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

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.

Keywords

Cheese rind, bacteria, genome, quantitative PCR, Advenella, Psychrobacter, Psychroflexus

Disciplines

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1 **Abundance and potential contribution of Gram-negative cheese rind bacteria from**

2 **Austrian artisanal hard cheeses**

3

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17 Psychroflexus

18

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.

42 **1. Introduction**

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

64 Vorarlberger Bergkäse (VB) is an artisanal long-ripened hard cheese with a protected designation
65 of origin (PDO) produced from raw cow's milk deriving exclusively from alpine pastures in the
66 western part of Austria (Vorarlberg). Highly similar types of hard cheese are produced in many
67 areas in the European alpine regions. Similar to other hard washed-rind cheeses, VB is
68 characterized by brining of cheese wheels either in a brine bath, by washing with brine or by dry
69 salting surface treatment. No external surface ripening cultures are applied during the ripening
70 process which lasts from three months to up to 18 months. We have previously characterized the
71 microbial communities on VB cheese rinds using 16S and 18S rRNA gene targeted cloning and
72 sequencing (Schornsteiner et al., 2014) and found Gram-negative bacteria to be highly abundant
73 on VB cheese rinds. To characterize the Gram-negative surface flora of VB cheeses in more detail,
74 we determined the abundance of *Psychrobacter*, *Psychroflexus* and *Advenella* on cheese rinds
75 during the ripening process using quantitative real-time PCR (qPCR) assays. We selected
76 *Psychrobacter*, *Psychroflexus* and *Advenella*, because they were highly abundant on VB cheese
77 rinds in our previous study (Schornsteiner et al., 2014) and until now, no genome data was
78 available for these strains. To further characterize the potential contribution of these strains to
79 cheese ripening, we have isolated bacteria from VB cheese rinds and determined and analyzed
80 draft genome sequences of three *Psychrobacter*, *Psychroflexus* and *Advenella* strains.
81
82 **2. Material and methods**
83 **2.1.Cheese rind sampling**
84 Cheese rind samples from VB, an Austrian artisanal hard cheese, were taken from ripening cellars
85 of two different cheese production plants (A, B) in Vorarlberg (Austria) in March 2014. Samples
86 were taken from five different ripening time points: directly after production (day 0) as well as

87 after 14, 30, 90 and 160 days of ripening. For each time point, 20 cheese rinds samples were
88 obtained from different cheese wheels by scraping the entire surface of each cheese wheel with
89 sterile scalpels, resulting in 200 samples. pH of cheese rinds was measured directly after sampling
90 with a portable pH-meter (WTW pH 3210, Germany). The a_w values of the cheese rinds were
91 measured with *Lab*Master-*aw* (Novasina AG, Switzerland) and are shown in Supplementary Table
92 1. Samples were stored on ice during transport to the laboratory and processed immediately. These
93 samples were used for cultivation and DNA extractions for quantitative PCR assays (see below).

94 **2.2.Isolation of strains from cheese rinds**

95 Two g from each sample were taken in duplicate and homogenized separately in 20 ml sterile
96 Ringer Solution with a Stomacher 400 blender (Steward, London, UK). 100 µL aliquots were
97 serially diluted in 10-fold steps in sterile Ringer Solution (Fresenius Kabi, Graz, Austria) and
98 plated on modified Plate Count Agar (PCA): 22.5g/L PCA (Oxoid), skim milk 1 g/L, vancomycin
99 5 mg/L, crystal violet 5 mg/L containing either 0% or 5% (wt/vol) NaCl. All incubations were
100 done under aerobic conditions at 37°C. To enrich for Gram-negative bacteria, vancomycin and
101 crystal violet were used as described in (Coton et al., 2012). From each plate type, colonies with
102 different morphologies (n=143) were randomly picked and re-cultivated on agar plates of the same
103 type. For taxonomic identification of the isolates, extraction of genomic DNA was performed by
104 using the NucleoSpin® Tissue DNA Extraction Kit (Macherey-Nagel, Germany) according to the
105 manufacturer's recommendations. Genomic DNA was used as a template for 16S rRNA gene PCR
106 with primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'- GGY TAC CTT
107 GTT ACG ACT T -3'). Amplification was performed in a standard thermocycler after initial
108 denaturation at 95°C for 5 min, followed by 30 cycles at 94°C for 40 sec, at 52°C for 40 sec, at
109 72°C for 60 sec and final extension at 72°C for 7min. PCR amplicons were purified (GeneJET

110 PCR Purification Kit; Thermo Fisher Scientific, Vienna, Austria) and sequenced with Sanger
111 sequencing to obtain near full-length 16S rRNA gene sequences (LGC Genomics, Berlin,
112 Germany).

113 **2.3.Genome sequencing, assembly, and analysis of selected cheese rind bacteria isolates**

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

128 **2.4.Nucleotide sequence accession numbers**

129 This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the
130 accession numbers NEXQ00000000 (*Psychroflexus* S27), NEXR00000000 (*Psychrobacter* L7),
131 and NEXS00000000 (*Advenella* S44). The versions described in this paper are versions
132 NEXQ00000000, NEXR00000000, and NEXS00000000. The raw reads were submitted to the

133 NCBI Sequence Read Archive (SRA) with the Bioproject accession numbers PRJNA385501
134 (*Psychrobacter* L7), PRJNA385495 (*Psychroflexus* S27), PRJNA385498 (*Advenella* S44). 16S
135 rRNA gene sequences are available under accession numbers LT844562 to LT844564.

136 **2.5.qPCR assays to determine the abundance of cheese rind bacteria based on 16S rRNA**
137 **gene copy numbers**

138 **2.5.1. DNA extraction**

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

145 **2.5.2. qPCR analysis of 16S rRNA genes**

146 To determine abundance differences between cellars from the two dairy production plants as well
147 as shifts in the absolute abundance at different time points along the ripening process, the levels
148 of total bacteria and of *Advenella*, *Psychrobacter* and *Psychroflexus*, for which we determined also
149 the genome sequences, were quantified with qPCR. The 16S rRNA gene PCR quantification of
150 total bacterial communities in cheese rind samples followed protocols described in Metzler-Zebeli
151 et al. (2013). Briefly, DNA samples were assayed in duplicate in a 20 µL reaction mixture
152 containing 10 µL of 2 × Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent, Vienna,
153 Austria), 2 µL of each primer (10 µM), 5 µL nuclease-free water, and 1 µL DNA template.
154 Amplification was conducted with 1 cycle at 95°C for 3 min and 40 cycles of 95°C for 5 s, followed
155 by 20 s at 61°C for total bacteria. Primers were designed with Primer 3 (Untergasser et al., 2012)

156 to amplify a 196 bp, 265 bp, and 160 bp target region of the 16S rRNA genes of *Advenella*,
157 *Psychrobacter* and *Psychroflexus*, respectively (Table 1). Primers were tested for specificity prior
158 to the qPCR analysis using the Ribosomal Database Project (RDP) probe match tool (Cole et al.,
159 2014). To determine the specificity of the amplifications, dissociation curves after each reaction
160 were recorded and carried out at 95°C for one min, followed by complete annealing at 50° C for
161 30 s, and a gradual increasing temperature up to 95°C. Post-run melting curves were checked for
162 the presence of multiple peaks due to primer-dimers or nonspecific amplification. Additionally, to
163 check for the presence of non-specific products and size of the amplicons, aliquots of qPCR
164 products were analyzed by agarose gel electrophoresis. Negative controls without template were
165 included in each qPCR reaction. The specificity of the amplicons was verified by DNA sequencing
166 of the PCR products (LGC Genomics), showing 99 to 100% identity to the 16S rRNA gene
167 sequences of their respective target organisms. qPCR conditions and primers were optimized to
168 obtain high PCR amplification efficiency of the target included in the qPCR assay as described in
169 Bustin et al. (2009) (Supplementary Table 2). Each optimized qPCR reaction was run in duplicate
170 with a final volume of 25 µL, using MicroAmp 0.2 mL optical tubes sealed with MicroAmp optical
171 8-cap strips (Applied Biosystems, Foster City, CA, USA). Single amplification reactions for
172 *Advenella*, *Psychrobacter* and *Psychroflexus* qPCRs consisted of 12.2 µL diethylpyrocarbonate
173 (DEPC) -treated water, 2.5 µL 10×buffer, 1.5 µL 3 mM $MgCl_2$, 1 µL 400 nM of each primer, 0.5
174 µL undiluted EvaGreen® fluorescent DNA stain (Jena Bioscience, Jena, Germany), 1 µL 200 mM
175 of each dNTP, 0.3 µL 1.5U of Platinum® *Taq* DNA polymerase (Thermo Fisher Scientific,
176 Vienna, Austria) and 5 µL template (genomic or plasmid DNA). The quantification of DNA was
177 performed in Mx3000P™ qPCR instrument (Stratagene, La Jolla, CA, USA) (software v.4.10)
178 after initial denaturation at 94°C for two min, followed by 45 cycles of 94°C for 30 sec, 60°C for

179 one min. Values for limits of quantifications and limits of detection are shown in Supplementary
180 Table S2.

181 **2.5.3. qPCR standard curves**

182 Copy numbers of the standard curves with genomic DNA (L7; S27) or plasmid DNA (S44)
183 templates were calculated using the following equation: DNA (molecules/μL) = [6.02 × 10^{23}
184 (molecules/mol) × DNA amount (g/μL)]/[DNA length (bp) × 660 (g/mol/bp)]. Based on the
185 assembly sizes of 2.98 Mbp and 2.67 Mbp of the sequenced *Psychrobacter* L7 and *Psychroflexus*
186 S27 strains, 3.11×10^5 and 3.46×10^5 genomes per ng DNA were calculated, respectively. For the
187 *Advenella* S44 template, containing plasmid-cloned target sequences of 196 bp, 2.04×10^8 copies
188 per ng were calculated. For the "total bacteria" template, containing plasmid cloned target
189 sequences of 189 bp, 2.05×10^8 copies per ng were calculated. The 16S rRNA gene copy numbers
190 (*Psychrobacter* and *Psychroflexus*: three copies, *Advenella*: two copies; total bacteria: four copies
191 (Vetrovsky and Baldrian, 2013)) were taken into account when extrapolating BCE (bacterial cell
192 equivalents) from qPCR. Genomic DNA and plasmid DNA standard curves were generated by
193 plotting the quantification cycle (Cq) values against the log initial quantities of the 10-fold dilution
194 series of purified DNA. Plasmid DNA was isolated with the QIAprep® Spin MiniPrep kit (Qiagen,
195 Hilden, Germany). Genomic DNA was isolated from sequenced cheese rind isolates with a
196 NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) according the manufacturer's
197 instructions. DNA concentrations were determined using a Qubit® 2.0 Fluorometer (Thermo
198 Fisher Scientific, Vienna, Austria).

199 **2.5.4. Statistical analysis**

200 The qPCR data (BCE per 0.5 g cheese rind) were analysed and compared using R (version 3.2.5,
201 psych package 1.6.12). The dataset was divided into 40 different subsets based on the location (A,

202 B), target (total bacteria, *Advenella*, *Psychrobacter*, *Psychroflexus*) and days of ripening (0, 14,
203 30, 90 and 160). Because the Shapiro-Wilk test showed normal distribution only for 13 of the 40
204 subsets, all subsets were described by Median and interquartile ranges (IQR). Furthermore, the
205 Wilcoxon Signed-Rank test was used to determine statistical differences between subsets with the
206 same location and same target based on days of ripening.
207

208 **3. Results and Discussion**

209 **3.1.qPCR results**

210 To determine the abundance of *Advenella*, *Psychrobacter* and *Psychroflexus* on VB cheese rinds
211 during the ripening process, qPCR assays were performed. Interestingly, all three bacteria were
212 already found in relatively high BCEs already at the first day of cheese production (Figure 1,
213 Supplementary Table 3), suggesting that these bacteria were present in the raw milk used for
214 cheese production. *Psychrobacter* has been found in raw cow milk before (Kable et al., 2016;
215 Quigley et al., 2013); to the best of our knowledge, no data on the presence of *Psychroflexus* and
216 *Advenella* in milk are currently available. In general, the most pronounced differences in
217 abundance were found during the first 30 ripening days (Supplementary Tables 3 and 4). The 16S
218 rRNA gene copy numbers of total bacteria decreased from 1.8+10 to 4.5e+08 for A, and from
219 5.0e+9 to 6.9e+8 for B during the first 30 days and remained relatively constant during the rest of
220 the ripening time. *Advenella* BCEs were decreasing significantly (A, $p<0.01$; B, $p<0.01$) from day
221 0 to day 14 and then increasing significantly (A, after 14 days, $p<0.01$; B, after 30 days, $p<0.01$)
222 during the remaining ripening time. In contrast, *Psychrobacter* BCEs increased significantly
223 ($p<0.01$) from 5.3e+5 and 8.9e+6 at day 0 to 1.2e+7 and 2.0e+8 at day 14 in dairy plant A and B,
224 respectively, and decreased significantly (A, $p<0.05$; B, $p<0.01$) after day 14. Similarly,
225 *Psychroflexus* BCEs also increased from 1.2e+05 and 3.3e+04 at day 0 to 1.2e+05 and 5.4e+05 at

226 day 14 (A, and B, respectively) and decreased after day 14. Our results show that particularly
227 *Psychrobacter* and – to a lesser degree - *Advenella* and *Psychroflexus* are abundant during ripening
228 of VB and that these changes in abundance may reflect important adaptations in community
229 composition during cheese ripening as these differences were consistently found within a single
230 ripening cellar and also in two different dairy plants. It is interesting to note that the significant
231 abundance increase of *Advenella* after day 30 (for plant A already after 14 days) suggests that
232 *Advenella* might be particularly well-adapted to growth at later ripening stages which are
233 characterized by less frequent washing of cheese rinds with lower salt concentrations. Until now,
234 only few data investigating the abundance of cheese rind bacteria during ripening are available
235 focusing on well-characterized cheese rind bacteria such as *Arthrobacter*, *Corynebacterium* or
236 *Brevibacterium* (Monnet et al., 2006; Monnet et al., 2013). *Advenella*, *Psychrobacter* and
237 *Psychroflexus* have been detected in cheese rinds in a number of previous studies using cultivation
238 or cultivation-independent approaches (Almeida et al., 2014; Coton et al., 2012; Delcenserie et al.,
239 2014; Dugat-Bony et al., 2016; O'Sullivan et al., 2015; Quigley et al., 2012; Schornsteiner et al.,
240 2014; Wolfe et al., 2014), although their abundance was not determined quantitatively. Our data
241 are the first quantitative data on the abundance of *Advenella*, *Psychrobacter* and *Psychroflexus* in
242 cheese or cheese rinds during ripening.

243 **3.2. Isolation of cheese rind bacteria from VB**

244 The contribution of Gram-negative bacteria to cheese ripening is currently still largely unknown.
245 To obtain cheese rind bacteria isolates which can be used for functional characterization such as
246 genome sequencing, we performed a cultivation approach focusing mainly on Gram-negative
247 bacteria yielding a total number of 143 isolates. Based on 16S rRNA gene sequence similarities,
248 these isolates belonged to 16 genera (data not shown). Out of the isolates, eight were identified as

249 *Psychrobacter*, eight as *Advenella* and six as *Psychroflexus*. From each of these genera, one strain
250 was selected for whole genome sequencing. *Advenella* S44 was isolated from three month old
251 cheese rinds using PCA containing 0% NaCl, *Psychroflexus* S27 and *Psychrobacter* L7 were
252 isolated from two week old cheese samples on PCA containing 5% NaCl.

253 **3.2.1. Similarity of VB cheese rind isolates to OTUs from Schornsteiner et al (2014)**

254 The full-length 16S rRNA sequences of S44, L7 and S27 were aligned with their representative
255 near full-length 16S rRNA clone sequences from each OTU from our previous study analyzing
256 VB produced at the same cheese production plants (Schornsteiner et al., 2014) using BLASTn.
257 The comparison showed high similarities between the isolates and the clones (OTUs) from our
258 previous study: 99.1%, (OTU5) for *Advenella* S44, 99.6% (OTU42) for *Psychroflexus* S27, and
259 98.3% (OTU9) for *Psychrobacter* L7.

260 **3.3. Genome sequencing of cheese rind bacteria**

261 After assembly, between 17 and 27 contigs remained for each strain with an average coverage of
262 170x for *Advenella* S44, 200x for *Psychroflexus* S27, and 214x for *Psychrobacter* L7 (Tables 2-
263 4).

264 **3.3.1. *Advenella***

265 *Advenella* S44 had an assembly size of 4.31 Mbp, which is smaller than other currently available
266 *Advenella* genomes. *Advenella* S44 probably contains three plasmids with approx. sizes of 46 kb,
267 32 kb and 25 kb. The plasmids show only limited similarity to described plasmids in other
268 *Advenella* strains such as pMIM24 and pBTK445, which are involved in biodegradation. The S44
269 plasmid contigs contain multiple putative toxin-antitoxin systems, magnesium, ornithine, as well
270 as uncharacterized transporters, and ornithine and tartrate decarboxylases. All *Advenella* strains
271 used for comparison in this study, share more than 99% 16S rRNA gene similarity (Table 2).

However, the genomes share only moderate average nucleotide identity (ANI, <84%), *Advenella* S44 shares highest ANI with the cheese isolate *Advenella* 3TF5 (84.3%). The different *Advenella* strains in this study thus belong to different species based on the ANI being lower than 95% (Chun and Rainey, 2014; Thompson et al., 2013).

Sulfur containing amino acids are important precursors for volatile sulfur compounds involved in flavor production in cheeses (Bonnarme et al., 2000; Liu et al., 2008; Yvon and Rijnen, 2001). The *Advenella* S44 genome harbors two methionine aminopeptidases (EC 3.4.11.18), a methionine transporter and two methionine aminotransferases (EC 2.6.1.88). *Advenella* S44 contains three putative Cystathionine beta-lyases (EC 4.4.1.8, CBL). CBLs have been characterized in a number of cheese bacteria and been shown to have broad substrate specificities and to be important for flavor production by converting methionine to methanethiol (Bonnarme et al., 2000; Dobric et al., 2000; Fernandez et al., 2000; Liu et al., 2008; Yvon and Rijnen, 2001). Proline is among the most abundant amino acids in casein and can be abundant in different cheeses (Gordon et al., 1950; Hintz et al., 1956; Mikulec et al., 2013). *Advenella* S44 encodes two proline iminopeptidases (EC 3.4.11.5) and a Xaa-Pro aminopeptidase (prolidase, PepQ, EC 3.4.11.9) to release proline from casein. Proline iminopeptidases and prolidases have been shown to be important for proteolysis during cheese production (Gobbetti et al., 2001; Morel et al., 1999; Smacchi et al., 1999). In addition, the *Advenella* S44 genome harbors also a bifunctional proline dehydrogenase encoded by the *putA* gene (EC 1.5.99.8 and 1.5.1.12) which converts proline to glutamate. Glutamate is another abundant amino acid in casein and many cheeses (Abellán et al., 2012; Mikulec et al., 2013; Møller et al., 2013). *Advenella* S44 can utilize glutamate by three GltLKJI transporters for glutamate import and by two glutamate dehydrogenases (EC 1.4.1.2 and 1.4.1.4).

294 Aminotransferases are responsible for amino acid transamination reactions which are key steps in
295 the conversion of amino acids into their corresponding alpha-keto acids and to aroma compounds.
296 Various aminotransferases have been characterized in cheese bacteria such as (i) aromatic amino
297 acid transferases (EC 2.6.1.57), (ii) aspartate aminotransferases (EC 2.6.1.1), (iii) branched-chain
298 amino acid transferases (EC 2.6.1.42), and (iv) methionine aminotransferases (EC 2.6.1.88)
299 (Bonnarme et al., 2000; Liu et al., 2008; Rijnen et al., 1999; Yvon et al., 2000; Yvon and Rijnen,
300 2001). The *Advenella* S44 genome encodes - among others - two putative methionine, two
301 aspartate and one branched-chain and aromatic amino acid aminotransferase.
302 Some *Advenella* strains were formerly classified as *Tetrathiobacter* and have been found in a
303 variety of habitats including soil, sewage, compost and sediments and humans; various *Advenella*
304 strains have the capability to degrade different organic pollutants (Gibello et al., 2009; Wubbeler
305 et al., 2014). In addition to this, *Advenella* strains have also been found on cheese rinds before
306 (Coton et al., 2012) and also on VB in our previous study (Schornsteiner et al., 2014). Although
307 the five *Advenella* genomes analyzed in this study derive from isolates from different environments
308 including two strains from cheese (S44 and 3TF) and three environmental strains, enzymes which
309 have been shown to be important for cheese ripening are highly conserved in all *Advenella* strains
310 analyzed here. This suggests that many *Advenella* strains may have the potential to contribute to
311 cheese ripening if present in a suitable environment such as a ripening cellar or other areas in a
312 cheese production plant. Based on the genome data from our study, a contribution on *Advenella* to
313 flavor production due to the presence of at least two copies of CBL and of proline iminopeptidases
314 is conceivable. More detailed comparative genome analyses using more genome sequences from
315 both environmental and cheese isolates and experimental verification will be necessary to verify
316 this hypothesis.

317 **3.3.2. *Psychrobacter***

318 The *Psychrobacter* L7 genome was assembled into 20 contigs with an assembly size of 2.98 Mbp
319 which is in the range of those described for other *Psychrobacter* genomes (Moghadam et al., 2016).
320 The L7 assembly contains at least four putative plasmid contigs with sizes of 3.2, 3.6, 4.3, and
321 12.5 kbp which probably represent three different plasmids based on the presence of three distinct
322 putative plasmid replication proteins. The *Psychrobacter* L7 plasmid contigs encode mostly for
323 uncharacterized proteins. *Psychrobacter* L7 shares highest 16S rRNA similarity (99.3%) with
324 *Psychrobacter celer* 91 also isolated from cheese (Almeida et al., 2014); the 16S rRNA similarity
325 to other *Psychrobacter* genomes analyzed here ranges from 97.8 to 98.8% (Table 3). Similarly,
326 *Psychrobacter* L7 shares highest ANI (95.5%) and average amino acid identity (93.7%) with
327 *Psychrobacter celer* 91. These two strains thus most likely belong to the same species. The ANI
328 to other *Psychrobacter* genomes is significantly lower. A high variability in genome size and in
329 the presence and number of plasmids has also been described for 22 *Psychrobacter* genomes
330 recently (Moghadam et al., 2016).

331 The *Psychrobacter* L7 genome harbors a methionine transporter and one methionine
332 aminopeptidase (EC 3.4.11.18). All but two of the *Psychrobacter* genomes analyzed here contain
333 one putative Cystathionine beta-lyase (EC 4.4.1.8). All *Psychrobacter* genomes harbor a putative
334 bifunctional proline dehydrogenase encoded by the *putA* gene (EC 1.5.99.8 and 1.5.1.12). Proline
335 can also be utilized by a Xaa-Pro aminopeptidase (PepQ, EC 3.4.11.9). *Psychrobacter* strains can
336 utilize glutamate by transporters for glutamate import such as GltLKJI and two sodium/glutamate
337 symporters and by two glutamate dehydrogenases (EC 1.4.1.2 and 1.4.1.4). The *Psychrobacter*
338 genomes encode - among others - one aspartate and one branched-chain and aromatic amino acid
339 aminotransferase.

340 Members of the genus *Psychrobacter* have been detected in diverse habitats including animals,
341 humans, cold marine and terrestrial environments and also in food and food production
342 environments (Bakermans et al., 2006; Jung et al., 2005; Lee et al., 2016; Yoon et al., 2005). In
343 spite of the described variability in genome size and other features, the ability of *Psychrobacter*
344 strains to being able to contribute to cheese ripening seems to be present in most *Psychrobacter*
345 genomes deriving from various sources, as many important enzymes for cheese ripening are
346 present in the genomes of different *Psychrobacter* strains. The currently available genome data of
347 six *Psychrobacter* strains from cheese suggests that many different and distinct (based on the low
348 ANI) species within the genus *Psychrobater* have the ability to grow on cheese and to contribute
349 to cheese ripening. Similar to what has been described for other *Psychrobacter* genomes
350 (Moghadam et al., 2016), the cheese *Psychrobacter* genomes also show high variability in genome
351 size. *Psychrobacter* strains have often been found to be abundant on cheese and particularly cheese
352 rinds during the last few years (Almeida et al., 2014; Coton et al., 2012; Delcenserie et al., 2014;
353 Dugat-Bony et al., 2016; O'Sullivan et al., 2015; Quigley et al., 2012; Schornsteiner et al., 2014;
354 Wolfe et al., 2014). Furthermore, different *Psychrobacter* isolates have been shown to harbor
355 important properties for cheese ripening such as the ability to form aroma compounds (Deetae et
356 al., 2007; Irlinger et al., 2012) and to contribute significantly to proteolysis and lipolysis
357 (Ozturkoglu-Budak et al., 2016). *Psychrobacter* spp. have been shown to produce high amounts
358 of volatile sulfur compounds which can be explained by the presence of CBL genes in most of the
359 *Psychrobacter* strains analyzed here. Additionally, the methionine aminopeptidases might also
360 contribute to aroma production by *Psychrobacter*. A number of *Psychrobacter* strains have been
361 shown to harbor cold and salt-adapted lipolytic enzymes (Santiago et al., 2016). The
362 *Psychrobacter* L7 genome harbors homologs of three experimentally characterized

363 esterases/lipases, which derive from *Psychrobacter* strains which are not cheese-associated,
364 showing 66% to 87% amino acid identity to their characterized homologs (Kulakova et al., 2004;
365 Wu et al., 2015; Wu et al., 2013). Whether these esterases or lipases can contribute to cheese
366 ripening is currently unknown. The genome data presented here thus provide additional evidence
367 for a likely positive contribution of *Psychrobacter* strains to cheese ripening which is supported
368 by our observation that *Psychrobacter* L7 has lipolytic and proteolytic activity (data not shown).

369 **3.3.3. *Psychroflexus***

370 The *Psychroflexus* S27 genome was assembled into 27 contigs with an assembly size of 2.67 Mbp.
371 The genome sizes of the *Psychroflexus* S27 and *Psychroflexus halocasei* WCC 4520 are smaller
372 than other currently available *Psychroflexus* genomes which show a high variability in genome
373 sizes varying from approx. 2.7 Mbp to 4.3 Mbp (Table 4). This variability in genome sizes might
374 be attributed to a high level of species-specific genes and to large genomic regions with no
375 significant nucleotide similarity (Feng et al., 2014). In line with this, *Psychroflexus* genomes share
376 only relatively low levels of ANI of approx. 68%. Only the two cheese *Psychroflexus* isolate
377 genomes show high 16S rRNA similarity (99.1%) and high ANI (93.9%) to each other. Based on
378 ANI, the two cheese *Psychroflexus* isolates might belong to different species because their ANI is
379 lower than 95% (Chun and Rainey, 2014; Thompson et al., 2013). In addition, also the average
380 amino acid identity between these two bacteria, which share 2209 proteins, is 93.5% and also
381 indicative of different species. The *Psychroflexus* genomes harbor a methionine transporter and
382 one methionine aminopeptidase (EC 3.4.11.18). All *Psychroflexus* genomes analyzed here contain
383 one putative cystathionine gamma-lyase (EC 4.4.1.1).A putative methionine gamma-lyase (EC
384 4.4.1.11) is found only in the two cheese strains, suggesting that the MGL homologs may be
385 particularly important for cheese ripening. The *Psychroflexus* S27 homologs show 55% and 32%

386 amino acid identity to the experimentally characterized CGL from *Lactococcus lactis* and MGL
387 from *Brevibacterium linens*, respectively (Amarita et al., 2004; Dobric et al., 2000; Fernandez et
388 al., 2000). For utilization of glutamate, the *Psychroflexus* genomes encode a sodium/glutamate
389 symporter and a glutamate dehydrogenase (EC 1.4.1.3). The *Psychroflexus* genomes do not encode
390 a proline iminopeptidase or proline dehydrogenase, but two PepQ Xaa-Pro aminopeptidases (EC
391 3.4.11.9) and a secreted prolyl endopeptidase (EC 3.4.21.26). The PepQ aminopeptidases are only
392 present in the cheese *Psychroflexus* isolates, they might thus be of particular importance for cheese
393 ripening. All *Psychroflexus* genomes analyzed harbor a cluster of genes possibly involved in
394 phenylacetate degradation. Phenylacetate has been described to be responsible for off-flavor in
395 Cheddar (Gummalla and Broadbent, 2001), but also for key flavor production in Swiss-type
396 cheeses (Helinck et al., 2004). The *Psychroflexus* genomes encode two aspartate aminotransferases
397 (EC 2.6.1.1) and one branched-chain amino acid transferase (EC 2.6.1.42).
398 All analyzed *Psychroflexus* genomes encode a gene cluster predicted to be responsible for
399 carotenoid production. Yellow to orange to red pigmentation has been described for other
400 *Psychroflexus* strains (Bowman et al., 1998; Chun et al., 2014; Donachie et al., 2004; Seiler et al.,
401 2012). *Psychroflexus* S27 might thus contribute to the development of the characteristic yellow-
402 orange rind of VB.
403 Members of the genus *Psychroflexus* have been found in a variety of environments, including sea
404 ice, hypersaline or saline lakes, solar salterns and cheese (Bowman et al., 1998; Chun et al., 2014;
405 Donachie et al., 2004; Feng et al., 2014; Seiler et al., 2012). So far, *Psychroflexus* have been
406 described a few times to be present in cheese: *Psychroflexus halocasei* WCC 4520 has been
407 isolated from the surface of Raclette cheese (Seiler et al., 2012), and *Psychroflexus* sequences have
408 been found on the surface of Belgian Herve hard cheese (Delcenserie et al., 2014), a variety of

409 French cheeses (Dugat-Bony et al., 2016) and also on the surface of VB in our previous study
410 (Schornsteiner et al., 2014). Whether *Psychroflexus* contribute positively to cheese ripening, is
411 currently unknown. Our qPCR abundance and genome sequencing data – particularly the presence
412 of both a putative MGL and CGL - and the presence of *Psychroflexus* on different cheeses do
413 suggest that also *Psychroflexus* is able to contribute to cheese ripening in a positive way. In line
414 with this, we found that *Psychroflexus* S27 was positive for lipolytic and proteolytic activity (data
415 not shown).
416
417 **4. Conclusion**
418 The abundance data during ripening of VB and the genome sequences provide first evidence for
419 an important and positive contribution of Gram-negative *Advenella* and *Psychroflexus* to cheese
420 ripening and provides candidate genes for the contribution of *Psychrobacter* to cheese ripening.
421 The availability of their genome sequences might allow for the development of surface ripening
422 strains for specific cheeses such as long-ripened washed rind cheeses. Our analyses show that the
423 genomic capability to contribute to cheese ripening seems to be present in most of the strains of a
424 given genus and did not evolve in the cheese-adapted strains only. The availability of these genome
425 sequences will allow for a more detailed characterization of these cheese rind bacteria in the future.
426

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431

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627 **Table 1: 16S rRNA gene-targeted primers used for qPCR**

Target (Primer name)	Amplicon length (bp)	Sequence (5’-3’)	Annealing temperature	Reference
Total bacteria (341Fwd)	189	CCTACGGGAGGCAGCAG	61°C	(Metzler-Zebeli et al., 2013)
Total bacteria (534Rev)		ATTACCGCGGCTGCTGG		
Genus *Advenella* (Adv_Fwd)	196	TATGTCAGAGGGGGGTGGAATTC	60°C	This study
Genus *Advenella* (Adv_Rev)		CTAAGCCCCGAAGGGCCCAAC		
Genus *Psychrobacter* (Psychrob_Fwd)	265	GCGCGCGTAGGTGGCTTGATAAG	60°C	This study
Genus *Psychrobacter* (Psychrob_Rev)		GACCCAACGACTAGTAGACAT		
Genus *Psychroflexus* (Psychrofl_Fwd)	160	TAGTTGCCAGCGAGTCATGT	60°C	This study
Genus *Psychroflexus* (Psychrofl_Rev)		TTCGCTCCTGGTCACCCA		

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630 **Table 2: General features of selected *Advenella* genomes from different habitats**

	***Advenella* S44**	***Advenella* 3T5F**	***Advenella* DPN7**	***Advenella* WT001**	***Advenella* W13003**
Source	Cheese rind, hard washed-rind cheese, Austria	Soft smear-ripened cheese, France	Compost, Germany	Orchard soil, India	Hydrocarbon contaminated marine sediment, China
Assembly size/genome size	4.31 Mbp	4.77 Mbp	4.74 Mbp	4.37 Mbp	4.8 Mbp
No of contigs	17	155	1	1	15
16S rRNA gene similarity to *Advenella* S44		99.7%	99.2%	99.4%	98.9%
ANI to *Advenella* S44		84.3%	81.2%	82.8%	78.3%
GC content	54.4%	54.6%	54.2%	54.9%	55.1%
Cystathionine beta-Lyase (EC 4.4.1.8)	+ (3 copies)	+ (3 copies)	+ (3 copies)	+ (2 copies)	+ (3 copies)
Proline iminopeptidase (EC 3.4.11.5)	+ (2 copies)	+ (2 copies)	+ (2 copies)	+ (2 copies)	+ (2 copies)
Proline dehydrogenase PutA (EC 1.5.99.8 and 1.5.1.12)	+	+	+	+	+
Glutamate dehydrogenase (EC 1.4.1.2 and 1.4.1.4)	+ (2 copies)	+ (2 copies)	+ (2 copies)	+ (2 copies)	+ (2 copies)
Methionine aminopeptidase (EC 3.4.11.18)	+ (2 copies)	+ (2 copies)	+ (2 copies)	+ (2 copies)	+ (2 copies)
Reference	This study	(Almeida et al., 2014)	(Wubbeler et al., 2014)	(Ghosh et al., 2013)	(Wang et al., 2014)

631

632 **Table 3: General features of selected *Psychrobacter* genomes from different habitats**

	***Psychrobacter* L7**	***P. celer* 91**	***P. aquimaris* ER15 174 BHI7**	***P. immobilis* PG1**	***P. namhaensis* 1939**	***P. faecalis* H5**	***P. cryohalolentis* K5**	***P. arcticus* 273-4**
source	Cheese rind, hard washed-rind cheese, Austria	Mould-ripened soft cheese, raw milk, France	Semi-hard cheese from cow milk, France	Soft smear-ripened cheese, France	Mould-ripened soft cheese with raw milk, France	Soft smear-ripened cheese, France	Permafrost, Russia	Permafrost, Russia
Assembly/genome size	2.98 Mbp	2.99 Mbp	3.34 Mbp	3.48 Mbp	2.95 Mbp	3.26 Mbp	3.06 Mbp	2.65 Mbp
No of contigs	20	140	126	99	86	156	1	1
GC content	47.2%	46.9%	42.9%	42.9%	44.8%	43.4%	42.3%	42.8%
16S rRNA similarity to *Psychrobacter* L7		99.3%	98.4%	98.3%	98.6%	97.8%	98.3%	98.8%
ANI to *Psychrobacter* L7		95.5%	78.5%	78.5%	78.6%	79.2%	77.3%	77.8%
Cystathionine beta Lyase (EC 4.4.1.8)	+	+	+	+	-	+	+	-
Proline dehydrogenase PutA (EC 1.5.99.8 and 1.5.1.12)	+	+	+	+	+	+	+	+
Glutamate dehydrogenase (EC 1.4.1.2 and 1.4.1.4)	+	+	+	+	+	+	+	+
Methionine aminopeptidase (EC 3.4.11.18)	+	+	+	+	+	+	+	+
Reference	This study	(Almeida et al., 2014)	(Almeida et al., 2014)	(Almeida et al., 2014)	(Almeida et al., 2014)	(Almeida et al., 2014)	GenBank accession number: CP000323	(Ayala-del-Rio et al., 2010)

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Table 4: General features of selected *Psychroflexus* genomes from different habitats

	***Psychroflexus* S27**	***P. halocasei* WCC4520**	***P. torquis* ATCC700755**	***P. gondwanensis* ACAM44**	***P. tropicus* DSM15496**
Source	Cheese rind, hard washed-rind cheese, Austria	Raclette cheese, Austria	Sea ice, Antarctica	Hypersaline lake, Antarctica	Hypersaline lake, Hawaii
Assembly/genome size	2.67 Mbp	2.67 Mbp	4.32 Mbp	3.32 Mbp	3.0 Mbp
No of contigs	27	44	1	62	28
GC content	33.6%	33.4%	34.5%	35.7%	36.5%
16S rRNA similarity to *Psychroflexus* S27		99.1%	95.4%	95.1%	94.0%
ANI to *Psychroflexus* S27		93.9%	68.4%	68.5%	68.2%
Cystathionine gamma Lyase (EC 4.4.1.1)	+	+	+	+	+
Glutamate dehydrogenase (EC 1.4.1.2 and 1.4.1.4)	+	+	+	+	+
Methionine aminopeptidase (EC 3.4.11.18)	+	+	+	+	+
Methionine gamma Lyase (EC 4.4.1.11)	+	+	-	-	-
Xaa-Pro aminopeptidase PepQ (EC 3.4.11.9)	+ (2 copies)	+ (2 copies)	-	-	-
Reference	This study	(Seiler et al., 2012), PRJNA329844	(Feng et al., 2014)	(Feng et al., 2014)	(Donachie et al., 2004)

635

636 **Figure legend**

637 **Figure 1: Abundance of cheese rind bacteria during ripening of Vorarlberger**
638 **Bergkäse in two different cheese production plants determined by qPCR.** Bacterial
639 cell equivalents (BCE) per 0.5 g cheese rind during ripening in two different cheese
640 production facilities are shown. Graph shows median and interquartile ranges for the 20
641 samples from each plant (A, B) for each analyzed day of ripening (0, 14, 30, 90 and 160
642 days). The BCE values are shown in Supplementary Table 3.

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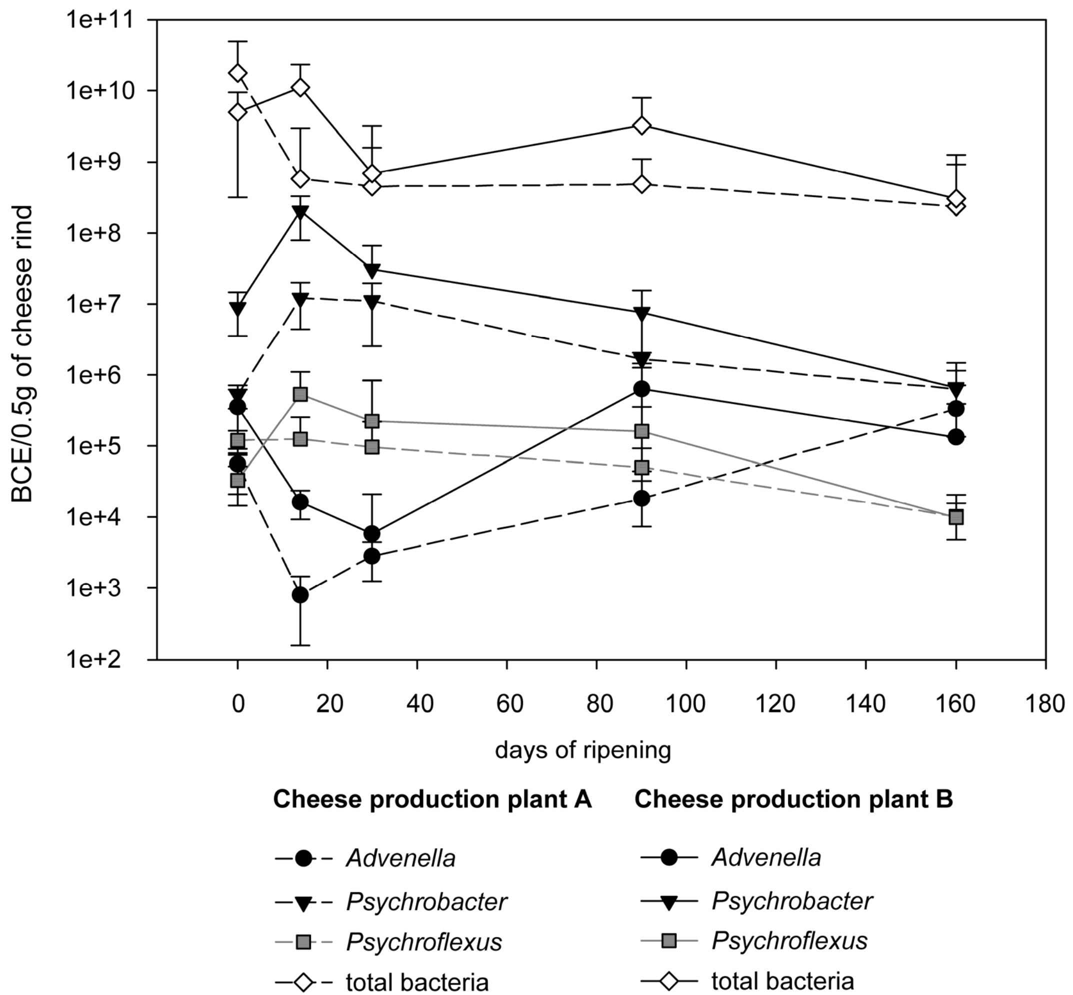

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648 **Supplementary Table 1. Physical parameters during ripening of Voralberger Bergkäse.**

649 **Abbreviations: SD, standard deviation; aw, water activity**

	Cheese production plant A				**Cheese production plant B**			
Day of ripening time	pH[a]	SD	a_w[b]	SD	pH[a]	SD	a_w[b]	SD
0	7.02	0.05	0.89	0.00	7.24	0.03	0.86	0.00
14	7.35	0.08	0.86	0.04	7.16	0.14	0.89	0.01
30	7.17	0.04	0.87	0.07	7.32	0.05	0.90	0.01
90	6.96	0.04	0.88	0.01	7.16	0.03	0.88	0.00
160	7.10	0.02	0.89	0.00	7.21	0.06	0.87	0.00

[a] and [b]: values are expressed as means

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652 **Supplementary Table 2: MIQE guidelines for qPCR.**

Item	Importance	Status	Remarks
Experimental design			
Definition of experimental and control groups	E[1]	✓	Two cheese diary plants, A and B
Number within each group	E	✓	Number of samples within each group (n=100), number of subgroups (n=5): ripening days 0; 14; 30; 90; 160
Assay carried out by core lab or investigator's lab?	D[2]	✓	Investigator's lab
Acknowledgement of authors' contributions	D	✓	See main manuscript
Sample			
Description	E	✓	DNA isolated from cheese rinds
Volume/mass of sample processed	D	✓	250 mg pellet of the homogenized cheese rind sample in duplicate
Microdissection or macrodissection	E	✓	Not relevant
Processing procedure	E	✓	DNA isolation using PowerSoil™ DNA Isolation kit
If frozen - how and how quickly?	E	✓	Samples were stored on ice during transport to the laboratory and processed immediately. After DNA isolation, samples were frozen within 10 minutes at -80°C.
If fixed - with what, how quickly?	E	✓	Samples were not fixed
Sample storage conditions and duration	E	✓	Cheese rind samples were processed immediately. DNAs were stored 1-6 months at -80°C, after thawing on ice, DNA samples were applied to the qPCRs within 5min
Nucleic acid extraction			
Procedure and/or instrumentation	E	✓	PowerSoil™ DNA Isolation kit, mechanical lysis
Name of kit and details of any modifications	E	✓	PowerSoil™ DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA), no modifications
Source of additional reagents used	D	✓	DNA was eluted in DEPC-treated water
Details of DNase or RNase treatment	E	✓	No treatment
Contamination assessment (DNA or RNA)	E	✓	NTCs included to DNA isolation were analyzed with qPCR
Nucleic acid quantification	E	✓	Qubit® 2.0 Fluorometer
Instrument and method	E	✓	Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Vienna, Austria)
Purity (A_{260}/A_{280})	D	✓	
Yield	D	✓	
RNA integrity method/instrument	E	✓	Not relevant
RIN/RQI or Cq of 3' and 5' transcripts	E	✓	Not relevant
Electrophoresis traces	D	✓	Aliquots of qPCR products were analyzed by agarose gel electrophoresis

Inhibition testing (Cq dilutions, spike or other)	E	✓	No inhibition determined (dissociation curves, gel electrophoresis analysis, specificity of the amplicons verified by DNA sequencing of the PCR products)
Reverse transcription			
Reaction conditions	E		Not relevant
Amount of RNA and reaction volume	E		Not relevant
Priming oligonucleotide and concentration	E		Not relevant
Reverse transcriptase and concentration	E		Not relevant
Temperature and time	E		Not relevant
Manufacturer of reagens and catalogue numbers	D		Not relevant
Cqs with and without RT	D		Not relevant
Storage conditions of cDNA	D		Not relevant
qPCR target information			
Gene symbol	E	✓	16S rRNA gene
Sequence accession number	E	✓	16S rRNA gene for total bacteria - V3 of bacterial 16S rRNA, 341–534 in *E. coli* 16S rRNA *Advenella* sp. S44 partial 16S rRNA gene, isolate S44 - Accession: LT844562 *Psychrobacter* sp. L7 partial 16S rRNA gene, isolate L7 - Accession: LT844563 *Psychroflexus* sp. S27 partial 16S rRNA gene, isolate S27- Accession: LT844564
Location of amplicon	D	✓	
Amplicon length	E	✓	16S rRNA gene total bacteria - 189bp 16S rRNA gene genus *Advenella* - 196bp 16S rRNA gene genus *Psychrobacter* - 265bp 16S rRNA gene genus *Psychroflexus* - 160bp
In silico specificity screen (blast, etc)	E	✓	Ribosomal Database Project (RDP) probe match tool, Primer3, NCBI primer designing tool, TestPrime arb-SILVA
Pseudogenes, retropseudogenes or other homologs?	D		Not relevant
Sequence alignment	D		Not relevant
Secondary structure analysis of amplicon	D	✓	
Location of each primer by exon or intron (if applicable)	E		Not relevant
What splice variants are targeted?	E		Not relevant
qPCR oligonucleotides			
Primer sequences	E	✓	See Table 1
RTPrimerdb identification number	D	✓	Not done, beside 16S rRNA gene for total bacteria, all of them are unpublished newly designed primers
Probe sequences	D	✓	Not done, as no probes were used

Location and identity of any modifications	E	✓	No modifications
Manufacturer of oligonucleotides	D	✓	Microsynth
Purification method	D	✓	Desalted
qPCR protocol			
Complete reaction conditions	E	✓	See main manuscript
Reaction volume and amount of cDNA/DNA	E	✓	For total bacteria: 20 µl reaction volume (incl. 1µl DNA), for other targets:25 µl reaction volume (incl. 5 µl DNA)
Primer, (probe), Mg++ and dNTP concentrations	E	✓	See main manuscript
Polymerase identity and concentration	E	✓	1.5U of Platinum® Taq DNA polymerase (Thermo Fisher Scientific, Vienna, Austria)
Buffer/kit identity and manufacturer	E	✓	See main manuscript
Exact chemical constitution of the buffer	D	✓	See manual of the Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent, Vienna, Austria)
Additives (SYBR green I, DMSO, etc.)	E	✓	For total bacteria: SYBR Green is contained in the mastermix; no further additives
Manufacturer of plates/tubes and catalog number	D	✓	MicroAmp optical tube (0.2 µl; Applied Biosystems by life technologies)
Complete thermocycling parameters	E	✓	Total bacteria: 95°C for 3 min and 40 cycles of 95°C for 5 s followed by 20 s at 61°C, melting curve 50°C to 90°C; for other targets: 94°C for 2 min and 45 cycles of 94°C for 30 s followed by 1min at 60°C, melting curve 50°C to 90°C
Reaction setup (manual/robotic)	D	✓	Manual
Manufacturer of qpcr instrument	E	✓	Stratagene Mx3000P real-time PCR System (Agilent Technologies, Santa Clara, USA)
qPCR validation			
Evidence of optimisation (from gradients)	D	✓	Concentrations of primers in range of 200-400 nM, $MgCl_2$ ranging from 2 to 3.5 mM, as well as annealing/extension temperature, ranging from 60°C to 64°C were tested
Specificity (gel, sequence, melt, or digest)	E	✓	Gel, sequence, melting curve
For SYBR green, Cq of the NTC	E	✓	No amplification
Standard curves with slope and y-intercept	E	✓	Done
PCR efficiency calculated from slope	E	✓	*Advenella* 95.3% *Psychrobacter* 97.7% *Psychroflexus* 92.8% Total bacteria 98.2%
Confidence interval for PCR efficiency or standard error	D		-
r^2 of standard curve	E	✓	Between 0.997 and 1 for all primer pairs
Linear dynamic range	E	✓	Determined, 8 log scales tested
Cq variation at lower limit	E	✓	Less than 5% within replicates
Confidence intervals throughout range	D	✓	

Evidence for limit of detection (LOD)	E	✓	*Advenella* 6.9e+01 BCE per 0.5g cheese rind *Psychrobacter* 1.3e+03 BCE per 0.5g cheese rind *Psychroflexus* 8.9e+02 BCE per 0.5g cheese rind
If multiplex, efficiency and LOD of each assay.	E		Not done, no multiplexing
Data analysis			
qPCR analysis program (source, version)	E	✓	Stratagene Mx3000P real-time PCR System (Agilent Technologies, Santa Clara, USA)
Method of Cq determination	E	✓	Stratagene Mx3000P real-time PCR System settings (baseline subtracted curve fit, single threshold, automatically calculated). Threshold manually curated for maximum efficiency within linear range for each plate
Outlier identification and disposition	E	✓	Done
Results of NTCs	E	✓	No amplificates
Justification of number and choice of reference genes	E		Not done
Description of normalisation method	E		Not done
Number and concordance of biological replicates	D	✓	2 biological replicates
Number and stage (RT or qPCR) of technical replicates	E	✓	2 technical replicates for all samples and standards
Repeatability (intra-assay variation)	E	✓	Repeatable
Reproducibility (inter-assay variation, %CV)	D	✓	Not determined (strongly recommended for clinical/diagnostic applications, but not other assays)
Power analysis	D	✓	Done
Statistical methods for result significance	E	✓	Wilcoxon Signed-Rank test
software (source, version)	E	✓	R (version 3.2.5, psych package 1.6.12).
Cq or raw data submission using RDML	D	✓	

653 [1]E = essential
654 [2]D = recommended
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657 **Supplementary Table 3. 16S rRNA gene-based qPCR copy numbers of cheese rind**
658 **bacteria on VB. The copy numbers are shown as bacterial cell equivalents (BCE) per 0.5**
659 **g cheese rind.**

Cheese production plant	Ripening days	Target	Median	Min	Max	IQR[a]
A	0	total bacteria	1.78E+10	4.71E+09	2.07E+11	3.12E+10
	14		5.77E+08	2.81E+08	4.97E+09	2.40E+09
	30		4.45E+08	1.92E+08	3.85E+09	1.14E+09
	90		4.79E+08	2.49E+08	3.77E+09	6.11E+08
	160		2.36E+08	1.36E+08	5.19E+10	1.03E+09
	0	*Advenella*	5.57E+04	2.83E+04	2.13E+05	3.46E+04
	14		8.01E+02	2.79E+02	1.91E+03	6.44E+02
	30		2.81E+03	8.12E+02	6.78E+03	1.57E+03
	90		1.82E+04	7.53E+03	2.73E+05	2.58E+04
	160		3.33E+05	1.13E+05	5.84E+06	3.93E+05
	0	*Psychrobacter*	5.26E+05	2.18E+05	1.17E+06	1.88E+05
	14		1.21E+07	5.88E+06	6.04E+07	7.72E+06
	30		1.10E+07	2.67E+06	2.51E+07	8.44E+06
	90		1.66E+06	1.13E+06	2.68E+06	4.04E+05
	160		6.37E+05	3.41E+05	1.49E+06	5.02E+05
	0	*Psychroflexus*	1.20E+05	3.47E+04	2.86E+05	4.43E+04
	14		1.24E+05	4.69E+04	1.69E+06	1.33E+05
	30		9.59E+04	6.35E+03	1.04E+06	1.22E+05
	90		4.99E+04	2.03E+04	1.18E+05	4.26E+04
	160		1.02E+04	6.37E+03	2.71E+04	5.43E+03
B	0	total bacteria	4.97E+09	6.83E+08	1.64E+10	4.66E+09
	14		1.10E+10	1.86E+09	3.87E+10	1.25E+10
	30		6.90E+08	1.54E+08	7.02E+09	2.49E+09
	90		3.27E+09	1.30E+08	1.10E+10	4.81E+09
	160		2.99E+08	1.20E+08	2.27E+09	6.11E+08
	0	*Advenella*	3.53E+05	8.70E+04	8.25E+05	2.74E+05
	14		1.65E+04	6.75E+03	3.32E+04	7.17E+03
	30		5.86E+03	2.66E+03	3.92E+04	1.51E+04
	90		6.32E+05	5.33E+04	4.15E+06	8.23E+05
	160		1.33E+05	1.14E+04	1.37E+06	2.61E+05
	0	*Psychrobacter*	8.94E+06	3.74E+06	1.83E+07	5.39E+06
	14		2.03E+08	7.54E+07	3.23E+08	1.24E+08
	30		3.05E+07	5.60E+06	1.24E+08	3.61E+07
	90		7.62E+06	8.45E+05	1.69E+07	7.59E+06
	160		6.67E+05	1.09E+05	1.53E+06	8.14E+05
	0	*Psychroflexus*	3.30E+04	1.40E+04	8.44E+04	1.85E+04
	14		5.38E+05	1.60E+05	2.34E+06	5.65E+05
	30		2.24E+05	4.04E+04	3.56E+06	6.10E+05
	90		1.61E+05	1.41E+04	3.40E+05	1.96E+05
	160		9.86E+03	6.27E+03	5.83E+04	1.09E+04

660 [a]: IQR Interquartile range

661

662 **Supplementary Table 4. Statistical significance of the abundance differences of qPCR**
663 **data using the Wilcoxon Signed-Rank Test.** Statistical differences between subsets (n=40)
664 within the same cheese production plant (A or B) and the same target (16S rRNA for total
665 bacteria, 16S rRNA for *Advenella*, *Psychrobacter* and *Psychroflexus*) during ripening (0, 14,
666 30, 90 and 160 days of ripening) were calculated using the Wilcoxon Signed-Rank Test. *P*-
667 values less than 0.05 are interpreted as statistically significant and are written in bold.

Cheese production plant	Target	Comparison (*p*-value)*				
		0 vs.14	0 vs. 30	14 vs. 30	30 vs. 90	90 vs.160
A	total bacteria	**<0.01**	**<0.01**	**0.01**	0.11	**<0.01**
B		**<0.01**	**<0.01**	**<0.01**	**0.04**	**<0.01**
A	*Advenella*	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
B		**<0.01**	**<0.01**	**0.04**	**<0.01**	**<0.01**
A	*Psychrobacter*	**<0.01**	**<0.01**	**0.03**	**<0.01**	**<0.01**
B		**<0.01**	**0.02**	**<0.01**	**<0.01**	**<0.01**
A	*Psychroflexus*	0.06	0.96	0.16	0.26	**<0.01**
B		**<0.01**	**<0.01**	0.16	0.35	**<0.01**

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