Delayed differentiation of vaginal and uterine microbiomes in dairy cows developing postpartum endometritis

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Abstract

Bacterial infection of the uterus is a normal event after parturition. While the healthy cow achieves uterine clearance early postpartum, cows unable to control the infection within 21 days after calving develop postpartum endometritis. Studies on the Microbial Ecology of the bovine reproductive tract have focused on either vaginal or uterine microbiomes. This is the first study that compares both microbiomes in the same animals. Terminal Restriction Fragment Length Polymorphism of the 16S rRNA gene showed that despite large differences associated to individuals, a shared community exist in vagina and uterus during the postpartum period. The largest changes associated with development of endometritis were observed at 7 days postpartum, a time when vaginal and uterine microbiomes were most similar. 16S rRNA Pyrosequencing of the vaginal microbiome at 7 days postpartum showed at least three different microbiome types that were associated with postpartum endometritis. All three microbiome types featured reduced bacterial diversity. Taken together, the above findings support a scenario where disruption of the compartmentalization of the reproductive tract during parturition results in the dispersal and mixing of the vaginal and uterine microbiomes, which subsequently are subject to differentiation. This microbial succession is likely associated to early clearance in the healthy cow. In contrast, loss of bacterial diversity and dominance of the microbiome by few bacterial taxa were related to a delayed succession in cows developing endometritis at 7 DPP.

50 # Introduction

51 Uterine infection is a common event in the postpartum period in cattle [1]. Early postpartum
52 endometrial inflammation has been shown to occur in response to infection and tissue damage,
53 and as a pre-requisite for uterine clearance and involution in preparation for a future pregnancy
54 [2,3]. Failure or delay in resolving infection has implications for the reproductive health and
55 fertility of cows. Postpartum endometritis is clinically defined as a persistent uterine infection
56 with purulent discharge beyond 21 days postpartum (DPP) and is a major cause of infertility and
57 economic loss in the dairy industry worldwide [4]. A scoring system based on the
58 characterization of vaginal mucus, which reflects the level of bacterial infection in the uterus,
59 has been used as a diagnostic tool for postpartum endometritis [5].

60 Early studies to identify possible aetiological agent(s) of postpartum uterine infection were
61 focused on the isolation of bacteria from diseased animals [5–8]. Although postpartum
62 endometritis often results from non-specific infections [8], the most common pathogens
63 associated with the uterus of endometritic animals are *Escherichia coli*, *Trueperella pyogenes*,
64 *Prevotella melaninogenicus* and *Fusobacterium necrophorum* [5,9]. These bacteria are often
65 associated in mixed infections of the uterus, with evidence pointing to a succession in which *E.*
66 *coli* is most prevalent in metritic cows during the first week postpartum. Its presence then
67 increases the subsequent risk of infection by *T. pyogenes* in postpartum weeks 2 and 3, which in
68 turn has been associated with postpartum endometritis [9,10].

69 A deeper insight into the bovine uterine microbiome at the postpartum period was recently
70 gained using cultivation-independent molecular techniques [11–18]. Temporal analysis showed
71 the occurrence of a bacterial succession in the uterine microbiome, with changes in the

72 composition of cows with uterine disease reported from calving until late postpartum
73 [12,15,17]. The vaginal microbiome has become the subject of analysis using culture-dependent
74 and culture-independent approaches [19–24]. However, a missing piece in the microbiology of
75 the postpartum period is the comparison of the vaginal and uterine microbiomes. This study
76 addresses whether there are consistently distinct communities in these compartments of the
77 reproductive tract and how they are affected by postpartum disease, specifically by postpartum
78 endometritis. In addition, given the differential ability to clear postpartum uterine infection, we
79 hypothesize that the disruption of the compartmentalization in the reproductive tract during
80 parturition results in the mixing of vaginal and uterine microbiota and that differentiation of
81 microbial communities between vagina and uterus will differ between healthy cows and cows
82 developing postpartum endometritis.

Materials and Methods

Animals

Three Irish dairy farms participated in this study. Each farm was selected based on high animal welfare standards. From the three farms, 113 cows were recruited onto the study: 42 from Farm A, 37 from Farm B and 34 from Farm C. Sixteen cows were excluded from the study for the following reasons: caesarean operation (1), twin birth (4) uterine wash immediately after calving (1), died (5), sold (3) and dried off (2). A total of 97 cows were included in the study. All procedures were carried out under authorisation of the Irish Department of Health and Children in compliance with the Cruelty to Animals Act 1876 (as amended by EU directive 86/609/EC), and all experimental protocols were approved by the University College Dublin Animal Research Ethics Committee (AREC-P-10-53).

Vaginal mucus assessment and uterine health diagnosis

Uterine health was assessed by weekly vaginal mucus scoring [5]. The vulva was thoroughly cleaned by spraying a solution of Hibiscrub (BCM Ltd, UK) and subsequent drying with a paper towel (Wypall L35; Kimberley Clark, UK). A clean, lubricated, gloved-hand was then inserted through the vulva. In each cow, the lateral, dorsal and ventral walls of the vagina and the external cervical os were palpated, and the mucus contents of the vagina withdrawn manually for examination. The vaginal mucus was assessed for colour, proportion and volume of pus, and a character score assigned as follows: 0 = clear or translucent mucus; 1 = mucus containing flecks of white or off-white pus; 2 = < 50 ml exudate containing ≤ 50% white or off-white mucopurulent material; and 3 = > 50 ml exudate containing purulent material, usually white or yellow, but occasionally sanguineous. The vaginal mucus was also assessed by odour, and given a score 0 for normal odour or a score of 1 if a fetid odour was detected. Animals were assessed weekly from 7 to 21 DPP and again prior to first breeding (at 50 DPP). They were

106 diagnosed as healthy if the vaginal mucus character score was 0 or 1 and there was no fetid odour
107 present at *every* time point. Animals were diagnosed as having postpartum endometritis if the vaginal
108 mucus character score was 0 or 1 and there was no fetid odour present on Days 7 and 14 postpartum
109 but then subsequently had purulent mucus i.e. character score of 2 or 3 with or without a fetid odour,
110 on day 21 postpartum or prior to breeding [25].
111

112 Uterine and vaginal swab collection

113 A double-guarded instrument (Labstock, Co. Meath, Ireland) was used to collect uterine and vaginal
114 swabs in duplicate from each cow. Uterine swabs were collected from the uterine body using a validated
115 method [5,26]. Briefly, the vulva was thoroughly cleaned as described above. A double guarded
116 instrument containing the swab was then inserted through the vagina and cervical canal into the lumen
117 of the uterus, guided by palpation per rectum. Within the uterine body, the cotton swab was extruded
118 from the double guard tube and brought into firm contact with the endometrium by gentle pressure per
119 rectum, about 2cm from the bifurcation of the horns, before being withdrawn into the guard. Vaginal
120 swabs were collected using a similar protocol. Briefly, the double guarded instrument was inserted into
121 the vagina, the swab was extruded from the guard and rotated gently against the vaginal wall before
122 being drawn back into the guard. Swab samples were collected before vaginal examination and mucus
123 assessment to avoid the possibility of introducing bacterial contaminants into the vagina and/or
124 disrupting the equilibrium of the reproductive tract prior to sample collection.
125 The tip of each swab was cut off and placed into a 1.5 ml polypropylene tube containing 300 µl
126 of TE buffer (20 mM TrisHCl, pH 8.0, 2 mM sodium EDTA), snap-frozen in liquid Nitrogen and
127 shipped in dry ice for molecular analysis.
128

129 ## DNA extraction

130 Swab samples were vortexed to disperse cells from the cotton tip and centrifugated for 8 min
131 at 8000 x *g*. Metagenomic DNA was extracted using the Qiagen DNeasy Blood and Tissue kit
132 following the manufacturer's instructions for Gram positive bacteria (Qiagen). Briefly, pellets
133 were suspended by vortexing in 90 µl TET buffer (TE supplemented with 0.2% [v/v] TritonX-
134 100). 90 µl of TET supplemented with 40 mg/ml egg white Lysozyme were added and incubated
135 at 37°C for 2h. Proteinase K digestion of the sample was performed at 56°C for 1h. Samples
136 were further incubated at 90°C for 5 min and after adding AL buffer, they were loaded in the
137 Qiagen column. Elution was performed in 50 µl of AE buffer. Extracted DNA was stored at -80°C
138 until further use.

139

140 ## Terminal-Restriction Fragment Length Polymorphism.

141 Terminal-restriction fragment length polymorphism (T-RFLP) was used to obtain fingerprints of
142 the microbial communities associated with vaginal and uterine DNA samples as previously
143 described [27]. Briefly, amplicons of the 16S rRNA genes were obtained by nested PCR, first by
144 15 amplification cycle with primers 27F-CM (5'-AGAGTTTGATCMTGGCTCAG) and 1492R (5'-
145 TACGGYTACCTTGTTACGACTT) [28,29], followed by a second amplification of a ~1kb
146 fluorescently-labelled product using primers 6FAM-27F-CM and U1052R (5'-
147 GARCTGRCGRCRRCCATGCA) [30]. *Msp*I digested products were ethanol precipitated,
148 resuspended in Hi-Di Formamide (final concentration 50 ng/µl) containing GeneScan-500 LIZ

149 Size Standard (Applied Biosystems) and separated by capillary electrophoresis using a 3130*xl*
150 capillary array (36 cm) in an ABI 3130*xl* Genetic Analyzer (Applied Biosystems).
151

152 Pyrosequencing

153 A fragment of ~507 bp covering V1-V3 regions of the 16S rRNA gene (*E. coli* position 27 to 534)
154 was selected as target for pyrosequencing. Libraries for pyrosequencing were obtained by
155 nested PCR. In a first step, ~1.5 kb amplicons (10 µL) were produced from each DNA sample
156 with primers 27F-CM and 1492R using the Phusion HF DNA polymerase (New England Biolabs).
157 Then, 1 µL of the resulting amplicon was used as template for the nested PCR (20 µL) using
158 primers A-MID-27F
159 (5'CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNAGAGTTTGATCMTGGCTCAG) and B-
160 534R (5'CCTATCCCCTGTGTGCCTTGGCAGTCTCAGATTACCGCGGCTGCTGG). Amplicons were
161 barcoded by introducing a 10 bp multiplex identifier (MID) represented by Ns in the primer A-
162 MID-27F. Underlined sequences at the 5' of the primers correspond to the pyrosequencing
163 adapters A and B, respectively (454 Life Sciences). PCR reactions contained 1X Phusion HF
164 buffer, 0.2 mM dNTPs, 0.5 µM each of primer (27F-CM and 1492R), 3% DMSO and 0.2 units of
165 Phusion HF DNA polymerase as recommended by the manufacturer (New England Biolabs). The
166 amplification program consisted of an initial denaturation step at 98°C 30 sec, followed by
167 either 15 or 27 cycles of 8 sec melting at 98°C; 20 sec of annealing at 65°C and 45 sec of
168 extension at 72°C for the first and second PCR, respectively. A final extension was carried out
169 for 5 minutes at 72°C. Duplicate PCR amplicons originated from the same sample were pooled
170 and purified with the Agencourt AMPure XP PCR Purification system following the

171 manufacturer's instructions (Agencourt Bioscience Corporation, Beckman Coulter). The
172 quantification of purified PCR amplicons was assessed in black 96-well plates on a Varioskan
173 spectrofluorometer (Thermo Electron Corporation) using PicoGreen dsDNA Quantitation Kit
174 (Invitrogen). Purified amplicons (1 µL) were also visualized on 2% agarose gel. Equimolar
175 amounts of amplicons obtained from different samples were pooled. Emulsion PCR and 454
176 library generation were performed at the 454 Sequencing Centre (Branford, USA). Sequencing,
177 starting from the A adapter end by using lib-L annealing beads, was performed on a Roche/454
178 GS-FLX Titanium system at the 454 Sequencing Centre (Branford, USA).
179

180 Bioinformatics

181 T-RFLP fragment sizes were determined using GeneMapper v4.0 (Applied Biosystems). Merging
182 of biological replicates and multiple alignment of T-RFLP profiles was performed with T-Align
183 [31]. Only fragments present in both biological replicates and contributing at least 0.5% of the
184 total fluorescence signal were included in the analysis. Terminal restriction fragments and Bray-
185 Curtis resemblance matrix are provided as supporting information (Table S1). Heatmaps of
186 relative abundance obtained from the fluorescent signal of terminal restriction fragments were
187 made using the conditional formatting tool in Excel 2010. Cells were formatted depending on
188 their value using a 3-colour scale where the midpoint was set as the 95 percentile. Data were
189 analysed using Primer6 v6.1.13 and Permanova+ v1.0.3 [32]. Briefly, square root transformed
190 relative abundances were used to obtain a resemblance matrix based on the S17 Bray-Curtis
191 similarity. The above matrix was then used into downstream analysis including hierarchical
192 cluster analysis and non-metric multidimensional scaling (nMDS). Group centroids were

193 determined from the above Bray-Curtis resemblance matrix and used to generate a new
194 resemblance matrix of distances between groups. A network focusing on the high frequency
195 OTUs detected in both vagina and uterus was generated with QIIME 1.8 and implemented in
196 Cytoscape 3.2.0.
197 Pyrosequencing data was analysed with the Quantitative Insights Into Microbial Ecology (QIIME
198 1.8) [33]. Multiplexed sequences were assigned to samples based on their unique nucleotide
199 barcode while any low quality or ambiguous reads were removed. In order to reduce the
200 amount of erroneous Operational Taxonomic Units (OTUs), denoising of the dataset was
201 performed using denoise_wrapper.py [34] in two hi1.4x large instances in EC2 Amazon Web
202 Services. Chimeras were detected with ChimeraSlayer [35] and removed from the dataset.
203 Datasets were deposited into the Sequence Read Archive (SRA) under accession numbers
204 SRX3849466 and SRX3849984. *De novo* picking of OTUs, at 97% of sequence identity, was
205 carried out with uclust [36]. Representative sequences were aligned to the best matching
206 sequence in the Greengenes 13_8 core reference alignment using the PyNAST method [37].
207 Taxonomic affiliations were assigned with uclust and a phylogenetic tree constructed using
208 FastTree [38]. Jackknifed-supported UPGMA trees of samples was constructed from rarefied
209 OTU tables using UniFrac distances [39].
210

211 # Results

212 ## Incidence of postpartum endometritis

213 In total, 26 out of 97 animals were diagnosed as healthy (26.8%) and 24 were diagnosed with
214 postpartum endometritis (24.7%). The remaining 47 (48.5%) animals presented with metritis;
215 short term, acute uterine disease characterised by the presence of purulent vaginal mucus on 7
216 and/or 14 DPP but that had resolved by 21 DPP. Given that the focus of the present work was
217 on comparing the microbiomes of healthy cows and cows that developed postpartum
218 endometritis, animals diagnosed with metritis were not further included. Samples that failed to
219 produce PCR products or whose replicates produced highly variable TRFLP profiles were also
220 excluded. Table 1 shows the number of samples remaining for healthy and endometritic cows
221 at different times postpartum.

Table 1. Summary of vaginal and uterine sampling and clinical assignments*

Days postpartum	Healthy		Endometritic	
	Vagina	Uterus	Vagina	Uterus
Precalving**	18	-	16	-
7	21	14	15	12
21	11	14	15	11
50	9	12	12	11

* Vaginal and uterine swab samples were taken in duplicate on 7, 21 and 50 DPP.
** Precalving samples were only taken from the vagina at -7 DPP.

222

223 ## Comparison of vaginal and uterine microbiomes in the reproductive
224 tract of healthy cows postpartum.

225 With the aim of comparing vaginal and uterine microbiomes, duplicate vaginal and uterine
226 swabs were collected from dairy cows on days 7, 21 and 50 postpartum (Table 1) and analysed

227 by T-RFLP of the 16S rRNA gene. Overall, 311 OTUs were observed (Fig 1A) at different
228 frequencies among the sampled animals (Fig 1B). Approximately 50% of the OTUs were
229 observed in 5% or less of the cows, showing an important degree of variation among
230 individuals. OTUs of medium representation, shared by 7 to 23% of the cows, constituted about
231 40% of the microbiome. The remaining 10% of the OTUs were detected in 23 to 68% of the
232 animals. Vaginal and uterine samples produced similar T-RFLP profiles (Fig 1A), OTU
233 distributions (Fig 1B) and shared highly represented OTUs, as visualised in an OTU network (Fig
234 1C). Unsurprisingly, ordination of samples by non-metric multidimensional scaling (nMDS) failed
235 to form clusters by site of sampling (Fig 1D). In addition, the data failed to show a significant
236 temporal variation in the microbial communities at different times postpartum (Permanova P =
237 0.087). Taken together, these results indicate that although there is a high degree of variation
238 among individuals, a core microbiome exists in the postpartum reproductive tract, not only
239 among different animals but also between the vaginal and uterine microbiomes as no
240 significant differences were detected (Permanova P = 0.38).

241

242 **Figure 1. T-RFLP-based comparison of the vaginal and uterine microbiomes of healthy cows**
243 **postpartum.** PCR amplicons of the 16S rRNA were obtained from DNA samples using primers 6-
244 FAM-labeled 27F and 1052R and digested with *Msp*I restriction enzyme. Fluorescently labeled
245 terminal restriction fragments (TRFs) were resolved in an ABI 3730xl genetic analyzer (Applied
246 Biosystems). GeneScan Liz 600 size standard was used for fragment sizing using GeneMapper
247 v4.0 (Applied Biosystems). Operational Taxonomic Units (OTUs) were assigned in T-Align from
248 TRFs representing at least 0.5% of the total signal and consistently found in duplicated samples.
249 **A)** Heat map representing community profiles obtained from postpartum vaginal and uterine
250 samples. The profile of each sample (rows) is populated by OTUs of varying lengths (columns).
251 The relative abundance of the OTUs in a given profile is indicated by the colour key on the top
252 where black and red represent the lowest and highest values and the midpoint (yellow) was set
253 as the 95 percentile. The heat map was produced in Excel 2010 using the conditional
254 formatting function and subsequently imported as image into CorelDraw X4. **B)** Distribution of
255 OTUs in the reproductive tract of healthy cows postpartum. Black, vagina; Red, uterus. The

256 regions marked on top show the contribution of OTUs to the total number of observed OTUS. i,
257 50%; m, 40%; s, 10%. **C)** Network showing high frequency OTUs (green circles) present in both
258 vaginal (black triangles) and uterine (red circles) samples. OTUs and samples are connected
259 through edges. The size of the green circles represents the OTU frequency among sampled
260 animals. Samples with higher number of connections are displayed closer to the OTUs. **D)**
261 Ordination of samples by non-metric multidimensional scaling (nMDS) based on the Bray-Curtis
262 resemblance of the samples. Vaginal and uterine samples are represented by black triangles
263 and red circles, respectively.
264

265 Community changes associated with postpartum endometritis.

266 This study hypothesises that the microbiome of the bovine reproductive tract is related to the
267 reproductive health status of the animal and that changes in microbial community structure
268 would be especially relevant in animals affected by postpartum endometritis. Comparison of T-
269 RFLP datasets showed that the microbiome of the reproductive tract of cows that subsequently
270 developed postpartum endometritis is significantly different to that of healthy cows
271 (Permanova P = 0.001) and that the microbial populations change over time (Permanova P =
272 0.014). Furthermore, a change in community structure on 7 DPP is associated with the
273 subsequent development of endometritis (Fig 2). The community change included both a
274 decline in OTUs that were otherwise highly represented in healthy animals, as well as the
275 appearance of a sub-community associated with postpartum endometritis that is observed in
276 both vagina and uterus (Fig 2A and 2B). Hierarchical cluster analysis of group centroids
277 separated the sample groups into two major clusters. Cluster 1 (Branch 1 in Fig 2C) contains
278 both vaginal and uterine populations from cows developing postpartum endometritis at 7 DPP
279 (V7E and U7E). Cluster 2 is formed by two branches (Branches 2a and 2b in Fig 2C). Branch 2a
280 contains healthy cows at 7 DPP (V7C and U7C), uterine microbiomes of healthy cows at 21 DPP
281 (U21C) and both vaginal and uterine microbiomes of endometritic cows at 21 and 50 DPP

282 (V21E, U21E, V50E and U50E). Branch 2b contains the precalving groups (VPcC and VPcE), the
283 group of vaginal microbiomes from healthy animals at 21 DPP (V21C), and both vaginal and
284 uterine microbiomes at 50 DPP (V50C and U50C). Recovery of the community structure in cows
285 that developed postpartum endometritis was evident at 21 DPP and continued at 50 DPP (Fig
286 2). Highly represented OTUs in healthy animals that were lost at 7 DPP in animals developing
287 postpartum endometritis, started to reappear while endometritis-associated OTUs decreased
288 (Fig 2A and Fig 2B). These results suggest a succession in the microbial communities as a
289 consequence of the disturbance in the reproductive tract during calving. In addition, they show
290 that the disturbance in healthy animals is lower, which may be associated with a faster
291 clearance than in cows developing postpartum endometritis.

292

293 **Figure 2. Temporal analysis of the microbiome associated with the reproductive tract of cows**
294 **pre- and postpartum. A)** T-RFLP-based analysis of the vaginal (triangles at the right of the
295 heatmaps) and uterine (circles) microbiomes of healthy (empty symbols) and endometritic
296 (solid symbols) cows pre-calving (Pc) and at different times postpartum (7, 21 and 50 DPP). The
297 profile of each sample (rows) is populated by OTUs of varying lengths (columns). Arrowheads at
298 the top of the heatmap indicate some of the changes associated with animals developing
299 postpartum endometritis. Black arrowheads, loss of highly represented OTUs; Red arrowheads,
300 appearance of OTUs. The relative abundance of the OTUs in a given profile is indicated by the
301 colour key at the top right corner. Amplicons of the 16S rRNA were obtained from DNA samples
302 by nested PCR and as described in Materials and Methods section. Fluorescently labeled
303 terminal restriction fragments (TRFs) were resolved in an ABI 3730xl genetic analyzer.
304 GeneScan Liz 600 size standard was used for fragment sizing using GeneMapper v4.0. OTUs
305 were assigned in T-Align from TRFs representing at least 0.5% of the total signal and
306 consistently found in duplicated samples. The heat maps were produced in Excel 2010 using the
307 conditional formatting function. **B)** Differential OTUs in the reproductive tract of dairy cows
308 postpartum. The dE_t value accounts for the difference in frequency of a given OTU between
309 endometritic and healthy cows ($dE_t = f_e - f_h$) at a given time postpartum (denoted by t). Thus,
310 the frequency of OTUs with positive values of dE is increased in endometritic animals while
311 negative values show increased frequency in healthy cows at a given time postpartum. Left to
312 right plots respectively correspond to 7, 21 and 50 DPP in vagina (Black plots) and uterus (Red
313 plots). **C)** Complete linkage hierarchical cluster analysis (Top panel) and non-metric
314 multidimensional scaling (Bottom panel) of groups. Distances are based on Bray-Curtis

315 dissimilarity of group centroids. Each group is identified using the same symbols showed at the
316 right of the heat maps in A. The analysis was performed in PRIMER6 and PERMANOVA+ with
317 group centroids obtained from a resemblance matrix generated from square root transformed
318 T-RFLP data. Figures were re-drawn using CorelDraw X4.
319

320 Vaginal and uterine microbiomes are most similar in cows developing 321 postpartum endometritis at 7 DPP.

322 So far, we have shown that the most important changes in the community structure of the
323 reproductive tract microbiome happen at 7 DPP (Fig 2), that there is a strong component of the
324 microbiome associated with individual-specific OTUs (Fig 1B) and that vaginal and uterine share
325 a core microbiome in healthy animals (Fig 1C and 1D). Regression analysis of shared OTUs in
326 paired samples of vagina and uterus at 7 DPP provided evidence that the observed similarity is
327 based not only on the presence of shared OTUs but also on their relative abundances (Fig 3A
328 and 3B). The probability of detecting OTUs in a given microbiome due to neutral processes,
329 such as bacterial dispersion, is proportional to the abundance of the same OTU in a source
330 microbiome [40]. The results presented in Fig 3C and 3D show that the higher the relative
331 abundance of shared OTUs in the vagina, the higher their frequency of detection in uterus. This
332 is consistent with the occurrence of a neutral process that results from the homogenization of
333 the vaginal and uterine microbiomes due to the disruption of the compartmentalization of the
334 reproductive tract during parturition. In addition, higher coefficients of determination were
335 observed in cows developing postpartum endometritis as compared with cows that achieved
336 clearance suggesting a delayed differentiation of the vaginal and uterine microbiomes in the
337 former group.

338

339 **Figure 3. Disruption of the compartmentalization in the reproductive tract during parturition**
340 **results in homogenization of vaginal and uterine microbiomes. A and B)** Scatter plots of the
341 abundance of OTUs observed in both vagina and uterus of dairy cows at 7 DPP. OTUs from each
342 cow are displayed in different colour. The line of best fit (solid line) was obtained by least
343 squares regression. The coefficient of determination shows the goodness of fit. The dotted line
344 indicates the expected line assuming perfect correlation. **C and D)** Frequency of OTU detection
345 in the uterus of dairy cows at 7 DPP as a function of their relative abundance in vagina. The
346 relative abundance of a given OTU in the community of vagina was calculated as the average
347 fluorescence signal associated to the OTU in the vagina of sampled animals. The observed
348 frequency of detection for each OTU in uterus was calculated as number of cows in which the
349 OTU was detected / total number of cows. Data was fitted to a 3-parameter sigmoid using the
350 dynamic fit wizard of SigmaPlot 13. The higher the coefficient of determination the best the
351 overall best-fit solution. **A and C)** Healthy cows; **B and D)** Cows developing postpartum
352 endometritis.
353

354 In agreement with the above results, comparison of the similarity between paired vaginal and
355 uterine microbiomes showed that, at 7 DPP, the vaginal and uterine microbiomes of cows
356 developing postpartum endometritis are more similar than in animals that cleared the transient
357 infection postpartum (61.9 ± 15.0% vs 25.7 ± 12.7%, $P < 0.001$) (Fig 4A). Differentiation between
358 vaginal and uterine communities was evident from a decreased similarity in paired samples
359 over time (38.9 ± 22.1 % at 21 DPP and 27.8 ± 13.1% at 50 DPP) (Fig 4B-4D). Taken together,
360 these results suggest that both differences among individuals and the presence of shared OTUs
361 in vagina and uterus mask differences in the composition of vaginal and uterine microbiomes
362 that only become evident when comparing paired samples. In addition, they also suggest that
363 the microbial succession in animals developing postpartum endometritis is delayed as
364 compared with animals capable of clearing the infection postpartum, whose vaginal and uterine
365 microbiomes differentiated as early as 7 DPP.

366

367 **Figure 4. Differentiation of the vaginal and uterine microbiomes. A-C)** Non-metric
368 multidimensional scaling of vaginal (squares) and uterine (circles) samples collected at **A)** 7, **B)**
369 21 and **C)** 50 days postpartum from animals that cleared the transient infection (black symbols)
370 or that developed postpartum endometritis (red symbols). Vaginal and uterine samples
371 originating from the same animal are linked. The colour of the link represents the Bray-Curtis
372 similarity between the microbiomes associated with each compartment of the reproductive
373 tract. Links between paired samples were added in CorelDraw X4. **D)** Average and standard
374 deviation of the Bray-Curtis similarities of paired vaginal and uterine samples at 7, 21 and 50
375 days postpartum from healthy (black bars) and endometritic cows (red bars). Asterisks above
376 the horizontal lines show the presence of significant differences between groups: *, $P < 0.05$;
377 **, $P < 0.001$.
378

379 The above results prompted the question whether the prepartum vaginal communities in dairy
380 cows could differentiate between healthy animals and those that will develop endometritis.
381 Comparison of T-RFLP datasets obtained from 35 cows indicated that the vaginal microbial
382 community of the pre-calving cow is not related to postpartum uterine health (Permanova P =
383 0.7655) (Fig S1).
384

385 Pyrosequencing of the vaginal microbiome of cows at 7 days
386 postpartum.

387 So far, the largest differences in the microbiomes of healthy cows and cows developing
388 postpartum endometritis were observed at 7 DPP. Interestingly, those differences are
389 contained in the vaginal microbiome. Thus, we decided to further analyse the vaginal
390 microbiome of 30 cows, 20 healthy and 10 developing postpartum endometritis, by
391 pyrosequencing an amplicon containing the variable regions v1 to v3 of the 16S rRNA. A dataset
392 of 701189 high-quality, non-chimeric, sequences was obtained with an average of 23373 ± 6898
393 sequences per animal. A representative set was generated by clustering sequences in (OTUs) at

394 97% of identity. In total, 8504 non-chimeric OTUs were found with an average 933 ± 614 per
395 sample. Table S2 shows a summary of the metrics for each sample. The Chao1 metric estimated
396 that the average number of species was 1410 ± 860 per sample. The current sequencing effort
397 was sufficient to cover 95.47 ± 3.56% of the species as determined by Good's estimator of
398 coverage. The diversity was observed in the range of *H'* 0.61 to 6.29 with an average of *H'* 3.82
399 ± 1.75 and the evenness ranging from *J'* 0.1 to 0.84 (Table S2). In agreement with the T-RFLP
400 data, the wide range in the values of these metrics show an important difference in the
401 communities associated with individual samples.
402 A total of 7576 out of 8504 OTUs had representative sequences in the Greengenes 13_8
403 database and were distributed into 21 phyla, 52 classes, 90 orders, 174 families and 379
404 genera. Overall, the six most abundant phyla were Firmicutes (64%), Bacteroidetes (27.7%),
405 Fusobacteria (2.9%), Proteobacteria (1.8%), Tenericutes (1.3%) and Actinobacteria (1.1%). The
406 remaining 928 OTUs were only assigned within the kingdom Bacteria. In spite of accounting for
407 a large percentage of the OTUs, their contribution to the total abundance was relatively minor
408 as only 4683 sequences (ie. 0.67% of the observations) were associated with these.
409

410 Dysbiosis in the vaginal microbiome of cows developing postpartum
411 endometritis.

412 In agreement with the results yielded by T-RFLP, pyrosequencing data showed distinct
413 microbiomes at 7 DPP between healthy cows and in cows subsequently developing postpartum
414 endometritis. Analysis of principal coordinates showed that 33% of the variation of the data

415 was explained by the first principal coordinate (PC1), which separated healthy from
416 endometritic cows (Fig 5A). PC2, accounting for 17% of the variation, was most likely related to
417 differences among individuals. These changed communities featured a significant reduction in
418 the number of observed OTUs ($P < 0.0001$), bacterial diversity (*H'*, $P < 0.0001$) and species
419 evenness (*J'*, $P = 0.0003$) as compared to healthy animals (Fig 5B and Table S2). The collapse of
420 bacterial diversity was evident in a rarefaction curve where the number of observed species in
421 cows that developed postpartum endometritis approached the asymptote much faster than
422 cows that cleared the postpartum infection (Fig 5C). The total number of estimated species by
423 the Chao1 metric was 649.6 ± 322.14 and 1789.95 ± 789.58 for endometritic and healthy cows,
424 respectively (Fig 5A and Table S2).
425

426 **Figure 5. Collapse of the vaginal microbiome in cows developing postpartum endometritis.**
427 Differences in the vaginal microbiome of cows developing postpartum endometritis (red) and
428 healthy cows (blue) were captured by 454 pyrosequencing of the v1-v3 16S rRNA at 7 days
429 postpartum. OTUs at 97% of identity were generated in QIIME 1.8 using the
430 pick_de_novo_otus.py pipeline. **A)** Principal components analysis showing a clear separation of
431 samples by health status. **B)** Box plot summary of the diversity metrics of the vaginal
432 microbiome showing lower richness and lower diversity in animals that developed postpartum
433 endometritis (See Table S2). **C)** Rarefaction analysis of observed species. The curves represent
434 the average of each group. Error bars are the standard error of the corresponding groups.
435

436 Taxonomic assignments of OTUs at phylum level showed that the microbiome of healthy
437 animals displays high content of Firmicutes while most cows that developed postpartum
438 endometritis had an increased representation of Bacteroidetes (Fig 6 and Fig S2A). The median
439 Firmicutes to Bacteroidetes ratio (F/B) in the healthy group was 4.02 while cows that developed
440 postpartum endometritis displayed a median F/B of 0.64.

441

442 The vaginal microbiome displays different community types in both 443 healthy and endometritic animals.

444 Hierarchical cluster analysis using a weighted UniFrac metric resulted in four clusters of samples
445 (Fig 6A). Cluster I is formed by 16 out of the 20 samples collected from healthy animals, whose
446 microbiomes featured the larger number of observed OTUs in the dataset (1297.5 ± 584.4),
447 high diversity (*H'* 5.25 ± 0.60) and evenness (*J'*, 0.75 ± 0.07) (Table 2). Cluster IV was formed by
448 two healthy cows that displayed a microbiome with low diversity but intermediate species
449 richness (*H'* = 1.66 and *J'* = 0.24, Table S2). The phylum Firmicutes constituted the 74.9 ± 7%
450 and 95.1 ± 1 % of the total abundance of the microbiomes of respectively clusters I and IV (Fig
451 6A and Table 2). An important difference between clusters I and IV is that while the most
452 abundant OTU in animals of cluster I constituted the 12.5 ± 7.5% of the microbiome, a single
453 OTU accounted for more than 70% of the microbiomes of cows in cluster IV.

454

455 **Figure 6. Taxonomic composition of the vaginal microbiome of cows at 7 DPP. A)** Whole-
456 microbiome weighted UniFrac hierarchical cluster. Jackknife support of internal tree nodes is
457 colour coded. Red, 75-100%; orange, 50-75%. The identity of the samples is given to the right of
458 the branch tips. Scale bar represents 0.1 distance. Cluster membership as defined by the
459 branching pattern of this dendrogram is shown at the far right of the figure and is based upon a
460 distance cut off displayed by a dashed line. **B) and C)** Taxonomic composition at phylum and
461 genus levels, respectively. Each bar represents the complete microbiome as observed in each of
462 the vaginal samples. **D)** Colour key of selected phyla (within box) and genera from B and C.
463 Superscripts: [a] OTUs with ambiguous assignment below the indicated taxonomic level; [b] OTUs
464 that although matching reference sequences in the Greengenes 13_8 database, no taxonomic
465 name has been defined. In these cases, the lowest taxonomic name is provided; [c] OTUs
466 matching reference sequences for which taxonomic changes above the rank of genus have
467 been recommend by Greengenes based on whole genome phylogeny; [d] OTUs matching Genus

468 name contested. Complete taxonomy plots derived from QIIME are shown as supporting
469 information (Fig S3).
470

Table 2. Summary of metrics for clusters obtained by Weighted Unifrac Hierarchical analysis

Cluster	Sequences	OTUs	*H'*	*J'*	Chao1	Coverage	Characteristics
I	20752.1 (4284.6) [a]	1297.5** (584.4)	5.25*** (0.6)	0.75*** (0.07)	1901.6** (843)	93.4** (3.5)	Healthy, high richness, high diversity
IIa	27010.7 (15756.5)	523 (160.7)	1.63 (0.7)	0.26 (0.11)	776.6 (127.5)	99.3 (0.3)	Endometritis, low richness, low diversity
IIb	24998 (6137.7)	319.5 (21.9)	3.23 (0.04)	0.56 (0.01)	429.8 (35.6)	99.7 (0.1)	Endometritis, low richness, high diversity
III	26652.4 (6177.1)	435.4 (325.3)	2.28 (1.18)	0.38 (0.16)	755.9 (491)	98.3 (1.5)	Endometritis, low richness, low diversity
IV	25780.5 (9975.2)	985.5* (99.7)	1.66 (0.51)	0.24 (0.08)	1413.6* (270.8)	95.9* (1.2)	Healthy, high richness, low diversity

[a] Standard deviations are given in brackets
* P < 0.05; ** P < 0.01; *** P < 0.0001 indicate significantly different as compared to the same metric of non-starred clusters.

471

472 Cows that developed postpartum endometritis branched in clusters II and III. Interestingly, both
473 clusters displayed similar metrics. In contrast to cluster I, both cluster II and III were
474 characterised by a lower species richness and lower diversity (Table S2). Interestingly, the
475 vaginal microbiomes of cows N495 and N233, both classified as healthy by inspection of the
476 vaginal mucus score, were most similar to the microbiomes of cows that developed postpartum
477 endometritis and branched in clusters II and III, respectively (Fig 6A and Table S2). The latter
478 was characterised by high content of Bacteroidetes (65 ± 14.7%, F/B = 0.49) (Fig S2B). In
479 contrast, cluster II was formed by two branches differing in the number of Firmicutes and
480 Bacteroidetes. The Firmicutes to Bacteroidetes ratio branch IIb was low (F/B = 1.46) while
481 samples in branch IIa had very low representation of Bacteroidetes and therefore displayed a
482 F/B of 78.83. At the phylum level both cluster IIa and IIb had a high content of Fusobacteria
483 (~12%) relative to other clusters (Fig S2B).

484 Firmicutes in cluster I comprised OTUs belonging to classes of Clostridia and Bacilli. A single
485 order, Clostridiales, was represented in the first, whereas the second was represented by the
486 orders of Lactobacillales (72.2%), Bacillales (21%) and Gemellales (6.2%). The most abundant
487 taxon in cows of cluster I, formed by OTUs assigned to the family Ruminococcaceae, which
488 accounted for 19.4 ± 7.4% of the abundance of the microbiome and 41.4% of Clostridia (Fig
489 S2B). Moreover, in contrast to the low diversity in clusters IIa, III and IV, in which microbiomes
490 were dominated by a single OTU, the combined abundance of the top five OTUs of the family
491 Ruminococcaceae in cows of cluster I accounted for 17.1 ± 11.2% of the taxon. Although this
492 taxon was found in 29 out of the 30 animals under study, its relative abundance was lower in
493 endometritic cows of clusters IIa, IIb and III ($P < 0.01$). Interestingly, the representation of this
494 taxon in cow N233, who was healthy but branched in cluster III was in similar abundance as in
495 cows of cluster I, while OTUs affiliated to Ruminococcaceae in the two healthy cows of cluster
496 IV constituted 4.5% and 11.5% of their microbiomes. Similar results were observed for the
497 family of Lachnospiraceae. In addition, while single OTUs dominated the diversity of
498 Bacteroidetes in cows that developed postpartum endometritis, healthy animals of cluster I
499 displayed higher diversity and evenness indexes of Bacteroidetes than cows in clusters IIb and
500 III ($P < 0.001$).
501

502 The loss of diversity in vaginal microbiome of cows developing
503 postpartum endometritis is characterised by the presence of dominant
504 OTUs at genus level.

505 At genus level, the two cows in cluster IV produced 26 and 34 OTUs of the genus Streptococcus,
506 which comprised 76.8% and 87.2% of their microbiomes. From these, a single OTU in each cow
507 dominated at least 98% of the representation of the above genus explaining the sharp decrease
508 in bacterial diversity (Fig 6C and Fig S2B). The high content of Bacteroidetes in 5 out of the 7
509 cows of cluster III was due to the presence of a highly dominant OTU of the genus Bacteroides
510 contributing 52.5 ± 24.2% of the total vaginal microbiome in cows that developed postpartum
511 endometritis. In addition, OTUs of Porphyromonas constituted 26.3 and 38% of the
512 microbiomes of two cows in cluster III (Fig 6C). Cluster IIa featured a high content of Firmicutes
513 (>80%). However, in contrast to cluster I that showed high indexes of diversity and evenness,
514 the microbiomes of cows in cluster IIa were dominated by OTUs of the family Tissierellaceae
515 (69.9%), of which 96% were affiliated to the genus Helcococcus (Fig S2B). The phylum of
516 Firmicutes in cluster IIb also displayed high proportion of Tissierellaceae (39.6%). However, in
517 contrast to cluster IIa, genera ph2, Sporanaerobacter and Parvimonas were in similar
518 proportions and constituted 90.8% of the above family. Another important characteristic of
519 clusters IIa and IIb is the high content of Fusobacteria. The major OTUs within this phylum had
520 close relatives from the family Leptotrichiaceae (99% identity) and genus Fusobacterium (100%
521 identity). Similar to cluster III, the OTUs with largest abundance in cows of cluster IIb were
522 Bacteroidetes from the genus Bacteroides and Porphyromonas, respectively.
523

524 # Discussion

525 This study revealed early signatures in the microbiome of cows that subsequently developed
526 postpartum endometritis. T-RFLP analysis showed that these signatures were characterised by
527 the appearance of a community associated with endometritis as well as the decline of OTUs
528 highly represented in healthy animals. Significantly changed communities were evident at
529 different time points between 7 DPP and 50 DPP. The occurrence of bacterial succession during
530 the postpartum period was previously reported for bovine uterine microbiota [12,15,17]. Here,
531 we show that this succession also includes the vaginal microbiota. The greatest differences in
532 microbiome composition were observed at 7 DPP between cows that achieved uterine
533 clearance and those that developed postpartum endometritis. Previous studies of either the
534 vaginal [19–24] or uterine microbiome [11–18] have been reported but to date no study has
535 compared the vaginal and uterine microbiomes in the same cows. Analysis of paired vaginal and
536 uterine microbiomes at 7 DPP suggested the mixing of bacteria due to neutral processes during
537 calving. Presented data also suggested a differentiation of the vaginal and uterine microbiomes
538 that was most evident in cows developing postpartum endometritis.

539 Despite widespread use of vaginal mucus assessment for the clinical evaluation and
540 classification of the reproductive health status of cows, the bovine vaginal microbiome has only
541 recently become the focus of analysis [19–24]. Using pyrosequencing of an amplicon
542 containing the V1 - V3 hypervariable regions of the 16S rRNA, we showed at 7DPP the presence
543 of a complex microbiome in the vaginas of healthy cows and a dysbiotic microbiome in cows
544 developing postpartum endometritis. High content of Firmicutes, high Firmicutes to
545 Bacteroidetes ratio and a high diversity index were some of the most prominent features of the

546 vaginal microbiome in healthy cows. A major reduction in the vaginal bacterial diversity of cows
547 that subsequently developed postpartum endometritis was associated with an increased
548 abundance of OTUs of *Bacteroides*, *Helcococcus,* and *Fusobacterium*, among other genera.
549 Similar results were recently shown in the vagina of cows [21,23]. In agreement with our data, a
550 recent study showed that at 7 DPP the number of Bacteroidetes is significantly higher in the
551 vagina of cows that were subsequently diagnosed with endometritis at 35 days in milk (DIM)
552 [24]. However, other studies have found different results. For example, in contrast to major
553 changes in the microbiome of cows developing postpartum endometritis observed in this work,
554 the most significant difference between metritic and healthy cows was an increased rate of
555 isolation of *E. coli* in infected cows [22]. The authors suggested a lack of a stable microbiota in
556 the bovine vagina and concluded that vaginal bacteria were likely contaminants from different
557 sources, including skin, faeces and/or from the environment. Although our data supports the
558 conjecture of an unstable (i.e. changing) microbiota during the postpartum period, the bias
559 introduced by enrichment in culture-dependent approaches is well known, as only a very small
560 fraction of the microbiome can be cultured on any given media and growth conditions [41]. A
561 study using denaturing gradient gel electrophoresis (DGGE) and clone libraries of 16S indicated
562 that there is a lower bacterial diversity in the vaginal microbiome of healthy cows as compared
563 to cows with endometritis at 30-40 DPP. Dominant taxa included *Lactobacillus* and *Weissella*
564 while endometritic animals did not show any dominant species [20]. Interestingly, while
565 *Bacteroides*, *Prevotella*, and *Clostridium perfringens* strains were equally prevalent in healthy
566 and endometritic cows, *Fusobacterium*, *Enterococcus* and *E. coli* were found in higher numbers
567 in diseased animals as determined by qPCR [20]. Another study using Ion Torrent showed

568 evidence of distinct communities in the healthy and diseased groups. *Bacteroides* and
569 *Enterobacteriacea* were the largest taxa in both groups [19]. However, the number of sequence
570 reads was highly dissimilar between groups: 31,000 and less than 1000 for the endometritic and
571 healthy groups, respectively, making difficult any comparison between the microbiomes
572 associated with groups of different health status. While some of the varying findings among the
573 above studies may be related to the employment of different technologies, sampling times or
574 other factors intrinsic to each study, our findings showed that postpartum endometritis may be
575 associated with microbiomes of varying composition. In fact, our pyrosequencing analysis
576 showed at least three different vaginal microbiome types associated with cows developing
577 postpartum endometritis. Microbiome type IIa was dominated by OTUs of the genus
578 *Helcococcus* while types IIb and III were characterised by a large content of *Bacteroides*.
579 *Fusobacteria* was higher in vaginal microbiome types IIa and IIb than in type III and rare in
580 healthy animals. Whether different microbiome types correspond to different types of
581 postpartum endometritis or represent intermediate states of recovery into a healthy
582 microbiome is yet to be determined.
583 In agreement with our T-RFLP data, suggesting an arrest of the differentiation of the vaginal
584 and uterine microbiomes in cows developing postpartum endometritis, *Bacteroides* and
585 *Fusobacterium* have been found to be most abundant in the uterine microbiome of cows with
586 uterine infection at different times postpartum [11,14,16–18,42,43]. Sequencing of the V4
587 hypervariable region of the 16S rRNA showed the progression of the uterine microbiome of
588 dairy cows during the first 6 DPP [17]. A rapid succession resulted in a shift from Proteobacteria
589 to Bacteroidetes and Fusobacteria as the most abundant phyla in the uterus of metritic cows.

590 Similar to our findings in vagina, diseased cows displayed lower uterine bacterial richness and
591 diversity indices related to an increased abundance of OTUs of *Bacteroides, Porphyromonas*
592 and *Fusobacterium* [17,18]. Failure to cure metritis, either spontaneously or with antibiotic
593 treatment, was related to increased relative abundances of the above genera and a
594 corresponding decrease in bacterial diversity in uterus [18]. In a study relying on DGGE and
595 analysis of clone libraries, the uterine fluid of two healthy and two metritic cows at 10 DPP
596 showed that the most abundant OTUs in metritic cows belonged to the phylum Fusobacteria
597 followed by Bacteroidetes [11]. Another study based on pair-ended MiSeq sequencing of an
598 amplicon containing the V1 and V2 hypervariable regions of the 16S DNA of uterine fluids
599 obtained from cows with pyometra, slaughtered at no less than 22 DPP, found that the five
600 most abundant OTUs in uterine fluids belonged to families of Fusobacteriaceae,
601 Bacteroidaceae, Pasteurellaceae and Porphyromonadaceae [16]. A higher prevalence of
602 *Bacteroides* and *Fusobacterium* was also reported upon pyrosequencing analysis of V1 and V2
603 hypervariable regions of the microbiota of uterine lavages of cows with severe endometritis at
604 35 DIM as compared to either the healthy group or to cows with mild endometritis [14]. In
605 another study, Ruminococcaceae, Bacteroidaceae, and an unclassified family that belonged to
606 class Bacteroidia were the three most abundant families in endometrial biopsies from healthy
607 cows at 4 weeks postpartum (WPP) as well as from both healthy and endometritic cows at 7
608 WPP [15]. Interestingly, our pyrosequencing analysis showed that Ruminococcaceae and
609 Bacteroidaceae were the top one and six most abundant families in the vaginal microbiome of
610 healthy cows at 7 DPP. In addition, the T-RFLP data showed that the differentiation between
611 vaginal and uterine communities of endometritic cows was evident from a decreased similarity

612 in paired samples. Taken together, our data and those of others are consistent with a bacterial
613 succession in the reproductive tract in which the differentiation of vaginal and uterine
614 microbiomes towards the recovery of their native states are conducive to reproductive health
615 and the achievement of a new pregnancy. Conversely, a delayed differentiation of vaginal and
616 uterine microbiomes is in line with impaired uterine clearance, decreased conception rates and
617 lower success of first service pregnancy rates in the endometritic cow [44]. Interestingly, high
618 prevalence of *Bacteroides*, *Ureaplasma*, *Fusobacterium* and *Arcanobacterium* were still
619 observed in the uterine microbiome of cows that failed to become pregnant after 200 DIM [14],
620 strengthening the link between a severe arrest in the differentiation of the uterine microbiome
621 and poor fertility.
622 Bacterial infection and tissue damage is a normal event occurring in the postpartum period.
623 Involution of the postpartum uterus is a highly regulated process in which an early
624 inflammatory response early postpartum is followed by a stage of proliferation and repair [2,3].
625 Comparison of RNAseq profiles of the endometrium at 7 and 21 DPP unveiled that the above
626 transition was arrested in cows with cytological endometritis [27]. Sustained inflammation was
627 also observed in the endometrium of cows with postpartum endometritis in the same time
628 frame [45]. The observed collapse in the diversity of the vaginal microbiome of endometritic
629 cows at 7 DPP and the arrested differentiation of vaginal and uterine microbiomes in cows
630 developing postpartum endometritis are in line with those data. A likely scenario implies
631 different metabolic landscapes in the reproductive tract of healthy and endometritic cows early
632 after calving, resulting from their distinct microbiotas. Support for the above scenario comes
633 from recent metagenomic analyses of the microbiome of the bovine uterus where significant

634 differences in the repertoire of functional gene categories were observed in healthy and
635 metritic cows within 3 and 12 DIM [42] and cows with purulent vaginal discharge between 25
636 and 35 DPP [43]. These studies showed that adhesins, bacteriocins and antibacterial peptides
637 and tolerance to colicin E2 are produced only by the uterine microbiota of healthy cows early
638 postpartum and continues until at least 35 DPP. In contrast, the uterine microbiota of metritic
639 and PDV cows appears to change from cold shock and acid stress in the former to increased
640 modification of lipid A and production of toxins in the later [42]. Changes in the composition of
641 the microbiome, and therefore in the metabolic landscape of the reproductive tract, may
642 impact uterine involution as a result of the dysregulated inflammatory response induced by the
643 presence of highly abundant bacteria activating specific signalling pathways and thus causing a
644 different pattern of endometrial gene expression [46]. This in turn is likely to affect the
645 transition from the inflammatory to proliferation and repair stage of the postpartum uterus
646 observed in healthy cows [2,27]. It is difficult to establish causality between dysbiosis and
647 inflammation [47]. Thus, it is not clear if changes in the composition of the microbiome precede
648 the inability of the cow to regulate the immune response or whether both the microbiome and
649 the dysregulated inflammatory response are factors predisposing the onset of postpartum
650 endometritis. In any case, a synergistic effect may occur where failure of each of these factors
651 exacerbates the other. In other words, failure to clear a highly changed microbiome
652 characterised by a low bacterial diversity and dominated by few bacterial taxa may set an
653 innate immune response in overdrive. Alternatively, an excessive inflammatory response may
654 contribute to the differential elimination of bacterial species, allowing the overgrowth of
655 bacteria able to avoid the innate immune response.

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805 # Supporting information

806 **Table S1. Dataset of Terminal Restriction Fragment Length Polymorphism**. (DOCX)

807 **Table S2. Diversity metrics of the vaginal microbiome of dairy cows at 7 days postpartum**.
808 (DOCX)

809 **Figure S1. Hierarchical cluster analysis of pre-calving dairy cows based of their vaginal**
810 **microbiomes.** The microbiomes associated with vaginal samples obtained from cows before
811 calving were compared in a resemblance matrix based on the Bray-Curtis similarity. The health
812 status of each cow was assessed depending on the outcome of the transient postpartum
813 infection. The tips of the branches are colour coded according to the outcome of postpartum
814 health status: Black, non-susceptible; Red, susceptible to postpartum endometritis. Community
815 profiles were determined by T-RFLP of the 16S rRNA as described in the section of Materials
816 and Methods. Analysis was performed in PRIMER6 and the figure was re-drawn in CorelDraw
817 X4. (TIF)

818 **Figure S2. Category-based taxonomic composition of the vaginal microbiome of cows at 7**
819 **DPP.** Taxonomic composition at phylum and genus levels, respectively. Each bar represents the
820 average of the vaginal microbiome in each of the following categories: **A)** Clinical assignment. h,
821 healthy; e, endometritis **B)** Cluster as defined in Figure 6A, **C)** Farm of collection. The colour key
822 of selected phyla (within box) and genera from A, B and C is placed at the right of the figure.
823 Superscripts: [a] OTUs with ambiguous assignment below the indicated taxonomic level; [b] OTUs
824 that although matching reference sequences in the Greengenes 13_8 database, no taxonomic

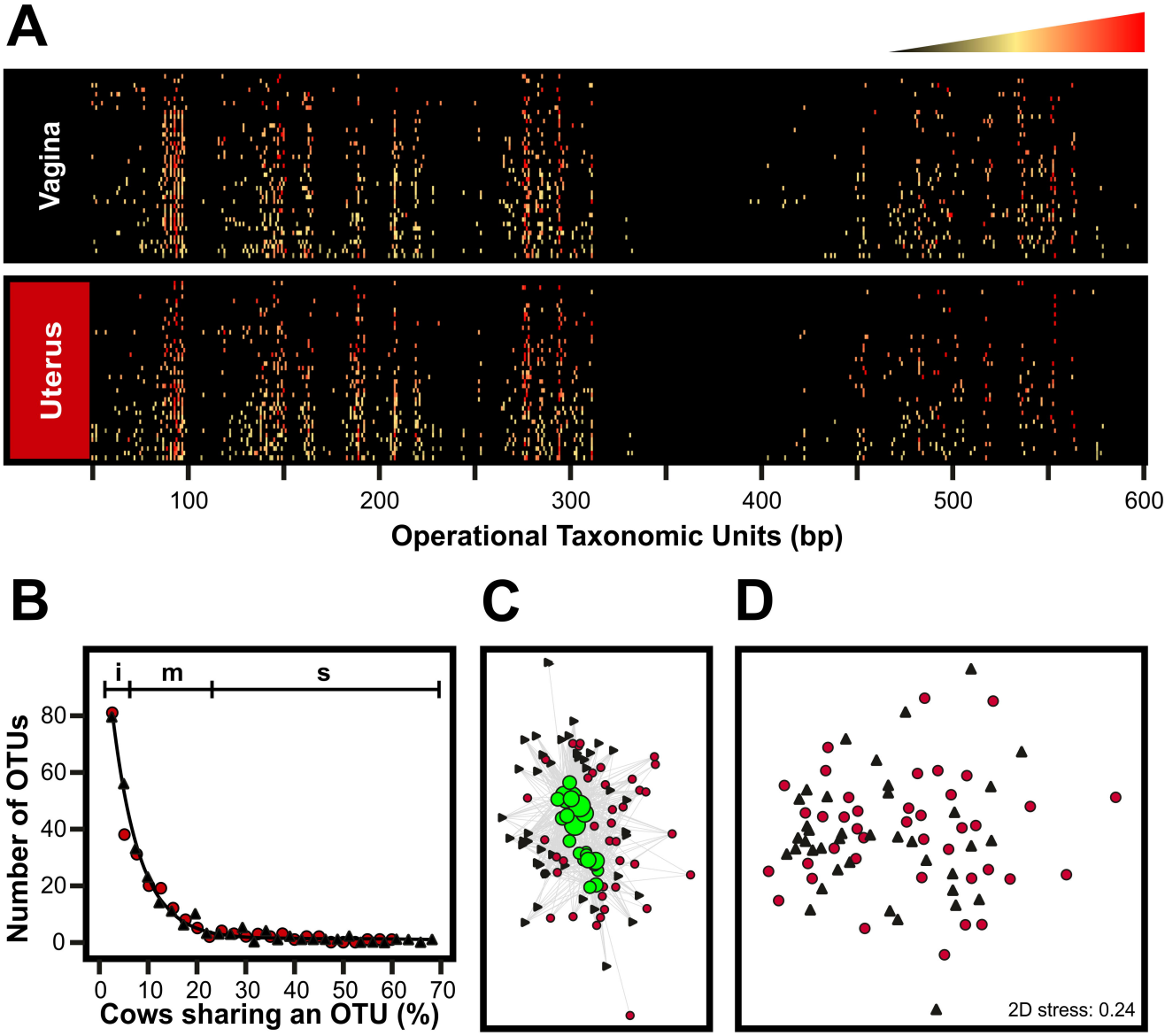
825 name has been defined. In these cases, the lowest taxonomic name is provided; [c] OTUs
826 matching reference sequences for which taxonomic changes above the rank of genus have
827 been recommend by Greengenes based on whole genome phylogeny; [d] OTUs matching Genus
828 name contested. (TIF)

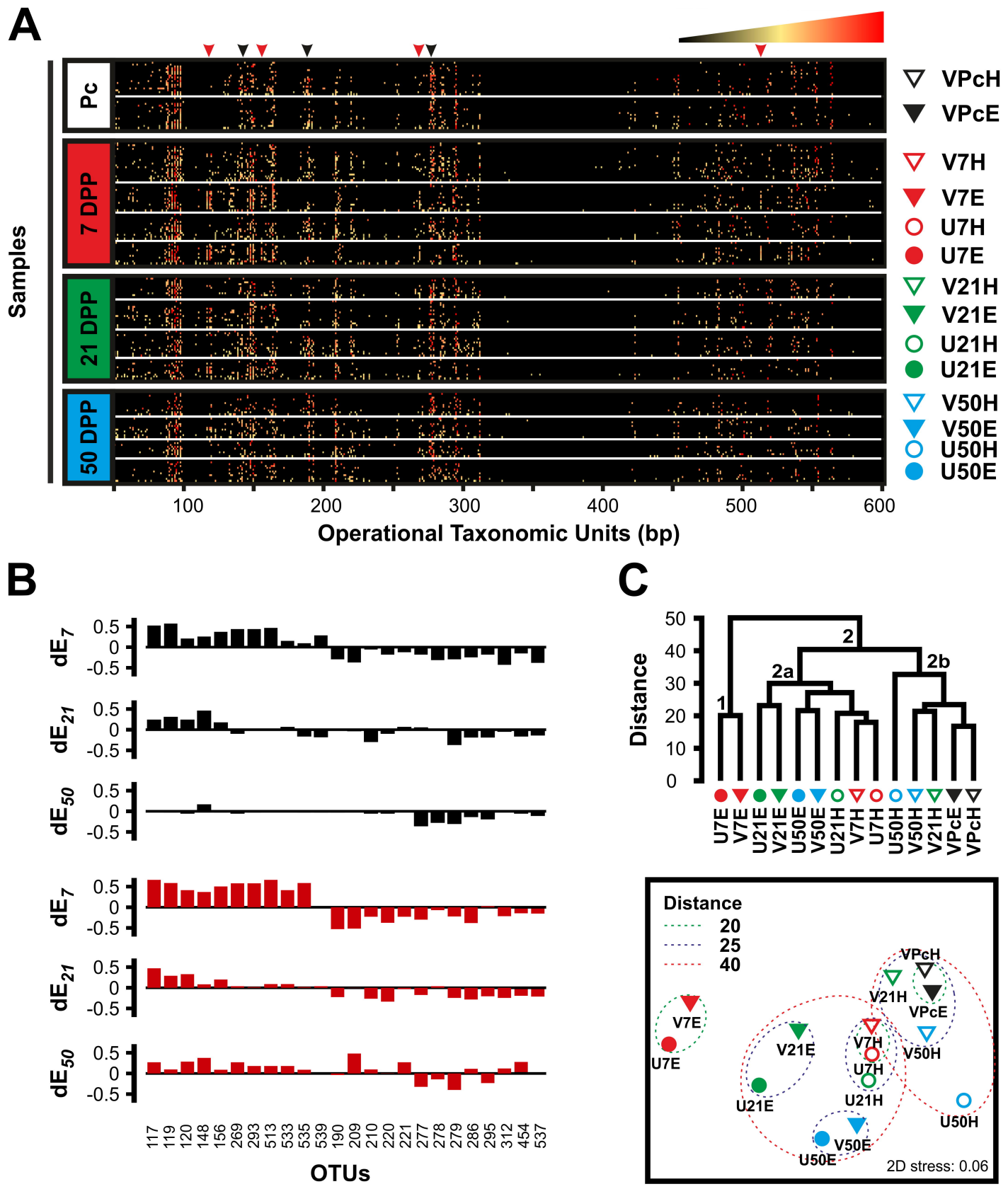
829 **Fig S3. Summary of the taxonomic composition of the vaginal microbiome of cows at 7 DPP.**
830 (HTML) Original output generated by QIIME. To visualise it double click on bar_charts.html.

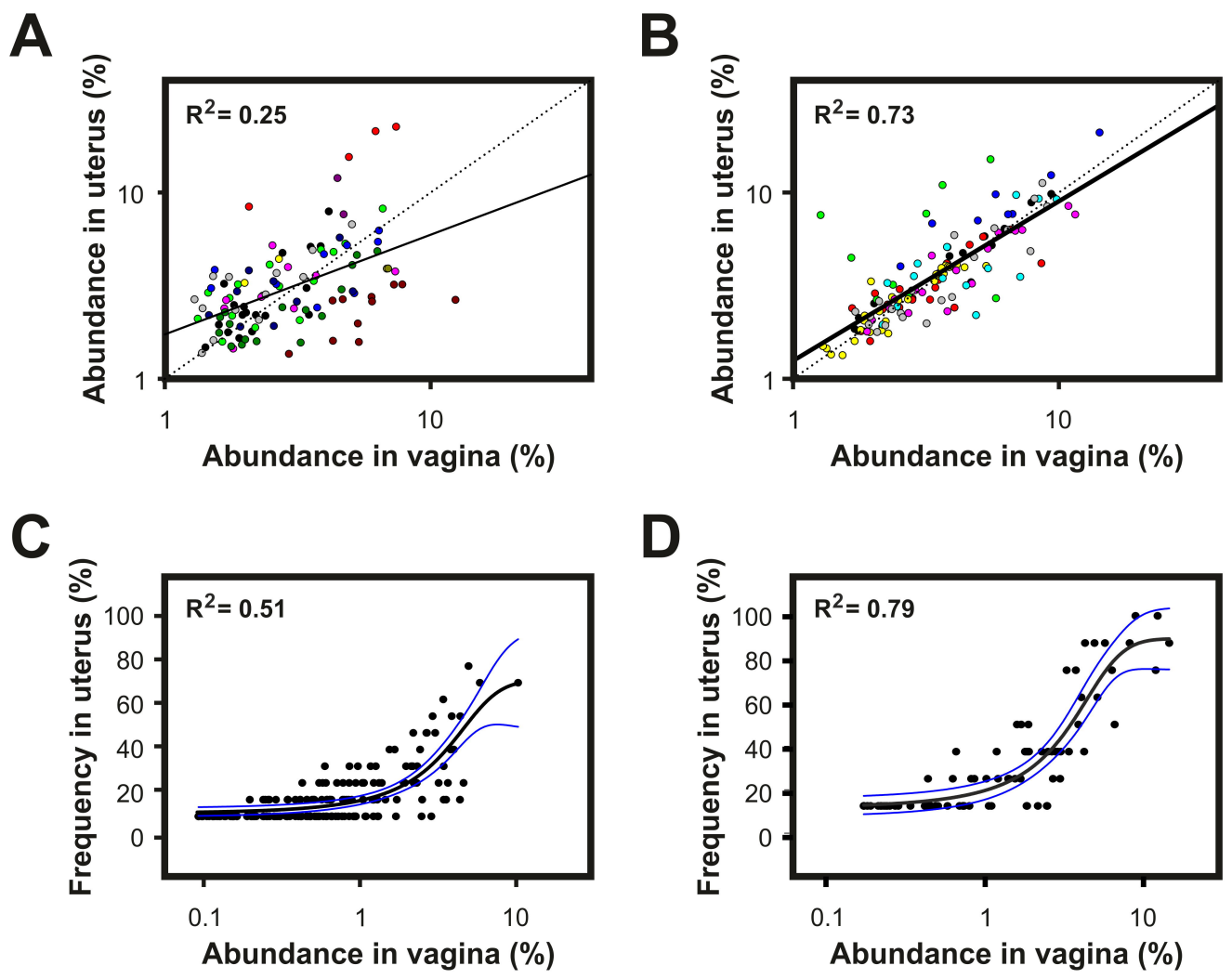
831

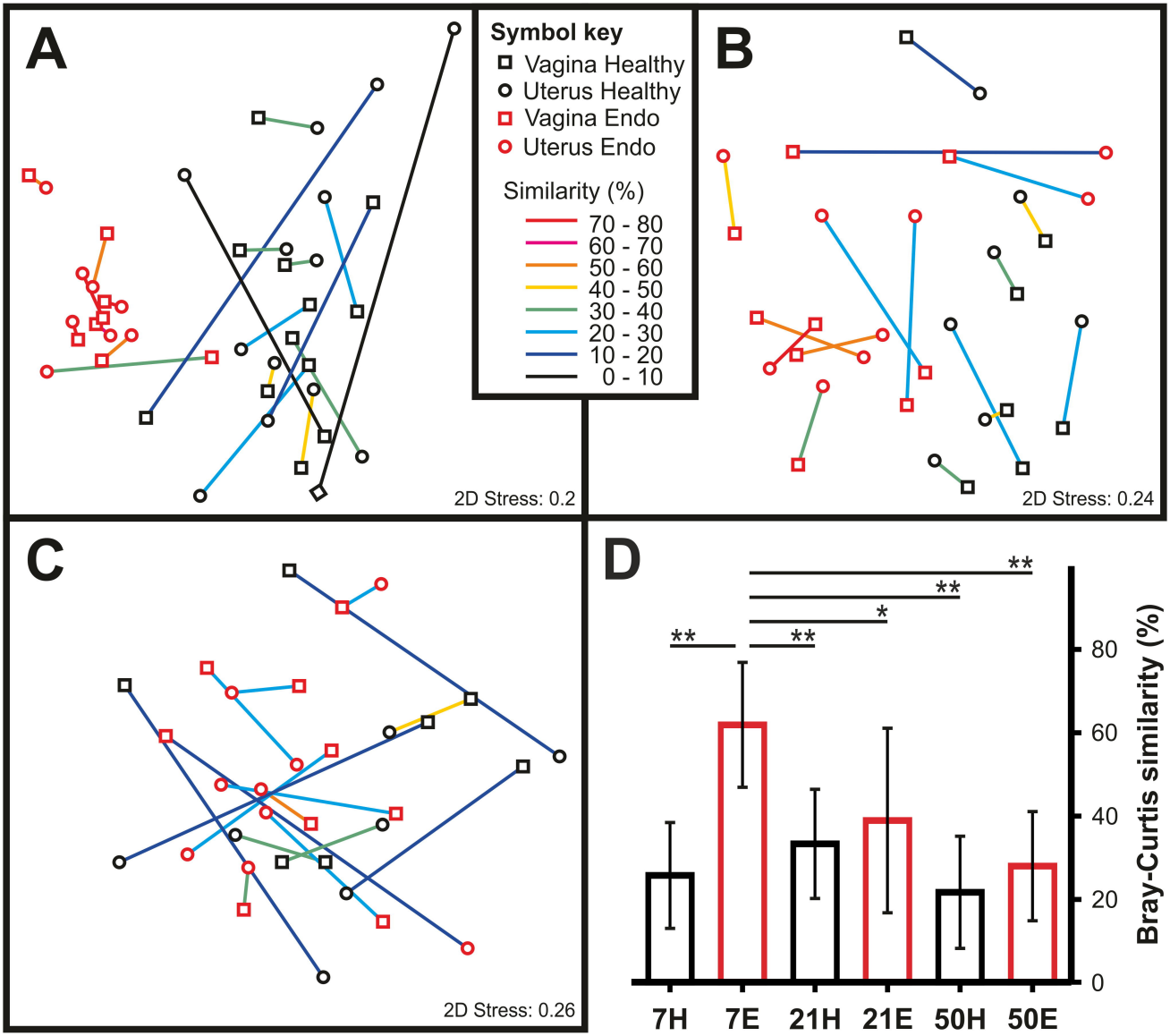
832

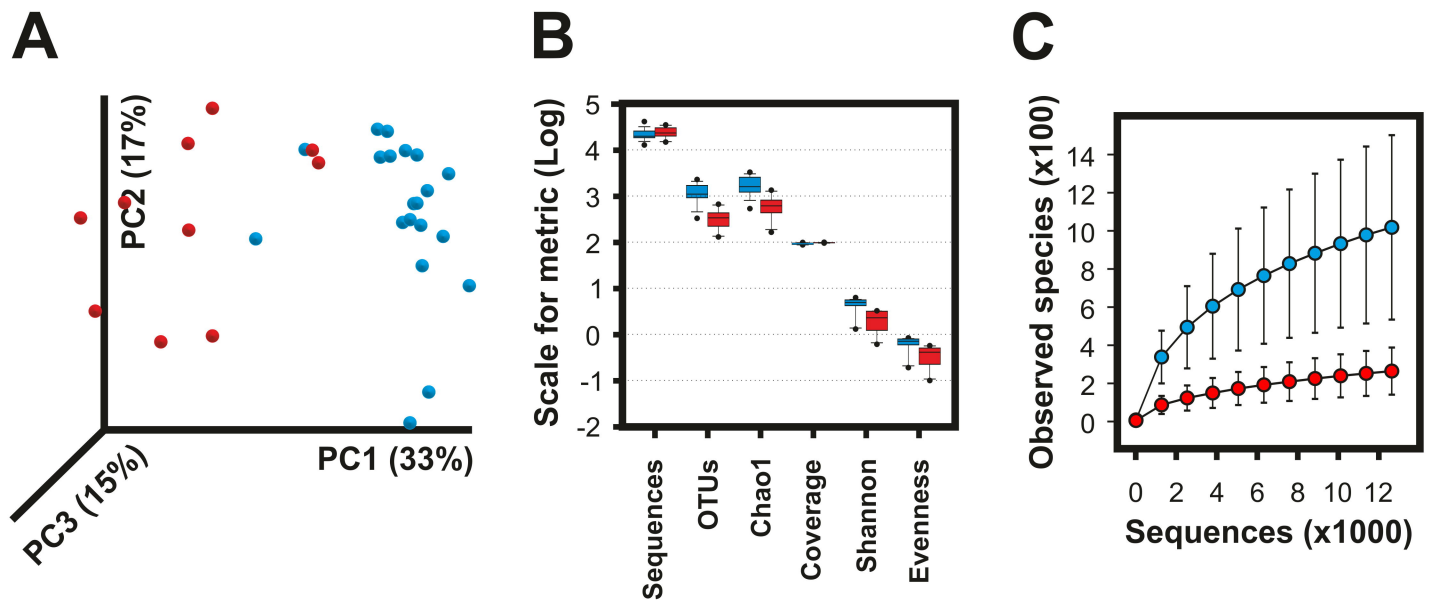
833

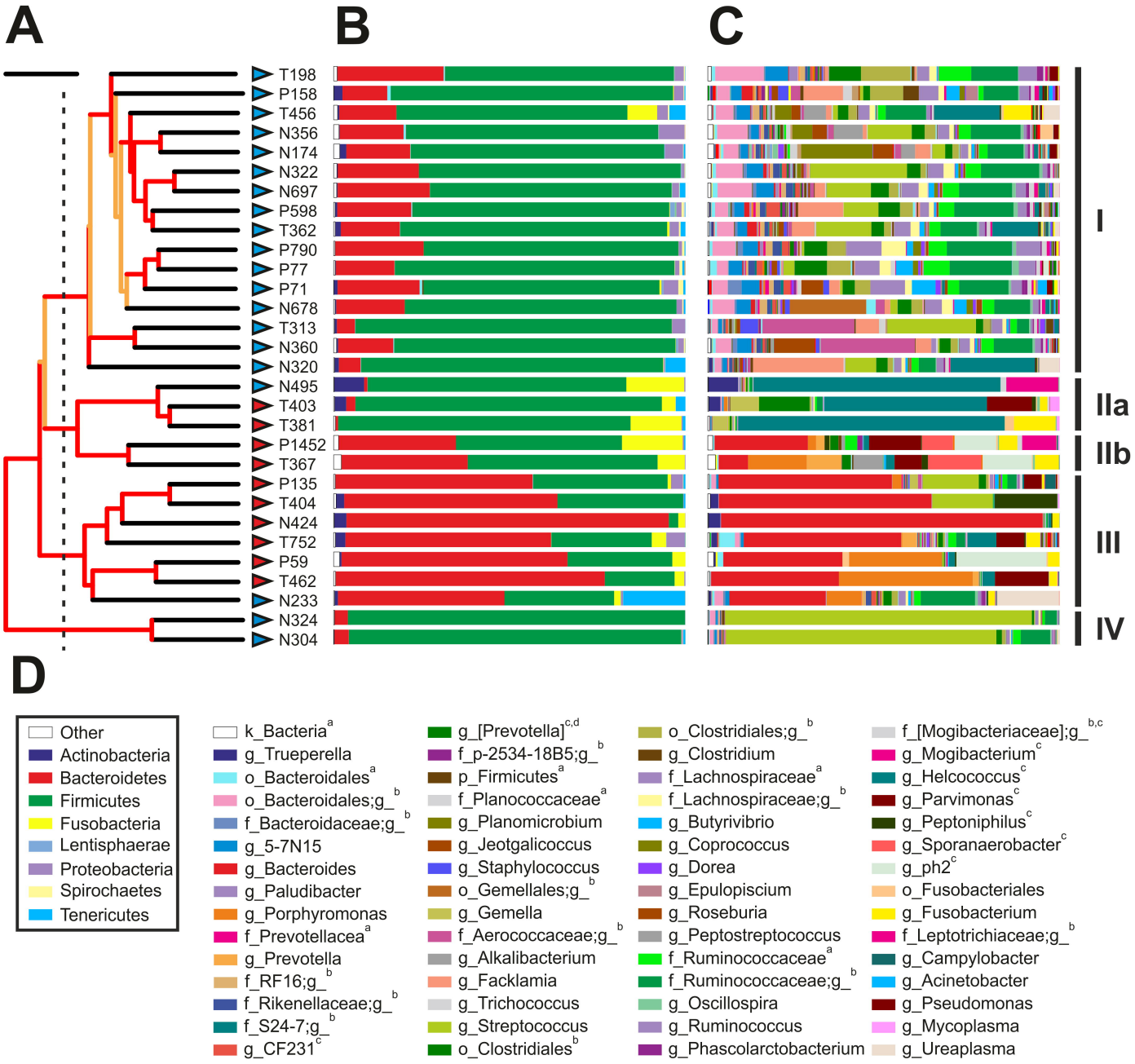

A
Vagina
Uterus
100
200
300
400
500
600
Operational Taxonomic Units (bp)
B
i
m
s
Number of OTUs
80
60
40
20
0
0
10
20
30
40
50
60
70
Cows sharing an OTU (%)
C
D
2D stress: 0.24


A
Samples
Pc
7 DPP
21 DPP
50 DPP
VPcH
VPcE
V7H
V7E
U7H
U7E
V21H
V21E
U21H
U21E
V50H
V50E
U50H
U50E
100
200
300
400
500
600
Operational Taxonomic Units (bp)
B
dE7
dE21
dE50
0.5
0
-0.5
117
119
120
148
156
269
293
513
533
535
539
190
209
210
220
221
277
278
279
286
295
312
454
537
OTUs
C
Distance
50
40
30
20
10
0
1
2
2a
2b
U7E
V7E
U21E
V21E
U50E
V50E
U21H
V7H
U7H
U50H
V50H
V21H
VPcE
VPcH
Distance
20
25
40
2D stress: 0.06


A
R2= 0.25
Abundance in uterus (%)
Abundance in vagina (%)
1
10
B
R2= 0.73
Abundance in uterus (%)
Abundance in vagina (%)
C
R2= 0.51
Frequency in uterus (%)
Abundance in vagina (%)
0.1
D
R2= 0.79
Frequency in uterus (%)
Abundance in vagina (%)


A
Symbol key
Vagina Healthy
Uterus Healthy
Vagina Endo
Uterus Endo
Similarity (%)
70 - 80
60 - 70
50 - 60
40 - 50
30 - 40
20 - 30
10 - 20
0 - 10
2D Stress: 0.2
B
2D Stress: 0.24
C
2D Stress: 0.26
D
Bray-Curtis similarity (%)
0
20
40
60
80
7H
7E
21H
21E
50H
50E
**
**
*
**
**


A
PC2 (17%)
PC1 (33%)
PC3 (15%)
B
Scale for metric (Log)
5
4
3
2
1
0
-1
-2
Sequences
OTUs
Chao1
Coverage
Shannon
Evenness
C
Observed species (x100)
14
12
10
8
6
4
2
0
0 2 4 6 8 10 12
Sequences (x1000)


A
B
C
T198
P158
T456
N356
N174
N322
N697
P598
T362
P790
P77
P71
N678
T313
N360
N320
N495
T403
T381
P1452
T367
P135
T404
N424
T752
P59
T462
N233
N324
N304
I
IIa
IIb
III
IV
D
Other
Actinobacteria
Bacteroidetes
Firmicutes
Fusobacteria
Lentisphaerae
Proteobacteria
Spirochaetes
Tenericutes
k_Bacteria[a]
g_Trueperella
o_Bacteroidales[a]
o_Bacteroidales;g_[b]
f_Bacteroidaceae;g_[b]
g_5-7N15
g_Bacteroides
g_Paludibacter
g_Porphyromonas
f_Prevotellacea[a]
g_Prevotella
f_RF16;g_[b]
f_Rikenellaceae;g_[b]
f_S24-7;g_[b]
g_CF231[c]
g_[Prevotella][c,d]
f_p-2534-18B5;g_[b]
p_Firmicutes[a]
f_Planococcaceae[a]
g_Planomicrobium
g_Jeotgalicoccus
g_Staphylococcus
o_Gemellales;g_[b]
g_Gemella
f_Aerococcaceae;g_[b]
g_Alkalibacterium
g_Facklamia
g_Trichococcus
g_Streptococcus
o_Clostridiales[b]
o_Clostridiales;g_[b]
g_Clostridium
f_Lachnospiraceae[a]
f_Lachnospiraceae;g_[b]
g_Butyrivibrio
g_Coprococcus
g_Dorea
g_Epulopiscium
g_Roseburia
g_Peptostreptococcus
f_Ruminococcaceae[a]
f_Ruminococcaceae;g_[b]
g_Oscillospira
g_Ruminococcus
g_Phascolarctobacterium
f_[Mogibacteriaceae];g_[b,c]
g_Mogibacterium[c]
g_Helcococcus[c]
g_Parvimonas[c]
g_Peptoniphilus[c]
g_Sporanaerobacter[c]
g_ph2[c]
o_Fusobacteriales
g_Fusobacterium
f_Leptotrichiaceae;g_[b]
g_Campylobacter
g_Acinetobacter
g_Pseudomonas
g_Mycoplasma
g_Ureaplasma