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Biofilms formed by microbiota recovered from fresh produce: Bacterial biodiversity, and inactivation by benzalkonium chloride and enterocin AS-48

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ABSTRACT

Microbiota recovered from fresh produce (Romaine lettuce, endives and cucumbers) was allowed to form biofilms on stainless steel coupons. The formed biofilms were treated with benzalkonium chloride (BC) at three different concentrations (0.01, 0.1, and 1.0 g/l), enterocin AS-48 (50 μg/ml) and combinations of BC and enterocin AS-48. The single treatment with bacteriocin had no effect on viability of sessile bacteria. A high concentration of BC (1.0 g/l) was required to achieve 4.1 logs reduction of viable cell counts. The combination of BC (1.0 g/l) and enterocin AS-48 reduced viable cell counts below detectable levels. High-throughput sequencing analysis revealed that the formed biofilms were composed mainly by *Proteobacteria* of the genera *Pseudomonas* and *Stenotrophomonas*. Treatments with enterocin AS-48 and BC at sub-inhibitory concentrations only induced minor changes in the relative abundance of the different bacterial groups associated with biofilms.

1. Introduction

Biofilms are surface-associated, three-dimensional multicellular structures whose integrity depends upon the extracellular matrix produced by their constituent bacterial cells (Branda, Vik, Friedman, & Kolter, 2005; Jefferson, 2004). Biofilm formation has serious implications in industrial, environmental, public health and medical situations (Gilbert, McBain, & Rickard, 2003; Hall-Stoodley, Costerton, & Stoodley, 2004). The occurrence of biofilms in food-processing environments can cause post-processing contamination leading to lowered shelf-life of products and contamination by pathogens such as *Escherichia coli* O157:H7, *Salmonella enterica* or *Listeria monocytogenes* (Jessen & Lammert, 2003; Yaron & Römling, 2014). Sessile micro-organisms are more difficult to mechanically remove from food-contact surfaces and are also more resistant to disinfectants compared with planktonic forms (Gilbert, Das, Jones, & Allison, 2001; Morton, Greenway, Gaylarde, & Surman, 1998; Van Houdt & Michiels, 2010). In the case of vegetable foods, there is little information concerning biofilm formation in food processing facilities or on food contact surfaces.

Among the different approaches proposed to inactivate sessile bacteria is the use of bacteriocins in combination with biocides. Enterocin AS-48 is one of the best studied bacteriocins. It is a cyclic antimicrobial peptide with broad spectrum of antibacterial activity against Gram positive bacteria. The molecular structure, mode of action and genetic determinants of this bacteriocin have been deciphered, and its effects on planktonic bacterial cells have been studied in different food systems (Abriouel, Lucas, Ben Omar, Valdivia, & Gálvez, 2010; Grande Burgos, Pérez Pulido, López Aguayo, Gálvez, & Lucas, 2014; Maqueda et al., 2004). Previous studies showed that enterocin AS-48 could improve the efficacy of biocides against planktonic and sessile *Listeria monocytogenes* (Caballero Gómez, Abriouel, Grande, Pérez Pulido, & Gálvez, 2012), *Bacillus cereus* (Caballero Gómez, Grande, Pérez Pulido, Abriouel, & Gálvez, 2013a), *Staphylococcus aureus* (Caballero Gómez, Abriouel, Grande, Pérez Pulido, & Gálvez, 2013b) and *Salmonella enterica* (Grande Burgos, Pérez Pulido, López Aguayo, Gálvez, & Lucas, 2012).

Biocides derived from quaternary ammonium compounds are widely used. Among them, benzalkonium chloride is a disinfectant and cationic surface active agent used for sanitation in food processing lines and surfaces in the food industry (Krysinski, Brown, & Marchisello, 1992; Kuda, Yano, & Kuda, 2008; Ueda & Kuwabara, 2007). The purpose of the present study was to determine biofilm

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formation by microbiota recovered from fresh produce, and to determine the efficacy of enterocin AS-48, benzalkonium chloride and combinations of both antimicrobials on inactivation of biofilm bacteria. Since there is also little information on the composition of bacterial biofilms from vegetable foods, the study also aimed at determining the bacterial diversity in the formed biofilms by using high-throughput sequencing technology.

2. Materials and methods

2.1. Preparation of bacterial suspensions

Romaine lettuce, endives and cucumbers were purchased at a local supermarket. All were fresh vegetables with no signs of spoilage. The outermost leaves of lettuce were removed and discarded. For each vegetable type, three pieces were cut in small pieces (approx. 3 cm long) with a sterile knife and mixed. The cut vegetables (200 g lettuce; 210 g endives; 183 g cucumber) were placed separately inside sterile stomacher bags and mixed each one with 200 ml sterile buffered peptone water (BPW, Panreac, Barcelona, Spain). Mixing was done by hand-rubbing for 5 min at ambient temperature. Then, the resulting BPW suspensions were removed from the stomacher bags and centrifuged (3.500×*g* for 30 min at 4 °C). The resulting sediments containing the microbial cells recovered from each vegetable food were resuspended in 10 ml BPW each, mixed together in one 50 ml test tube, and washed by centrifugation (3.500×*g* for 30 min, 4 °C) with 50 ml BPW. The resulting sediment was resuspended in 10 ml BPW and stored at 4 °C (for not more than 2 h) until use as inoculum for biofilm formation.

2.2. Antimicrobials

Enterocin AS-48 was obtained from cultured broths of the producer strain *Enterococcus faecalis* A-48-32 after concentration by cation exchange chromatography as described elsewhere (Abriouel, Valdivia, Martínez-Bueno, Maqueda, & Gálvez, 2003). Bacteriocin concentrates were filtered through 0.22 μm pore size low protein binding filters (Millex GV; Millipore Corp., Belford, MA, USA) under sterile conditions. Bacteriocin concentrates were diluted 20-fold in sterile saline solution (SS) or in biocide solutions in order to achieve the desired final bacteriocin concentration of 50 μg/ml. Benzalkonium chloride (BC) commercial solution (Sigma-Aldrich, Madrid, Spain) contained 50% (wt/v) of the active compound.

2.3. Biofilm formation and antimicrobial treatments

Stainless steel coupons (1.5 by 4.0 cm, type 304 with a no. 4 finish) were sonicated in distilled water for 2 min, immersed in 70% ethanol for 10 min, rinsed with sterile distilled water and then dried in a biosafety cabinet for 4 h and sterilized by autoclaving. Coupons were placed individually inside 50 ml sterile Falcon test tubes with conical bottom containing 20 ml BPW inoculated (1%, vol/vol) with the bacterial cell suspension obtained from vegetable foods as described above. Two replicates consisting of 25 tubes each were prepared. After 48 h incubation at 30 °C, the liquid was removed from test tubes, and the formed biofilms were washed twice with 20 ml BPW. Washing was carried out gently by immersion in order to avoid disturbing the formed biofilms.

Following the washing steps, 20 ml of SS (controls) or antimicrobial solutions were added to each of the 50 ml Falcon tubes containing coupons. The following antimicrobial solutions were added: enterocin AS-48 at 50 μg/ml in SS, BC at final concentrations of 0.01, 0.1, and 1.0 g/l in SS, or combinations of the above-mentioned BC solutions plus 50 μg/ml enterocin AS-48. Coupons were left immersed in the antimicrobial solutions for one hour at 22 °C. After treatments, the biocidal solutions were removed and the coupons were washed twice with 25 ml of D/E Neutralizing broth (Difco, Barcelona) followed by 25 ml BPW. Coupons were transferred to new Falcon test tubes containing 15 ml BPW. In order to resuspend the microbial cells attached to the biofilms, the coupons were rubbed on both sides with sterile cotton swabs followed by vortexing for 30 s. For each treatment replicate, the bacterial suspensions obtained from triplicate coupons were pooled together, centrifuged (3.500 × *g*, 30 min) and resuspended in a final volume of 5 ml SS. The resulting bacterial suspensions were serially diluted in SS and plated in triplicate on Trypticase soy agar (TSA, Scharlab, Barcelona). Viable cell counts obtained after 24 h incubation at 37 °C were used to calculate the average numbers of viable cells per ml.

2.4. DNA extraction, amplicon library preparation and sequencing

Aliquots (1.5 ml) of bacterial suspensions recovered from biofilms as described above were transferred to 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 SS each. Then, Propidium Monoazide (PMA™, Biotium, UK) was added to block subsequent PCR amplification of the genetic material from dead cells as described by Elizaquivel, Sánchez, and Aznar (2012). DNA from PMA-treated cells was extracted by using a GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Madrid), following instructions provided by the manufacturer. Briefly, cell pellets were treated with lysozyme solution (40 mg/ml) for 30 min at 37 °C and then incubated with the extraction kit lysis solution. DNA from the lysate was purified on the extraction kit purification columns and finally eluted with 100 μl of the Tris-EDTA buffer. 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). Polymerase chain reactions (PCR) 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 bovine serum albumin (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 emulsion-based clonal amplification (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.