International Journal of Food Microbiology 128 (2009) 484–490

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Polyphasic characterization of bacterial community in fresh cut salads

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ARTICLE INFO

Article history:
Received 26 February 2008
Received in revised form 14 October 2008
Accepted 15 October 2008

Keywords:
Fresh cut salads
Bacterial community
PCR-DGGE
Clone library

ABSTRACT

In the present work we describe a polyphasic study of bacterial community in fresh cut salads packaged under ordinary and modified atmospheres. Samples of fresh cut salads were aseptically collected at 0, 3, and 6 days of storage and analysed both by culture-dependent and -independent methods. DNA extracted from fresh cut salad samples was used as a template for PCR amplification of 16 S rRNA gene; the PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE); finally, clone libraries of 16 S rRNA gene from the fresh cut salad was constructed. Results of plating count revealed a significant increase of all microbial loads in fresh-cut salad samples packaged in OA and that the microbial growth of the different groups was significantly affected by the conditions applied for MA packaging. A constant presence, throughout storage, of the pathogenic bacteria in all the fresh-cut salads samples was highlighted by PCR-DGGE analysis. Therefore, the polyphasic approach used in the present study allowed us to characterize the main species involved in the fresh cut salad products and to better understand their dynamics throughout storage.

1. Introduction

Fresh cut salad (FCS) sold in ready-to-eat or ready-to use form have become a very important area of potential economic growth for fresh cut industry. Today these products have gained popularity mainly because consumers perceive such products, besides their well known nutritional qualities, as fresh, healthy, convenient, tasty and easy to use (Garret et al., 2003).

Fresh cut processing includes unit operations such as peeling, trimming or cutting that alter the integrity of the commodity's tissues and induce wounding stress. For these reasons, these products have a limited shelf-life (≤12 days), which is one of the greatest problems faced by commercial marketers (Soliva-Fortuny and Martín-Belloso, 2003). Although FCS products are lightly acidic foods (pH≤6.0), their high humidity and the large number of cut surfaces can create ideal conditions for several microrganisms growth consequently leading to shelf-life reduction (Willocx et al., 1993). Bacterial species on the outer plant surface are often related to soil bacteria, which are members of *Pseudomonas*, *Enterobacter* or *Erwinia* genera, lactic acid bacteria (LAB) and yeast species (Nguyen-The and Carlin, 1994; Bennik et al., 1998; Beuchat, 2002; Tournas, 2005). Moulds are less important in FCS products due to the intrinsic properties such as slightly acid to neutral pH favouring bacteria and yeasts which will overgrow moulds (Lund, 1992; Moss, 1999; Giménez et al., 2003; Tournas, 2005). Among spoilage microrganisms, *Pseudomonas fluorescens*, *Pantoea agglomerans*, and *Rahnella aquatilis*, have frequently been isolated from minimally processed lettuce. These microrganisms have been also found to dominate the microbial population of FCS products at the end of shelf-life under refrigerated conditions (Zagory, 1999; Ragaert et al., 2007). Moreover, several opportunistic pathogens, such as *Escherichia coli*, *Salmonella* and *Shigella* spp., *Aeromonas hydrophila*, *Yersinia enterocolitica*, *Listeria monocytogenes* and *Campylobacter* spp. may also grow in FCS products, leading to a safety problem (Gleeson and O'Beirne, 2005; Abadias et al., 2008). Preservation techniques (e.g. chlorine-containing compounds, irradiation, light or electric pulses, ClO_2 with cysteine) alone or in combination with active packaging and low temperature both during production and storage could minimise microbial development ensuring the shelf-life of the products (Everis, 2004; Gomez-Lopez et al., 2005, 2008). Nevertheless, microbial load reduction depends on the sensitivity of the microrganisms and the accessibility of the agent or the treatment to the microbial population (Ragaert et al., 2007). Hence, a better understanding of microbial ecosystems of the FCS products would be extremely useful in order to prevent the growth of pathogens, and to reduce spoilage microrganisms at the various stages of processing and storage.

Present knowledge of microbial population in FCS products is based mainly on bacterial cultivation, and on the identification of the dominant microorganisms; both methods are time-consuming and poorly reproducible. Moreover, most microrganisms widespread in nature are not cultivable under conditions typically used; microbial diversity can be over- or underestimated with media that are not sufficiently selective (Randazzo et al., 2002). Thus, molecular approaches, especially those based on the use of rRNA genes (DNA), like denaturing gradient gel electrophoresis (DGGE), and related technique, have provided the opportunity to analyze complex

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doi:10.1016/j.ijfoodmicro.2008.10.019

communities on the basis of sequence diversity (Muyzer et al., 1993). Bacterial species can be identified by generating clone libraries of the 16S rDNA followed by sequencing and comparison with databases containing thousands of ribosomal sequences to allow a phylogenetic affiliation to cultured, as well as uncultured microorganisms. DGGE is now frequently applied in food microbial ecology to compare the compositions of complex microbial communities and to study their dynamics (Randazzo et al., 2002; Ercolini, 2004).

To obtain knowledge on the bacterial communities of fresh cut salad, packaged under ordinary and modified atmosphere, in the present study a polyphasic characterization mainly based on molecular approaches, was applied. Whole bacterial community diversity was detected using PCR-DGGE analysis whereas the dominant bacteria were identified throughout the clone libraries of the 16 S rRNA gene amplified fragments.

2. Materials and methods

2.1. Salad composition and processing

In the present study samples of fresh cut salads, containing from 3 to 6 ingredients such as curly endive (*Cichorium endivia*), white cabbage (*Brassica oleracea* var. *botrytis*), chicory (*Cichorium intybus*), carrot (*Daucus carota* L.), radish (*Cichorium intybus*) and sugar loaf (*Cichorium intybus* var. *foliosum*) were investigated. The whole vegetables, field-grown under Mediterranean climate (Catania, Sicily, Italy), were harvested at the beginning of April, 1–3 days before the processing, transported to laboratory and stored at 2–4 °C. Then, the vegetables were sorted, cut manually and rinsed in potable cold water with 5–20 ppm active chlorine, washed in cold water, centrifuged (5590×*g* for 2 min) to remove excess water and mixed at different ratio.

Portion of 250 g of fresh cut salads previously described, was packaged under ordinary atmosphere (OA) using an 18 mm of polypropylene (PP) film bags (17×21 cm) (Tecnoplast, Reggio Emilia, Italy). The PP permeability at 23 °C and 75% RH was 730 mL/m^2 day atm for O_2 and 2460 mL/m^2 day atm for CO_2. For the modified atmosphere packaging (MA) a 90 mm polyamide-polyethylene (PA/PE) film bags (17×21 cm) (Niederwieser Bolzano, Italy) was used. The PA/PE permeability at 23 °C and 75% RH was 50 mL/m^2 day atm for O_2 and 150 mL/m^2 day atm for CO_2. The permeability of the film (PA/PE) at 23 °C and 85% RH was 2.8 g/m^2 day atm for water vapour (data provided by Niederwieser, Bolzano, Italy). For the MA the bags inside packaging were flushed for ca. 5 s with a gas mixture containing 90% nitrogen, 6% carbon dioxide, and 3% oxygen (Delta 30, Delta, Brindisi, Italy). A total of 72 fresh cut salad samples were packaged and stored at 4 °C for 6 days.

2.2. Microbiological analysis

All 72 samples were subjected to microbiological analysis in order to monitor the microbial population dynamics throughout storage. Ten grams of each fresh cut vegetable samples were added to 90 mL of sterile physiological solution (0.9% NaCl) (1:10 [wt/v]) in stomacher Lab-Blender 400 (Brinkmann, Westbury, NY, USA) for 5 min. Serial dilutions of the suspension were pour plated in duplicate on the following media: De Man Rogosa Sharpe (MRS) (Oxoid Basingstoke, UK) agar for lactic acid bacteria (LAB); Plate Count Agar (PCA) (Oxoid) for psychrotrophic bacteria; Violet Red Bile Glucose Agar (VRBGA) (Oxoid) for *Enterobacteriaceae*; Saboraud Dextrose Agar (SDA) (Oxoid) for yeasts. VRBGA and MRS agar plates were incubated at 32 °C for 24–48 h in aerobic and anaerobic conditions, respectively. PCA plates were incubated at 21 °C for 2–5 days, while for yeast counts the plates were incubated at 25 °C at least for 2–4 days.

2.3. Measurements of CO_2, O_2 and pH

The CO_2 and O_2 concentrations in the packages during the storage were measured using the gas analysing equipment CheckPoint (PBI Dansensor, Denmark). The samples were drawn through septa with a syringe to prevent gas leakage.

The pH values of the homogenized vegetables were measured, during storage, using a pH meter HI 9017 (Hanna Instrument, Italy) according to the manufacturer's recommendations.

2.4. Statistical analysis

All experiments were performed on duplicate and the experimental data were reported as average values and provided with Standard Deviation. Statistical ANOVA ($P<0.01$) and Duncan test were performed using XLSTAT PRO 5.7 (Addinsoft, New York, USA). Statistical ANOVA was carried out to evaluate the effect both of the storage at 4 °C and of the atmospheres (OA and MA) used for packaging for each individual time (to, t3, ad t6) on the microbial growth on different media.

2.5. Nucleic acid isolation from FCS samples

The suspension, previously used for microbiological analysis, was centrifuged for 15 min at 10,000 rpm, and the pellets were suspended in 1 mL of TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0), recentrifuged and resuspended in 0.5 mL of TE buffer. Then, the supernatant fluid was removed and transferred to a sterile screw-cap tube containing 0.3 g of zirconium beads (diameter, 0.1 mm). The samples were treated at 5000 *g* for 180 s in a bead beater (Biospec products, Bartlesville, Ok). After the addition of 150 µL of CI solution, made of chloroform and isoamylic alcohol at a 24:1 (vol/vol) ratio, the tubes were briefly vortexed and centrifuged at 9447×*g* for 5 min. The supernatant fluid was transferred to a sterile tube and stored at −20 °C until use.

2.6. PCR amplification

PCR amplification was performed with the *Taq* DNA polymerase kit from Life Technologies. Reaction mixtures consisted in a volume of 50 µL of 20 mmol/L Tris HCl (pH 8.4), 50 mmol/L KCl, 3 mmol/L $MgCl_2$, 50 µmol/L each deoxynuclotide triphosphate, 1.25 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 5 pmol of each primer and 1 µg of DNA template.

To investigate the dominant bacterial communities by DGGE analysis, PCR products were generated with PCR primers U968-GC and L1401-r to amplify the V6 to V8 region of the 16S rRNA gene (Nubel et al., 1996). The samples were amplified in a Perkin-Elmer Applied Biosystems (Foster City, CA) GenAmp PCR System 9700 programmed as follows: initial denaturation of DNA for 5 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 56 °C, and 40 s at 68 °C; and extension of incomplete products for 7 min at 68 °C.

PCR was performed with primer pairs (7-f and 1510-r) (Lane, 1991) (T7 and Sp6) (Promega Corporation, Madison, USA) to amplify the bacterial 16 S rDNA prior to cloning and sequence analysis by using the cell lysates as template to confirm an insert of the correct size.

DNA amplification was carried out with the reaction mixtures as described above under the following conditions: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 68 °C for 1.5 min; and finally 68 °C for 7 min.

PCR products were quantified by electrophoresis on a 1.2% (wt/vol) agarose gel containing ethidium bromide and, where necessary, were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

2.7. DGGE analysis

The DCode Universal Mutation Detection system (Bio-Rad, Hercules, California) was used for the separation of the PCR products. Electrophoresis was performed in a 0.8-mm-thick polyacrylamide gel