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Highlighting the microbial diversity of 12 French cheese varieties

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ABSTRACT

Surface-ripened cheeses host complex microbial communities responsible for the transformation of milk into cheese as well as the development of important properties in terms of texture, color and sensory perception. In this study, we used high-throughput amplicon sequencing to decipher the bacterial and fungal diversity of 60 cheeses belonging to 12 popular French cheese varieties. Using this approach, 76 bacterial and 44 fungal phylotypes were identified. Major differences were observed between rind and core samples and also according to cheese varieties and manufacturing processes. Occurrence analysis revealed the presence of widespread taxa as well as operational taxonomic units (OTUs) specific to one or several cheese varieties. Finally, we observed patterns specific to the cheese production facility, supporting the importance of indigenous microorganisms for the microbial assemblage of cheese microbiota.

1. Introduction

Cheese is one of the oldest fermented food products. The cheese fermentation process is deeply related to culture and tradition, especially in rural households and village communities. Cheese has been produced and consumed for thousands of years and has been adapted to the technical, social and economic conditions in various parts of the world. Moreover, the technological processes associated with the manufacturing environment shape the chemistry and microbiology of local cheeses, which subsequently develop the visual, odor and texture characteristics that express and embody the identity of traditional cheese. There are over 1400 traditional cheese varieties identified around the world, displaying an amazing diversity of textures, aromas and flavors. France alone represents almost 1000 different cheese varieties, including 45 registered under the Protected Designation of Origin (PDO), a European protection program that covers traditional cheese practices (Donnelly, 2013). One of the main characteristics of PDO cheeses produced, processed and prepared from raw or heat-treated milk is that they harbor a complex microbial community. Through their growth and metabolic activities during the cheese-making process and ripening period, these microbial communities contribute to the transformation of milk into final products that are recognized, appreciated and envied by consumers worldwide (Montel et al., 2014). Thus, microbial communities appear to be major players in the expression and perception of cheese organoleptic properties as well as in terms of safety since they act as a biopreservative shield against microbial pathogenic and spoilage populations (Grattepanche et al., 2008; Marcelino, 2013).

Over the past 15 years, microbial diversity studies of several varieties of European cheeses that combine both genotypic and phenotypic approaches have partly described the complexity of such communities (Irlinger et al., 2015). It has been shown that each cheese has a specific, dense microbiota (2–3 × 10^9 cells/g cheese) composed of a few to several dozen species. Importantly, more than half of the microorganisms detected were not deliberately inoculated as starters but originated from the milk itself and/or the manufacturing environment (Feurer et al., 2004; Goerges et al., 2008). The molecular approaches based on the use of metagenomics combined with high-throughput sequencing (HTS) technologies now offer the unprecedented opportunity to profile dominant as well as subdominant cheese microbial populations on a large scale (Ercolini, 2013; Parente et al., 2016). For example, Wolfe et al. (2014) and Quigley et al. (2012) used such approaches to reveal the microbial composition of 137 cheese rind communities sampled in ten different countries and from 62 Irish artisanal cheeses, respectively. These pioneer studies allowed, for example, the correlation between abiotic factors such as moisture and rind type (natural, bloomy and washed) with microbial community composition. Such an approach was also used for identifying possible microbial reservoirs in two American cheese production facilities (Bokulich and Mills, 2013). Most HTS studies limit taxonomic affiliation to the genus level due to technological (e.g., sequence length) and/or data processing issues (e.g., sequence database availability). Currently, there is a need for increasing

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taxonomic resolution in order to study possible relationships that exist between cheese species composition and technological/environmental parameters.

The objective of this study was to investigate the specificity and diversity of cheese microbiota associated with 60 cheeses belonging to 12 traditional French cheese varieties manufactured from cow's milk, and including nine PDO cheese varieties. These varieties were selected from among uncooked washed-rind and natural-rind cheeses and covered a wide range of technological processes (Fig. 1). We used high-throughput amplicon sequencing (V3-V4 region of the 16S rRNA gene and ITS2) to characterize both the bacterial and fungal community composition and to propose sequence affiliation down to the species level whenever possible. Specifically, we aimed to determine whether microbial profiles can be used as a signature for a technological process, a cheese variety or even a cheese factory.

2. Material and methods

2.1. Sample collection and DNA extraction

Ready-to-consume cheeses were purchased from a master cheese ripener (Fromagerie Beaufils, Paris, France, http://www.fromagerie-beaufils.com/) who is directly supplied by different carefully-selected cheese producers, from local producers or from the local supermarket. A total of 60 cheeses were collected, encompassing 12 distinct cheese varieties and diverse manufacturing processes (Fig. 1). Regarding the Saint-Marcellin, Maroilles, Soumaintrain, Langres, Pont l'Evêque, Reblochon, Abbaye de Citeaux and Mont d'Or varieties, three independent cheeses were purchased at the same date. Rind was gently separated from the core using sterile knives and only the rind fraction was analyzed. Regarding Epoisses, Munster, Livarot and Saint-Nectaire, which were chosen to cover four contrasted manufacturing processes (coagulation mostly due to lactic acidification, mixed or mostly due to rennet action and for the last one without pressure or with high pressure), the sampling effort was greater. For each variety, three independent cheeses were purchased at the same date from three different producers (named Factories 1, 2 and 3; different factories were selected for each cheese variety), for a total of nine cheeses per variety, in order to evaluate variation observed within cheese varieties. Rind was gently separated from the core using sterile knives and both rind and core fractions were analyzed in order to obtain a more detailed picture of the microbial diversity for those cheeses. Cheese samples were diluted 1:10 (w/v) in sterile distilled water and homogenized with an Ultra Turrax® (Labortechnik) at 8000 rpm for 1 min. DNA extraction was performed on 0.5 g of the mixture using the bead beating-based protocol detailed in a previous study (Dugat-Bony et al., 2015). DNA concentration was determined using a Qubit fluorometer (Life Sciences) according to the Broad Range DNA assay kit protocol.

2.2. Bacterial 16S rRNA gene amplification and sequencing

PCR amplification, library preparation and sequencing were performed at the GeTPlaGe platform (Toulouse, France). Briefly, the V3-V4 region of the 16S rRNA gene (nearly 460 bp long) was amplified from 10 ng of purified genomic DNA using the primers F343-TACGGRAGGCAGCAG (Liu et al., 2007) and R784-TACCAGGGTA TCTAATCCT (Andersson et al., 2008), as previously described (Lazuka et al., 2015). The resulting libraries were purified using Ampure beads (Beckman Coulter Genomics) and loaded onto the Illumina MiSeq cartridge according to the manufacturer's instructions. Each paired-end sequence was assigned to its corresponding sample according to a previously integrated index.

2.3. Fungal ITS2 amplification and sequencing

PCR amplification, library preparation and sequencing were performed at Genoscreen (Lille, France). Briefly, the Illumina sequencing MiSeq ITS2 libraries were prepared by PCR from 10 ng of purified genomic DNA according to the GenoScreen protocol, using the primers ITS3-GCATCGATGAAGAACGCAGC (White et al., 1990) and ITS4_KYO1-TCCTCCGCTTWTTGWTWTGC (Toju et al., 2012), generating amplicons with a size ranging from 240 to 400 bp. According to Illumina (personal communication), this difference (<200 bp) don't bias the results. The resulting libraries were purified using Ampure beads (Beckman Coulter Genomics) and loaded onto the Illumina MiSeq cartridge according to the manufacturer's instructions. Each single-end sequence

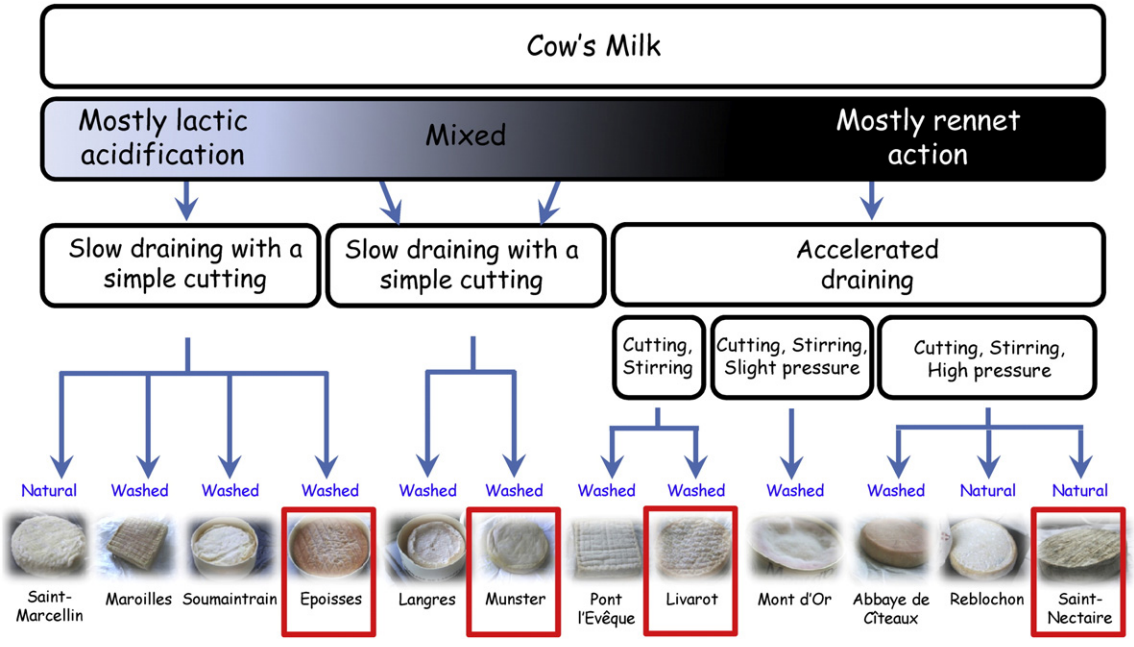

Fig. 1. Classification of the 12 studied cheese varieties according to their manufacturing process. For all of the cheese varieties, three independent cheeses were sampled and the rind was analyzed. For four cheese varieties, highlighted in red, three independent cheeses were sampled in three different factories and both rind and core were analyzed. Rind type is written in blue.