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Cultivation-independent analysis of microbial communities on Austrian raw milk hard cheese rinds

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ABSTRACT

"Vorarlberger Bergkäse" (VB) is an Austrian artisanal hard cheese produced from raw cow's milk. The composition of its rind microbiota and the changes in the microbial communities during ripening have not previously been investigated. This study used 16S and 18S rRNA gene cloning and sequencing to characterize the bacterial and fungal communities of seven pooled cheese rind samples taken in seven different ripening cellars of three Austrian dairy facilities. A total of 408 clones for 16S and 322 clones for 18S rRNA gene libraries were used for taxonomic classification, revealing 39 bacterial and seven fungal operational taxonomic units (OTUs). Bacterial OTUs belonged to four different phyla. Most OTUs were affiliated to genera often found in cheese, including high numbers of coryneforms. The most abundant OTU from 16S rRNA gene libraries showed highest similarity to *Halomonas*. Young cheese rinds were dominated by *Actinobacteria* or *Proteobacteria*, particularly by *Halomonas* and *Brevibacterium aurantiacum*, while *Staphyloccocus equorum* was most abundant in old cheeses. The most abundant 18S rRNA OTU had highest similarity to the filamentous fungus *Scopulariopsis brevicaulis*. Pairwise correlation analyses revealed putative co-occurrences between a number of OTUs. It was possible to discriminate the different cheese rind microbiota at the community-level by facility affiliation and ripening time. This work provides insights into the microbial composition of VB cheese rinds and might allow the processing- and ripening conditions to be improved to enhance the quality of the product.

1. Introduction

Cheese production is characterized by dynamic changes in the complex cheeses microbiome, which consists of both bacteria and fungi. An important period in cheese production is the ripening process, during which the cheese surface microbial community establishes itself and matures, facilitating the development of the cheese's organoleptic and textural properties. Bacteria and fungi on the cheese surface contribute to ripening due to their proteolytic and lipolytic activities and the production of volatile sulfur compounds as well as of ammonia. The metabolic activity of both yeasts and bacteria on the cheese surface, which either naturally develop during contact with air, or are intentionally inoculated during the ripening process, are of great importance (Irlinger and Mounier, 2009; Montel et al., 2014). From the perspective of microbial ecology, the cheese surface and core are fundamentally different environments; the latter becomes anaerobic during ripening, whereas the surface remains aerobic. Particularly surface-ripened cheeses, which are defined by additional ripening from the cheese surface to the interior, develop their flavor in this period. Surface-ripened cheeses can be divided into bacteria-ripened (e.g. Limburger, Munster, Tilsit, and Appenzeller) and mold-ripened (e.g. Camembert, Brie) cheeses (Brennan et al., 2002; Dolci et al., 2009; Mounier et al., 2005). Due to extensive washing of the cheese surfaces with brine during ripening, bacteria-ripened cheeses are also known as washed-rind cheeses and develop a red-brown glistening appearance. At the beginning of the ripening process lactate, produced by lactic acid bacteria, is metabolized by yeasts, primarily *Debaryomyces hansenii* and *Geotrichum candidum*, resulting in an increase in the pH of the cheese surface (Bockelmann et al., 2005; Irlinger and Mounier, 2009; Montel et al., 2014). These processes and the metabolism of yeast growth factors facilitate the growth of coagulase-negative staphylococci such as *Staphylococcus equorum* and salt-tolerant bacterial communities – mainly coryneforms – such as *Corynebacterium* spp., *Arthrobacter* spp., *Micrococcus* spp., *Brevibacterium* spp. and *Brachybacterium* spp. Finally, bacteria and yeasts cover the entire surface of the cheeses. Coryneform bacteria are particularly abundant in cheese rinds (Delbes et al., 2007; Gori et al., 2013; Maoz et al., 2003; Rea et al., 2007). In addition, Gram-negative bacteria such as *Halomonas* spp., *Vibrio* spp., and *Hafnia alvei* have been detected on cheese surfaces (Brennan et al., 2002; Coton et al., 2012; Feurer et al., 2004a; Gori et al., 2013; Irlinger and Mounier, 2009; Ishikawa et al., 2007; Maoz et al., 2003; Mounier et al., 2005; Rea

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et al., 2007). Austrian Vorarlberger Bergkäse (VB) with a protected designation of origin (PDO) is an artisanal long-ripened hard cheese produced in a long-established cheese producing area in the western part of Austria (Vorarlberg). Cheese production and cheese treatment relies exclusively on traditional techniques including the use of raw cow's milk, the addition of a defined combination of starter cultures, and the brining of cheese wheels either in a brine bath or by dry salting surface treatment (Table 1). Except for the treatments that allow the development of the characteristic yellow-orange rind, no other treatment is applied during the ripening process. Based on market demand and customer requirement, ripening takes at least three months and may last for up to 18 months. Highly similar types of hard cheese, e.g. "Tiroler Bergkäse PDO" or "Allgäuer Bergkäse PDO" are produced in neighboring regions of the Alps. There have been a number of studies of smear- and mold-ripened soft or semi-soft cheeses. However, the structure of microbial communities of raw milk hard washed-rind cheeses such as VB has received only limited attention (Irlinger and Mounier, 2009; Jany and Barbier, 2008; Montel et al., 2014; Ndoye et al., 2011; Quigley et al., 2011). Some recent studies have explored the community structure of cheeses and cheese rinds by high-throughput pyrosequencing (Alegria et al., 2012; Masoud et al., 2011; O'Sullivan et al., 2013; Quigley et al., 2012) but, the cheeses investigated differed from VB. Despite the advantages in terms of throughput and sequencing depth of the novel sequencing technologies, we used rRNA cloning and Sanger sequencing to provide the longest possible sequences and thus the best possible taxonomic assignments, as only few rRNA data are currently available on VB and similar cheeses.

We have examined the diversity of bacteria and fungi in rind samples of Austrian VB cheese and the alterations in species abundances associated with different ripening times and production facilities. We hypothesized that VB cheese rinds harbor a high bacterial diversity and that there are significant community shifts with longer ripening times. Based on 16S and 18S rRNA gene cloning and sequencing, we characterized the microbial rind communities from seven pooled cheese rind samples belonging to three cheese producing facilities.

2. Materials and methods

2.1. Cheese production

VB cheeses were produced from a single morning milking of raw cow's milk. A defined combination of starter cultures (*Streptococcus thermophilus*, *Lactococcus delbrueckii* ssp. *lactis* and *Lactobacillus casei*) was added to the preheated milk before calf's rennet was added. Curd was cut by using a cheese harp while temperature was gradually raised to 48–50 °C. After a short rest in whey, curd was collected, molded and pressed to drip off residual whey. Cheese wheels were stored in 20–22% brine for 48 h to 60 h before cheeses were stored in the ripening cellars. Except in ripening cellar B1, in which dry salting was used during the first week of ripening, cheese wheels were treated with a distinct concentration of brine only at scheduled intervals and no ripening cultures were added (Table 1). VB ripens for at least three months before it is ready for sale but it is often ripened for six months or longer. The final cheese wheels have a diameter of 50 to 55 cm and a weight of approximately 30 kg. The external appearance of a ready-for-sale product – depending on the ripening time – is a yellow to orange cheese rind with a hard cheese body texture. Although slightly different dairy technologies are used during cheese production and ripening, the cheeses from all three facilities are sold as VB PDO with the main organoleptic and textural differences attributable to differences in ripening time.

2.2. Cheese rind sampling

Seven pooled cheese rind samples (A1, A2, A3, B1, B2, C1, C2) from three different cheese producing facilities A, B and C consisting of two to three different ripening cellars in Vorarlberg, Austria were taken in July 2012. For each pooled sample, 25 to 30 different cheese wheels within each ripening cellar were sampled by scraping cheese rinds with sterile scalpels. The entire surface of each cheese wheel at the end of the ripening stage was sampled by a single person. Samples were stored on ice at 4 °C for transport to the laboratory.

2.3. DNA extraction from cheese rind samples

Six DNA extractions were performed for each of the pooled samples. The samples were homogenized before DNA extraction. DNA was extracted from 200 mg of pooled cheese rind with the PowerSoil™ DNA Isolation kit (MoBio Laboratories, Carlsbad, California, USA), used in accordance with the manufacturer's instructions. The DNA concentration was measured with a Qubit® fluorometer (Invitrogen, Vienna, Austria). 50 μl of each DNA sample was pooled and precipitated with 3 M sodium acetate and ethanol (96%). The DNA pellet was resuspended in 10 mM Tris–HCl (pH 8) and stored at −20 °C.

2.4. 16S and 18S rRNA gene amplification

To detect the bacterial microflora of cheese rind samples, 16S rRNA gene PCR was performed using the primers 616 F (5′-AGA GTT TGA TYM TGG CTC-3′, *E. coli* 16S rRNA positions 8 to 27) (Juretschko et al., 1998) and 1492R (5′-GGY TAC CTT GTT ACG ACT T-3′, *E. coli* 16S rRNA positions 1492 to 1510) (Lane, 1991). Each PCR reaction was performed in a final volume of 50 μl, containing 0.2 pmol/μl of each primer, 0.8 mM dNTP-mix (TaKaRa, Saint-Germain-en-Laye, France), 1 × Ex Taq Buffer (TaKaRa, Saint-Germain-en-Laye, France), 0.025 U TaKaRa Ex Taq HS (TaKaRa, Saint-Germain-en-Laye, France), 1 μl DNA template and DEPC-treated water (Thermo Scientific, Vienna, Austria). 16S rRNA gene PCR was performed under the following conditions: initial denaturation at 95 °C for 5 min, 25 cycles at 94 °C for 40 s, annealing at 52 °C for 40 s, elongation at 72 °C for 1 min and a final elongation at 72 °C for 7 min.

Table 1
Properties of the ripening cellars, cheese treatment and starter cultures.

Facility	Ripening cellar	Sample designation	Ripening age [months]	Temperature [°C]	Humidity [%]	Cheese treatment with brine	Brine concentration [%]	Starter cultures for cheese production
A	A1	A1	0–4	13.5	96	Daily	20	*Streptococcus thermophilus, Lactococcus delbrueckii ssp. lactis, Lactobacillus casei*
	A2	A2	0–4	13.5	96	Daily	20	
	A3	A3	4–18	10	95–96	Weekly	10	
B	B1	B1	0–6	13	93–94	2–3 times a week	10	*Streptococcus thermophilus, Lactococcus delbrueckii ssp. lactis, Lactobacillus casei*
	B2	B2	6–10	13	93–94	Weekly	15	
C	C1	C1	6–14	11	93	Weekly	2–3	*Streptococcus thermophilus, Lactococcus delbrueckii ssp. lactis, Lactobacillus casei*
	C2	C2	0–6	13	94	3–4 times a week	22	

Brine washing of cheese surfaces was performed in all cellars. Dry salting of cheese surfaces was only applied in ripening cellar B1 (during day 0 to 6; each side three times with 45 to 50 g NaCl per side).
Starter cultures were obtained from the Federal Institute for Alpine Dairying, Rotholz, Austria.