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Changes in bacterial diversity of refrigerated mango pulp before and after treatment by high hydrostatic pressure

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ABSTRACT

The purpose of the present study was to determine the effect of high hydrostatic pressure treatment (HHP) on the microbiota of mango pulp. Mango pulp was artificially contaminated with its own epiphytic microbiota to simulate a worst-case scenario of contamination during pulp preparation. Controls and samples treated by HHP (600 MPa, 8 min) were chill stored for 30 days. HHP treatment significantly ($P < 0.05$) reduced viable cell counts. Pyrosequencing analysis of the bacterial community revealed that the relative abundances found in the starting control samples for *Actinobacteria* (45.63%), *Firmicutes* (42.55%), *Proteobacteria* (10.68%) and *Bacteroidetes* (1.0%) changed during storage, with a strong increase of *Proteobacteria*. HHP treatment also induced a strong increase in *Proteobacteria*, followed by a late recovery of *Firmicutes* and to a less extent *Actinobacteria*. *Lactobacillus* was the main operational taxonomic unit (OTU) detected both in controls during early storage and in HHP-treated samples during late storage. Results from the present study indicate how bacterial populations of both controls and HHP-treated mango pulp samples undergo complex changes during refrigeration storage.

1. Introduction

The mango (*Mangifera indica* L. Anacardiaceae) is one of the most commercialised fruits in tropical countries (Muchiri, Mahungu, & Gituanja, 2012). Mango puree, slices in syrup, nectar, leather, pickles, canned slices and chutney are the main industrial products obtained from mango fruits (Jahurul et al., 2015). Mango fruit is an excellent source of antioxidants and vitamins (Abbasi et al., 2015; USDA, 2010). Mango polyphenolics including gallic acid and gallotannins have been shown to elicit cytotoxic and anti-inflammatory properties (Banerjee, Kim, Krenek, Talcott, & Mertens-Talcott, 2015; Kim et al., 2016).

The marketability of fresh mango is limited by its short shelf-life. Mango is usually processed and preserved by thermal pasteurization, which often leads to deterioration of organoleptic attributes and loss of nutritional quality (Ahmed, Shivhare, & Kaur, 2002; Miller & Silva, 2012; Ndiaye, Xu, & Wang, 2009). High hydrostatic pressure treatments (HHP) do not affect covalent bonds, and have been reported to only cause minor loss of low molecular weight compounds in vegetables, such as pigments, vitamins, and flavor substances (Butz et al., 2002). HHP processing of mango has been reported to result in moderate to non-significant losses in ascorbic acid content (Kaushik, Kaur, Rao, & Mishra, 2014; Liu, Li, Wang, Bi, & Liao, 2014), while its measurable carotenoid content was not affected or even increased (Liu, Wang, Li, Bi, & Liao, 2013; Liu et al., 2014). HHP treatment inactivates *Escherichia coli* and *Aspergillus niger* in mango nectar (Bermúdez-Aguirre, Guerrero-Beltrán, Barbosa-Cánovas, & Welti-Chanes, 2011; Tribst, Franchi, Cristianini, & de Massaguer, 2009) and prolongs the product shelf life (Jacobo-Velázquez, Ramos-Parra, & Hernández-Brenes, 2010). HHP treatment at 600 MPa has been reported to achieve maximum reduction in microflora and moderate changes in quality attributes in fresh mango pulp (Kaushik et al., 2014).

Most studies on the effects of HHP processing on the food microbiota have been based on culture-dependent methods. Recently, high-throughput sequencing (HTS) technology has been applied to identify changes in microbial populations during the shelf-life of HHP-processed foods stored under different conditions (Pérez Pulido, Toledo, Grande, Gálvez, & Lucas, 2015; Toledo del Árbol et al., 2016). Moreover, HTS can also provide insights into the microbiota of raw materials and sources of contamination (Cocolin & Ercolini, 2015; Ercolini, 2013), and to study the bacterial communities associated with the surfaces of fruits and vegetables

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(Leff & Fierer, 2013; Lopez-Velasco, Carder, Welbaum, & Ponder, 2013). However, there are no previous studies on the bacterial diversity of mango pulp processed by HHP.

The purpose of the present study was to determine the effects of HHP treatments on the bacterial diversity of mango pulp stored under refrigeration. Mango pulp was inoculated with its own epiphytic microbiota in order to simulate a worst-case surface contamination scenario during processing. In order to determine the effects of HHP treatments on the microbiota of mango pulp, high-throughput sequencing of the 16S rRNA gene was carried out after treatment and during sample storage.

2. Materials and methods

2.1. Sample preparation

Mangoes (*Mangifera indica* L. var Kent) were purchased from four different local food stores and kept under refrigeration until processing (for no more than 24 h). A microbial suspension from mango surfaces was obtained by washing 10 representative mango units (4230 g total weight) with sterile saline solution. The obtained suspension was washed with sterile saline solution, resuspended in 5 ml saline and stored under refrigeration for no longer than 18 h before it was inoculated into freshly-made mango pulp. For preparation of mango pulp, mangoes were peeled and sliced under aseptic conditions with a sterile knife. Slices were processed with a Braun Vario 350W blender (Braun GmbH, Germany) to obtain the final mango pulp.

Mango pulp (250 g) was inoculated (2%, vol/vol) with the suspension of epiphytic microbiota obtained as described above to yield a final concentration of 4.4 $\log_{10}$ CFU/g total aerobic mesophilic counts and distributed in 10-ml aliquots in sterile zip-lock bags. Then, samples were placed individually inside polyethylene-polyamide bags and sealed under vacuum. Ten bags were used as controls and ten treated by HHP as will be described below.

2.2. High hydrostatic pressure treatments

High hydrostatic pressure (HHP) treatments were carried out by using a Stansted Fluid Power LTD HHP equipment (SFP, Essex, UK) suited with a 2.5 l vessel capable of operating in a pressure range of 0–700 MPa. Mango pulp samples were pressurized at 600 MPa for 8 min. Come-up speed was 75 MPa/min. Decompression was almost immediate. Pressurization fluid was water with added 10% propylenglycol. The temperature inside the vessel during treatments ranged between 23 and 27 °C. All samples (treated or not by HHP) were stored at 4 °C for up to 30 days.

After treatments and also at desired times during storage (1, 7, 15 and 30 days), two bags from controls and two from HHP-treated samples were removed. Each bag was mixed with sterile saline solution (10 ml per bag) and pummeled in stomacher bags for 2 min. The obtained pulp suspension was serially diluted in sterile saline solution and plated in triplicate on tryptic soya agar (TSA, Scharlab, Madrid) for total aerobic mesophilic counts and on Yeast Mannitol Agar (Scharlab) with added Chloramphenicol (Sigma Aldrich, Madrid) at 100 mg/l (YMA-CM) for yeasts and molds. Plates were incubated at 30 °C for 24 h (TSA) or 48 h (YMA-CM). The pH of pulp suspensions was measured with a pH meter (Crison Instruments, S.A., Barcelona, Spain).

2.3. DNA extraction, amplicon library preparation and sequencing

Aliquots (1.5 ml) of homogenized mango samples obtained as described above were centrifuged at 600×*g* for 5 min in an Eppendorf centrifuge in order to remove pulp solids. The supernatants were transferred to new Eppendorf test tubes and centrifuged at 13,500×*g* for 5 min to recover microbial cells. The pellets obtained from each sample were resuspended in 0.5 ml sterile saline solution each. Then, Propidium Monoazide (PMA™, Biotium, UK) was added to block subsequent PCR amplification of the genetic material from dead cells (Nocker, Cheung, & Camper, 2006; Nocker, Sossa-Fernandez, Burr, & Camper, 2007) as described by Elizaquivel, Sánchez, and Aznar (2012) and Toledo del Árbol et al. (2016). DNA from PMA-treated cells was extracted by using a GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Madrid), following instructions provided by the manufacturer. DNA recovered from duplicate samples was then pooled into a single sample. DNA concentration and quality were measured with a NanoDrop spectrophotometer (Thermo Scientific, United Kingdom).

For pyrosequencing, V3–V5 region of the 16S rRNA gene was amplified using key-tagged bacterial primers prepared by Life-sequencing S.L. (Valencia, Spain) based on Sim et al. (2012). PCR reactions were performed with 20 ng of community DNA, 200 μM of each of the four deoxynucleoside triphosphates, 400 nM of each primer, 2.5 U of FastStart HiFi Polymerase, and the appropriate buffer with $MgCl_2$ supplied by the manufacturer (Roche, Mannheim, Germany), 4% of 20 mg/ml BSA (Sigma, Dorset, United Kingdom), and 0.5 M Betaine (Sigma). Thermal cycling consisted of initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 20 s, annealing at 50 °C for 30 s, and extension at 72 °C for 5 min. To obtain sufficient material, PCR reactions were repeated in triplicate and pooled prior to purification by running the PCR amplicons on 1% (w/v) agarose gels. Amplicons were quantified using the PicoGreen assay (Quant-iT, PicoGreen DNA assay, Invitrogen) and combined in a single tube in equimolar concentrations. The pooled amplicon mixture was purified twice (AMPure XP kit, Agencourt, Takeley, United Kingdom) and the cleaned pool requantified with PicoGreen assay. Amplicons were submitted to the pyrosequencing services offered by Life Sequencing S.L. (Valencia, Spain) where EmPCR was performed and subsequently, unidirectional pyrosequencing was carried out on a 454 Life Sciences GS FLX+ instrument (Roche) following the Roche Amplicon Lib-L protocol.

2.4. Bioinformatic analysis

Bioinformatic analysis was carried out by Life Sequencing S.L. services. Raw reads were first filtered according to the 454 processing pipeline. Pyrosequencing reads were filtered with Q20 FASTX_tool_kit version 0.0.14, and reads were excluded from the analysis if they had an average quality score < Q20 and if there were ambiguous base calls (Ns). Reads were trimmed for adaptors and PCR primers, and only reads greater than 300 nts were retained for analysis. Chimera were eliminated using the Uchime algorithm under default mode (UCHIME version 4.2.40). The average final lengths of reads ranged from 559 to 588 nt. Sequences were compared with NCBI 16 S rRNA database using BLASTN. Operational taxonomic units (OTUs) were defined by a 97% similarity. The read clusters were further assigned to taxonomies using the RDP classifier.

2.5. Statistical analysis

All experiments were carried out in duplicate. The average data ± standard deviations from viable cell counts were determined with Excel programme (Microsoft Corp., USA). A *t*-test was performed at the 95% confidence interval with Statgraphics Plus version 5.1 (Statistical Graphics Corp, USA), in order to determine the statistical significance of viable cell count data. Data on the