

1 Running Title: Halophilic and halotolerant bacteria in cheese

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3 **Unraveling the world of halophilic and halotolerant bacteria in cheese by**
4 **combining cultural, genomic and metagenomic approaches**

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13

14 **ABSTRACT**

15 Halophilic and halotolerant bacteria are generally assumed to live in natural
16 environments, although they may also be found in foods such as cheese and seafood. These
17 salt-loving bacteria have only been occasionally characterized in cheese, and studies on their
18 ecological and technological functions are still scarce. We therefore selected 13 traditional
19 cheeses in order to systematically characterize these microorganisms in their rinds via
20 cultural, genomic and metagenomic methods. Using different salt-based media, we identified
21 35 strains with unique 16S rRNA and *rpoB* gene sequences, whose whole genome was
22 sequenced. The most frequently isolated species are the halotolerant Gram-positive bacteria
23 *Brevibacterium aurantiacum* (6) and *Staphylococcus equorum* (3), which are also frequently
24 added as starters. Their genomic analyses confirm the high genetic diversity of *B.*
25 *aurantiacum* and reveal the presence of two subspecies in *S. equorum*, as well as the genetic
26 proximity of several cheese strains to bovine isolates. Additionally, we isolated 15 Gram-
27 negative strains, potentially defining ten new species of halophilic cheese bacteria, in
28 particular for the genera *Halomonas* and *Psychrobacter*. The use of these genomes as a
29 reference to complement those existing in the databases allowed us to study the
30 representativeness of 66 species of halophilic and halotolerant bacteria in 74 cheese rind
31 metagenomes. The Gram-negative species are particularly abundant in a wide variety of
32 cheeses with high moisture, such as washed-rind cheeses. Finally, analyses of co-occurrences
33 reveal assemblies, including the frequent coexistence of several species of the same genus,
34 forming moderately complex ecosystems with functional redundancies that probably ensure
35 stable cheese development.

36

37 **IMPORTANCE**

38 Salt is commonly added to food to avoid the growth of pathogens by lowering water
39 activity, resulting in profound changes in the medium that lead to the development of
40 particular ecosystems dominated by halophilic and halotolerant bacteria, communities that
41 probably originate in the natural environment. In order to explore these communities that have
42 been poorly studied in food up until now, we developed a combined approach that includes
43 cultures, genomics and metagenomics to deconstruct these ecosystems in cheese rinds. This
44 approach allowed us to isolate 26 different species, ten of which belong to still undescribed
45 species that could be used as references to promote advances in functional studies of this
46 particular world. The metagenomic scan of 74 cheese rind samples for the assembly of 66
47 halophilic and halotolerant species showed that these bacteria are widely distributed and form
48 moderately complex ecosystems where related species coexist and probably jointly contribute
49 to safe and efficient cheese development.

50

51 **Keywords:** salt, cheese surface, microbial diversity, halophile, ecology.

52

53 **Introduction**

54 Occurring naturally in nature, salt has been harvested since ancient times on the shores of
55 lakes, seacoasts and oases. There is even evidence that it was produced industrially during
56 protohistory, in the 5-6th millenium BC [1-3]. Regardless of its source (rock salt, sea salt,
57 spiced salt, etc.), raw salt serves as a flavor enhancer and increases the palatability of food,
58 while allowing the preservation of certain foodstuffs, especially meat and fish.

59 Salt is effective as a preservative because it reduces water activity in foods [4, 5]. Its
60 addition can reduce the rate of microbial growth in foods due to osmotic shock and other
61 disorders that interfere with cell enzymes or that expend energy to exclude sodium ions from
62 the microbial cell [6]. Although high levels of salt totally inhibit the growth of most

63 microorganisms, moderate levels create an inhospitable environment for the majority of
64 pathogens and promote the growth of certain microorganisms in various food products.
65 Despite advances in food processing, storage, packaging and transportation have largely
66 diminished this role, and salt is still widely used as one of the multiple means to control food
67 safety. Moreover, it frequently plays a central role in the production of fermented foods (i.e.,
68 pickles, sauerkraut, cheeses, Asian seafood, fermented meats, etc.), not only contributing to an
69 extended shelf life, but also leading to the development of particular aroma, texture,
70 nutritional and beneficial health properties, eventually becoming part of the cultural
71 patrimony in many countries. Whereas many studies have focused on lactic fermentation in
72 recent decades, the interest of the salt-driven development of non-pathogens in fermented
73 food is more recent and at its beginning stages.

74 Microorganisms that grow in the presence of salt may be subdivided into two categories:
75 (1) halotolerants, which are able to grow in the presence or absence of salt; and (2) halophiles,
76 which require salt to develop [7, 8]. The precise definition of a halophile diverges depending
77 on the authors, some of whom include any organism that requires percentages of around 3.5%
78 of salt, as in seawater [7, 9], while others consider only those that grow optimally at 5% or
79 above, and tolerate at least 10% of salt [10]. Such halophilic bacteria have been widely
80 described and isolated in different natural ecosystems such as soil, salt lakes and seas, and are
81 of interest as producers of pigments and antibacterial activities [11, 12], as well as a variety of
82 bioactive compounds [13, 14]. Halophilic and halotolerant bacteria have also been isolated
83 from foods such as salt meat, shrimp, fermented fish sauce, sea food, poultry and cheese [15-
84 18]. Several strains found in these foods have already been characterized, but their functions
85 are still unknown. Certain studies consider that halophilic bacteria are involved in food
86 spoilage and are thus considered to be undesirable in food processing environments [16, 17,
87 19]. Other authors report that several species produce significant quantities of volatile

88 compounds such as sulfides, acetone, ammonia and ethanol, suggesting that they have a
89 potential function in aroma production [15, 20].

90 Overall, studies on halophilic and halotolerant bacteria in food are still scarce. A
91 comprehensive overview of all ecosystem agents is therefore necessary in order to understand
92 their functions, manage their evolution in the process and, eventually, understand the
93 association of these microorganisms with the shelf life of food products. Cheese is part of
94 these ancient fermented foods in which salt addition may lead to major changes in its
95 processing. There are more than 1,000 distinct cheese types worldwide, with a variety of
96 textures, appearances, aromas and flavors that can be attributed to the technological
97 development of complex and specific microbial communities, as well as to local factors such
98 as milk source and farming practices [21-23]. The salting process might thus be one of the
99 factors that strongly influence the way a microbial community will develop in cheese. Salt can
100 be added in different ways - in crystal form or in solution, by brining or applying brine
101 directly to the curd, by rubbing, smearing or scraping the surface, with clear salt solutions or
102 historical old brines - and each of these processes may be applied once or several times and at
103 different time scales (**Fig. 1**). Indeed, recent studies based on non-cultivable methods have
104 shown that halophilic bacteria may be the dominant microbiota on cheese rinds, suggesting
105 that their role has been underestimated until now [24, 25]. A better understanding of their
106 origin and how these organisms evolve during the different stages of cheese development
107 could be of major interest to determine the role of salt in cheese ecosystem organization.

108 We therefore decided to enlarge the data repository of these salt-tolerant and salt-
109 dependant bacteria in cheese in order to determine and produce a precise overview of their
110 presence in cheeses produced using different technologies. For this purpose, we used culture
111 methods to isolate and identify - in a systematic way - halophilic and halotolerant bacteria in
112 13 artisanal cheese rinds produced by different salting processes. We then combined genomic

113 and metagenomic approaches that revealed potential new species, a wide diversity of
114 halophilic and halotolerant bacteria in cheese rinds, and the coexistence of these species in
115 this food ecosystem.

116

117 **Results**

118 **Abundance of halophilic and halotolerant bacteria in cheese rinds**

119 For this study, we selected 13 artisanal cheese rinds whose main technological features are
120 presented in **Table 1**, in order to systematically characterize their halophilic and halotolerant
121 microorganisms by culture methods. Different cheese technologies were included: lactic
122 paste, unpressed or pressed, uncooked or cooked, soft or semi-hard and blue cheeses.
123 Moreover, the cheeses involved different salting processes such as brining, dry salting, rind
124 washing or smearing.

125 The halophilic and halotolerant bacterial population level was estimated by plate counts
126 on several media and temperatures in order to optimize their growth and maximize the
127 potential to isolate diverse strains. Overall, our results demonstrated the presence of dense
128 bacterial populations (around 7-8 log CFU/g) on the surface of most cheese rind samples, with
129 a minimum of 4.0 and a maximum of 9.5 log CFU/g (**Figure S1**). No major differences in
130 bacterial counts were found between LH and MB media, whereas lower counts were obtained
131 with HM medium for most cheeses, in particular Cheeses A, B and D. The effect of increasing
132 salt was tested on MB medium and its increase negatively affected bacterial counts, in
133 particular for Cheeses A, B and F. Interestingly, counts in MB+8% salt do not present major
134 differences with those obtained in HM. The lower counts obtained in these two media,
135 compared with other ones, could be due to a higher salt content: 8-10%, vs. 1 and 2% in LH
136 and MB, respectively. Finally, the counts were similar at the three incubation temperatures
137 tested (20, 25 and 30°C).

138

139 **Defining potential new food halophilic species**

140 Two marker genes, 16S rRNA and *rpoB*, were used to identify and differentiate the
141 bacterial isolates. For each cheese, representative isolates differing in at least one of these
142 markers were selected for further study, and those grown on MB or its derivative containing
143 higher salt content were preferentially chosen in the case of the identity of both markers.
144 From ~320 isolates, we finally obtained 35 strains tentatively assigned to species, as described
145 in **Additional File 1** and presented in **Table S1**. Of these strains, 20 belong to the Gram-
146 positive group and 15 to the Gram-negative group, 17 and three isolates of which are assigned
147 at the species level, respectively. To obtain reliable taxonomic data, we determined the
148 genomic sequences of the 35 strains (**Table S2**) and performed an ANI analysis with closely
149 related strains, as shown in **Table S3**.

150 Genomic analyses confirmed the assignment of the 17 Gram-positive bacteria carried
151 out by the marker genes, while three remaining isolates belong to undescribed species (**Table**
152 **2**). *Brachybacterium* strain FME24 displays an ANI value of 83.17% with its closest relative
153 *B. tyrofermentans*, indicating that it belongs to a new species. Similar analysis of
154 *Brevibacterium* strains FME17 and FME37 showed that these two strains and *Brevibacterium*
155 *sp.* 239c share an ANI of over 97% of each other (**Table S3**), but less than 87% with the
156 closest reference species, indicating that they belong to a new species.

157 Concerning Gram-negative species, ANI analysis could assign only five isolates, while
158 ten remained ambiguously or not assigned (**Table 2**). *Advenella sp.* FME57, isolated in this
159 study, and *Advenella sp.* 3T5F (formerly referred to as *kashmirensis*) appear to belong to the
160 same species (ANI=98.57%) but significantly differ from the *A. kashmirensis* type strain
161 (WT001^T) with which they share an ANI < 90% (**Table S3**). These two strains should
162 therefore belong to a new species. Concerning the *Halomonas* genus, ANI analysis showed

163 that none of the strains isolated here could be assigned to an already described species,
164 including the FME20 strain whose 16S rRNA and *rpoB* analyses suggested its assignment to
165 *H. zhanjiangensis*. Indeed, their ANI of 93.22% is below the threshold of 95% (**Table 2**).
166 Results of marker analyses of the two *Pseudoalteromonas* strains (FME14 and FME53)
167 remained ambiguous due to multiple hits with similar identities with different species
168 (**Additional File 1**). The ANI analysis demonstrated that the FME14 strain could be assigned
169 to *P. prydzensis* (ANI=95.96%) and FME53 to *P. nigrifaciens* (best score ANI=98.31%)
170 (**Table 2**). Furthermore, *Proteus sp.* FME41 belongs to a new species since it shares only
171 88.62% ANI with its close relative *P. cibarius* JCM 30699^T. Similarly, *Pseudomonas* strain
172 FME51 does not belong to *Pseudomonas litoralis* since it shares an ANI of only 87.70%
173 (**Table 2**). Regarding the *Psychrobacter* genus, FME2 shares ANI~95% with five strains of
174 *Psychrobacter*, including *P. immobilis* and *P. cibarius* type strains (**Table S3**), leaving its
175 assignment unresolved. Finally, *Psychrobacter* strains FME5, FME6 and FME13 could not be
176 assigned to any already known species by both markers and ANI analyses (**Table S1** and
177 **Table S3**). Since FME5 and FME6 strains display an ANI of 98%, these three strains may
178 represent two new species.

179 Therefore, from these 35 isolates, we obtained strains belonging to 26 different
180 species, ten of which potentially belong to new species: two to the Gram-positive group and
181 eight to the Gram-negative group.

182

183 **Genomic diversity of *B. aurantiacum* and *S. equorum***

184 Among the Gram-positive bacteria, we isolated six *B. aurantiacum* strains from five
185 different cheeses (**Table 2**), which, in addition to the 19 already sequenced genomes available
186 in the NCBI database (**Table S4**), gave a total of 25 genomes. ANI analysis shows that they
187 share over 97% identity and clustering analysis indicates the presence of four groups

188 (indicated as A, B, C and D; **Fig. 2A**). To further determine their genetic diversity, we
189 performed a pan-genome analysis that revealed an open pan-genome for 25 strains of *B.*
190 *aurantiacum*, with a total of 10,823 genes (**Fig. S2A**). We also analyzed the number of genes
191 present in different numbers of k genomes, yielding two major groups. The first corresponds
192 to the core genome (k = 25 genomes) and the second to the orphan genes (k = 1 genome),
193 with 1,871, and 4,000 genes, respectively (**Fig. S2B**). Furthermore, we constructed the
194 maximum likelihood tree from the accessory genome elements, which makes it possible to
195 visualize the relatedness of strains based on their pan-genome composition and genes shared
196 by different strains (**Fig. 2B**). This analysis indicates that strains belonging to groups B, C and
197 D are also clustered together and shows that SMQ-1335 and 862_7 strains are very closely
198 related, differing only by a few genes (**Fig. 2B**) and presenting an ANI of 99.94% (**Fig. 2A**).
199 Both strains were isolated from cheese made in different regions (**Table S4**). Moreover,
200 FME43 and FME45 strains, both isolated from the same cheese in this study, belong to group
201 B with an ANI of ~98.8%, while their gene content differs by about 10%. These strains are
202 thus closely related but their pan-genome differs significantly.

203 In previous studies, potential horizontal gene transfer events were proposed to have
204 occurred between *Brevibacterium* and several Actinobacteria [26-28]. From these studies, we
205 selected ten regions containing genes involved in different metabolic functions and studied
206 their distribution within the 25 *B. aurantiacum* genomes available (**Fig. 2B**). This analysis
207 shows that, except for islands 3, 4 and 5, which are present in only one strain each, the other
208 islands are widely spread out in the different *B. aurantiacum* strains. Concerning iron
209 transport systems, which are carried out by islands 1 and 2, they are distributed in six and nine
210 strains, respectively, and seem to exclude each other (**Fig. 2B**), probably to avoid the cost of
211 their overload [29]. Eleven strains do not contain any of these additional genes, indicating that
212 although they may confer a selective advantage, alternative systems exist.

213 Finally, we characterized three *S. equorum* strains, which, together with the other
214 genomes of this species available in the NCBI database (**Table S4**), make a total of 43 strains.
215 The tree based on ANI analysis revealed two well-separated groups of 39 and four strains,
216 indicated as Groups I and II, respectively (**Fig. 3A**). Strains belonging to Groups I and II
217 display an ANI > 98% within their group, but an ANI of ~95% with those of the other group.
218 Group I, which is the largest, may be subdivided into three subgroups (A, B and C), sharing
219 an average of 99.5% ANI intra-subgroups and differentiated by over 98.7% ANI inter-
220 subgroups. While Group II contains only dairy strains, Group I also contains strains of cattle
221 and several other environments (**Fig. 3A, Table S4**). Pan-genome analysis of *S. equorum*
222 indicated an open pan-genome with up to 7,000 genes (**Fig. S2C**). The analysis of the number
223 of genes present in different numbers of k genomes revealed two major groups consisting of
224 the core genome (k = 43 genomes) and the orphan genes (k = 1 genome), with 1,868, and
225 2,500 genes, respectively (**Fig. S2D**), which reflects a moderate level of genetic diversity in
226 this species. Pan-genome clustering produced several groups, which were tentatively linked to
227 metadata and ANI (**Fig. 3B**). First, it confirmed the distinction of Group II (FME19,
228 White_SAM, OffWhite_SAM and BC9), whose strains mainly differ from each other by their
229 content of mobile elements (prophages, potential plasmids, etc.), while the rest of their
230 genome is nearly identical (2,292 genes > 99.9% identity). Furthermore, seven strains isolated
231 in different cheeses and countries (France and the U.S.) appear to be highly related (908_10,
232 Mu_2, 876_5, 862_5, 962_6, 947_12 and 738_7; **Fig. 3B**). They share ~2,500 almost
233 identical proteins (> 99.7%), compared to ~1,400 with the other strains of this subgroup.
234 Their pan-genomes mainly differ by mobile elements, including potential prophages, plasmids
235 and the number of hypothetical proteins. Finally, while several cattle strains may also form
236 distinct groups such as those of the ANI group B, several cheese and cattle strains appear to
237 be related.

238

239 **Overview of halophilic species in cheese rind metagenomes**

240 In this study, we isolated and characterized the genomes of strains belonging to 26
241 different species, ten of which are potentially new species. The availability of their genome
242 sequences as references opens the possibility to detect and quantify their presence in shotgun
243 metagenomic data. We therefore selected a set of 74 metagenomic samples corresponding to
244 cheese rinds from different types, including 49 from this study and 25 from former studies
245 [24, 25] (**Table S5**). In order to provide a comprehensive overview of halophilic and
246 halotolerant species in these samples, we completed our set with 40 supplementary genomes
247 of related species isolated from cheese in previous studies. The percentages of reads
248 corresponding to the 66 reference genomes, which were mapped on the 74 metagenomic
249 samples, are presented in **Table S6**. Among the 74 cheese rinds analyzed, only five have no
250 detectable level of halophilic or halotolerant bacteria. Interestingly, more than half of the
251 samples (42) present more than 10% of reads from these bacteria, showing their importance in
252 cheeses.

253 Among Gram-positive bacteria, *Brevibacterium* and *Brachybacterium* species are
254 widely distributed, especially in natural and washed cheese rinds (**Fig. 4**). *B. aurantiacum* is
255 the most frequently detected, in about 70% of the samples, and its amount exceeds 10% in six
256 cheese rinds. Interestingly, the new species of *Brevibacterium* isolated here, represented by
257 FME37, is also frequently detected in cheese rind metagenomes (half of the samples) and
258 exceeds 5% of the reads in three cheese rinds (**Table S6**). Similarly, the potential new species
259 represented by *Brachybacterium* sp. FME24 is detected in 12% of the samples, showing the
260 potential relevance of this species in cheese ecosystems. Both species of *Corynebacterium*, *G.*
261 *arilaitensis*, *A. casei* and *M. gubbeenense* are also frequent (present in more than 17% of the
262 samples) and sometimes abundant (more than 5% of the reads) in our dataset (**Table S6**).

263 Additionally, coagulase-negative *Staphylococci* are particularly present in several natural
264 cheese rinds (**Fig. 4**). Among the four species, *S. equorum* is the most frequent (present in
265 31% of the samples) and abundant one (more than 5% of the reads in two samples). Finally,
266 several other Gram-positive species are detected at low frequency (between 1 and 13% of the
267 sample) and at low abundance (less than 1% of the reads) in our dataset (**Table S6**).

268 Furthermore, several Gram-negative species appear to be frequent and dominant in a
269 significant number of samples, especially in washed cheese rinds (**Fig. 4**). In particular,
270 different species of *Halomonas*, *Pseudoalteromonas* and *Psychrobacter* are detected in 13 to
271 32% of the samples, and several species, including new species isolated in our study, exceed
272 10% of the reads (**Table S6**). Three additional Gram-negative species, *V. casei*, *V. littoralis*
273 and *H. alvei*, are also relatively frequent (11 to 18% of the samples) and sometimes abundant
274 (more than 5% of the reads mapped). Both *Vibrio* species are mainly found in washed rinds,
275 whereas *H. alvei* is present in bloomy rinds (**Fig. 4**). Additionally, the *Pseudomonas* FME51-
276 like species is only detected in the cheese sample it was isolated from (Sample 3), while *P.*
277 *helleri* and *P. ludensis* are detected in seven and four samples, respectively, and sometimes at
278 high levels (up to 25% of the reads, **Table S6**). Finally, the other Gram-negative species are
279 detected at low frequency (between 1 and 8% of the samples) and at low abundance.

280

281 **Co-occurrence relationships among bacterial species**

282 Exploratory network and correlation analyses were performed to investigate the co-
283 occurrence among cheese halophilic and halotolerant bacteria in order to identify
284 combinations of species and ecosystem structuration (**Fig. 5**). Only species present > 1% in at
285 least two cheese sample were plotted. As previously demonstrated (**Fig. 4**), Gram-positive
286 species are more closely related in cheese with natural and washed rinds, while Gram-
287 negative species are more closely related in cheese with washed rinds (**Fig. 5A**). Overall, the

288 different species appear to present a higher level of co-occurrence within their group than
289 outside (**Fig. 5A and 5B**; P value < 0.05). We observed that species belonging to the same
290 genus are often found together, such as *Brevibacterium*, *Corynebacterium*,
291 *Pseudoalteromonas*, *Halomonas*, *Psychrobacter*, *Pseudomonas* and *Vibrio* species (**Fig. 5B**).
292 Additionally, we noted a positive correlation with *Brevibacterium* species, *Brachybacterium*
293 *tyrofermentans* and *Agrococcus casei*. Finally, we highlight here the high level of co-
294 occurrence between some species of *Psychrobacter* and *Vibrio*, as well between several
295 species of *Halomonas* and *Pseudoalteromonas* (**Fig. 5B**; P value < 0.05).

296

297 **Discussion**

298 We isolated halophilic and halotolerant bacteria from the rinds of 13 traditional French
299 cheeses by selecting colonies of different morphotypes on media containing 1 to 10% salt.
300 Total halophilic population count was evaluated on media with three different basic
301 compositions (HM, LH and MB), usually used in the study of environmental halophiles, and
302 the effect of increased amounts of salt was tested on MB. Counts obtained with MB and LH
303 media were similar, whereas they were lower on HM and MB+8% NaCl (**Figure S1**),
304 possibly due to its higher salt concentration. Moreover, incubation temperatures in the range
305 of 20 to 30°C had no effect on global bacterial counts. In most rind samples, halophilic and
306 halotolerant populations were 7-8 log CFU/g on the former media, which is a range similar to
307 those reported in other studies using different culture media supplemented with salt, such as
308 Milk Plate Count Agar (MPCA) with 5% salt [30, 31], Trypticase Soy Agar (TSA)
309 supplemented with 4% NaCl [32] and Brain Heart Infusion (BHI) [33]. These data, together
310 with the fact that most isolates were able to grow on all tested salt-based media, would
311 indicate that the choice of the media is not determinant in the study of halophilic and
312 halotolerant bacteria in cheese. Nevertheless, we did not always obtain the same species in the

313 parallel plate isolation, suggesting that the use of different media could favor, differently and
314 sufficiently (without being sharply selective), the growth of different populations and thus
315 allow a greater variety of species to be isolated. From ~320 isolates, we selected 35 strains
316 with unique 16S-*rpoB* sequences corresponding to 20 Gram-positive and 15 Gram-negative
317 strains (**Table 2**). Their genome sequences were determined and ANI analysis showed that 12
318 of these isolates belong to two and eight potentially new species of Gram-positive and Gram-
319 negative bacteria, respectively.

320

321 **Halophilic and halotolerant as food bacteria**

322 The availability of the genomic sequences of halophilic and halotolerant cheese
323 bacteria offers the opportunity to reliably detect and quantify their corresponding populations
324 in cheese metagenomic samples at a level of 0.1% of DNA. This relative abundance level
325 corresponds to subdominant populations, which may reach 10^5 - 10^6 CFU/g for cultivable
326 species in several types of cheeses, a level compatible with a significant metabolic activity
327 that could impact cheese technology.

328 The development of Gram-positive species belonging to Actinobacteria and
329 Firmicutes in cheese rinds during ripening is well established and has been previously
330 reported in different types of cheeses all over the world (**Additional File 2**). A metagenomic
331 search showed that species like *B. tyrofermentans*, *B. aurantiacum*, *Corynebacterium* species
332 and *G. airilaitensis* were detected in 25 to 70% of samples at maximal levels of 6 to 45% of
333 the reads (**Table S6**), confirming their wide distribution in cheese rinds and their potential
334 importance in cheese technology. Interestingly, we isolated a new species of *Brevibacterium*
335 (FME37 strain) that is widely distributed in metagenomic samples and sometimes detected at
336 high levels (> 20%), suggesting a role in cheese.

337 *B. aurantiacum* is the most frequent and abundant Actinobacteria found in our
338 metagenomic samples. This frequency was probably boosted by the over 40-year-old history
339 of the use of this bacteria as an adjunct to take advantage of its various technological
340 properties [34, 35], although the persistense of adjuncted strains is questioned [36, 37]. This
341 species was subject to the most detailed genomic analysis, and 19 cheese genome sequences
342 were available at the time of this study. The comparison of these genomes, in addition to the
343 six provided here, confirmed the substantial genetic diversity within *B. aurantiacum* and its
344 plasticity, which might be reflected in the diversity of color, aroma, lipolytic and other
345 technological factors described for this bacteria. Finally, the study of the different genomic
346 islands - characterized earlier – suggests that their roles are not crucial for the development of
347 this species in cheese since a significant number of strains are not concerned by these
348 additional factors. In particular, from the two strains FME43 and FME45 (isolated from the
349 same cheese), only one contains iron acquisition genes on ISL2 (**Fig. 2B**), which were
350 described as being important to develop within the cheese surface habitat [27, 38]. The
351 alternative distribution of the two different iron acquisition systems on ISL1 and ISL2 in
352 about half of the strains may suggest that their presence is also a metabolic load, whereas
353 alternative strategies probably exist.

354 Furthermore, we isolated three coagulase-negative staphylococci species, a type of
355 bacteria commonly isolated from cheese. In agreement with a previous metagenomic study
356 [39], we found that *S. equorum* is the most frequent and abundant species, while *S. succinus*
357 and *S. vitulinus* are more scarce (**Fig. 4**). *S. equorum*, which is also used as an adjunct in
358 cheese to improve its texture and contribute particular flavors (**Additional File 2**), has been
359 extensively studied for safety reasons, and 40 genomes from food, cattle and clinical samples
360 were available at the time of this study. Their ANI analyses, together with our three isolates,
361 suggest that *S. equorum* could be subdivided into two subspecies (**Figure 3A**), the second one

362 being represented by four cheese strains. The combined ANI and pan-genome analyses of
363 these strains (**Fig. 3A and 3B**) indicates that they mainly differ by their mobile element
364 content (prophages and potential plasmids; **Fig. 3B**), whereas the rest of their genomes are
365 nearly identical, suggesting a recent common origin for their use in cheese. Further
366 investigations will be required to determine if the two potential subspecies express different
367 technological properties, including phage resistance. In the major group of strains (Group IA),
368 many isolates from cheese could not be clearly differentiated from those from cattle,
369 suggesting their animal origin, except a group of seven cheese strains (**Fig. 3B**). The latter
370 ones mainly differ by their mobile elements, which could be the result of starter culture
371 selection. However, further studies will be necessary to demonstrate that these strains
372 followed a drift and are now specifically growing in cheese rinds.

373 Additionally, we isolated three other less documented Firmicutes, including
374 *Marinilactibacillus psychrotolerans* and *Carnobacterium mobile* (occasionally found in
375 cheese), and *Oceanobacillus oncorhynchi*, a halotolerant bacteria sometimes isolated from
376 Asian salted food (**Additional File 2**). Whereas *M. psychrotolerans* was detected in around
377 13% of our metagenomic data with a maximum of 4% of the reads, *C. mobile* and *O.*
378 *oncorhynchi* were not detected, including the samples from which they were isolated (**Table**
379 **S6**). These results indicate that the size of the population of these two species was very small
380 and that they may not play a significant role in cheese technology.

381 In addition to these halotolerant Gram-positive bacteria, we obtained several species of
382 Gram-negative bacteria that have not yet aroused keen interest in technological developments.
383 Remarkably, Gammaproteobacteria from the genera *Halomonas*, *Pseudoalteromonas* and
384 *Psychrobacter* were the most frequently isolated (representing 60% of the Gram-negative
385 isolates obtained in this study), and they often represent high relative abundance in washed
386 cheese rinds (**Fig. 4**). This observation is in agreement with recent culture-independent

387 analyses [24, 25], and their presence in cheeses of good quality supports the possibility that
388 they may have a positive impact during ripening, while several authors consider them as
389 contaminants (**Additional File 2**). Nevertheless, the characterization of the potential role of
390 these genera in cheese technology remains to be explored. Finally, we isolated species of
391 *Pseudomonas*, *Proteus*, *Hafnia*, *Vibrio* and *Advenella*. All these genera have already been
392 reported in cheese rind communities and the abundance of several of these species may
393 support further interest for their role in cheese ripening (**Additional File 2**). Currently, *Hafnia*
394 *alvei* is the only Gram-negative bacterium used as a ripening starter in the cheese-making
395 process and it was detected here at level of 4.5 and 8.5% in two bloomy cheese rinds (**Table**
396 **S6**), where it was probably added as an adjunct culture.

397

398 **New insights into cheese microbial ecology**

399 Interestingly, the development of Gram-negative bacteria appears to be greater in soft
400 cheese, and also favored by the washing and smearing processes of the rind, which
401 correspond to cheeses displaying higher moisture [40], as roughly depicted in **Figure 1**.

402 Remarkably, the present metagenomic analysis discriminates populations at the level
403 of species, which is not always possible with amplicon sequencing that is limited by the lack
404 of significant divergence, especially for Gram-negative species such as those presented in this
405 study. Consequently, the present set of data uncovers the world of halophilic and halotolerant
406 bacteria in cheese rinds with a high level of resolution, and reveals, in particular, the co-
407 occurrence of a number of species, including closely related species hardly distinguishable by
408 marker gene analysis. For example, species of *Brevibacterium*, *Corynebacterium*, *Halomonas*,
409 *Psychrobacter*, *Pseudoalteromonas* and *Vibrio* are found co-occurring with at least another
410 species of the same genus (**Fig. 5B**). The coexistence of such a variety of species reflects the
411 fact that these ecosystems are open to the microbial environment, which is likely to be

412 resilient to their production conditions and develop with salt as a key driver. The presence of
413 related species, which are thus likely to carry out similar metabolic functions, will lead to a
414 functional redundancy, a factor that was proposed to be of primary importance in the the
415 resilience of ecosystems submitted to changes or pressures [41, 42]. In the context of
416 traditional productions, changes could encompass modification of milk quality, technological
417 issues and phage attacks, thus structuring the ecosystem and maintaining a variety of
418 microorganisms. However, while the presence of diverse microorganisms in the processing
419 environment may increase the ecosystem's capacity to respond to changes, it may also modify
420 the organoleptic properties of cheeses [43]. Further studies will be required to understand the
421 interactions that occur between these microorganisms and their role in the development of
422 cheese, as well as the development of the rich and various organoleptic properties of
423 traditional products.

424 The present study made it possible to isolate 26 different species, ten of which belong
425 to still undescribed species, although they are frequent and abundant in various cheeses. These
426 strains could be used as references to promote advances in functional studies of this particular
427 world driven by salt addition and to jointly contribute to safe and efficient cheese
428 development.

429

430 **Materials and methods**

431 **Cheese sampling**

432 A total of 13 cheese samples were selected in this study (**Table 1**). The cheeses were
433 purchased from a local supermarket and their rinds were sampled in portions (1 g) with a
434 sterile knife and frozen at -20°C until further analysis.

435

436 **Enumeration and isolation of halophilic bacteria from cheeses**

437 To enumerate and isolate halophilic strains, we used Marine Broth (MB; Difco, Sparks,
438 USA), Long and Hammer Agar (LH; [44]) and *Halomonas* Medium (HM; [45]). Different
439 concentrations of salt were supplemented in MB (0, 4, 6 and 8% NaCl). In order to prevent
440 fungal growth, Amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) was added to a final
441 concentration of 20 µg/ml (50 mg/ml stock solution of Alfa Aesar™ Amphotericin B from
442 *Streptomyces nodosus* in DMSO). Cultivable bacterial strains were enumerated using serial
443 dilutions of homogenized cheese samples in sterile 0.9% NaCl solution. Population counts of
444 cheese rinds were determined by 10⁻³ to 10⁻⁷ dilutions and incubated 48-72 h at 20, 25 and
445 30°C. For each cheese, the plates with a bacterial count comprised between 20 and 200 clones
446 were selected for isolate characterization. An initial selection of apparently different isolates
447 (morphotypes) was performed based on colony morphology (color, shape, elevation,
448 pigmentation and opacity). A representative of each morphotype was then restreaked on a
449 new plate for subsequent DNA extraction.

450

451 **Identification of isolated morphotypes**

452 The selected clones were collected with a sterile loop and mixed in a tube containing 300
453 µl biomol water, 100 mg of 0.1 mm-diameter zirconium beads and 100 mg of 0.5 mm-
454 diameter (Sigma, St. Louis, MO, USA). The tube was then vigorously shaken in a bead-beater
455 (FastPrep-24, MP Biomedicals Europe, Illkirch, France) for 20 s at 4.5 m/s. The supernatant
456 of this lysis was used directly for DNA amplification. The species assignment was performed
457 by sequencing the 16S rRNA and the *rpoB* genes. The 16S rRNA gene was amplified using
458 27-F (5'-AGAGTTTGATCATGGCTCA-3') and 1492-R (5'-
459 TACGGTTACCTTGTTACGACTT-3') [46]. The *rpoB* gene was amplified using primers

460 VIC4 (5'-GGCGAAATGGCDGARAACCA-3') and VIC6 (5'-
461 GARTCYTCGAAGTGGTAACC-3') [24, 47].

462 Thermal cycling conditions applied for both were (i) 1 min at 94°C to initial
463 denaturation; (ii) 30 cycles of 1 min at 94°C to denaturation, 0.5 min at 56°C to primer
464 annealing, 1.5 min at 72°C to initial elongation; and (iii) 5 min at 72°C to final elongation.
465 DNA amplifications were separated on 0.8% agarose gel. The PCR products were purified
466 using the ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) and sent for
467 sequencing to the service provider (Eurofins Genomics, Ebersberg, Germany). Once received,
468 the sequences were analyzed via the NCBI BLAST tool [48] to obtain a taxonomic
469 classification for each isolate.

470

471 **Genomic DNA extraction and sequencing**

472 For each unique isolate, after cultivation in MB for 48 h at 25°C, DNA was extracted
473 from the bacterial cells according to the protocol described by Almeida *et al.* [24] with some
474 modifications. Briefly, we used an enzymatic lysis step followed by protein precipitation by
475 adding potassium acetate. DNA was precipitated at -20°C after the addition of 0.1 volume of
476 3 M sodium acetate and two volumes of cold absolute ethanol to the upper phase. After
477 centrifugation (30 min at 12,000 *g* and 4°C), the DNA was dried in a laboratory hood and
478 resuspended in TE 1X buffer. The DNA concentration and quality was evaluated using a
479 NanoDrop ND-1000 spectrophotometer (NanoDrop Technology Inc., Wilmington, DE, USA).
480 Additionally, 5 µL of DNA were loaded on 0.8% agarose gel and visualized after migration
481 by ethidium bromide staining.

482 DNA sequencing was carried out on an Illumina HiSeq at GATC-Biotech (Konstanz,
483 Germany) in order to generate paired-end reads (150 bases in length). For each strain, the
484 paired-end reads were merged and *de novo* assembly was performed using SPAdes, version

485 3.9 [49]. Only contigs with length \geq 300 bp and coverage $>$ 100 were considered for further
486 study. Annotations were performed using the Rapid Annotation using Subsystem Technology
487 server [50].

488

489 **Phylogenetic analysis**

490 For species assignment, evolutionary trees were built using the 16S rRNA and *rpoB*
491 genes. To delineate species, we used a threshold of over 99% identity for 16S rRNA genes
492 with type or well-defined strains [51], and of above 97.7% identity for *rpoB* nucleotide
493 sequences [52]. Further phylogenetic analyses were performed using ClustalX 2.1 [53] and
494 MEGA7 [54]. The trees were built using the Neighbor-Joining method [55] with 1,000
495 bootstrap replicates [56]. Lastly, using genomic sequences, we determined the Average
496 Nucleotide Identity (ANI) using JSpeciesWS [57] to confirm speciation of the different
497 isolates. For new species delineation, we used the recommended cut-off point of 95% ANI
498 [58].

499

500 **Pan-genome of *Brevibacterium aurantiacum* and *Staphylococcus equorum***

501 ANI and pan-genome analyses were estimated for 25 genomes of *Brevibacterium*
502 *aurantiacum* and 43 genomes of *Staphylococcus equorum* (**Table S4**). The ANI was
503 performed using the ANIm method described by Richter *et al.* [59] and implemented in the
504 Python module PYANI (version 0.2.6) (<https://github.com/widdowquinn/pyani>). The pan-
505 genomes of both species were performed with Roary software (version 3.11.2; [60]) and the
506 gene-based genome-wide association using Scoary [61]. Interactive visualization of genome
507 phylogenies was done with Phandango (version 1.3.0; [62]).

508

509 **Cheese DNA extraction and sequencing**

510 From the 13 samples used to isolate halophilic and halotolerant bacteria, ten were selected
511 to analyze their total DNA. The DNAs were prepared from cell pellets obtained from each
512 cheese sample, following a method that combines enzymatic and mechanical treatments for
513 cell lysis and treatment with phenol/chloroform/isoamyl alcohol to extract and purify
514 DNA, as previously described by Almeida *et al.* [24]. The ten DNA samples from cheese
515 were sequenced using Illumina HiSeq2500 technology at GATC-Biotech (Konstanz,
516 Germany), which yielded between six and eight million paired-end reads of 150-nucleotide
517 length. Moreover, 39 additional samples from different types of cheese were sequenced using
518 SOLiD technology, which yielded between 11-19 million single reads of 50-nucleotide
519 length. The raw read data for all samples are available under the accession numbers listed in
520 **Table S5**.

521

522 **Quantification of species in metagenomic samples**

523 First, species present in each metagenomic sample were identified with the Food-
524 Microbiome Transfert tool, an in-house designed tool managing the following tasks. Each of
525 the 66 reference genomes (one genome per species) was mapped on the metagenomic samples
526 with Bowtie [63] (adapted to SOLiD data, parameters were adapted to take into account intra-
527 species polymorphisms and choose at most one mapping position per read: first 35
528 nucleotides mapped; 3 mismatches allowed; --all --best --strata -M 1). In order to discard
529 reads that could have been aligned on conserved regions on a more distant genome (same
530 genus for example) or repeated regions, BEDtools [64] and SAMtools [65] were used to filter
531 reads and compute genome coverage. Reads mapping genomic regions that were less
532 informative and/or that could have been acquired by gene transfer (intergenic regions, tRNA,
533 rRNA, genes annotated as “transposase”, “integrase”, “IS”, “phage/prophage” or “plasmids”)

534 were not taken into account. In order to select genomes whose species is present in the
535 sample, we selected genomes with at least 50% of their genes covered by at least one read.
536 Food-Microbiome Transfert tool was used via a web interface developed via the Python
537 Django framework as well as web technologies such as HTML and JavaScript. Genome,
538 metagenome and analysis data are stored on a PostgreSQL relational database. Computations
539 were performed on the Migale platform's calculation cluster via the Bioblend API and the
540 Galaxy portal.

541 Then, to determine the abundance of the different halophilic and halotolerant species,
542 metagenomic reads were mapped on a database containing all 66 reference genomes with
543 Bowtie (same parameters). We selected only reads mapping on genomes selected at the
544 previous step. Quantification was done by counting the number of read for each genome. In
545 order to obtain comparable results between metagenomes, we downsized the samples to 5
546 million reads. The metadata of metagenomic samples are presented in **Table S5**.

547

548 **Statistical analysis of co-occurrence relationships**

549 The relationship between the halophilic species was examined by performing a
550 correlation matrix using Pearson's test. The function 'rcorr' (in 'Hmisc' package) was used to
551 compute the significance levels ($P < 0.05$) and the graph were plotted using the 'corrplot'
552 package for R. Only species with a relative abundance $\geq 1\%$ were used to generate matrix and
553 network correlations. Bacterial networks were explored and visualized using Gephi software
554 0.9.2 [66].

555

556 **Data availability**

557 Raw genomic reads were deposited to the European Nucleotide Archive under the
558 project accession number PRJNA501839, while Illumina and SoliD metagenomic reads under
559 PRJNA642396 and PRJEB39332, respectively.

560

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571

572 **Authors contributions**

573 PR conceived the study and its experimental design. BD and BFK collected samples, isolated
574 and identified strains. CIK performed genomic and metagenomic analysis. AB contributed to
575 genomic analysis. The Food Microbiome team supported overall cheese rind metagenomic
576 analysis. CIK and PR analyzed the data and wrote the manuscript. PR supervised the project.

577

578 **Competing interests**

579 The authors declare that they have no competing interests.

580

581 **Table and Figure Legends**

582 **Figure 1.** Cheese processing including examples of the salting process. Processes were
583 classified as proposed by Almena-Aliste & Mietton [40]. The effect of water activity
584 depending on the process, in particular, on drainage and temperature, is primarily dependent
585 on the moisture in non-fat substances (MNFS), whereas the salting level decreases this effect.
586 Examples of cheeses are given to illustrate this figure.

587

588 **Figure 2.** ANI and pan-genome analyses of 25 *B. aurantiacum* cheese strains. Strains from
589 this study are highlighted in bold. **(A)** Phylogeny based on ANI values showing the presence
590 of four groups marked in green (Group A), violet (Group B), orange (Group C) and red
591 (Group D). **(B)** Pan-genome analysis. Left panel: maximum likelihood tree constructed from
592 the accessory genome elements; middle panel: distribution of several horizontal gene transfer
593 (HGT) regions described as islands [26]; Right panel: gene presence-absence matrix showing
594 the presence (blue) and absence (white) of orthologous gene families.

595

596 **Figure 3.** ANI and pan-genome analyses of 43 *S. equorum* strains. The origin of the strain is
597 indicated by color: blue (dairy), red (cattle) and black (other). Strains from this study are
598 highlighted in bold. **(A)** Phylogeny based on ANI values showing the presence of two groups
599 potentially representing two subspecies; four subgroups in Group I (A, B, C and D) are
600 presented by colored lines. **(B)** Pan-genome analysis. Left panel: maximum likelihood tree
601 constructed from the accessory genome elements; right panel: gene presence-absence matrix
602 showing the presence (blue) and absence (white) of orthologous gene families.

603

604 **Figure 4.** Heatmap depicting the relative abundance (%) of halophilic and halotolerant
605 species in 74 cheeses. Samples are ordered according to rind types, as indicated by upper
606 labels (bloomy, natural and washed). Bacterial species are ordered according to their
607 taxonomical class and whether they belong to the Gram-positive or Gram-negative groups, by
608 the color of their name, black and blue, respectively.

609

610 **Figure 5.** Relationships between halophilic and halotolerant bacteria detected by
611 metagenomic analysis in cheese rinds. **(A)** Network summarizing the relationships between
612 bacterial species and 74 cheese rind samples. Nodes represent species and cheese samples.
613 For sample nodes, different colors (green, orange and red) are used to differentiate cheese
614 rinds (natural, washed and bloomy, respectively). For species nodes, grey and blue are used to
615 differentiate Gram-positive and Gram-negative strains, respectively. Connecting edges
616 indicate the detection of given species in the samples and are colored according to whether
617 they belong to the Gram-positive or Gram-negative group. Only edges corresponding to
618 species detected at 1% relative abundance in the samples are shown. Sizes for cheese nodes
619 and edges are proportional to in-degree (i.e., total occurrence of a species in the whole
620 dataset). **(B)** Correlation matrix between halophilic and halotolerant species described in this
621 study. Bacterial species are ordered according to their taxonomical class and whether they
622 belong to the Gram-positive or Gram-negative groups, by the color of their name, black and
623 blue, respectively. Only species present > 1% in at least two samples of cheese rind were
624 plotted. Measurements are computed with Pearson's test (P value < 0.05) and coefficient
625 values are depicted using the following color gradient scale: red indicates negative
626 correlations and blue indicates positive correlations.

627

628 **Table 1:** Metadata describing the 13 cheese rind samples.

629

630 **Table 2.** The 35 isolates and their cheese origin, media and temperature of isolation, as well
631 as the closest species with their respective ANI values.

632

633 **Supplementary Material**

634 **Additional File 1.** This supplementary information documents the identification of halophilic
635 and halotolerant isolates using the phylogenetic analysis of 16S rRNA and *rpoB* genes.

636

637 **Additional File 2.** This supplementary information documents the main bacterial species of
638 halophilic and halotolerant bacteria in food and their potential roles.

639

640 **Figure S1.** Total viable counts in different salt media and temperatures in log CFU/g from 13
641 cheese rind samples.

642

643 **Figure S2.** Pan-genome analyses **(A)** Accumulated number of new genes in the pan-genome
644 and genes of *B. aurantiacum* attributed to the core-genome are plotted against the number of
645 added genomes. **(B)** Accumulated number of genes in k genomes are plotted against the
646 number of k genomes. The number of core genes present in all 25 *B. aurantiacum* genomes
647 can be observed with k = 25 genomes. **(C)** Accumulated number of new genes in the pan-
648 genome and genes of *S. equorum* attributed to the core-genome are plotted against the number
649 of added genomes. **(D)** Accumulated number of genes in k genomes are plotted against the
650 number of k genomes. The number of core genes present in all 43 *S. equorum* genomes can be
651 observed with k = 43 genomes.

652

653 **Table S1.** Potential assignment of 35 representative isolates using 16S rRNA and *rpoB* genes.

654

655 **Table S2.** General features of the 35 genomes sequenced in this study.

656

657 **Table S3.** Average Nucleotide Identity (ANI) with close relatives of strains that marker genes

658 (16S rRNA and *rpoB*) were unable to reliably assign to a described species. ANI values

659 greater than or equal to 95% are shown in green.

660

661 **Table S4.** Metadata of 25 *Brevibacterium aurantiacum* and 43 *Staphylococcus equorum* used

662 for pan-genome analyses.

663

664 **Table S5.** Metadata of 74 cheese rind metagenomes used to map the halophilic and

665 halotolerant genomes selected in this study.

666

667 **Table S6.** Abundance (%) of each halophilic and halotolerant species in 74 cheese rinds. The

668 table includes the abundances corresponding to the taxonomical order of the species, the type

669 of bacteria (Gram-positive or Gram-negative) and the total halophilic and halotolerants in

670 each sample. Corresponding statistics on the number of samples containing more than 0.1,

671 0.5, 1, 5 and 10% are also available.

672

673 **References**

674 1. Weller, O. and G. Dumitroaia. *The earliest salt production in the world: an early*
675 *Neolithic exploitation in Poiana Slatinei-Lunca, Romania*. 2005.

676 2. Catherine, M., V. Bakhshaliyev, and S. Sanz. *Archaeological investigations on the salt*
677 *mine of Duzdagi (Nakhchivan, Azerbaïdjan)*. 2009.

678 3. McIntosh, J., *Handbook to life in prehistoric Europe*. 2009, Oxford ; New York:
679 Oxford University Press. xii, 404 p.

- 680 4. Fennema, O.R., *Food chemistry*. 3rd ed. Food science and technology. 1996, New
681 York: Marcel Dekker. xii, 1069 p.
- 682 5. Potter, N.N. and J.H. Hotchkiss, *Food science*. 5th ed. Food science texts series. 1995,
683 New York: Chapman & Hall. xiii, 608 p.
- 684 6. Davidson, P.M., J.N. Sofos, and A.L. Branen, *Antimicrobials in food*. 3rd ed. Food
685 science and technology. 2005, Boca Raton: Taylor & Francis. 706 p.
- 686 7. Larsen, H., *Halophilic and Halotolerant Microorganisms - an Overview and
687 Historical-Perspective*. Fems Microbiology Letters, 1986. **39**(1-2): p. 3-7.
- 688 8. Kushner, D., *Microbial life in extreme environments*. 1978, London ; New York:
689 Academic Press. xii, 465 p.
- 690 9. Ventosa, A., J.J. Nieto, and A. Oren, *Biology of moderately halophilic aerobic
691 bacteria*. Microbiology and Molecular Biology Reviews, 1998. **62**(2): p. 504-+.
- 692 10. Oren, A., *Microbial life at high salt concentrations: phylogenetic and metabolic
693 diversity*. Saline systems, 2008. **4**: p. 2-2.
- 694 11. Rodriguez-Saiz, M., et al., *Engineering the halophilic bacterium Halomonas elongata
695 to produce beta-carotene*. Applied Microbiology and Biotechnology, 2007. **77**(3): p.
696 637-643.
- 697 12. Vynne, N.G., et al., *Bioactivity, Chemical Profiling, and 16S rRNA-Based Phylogeny
698 of Pseudoalteromonas Strains Collected on a Global Research Cruise*. Marine
699 Biotechnology, 2011. **13**(6): p. 1062-1073.
- 700 13. Holmstrom, C., et al., *Pseudoalteromonas tunicata sp. nov., a bacterium that produces
701 antifouling agents*. International Journal of Systematic Bacteriology, 1998. **48**: p.
702 1205-1212.
- 703 14. Mai-Prochnow, A., et al., *Ecological advantages of autolysis during the development
704 and dispersal of Pseudoalteromonas tunicata biofilms*. Applied and Environmental
705 Microbiology, 2006. **72**(8): p. 5414-5420.
- 706 15. Broekaert, K., et al., *Volatile compounds associated with Psychrobacter spp. and
707 Pseudoalteromonas spp., the dominant microbiota of brown shrimp (Crangon
708 crangon) during aerobic storage*. International Journal of Food Microbiology, 2013.
709 **166**(3): p. 487-493.
- 710 16. Busconi, M., C. Zacconi, and G. Scolari, *Bacterial ecology of PDO Coppa and
711 Pancetta Piacentina at the end of ripening and after MAP storage of sliced product*.
712 International Journal of Food Microbiology, 2014. **172**: p. 13-20.
- 713 17. Ferrocino, I., et al., *RNA-Based Amplicon Sequencing Reveals Microbiota
714 Development during Ripening of Artisanal versus Industrial Lard d'Arnad*. Applied
715 and Environmental Microbiology, 2017. **83**(16).
- 716 18. Pacova, Z., E. Urbanova, and E. Durnova, *Psychrobacter immobilis isolated from
717 foods: characteristics and identification*. Veterinarni Medicina, 2001. **46**(4): p. 95-
718 100.
- 719 19. Stellato, G., et al., *Coexistence of Lactic Acid Bacteria and Potential Spoilage
720 Microbiota in a Dairy Processing Environment*. Applied and Environmental
721 Microbiology, 2015. **81**(22): p. 7893-7904.

- 722 20. Deetae, P., et al., *Production of volatile aroma compounds by bacterial strains*
723 *isolated from different surface-ripened French cheeses*. Applied Microbiology and
724 Biotechnology, 2007. **76**(5): p. 1161-1171.
- 725 21. Delbès C., M.C., Irlinger F., *Des communautés microbiennes au service de la qualité*
726 *des fromages: Diversité et dynamique adaptative et fonctionnelle des populations*
727 *endogènes etensemencées* Innovations Agronomiques, 2015. **44**: p. 69-86.
- 728 22. Irlinger, F., et al., *Cheese rind microbial communities: diversity, composition and*
729 *origin*. Fems Microbiology Letters, 2015. **362**(2).
- 730 23. Bokulich, N.A. and D.A. Mills, *Facility-Specific "House" Microbiome Drives*
731 *Microbial Landscapes of Artisan Cheesemaking Plants*. Applied and Environmental
732 Microbiology, 2013. **79**(17): p. 5214-5223.
- 733 24. Almeida, M., et al., *Construction of a dairy microbial genome catalog opens new*
734 *perspectives for the metagenomic analysis of dairy fermented products*. BMC
735 Genomics, 2014. **15**.
- 736 25. Wolfe, B.E., et al., *Cheese Rind Communities Provide Tractable Systems for In Situ*
737 *and In Vitro Studies of Microbial Diversity*. Cell, 2014. **158**(2): p. 422-433.
- 738 26. Levesque, S., et al., *Mobilome of Brevibacterium aurantiacum Sheds Light on Its*
739 *Genetic Diversity and Its Adaptation to Smear-Ripened Cheeses*. Frontiers in
740 Microbiology, 2019. **10**.
- 741 27. Pham, N.P., et al., *Comparative genomic analysis of Brevibacterium strains: insights*
742 *into key genetic determinants involved in adaptation to the cheese habitat*. BMC
743 Genomics, 2017. **18**.
- 744 28. Bonham, K.S., B.E. Wolfe, and R.J. Dutton, *Extensive horizontal gene transfer in*
745 *cheese-associated bacteria*. Elife, 2017. **6**.
- 746 29. Mackenzie, E.L., K. Iwasaki, and Y. Tsuji, *Intracellular iron transport and storage:*
747 *From molecular mechanisms to health implications*. Antioxidants & Redox Signaling,
748 2008. **10**(6): p. 997-1030.
- 749 30. Rea, M.C., et al., *Stability of the biodiversity of the surface consortia of Gubbeen, a*
750 *red-smear cheese*. J Dairy Sci, 2007. **90**(5): p. 2200-10.
- 751 31. Mounier, J., et al., *Surface microflora of four smear-ripened cheeses*. Applied and
752 Environmental Microbiology, 2005. **71**(11): p. 6489-6500.
- 753 32. Gori, K., et al., *Isolation and Identification of the Microbiota of Danish Farmhouse*
754 *and Industrially Produced Surface-Ripened Cheeses*. Microbial Ecology, 2013. **65**(3):
755 p. 602-615.
- 756 33. Mounier, J., et al., *Assessment of the microbial diversity at the surface of Livarot*
757 *cheese using culture-dependent and independent approaches*. International Journal of
758 Food Microbiology, 2009. **133**(1-2): p. 31-37.
- 759 34. Ades, G.L. and J.F. Cone, *Proteolytic Activity of Brevibacterium Linens during*
760 *Ripening of Trappist-Type Cheese*. Journal of Dairy Science, 1969. **52**(7): p. 957-&.
- 761 35. Sharpe, M.E., et al., *Methanethiol Production by Coryneform Bacteria - Strains from*
762 *Dairy and Human-Skin Sources and Brevibacterium-Linens*. Journal of General
763 Microbiology, 1977. **101**(Aug): p. 345-349.

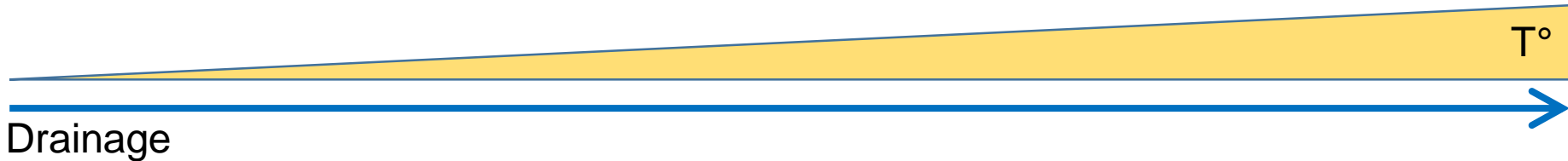
- 764 36. Brennan, N.M., et al., *Biodiversity of the bacterial flora on the surface of a smear*
765 *cheese*. Applied and Environmental Microbiology, 2002. **68**(2): p. 820-830.
- 766 37. Feurer, C., et al., *Assessment of the rind microbial diversity in a farm house-produced*
767 *vs a pasteurized industrially produced soft red-smear cheese using both cultivation*
768 *and rDNA-based methods*. Journal of Applied Microbiology, 2004. **97**(3): p. 546-556.
- 769 38. Monnet, C., et al., *Growth and adaptation of microorganisms on the cheese surface*.
770 Fems Microbiology Letters, 2015. **362**(1).
- 771 39. Kastman, E.K., et al., *Biotic Interactions Shape the Ecological Distributions of*
772 *Staphylococcus Species*. Mbio, 2016. **7**(5).
- 773 40. Almena-Aliste, M. and B. Mietton, *Cheese Classification, Characterization, and*
774 *Categorization: A Global Perspective*. Microbiol Spectr, 2014. **2**(1): p. CM-0003-
775 2012.
- 776 41. Louca, S., et al., *Function and functional redundancy in microbial systems*. Nature
777 Ecology & Evolution, 2018. **2**(6): p. 936-943.
- 778 42. Pan, Q.M., et al., *Effects of functional diversity loss on ecosystem functions are*
779 *influenced by compensation*. Ecology, 2016. **97**(9): p. 2293-2302.
- 780 43. Gladstone-Gallagher, R.V., et al., *Linking Traits across Ecological Scales Determines*
781 *Functional Resilience*. Trends in Ecology & Evolution, 2019. **34**(12): p. 1080-1091.
- 782 44. Broekaert, K., et al., *Seafood quality analysis: Molecular identification of dominant*
783 *microbiota after ice storage on several general growth media*. Food Microbiology,
784 2011. **28**(6): p. 1162-1169.
- 785 45. Maskow, T. and W. Babel, *Calorimetrically obtained information about the efficiency*
786 *of ectoine synthesis from glucose in Halomonas elongata (vol 1527, pg 4, 2001)*.
787 Biochimica Et Biophysica Acta-General Subjects, 2001. **1528**(1): p. 60-60.
- 788 46. Acinas, S.G., et al., *Fine-scale phylogenetic architecture of a complex bacterial*
789 *community*. Nature, 2004. **430**(6999): p. 551-554.
- 790 47. Tayeb, L.A., et al., *Comparative phylogenies of Burkholderia, Ralstonia, Comamonas,*
791 *Brevundimonas and related organisms derived from rpoB, gyrB and rrs gene*
792 *sequences*. Research in Microbiology, 2008. **159**(3): p. 169-177.
- 793 48. Camacho, C., et al., *BLAST plus : architecture and applications*. BMC Bioinformatics,
794 2009. **10**.
- 795 49. Bankevich, A., et al., *SPAdes: A New Genome Assembly Algorithm and Its*
796 *Applications to Single-Cell Sequencing*. Journal of Computational Biology, 2012.
797 **19**(5): p. 455-477.
- 798 50. Aziz, R.K., et al., *The RAST server: Rapid annotations using subsystems technology*.
799 BMC Genomics, 2008. **9**.
- 800 51. Stackebrandt, E. *Taxonomic parameters revisited : tarnished gold standards*. 2006.
- 801 52. Adekambi, T., M. Drancourt, and D. Raoult, *The rpoB gene as a tool for clinical*
802 *microbiologists*. Trends in Microbiology, 2009. **17**(1): p. 37-45.
- 803 53. Thompson, J.D., et al., *The CLUSTAL_X windows interface: flexible strategies for*
804 *multiple sequence alignment aided by quality analysis tools*. Nucleic Acids Research,
805 1997. **25**(24): p. 4876-4882.

- 806 54. Kumar, S., G. Stecher, and K. Tamura, *MEGA7: Molecular Evolutionary Genetics*
807 *Analysis Version 7.0 for Bigger Datasets*. Molecular Biology and Evolution, 2016.
808 **33**(7): p. 1870-1874.
- 809 55. Saitou, N. and M. Nei, *The Neighbor-Joining Method - a New Method for*
810 *Reconstructing Phylogenetic Trees*. Molecular Biology and Evolution, 1987. **4**(4): p.
811 406-425.
- 812 56. Felsenstein, J., *Confidence-Limits on Phylogenies - an Approach Using the Bootstrap*.
813 Evolution, 1985. **39**(4): p. 783-791.
- 814 57. Richter, M., et al., *JSpeciesWS: a web server for prokaryotic species circumscription*
815 *based on pairwise genome comparison*. Bioinformatics, 2016. **32**(6): p. 929-931.
- 816 58. Goris, J., et al., *DNA-DNA hybridization values and their relationship to whole-*
817 *genome sequence similarities*. International Journal of Systematic and Evolutionary
818 Microbiology, 2007. **57**: p. 81-91.
- 819 59. Richter, M. and R. Rossello-Mora, *Shifting the genomic gold standard for the*
820 *prokaryotic species definition*. Proceedings of the National Academy of Sciences of
821 the United States of America, 2009. **106**(45): p. 19126-19131.
- 822 60. Page, A.J., et al., *Roary: rapid large-scale prokaryote pan genome analysis*.
823 Bioinformatics, 2015. **31**(22): p. 3691-3693.
- 824 61. Brynildsrud, O., et al., *Rapid scoring of genes in microbial pan-genome-wide*
825 *association studies with Scoary (vol 17, 238, 2016)*. Genome Biology, 2016. **17**.
- 826 62. Hadfield, J., et al., *Phandango: an interactive viewer for bacterial population*
827 *genomics*. Bioinformatics, 2018. **34**(2): p. 292-293.
- 828 63. Langmead, B., et al., *Ultrafast and memory-efficient alignment of short DNA*
829 *sequences to the human genome*. Genome Biology, 2009. **10**(3).
- 830 64. Quinlan, A.R. and I.M. Hall, *BEDTools: a flexible suite of utilities for comparing*
831 *genomic features*. Bioinformatics, 2010. **26**(6): p. 841-842.
- 832 65. Li, H., et al., *The Sequence Alignment/Map format and SAMtools*. Bioinformatics,
833 2009. **25**(16): p. 2078-2079.
- 834 66. Bastian, M., S. Heymann, and M. Jacomy, *Gephi: an open source software for*
835 *exploring and manipulating networks*. 2009. p. 361--362.
- 836

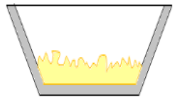
Cheese	Cheese Technology	Animal	Rind Type	Origin (France)	Salting	Ripening*
A	Lactic paste	Cow	Washed	Bourgogne Champagne	Washed one to three times a week in brine	short
B	Lactic paste	Cow	Washed	Bourgogne Champagne	Washed one to three times a week in brine	short
C	Unpressed uncooked soft	Cow	Washed	Alsace-Lorraine	Washed three times a week in brine	short
D	Pressed uncooked soft	Cow	Washed	Savoie	Dry salt or brine before ripening	short
E	Pressed uncooked semihard	Ewe	Washed	Aquitane Midi-Pyrenées	Turned and brushed with dry salt or brine	medium
F	Lactic paste	Ewe	Washed	Méditerranée	Unkown	short
G	Blue cheese	Cow	Natural	Auvergne	Dry salt Washed several times in brine and regularly returned	medium
H	Pressed uncooked soft	Cow	Washed	Auvergne	returned	medium
I	Pressed uncooked soft	Cow	Natural	Franche-Comté	Dry salt	medium
J	Lactic paste	Goat	Bloomy	Méditerranée	Exclusively with dry salt	short
K	Pressed uncooked soft	Cow	Washed	Alsace-Lorraine	Washed twice a week in brine	medium
L	Pressed uncooked semihard	Ewe	Washed	Aquitane Midi-Pyrenées	Turned and brushed with dry salt or brine	medium
M	Pressed cooked hard	Cow	Washed	Franche-Comté	Turned and brushed with dry salt or brine	long

*short: several weeks; medium: several months; long: over 6 months

	Isolate in this study	Cheese	Media	Temperature isolated (°C)	Closest reference strains of whole-genome	ANiB score (%)
Gram-positive strains	<i>Brachybacterium</i> sp. FME24	K	MB+6%NaCl	30	<i>Brachybacterium tyrofermentans</i> CNRZ926 ^T	83.17
	<i>Brachybacterium tyrofermentans</i> FME25	J	MB+8%NaCl	25	<i>Brachybacterium tyrofermentans</i> CNRZ926 ^T	95.89
	<i>Brevibacterium aurantiacum</i> FME34	G	MB+8%NaCl	20	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	96.27
	<i>Brevibacterium aurantiacum</i> FME43	K	MB+8%NaCl	30	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	96.29
	<i>Brevibacterium aurantiacum</i> FME45	K	HM	25	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	96.62
	<i>Brevibacterium aurantiacum</i> FME48	C	HM	25	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	96.67
	<i>Brevibacterium aurantiacum</i> FME49	L	MB+4%NaCl	20	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	96.69
	<i>Brevibacterium aurantiacum</i> FME9	J	MB	25	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	96.17
	<i>Brevibacterium</i> sp. FME17	M	MB+8%NaCl	20	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	85.7
	<i>Brevibacterium</i> sp. FME37	K	MB	25	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	86.18
	<i>Corynebacterium casei</i> FME59	L	MB+4%NaCl	25	<i>Corynebacterium casei</i> LMG S-19264 ^T	97.92
	<i>Glutamicibacter arilaitensis</i> FME22	H	MB	25	<i>Glutamicibacter arilaitensis</i> Re117 ^T	98.05
	<i>Carnobacterium mobile</i> FME4	A	MB	25	<i>Carnobacterium mobile</i> DSM 4848 ^T	97.42
	<i>Marinilactibacillus psychrotolerans</i> FME56	C	MB+4%NaCl	25	<i>Marinilactibacillus psychrotolerans</i> NBRC 100002 ^T	97.07
	<i>Oceanobacillus oncorhynchi</i> FME55	A	MB+8%NaCl	30	<i>Oceanobacillus oncorhynchi</i> Oc5	98.04
	<i>Staphylococcus equorum</i> FME18	H	MB	25	<i>Staphylococcus equorum</i> NCTC 12414 ^T	99.45
	<i>Staphylococcus equorum</i> FME19	G	MB+6%NaCl	25	<i>Staphylococcus equorum</i> NCTC 12414 ^T	94.85
	<i>Staphylococcus equorum</i> FME58	C	MB+8%NaCl	25	<i>Staphylococcus equorum</i> NCTC 12414 ^T	99.05
	<i>Staphylococcus vitulinus</i> FME39	F	HM	25	<i>Staphylococcus vitulinus</i> DSM 15615 ^T	98.67
	<i>Staphylococcus succinus</i> FME10	D	MB	25	<i>Staphylococcus succinus</i> DSM 14617 ^T	97.77
Gram-negative strains	<i>Advenella</i> sp. FME57	I	MB	25	<i>Advenella incenata</i> DSM 23814 ^T	90.42
	<i>Hafnia alvei</i> FME31	B	MB	25	<i>Hafnia alvei</i> ATCC 13337 ^T	99.58
	<i>Halomonas</i> sp. FME1	I	MB	25	<i>Halomonas boliviensis</i> LC1 ^T	79.97
	<i>Halomonas</i> sp. FME16	F	HM	25	<i>Halomonas zhanjiangensis</i> DSM 21076 ^T	82.23
	<i>Halomonas</i> sp. FME20	H	MB+4%NaCl	25	<i>Halomonas zhanjiangensis</i> DSM 21076 ^T	93.22
	<i>Proteus</i> sp. FME41	C	LH	20	<i>Proteus cibarius</i> JCM 30699 ^T	88.62
	<i>Pseudoalteromonas prydzensis</i> FME14	H	MB	25	<i>Pseudoalteromonas prydzensis</i> DSM 14232 ^T	95.96
	<i>Pseudoalteromonas nigrifaciens</i> FME53	B	MB+6%NaCl	25	<i>Pseudoalteromonas nigrifaciens</i> NCTC 10691 ^T	98.31
	<i>Pseudomonas lundensis</i> FME52	B	MB	25	<i>Pseudomonas lundensis</i> DSM6252 ^T	98.33
	<i>Pseudomonas</i> sp. FME51	C	LH	20	<i>Pseudomonas litoralis</i> 2SM5 ^T	87.7
	<i>Psychrobacter</i> sp. FME13	F	MB	25	<i>Psychrobacter fozi</i> CECT 5889 ^T	82.65
	<i>Psychrobacter</i> sp. FME2	A	MB	25	<i>Psychrobacter cibarius</i> JG-219 ^T	95.58
	<i>Psychrobacter</i> sp. FME5	G	MB	25	<i>Psychrobacter faecalis</i> Iso-46 ^T	80.7
	<i>Psychrobacter</i> sp. FME6	J	MB	25	<i>Psychrobacter faecalis</i> Iso-46 ^T	80.5
	<i>Vibrio casei</i> FME29	A	MB+4%NaCl	25	<i>Vibrio casei</i> DSM 22364 ^T	99.87

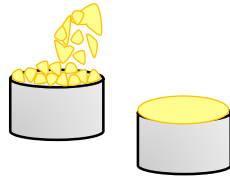


Lactic coagulation
(slow coagulation)



Lactic paste

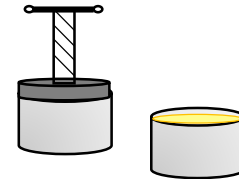
Mixed and enzymatic coagulation
(fast coagulation)



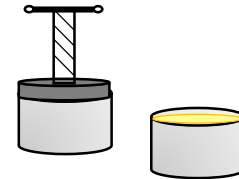
Unpressed uncooked soft cheese



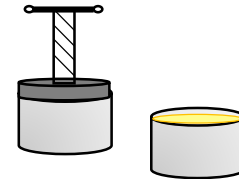
Blue cheese



Pressed uncooked soft/semi-hard cheese



Pressed semi-cooked/semi-hard cheese



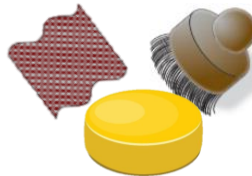
Pressed cooked semi-hard/hard cheese

MNFS

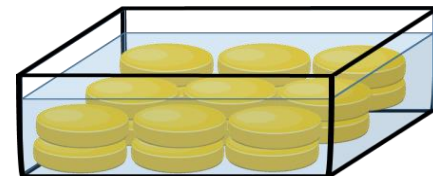
Dry salting



Smearing



Brining



Bloomy rind

Chaource
Selles sur Cher

Washed or mixed rind

Époisses
Langres
Picodon

Bloomy rind

Camembert
Brie

Washed or mixed rind

Munster
Livarot

Roquefort
Gorgonzola

Washed or mixed rind

Reblochon
Mont D'or
Morbier

Surface Mold

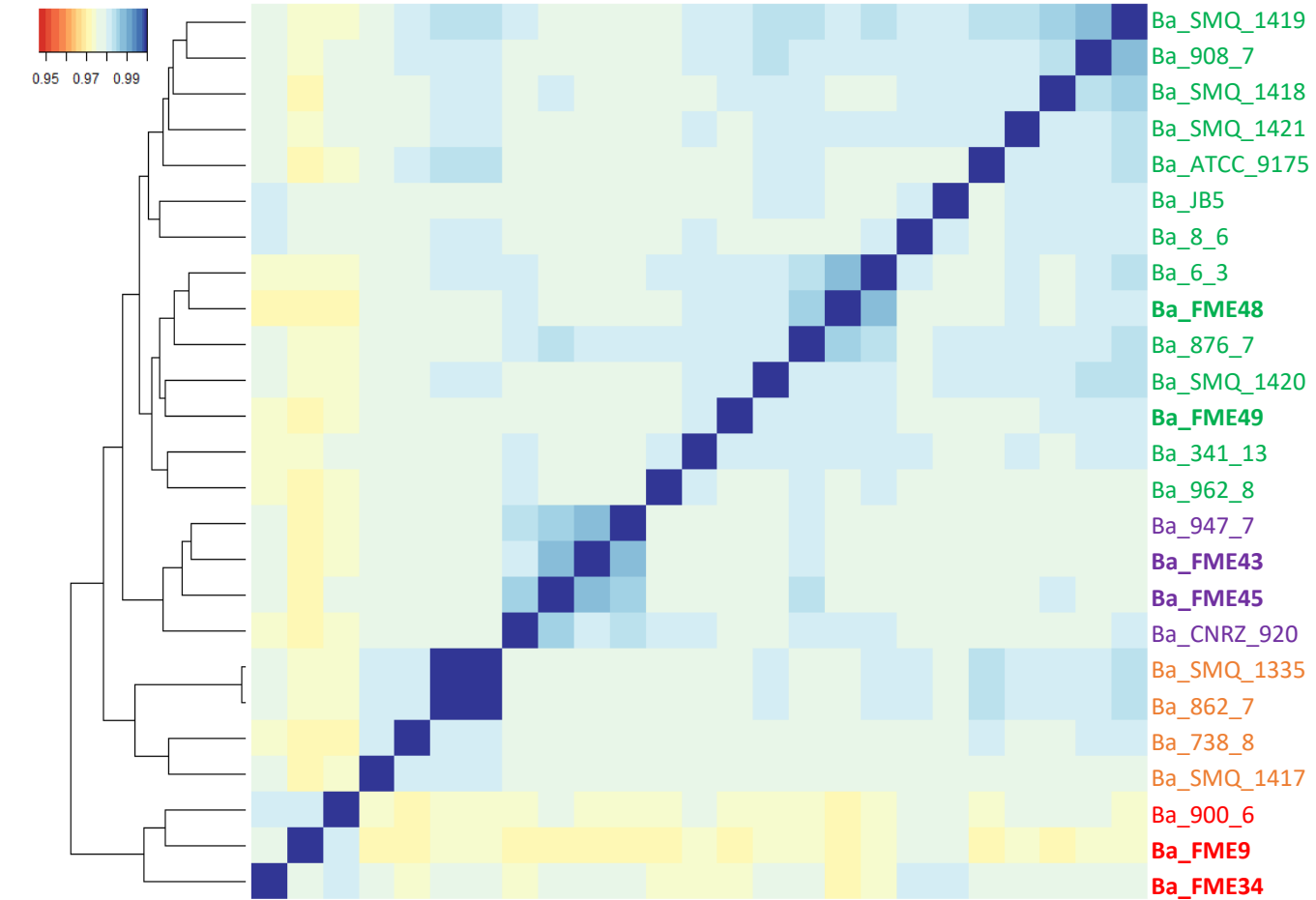
Saint Nectaire

Milled curd

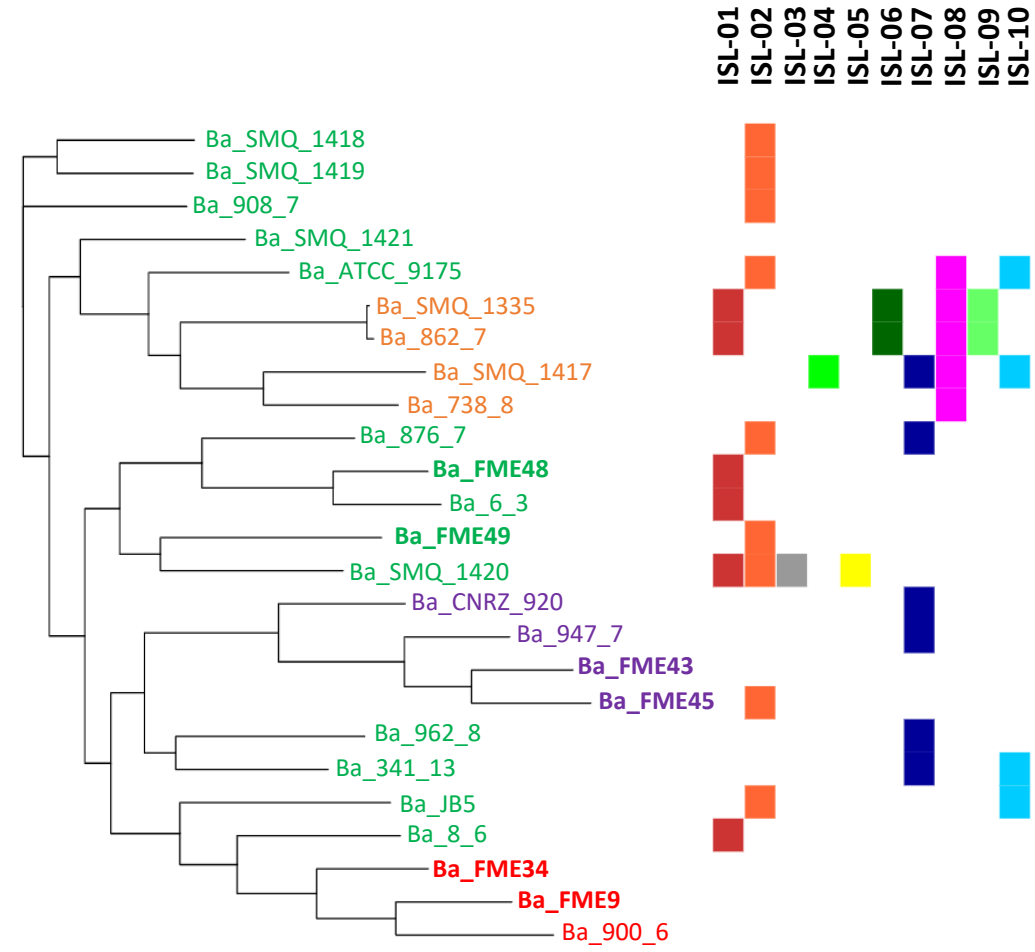
Cantal
Salers
Cheddar

Abondance
Asiago
Appenzeller

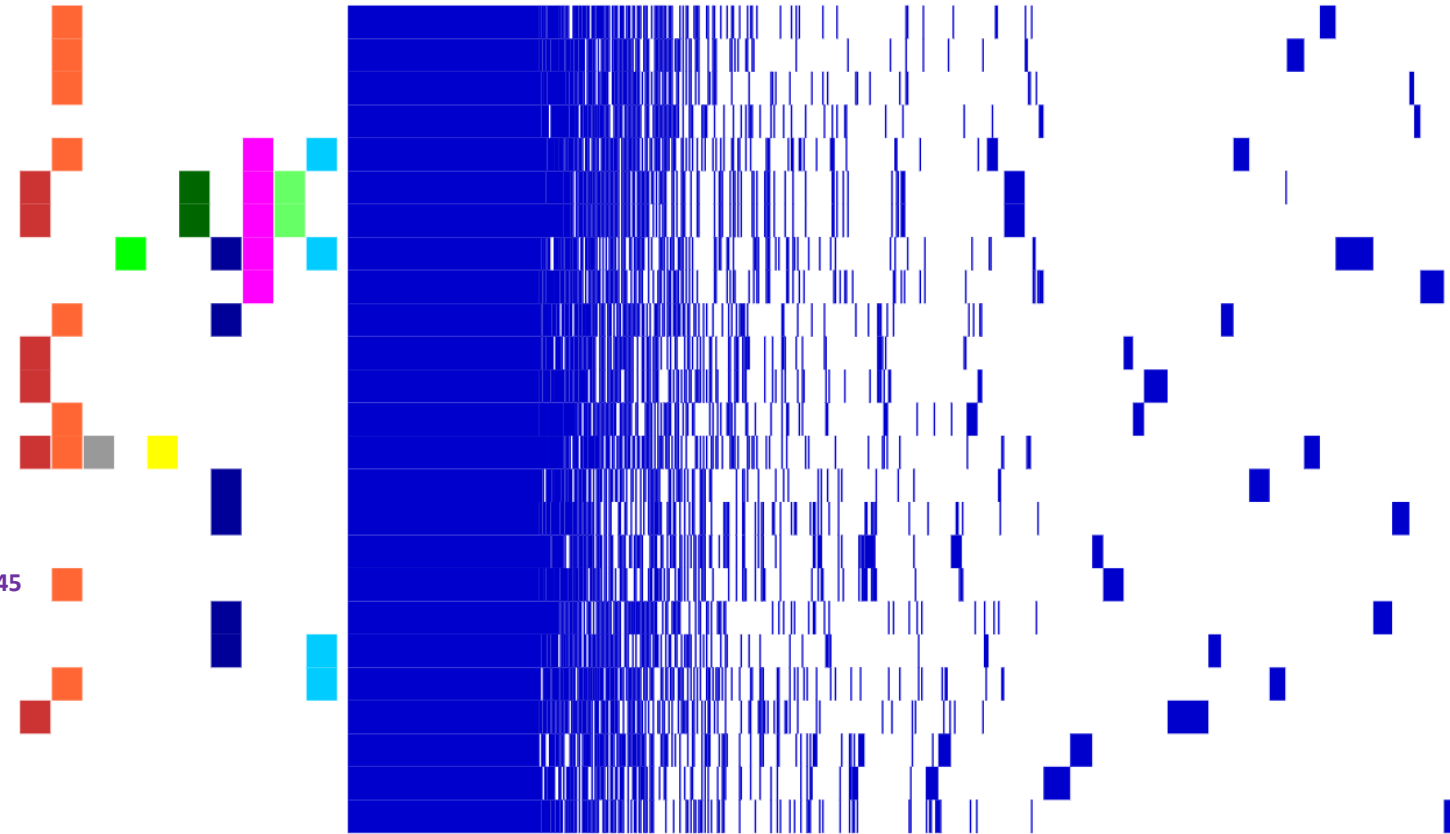
Beaufort
Pecorino
Emmental



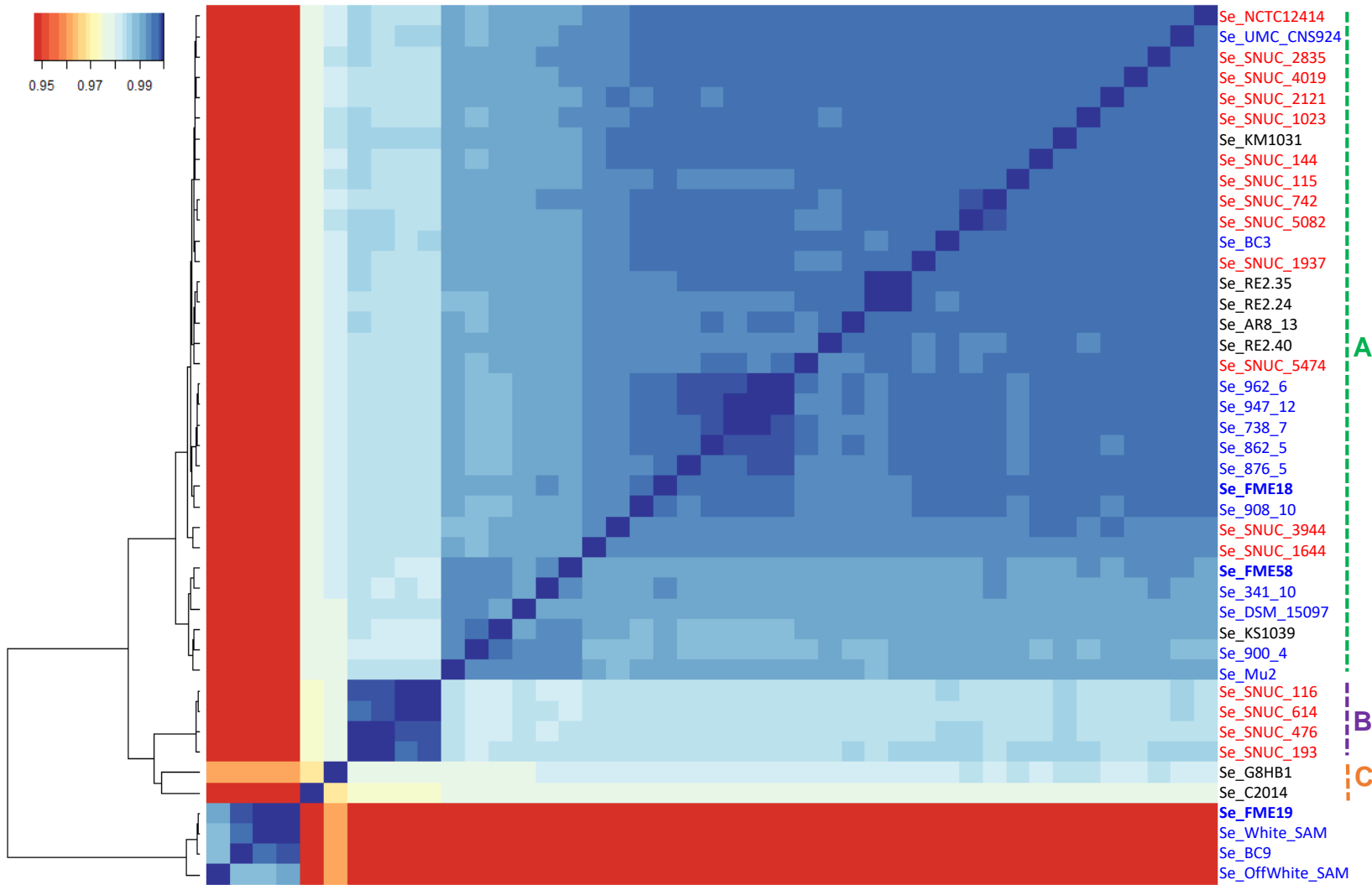
(A)



ISL-01
ISL-02
ISL-03
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ISL-08
ISL-09
ISL-10



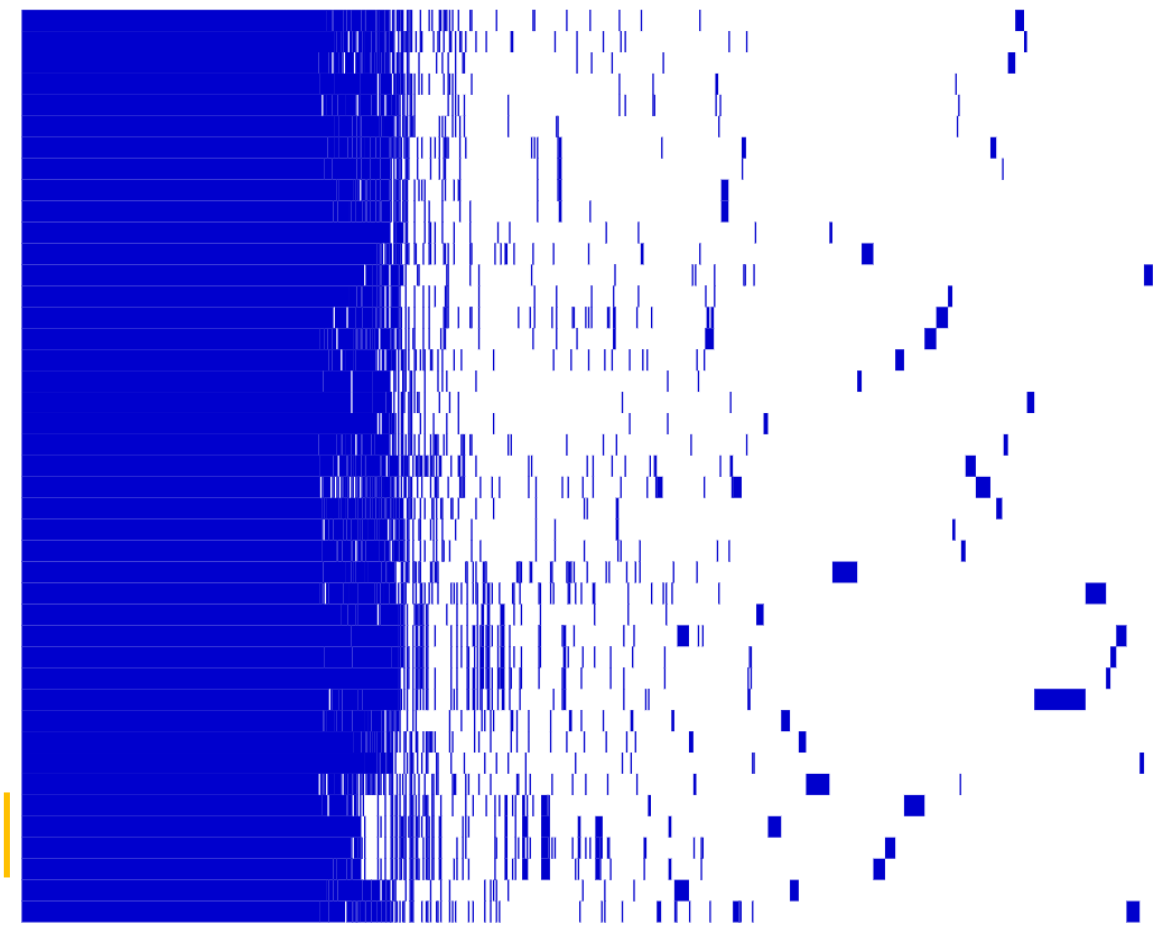
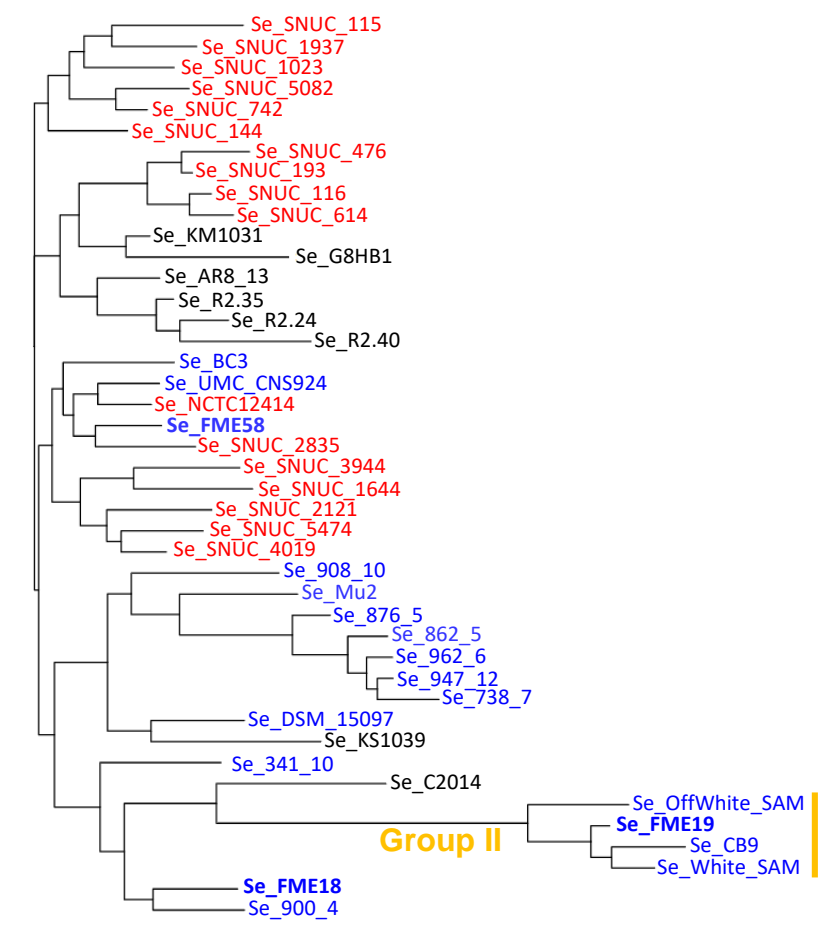
(B)



(A)

Group I

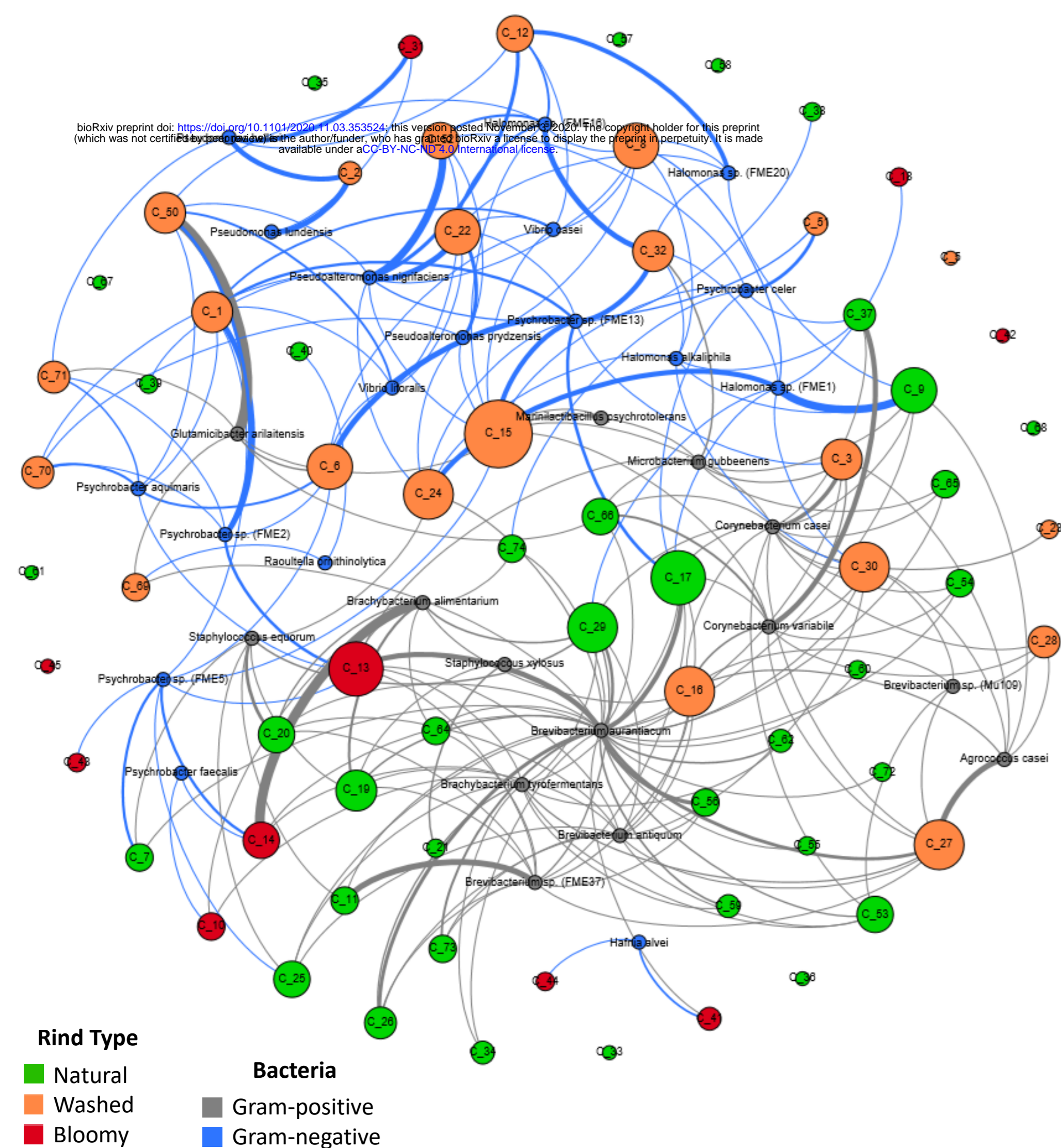
Group II



(B)



(A)



(B)

