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Development of spoilage bacterial community and volatile compounds in chilled beef under vacuum or high oxygen atmospheres

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ABSTRACT

Research into microbial community development and metabolism is essential to understand meat spoilage. Recent years have seen the emergence of powerful molecular techniques that are being used alongside conventional microbiology approaches. This enables more accurate studies on meat spoilage. The aim of this study was to investigate the influence of packaging (under vacuum and in high oxygen atmosphere) on the development of microbial communities and metabolic activities at 6 °C by using culture-dependent (cultivation, ribotyping) and culture-independent (amplicon sequencing) methods. At the beginning of shelf life, the microbial community mostly consisted of *Carnobacterium* and *Lactobacillus*. After two weeks of storage, *Lactococcus* and *Lactobacillus* were the dominant genera under vacuum and *Leuconostoc* in high oxygen meat packages. This indicates that oxygen favoured the genus *Leuconostoc* comprising only heterofermentative species and hence potential producers of undesirable compounds. Also the number of volatile compounds, such as diacetyl, 1-octen-3-ol and hexanoic acids, was higher in high oxygen packages than under vacuum packages. The beef in high oxygen atmosphere packaging was detected as spoiled in sensory evaluation over 10 days earlier than beef under vacuum packaging. *Leuconostoc gelidum*, *Lactococcus piscium*, *Lactobacillus sakei* and *Lactobacillus algidus* were the most common species of bacteria. The results obtained from identification of the isolates using ribotyping and amplicon sequencing correlated, except for *L. algidus*, which was detected in both types of packaging by amplicon sequencing, but only in vacuum packaged samples using the culture-based technique. This indicates that *L. algidus* grew, but was not cultivable in high oxygen beef using the Nordic Committee on Food Analysis standard method.

1. Introduction

Spoilage of raw meat is a combination of biological and chemical activities. The microbial communities occurring frequently in fresh meat belong to the genera of *Acinetobacter*, *Pseudomonas*, *Brochothrix*, *Flavobacterium*, *Psychrobacter*, *Moraxella*, *Staphylococcus*, *Micrococcus*, lactic acid bacteria (LAB) and the family of *Enterobacteriaceae* (Dainty et al., 1983; Doulgeraki et al., 2012; Enfors et al., 1979; Erichsen and Molin, 1981). A set of organisms interacting to spoil the products is called specific spoilage organisms (SSO) (Gram et al., 2002). These SSO form only a minor part of the initial microbial community (Huis in't Veld, 1996), but during storage certain extrinsic and intrinsic factors favour their growth and develop into a major fraction of the microbiota. Temperature and packaging atmosphere are the most important extrinsic factors which determine the development of microbial communities. Combining refrigeration with modified-atmosphere packaging (MAP) or vacuum packaging (VP) favours the growth of *Pseudomonas* spp., *Enterobacteriaceae*, *Brochothrix thermosphacta*, and LAB (Ercolini et al., 2011). In low storage temperature, such as −1.5 °C, the *Clostridium* spp. has been described as a major SSO in VP beef (Hernandez-Macedo et al., 2012; Silva et al., 2012).

An understanding of microbial community development is a key factor for meat quality, but the microbial ecology alone might not explain meat spoilage in general (Doulgeraki et al., 2012). Spoilage usually occurs when SSO grow to unacceptable levels and the spoilage potential of SSO depends on their ability to produce metabolites, such as aldehydes, ketones, esters, alcohols, organic acids, amines, and sulphur compounds, which determine the sensory spoilage characteristics of meat.

Abbreviations: GC, Gas chromatography; HS-SPME, Headspace solid phase microextraction; LAB, Lactic acid bacteria; MAP, Modified atmosphere-packaging/packaged; MS, mass spectrometry; OTU, Operational taxonomic units; SSO, Specific spoilage organism; VP, Vacuum packaging/packaged; VOC, Volatile Organic Compounds.

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In this study, we used both culture-dependent (cultivation, ribotyping) and culture-independent (amplicon sequencing of the V1–V3 regions of the 16S ribosomal RNA gene) methods to study the development of microbial communities in beef during chilled storage in high oxygen MAP and VP. In our study, the abundance of other SSO than LAB was lower than in previous studies (De Filippis et al., 2013; Ercolini et al., 2011; Pennacchia et al., 2011). The microbiological data were combined with the results from the volatile organic compound (VOC) analysis and sensory evaluation to detect factors limiting shelf life. This is the one of a few articles about spoilage of beef in which all above analyses have been combined. The study showed that the two most common meat packaging technologies affect both bacterial community composition and metabolism. High oxygen MAP favoured mainly heterofermentative species and the number of VOCs was higher compared to VP.

2. Materials and methods

2.1. Meat storage and sampling

The meat manufacturer packaged the cubes of beef studied (48 h after slaughtering, stored 0 °C) either under VP or in high oxygen MAP (80% O_2: 20% CO_2). In the laboratory the storage temperature was 6 °C, and sampling was done after 2, 4, 7, 9, 11 and 14 days. Additionally, the VP cubes were analysed on days 22, 24 and 26. The 6 °C temperature is the highest recommended storage temperature in retail storage for raw meat in Finland. Both products had a 10-day shelf life after packaging. A total of three of the aforementioned time series were analysed, with duplicates.

2.2. Microbial enumeration

Colony counts of an individual package at each time of sampling were performed by homogenizing for 1 min (Stomacher, Seward, Worthing, UK) 25 g of beef with 0.1% peptone saline (225 ml). 10-fold dilutions were plated on Plate Count Agar (PCA, Oxoid) for total aerobic colony counts, Violet Red Bile Glucose Agar (VRBGA, Oxoid) for the *Enterobacteriaceae*, de Man-Rogosa-Sharpe (MRS, Oxoid) for LAB, Streptomycin-thallous acetate-actidione Agar (STAA, Oxoid) for *B. thermosphacta* and Pseudomonas isolation agar (PIA; Merck, Darmstadt, Germany) for *Pseudomonas*. The PCA and MRS plates were incubated at 25 °C for 5 days, VRBG and STAA at 25 °C for 2 days, and PIA 37 °C for 2 days. The MRS plates were incubated anaerobically (Anaerogen, Oxoid, Basingstoke, UK) in jars and the other plates aerobically. The typical *Pseudomonas* colonies were confirmed by oxidation test (Sigma-Aldrich Co, St. Louis, MO, USA).

2.3. Physical parameters

Gas compositions in the high oxygen MAP were measured using a gas sensor (Checkpoint, PBI Dansensor, Ringstedt, Denmark). The pH of beef was measured after 1 min Stomacher homogenization with 0.1% peptone saline at a ratio of 1:10 by pH meter (Inolab 720, WTW, Weilheim, Germany).

2.4. Sensory analyses

Sensory analyses were performed by a trained panel of at least five individuals. For these analyses, the beef samples were equilibrated at room temperature. Beef from the same meat lots was stored fresh (day 0) in the freezer (−20 °C) and used as a reference. The panellists evaluated the odour and the appearance of the samples using a five-point scale (1 = severe defect, spoiled, 2 = clear defect, spoiled, 3 = mild defect, satisfactory, 4 = good, 5 = excellent); the observed deficiencies were described. The sample was considered spoiled when the median of the grades given was 2 or less.

2.5. 16S rRNA amplicon sequencing

2.5.1. DNA extraction

DNA was extracted directly from the meat samples as in Hultman et al. (2015), by using 15 ml of the 1:10 homogenate of microbial dilute. Briefly, the majority of the meat cells were removed by centrifugation (3 min, 200 rcf, Eppendorf 5810 R, Eppendorf AG, Hamburg, Germany) after which the bacterial cells from the supernatant were collected by a second round of centrifugation (3 min, 10 000 rcf). DNA was extracted from the cell pellets by bead beating a GES-phenol-chloroform extraction with FastPrep Lysing matrix E tubes (MP Biomedicals, Santa Ana, CA, USA). The lysing matrix tubes were bead beaten for 40 s at 5.5 m/s in a FastPrep-24 instrument (MP Biomedicals), after which the tubes were incubated on ice for 5 min and centrifuged for 10 min at 13 000 rcf (Eppendorf, Hamburg, Germany). 500 μl of chloroform was added to the upper layer and the tubes were centrifuged for 10 min after vortexing. The nucleic acids were precipitated with 1/10 volume of 3 M sodium acetate, 1 μl of glycoblue (Invitrogen, Carlsbad, CA, USA) and 3× ethanol. The pellets were washed with 70% ethanol and eluted in 50 μl of sterile nuclease free water.

2.5.2. PCR amplification and 16S rRNA sequencing

To characterise the microbiome, the V1–V3 regions of the 16S rRNA gene were amplified through PCR with primers 8f and 518r (Edwards et al., 1989). The PCR reaction consisted of 1 × Phusion GC buffer, 200 μM of dNTP, 0.2 μM of each primer, 2.5% of DMSO and 50–250 ng of community DNA. After heating the PCR mix to 98 °C, 1 U of Phusion polymerase (Life Technologies, Carlsbad, CA, USA) was added to the reaction. The PCR programme was as follows: denaturation at 98 °C for 30 s, 20 cycles at 98 °C for 10 s, 65 °C for 30 s and 72 °C for 10 s followed by 72 °C for 5 min and cooling down to 4 °C. The PCR products were purified using 0.9× Ampure beads (Beckman Coulter, Pasadena, CA, USA) and eluted in 40 μl of 0.1× TE buffer. The sample specific barcodes and sequencing adapters were added to the amplified PCR fragments in a second PCR with a 1 × Phusion GC buffer, 200 μM of dNTP, 0.05 μM of each primer, 2.5% of DMSO and approximately 50 ng of purified PCR product. Again, the Phusion polymerase (1 U) was added after heating the reaction to 98 °C. The reverse primer had the adapter A attached and the forward primer included the B adapter with a sample specific barcode (8 bp). Three replicate PCR reactions were done per sample and the products were pooled prior to purification. PCR product purification and sequencing were conducted at the Institute of Biotechnology, University of Helsinki, using the Roche 454 Titanium FLX protocol. The raw sequence reads have been deposited to SRA under accession number PRJNA293921.

2.5.3. Data analysis

The sequences were analysed using QIIME (Caporaso et al., 2010). The sequence reads were filtered for quality and reads with a length of less than 200 bp, containing ambiguous bases, with a quality score of below q30 or mismatches in the primer sequence were discarded. The remaining reads were assigned to samples based on the sample specific barcode. OTUs (operational taxonomic units) were picked with 97% similarity using the UCLUST (Edgar, 2010) algorithm and the representative sequence read from each OTU were taxonomically classified through a QIIME-based wrapper of BLAST (Altschul et al., 1990) against the Greengenes database (Version 13.8.2014) (DeSantis et al., 2006).

2.6. Ribotyping

To identify the LAB species associated with spoilage, up to 10 colonies per sample were picked randomly from the MRS plates sampled