Purified avocado seed acetogenins: Antimicrobial spectrum and complete inhibition of \textit{Listeria monocytogenes} in a refrigerated food matrix

Raúl Villarreal-Lara\textsuperscript{a}, Dariana Graciela Rodríguez-Sánchez\textsuperscript{b}, Rocio I. Díaz De La Garza\textsuperscript{a}, María Isabel García-Cruz\textsuperscript{b}, Alejandro Castillo\textsuperscript{b}, Adriana Pacheco\textsuperscript{c} and Carmen Hernández-Brenes\textsuperscript{a}

\textsuperscript{a}Tecnológico de Monterrey, Escuela de Ingeniería y Ciencias, Monterrey, N.L., México; \textsuperscript{b}Tecnológico de Monterrey, Escuela de Medicina y Ciencias de la Salud, Monterrey, N.L., México; \textsuperscript{c}Department of Animal Science, Texas A&M University, College Station, TX, USA

1. Introduction

Avocado (\textit{Persea americana} Mill.) is a native fruit of Mexico and Central America (Karamac, Estrella, Herna, & Dykes, 2012). Worldwide, Mexico is the main avocado producer with near 30\% of the global annual production (FAO, 2015; Secretaría de Economía, 2012). A major waste product of avocado processing is the seeds, which represents 8–25\% w/w of the fruit total weight (Weatherly & Sorber, 1931). Around 300,000 tons of avocado seed waste are generated in Mexico each year (Secretaría de Economía, 2012). Because of large volume and low biodegradability of avocado seeds, management of such waste is a challenge for the industry (Bhaumik, Choi, Seopela, McGrindle, & Maity, 2014). Different approaches have been explored to identify alternative uses for avocado seeds and generate products of commercial value.

Compounds in avocado seed have been reported as effective antimicrobials with specific activities (Salinas-Salazar, 2016). Since 1951, crude extracts and a few isolated molecules from avocado seeds are known to exhibit inhibitory properties against vegetative bacteria, yeast, fungi and protozoa (Dharmaratne, Tekwani, Jacob, & Nanayakkara, 2012; Domergue, Helms, Prusky, & Browse, 2000; Lu, Chang, Peng, Lin, & Chen, 2012; Neeman, Lifshitz, & Kashman, 1970; Prusky, Keen, Sims, & Midland, 1982; Rodríguez-Carpena, Morcuende, Andrade, Kylli, & Estévez, 2014).
A significant number of scientific reports have evidenced the potential application of avocado extracts to control growth of relevant human food-borne bacterial pathogens and spoilage microorganisms, such as *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Listeria mono cytogenes*, *Staphylococcus aureus*, *Salmonella serovar Typhi*, *Shigella dysenteriae*, *Candida albicans*, *Saccharomyces cerevisiae*, *Leishmania donovani* and *Mycobacterium tuberculosis*, among others (Dharmaratne et al., 2012; Lu et al., 2012; Neeman et al., 1970; Rodríguez-Carpena et al., 2011). However, most of the previous knowledge was generated using crude extracts (mixtures of molecules) and very few studies have isolated, identified and linked chemical features with specific antimicrobial activities (Salinas-Salazar et al., 2016).

In alignment with consumer demands for natural food additives (Erickson & Doyle, 2017), our research group conducted bioassay-guided isolation studies to identify specific compounds with inhibitory activity against bacterial endospores, using *Clostridium sporogenes* as surrogate for *C. botulinum* (Rodríguez-Sánchez et al., 2013). The study led to the isolation and identification of six lipid derivatives of the family of acetogenins as responsible for the inhibitory activity against endospores and vegetative cells of *C. sporogenes* (Rodríguez-Sánchez et al., 2013). Subsequent work was conducted to validate the effectiveness of a food-grade acetogenin enriched extract (Avosafe®) obtained from avocado seed to control endospore germination (targeting *C. sporogenes*) within a model food matrix; and to determine its stability under food processing conditions (Pacheco et al., 2017). The food-grade acetogenin enriched extract used in prior works has been called Avosafe® as a scientific research brand to facilitate its reference, when compared to other commercially used food antimicrobial agents. Avosafe®’s activity against *L. monocytogenes* was characterized in a prior study in bacterial growth medium at 4°C and 37°C; and results demonstrated a bactericidal effect (MBC values from 15.63 to 31.2 mg L<sup>−1</sup>) for both temperatures) accompanied with a lytic effect on *Listeria* cells, attributed to a potential increase in membrane permeability (Salinas-Salazar et al., 2016).

This study aimed to characterize the antimicrobial spectrum of a purified avocado seed extract enriched in acetogenins, Avosafe®, including a group of food-borne bacterial pathogens and spoilage microorganisms; and to compare acetogenins’ efficacy against two commercial food additives (Nisaplin® and Mirenat®). The study also aimed to evaluate the inhibitory properties of avocado seed acetogenins against the growth of *L. monocytogenes*, for the first time, to the best of our knowledge, in a more complex system than laboratory culture media; a food system containing meat stored under refrigerated storage (4°C) and at accelerated microorganism growth conditions (20°C).

## 2. Materials and methods

### 2.1. Materials

*Reagent grade solvents (dichloromethane and distilled water, dH<sub>2</sub>O) were acquired from DEQ (San Nicolas de los Garza, NL, Mexico). HPLC grade solvents (methanol, isopropanol, water) were purchased from Fisher Scientific (Springfield, NJ, USA). Analytic standards of acetogenins were purified from avocado seed in our laboratory (>97% purity) verifying their identity by MS-TOF (Rodríguez-López, Hernández-Brenes, & de la Garza, 2015; Rodríguez-Sánchez et al., 2013). Standards included 1-acetoxy (AcO)-avocadonine (1), 1-acetoxy-2,4-dihydroxy-heptadec-12-en-16-yn; AcO-avocadene (2), (2S,4S)-1-acetoxy-2,4-dihydroxy-n-heptadeca-16-ene; UPA (3) unknown putative acetogenin; personene C (5), (2R,5E,16E)-1-acetoxy-2-hydroxy-4-oxononadeca-5,16-diene; personene A (6), (2R,5E,12Z,15Z)-1-acetoxy-2-hydroxy-4-oxoheneicos-5,12,15-triene; persin (7), 2R,12Z,15Z)-1-acetoxy-2- hydroxy-4-oxoheneicos-12,15-diene; and personene B (8), (5E)-1-acetoxy-2-hydroxy-4-oxononadeca-5-ene. Persediene (4) was quantified in personene C (5) equivalents (Salinas-Salazar et al., 2016). Numbers near their chemical names (1–8) were assigned based on their chromatographic elution order, and additional structural information can be found in Table S1. Avosafe®, a food-grade acetogenin enriched extract, was kindly provided by Tecnologico de Monterrey, Centro de Biotecnologia-FEMSA (Monterrey, NL, Mexico). Mirenat® (14.5 wt% lauroyl arginate ethyl ester) and Nisaplin® (2.5 wt% nisin from *Lactococcus lactis*), used commercial antimicrobial controls, were obtained from LAMIRSA (Terrassa, Barcelona, Spain) and Sigma Aldrich (St. Louis, MO, USA), respectively. Tween 20 (≥40% lauric acid, saponification value 40–50, hydroxyl value 96–108 mg g<sup>−1</sup>), food-grade propylene glycol (PG) and lysozyme were purchased from Sigma Aldrich (St. Louis, MO, USA). All culture media and microbiology reagents were purchased from Difco Laboratories (Sparks, MD, USA).

### 2.2. Acetogenin characterization of a food grade avocado seed extract (Avosafe®)

Avosafe® (3 mg) was incorporated into a biphasic system containing 3.5 mL of dichloromethane (saturated with water) and 3.5 mL of dH<sub>2</sub>O (saturated with dichloromethane). Then, system was vortexed 30 s and phases were separated by centrifugation (5000 rpm, 5 min). The organic phase (lower phase) was recovered, washed again with 3.5 mL of deionized water (saturated with dichloromethane) and recovered as mentioned. Organic phases were combined and dried under N<sub>2</sub> gas, further solubilized in 1 mL of isopropanol and passed through a PTFE filter (0.45 μm) before being subjected to HPLC analysis. Acetogenins in samples were quantified as reported by Salinas-Salazar et al. (2016).

### 2.3. Study I: Characterization of in vitro antimicrobial activity of a food grade avocado seed extract (Avosafe®)

#### 2.3.1. Inoculum preparation

Eight Gram-positive bacteria, two Gram-negative bacteria, and two yeasts were obtained from the American Type Culture Collection (Manassas, VA, USA). General characteristics and growth conditions of each microorganism are described in Table 1. Vegetative cells of each microorganism were grown up to the exponential phase (7–8 h) in their corresponding media and optimum growth conditions (Table 1). Anaerobic jar (GasPak™ Difco Laboratories Becton Dickinson, Sparks, MD, USA) was used to grow anaerobic bacteria. Stocks of
vegetative cells were then obtained by centrifugation for 20 min at 2862 × g and 22°C, and were preserved at −80°C in fresh culture medium containing glycerol (20% v/v). For experimentation, frozen cell stocks of all strains were cultured individually in 2 mL of medium and incubated for 24 h, at optimum growth conditions (Table 1). Then, an aliquot (10% v/v) from these cultures was inoculated in fresh medium and grown under the same conditions to late exponential phase (7–8 h), generating an acclimated and active inoculum for experimentation. Inocula were adjusted to 8 × 10^6 CFU mL^-1 using fresh culture medium.

To induce sporulation of Clostridium sporogenes, this microorganism was cultured in reinforced clostridial medium (RCM; Difco Laboratories, Sparks, MD, USA) at 37°C for 14 d, using an anaerobic jar, as described by Rodríguez-Sánchez et al. (2013). Further treatment with 500 ppm lysozyme in 1X phosphate-buffered saline (PBS), ultrasonde (15 min), followed by 2 h incubation at 37°C, was applied to separate endospores from sporangia and to digest vegetative cells. This suspension was then washed (with 1X PBS) and pelleted by centrifugation at 2862 × g for 20 min at 22°C, 10–14 times, and finally adjusted to 3.25 × 10^9 CFU mL^-1. Purity of endospore stock suspension was verified by phase contrast microscopy. For experimentation, endospore suspension was adjusted to the required cell density (8 × 10^6 CFU mL^-1) and, to inactive any remaining vegetative cells and activate endospores, it was heat-shocked at 80°C for 15 min and kept in ice until inoculation (Mah, Kang, & Tang, 2008).

2.3.2. Disc diffusion assay
Initially, Avosafe® was tested against the microorganisms described in Table 1 and, subsequently, Mirenat® and Nisaplin® were evaluated against those microorganisms susceptible to Avosafe® for comparative purposes. Vegetative cell suspensions of all strains or C. sporogenes were evaluated against Avosafe® for comparative purposes. Vegetative cell suspensions were evenly spread on independent sterile plates vehicle controls (PG for Avosafe® and dH2O for Nisaplin® and Mirenat®) and positive controls (Mirenat® and Nisaplin®) were also included in each plate. Diameters (in cm) of inhibition zones around each disc (including the disc (CCLS [Clinical and Laboratory Standards Institute, 2013]) were measured after 24 h incubation at each microorganism specific growth condition (Table 1). Antimicrobial solutions were kept at −80°C and protected from light until use. Prior experimentation, solutions were allowed to acclimatize to room temperature and subjected to frequent sonication (30 s, Branson 2510; Branson Ultrasonics, Danbury, CT, USA) and vortexing (5 min, Model VX-200; Labnet, Edison, NJ, USA) cycles during test preparation, to assure homogenous dispersion.

Table 1. General characteristics, oxygen requirement and growth conditions of studied microorganisms at 15 µg disc^-1 of acetogenins present in Avosafe®.

| Microorganism                          | ATCC | General characteristics | Oxygen requirement | Culture media | Avosafe® antimicrobial effect |
|----------------------------------------|------|--------------------------|--------------------|--------------|-----------------------------|
| Clostridium sporogenes                  | 7955 | Bacteria, Gram +, SFB    | anaerobic          | Tryptic soy agar | Yes                         |
| Clostridium perfringens                | 14810| Bacteria, Gram +, SFB    | anaerobic          | Tryptic soy agar | Yes                         |
| Allobacillus acidocaldarius            | 27009| Bacteria, Gram +, SFB    | aerobic            | MRS at 35°C    | Yes                         |
| Bacillus subtilis                      | 11774| Bacteria, Gram +, SFB    | aerobic            | NB at 30°C     | Yes                         |
| Listeria monocytogenes                 | 35152| Bacteria, Gram +         | aerobic            | BHI at 37°C    | Yes                         |
| Staphylococcus aureus                  | 6538 | Bacteria, Gram +         | aerobic            | TSA at 37°C    | Yes                         |
| Lactobacillus plantarum                | 8014 | Bacteria, Gram +         | anaerobic          | MRS at 37°C    | No                          |
| Leuconostoc mesenteroides              | 10830a| Bacteria, Gram +         | anaerobic          | MRS at 37°C    | No                          |
| Escherichia coli                       | 35218| Bacteria, Gram –         | aerobic            | NB at 37°C     | No                          |
| Pseudomonas aeruginosa                 | 29260| Bacteria, Gram –         | aerobic            | TSA at 37°C    | No                          |
| Saccharomyces cerevisiae               | 24858| Yeast                    | anaerobic facultative | YM at 37°C    | No                          |
| Candida parapsilosis                   | 22019| Yeast                    | aerobic            | YM/PDA at 37°C | No                          |

SFB: Spore-forming bacteria. *Tryptone-peptone-yeast extract-glucose [17].
* Nutrient broth. * Brain heart infusion. * Tryptic soy agar. * De Man, Rogosa and Sharpe. * Yeast extract-malt extract. * Potato dextrose agar.

* C. sporogenes was tested with both vegetative cells and endospore germination. * SFB: bacteria formadoras de esporas. * Extracto de levadura peptona dextrosa [17]. * Caldo de nutrientes. * Infusión cerebro-corazón. * Agar de soya tripético. * De Man, Rogosa y Sharpe. * Extracto de levadura – extracto de melá. * Agar papa dextrosa.

2.3.3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)
Avosafe® minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for Clostridium sporogenes, Listeria monocytogenes and Allobacillus acidocaldarius were determined as described by Rodríguez-Sánchez et al. (2013). A 3000 ppm working solution of Avosafe® in PG was used to prepare twofold serial dilutions ranging from 125 to 3.9 ppm (1 mL final volume) in test tubes with culture medium adequate for each microorganism. To account for the effect of the vehicle in all tested concentrations, they were adjusted to 4.1% v/v PG, having previously verified that this PG level did not affect microorganism growth. Each tube was then inoculated with the corresponding bacterial suspension (100 µL, containing 8 × 10^6 CFU mL^-1) and incubated for 24 h according to conditions shown in Table 1. A growth control (without antimicrobial addition), an aseptic techniques control (without inoculum addition) and positive controls (with Nisaplin® or Mirenat®) were also included. MIC was defined as the lowest concentration at which no growth was visually obvious. Then, cells from dilutions at and above the MIC were recovered by centrifugation (2862 × g, 20 min at 22°C), washed two times with fresh culture medium and sub-cultured as
described above (in absence of antimicrobial). The MBC was defined as the lowest concentration where growth is not visually detected. Antimicrobial stock solutions were stored at −80°C until testing and acclimatized as previously described.

2.4. Study II: evaluation of antilisterial efficacy of acetogenins in food system containing meat

2.4.1. Inoculum preparation
Frozen stocks of *Listeria monocytogenes* (ATCC 35152) were first activated in brain heart infusion (BHI) for 13 to 15 h at 37°C. A subculture was prepared under the same growth conditions to late exponential phase (6–7 h). At this stage, cells were adjusted with BHI to an initial absorbance at 600 nm of 0.15, which corresponded to $8 \times 10^6$ CFU mL$^{-1}$ and served for further inoculation of the food system containing meat.

2.4.2. Preliminary evaluation of antilisterial efficacy of acetogenins at 7,500 ppm in a food system containing meat stored at 20°C for 96 h
Sterile commercial baby food puree (Gerber Nestle, CDM, Mexico) was purchased at a local supermarket, its ingredient label declared to contain mainly beef, vegetables and rice (water, 13% beef, 11% carrots, 7% green beans, 3% rice, vegetable oil mix – sunflower, coconut and soybeans, 1% tomato paste, 1% dehydrated potato, 1% dehydrated peas, rice flour, butter, natural flavorings (beef, garlic and onion), iodized salt, chicken fat, iron, zinc and folic acid). The nutritional label of the food puree declared the contents as (113 g): total fat 4.0 g, saturated fat 1.1 g, total carbohydrates 8.0 g, dietary fiber 0.45 g, sugars 1.5 g, protein 3.5 g, sodium 90 mg, iron 15 mg, zinc 1.1 mg, and folic acid 11 µg.

For precise and aseptic incorporation of all ingredients, an analytical balance was introduced into a biological safety cabinet and Avosafe® (formulated as 80% w/w avocado oil, containing 38.1% acetogenins, and 20% w/w PG) and Tween 20 were individually filter-sterilized (0.2 µm cellulose acetate membrane; Corning, NY, USA). Ingredients were added in the following order and were vigorous stirred with a spatula to ensure adequate homogenization of the system: i) baby food puree (10% w/w), ii) Avosafe® (1.97% w/w), iii) baby food puree (86.52%) containing 0.034% Tween 20 and iv) microbial inoculum (1% v/w). Positive and negative controls were also included. After incubation at 4 ± 1°C, samples were taken in duplicate at 0, 125, 20, 40, 60 and 72 days and microbial counts performed as described above.

2.4.3. Evaluation of antilisterial efficacy of acetogenins at 10,000 ppm in a food system containing meat stored at 4°C for 72 days
As described in section 2.4.2., 16 g of the food system containing meat were prepared at a concentration of 10,000 ppm acetogenins and divided into 1 g samples. To achieve this concentration, Avosafe® was formulated as 84% w/w avocado oil (containing 40.3% acetogenins) and 16% w/w PG. Ingredients were added as previously described and at the following proportions: i) baby food puree (10% w/w), ii) Avosafe® (2.5% w/w), iii) baby food puree (86.52%) containing 0.034% Tween 20 and iv) microbial inoculum (1% v/w). Positive and negative controls were also included. After incubation at 4 ± 1°C, samples were taken in duplicate at 0, 125, 20, 40, 60 and 72 days and microbial counts performed as described above.

2.5. Statistical analysis
All samples per experiment were analyzed with technical triplicates, unless specified, independent measures associated with protocols or equipment, and data presented as mean ± standard deviation (SD). One-way ANOVA and post hoc contrasts (Tukey HSD, $p < 0.05$) were performed using JMP® software version 13.0.0 (SAS Institute Inc., Cary, NC, USA) to compare the difference between group means.

3. Results and discussion

3.1. Study I: Antimicrobial activity of a food grade avocado seed extract

3.1.1. Acetogenin characterization of a food grade avocado seed extract (Avosafe®)
Eight acetogenins were detected and quantified as present in the avocado seed extract (Avosafe®) and numbers from (1) to (8) were assigned to each of them, based on their chromatographic elution order (Table S1). Mass spectra of the eight compounds consistently presented a molecular ion accompanied with its corresponding sodium adduct and fragments representing losses of 18, 78, 60 mass units, that correlate with losses of H$_2$O and/or acetic acid present in their structures. This fragmentation pattern has been reported to be characteristic of acetogenins (Degenhardt & Hofmann, 2010; Domergue et al., 2000; Rodríguez-López et al., 2015; Rodríguez-Sánchez et al., 2013). Then, by comparing their mass spectrum and retention time to those of analytical standards (whose identity has been previously confirmed by MS-TOF and NMR (Rodríguez-Sánchez et al., 2013), chemical identity of compound (2) and (4) to (8) was determined as: AcO-avocadene (2), persediene (4), persenone C (5), persenone A (6), persin (7), and persenone B (8). Based on similarities of the ion pattern of compound (1) and that of an acetogenin previously reported by Ramos-Jeriz (2007), it was tentatively identified as 1-acetoxy-2,4-dihydroxy-heptadec-12-en-16-yno (AcO-avocadene) (Rodríguez-López et al., 2015). While compound (3) was referred as an unknown putative acetogenin (UPA) since its mass and spectrum correspond to that of three acetogenin mesophiles, as the food matrix was previously confirmed to be sterile. Plates were incubated for 36 h at 37°C.
isomers previously described (Ramos-Jerz, 2007; Rodríguez-López et al., 2015), not being possible to assign an definite identity.

As shown in Figure 1, Avosafe® contained a total acetogenin concentration of 71 ± 0.78% w/w that represented an enrichment of 140-fold from concentrations naturally present in avocado seed from cultivar Hass (5048 ppm in a fresh weight (FW) basis) as previously reported by Salinas-Salazar et al. (2016). Concentrations of AcO-avocadene (2), and persenone A (6) were 22.05 ± 1.84 and 20.14 ± 1.40% w/w, respectively, as reported in Figure 1, and were significantly higher than other acetogenins found in the acetogenin-enriched extract (Avosafe®). Persin (7) was next in abundance (11.08 ± 0.66% w/w). Rodriguez-López et al. (2015) reported similar acetogenin profiles for avocado seed extracts from 22 different cultivars.

3.1.2. Screening of antimicrobial spectrum of a food grade avocado seed extract (Avosafe®)

A screening study was conducted against eight Gram-positive bacteria, two Gram-negative bacteria and two yeasts, and results are shown in Table 1. Avosafe® presented higher selectivity against Gram-positive bacteria (Table 1); since, at evaluated concentrations (15 µg disc⁻¹ of total acetogenins), data indicated that the extract inhibited most microorganisms in that particular group. Among Gram-positive bacteria, all the spore formers were inhibited, while no inhibition was observed for two species of non-spore formers (Lactobacillus plantarum and Leuconostoc mesenteroides) at the concentrations evaluated. Observations were in agreement with previous studies, in which Avosafe® was effective against endospore germination and vegetative cell growth of C. sporogenes (Pacheco et al., 2017).

In the present work (Table 1), no growth inhibition was observed for Gram-negative bacteria (Escherichia coli and Pseudomonas aeruginosa), at the evaluated acetogenin concentration. Conversely, Rodríguez-Carpena et al. (2011) reported inhibition of Gram-negative bacteria in their antimicrobial characterization of crude extracts from different avocado tissues (seed, pulp, and peel) of commercial varieties Hass and Fuerte. Gram-negative bacteria tested in their work included E. coli and P. aeruginosa, among other microorganisms (Rodríguez-Carpena et al., 2011). In general, the authors observed that P. aeruginosa was the most resistant bacteria of the evaluated microorganisms, since only peel extracts from both varieties were able to inhibit its growth. While pulp and seed extracts from Fuerte variety were active only against E. coli, and pulp, and seed extracts from Hass variety were inactive against both microorganisms. The crude extracts evaluated in their work were obtained with acetone, and due to the polarity of the solvent used, authors attributed the observed antimicrobial properties to phenolic compounds (such as catechins, procyanidins and hydroxycinnamic acids) that were present in all their extracts. Peel extracts from both varieties were also reported by the authors to be particularly high in phenolic concentrations. Based on the non-polar nature of acetogenins, they are less soluble in acetone than phenolic compounds are, therefore, it can be hypothesized that they were not the main compounds responsible for the activity against Gram-negative bacteria observed by Rodriguez-Carpena et al. (2011). More in agreement with our observations, Neeman et al. (1970) also reported that purified AcO-avocadene (2) was inactive against E. coli and P. aeruginosa and other two Gram-negative bacteria (Salmonella typhi and Shigella dysenteriae), when evaluated at 50 µg pure compounds disc⁻¹; while, its natural occurring analog, differing only in the presence of a hydroxyl group at C-1, was very active against these microorganisms. However, it is possible that acetogenins may exert antimicrobial activity against Gram-negative microorganisms at higher concentrations than those tested in prior studies.

Also shown in Table 1, no inhibition of acetogenins was observed towards the growth of yeast (Saccharomyces cerevisiae and Candida parapsilosis), at the concentrations tested. Contrary to our results, Neeman et al. (1970) reported that purified AcO-avocadene (2) and two of its structural analogs were able to inhibit Saccharomyces cerevisiae and Candida

![Figure 1](image-url)

**Figure 1.** Relative concentration of individual acetogenins present in Avosafe®, a food-grade avocado seed extract enriched in acetogenins. Bars represent the mean ± standard deviation of technical replicates (n = 3). *Numbers in parenthesis for each acetogenin refer to their chromatographic elution order, additional structural information can be found in Table S1. **Different letters indicate significant differences between each individual acetogenin concentration (Tukey HSD test, a = 0.05). (g) Quantified in persenone C (5) equivalents (Salinas-Salazar et al., 2016). *UPA: Unknown putative acetogenin.

**Figura 1.** Concentración relativa de acetogeninas individuales presentes en Avosafe®, un extracto grado alimenticio de semilla de aguacate, enriquecido en acetogeninas. Las barras representan la media ± desviación estándar de las repeticiones técnicas (n = 3). *Los números entre parénthesis para cada acetogenina se refieren a su orden de elución cromatográfica, la información adicional sobre su estructura se puede encontrar en la Tabla complementaria S1. **Letras diferentes indican diferencias significativas entre la concentración individual de cada acetogenina (Tukey HSD, α = 0.05). (g) Cuantificado en equivalentes de persenona C (5) (Salinas-Salazar et al., 2016). *UPA: acetogenina putativa desconocida.
albicans. Chemical analogs tested, differed among them in the presence of a hydroxyl group at C-1 for one, and of a hydroxyl group at C-1 combined with a terminal carbon-carbon triple bond for the other (Neeman et al., 1970). The differences between our results and those reported by Neeman et al. (1970) may be related to the evaluated concentrations; as they tested 50 µg pure compounds disc\(^{-1}\), compared to 15 µg of acetogenins disc\(^{-1}\) in our work, which corresponded to 3.3 µg AcO-avocadone (2) disc\(^{-1}\) (based on results described in section 3.1.1). Further work is needed to evaluate the antimicrobial activity of acetogenins at higher concentrations and, if needed, determine the MIC against the growth of Gram-negative bacteria and yeast.

Antimicrobial properties of lipids, other than avocado acetogenins, have been reported for molecules such as long-chain fatty acids. Likewise acetogenins, it has been observed that Gram-positive bacteria appear to be more sensitive to lipophilic compounds than their Gram-negative counterparts (Inoue & Horikoshi, 1991; Shelef, Naglick, & Bogen, 1980; Umerska et al., 2016). It is known that outer cell structures of Gram-positive bacteria are comprised of thick layers of peptidoglycans followed by the cytoplasmic membrane, whereas Gram-negative bacteria have an extra outer membrane with lipopolysaccharides (LPS), followed by a thin layer of peptidoglycan and, lastly, the cytoplasmic membrane (Silhavy, Kahne, & Walker, 2010). The (LPS)-phospholipid region is highly impermeable to hydrophobic compounds, but may allow transit of small hydrophilic solutes through porins transmembrane channels (Umerska et al., 2016). Therefore, it has been suggested that the outer membrane of Gram-negative bacteria confers them a strong resistance to antimicrobials of non-polar nature that may be very toxic to Gram-positive bacteria, such as long-chain fatty acids (Knapp & Melly, 1986; Umerska et al., 2016). Considering that acetogenins are fatty acid derivatives (Rodriguez-Saona & Trumble, 2000) with a long odd unsaturated aliphatic chain (C19-C23) and a highly oxygenated end (Table S1), lack of antimicrobial activity against Gram-negative bacteria might indicate that the outer membrane prevents permeability into the interior of the cell.

As observed in Figure 2, at the tested concentration, Avosafe\(^{®}\) inhibited all evaluated Gram-positive species, presenting significantly higher or comparative antimicrobial activity to commercial food additives, except for Staphylococcus aureus, for which Mirenat\(^{®}\) resulted more efficient. At equivalent concentrations (w/w), Nisaplin\(^{®}\) did not display inhibition against Listeria monocytogenes; however, it is possible that higher levels of the food additive are required to observe the effect, since L. monocytogenes inhibition by Nisaplin\(^{®}\) has been previously documented (Davies, Bevis, & Delves-Broughton, 1997). The active ingredient of Mirenat\(^{®}\) is lauroyl arginate ethyl (LAE), a cationic surfactant synthetically produced from fatty acid lauric acid and arginine (Ruckman, Rocabayera, Borzelleca, & Sandusky, 2004). While nisin, Nisaplin\(^{®}\)s bioactive component, is a small gene-encoded antimicrobial peptide produced by Lactococcus lactis (Gao, Abee, & Konings, 1991).

Besides the observed specificity of acetogenins towards Gram-positive bacteria, another relevant remark from the results shown in Figure 2 was the specificity against endospore-forming bacteria such as C. sporogenes, A. acidocaldarius and B. subtilis. These species presented significantly higher diameters of inhibition when compared to Nisaplin\(^{®}\) and Mirenat\(^{®}\). In accordance with our previous results (Pacheco et al., 2017), acetogenins can inhibit endospore germination and vegetative cell growth of C. sporogenes effectively. Although mechanisms behind the observed sporicidal activity should be further studied, observations of the present work continue to strengthen the antimicrobial properties of acetogenins as endospore germination and vegetative cell growth inhibitors of different pathogenic and spoilage bacteria.

In addition to advancing scientific knowledge on acetogenins inhibitory properties against vegetative cell growth

---

**Figure 2.** Diameter of inhibition zones of Avosafe\(^{®}\), Mirenat\(^{®}\) and Nisaplin\(^{®}\) against the growth of different Gram-positive microorganisms. All antibacterial agents were tested at 15 µg disc\(^{-1}\) of their active ingredients (total acetogenins, ethyl lauroyl arginate (LAE) and nisin for Avosafe\(^{®}\), Mirenat\(^{®}\) and Nisaplin\(^{®}\), respectively). Values represent mean ± standard deviation of technical replicates (n = 2–3). *Different letters indicate significant differences between antibacterial agents for the same microorganism (Tukey HSD test, α = 0.05, n = 6–9). **Cs veg: C. sporogenes vegetative cells. Cs end: C. sporogenes endospore germination. Cp: C. perfringens. Aa: A. acidocaldarius. Bs: B. subtilis. Lm: L. monocytogenes. Sa: S. aureus. * ND: Not detected.

---

**Figura 2.** Diámetro de zonas de inhibición de Avosafe\(^{®}\), Mirenat\(^{®}\) y Nisaplin\(^{®}\) contra el crecimiento de diferentes microorganismos Gram positivos. Todos los agentes antibacterianos se probaron a 15 µg de disco \(^{-1}\) de sus ingredientes activos (acetogeninas totales, etil laurilo arginato (LAE) y nisina para Avosafe\(^{®}\), Mirenat\(^{®}\) y Nisaplin\(^{®}\) respectivamente). Los valores representan la media ± desviación estándar de las repeticiones técnicas (n = 2–3). *Letras diferentes indican diferencias significativas entre los agentes antibacterianos para el mismo microorganismo (Tukey HSD, α = 0.05, n = 6–9). **Cs veg: células vegetativas de C. sporogenes. Cs end: germinación de endosporas de C. sporogenes. Cp: C. perfringens. Aa: A. acidocaldarius. Bs: B. subtilis. Lm: L. monocytogenes. Sa: S. aureus. * ND: No detectado.
and endospore germination of various microorganisms (Table 1 and Figure 2), the present work also strengthened acetogenins’ potential industrial applications. Particularly as alternatives to existing (natural and synthetic) preservatives, since food additives from natural sources are being sought (Erickson & Doyle, 2017). Specifically, spore inhibitors, since it is known that endospores are highly resistant to adverse conditions, remaining dormant for extended periods of time, until they find a favorable environment to restart growth in a so-called germination and outgrowth process (Ciarcialini et al., 2000). Therefore, spore control or destruction represents a big challenge for food processors that need to avoid safety concerns (toxin production) and spoilage of products (Erickson & Doyle, 2017). For instance, in minimally processed foods, both vegetative cells and spores can survive, as a result, cooling, pH reduction and/or application of preservatives is required to control bacterial outgrowth. On the other hand, spore-forming bacteria represent a safety concern in non-acidic food products subjected to pasteurization process, aimed to destroy only vegetative cells, since they rely on the temperature hurdle (4°C) to keep spores from germinating. Nevertheless, in low pH pasteurized food products (pH <4.6), where pathogenic spore formers are not a concern, since their spores cannot germinate at those pH values, acidophilic spoilage spore formers (such as Allicyclobacillus acidoterrestris and some Bacillus species) can grow, and affect their acceptability and shelf-life (Wells-Bennik et al., 2016). Particularly, A. acidoterrestris, with unique physiological characteristics (growth from pH 2.5–6.0, with optimum around pH 3.5–5.0, and temperatures from 20°C to 60°C, with optimum at 42–53°C), may cause spoilage of fruit juices. A. acidoterrestris has been associated with different quality defects, but the most important fault is the development of strong medicinal or antiseptic flavor by the production of guaiacol and other secondary metabolites (N. Jensen & Whitfield, 2003).

3.1.3. Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of the food grade avocado seed extract (Avosafe®)

MIC and MBC values against vegetative cell growth of A. acidocaldarius and L. monocytogenes, and endospore germination of C. sporogenes were also characterized for Avosafe® (Table 2). MIC values were found to be in the range of 3.9 to 15.6 ppm; levels that were 200 to 795 times lower than concentrations of acetogenins found in avocado pulp (the edible part of the fruit) (Salinas-Salazar et al., 2016). Ahn, Shin, Baek, Seong, and Woo (2001) established that the MIC value of linolenic acid for L. monocytogenes was 20 ppm (Ahn et al., 2001). Compared to the present results, Avosafe® showed similar or slightly higher inhibitory properties against L. monocytogenes (MIC 15.6 ppm) than linolenic acid, structurally similar to acetogenins and proposed as its precursor in planta (Rodríguez-Saona & Trumble, 2000). Slightly higher MICs (25 ppm) have been reported for LAE against Listeria innocua (Becerril, Manso, Nerín, & Gómez-Lus, 2013). Whereas for C. sporogenes endospores, Avosafe®’s MIC values were the lowest (3.9 ppm) suggesting that acetogenins have certain specificity against that form of life. Moreover, Avosafe® MBC/MIC ratios observed in this study for A. acidocaldarius vegetative cells and C. sporogenes endospores were >4, therefore a bacteriostatic and sporostatic of action can be described (Levison, 2004), respectively. While in presence of Avosafe®, at 4°C or 37°C, MBC/MIC ratios against L. monocytogenes were ≤4, indicating a bactericidal activity.

Mechanisms of acetogenin inhibition towards Gram-positive bacterial endospore germination and vegetative cell growth remain unknown. However, it has been observed that when tested as individual acetogenin molecules, not in a mixture like Avosafe®, the trans-enone group present in some acetogenins was a structural feature that favored antimicrobial activity against C. sporogenes endospores (Rodríguez-Sánchez et al., 2013). As observed in Table S1, from acetogenins present in Avosafe®, persenone A (6), persenone B (8), and persenone C (5), contained the trans-enone structural feature. Moreover, the presence of multiple unsaturations in their aliphatic chains was an additional structural feature that enhanced acetogenin antilisterial activity (Salinas-Salazar et al., 2016). From the structures shown in Table S1, persenone C (5), persenone A (6), and AcO-avocadoenypen (1) containing unsaturations in their aliphatic chains, were previously linked to antilisterial properties (Salinas-Salazar et al., 2016). A lytic effect caused by acetogenins was observed by Salinas-Salazar et al. (2016), when L. monocytogenes cells (at late exponential phase) were exposed to 10 and 100 times MIC value (156.3 and 1563 ppm, respectively) for 24 h at 37°C. Similarly, Desbois and Smith (2010) observed that the antibacterial activity and potential antimicrobial mechanisms of fatty acids were determined by their structure and shape (linked to number and type of unsaturations). Considering structural similarities between acetogenins and fatty acids, it is possible that structural features also can influence their mechanisms of action. For instance, physically disruption of cell membranes has been linked to the insertion of fatty acids. Furthermore, Cis double bond unsaturations in their structures results in fixed bends that allow them to occupy a greater space.

### Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of acetogenins present in Avosafe® against vegetative cell growth (A. acidocaldarius, L. monocytogenes) and endospore germination (C. sporogenes).

| Microorganism | MIC (ppm) | MBC (ppm) | Ratio MBC/MIC | Reference |
|---------------|-----------|-----------|---------------|-----------|
| A. acidocaldarius | 15.63 | >125 | >8 | In this study |
| Listeria monocytogenes (4°C) | 7.8 to 15.63 | 15.6 to 31.25 | 2 | [17] |
| Listeria monocytogenes (37°C) | 15.63 | 31.25 | 2 | [17] |
| Clostridium sporogenes | 3.91 | >125 | >32 | [16] |

1Tests were performed in triplicate. 2MBC/MIC ratio ≤ 4 indicates bacteriostatic activity and >4 sporostatic or bacteriostatic activity [37]. 3Incubation conditions are presented in Table 1. 4Las pruebas se realizaron por triplicado. 5La relación MBC/MIC ≤ 4 indica actividad bactericida y > 4 actividad esporostática o bacteriostática [37]. 6Las condiciones de incubación se presentan en la Tabla 1.
within the membranes, therefore introducing generalized disorganization, higher membrane fluidity and eventual cell disintegration (Avis & Bélanger, 2001).

Other authors have also documented the relationship between structural features and antimicrobial activities. van Melis et al. (2012) using Bacillus cereus as model microorganism, demonstrated that lipophilic nature of medium-chain organic acids and their structurally related alcohols were relevant factors that influenced their endospore germination inhibitory activity. Their findings indicated that most lipophilic compounds inhibited germination more efficiently, and hypothesized two possible mechanisms of action. The first related to accumulation of lipophilic molecules in the inner membrane of endospores, which disrupted phospholipid layers and affected integrity (van Melis, Almeida, Kort, Groot, & Abeel, 2012). Results that were consistent with other authors (Ababouchl, Chaib, & Busta, 1992), which previously reported that linoleic and linolenic acid inhibited B. cereus endospore germination and outgrowth. Higher inhibition was observed for linolenic acid, which contains an additional unsaturation and therefore possibly caused greater disruption due to a greater occupied area in the inner membranes (Ababouchl et al., 1992). The second mechanism proposed by the authors (van Melis et al., 2012), was the accumulation of foreign compounds in endospore inner membrane, which produced volume adjustments and influenced protein–protein interactions, without affecting membrane integrity. The conservation of protein–protein interaction is important in view of the existence of inter-dependent clusters of proteins located in close proximity within endospore inner membrane, being crucial for nutrient-induced germination (known as germinosomes) (Griffiths, Zhang, Cowan, Yu, & Setlow, 2011). Further tests are needed in order to confirm the above hypotheses for acetogenins and elucidate their mechanism of action.

### 3.2. Study II: Evaluation of antilisterial efficacy of acetogenins in a food system containing meat

#### 3.2.1. Preliminary evaluation of antilisterial efficacy of acetogenins at 7,500 ppm in a food system containing meat and stored at 20°C for 96 h

A commercially sterilized meat based puree was selected as a food system to explore the inhibitory properties of the avocado seed acetogenin enriched extract (Avosafe®) against the growth of Listeria monocytogenes in a more complex matrix. Preliminary studies were conducted in order to confirm the ability of the food matrix to support L. monocytogenes growth.

As shown in Figure 3, target inoculation of 3-log of L. monocytogenes was attained in the food system containing meat. Inoculation level was selected to represent counts likely to occur in commercial RTE products (Wong, Carey-Smith, Hollis, & Hudson, 2005) and inoculation counts were also within the suggested levels (10^2–10^5 CFU g^-1) for the validation of post-processing lethality or efficacy of antimicrobial agents (Health Canada, 2012; Scott et al., 2005). Figure 3 also shows results from the preliminary experiment conducted to test the antimicrobial effects of Avosafe® (7500 ppm) under accelerated conditions (20 ± 2°C). Avosafe® generated a 2-log reduction, from the original inoculated L. monocytogenes level, after 3 h of incubation. After 24 h, Listeria counts were around those of initial inoculum (3.60 ± 0.06 log). However, when compared to growth of control treatments, Avosafe® presented 3 and 1.3 log lower Listeria counts after 24 and 96 h (4 d) of incubation, respectively.

Since acetogenins are lipophilic molecules, PG (20%) was used as vehicle to generate translucent solutions, improve solubility of acetogenins, and to facilitate their incorporation into the food system containing meat. Tween 20 was added as a surfactant; however, it was directly added to the food system containing meat, not to the PG-Avosafe® mixture, and its final concentration in the food matrix maintained at minimum levels (0.03%). The latter precautions were taken since it is known that antimicrobial activity of lipidic substances can be reduced by the direct incorporation of surfactants at high concentrations (Hammer, Carson, & Riley, 1999; Ma, Davidson, & Zhong, 2016). When they come in direct contact, at high concentrations, Tween molecules are thought to bind hydrophobically to lipidic molecules, reducing the interaction with bacterial cells (Ma et al., 2016). In contrast, low concentrations of Tween appear to increase antimicrobial concentration in the continuous phase of the emulsion, facilitating access through cell membranes, and improving interactions between cells and antimicrobial molecules (Gayinsky, Taylor, Davidson, Bruce, & Weiss, 2007).

#### 3.2.2. Antilisterial efficacy of acetogenins at 10,000 ppm in a food system containing meat stored at 4°C for up to 72 days

An acetogenin concentration of 10,000 ppm was selected for the refrigerated study (4°C), based on observations from the preliminary study (conducted at 20°C, Figure 3) in which a bacteriostatic effect but not a listericidal effect was observed. Refrigerated study was designed to describe the growth of Listeria, as a psychrophilic pathogen, during the shelf-life of a food product that contained meat. As shown in Figure 4 Listeria counts decreased from 3 log to undetectable levels, after 3 h (0.125 days) of exposure to the antimicrobial, indicating that an antilisterial effect was observed.
at the lower growth temperature and higher acetogenin concentration (1.3X). As described in the previous section, at 20 ± 2°C and 7,500 ppm, only a 2 log reduction was observed after 3 h (0.125 days) of exposure and a lysteriostatic effect was attained (Figure 3). Conversely, in the refrigerated study (4°C) the microbial population was undetectable during the whole storage period (72 d) (Figure 4); whereas the positive control counts were 8.90 ± 0.01 log at 20 days and maximum counts (9.49 ± 0.01 log) were reached at 40 days of storage. A slight, but non-significant, decrease in counts was observed towards the end of storage period. In a previous study using two storage temperatures, the sensitivity of *L. monocytogenes* to the bacteriocin produced by *Lactobacillus bavaricus* was described. Authors observed that the bacteriocin inhibition was more intense at 5°C than at 19°C, suggesting that a multiple-hurdle system is the best approach to inhibit the growth of the pathogen (Winkowski, Crandall, & Montville, 1993). Although the complete inhibition induced by acetogenins (10,000 ppm) in a food matrix was a relevant observation, further work is needed for the determinations of MIC and MBC values in real food matrices.

An additional observation of the present work was the increase in concentrations required to cause the complete inhibition of *L. monocytogenes* in bacterial growth media versus the food system containing meat. At refrigeration temperatures (4 ± 1°C), acetogenins produced a listericidal effect at 7.8–15.6 ppm when evaluated in BHI liquid medium (Table 2), whereas 10,000 ppm were required to observe a similar listericidal effect when they were incorporated into a model food system (Figure 4). Other authors have also reported that the efficacy of antimicrobials evaluated in growth media is greater than when compounds are incorporated into food matrices (Glass & Johnson, 2004). For example, Soni, Nannapaneni, Schilling, and Jackson (2010) observed that LAE (Mirenat’s active ingredient) when added at 200 ppm completely reduced 4 log of *L. monocytogenes* after 30 min in growth media, and counts remained undetectable during 15 days at 4°C after treatment. However, a complete growth inhibition was not observed in skim and whole milks at the same concentration, although populations after 24 h were lower than the untreated samples, 1.6 and 1.3 log10 CFU mL−1, respectively (Soni et al., 2010). Possible explanations, for the differences in the antilisterial activities observed in laboratory growth media when compared to food matrices have been attributed to the higher nutrient-density and water activity of liquid broths, providing better conditions for the recovery of damaged cells exposed to the antimicrobial agent (Pattanayaiying, H-Kittikun, & Cutter, 2014). As well as the presence of other substances in the matrix that may neutralize antimicrobial activity, including fat, protein and oxidants (Soni et al., 2010). In the case of LAE, it has been proposed that it may partition into the lipid phase of 2% reduced fat milk, which can result in less LAE molecules available in the matrix for direct contact with *L. monocytogenes* (Ma, Davidson, & Zhong, 2013). Since LAE and acetogenins from Avosafe® are fatty acids derivatives (synthetic and natural occurring, respectively), a similar interaction with fats within food systems can be hypothesized.

As result of the frequency and magnitude of food recalls, as well as the severity of infections caused by *L. monocytogenes*, the USDA Food Safety and Inspection Service (USDA/FSIS) established the so-called Listeria first rule for RTE meat and poultry manufacturers to better control *L. monocytogenes* in their products (FSIS, 2014). This rule states that antimicrobial agents used in formulation should not allow more than 2 log of growth over the shelf-life of the product (FSIS, 2014). At low temperatures (<30°C), *L. monocytogenes* expresses flagella and displays swimming motility as a mechanism to access nutrients and cause infection (Gray, Freitag, & Boor, 2006). Therefore, the study of antimicrobial properties at low temperatures is relevant, as shown in Figure 4, acetogenin molecules contained in Avosafe® were able to inhibit the growth completely. Therefore, Avosafe® shows potential as an alternative to comply with the regulatory requirement; however, further studies are needed to evaluate acetogenins in RTE meat products, and in the presence of other *L. monocytogenes* strains.

The present work generated new information on the antilisterial properties of avocado acetogenins (contained in the Avosafe® extract) within a food system containing meat; nevertheless, further research on their safety and sensory properties is needed before it can be used as a commercial food additive. To date, the safety of acetogenin molecules shown in Figure 1 and contained in Avosafe®, has not been evaluated. To the best of our knowledge, only four studies with pure acetogenin molecules and with experimental animal models (in vivo) have been conducted (Butt et al., 2006; Kawagishi et al., 2001; Oelrichs et al., 1995; Rodriguez-Sanchez et al., 2015). As previously discussed by Salinas-Salazar et al. (2016), these few in vivo tests showed contradictory results since they reported both, toxic effects and health-promoting bioactivities at similar doses (ranging from 60 to 100 and 25–100 mg Kg−1 of body weight (bw), respectively). Particularly, negative effects, such as necrosis of secretory mammary gland and affection of the myocardial tissue (at 60–100 mg kg−1 bw) have been associated to persin (7) (Butt et al., 2006; Oelrichs et al., 1995). While health-promoting properties have also been reported.
in vivo, without negative side effects, including protection against D-galactosamine-induced liver injury reported for persin (7), at 100 mg kg$^{-1}$ bw (Kawagishi et al., 2001); and cardioprotective effects by increasing blood clotting times and reducing thrombus formation, reported for persenone A (6), at 25 mg kg$^{-1}$ bw (Rodriguez-Sanchez et al., 2015). Interestingly, persin (7) has also been reported to be consistently present in the pulp and seed of over 20 avocado cultivars, at concentration ranges of 118–1416 and 213–768 ppm (mg kg$^{-1}$) fresh weight, respectively (Rodriguez-López et al., 2015; Salinas-Salazar et al., 2016). Furthermore, persin (7) concentrations in the food system that contained Avosafe$^\text{®}$ shown in Figure 4 were within the range of those previously reported for avocado pulp; since the food system contained 10,000 ppm of Avosafe$^\text{®}$ of which 1,108 ppm was quantified to be persin (7) (based on Figure 1).

As previously stated further scientific knowledge is required on the safety of avocado acetogenins. To date, the only information available is that humans have already been exposed to the consumption of avocado acetogenins contained in Avosafe$^\text{®}$ through the ingestion of products obtained from the processing of avocado pulp. Particularly when avocado pulp has been exposed to treatments that disrupt its cells, such as avocado oil extraction or microwave treatments (Brown, 1972; Guzmán-Gerónimo & Dorantes, 2008), however bioavailability and safety of acetogenins to humans remains unknown.

Although sensory properties of Avosafe$^\text{®}$ were not evaluated in the present work, previous authors have studied the sensory properties of acetogenins and their de-acetylated natural occurring analogues, which were isolated and purified from avocado seed, pulp and pulp’s oil (Brown, 1972; Degenhardt & Hofmann, 2010; US 2010/0034944 A1, 2010; WO 2011/020908, 2010). Some of the evidence have linked both families of molecules to the perception of bitter taste (Brown, 1972; Degenhardt & Hofmann, 2010; US 2010/0034944 A1, 2010; WO 2011/020908, 2010); and some acetogenins (including AcO-avocadene (2), persenone A (6), and persin (8)) have also been found to induce umami taste (Degenhardt & Hofmann, 2010) and a strong mouthfulness enhancing perception, known as kokumi flavor (Degenhardt & Hofmann, 2010). Further research needs to be done in order to characterize the sensory properties of Avosafe$^\text{®}$ within different food matrices, and at the potential usage levels as an antimicrobial food additive.

4. Conclusions

Eight acetogenins were identified and quantified in Avosafe$^\text{®}$; the major components were AcO-avocadene and persenone A (22.05 ± 1.84 and 20.14 ± 1.40% w/w, respectively), and the extract contained a total acetogenin concentration of 71.3% w/w. Antimicrobial screening was conducted against 13 microorganisms. At evaluated concentrations (15 μg disc$^{-1}$), Avosafe$^\text{®}$ inhibited Gram-positive bacteria and showed higher efficacy towards spore-forming bacteria (C. sporogenes, C. perfringens, Bacillus subtilis, and Allicyclobacillus acidocaldarius). Inhibition properties against Gram-positive bacteria were also contrasted for Avosafe$^\text{®}$, Nisaplin$^\text{®}$ and Mirenat$^\text{®}$ at the same concentration (15 μg additive disc$^{-1}$). Avosafe$^\text{®}$ showed larger inhibition zones (2–4 times) than Nisaplin$^\text{®}$ and Mirenat$^\text{®}$ for all bacteria tested, with the exception of S. aureus for which Nisaplin$^\text{®}$’s inhibition was significantly higher.

During refrigerated storage (4°C for 72 days), Avosafe$^\text{®}$ showed a strong inhibition of L. monocytogenes by decreasing initial concentration (3 log) to undetectable levels within 3 h after inoculation, and maintaining undetectable counts until the end of the experimental period. To the best of our knowledge, this is the first work that demonstrated control of Listeria monocytogenes by avocado acetogenins in a refrigerated food matrix, strengthening their potential use as a natural antimicrobial food additive. However, further efficacy evaluations are required within RTE products inoculated with multiple L. monocytogenes strains, as well as the assessment of the safety, sensory, and quality effects of acetogenins when incorporated into different food products.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Funding was provided by Tecnologico de Monterrey, Micronutrients [CAT-196] and Emerging Technologies [GIEE EICIMO] Research Chair Funds, and by the Mexican National Science and Technology Council (CONACYT) [Scholarship to R. Villarreal-Lara no. 359813 and FINNOVA-CONACYT Project no. 209050].

ORCID

Adriana Pacheco © http://orcid.org/0000-0002-9512-7674

References

Ababouchl, L., Chaila, A., & Busta, F. F. (1992). Inhibition of bacterial spore growth by fatty acids and their sodium salts. Journal of Food Protection, 55(December), 980–984.
Ahn, Y. S., Shin, D. H., Baek, N. I., Seong, R. S., & Woo, G. J. (2001). Isolation and identification of antimicrobial active substance from Mallotus japonicus Muell on Listeria monocytogenes. Korean Journal of Food Science and Technology, 33(2), 271–277.
Avis, T. J., & Bélanger, R. R. (2001). Specificity and mode of action of the antifungal fatty acid cis-9-heptadecenoic acid produced by Pseudomyza floculosa. Applied and Environmental Microbiology, 67(2), 956–960.
Becerril, R., Manso, S., Nerin, C., & Gómez-Lus, R. (2013). Antimicrobial activity of lauroyl arginate ethyle (LAE), against selected food-borne bacteria. Food Control, 32(2), 404–408.
Beyazova, M., MI, D., Gravina, S., Kenneth, K., Beach, U., Trinnaman, L., & Island, S. (2010). US 2010/0034944 A1. US. US 2010/031130A1
Bhuamik, M., Choi, H. J., Seopella, M. P., McCrindle, R. I., & Maity, A. (2014). Highly effective removal of toxic Cr(VI) from wastewater using sulfuric acid-modified avocado seed. Industrial and Engineering Chemistry Research, 53(3), 1214–1224.
Brown, B. I. (1972). Unpleasant flavor compounds. Journal of Agricultural and Food Chemistry, 20(4), 753–757.
Butt, A. J., Roberts, C. G., Seawright, A. A., Oelrichs, P. B., Macleod, J. K., Llai, T. Y. E., … Sutherland, R. L. (2006). A novel plant toxin, persin, with in vivo activity in the mammary gland, induces Bim-dependent apoptosis in human breast cancer cells. Molecular Cancer Therapeutics, 5(9), 2300–2309.
Ciarcigliani, G., Hill, P. J., Davies, K., McClure, P. J., Kilsby, D., Brown, M. H., & Coote, P. J. (2000). Germination-induced bioluminescence, a route to determine the inhibitory effect of a combination preservation...
treatment on bacterial spores. Applied and Environmental Microbiology, 66(9), 3735–3742.

CLSI (Clinical and Laboratory Standards Institute). (2013). Performance Standards for Antimicrobial Susceptibility Testing. CLSI Approved Standard M100-S23.

Davies, E. A., Bevis, H. E., & Delves-Broughton, J. (1997). The use of the bacteriocin, nisin, as a preservative in ricotta-type cheeses to control the food-borne pathogen Listeria monocytogenes. Letters in Applied Microbiology, 24(5), 343–346.

Degenhardt, A. G., & Hofmann, T. (2010). Bitter-tasting and kokumi-enhancing molecules in thermally processed avocado (Persea americana Mill.). Journal of Agricultural and Food Chemistry, 58(24), 12906–12915.

Desbois, A. P., & Smith, V. J. (2010). Antibacterial free fatty acids: Activities, mechanisms of action and biotechnological potential. Applied Microbiology and Biotechnology, 85(6), 1629–1642. 

doi:10.1007/s00253-009-2355-7.

Dharmaratne, H., Tekwani, B., Jacob, M., & Nanayakkara, N. (2012). Anti microbial and antielieishmanial active aceticinogen from Avocado (Persea americana) fruits. Panta Med, 78, 34.

Domergue, F., Helms, G. L., Prusky, D., & Browse, J. (2000). Antifungal compounds from idlioloblast cells isolated from avocado fruits. Phytochemistry, 54(2), 183–189. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC872209.

Erickson, M. C., & Doyle, M. P. (2007). Mechanism of action of the peptide antibiotic nisin in liposomes and cytochrome c oxidase-containing proteoliposomes. Applied and Environmental Microbiology, 75(8), 2164–2170.

Gayniszky, S., Taylor, T. M., Davidson, P. M., Bruce, B. D., & Weiss, J. (2007). Antimicrobial efficacy of Eugenol microemulsions in milk against Listeria monocytogenes and Escherichia coli O157:H7. Journal of Food Protection, 70(11), 2631–2637.

Glass, K. A., & Johnson, E. A. (2004). Antagonistic effect of fat on the antibotulinal activity of food preservatives and fatty acids. Food Microbiology, 21, 675–682.

Gray, M. J., Freitag, N. E., & Boor, K. J. (2006). How the bacterial pathogen listeria monocytogenes mediates the switch from environmental Dr. Jekyll to Pathogenic Mr. Hyde. Infection and Immunity, 74(5), 2505–2512.

Griffiths, K. K., Zhang, J., Cowan, A. E., Yu, J., & Setlow, P. (2011). Germination proteins in the inner membrane of dormant Bacillus subtilis spores colocalize in a discrete cluster. Current Biology, 21, 1061–1077.

Guzmán-Gerónimo, R. I., & Darantes, L. (2008). Changes in the perfil of acidos grasos microestructura de aguacate hass tratado con microondas. Archivos latinoamericanos de nutricion, 58(3), 298–302.

Hammer, K. A., Carson, C. F., & Riley, T. V. (1999). Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology, 86(6), 985–990.

Health Canada. (2012). Listeria monocytogenes challenge testing of refrigerated ready-to-eat foods. Retrieved from http://www.hc-sc.gc.ca/fn-an/legislation/pol/listeria_monocytogenes-test-eng.php.

Hofmann, T., & Degenhardt, A. G. (2010). GO 2011/020908.

Inoue, A., & Horikoshi, K. (1991). Estimation of solvent-tolerance of bacteria by the solvent parameter log P. Journal of Fertilization and Bioengineering, 71(3), 194–196.

Jensen, L. B. (1951). US2550254A. US2550254A. U.S. Retrieved from https://patents.google.com/patent/US2550254A/en.

Jensen, N., & Whitfield, F. B. (2003). Role of Acylcyclobacillus acidoterress in the development of a disinfectant taint in shelf-stable fruit juice. Letters in Applied Microbiology, 36(1), 9–14.

Karamac, M., Estrella, I., Herna, T., & Dykes, G. A. (2012). Phenolic compound profiles and antioxidant capacity of Persea americana Mill. Peels and seeds of two varieties. Journal of Agricultural and Food Chemistry, 60, 4613–4619.

Kawagishi, H., Fukushima, Y., Hatakeyama, M., He, P., Arimoto, H., Matsuzawa, T., … Sugiyama, K. (2001). Liver injury suppressing compounds from avocado (Persea americana). Journal of Agricultural and Food Chemistry, 49(5), 2215–2221.

Knapp, H. R., & Melly, M. A. (1986). Bactericidal effects of polyunsaturated fatty acids. The Journal of Infectious Diseases, 154(1), 84–94.

Levison, M. E. (2004). Pharmacodynamics of antimicrobial drugs. Infectious Disease Clinics of North America. 10.1016/j.idcl.2004.04.012.

Luo, Y.-C., Chang, H.-S., Peng, C.-F., Lin, C.-H., & Chen, I.-S. (2012). Secondary metabolites from the unripe pulp of Persea americana and their anticytobacterial activities. Food Chemistry, 135(4), 2904–2909.

Ma, Q., Davidson, P. M., & Zhong, Q. (2013). Antimicrobial properties of lauric arginate alone or in combination with essential oils in tryptic soy broth and 2% reduced fat milk. International Journal of Food Microbiology, 166(1), 77–84.

Ma, Q., Davidson, P. M., & Zhong, Q. (2016). Antimicrobial properties of microemulsions formulated with essential oils, soybean oil, and Tween 80. International Journal of Food Microbiology, 226, 20–25.

Mah, J.-H., Kang, D.-H., & Tang, J. (2008). Effects of minerals on sporulation and heat resistance of Clostridium sporogenes. Elsevier, 128, 385–389. Retrieved from https://www.sciencedirect.com/science/article/pii/S016816050800537X.

Neeman, I., Lifshitz, A., & Kadochman, Y. (1970). New antibacterial agent isolated from the avocado pear. Applied Microbiology, 19, 3. Retrieved from http://aem.asm.org/content/19/3/470.short.

Oelrichs, P. B., Ng, J. C., Seawright, A. A., Ward, A., Schäffler, L., MacLeod, J. K., … MacLeod, J. K. (1995). Isolation and identification of a compound from avocado (Persea americana) leaves which causes necrosis of the acinar epithelium of the lactating mammary gland and the myocardin. Natural Toxins, 3(5), 344–349.

Pacheco, A., Rodríguez-Sánchez, D. G., Villarreal-Lara, R., Navarro-Silva, J. M., Senés-Guerrero, C., & Hernández-Brenes, C. (2017). Stability of the antimicrobial activity of aceticinogens from avocado seed, under common food processing conditions, against Clostridium sporogenes vegetative cell growth and endospore germination. International Journal of Food Science and Technology, 52(11), 2311–2323.

Pattanayaying, R., H-Kittikun, A., & Cutter, C. N. (2014). Effect of lauric arginate, nisin Z, and a combination against several food-related bacteria. International Journal of Food Microbiology, 188, 135–146.

Prusky, D., Keen, N., Sims, J., & Midland, S. (1982). Possible involvement of an antifungal diene in the latency of Colletotrichum gloeosporioides on unripe avocado fruits. Phytopathology, 1578–1582. Retrieved from http://www.apsnet.org/publications/phytopathology/backissues/Documents/1982Articles/Phyto72n12_1578.pdf.

Ramos-Jerz, M. D. R. (2007). Phytochemical analysis of Avocado seeds (Persea americana Mill, cv Hass). Göttengen: Cuviller Verlag.

Rodríguez-Carpena, J.-G., Morcuende, D., Andrade, M.-J., Kyll, P., & Estévez, M. (2011). Avocado (Persea americana Mill.) phenolics, in vitro antioxidant and antimicrobial activities, and inhibition of lipid and protein oxidation in porcine patties. Journal of Agricultural and Food Chemistry, 59(10), 5625–5635.

Rodríguez-López, C. E., Hernández-Brenes, C., & de la Garza, R. I. D. (2015). A targeted metabolomics approach to characterize aceto genin profiles in avocado fruit (Persea americana Mill.). RSC Advances, 5(128), 106019–106029.

Rodriguez-Sánchez, D. G., Flores-Gómez, M., Silva-Platas, C., Rizzo, S., Torre-Amione, G., De la Peña-Díaz, A., Garcia-Rivas, G. (2015). Isolation and chemical identification of lipid derivatives from avocado (Persea americana) pulp with antiplatelet and antithrombotic activities. Food & Function, 6(1), 193–203.

Rodríguez-Sánchez, D. G., Pacheco, A., García-Cruz, M. I., Gutiérrez-Uribe, J. A., Benavides-Lozano, J. A., & Hernández-Brenes, C. (2013). Isolation and structure elucidation of avocado seed (Persea americana) lipid derivatives that inhibit Clostridium sporogenes endospore germination. Journal of Agricultural and Food Chemistry, 61(30), 7403–7411.

Rodríguez-Saona, C., & Trumble, J. (2000). Biologically active alphatic aceto genins from specialized idioblast oil cells. Current Organ Cover Chemistry, 41(2), 1249–1260.
Ruckman, S. A., Rocabayera, X., Borzelleca, J. F., & Sandusky, C. B. (2004). Toxicological and Metabolic Investigations of the Safety of N-o-Lauroyl-l-arginine Ethyl Ester Monohydrochloride (LAE). Food and Chemical Toxicology, 42(2), 245–259.

Salinas-Salazar, C. L., Hernández-Brenes, C., Rodríguez-Sánchez, D., Castillo, E., Navarro-Silva, J., & Pacheco, A. (2016). Inhibitory activity of Avocado seed fatty acid derivatives (Acetogenins) against Listeria monocytogenes. Journal of Food Science, 82(1), 134–144.

Scott, V. N., Swanson, T. A., Freier, T. A., Payton, P. J., Sveum, W. H., Hall, P. A., … Brown, D. G. (2005). Guidelines for conducting Listeria monocytogenes challenge testing of foods. Food Protection Trends, 25(11), 818–825. Retrieved from: http://agris.fao.org/agris-search/search.do?recordID=US201301047377

Secretaría de Economía. (2012). Monografía del sector aguacate en México: Situación actual y oportunidades de mercado. Dirección General de Industrias Básicas, Mexico.

Secretaría de Salud. (2005). Official Mexican standard NOM-213-SSA1-2002. Products and Services. Meat Process Products. Sanitary Specifications. Methods of Test. Issued on the Official Gazette on July 11, 2005.

Shelef, L. A., Naglick, O. A., & Bogen, D. W. (1980). Sensitivity of some common food-borne bacteria to the spices sage, rosemary, and allspice. Journal of Food Science, 45(4), 1042–1044.

Silhavy, T. J., Kahne, D., & Walker, S. (2010). The bacterial cell envelope. Cold Spring Harb Perspect Biology, 2(5), 1–16.

Soni, K., Nannapaneni, R., Schilling, M. W., & Jackson, V. (2010). Bactericidal activity of lauric arginate in milk and Queso Fresco cheese against Listeria monocytogenes cold growth. Journal of Dairy Science, 93(10), 4518–4525.

Umerska, A., Cassisa, V., Matougui, N., Joly-Guillou, M. L., Eveillard, M., & Saulnier, P. (2016). Antibacterial action of lipid nanocapsules containing fatty acids or monoglycerides as co-surfactants. European Journal of Pharmaceutics and Biopharmaceutics, 108, 100–110.

Valerí, A., & Gimeno, N. (1954). Estudio fitoquímico toxicológico del pericarpio del aguacate (Persea americana). Revista De Medicina Veterinaria Y Parasitología (Maracay), 37, 37–58.

van Melis, C. C. J., Almeida, C. B., Kort, R., Groot, M. N. N., & Abeel, T. (2012). Germination inhibition of Bacillus cereus spores: Impact of the lipophilic character of inhibiting compounds. International Journal of Food Microbiology, 160(2), 124–130.

Weatherby, L. S., & Sorber, G. (1931). Composition of Avocado seed. Industrial and Engineering Chemistry, 23(12), 1421–1423.

Wells-Bennik, M. H. J., Eijlander, R. T., Den Besten, H. M. W., Berendsen, E. M., Warda, A. K., Krawczyk, A. O., … Abeel, T. (2016). Bacterial spores in food: Survival, emergence, and outgrowth. Annual Review of Food Science and Technology, 7(1), 457–482.

Winkowski, K., Crandall, A. D., & Montville, T. J. (1993). Inhibition of Listeria monocytogenes by Lactobacillus bavaricus MN in beef systems at refrigeration temperatures. Applied and Environmental Microbiology, 59(8), 2552–2557.

Wong, T. L., Carey-Smith, G. V., Hollis, L., & Hudson, J. A. (2005). Microbiological survey of prepackaged pate and ham in New Zealand. Letters in Applied Microbiology, 41(2), 106–111.