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Characterization of psychrotrophic bacterial communities in modified atmosphere-packed meat with terminal restriction fragment length polymorphism

T.T. Nieminen [a,*], E. Vihavainen [a], A. Paloranta [a], J. Lehto [a], L. Paulin [b], P. Auvinen [b], M. Solismaa [a], K.J. Björkroth [a]

[a] Department of Food Hygiene and Environmental Health, PO Box 66, FI-00014, University of Helsinki, Finland
[b] DNA Sequencing and Genomics Laboratory, PO Box 56, FI-00014, University of Helsinki, Finland

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ABSTRACT

Characterization of psychrotrophic lactic acid bacteria (LAB) and *Brochothrix thermosphacta* communities is needed to understand the microbial ecology of spoilage of modified atmosphere-packed (MAP) meats. To overcome the limitations of the currently used methods for the characterization of psychrotrophic bacterial communities in meat, we developed a culture-independent, 16S rRNA gene-targeted terminal restriction fragment length polymorphism (T-RFLP) method. An identification library consisting of 100 Gram-positive and 30 Gram-negative meat-associated bacterial strains was set up to identify the terminal restriction fragments derived from the communities. The taxonomic resolution level of the T-RFLP method was in between genus and species within the investigated LAB strains and within family and genus within the investigated Gram-negative strains. The established library was applied to identify the members of bacterial communities in MAP minced meat at the end of the shelf life. The T-RFLP results and plate counts on Man-Rogosa-Sharpe, Violet Red Bile Glucose, and Streptomycin sulfate thallium acetate actidione agars indicated that LAB and *B. thermosphacta* predominated in meat. The bacterial taxa associated with the T-RFLP results were compared to those identified among plate-grown LAB isolates by numerical ribopattern analysis. Both methods agreed that *Leuconostoc* spp. and *Carnobacterium* spp. prevailed in the LAB community in minced meat followed by *Lactobacillus algidus*, *Lactococcus* spp. and *Weissella* spp. Colony identification revealed that *Leuconostoc gasicomitatum*, *L. gelidum*, *Carnobacterium divergens* and *C. maltaromaticum* were the predominant LAB species. The T-RFLP results were shown to correlate with viable counts of *Leuconostoc* spp. and *B. thermosphacta*. The T-RFLP method was found to be a useful tool enabling rapid and high-throughput characterization of psychrotrophic bacteria prevailing in MAP meat.

1. Introduction

Psychrotrophic lactic acid bacteria (LAB) and *Brochothrix thermosphacta* are typical spoilage organisms of various cold-stored, modified atmosphere-packed (MAP) raw meats due to their ability to grow at low temperatures under CO_2-rich atmosphere (Borch et al., 1996; Samelis, 2006). The LAB most often detected in meat belong to the genera *Carnobacterium, Lactobacillus, Leuconostoc* and *Weissella* (Nychas et al., 2007). LAB show varying potential to cause spoilage (Laursen et al., 2006; Samelis 2006; Schillinger et al., 2006; Schirmer et al., 2009), e.g. by discoloration (Vihavainen and Björkroth, 2007), slime production (Korkeala et al., 1988) or production of off-odorous compounds (Egan, 1983). Hence, characterization of LAB communities is needed to understand the microbial spoilage of MAP meat.

Currently, psychrotrophic bacterial communities in meat are typically characterized by identification of colonies plated on solid medium. The method provides a quantitative estimate of the cultivable community structure and enables high taxonomic resolution of the isolates chosen for identification. However, colony identification is time-consuming, expensive and laborious and thus unsuitable for characterization of large number of communities. Tens of colonies typically chosen for identifications may not be a representative sample of the community. Furthermore, selectivity of growth medium may distort the results.

To overcome the limitations, psychrotrophic bacterial communities in meat have been characterized culture independently using 16S rRNA gene-targeted denaturing gradient gel electrophoresis (DGGE) (Brightwell et al., 2009; Cocolin et al., 2007; Ercolini et al., 2006). The method requires simple equipment but suffers from a number of technical drawbacks, including gel-to-gel variation, labor intensity and co-

Abbreviations: DGGE, Denaturing gradient gel electrophoresis; LAB, Lactic acid bacteria; MAP, Modified atmosphere-packed; MRS, Man-Rogosa-Sharpe; STAA, Streptomycin sulfate thallium acetate actidione; T-RFLP, Terminal restriction fragment length polymorphism; TRF, Terminal restriction fragment; VRBG, Violet Red Bile Glucose.

* Corresponding author. Tel.: +358 9 191 57458; fax: +358 9 191 57101.
E-mail address: timo.t.nieminen@helsinki.fi (T.T. Nieminen).

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migration of dissimilar fragments (Justé et al., 2008). Terminal restriction fragment length polymorphism (T-RFLP) (Avaniss-Aghajani et al., 1994) is a culture-independent method for community characterization employing restriction digestion of PCR-amplified DNA fragments. One or both of the PCR primes is fluorescently labeled allowing the detection of terminal restriction fragments (TRFs). T-RFLP has been widely applied to bacterial community characterization in various environments (Schüette et al., 2008) including sugar thick juice (Justé et al., 2008), grass silage (McEniry et al., 2008) and fish (Reynisson et al., 2009, Tanaka et al., 2010). The method has been shown reproducible (Blackwood et al., 2003) and has potential for higher throughput than DGGE.

In this study we developed a T-RFLP method for the characterization of psychrotrophic bacterial communities in MAP minced meat. The results obtained by the T-RFLP method were evaluated by comparing them to colony counts on selective media and identification of LAB isolates by numerical ribopattern analysis.

2. Materials and methods

2.1. Meat

Forty-one packages of minced meat (80% of pork and 20% beef) packed under modified atmosphere containing 65% O_2, 25% CO_2, and 10% residual air were obtained from a commercial processing plant for this study.

Altogether 20 meat packages from five lots, four packages from each, were used for the first subset of bacterial community characterization experiment by T-RFLP and colony identification methods. Manufacturer-defined shelf life was 8 days for lot 1, 9 days for lots 2, 3, 5 and 10 days for lot 4. The packages were stored at +6 °C and analyzed one day after the end of shelf life except the packages from lot 1, which were analyzed one day before the end of shelf life.

For the determination of *B. thermosphacta*/LAB colony count and T-RFLP ratios in the second subset of community characterization experiments, 21 meat packages were collected from a single lot. The packages were stored for 19 days at +4 °C. Meat was sampled 8, 11, 12, 13, 14, 16 and 19 days after packaging. Three packages were analyzed on each time point.

2.2. Colony counts and pH

From each 41 packages, 25 g of meat were homogenized with 225 ml of peptone saline solution (0.85% NaCl and 0.1% peptone in distilled water) for 1 min in a stomacher blender (Lab Blender 400, Seward, Worthing, UK). The resulting meat homogenate was serially diluted in peptone saline solution and appropriate dilutions were spread on Man-Rogosa-Sharpe agar (MRS, Oxoid, Basingstoke, UK) for determination of psychrotrophic LAB. Plates were incubated anaerobically (Anaerogen, Oxoid) at 25 °C for 5 days. For determination of psychrotrophic enterobacteria, sample dilutions were poured in Violet Red Bile Glucose agar (VRBG, Lab M, Bury UK) and incubated aerobically at 25 °C for 2 days. Streptomycin sulfate thallium acetate actidione agar (STAA Oxoid, Basingstoke, UK) was used to determine *B. thermosphacta* plate counts from the 21 meat packages from lot 6 in the second subset of community characterization experiments. Plates were incubated at 25 °C for 2 days. Ten presumptive *B. thermosphacta* colonies per sample were streaked on tryptic soy agar (Difco Laboratories, Sparks, MD, USA) and tested for oxidase-negativity.

From each meat homogenate, pH was measured by an inoLab pH 720 (WTW GmbH, Weilheim, Germany) instrument.

2.3. Terminal restriction fragment polymorphism analysis

2.3.1. Terminal restriction fragment identification library

The library consisted of 130 meat-associated bacterial strains, including 92 LAB and 30 Gram-negative strains (Table 1). DNA for the T-RFLP analysis was extracted as described by Vihavainen and Björkroth (2007).

2.3.2. Community DNA isolation

A 50-ml portion of meat homogenate was centrifuged at 800 *g* for 5 min at +4 °C. Thirty-five milliliters of supernatant were collected and centrifuged at 12 000 *g* for 10 min at +4 °C. Supernatant was discarded, and the pellet suspended in 1.5 ml of 0.15 M NaCl. Suspension was transferred to 1.5 ml microcentrifuge tube and centrifuged at 8000 *g* for 5 min at RT. Supernatant was discarded and the pellet stored at −70 °C for a maximum of 1 month. The frozen pellet was suspended in 1 ml of 1.05 strength Percoll (Sigma-Aldrich, St. Louis, MO) density gradient matrix and centrifuged at 4500 *g* for 15 min at RT. Top layer including the solids was removed leaving a 0.3-ml bottom layer which was mixed with 1.2 ml of 0.15 M NaCl and centrifuged at 10 000 *g* for 2 min at RT. Supernatant was discarded and the remaining pellet washed with 1.2 ml of 0.15 M NaCl. DNA was extracted from pellet using a commercial kit (Genelute bacterial DNA, Sigma-Aldrich) as described in the instructions for Gram-positive bacteria. The DNA concentration was measured by Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltman, MA).

2.3.3. PCR amplification, restriction digestion and capillary electrophoresis

A fragment of 16S rRNA gene, approximately 940 bp in size, was PCR-amplified from the community DNA with forward primer Bact-8F (5′-AGA GTT TGA TCC TGG CTC AG) (Weisburg et al., 1991), 5′ end-labeled with phosphoramidite fluorochrome 6-carboxy fluorescein (6-FAM), and reverse primer 926r (5′-CCG TCA ATT CMT TTG AGT TT) (Muyzer et al., 1995). Primers were synthesized by Oligomer Oy (Helsinki, Finland). PCR reactions (50 µl) were as follows: 1.0 U Dynazyme II DNA polymerase (Finnzymes, Espoo, Finland), 1 × Dyna-buffer, 5 nmol each dNTP, 15 pmol forward and reverse primers and 20 to 30 ng of template DNA. Thermal cycling program was: initial denaturation at 95 °C for 2 min, 30 cycles of amplification (94 °C for 15 s, 55 °C for 40 s, 72 °C for 90 s), final extension 72 °C for 8 min. PCR product size was visually checked with agarose gel electrophoresis. PCR products were purified with silica spin columns (DNA clean and concentrator-5 kit, Zymo Research, Orange, CA) and the purified product quantified with the spectrophotometer.

Purified PCR product was digested with restriction enzymes *Hin*P1I, *Hpy*8I and *Nla*IV (Fastdigest, Fermentas, Vilnius, Lithuania) at 37 °C for 1 h. The enzymes were selected on the basis of *in silico* digestion of 16S rRNA gene sequences to get maximum number of different TRF length combinations from LAB listed in Table 1. Reagent concentrations in digestions (15 µl) were as follows: 1 × Fastdigest buffer, 3–5 ng/µl of purified PCR product, 0.033 U/µl of restriction enzyme. Enzyme was thermally inactivated at 80 °C for 10 min. One µl of digestion mix was mixed with 20 µl of HiDi-formamide (Applied Biosystems, Foster City, CA) and 0.15 µl size standard (MapMarker 1000, Bioventures, Murfreesboro, TN) before fragments were size-separated and detected with 3130xl Genetic Analyzer capillary electrophoresis instrument (Applied Biosystems). The samples were injected at 3.2 kV for 25 s and run at 15 kV for 1800 s. Correlation between size standard fragment length and migration time was nearly linear for fragments 100–600 bp.

2.3.4. Data analysis

Electropherograms were imported to Bionumerics software (version 5.1.0., Applied Maths, Sint-Martens-Latem, Belgium) and normalized according to the internal size standards. Electropherogram peaks were recognized as TRFs using the following settings: a peak minimum profiling 1% and minimum height 1% relative to maximum value of the electropherogram. Shoulder sensitivity was set to 1. TRF lengths in bp were determined by comparing them to the internal standards using cubic spline fit. Peaks shorter than 100 bp were excluded from further