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Diversity of bacterial communities in French chicken cuts stored under modified atmosphere packaging

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ABSTRACT

Poultry meat, the second most consumed meat in France, is commercialized mainly as portions of chicken cuts with various quality labels, stored under various modified atmosphere packaging (MAP), with shelf-life ranging from 9 to 17 days. We used 16S rDNA pyrosequencing to describe microbiota of chicken legs. Ten samples representing a wide diversity of labels and MAP available on the market were collected from local supermarkets and stored at 4 °C. Microbiota were collected, total DNA was extracted, and V1-V3 fragment of 16S rRNA genes were amplified and sequenced. For data analysis several pipelines were compared. The Qiime pipeline was chosen to cluster reads and we used a database previously developed for a meat and fish microbial ecology study. Variability between samples was observed and a listing of bacteria present on chicken meat was established. The structure of the bacterial communities were compared with traditional cultural methods and validated with quantitative real time PCR. *Brochothrix thermosphacta*, *Pseudomonas* sp., and *Carnobacterium* sp. were dominant and the nature of the gas used for packaging influenced the relative abundance of each suggesting a MAP gas composition dependent competition between these species. We also noticed that slaughterhouse environment may influence the nature of the contaminants.

1. Introduction

Richness and abundance of microbiota present in food products, and especially meats, play an important role in the shelf life of the products, their microbial safety, and therefore the consumer health. Unlike fermented food, where unwanted bacteria are controlled by the addition of bacterial starters that become dominant during the process, fresh meat contamination is more diversified. Sources of contamination are the animal and the environment microbiota, and depend on the farming and slaughtering process (Chaillou et al., 2015). Poultry meat can host very diverse microbial communities varying with seasonal changes (Cohen et al., 2007) among which spoilage bacteria (Doulgeraki et al., 2012) or pathogens such as *Campylobacter* (Gruntar et al., 2015) and Salmonella (Rasschaert et al., 2008) which must be controlled to ensure safety of the products (Álvarez-Astorga et al., 2002).

The use-by-date (UBD) of fresh poultry meat is determined as the time period during shelf life for bacterial contamination to reach around 7 log CFU.g^{-1} (Okolocha and Ellerbroek, 2005). It usually varies from 4 to 15 days depending notably on the type of gas used for packaging, *i.e.* air or modified atmosphere packaging (MAP). In France, the chicken cuts most commonly sold in supermarkets are packed under various MAP, either enriched or devoid of O_2 and the shelf-life can reach 17 days (Rouger et al., 2016). In addition a large panel of quality labels (standard, organic, halal, free range) is available and various breeding or farming practices exist, that may influence the bacterial loads present on meat.

Most of the information dealing with fresh meat product bacterial contamination is issued from cultural methods (for a review see Doulgeraki et al., 2012). These cultural methods use selective media for bacteria detection and quantification such as total viable counts, lactic acid bacteria, Enterobacteria, *Pseudomonas* sp., *Brochothrix* (Mead, 2004). In a previous study, we used such plating methods to determine the contamination level of chicken legs and a large variation of total aerobic counts between samples (from 3 to 8 log CFUg^{-1}) was observed (Rouger et al., 2016). We also noticed that the ratio between lactic acid bacteria, *Pseudomonas*, Enterobacteria, and *Brochothrix thermosphacta* loads differed within

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samples. However, we did not observe any correlation between these variations and meat quality labels or MAP gas composition. Nevertheless a competition between bacterial contaminants exists during poultry meat storage (Alonso-Hernando et al., 2012) and storage conditions may influence food microbiota (Chaillou et al., 2015). With the development of high-throughput sequencing methods, the description of complex microbial communities of many environments has been revisited. Next generation sequencing (NGS) technologies are nowadays commonly used, in particular to investigate animal and environmental microbiota and In addition software and analysis pipelines are easily and freely available (Ercolini, 2013; Mayo et al., 2014). More recently, these have been also applied to food but mainly to fermented products which microbial diversity is less complex than that of fresh products.

Nevertheless few studies using sequencing approach have been reported on non-fermented meat products, most of them dedicated to beef or pork meat (Benson et al., 2014; Chaillou et al., 2015; De Filippis et al., 2013; Fougy et al., 2016; Hultman et al., 2015). To our knowledge, only two studies using NGS focused on poultry meat, a comparison of microbiota present in marinated *vs* non marinated Finnish chicken breast (Nieminen et al., 2012) and the analysis of the contamination along the production chain in USA, from broiler chicken production to carcasses, which are rinsed in a chlorinated solution (Oakley et al., 2013).

In the present study, we describe the diversity of the microbiota of chicken legs from 10 different samples collected from French supermarkets and stored under various MAP, by a 16S rRNA gene pyrosequencing approach.

2. Materials and methods

2.1. 16S rRNA gene pyrosequencing

2.1.1. DNA extraction from meat microbiota

In a previous study bacterial communities were collected from 23 chicken leg samples stored at 4 °C. After collection each microbiota was stored at −80 °C with glycerol 15%, and bacterial DNA was extracted from 10 out of these communities (Rouger et al., 2016). Briefly, after thawing tubes, bacteria were collected by centrifugation at 10000 × *g* for 10 min at 4 °C. DNA was extracted with Mobio Power Food Microbial DNA isolation kit which combines mechanical (beat beating) and chemical lysis of the cells with a prior step of incubation in an ultrasonic bath (see Rouger et al., 2016).

2.1.2. Pyrosequencing PCR conditions

The V1-V3 region of the 16S rRNA gene (567 bp) was amplified by PCR with 27F CGTATCGCCTCCCTCGCGCCATCAGxAGAGTTTGATCCTGGCTCAG and 534R CTATGCGCCTTGCCAGCCCGCTCAGxATTACCGCGGCTGCTGG with x representing the barcodes specific for each of the 10 samples (see Table 1). The 50 μL PCR mixture was composed of 2.5 U of high fidelity Pwo DNA polymerase (Roche Diagnostics, France), 1X Pwo buffer (100 mM Tris−HCl, 250 mM KCl, 50 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, pH 8.85), 0.2 mM dNTP (New England Biolabs, USA), 0.6 μM of each primers, and 2.5 μL of the DNA solution. All PCR amplifications were performed in a PTC-100 Thermocycler (MJ Research Inc., USA). The PCR protocol encompassed an initial denaturation step (94 °C for 2 min) followed by 30 or 35 cycles comprising a denaturation step (94 °C for 30 s), primer annealing steps using a temperature gradient (60 °C for 30 s, −0.5 °C per cycle), and an extension step (72 °C for 1 min). At the end a final extension at 72 °C for 7 min was performed. Two PCR amplifications were performed per sample, with either 30 or 35 cycles.

2.1.3. DNA quantification and quality control

PCR fragments were visualized on 1% (w/v) agarose gels. PCR products were purified with the QIAquick kit (Qiagen SA, France) according to the manufacturer's procedure, then concentrated in a SpeedVac system (Thermofisher scientific, France) to obtain a final volume of 30 μL purified DNA. DNA concentration was measured with a Qubit fluorimeter (Invitrogen, CA, USA), quality and quantity parameters were checked on Experion DNA 12K chips (Biorad, France) prior sequencing.

2.1.4. Sequencing and data analysis

For each sample, the DNA amplified after 30 and 35 cycles were pooled and sequenced in single end by Eurofins MWG (Ebersberg, Germany) using 454 GS-FLX++ Titanium Technologies (454 Life Technologies, USA).

Different strategies were compared for data analysis: the FROGS pipeline (Find Rapidly OTUs with Galaxy Solution) (Escudie et al., 2015) or the protocol designed in previous study (Chaillou et al., 2015) were tested. In addition the pipeline using Qiime software currently found in the literature for metabarcoding data sets (Caporaso et al., 2010). Those were combined to different databases. The main features of the strategies tested are summarized Table 2.

FROGS is a pipeline developed to run in a reasonable time in an user-friendly under Galaxy environment. The pipeline includes demultiplexing, and a pre process step to filter and delete sequences with unexpected lengths, with ambiguous bases (N) and which do not contain primer sequence at both 3′- and 5′-ends. The clusterization is performed with Swarm, a robust and fast clustering method for amplicon-based studies without global threshold and independent of sequence order (Mahe et al., 2014). After clustering, detection of chimeras is performed with a specific removal method of FROGS (Vsearch and cross-validation). After filtering multi-affiliation with 2 taxonomy affiliation procedures were performed. FROGS pipeline includes also statistics tools.

The protocol design by Chaillou et al. (2015) uses different software, reads were demultiplexed according to barcode sequences with cutadapt and quality of the sequencing is checked using FastQC software (Babraham Bioinformatics). The reads are trimmed and filtered with quality score threshold of 20. Chimeric sequences are detected using Decipher web server (Wright et al., 2012) and are removed from the dataset prior any bioinformatic analysis (Haas et al., 2011). Software used for clustering, initially designed for genome assembly, is used here to cluster 16S rDNA sequences. The clustering is performed with Qiime software (Caporaso et al., 2010) using the longest reads as reference for each operational taxonomic unit (OTU) whereas in the strategy developed by Chaillou et al. (2015) a consensus sequence of each OUT is used as reference. The reference sequences of each OTU are blasted against the Ribosomal Database Project database (RDP II) (Cole et al., 2005) and the EBP/silva database designed by Chaillou et al. (2015) for taxonomic assignation. Relative abundances are estimated by counting the number of reads mapped on OTUs sequences. For both Qiime and EBP methods statistical analysis are performed manually.

2.1.5. Statistical analysis

The rarefaction curves were designed using command citation ("vegan") in R (Oksanen et al., 2016) and Qiime was used to calculate diversity and richness indices (Caporaso et al., 2010). To establish OTU relative abundance, the numbers of reads were normalized to the median value of total reads as described by Chaillou et al. (2015). For each sample read counts were divided by a normalization factor corresponding to the number of reads in the sample divided by the median value of total reads obtained for the 10 samples.