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Autochthonous facility-specific microbiota dominates washed-rind Austrian hard cheese surfaces and its production environment

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ABSTRACT

Cheese ripening involves the succession of complex microbial communities that are responsible for the organoleptic properties of the final products. The food processing environment can act as a source of natural microbial inoculation, especially in traditionally manufactured products. Austrian Vorarlberger Bergkäse (VB) is an artisanal washed-rind hard cheese produced in the western part of Austria without the addition of external ripening cultures. Here, the composition of the bacterial communities present on VB rinds and on different processing surfaces from two ripening cellars was assessed by near full length 16S rRNA gene amplification, cloning and sequencing. Non-inoculated aerobic bacteria dominated all surfaces in this study. VB production conditions (long ripening time, high salt concentration and low temperatures) favor the growth of psychro- and halotolerant bacteria. Several bacterial groups, such as coryneforms, *Staphylococcus equorum* and *Halomonas* dominated VB and were also found on most environmental surfaces. Analysis of OTUs shared between different surfaces suggests that VB rind bacteria are inoculated naturally during the ripening from the processing environment and that cheese surfaces exert selective pressure on these communities, as only those bacteria better adapted flourished on VB rinds. This study analyzed VB processing environment microbiota and its relationship with VB rinds for the first time, elucidating that the processing environment and the cheese microbiota should be considered as microbiologically linked ecosystems with the goal of better defining the events that take place during cheese maturation.

1. Introduction

Cheese is one of the oldest fermented foods. There is proven evidence for cheese making in the sixth millennium B.C. and its manufacture is strongly related with regionally different technical, social and economic conditions (Irlinger et al., 2015; Salque et al., 2013). Particularly in long-ripened raw milk cheeses, cheese fermentation involves a complex and dynamic microbiome that establishes through the ripening process and strongly influences the characteristics of the final products including also food safety, as cheese microbiota can act as a natural barrier for pathogens and spoilage microorganisms (reviewed by Boldyreva et al., 2016).

Cheese rinds microbiota differs largely from core cheese microbiota and can develop either by direct inoculation of external ripening cultures and/or by natural colonization of microbes from the processing environment, which is in continuous contact with cheese rinds (Bokulich and Mills, 2012; Irlinger et al., 2015; Monnet et al., 2015). The processing environment encompasses many surfaces such as equipment, brine tanks, ripening rooms, vats, benches, clothes and human skin that can act as potential sources of microorganisms (Kousta et al., 2009; Montel et al., 2014). The successful colonization of these autochthonous microbial communities, summed up in the term “house microbiota”, relies on their ability to cope with environmental conditions such as low temperature, high salt content, humidity, pH, moisture control (brining, pressing), cleaning procedures and competition against other microbes (Bokulich and Mills, 2013; Mounier et al., 2006). Deacidification of the cheese surface by yeasts and molds which consume lactate and produce ammonia as well as extensive washing of cheeses with salt favors the establishment of acid-sensitive and salt- and psychro-tolerant bacteria, such as coagulase-negative cocci (CNC, mainly *Staphylococcus equorum*), Gram-positive coryneforms (*Brevibacterium*, *Corynebacterium*) and Gram-negative bacteria such as *Halomonas* (Coton et al., 2012; Delbès et al., 2007; Gori et al., 2013; Irlinger and Mounier, 2009; Mounier et al., 2005; Rea et al., 2007). Microbial

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populations involved in cheese ripening are often also found in the processing environment and it has been demonstrated that facility-specific "house" microbiota is implicated in the development of brand-specific characteristics of many traditionally manufactured cheeses (Bokulich and Mills, 2013; Dolci et al., 2009; Mounier et al., 2006; Van Hoorde et al., 2010). Moreover, exploring the relationships between ripening and production facility environment can be very useful to understand the impact of the house microbiota on the hygienic safety of the processed cheese products (Bokulich and Mills, 2013; Calasso et al., 2016; Mounier et al., 2006; Stellato et al., 2015). In contrast to our current study, prior studies have mainly focused on short-ripened soft or semi-soft cheeses. Washed-rind technology is used to homogenously spread microorganisms on the cheese surface to favor implantation of desirable bacteria, but is also sensitive to cross-contamination events due to the magnitude of manipulative steps applied.

Austrian Vorarlberger Bergkäse (VB) is an artisanal hard cheese produced in the western part of Austria (Vorarlberg) and has a protected designation of origin (PDO). Highly similar types of cheeses are produced in the adjacent regions of Austria and Germany. Its manufacture consists of traditional techniques including the use of raw cow milk exclusively from alpine pastures with the addition of starter cultures. VB is brined either in a brine bath or by dry salting surface treatment and no other treatment, such as the addition of external ripening cultures, is applied during ripening, which last from three to up to 18 months.

In a previous study, the bacterial and fungal composition of the rind microbiota of VB was assessed by using 16S and 18S rRNA cloning and Sanger sequencing, revealing a high diversity on VB cheese rinds with Gram-negative bacteria being particularly abundant (Schornsteiner et al., 2014). The question remained vital how the surface of the brined cheese is influenced by yet uncharacterized microbial communities originating from food-contact and non-food-contact surfaces in the ripening cellar.

We aimed to characterize the microbial communities present in VB rinds and in environmental surfaces including air filters, floors, racks, shelves and walls from two different ripening cellars from the same cheese production facility to identify shared bacteria between the different environments in this dairy plant.

2. Material and methods

2.1. Dairy plant, cheese production and sampling

For this study, two ripening cellars (A2 and A3) in an Austrian dairy plant have been sampled in November 2012. Cellar A2 contains VB from 0 to 4 months ripening time (short-ripening cellar), cellar A3 contains VB aged from 4 to 18 months (long-ripening cellar). Temperature in short- and long-ripening cellars was 13.5 °C and 10 °C, respectively. VB undergo different brining treatments in each cellar: daily treatment with 20% NaCl solution for VB in the short ripening cellar and weekly treatment with 10% NaCl solution in the long ripening cellar. For more details regarding the ripening cellars and the dairy plant, see Schornsteiner et al. (2014). Surface swab samples were taken during ripening with sterile dry sponge sticks (3 M) from the wooden shelves, racks, walls, floor and air filters, sampling an area of approximately one m^2 for each surface. With the exception of the air filters, within each ripening cellar, three different areas (i.e. shelves, racks, walls and floor) were sampled at the same time. For the cheese rinds, the surface of 25 to 30 cheese wheels was sampled with sterile scalpels and pooled for DNA extraction.

2.2. DNA extraction, 16S rRNA gene amplification, cloning and sequencing

100 mL sterile 1 × PBS buffer was added to the sponges in a sterile bag and homogenized for seven min in a lab blender (Stomacher 3500 Seward, UK). Subsequently, the cells were pelleted at 11,000 × *g* for 20 min at 4 °C. The three corresponding samples (i.e. from shelves, racks or walls) were then pooled and DNA isolation was performed from 250 mg of the remaining pellet using the MoBio PowerSoil Kit according to the manufacturer's instructions. To investigate the bacterial microbiota in the different environmental samples, 16S rRNA gene PCR was performed using the primers 616F (Juretschko et al., 1998) and 1492R (Lane, 1991). Each PCR reaction was performed in a final volume of 50 µL, containing 0.2 pM of each primer, 0.8 mM dNTP-mix (TaKaRa, Saint-Germain-en-Laye, France), 1 × Ex Taq Buffer (TaKaRa), 0.025 U TaKaRa Ex Taq HS (TaKaRa), 5 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, at 52 °C for 40 s, and at 72 °C for 1 min and a final elongation at 72 °C for 7 min.

PCR amplicons were ligated into the pSC-A-amp/kan PCR cloning vector using the StrataClone PCR Cloning Kit (Agilent Technologies, Vienna, Austria) and transformed into competent cells following the manufacturer's instructions.

One gene library was created for each environment (air filter, floor, shelves, racks and wall; 96 clones per environment) and for cheese rinds (48 clones per rind) from both cellars. In total, 1056 clones were sequenced using the vector-specific primers M13F and M13R, yielding approximately 1500 bp high-quality 16S rRNA gene sequences per clone. Sequencing was performed at LGC Genomics (Berlin, Germany). Fifty-four and 58 clone sequences from cheese rinds from short- and long-ripening cellars, respectively, were taken from our previous study (Schornsteiner et al., 2014) and included in the sequence analysis.

2.3. Sequence analysis

All sequences were analyzed using mothur (Schloss et al., 2009) with the following parameters: minimum sequence length of 900 bp and the maximal number of ambiguities set to five. Chimeric sequences were excluded with "chimera.uchime" and "chimera.bellerophon". After quality control and removal of chimeric sequences, 1059 sequences remained and were aligned to the SILVA SSURef 119 reference database (Pruesse et al., 2007). Sequences were clustered into operational taxonomic units (OTUs) using a distance limit of 0.01 (= 99% similarity). This resulting OTU classification was used for all further analyses on all taxonomic levels. Representative sequences of OTUs with an abundance higher than 0.1% were double checked for correct taxonomic assignment against type strains by using the RDP seqmatch tool (Cole et al., 2014). Plotting was carried out in R environment (https://www.r-project.org/) by using the *enveomics.R*, *factoextra* and *vegan* packages. Heatmaps were created using JColorGrid (Joachimiak et al., 2006). The OTU table was formatted to a BIOM file using mothur ("make.biom") and used to create a bipartite graph ("make_otu_network.py") by using QIIME 1.9.1 software (Caporaso et al., 2010). OTU networks were visualized using Cytoscape 3.3.0 (Shannon et al., 2003). Nodes represent either samples or bacterial OTUs. Connections were drawn between OTUs and the samples they belong, with edge weights defined as the number of sequences from each OTU that occurred in each sample. The BIOM file was also used to calculate Weighted and Unweighted Unifrac with QIIME 1.9.1 software and imported to R environment to perform Principal Coordinates Analysis (PCoA). Venn diagrams were obtained by using Bioinformatics and Evolutionary Genomics software (Shade and Handelsman, 2012).

2.4. Accession numbers

The 16S rRNA gene clone sequences have been submitted to GenBank and are available under accession numbers MF091711 to MF092719.