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CPS 2021 RFP
FINAL PROJECT REPORT

Project Title
Survival of *Listeria monocytogenes* and *Salmonella* on surfaces found in the dry packinghouse environment and effectiveness of dry-cleaning processes on pathogen reduction

Project Period
January 1, 2022 – December 31, 2022 (extended to February 28, 2023)

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Objectives

1. *Determine die-off curves of dry surface-formed biofilms and dried planktonic cells of Listeria monocytogenes and Salmonella (in single and mixed cultures with environmental isolates) on surfaces commonly found in the packinghouse.*
2. *Test the efficacy of commonly used dry cleaning and sanitation methods on dry surface biofilms and desiccated planktonic cells of L. monocytogenes and Salmonella on surfaces found in the packinghouse.*
3. *Validate, in pilot plant trials*, the laboratory die-off rates of the surface-associated microflora isolated from the dry areas of the packinghouse, and the inactivation through dry cleaning/sanitation methodology.* *Objective 3 was modified to remove pilot plant trials; instead, to determine the specific variables that are associated with the microflora on dry surfaces, two large packinghouse facilities in the Central Valley of California were sampled over the summer 2022, before and after sanitation.

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FINAL REPORT

Abstract
Fresh fruit processing is done through a series of steps such as washing, waxing, drying, sorting, and packaging. If the wash/wax part takes place in the wet area of the packinghouse, then the sorting and packaging are completed in a part of the facility that is typically dry. The lack of available water for microbial growth would prompt the hypothesis that the dry conditions can help eliminate foodborne pathogens such as *Listeria monocytogenes* (*L monocytogenes*) and *Salmonella*, that are microorganisms of concern for the fresh produce industry. Results from this research indicate that both *L monocytogenes* and *Salmonella* can survive for extended times (days and weeks) in relative humidity (RH) as low as 25%. Microbial viability is influenced by several factors such as the physiological state, e.g., if cells dry on surfaces as free-floating planktonic cells or in the form of biofilms, the presence of compatible solutes, and the combination RH-temperature present in the environment. Project results showed that the dry environment by itself was deleterious only for microorganisms present as planktonic cells without the protection of compatible solutes (survived for hours). In the presence of compatible solutes, the viability of planktonic cells increased dramatically (viable for days). Most notable was the long persistence of biofilms in dry conditions. For example, *L monocytogenes* biofilms incubated at 30-32°C and 70-72% RH, a combination of environmental conditions frequently found in the summer in the packinghouse, survived for a month. The efficacy of 3 EPA-approved sanitizers for the dry environment was tested against planktonic cells and biofilms. The tested dry sanitation compounds were effective against planktonic cells; in most cases cells were below the detection limit of the assay (1.3 log CFU/test surface) after 1 minute of exposure. However, the sanitation agents failed to achieve complete elimination of the pathogens except in the case of *Salmonella* biofilms. The most efficient sanitizer was hypochlorous acid followed by quaternary ammonium. The sanitizers' efficacy increased on samples incubated at higher humidity (65% RH). Finally, sampling of two major stone fruit packinghouses in the Central Valley of California indicated that environmental microorganisms survive in the dry area of the plant for an extended time. These strains withstand the sanitation treatment, and the *in vitro* biofilm experiments showed that they are more resistant to the sanitizers than the laboratory strains. Some of these microorganisms persist on surfaces as biofilms, which can protect the incidental *L monocytogenes* or *Salmonella*.

Background
The increased consumption of fresh fruits and vegetables in the past decade has also brought increased scrutiny over microbial safety of fresh produce. Microbial contamination is problematic since these products have no kill step and are consumed with minimal preparation and limited traditional preservation techniques. Microorganisms including foodborne pathogens can enter the packing facility through the raw commodities, human carriers, tools, and utensils. Produce can carry the environmental, generally non-pathogenic, microflora but also the incidental foodborne pathogens. In the packinghouse the typical processing of fruits and produce includes cooling, washing, drying/waxing if applicable, then sorting and packaging. While some of the packing operations occur in the wet area of the plant (washing), sorting and packaging are carried out in the dry section of the packinghouse. Based on the lack of available water and nutrients, it is reasonable to hypothesize that the dry conditions in the packing area of the facility may be detrimental for microbial survival and this area of the facility can be subjected only to dry cleaning and sanitation.

Most microorganisms grow in the 0.98–0.995 range of water activity, and the minimum water activity required for bacterial survival is 0.88–0.91. Microbial survival in dry conditions is not only dependent on the water activity of the environment or type of food product but also on other factors, such as the presence of organic compounds in the microenvironment (e.g., compatible solutes), microbial physiological state and environmental temperature. Typically, microbial exposure to air or desiccation, also known as matric stress, can result in an increase in the osmotic stress with deleterious effects on bacteria. Drying is associated with an efflux of cellular water (removal of bulk and sorbed water) leading to decreased cytoplasmic volume and turgor pressure, physical changes of cell membrane with loss of membrane integrity and cytoplasmic leakage, loss of proton motive force, loss of protein structure and function and finally cell death. Organisms that survive complete dehydration, or anhydrobiotes, are rare among prokaryotes, and many of them are found in extreme environments. Anhydrobiotes can remain dehydrated for years, but when contacted with water they quickly recommence metabolic activity.

While foodborne pathogens such as *L monocytogenes* and *Salmonella* are not considered anhydrobiotes, some are able to survive for long times in foods and food ingredients with water activity below 0.85 as well as in the dry processing environments. For example, *Salmonella* species are known to tolerate low or high temperatures, low moisture, and desiccation conditions and consequently may persist in the packinghouse environment. There have been a number of *Salmonella* foodborne outbreaks involving cantaloupes, tomatoes, seed spouts, and unpasteurized orange juice. The dried or desiccated *Salmonella* cells are more resistant to other environmental stressors including sanitizing agents such as ethanol, sodium hypochlorite and hydrogen peroxide.

There are specific repair mechanisms against desiccation in bacteria. For example, the synthesis of *de novo*, or the active cellular uptake of compatible solutes and ions (trehalose or sucrose, potassium and magnesium ions) or other osmoprotectants (glycine, betaine) has been documented, including for *Salmonella* and *L monocytogenes*. Compatible solutes are organic or inorganic compounds that essentially aid microorganisms to withstand dry conditions. There are different mechanisms, including a well-described “water-replacement” process that reestablish cell differential pressure and preserve proteins and lipid membranes. The inadvertent presence of compatible solutes in foods or in residues on surfaces in the plant environment may assist pathogens to overcome physiological barriers and survive dry conditions and desiccation.

In addition to surviving in dry conditions, bacteria can form microbial biofilms, or complex communities with intra- and interspecies interactions, that can develop on food contact surfaces. Biofilms can be defined as surface-attached aggregates of bacteria (single or multi species) in which cells are embedded in a self-produced extracellular polymeric substance (EPS). The EPS matrix serves as a reserve for nutrients, protection against shear forces and antimicrobials and retains moisture for biofilm survival. From the microbial physiology standpoint, the opposite of biofilms are planktonic cells that can be defined as free-floating individual cells that follow logarithmic growth according to their specific growth rate. Typically, physical characteristics of biofilms (presence of exopolymers, the presence of cells in groups versus individual microorganisms) offer better protection against environmental stresses than planktonic growing cells. The switch from planktonic to sessile growth type, and the microbial adaption to physicochemical conditions surrounding the cells (matric stress), require physiological cellular changes which occur through the regulation of gene expression in response to various environmental signals. Typically, these genetic and metabolic adaptations require an aqueous environment, and it is unclear if these changes occur in the dry environment of the packinghouse.

In the packinghouse, dry sanitizers can be used for food contact surfaces, or Zone 1 following the dry-cleaning techniques (vacuum, scraping). The rationale is that the presence of water is a significant risk for foodborne pathogen growth and cross-contamination. The dry cleaning, coupled with the deleterious effects of the lack of available water for microorganisms' survival, could limit the presence of bacteria on packinghouse surfaces. *Salmonella* is traditionally associated with dry surfaces in the packinghouse, however there is limited information on *L monocytogenes* survival in the dry environment. Typically, dry environments are lethal for planktonic (nonattached) microorganisms but there is limited information regarding biofilm survival in dry conditions. In this research we determined (i) survival of planktonic and biofilms of *L monocytogenes* and *Salmonella* on dry surface and conditions typically found in a dry packing area, (ii) efficacy of some dry-cleaning sanitizers approved by EPA, and (iii) performed large-scale packinghouse sampling to validate our findings.

Research Methods and Results

Microbial strains and growth conditions:
In drying experiments, *Listeria monocytogenes* Petite Scott A (ATCC 49594), a derivative expressing green fluorescent protein (GFP), and a previously isolated *Salmonella* were used for planktonic and biofilm experiments. 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 *L monocytogenes, Salmonella* and packinghouse strains were grown in Listeria Synthetic Media (LSM). LSM is a chemically defined medium, in which microorganisms utilize glucose as the main carbon source and glutamate as the nitrogen source. Inoculums and subsequent biofilm growth were prepared in 2x LSM. The minimal media supports the growth for both strains, *L monocytogenes* and *Salmonella*, as planktonic cells and biofilms.

Objective 1. *Determine die-off curves of dry surface-formed biofilms and dried planktonic cells of Listeria monocytogenes and Salmonella (in single and mixed cultures with environmental isolates) on surfaces commonly found in the packinghouse.*

For inoculation and enumeration of viable planktonic cells, that survive in low relative humidity, the tested microorganisms were grown first in TSB and then in 2x LSM to early stationary phase. Cells were harvested by centrifugation and washed twice in sterile phosphate buffer (PB to eliminate the residual media. Cells were then resuspended in the resuspension media (sterile water, 1.5% trehalose, 2x LSM, 1.5% proteose peptone 3 to simulate the presence of organic matter, **Table 1**). Five microliters of suspension were spotted on the surface of test surfaces (**Supplemental Figure 6A**). The test surfaces used through experiments were round-cut PVC coupons – ½ inch rounds cut from a transport belt previously obtained from a packinghouse. Prior to inoculation, the coupons were sterilized with 75% ethanol, rinsed with sterile distilled water, and dried in a safety cabinet. Inoculated test surfaces were then placed in humidity-controlled cabinets for storage at 25, 65, 70 and 95% relative humidity.

Samples were removed at time 0 (immediately after spotted droplets were visibly dried – drying took approximately 30 min for 25%RH and 2 hours for 65%RH) and then at select intervals samples were removed to recover and enumerate viable bacteria. Test surfaces were placed in sterile culture tubes with 1 ml phosphate buffer supplemented with 5% sodium pyruvate (to quench oxidative radicals and help in microbial recovery). Cells were allowed to recover in the buffer for 30 minutes and then serial dilutions were made in PBS and plated on

TSA. Each plotted die-off curve is the average of 3 independent experiments. Kinetic curves were modelled in GInaFit that fitted the data with the Weibull/modified Weibull models.

To confirm the plate count and verify the possible presence of viable but not culturable (VBNC) organisms, select conditions were verified for viable cells using quantitative PCR (qPCR). Briefly, for a separate set of samples recovered in the PBS buffer that contained 1x final concentration PMAxx™ viability dye, DNA was extracted with PureLink™ Genomic DNA Mini Kit. The total genomic DNA was used as template for qPCR. The target genes were *invA* for *Salmonella* and *hly* for *L monocytogenes*. PCR conditions were 95°C for 2 minutes and then 40 cycles of denaturation at 95°C for 10 seconds, then extension at 60°C for 30 seconds. The melt curves were then generated from 55°C to 98°C, and qPCR was performed on a Roche LightCycler 96. For the absolute quantification of viable cells, a standard curve was generated for each qPCR experiment from serial dilutions of microbial cultures with known number of bacteria. The instrument software was used to calculate R^2, slope and y-intercept of the standard curve, which in turn were used to calculate the number of viable microorganisms present on test surfaces.

The effects of desiccation on microbial survival were also evaluated on dry (colony) biofilms. The biofilms were grown on polycarbonate membranes placed on the top of LSM media supplemented with 3% agar. These biofilms were already exposed to air because the polycarbonate membrane allows only limited transfer of nutrients and water (**Supplemental Figure 6B and 6C**). Preliminary experiments – cell numbers from plate counts and confocal microscopy – were performed to determine the growth rate of the dry biofilms. We determined that mature biofilms were reached after 48 hours of growth for *Salmonella* and 72 hours for *L monocytogenes*. These growth parameters were used for all experiments. After they were allowed to develop on the polycarbonate membranes, the biofilms were incubated in the same temperature and relative humidity conditions as the planktonic cells. Microbial viability over time was determined as described above.

We tested 2 distinct scenarios that could occur on surfaces in the dry area of the packinghouse: (i) cells drying as free-floating planktonic microorganisms and, (ii) the possibility that microorganisms were allowed to form biofilms that subsequently dried on surfaces. In addition, organic and inorganic compounds that are inherently associated with the produce and or packing operations could be present on the soft, porous food-contact surfaces. One can hypothesize that some of these compounds or organic residuals from fruit surface could act as compatible solutes. In the planktonic cell viability testing we included trehalose, the most studied compatible solute that is a cell membrane and enzyme stabilizer, and proteose peptone 3 as a compatible solute to simulate the presence of organic matter. The list of microbial compatible solutes is by no means limited to those two compounds. We also included LSM in planktonic cell trials because it was the growth environment for biofilm development.

Planktonic cells of *L monocytogenes* and *Salmonella* without protection survived for a limited time on dry surfaces (**Table 1** and **Supplemental Figure 7 and 8**) specifically, the limit of detection (1.3 log CFU/test surface) was reached in less than 20 hours in the tested experimental conditions of relative humidity and temperature. The low numbers of the surviving populations were observed for both low (25%) and higher (65 and 80%) RH. Temperature (20°C versus 30°C) did have a negative effect on the planktonic cell decline; planktonic cells survived for less than 15 hours when incubated at 25% ± 5% RH (**Supplemental Figure 9**). If only planktonic cells with no extracellular protection were present on the packinghouse surfaces, then the lack of moisture would severely affect their survival.

The survival time for both pathogens increased dramatically when cells were protected in their microenvironment by compatible solutes. For example, *L monocytogenes* at 25% RH

microbial viability declined to reach the assay detection limit after 96, 260 and more than 260 hours when dried in the presence of trehalose, proteose peptone 3 and 2x LSM, respectively. In contrast, samples incubated at 65%RH reached the low limit of detection in less than 24, 250 and less than 260 hours when dried in the presence of trehalose, proteose peptone 3 (PP3) and 2x LSM, respectively (Table 1 and Supplemental Figure 7 and 8). Compared with 25% RH, *Salmonella* population dried at 65% RH dropped by 4 log after 144 hours for biofilm media resuspended samples and 74 hours for samples resuspended in PP3 (Supplemental Figure 8). Overall, the low relative humidity (25% RH) preserved the cells better than the higher humidity (65% RH). Experiments were also performed for samples stored at higher RH (95% RH) but in this instance, there was no microbial reduction for both pathogens.

We investigated if the dried cells were attached to surfaces and/or had the capability to transition to biofilms. Confocal microscopy of the inoculated droplets that were stained with Live/Dead stain indicated that cells were not attached when the droplet was dried at 25 or 65% RH. These cells were simply removed by the sheer force of another water droplet. Microbial attachment occurred at humidity higher than 90% RH. This avenue was not pursued further since the high relative humidity, over 90% RH, is unlikely to be found in a dry room.

Microbial survival increased dramatically when both pathogens were present on surfaces as biofilms. When *L monocytogenes* was grown as a biofilm in a limited nutrient and water environment, the pathogen had a 4-log reduction in viable cells after 165 hours when biofilms were stored at 25% RH, and after 124 hours when dried and stored at 65% RH. In contrast, *Salmonella* biofilm lost 4 log after 65 hours in low relative humidity and after 92 hours at 65% RH, respectively (Table 1). In all tested conditions, viable microorganisms were detected for at least 120 hours of incubation in dry conditions. There were differences in biofilm viability depending on the incubation temperature. *L monocytogenes* biofilms survived for longer time when incubated at low RH (25%) than the higher RH (65%). *Salmonella* biofilms showed a similar effect. However, when biofilms were incubated at 30-32°C, the temperature-RH combination has a different effect on cell viability. Biofilms incubated at 30-32°C and 70% RH (conditions simulating the packinghouse environment) were viable and cells could be recovered for extended time: a month for *L monocytogenes* biofilms and three weeks for *Salmonella* biofilms (**Supplemental Figure 12**). These were die-off curves; the pathogens did not grow as biofilms or planktonic cells in the experimental conditions; however, the extended microbial survival is concerning from the food safety standpoint.

Objective 2. Test the efficacy of commonly used dry cleaning and sanitation methods on dry surface biofilms and desiccated planktonic cells of L. monocytogenes and Salmonella on surfaces found in the packinghouse.

In Objective 1, we determined that cells can survive without available water on surfaces and that the dry environment alone is not effective to completely eliminate them. In Objective 2, inactivation studies with antimicrobials approved for use in dry cleaning were performed for planktonic cells and biofilms *of L monocytogenes* and *Salmonella*. The inactivation agents along with their EPA registration number are listed in **Table 2**. Planktonic cells and biofilms for *L monocytogenes* and *Salmonella* were grown and incubated in conditions described in Objective 1. In addition, two microbial mixed samples isolated from packinghouses were included in the inactivation studies. The microorganisms from samples labeled S1 (isolated from Facility A) and S2 (isolated from Facility B) were grown as biofilms on polycarbonate surfaces as described in Objective 1. The samples were exposed to the respective sanitizer for 1 min following manufacturer's instructions, and no previous scrubbing or cleaning was performed. After the treatment with sanitizer, cells were recovered as described in Objective 1. Data resulted from

three independent experiments, each performed in triplicate (n=9) except the packinghouse biofilms experiments which were performed only twice.

The planktonic cells were easily inactivated by the tested sanitizers. No viable microorganisms were recovered from hypochlorous acid and quaternary ammonium sanitizers. The ethanol-based sanitizer resulted only in microbial recovery near the detection limit of the plate count (1.3 log CFU) as shown in **Tables 3 and 4**. In contrast with planktonic inoculated coupons, microorganisms were recovered from the treated biofilm samples. Hypochlorous acid was the most effective dry-cleaning sanitizer for both *L monocytogenes* and *Salmonella* biofilms, and the differences with the quaternary ammonium compound sanitizer are probably not sufficient for practical importance (Tables 3 and 4). The efficacy of both sanitizers was enhanced in higher relative humidity (65% RH).

In contrast, the ethanol-based sanitizer failed to achieve a 5-log reduction for the biofilm treated samples for both *L monocytogenes* and *Salmonella*. When packinghouse-isolated environmental strains were exposed to the dry sanitizers, none of them achieved a 5-log reduction of the biofilm. The notable difference between the environmental and laboratory strains in their susceptibility to the dry sanitizers could be explained by the repeated exposure of the environmental strains to the cleaning/sanitation that typically occurred in the facility. The sanitizer that achieved the most reduction in the microbial count, hypochlorous acid, was most effective with samples incubated at 65% RH. The S1 and S2 microbial mixes were also visualized with confocal microscopy, after staining with Bac Live/Dead™. Morphologically, the biofilms formed S1 and S2 were different. S1 formed a slow growing biofilm with a compact structure, while S2 had a thick biofilm with high cell turnover (**Supplemental Figure 13**).

Objective 3.

Results from Objective 1 and 2 demonstrated that in the laboratory, pathogen survival in dry conditions is influenced by multiple variables (physiological state, the presence of extracellular matter and compatible solutes) and that the dry sanitation treatment may not eliminate them. Since the specific laboratory conditions and experimental variables were not similar to the packinghouse environment, we decided to modify and expand Objective 3.

The following were the rationale for changing objective 3: (i) to perform a long-term study to determine the number of microorganisms that typically reside on surfaces in the dry area of the plant during the packing season, (ii) investigate the physiological state of the bacteria that resides in the dry area of the packinghouse surfaces through the packing season, (iii) determine the efficacy of the cleaning/sanitation on the microorganisms found in the dry packinghouse. Specifically, instead of a more limited and seasonal validation trial, a wider approach was designed. The objective main goal was to determine the actual microbial number on dry surfaces in the packinghouse that can survive dry conditions and determine microbial composition (species present on surfaces) and their physiological state. In order to determine the specific variables that are associated with the microflora on dry surfaces, 2 large facilities in the Central Valley of California were sampled over the stone fruit packing season in 2022. The sampling plan in both packinghouses consisted of swabbing of the same 15 points across the packing line in each facility, with emphasis on the dry area.

These 15 points were sampled before sanitation (after packing) and after sanitation. In facility A, sanitation in the facility was typically performed immediately after finishing the daily operations, therefore most of the surfaces would be dry for the after-sanitation sampling. The brush beds were removed often and cleaned out of place. Facility A processed a variety of stone-fruit (peaches, nectarines, plums, pluots). In contrast, Facility B performed sanitation right before the start of the packing for the day. The samples before sanitation in this case would be

collected at the end of the workday and the before-sanitation samples would come from dry surfaces. The after-sanitation samples were collected from wet surfaces, since the sanitation crew worked on the line right before the workday. Facility B processed mostly peaches and in the facility the brush beds were typically cleaned in place.

Sampling in Facility A and B was performed every 2 weeks, starting on May 31 through September. The September samples consisted only of after sanitation samples (season almost over) and the Facility B samples were collected 10 days after the plant stopped packing. Collected environmental samples were shipped overnight and they were processed in the laboratory within 48 hours. Typically, 10 ml of sterile TSB was added to the sponge samples, and they were massaged manually for 1 minute to release all trapped microorganisms. We found that stomaching or any other mild agitation did not release the microorganisms trapped in the spongicles. In the case of Q swabs, 1 ml of TSB was added and vortexed at maximum speed for 1 minute. The following were performed in the lab: (i) aerobic plate count to determine the microbial count, (ii) a short (6-hour growth) crystal violet biofilm assay to determine the physiological state of the microbiota isolated from surfaces, (iii) genomic DNA extraction for select samples, and (iv) freezer stock is glycerol for later use. Metagenomic sequencing and analysis of the V3-V4 region of the 16S rRNA for select samples followed the protocol described by Schloss (https://github.com/SchlossLab/MiSeq_WetLab_SOP/blob/master/MiSeq_WetLab_SOP.md#50-detailed-methods).

Boxplots were generated in IBM SPSS for the plate count and biofilm assays and are presented in **Figures 1 and 3** for plate counts and **Figures 2 and 4** for biofilm results. The whiskers of the plots represent the minimum/maximum, the boxes are the first quartile, and third quartile and the horizontal line is the median. There were variables inherently associated with large-field trials that influenced the results. For example, in Facility A one collection of the after-sanitation samples happened 2 days after the sanitation (sanitation on Saturday and sampling of the clean line on Monday morning clean right before packing). Due to the large interval between the sanitation and after-sanitation sampling, the plate counts for after sanitation were similar to the before-sanitation samples. This suggests that residual organics are present on surfaces in sufficient concentration for microorganisms to survive and even replicate on packinghouse surfaces.

One important finding is that cleaning/sanitation in the packinghouse does not completely eliminate environmental microorganisms on surfaces. On surfaces in the dry area of the plant the before-sanitation samples indicated that highest microbial counts were found on transport belts, sizer drop (chute) and rotary accumulator. In Facility A, the before-sanitation biofilm data did not always correlate with the high plate count, suggesting that some of the microorganisms were present in the planktonic form. In the dry area of the plant, the after-sanitation samples in Facility A were typically lower in aerobic plate count (average difference 2.5 log CFU) than the before sanitation (Figure 1). The biofilm crystal violet data indicated lower biofilm-former in the after-sanitation than before-sanitation samples (Figure 3). Overall data from Facility A suggests that: (i) sanitation removes microorganisms from surfaces but there are residuals persisting on dry surfaces, and (ii) microorganisms can persist as planktonic cells.

By contrast, in Facility B the total number of aerobic microorganisms was similar or even higher in the after-sanitation than before-sanitation samples (**Figure 2**). The average after-sanitation plate count in the dry area was approximately 1 log higher than the before-sanitation samples. This suggests that microorganisms multiplied in the interval after packing (before sanitation) and the after-sanitation interval. The surfaces with high plate count in the dry area were delivery belts, sizer drop and sizer carriers. The trend was also reflected in the biofilm data (**Figure 4**). In general, there were more biofilm formers in Facility B than in Facility A, and in

some samples higher biofilm results were obtained from the after-sanitation samples than before sanitation.

Since these are the regular inhabitants of the packing environment, we focused on a series of experiments on the survival of mixed planktonic or biofilms that were co-inoculated with *L monocytogenes* or *Salmonella*. We tested two sample mixes from the packinghouse environmental swabs, and developed *in vitro* biofilms that were "spiked" with *L monocytogenes* or *Salmonella*. Mixed biofilms were incubated at 25% and 65% RH and the pathogens were enumerated. The relative humidity did not affect *L monocytogenes* viability in the mixed biofilms (**Supplemental Figure 14**). The *L monocytogenes* cell number decreased only by 4 log in 10 days of incubation (Supplemental Figure 14). The high relative humidity had an inhibitory effect on the mixed *Salmonella* biofilms. The *Salmonella* plate count was below the limit of detection on 65% RH samples after 245 hours; however for the same samples stored at 25% RH, viable counts were obtained after more than 300 hours. The results suggest that packinghouse microorganisms survive on dry surfaces not only through the dry conditions but also the sanitation process and if the pathogens incidentally enter the packinghouse can form mixed biofilms with the "native" microflora. Metagenomic sequencing and analysis of the V3-V4 of the 16S rRNA segment of the before and after sanitation samples collected from the dry area indicated that (i) before and after sanitation environmental samples in the dry area have multiple taxonomic units, and (ii) the community structure was preserved for the before and after sanitation samples (**Figure 5**). The population composition, however, was different between facilities. For example, dominant taxa on dry samples at Facility A are *Acidobacteria, Proteobacteria* and *Actinomycetales,* while Facility B has *Proteobacteria*, *Gammaproteobacteria* and *Actinobacteria*.

Outcomes and Accomplishments

We had some challenges in accomplishing the goals of the project. First, there were major delays resulting from the non-availability of the needed research supplies. Frequently, in the case of backlogged materials, the search and ordering of alternate items resulted in being placed on another backlogged list. We also had some technical difficulties with the new humidity chamber.

The experimental field trial (Objective 3) was changed partway through the project. We decided to extend the sampling and follow the packing season. Our goal was to determine the presence and number of the microbiota of dry surfaces in the packinghouse. Knowing how many and what they are and then working with them in tandem with the pathogens could deliver results applicable and close to the industry conditions. Sampling was performed for before and after sanitation surfaces to indicate the type of microbiota and physiological state on dry surfaces. The biofilm assay used to determine the physiological state can be developed into a quick, qualitative, inexpensive test that can assist the stone fruit industry in assessing the presence of residual biofilms on surfaces after sanitation and quickly address the problem. The assay can be tailored for dry and wet parts of the plant. While the goal of the project was not to assess the growth of the packinghouse biofilms, on three sample sets collected this year we observed that environmental strains prevented *L monocytogenes* from growing in the mixed biofilm. The reduction compared to the control biofilm was around 4 log. Competitive exclusion is a common mechanism of survival in adverse conditions.

Summary of Findings and Recommendations

Listeria monocytogenes is a pathogen of concern in dry environments since its survival rates were comparable with *Salmonella* viability on dry surfaces. *L monocytogenes* and *Salmonella* can survive for extended times (days and weeks) in relative humidity as low as 25%. Microbial viability is influenced by the physiological state: e.g., if cells dry on surfaces as free-floating planktonic cells or in the form of biofilms, as polymer-embedded groups of cells. Most notable is the long persistence of more than a month of *L monocytogenes* biofilms at 30-32°C and 70-72% RH, a combination of environmental conditions frequently found in the summer in the packinghouse. The dry environment by itself was deleterious only for microorganisms present as planktonic cells without the protection of compatible solutes. The tested dry sanitation compounds were effective against planktonic cells; in most cases cells were below the detection limit of the assay (1.3 log CFU/test surface) after 1 minute of exposure. However, the sanitation agents failed to achieve complete elimination of the pathogens except in the case of *Salmonella* biofilms. The most efficient sanitizer was hypochlorous acid followed by quaternary ammonium. The sanitizers' efficacy increased on samples incubated at 65% RH. Our findings suggest that application of the sanitizing agent should be accompanied by some physical cleaning (e.g., scraping) to remove microbial biomass. Finally, sampling of the major facilities in the Central Valley of California indicated that microorganisms survive in the dry area of the plant. These strains withstand the sanitation treatment and the *in vitro* biofilm experiments showed that they are more resistant to the sanitizers than the laboratory strains. Some of these microorganisms persist on surfaces as biofilms, which can protect the incidental *L monocytogenes* or *Salmonella*

APPENDICES

Publications and Presentations
Not at the time of submitting the final report.

Budget Summary
This project received a total of $190,272 in grant funds and to date has spent ~$180,000; some of the remaining funds will be spent on travel to the CPS Research Symposium in June 2023. Funding was sufficient for project completion.

Suggestions to CPS
We would like to thank CPS for their support and the two-month extension needed for project completion. We would also like to thank Mr. George Nikolich for collecting packinghouse environmental samples from May through September 2022. The principal investigator, the co-PI and the postdoctoral fellow would like to acknowledge and thank the management of Facilities A and B and that allowed access for sampling and participated in the study. Clemson University is acknowledged for generous allotment of compute time on the Palmetto cluster.

Tables 1–5 and Figures 1–5 & Supplemental Figures 6–14 (see below)

Table 1. Summary of *L monocytogenes* and *Salmonella* survival (planktonic cells and biofilms) in dry conditions. Survival was determined based on plate count (limit of detection, 1.3 log CFU/ test surface). The inoculated test surfaces with planktonic cells contained 4.5-6.0 log viable CFU (cells recovered after drying) per coupon while the biofilm surfaces contained 8.0-9.0 log CFU/coupon. Viability curves are presented in Supplemental figures (Figures 7 through 12).

Temperature (°C)	Microorganism and physiological state	Relative humidity (%)	Compatible solutes	Time to reach limit of detection (hours)
20-22°C	*L monocytogenes* planktonic	25 ± 5	None	16 h
			Trehalose	96 h
			Proteose peptone 3	260 h
			LSM	Not reached after 260 hours
		65 ± 5	None	10 h
			Trehalose	Less than 24 h
			Proteose peptone 3	250 h
			LSM	Less than 260 h
	L monocytogenes biofilm	25 ± 5	N/A	Not reached, 4-log reduction after 165 hours
		65 ± 5	N/A	Not reached, 4-log reduction after 124 hours
	Salmonella planktonic	25 ± 5	None	Less than 12 h
			Trehalose	Not reached after 100 hours
			Proteose peptone 3	More than 250 hours
			LSM	Less than 180 hours
		65 ± 5	None	Less than 10 hours
			Trehalose	18 hours
			Proteose peptone 3	Less than 96 hours
			LSM	150 hours
	Salmonella biofilm	25 ± 5	N/A	180 hours; 4-log reduction after 64 hours
		65 ± 5	N/A	Not reached, 4-log reduction after 92 hours
30-32°C	*L monocytogenes* planktonic	25 ± 5	N/A	12 hours
		70 ± 2	N/A	24 hours
	L monocytogenes biofilm	25 ± 5	N/A	Not reached after 120 hours; in 120 hours lees than 4 log loss in viability
		70 ± 2	N/A	Not reached after 600 hours (viable bacteria were recovered over a month)
	Salmonella planktonic	25 ± 5	N/A	Limit of detection in less than 8 hours
		70 ± 2	N/A	Limit of detection reached in less than 12 hours
	Salmonella biofilm	25 ± 5	N/A	Not reached after 120 hours; less than 4 log loss in viability
		70 ± 2	N/A	Limit of detection reached after 504 hours (viable cells recovered for 21 days)

Table 2. Antimicrobials used for microbial inactivation with dry-cleaning agents in Objective 2.

EPA Reg number	Active ingredient	Commercial product name	Recommended concentration	Surface type and contact time (recommended)
98003-1	Hypochlorous acid	NovaBay Hard Non-Porous Surface Pro	Spray, 338 ppm active chlorine, needs chlorine test before use	10 min food contact no rinse, hard non-porous surface
9480-13	Ethanol	Salsa (alt name Purell)	Ethyl alcohol 29.4%	1 min or less hard non-porous surfaces
9480-13	Quaternary ammonium	Backspin No-Rinse, FCSS	Total QUAT 0.038%	1 min, no rinse, hard non-porous surfaces

Table 3. Inactivation of *L monocytogenes* planktonic and biofilms by dry-cleaning agents NovaBay, Salsa and Backspin. The plate counts represent the means and standard deviations from three independent experiments. ND – not detected. Means statistically different than the control samples (t-test) are indicated by asterisk.

Strain/conditions	***Time (h)***	***Temperature / %RH***	***Plate count (log CFU/sample)***			
			Control	Treated Salsa (Ethanol 29.4%)	Treated Surface Pro (Hypochlorous acid)	Treated Backspin No-Rinse (quaternary ammonium)
LM planktonic cells	T0	20-22°C / 25% ± 5%	5.66 ± 0.2*	1.33 ± 0.04**	2.03 ± 0.06**	ND
	24	20-22°C / 25% ± 5%	4.48 ± 0.26	ND	ND	ND
	24	20-22°C / 65% ± 5%	3.98 ± 0.36	ND	ND	ND
LM biofilm	T0	20-22°C / 25% ± 5%	8.68 ± 0.38*	5.13 ± 0.6**	4.33 ± 1.1**	3.01 ± 0.2**
	24	20-22°C / 25% ± 5%	7.37 ± 0.25*	3.23 ± 0.45**	3.03 ± 0.11**	2.67 ± 0.15**
	24	20-22°C / 65% ± 5%	6.82 ± 0.28*	2.56 ± 0.51**	1.96 ± 0.3**	2.01 ± 0.31**

Table 4. Inactivation of *Salmonella* planktonic and biofilms by dry-cleaning agents NovaBay, Salsa and Backspin. The plate counts represent the means and standard deviations from three independent experiments. ND – not detected. Means statistically different than the control samples (t-test) are indicated by asterisk.

Strain/conditions	***Time (h)***	***Temperature/RH %***	***Plate count (log CFU/sample)***			
			Control	Treated Salsa (Ethanol 29.4%)	Treated Surface Pro (Hypochlorous acid)	Treated Backspin No-Rinse (quaternary ammonium)
Salmonella planktonic cells	T0	20-22°C / 25% ± 5%	5.89± 0.29*	1.3 ± 0.03*	ND	ND
	24	20-22°C / 25% ± 5%	4.33± 0.28	ND	ND	ND
	24	20-22°C / 65% ± 5%	3.41 ± 0.29	ND	ND	ND
Salmonella biofilm	T0	20-22°C / 25% ± 5%	8.58± 0.4*	5.53 ± 0.58**	3.68 ± 0.4**	3.22 ± 0.4**
	24	20-22°C / 25% ± 5%	7.15 ± 0.3*	3.23 ± 0.45**	2.29 ± 0.21**	2.18 ± 0.2**
	24	20-22°C / 65% ± 5%	6.03 ± 0.42*	2.56 ± 0.51**	ND	ND

Table 5. Inactivation of packinghouse strain isolated in Objective 3- *in vitro* biofilms by dry-cleaning agents NovaBay, Salsa and Backspin. The plate counts represent the means and standard deviations from three independent experiments. ND – not detected. Means statistically different than the control samples (t-test) are indicated by asterisk.

Strain/conditions	***Time (h)***	***Temperature/%RH***	***Plate count (log CFU/sample)***			
			Control	Treated Salsa (Ethanol 29.4%)	Treated Surface Pro (Hypochlorous acid)	Treated Backspin No-Rinse (quaternary ammonium)
Mix 1 (from Facility A) biofilms	T0	20-22°C / 25% ± 5%	8.96± 0.15*	6.63 ± 0.40**	6.28 ± 0.31**	5.96 ± 0.1**
	24	20-22°C / 25% ± 5%	7.23 ± 0.85*	4.76 ± 0.7**	5.52 ± 0.23**	5.13 ± 0.02**
	24	20-22°C / 65% ± 5%	6.83 ± 0.85*	3.21 ± 0.8**	4.45 ± 0.2**	4.01 ± 0.14**
Mix 1 (from Facility B) biofilms	T0	20-22°C / 25% ± 5%	7.86 ± 0.95*	5.53 ± 0.45**	5.59 ± 0.11**	4.69 ± 0.2**
	24	20-22°C / 25% ± 5%	6.85 ± 0.85*	5.03 ± 0.4**	4.33 ± 0.1**	3.78 ± 0.05**
	24	20-22°C / 65% ± 5%	7.26 ± 0.71*	3.6 ± 0.87**	2.27± 0.3**	2.95 ± 0.13**

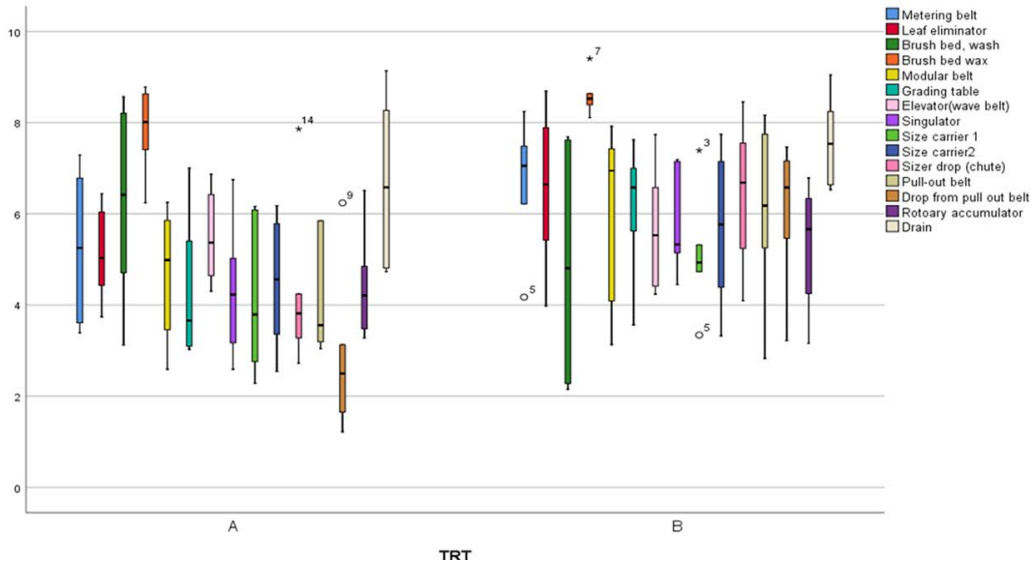


Figure 1. Box plot of aerobic plate count results from Facility A. The sampling points are described in the legend. A-after sanitation samples and B-before sanitation samples. The before-sanitation samples were collected at the end of the workday and the after-sanitation samples before the start of the nest workday (sanitation overnight).

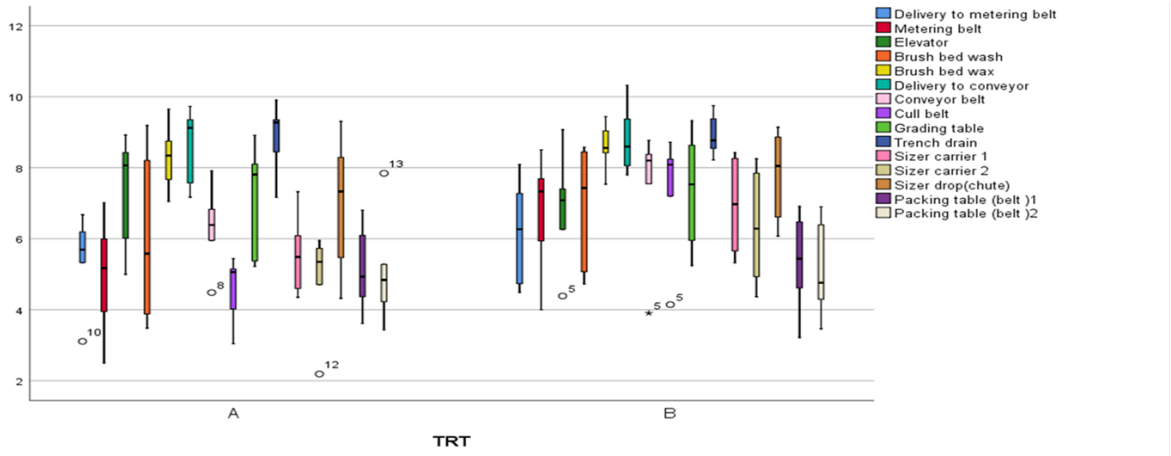


Figure 2 Box plot of aerobic plate count results from Facility B. The sampling points are described in the legend. A-after sanitation samples and B-before sanitation samples. The before-sanitation samples were collected at the end of the workday. After finishing packing, the surfaces were left undisturbed and the sanitation crew performed sanitation right before the start of the packing for the day.

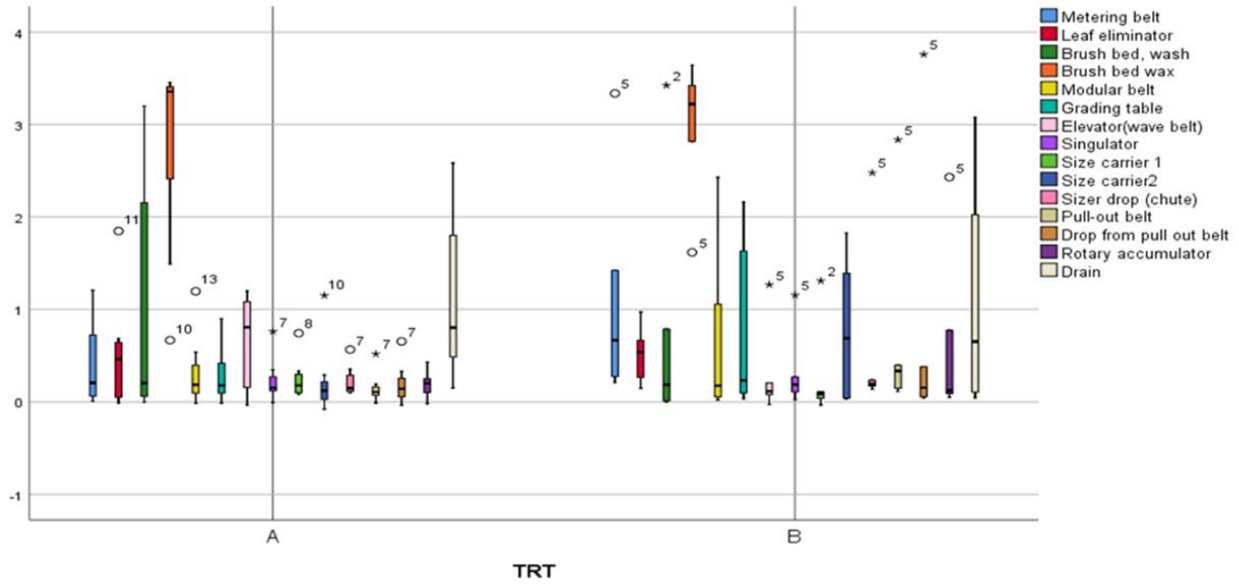


Figure 3. Box plot of crystal violet quantitative results from Facility A. The sampling points are described in the legend. A-after sanitation samples and B-before sanitation samples.

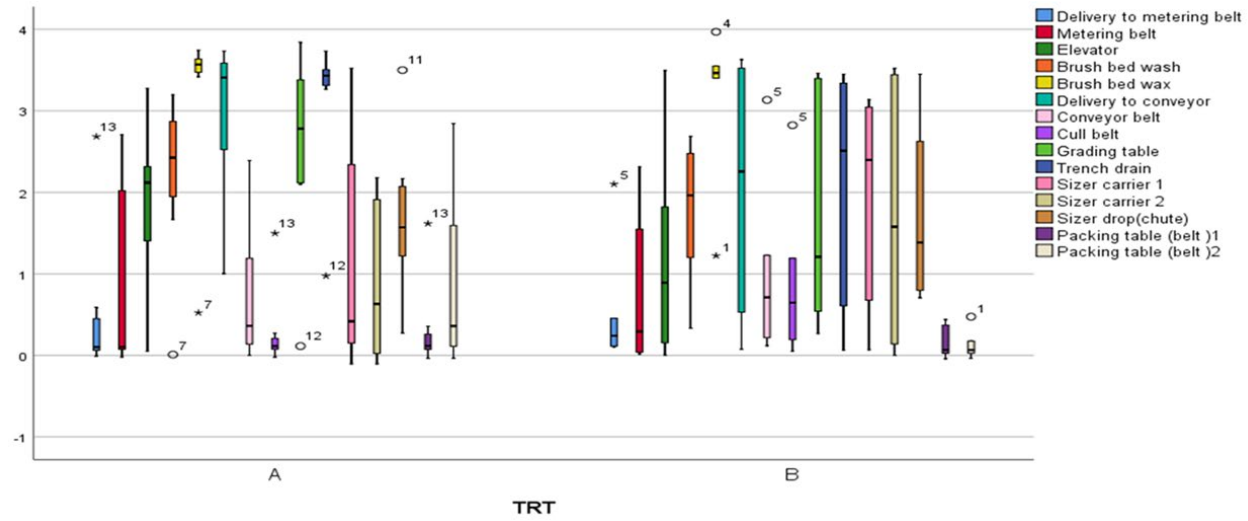
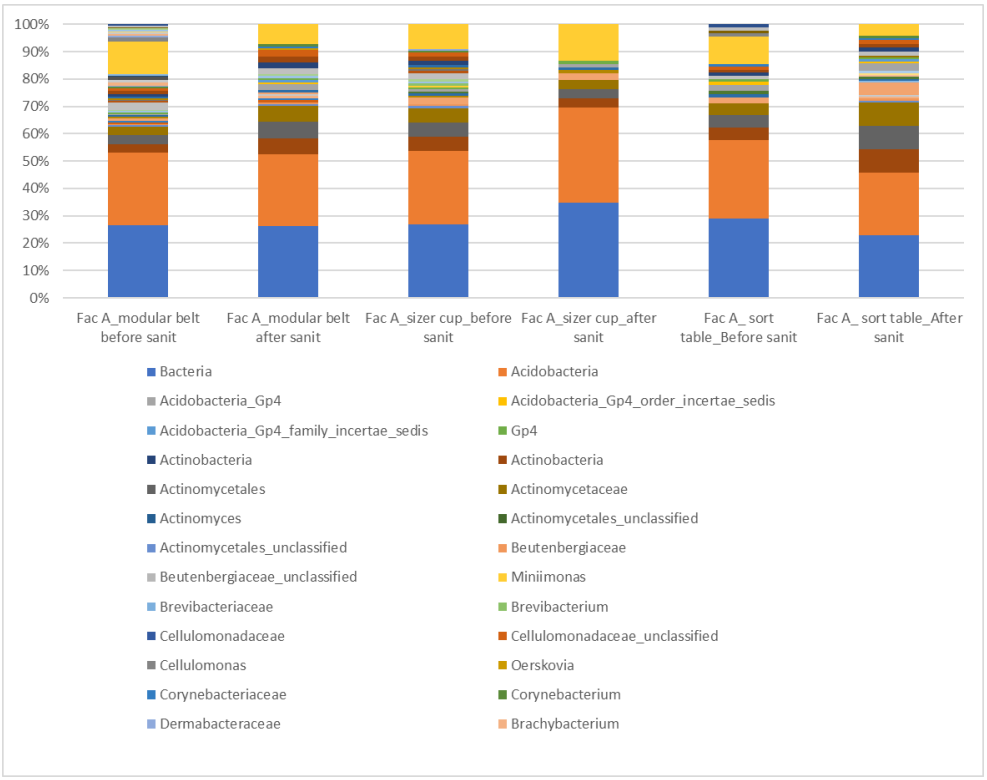


Figure 4. Box plot of crystal violet quantitative results from Facility B. The sampling points are described in the legend. A-after sanitation samples and B-before sanitation samples.



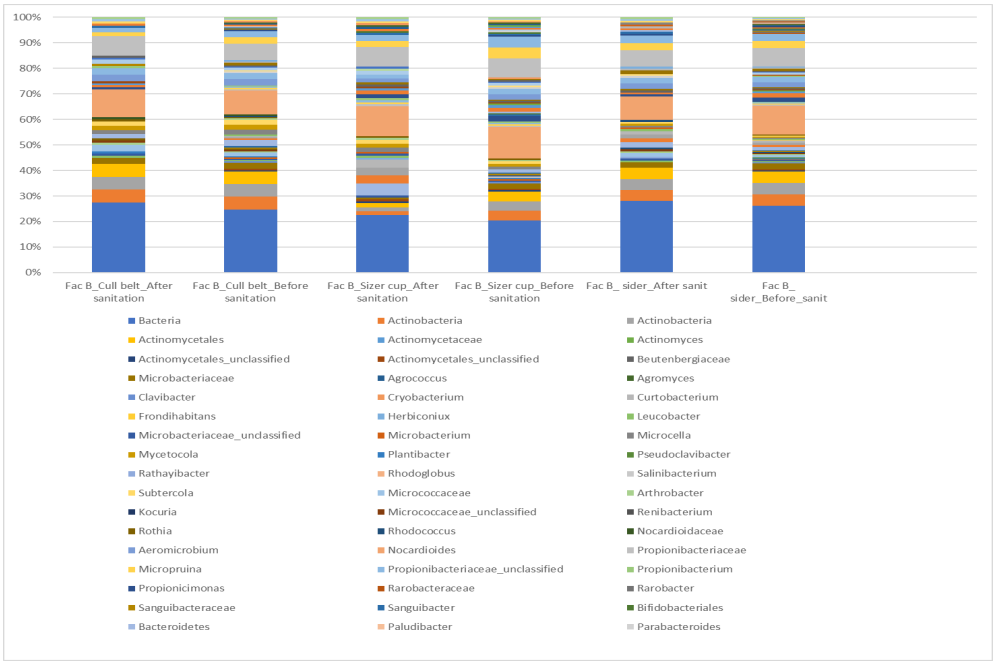


Figure 5. The relative abundance of total sequences of bacterial 16S rRNA OTUs at the class level. Samples were collected from Facility A (top) and facility B (bottom figure) in the dry area. Samples are represented as before and after sanitation.

Supplemental Figures:

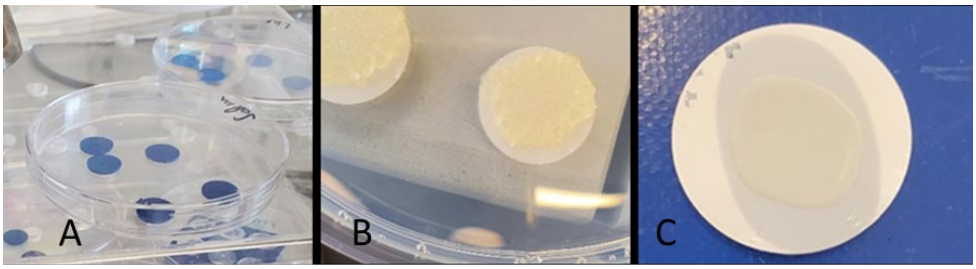


Figure 6. Test surfaces and biofilms grown for testing their susceptibility to dry conditions. A. Planktonic cells inoculated as droplets and dried in controlled relative humidity conditions. B. Dry (colony) biofilms grown on sterile polycarbonate membranes on LSM agar plates. Biofilms develop at the air-water interface on the agar surface. C. Example of mature biofilm on polycarbonate membrane transferred on the test surface and dried in controlled relative humidity conditions.

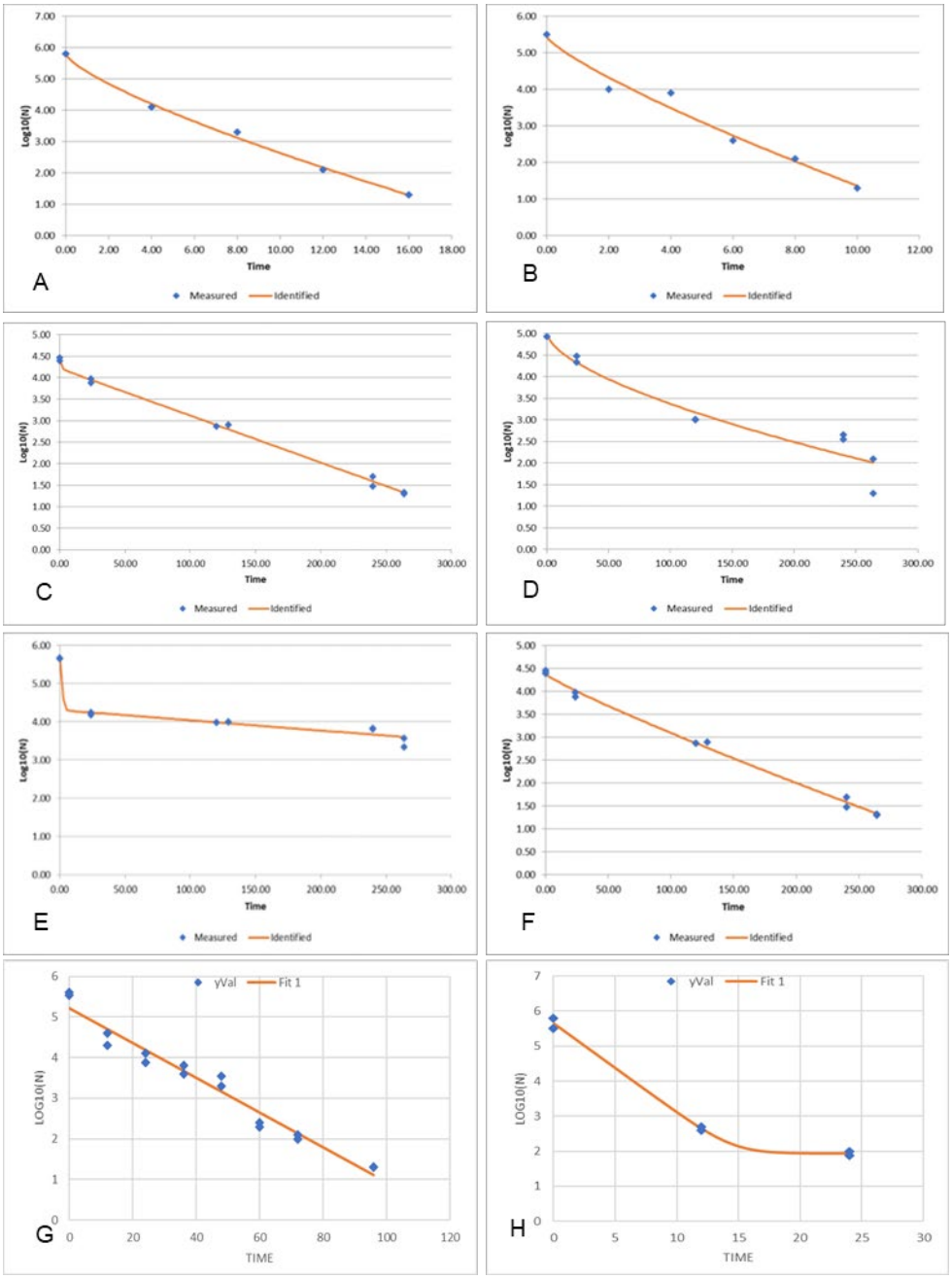


Figure 7. Survival of *L monocytogenes* planktonic cells at 20-22°C and 25% RH (left column) and 65% RH (right column). A and B, survival of *L monocytogenes* cells (no compatible solutes) at 25% and 65% RH. C and D, survival of *L monocytogenes* planktonic cells dried with 1.5% proteose peptone 3 at 25% and 65% RH. E and F, survival of *L monocytogenes* planktonic cells dried in the presence of 2x LSM at 25% and 65% RH, and G and H, survival of *L monocytogenes* dried in the presence of 1.5% trehalose.

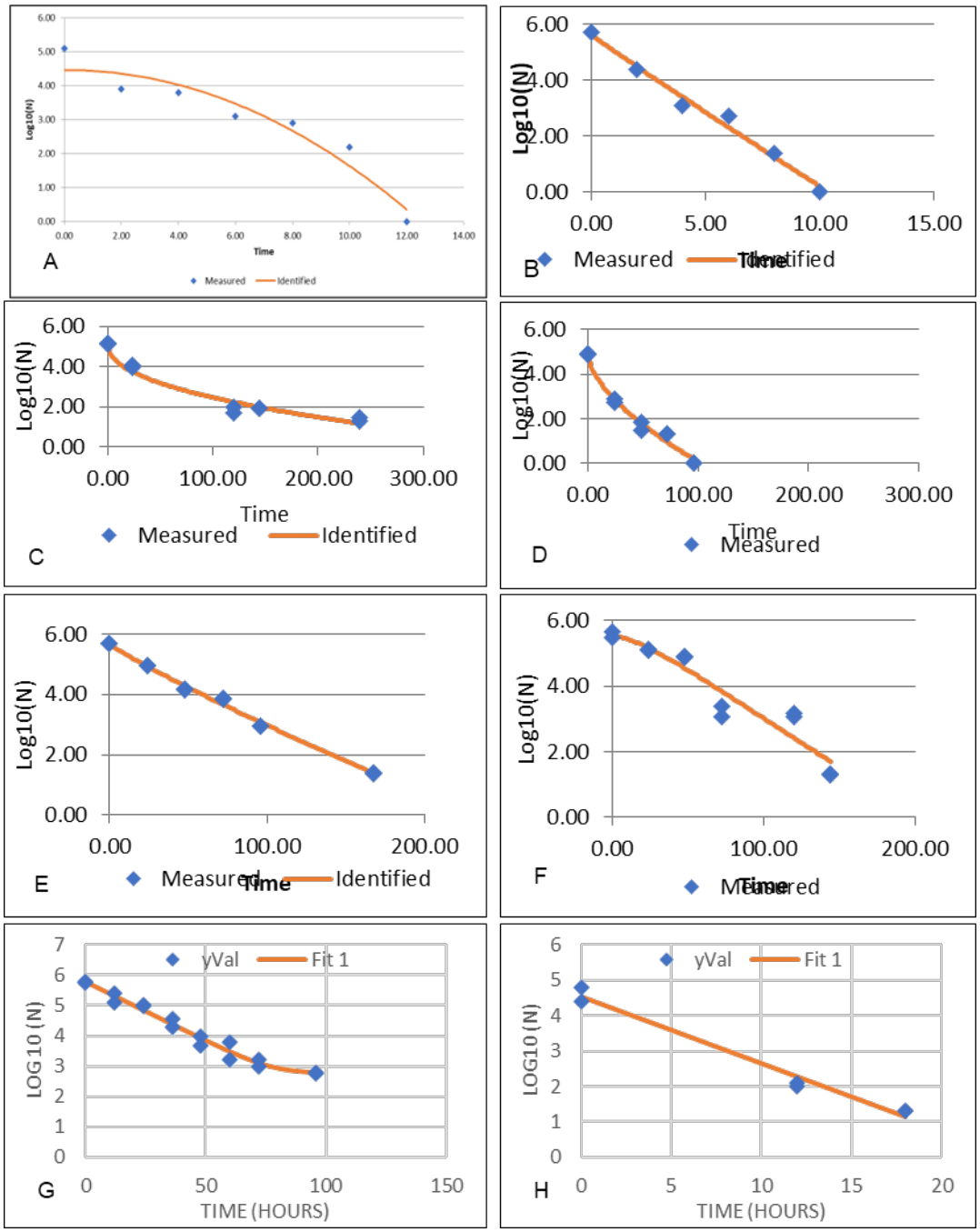


Figure 8. Survival of *Salmonella* planktonic cells at 20-22°C and 25% RH (left column) and 65% RH (right column). A and B, survival of *Salmonella* cells (no compatible solutes) at 25% and 65% RH. C and D, survival of *Salmonella* planktonic cells dried with 1.5% proteose peptone 3 at 25% and 65% RH. E and F, survival of *Salmonella* planktonic cells dried in the presence of 2x LSM at 25% and 65% RH, and G and H, survival of *Salmonella* dried in the presence of 1.5% trehalose.

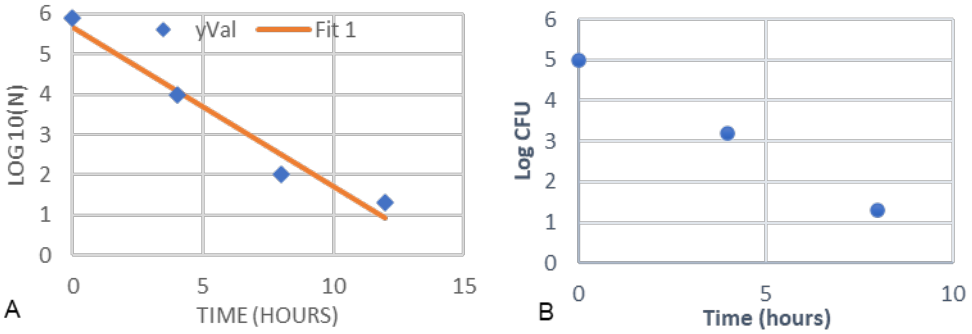


Figure 9. Survival of planktonic (A) *L monocytogenes* and (B) *Salmonella* incubated at 30-32°Ca and 25% ± 5% RH.

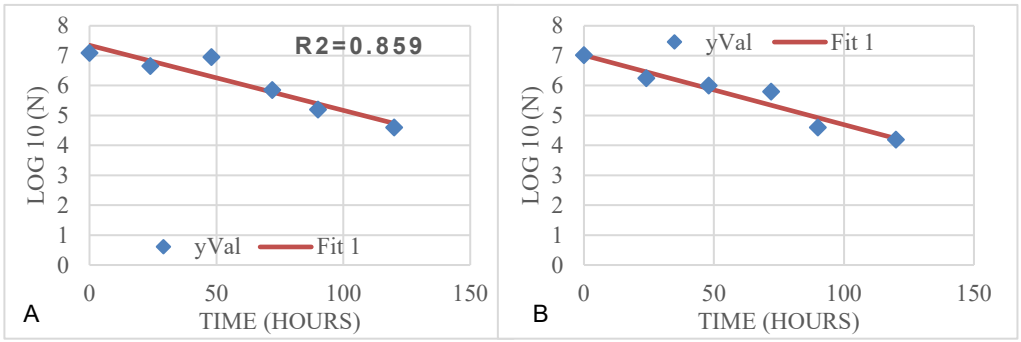


Figure 10. Survival of (A) *L monocytogenes* biofilms and, (B) *Salmonella* biofilms incubated at 30-32°C and 25% ± 5% RH.

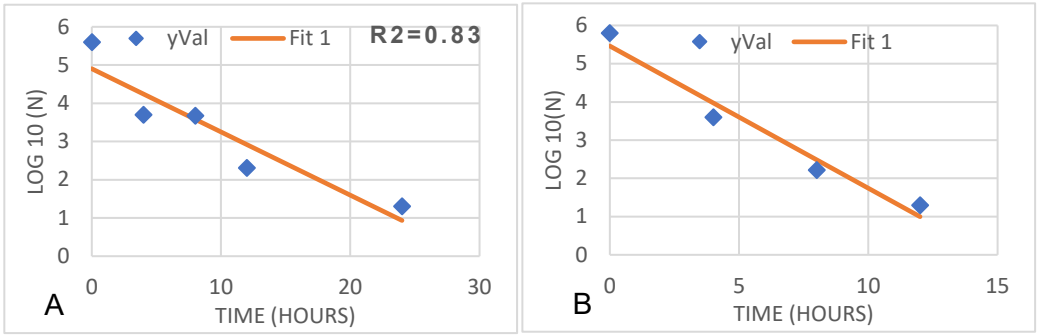


Figure 11. Survival of planktonic cells dried and incubated at 30-32°C and 70-72% RH. (A) *L monocytogenes* planktonic cells survived less than 30 hours and (B) *Salmonella* planktonic cells survived less than 15 hours.

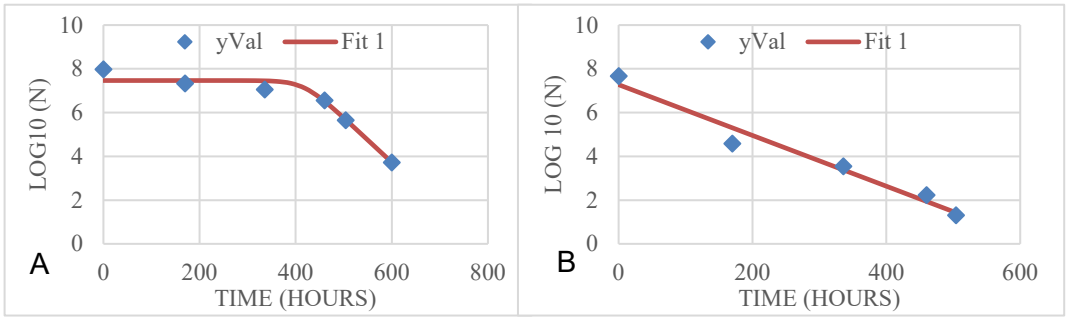


Figure 12. Survival of *L monocytogenes* biofilms (A) and *Salmonella* biofilms (B) when exposed to 30-32°C and 70% ± 2% RH. *L monocytogenes* biofilms were viable for a month, *Salmonella* cells were recoverable for 3 weeks.

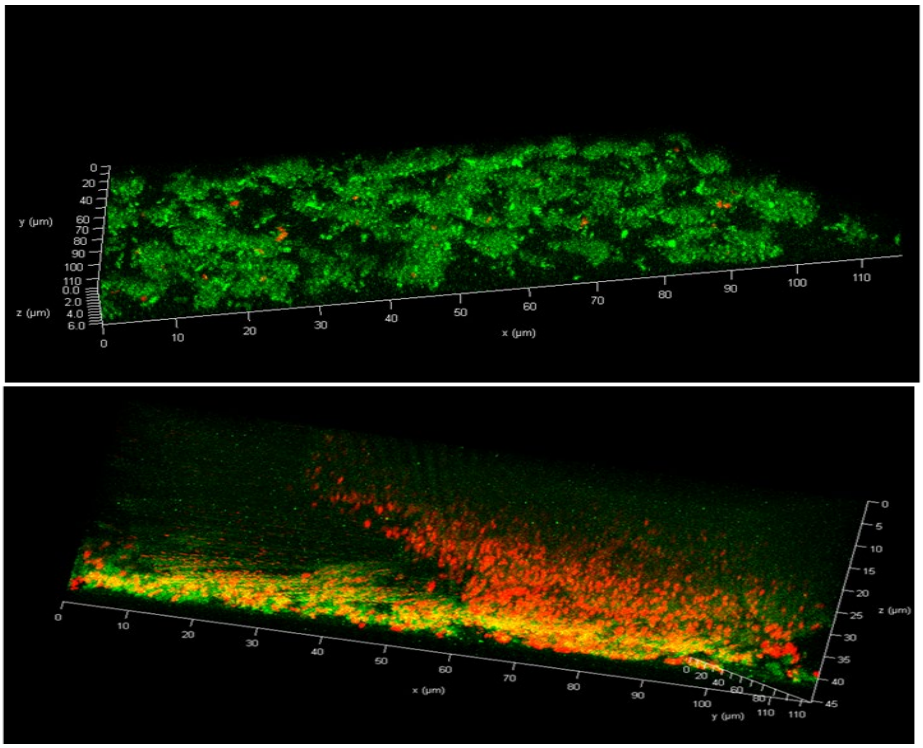
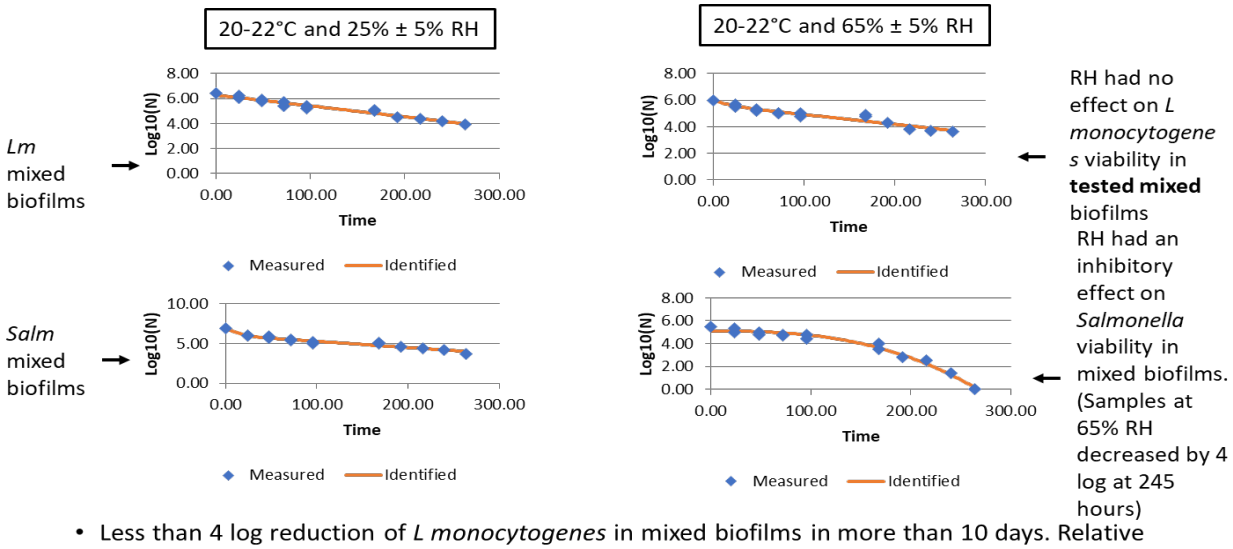


Figure 13. Confocal microscopy (100x magnification) of packinghouse samples S1 (top) and S2 (bottom). Green dots are live cells, the dead cell biomass is stained in red. Biofilm S1 is compact with slow growing biomass. Biofilm S2 grew actively close to the surface, with a high cell turn-over at the outer edge of the biofilm.



- Less than 4 log reduction of *L monocytogenes* in mixed biofilms in more than 10 days. Relative humidity level was not a factor in microbial viability (similar die-off curves for 25 and 65% RH.

Figure 14. *In vitro* biofilms of environmental packinghouse strains that were "spiked" with *L monocytogenes* and *Salmonella*. Pathogens were enumerated to determine their viability over time in mixed biofilm.