International Journal of Food Microbiology 266 (2018) 95–103

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Abundance and potential contribution of Gram-negative cheese rind bacteria from Austrian artisanal hard cheeses

Stephan Schmitz-Esser[a,*,1], Monika Dzieciol[a], Eva Nischler[a], Elisa Schornsteiner[a], Othmar Bereuter[b], Evelyne Mann[a], Martin Wagner[a]

[a] Institute for Milk Hygiene, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine, 1210 Vienna, Austria
[b] Landwirtschaftskammer Vorarlberg, 6900 Bregenz, Austria

ARTICLE INFO

Keywords:
Cheese rind
Bacteria
Genome
Quantitative PCR
Advenella
Psychrobacter
Psychroflexus

ABSTRACT

Many different Gram-negative bacteria have been shown to be present on cheese rinds. Their contribution to cheese ripening is however, only partially understood until now. Here, cheese rind samples were taken from Vorarlberger Bergkäse (VB), an artisanal hard washed-rind cheese from Austria. Ripening cellars of two cheese production facilities in Austria were sampled at the day of production and after 14, 30, 90 and 160 days of ripening. To obtain insights into the possible contribution of *Advenella*, *Psychrobacter*, and *Psychroflexus* to cheese ripening, we sequenced and analyzed the genomes of one strain of each genus isolated from VB cheese rinds. Additionally, quantitative PCRs (qPCRs) were performed to follow the abundance of *Advenella*, *Psychrobacter*, and *Psychroflexus* on VB rinds during ripening in both facilities. qPCR results showed that *Psychrobacter* was most abundant on cheese rinds and the abundance of *Advenella* decreased throughout the first month of ripening and increased significantly after 30 days of ripening ($p < 0.01$). *Psychrobacter* and *Psychroflexus* increased significantly during the first 30 ripening days ($p < 0.01$), and decreased to their initial abundance during the rest of the ripening time ($p < 0.05$). Genome sequencing resulted in 17 to 27 contigs with assembly sizes of 2.7 Mbp for *Psychroflexus*, 3 Mbp for *Psychrobacter*, and 4.3 Mbp for *Advenella*. Our results reveal that each genome harbors enzymes shown to be important for cheese ripening in other bacteria such as: Cystathionine/Methionine beta or gamma-lyases, many proteases and peptidases (including proline iminopeptidases), aminotransferases, and lipases. Thus, all three isolates have the potential to contribute positively to cheese ripening. In conclusion, the three species quantified were stable community members throughout the ripening process and their abundance on cheese rinds together with the results from genome sequencing suggest an important contribution of these bacteria to cheese ripening.

1. Introduction

Cheese production is a way to use microbial fermentation to convert perishable milk into less-perishable products. Microbes are essential for cheese production and the earliest documented usages of cheese production date back to 5000 BCE (Salque et al., 2013). Nowadays, cheeses are produced in many different ways, with different microbes being involved in different steps of cheese production. Some cheese varieties are characterized by long ripening times during which a surface microbiota establishes which contributes significantly to ripening including flavor formation and texture. These cheese rind microbial communities can either be inoculated artificially with surface ripening cultures during the manufacturing process as done for many soft cheeses such as Munster or Camembert or establish themselves independently from the microbial communities present in the ripening cellar during the ripening process (Irlinger et al., 2015; Monnet et al., 2015). Although a number of recent studies have described microbial communities on cheese rinds (Coton et al., 2012; Delcenserie et al., 2014; Dugat-Bony et al., 2016; O'Sullivan et al., 2015; Quigley et al., 2012; Schornsteiner et al., 2014; Wolfe et al., 2014), our knowledge on the function of many microbes involved in ripening of these surface-ripened cheeses is still limited. While some of these aforementioned studies are semi-quantitative, only very little quantitative data on cheese rind bacteria abundance is currently available. Furthermore, the contribution of particularly Gram-negative cheese rind microbes to cheese ripening is still largely unknown with the exception of studies showing aroma forming capabilities of *Psychrobacter*, *Hafnia*, and *Proteus* (Deetae et al., 2007; Irlinger et al., 2012). In addition, a recent

* Corresponding author at: Department of Animal Science, Iowa State University, 3222 NSRIC, 1029 North University Boulevard, Ames, IA 50011, USA.
E-mail address: sse@iastate.edu (S. Schmitz-Esser).
[1] Current address: Department of Animal Science, Iowa State University, Ames, IA 50011, USA.

https://doi.org/10.1016/j.ijfoodmicro.2017.11.013
Received 14 June 2017; Received in revised form 23 October 2017; Accepted 18 November 2017
Available online 26 November 2017

cheese rind metagenomics study revealed the presence of putative methionine gamma lyases related to *Pseudoalteromonas* (Wolfe et al., 2014).

Vorarlberger Bergkäse (VB) is an artisanal long-ripened hard cheese with a protected designation of origin (PDO) produced from raw cow's milk deriving exclusively from alpine pastures in the western part of Austria (Vorarlberg). Highly similar types of hard cheese are produced in many areas in the European alpine regions. Similar to other hard washed-rind cheeses, VB is characterized by brining of cheese wheels either in a brine bath, by washing with brine or by dry salting surface treatment. No external surface ripening cultures are applied during the ripening process which lasts from three months to up to 18 months. We have previously characterized the microbial communities on VB cheese rinds using 16S and 18S rRNA gene targeted cloning and sequencing (Schornsteiner et al., 2014) and found Gram-negative bacteria to be highly abundant on VB cheese rinds. To characterize the Gram-negative surface flora of VB cheeses in more detail, we determined the abundance of *Psychrobacter*, *Psychroflexus* and *Advenella* on cheese rinds during the ripening process using quantitative real-time PCR (qPCR) assays. We selected *Psychrobacter*, *Psychroflexus* and *Advenella*, because they were highly abundant on VB cheese rinds in our previous study (Schornsteiner et al., 2014) and until now, no genome data was available for these strains. To further characterize the potential contribution of these strains to cheese ripening, we have isolated bacteria from VB cheese rinds and determined and analyzed draft genome sequences of three *Psychrobacter*, *Psychroflexus* and *Advenella* strains.

2. Material and methods

2.1. Cheese rind sampling

Cheese rind samples from VB, an Austrian artisanal hard cheese, were taken from ripening cellars of two different cheese production plants (A, B) in Vorarlberg (Austria) in March 2014. Samples were taken from five different ripening time points: directly after production (day 0) as well as after 14, 30, 90 and 160 days of ripening. For each time point, 20 cheese rinds samples were obtained from different cheese wheels by scraping the entire surface of each cheese wheel with sterile scalpels, resulting in 200 samples. pH of cheese rinds was measured directly after sampling with a portable pH-meter (WTW pH 3210, Germany). The a_w values of the cheese rinds were measured with *Lab*Master-*aw* (Novasina AG, Switzerland) and are shown in Supplementary Table 1. Samples were stored on ice during transport to the laboratory and processed immediately. These samples were used for cultivation and DNA extractions for quantitative PCR assays (see below).

2.2. Isolation of strains from cheese rinds

Two g from each sample were taken in duplicate and homogenized separately in 20 mL sterile Ringer Solution with a Stomacher 400 blender (Steward, London, UK). 100 µL aliquots were serially diluted in 10-fold steps in sterile Ringer Solution (Fresenius Kabi, Graz, Austria) and plated on modified Plate Count Agar (PCA): 22.5 g/L PCA (Oxoid), skim milk 1 g/L, vancomycin 5 mg/L, crystal violet 5 mg/L containing either 0% or 5% (wt/vol) NaCl. All incubations were done under aerobic conditions at 37 °C. To enrich for Gram-negative bacteria, vancomycin and crystal violet were used as described in (Coton et al., 2012). From each plate type, colonies with different morphologies (n = 143) were randomly picked and re-cultivated on agar plates of the same type. For taxonomic identification of the isolates, extraction of genomic DNA was performed by using the NucleoSpin® Tissue DNA Extraction Kit (Macherey-Nagel, Germany) according to the manufacturer's recommendations. Genomic DNA was used as a template for 16S rRNA gene PCR with primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′- GGY TAC CTT GTT ACG ACT T -3′). Amplification was performed in a standard thermocycler after initial denaturation at 95 °C for 5 min, followed by 30 cycles at 94 °C for 40 s, at 52 °C for 40 s, at 72 °C for 60 s and final extension at 72 °C for 7 min. PCR amplicons were purified (GeneJET PCR Purification Kit; Thermo Fisher Scientific, Vienna, Austria) and sequenced with Sanger sequencing to obtain near full-length 16S rRNA gene sequences (LGC Genomics, Berlin, Germany).

2.3. Genome sequencing, assembly, and analysis of selected cheese rind bacteria isolates

Our main focus was Gram-negative cheese rind bacteria, as little information about their potential contribution to cheese ripening is currently available. One strain of the most abundant isolates affiliating to the genera *Psychrobacter* (L7), *Psychroflexus* (S27) and *Advenella* (S44) was randomly chosen and used for whole genome sequencing. DNA was isolated using the Qiagen Genomic-tip columns 20/G and buffers according to the recommendations of the manufacturer (Qiagen, Hilden, Germany). Genome sequencing was performed using Illumina MiSeq sequencing technology with paired-end sequencing chemistry and 300-bp read length, using one Illumina Nextera XT library with 1 kb insert size and one 3 kb mate-pair Nextera XT library for each genome. Library preparation and genome sequencing was performed by Microsynth (Balgach, Switzerland). Both libraries for each strain were assembled with ABySS (Simpson et al., 2009). The draft genome sequences of the strains were annotated and analyzed using the Rapid Annotation using Subsystem Technology (RAST) automated web service (Overbeek et al., 2014). The average nucleotide identity between genomes was determined using the JSpeciesWS webserver (Richter et al., 2016).

2.4. Nucleotide sequence accession numbers

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession numbers NEXQ00000000 (*Psychroflexus* S27), NEXR00000000 (*Psychrobacter* L7), and NEXS00000000 (*Advenella* S44). The versions described in this paper are versions NEXQ00000000, NEXR00000000, and NEXS00000000. The raw reads were submitted to the NCBI Sequence Read Archive (SRA) with the Bioproject accession numbers PRJNA385501 (*Psychrobacter* L7), PRJNA385495 (*Psychroflexus* S27), PRJNA385498 (*Advenella* S44). 16S rRNA gene sequences are available under accession numbers LT844562 to LT844564.

2.5. qPCR assays to determine the abundance of cheese rind bacteria based on 16S rRNA gene copy numbers

2.5.1. DNA extraction

Ten g of the cheese rind samples were homogenized in 30 mL sterile Ringer Solution (Fresenius Kabi, Graz, Austria). Genomic DNA was isolated in duplicate from 250 mg pellet of the homogenized cheese rind sample using the PowerSoil™ DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. Duplicate elutions (250 µL each) were pooled and DNA concentrations were determined with a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Vienna, Austria).

2.5.2. qPCR analysis of 16S rRNA genes

To determine abundance differences between cellars from the two dairy production plants as well as shifts in the absolute abundance at different time points along the ripening process, the levels of total bacteria and of *Advenella*, *Psychrobacter* and *Psychroflexus*, for which we determined also the genome sequences, were quantified with qPCR. The 16S rRNA gene PCR quantification of total bacterial communities in cheese rind samples followed protocols described in Metzler-Zebeli et al. (2013). Briefly, DNA samples were assayed in duplicate in a 20 µL