes biofilms can grow at low temperatures in conditions simulating the packinghouse if nutrients and moisture are available. In our experimental conditions, biofilms were developed for 16 days (Supplemental Figure 14). To compare the growth parameters among different conditions, the Gompertz equation was used to model the data. The resulting params account only for live bacteria growing in the biofilm, just as a nonlinear growth model.

Although *Listeria* biofilms grow well in refrigeration conditions, one common denominator in our results is the long time for the lag phase. The time required for cell adaptation to the new conditions can take up to 137 hours, and the lag phase could account for the long time required for *L. monocytogenes* biofilm development at low temperatures. Our observations indicated that biofilm slouching (detaching) probably occurred around 340 hours of growth (based on the decline in the plate counts recorded after 340 hours).

Biofilms grown on PVC required shorter time for the maximum growth rate and had longer lag phase compared with stainless steel biofilms (0.09 and 0.02 versus 137.31 and 84.78, respectively) The initial bacterial levels (y0 - in this case the value can be considered as initial attachment) were higher on the PVC coupons than stainless steel (2.65 and 2.55 versus 1.78 and 2.01, respectively), suggesting that perhaps *Listeria* attached in higher number on the PVC coupons and biofilm development was different. The maximal population (yEnd) was also lower on the PVC coupons than stainless steel suggesting biofilm detachment on these surfaces. The presence of mineral oil-based coating did not seem to have an influence on biofilm formation.

Growth kinetics of the Listeria monocytogenes in mixed cultures on simulated packinghouse surfaces in refrigeration conditions:
L. monocytogenes biofilms were grown in mixed culture with previously isolated packinghouse cold room microflora and developed for 16 days (Supplemental Figure 15). The mixed culture biofilms showed overall longer lag phase (between 103.91 and 122.31) than monoculture biofilms and faster maximal growth rate (between 0.05 to 0.09) for *Listeria* than the monoculture biofilms. There were no differences regarding the presence or absence of mineral oil-based coating. Similar to the monoculture experiments, the PVC-grown biofilms had a lower maximal population (yEnd for stainless steel was 10.07 log CFU versus 7.24 log CFU for PVC). This suggests that the biofilm configuration on PVC is probably different and the biofilm detachment occurred before the biofilm grown on stainless steel.

The films from the mixed cultures were also plated on non-selective media to estimate the cell number for the co-cultures. The co-inoculated bacteria did not grow, their biofilm numbers maintained around 3.5 log CFU/coupon.

Objective 3. *Validate the biofilm development parameters in a pilot plant study; build a mathematical model of biofilm growth and detachment and develop an Excel add-in to predict optimal sampling time and sanitation schedule.*

Laboratory experiments to determine the growth parameters were performed for this objective and a pilot plant study was not performed. The study was to take place at a South Carolina packinghouse in December 2021 with imported stone fruit from Chile, since the trials were out of the domestic fruit season. Another major roadblock was the lack of a clear procedure on inoculating large food-contact surfaces with environmental microflora that would have been already subcultured a number of times. In collaboration with the CPS Technical Committee, it was decided that the trial should be postponed and run concurrently with a California packing-line trial that is scheduled for the research team's 2022 CPS project (in September-October 2022). To avoid bias in calculating the growth rates, the California trial will involve a packing line in a facility that has been used extensively for the packing season. The packing line will already have the microorganisms present on surfaces; in addition, the biofilm buildup will be monitored through the packing season. With this experimental setup, we would avoid bias from both a faulty inoculation procedure and working in almost sterile conditions that are typically found in the standard pilot plant. This experimental plan would require periodical sampling of the packing line throughout the season (April-September) to monitor microbial population over time. The trial will be performed as described, except ATP swabs will be included in the sample collection as explained below.

Experiments were performed in the laboratory to determine the influence of the growth substrate (media) and the presence of additional substances (wax) on packinghouse surfaces. For this objective we focused mostly on the brush area. Three types of media were compared for the maximal cell biomass for *L. monocytogenes* and mixed environmental strains (**Table 3**). Plate counts from inoculated cultures indicated that microbial growth is dependent on the availability of nutrients, for both *Listeria* and environmental strains. Surprisingly, the PE supports the growth of *L monocytogenes*; our results indicate that PE can support growth for *L. monocytogenes* (more than 6 log CFU over 48 hours). The addition of fruit wax (1:10 dilutions were made in the respective growth media – TSB, LSM and PE – of Prima Fresh 220, DuraFresh and Lustr) did not result in significantly different plate counts (no growth or inhibition of *Listeria* or environmental isolates).

The cell growth is correlated with biofilm formation of *L monocytogenes* and the environmental strains in PE (**Figure 3**). The tested *L. monocytogenes* and environmental groups formed biofilms when grown in PE for 48 hours (longer growth time than LSM where the typical biofilms for crystal violet were formed in 24 hours). To determine the biofilm growth rate (goal for prediction of their growth in the packinghouse) a series of experiments were performed where *L. monocytogenes* and or the environmental strains groups (Table 3) were inoculated and grown in defined conditions (e.g., flow cells in laminar flow conditions). The inoculated surfaces and the bulk phase of the biofilms were sampled by optical density and plate count (selective and non-selective media). To obtained accurate counts biofilm cells were disrupted by vortexing with 1 gram of sterile glass beads (acid washed ≤ 106 µm).

In defined conditions, the comparison of the log CFU means from the plate counts indicates that environmental strains have higher growth rate than the *Listeria* alone biofilms at the same time intervals (Supplemental Figure 17). The plate counts from *Listeria* alone and the *Listeria* isolated from the mixed biofilms were not statistically different (except for the 12-hour sampling point), which indicates that the pathogen can grow within the biofilm matrix formed by environmental microflora, just as a single-species biofilm.

The mixed biofilms experiments in flow-through enclosures (*Listeria*, environmental microflora, and mixed environmental and *Listeria*) have been performed for 10 select samples from those submitted to genomic sequencing (**Table 4**).

Growth rates of biofilms were calculated by dividing the number of newly divided cells (estimated in the bulk phase and biofilm biomass) produced per hour at steady state by the estimated adherent cell population (determined by obtaining the viable counts of the attached resuspended biofilms). All biofilms were grown for at least 48 h under steady-state conditions (laminar flow 0.5 ml min -1). *L. monocytogenes* had a maximal growth rate (μ) of 1.7 h-1 for the first 12 hours, then 1.49 after 24 hours and then growth rate declined to 1.39 h-1 for the rest of 72 hours of growth. The growth rate of the environmental strains in 0.5x LSM in steady condition is shown in Table 4. For example, the FTBP7 (sample from the drying sponge in Supplemental Figure 16) has a maximal growth rate of 2.05 h-1 at least for the first 24 hours of growth. Because of physical constraints of the biofilm the growth rate declined to 1.73 h-1 (physical boundaries: the flow cell filled up with extracellular polymers).

Since we tested mostly environmental samples from brush beds and drying sponges, we attempted to inoculate mock brushes in our laboratory. The brushes were immersed in PE and then the environmental samples were inoculated on the surface by spraying under a laminar flow hood with a low microbial suspension (approx. 4 log CFU/ml). The microbial populations were recovered over the next 24 hours, but the inoculum failed to grow and develop a biofilm biomass comparable with the packinghouse. Since the microbial growth of cells and biofilms is dependent on the availability of nutrients, the presence of a conditioning layer, the above formula and the Excel predictor will be fully evaluated with the field trial data. The biofilm composition does not seem to influence the overall growth rate. The growth rate in the packinghouse seems to be influenced mostly by the sanitation schedule and the management practices. An accurate prediction would require the input of residual biomass after cleaning/packing (start point for microbial growth). Since plate count is not a feasible choice for packinghouses, and there are differences in the sanitation practices, another choice would be to estimate the start point of the microbial community with ATP swabs. The step will be included in the field trials in September-October 2022 (for the next project).

Outcomes and Accomplishments

More than 800 environmental samples were collected from stone fruit packinghouse facilities in California and South Carolina. The samples were collected before and after packing operations and were evaluated by total plate count, biofilm formation capability, and for select samples the multi-species present on the sampling points were identified at the genus level16S rRNA. The microbial plate counts and metagenomics indicated three different scenarios: (i) the plate count and microbial diversity were lower for the before packing than the after packing operations, (ii) plate counts and microbial composition were higher in the samples collected before packing than after, and (iii) for one facility there were no significant differences in the before and after samples. Metagenomics indicated more diverse microbiota in the wash/wax areas of the packinghouse (wet surfaces) than in the sorting area (dry surfaces). The variation in results can be explained by differences in the packinghouse design and plant sanitation practices. The environmental strains are robust biofilm-formers, suggesting that the packinghouse environment (packing, cleaning, sanitation) is a selection factor. *L. monocytogenes* can form single species biofilms, but in all performed experiments the pathogen integrated in the environmental biofilm matrix. The environmental isolates have a faster growth rate than the pathogen, probably adaptation to the packinghouse environment.

Summary of Findings and Recommendations

Prevention of biofilm formation on food-contact surfaces is important in maintaining food safety. In general, recalls involve the detection of foodborne pathogens and not plant environmental bacteria. However, the presence of these plant isolates should not be overlooked since they are robust biofilm formers, outcompete and at the same time incorporate and protect the pathogen in the mixed biomass.

APPENDICES

Publications and Presentations

- CPS 2020 Annual Symposium (virtual meeting). Poster presentation. Paul Dawson and Claudia Ionita. Verification and validation of environmental monitoring programs for biofilm control in the packinghouse
- CPS 2021 Annual Symposium (virtual meeting). Poster presentation. Paul Dawson and Claudia Ionita. Verification and validation of environmental monitoring programs for biofilm control in the packinghouse.
- Two manuscripts are in preparation.

Budget Summary

Due to numerous COVID-19 restrictions, the initial one-year project was extended to a two-year project. The project was awarded a total of $161,182 in research funds. Expenditures were primarily for salaries and fringe benefits, supplies, and some travel. Funding was sufficient to complete the project.

Suggestions to CPS

We thank CPS for their support to complete the project, and their help in overcoming certain technical difficulties. We would also like to thank Mr. George Nikolich for his assistance in the collection of the packinghouse environmental samples and obtaining the peach wash water. The principal investigator and the postdoctoral fellow are grateful to and would like to thank the packinghouse management of the facilities that participated in the study. Clemson University is acknowledged for generous allotment of compute time on the Palmetto cluster.

Tables 1–4, Figures 1–3, and Supplemental Figures and Tables (see below)

Table 1. Summary of paired-sample t test for the total plate count of environmental samples collected from stone fruit packinghouses

Facility	Group of samples		*t*	df	Sig (2 tailed)
	Mean log CFU before pack	Mean after pack			
Plant 1	6.730 (N=66)	9.5873 (N=66)	-12.931	65	0.001
Plant 2	10.194 (N=42)	8.942 (N=42)	5.889	41	0.001
Plant 3	9.708 (N=34)	9.466 (N=34)	1.678	33	0.103
Plant 4	10.747 (N=40)	9.645 (N=40)	4.240	39	0.001
Plant 5	6.971(N=56)	8.139 (N=56)	-9.212	55	0.001
Plant 6	6.381 (N=58)	8.020 (N=58)	-12.753	57	0.002
Plant 7	4.905 (N=50)	7.821 (N=50)	-17.755	49	0.001

Table 2. Number of OTUs identified for before and after packing and the percentage of OTUs shared for the before and after packing samples

Facility	OTU number species group				
	Before pack (BP)	After pack (AP)	Shared sequences BP/AP	Percentage of OTUs shared	Total richness all groups
1	4532	4477	893	11.003	8116
2	2720	230	117	4.12	2833
3	3368	4555	897	12.76	7026

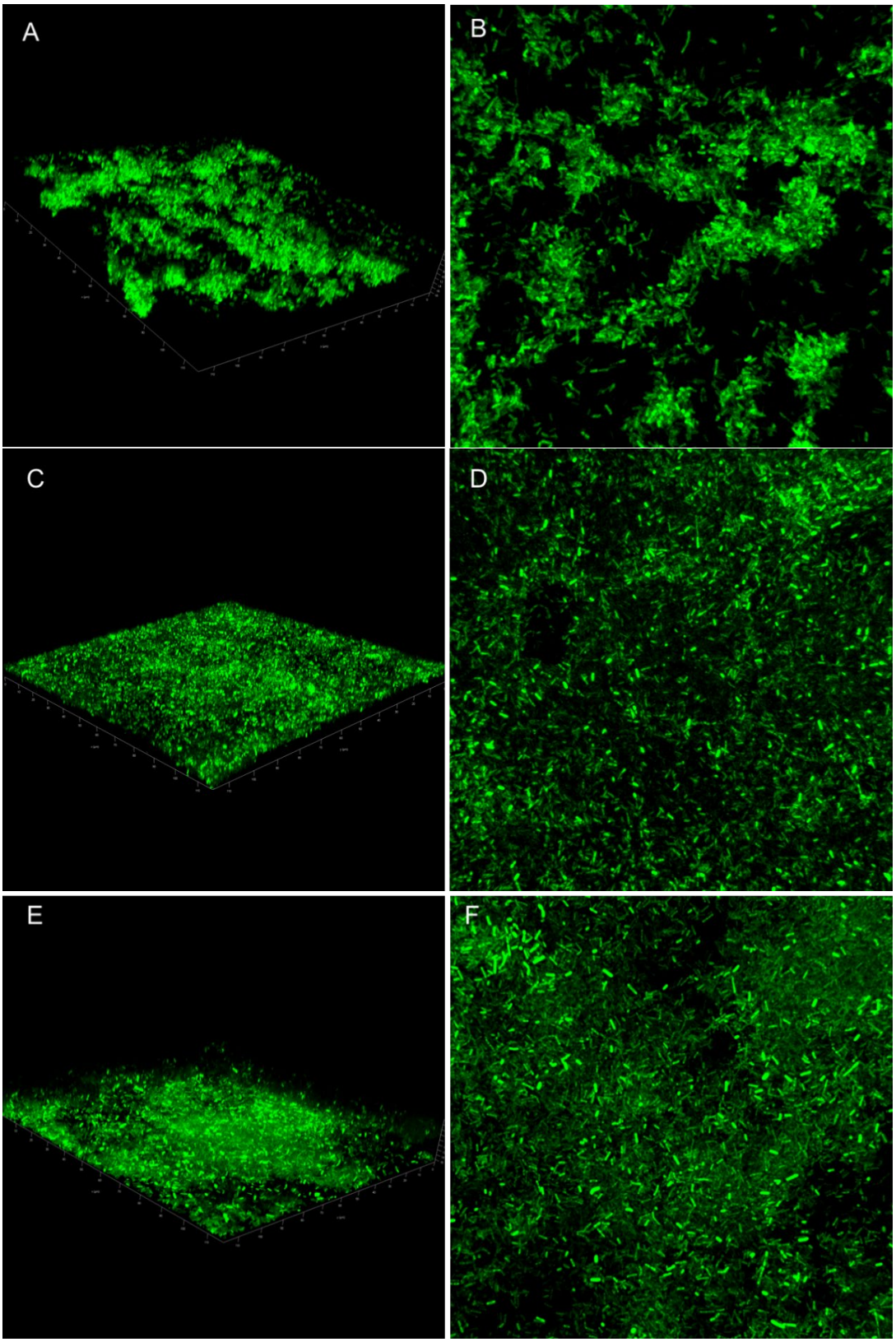


Figure 1. Biofilms of *L monocytogenes* Petite Scott A grown for 24 hours (A and B), 48 hours (C and D) and 72 hours (E and F). Biofilms were imaged with a Leica SP8 confocal microscope under 20x objective (panels A, C and E) and 100x (panels B, D and F).

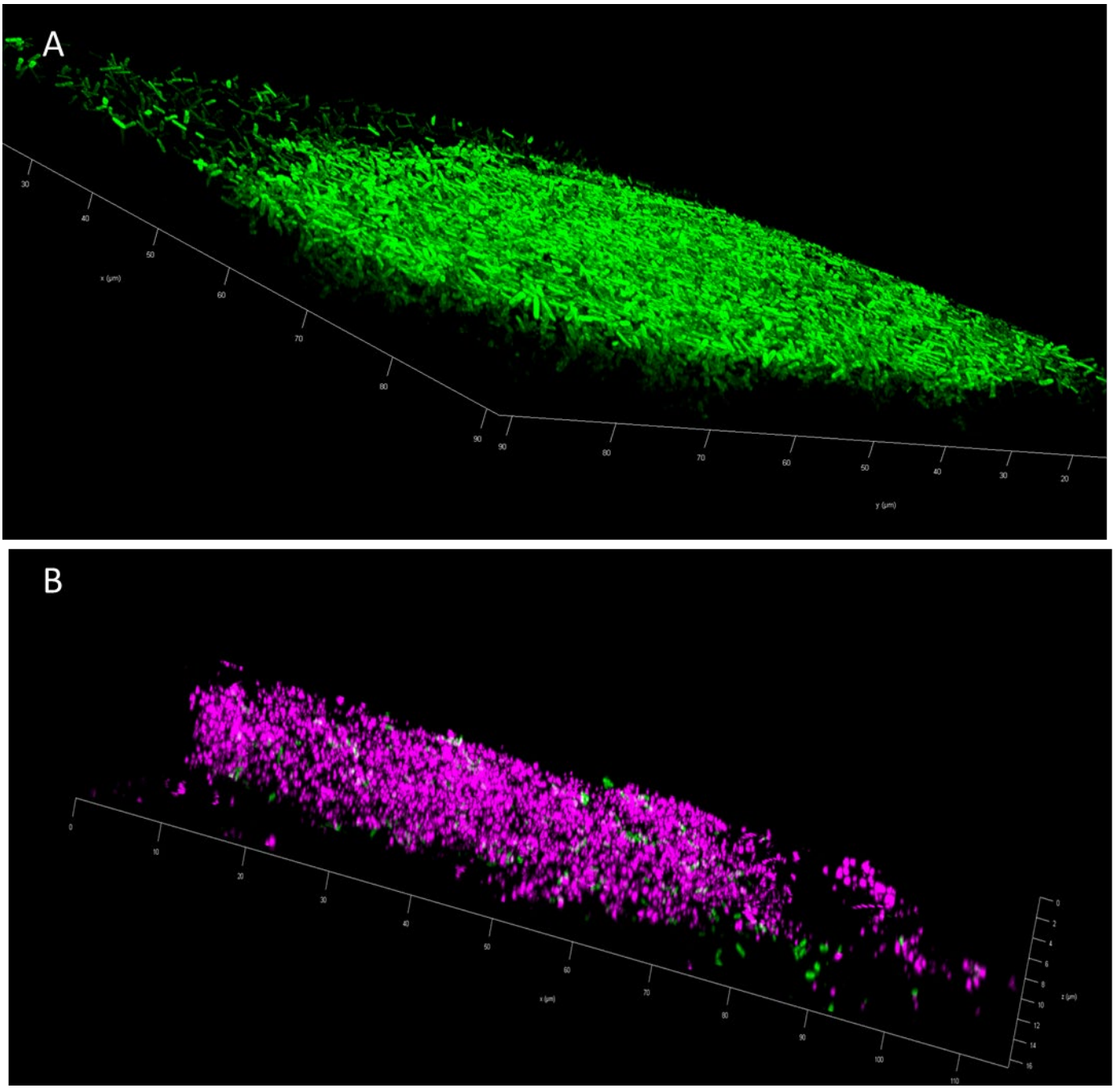


Figure 2. Side view of a *L monocytogenes* biofilm (A) and a mixed biofilm (Listeria co-inoculated with packinghouse environmental strains- panel B). *Listeria* cells appeared green (GFP protein) and the environmental isolates have been stained red with 5-Cyano-2,3-Ditolyl Tetrazolium Chloride (CTC), an indicator of microbial viability.

Table 3. Maximal cell biomass (plate counts) of *L. monocytogenes* and 10 select environmental mixed species in PE, LSM and TSB. Growth was monitored spectrophotometrically and at the end of the 24 hours growth time samples were plated for microbial count except for PE samples. The maximal growth for the PE grown isolates was measured at the 48 hours. Numbers represent the means from 2 different experiments.

	PE	**LSM**	**TSB**
Strain/Group	Log CFU/ml	Log CFU/ml	Log CFU/ml
L monocytogenes	6.29	7.1	7.75
WBP4-green brush	5.26	8.4	8.52
WBP7-red brush	6.44	8	8.58
WBP8-black brush	6.87	7.67	8.02
WBP9-wax brush	6.03	7.67	7.59
FTBP4-green brush	6.85	8.08	7.95
FTBP5-white brush	6.76	7.04	7.7
FTBP7-sponge	6.61	7.45	8.03
FTBP8-wax brush	6.89	7.55	8.08
HMCBP6	6.31	7.89	8.14
HMCBP7	6.42	8.05	8.48

Strain/group	**Absorbance @590nm ±SD**
L monocytogenes	1.26 ± 0.52
WBP4-green brush	2.37 ± 0.2
WBP7-red brush	0.88 ± 0.11
WBP8-black brush	2.15 ± 0.17
WBP9-wax brush	1.97 ± 0.52
FTBP4-green brush	2.11 ± 0.62
FTBP5-white brush	1.94 ± 0.4
FTBP7-sponge	3.91 ± 0.86
FTBP8-wax brush	3.27 ± 0.93
HMCBP6	3.02 ± 0.42
HMCBP7	2.91 ± 0.77

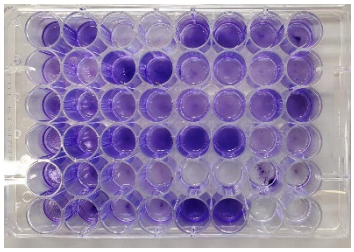
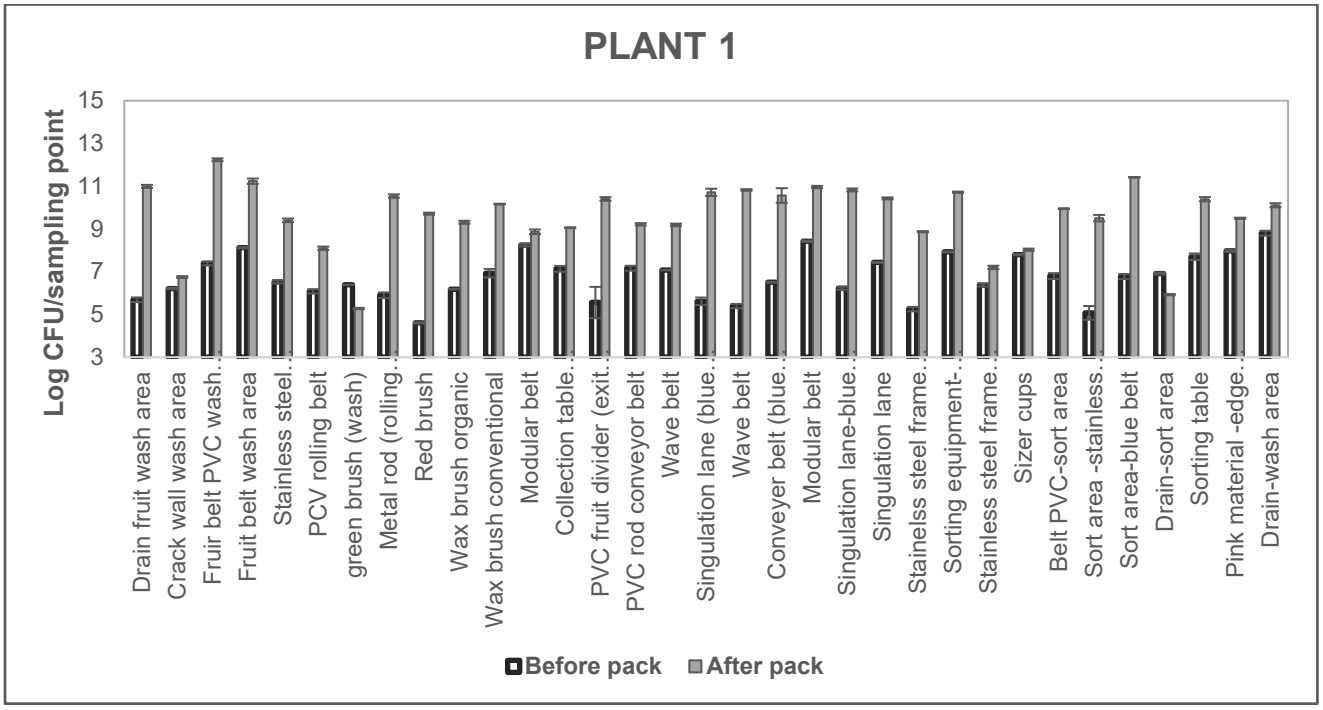


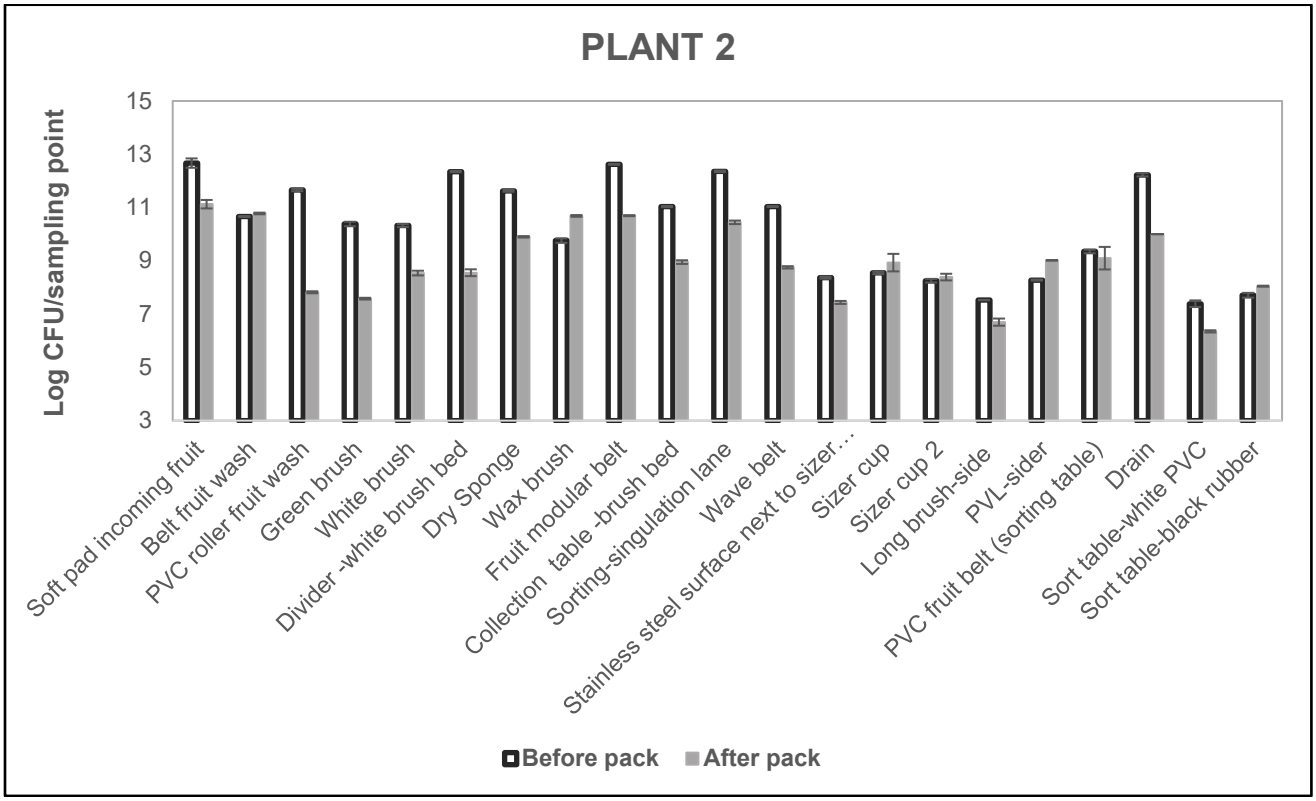
Figure 3. Example of 48-well plate stained with 0.1% crystal violet. Strains were grown in PE for 48 hours and then washed and stained as described above. The table values indicate stained biofilm biomass (absorbance at 590nm) for *L monocytogenes* and the environmental groups.

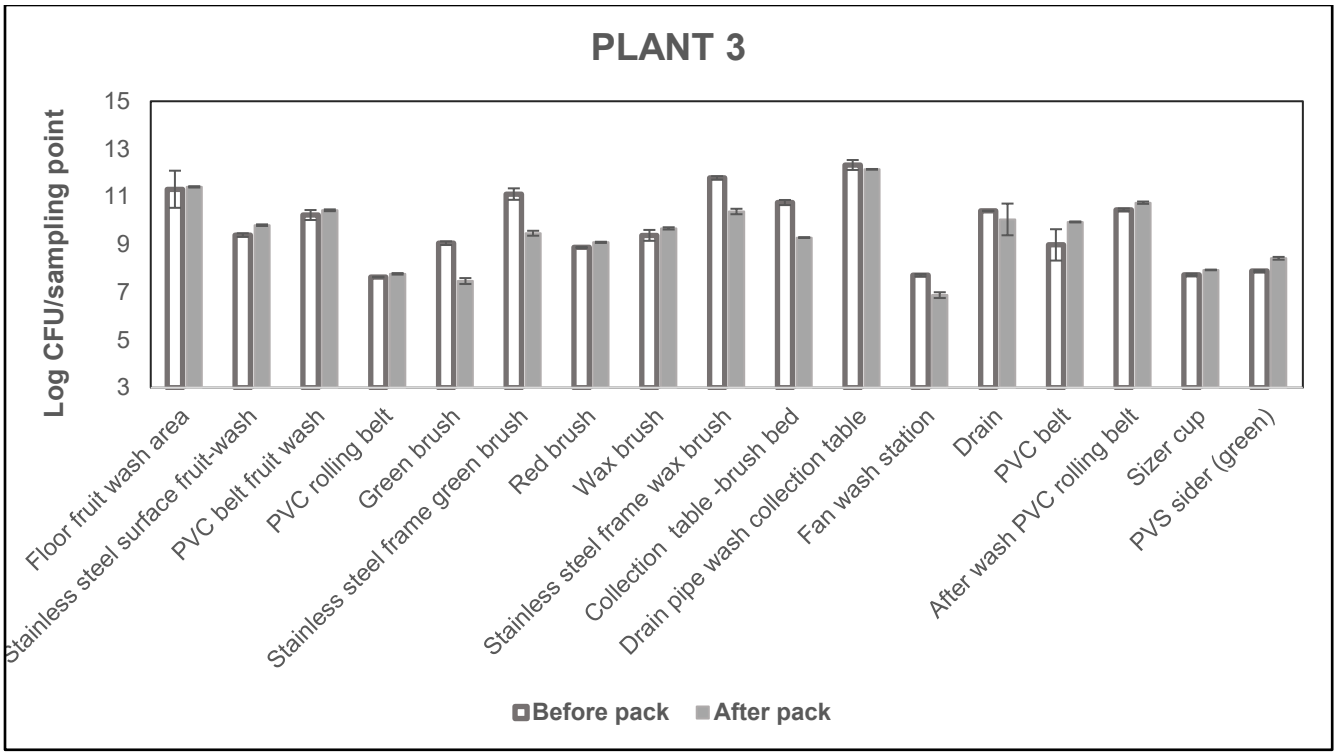
Table 4. Averaged growth rates for the select environmental isolates

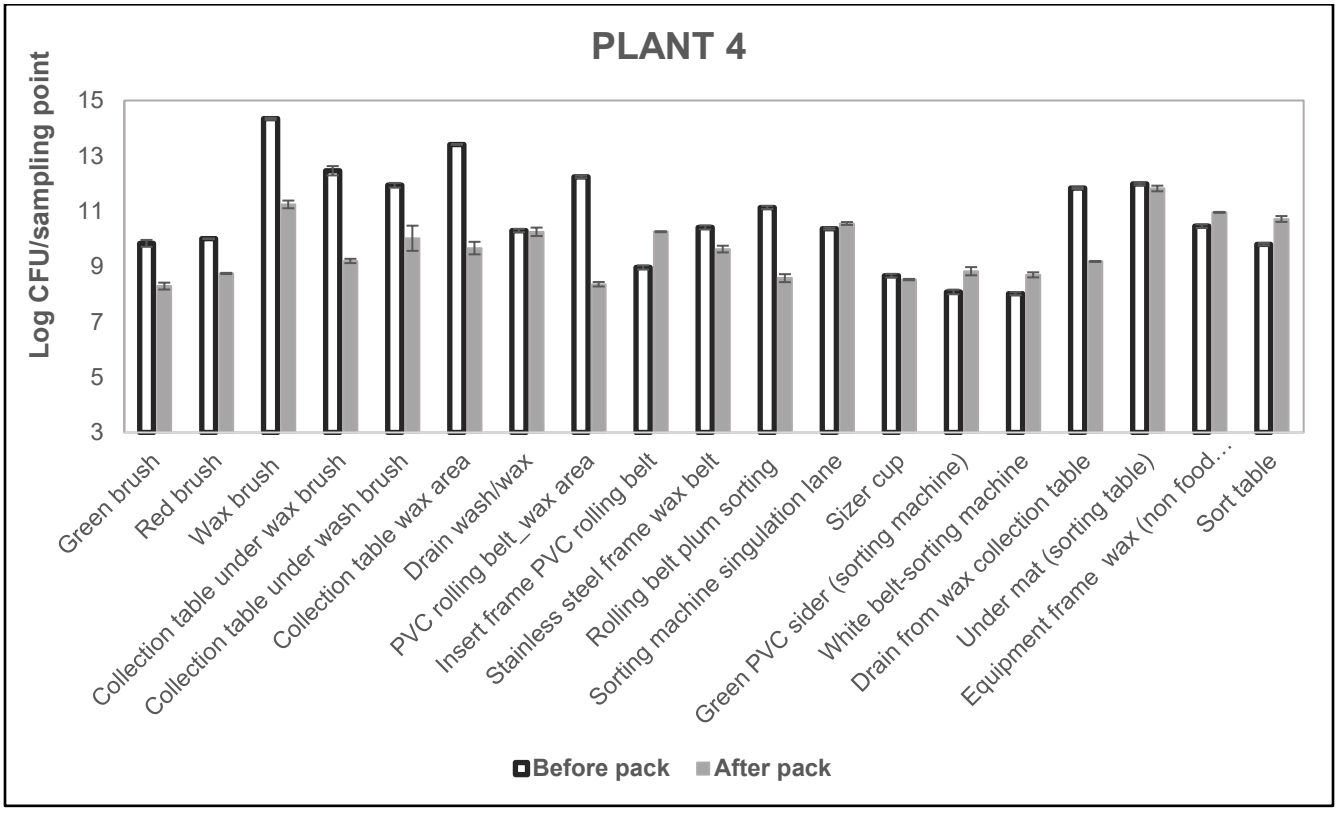
Strain/Group	**Average growth rate (μ h-1)**	**Consideration biofilm growth for calculation purposes (h)**
WBP4-green brush	2.02	24
WBP7-red brush	1.97	24
WBP8-black brush	1.84	24
WBP9-wax brush	1.99	24
FTBP4-green brush	2.07	24
FTBP5-white brush	2.18	24
FTBP7-sponge	2.05	24
FTBP8-wax brush	2.13	24
HMCBP6	2.06	24
HMCBP7	2.09	24

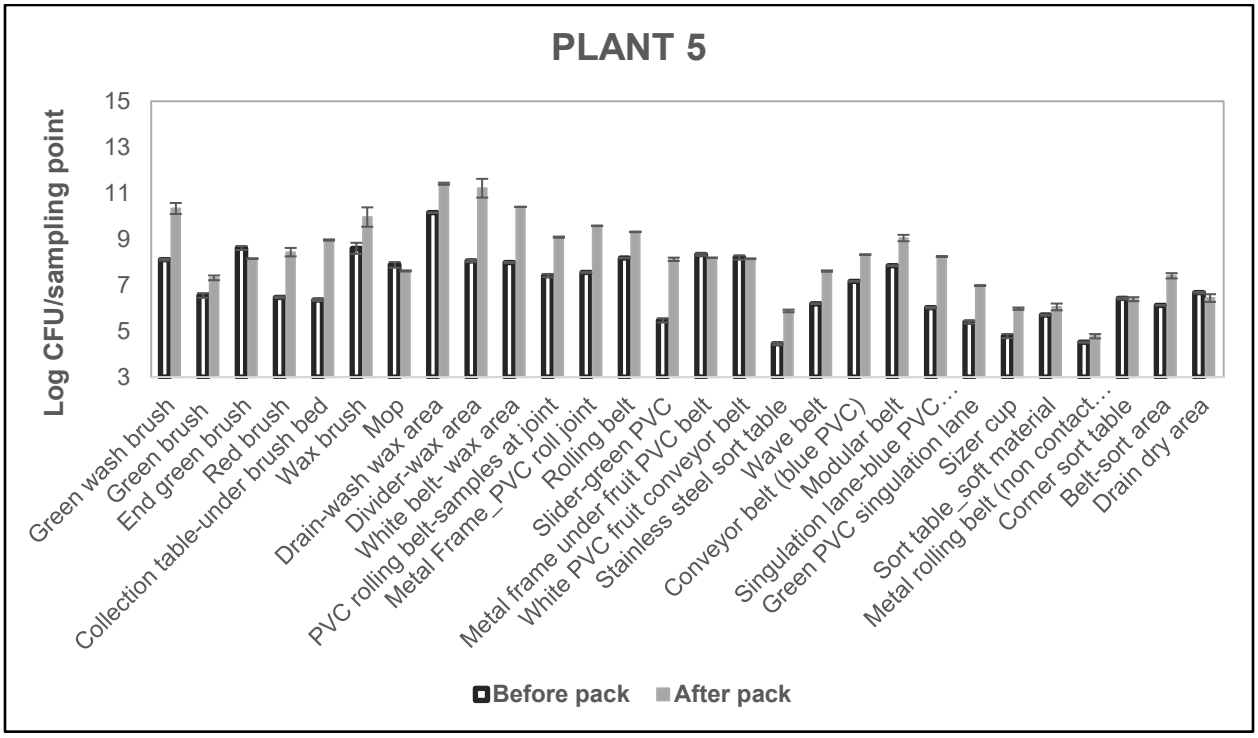
Supplemental Figures and Tables

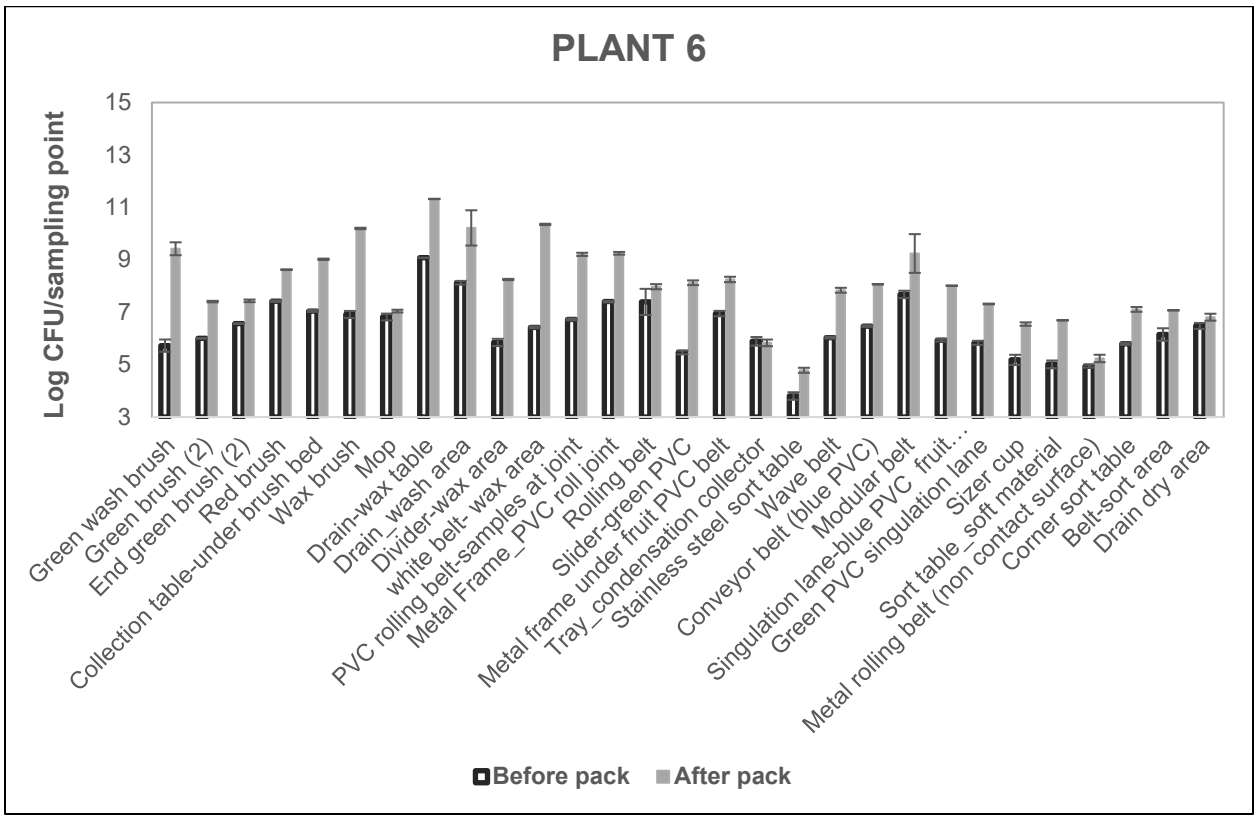


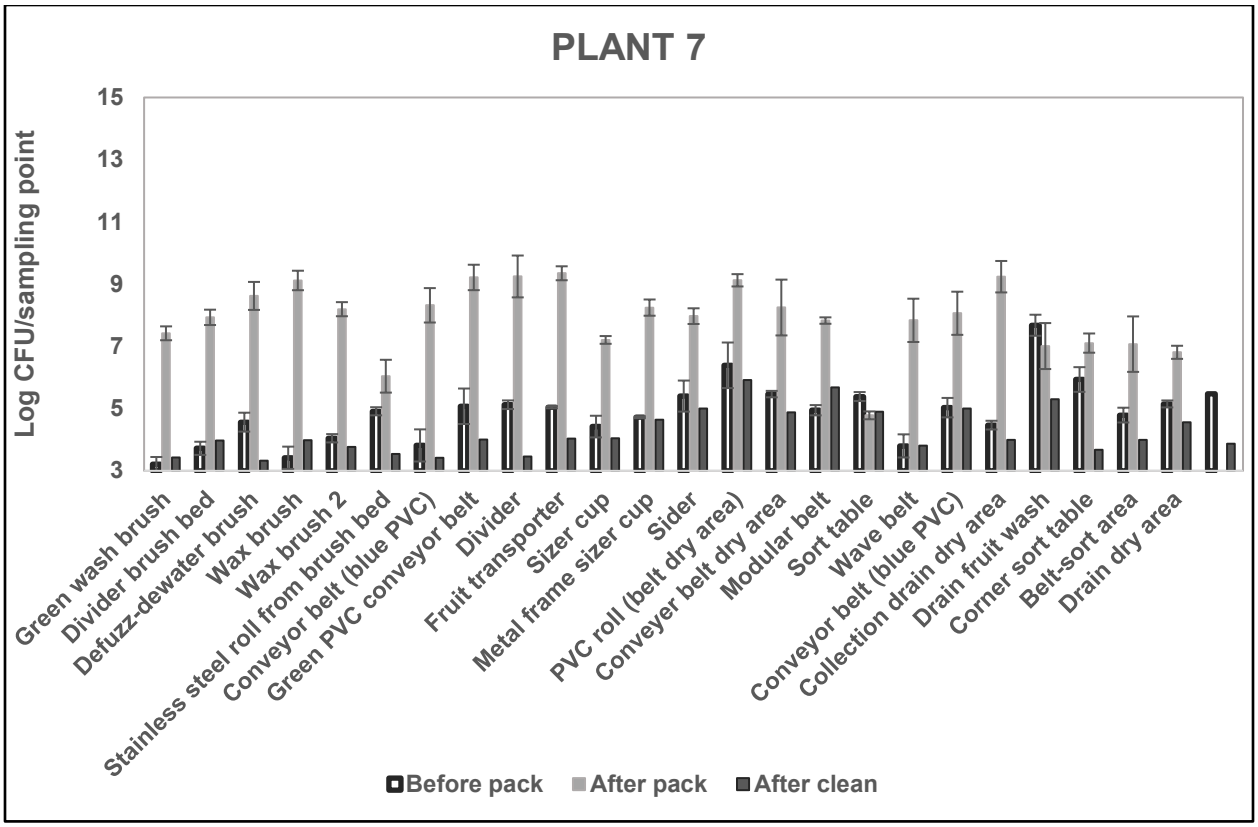



PLANT 3
Log CFU/sampling point
15
13
11
9
7
5
3
Floor fruit wash area
Stainless steel surface fruit-wash
PVC belt fruit wash
PVC rolling belt
Green brush
Stainless steel frame green brush
Red brush
Wax brush
Stainless steel frame wax brush
Collection table -brush bed
Drain pipe wash collection table
Fan wash station
Drain
PVC belt
After wash PVC rolling belt
Sizer cup
PVS sider (green)
Before pack
After pack


PLANT 4
Log CFU/sampling point
15
13
11
9
7
5
3
Green brush
Red brush
Wax brush
Collection table under wax brush
Collection table under wash brush
Collection table wax area
Drain wash/wax
PVC rolling belt_wax area
Insert frame PVC rolling belt
Stainless steel frame wax belt
Rolling belt plum sorting
Sorting machine singulation lane
Sizer cup
Green PVC sider (sorting machine)
White belt-sorting machine
Drain from wax collection table
Under mat (sorting table)
Equipment frame wax (non food…
Sort table
Before pack
After pack


PLANT 5
Log CFU/sampling point
15
13
11
9
7
5
3
Green wash brush
Green brush
End green brush
Red brush
Collection table-under brush bed
Wax brush
Mop
Drain-wash wax area
Divider-wax area
White belt- wax area
PVC rolling belt-samples at joint
Metal Frame_PVC roll joint
Rolling belt
Slider-green PVC
Metal frame under fruit PVC belt
White PVC fruit conveyor belt
Stainless steel sort table
Wave belt
Conveyor belt (blue PVC)
Modular belt
Singulation lane-blue PVC…
Green PVC singulation lane
Sizer cup
Sort table_soft material
Metal rolling belt (non contact…
Corner sort table
Belt-sort area
Drain dry area
Before pack
After pack


PLANT 6
Log CFU/sampling point
15
13
11
9
7
5
3
Green wash brush
Green brush (2)
End green brush (2)
Red brush
Collection table-under brush bed
Wax brush
Mop
Drain-wax table
Drain_wash area
Divider-wax area
white belt- wax area
PVC rolling belt-samples at joint
Metal Frame_PVC roll joint
Rolling belt
Slider-green PVC
Metal frame under fruit PVC belt
Tray_ condensation collector
Stainless steel sort table
Wave belt
Conveyor belt (blue PVC)
Modular belt
Singulation lane-blue PVC fruit…
Green PVC singulation lane
Sizer cup
Sort table_soft material
Metal rolling belt (non contact surface)
Corner sort table
Belt-sort area
Drain dry area
Before pack
After pack



Supplemental Figure 1. Plate count data (total log CFU/surface swabbed) for the sampled facilities in California and South Carolina. Plants 1-6 were sampled one round each before and after packing. Facility number 7 was sampled before and after packing, and after cleaning operations on two separate sampling sessions.

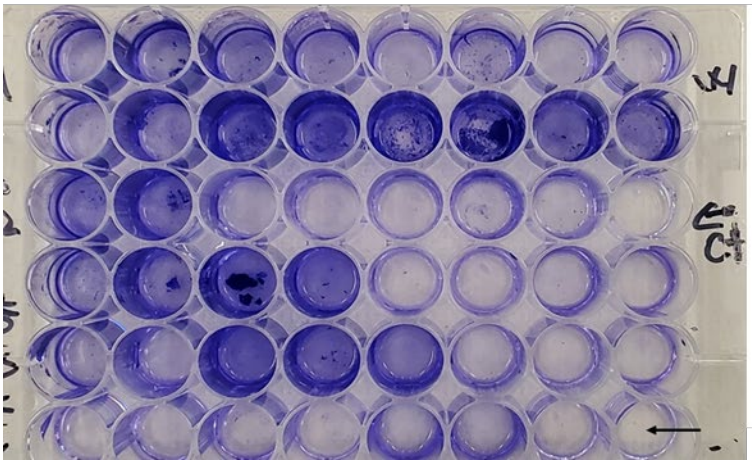


Figure 2. Example of crystal violet-stained biofilms formed on 48-well plates. Arrow points out to the negative control (no inoculum) wells.

Supplemental Table 1. Description of samples selected for genetic sequencing-Facility 1

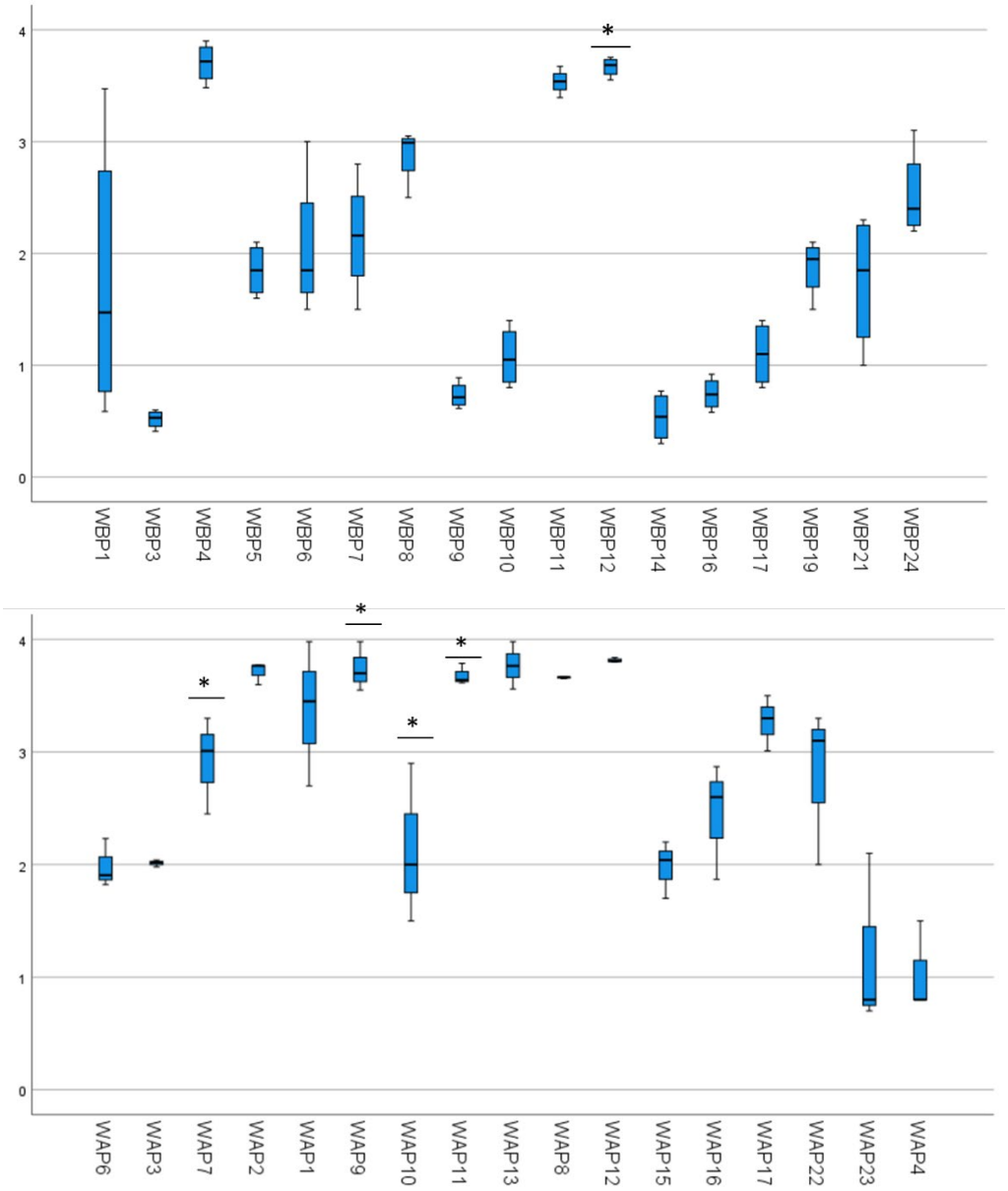
BEFORE PACKING			***AFTER PACKING***		
Sample number	Sample code	Location	Sample number	Sample code	Location
1	WBP1	Fruit belt	1	WAP6	Fruit belt
2	WBP3	PVC rod fruit conveyor	2	WAP3	PVC rod fruit conveyor
3	WBP4	Green brush	3	WAP7	Green brush
4	WBP5	Wash area-wall	4	WAP2	Wash area-wall
5	WBP6	Wash area drain	5	WAP1	Wash area drain
6	WBP7	Red brush	6	WAP9	Red brush
7	WBP8	Wash brush	7	WAP10	Wash brush
8	WBP9	Wax brush	8	WAP11	Wax brush
9	WBP10	Collection table (wax brush)	9	WAP13	Collection table (wax brush)
10	WBP11	Metal rod-packaging conveyor	10	WAP8	Metal rod-packaging conveyor
11	WBP12	Modular belt	11	WAP12	Modular belt
12	WBP14	Dry-PVC rod conveyor belt	12	WAP15	Dry-PVC rod conveyor belt
13	WBP16	Wave belt	13	WAP16	Wave belt
14	WBP17	Sorting machine-singulation lane	14	WAP17	Sorting machine-singulation lane
15	WBP19	Sizer cup	15	WAP22	Sizer cup
16	WBP21	Dry fruit belt	16	*WAP23*	*Dry fruit belt*
17	WBP24	Dry sorting table		*WAP4*	*Dry sorting table*

Supplemental Table 2. Description of samples selected for genetic sequencing-Facility 2

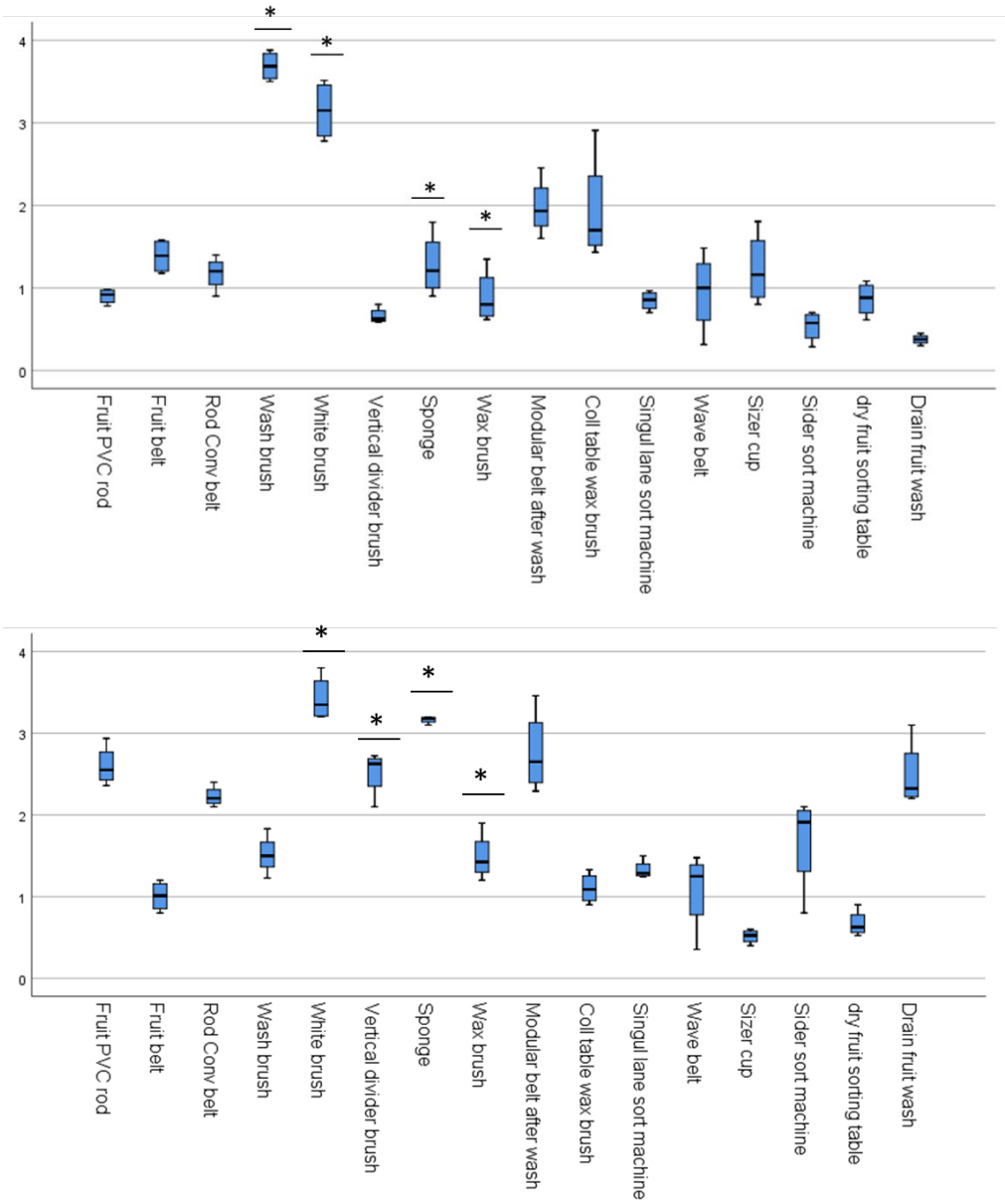
BEFORE PACKING			***AFTER PACKING***		
Sample number	Sample code	Location	Sample number	Sample code	Location
1	FTBP1	PVC rod fruit conveyor	1	FTAP1	PVC rod fruit conveyor
2	FTBP2	Fruit belt	2	FTAP2	Fruit belt
3	FTBP3	Rod conveyor belt	3	FTAP3	Rod conveyor belt
4	FTBP4	Wash brush	4	FTAP4	Wash brush
5	FTBP5	White brush	5	FTAP5	White brush
6	FTBP6	Divider brush bed	6	FTAP6	Divider brush bed
7	FTBP7	Sponge	7	FTAP7	Sponge
8	FTBP8	Wax brush	8	FTAP8	Wax brush
9	FTBP9	Modular belt after wax	9	FTAP9	Modular belt after wax
10	FTBP10	Collection table (wax brush)	10	FTAP10	Collection table (wax brush)
11	FTBP11	Sort machine-singulation lane	11	FTAP11	Sort machine-singulation lane
12	FTBP12	Wave belt	12	FTAP12	Wave belt
13	FTBP14	Sizer cup	13	FTAP14	Sizer cup
14	FTBP17	Sort machine-sider	14	FTAP17	Sort machine-sider
15	FTBP18	Sorting table-dry area	15	FTAP18	Sorting table-dry area
16	FTBP20	Drain fruit wash	16	FTAP19	Drain fruit wash

Supplemental Table 3. Description of samples selected for genetic sequencing-Facility 3

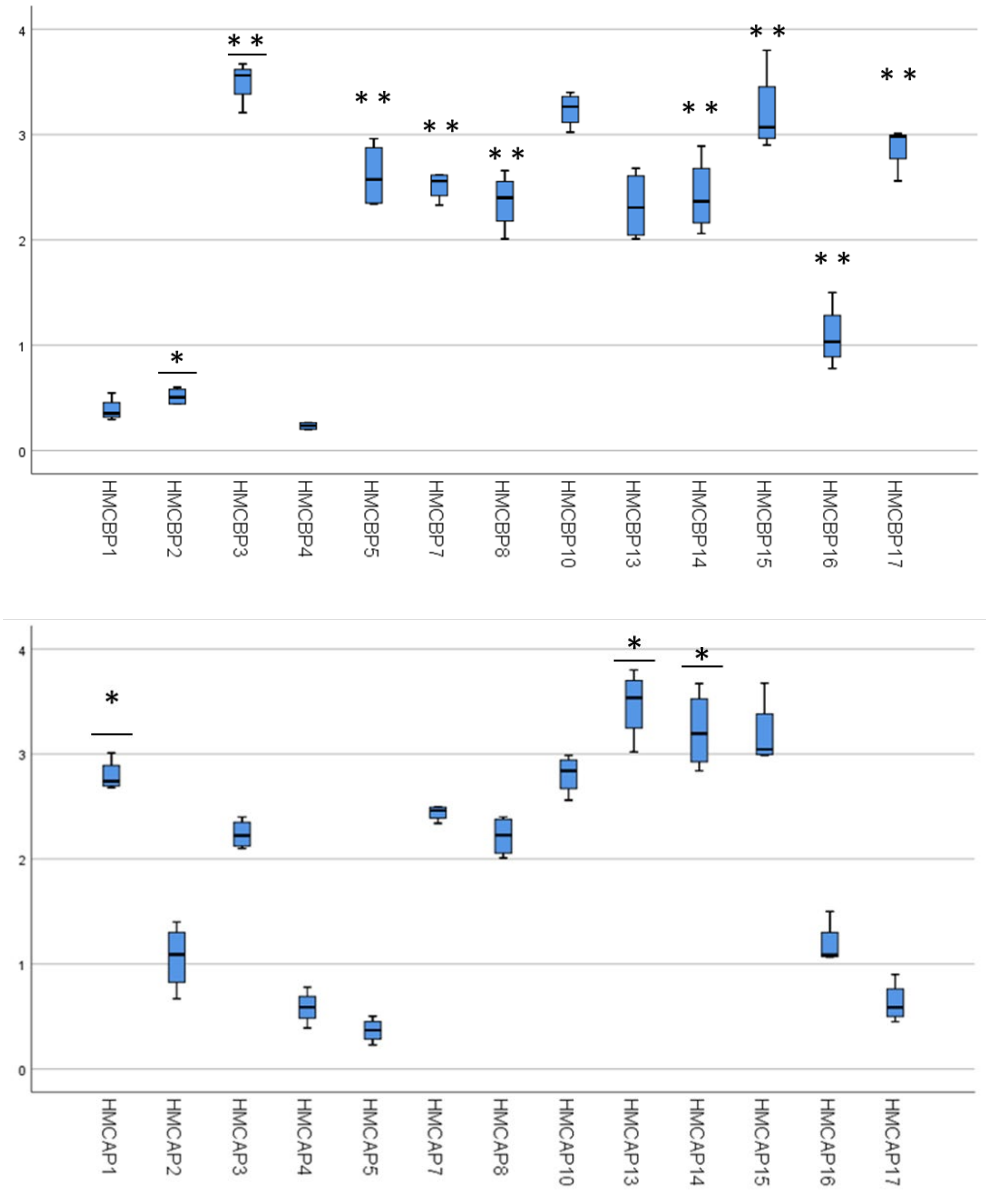
BEFORE PACKING			***AFTER PACKING***		
Sample number	Sample code	Location	Sample number	Sample code	Location
1	HMCBP1	Floor under collection table	1	HMCAP1	Floor under collection table
2	HMCBP2	Stainless steel platform-incoming fruit	2	HMCAP2	Stainless steel platform-incoming fruit
3	HMCBP3	Belt before wash	3	HMCAP3	Belt before wash
4	HMCBP4	PVC roll conveyor before wash	4	HMCAP4	PVC roll conveyor before wash
5	HMCBP5	Green brush	5	HMCAP5	Green brush
6	HMCBP7	Red brush	6	HMCAP7	Red brush
7	HMCBP8	Wax brush	7	HMCAP8	Wax brush
8	HMCBP10	Collection table wax brush	8	HMCAP10	Collection table wax brush
9	HMCBP13	Drain fruit wax	9	HMCAP13	Drain fruit wax
10	HMCBP14	Fruit belt after wash	10	HMCAP14	Fruit belt after wash
11	HMCBP15	PVC roll conveyor after wash	11	HMCAP15	PVC roll conveyor after wash
12	HMCBP16	Sizer cup	12	HMCAP16	Sizer cup
13	HMCBP17	Sorting machine sider	13	HMCAP17	Sorting machine sider



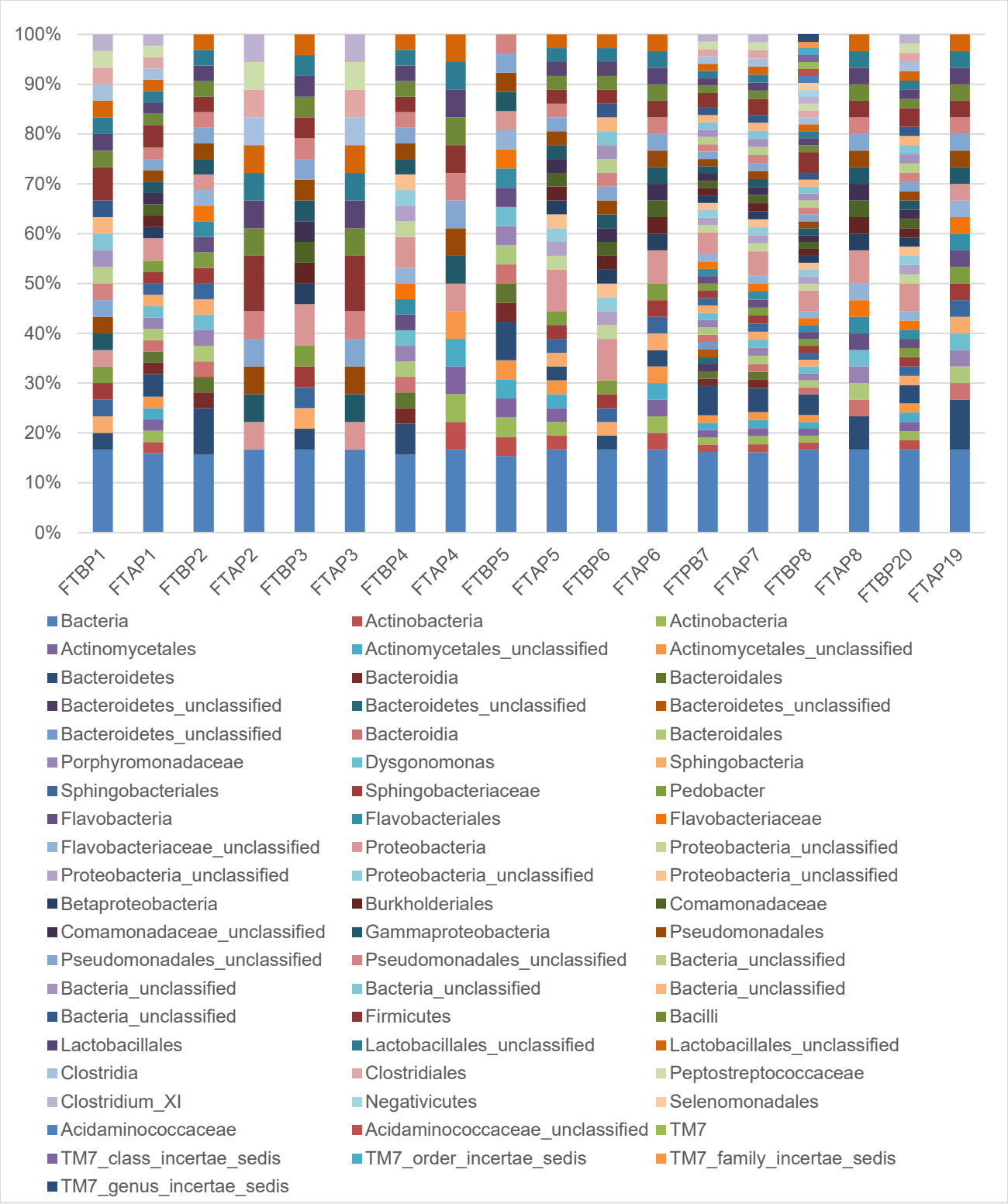
Supplemental Figure 3. Boxplot representing crystal violet staining of the biofilm biomass of the isolated bacterial strains.
Panel A represents the biofilms from bacterial strains that were isolated from the swabs and sponges at plant 1 before packing (WTB). Panel B-after pack samples.



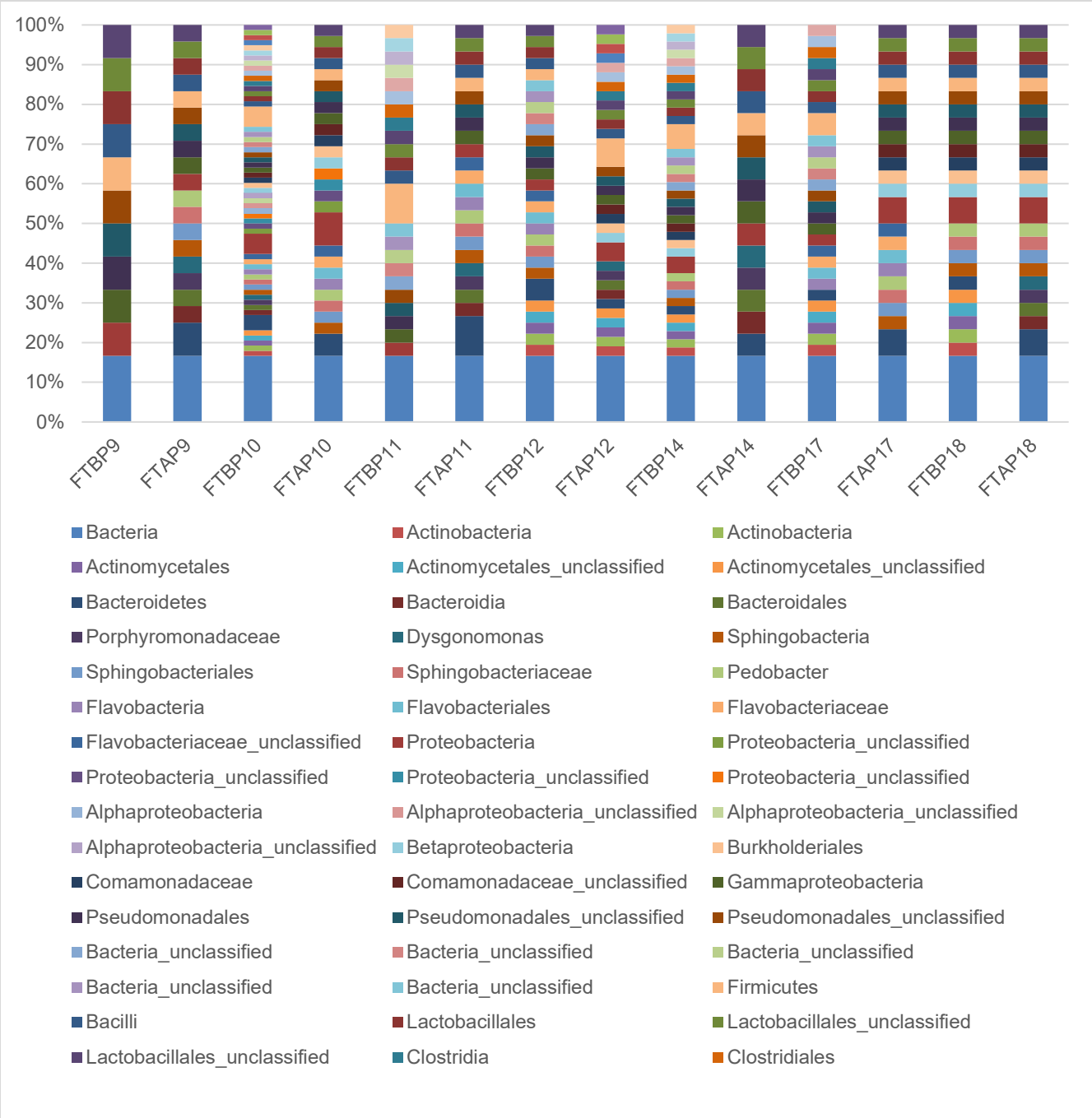
Supplemental Figure 4. Boxplot of crystal violet staining of environmental samples isolated from plant 2. Panel A samples collected before packing, panel (B) samples collected after packing operations.



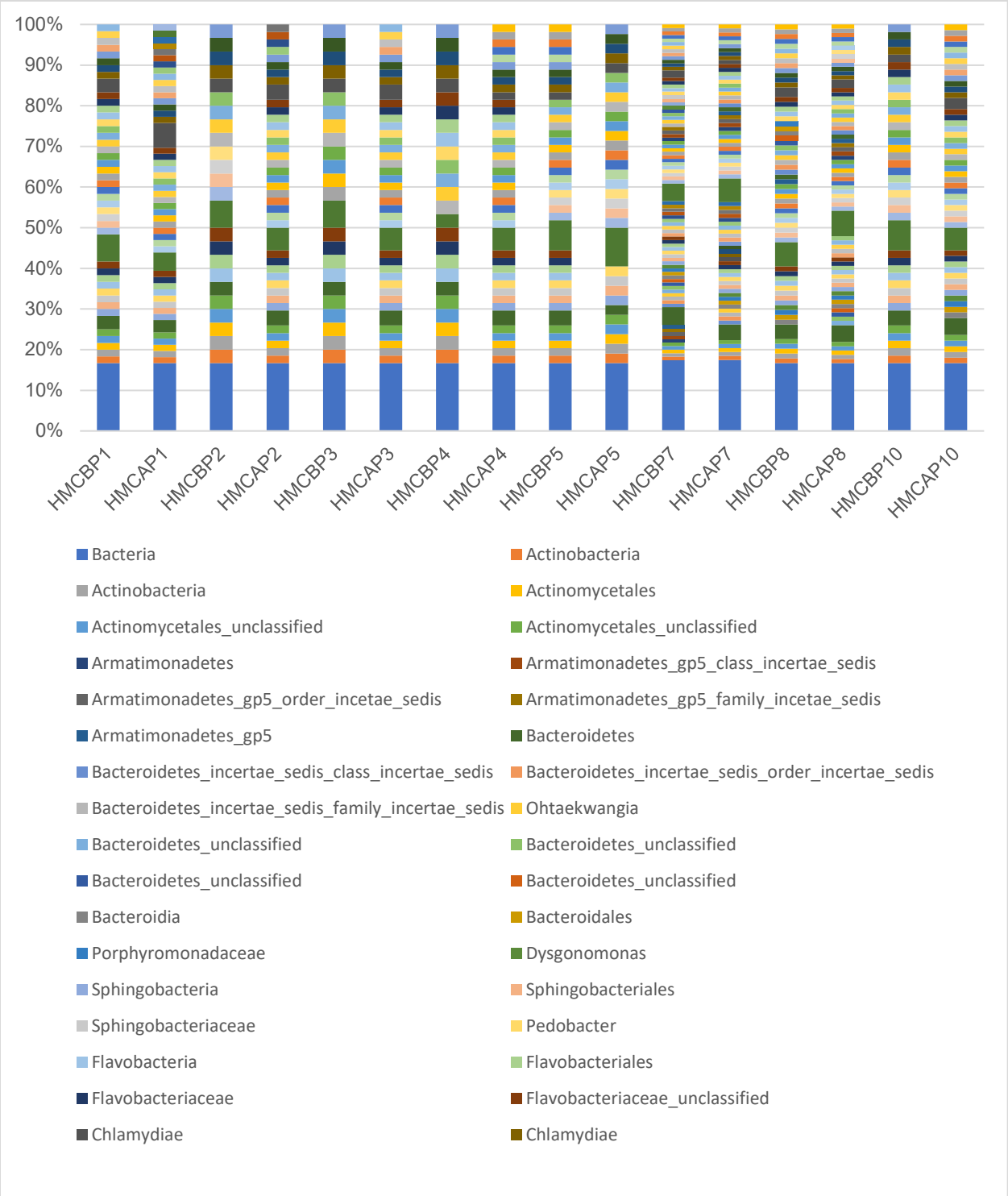
Supplemental Figure. 5. Boxplot of environmental samples isolated from plant 3. Panel A, samples isolated before packing operations, panel B samples collected approximately from the same locations after the packing operations.



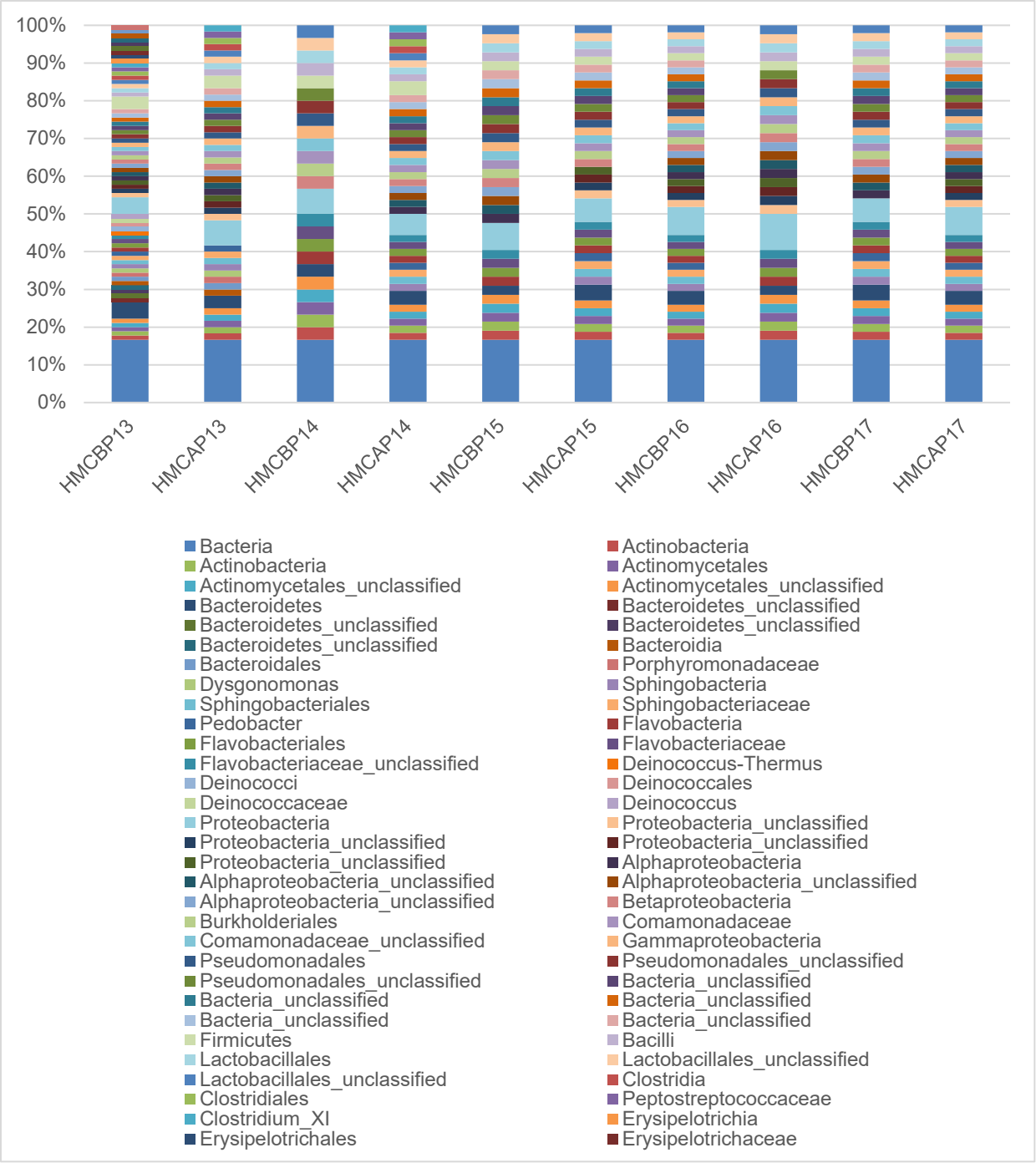
Supplemental Figure 6. The relative abundance of total sequences of bacterial 16S rRNA OTUs at the class level. Samples were collected from Facility 2, in the wash/wax area (wet surfaces).



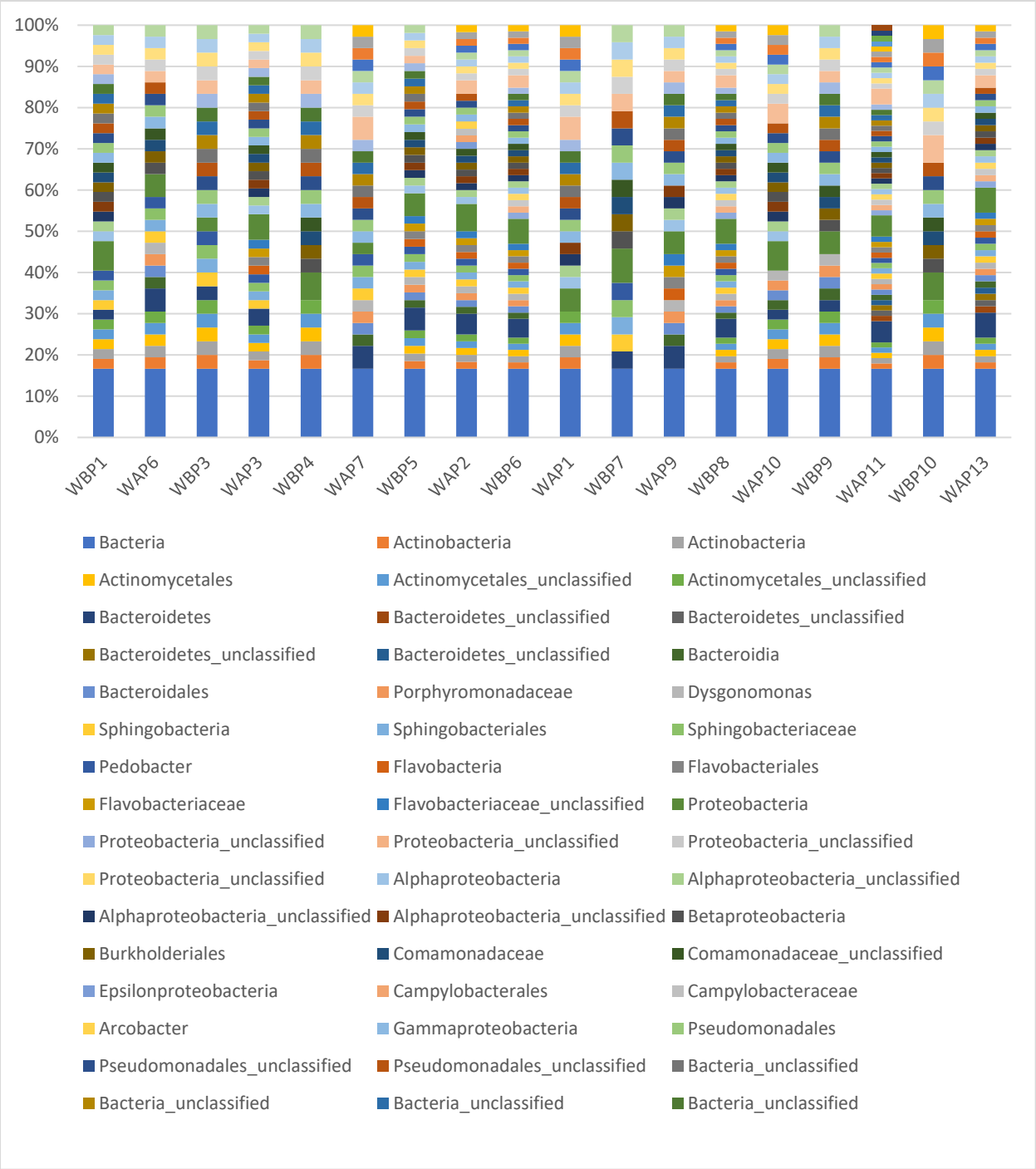
Supplemental Figure 7. The relative abundance of total sequences of bacterial 16S rRNA OTUs collected at facility 2 in the sorting area of the packinghouse (dry area)



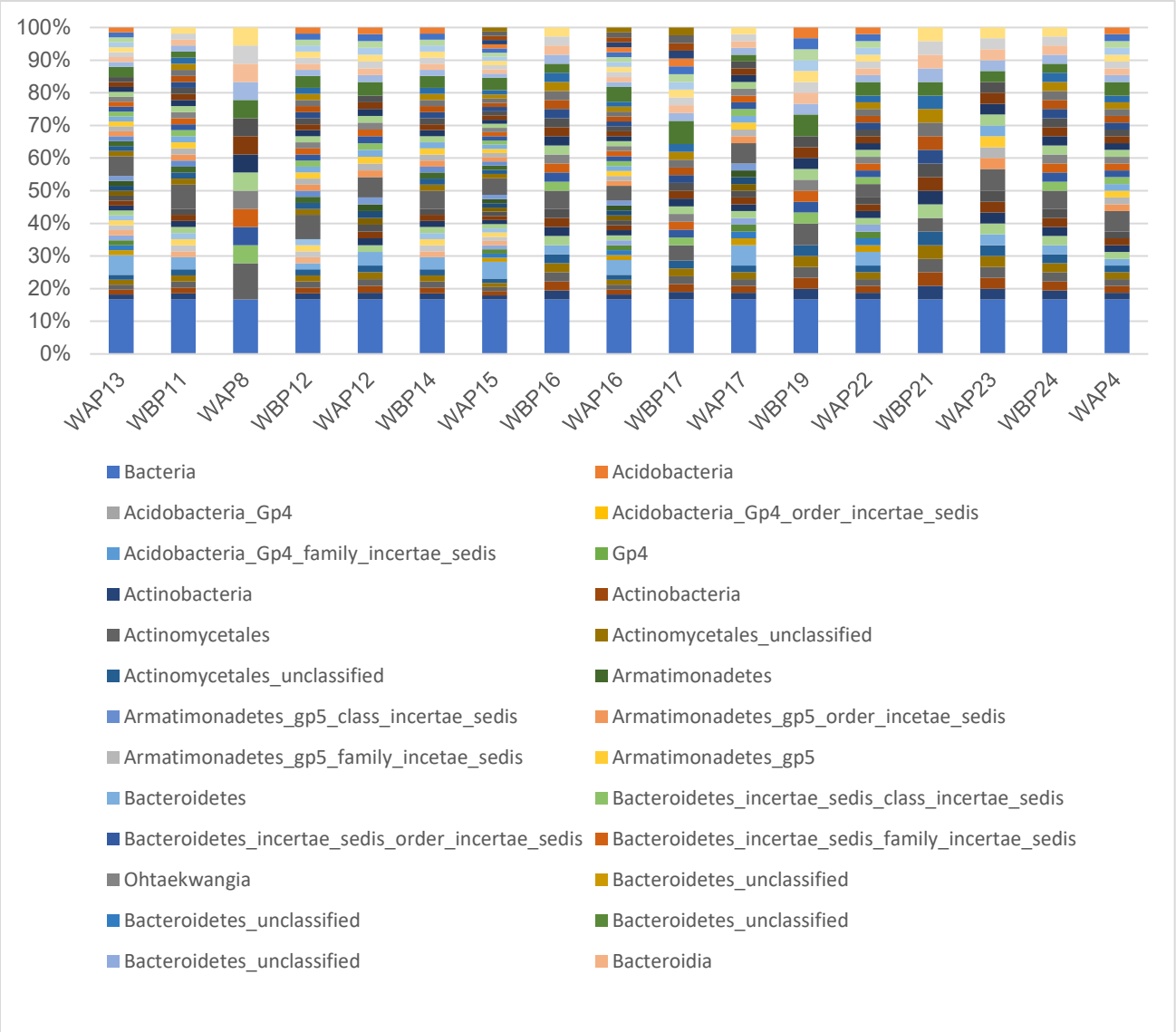
Supplemental Figure 8. The relative abundances of the total sequences of bacterial 16S rRNA OTUs collected in facility 3 in the wash/wax area (wet surfaces).



Supplemental Figure 9. The relative abundances of the total sequences of bacterial 16S rRNA OTUs collected in facility 3 in the sorting area (dry surfaces). Before and after samples of the same location are shown side by side.

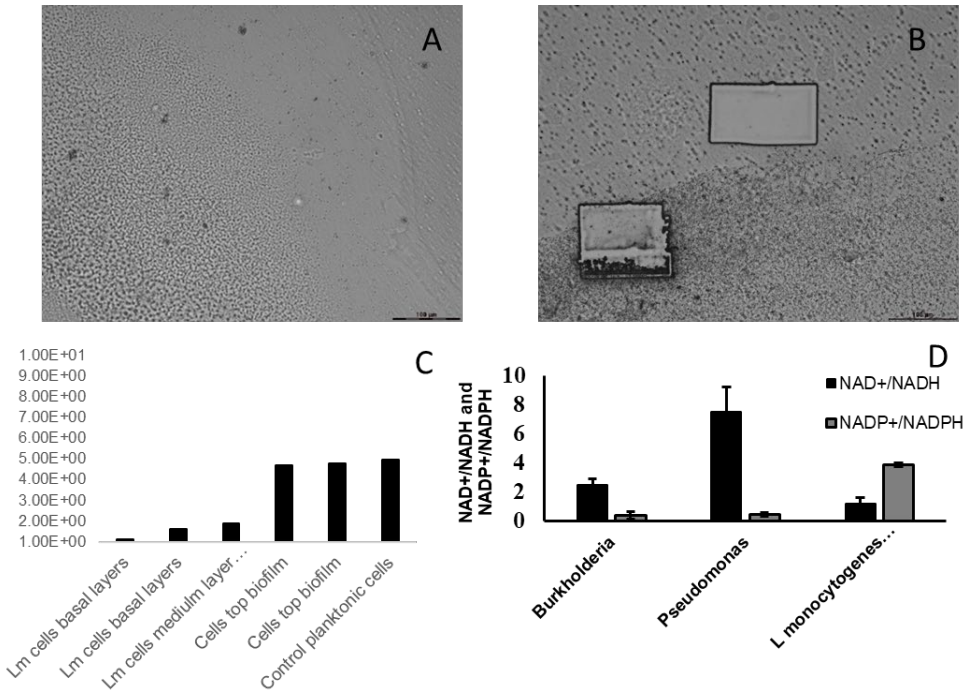


Supplemental Figure 10. The relative abundances of the total sequences of the 16S rRNA OTUs collected in facility 1 in the wax/wash area (wet surfaces). Samples collected before and after packing are shown together.

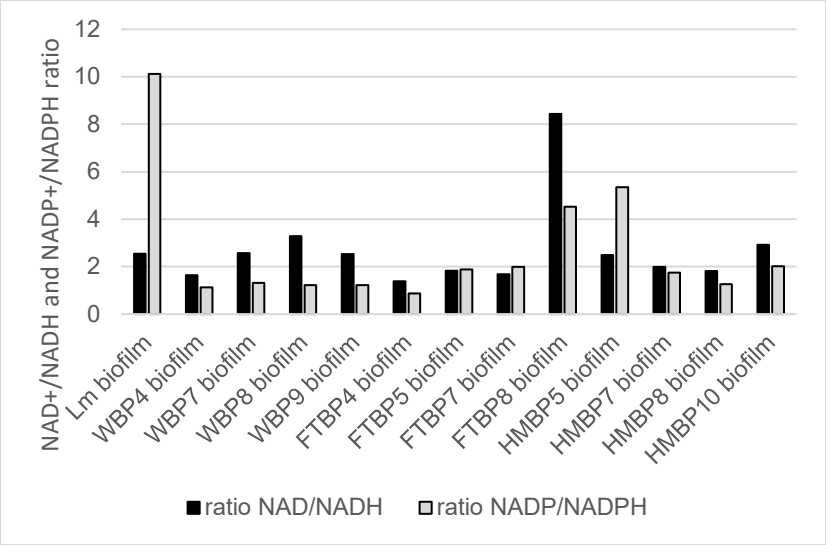


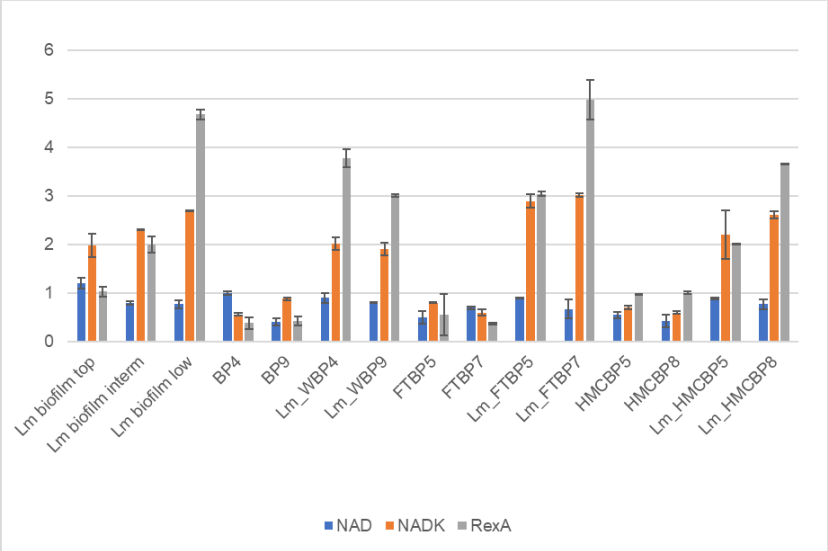
Supplemental Figure 11. The relative abundances of the 16S rRNA OTUs collected from facility 1 in the sorting area (dry surfaces). Before and after samples are shown together.

Objective 2. Supplemental information

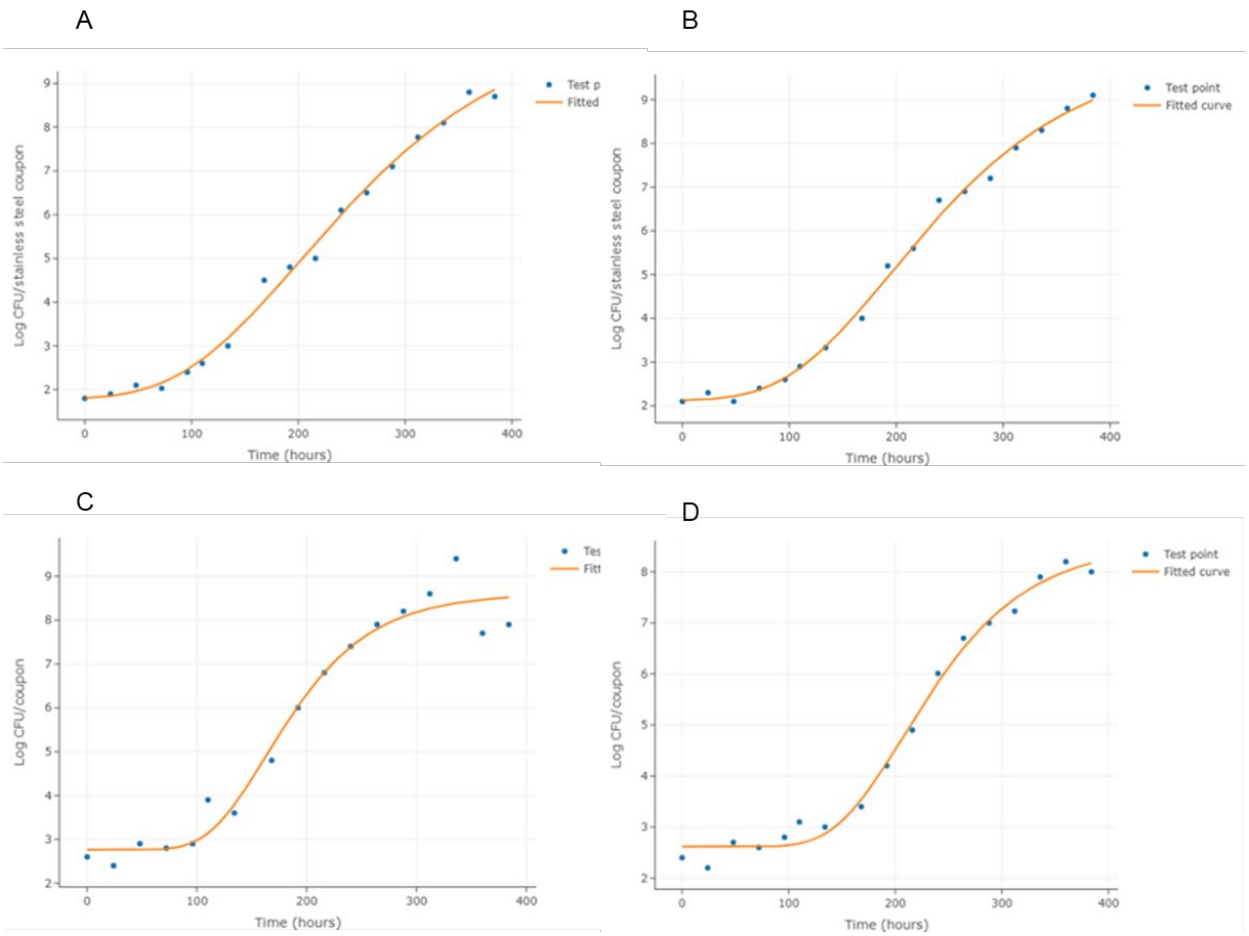


Supplemental Figure 12. Section of biofilm cut with cryomicrotome for laser dissection. (A) biofilm on PEN membrane, (B) rectangular sections cut with the laser (20X magnification). (C) Average mean normalized qRT-PCR of the rexA transcript present in RNA extracted from cells cut from different layers of the biofilm. The reduced activity in the basal parts of the biofilm indicate localized metabolic activity of *L. monocytogenes* biofilms. Bioluminescence assays of cells from biofilms of *Bulkholderia* spp., *Pseudononas* spp. and *L. monocytogenes*. Ratio of NAD+/NADH and NADP+/NADPH were derived from relative light units measured with a benchtop luminometer (Promega). The cell preparation and extraction of NAD and NADP complexes were performed according to manufacturer instructions (Promega). Results from 3 experiments are expressed as means of ratios and vertical bars represent standard deviations.



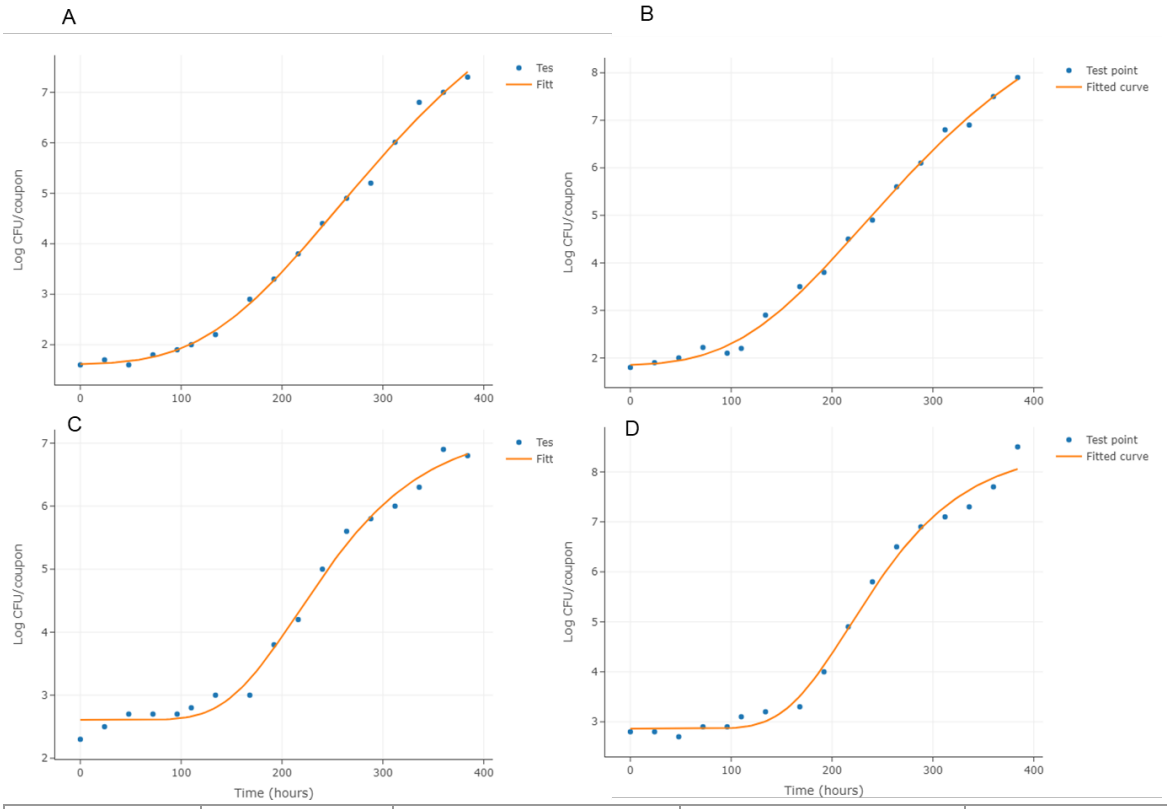


Supplemental Figure. 13. Panel A represents the NAD+/NADH and NADP+/NADPH ratio of cells obtained from *L. monocytogenes* biofilms and environmental samples. The reductase activity was determined with a NAD/NADP Glo Assay (Promega). Panel B- normalized RT PCR transcript analysis of the RNA collected from different locations of *L. monocytogenes* biofilms and environmental isolates. The transcript of *nad*, *nadk*, and *lexA* were normalized against housekeeping genes such as *rpoS*.



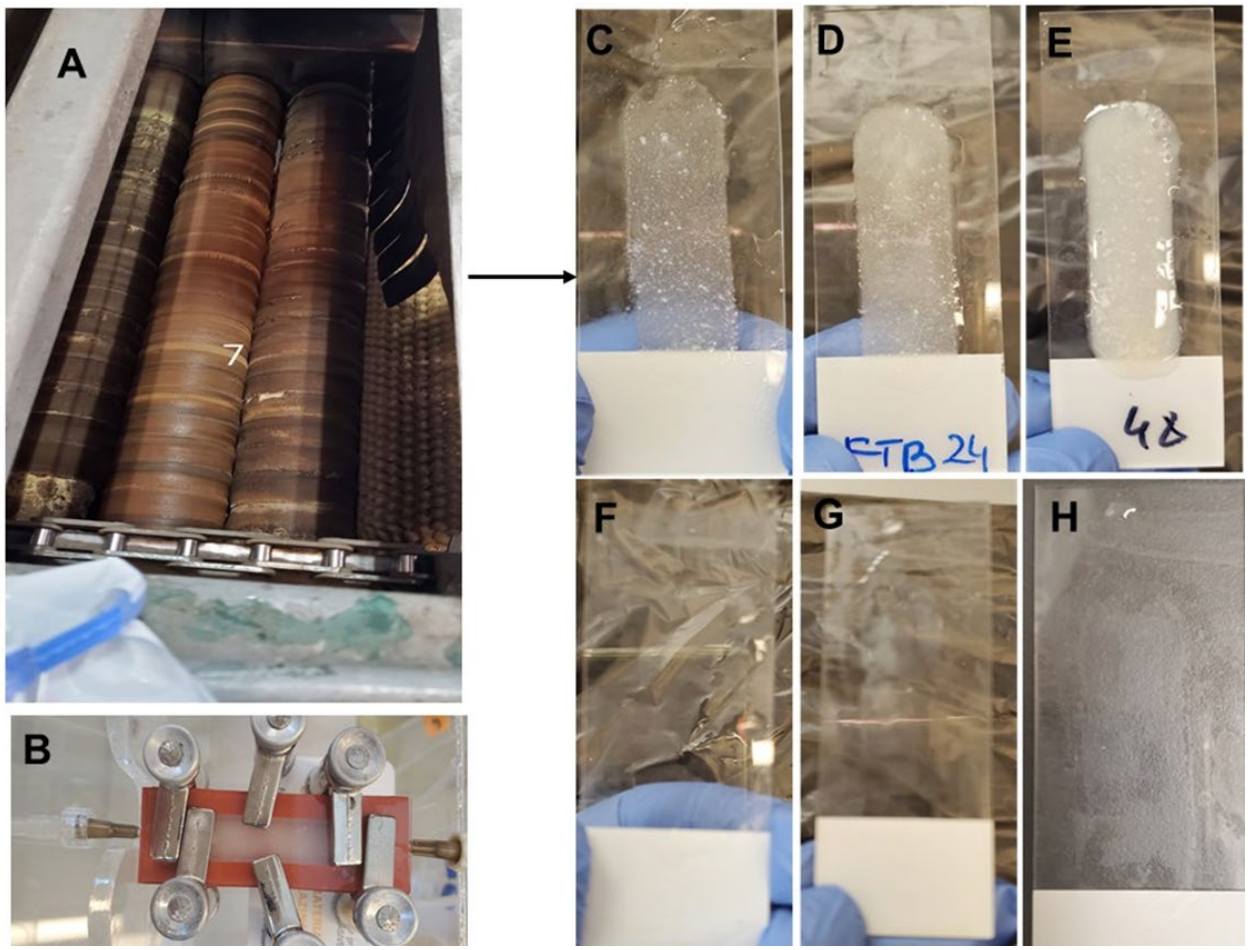
Parameters	Stainless steel	Stainless steel coated with fruit wax	PVC	PVC coated with fruit wax
y0 (logCFU)	1.78	2.01	2.65	2.55
yEnd (logCFU)	9.03	9.16	8.37	8.04
Lag time (hours)	83.92	84.78	104.24	137.31
μmax/h	0.02	0.02	0.09	0.08
Model R2	0.993	0.992	0.969	0.988
Model RMSE	0.49	0.42	1.11	0.54

Supplemental Figure 14. Experimental data sets (blue dots) indicating the growth of *L. monocytogenes* **monoculture** biofilms are fitted with the Gompertz model (red line fitted curve). A). Growth of *L. monocytogenes* on stainless steel coupons, B). Growth of *L. monocytogenes* on stainless steel coupons pretreated with mineral oil-based coating, C). Growth of *L monocytogenes* on PVC coupons and, D). Growth and model of *L monocytogenes* on PVC coupons pretreated with mineral oil-based coating. The table lists the values of maximal growth rate (μmax), lag time and model fitness.

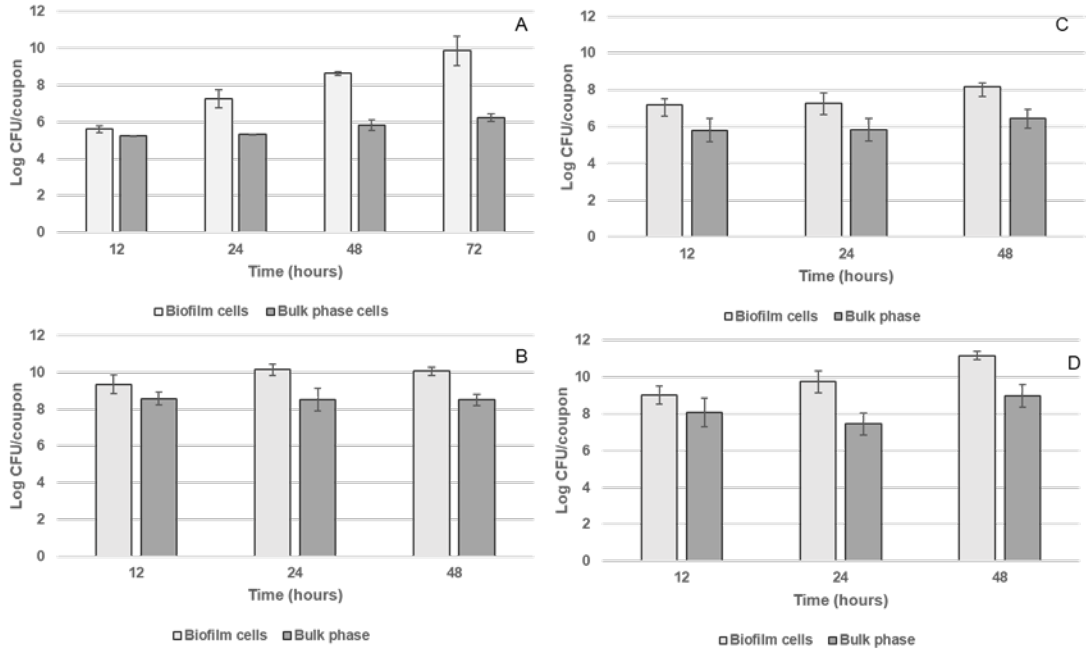


Parameters	Stainless steel	Stainless steel coated with fruit wax	PVC	PVC coated with fruit wax
y0	1.62	1.85	2.51	2.69
yEnd	10.07	8.29	7.24	ND
Lag time (hours)	116.67	103.91	122.70	119.85
µmax/h	0.05	0.09	0.06	0.07
Model R2	0.996	0.996	0.98	0.976
Model RMSE	0.28	0.31	0.42	0.51

Supplemental Figure 15. Experimental data sets indicating the growth of *L. monocytogenes* **mixed culture** biofilms (blue dots) are fitted with the Gompertz model (red line fitted curve). A) Growth of *L. monocytogenes* on stainless steel coupons, B) Growth of *L. monocytogenes* on stainless steel coupons pretreated with mineral oil-based coating, C) Growth of *L. monocytogenes* on PVC coupons, and D) Growth and model of *L. monocytogenes* on PVC coupons pretreated with mineral oil-based coating. The table lists the values of maximal growth rate (µmax), lag time and model fitness.
ND, value could not be determined, probably faster biofilm sloughing.



Supplemental Figure 16. Environmental sample collected from a rolling drying sponge (FTB7) for washed fruit (panel A) was cultured in the laboratory and grown as biofilm in flow cells (panel B) in continuous laminar flow on sterile microscope glass slides. Glass slides are environmental sample biofilms removed after 12 hours of growth (C), 24 hours of growth (D) and 48 hours of growth (E). To compare the *L monocytogenes* biofilm growth rate channels were inoculated with *L monocytogenes* Petite Scott A and samples were removed after 12 hours (F), 24 hours of growth (G) and mature biofilm at 72 hours (H).



Supplemental Figure 17. Example of experiment designed to calculate the maximal growth rate for *L. monocytogenes* and environmental isolates (FTB7) in steady-state conditions. Plate counts obtained from *L monocytogenes* and environmental packinghouse strains in flow cells. (A) *L. monocytogenes* biofilms, (B) Environmental isolates (FTB7-sponge), (C) Listeria counted from a mixed biofilm (*Listeria* and FTB7 inoculated 1:1 ratio) and (D) plate counts of the FTB7 strains from the *Listeria* mixed biofilm. The environmental strains and the mixed biofilms could not be grown for more than 48 hours (biomass filled the flow-through enclosure).

Supplemental Table 4. Listeria Synthetic Media (LSM) composition

Component	Stock name	Stock concentration M or (µM)	Final concentration 2X (g/L)
MOPS	MOPS PH 7.5	0.1	20.93
Glucose	Glucose	0.0555	10
KH2PO4	Phosphate	0.0048	0.656
Na2HPO4 • 7H2O		0.0115	3.096
MgSO4 • 7H2O	Magnesium	0.0017	0.409
Biotin	Micronutrients	(2.05)	0.0005
Riboflavin		(1.33)	0.0005
Lipoic Acid		(0.02)	0.000005
Niacinamide/Nicotinamide		(8.19)	0.001
Thiamine • HCl		(2.96)	0.001
L-Arginine • HCl	Minimum amino acids	0.0005	0.1
L-Histidine • HCl • H2O		0.0005	0.1
DL-Isoleucine		0.0008	0.1
L-Leucine		0.0008	0.1
DL-Methionine		0.0007	0.1
L-Phenylalanine		0.0006	0.1
L-Tryptophan		0.0005	0.1
DL-Valine		0.0009	0.1
Adenine		(18.50)	0.1
L-Cysteine * 2HCL		(63.44)	0.1
L-Glutamine		(41.65)	0.6

Supplemental Table 5. TaqMan probes for RT PCR.

Target gene	*Type*	*Sequence (5'-3')*
NAD	Forward Primer	GTTGGTGACCTTGCTTACTTTG
	Probe	AACTCCGCAAATCGTTGAAGGTGC
	Reverse Primer	TACCGCTCATTTCCACGATAAT
NADK	Forward Primer	TGGTTGGACTTCTCGTTATGG
	Probe	TGGTTGGTTCTGGATTGACGAAGCT
	Reverse Primer	CCCATGTAGTTTCACCGTAGAT
rpoB	Forward Primer	CCAGCTTCTATCGCACTTCTAA
	Probe	ATCAAGACCAAATGTGCGTCCAGC
	Reverse Primer	GCTTACCGTACCACCAATGA
bglA	Forward Primer	CTGATAACGTGACGCATGTAGA
	Probe	ACAACAGAGCACGGTAGTTATCCGT
	Reverse Primer	CATTAGGTTCTCCGCCCATAC
RexA	Forward Primer	TGGCTTGTAAGTATCGGAAGTG
	Probe	ATTTGTCCCAACCAAGCATAGCGC
	Reverse Primer	GAAGTGCGATAGAAGCTGGTAA