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Short communication

Fungal diversity of "Tomme d'Orchies" cheese during the ripening process as revealed by a metagenomic study

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ABSTRACT

Tomme d'Orchies is an artisanal pressed and uncooked cheese produced and marketed in the north of France. This study aimed at showing the fungal microbiota evolution of this cheese using a metagenetic based Illumina technology targeting the ITS2 domain of 5.8S fungal rDNAs. To this end, samples were taken from the rind and the core of different cheeses, after 0, 1, 3, 7, 14 and 21 days of ripening. The data underpinned the prevalence of *Yarrowia lipolytica* and *Galactomyces geotrichum* for both microbiotas. Unusual species including *Clavispora lusitaniae*, *Kazachstania unispora* and *Cladosporium cladosporioides* were also detected, but their origins remain to be ascertained. The metagenomic revealed also the presence of *Kluyveromyces* and *Debaryomyces* species.

1. Introduction

Advances in genomic and metagenomic sequencing are providing researchers and industrialists with insightful informations on the bacterial, fungal and viral diversities in many traditionally produced foods. DNA sequencing based technologies applied for different habitats provided a snapshot of the microbial diversity within and across environments (Lozupone and Knight, 2007). Large differences from one environment to another one were reported, and the number of species occupying such niches can reach hundreds even thousands (Lozupone and Knight, 2007). Complex ecosystems are not easy to rebuild *in vitro* because of the non-cultivable hallmark of some species (Wolfe and Dutton, 2015). This drawback avoids therefore to understand the role of each species. Cheese microbiota is a complex ecosystem contributing tightly for cheese flavor, aroma, texture, and appearance (Beresford et al., 2001; Irlinger and Mounier, 2009; Ogier et al., 2002). Fungal species found in cheese are known to exert positive effects, but some species as *Penicillium commune* (Lavoie et al., 2012), or *Debaryomyces hansenii* at high concentration (Fleet, 2011) could be deleterious. These microorganisms are thought to be issued from raw milk, and other sources as the environment of production and ripening (Atanassova et al., 2016; Wojtatowicz et al., 2001). Interestingly, Wolfe et al. (2014) sequenced 137 different rind microbiotas from 10 countries, and concluded that at least 60% of the bacterial, and 25% of the fungal communities were other than the starter cultures.

Studies of fungal communities based on the amplification and sequencing of the ITS1-5.8S-ITS2 regions are suitable and adequate for the next generation sequencing methods, or NGS (Schoch et al., 2012). The 5.8S rDNA is highly conserved and could permit accurate taxonomic information, conversely to the ITS1 and ITS2 regions, which are highly variable give the species status (Lindahl et al., 2013). The NGS Illumina sequencing allow high throughput sequencing of total DNA without any prior culture, and provide therefore data on the viable-but-not-cultivable strains (Bokulich and Mills, 2012). The NGS Illumina technology is anticipated to evolve and become likely a routinely used method because of its swiftness and ease of utilization (Delcenserie et al., 2014; Franzosa et al., 2015). This technology leads to DNA fragments of about 35 to 150 bp, which are smaller than those reported for the ITS1-5.8S-ITS2 rDNA; an approach tightly recommended for yeast and fungi identifications (Schoch et al., 2012). According to Lindahl et al. (2013), the sequencing of the ITS2 domain could be used for fungal communities identification. Notably, the ITS2 region is smaller than the ITS1 domain, allowing more compatibility with Illumina technology.

The "Tomme d'Orchies" is a French artisanal cheese manufactured and marketed in the North of the country for less than 50 years. This uncooked pressed cheese, which is washed twice during ripening, is made with a local raw milk of Holstein cows. The use of a culture-dependent approach (Ceugniez et al., 2015), and a metagenomic technology (Ceugniez et al., 2016) permitted to draw the first yeast and bacterial snapshots. Besides, the aforementioned reports portrayed some unusual bacterial species, and yeasts with beneficial properties.

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Table 1
Fungal microbiota distribution, at the species level, in the core of the "Tomme d'Orchies" cheese, according to the period of ripening. Grey shading correspond to strain added as starter. Bold writing were molds, fine writing were yeasts.

		Ripening (Day)						Average
OTU		0	1	3	7	14	21	
Yarrowia	*lipolytica*	74.75%	72.92%	85.18%	85.03%	89.67%	86.42%	83.33%
Galactomyces	*geotrichum*	24.04%	26.36%	13.96%	14.31%	9.32%	13.12%	16.85%
Saccharomyces	*cerevisiae*	0.25%	0.23%	0.10%	0.14%	0.08%	0.08%	0.15%
Aspergillus	***niger***	0.10%	0.16%	0.12%	0.08%	0.10%	0.06%	0.11%
Cryptococcus	sp.	0.08%	0.02%	0.08%	0.12%	0.17%	0.12%	0.10%
	curvatus	-	-	-	-	0.02%	0.02%	0.01%
Kazachstania	*unispora*	0.23%	0.04%	0.08%	-	0.10%	0.02%	0.08%
Candida	*rugosa*	0.13%	-	0.04%	0.02%	0.12%	0.02%	0,06%
	intermedia	-	-	0.02%	0.06%	0.08%	0.02%	0,03%
	parapsilosis	-	-	-	-	0.02%	-	<0.01%
Debaryomyces	*hansenii*	0.08%	0.06%	0.04%	0.02%	0.04%	0.02%	0.05%
Kluyveromyces	*marxianus*	0.04%	0.02%	0.06%	0.04%	0.06%	0.04%	0,05%
Torulaspora	*delbrueckii*	0.02%	0.06%	0.02%	0.06%	0.02%	-	0,03%
Trichosporon	sp.	0.06%	0.04%	-	-	0.06%	-	0.03%
	ovoides	0.02%	-	0.06%	0.02%	-	-	0.02%
	montevideense	-	-	0.02%	-	0.04%	-	0.01%
Pichia	*fermentans*	0.04%	-	0.06%	-	0.02%	0.02%	0,02%
Lewia	***infectoria***	0.02%	0.02%	0.04%	-	0.02%	-	0,02%
Clavispora	*lusitaniae*	0.02%	-	0.02%	-	-	0.02%	0,01%
Cladosporium	***cladosporioides***	0.02%	-	-	0.02%	-	-	0,01%
Myrothecium	**sp.**	-	0.02%	-	-	-	-	<0.01%
Nectria	**sp.**	-	-	0.02%	-	-	-	<0.01%
Unidentified		0.08%	0.04%	0.06%	0.04%	0.06%	-	0.05%

To gain more insights on the fungal microbiota, we analyzed samples withdrawn from different cheeses along ripening, and drawn a comprehensive overview on yeast and mold diversities of "Tomme d'Orchies" cheese.

2. Materials and methods

2.1. Cheese manufacturing and sampling

Tomme d'Orchies cheese was obtained from "G.A.E.C de la Motte" farm, located in Nomain (north of France). Cheese samples used here served as well to establish the bacterial metagenomic analysis (Ceugniez et al., 2016). Notably, *D. hansenii* PAL DH D purchased from Standa laboratories (Caen, France) was the only yeast employed as starter. To follow fungal diversity evolution along ripening, six different cheeses were sampled at 0, 1, 3, 7, 14 and 21 days. One sample was taken from each cheese, and each cheese corresponds to one time of ripening. Afterwards, each sample was divided into rind and core before analysis, without replicates.

2.2. DNA extraction and purification

Cheese samples were centrifuged (5000*g*, 10 min, 4 °C), and the resulting pellets were treated for 1 h at 37 °C with a lysing buffer containing Tris-HCl (20 mM), EDTA (2 mM), Triton X-100 (1.2%) and lysozyme (20 g/L). Total DNA was extracted from each primary suspension with the DNeasy Blood & Tissue DNA extraction kit (Qiagen, Venlo, the Netherlands), using the manufacturer's recommendations. Total DNA was then eluted into DNAse/RNAse-free water and quantified with a NanoDrop ND-1000 spectrophotometer (Isogen, St-Pieters-Leeuw, Belgium). Pure DNA samples were stored at − 20 °C until to be used for 5.8S-ITS2 rDNA sequencing.

2.3. 5.8S-ITS2 rDNA gene library construction and sequencing

The 5.8S-ITS2 rDNA PCR libraries were prepared for each cheese sample using universal primers with Illumina overhand adapters targeting the ITS2 region. The forward primer ITS3KYO2 (5′-GATGAAGAACGYAGYRAA-3′), and the reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used for their high coverage of fungi taxon (Toju et al., 2012). Each PCR product was purified with the Agencourt AMPure XP beads kit (Beckman Coulter, Pasadena, USA). A second indexing PCR was performed with the Nextera XT index primers 1 and 2 (Illumina, San Diego, USA). The PCR products were purified as indicated above. Quantifications were made with the Quant-IT PicoGreen (ThermoFisher Scientific, Waltham, USA). To constitute a library, each PCR product, after quantification, was diluted to 10 ng/μL with Tris 10 mM Tween 20 0.05% and all PCR products were mixed together. A 1% agarose gel permitted to check, if the library was free of unwanted bands, if not, a new purification was performed with AMPure XP beads. A precise quantification, by qPCR, of each sample in the library was performed using the KAPA SYBR® FAST qPCR Kit (Kapa-Biosystems, Wilmington, USA) before normalization, pooling and sequencing on a MiSeq sequencer using v3 reagents (Illumina, USA).

2.4. Bioinformatics analysis

Sequence reads processing were used as reported by Rodriguez et al. (2015) using MOTHUR software package v1.35 (Schloss et al., 2009), and UCHIME algorithm (http://drive5.com/usearch/manual/uchime_

Table 2
Fungal microbiota distribution, at the species level, on the rind of the "Tomme d'Orchies" cheese, according to the period of ripening. Grey shading correspond to strain added as starter. Bold writing were molds, fine writing were yeasts.

		Ripening (Day)						Average
OTU		0	1	3	7	14	21	
Yarrowia	*lipolytica*	79.70%	92.63%	60.47%	76.49%	61.43%	81.47%	75.37%
Galactomyces	*geotrichum*	19.51%	6.64%	38.10%	22.67%	34.98%	17.45%	23.23%
Aspergillus	***niger***	0.12%	0.15%	0.47%	0.15%	0.76%	0.17%	0.30%
Saccharomyces	*cerevisiae*	0.17%	0.06%	0.37%	0.17%	0.52%	0.23%	0.25%
Cryptococcus	sp.	0.12%	0.08%	0.06%	0.12%	0.41%	0.04%	0.14%
	curvatus	--	-	-	-	0.02%	-	<0.01%
Kluyveromyces	*marxianus*	0.02%	0.06%	0.08%	0.04%	0.29%	0.10%	0.10%
	lactis	-	-	-	0.02%	-	-	<0.01%
Kazachstania	*unispora*	0.12%	-	0.08%	0.06%	0.21%	0.02%	0.08%
Candida	*rugosa*	0.04%	0.04%	0.04%	0.02%	0.16%	0.08%	0.07%
	intermedia	-	0.02%	0.02%	-	0.27%	0.02%	0.06%
	zeylanoides	-	-	-	-	0.02%	-	<0.01%
Pichia	*fermentans*	0.04%	0.02%	0.02%	0.04%	0.04%	0.06%	0.04%
Torulaspora	*delbrueckii*	-	0.02%	0.02%	-	0.06%	0.10%	0.04%
Trichosporon	*ovoides*	-	-	0.08%	0.04%	0.04%	0.04%	0.03%
	gracile	-	-	0.10%	-	0.02%	-	0.02%
	sp.	-	-	-	0.02%	0.06%	0.02%	0.02%
	asahii	-	0.02%	-	-	-	-	<0.01%
	guehoae	-	-	-	-	0.02%	-	<0.01%
	montevideense	-	-	-	-	0.02%	-	<0.01%
Cladosporium	***cladosporioides***	-	0.02%	-	0.02%	0.14%	0.02%	0.03%
Debaryomyces	*hansenii*	-	0.02%	0.02%	0.02%	0.06%	0.02%	0.02%
Clavispora	*lusitaniae*	0.02%	0.02%	-	0.04%	0.02%	0.02%	0.02%
Malassezia	*restricta*	0.02%	-	-	0.02%	0.02%	0.02%	0.01%
Lewia	***infectoria***	-	0.02%	-	-	0.04%	-	0.01%
Exophiliala	*phaeomuriformis*	-	0.02%	-	-	-	-	0.01%
Nectria	**sp.**	-	0.02%	-	-	-	-	<0.01%
Mucor	***circinelloides***	0.02%	-	-	-	-	-	<0.01%
Unidentified		0.08%	0.12%	0.04%	0.04%	0.37%	0.08%	0.12%

algo.html). The clustering distance for operational taxonomic unit (OTU) is 0.03. ITS2-5.8S rDNA reference alignment, and taxonomical assignation were based upon the UNITE database v6 (Kõljalg et al., 2013) of full-length ITS1-5.8S-ITS2 rDNA sequences, with an average length of 351 bp. All the biosample raw reads were deposited at the National Center for Biotechnology Information (NCBI) and now available under the Bioproject ID PRJNA355071.

3. Results

3.1. Global cheese content

The metagenetic study underpinned fungal OTUs encompassing family, genera and species taxonomic levels. A total of 30 OTUs containing 4 genera and 26 species were observed for this cheese. Molds were composed of 2 genera and 4 species. Notably, 0.05% of the sequences obtained from the core, and 0.12% from the rind were not identified (Tables 1 and 2).

3.2. Core content

The core of this cheese appeared to be dominated by *Yarrowia lipolytica* and *Galactomyces geotrichum*. At the beginning of the ripening (day 0), we noted that 74.75% of the sequences were allocated to *Y. lipolytica*, afterwards these sequenced had increased to 86.42%, after 21 days of ripening (Table 1). On the other hand, *G. geotrichum* was present at 24.04% at the beginning of ripening before decreasing to 13.12%, at the end of the process. These two species represent noteworthy 98.79% to 99.55% of the total sequences allocated for the fungal content. The sequences corresponding to the starter *D. hansenii* were ranging from 0.08% (at the beginning) to 0.02% (at the end) of the repining. The unidentified sequences were present at 0.08% at the beginning of the ripening before disappearing at the end of the ripening (Table 1). The core contains specific OTUs fitting with *Candida parapsilosis* and *Myrothecium* sp. Thus, we noticed 5 genera, including *Aspergillus*, *Lewia* (also known as *Alternaria*), *Cladosporium*, *Myrothecium* and *Nectria*. Notably, *Aspergillus niger* resulted to be the upmost prevalent species.

3.3. Rind content

Y. lipolytica and *G. geotrichum* species were also prevalent in the rind. The sequences allocated to *Y. lipolytica* ranged from 79.70% (at the beginning) to 81.47% (at the end) of the ripening (Table 2), while

sequences of *G. geotrichum* remained stable along ripening. Indeed, at the beginning, *G. geotrichum* sequences were estimated to 19.51%, and decreased slightly to 17.45% at the end of ripening (Table 2). Similarly, these two species are also prevalent in the rind and represent 99.21% of total sequences at the beginning, and decreased slightly to 98.92% at the end of the ripening. Specific OTUs corresponding to *Candida zeylanoides*, *Exophiala phaeomuriformis*, *Kluyveromyces lactis*, *Malassezia restricta*, *Trichosporon asahii*, *T. gracile*, *T. guehoae* and *Mucor circinelloides* were also evidenced here. Furthermore, sequences allocated to starter *D. hansenii* were estimated to 0.1%. The rind microbiota appeared to be dominated by *Y. lipolytica*, *G. geotrichum* species, and at a lesser extent *D. hansenii*. Moreover, 18 different OTUs were evidenced. In this case, each OTU contained more than 0.01% of the total sequences. Therefore, the sequences resulting from these additional 18 OTUs were estimated to 0.743%, (at the beginning) and 1.08%, at the end of the ripening. Nevertheless, a peak of 3.13% of the sequences was observed after 14 days of ripening.

Molds potentially present in rind microbiota include the following genera: *Aspergillus*, *Cladosporium*, *Lewia*, *Nectria*, and *Mucor*. The most abundant species were *A. niger* and *Cladosporium cladosporioides*.

In the rind, we detected 0.08% of total sequences without any allocation. These non-identified sequences were remained overall stable along ripening of cheese (Table 2).

4. Discussion

Recently, we established the yeast diversity of “Tomme d'Orchies” using a culture-dependent approach (Ceugniez et al., 2015), to gain more insights on the fungal microbiota of this cheese, we carried out a metagenomic study based on the Illumina technology. Nevertheless these approaches were not fully concordant. While these methods permitted accurate identification of *Clavispora lusitaniae*, *D. hansenii*, *Kluyveromyces marxianus*, *K. lactis* and *Y. lipolytica*, the culture-dependent approach, but not the metagenetic one, allowed adventitious identification of *Saturnispora mendoncae*. This discrepancy could be explained by the absence of ITS2-5.8S rDNA sequences in the UNITE database matching with this species. On the other hand, the metagenetic technology permitted identification of a large panel of species including *Candida intermedia*, *C. parapsilosis*, *C. rugosa*, *C. zeylanoides*, *Cryptococcus curvatus*, *E. phaeomuriformis*, *G. geotrichum*, *Kazachstania unispora*, *M. restricta*, *Pichia fermentans*, *Saccharomyces cerevisiae*, *T. asahii*, *T. gracile*, *T. guehoae*, *T. montevideense*, *T. ovoides* and *Torulaspora delbrueckii*. The Illumina based metagenomic approach permitted to identify *A. niger*, *C. cladosporioides*, *M. circinelloides*, *L. infectoria*, *Myrothecium* sp., and *Nectria* sp.

In this analysis, we noticed the presence of *Malassezia*; a species which usually is isolated from human skin. Delavenne et al. (2011) underpinned the adventitious presence of *M. restricta* species in cow's and ewe's milk, while Guého-Kellermann et al. (2011) designed the manufacturer's skin as the main source. To the best of our knowledge, this is the first report underlining the presence of this fungal species in an artisanal cheese.

The culture-dependent approach recently conducted, and the current metagenetic analysis permitted to detect *Y. lipolytica* at a high proportion. The presence of this species in cheese is thought to be mediated in some strains by galactose and broadly by lactic acid (Kurtzman, 2011). *Y. lipolytica* has been reported as an emerging opportunistic pathogen specifically when afflicting immunodeficient patients (Jacques and Casaregola, 2008).

In turn, *G. geotrichum* might be originated from raw cow milk, as previously reported (Boutrou et al., 2006). Regarding *D. hansenii*, we noticed a weak proportion in “Tomme d'Orchies” along ripening, in contrary to Banjara et al. (2015) who indicated its prevalence in different cheeses.

A low fungal presence, with more than 0.11% of the total sequences, was registered for *S. cerevisiae*, *A. niger* and *Cryptococcus* sp. (Tables 1 and 2). Usually rinds are composed of a body of bacterial and fungal species issued from raw milk, starter cultures added by the cheesemakers, the aging environment, and likely other unknown sources (Fox et al., 2004; Quigley et al., 2013). The presence of *S. cerevisiae* in the rind could be explained by the rubbing of this cheese in a mix of brown beer. Nevertheless, the presence of *Cryptococcus* is not unusual as this species was reported as key species in the surface-ripened cheese (Corsetti et al., 2001), and other dairy products (Jakobsen and Narvhus, 1996; Lopandic et al., 2006). The low levels of both *K. marxianus* and *K. lactis* was noticed in previous studies (Delavenne et al., 2011; Cocolin et al., 2002). Other identified OTUs were detected at under 0.02% and 1.9% in the rind and in the core, respectively. The unusual cheese contaminant *C. lusitaniae* (Jacques and Casaregola, 2008; Jakobsen and Narvhus, 1996) was also found here. On the other hand, *C. rugosa*, *P. fermentans* and *T. delbrueckii* were found in raw milk (Cocolin et al., 2002; Delavenne et al., 2011; Fadda et al., 2004), whereas *Trichosporon ovoides* in human and animal sources (Fadda et al., 2004).

C. intermedia was found in different surface-ripening cheeses (Atanassova et al., 2016; Corsetti et al., 2001; Jacques and Casaregola, 2008), and was also reported as a contaminant of artisanal products (Lachance et al., 2011). Finally, *Kazachstania unispora* which is an unusual species, was encountered in fermented mare milk, semi-hard cow's milk, and other cheeses (Lopandic et al., 2006; Vaughan-Martini et al., 2011).

To sum-up, the “Tomme d'Orchies” possesses a fungal diversity quite similar to the previously reported for semi-soft cheeses (Corbo et al., 2001; Corsetti et al., 2001; Lopandic et al., 2006; Minervini et al., 2001). However the “Tomme d'Orchies” cheese contains some usual species at proportions that are usually different from other cheeses (data not shown). Notably for *Y. lipolytica* and *G. geotrichum*, resulted to be the most abundant species in both core and rind parts of the cheese.

The Illumina technology targeting the ITS2-5.8S rDNA permitted us to gain insights on the fungal diversity of this artisanal cheese and show its capabilities to described fungal cheese microbiota. However, some adjustment in the database content was needed to be totally functional.

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