CPS 2023 RFP – FINAL PROJECT REPORT

Project Title:

Control of *Listeria monocytogenes* on avocado and avocado contact surfaces during dry packing by acetogenins naturally present in avocado seed

Project Period:

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Objectives:

1. Evaluate the ability of the acetogenin-enriched (AFA) extract to reduce *Listeria monocytogenes* (*Lm*) on avocado surfaces in a simulated dry packing operation.
2. Evaluate the effectiveness of the AFA extract at reducing cross-contamination with *Lm* after contact with contaminated packing equipment.
3. Study metabolic responses of avocados to the coating with AFA extract.

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FINAL REPORT

Summary of Findings and Recommendations

Objective 1. Effectiveness of avocado fatty alcohols (AFA) coatings at reducing *Lm* on avocados.

- A coating composed of AFA extracted from avocado seeds, in a vehicle composed of propylene glycol (PG) and avocado oil resulted in a 99-99.9% reduction in *Listeria monocytogenes (Lm).* The antilisterial activity was attributed to the combination of AFA and PG.
- The initial numbers of *Lm* inoculated onto the avocado surface were rapidly reduced after inoculation in the fruits treated with the AFA coatings. This initial reduction resulted in a low *Lm* concentration during slow growth during the storage time (4 weeks).
- Green Skin avocados were more perishable and permitted more *Lm* growth on the rind compared to Hass avocados.

Objective 2. Effect of AFA coatings on the reduction of cross-contamination from contaminated food-contact surfaces to the avocado surface.

- Approximately 55% of the *Lm* cells inoculated on a PVC conveyor belt surface developed strong attachment within 1 h after inoculation, and this proportion of strongly attached cells did not increase with time extended up to 8 h. Results indicated that *Lm* could develop strong bonds and firmly attach onto the food-contact surfaces.
- In a simulated scenario of cross-contamination of avocados from contaminated food-contact surfaces, about 1% of the bacteria that were inoculated on the surface of the PVC conveyor belt were transferred to the avocado surfaces when the fruits were not coated, but the counts of transferred cells on avocados were about 2 log cycles lower for the AFA coated avocados.
- *Lm* showed capacity to form biofilm when inoculated on avocado rinds, and the biofilm formation did not seem to be interrupted by coating with AFA prior to contamination.

Objective 3. Metabolic responses of avocados to the coating with AFA.

- AFA antimicrobial effects resulted in the extension of the fruit shelf-life as evidenced by physicochemical parameters (lower decay grey areas and higher firmness) and changes in metabolites induced by decay (e.g., carbohydrates and amino acids).
- Changes in desirable metabolites, including fatty acid and phytosterols, assessed during storage indicated that AFA antimicrobial coatings did not have a significant impact on the characteristic nutritional quality of ripe avocados.
- Metabolomic signatures of the coated fruits during storage allowed us to study changes in a total of 205 avocado pulp metabolites, including fatty acids and phytosterols (targeted analyses) and non-volatile flavor and others (untargeted analyses); of which 79 metabolites were associated with the corresponding avocado quality determinations and with changes induced by the antimicrobial effects of the AFA coating.

Abstract

Avocado fatty alcohols (AFAs) are unique molecules of avocado fruit that have a range of biological benefits, including antimicrobial activity against *Listeria monocytogenes (Lm)*. AFA extracts from seeds or low-quality fruits offer a novel technology for the avocado industry to enhance avocado packing safety while adding value to avocado waste. This proof-of-concept study aimed to determine the feasibility of reducing *Lm* in avocados with AFA coatings by conducting challenge tests. Coatings containing AFAs were formulated in food-grade propylene glycol (PG) and avocado oil (AO). Coating formulations were standardized to deliver 7,000, 10,000 and 14,000 ppm of AFA molecules. After their inoculation, the prototypes containing AFA showed a rapid reduction of *Lm* numbers, resulting in lower *Lm* growth during storage time (4 weeks at 25°C). AFA coatings reduced *Lm* by 99-99.9%, where the antilisterial activity was attributed to the combination of AFA and PG. On the other hand, avocado skin topography affected AFA coating antilisterial effectiveness since a higher *Lm* growth rate was observed in Green Skin avocados compared to Hass avocados. Cross-contamination tests in a PVC conveyor inoculated with *Lm* indicated that counts of transferred cells on AFA-coated avocados were about 2 log cycles lower than on uncoated avocados. AFA antimicrobial coatings also extended avocado fruit shelf-life as evidenced by physicochemical parameters (lower decay grey areas and greater firmness) and changes in metabolites induced by decay (e.g., carbohydrates and amino acids). As evidenced by fatty acid and phytosterol profiles, avocado nutritional quality was not altered by AFA coatings.
Although further work is needed to optimize formulations and usage levels, proof-of-concept experiments indicated that AFA coatings could be implemented as a complementary measure for controlling *Lm* growth in joint efforts with the strict sanitation and verification programs already existing in the avocado packing industry.

Background

The safety of avocados is a growing concern due to the large volumes of avocados that are traded worldwide, which have been steadily increasing over the last five years. The world market value is projected to grow from 10.9 billion dollars in 2024 to 19.9 billion dollars in 2026 (Shahbandeh, 2022). It is important to note that fresh avocados have not been linked to any case of foodborne listeriosis. Although a recent study reported molecular and epidemiological indications of a possible outbreak of listeriosis linked to avocado consumption (Pomeroy et al., 2021), the amount of data available and the reliability of some of these data made the association between listeriosis and avocado consumption extremely weak. Another source of concern in the US was the finding of *Lm* in fresh avocados, since 17.7% of the samples were found to contain *Lm* on their skin, and 3 (0.24%) of 1,615 samples tested positive for the presence of *Lm* in the edible portion of the avocados (FDA, 2018). The presence of *Lm* inside the avocado may be related to defective washing procedures, which are known to promote the internalization of pathogens inside a variety of fruits, including avocados (Chen et al., 2016). To prevent any potential food safety issues resulting from postharvest washing, the avocado exporters in Mexico have adopted a no-wash policy in their operations. Since most of the fresh avocados consumed in the US are imported (ERS, 2025), and almost 90% of these imports are from Mexico (ERS, 2025), this measure is expected to have a great impact on the overall avocado safety in the US. However, a small residual risk may still occur in relation with *Lm* potentially present on the avocado skin surface. The limitations of postharvest disinfection treatments using aqueous sanitizing solutions has been extensively studied (Yoon and Lee, 2018). Furthermore, the use of some chemical sanitizers may be against the requirements of the National Organic Program, needing the use of natural compounds.

Among natural compounds, lauraceous acetogenins, which will be referred to here as avocado fatty alcohols (AFA)[1], are a group of compounds found in avocados, which possess biological activity (Rodriguez-Saona and Trumble, 2000). These compounds were first described as early as 1969 (Kashman et al., 1969) and were further found to be part of the complex pool of bioactive compounds produced uniquely by avocado fruit in idioblastic oil cells. These cells are believed to be the place where some plants produce compounds acting as mechanisms for protection against various stressors (Rodriguez-Saona and Trumble, 2000). Although the AFA are naturally present in various structures of avocado plants, the seed is the part of the fruit where the greatest concentration has been found (Rodriguez-Lopez et al., 2017). Particularly, AFAs have been reported to possess bioactivities potentially beneficial for human health, from tumor reducing to antimicrobial activity against a variety of microorganisms (Chia and Dykes, 2010; D'Ambrosio et al., 2011; Domergue et al., 2000; Echenique-Martinez et al., 2021; Leon et al., 2011; Lu et al., 2012; Neeman et al., 1970). Using a precise metabolomics approach, Rodriguez-Lopez et al. (2015) were able to classify the avocado acetogenins in 3 different families, all with bioactive traits. The potential application of acetogenins as a food additive has been intensively studied in Co-PI Hernandez-Brenes' laboratory over the last decade, especially as antioxidant ingredient (Rodriguez-Sanchez et al., 2013a), as spore inhibitor with potential application in processed meats (Pacheco et al., 2017; Rodriguez-Sanchez et al., 2013) and more recently, as antilisterial with potential application to a variety of ready-to-eat (RTE) foods (Rodriguez-Sanchez et al., 2019; Salinas-Salazar et al., 2017; Villarreal-Lara et al., 2019). In these studies, the acetogenins obtained from avocado seed showed a specific effect against Gram-positive bacteria in comparison to Gram-negatives, with a specific listericidal effect, attributed to lysis of *Lm* cells and increase in membrane permeability (Salinas-Salazar et al., 2017). The major focus of ongoing studies has been on fully cooked RTE meat products, but the potential applications are not restricted to meat products. For produce packing, the oily nature of the AFA would make this compound particularly attractive when a washing step needs to be avoided, such as the dry packing of avocados. Although the natural concentration of AFA in avocado (< 1%), may be too low to have an antimicrobial effect that would be practical in the industry as antilisterial, using a clean extraction technology developed in Dr. Hernandez-Brenes' laboratory (Hernandez-Brenes et al., 2012) it is possible to increase the AFA concentration to levels sufficiently high to show a significant reduction in *Lm,* and adjust it to accurately match any research or industry needs*.* More recent works in meat matrices (unpublished) have demonstrated that avocado AFAs at 8,900 ppm in a sausage paste showed a listericidal effect (absent in the product) at time 0 and after storage for 7 days at 4 °C, with a reduction of the initial *Lm* inoculum of 2.78 log. Listeriostatic activity was also observed at concentrations of 2,200 mg/kg AFAs at 7 days, 4 °C. Prior works on stability have shown molecules are stable to temperatures up to 100 °C, and remain unchanged in a meat matrix and refrigerated storage for up to 42 days, with losses that range from 8-20% at the end of shelf-life (Pacheco et al., 2017). The AFAs are then mixed with avocado oil to adjust consistency and applicability. This is a food-grade extract that features specific listericidal activity (Salinas-Salazar et al., 2017; Villarreal-Lara et al., 2019).

[1] Note: The acetogenins used to prepare acetogenin-enriched oil extract (ACE) in this study were extracted from avocado seed. The avocado (lauraceous) acetogenins are different in bioactivity and chemical structure from other acetogenins present in Annonaceaea. Concerns about neurotoxicity have been reported for Annonacenous acetogenins, whereas safety of lauraceous acetogenin consumption (fatty alcohols) has been assessed in a clinical trial (up to 200 mg d^{-1} for 60 d), and avocado fatty alcohols (AFAs) were well-tolerated and not associated with toxicity effects (Ahmed et al*.,* 2019). **To prevent any confusion, we will refer to the avocado acetogenins as avocado fatty alcohols (AFA) throughout the final report presented below.**

Regarding safety of these compounds, Rodriguez-Sanchez et al. (2019) used propylene glycol as vehicle to test the safety of acetogenin-enriched oil extracts for human consumption. Their study also included the use of an independent laboratory in the UK for conducting mutagenicity and acute oral toxicity of an avocado seed extract under protocols approved for European Union regulations. These authors concluded that the avocado AFA extracts were non-mutagenic and with safe consumption at very high levels (LD_{50}>2000 mg/kg) which classified it in the lowest toxicity category of the Globally Harmonized System (GHS). Other authors have reported similar results (Ahmed et al., 2019).

Using avocado seeds for isolation of bioactive compounds offers a new opportunity for the avocado industry to obtain effective, natural antimicrobial compounds that can be used as natural antimicrobials for adding an extra element of safety to avocado packing, while adding value to a component commonly perceived as waste from avocado processing, as occurs with other agro-industrial waste items (Shirahigue and Ceccato-Antonini, 2020). Avocado packers could use the AFA extract as a natural, food-grade compound with specific activity against *Lm* and as a complement rather than a replacement to the strict sanitation and verification via environmental monitoring as the already existing programs to control *Lm* in avocado packing. This is a non-aqueous compound and therefore should not interfere with the dry packing system currently used by Mexican avocado packing plants that process avocado for export to the US. This proof-of-concept project aimed to evaluate the effect of the antilisterial lipid derivatives contained in the AFA extract applied as a coating on avocados, on the reduction and prevention of cross-contamination of avocado surfaces with *Lm* and to develop an effective method to apply the AFA coating onto avocados during dry packing.

The prototype of the AFA coating used in this proof-of-concept study contained relatively high concentrations of AFA obtained from avocado seed extracts and propylene glycol (PG) as a vehicle, which has reported antimicrobial activity on its own. This coating was tested as a potential antilisterial compound to be applied on fresh avocados as a non-water based, natural compound to add one more hurdle in the prevention of *Lm* during avocado packing.

Research Methods and Results

For Objective 1: Evaluate the ability of the acetogenin-enriched (AFA) extract to reduce Listeria monocytogenes (Lm) on avocado surfaces in a simulated dry packing operation:

Methods

Preparation of the AFA extract and formulation prototypes

AFA extraction and quantification. Avocado seeds were sourced from Avodehy S.A. de C.V., Mexico and transported to the laboratories at ITESM. The seeds were dried and ground. Extracts were purified by centrifuge partition chromatography as described by Rodriguez-Sanchez et al. (2013) in a pilot-scale process with food-grade solvents. Following solvent evaporation, the fractionated oil enriched in AFAs was chemically characterized to confirm the concentration of individual compounds and purity (47.9% of AFAs). AFA molecules were quantified by liquid chromatography (HPLC-PDA and LSD) as previously described by Rodriguez-Sanchez et al. (2019).

Formulation and standardization of AFA extract coatings for their application on fruit surfaces. Coatings containing AFAs were formulated in food-grade propylene glycol (PG) and avocado oil (AO). Coating formulations were standardized to deliver 7000, 10000, and 14000 ppm of AFA molecules. AFA

coatings were shipped overnight to PI Castillo's lab for experiments of Objectives 1 and 2 and used at ITESM for the experiments of Objective 3. For shipment, the formulations were vacuum packaged in low-permeability films, frozen at -20 °C, and then the packages were placed in coolers with ice packs. Upon arrival, formulations were stored at 4 °C until use.

_Lm_ challenge studies

Sourcing and selecting avocados. Fresh Hass and Green Skin (cv Bacon) avocados were graciously donated by Giumarra Company in Ventura, California. The avocados were packed after harvesting without being subjected to any postharvest treatment such as cleaning, brushing, or washing, and immediately shipped to ensure arrival at the laboratory in Texas A&M within two business days after the shipping date to maintain freshness and prevent damage and ripening of the fruit. Upon arrival, damaged avocados were inspected to separate and discard damaged units, and the remaining avocados were stored at refrigeration (4 °C) until use.

Microorganisms and inoculation. The avocados were spot-inoculated with a cocktail of 5 strains of *Lm* isolated from avocado packing environments during a previous study corresponding to strains Lm-Ag -251, Lm-Ag-333, Lm-Ag-336 isolated from the skin of retail avocados and EI-P44-497, EI-S263-51 isolated from non-food contact surfaces in avocado packing plants. These isolates were supplied by Drs. Ofelia Rodriguez, Nanci Martinez and Ramon García-Frutos of University of Guadalajara, and were confirmed as *Lm* by biochemical and DNA characterization prior to shipment. All necessary permits were cleared to enable receiving these isolates. For each avocado, three circles slightly larger than 10-cm^2 in area were marked on the avocado rind using a white/metallic permanent marker. From the *Lm* cocktail, 100 µl was transferred using a pipette and placed onto each of the three spots, then evenly distributed with a sterile loop. Inoculated avocados were allowed to dry for two hours inside the biosafety cabinet. The target concentration of *Lm* on the inoculated surfaces was ~5 log CFU/cm^2, and this inoculum level was verified by plate count every time the experiments were executed.

The above inoculation method was used for Tasks 1.1 and 1.2 of this objective.

Task 1.1. Effect of the acetogenin concentration in the AFA extract on the reduction of _Lm_ on the skin of Hass avocado

This task used one single variety of avocados (Hass) to avoid the introduction of one more effect (surface topography), which could work as a confounding factor in the experimental design. The effect of the surface this effect was tested in Task 1.2 and is described below. This task (1.1) also included testing different methods for delivery of the AFA coating to select a method that may provide the most realistic and efficient treatment application.

Application of treatments. The inoculated Hass avocados were separated in 4 groups, each for application of the coatings containing 7,000, 10,000, or 14,000 mg/kg of AFA. These concentrations were achieved by formulating with propylene glycol (PG), avocado oil (AO, Chosen Foods, San Diego, CA), and AFA from avocado seed extract as follows:

- For 7,000 mg/kg: 95% w/w of PG, 3.5% w/w of AO, and 1.5% w/w of AFA
- For 10,000 mg/kg: 95% w/w of PG, 3% w/w of AO, and 2% w/w of AFA.
- For 14,000 mg/kg: 95% w/w of PG, 2% w/w of AO, and 3% w/w of AFA.
- For 0 mg/kg: 100% AO (untreated control).

The brand of AO used for the coatings was selected from several brands previously tested in Co-PI Brenes' laboratory to ensure that it had no detectable levels of AFA naturally present.

After preparing the coatings at different AFA concentrations (including untreated controls), avocados in each group were subdivided into 3 groups, each assigned to a different method of AFA coating application, i.e. spraying (misting), brushing, and rolling. The application of coatings was conducted in a biosafety cabinet as follows:

Misting. An oil sprayer with a rotating nozzle (Flairosol Olivia FL1002, New York, NY) was used to create a fine mist to cover the entire surface of the avocados. An independent oil sprayer was used for spreading the AFA coating at each concentration, including AO. To apply the coating, the fruit was placed on a light duty gravity conveyor with 1.5-in-diameter galvanized steel rollers (Ultimation Store, Roseville, Michigan) and manually operated to simulate the conveyor belt on an avocado packing plant.

Brushing. A nylon cylinder brush (Model: CB3510 Grainger, TX) was attached to a light-duty gravity conveyor with 1.5-in-diameter galvanized steel rollers (Ultimation Store, Roseville, MI). The AFA coatings at the corresponding concentration (including 0%) were dripped onto the brush, and the avocados were placed on the rollers and manually passed through the rotating brush.

Coating by rollers. Stainless-steel rollers were wrapped with food industry high-temperature silicone foam sheets with adhesive back (McMaster-Carr, Chicago, IL). These wrapped rollers were assembled to a light-duty gravity conveyor (Ultimation Store, Roseville, MI) inside the biosafety cabinet. The AFA coating or AO was added to the sponge before placing the avocados. The coated rollers were rotated as the avocados were in contact to ensure an even coating of the fruits.

After application of their respective coating, the avocados were left to dry overnight inside a biosafety cabinet. After this time, samples of avocado skin were collected from each treatment group and subjected to microbiological testing as described in the procedures below.

Task 1.2 Effect of the skin topography on the ability of AFA coating to reduce *Lm* on the avocado rind and its survival during storage

For this task, Hass (rough skin) and Green Skin (smooth skin) were used to compare the effect of surface topography on the antimicrobial effect of the AFA coating.

Inoculation and treatment application. From the data collected during Task 1.1, the AFA coating at a concentration of 7,000 mg/kg AFA and coating by misting were determined to be the most convenient conditions for AFA coating. Avocados were inoculated as described above and then separated in 3 groups for each avocado variety. One group consisted of non-treated avocados (positive control), while one of the other groups was coated with AO only (0 mg/kg AFA) and the other with the AFA coating at 7,000 mg/kg). For both AO and AFA coating, the treatment was applied by misting as described in Task 1.1. This inoculation and treatment procedure was followed for the 2 experiments described as follows:

Experiment 1: Effect of the skin roughness. After inoculation and application of treatments, avocados from the two treatment groups (0 mg/kg and 7,000 mg/kg) for each variety were left to dry inside the biosafety cabinet overnight (approximately 8 h), whereas the untreated avocados were immediately sampled for bacterial counts as described above. After drying, all avocados were subjected to skin sampling and tested for counts of *Lm.* These counts were later compared to determine whether the roughness of the surface affected the ability of *Lm* to survive after being treated.

Experiment 2: Effect of AFA coating on survival of Lm during storage. For this experiment, the survival of *Lm* during refrigerated storage was tested as a function of the type of avocado surface. Subjects consisted of inoculated Hass and Green Skin avocados treated with AFA coating (7,000 mg/kg), AO (0 mg/kg), and no treatment (positive control). Inoculation of the fruit and coating was carried out as described above. After treatment, all avocados were stored at 4-5 °C for 4 weeks. Sample collection started after 4-5 hours of refrigeration, representing the initial data point of refrigeration storage (zero time). Samples were also collected at weeks 1, 2, 3, and 4 of storage. For each sampling time point, the sample collection and microbiological testing procedures were followed as described below.

Firmness tests. A set of non-inoculated coated and non-coated avocados was over 28 days. At 0, 7, 14, 21 and 28 days, the firmness was measures using a FT 444 penetrometer (QA supplies, Norfolk, VA), and the firmness in N/mm^2 was recorded and plotted.

Sampling and testing. In all the experiments and tasks described above, sample collection started after overnight storage (~8 h) of coated avocados, whereas the positive controls were sampled 2 h after inoculation to establish the baseline *Lm* population in the avocados.

Sample collection and testing. Each individual avocado was sampled by excising 10-cm^2 core samples from within the inoculated areas previously marked. These core samples were collected using a flamed stainless-steel borer to excise a 10-cm^2 circular sample of the inoculated skin from each of the three marked circles. Using sterile forceps and scalpel, the samples were separated and placed into a sterile bag (Whirl-Pak, Chicago, IL) containing 50 ml of sterile PBS supplemented with 10% Tween 80 (PBS+T80) to deactivate any residual antilisterial effect of the AFA coating, which could interfere with colony growth on the counting media.

For plate counts, each sample was homogenized in a stomacher and then subject to decimal dilutions, then spread-plated onto modified oxford agar (MOX) Plates were incubated for 48 hours at 35 °C following the Bacteriological Analytical Manual (BAM) (Hitchins et al., 2022) prior to count typical colonies. To ensure that the colony counts were *Lm*, a representative number of these colonies was picked and subjected to confirmation by preliminary tests followed by confirmation using BAX system.

Every treatment was tested in triplicate and repeated across three independent trials, except in Experiment 2 of Task 1.2, where each treatment was tested in duplicate and repeated across three independent trials.

Results – Objective 1

Characterization of the acetogenin-enriched (AFA) extract.

The avocado fatty alcohol-enriched extract was obtained from avocado seeds and characterization result indicated a purity of 47.9% (w/w) of total AFAs. Individual molecules in the profile included 24.31% persin (C23:2n-6), 22.79% persenone A (C23:3n-6), and 18.00% AcO-avocadene (C19:2n-1). Other molecules in the profile were 12.00% persenone B (C21:1n-14), 10.36% AcO-avocadyne (C19:2n-1), 5.40% persenone C (C21:2n-3), 3.56% AcO-avocadenyne (C19:3n-1), 1.93% AcO-avocadiene B (C19:2n-1), and 1.66% persediene (C21:2n-1). As described in the methodology section, coating formulations contained food-grade propylene glycol (PG) and avocado oil (AO) as excipients. Formulations were standardized to contain 7000, 10000, and 14000 ppm of AFA molecules.

Task 1.1. Effect of the acetogenin concentration in the AFA coating and of the method of coating application on the reduction of Lm on avocado skin

AFA coatings with all combinations of AFA concentration and coating method resulted in Lm counts significantly lower than the *Lm* counts of the control (0 mg/kg AFA). *Lm* was reduced by 2.5-3.0 log cycles as a result of coating avocados with the propylene glycol-AO vehicle containing AFA at 7,000, 10,000 or 14,000 mg/kg when applied by brushing or misting (spray). There were no differences between these two methods ($P > 0.05$). Thus, when the coating was applied by sponge rollers, the reductions in *Lm* were significantly smaller than those obtained by brushing or misting ($P < 0.05$) at 7,000 mg/kg AFA. These data are shown in **Table 1** in the Tables and Figures Appendix. The largest reductions in *Lm* (lowest counts) were observed when the AFA coating was applied by brushing or misting. Since the brushes have been seen in previous work to be a major source of potential contamination (Castillo et al., 2024), a coating containing 7,000 mg/kg AFA applied by misting (spraying) was selected as the method of choice for further tests.

Task 1.2. Effect of the skin topography on the reduction of *Lm* on AFA extract-coated avocado and its survival during storage

In Experiment 1 of this task, coating avocados with the PG-based AFA coating at 7,000 mg/kg resulted in *Lm* counts of 0.7 and 0.5 log CFU/cm^2 on Hass and Green Skin avocados, respectively. These counts were significantly smaller ($P < 0.05$) than those for non-treated or AO-coated avocados (AO-coated only included the excipient). For all AFA treatments, there were no differences between Hass and Green Skin avocados, suggesting that the antimicrobial effect of the AFA coating was not affected by the roughness of the avocado skin. In addition, the numbers of *Lm* on non-treated vs. AO-treated avocados were not different, proving that the AO excipient did not have any AFAs present. In reference to the counts of non-treated and AO-coated avocados, the reductions of *Lm* by the AFA coating agent were of 3.4 and 3.7 log cycles on the surface of Hass and Green Skin avocados respectively; and the magnitude of these reductions did not differ between avocado varieties ($P > 0.05$). The data are shown in **Figure 1**.

In Experiment 2, *Lm* was able to grow from 5 log CFU/cm^2 to final levels of ~6 log CFU/cm^2 over 4 weeks of refrigerated storage (4-5 °C) on avocados that received no treatment or that were coated with AO excipient alone. In contrast, under the same storage conditions *Lm* showed minimal growth (1.2 to 1.9 log CFU/cm^2) on avocados that were covered with the AFA coating. Lastly, Green Skin avocados showed more active growth, from 1.7 to 4.4 log CFU/cm^2 by 3 weeks of storage (2.7 log increase, **Figure 2**). Generation (doubling) times (GT) for *Lm* on Hass and Green Skin avocados are shown in **Table 2**. These simplified values were estimated based on mean *Lm* counts during growth. A faster growth of *Lm* on Green Skin seemed to be favored by the more rapid decay of Green Skin, where the rind was visibly degraded, after as soon as 2 weeks, perhaps exposing structures that provided more support for microbial growth. However, a plausible explanation for the shortest GT (1 doubling every 1.4 days at exponential phase) for Green Skin avocados treated with AFA coating is still needed. From these data, it was concluded that the PG-based AFA coating caused an initial reduction in both avocado varieties, and as shown at least for Hass avocados, this reduction permitted a low level of *Lm* to be maintained during storage, even though the growth rate may not have been lowered.

The trials to determine loss of firmness in the avocados showed that the firmness decreased significantly during storage, and the loss of firmness was affected by the avocado variety, treatment and days of storage, as well as interactions variety*treatment and variety*days of storage ($P < 0.05$). The data showing the decreases in firmness are shown in **Figure 3**.

<u>For Objective 2</u>: Evaluate the effectiveness of the AFA extract at reducing cross-contamination with Lm after contact with contaminated packing equipment.

Methods

To achieve this objective, the transfer of *Lm* from a contaminated food-contact surface to the surface of avocados, was tested, simulating the normal avocado contact with surfaces during normal packing operations. Furthermore, coated and non-coated avocados were inoculated to determine the ability of Lm to attach onto the surface and form biofilms. The data allow determining the effect of factors commonly found in the industry on the avocado cross contamination with *LM* via contaminated food-contact surfaces to the avocados, and the effect of AFA extracts to prevent or reduce such cross-contamination.

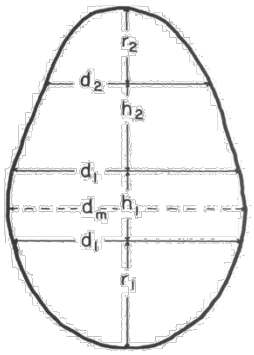
Avocado surface area measurement. Since this task included the collection of the entire avocado rind for testing, a set of avocados was subjected to measurements to calculate the area of the surface of Hass and Green Skin avocados as described by Erickson and Kikuta (1965). Briefly, for each avocado variety a set of similar size and shape avocado fruits was subjected to height and diameter measurements at the avocado regions shown in the diagram below, and then using the following equation for calculating the area:

$$\pi\left[\frac{d_1^2+d_2^2}{2}+dh_1+(r_1+r_2)\sqrt{h_2^2+(r_1-r_2)^2}\right]$$

where d is the diameter of the cylinder obtained from averaging d1 and d_m, and the rest of the values are the diameter, height or radius of the measurement locations shown in the diagram below.

Task 2.1. Testing of avocados after contact with contaminated surfaces

Preparation of avocados. Hass and Green Skin avocados supplied by Giumarra, were separated in two groups. One group was covered with AFA coating containing 7,000 mg/kg AFA applied by misting, as described in the Methods for Objective 1 above. The second group was not subjected to any coating.



Source: Erickson and Kikuta, 1965

Inoculum and inoculation of food-contact surfaces. A 5-strain *Lm* cocktail was grown, washed by centrifugation and adjusted to a target concentration as described for **Objective 1 – Microorganisms and inoculation**, adjusting the concentration of the bacterial suspension to 7-8 log CFU/ml. To inoculate, a 5-cm foam brush was dipped into the cocktail suspension, allowed to drain and the remaining liquid was spread over a 20 x 40 cm area (800 cm^2) collected from a food conveyor belt made of polyvinyl chloride (the same material used in avocado packing facilities), to simulate a food-contact surface in the avocado packing line. This procedure was determined by preliminary experiments to deliver target *Lm* inoculum level of ~6 log CFU/cm^2 on the food-contact surface. This relatively high target level was selected because of the irregularity and absorbent condition of these surfaces, which may increase the chances for some of the inoculum to be lost or out of reach for contact with avocados. After inoculation, the actual *Lm* numbers were determined on the conveyor belt surface for later comparison with the numbers that were detected on avocados after contacting this surface. A set of 6 non-inoculated avocados was tested at every trial to

verify that no detectable listerias were found, which would have interfered with the outcome of the experiments. The inoculated surface was allowed to dwell for 1, 4 and 8 h inside the biosafety cabinet to promote bacterial attachment.

Transfer from contaminated food-contact surfaces to avocados. After each time, coated and non-coated avocados (non-inoculated) were rolled over the inoculated surface for 15 s to promote bacterial detachment from equipment surfaces and transfer to the avocado fruits. After rolling over the inoculated conveyor belt surface, the entire skin of each avocado was removed with a sterile scalpel and forceps and then mixed in a stomacher bag with 99 ml of PBS+T80, gently rotating to allow full contact and AFA neutralization and then preparing decimal dilutions for spread plating onto MOX. The numbers of *Lm* found on the avocado surfaces, were assumed to have been transferred from the inoculated conveyor belt. This experiment was conducted in 3 independent repetitions, each using triplicate samples per treatment.

Task 2.2. Attachment and biofilm formation on coated avocado surface

In separate experiments, coated and non-coated avocados were tested for ability to exposed to inoculated equipment for the 3 dwell times were separated in two groups. One group was used for determination of strength of attachment and the other for preparation of samples for examination under the scanning electron microscope (SEM) for potential biofilm formation.

Effect of AFA coating on strength of attachment. A PVC conveyor belt surface was prepared and inoculated as described for Task 2.1 simulating contamination of food-contact surfaces and then left to dwell for up to 8 h at room temperature. After 1, 4 and 8 h dwell time, coated and non-coated Hass and Green Skin avocados were manually rolled over the contaminated food-contact surface for 15 s. The rolling procedure was performed without applying pressure, moving the avocado from one side of the conveyor belt to the other approximately 5 times during the 15 s. The avocados then were to stand in the biosafety cabinet allowed for 60 min to promote bacterial attachment prior to sample collection. After this time, each whole avocado was submerged in a sterile bag (Whirl-Pak, Chicago, IL) with 100 ml of PBS+T80 and gently shaken for 15 s to remove *Lm* cells that had not developed strong attachment onto the avocado surface. There are referred to as loosely attached (LA) cells. The PBS+T80 rinsate then was tested for numbers of *Lm* as described below. After sampling for LA cells, the same avocado was carefully transferred into a separate sterile bag with 100 ml of PBS+T80 and subjected to sonication (2 min, 40 kHz) using a Branson CPX-952-217R sonicator to detach strongly attached (SA) cells, and the resulting suspension was subjected to *Lm* enumeration as described below. Additionally, samples of inoculated conveyor belt were also collected by cutting and tested for LA and SA *Lm* counts. For avocado and conveyor belt surface samples, the numbers of *Lm* without conversion to log values were used to calculate the strength of attachment (S_R) of *Lm* to the surface of coated and non-coated avocados by the method of Dickson et al. (1989) applying the formula:

$$S_R = \frac{\text{SA (CFU)}}{\text{SA (CFU) + LA (CFU)}}$$

Then multiplying the S_R x 100 to express it as percent cells with strong attachment. This experiment was repeated in three independent trials with duplicate or triplicate samples per treatment.

Effect of AFA coating on biofilm formation on avocado surfaces. The ability of *Lm* to form biofilm on the surface of AFA-coated and non-coated avocados was visually tested by examination of inoculated surfaces avocados under SEM. A 5-cm^2 core avocado rind sample was excised from each of 15 coated and 15 non-coated Hass and Green Skin avocados using a sterile circular sharp stainless-steel borer with a 2.5 cm in diameter and forceps. Each core sample was inoculated with 50 μl of a suspension

containing a cocktail of 5 *Lm* strains prepared as described above and placed in an enclosed glass chamber at a 95% relative humidity (RH) at room temperature (average 21.5 °C) over 14 days. At 0.25, 4 and 14 days, samples of inoculated rinds corresponding to all varieties and treatments were collected and three times with PBS for 15 min to remove excess bacteria, fixed by submerging in 2.5 % glutaraldehyde in 10 mM PBS with a pH of 7.5 for 4 h. Following fixation, samples were rinsed with PBS to remove the remaining glutaraldehyde and dehydrated by submerging in 20%, 40%, 60%, and 80% ethanol, each for 10 min to remove water content. These steps were performed inside the biosafety cabinet. The samples were then incubated in 100% ethanol for 1 hour at 37 °C and finally, individually placed in sterile labeled petri dishes, sealed with parafilm, and packaged for transportation to the Texas A&M Microscopy and Imaging Center Core Facility. Imaging was performed at this facility using a FEI Quanta 600 F SEM. The best quality images that were representative of all observations were selected to for comparison between treatments.

Results – Objective 2

Task 2.1. Testing of avocados after contact with contaminated surfaces

Transfer from contaminated surfaces to avocados. The estimated the area of the entire avocado surface, was, 137 cm^2 and 277 cm^2 for Hass and Green Skin avocados, respectively. This resulted in a limit of detection of the plate count method of -0.1 log CFU/cm^2 for Hass and -0.4 log CFU/cm^2 of Green Skin avocados when sampling the entire rind of each avocado. Under the cross-contamination scenario simulated in this experiment, the mean *Lm* counts obtained for avocados rolled over the inoculated conveyor belt 1 h after inoculation were 3.4 and 1.5 log CFU/cm^2 respectively for non-coated and AFA-coated Hass avocados, whereas for Green Skin these counts were 2.9 log CFU/cm^2 for non-coated and 2.0 log CFU/cm^2 for coated. These differences were significantly ($P < 0.05$) for Hass but not for Green Skin avocados. This indicates that the AFA coating may have shown protective effect against *Lm,* reducing them upon contact at least on Hass avocados. It is uncertain whether the roughness of the skin played a role in the better performance of AFA coating in Hass compared to Green Skin varieties. Rolling avocados over the contaminated surface at 4 and 8 h after inoculation resulted in minimal transfer of *Lm* onto the avocado surfaces, with counts close to or below the detection limit of the plate counting method, and there were no differences in counts between coated and non-coated on either avocado variety. These results are shown in **Table 3**. This table also shows the percentage of the numbers without log conversion, of *Lm* that were transferred onto the avocado skin in relation to the numbers on the contaminated conveyor belt. The maximum transfer rate for all avocados and treatments was between 0.8–1% and only occurred for avocados that were exposed to cross-contamination 1 h after inoculation of the conveyor belt. Those rolled 4 and 8 h after inoculation showed % transfers between 0 and 0.0008. This may be attributed to a potential increase in the number of strongly attached cells as the time of cell contact with the conveyor belt surface increases, where cells may not have been detached during contact with the avocado fruits.

Task 2.2. Attachment and biofilm formation of *Lm* on coated avocado surface

Effect of AFA coating on strength of attachment. When a piece of PVC conveyor belt was inoculated with *Lm,* the counts of this pathogen were gradually reduced during dwelling of this material at room temperature. As seen in Table 4, the counts of loosely attached (LA) cells were 4.1, 3.1 and 1.6 log *Lm* CFU/cm^2, after 1, 4 and 8 h dwelling post inoculation, respectively, and these counts were significantly different between 1 and 8 h ($P < 0.05$). For strongly attached (SA) cells, these counts were 4.3, 2.9 and 1.4 at 1, 4 and 8 h dwell times, and all counts were different from each other ($P < 0.05$). Further research should permit elucidate whether the reduction in counts during dwell time is caused by a gradually increasing strong attachment of cells, which makes them hard to detach during sampling, due to absorption of inoculum by the PVC material or it is due to poor survival of *Lm* on the PVC surface. The S_R

x 100 values calculated from the SA and LA counts showed a decreasing trend in S_R after 4 h dwell time compared to 1 h (see **Figure 4**). However, the statistical analysis did not show any differences in S_R x 100 between all the sampling times.

The S_R x 100 for *Lm* that were transferred to the avocados during contact with the contaminated conveyor belt surface, was difficult to calculate due to many samples showing no detectable counts. The low transfer rate during simulated cross-contamination had been seen in the previous experiments (see Table 3). In this experiment, there were samples with detectable counts only after 1 h dwell time. Because of this issue, the S_R x 100 was only possible to be calculated for 1 h, as all samples collected after 4 and 8 h dwell time yielded counts below the detection limits (**Table 5**) even though we established a very low limit of detection to increase the chances for detection to enable S_R calculation. **Figure 5** shows that the cells that developed strong attachment within 1 h after inoculation were not affected by the application of AFA coating. In this study, a polyvinyl chloride conveyor belt piece was inoculated and used as a food-contact surface for cross-contamination.

Effect of AFA coating on biofilm formation on avocado surfaces. Hass and Green Skin avocado rinds that were not coated and those that were coated with the AFA coating compound all showed that *Lm* could attach and produce biofilm. In our study we inoculated the actual avocado rind and did not induce special conditions for biofilm other than a high RH in the chamber to create an environment that simulates the avocado storage as much as possible. Due to these conditions, we could not test biofilm formation in microplates but recurred to SEM examination. **Figures 6** and **7** show SEM images of *Lm* inoculated on Hass and Green Skin avocado rinds, respectively, collected from non-coated avocados, **Figures 8** and **9** show *Lm* biofilm on avocado rinds collected from avocados that were coated prior to inoculating. In all images (Figures 6-9), image A was taken 6 h after inoculation and storage, B corresponds to samples prepared after 5 days of storage and C to samples prepared after 14 days. Image D is the same preparation as in D (14 days biofilm maturation) but with lower magnification to have a wider view of the biofilm distribution. At 6 h, all images show individual rod-shaped cells, and in Figure 8 (coated Hass avocado rinds) the cells show incipient development of filaments consistent with exopolysaccharide (EPS) production. On day 5 (B for Figures 6-9), This the biofilm development was observed on rinds from both avocado varieties regardless of treatment (coating vs. no coating). The cells clearly look covered with structure typical of biofilm images. Finally, at day 14 the images (C) and (D) show mature biofilm. It is of interest to note that in the non-coated Green Skin avocado rinds (Figure 7), the image with the lower magnification (D) showed bacteria of coccoid shape together with hyphae alongside the rod-shaped bacteria consistent with the inoculated *Lm.* Hyphae were also observed at 14 days for non-treated Hass avocados (Figure 6), with no coccoid cells. This observation in the non-coated avocado images may be an indication that the AFA coating may be preventing the growth of concomitant microbiota, another possible beneficial effect of AFA coatings. However, with regards to *Lm* biofilm, the SEM images showed that biofilm formation occurred in all samples, regardless of whether they were coated or not. Indicating that AFA- coating did not prevent Lm biofilm formation. Therefore, the effectiveness of the AFA coating at reducing *Lm* in the surface of the avocado observed in the challenge experiments, did not influence *Lm* biofilm reduction.

Statistical analysis. *Lm* counts were analyzed and converted to log values in Microsoft Excel. The distribution of the data was tested using the log CFU/cm^2 and their residuals, and log normality was assessed by fitting the data to a normal distribution and checking the goodness of fit, using Shapiro-Wilk test. Since the data followed a normal distribution, JMP software (JMP Pro, v16.0, SAS Institute Inc, Cary, NC) was used for further analysis. The analysis of all tasks included a multifactorial analysis of variance, with mean separation using Tukey´s method at $P < 0.05$ to compare the effects of each sample group between experiments. Results from JMP software were used to generate figures and tables in Microsoft Excel.

For Objective 3: Study metabolic responses of avocados to the coating with AFA extract (rich in acetogenins) that are uniquely present in the same avocado and their effect on ripening.

Methods

Task 3.1. Assessment of potential changes in avocado fruit commercial maturation procedures

Sourcing and shelf-life studies. For the experiments of Objective 3, Hass avocados were sourced in collaboration with the *Asociación de Productores y Empacadores Exportadores de Aguacate de Mexico* (APEAM) from the Michoacan Region in Mexico. Avocados were harvested and shipped overnight to the ITESM laboratory. Avocados were randomly divided into four treatment groups: Group 1 included avocados coated with formulations that contained AFAs at concentrations of 7000 ppm (AFA 1) and at 14000 ppm (AFA 2) for Group 2. Group 3 included avocados coated with formula excipients (PG and avocado oil excipient), and Group 4 included uncoated avocados. As shown in **Figure 10a**, coated and uncoated fruits were stored at 4 °C for 28 days, sampled on days 8, 14, 20, and 28, and subsequently stored at 25 °C for 7 days for ripening. After ripening, physiochemical analyses were performed, and pulps were freeze-dried for metabolite analyses.

Physicochemical analysis. Instrumental color and texture analyses were performed on ripe avocados. Color was analyzed in the avocado mesocarp (inner and outer locations) using a Colorimeter CR-400 (Konica Minolta, Tokyo, Japan), and measurements were performed at six different points for each section. Texture measurements were conducted at 6 points of the avocado pulp using a 5 mm diameter flat cylinder tipped puncture probe at a constant speed of 2 mm s^{-1} in a TA-XT2 texture analyzer (Stable Micro Systems, Surrey, United Kingdom). Respiration rate was measured after being stored at 4 °C (on sampling days 8, 14, 20, and 28) and again after ripening at 25 °C (on sampling days 15, 21, 27, and 35) using an F-950 gas analyzer (Felix Instruments, Camas, WA).

Task 3.2. Assessment of potential changes in avocado fruit desirable metabolites

***Testing avocado fatty acids and phytosterols (targeted metabolomics)*.** Chemical quantifications of avocado metabolites were performed on the freeze-dried pulps after ripening at 25 °C for days 21 and 35 and in unripe avocados (day 0, after 24 h of harvesting and shipping).

Fatty acid profile analysis. The fatty acids profiles were determined by AOCS method Ce 2-66 (AOCS, 2017b). Individual compounds were quantified in an Agilent 8860 gas chromatograph coupled with a flame ionization detector (Agilent Technologies Inc., CA). Separation was performed in a fused silica SP-2380 (Supelco, PA) capillary column (100 m × 0.25 mm i.d., 0.2 µm film thickness) and the oven temperature ramp ranged from 140 to 240 °C at a rate of 4 °C min^{-1} and a hold time of 15 min, resulting in 45 min of total run time. The injector temperature was 260 °C. Helium was used as carrier gas at a flow rate of 1.5 mL min^{-1}. FAMEs profiles were calculated with the peak area of individual fatty acids.

Phytosterol profile analysis. The official method for the quantification of sterols and stanols in foods and dietary supplements containing added phytosterols Ce 12-16 was followed (AOCS, 2017a). GC-FID analyses were conducted on an Agilent 8860 GC (Agilent Technologies). An HP-5 equivalent column was used, the XTI-5 Column 30 m × 0.25 mm ID, 0.5 µm (Restek, Chromatographic Specialties Inc., Brockville, ON, CA) was used to separate phytosterols. The injection volume was 2 µL and helium was used as a carrier gas at a flow rate of 1.2 mL·min^{-1}. The injector temperature was 280 °C at a split ratio of 5. The GC oven program was initially held at 150 °C for 1 min, then ramped at 15 °C·min^{-1} at 295 °C·min^{-1} and held until the total run time was 40 min. The FID temperature was 300 °C. The detector gas consisted of hydrogen (flow rate: 400 mL·min-1), air (flow rate: 30 mL·min-1), and helium gas (flow rate: 30 mL·min^{-1}). Sterol quantifications were performed using the peak area and concentration of epicoprostanol as the internal standard. Individual desmethylsterols were expressed as the percent of total sterols.

Untargeted metabolomic analyses (non-volatile flavor metabolites and others). Extracts were obtained from 10 mg of freeze-dried pulps, and samples were kept on ice. Briefly, 1 mL 3:3:2 (v/v/v) acetonitrile/ isopropanol/ water and 10 glass beads were added to each sample. Lysis was performed in the FastPrep®-24 Homogenizer (MP Biomedicals, CA) at 6 m s^{-1} x 40 s. Then, the solutions were vortexed (10 s), shaken for 5 min at 4°C, vortexed (30 s), and sonicated (1 min). The tubes were centrifuged at 14000 g for 2 min. Aliquots of 225 µL of the supernatant were transferred into 1.5 mL Eppendorf tubes and 600 µL kept as a backup sample. Pools QC were generated by transferring 50 µL of the remaining supernatant from each sample to a 15 mL tube. Samples were dried at 55 ˚C using a CentriVap Concentrator (LABCONCO, MO), and the extracts were stored at 80 °C until further analyses. Extracts were analyzed using Gas Chromatography–Time-of-Flight Mass Spectrometry (GC-TOF MS) system equipped with an Rtx-5Sil MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Restek Corporation, Bellefonte, PA). Helium was used as carrier gas at a constant flow rate of 1.0 mL min^{-1}. The oven temperature was set at an initial temperature of 50°C for 1 min, then ramped to 300 °C at 20 °C min^{-1}, and held constant for 5 min. The mass spectrometer was set to acquire a unit mass resolution at 17 spectra s^{-1} from 80-500 Da at -70 eV ionization.

Statistical analysis. The R programming language was used for all statistical analyses. For untargeted metabolomics, data were log-transformed and quantile-normalized. Two-way ANOVA with Tukey´s post hoc test (p-value < 0.05) was conducted to establish significant differences among treatments and storage time. The principal component analyses (PCA) were conducted using the *ggplot()* function from the ggplot2 library and heatmaps sing *Heatmap()* function from the ComplexHeatmap library.

Results – Objective 3

Task 3.1. Assessment of potential changes in avocado fruit commercial maturation procedures

The study of metabolic responses included fruit quality physicochemical changes in instrumental color and texture, whole avocado weight loss and respiration rate. Chemical analyses included phytonutrients that are part of avocado composition standards (fatty acids and phytosterols). Moreover, the profiles of non-volatile flavor metabolites and other relevant fruit components were characterized using an untargeted metabolomics approach. The results are described below.

Effects of the acetogenin-enriched (AFA) coatings on avocado physicochemical properties. Avocado weight losses of the coated fruits were lower than those of uncoated fruits (P<0.05), indicating reduced dehydration (**Figure 11a**). The firmness of the pulp was higher in the coated avocados, with noticeable effects for the AFA 2 coating at 35 days (**Figure 11b**). Pulp grey areas were also measured as an indirect assessment of fruit decay (**Figure 10b**). On day 35 (28 d cold storage plus 7 d at 25 °C), uncoated avocados showed larger grey areas (up to 76%) than avocados coated with AFAs (P < 0.05*)*, followed by excipient treatment (up to 29%), while small to no grey areas were observed in AFA coatings (<10%) (Figure 10b). The grey areas possibly indicated fruit spoilage. Therefore, results suggested that AFA coatings could extend shelf-life up to 35 days (until ripening). The respiration rate was also lower in coated avocados, with a higher decrease in magnitude for the 14,000 ppm AFA 2 treatment.

Task 3.2. Assessment of potential changes in avocado fruit desirable metabolites

Effects of the acetogenin-enriched (AFA) coatings on the characteristic avocado pulp fatty acid and phytosterol profiles. Fatty acid and phytosterol profiles were included in a recently approved composition standard for avocado oils CXS 210-1999 (Codex Alimentarius Commission, 2025). In the present work both families of molecules were quantified to explore the potential effects of the AFA coatings on the characteristic avocado nutritional profile.

Fatty acids profiles – The results from the major fatty acids agreed with the composition standard and were not affected by the AFA treatments. The major fatty acids in the profile were oleic acid (50.4 - 55.1%), followed by palmitic acid (14.1 - 16.6%), palmitoleic acid (5.3 - 6.6%), vaccenic acid (6.2 - 7.1%), linoleic acid (14.2 - 18.1%), a-linoleic acid (1.0 - 1.5%), and stearic acid (0.4 - 0.5%) (**Figure 12a**). Overall ranges were 14.7 - 17.3% for saturated fatty acids, 64.1 - 67.6% for monounsaturated fatty acids, and 15.2 - 19.5% for polyunsaturated fatty acids. The AFA treatments only featured significant differences for three minor FAs including arachidic acid, behenic acid, and lignoceric acid ($P<0.05$). The fatty acid PCA biplot shown in **Figure 12b** shows that principal components 1 and 2 explained 42.01% of total data variability. However, no clear separation in the treatment nor time factors was observed. Therefore, results suggest that the AFA coating or storage time did not significantly alter avocado fatty acids composition.

Phytosterol profiles – Desmethylsterols are a common type of phytosterols found in plants, comprising about 80% of the total sterols in avocados (Fernandes et al., 2018). Important desmethylsterols found in this study's avocados were β-sitosterol (83.2 - 85.6%), followed by campesterol (5.3 - 6.1%), Δ-5 avenasterol (4.2 - 5.8%), clerosterol (1.8 - 2.0%) and stigmasterol (0.7 - 0.9%) (**Figure 12c**). Significant differences in sterol profiles were found per treatment per storage time. The AFA 2 treatment on day 35 presented higher levels of β-sitosterol (85.6%) than the AFAs, uncoated, and excipient groups on day 21 (83.3 - 84.2%). Other minor sterols such as 24-methylene cholesterol and Δ-5,24 stigmastadienol were found to be significantly different. PCA suggested that some phytosterol levels were slightly affected by storage time and treatments (**Figure 12d**). Principal component 1 explained 34.38% of total data variability. The control group loaded on the negative side, while most excipient samples loaded on the positive side. Additionally, the AFA 2 treatment seems to be closer to the control group than the other samples, suggesting that the high AFAs concentration of (14,000 ppm) appeared to slowdown phytosterol decline.

Effects of the acetogenin-enriched (AFA) coatings non-volatile flavor metabolites and others.
Untargeted metabolomic analyses were performed in the pulp of ripe avocados to determine the effects of AFA coating on fruit metabolism during storage (**Figure 13**). A total of 175 annotated metabolites from primary metabolism were analyzed, which included carbohydrates, organic acids, lipids, amino acids, phenolic compounds, vitamins, among others. In the PCA biplot, unripe avocados from day 0 (controls) were separated from most ripe avocados after storage times 21 and 35 days by the principal component 1 (explaining 19.68% of data variability) (**Figure 13a**). Principal component 2, explained 10% of data variability, and grouped all avocados coated with 14,000 ppm AFAs apart from controls (day 0), and avocados coated with excipients and uncoated avocados on day 35. Interestingly, avocados coated with 14,000 ppm on day 35 were grouped with avocados from all other treatments and uncoated on day 21. Since, on day 21, avocados still preserved high-quality parameters (firmness and color), results suggested that AFAs retained the characteristic fruit profile up to day 35.
Data showed that treatments and storage times resulted in significant differences for various metabolite families (**Figure 13b**). The heatmap in **Figure 13c** shows the normalized intensities of significantly different metabolites among treatments and storage time ($P < 0.05$). Results indicated that 79 metabolites were possibly associated to avocado quality and to the antimicrobial effects of the AFA coating.

Outcomes and Accomplishments

Objective 1 and 2 were conducted at Texas A&M University under the supervision of Co-PI Castillo. Objective 1, aimed at determining the effect of AFA extracted from avocado seeds as a potential processing aid for reducing *Lm* on the surface of avocados as a postharvest control measure, and Objective 2 aimed at testing whether AFA coating provided protection against attachment and biofilm formation by Lm on avocados. Relevant outcomes and accomplishments included:

- A recommendation of a possible method of application of AFA coatings during avocado packing operations, if this method is applied in commercial packing plants. A fine spray (mist) was sufficient to achieve adequate coverage and was comparable to coating using brushes.
    - Brushes may be an important source of potential contamination in fruit packing due to their design and materials used, which make brushes difficult to clean and disinfect. Brushes may be replaced by misting the AFA coatings and other cleaning activities, reducing opportunities for potential contamination.
- Confirmation of antilisterial effect of AFA diluted in PG applied as a coating on fresh avocados.
    - In the prototype coating used in this proof-of-concept project, PG served as non-aqueous vehicle for the AFA, but during the studies it was evident that PG had antilisterial activity of its own and potentiated the proven antimicrobial activity of AFA. The antimicrobial effect of PG has been reported before, but this is the first report of PG against *Lm.* As an outcome, we have been contacted by a company that develops various coatings for the food industry and they are interested in collaborating in further research.
- This antilisterial effect was useful at reducing *Lm* cross-contamination after contact of avocados with contaminated food-contact surfaces to the avocado surfaces, probably as a result of the antilisterial effect rather than preventing the physical transfer from the contaminated food-contact surface to the avocado surface.
- From the academic viewpoint, this project also provided the opportunity for a graduate student to obtain her M.Sc. degree.
- One manuscript is under preparation and another planned.

In Objective 3, conducted under the supervision of Co-PI Hernandez Brenes at ITESM, the project aimed to study the metabolic responses of the coating with AFA on the characteristic profile of avocado phytonutrients and to assess potential impacts on the fruit quality during ripening. Relevant outcomes included:

- AFA antimicrobial effects resulted in the extension of the fruit shelf-life as evidenced by physicochemical parameters (decay grey areas and firmness) and changes in metabolites induced by decay (e.g., carbohydrates and amino acids).
- Changes in fatty acid and phytosterols over time indicated that AFA antimicrobial coatings did not have a significant impact on the characteristic nutritional quality of ripe avocados.
- Metabolomic signatures of the coated fruits during storage allowed us to study changes in a total of 205 avocado pulp metabolites, including fatty acids and phytosterols (targeted analyses) and non-volatile flavor and others (untargeted analyses); of which 79 metabolites were associated with the corresponding avocado quality determinations and with changes induced by the antimicrobial effects of the AFA coating.
- The project had a positive impact on the training of two master of science students and three undergraduate students from ITESM.

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APPENDICES

Publications and Presentations

- Gonzalez de Cossio, M. 2024. Control of *Listeria monocytogenes* on Whole Avocados During Dry Packing by Applying an Avocado Oil Coating Enriched With Fatty Alcohols Naturally Present in Avocado Seed. Thesis (M.Sc. in Food Science and Technology). Texas A&M University.
  - **Gonzalez de Cossio**, **M.,** Carrete, C., Hernandez-Brenes, C and Castillo, A. 2024. Application of a Natural Antimicrobial Coating on Hass Avocados and Mangos for the Control of *Listeria monocytogenes* during Packaging. Poster P2-08. International Association for Food Protection Annual Meeting. Long Beach, CA. July 14-17, 2024.
- Gonzalez de Cossio, M., Mora-Godinez, S., Hernandez-Brenes, C. and Castillo, A. Application of propylene glycol-based coatings containing avocado fatty acids for reducing *Listeria monocytogenes* on fresh avocados. **Manuscript under preparation. Expected submission to CPS for approval April, 2025.**
- One manuscript in preparation from Objective 3.

Budget Summary

This project received a total of $61,486 in research funds, and the majority of funds were spent.

Tables and Figures (see below)

Table 1. Counts of *Listeria monocytogenes* on AFA-coated Hass avocados as affected by the AFA concentration and the method of coating application.

AFA concentration[a]	Method of application	Log CFU/cm^{2}[b]
0	Brush	4.2 A[c]
	Mist	4.6 A
	Rollers	4.5 A
7,000	Brush	1.3 DE
	Mist	1.7 CDE
	Rollers	2.6 B
10,000	Brush	1.2 E
	Mist	2.0 BCDE
	Rollers	2.2 BC
14,000	Brush	1.2 E
	Mist	2.1 BCD
	Rollers	1.6 CDE

[a] Concentration in mg/kg

[b] Standard error = 0.17

[c] Means followed by the same letter are not different (P >0.05)

Table 2. Doubling times (days)[a] for *Lm* on Hass and Green Skin avocados coated with avocado oil (AO) or AFA-enriched coating agent (AFA coating) and stored at 4–5 °C.

	Doubling times (days) by type of coating applied		
Variety	Control	Avocado oil	AFA coating
Hass	3.0[b]	5.3	5.3
Green Skin	3.0	4.2	1.4

[a] To calculate the doubling times, 1 week was assumed to be = 7 days.
[b] Generation (doubling) times calculated from the data in Figure 2.

Table 3. Numbers of *Listeria monocytogenes (Lm)* on Hass and Green Skin avocados after contact with an inoculated conveyor belt.

Avocado variety	Coating[a]	Contact Time (h)[b]	Log CFU/cm^2 *Lm* $\pm$ SERR	% Transfer[c]
Hass	Without coating	1	3.4 $\pm$ 0.22 A[d]	1.0448
		4	0.5 $\pm$ 0.19 DE	0.0008
		8	0.2 $\pm$ 0.21 EF	0.0008
	With coating	1	1.5 $\pm$ 0.21 CD	0.0719
		4	0.1 $\pm$ 0.19 EF	0.0004
		8	< -0.4 EF[e]	0.0000
Green Skin	Without coating	1	2.9 $\pm$ 0.19 AB	0.7936
		4	0.1 $\pm$ 0.19 EF	0.0005
		8	0.1 $\pm$ 0.19 EF	0.0003
	With coating	1	2.0 $\pm$ 0.19 BC	0.1246
		4	-0.3 $\pm$ 0.19 EF	0.0001
		8	< -0.7 F[e]	0.0000

[a] Coating consisted of a PG+AO supplemented with 7,000 mg/kg AFA. Coated and non-coated avocados were rolled onto an inoculated piece of conveyor belt material simulating packing operations.

[b] Hours allowed for *Lm* to dwell on the conveyor belt before rolling avocados.

[c] % transfer is the percent of the *Lm* count in CFU/cm^2 on the avocados with respect to the mean count on the contact surface (4.8 x 10^5 CFU/cm^2).

[d] Means followed by the same letter are not significantly different ($P < 0.05$).

[e] No colonies detected at a limit of detection of -0.4 log CFU/cm^2 for Hass avocados and -0.7 log CFU/cm^2 for Green Skin avocados. To include samples with non-detectable counts in the statistical analysis, these samples were assigned a count corresponding to one-half of the limit of detection.

Table 4. Numbers of *L. monocytogenes* cells that were loosely and strongly attached after different times of contact with a conveyor belt surface.

	Log CFU/cm^{2}[a]	
Contact time (h)	LA[b]	SA
1	4.1 AB[c]	4.3 A
4	3.1 AB	2.9 B
8	1.6 C	1.4 C

[a] Values represent LSMeans with SERR of 0.28.

[b] LA means loosely attached cells; SA means strongly attached cells.

[c] Values followed by the same letter are not significantly different.

Table 5. Numbers of loosely attached (LA) and strongly attached (SA) cells of *Lm* on the surface of Hass and Green Skin avocados after cross-contamination with a contaminated food-contact surface.

			Log CFU/cm^2 ± STDEV	
Avocado variety	Treatment	Dwell time (h)	LA	SA
Hass	Coated[a]	1	1.2 ± 0.11 A[c]	0.1 ± 0.10 B
		4	< -0.4[d] ± 0.10 CD	< -0.4 ± 0.17 BCD
		8	< -0.4 ± 0.10 CD	< -0.4 ± 0.10 CD
	Non-coated[b]	1	1.5 ± 0.11 A	< -0.4 ± 0.10 BC
		4	< -0.4 ± 0.10 CD	< -0.4 ± 0.10 CD
		8	< -0.4 ± 0.10 CD	< -0.4 ± 0.10 CD
Green Skin	Coated	1	0.9 ± 0.10 A	0.1 ± 0.10 B
		4	< -0.7 ± 0.10 D	< -0.7 ± 0.10 D
		8	< -0.7 ± 0.10 D	< -0.7 ± 0.10 D
	Non-coated	1	1.2 ± 0.10 A	0.2 ± 0.10 B
		4	< -0.7 ± 0.29 BCD	< -0.7 ± 0.10 D
		8	< -0.7 ± 0.10 D	< -0.7 ± 0.10 D

[a] Coated with AFA coating with a 7,000 mg/kg AFA concentration.

[b] Non-coated avocados, inoculated, no additional treatment.

[c] Means followed by the same letter are not significantly different (P< 0.05).

[d] Means < -0.4 or < -0.7 indicate no colonies detected at a limit of detection of -0.4 log CFU/cm^2 for Hass avocados and -0.7 log CFU/cm^2 for Green Skin avocados. To include samples with non-detectable counts, in the statistical analysis, these samples were assigned a count corresponding to one-half of the limit of detection.

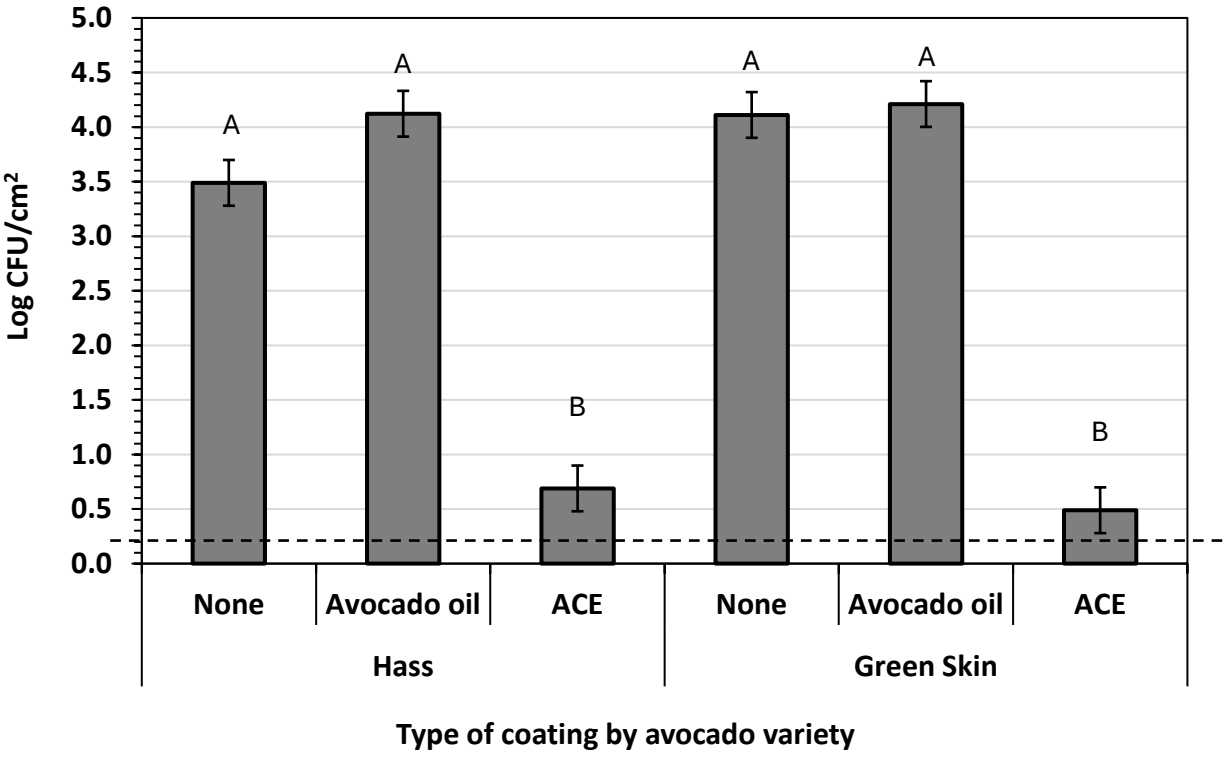

Figure 1. Counts (log CFU/cm²) of* Listeria monocytogenes *on Hass and Green Skin avocados after coated with avocado oil or ACE. Columns topped with same letter are not different (P >0.05). Error bars represent standard error. The dotted line indicates the limit of detection of the plate count method (0.2 log CFU/cm²).

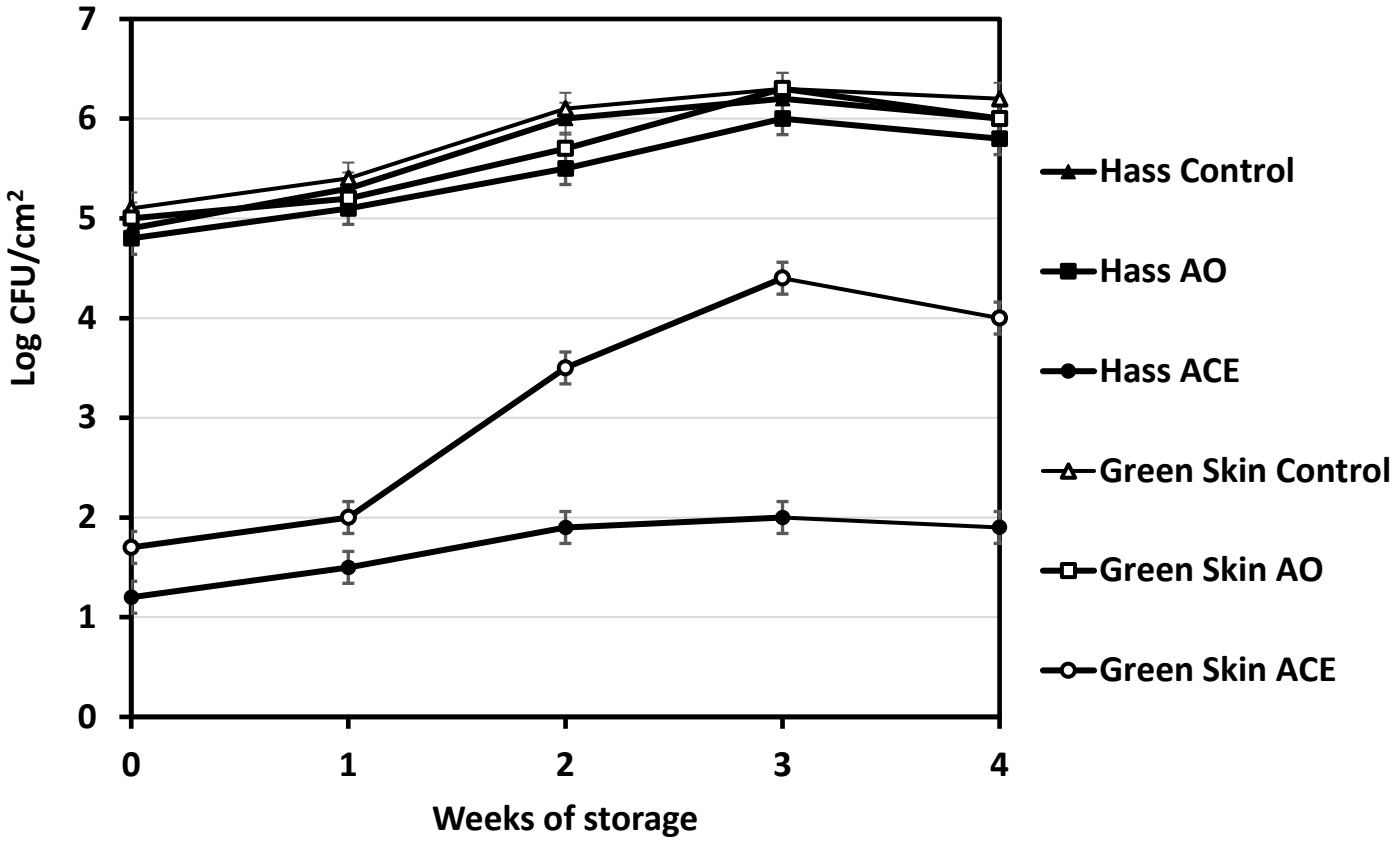

Figure 2. Effect of the application of AFA coating on the activity of* Listeria monocytogenes *on Hass and Green Skin avocados over four weeks at 4–5 °C. Control means avocados that were not coated with any agent, AO means avocados that were coated with avocado oil alone, and ACE means avocados coated with PG-based coating containing 7,000 mg/kg avocado fatty alcohols.

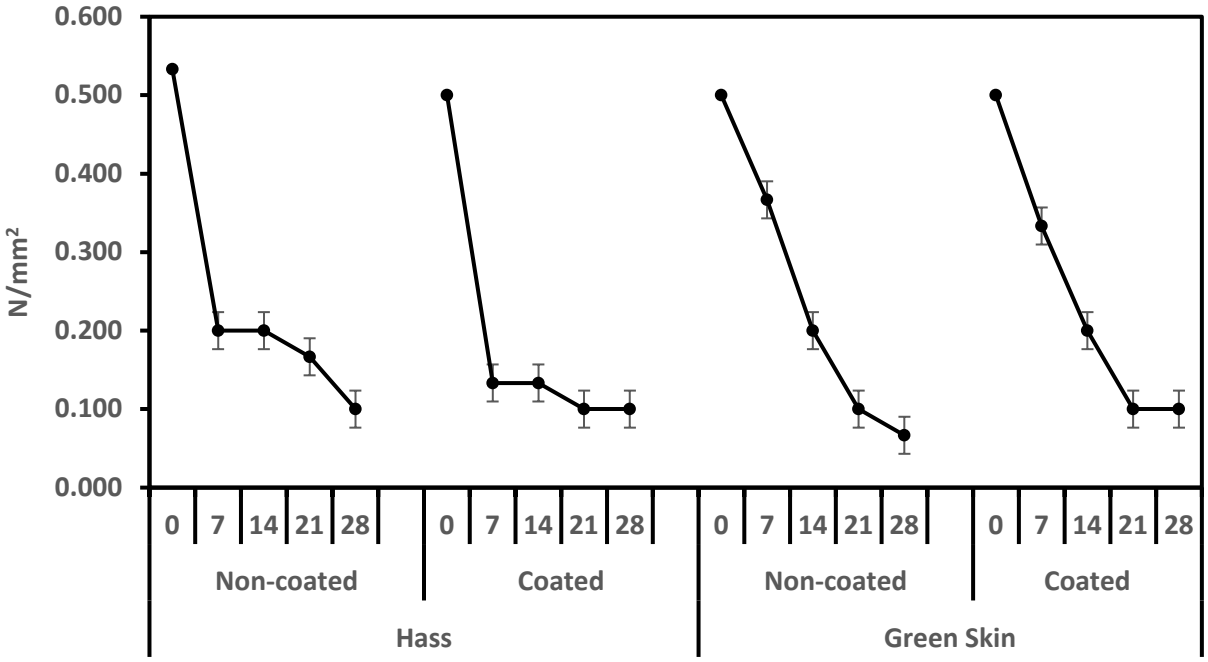

Figure 3. Firmness (N/mm²) of coated and non-coated avocados during storage at room temperature. Variables that had significant effect on firmness were the avocado variety, treatment and days of storage, as well as interactions variety*treatment and variety*days of storage (P < 0.05). SERR = 0.02.

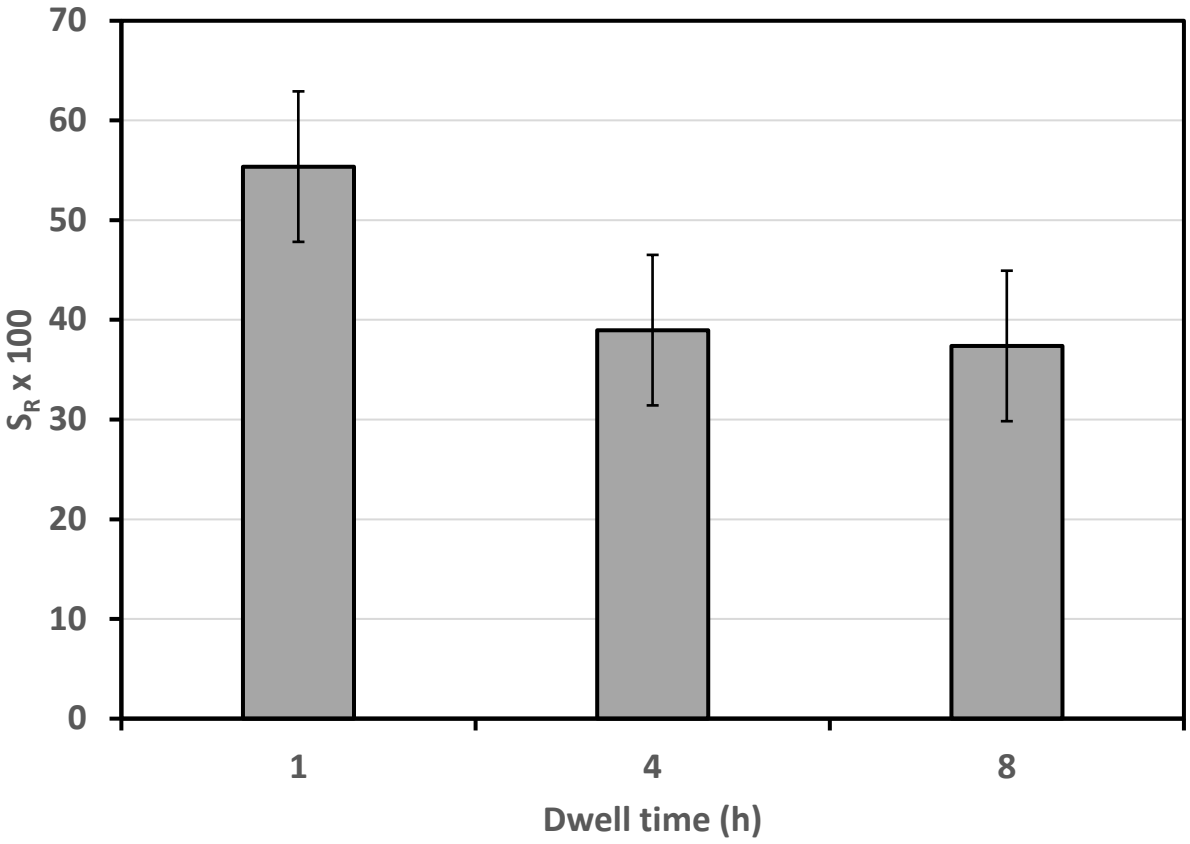

Figure 4. Strength of attachment (S_R) expressed in percent (S_R x 100) of* Listeria monocytogenes *on PVC conveyor belt material after various contact (dwell time) after inoculation. Error bars indicate standard error. There were no significant differences between dwell times (P > 0.05).

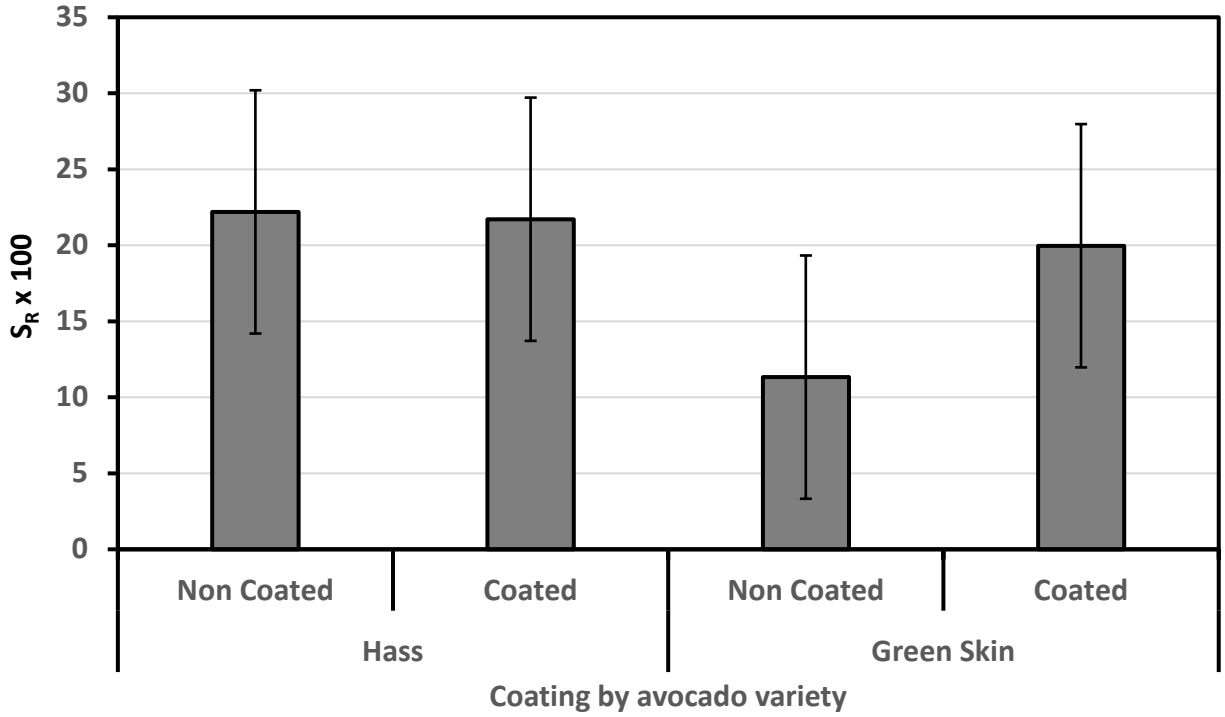

Figure 5. Strength of attachment (S_R) expressed in percent (S_R x 100) of* Listeria monocytogenes *cells on the skin of coated and non-coated Hass and Green skin avocados after contact with a contaminated food-contact surface 1 h after being inoculated. Error bars indicate standard error. Coating consisted of AFA at 7,000 mg/kg in a PG-based solution.

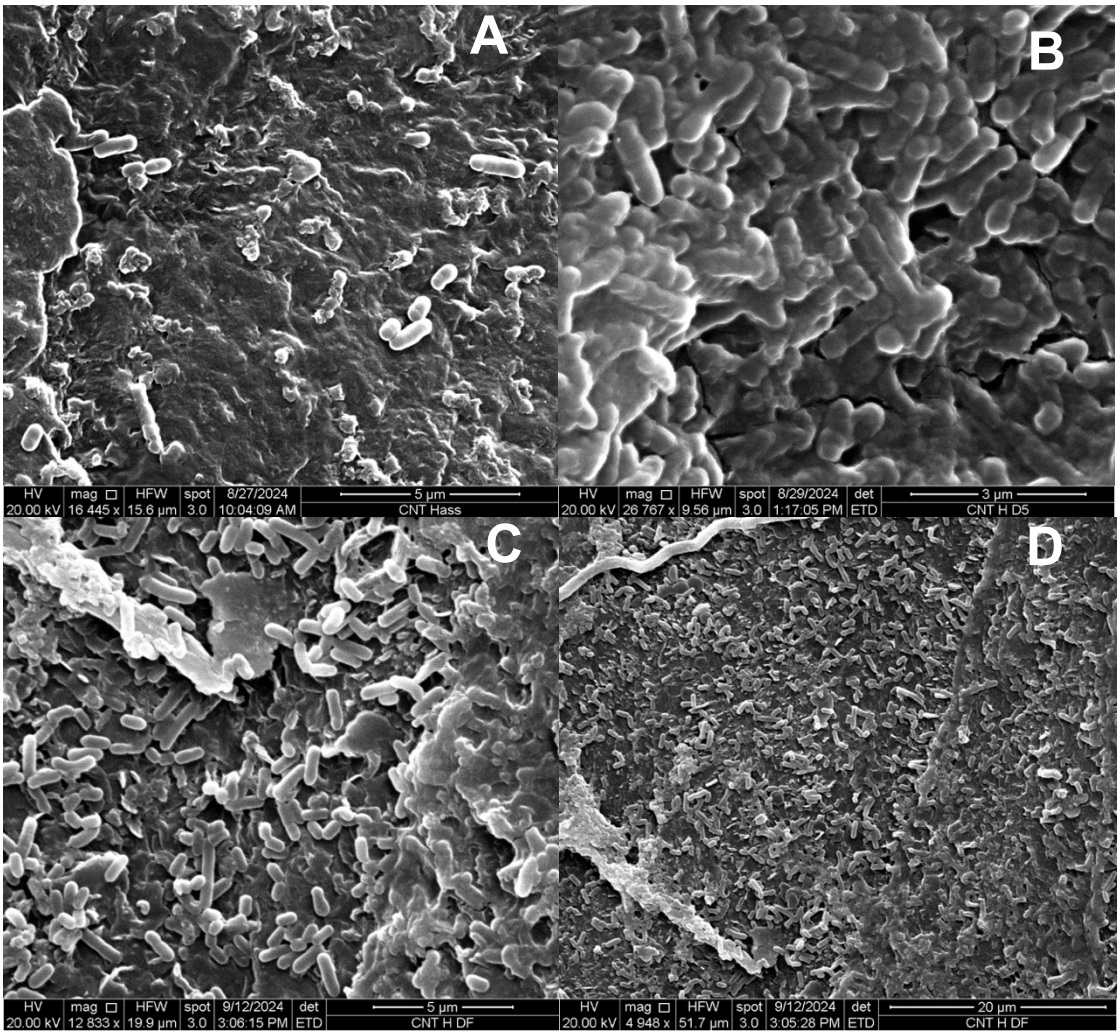

Figure 6. SEM images of* Listeria monocytogenes *on rind of non-treated Hass avocado inoculated and stored in a chamber with 95% RH at 21.5 °C for 6 h, (A), 5 days (B), and 14 days (C) at 16,000-20,000 X magnification. Image D is the same as C shown at 5,000 X magnification.

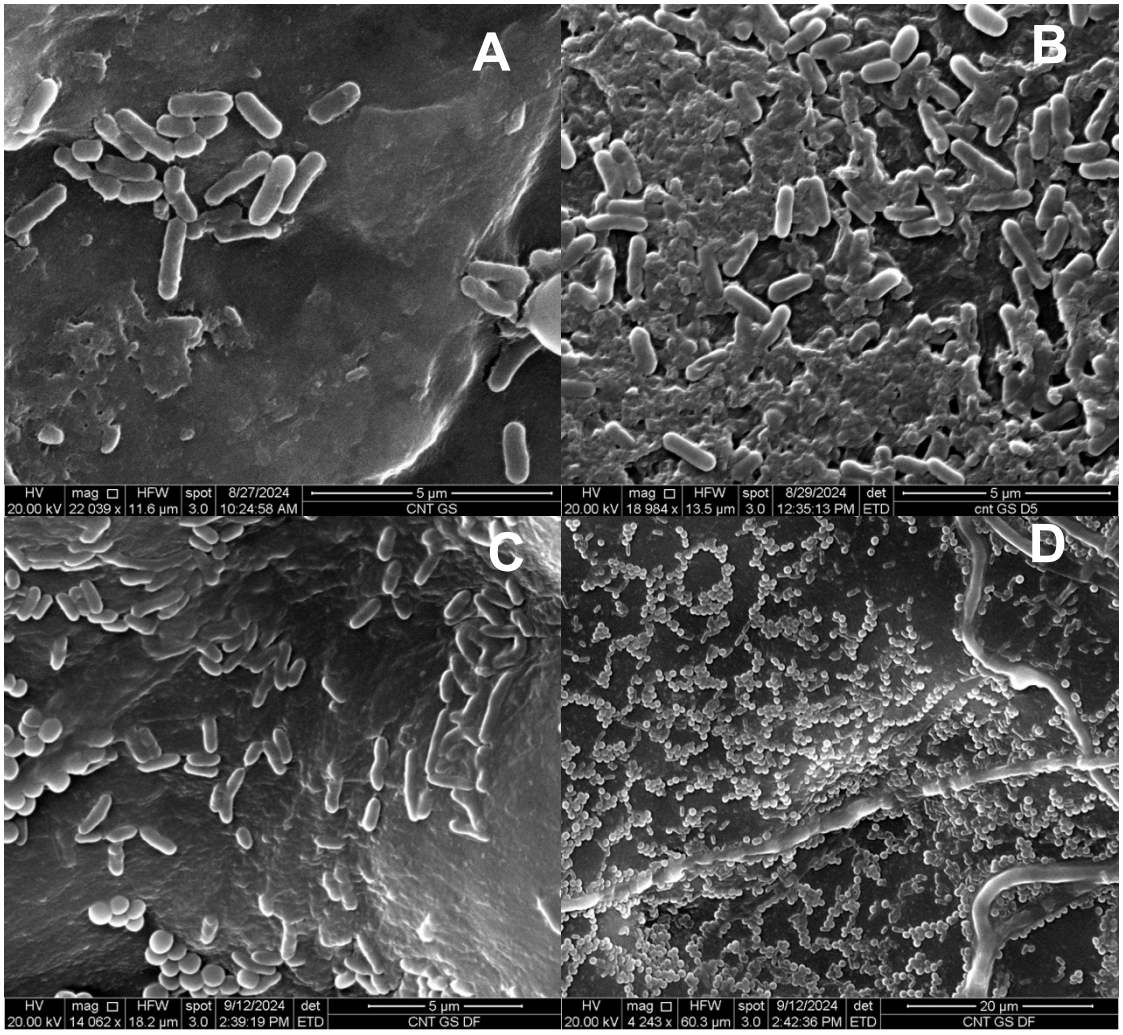

Figure 7. SEM images of* Listeria monocytogenes *on rind of non-treated Green Skin avocados inoculated and stored in a chamber with 95% RH at 21.5 °C for 6 h, (A), 5 days (B), and 14 days (C and D, the latter shown at 4 X lower magnification than C). See Figure 6 caption for details about magnifications.

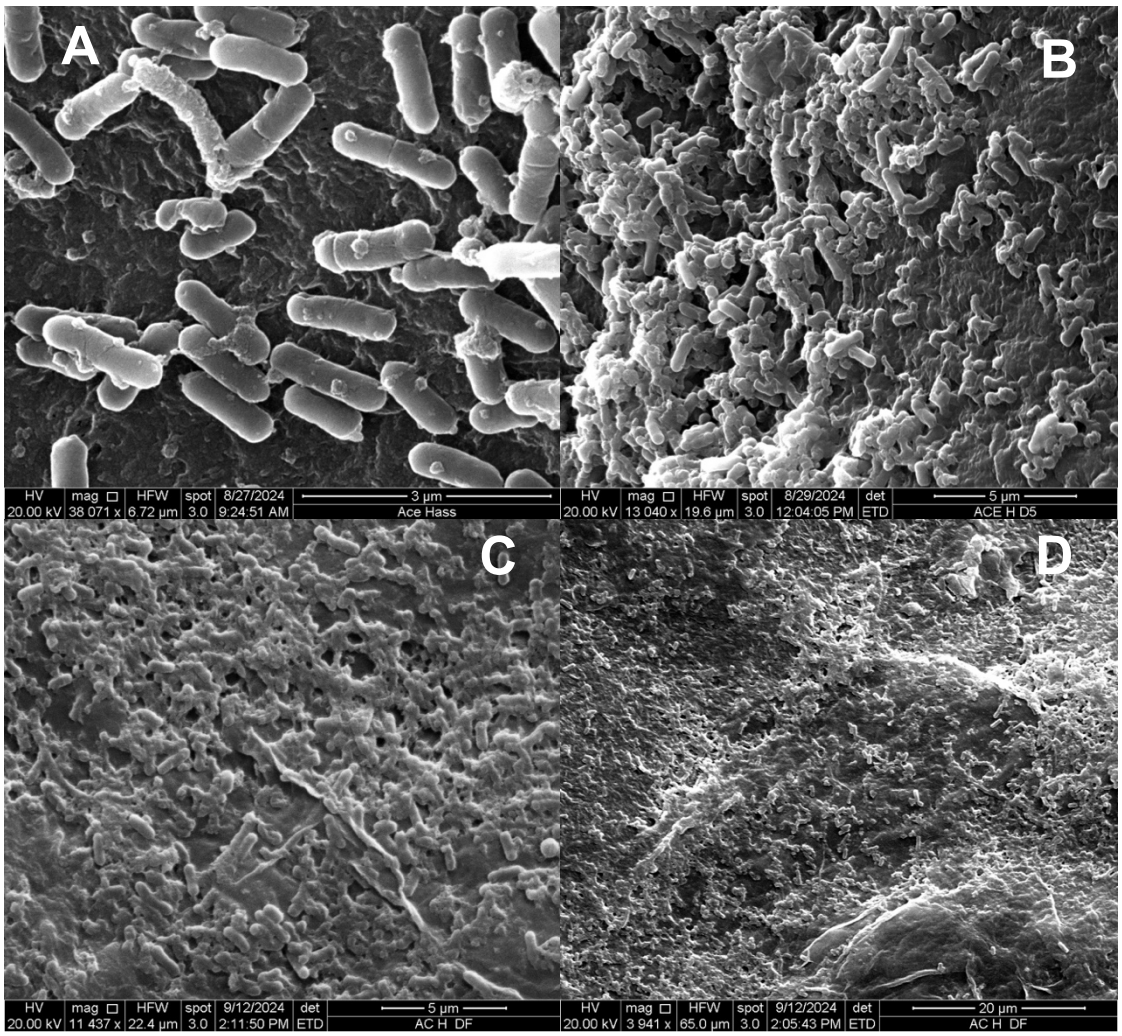

Figure 8. SEM images of* Listeria monocytogenes *on rind of Hass avocado after treating with AFA coating stored in a chamber under 95% RH at room temperature 21.5 °C for 6 h, (A), 5 days (B), and 14 days (C and D). Image D is the same as C shown at a lower magnification. AFA coating contained 7,000 mg/kg AFA in PG and was applied by misting.

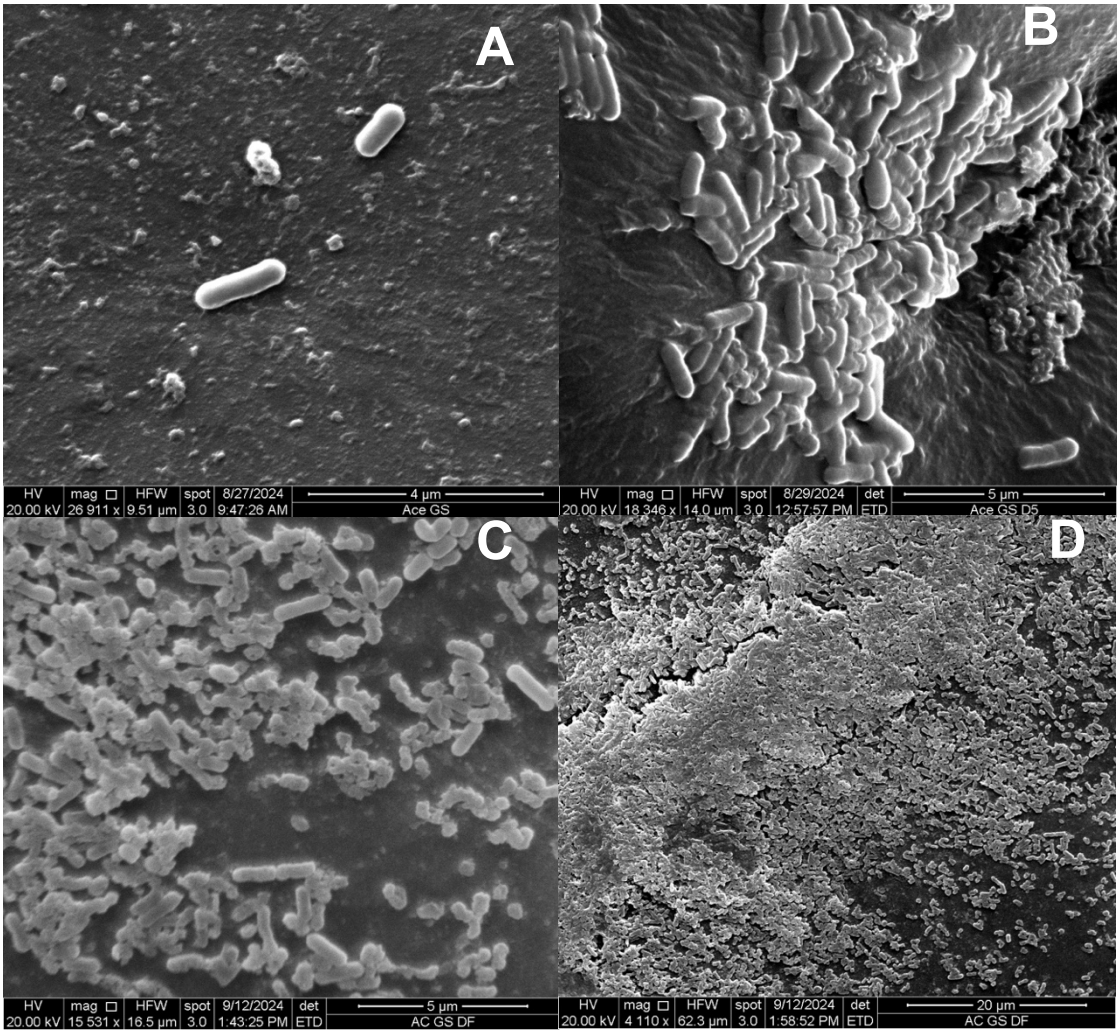

Figure 9. SEM images of* Listeria monocytogenes *on rind of Green Skin avocado after treating with AFA coating stored in a chamber under 95% RH at room temperature 21.5 °C for 6 h, (A), 5 days (B), and 14 days (C and D). Image D is the same as C shown at a lower magnification. See Figure 6 for details about the coating and its application.

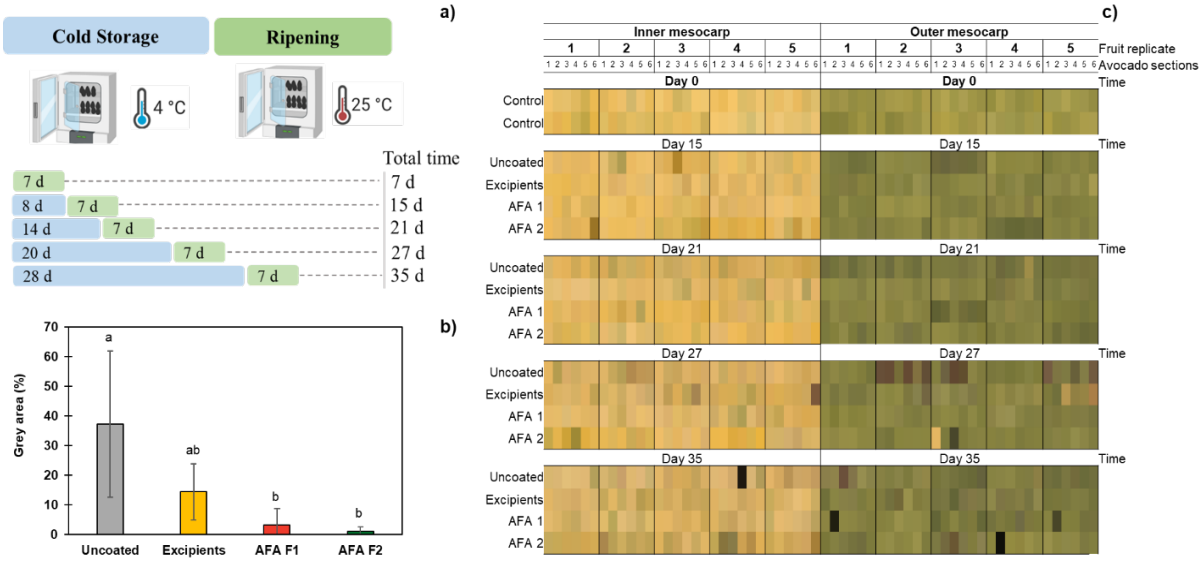

Figure 10. Export simulation design, including cold storage (4 °C) and ripening (25 °C) stages (a). Avocados were coated with prototypes containing AFAs at 7000 ppm (AFA 1) and 14 000 ppm (AFA 2), and formula excipients (PG and avocado oil). The percentage of gray areas in the outer mesocarp after 35 days of storage was determined (b). Instrumental color determination of inner and outer mesocarp (c).

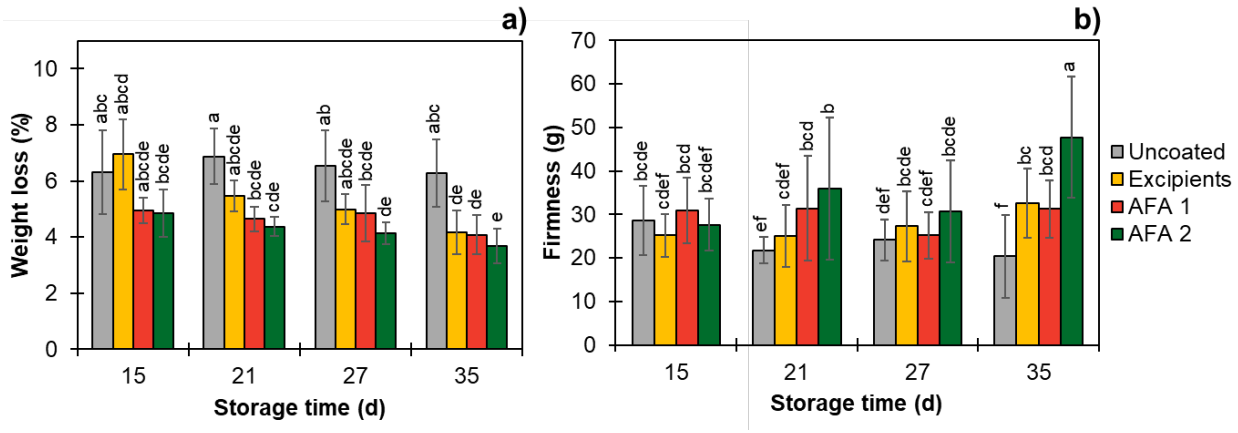

Figure 11. Avocado weight loss (a) calculated as the weigh difference of avocados after ripening (7 days at 25 °C) and weight of the unripe avocado (stored at 4 °C for various times); and instrumental firmness (b) of avocados also after ripening at 25 °C.

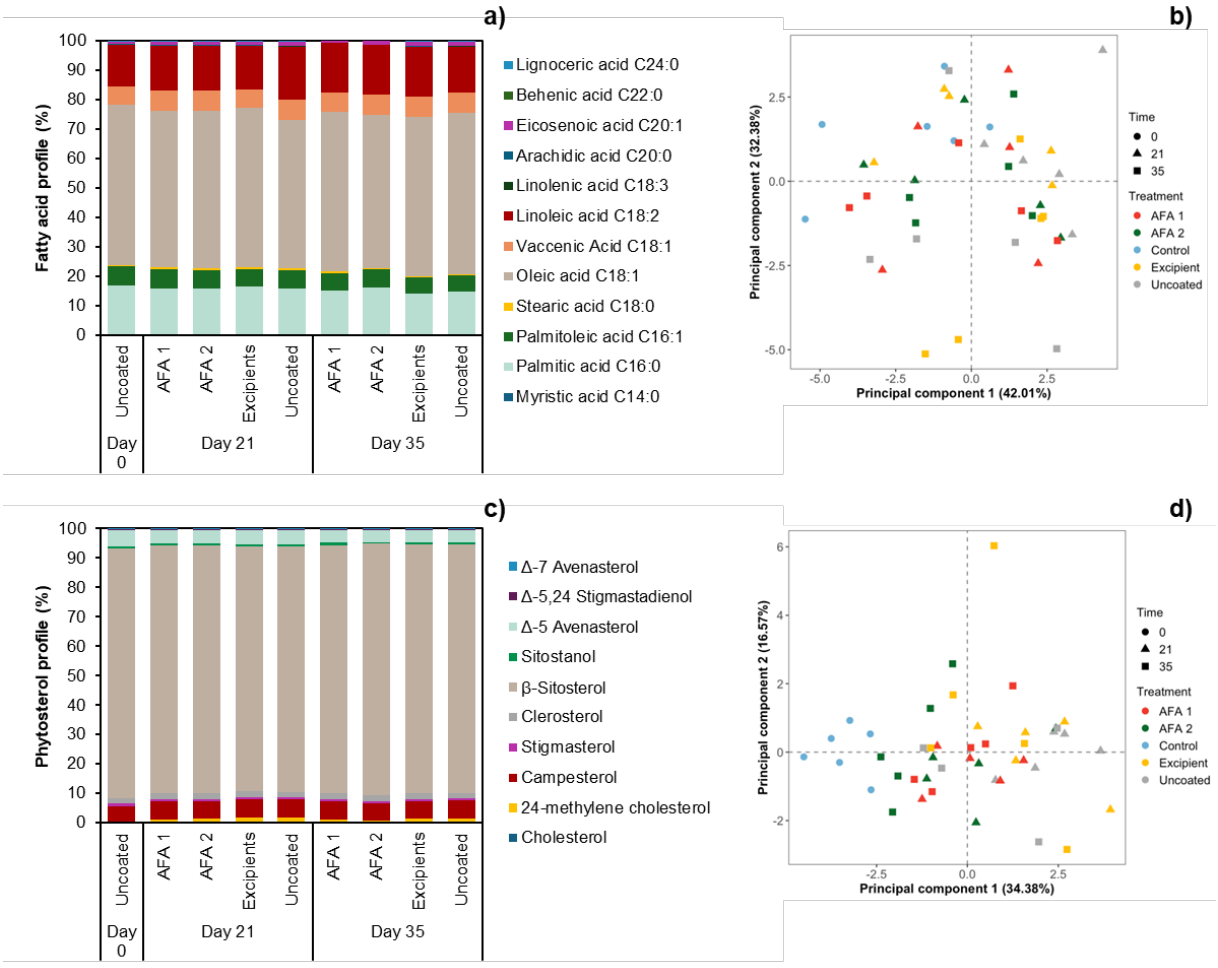

Figure 12. Nutritional quality indicators in ripe avocados over time after cold storage and ripening stages. Profile (a, c) and principal component analysis (b, d) of fatty acids alcohols (a, b) and phytosterols (c, d) in pulp of avocado coated with AFAs at 7000 ppm (AFA 1) and 14 000 ppm (AFA 2), and formula excipients (PG and avocado oil), and uncoated avocados after ripening at 25 °C (days 21 and 35) and unripe controls (day 0).

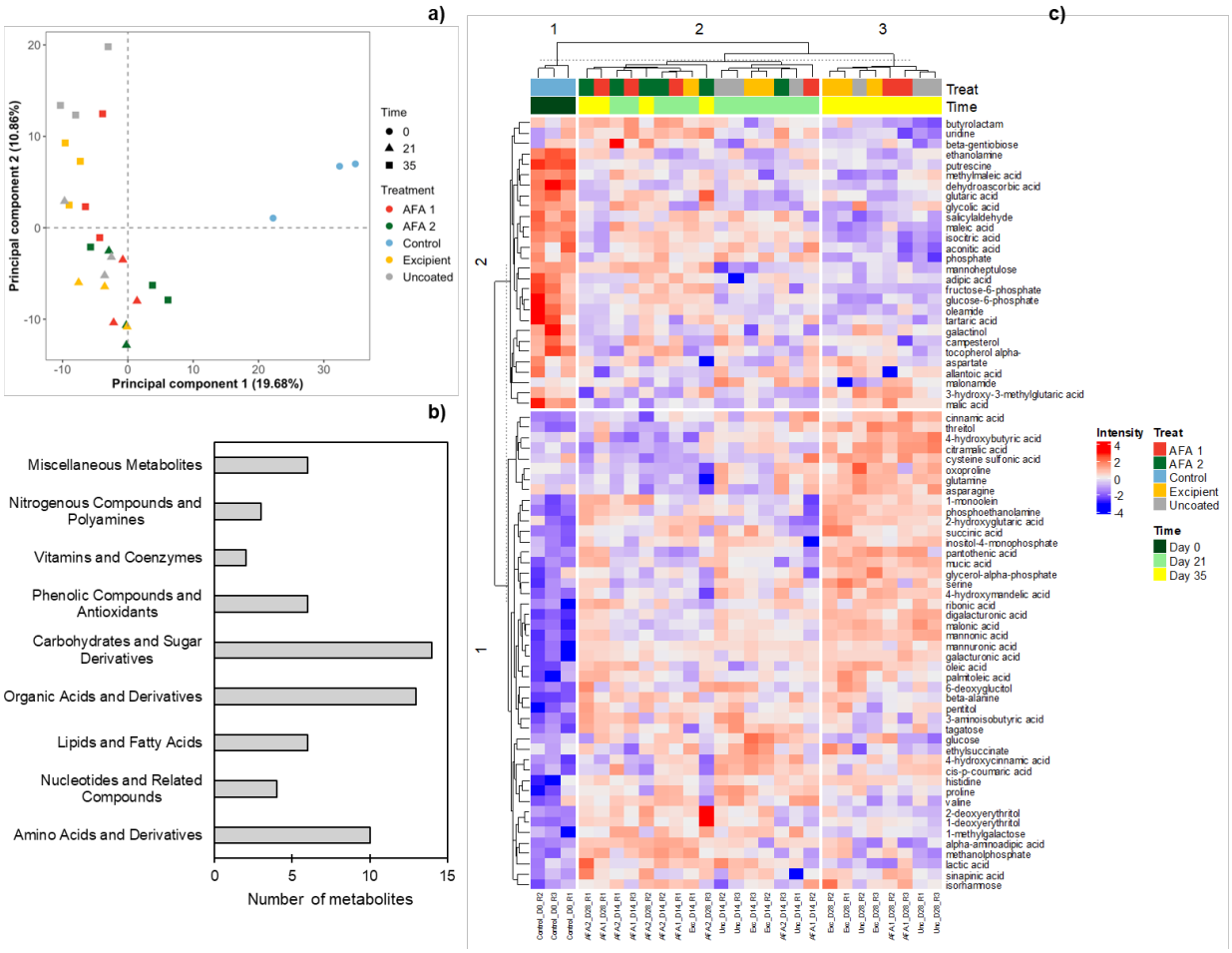

Figure 13. Analysis of primary metabolites (e.g., organic acids, carbohydrates and amino acids) by untargeted metabolomic avocado pulp analyses of fruits coated with AFAs at 7000 ppm (AFA 1) and 14000 ppm (AFA 2), and formula excipients (PG and avocado oil), and uncoated avocados after ripening at 25 °C (days 21 and 35) and unripe controls (day 0). Principal component analysis (a). Family annotation of significantly different metabolites among groups (b). Heatmap showing the normalized intensities of significantly different metabolites among groups (ANOVA, P<0.05) (c).