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Characterization of the heterotrophic bacteria from a minimally processed vegetables plant

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ABSTRACT

The knowledge on the microorganisms present in an industrial process is crucial to delineate the best strategy for their effective control. The aims of the present work were to isolate, identify and characterize (in terms of production of proteases, gelatinases and siderophores, quorum-sensing inhibition and biofilm formation) the resident heterotrophic bacteria present in a minimally processed vegetables (MPV) plant where sodium hypochlorite was used for decontamination. A total of 47 isolates were obtained with 49% belonging to the *Pseudomonas* genera. Twenty different bacterial species were identified and the conveyor belt in the high care area was found to be a significant source of contamination. Most of the isolates were capable of producing virulence related molecules and all isolates were able to form biofilm. *Pseudomonas* was the genera with the highest biofilm formation ability, being the predominant microflora along the process chain. Even if no relevant foodborne pathogen was isolated, the results clearly propose that improvements in decontamination during processing are required to effectively control microbial presence in the final product.

1. Introduction

Minimally processed vegetables (MPV) are defined as fresh produce that are pre-washed and cut, to a minimal level, and packaged (Ragaert, Verbeke, Devlieghere, & Debevere, 2004). The consumption of MPV has an increasing trend as they are a natural source of nutrients, are easy to use and have already been washed; thus reducing water consumption by the consumer (Seiber, 2012). This kind of product does not have a long shelf-life as current decontamination procedures of the produce have reduced efficiency and the cutting processes increase the availability of nutrients for the microorganisms (Siroli, Patrignani, Serrazanetti, Gardini, & Lanciotti, 2015). However, with the increasing consumption of MPV the illness outbreaks associated to human pathogens have also increased (Foong-Cunningham, Verkaar, & Swanson, 2012; Olaimat & Holley, 2012). The major microorganisms responsible for foodborne illness outbreaks in fresh produce are *Escherichia coli* O157:H7 and *Salmonella* spp. (Warriner, Huber, Namvar, Fan, & Dunfield, 2009). Nevertheless, human infections can be caused by other foodborne pathogens, e.g. *Listeria monocytogenes*, *Clostridium botulinum*, *Bacillus cereus* (Seiber, 2012; Warriner et al., 2009) and norovirus (Van Boxstael et al., 2013). Furthermore, MPV are also susceptible to colonisation by spoilage microorganisms (Olaimat & Holley, 2012). Spoilage is any modification in the food that causes a change in the texture, colour, odour and flavour, altering the organoleptic properties. These modifications can be caused by physical damages, chemical or microbiological reactions (Degl'Innocenti, Guidi, Pardossi, & Tognoni, 2005; Gram et al., 2002; Madigan, Martinko, Dunlap, & Clark, 2009). All microorganisms that are present in the food and persist after decontamination can be considered spoiling, since they were able to resist decontamination and can cause changes in the organoleptic properties of the product (Gram et al., 2002). As the disinfection and decontamination of the produce is minimal, pathogenic and spoilage microorganisms are not always eliminated with chemical products (Foong-Cunningham et al., 2012; Olaimat & Holley, 2012). In the MPV industry, decontamination of the produce and sanitization of the food-contact surfaces can be achieved by applying the adequate decontaminants/disinfectants, typically chlorine. Chlorine is effective against a wide range of microorganisms, has a low price and can be easily applied (Ramos, Miller, Brandão, Teixeira, & Silva, 2013). However, its action is not

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List of abbreviations

AHLs	*N*-acyl homoserine lactones
BPW	Buffered peptone water
C4-HSL	*N*-butanoyl homoserine lactone
C6-HSL	*N*-hexanoyl homoserine lactone
CAS	Chrome azurol S
CFU	Colony forming units
EPS	Exopolymeric substances
HSL	Homoserine lactone
LB	Luria-Bertani broth
MHB	Mueller–Hinton broth
MPV	Minimally processed vegetables
MRS	Man, Rogosa and Sharpe broth
O.D.	Optical density
$O.D._{c}$	Optical density of the cut-off value
PCA	Plate count agar
QS	Quorum-sensing
QSI	Quorum-sensing inhibition
RNA	Ribonucleic acid
TSA	Tryptic soy agar
TSB	Tryptic soy broth

suitably effective in pathogens elimination (Yaron & Romling, 2014) and it interacts strongly with organic matter originating carcinogenic and mutagenic organochlorinated by-products (Bull et al., 2011). Chlorine replacement with other antimicrobial agents has been evaluated in order to reduce the environmental impact and also to increase produce safety to the consumer (Gopal, Coventry, Wan, Roginski, & Ajlouni, 2010; Meireles et al., 2015). To develop efficient food decontamination and food-contact surfaces sanitization, it is important to identify the target/more resistant microorganisms persisting along the processing chain and their characteristics of potential concern to the quality of the produce and to the public health (Corbo, Speranza, Campaniello, D'Amato, & Sinigaglia, 2010). Consequently, this work aimed to characterize the heterotrophic bacteria contaminating a MPV processing plant. To understand the putative resilient properties the isolated bacteria were characterized in terms of biofilm formation, production of proteases, gelatinases and siderophores and also the ability to inhibit quorum-sensing.

2. Materials and methods

2.1. Bacteria isolation and identification

The sampling was performed in a MPV industry in Portugal, processing 2500 tons of salad leaves per year. The scheme of the processing line studied can be observed in Fig. 1. The washing room comprises the washing and sanitizing tank. The high care area englobes the optical sorting and packaging. Both rooms are at 5 °C and are separated from a hole in the wall where the conveyor belt goes through. The washing tank is filled with tap water and allows the removal of dirt and debris. The sanitizing tank has tap water and sodium hypochlorite (9.4×10^{-4}–1.2×10^{-3} mol/L) allowing the microbial load reduction. The water was changed when the facilities (equipment and tanks) were sanitized (after operating continuously for 18 h) with Tego 2000® (JohnsonDiversey, Northampton, United Kingdom) for 2 h.

The samples were collected from food contact surfaces (before the sanitization procedure - during processing - and after the sanitization procedure with Tego 2000®), from baby green Batavia leaves (during processing) and from the surrounding air (during the sanitization procedure with Tego 2000®). Both washing room and high care area of the MPV industry were sampled (Fig. 1).

The sampling from the food-contact surfaces was done by cotton swabbing, already proposed as reliable sampling method by Verran, Redfern, Smith, and Whitehead (2010). The swabbing was done 5 cm above and below the line of water (on a surface area of 10 cm^2) and the collection swab was placed in 50 mL tubes with 25 mL of medium in each tube. The procedure was repeated three times in order to place a cotton swab in each medium used (Man, Rogosa and Sharpe broth (MRS; Merck, Gernsheim, Germany), tryptic soy broth (TSB; Merck, Gernsheim, Germany) and buffered peptone water (BPW; Merck, Gernsheim, Germany)). To place the cotton swab in the tubes with medium they were opened under sterile conditions using a fire flame. For all the samples collected in the 50 mL tubes it was applied the spread-plate method. In this method 100 μL of each sample was spread in agar plates containing the same medium of the tube, except for the tubes with buffered peptone water, where it was used plate count agar (PCA; Oxoid, Hampshire, England). This procedure was done in duplicate. The plates were grown at 30 °C during 72 h.

Baby green Batavia leaves (6 cm) were placed in 50 mL tubes with 25 mL of each medium in each tube for the three different media (three leaves in each tube). Samples of 100 μL were retrieved from each tube with different medium and were spread in agar plates of MRS (Merck, Gernsheim, Germany), TSB (Merck, Gernsheim, Germany) and PCA (Oxoid, Hampshire, England). This procedure was done in duplicate. The plates were grown at 30 °C during 72 h.

The air sampling was performed based on the microbial fallout on to Petri dishes left open to the air according to Pasquarella, Pitzurra, and Savino (2000), using plate count agar (PCA) (Oxoid, Hampshire, England) plates. The plates were dispersed over the two areas (6 plates in the washing room and 3 plates in the high care) and left opened for 2 h. The plates from air sampling were left to incubate for 72 h at 30 °C.

Each colony obtained from the sampling was then streaked to a new plate from the same medium as the one used in the first steps of isolation (except for BPW where PCA was used). The isolated bacteria were grown at 30 °C. The diverse isolates were identified by 16S rRNA sequencing using four primers 27F, 518F, 800R e 1492R (Nuobariene et al., 2015).

2.1.1. Phylogenetic tree construction

The DNA sequences were aligned with ClustalW and the phylogenic tree was constructed with MEGA 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) using the Neighbor-Joining method (Saitou & Nei, 1987). The tree was analysed with bootstrap test (1000 replicates) (Felsenstein, 1985). The evolutionary distances were computed using the p-distance method (Nei & Kumar, 2000).

2.2. Biofilm formation

Bacteria were obtained from overnight cultures incubated at 30 °C and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Göttingen, Germany) and grown in 50 mL tubes with 20 mL of TSB. An optical density ($O.D._{600nm}$) of 0.1 was achieved with TSB and the cells were placed in 96-well flat-bottomed PS tissue culture plates with a lid (Orange Scientific, Braine-l'Alleud, Belgium) using a total volume of 200 μL (Borges, Saavedra, Simões, 2012). After an incubation period (30 °C and 120 rpm) of 24 h the biofilm mass was quantified by crystal violet staining according to a method adapted from Stepanović, Vuković, Dakić, Savić, and Švabić-Vlahović (2000). The