

1 **Longitudinal metagenomic profiling of bovine milk to assess the impact of**
2 **intramammary treatment using a third-generation cephalosporin**
3 **Erika K. Ganda¹, Rafael S. Bisinotto^{1,2}, Svetlana F. Lima¹, Kristina Kronauer¹, Dean**
4 **H. Decter¹, Georgios Oikonomou^{1,3}, Ynte H. Schukken¹, and Rodrigo C. Bicalho^{1,*}**

5
6 ¹ Department of Population Medicine and Diagnostic Sciences, College of Veterinary
7 Medicine, Cornell University, Ithaca, NY, USA

8 ² Current address: Department of Veterinary Population Medicine, College of Veterinary
9 Medicine, University of Minnesota, St. Paul, MN, USA

10 ³ Epidemiology and Population Health, Institute of Infection and Global Health,
11 University of Liverpool, Liverpool, UK

12 * Correspondence and requests for materials should be addressed to R.C.B. (email:
13 rcb28@cornell.edu).

14

15

16 Conflict of interest statement: The authors declare no conflict of interest.

17

18

19 **ABSTRACT**

20 Antimicrobial usage in food animals has a direct impact on human health, and
21 approximately 80% of the antibiotics prescribed in the dairy industry are used to treat
22 bovine mastitis. Here, we provide a longitudinal description of the changes in the
23 microbiome of milk that are associated with mastitis and antimicrobial therapy. Next-
24 generation sequencing, 16S rRNA gene quantitative real-time PCR, and aerobic
25 culturing were applied to assess the effect of disease and antibiotic therapy on the milk
26 microbiome. Cows diagnosed with clinical mastitis associated with Gram-negative
27 pathogens or negative aerobic culture were randomly allocated into 5 days of Ceftiofur
28 intramammary treatment or remained as untreated controls. Serial milk samples were
29 collected from the affected quarter and the ipsilateral healthy quarter of the same
30 animal. Milk from the mastitic quarter had a higher bacterial load and reduced microbial
31 diversity compared to healthy milk. Resolution of the disease was accompanied by
32 increases in diversity indexes and a decrease in pathogen relative abundance.
33 *Escherichia coli*-associated mastitic milk samples had a remarkably distinct bacterial
34 profile, dominated by Enterobacteriaceae, when compared to healthy milk. However, no
35 differences were observed in culture-negative mastitis samples when compared to
36 healthy milk. Antimicrobial treatment had no significant effect on clinical cure,
37 bacteriological cure, pathogen clearance rate or bacterial load.

38

39 INTRODUCTION

40 Production of animal protein to support the world's growing human population is
41 one of the main challenges facing humankind. Concerns related to food safety and
42 development of antimicrobial resistance may lead to decreased availability of antibiotics
43 for use in food animals and thereby limit our ability to control disease in agricultural
44 animal species. Such a change in antibiotic usage in food animals could also alter
45 perspectives on food security as it relates to public health concerns regarding antibiotic
46 use in food animals. Thus, in-depth understanding of disease mechanisms is critical to
47 promote animal health and at the same time encourage judicious use of antibiotics in
48 livestock. Mastitis is one of the most common diseases in dairy herds, and
49 approximately 20% to 30% of dairy cows develop clinical mastitis at least once during
50 lactation ^{1,2}. Not surprisingly, prophylaxis and treatment of mastitic cows are the major
51 reasons for antibiotic usage in dairy farms ^{3,4}.

52 Maternal milk harbors a rich microbial community that is vital for establishment of
53 the gut microbiome and immune tolerance in neonates ^{5,6}. The same microbial
54 community in the mammary gland may provide an environment that aids the host in
55 preventing mammary infections. For instance, commensal bacteria present in human
56 milk inhibit proliferation of *Staphylococcus aureus*⁷, which is also a pathogen commonly
57 associated with mastitis in dairy cows ⁸. Considering that mastitis possibly reflects a
58 dysbiosis within the mammary gland⁹⁻¹¹, a detailed assessment of the milk microbiome
59 during active stages of clinical disease, spontaneous recovery, treatment and post-
60 treatment is essential to further elucidate this pathological condition.

61 The multifactorial etiology of mastitis presents a major challenge for disease
62 prevention and treatment of affected animals. Implementation of programs for mastitis
63 control has reduced the prevalence of important contagious pathogens, and
64 approximately 40% of clinical cases of mastitis are associated with opportunistic Gram-
65 negative bacteria such as *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., and
66 *Pasteurella* spp.^{8,12,13}. Although current guidelines do not recommend the use of
67 intramammary antibiotics for cows diagnosed with Gram-negative mastitis^{3,14}, improved
68 bacteriological and clinical outcomes have been shown in mastitic cows treated with
69 third-generation cephalosporins compared with other antimicrobials or untreated
70 controls^{15,16}. However, the impact of these broad-spectrum antibiotics on the milk
71 microbiota (other than major pathogens) remains unknown. In fact, currently, no data
72 concerning the effect of antibiotic therapy on the mammary microbiota are available in
73 either humans or animals. Routine methods used to assess responses to intramammary
74 treatments overlook numerous microorganisms, which is supported by the fact that 40%
75 of milk samples collected from cows with clinical mastitis yield negative results by
76 aerobic culture¹³. Later-generation cephalosporins have broad-spectrum antibacterial
77 activity, so their use could unintentionally disrupt general mammary microbial
78 populations and also increase the risk of antimicrobial resistance if not used in a
79 judicious manner¹⁷. Understanding the dynamics of the mammary microbiota upon
80 antibiotic therapy is essential not only for development of effective treatment strategies,
81 but also to facilitate the process of restoring a healthy mammary microbiota.

82 State-of-the-art technologies have allowed the investigation of microbial
83 communities in milk without some of the limitations imposed by culture methods^{9,18,19}.

84 Therefore, the specific objectives of the present study were: 1) to use high-throughput
85 DNA sequencing to describe the microbiome of milk in dairy cows affected by clinical
86 mastitis associated with Gram-negative bacteria or negative culture; 2) to determine the
87 bacterial load based on PCR quantification of 16S rRNA gene copies, and compare
88 microbial populations of affected and healthy quarters; and 3) to assess the effect of
89 intramammary treatment with ceftiofur hydrochloride on the milk microbiome, bacterial
90 load, and clinical cure in quarters affected with clinical mastitis.

91

92 **METHODS**

93 **Ethics Statement**

94 The research protocol was reviewed and approved by the Cornell University
95 Institutional Animal Care and Use Committee (protocol number 2013-0056). The
96 methods were carried out in accordance with the approved guidelines.

97

98 **Animals, Enrollment Criteria, and Treatments**

99 Milk samples were collected from lactating Holstein cows diagnosed with clinical
100 mastitis between April and June, 2014. All cows were housed in a single herd located in
101 upstate New York which milked approximately 2,800 cows thrice daily during the
102 experimental period. Clinical mastitis was defined as the presence of at least visually
103 abnormal milk (i.e. presence of flakes, clots, or serous milk) during forestripping
104 performed at the milking parlor. Once mastitis was diagnosed, the initial milk sample for
105 mastitis pathogen identification was collected by trained farm personnel according to
106 National Mastitis Council guidelines. These samples were defined as day 0 samples.

107 Teats were cleaned and disinfected using 70% ethanol (v/v), the initial three streams
108 were discarded, and approximately 5 mL of milk was collected into a sterile plastic tube
109 without preservative (Corning Life Sciences, Tewksbury, MA). Milk samples were
110 cultured using an on-farm chromogenic culture system for fast identification of causal
111 agents (Accumast[®], FERA Animal Health LCC, Ithaca, NY) according to the
112 manufacturer's recommendations, and then submitted for analysis at the Quality Milk
113 Production Services laboratory (**QMPS**; Cornell University, Ithaca, NY) to ensure the
114 accuracy of on-farm culture. Disagreement between methods was observed in only two
115 samples, which were excluded from further analyses after the results from QMPS were
116 received.

117 Cows diagnosed with clinical mastitis associated with Gram-negative bacteria or
118 negative on-farm culture and that had not been treated with intramammary
119 antimicrobials in the 14 days preceding diagnosis were deemed eligible for enrollment.
120 On study day 1, eligible cows were randomly allocated into one of two treatments based
121 on a list of numbers generated using the RAND function of Excel (Microsoft, Redmond,
122 WA), blocked by aerobic culture results. Clinical score was assessed on days 1, 8, 10
123 and 14 according to Wenz et al ^{20,21}. Milk appearance, mammary gland appearance and
124 systemic signs of disease (i.e. rectal temperature ≥ 39.5 °C, dehydration and
125 depression) were evaluated for classification of clinical score. A clinical score of 'mild'
126 was assigned if only abnormal milk was observed. A 'moderate' score was assigned
127 when abnormal milk and inflammation of the mammary gland were present. A 'severe'
128 score was assigned if abnormal milk, local inflammation and one or more of the
129 systemic signs of the disease were observed.

130 Cows assigned to the treatment group received daily intramammary infusions
131 containing 125 mg of ceftiofur equivalents (as ceftiofur hydrochloride; Spectramast LC[®],
132 Zoetis, Florham Park, NJ) only on the mastitic quarter for five consecutive days,
133 whereas those assigned to the control group did not receive intramammary therapy.

134

135 **Sample and Data Collection**

136 Serial milk samples were collected by a trained veterinarian member of the
137 research team from each cow on study days 1, 2, 3, 4, 5, 8, 10, and 14, from both the
138 mastitic quarter and the ipsilateral healthy quarter of the same cow. For cows in the
139 treated group, sampling on days 1 through 5 was performed after milk out of the quarter
140 in untreated cows, whereas treated cows were sampled immediately before
141 intramammary treatments were applied. Teats were disinfected as described above and
142 10 mL of milk was harvested from each quarter into a sterile plastic tube without
143 preservative (Corning Life Sciences, Tewksbury, MA). Samples were immediately
144 refrigerated at 4 °C, transported to the laboratory on ice, and frozen at -20 °C until
145 assayed. Milk samples collected from mastitic quarters on days 10 and 14 were
146 submitted to the QMPS laboratory for bacterial identification using standard aerobic
147 culture.

148 Clinical cure was defined as cows without any clinical signs on both day 10 and
149 day 14. Bacteriological cure was defined as both the samples taken on day 10 and day
150 14 being negative for the organism present on day 0. In all other cases the quarter was
151 considered to be a non-cure or treatment failure. Bacteriological cure can only be
152 evaluated in quarters that were culture positive on day 0.

153 **DNA Isolation and Purification**

154 Milk samples were thawed, homogenized by inverting the tubes, and a 6-mL
155 aliquot was taken for DNA isolation and purification. Milk samples were centrifuged at 4
156 °C and 16,100 × *g* for 3 minutes and the supernatant was discarded. Genomic DNA was
157 isolated from the remaining pellet using a commercially available kit (PowerFood DNA
158 Isolation Kit, MO BIO Laboratories Inc., Carlsbad, CA) as described previously ²².
159 Concentration and purity of isolated DNA were evaluated based on optical density at
160 230, 260 and 280 nm wavelengths (NanoDrop ND-1000, NanoDrop Technologies,
161 Wilmington, DE).

162

163 **Amplification of the V4 Hypervariable Region of the Bacterial 16S rRNA Gene,**
164 **Library Preparation, and 16S rRNA Gene Sequencing**

165 The V4 hypervariable region of the bacterial 16S rRNA gene was amplified from
166 genomic DNA by PCR utilizing the primers 515F and 806R optimized for the Illumina
167 MiSeq platform (Illumina Inc., San Diego, CA) ²³ as described previously ²⁴.

168 Equimolar libraries were sequenced in six runs using the MiSeq reagent kit V2
169 for 300 cycles on the MiSeq platform (Illumina). Each run included 279 samples and a
170 sequencing control that consisted of the purified barcoded PCR product of DNA
171 extracted from *Staphylococcus aureus* (ATCC 25923). Gene sequences were
172 processed using the 16S Metagenomics workflow in the MiSeq Reporter analysis
173 software version 2.5 based on quality scores generated by real-time analysis during the
174 sequencing run. Quality-filtered indexed reads were demultiplexed for generation of
175 individual FASTQ files and aligned using the banded Smith-Waterman method of the

176 Illumina-curated version of the Greengenes database for taxonomic classification of milk
177 microbes. Resulting FASTQ files were uploaded into the open-source pipeline
178 Quantitative Insights into Microbial Ecology (**QIIME**) version 1.9.1²⁵. Sequences were
179 filtered based on quality as described previously²⁶ and assigned to operational
180 taxonomic units (**OTUs**) with 97% identity using UCLUST²⁷. The OTU database was
181 rarefied using the command `single_rarefaction.py` from QIIME and the number of OTUs,
182 as well as Chao1 and Shannon indexes, was calculated for each sample at a
183 rarefaction level of 5,000 reads per sample.

184 **Quantification of 16S rRNA Copies by qPCR**

185 The number of 16S rRNA copies was used as a proxy to determine bacterial load
186 in milk samples collected on days 1, 3, 8 and 14. 16S rRNA gene copies were
187 quantified by qPCR as described previously²⁸. Reactions were performed using Unibac
188 primers (forward: 5'-TGG AGC ATG TGG TTT AAT TCG A-3'; reverse: 5'-TGC GGG
189 ACT TAA CCC AAC A-3'; 50 pmol/reaction), 1X iQTM SYBR® Green Mastermix (Bio-
190 Rad Laboratories, Hercules, CA), and 1.5 µL of sample DNA. A standard curve was
191 built using plasmid DNA quantified by spectrophotometry. All samples were assayed in
192 duplicate using an iQTM5 Real-time PCR system (Bio-Rad Laboratories, Hercules, CA)
193 set to perform denaturation at 95 °C for 3 minutes, then 40 cycles of amplification (95 °C
194 for 10 seconds and 55 °C for 30 seconds), one cycle at 95 °C for 60 seconds, one cycle
195 at 55 °C for 60 seconds, and a melting curve determination.

196

197 **Statistical Analyses**

198 Descriptive analyses on sequencing results were performed using the
199 UNIVARIATE procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC). Differences
200 in the relative abundance of bacteria between quarters with clinical mastitis and healthy
201 counterparts were evaluated at the phylum and family levels using JMP Pro 11 (SAS
202 Institute Inc., Cary, NC). Cows were categorized according to the main pathogen
203 identified on samples taken on study day 0 through standard culture methods into four
204 groups, namely *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., and negative culture. Within
205 each group, the effect of clinical mastitis on the relative abundance of each of the ten
206 most prevalent phyla was evaluated by ANOVA. The prevalences of all remaining phyla
207 were combined into a single cluster. The fixed effect of disease (healthy vs. mastitic
208 quarters) was included in the statistical models as an independent variable. Response
209 screening was performed to assess the effect of clinical mastitis on the relative
210 abundance of the 100 most prevalent families in each pathogen group. *P*-values were
211 adjusted for false discovery rate (**FDR**²⁹) and presented as FDR LogWorth (i.e. $-\log_{10}P$).
212 The mean relative abundance for each family observed across all healthy quarters was
213 used as a reference for calculation of fold-changes.

214 Microbiome changes occurring over time and in response to intramammary
215 antibiotic therapy were described for the 25 most prevalent families in each pathogen
216 group using the tabulate function of JMP Pro 11. Relative abundances of all remaining
217 families were combined into a single cluster. The magnitude of change was scaled
218 uniformly within health status (healthy vs. mastitic quarters). The relative abundances of
219 major pathogens associated with clinical mastitis were evaluated within pathogen

220 groups by ANOVA for repeated measures using the GLIMMIX procedure of SAS.
221 Outcomes were the relative abundance of each pathogen and the explanatory variables
222 were treatment, time, health status (healthy vs mastitic quarter) and their two- and
223 three-way interactions. Cow was considered a random effect in all statistical models.
224 Time changes in the number of OTUs, Chao1 index, and Shannon index were analyzed
225 by ANOVA for repeated measures using the GLIMMIX procedure of SAS. Within each
226 pathogen group, two statistical models were built to evaluate the effects of mastitis (i.e.
227 fixed effects of mastitis, time, and interaction between mastitis and time) and treatment
228 (i.e. fixed effects of treatment, mastitis, time, and all two- and three-way interactions).

229 The effect of cure on the relative abundance of Enterobacteriaceae family
230 members and the Shannon diversity index was evaluated between cured and non-cured
231 cows with clinical mastitis associated with *E. coli* by ANOVA for repeated measures
232 using the Fit Model function on JMP Pro 11. Tests for normality of residuals and
233 homogeneity of variances were conducted for each dependent variable, and data that
234 did not fulfill ANOVA assumptions were transformed accordingly (i.e. 16S rRNA gene
235 copy numbers). The covariance structure with the smallest Schwarz's Bayesian
236 information criterion value was selected for each analysis. Differences with $P \leq 0.05$
237 were considered significant and those with $0.05 < P \leq 0.10$ were considered tendencies.
238 Results are presented as average and standard deviation (i.e. descriptive analyses of
239 sequencing results) or least square means followed by the respective standard error of
240 the mean.

241 Multivariate analysis of microbiome data was carried out in R (R Core Team,
242 Vienna, Austria)³⁰ and QIIME. Beta diversity was analyzed through analysis of

243 similarities (**ANOSIM**) using non-rarefied data normalized employing the packages
244 metagenomeSeq³¹ and vegan³² in R. Principal coordinate analysis (**PCoA**) was
245 performed using weighted Unifrac distances calculated in QIIME and visualized through
246 EMPeror³³.

247 **RESULTS**

248 ***Clinical and Bacteriological Cure***

249 Intramammary treatment with ceftiofur hydrochloride did not significantly improve
250 clinical and bacteriological cures of clinical mastitis compared with untreated controls
251 (**Table 1**). Of the 40 cows enrolled in the control group, 75% (n=30) experienced clinical
252 cure, whereas of the 40 cows that received intramammary antibiotic therapy, 77.5%
253 (n=31) experienced clinical cure (P -value = 0.79). Clinical cures for cows affected with
254 Gram-negative intramammary infections also did not differ between the treated (75%
255 cure rate) and control cows (73.9% cure rate) (P -value = 0.93). Bacteriological cure
256 followed the same pattern as for clinical cure, with 82.6% of the milk samples collected
257 from non-treated mastitic quarters being negative on days 10 and 14 for the organism
258 present on day 0, whereas in the treated group, 79.2% of the samples were considered
259 to be bacteriological cures (P -value = 1.00). Bacteriological cure was not altered by
260 treatment when the data were stratified and analyzed by each pathogen group (**Table**
261 **1**).

262 ***Real-time PCR Results***

263 Cows diagnosed with clinical mastitis caused by *E. coli* had a significantly
264 ($P=0.008$) lower number of 16S rRNA gene copies in healthy quarters compared to
265 mastitic ones on day 3 post diagnosis; however, no difference was observed in the

266 bacterial load as measured in 16S rRNA gene copies between healthy and mastitic
267 quarters at days 8 and 14. Intramammary treatment with Ceftiofur caused a significant
268 decrease in the bacterial load of mastitic quarters on day 3 ($P = 0.01$) compared to non-
269 treated mastitic quarters. Nonetheless, a treatment effect was no longer observed at
270 study day 8 (**Fig. 1a**).

271 In animals diagnosed with clinical mastitis yielding no bacterial growth upon
272 aerobic culture, the number of 16S rRNA gene copies was higher in mastitic quarters
273 compared to healthy ones. No treatment effect was observed on the bacterial load in
274 this group of animals. Mastitic and healthy quarters exhibited the same bacterial load by
275 study day 14 (**Fig. 1c**).

276 **Sequencing Results**

277 Quality-filtered reads were demultiplexed and a total of 67,413,334 sequences
278 was used for downstream analyses (mean=47,241.3 \pm SD=32,625.0 reads/sample).
279 The median length for all reads was 301 bp.

280 **Microbiome Changes Associated With Clinical Mastitis**

281 The mean relative abundance of bacteria from the phylum Proteobacteria was
282 greater ($P < 0.01$) in the milk from mastitic quarters infected by *E. coli* and
283 *Pseudomonas* spp. compared with that of healthy quarters (**Fig. 2a-b**). This was driven
284 mostly by greater abundances of Enterobacteriaceae ($P < 0.001$; **Fig. 3**) and
285 Pseudomonadaceae ($P = 0.03$; **Fig. S1**). On the other hand, the average abundance of
286 Firmicutes, Actinobacteria, Bacteroidetes, Tenericutes, Chlorobi, and the combination of
287 all remaining phyla was greater ($P < 0.05$) in healthy compared with mastitic quarters
288 infected by *E. coli* (**Fig. 2a**). A similar pattern was observed in cows with clinical mastitis

289 associated with *Pseudomonas* spp. (n = 2 quarters), in which the abundance of
290 Actinobacteria and Bacteroidetes was greater ($P < 0.05$) and that of Chlorobi tended to
291 be greater ($P = 0.08$) in healthy compared with mastitic quarters (**Fig. 2b**). The diversity
292 of milk microbial populations was reduced ($P < 0.0001$) in *E. coli* mastitis compared with
293 healthy quarters (**Fig. 1b**). The Shannon index was also influenced ($P < 0.0001$) by the
294 interaction between mastitis and time, as values increased from day 1 through 14 in
295 mastitic quarters, whereas no change was observed in healthy counterparts (**Fig. 1b**).
296 Likewise, the richness of microbial communities was reduced in *E. coli* mastitis
297 compared with healthy quarters (**Fig. S2 a**).

298 The relative abundances of Firmicutes ($P = 0.06$) and the remaining phyla ($P =$
299 0.02) were greater in healthy quarters compared with those infected by *Klebsiella* spp.
300 (**Fig. 2c**). Nevertheless, mastitis did not affect the relative abundances of other phyla or
301 individual families (**Fig. S3**). Clinical mastitis associated with *Klebsiella* spp. had
302 reduced ($P = 0.05$) Shannon values shortly after diagnosis but had no effect on the
303 Chao1 index (**Fig. S4 a-b**).

304 Shifts in the milk microbiome were less pronounced in cases of clinical mastitis
305 associated with a negative aerobic culture (**Fig. 2d**). The relative abundance of
306 Firmicutes was higher ($P = 0.08$), whereas those of Bacteroidetes ($P = 0.06$),
307 Tenericutes ($P = 0.05$), Spirochaetes ($P = 0.01$), and the combined remaining phyla ($P =$
308 0.08) were lower in mastitic quarters. Fluctuations in bacterial communities were not
309 associated with specific families (**Fig. 4**). Nevertheless, diversity was reduced ($P < 0.01$)
310 in mastitic compared with healthy quarters (**Fig. 1d**). The same trend was observed in

311 the Chao1 richness index on the first two days after diagnosis of clinical mastitis (**Fig.**
312 **S2 b**).

313 ***Effect of Intramammary Antibiotic Therapy on the Milk Microbiome***

314 In cows diagnosed with clinical mastitis caused by *E. coli*, microbiome dynamics
315 in healthy quarters did not change over time (**Fig. 5a**). On the other hand, the relative
316 abundance of Enterobacteriaceae decreased from study day 1 to 14 (62.6% vs. 9.7%),
317 whereas the relative abundances of other families increased in mastitic quarters (**Fig.**
318 **5b**). Changes in milk bacterial populations were not affected by intramammary therapy
319 with ceftiofur hydrochloride (**Fig. 5b**). Treatment and the interaction between treatment
320 and time did not affect the relative abundance of Enterobacteriaceae (**Fig. 6a**), or the
321 Shannon (**Fig. 1c**) and Chao1 (**Fig. S2 a**) indexes in mastitic quarters infected by *E.*
322 *coli*.

323 Similar patterns were observed in cows with clinical mastitis caused by *Klebsiella*
324 spp., as intramammary therapy did not impact the milk microbiome or the relative
325 abundance of Enterobacteriaceae (**Fig. S5 and S6**). The only two cows diagnosed with
326 clinical mastitis associated with *Pseudomonas* spp. presented an elevated abundance
327 of Pseudomonadaceae on day 1 (44.3%), which was reduced until day 8 (3.3%) and
328 then returned to initial values on day 14 (46.1%). Because both cows were assigned to
329 receive intramammary infusion with ceftiofur hydrochloride, the effect of treatment on
330 abundance of *Pseudomonas* spp. could not be assessed.

331 Changes in the milk microbiome over time were not observed in cows affected by
332 clinical mastitis associated with negative aerobic culture (**Fig. 7a**). Moreover,
333 intramammary treatment with ceftiofur hydrochloride in these quarters did not affect the

334 milk microbiome (**Fig. 7b**) or the measures of microbial diversity and richness (**Fig. 1d**),
335 (**Fig. S2 b**).

336 ***Microbiome Changes Associated With Clinical Mastitis Cure on the Mastitic*** 337 ***Quarters of Cows With Mastitis Caused by Escherichia coli***

338 In cows diagnosed with clinical mastitis caused by *E. coli*, microbiome dynamics
339 in mastitic quarters exhibited remarkable changes over time. Quarters that experienced
340 clinical cure by the end of the study period had significantly lower abundances of
341 Enterobacteriaceae family members in both control (**Fig. 6c**) and treated animals (**Fig.**
342 **6e**). Nevertheless, microbial diversity at diagnosis of clinical mastitis did not differ
343 between quarters that eventually became cured or not (**Fig. 6b**). However, microbial
344 diversity of quarters that eventually were cured showed increasing microbial diversities
345 in both the control (**Fig. 6d**) and treated groups (**Fig. 6f**) relative to quarters that did not
346 show bacteriological cure during the study period. Similar patterns were observed for
347 bacteriological cure (**Fig. S7**).

348

349 ***Multivariate Analysis of Microbiome data from Healthy and Mastitic Quarters***

350 Analysis of similarities revealed that mastitic quarters were significantly different
351 from healthy quarters at the first day after diagnosis of clinical mastitis in cows with
352 clinical mastitis associated with *E. coli* (**Fig. 8a**), and negative culture (**Fig. 8b**). A clear
353 separation between mastitic and healthy quarters was observed in the principal
354 coordinate analysis of Unifrac distances in animals with clinical mastitis associated with
355 *E. coli* (**Fig. 8a**); however the same could not be observed in animals with mastitis
356 associated with negative culture (**Fig. 8b**). At the end of the study, namely day 14 after

357 diagnosis of clinical mastitis, the microbiome of quarters that had been cured from
358 clinical mastitis did not differ from the one of healthy quarters in either ANOSIM or
359 Unifrac PCoA in cows previously identified with clinical mastitis associated with *E. coli*
360 **(Fig. 8e)** nor in animals with clinical mastitis yielding negative aerobic culture **(Fig. 8f)**.
361 Interestingly, when the microbiome of quarters that remained with abnormal milk by the
362 end of the study was included in the analysis, a significant difference could be observed
363 in both ANOSIM and Unifrac PCoA on the microbiome of milk from cows identified with
364 clinical mastitis associated with *E. coli* **(Fig. 8c)**. No separation between mastitic,
365 healthy, and cured quarters could be observed when the first three components of
366 Unifrac PCoA were plotted in animals with mastitis associated with negative culture
367 **(Fig. 8d)**.

368

369 **DISCUSSION**

370 In an endeavor to better understand the effect of a third-generation
371 cephalosporin (ceftiofur) in Gram-negative and culture-negative bovine mammary
372 infections, we used high-throughput DNA sequencing to assess longitudinal changes in
373 the microbiome of mastitic and healthy milk in a randomized clinical trial. Our data
374 demonstrate that antimicrobial treatment did not significantly affect total bacterial load in
375 the infected quarters by the end of the treatment period, nor was it able to increase the
376 rate of pathogen clearance within the mammary gland. Moreover, this is the first study
377 to document in depth the dynamics of the milk microbiota longitudinally using state-of-
378 the art technology.

379 Treatment with ceftiofur did not affect clinical or bacteriological cure and did not
380 have long-lasting effects on the milk microbiome. Our results are in disagreement with
381 those of Schukken et al. (2011)³⁴, who reported 38% bacteriological cure in non-treated
382 cows and 73% bacteriological cure in treated animals. Their 5-day intramammary
383 treatment regime with ceftiofur resulted in a significant increase in bacteriological cure,
384 particularly in animals infected with *E. coli*, whereas our results demonstrate no
385 difference between treated and untreated animals in this aspect. However, our results
386 are in agreement with those of a landmark study conducted by Lago et al. (2011)^{35,36},
387 which demonstrated that selective antimicrobial treatment of mastitic cows can lead to a
388 considerable reduction in antimicrobial use without any significant differences in days to
389 clinical cure, bacteriological cure risk, new intramammary infection risk or treatment
390 failure. In that study, cows diagnosed with mastitis associated with *E. coli* either
391 received two intramammary doses of cephapirin sodium 12 hours apart or did not
392 receive antimicrobial treatment. The investigators were not able to detect any
393 differences between treated and untreated animals in either clinical or bacteriological
394 cures. It is important to acknowledge that the treatment applied in that study utilized a
395 first-generation cephalosporin, which has a lower effectiveness against Gram-negative
396 pathogens compared to ceftiofur¹⁶.

397 Despite the observed effect of intramammary infusion of ceftiofur in reducing the
398 total bacterial load measured by qPCR of the V4 region of the 16sRNA gene in the
399 affected quarter on day 3, we failed to detect any differences between treated and
400 untreated quarters at days 8 and 14 post diagnosis. Furthermore, our data revealed that
401 ceftiofur therapy had no effect on total bacterial load 3 days after cessation of treatment.

402 We also assessed the longitudinal effect of antibiotic therapy on the relative abundance
403 of the causal mastitis pathogens between treated and untreated cows; again, no
404 differences were observed between the treatment and control groups. The observation
405 that pathogen load was not affected by antimicrobial treatment is substantiated by a
406 consistent decrease in the relative abundance of Enterobacteriaceae at 14 days post
407 diagnosis in both treatment arms. Lastly, regardless of the treatment group, milk
408 samples obtained on day 14 from all quarters deemed as mastitic on day 0 and that had
409 normal milk on day 14 all presented a similar, more diverse bacterial profile, one
410 remarkably comparable to that in healthy milk. Our data demonstrate that antimicrobial
411 therapy does not improve cure rates for mastitis caused by *E. coli*, given the similar
412 patterns of reduction in the percentage of pathogens over time in treated versus non-
413 treated animals, which is in line with the results of Leininger et al. (2003)³⁷ and the
414 recommendations of Suojala et al. (2013)¹⁴.

415 We have demonstrated how the microbiome of mastitic quarters associated with
416 Gram-negative pathogens dynamically changes over time. More interestingly, quarters
417 that were not cured by the end of the study period had diverging abundances of
418 Enterobacteriaceae and microbial diversities over time when compared to mastitic
419 quarters that became healthy by the end of the study period. Reduced bacterial diversity
420 has also been reported in other studies comparing samples derived from healthy and
421 diseased mammary environments^{9,11,38}. Although, most mastitis cases caused by *E.*
422 *coli* are of an acute/peracute nature and have a high self-cure rate, chronic cases have
423 been reported in the literature^{39,40}. Further research is needed to understand the host

424 and pathogen idiosyncrasies that are associated with the chronification of these *E. coli*-
425 related mastitis cases.

426 Elucidating the milk microbiome has been a daunting task⁴¹, particularly in clinical
427 mastitis with negative culture results¹⁰. Various reasons could explain why a negative
428 result might be obtained from a mastitic milk culture: the microorganisms associated
429 with the infection might be shed intermittently; or the number of viable bacterial cells are
430 small; finally, the cow's immune system might have eliminated the pathogen, and the
431 observation of abnormal milk could be a consequence of the inflammatory process that
432 occurred during destruction of the pathogen⁴². Nevertheless, mastitis has also been
433 reported to be caused by mechanical or chemical injury, as well as by non-bacterial
434 infectious agents such as viruses⁴³ and yeasts⁴⁴. Although infrequent, it is important to
435 acknowledge that a portion of these culture negative mastitis cases can be result of a
436 viral infection playing a role in clinical mastitis. In fact, the historical role of viruses in
437 mastitis might have been underestimated, given that the practice of laboratory diagnosis
438 of viruses in mastitis cases is unusual⁴³.

439 In our study, mastitic quarters yielding a negative aerobic result differed in
440 bacterial load compared to their healthy counterparts. This is a very interesting finding,
441 as we were not able to identify in the microbial profiles any specific bacterial family that
442 could be associated with these mastitis cases. The identification of a higher bacterial
443 load not linked to a specific group of pathogens might indicate that dysbiosis occurs not
444 only with changes in the composition of the mammary microbiota, but also with a simple
445 nonspecific increase of intramammary bacterial load, leading to clinical signs of mastitis.
446 It is true that the number of 16S rRNA copies in the genome is variable, which can

447 impact bacterial community analysis⁴⁵; however, the quantification of 16S rRNA gene
448 has been proved to be useful as a proxy for estimating bacterial load⁴⁶. In our study,
449 mastitic quarters exhibited significantly lower microbial diversity upon diagnosis
450 compared to healthy quarters, which could indicate that fewer microbes were
451 dominating the milk microbiome. Our results are in line with those of Kuehn et al. (2013)
452 ¹⁰, who identified that the microbiome of mastitic quarters is less diverse than healthy
453 ones in culture-negative mastitis cases. Recent work by Falentin et. al. (2016)⁴⁷ has
454 raised an interesting discussion when it comes to microbial diversity, dysbiosis and
455 disease. The investigators demonstrated that animals presenting normal milk at
456 sampling, but with different histories in regards of clinical and subclinical mastitis had
457 remarkably different bacterial diversity, as well as an altered microbial profile far from an
458 episode of clinical mastitis. Research is warranted to determine the relationship
459 between changes of the mammary microbiota and timing of clinical mastitis, and
460 elucidate if a shift in the microbial profile predisposes to clinical mastitis, or if an active
461 colonization of a rather normal microbiome is to be held accountable for both the clinical
462 episode and the lasting effect on the alteration of the milk microbiome. Koskinen et al.
463 (2010)⁴⁸ evaluated the use of a pathogen-specific real-time PCR assay for identification
464 of mastitis bacteria and reported that 76% of culture-negative clinical mastitis samples
465 were positive for various mastitis pathogens, including members of the Streptococci,
466 Staphylococci and Enterobacteriaceae families. However, it has previously been
467 reported that such bacteria are found in the microbiome of healthy milk of both humans
468 and cows ^{11,18,49}. Although infrequent, mastitis caused by different species of
469 *Mycobacterium* has been reported in bovines, alpacas and dogs ⁵⁰⁻⁵³. *Mycobacterium* is

470 often misidentified as a negative culture due to its slow growth characteristics and
471 because it is a facultative anaerobic microbe ^{50,54}. In our results, we observed a non-
472 significant increase in the relative abundance of Mycobacteriaceae in mastitic animals
473 yielding negative aerobic culture results. Identifying which microorganisms are
474 associated with culture-negative mastitis does not justify the use of antimicrobial
475 treatment; however, it does shed light on the bacterial etiology of the disease, facilitating
476 decision-making regarding mastitis prevention strategies.

477 Differences in the microbiome of healthy and mastitic milk samples have also
478 been reported for cows ⁹⁻¹¹ and humans ^{18,38}. However, a unique feature of the research
479 presented here is that we used a controlled randomized clinical trial approach to
480 longitudinally describe the differences between milk from mastitic mammary glands and
481 from healthy ones and the impact of antibiotic therapy on the microbiome from the onset
482 of disease until its resolution. To our knowledge, this is the first study to longitudinally
483 evaluate the effect of antimicrobial therapy using the combination of quantitative PCR
484 and next-generation DNA sequencing in dairy cows. Bovine milk, similarly to human
485 milk, exhibits a complex and dynamic microbial ecology ^{9-11,18,38,55}. Nevertheless,
486 significant efforts have been recently undertaken using culture-independent techniques
487 to evaluate the effects of antibiotic therapy in swine ⁵⁶, horses ⁵⁷, gorillas ⁵⁸, and
488 humans ⁵⁹⁻⁶³.

489 Antimicrobial use in the food industry could potentially impact human health,
490 warranting its judicious use ^{64,65}. Ceftiofur is the only FDA-approved third-generation
491 cephalosporin for use in food-producing animals and has been classified by the World
492 Health Organization as one of the critically important antimicrobials for human medicine

493 ⁶⁶. In summary, our work corroborates the existing literature and also provides novel
494 evidence that the use of intramammary ceftiofur therapy for the treatment of mild and
495 moderate cases of *E. coli*-caused and culture-negative mastitis is ineffective. More
496 importantly, it suggests that antimicrobial stewardship in food animals can be achieved
497 in certain situations without compromising the health of the animals. Additionally, the
498 combined use of quantitative PCR and sequencing of the 16s rRNA gene is an effective
499 approach to evaluate the usefulness of antibiotic therapy.

500

501 **ACKNOWLEDGEMENTS**

502 This project was supported by Agriculture and Food Research Initiative Competitive
503 Grant No 2013-67015-21233 from the USDA National Institute of Food and Agriculture.

504

505 **AUTHOR CONTRIBUTIONS**

506 Conceived and designed the experiments: RCB, GO, YHS. Performed the experiments:
507 EKG, SFL, KK, DHD. Analyzed the data: EKG RSB RCB. Wrote the paper: EKG RSB
508 RCB, Revised the manuscript: YHS, GO. All authors reviewed the manuscript.

509

510 **COMPETING FINANCIAL INTERESTS**

511 The authors declare no conflict of interest.

512 REFERENCES

- 513 1 Sargeant, J. M., Scott, H. M., Leslie, K. E., Ireland, M. J. & Bashiri, A. Clinical mastitis in dairy
514 cattle in Ontario: frequency of occurrence and bacteriological isolates. *Can Vet J* **39**, 33-38
515 (1998).
- 516 2 Hertl, J. A., Schukken, Y. H., Welcome, F. L., Tauer, L. W. & Grohn, Y. T. Effects of pathogen-
517 specific clinical mastitis on probability of conception in Holstein dairy cows. *J Dairy Sci* **97**, 6942-
518 6954, doi:10.3168/jds.2014-8203 (2014).
- 519 3 Erskine, R. J., Wagner, S. & DeGraves, F. J. Mastitis therapy and pharmacology. *Vet Clin North*
520 *Am Food Anim Pract* **19**, 109-138, vi (2003).
- 521 4 Pol, M. & Ruegg, P. L. Treatment practices and quantification of antimicrobial drug usage in
522 conventional and organic dairy farms in Wisconsin. *J Dairy Sci* **90**, 249-261,
523 doi:10.3168/jds.S0022-0302(07)72626-7 (2007).
- 524 5 Martin, R. *et al.* Human milk is a source of lactic acid bacteria for the infant gut. *J Pediatr* **143**,
525 754-758, doi:10.1016/j.jpeds.2003.09.028 (2003).
- 526 6 Bertelsen, R. J. *et al.* Probiotic milk consumption in pregnancy and infancy and subsequent
527 childhood allergic diseases. *J Allergy Clin Immunol* **133**, 165-171.e161-168,
528 doi:10.1016/j.jaci.2013.07.032 (2014).
- 529 7 Heikkila, M. P. & Saris, P. E. Inhibition of *Staphylococcus aureus* by the commensal bacteria of
530 human milk. *J Appl Microbiol* **95**, 471-478 (2003).
- 531 8 Olde Riekerink, R. G., Barkema, H. W., Kelton, D. F. & Scholl, D. T. Incidence rate of clinical
532 mastitis on Canadian dairy farms. *J Dairy Sci* **91**, 1366-1377, doi:10.3168/jds.2007-0757 (2008).
- 533 9 Oikonomou, G., Machado, V. S., Santisteban, C., Schukken, Y. H. & Bicalho, R. C. Microbial
534 diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA.
535 *PLoS One* **7**, e47671, doi:10.1371/journal.pone.0047671 (2012).
- 536 10 Kuehn, J. S. *et al.* Bacterial community profiling of milk samples as a means to understand
537 culture-negative bovine clinical mastitis. *PLoS One* **8**, e61959, doi:10.1371/journal.pone.0061959
538 (2013).
- 539 11 Oikonomou, G. *et al.* Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically
540 diseased quarters. *PLoS one* **9**, e85904 (2014).
- 541 12 Erskine, R. J., Eberhart, R. J., Hutchinson, L. J., Spencer, S. B. & Campbell, M. A. Incidence and
542 types of clinical mastitis in dairy herds with high and low somatic cell counts. *J Am Vet Med*
543 *Assoc* **192**, 761-765 (1988).
- 544 13 Botrel, M. A. *et al.* Distribution and antimicrobial resistance of clinical and subclinical mastitis
545 pathogens in dairy cows in Rhone-Alpes, France. *Foodborne Pathog Dis* **7**, 479-487,
546 doi:10.1089/fpd.2009.0425 (2010).
- 547 14 Suojala, L., Kaartinen, L. & Pyorala, S. Treatment for bovine *Escherichia coli* mastitis - an
548 evidence-based approach. *Journal of veterinary pharmacology and therapeutics* **36**, 521-531
549 (2013).
- 550 15 Schukken, Y. H. *et al.* Randomized clinical trial to evaluate the efficacy of a 5-day ceftiofur
551 hydrochloride intramammary treatment on nonsevere gram-negative clinical mastitis. *J Dairy Sci*
552 **94**, 6203-6215, doi:10.3168/jds.2011-4290 (2011).
- 553 16 Schukken, Y. H. *et al.* Noninferiority trial comparing a first-generation cephalosporin with a
554 third-generation cephalosporin in the treatment of nonsevere clinical mastitis in dairy cows. *J*
555 *Dairy Sci* **96**, 6763-6774, doi:10.3168/jds.2013-6713 (2013).
- 556 17 Dolejska, M. *et al.* Dissemination of IncFII(K)-type plasmids in multiresistant CTX-M-15-
557 producing Enterobacteriaceae isolates from children in hospital paediatric oncology wards. *Int J*
558 *Antimicrob Agents* **40**, 510-515, doi:10.1016/j.ijantimicag.2012.07.016 (2012).

559 18 Hunt, K. M. *et al.* Characterization of the diversity and temporal stability of bacterial
560 communities in human milk. *PLoS One* **6**, e21313, doi:10.1371/journal.pone.0021313 (2011).

561 19 Cabrera-Rubio, R. *et al.* The human milk microbiome changes over lactation and is shaped by
562 maternal weight and mode of delivery. *Am J Clin Nutr* **96**, 544-551, doi:10.3945/ajcn.112.037382
563 (2012).

564 20 Wenz, J. R., Barrington, G. M., Garry, F. B., Dinsmore, R. P. & Callan, R. J. Use of systemic disease
565 signs to assess disease severity in dairy cows with acute coliform mastitis. *J Am Vet Med Assoc*
566 **218**, 567-572 (2001).

567 21 Wenz, J. R., Garry, F. B. & Barrington, G. M. Comparison of disease severity scoring systems for
568 dairy cattle with acute coliform mastitis. *J Am Vet Med Assoc* **229**, 259-262,
569 doi:10.2460/javma.229.2.259 (2006).

570 22 Quigley, L. *et al.* A comparison of methods used to extract bacterial DNA from raw milk and raw
571 milk cheese. *J Appl Microbiol* **113**, 96-105, doi:10.1111/j.1365-2672.2012.05294.x (2012).

572 23 Caporaso, J. G. *et al.* Ultra-high-throughput microbial community analysis on the Illumina HiSeq
573 and MiSeq platforms. *Isme j* **6**, 1621-1624, doi:10.1038/ismej.2012.8 (2012).

574 24 Foditsch, C. *et al.* Oral Administration of Faecalibacterium prausnitzii Decreased the Incidence of
575 Severe Diarrhea and Related Mortality Rate and Increased Weight Gain in Preweaned Dairy
576 Heifers. *PLoS One* **10**, e0145485, doi:10.1371/journal.pone.0145485 (2015).

577 25 Caporaso, J. G. *et al.* in *Nat Methods* Vol. 7 335-336 (2010).

578 26 Bokulich, N. A. *et al.* Quality-filtering vastly improves diversity estimates from Illumina amplicon
579 sequencing. *Nat Methods* **10**, 57-59, doi:10.1038/nmeth.2276 (2013).

580 27 Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**,
581 2460-2461, doi:10.1093/bioinformatics/btq461 (2010).

582 28 Boutin, S. *et al.* Comparison of microbiomes from different niches of upper and lower airways in
583 children and adolescents with cystic fibrosis. *PLoS One* **10**, e0116029,
584 doi:10.1371/journal.pone.0116029 (2015).

585 29 Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful
586 approach to multiple testing. *J. Roy. Stat. Soc. B.* **57**, 289-300 (1995).

587 30 R Core Team. R: A language and environment for statistical computing. *R Foundation for*
588 *Statistical Computing, Vienna, Austria.* (2016).

589 31 Paulson, J. N., Stine, O. C., Bravo, H. C. & Pop, M. Differential abundance analysis for microbial
590 marker-gene surveys. *Nat Methods* **10**, 1200-1202, doi:10.1038/nmeth.2658 (2013).

591 32 Oksanen, J. *et al.* vegan: Community Ecology Package. R package version 2.4-1. (2016).

592 33 Vazquez-Baeza, Y., Pirrung, M., Gonzalez, A. & Knight, R. EMPERor: a tool for visualizing high-
593 throughput microbial community data. *Gigascience* **2**, 16, doi:10.1186/2047-217X-2-16 (2013).

594 34 Schukken, Y. H. *et al.* Randomized clinical trial to evaluate the efficacy of a 5-day ceftiofur
595 hydrochloride intramammary treatment on nonsevere gram-negative clinical mastitis. *Journal of*
596 *dairy science* **94**, 6203-6215 (2011).

597 35 Lago, A., Godden, S. M., Bey, R., Ruegg, P. L. & Leslie, K. The selective treatment of clinical
598 mastitis based on on-farm culture results: I. Effects on antibiotic use, milk withholding time, and
599 short-term clinical and bacteriological outcomes. *Journal of dairy science* **94**, 4441-4456 (2011).

600 36 Lago, A., Godden, S. M., Bey, R., Ruegg, P. L. & Leslie, K. The selective treatment of clinical
601 mastitis based on on-farm culture results: II. Effects on lactation performance, including clinical
602 mastitis recurrence, somatic cell count, milk production, and cow survival. *J Dairy Sci* **94**, 4457-
603 4467, doi:10.3168/jds.2010-4047 (2011).

604 37 Leininger, D. J., Roberson, J. R., Elvinger, F., Ward, D. & Akers, R. M. Evaluation of frequent
605 milkout for treatment of cows with experimentally induced Escherichia coli mastitis. *Journal of*
606 *the American Veterinary Medical Association* **222**, 63-66 (2003).

607 38 Jimenez, E. *et al.* Metagenomic Analysis of Milk of Healthy and Mastitis-Suffering Women. *J Hum*
608 *Lact* **31**, 406-415, doi:10.1177/0890334415585078 (2015).

609 39 Dogan, B. *et al.* Adherent and invasive Escherichia coli are associated with persistent bovine
610 mastitis. *Veterinary microbiology* **116**, 270-282 (2006).

611 40 Fairbrother, J. H. *et al.* Characterization of persistent and transient Escherichia coli isolates
612 recovered from clinical mastitis episodes in dairy cows. *Vet Microbiol* **176**, 126-133,
613 doi:10.1016/j.vetmic.2014.12.025 (2015).

614 41 Addis, M. F. *et al.* The bovine milk microbiota: insights and perspectives from -omics studies.
615 *Mol Biosyst*, doi:10.1039/c6mb00217j (2016).

616 42 Britten, A. M. The role of diagnostic microbiology in mastitis control programs. *The Veterinary*
617 *clinics of North America.Food animal practice* **28**, 187-202 (2012).

618 43 Wellenberg, G. J., van der Poel, W. H. & Van Oirschot, J. T. Viral infections and bovine mastitis: a
619 review. *Vet Microbiol* **88**, 27-45 (2002).

620 44 Dworecka-Kaszak, B., Krutkiewicz, A., Szopa, D., Kleczkowski, M. & Bieganska, M. High
621 prevalence of Candida yeast in milk samples from cows suffering from mastitis in poland.
622 *ScientificWorldJournal* **2012**, 196347, doi:10.1100/2012/196347 (2012).

623 45 Vetrovsky, T. & Baldrian, P. The variability of the 16S rRNA gene in bacterial genomes and its
624 consequences for bacterial community analyses. *PLoS One* **8**, e57923,
625 doi:10.1371/journal.pone.0057923 (2013).

626 46 Nadkarni, M. A., Martin, F. E., Jacques, N. A. & Hunter, N. Determination of bacterial load by
627 real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* **148**, 257-266,
628 doi:10.1099/00221287-148-1-257 (2002).

629 47 Falentin, H. *et al.* Bovine Teat Microbiome Analysis Revealed Reduced Alpha Diversity and
630 Significant Changes in Taxonomic Profiles in Quarters with a History of Mastitis. *Front Microbiol*
631 **7**, 480, doi:10.3389/fmicb.2016.00480 (2016).

632 48 Koskinen, M. T. *et al.* Field comparison of real-time polymerase chain reaction and bacterial
633 culture for identification of bovine mastitis bacteria. *J Dairy Sci* **93**, 5707-5715,
634 doi:10.3168/jds.2010-3167 (2010).

635 49 Martin, R. *et al.* Cultivation-independent assessment of the bacterial diversity of breast milk
636 among healthy women. *Res Microbiol* **158**, 31-37, doi:10.1016/j.resmic.2006.11.004 (2007).

637 50 Menard, L., Vanasse, C., Diaz, C. & Rivard, G. Mycobacterium chelonae Mastitis in a Quebec Dairy
638 Herd. *Can Vet J* **24**, 305-307 (1983).

639 51 Murai, A., Maruyama, S., Nagata, M. & Yuki, M. Mastitis caused by Mycobacterium kansasii
640 infection in a dog. *Vet Clin Pathol* **42**, 377-381, doi:10.1111/vcp.12056 (2013).

641 52 Richey, M. J., Foster, A. P., Crawshaw, T. R. & Schock, A. Mycobacterium bovis mastitis in an
642 alpaca and its implications. *Vet Rec* **169**, 214, doi:10.1136/vr.d5287 (2011).

643 53 Schultze, W. D. & Brasso, W. B. Characterization and identification of Mycobacterium smegmatis
644 in bovine mastitis. *Am J Vet Res* **48**, 739-742 (1987).

645 54 Franco, M. M. *et al.* Occurrence of mycobacteria in bovine milk samples from both individual
646 and collective bulk tanks at farms and informal markets in the southeast region of Sao Paulo,
647 Brazil. *BMC Vet Res* **9**, 85, doi:10.1186/1746-6148-9-85 (2013).

648 55 Bhatt, V. D. *et al.* Milk microbiome signatures of subclinical mastitis-affected cattle analysed by
649 shotgun sequencing. *J Appl Microbiol* **112**, 639-650, doi:10.1111/j.1365-2672.2012.05244.x
650 (2012).

651 56 Kim, J. *et al.* Effects of the Antibiotics Growth Promoter Tylosin on Swine Gut Microbiota. *J*
652 *Microbiol Biotechnol*, doi:10.4014/jmb.1512.12004 (2016).

653 57 Costa, M. C. *et al.* Changes in the equine fecal microbiota associated with the use of systemic
654 antimicrobial drugs. *BMC Vet Res* **11**, 19, doi:10.1186/s12917-015-0335-7 (2015).

655 58 Vlckova, K. *et al.* Effect of Antibiotic Treatment on the Gastrointestinal Microbiome of Free-
656 Ranging Western Lowland Gorillas (*Gorilla g. gorilla*). *Microb Ecol*, doi:10.1007/s00248-016-
657 0745-5 (2016).

658 59 Lax, S. *et al.* Longitudinal analysis of microbial interaction between humans and the indoor
659 environment. *Science* **345**, 1048-1052, doi:10.1126/science.1254529 (2014).

660 60 Zhou, Y. *et al.* Longitudinal analysis of the premature infant intestinal microbiome prior to
661 necrotizing enterocolitis: a case-control study. *PLoS One* **10**, e0118632,
662 doi:10.1371/journal.pone.0118632 (2015).

663 61 IHMP Research Network Consortium. The Integrative Human Microbiome Project: dynamic
664 analysis of microbiome-host omics profiles during periods of human health and disease. *Cell*
665 *Host Microbe* **16**, 276-289, doi:10.1016/j.chom.2014.08.014 (2014).

666 62 Zhao, J. *et al.* Decade-long bacterial community dynamics in cystic fibrosis airways. *Proc Natl*
667 *Acad Sci U S A* **109**, 5809-5814, doi:10.1073/pnas.1120577109 (2012).

668 63 Stressmann, F. A. *et al.* Long-term cultivation-independent microbial diversity analysis
669 demonstrates that bacterial communities infecting the adult cystic fibrosis lung show stability
670 and resilience. *Thorax* **67**, 867-873, doi:10.1136/thoraxjnl-2011-200932 (2012).

671 64 van Helden, P. D., van Helden, L. S. & Hoal, E. G. One world, one health. Humans, animals and
672 the environment are inextricably linked--a fact that needs to be remembered and exploited in
673 our modern approach to health. *EMBO Rep* **14**, 497-501, doi:10.1038/embor.2013.61 (2013).

674 65 Wittum, T. E. The challenge of regulating agricultural ceftiofur use to slow the emergence of
675 resistance to extended-spectrum cephalosporins. *Appl Environ Microbiol* **78**, 7819-7821,
676 doi:10.1128/AEM.01967-12 (2012).

677 66 WHO. Critically important antimicrobials for human medicine *World Health Organization 3rd rev*
678 (2012).

679

680 **Table 1.** Effects of intramammary treatment with ceftiofur hydrochloride on clinical mastitis cure in lactating dairy cows

Parameter	Clinical cure			Bacteriological cure ¹		
	Control	Ceftiofur	<i>P</i>	Control	Ceftiofur	<i>P</i>
	----- % (n/n) -----			----- % (n/n) -----		
Cure on day 10 and 14						
Gram negative	73.9 (17/23)	75.0 (18/24)	0.93	82.6 (19/23)	79.2 (19/24)	0.76
<i>Escherichia coli</i>	75.0 (15/20)	70.0 (14/20)	0.72	85.0 (17/20)	80.0 (16/20)	0.67
<i>Klebsiella</i> spp.	66.7 (2/3)	100.0 (2/2)	0.36	66.7 (2/3)	100.0 (2/2)	0.36
<i>Pseudomonas</i> spp. ²	---	100.0 (2/2)	---	---	50.0 (1/2)	---
Negative culture ³	76.5 (13/17)	81.3 (13/16)	0.73	NA	NA	NA
Overall	75.0 (30/40)	77.5 (31/40)	0.79	82.6 (19/23)	79.2 (19/24)	1.00

681 ¹ Based on standard laboratory culture methods for identification of milk pathogens.

682 ² All cows diagnosed with *Pseudomonas* spp. by laboratory culture were assigned to the Ceftiofur group; thus, evaluation
683 of treatment effect was not possible.

684 ³ Evaluation of bacteriological cure is not applicable to cows with negative culture. NA = non-applicable.

685

686 **FIGURES**

687 **FIG 1.** Effect of clinical mastitis and intramammary treatment with ceftiofur hydrochloride
688 (days 1-5) on the number of 16S rRNA gene copies in cows with clinical mastitis
689 associated with *Escherichia coli* (a) or negative culture (c), and microbial diversity in
690 cows with clinical mastitis associated with *Escherichia coli* (b) or negative culture (d).
691 Bars represent standard error of the mean. Asterisks represent significant differences at
692 $\alpha = 0.05$ between groups within the same study day. (a) Mastitic-Control had a
693 significantly greater bacterial load than Mastitic-Ceftiofur and healthy quarters on day 3.
694 (c) On day 1, both mastitic quarters had a significantly greater bacterial load when
695 compared to healthy quarters. On day 8: Mastitic-Control had a significantly greater
696 bacterial load than Mastitic-Ceftiofur and healthy quarters.

697 **FIG 2.** Relative abundance of phyla in quarters diagnosed with clinical mastitis (red
698 bars) and healthy quarters (blue bars) according to identification of milk pathogens by
699 laboratory culture. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.10$. Bars represent standard
700 error of the mean.

701 **FIG 3.** Comparison of the microbiome from quarters with clinical mastitis associated
702 with *Escherichia coli* and healthy quarters (i.e. reference for calculation of fold change).
703 Size of the circle is proportional to the overall prevalence of each family. Color of the
704 circle is associated with effect size. The graph plots log fold change in 16S rRNA gene
705 abundance in mastitic relative to healthy control quarters versus false discovery rate
706 (FDR) logWorth (i.e. $-\log_{10}P$). P-values are adjusted for FDR. The dashed line
707 represents the adjusted P -value = 0.01.

708 **FIG 4.** Comparison of the microbiome from quarters with clinical mastitis associated
709 with negative culture and healthy quarters (i.e. reference for calculation of fold change)
710 on day 0. Size of the circle is proportional to the overall prevalence of each family. Color
711 of the circle is associated with effect size. The graph plots log fold change in 16S rRNA
712 gene abundance in mastitic relative to healthy control quarters versus false discovery
713 rate (FDR) logWorth (i.e. $-\log_{10}P$). P-values are adjusted for FDR. The dashed line
714 represents the adjusted P -value = 0.05.

715 **FIG 5.** Effect of intramammary treatment with ceftiofur hydrochloride on relative
716 abundance of the 25 most prevalent families in milk from quarters with clinical mastitis
717 associated with *Escherichia coli*. Numbers indicate day after diagnosis of clinical
718 mastitis.

719 **FIG 6.** Effect of intramammary treatment with ceftiofur hydrochloride (day 1) or cure on
720 the relative abundance of Enterobacteriaceae and Shannon diversity index in cows with
721 clinical mastitis associated with *Escherichia coli*. (a) Effect of intramammary treatment
722 with ceftiofur hydrochloride (day 1) on the relative abundance of Enterobacteriaceae in

723 cows with clinical mastitis associated with *E. coli*. Effect of eventual clinical cure on the
724 relative abundance of Enterobacteriaceae in cows with clinical mastitis associated with
725 *E. coli* on control cows (c) and treated cows (e). Effect of cure on the Shannon index in
726 cows with clinical mastitis associated with *E. coli* (b), on control cows (d) and treated
727 cows (f). Asterisks represent significant differences at $\alpha = 0.05$ between groups within
728 the same study day.

729 **FIG 7.** Effect of intramammary treatment with ceftiofur hydrochloride on relative
730 abundance of the 25 most prevalent families in milk from quarters with clinical mastitis
731 associated with negative culture. Numbers indicate day after diagnosis of clinical
732 mastitis.

733 **FIG 8.** Principal coordinate analysis of weighted Unifrac distances and ANOSIM
734 analysis comparing the microbiome data of samples from healthy and mastitic quarters
735 on day 1 (a and b) and day 14 (c, d, e, and f). Samples from quarters with clinical
736 mastitis associated with *E coli* are depicted in sections a, c and e. Samples from
737 quarters with clinical mastitis associated with negative culture are shown in sections b,
738 d, and f.

739

740