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1 **Characterization of bacterial communities of donkey milk by high-throughput**
2 **sequencing.**

3

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25

26

27 **Abstract**

28 The interest in donkey milk (DM) is growing because of its functional properties and
29 nutritional value, especially for children with allergies and food intolerances. However, most
30 of the available reports of DM microbiota are based on culture-dependent methods to
31 investigate food safety issues and the presence of lactic acid bacteria (LAB).

32 The aim of this study was to determine the composition of DM bacterial communities using a
33 high-throughput sequencing (HTS) approach.

34 Bulk milk samples from Italian donkey dairy farms from two consecutive years were analysed
35 using the MiSeq Illumina platform. All sample reads were classified into **five phyla**:
36 *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Verrucomicrobia*. The most
37 prevalent genera—*Pseudomonas*, *Ralstonia*, *Acinetobacter*, *Cupriavidus*, *Citrobacter* and
38 *Sphingobacterium*—**were** gram-negative bacteria.

39 The core microbiota was composed of genera that comprise commonly associated milk
40 bacteria, LAB and species normally found in soil, water and plants. Reads assigned to LAB
41 genera—*Streptococcus*, *Lactococcus*, *Enterococcus*, *Leuconostoc*, *Lactobacillus*, and
42 *Carnobacterium*—corresponded on average to 2.55% of the total reads per sample. Among
43 these, the distribution of reads assigned to coccus- and bacillus-shaped LAB was variable
44 between and within the farms, confirming their presence and suggesting a complex population
45 of these bacteria in DM.

46 The present study represents a general snapshot of the DM microbial population, underlining
47 its variability and motivating further studies for the exploitation of the technological potential
48 of bacteria naturally present in DM.

49

50

51

52 **Keywords:**

53 donkey milk, bacterial communities, high-throughput sequencing

54

55 **Highlights:**

56 Bulk milk samples of donkey milk were studied with a HTS approach.

57 Microbial population of DM is complex, diverse, variable

58 The most prevalent genera are Gram negative bacteria.

59

60 **1. Introduction**

61 Donkey milk (DM) has recently received growing interest since it has been reported to be an
62 adequate replacement for children with cow milk protein allergy, mainly due to its
63 tolerability, nutritional contents and good taste (Monti et al., 2012). In fact, studies have
64 demonstrated a number of qualities that make DM more favourable than cow milk: better
65 digestibility (Tidona et al., 2011), lower allergenicity (Vincenzetti et al., 2008) and a set of
66 unique nutritional and physicochemical characteristics (Guo et al., 2007).

67 Following the growing demand for DM, several new dairy farms have opened in the last few
68 years. Italian donkey dairies are generally small, with 20 to 25 milking jennies and one or two
69 stallions; their overall average daily production is approximately 2,000 litres, for a total of
70 700,000 litres per year (Milonis and Polidori, 2011). The production is mainly used for direct
71 human consumption, while a smaller part is destined for the cosmetics and food industries.
72 Pasteurized donkey milk is usually sold directly from the farms. However, considering its
73 target consumers and nutritional properties, it can be sold raw, with 3 days of shelf life
74 (similar to raw bovine milk) (Giacometti et al., 2016).

75 The composition of DM is closer to human milk than to cow milk and has been fully
76 described (Salimei and Fantuz, 2012). It contains high levels of lactose and essential amino
77 acids (Guo et al., 2007) as well as low concentrations of β -lactoglobulin and casein—the most
78 common allergens in cow milk (Vincenzetti et al., 2008). One of the main characteristics of
79 DM is its high concentration of lysozyme: from 1300 to 4000 mg/l, compared to 0.09 mg/l in
80 cow milk and 40–200 mg/l in human milk (Carminati et al., 2014; Chiavari et al., 2005;
81 Vincenzetti et al., 2008). This enzyme has bactericidal properties; it hydrolyses the **murein** of
82 bacterial cell walls, causing lysis of sensitive bacteria (Chiavari et al., 2005). Currently, there
83 is no confirmed hypothesis as to why DM is so rich in lysozyme, but it seems to positively
84 affect the animals, defending against infections in both the mammary gland and the foal. In

85 addition to lysozyme, DM lactoferrin concentration is twice as high as in bovine milk
86 (Malacarne et al., 2002), and other components have been described, such as
87 immunoglobulins, free fatty acids and members of the lactoperoxidase peroxide system
88 (Zhang et al., 2008), that might act synergistically against specific bacteria (Šarić et al., 2012).
89 Traditional microbiological tests and biomolecular culture-dependent methods have been used
90 to study the bacterial population of DM, mainly focusing on hygienic conditions and/or the
91 presence of lactic acid bacteria (LAB) (Cavallarin et al., 2015; Pilla et al., 2010; Zhang et al.,
92 2008; Šarić et al., 2012). Moreover, in the last few years, culture-independent methods, based
93 on the direct analysis of DNA without a culturing step, have also been used to characterize the
94 milk of different species (Quigley et al., 2013). PCR-denaturing gradient gel electrophoresis
95 (PCR-DGGE), for example, has been successfully applied to the study of the microbiota of
96 milk and dairy products (Delgado et al., 2013). However, limitations in the resolution still
97 need to be overcome, especially for the analyses of matrices with diverse microbial
98 communities (Ogier et al., 2004). Recently, rapid developments of high-throughput
99 sequencing (HTS) methods have allowed a deeper and more precise evaluation of the milk
100 microbiota from different animals, including cattle, goat, sheep, buffalo and humans (Quigley
101 et al., 2013).

102 Notwithstanding the extensive literature on DM, no high-throughput analysis of its bacterial
103 population has yet been performed, despite ever-increasing interest from both technological
104 and commercial points of view. For this reason, the present study aimed to contribute to the
105 knowledge of DM by characterizing its microbiota using an HTS approach.

106

107 **2. Materials and Methods**

108

109 **2.1 Milk sampling and DNA extraction**

110 Five donkey dairy farms (A, B, C, D, E) in the northwest part of Italy were sampled during
111 the spring (March) of 2013 (samples A.2013, B.2013, C.2013, D.2013, E.2013) and 2014
112 (samples A.2014, B.2014, C.2014, D.2014, E.2014); in the second year, an additional farm
113 was included (F; sample F.2014). These are small dairies, with a few milking jennies, family-
114 run and with a limited production (around one litre per day, per animal); the general
115 characteristics of the surveyed farms are summarized in Table S1. The biochemical
116 characterization, the shelf life and the safety of the samples have been reported in a previous
117 work (Cavallarin et al., 2015).

118 Bulk milk samples from healthy jennies, collected in sterile tubes, were transported to the
119 laboratory immediately after sampling in cool conditions and stored at -20 °C until DNA
120 extraction. Samples were treated as reported elsewhere (Dalmaso et al., 2011), and DNA was
121 extracted from 3 ml of milk following the manufacturer protocol of the Dneasy Blood &
122 Tissue kit (Qiagen) and quantified with a Nanodrop 2000 (Thermo Fisher Scientific). To
123 minimize the bias associated with single extractions, triple extractions of each sample were
124 done in parallel and mixed in a final pool.

125

126 **2.2 High-throughput sequencing**

127 Illumina libraries were prepared following the protocol described by Dalmaso et al. (2016)
128 with the NEXTFlex 16S V4 Amplicon-Seq Kit (Bioo Scientific, Austin, USA). Briefly, the
129 bacterial V4 region of the 16S ribosomal gene was amplified from 50 ng of DNA for each
130 sample. The universal primers 515F and 806R tailed with Illumina barcoded adapters were
131 used with the following touchdown PCR conditions: an initial 9 cycles (15 sec. at 95°C, 15
132 sec. at 68°C, 30 sec. at 72°C) and then another 23 cycles (15 sec. at 95°C, 15 sec. at 58°C, 30
133 sec. at 72°C). The PCR products were purified using Agencourt XP Ampure Beads (Beckman

134 Coulter). The quality of the final products was assessed with a Bioanalyzer 2100 (Agilent
135 Technologies).

136 The samples were quantified with Qubit (Invitrogen) and pooled in equal proportions for their
137 paired-end sequencing with Illumina MiSeq for 312 cycles (150 cycles for each paired read
138 and 12 cycles for the barcode sequence) at IGA Technology Services (Udine, Italy). To
139 prevent focusing and phasing problems due to the sequencing of “low diversity” libraries,
140 30% PhiX genome was spiked in the pooled library.

141

142 **2.3 Bioinformatics and data analyses**

143 Sequence reads were trimmed with the collection command line tools of FASTX-Toolkits
144 (http://hannonlab.cshl.edu/fastx_toolkit/) so that the quality score for each read was above 20
145 with more than 50 base pairs. The PRINSEQ standalone lite version (Schmieder and
146 Edwards, 2011) was used to check and prepare the data set for the downstream analyses.

147 Data were then analysed with the QIIME software, version 1.9.0 (Caporaso et al., 2012).
148 Using the uclust method (Edgar, 2010), sequences >97% identical were considered to
149 correspond to the same operational taxonomic unit (OTU). Representative sequences were
150 submitted to the RDPII classifier (Wang et al., 2007) to obtain the taxonomy assignment and
151 relative abundance of each OTU using the Greengenes 16S rDNA database v13.8 (McDonald
152 et al., 2012).

153 Alpha diversity was evaluated with QIIME to obtain the rarefaction curves. A rarefaction
154 curve shows the variation in the number of OTUs identified at a given percentage of identity
155 as a function of the number of sequence reads obtained per sample. Ideally, an optimal
156 coverage is identified by the plateau of the curve, which indicates that increasing the number
157 of reads does not change the number of OTUs that can be determined.

158 Moreover, Good's coverage (a sampling completeness indicator that indicates what percent of
159 the total species is represented in the sample), Chao1 and ACE (richness estimators that
160 calculate an approximate number of species in the samples using different methods), and
161 Shannon and Simpson indices (estimators of the samples' diversity taking into account the
162 approximated number of species and how evenly they are distributed) were determined.

163 Beta diversity was evaluated with the UniFrac method. Weighted UniFrac distance matrices
164 and OTU tables were used to plot the principal coordinate analysis (PCoA) and to perform
165 Adonis and Anosim statistical tests with the compare_category.py script of QIIME to evaluate
166 differences between the farms, their practices and their characteristics.

167 The core microbiota of the samples was obtained with the compute_core_microbiome.py
168 script in QIIME; OTUs present with more than 0.001% of the reads of each sample, in at least
169 9 samples, were included. The pseudo-heatmap was plotted with the gplots package in the R
170 environment (<http://www.r-project.org>) using the OTUs table generated by QIIME.

171

172 **3. Results and Discussion**

173

174 **3.1 Characteristics of the sequencing data**

175 We obtained a total of 5,225,689 raw sequences; after filtering, 3,743,291 high-quality 16S
176 rRNA gene sequences with an average length of 288 bp were recovered. Table 1 shows the
177 number of analysed reads per sample. The rarefaction curves of our data (Figure S1) suggest a
178 sufficient coverage; this consideration is further supported by the observed values of the
179 Good's coverage estimator **-higher than 0.99-** for all the samples (Table 1).

180

181 **3.2 Bacterial composition of donkey milk**

182 The sequences obtained from all the studied samples correspond to five phyla:
183 *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Verrucomicrobia* (Table 2) in

184 agreement with the main taxons found in raw milk from different animals (Dalmasso et al.
185 2016; Quigley et al., 2013). The total reads corresponded to 201 families and 314 different
186 genera (data not shown).

187 The most abundant genera observed in all the studied samples were gram-negative bacteria:
188 *Pseudomonas*, *Ralstonia*, *Cupriavidus*, *Acinetobacter*, *Citrobacter* and *Sphingobacterium*
189 (Figure 1, Table 2).

190 However, only the genus *Pseudomonas* reached high percentages in almost all the studied
191 samples. Furthermore, previous studies, using culture-dependent methods, had found that
192 *Pseudomonas* spp. is an important component of the DM microbiota (Cavallarin et al., 2015;
193 Giacometti et al., 2016). This observation is consistent with a previous report that indicated
194 *Pseudomonas* spp. to be the predominant microorganism in different milks (Quigley et al.,
195 2013); in raw bovine milk stored at low temperatures, *Pseudomonas* spp. may constitute up to
196 70-90% of the total microbial population (Sørhaug and Stepaniak, 1997). The abundance of
197 these microorganisms, which are the most common cause of milk spoilage (Ercolini et al.,
198 2009), mainly because of their proteolytic activity and psychrotolerant nature, leads to the
199 short commercial shelf life of the product (3 days). Given that raw DM is sold, is necessary to
200 focus attention not only on spoilage but also on hygienic safety. Cavallarin et al., (2015),
201 while characterizing DM by traditional microbiological methods, showed the absence of
202 pathogens. In our study, the limitations of the analytical approach (genus identification and
203 the impossibility of viability evaluation) did not allow us to infer the hygienic safety status.

204 The other genera (*Ralstonia*, *Cupriavidus*, *Acinetobacter*, *Citrobacter* and *Sphingobacterium*)
205 (Figure 1, Table 2), are considered environmental microorganisms since they are commonly
206 found in soil, water and dust. *Ralstonia* spp. and *Cupriavidus* spp. are phylogenetically related
207 to *Pseudomonas* spp., and they have only recently been reclassified (Balkwill, 2015;

208 Yabuuchi et al., 2015). Nevertheless, HTS studies have found them in human, bovine, goat
209 and buffalo milk (Quigley et al., 2013).

210 The composition of the DM core microbiota, i.e., those OTUs shared between the samples,
211 was also evaluated. This core contained 4 families and 24 genera that comprise commonly
212 associated milk bacteria, LAB and species normally found in soil, water and plants (Figure 2).
213 One compelling member of the core was the genus *Akkermansia* since the only species that
214 currently forms the genus, *Akkermansia muciniphila*, has been linked with intestinal health,
215 the metabolic status of obese and diabetic patients, and markers of inflammation and immune
216 responses (Reunanen et al., 2015). This potential probiotic bacterium uses mucin—a protein
217 amply present in milk—as its main source of carbon and nitrogen and has been detected in
218 human and animal gut environments (Belzer and de Vos, 2012), including in donkeys (Liu et
219 al., 2014). Additionally, this bacterium has been detected in breast milk using real-time PCR
220 (Collado et al., 2012), and just recently, Ottman (2015) reported its ability to grow in human
221 milk. Further studies are needed to isolate and characterize the probable *Akkermansia* species
222 present in DM; nonetheless, our observation creates a new perspective on this functional
223 microbe that has not yet been isolated from food matrices.

224 Subsequently, we analysed the differences in the distribution of the OTUs between and within
225 the farms, where some particular trends were observed. Beta diversity analyses, using the
226 UniFrac method, were performed to compare the samples between the dairies. We performed
227 Anosim and Adonis tests for all the different parameters of the dairies (farm area, altitude of
228 the farm, breed, milking practice, farming type and feeding), but none of them had a
229 significant ($P > 0.01$) influence on the variation observed in the DM microbiota (data not
230 shown). The only variable that resulted in significant differences was the sampling year,
231 indicating that the bacteria present in the samples from 2013 were different from those from
232 2014 (Figure S2 of the supplementary material). This very interesting result suggests that the

233 variability in the milk microbiota may derive from the individual components of each animal
234 and/or their lactation period. As the gestation period in donkeys is approximately one year
235 and jennies produce milk only for 6 months, we sampled milk from completely different
236 animals in each year. Moreover, the different stages of lactation of the milking jennies in each
237 farm would further contribute to the variability observed. These interindividual differences
238 have been amply described for breast milk (Cabrera-Rubio et al., 2012), and we can most
239 likely assume that they are also valid for other mammal milks; still, further studies are needed
240 to corroborate this presumption.

241 Additionally, the Chao1 richness estimator and the Shannon diversity index of Farm D (Table
242 1) and its rarefaction curves (Figure S1) demonstrated that this farm had the fewest number of
243 observed genera of all the tested farms. In particular, the most representative were *Ralstonia*
244 and *Cupriavidus* spp. (Figure 1). This low variability could be a consequence of the farming
245 practices since it is the only sampled farm run extensively; the animals are free to pasture and
246 are hand milked only when it is requested (Table S1). Moreover, Cavallarin et al. (2015)
247 showed that the samples from this dairy had lower total bacterial counts than those milked
248 automatically. This thesis could be further confirmed by i) the higher percentage of
249 *Streptococcus* spp. reads (Figure 3A), a genus considered skin-associated (Cogen et al.,
250 2007), and ii) the low percentage of *Pseudomonas* spp. reads (Figure 3B); members of this
251 genus are normally present in water, and they might derive from the water used to rinse the
252 milking machinery. The supposition that farm practices have a direct consequence in the milk
253 microbiota has also been supported by goat farm observations, where hand milking practices
254 resulted in lower total bacteria counts (Delgado-Pertíñez et al., 2003).

255

256 **3.3 Lactic acid bacteria in donkey milk**

257 Studies regarding the microbiota of DM have focused on the hygienic quality of DM (Pilla et
258 al., 2010; Zhang et al., 2008; Šarić et al., 2012). Only more recently have some authors
259 characterized the lactic bacteria for their probiotic activity and potential technological aspects
260 (Carminati et al., 2014; Soto del Rio et al., 2016). It is generally accepted that LAB are the
261 dominant population in milk from several species, independent of the methodology used for
262 study. Reports with an HTS approach in cow, sheep, buffalo and human milk have identified
263 LAB reads that corresponded to more than 40% of the total sequences (Quigley et al., 2013).
264 In our samples, we detected reads for the LAB genera *Carnobacterium*, *Enterococcus*,
265 *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus* (Figure 3A, Table 2) with an
266 average of 2.55%, ranging from 0.02% (zoomed in Figure 3B) to 15.85%, of the total reads
267 per sample, which is consistent with the low LAB count in these samples reported by
268 Cavallarin et al., (2015).

269 In this study, all the samples had sequences that corresponded to both coccus (*Enterococcus*,
270 *Lactococcus*, and *Streptococcus*)- and bacillus (*Carnobacterium*, *Lactobacillus*, and
271 *Leuconostoc*)-shaped genera (Figure 3), although in different proportions. This result is in
272 contrast with other studies, where the authors isolated and characterized only coccus-shaped
273 LAB (Carminati et al., 2014) or bacilli species (Soto del Rio et al., 2016). However, there was
274 important variability in the distribution of cocci/bacilli reads both within and between the
275 different farms (Figure 3). In particular, cocci were noticeably present only in Farms C and E
276 in both sampling years (Figure 3C), whereas sample D.2013 presented more cocci reads.
277 Sample A.2014 was characterized by a similar proportion of bacilli and cocci reads.
278 Regarding the bacilli, the sole sampling year of Farm F showed only bacilli reads (Figure 3C),
279 while in Farm B, their presence was not constant; in 2013, the prevalence of cocci was clear,
280 while the situation was reversed in the following year. It is relevant to note that these two

281 bacilli-rich samples (B.2014 and F.2014) were the ones that had higher percentages of LAB
282 reads from the total number of sequences (Figure 3A).

283 These results are relevant to the possible production of probiotic milks. Several authors have
284 proposed novel fermented DM beverages that used lactobacilli strains isolated from bovine
285 milk adapted to grow in DM (Chiavari et al., 2005; Perna et al., 2015). Consequently, having
286 available bacilli strains naturally adapted to DM might be notable from a biotechnological
287 point of view to facilitate the production of these beverages.

288 Overall, the results suggest that the LAB population of DM is complex, diverse, variable and
289 may depend upon several parameters, thus requiring further investigation.

290

291 **4. Conclusions**

292 The present survey provides a broad characterization of the bacterial composition of DM,
293 allowing a description of microorganisms not previously detected in this product. The
294 microbiota of DM is mainly composed of gram-negative bacteria. Unlike other milks, LAB
295 reads were present in low percentages, both cocci and bacilli, even though their growth is not
296 particularly favoured by the composition of DM. The HTS analysis of diverse farms allowed
297 the proposal of several genera as members of a core DM microbiota. The observed results
298 also support the premise that the microbial composition of DM may be influenced by
299 individual animal components.

300 The present study aimed to give a general picture of the bacterial communities present in DM,
301 and it has shown that this microbiota can be highly diverse. Further studies are needed to
302 better understand the dynamics between the bacterial population in this matrix and the
303 relationship between the milk components.

304

305

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307 The authors would like to thank the owners of the Piedmont donkey milk farms for their
308 availability and cooperation in the sampling.

309

310 **Figure 1.** Distribution of the most abundant genera in donkey milk. Percentages refer to the
311 total number of reads per sample.

312 **Figure 2.** Core microbiota of donkey milk. **A)** Taxonomic distribution of the OTUs present at
313 > 0.001% in at least nine samples. **B)** Pseudo-heatmap of the distribution (%) of the core
314 OTUs. Samples were clustered using Euclidean distance and the complete method.

315 **Figure 3.** Distribution of lactic acid bacteria detected in donkey milk samples. **A)** Abundance
316 of LAB genera found in the studied samples; percentages refer to the total number of reads.
317 **B)** Zoomed-in for the lower percent levels of LAB genera abundance in each sample **C)**
318 Relative abundance for the sum of the percentages of coccus-shaped (*Enterococcus*,
319 *Lactococcus*, *Streptococcus*) and bacillus-shaped (*Carnobacterium*, *Lactobacillus*,
320 *Leuconostoc*) LAB genera reads for each farm.

321

322 **Table 1.** Numbers of sequences analyzed, observed OTUs, coverage and diversity estimators
323 for all the studied samples.

324 **Table 2.** Percentages of the most abundant taxonomical groups of the sampled donkey milk
325 farms.

326

327 **Figure S1.** Rarefaction curves of the observed species for each studied sample.

328 **Figure S2.** Principal coordinate analysis (PCoA) of the surveyed donkey milk samples. The
329 plot was based on the weighted UniFrac distance matrix of the microbiota. The dots and
330 names in red correspond to the sampling of 2013, while the blue ones correspond to 2014.

331

332 **Table S1.** General characteristics of the surveyed donkey milk farms. Modified from
333 (Cavallarin et al., 2015)

334

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Table 1

Sample	Reads	Good's coverage	Observed OTUs	Chao 1	ACE	Shannon	Simpson
A.2013	294,557	0.994	5078	6875.19	6833.26	7.09	0.96
A.2014	188,349	0.993	3760	5616.95	5513.89	6.15	0.92
B.2013	203,091	0.993	4008	5410.35	5345.92	6.52	0.94
B.2014	223,728	0.993	4338	6002.83	6138.72	5.46	0.81
C.2013	279,374	0.993	5745	7880.04	7818.74	7.37	0.97
C.2014	850,529	0.998	7686	9465.44	9477.87	6.60	0.92
D.2013	172,717	0.996	2316	2965.35	2964.90	5.65	0.90
D.2014	220,559	0.997	2019	2928.77	2853.42	2.90	0.46
E.2013	254,323	0.994	3839	5474.76	5453.59	5.73	0.87
E.2014	501,861	0.997	5012	6989.73	7026.50	5.99	0.92
F.2014	554,203	0.997	5759	7702.27	7826.34	5.84	0.89

Table 2

Phylum	Genus	Farms										
		A.2013	A.2014	B.2013	B.2014	C.2013	C.2014	D.2013	D.2014	E.2013	E.2014	F.2014
Actinobacteria		0.18	0.33	2.00	0.38	2.04	0.22	2.17	0.17	0.31	0.02	0.40
	<i>Arthrobacter</i>	0.01	0.001	0.19	0.02	0.07	0.001	0.004	0.02	<0.000	0.001	0.17
	<i>Kocuria</i>	<0.000	0.01	0.01	0.004	0.69	0.04	0.001	0.002	ND	ND	0.001
	<i>Corynebacterium</i>	0.004	0.01	0.53	0.18	0.08	0.08	0.04	0.02	0.02	0.005	0.05
	<i>Pseudonocardia</i>	0.01	0.002	0.05	0.004	0.12	ND	0.39	0.004	0.02	<0.000	0.001
	<i>Rothia</i>	0.001	0.001	0.21	0.003	0.01	0.004	0.01	0.001	0.003	0.001	0.001
Bacteroidetes		24.15	2.52	1.00	0.93	0.95	1.37	0.81	0.05	0.60	0.70	0.78
	<i>Chryseobacterium</i>	3.42	1.31	0.002	0.002	0.36	0.36	0.01	0.005	0.01	0.002	0.23
	<i>Cloacibacterium</i>	0.02	0.002	0.21	0.004	0.19	<0.000	0.64	<0.000	0.09	<0.000	<0.000
	<i>Flavobacterium</i>	3.00	0.31	ND	0.001	0.01	0.05	0.01	0.001	0.48	0.58	0.23
	<i>Sphingobacterium</i>	17.34	0.70	0.16	0.69	0.33	0.88	0.004	0.01	<0.000	0.02	0.11
Firmicutes		0.43	0.93	8.09	17.39	2.59	0.76	6.38	0.33	0.89	0.08	9.80
	<i>Carnobacterium</i>	ND	0.002	0.002	0.003	0.002	0.004	0.001	0.01	<0.000	0.002	7.32
	<i>Enterococcus</i>	0.005	0.02	0.01	0.001	0.32	0.32	0.01	<0.000	0.001	ND	0.002
	<i>Lactobacillus</i>	0.03	0.21	0.04	3.16	0.05	0.01	0.02	0.01	0.003	0.002	0.003
	<i>Lactococcus</i>	0.07	0.03	0.65	1.01	0.06	0.08	0.04	ND	0.01	ND	0.001
	<i>Leuconostoc</i>	0.001	0.001	0.06	11.61	0.01	0.004	0.002	ND	0.001	<0.000	0.02
	<i>Streptococcus</i>	0.05	0.07	0.40	0.08	0.16	0.02	1.98	0.02	0.03	0.02	0.05
	<i>Veillonella</i>	0.04	0.31	0.16	0.03	0.01	0.01	1.98	0.01	0.003	0.01	0.02
Proteobacteria		74.92	91.09	87.86	75.54	93.99	94.01	89.64	93.89	98.05	92.13	84.85
	<i>Acinetobacter</i>	2.39	1.72	3.52	2.21	4.19	23.36	4.03	0.03	0.80	0.02	0.37
	<i>Agrobacterium</i>	0.06	0.28	0.02	0.003	0.04	0.11	0.001	0.003	<0.000	0.10	0.01
	<i>Citrobacter</i>	0.27	0.07	0.002	0.03	5.95	3.75	0.01	0.01	0.001	0.02	14.00
	<i>Cupriavidus</i>	ND	0.002	ND	6.57	ND	0.79	ND	86.96	ND	0.002	0.002
	<i>Janthinobacterium</i>	2.57	0.001	0.001	0.002	<0.000	0.07	0.003	0.03	<0.000	3.83	3.78
	<i>Mesorhizobium</i>	0.004	ND	0.04	ND	0.04	ND	0.08	0.001	0.02	ND	<0.000
	<i>Mycoplana</i>	0.14	0.17	0.001	<0.000	0.001	0.7	0.01	<0.000	0.001	0.01	0.001
	<i>Ochrobactrum</i>	0.03	0.02	0.001	0.001	0.31	0.37	ND	ND	ND	ND	<0.000
	<i>Pseudomonas</i>	54.48	84.22	24.18	57.96	25.52	24.70	0.11	0.26	72.57	76.19	59.53
	<i>Ralstonia</i>	4.18	ND	42.30	ND	28.65	ND	60.68	0.002	16.34	<0.000	0.001
	<i>Stenotrophomonas</i>	5.20	0.41	1.24	3.54	2.25	1.23	0.002	0.004	ND	0.02	0.004
	<i>Sphingomonas</i>	0.03	0.07	0.49	0.20	0.23	0.18	0.57	0.31	0.06	0.07	0.04
	<i>Yersinia</i>	0.01	0.001	ND	0.55	2.67	1.12	ND	0.002	ND	0.25	0.02
Verrucomicrobia		0.14	0.11	0.17	0.07	0.02	0.11	0.07	0.01	0.01	0.001	0.03
	<i>Akkermansia</i>	0.001	0.01	0.09	0.02	0.01	0.002	0.01	0.01	0.001	<0.000	0.02

ND stands for non detected reads in the sample for that particular taxon

Table S1

	Farm A	Farm B	Farm C	Farm D	Farm E	Farm F
Farm area (ha)	35	12	10	10	42	20
Altitude above sea level (m)	194	1110	395	600	183	430
Jennies ^a (no.)	45	40	40	70	32	32
Milking jennies ^a (no.)	7-10	7-10	8-10	30-33	6-10	6-10
Herd breed	Crossbreds	Martina Franca	Crossbreds	Crossbreds	Martina Franca, Ragusana, Crossbreds	Crossbreds
Milking practice	Automatic in milking room	Automatic in milking room	Automatic in cowshed	Hand milking	Automatic in milking room	Automatic in in milking room
Farming type	Semi-extensive	Semi-extensive	Semi-extensive	Extensive	Semi-extensive	Semi-extensive
Feed	Grazing - Hay	Hay - Bread – Protein supplementation	Grazing - Hay	Grazing - Hay	Grazing - Hay	Grazing - Hay
Milk use	Food - cosmetics	Food	Food - cosmetics	Food - cosmetics	Food - cosmetics	Cosmetics

^a counted during the visits

Figure 1

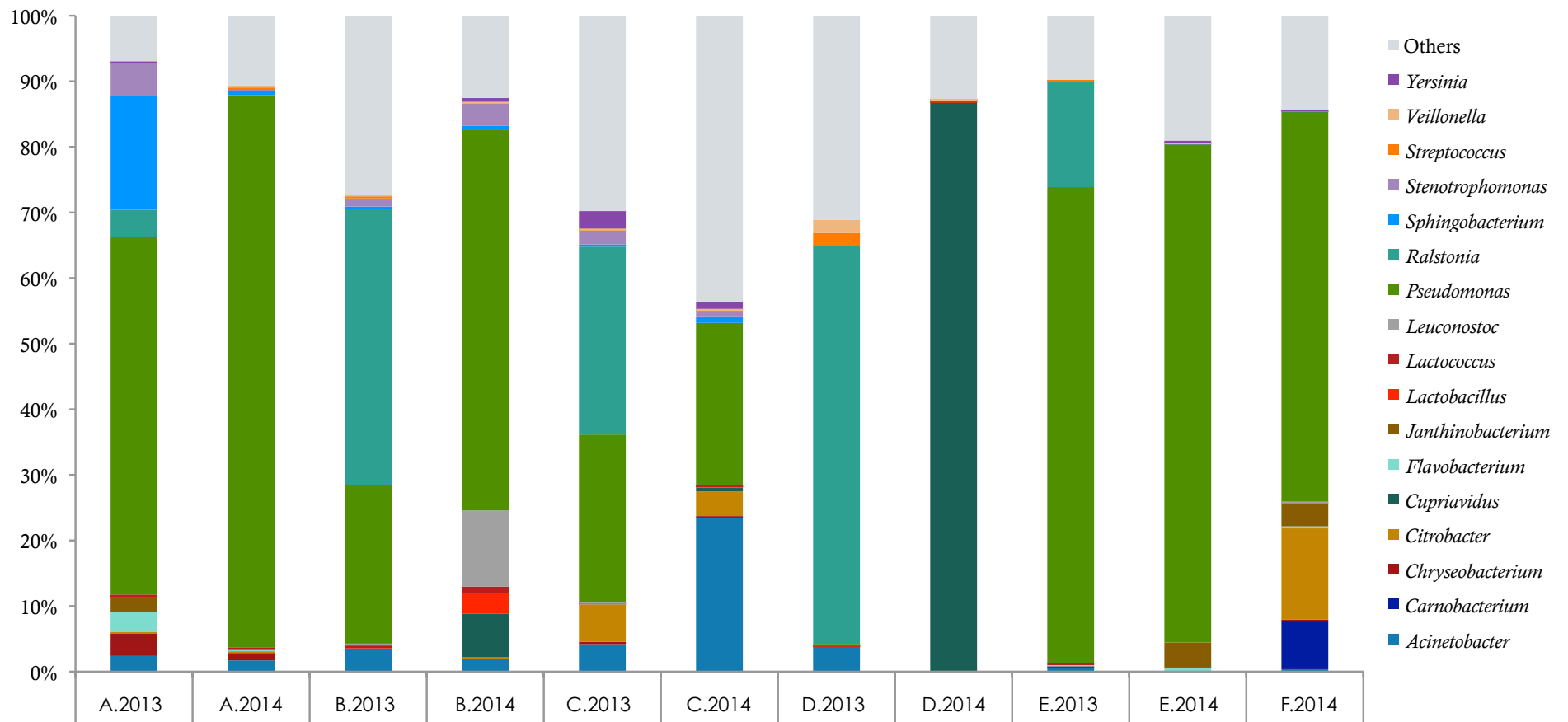


Figure 2

A)

Phylum	Class	Order	Family	Genus
			<i>Actinomycetaceae</i>	<i>Actinomyces</i>
			<i>Corynebacteriaceae</i>	<i>Corynebacterium</i>
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Micrococcaceae</i>	<i>Arthrobacter</i>
				<i>Rothia</i>
			<i>Mycobacteriaceae</i>	<i>Mycobacterium</i>
			<i>Nocardiopsaceae</i>	
<i>Bacteroidetes</i>	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Weeksellaceae</i>	<i>Cloacibacterium</i>
	<i>Shingobacteriia</i>	<i>Sphingobacteriales</i>	<i>Shpingobacteriaceae</i>	<i>Sphingobacterium</i>
<i>Cyanobacteria</i>	<i>Chloroplast</i>	<i>Streptophyta</i>		
		<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Bacillus</i>
			<i>Staphylococcaceae</i>	<i>Staphylococcus</i>
	<i>Bacilli</i>	<i>Gemellales</i>	<i>Gemellaceae</i>	
<i>Firmicutes</i>		<i>Lactobacillales</i>	<i>Lactobacillaceae</i>	<i>Lactobacillus</i>
			<i>Streptococcaceae</i>	<i>Streptococcus</i>
		<i>Clostridia</i>	<i>Veillonellaceae</i>	<i>Veillonella</i>
			<i>Bradyrhizobiaceae</i>	<i>Bradyrhizobium</i>
		<i>Rhizobiales</i>	<i>Methylobacteriaceae</i>	<i>Methylobacterium</i>
	<i>Alphaproteobacteria</i>		<i>Phyllobacteriaceae</i>	
			<i>Rhizobiaceae</i>	<i>Agrobacterium</i>
		<i>Sphingomonadales</i>	<i>Sphingomonadaceae</i>	<i>Sphingomonas</i>
			<i>Comamonadaceae</i>	<i>Acidovorax</i>
<i>Proteobacteria</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Oxalobacteraceae</i>	<i>Janthinobacterium</i>
		<i>Neisseriales</i>	<i>Neisseriaceae</i>	<i>Neisseria</i>
		<i>Enterobacteriales</i>	<i>Enterobacteriaceae</i>	<i>Citrobacter</i>
				<i>Escherichia</i>
	<i>Gammaproteobacteria</i>	<i>Pasteurellales</i>	<i>Pasteurellaceae</i>	
			<i>Moraxellaceae</i>	<i>Acinetobacter</i>
		<i>Pseudomonadales</i>	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i>
<i>Verrucomicrobia</i>	<i>Verrucomicrobiae</i>	<i>Verrucomicrobiales</i>	<i>Verrucomicrobiaceae</i>	<i>Akkermansia</i>

B)

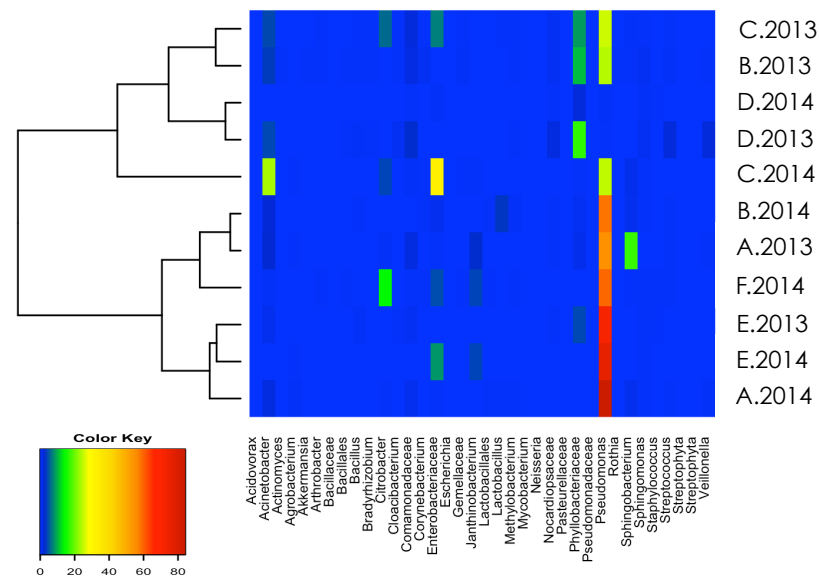


Figure 3

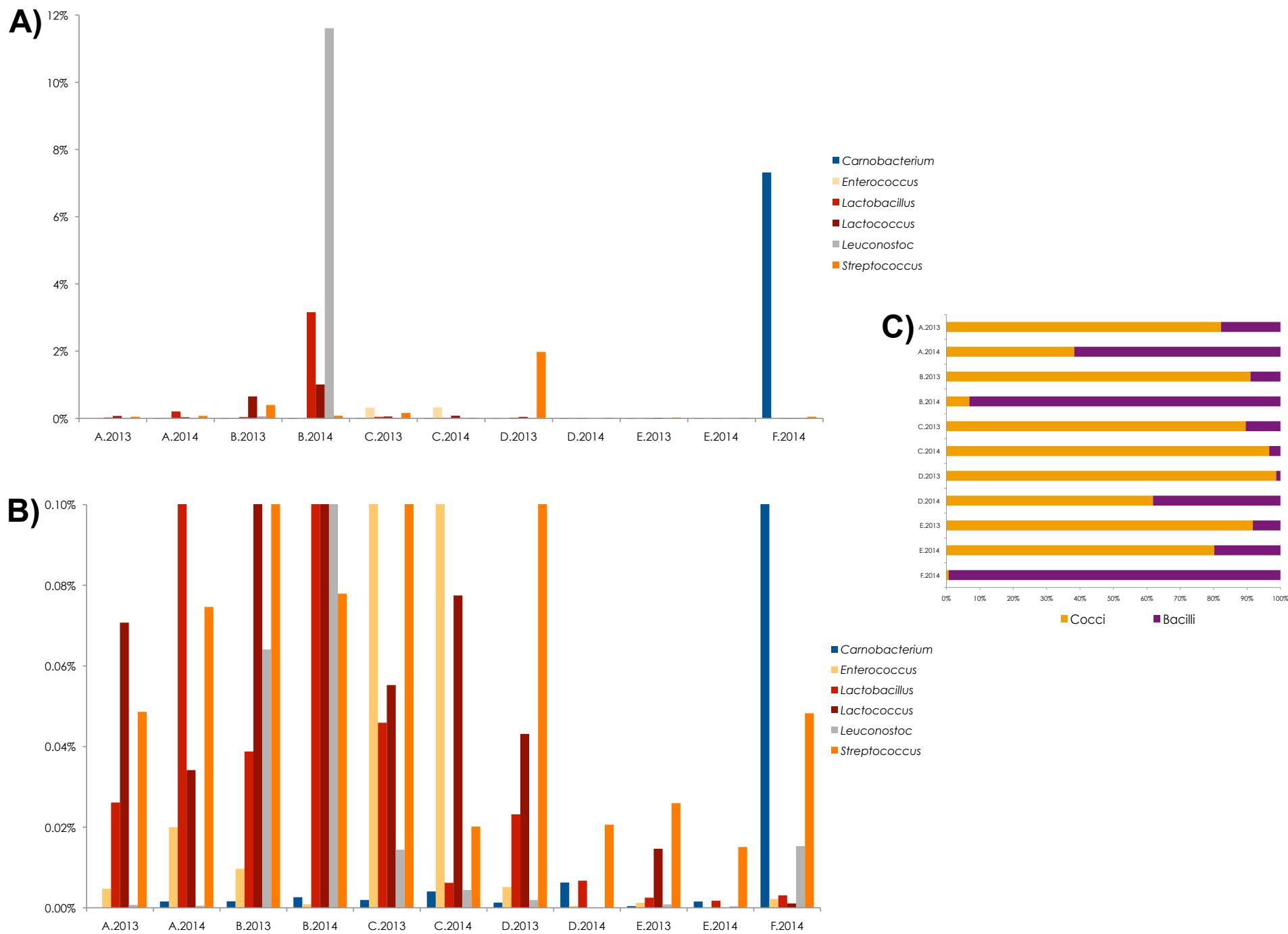


Figure S1

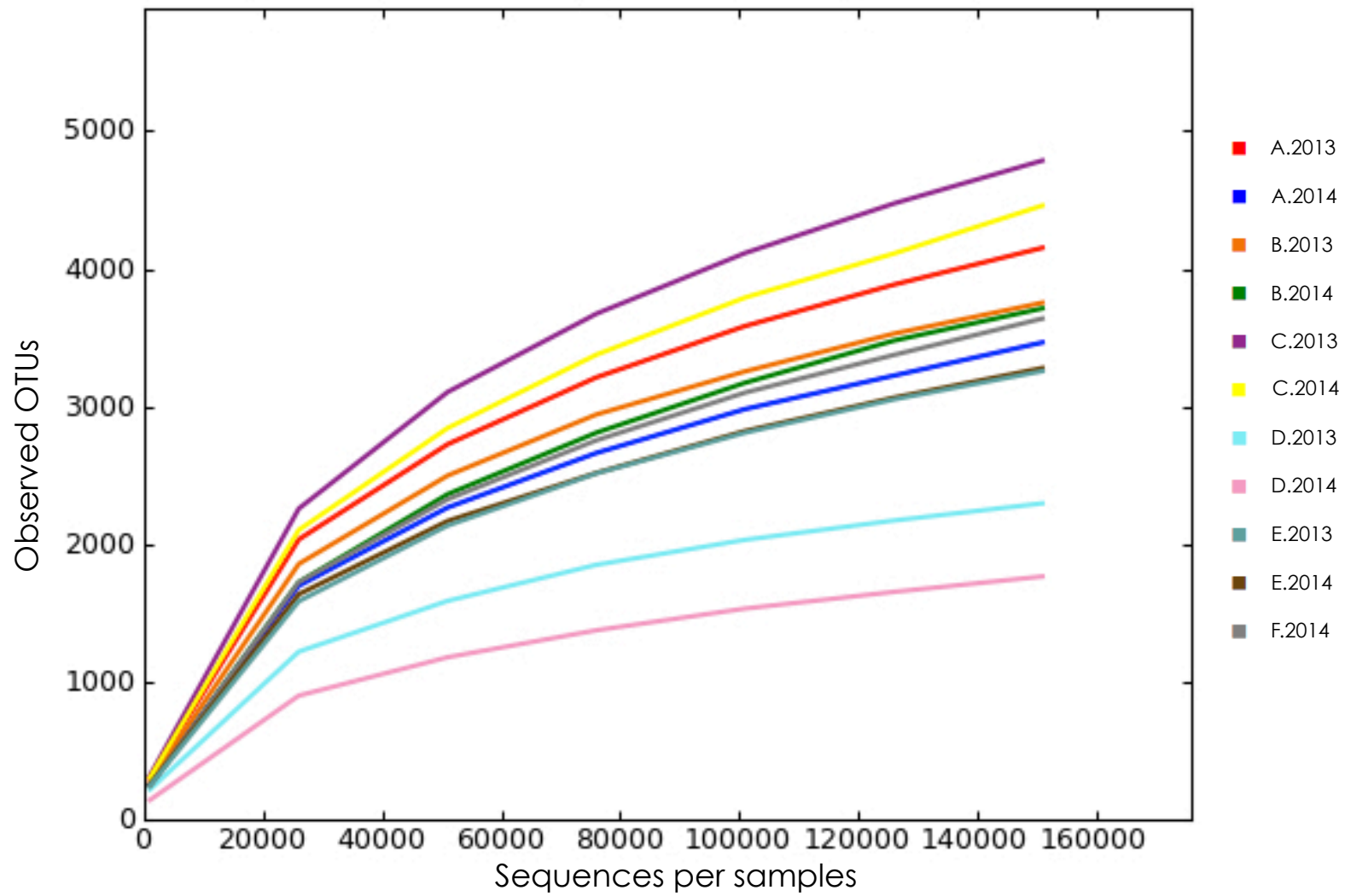


Figure S2

