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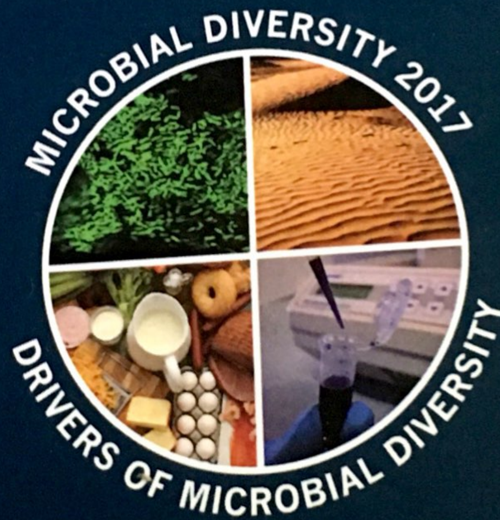

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16S rRNA GENE OLIGOTYPING REVEALS THE CORRELATION BETWEEN FOOD PROCESS AND FOOD QUALITY

FERROCINO Ilario (1)*, BOTTA Cristian (1), GIORDANO Manuela (1), RANTSIOU Kalliopi (1), COCOLIN Luca (1)

(1) DISAFA - Microbiology and Food Technology sector, University of Turin, Largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy

* Corresponding author: ilario.ferrocino@unito.it

Introduction

The new frontiers of microbial ecology are concerned with how microbes are involved in a complex ecosystem, such as food, and how the environmental conditions (e.g., changes in the process parameters, storage temperature, the addition of a starter culture or the use of antimicrobial agents) can affect the development and functioning of microbiota. High-throughput (HT) amplicon target sequencing is most frequently applied to study the microbiota (targeting the 16S gene) of the foods in order to find a correlation between the external perturbations and the evolution of the microbial composition (Ferrocino and Cocolin, 2017). The current methodology to analyze HT amplicon data is limited by the common use of a de novo clustering approach, relying on the clustering of sequences into operational taxonomic units (OTUs) at 97% or 99% of similarity threshold. Large areas of the 16S reference tree offer a poor taxonomic resolution due to lack of such isolated representatives and the clustering often resulting in phylogenetically mixed units. This approach makes it difficult to identify organisms in communities that differ from each other by a very small number of nucleotides. The 16S rRNA gene has limited specificity yet it is very sensitive, since a single nucleotide difference at the 16S rRNA gene level can predict remarkable genomic variation (Eren *et al.*, 2013). Unlike clustering methods, which compare all the positions in sequence reads to assess similarity, oligotyping utilizes the nucleotide positions that have been identified as the most information-rich by Shannon entropy calculations and allows resolution at a species level or even below (Eren *et al.*, 2013).

Recently oligotyping was used in environmental studies as a tool to identify subOTU level differences across samples (De Filippis *et al.*, 2016) or to discover the different adaptation efficiency of strains of the same species (Stellato *et al.*, 2017). In this study we expand the scope of oligotyping and demonstrate that it can successfully resolve key microbial diversity among numerically and ecologically important microbial taxa in the food matrix in order to have a better idea of the distribution of a specific taxon in a food-based system.

Materials and Methods

Three different food systems were analyzed through ripening or storage to assess shifts in the microbial community due to different process parameters (i), the use of antimicrobial agents (ii) and the addition of a starter culture.

i) Three farms producing Lard d'Arnad were selected. Two plants, reflecting the industrial process characterized either by low maturation temperature (plant A [10% NaCl, 2°C]) or by using a low NaCl concentration (plant B [2.5% NaCl, 4°C]) were selected, while the third was characterized by an artisanal process (plant C [30% NaCl, 8°C]). Lard samples were obtained at time 0 and after 7, 15, 30, 60, and 90 days of maturation (Ferrocino *et al.*, 2017).

ii) Beefsteaks pre-treated with aqueous ozone (AO) or electrolyzed water (EW) and vacuum packed. After storage at refrigerated condition the microbiota was analyzed over 15 days. Analysis of volatile compounds by GC-MS was also assessed (Botta *et al.*, 2017).

iii) *Lactobacillus plantarum* was used as a starter culture during Sataw-Dong fermentation (fermented stink bean in brine, made with *Parkia speciosa*). Two fermentations were performed: spontaneous (S) and plus starter (ST). The brine microbiota was analyzed throughout fermentation for 15 days by 16S HTS and by GC-MS (Jampaphaeng *et al.*, 2017).

Samples were collected across time/condition and were subjected to nucleic acid extraction followed by PCR amplification of the V3-V4 region of the 16S rRNA and then sequenced on a MiSeq Illumina platform. The sequences were quality filtered and an OTUs clustering approach was then applied to select the main OTUs. Reads from selected OTUs were then extracted and the Shannon entropy analysis was used to find only the significant discrimination in sub-species (oligotypes) by focusing on th variable sites. To minimize the noise, each oligotype was required to appe in at least 1 sample, occur in more than 1.0% of the reads for at least o

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sample, represent a minimum of 500 reads (M option) in all samples combined, and have a most abundant unique sequence with a minimum abundance of 50. Pairwise Wilcoxon tests were used in order to determine significant differences in specific oligotype abundance according to samples/time/treatment. Spearman correlation was also assessed between oligotype and VOCs.

Results

i) The main taxa identified in lard samples by sequencing were *Acinetobacter johnsonii*, *Psychrobacter*, *Staphylococcus equorum*, *S. sciuri*, *Pseudomonas fragi*, *Brochothrix*, *Halomonas* and *Vibrio* and differences in their relative abundance distinguished samples from the individual plants. The composition of the microbiota was more similar among plants A and B (industrialized process) and was characterized by the higher presence of taxa recognized as undesired bacteria in food processing environments. After Entropy analysis reads of *Acinetobacter* and *Halomonas* were extracted and analyzed. A higher number of oligotypes was found within *Acinetobacter*. 9 oligotypes were more abundant in A samples [10% NaCl, 2°C] (FDR <0.05), while 10 prevailed in B samples [2.5% NaCl, 4°C]. Only 4 oligotypes were characteristic of C samples. Seven *Halomonas* oligotypes were found more abundant in A samples while 4 characterized B samples and only 3 were associated with C samples [30% NaCl, 8°C].

ii) The targeted HT amplicon sequencing conducted on beefsteak identified *Pseudomonas fragi* as the most frequent species before and after the treatments with AO and EW, as well as in the untreated control. The tested treatments did not reduce its overall presence, but they did affect the intra-species distribution of its oligotypes. The oligotype analysis of *P. fragi* showed a total of 32 oligotypes, 9 of which associated with EW, 2 associated with AO and 3 with the control. When the correlation between *P. fragi* oligotype abundance and VOC profiles was analyzed, it was possible to observe several positive correlations between specific oligotypes and volatile compounds considered as a spoiler marker.

iii) The microbiota of Sataw-Dong during fermentation with the use of *L. plantarum* as a starter culture revealed the dominance of *L. plantarum* and *L. sanfranciscensis* indicating these taxa adapted well in brines. Using a starter markedly affected the microbiota after 1 day of fermentation and clearly influenced VOC profiles. Oligotyping of *L. plantarum* showed the presence of 53 oligotypes, however when comparing the relative abundance

only 12 were associated with spontaneous fermentation and 5 with the starter fermentation (FDR<0.05). The correlation analysis between oligotypes abundance and VOCs showed a strong correlation with oligotype associated with starter fermentation and esanoic and nonanoic acid as well as with disulfide, thus indicating a higher fermentation of carbohydrates and degradation of sulfur-containing amino acids. On the other hand oligotypes associated with spontaneous fermentation were positively correlated with esanal, nonanal, heptanal and pentanon. Sensorial analysis showed that starter-fermented Sataw-Dong significantly received a positive response in overall acceptance when compared with the natural ones.

Discussion

Several studies have clearly shown that environmental conditions induce a selection of the microbiota even at strain level and different biotypes can have different metabolic behaviors that can drastically affect the final characteristics of the products (Ercolini *et al.*, 2010). The use of oligotyping, a novel computational method, can elucidate subtle diversity within the final operational units of classification or clustering approaches by using the 16S data information. Here we used this approach to assess how changes in process parameters can affect the distribution of the oligotypes and how this difference can affect the final characteristics of the products. We found that changes in the process parameters (salt concentration and temperature) during lard d'Arnad manufacturing select the abundance of the oligotypes, clearly showing that the traditional method used to produce cured and ripened lard is effective in reducing the abundance of spoilage bacteria compared with industrial manufacturing. An oversimplified classification of taxa in a homogeneous OTU cannot disclose the possible strain-specific response to treatments, or the strain-specific relationship to VOCs. Analyzing two different food based systems (meat treated with antimicrobial agents and inoculated fermented beans) we clearly observed that different oligotypes from the same species showed distinctive correlation patterns with their volatilome. The selectiv development was therefore observed to be responsible for the sensori characteristics of the final products.

In conclusion this tool can also be easily applied to food-based studies order to ascertain an association between an oligotype and a process, or have a better idea of the distribution of a specific taxon in a food-ba system. A modulation of the main factors during food preparation

drastically affect the development of the microbiota and require an accurate study in order to preserve the food quality from the microbiota modulations.

Keywords
16S amplicon target sequencing, oligotyping, spoilage microbiota, fermentation, VOCs

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