

# Metagenomic signature of natural strongyle infection in susceptible and resistant horses

**Running head:** Parasitic disease and gut microbiome in horses

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## 31 Abstract

32 Gastrointestinal strongyles are a major threat to horses' health and welfare. Given that  
33 strongyles inhabit the same niche as the gut microbiota, they may interact with each other.  
34 These beneficial or detrimental interactions are unknown in horses and could partly explain  
35 contrasted susceptibility to infection between individuals. To address these questions, an  
36 experimental pasture trial with 20 worm-free female Welsh ponies (10 susceptible (S) and 10  
37 resistant (R) to parasite infection) was implemented for five months. Fecal egg counts (FEC),  
38 hematological and biochemical data, body weight and gut microbiota composition were  
39 studied in each individual after 0, 24, 43, 92 and 132 grazing days.

40  
41 The predicted R ponies exhibited lower FEC after 92 and 132 grazing days, and showed  
42 higher levels of circulating monocytes and eosinophils, while S ponies developed  
43 lymphocytosis by the end of the trial. Although the overall microbiota diversity remained  
44 similar between the two groups, R and S ponies exhibited sustained differential abundances in  
45 *Clostridium XIVa*, *Ruminococcus*, *Acetivibrio* and unclassified *Lachnospiraceae* at day 0.  
46 These bacteria may hence contribute to the intrinsic pony resistance towards strongyle  
47 infection. Moreover, *Paludibacter*, *Campylobacter*, *Bacillus*, *Pseudomonas*, *Clostridium III*,  
48 *Acetivibrio*, members of the unclassified *Eubacteriaceae* and *Ruminococcaceae* and fungi  
49 loads were increased in infected S ponies, suggesting that strongyle and fungi may contribute  
50 to each other's success in the ecological niche of the equine intestines. In contrast, butyrate-  
51 producing bacteria such as *Ruminococcus*, *Clostridium XIVa* and members of the  
52 *Lachnospiraceae* family decreased in S relative to R ponies. Additionally, these gut  
53 microbiota alterations induced changes in several immunological pathways in S ponies,  
54 including pathogen sensing, lipid metabolism, and activation of signal transduction that are  
55 critical for the regulation of immune system and energy homeostasis. These observations shed  
56 light on a putative implication of the gut microbiota in the intrinsic resistance to strongyle  
57 infection.

58  
59 Overall, this longitudinal study provides a foundation to better understand the mechanisms  
60 that underpin the relationship between host susceptibility to strongyle infection, immune  
61 response and gut microbiota under natural conditions in horses and should contribute to the  
62 development of novel biomarkers of strongyle susceptibility and provide additional control  
63 options.

64  
65 **Keywords:** cyathostomin, fungi, gut microbiome, immunity, horse

## 66 67 1. Introduction

68 Grazing horses are infected by a complex community of parasitic helminths, mainly strongyles  
69 (Bucknell et al., 1995; Corning, 2009; Kuzmina et al., 2016; Ogbourne, 1976). Like other  
70 worms, strongyles have a direct life cycle in which they can survive outside of its host in  
71 pastures as well as in the horse's intestines (Taylor et al., 2007). Infective larvae (L3 stage)  
72 are usually ingested and migrate to their preferred niche in the small or large intestine. After  
73 two molts, they will eventually become sexually mature adults and will lay eggs that are  
74 passed onto the pasture in the feces (Taylor et al., 2007).

75  
76 Equine strongyle species are classified into *Strongylinae* and *Cyathostominae*, which differ,  
77 among other criteria, by their respective size (Lichtenfels et al., 2008). *Strongylus vulgaris* is  
78 the most pathogenic of the large strongyles as a result of the intestinal infarction that larval  
79 stages can cause during their migration. Its prevalence has been drastically reduced since the  
80 release of modern anthelmintics (Nielsen et al., 2012). On the one hand, nearly all horses are

81 infected by small strongyles or cyathostomins throughout the world (Bucknell et al., 1995;  
82 Lyons et al., 1999; Ogbourne, 1976). Compared to *S. vulgaris*, small strongyles are  
83 responsible for milder symptoms, including weight loss or poor hair condition (Love et al.,  
84 1999). Infections are more common in immature animals meaning there is likely an immune  
85 component to infection susceptibility (Lyons et al., 1999). Furthermore, larval stages encyst  
86 into the colonic mucosa as part of their life cycle where millions can remain for years (Love et  
87 al., 1999; Matthews, 2014). Cyathostomin larvae encyst mostly in the autumn and winter in  
88 temperate zones of the northern hemisphere, accounting for up to 90% of the total worm  
89 burden (Matthews, 2014). The massive emergence of these larvae results in larval  
90 cyathostomiasis, which is characterized by abdominal pain and diarrhea (Love et al., 1999).  
91 Treatment failure in this case can result in the death of horses in at least a third of cases (Giles  
92 et al., 1985), underscoring the need for efficient anthelmintic compounds.

93  
94 Drug inefficacy reports have accumulated worldwide over the recent years and resistant  
95 isolates are now to be found in Europe (Geurden et al., 2014; Sallé et al., 2017), America  
96 (Smith et al., 2015) and Oceania (Scott et al., 2015). Additional control strategies are  
97 therefore required to alleviate selection pressure put on cyathostomin populations by  
98 anthelmintic treatments. One of the possible approaches is to exploit the over dispersion of  
99 strongyle infection in a herd to treat the only highly infected horses (Lester and Matthews,  
100 2014). Indeed, it has been estimated that 80% of the total worm burden is produced by 20% of  
101 horses (Wood et al., 2012) and that 21% of the inter-individual variation had a heritable  
102 component (Kornaś et al., 2015). However, the factors underpinning this phenotypic contrast  
103 (Lester et al., 2013; Sallé et al., 2015) still remain unclear.

104  
105 Equine strongyles are in close contact with a large community of microorganisms in the host  
106 intestines, estimated to reach a concentration of  $10^9$  microorganisms per gram of ingesta in  
107 the cecum alone (Mackie and Wilkins, 1988), spanning 108 genera (Mach et al., 2017;  
108 Steelman et al., 2012; Venable et al., 2017) and at least seven phyla (Costa et al., 2012, 2015;  
109 Mach et al., 2017; Shepherd et al., 2012; Weese et al., 2015). Bacterial populations differ  
110 greatly throughout the various compartments of the equine gastrointestinal tract (*e.g.*  
111 duodenum, jejunum, ileum and colon) due to differences in the gut pH, available energy  
112 sources, epithelial architecture of each region, oxygen levels and physiological roles (Costa et  
113 al., 2015; Ericsson et al., 2016). The gut microbiota promotes digestion and nutrient  
114 absorption for host energy production and provides folate (Sugahara et al., 2015), vitamin K<sub>2</sub>  
115 (Marley et al., 1986) and short chain fatty acids (SCFA) such as acetate, butyrate and  
116 propionate (Ericsson et al., 2016; Nedjadi et al., 2014). The gut microbiota also neutralizes  
117 drugs and carcinogens, modulates intestinal motility, protects the host from pathogens, and  
118 stimulates and matures the immune system and epithelial cells (reviewed by Nicholson et al.  
119 (2012)). Along with bacteria, both fungi and protozoa, comprise about 6-8% of the equine  
120 hindgut population (Dougal et al., 2013).

121  
122 The physical presence of helminths in the intestinal lumen can alter the gut microbiota  
123 activity and composition (Midha et al., 2017; Peachey et al., 2017). These perturbations have  
124 been demonstrated in various host-nematode relationships including mice infected by  
125 *Heligmosomoides polygyrus* (Reynolds et al., 2014b; Su et al., 2017), *Nippostrongylus*  
126 *brasiliensis* (Fricke et al., 2015), or *Hymenolepis diminuta* (McKenney et al., 2015),  
127 ruminants (El-Ashram and Suo, 2017; Li et al., 2011), and pigs (Li et al., 2012; Wu et al.,  
128 2012), although no universal modification has been observed across systems. It also remains  
129 unresolved whether nematode infection has a beneficial (Lee et al., 2014) or a detrimental  
130 (Houlden et al., 2015) impact on the gut microbiota diversity, richness and functions. The

131 mechanisms supporting these gut microbiota modifications also remain unclear and could  
132 arise indirectly because of the immune response helminths trigger in their host (Cattadori et  
133 al., 2016; Fricke et al., 2015; Reynolds et al., 2014b, 2015; Zaiss and Harris, 2016), such as  
134 regulatory T cell stimulation and lymphoid tissue modifications, alterations to the intestinal  
135 barrier (Boyett and Hsieh, 2014; Giacomini et al., 2016) or directly by the secretion of putative  
136 anti-bacterial compounds (Holm et al., 2015; Mcmurdie and Holmes, 2012) or modifications  
137 in the intestinal environment that supports them (D'Elia et al., 2009; Midha et al., 2017).

138

139 The putative interactions between equine strongyle infection and the gut microbiota and host  
140 physiology are unknown in horses. To address these questions, grazing ponies with extreme  
141 resistance or susceptibility toward natural strongyle infection were monitored over a five-  
142 month period. We aimed to provide insights into the host response and the gut microbiota  
143 composition associated with strongyle natural infection that should guide the development of  
144 new microbiota-based control strategies.

145

## 146 **2. Materials and methods**

### 147 **2.1. Animals selection**

148 Twenty female Welsh ponies (10 resistant, R, and 10 susceptible, S;  $5 \pm 1.3$  years old) from  
149 an experimental unit at the National Institute for Agricultural Research (INRA, UEPAO,  
150 Nouzilly, France) were selected from a set of 98 ponies monitored for fecal egg counts (FEC)  
151 since 2010. The FEC data were log-transformed to correct for over-dispersion and fitted a  
152 linear mixed model accounting for environmental fixed effects (month of sampling, year of  
153 sampling, time since last treatment, age at sampling). The individual was considered as a  
154 random variable to account for the intrinsic pony potential against strongyle infection.  
155 Estimated individual effects were centered and reduced to express each individual potential as  
156 a deviation from the mean on the logarithmic scale. Based on these values, two groups of the  
157 10 most extreme ponies balanced for age were selected resulting in a resistant R group (mean  
158 individual effect with -1.18 phenotypic deviation from the mean and average age of 5.6 years)  
159 and a susceptible S group (mean individual effect with +1.45 phenotypic deviation from the  
160 mean and average age of 4.7 years). At inclusion, the median FEC was 800 eggs/g for the S  
161 group, whereas the median egg counts /g feces was 0 for the R ponies (Figure S1A, S1B and  
162 S1C).

163

164 As it has been previously established that gut microbiota profiles might be shaped by host  
165 genetics (Goodrich et al., 2014; Lozupone et al., 2012), this information was considered to  
166 understand the individual variance underlying microbiota composition. The kinship2 R  
167 package was used to create the genetic relationship matrix to estimate the genetic parameters  
168 and predicts breeding values between every considered pony. Both pedigree tree and  
169 correlation structure matrix are depicted in Figure S1D and Figure S1E, respectively.

170

171 All the procedures were conducted according to the guidelines for the care and use of  
172 experimental animals established by the French Ministry of Teaching and Research and the  
173 regional Val de Loire Ethics Committee (CEEA VdL, no 19). The protocol was registered  
174 under the number 2015021210238289\_v4 in the experimental installations with the permit  
175 number: C371753. All the protocols were conducted in accordance with EEC regulation (n°  
176 2010/63/UE) governing the care and use of laboratory animals and effective in France since  
177 the 1<sup>st</sup> of January 2013.

178

### 179 **2.2. Longitudinal monitoring and sampling**

180 The 20 Welsh ponies were treated with moxidectin and praziquantel (Equest Pramox®,  
181 Zoetis, Paris, France, 400 µg/kg of body weight of moxidectin and 2,5 mg/kg of praziquantel)  
182 in March 2015 to clear any patent and pre-patent infection and kept indoors for 3 months until  
183 the end of the moxidectin remanence period. They were maintained under natural light  
184 conditions in a 240 m<sup>2</sup> pen with slatted floors, which precluded further nematode infections  
185 until they were moved to the experimental pasture. During housing, animals were fed with  
186 hay *ad libitum* and 600 g concentrate per animal per day. The concentrate (Tellus Thivat  
187 Nutrition Animale Propriétaire, Saint Germain de Salles, France) consisted of barley (150  
188 g/kg), oat bran (162 g/kg), wheat straw (184.7 g/kg), oats (200 g/kg), alfalfa (121.7 g/kg),  
189 sugar beet pulp (50 g/kg), molasses (30 g/kg), salt (7.3 g/kg), carbonate Ca (5.5 g/kg) and a  
190 mineral and vitamin mix (2 g/kg), on an as-fed basis. The mineral and vitamin mix contained  
191 Ca (28.5%), P (1.6%), Na (5.6%), vitamin A (500,000 IU), vitamin D<sub>3</sub> (125,000 IU), vitamin  
192 E (1,500 IU), cobalt carbonate (42 mg/kg), cupric sulfate (500 mg/kg), calcium iodate (10  
193 mg/kg), iron sulfate (1,000 mg/kg), manganese sulfate (5,800 mg/kg), sodium selenite (16  
194 mg/kg), and zinc sulfate (7,500 mg/kg) on an as-fed basis.

195  
196 Grazing started by the end of a 3-month moxidectin remanence period (mid-June 2015).  
197 Ponies grazed from mid-June to the end of October 2015 at the Nouzilly experimental station  
198 (France). The experimental pasture (7.44 ha) consisted of tall fescue, *Festuca arundinacea*;  
199 timothy-grass, *Phleum pratense*, meadow-grass, *Poa abbreviata*; soft-grass, *Holcus lanatus*;  
200 and cocksfoot, *Dactylis glomerata*). During all phases of the experimental period, ponies were  
201 provided *ad libitum* access to water.

202  
203 Moxidectin is known to have moderate efficacy against encysted cyathostomin larvae  
204 (Reinemeyer et al., 2015; Xiao et al., 1994). Therefore, residual egg excretion can occur at the  
205 end of the remanence period, as a result of encysted larvae completing their development into  
206 adults. To eliminate this residual excretion and to avoid any interference with strongyle  
207 infection at pasture, a treatment targeting the only luminal immature and adult stages  
208 (pyrantel embonate; Strongid® paste, Zoetis, Paris, France; single oral dose of 1.36 mg  
209 pyrantel base per Kg of body weight) was implemented at day 30.

210  
211 Every pony was subjected to a longitudinal monitoring of fecal strongyle egg excretion and  
212 microbiota was performed monthly, *e.g.* 0, 24, 43, 92 and 132 days after the onset of grazing  
213 (Figure 1).

214  
215 Fecal samples were collected from the rectum. Fecal aliquots for microbiota analysis were  
216 immediately snap-frozen in liquid nitrogen and stored at -80°C until DNA extraction, whereas  
217 fecal aliquots to measure the fecal egg counts were immediately sent to the laboratory.  
218 The pH in the feces was immediately determined after 10% fecal suspension (wt/vol) in saline  
219 solution (0.15 M NaCl solution).

220  
221 Blood samples were taken from each pony and collected in EDTA-K3-coated tubes (5 mL) to  
222 determine hematological parameters and heparin tubes (10 mL) to determine biochemical  
223 parameters. After clotting, the heparin tubes were centrifuged at 4000 rpm during 15 min and  
224 the harvested plasma was stored at -20°C until analysis. Additionally, blood collected in  
225 EDTA-K3-tubes was used to measure the different blood cells.

226  
227 For each individual, body weight and average daily weight gain were recorded until the end of  
228 the experiment.

229



230 None of the ponies received antibiotic therapy during the sampling period and diarrhea was  
231 not detected in any ponies.

232

### 233 **2.3. Fecal egg counts**

234 Fecal egg counts (eggs per gram of wet feces) was measured as a proxy for patent strongyle  
235 infection. FEC was carried out using a modified McMaster technique (Raynaud, 1970) on 5 g  
236 of feces diluted in 70 mL of NaCl solution with a density of 1.2 (sensitivity of 50 eggs/g). A  
237 Wilcoxon rank-sum test with Benjamini-Hochberg multiple test correction was used to  
238 determine whether there was a significant difference between groups across the experiment. A  
239  $q < 0.05$  was considered significant.

240

### 241 **2.4. Blood hematological and biochemical assays**

242 For blood hematological assays, blood were stirred at room temperature for good oxygenation  
243 during 15 min. Different blood cells were analyzed, including leucocytes (lymphocytes,  
244 monocytes, neutrophils, basophils and eosinophils), erythrocytes and different blood  
245 parameters such as hematocrit, mean corpuscular volume and the thrombocytes. The total  
246 blood cells were counted with a MS9-5 Hematology Counter® (digital automatic hematology  
247 analyzer, Melet Schloesing Laboratories, France).

248 The serum biochemical parameters (albumin, cholesterol, globin, glucose, phosphatase  
249 alkaline, total proteins and urea) were measured colorimetric method using Select-6V rings  
250 with the M-ScanII Biochemical analyzer (Melet Schloesing Laboratories, France).

251

252 Mixed-effects analysis of the variance (ANOVA) or Wilcoxon rank-sum tests were conducted  
253 for continuous variables fitting a normal or non-normal distribution respectively to delineate  
254 whether there was a significant difference between the average values of phenotype traits for  
255 the different groups, using a significance level of  $p < 0.05$ . Blood cell counts were corrected  
256 for the mild dehydration by fitting the hematocrit as a co-variable in the model.

257

### 258 **2.5. Weather data**

259 Daily precipitation and temperatures were recorded at a meteorological station located 14 km  
260 from the experimental area.

261

### 262 **2.6. Pasture contamination**

263 Pasture contamination was assessed before the entry to the pasture and during the experiment.  
264 The number of infective larvae (L3) per kg of dry herbage was measured by 100 x 4 random  
265 sampling of grass on pasture following previously described method (Gruner and Raynaud,  
266 1980). First, 600 g of grass were mixed with 20 mL of neutral pH soap diluted in 5L of tap  
267 water to allow larvae migration. This solution was subsequently left at room temperature prior  
268 to washing for larval recovery. Second, the washed material was passed through a coarse  
269 mesh sieve (20 cm of diameter) and collected in a container, then passed through both a 125  
270  $\mu\text{m}$  mesh sieve and a 20  $\mu\text{m}$  mesh sieve. Third, the solution of the filtered material was split  
271 into four 10 mL glass tubes before centrifugation at 2500 rpm for 5 min. Water was soaked  
272 and replaced by a dense solution (NaCl, density 1.18-1.2). Lastly, a cover slip was added on  
273 the top of each. Tubes were subsequently centrifuged gently at 1500 rpm for 8 min to allow  
274 larval material adhere onto the cover slip, before further examination under an optical  
275 microscope. This step was performed four times.

276

### 277 **2.7. Pasture chemical analysis**

278 Hay was sampled at day 0 via grab samples from multiple depths into a bale and then  
279 composited. Herbage samples were collected from three randomly selected zones in the

280 experimental pasture at days 24, 43, 92 and 132. At each sampling time, 10 hand-plucked  
281 samples simulating « bites » were taken from pasture. All samples were frozen until chemical  
282 analyses. Chemical compositions of samples were determined according to the “Association  
283 Française de Normalisation” (AFNOR) procedures: NF V18-109 (AFNOR, 1982) for dry  
284 matter (DM); NF V18-101 (AFNOR, 1977b) for crude ash; NF V18-100 (AFNOR, 1977a) for  
285 crude proteins; NF V03-040 (AFNOR, 1993) for crude fiber; NF V18-117 (AFNOR, 1977a)  
286 for crude fat; NF V18-122 (AFNOR, 1997) for neutral detergent fiber (NDF), acid detergent  
287 fiber (ADF) and acid detergent lignin (ADL). All of them were assayed with a heat stable  
288 amylase and expressed with exclusive of residual ash according to the method of Van Soest et  
289 al. (1991). Non-fiber carbohydrate (NFC), also called neutral detergent soluble carbohydrate  
290 (NDCS) were obtained by calculations:  $NFC = 1000 - CP - CF - Mm - NDF$ .

291

## 292 **2.8. Microorganisms DNA extraction from feces samples**

293 Total DNA was extracted from aliquots of frozen fecal samples (200 mg; 100 samples at  
294 different time points from 20 ponies), using E.Z.N.A.® Stool DNA Kit (Omega Bio-Tek,  
295 Norcross, Georgia, USA). The DNA extraction protocol was carried out according to the  
296 manufacturer’s instructions (Omega- Bio-Tek, Norcross, Georgia, USA).

297

## 298 **2.9. V3–V4 16S rRNA gene amplification**

299 The V3-V4 hyper-variable regions of the 16S rDNA gene were amplified with two rounds of  
300 PCR using the forward primer (5’-  
301 CTTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG-3’) and the reverse  
302 primer (5’- GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT-3’)   
303 modified in order to include Illumina adapters and barcode sequences which allow for  
304 directional sequencing. The first round of amplification was performed in triplicate in a total  
305 volume of 50 µL containing 10 ng of DNA, 2.5 units of a DNA-free Taq DNA Polymerase  
306 and 10X Taq DNA polymerase buffer (MTP Taq DNA Polymerase, Sigma). Subsequently, 10  
307 mM of dNTP mixture (Euromedex, Souffelweyersheim, France), 20 mM of each primer  
308 (Sigma, Lezennes, France) and Nuclease-free water (Ambion, Thermo Fisher Scientific,  
309 Waltham, USA) were added. Ultrapure Taq DNA polymerase, ultrapure reagents, and plastic  
310 were selected in order to be DNA-free. The thermal cycle consisted of an initial denaturation  
311 step (1 min at 94°C), followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min  
312 at 65°C) and 1 min of extension at 72°C. The final extension step was performed for 10 min  
313 at 72°C. Amplicons were then purified using magnetic beads (Clean PCR system, CleanNA,  
314 Alphen an den Rijn, The Netherlands) as follows: beads/PCR reactional volume ratio of 0.8X  
315 and final elution volume of 32 µL using Elution Buffer EB (Qiagen). The concentrations of  
316 the purified amplicons were checked using a NanoDrop 8000 spectrophotometer (Thermo  
317 Fisher Scientific, Waltham, USA).

318

319 Sample multiplexing was performed thanks to 6 bp unique indexes, which were added during  
320 the second PCR step at the same time as the second part of the P5/P7 adapters used for the  
321 sequencing step on the Illumina MiSeq flow cells with the forward primer (5’-  
322 AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGAC-3’) and reverse  
323 primer (5’-  
324 CAAGCAGAAGACGGCATAACGAGATNNNNNNGTACTGGAGTTCAGACGTGT-3’).  
325 This second PCR step was performed using 10 ng of purified amplicons from the first PCR  
326 and adding 2.5 units of a DNA-free Taq DNA Polymerase and 10X MTP TaqDNA  
327 polymerase buffer (Sigma). The buffer was complemented with 10 mM of dNTP mixture  
328 (Euromedex), 20 mM of each primer (Eurogentec, HPLC grade) and Nuclease-free water  
329 (Ambion, Life Technologies) up to a final volume of 50 µL. The PCR reaction was carried

330 out as follows: an initial denaturation step (94°C for 1 min), 12 cycles of amplification (94°C  
331 for 1 min, 65°C for 1 min and 72°C for 1 min) and a final extension step at 72°C for 10 min.  
332 Amplicons were purified as described for the first PCR round. The concentration of the  
333 purified amplicons was measured using Nanodrop 8000 spectrophotometer (Thermo  
334 Scientific) and the quality of a set of amplicons (12 samples per sequencing run) was checked  
335 using DNA 7500 chips onto a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA,  
336 USA). All libraries were pooled at equimolar concentration in order to generate equivalent  
337 number of raw reads with each library. The final pool had a diluted concentration of 5 nM to  
338 20 nM and was used for sequencing. Amplicon libraries were mixed with 15% PhiX control  
339 according to the Illumina's protocol. Details on sequencing, PhiX control and FastQ files  
340 generation are specified elsewhere (Luch et al., 2015). For this study, one sequencing run  
341 was performed using MiSeq 500 cycle reagent kit v2 (2x250 output; Illumina, USA).

342

## 343 **2.10. Sequencing Data Preprocessing**

344 Sequences were processed using the version 1.9.0 of the Quantitative Insights Into Microbial  
345 Ecology (QIIME) pipeline (Caporaso et al., 2010; Rideout et al., 2014) and by choosing the  
346 open-reference operational taxonomic units (OTU) calling approach (Rideout et al., 2014).  
347 First, forward and reverse paired-end sequence reads were collapsed into a single continuous  
348 sequence according to the 'fastq-join' option of the 'join\_paired\_ends.py' command in  
349 QIIME. The fastq-join function allowed a maximum difference within overlap region of 8%,  
350 a minimum overlap setting of 6 bp and a maximum overlap setting of 60 bp. The reads that  
351 did not overlap (~20% of the total) were removed from the analysis. The retained sequences  
352 were then quality filtered. De-multiplexing, primer removal and quality filtering processes  
353 were performed using the 'split\_libraries\_fastq.py' command in QIIME. We applied a default  
354 base call Phred threshold of 20, allowing maximum three low-quality base calls before  
355 truncating a read, including only reads with >75% consecutive high-quality base calls, and  
356 excluding reads with ambiguous (N) base calls (Navas-Molina et al., 2013).  
357 Subsequently, the sequences were clustered into OTUs against the GreenGenes database  
358 (release 2013-08: gg\_13\_8\_otus) (DeSantis et al., 2006) by using the uclust (Edgar, 2010)  
359 method at a 97% similarity cutoff. The filtering of chimeric OTUs was performed by using  
360 Usearch version 6.1 (Edgar et al., 2011) against the GreenGenes reference alignment  
361 (DeSantis et al., 2006). A phylogenetic tree was generated from the filtered alignment using  
362 FastTree (Price et al., 2010). Singletons were discarded from the dataset to minimize the  
363 effect of spurious, low abundance sequences using the 'filter\_otus\_from\_otu\_table.py' script  
364 in QIIME. To confirm the annotation, the resulting OTU representative sequences were then  
365 searched against the Ribosomal Database Project naïve Bayesian classifier (RDP 10 database,  
366 version 6 (Cole et al., 2009) database, using the online program SEQMATCH  
367 ([http://rdp.cme.msu.edu/seqmatch/seqmatch\\_intro.jsp](http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp)). Finally, consensus taxonomy was  
368 provided for each OTU based on the taxonomic assignment of individual reads using  
369 GreenGenes and RDP databases. Using OTU abundance and the corresponding taxonomic  
370 classifications, feature abundance matrices were calculated at different taxonomic levels,  
371 representing OTUs and taxa abundance per sample. The "Phyloseq" (Mcmurdie and Holmes,  
372 2012) and "Vegan" (Dixon, 2003) R package were used for the detailed downstream analysis  
373 on abundance matrix.

374

375 In the end, a total of 8,010,052 paired-end 250 bp reads were obtained, 6,428,315 of which  
376 were retained as high-quality sequences (Table S1). On average, a total of 58,015 sequences  
377 per sample were obtained in the study, with a mean length of  $441 \pm 15$  bp. These sequences  
378 were clustered into 15,784 OTUs using the reference-based OTU-picking process (Table S2).  
379 Among them, 12,069 were classified taxonomically down to the genus level (Table S2). OTU



380 counts per sample and OTU taxonomical assignments are available in Table S2. We filtered  
381 out unclassified taxa from the analysis because the main goal of the current study was to  
382 identify specific taxa related to host susceptibility to strongyle infection.  
383

384 The  $\alpha$ -diversity indexes (observed species richness, Chao1(Chao, 1984) and Shannon  
385 (Shannon, 1997)) were calculated using the “Phyloseq” R package (McMurdie and Holmes,  
386 2012). Shannon’s diversity index is a composite measure of richness (number of OTUs  
387 present) and evenness (relative abundance of OTUs). The nonparametric Wilcoxon rank-sum  
388 test was used to compare  $\alpha$ -diversity indexes between groups.  
389

390 Relative abundance normalization was applied, which divides raw counts from a particular  
391 sample by the total number of reads in each sample.  
392

393 To estimate  $\beta$ -diversity, un-weighted and weighted UniFrac distances were calculated from  
394 the OTU and genera abundance tables, and used in principal coordinates analysis (PCoA),  
395 correspondence analysis (CA), and non-parametric multidimensional scaling (NMDS) with  
396 the “Phyloseq” R package. The Permutational Multivariate Analysis of Variance  
397 (PERMANOVA), on un-weighted and weighted UniFrac distance matrices were applied  
398 through the Adonis function from “Vegan” R package to test for groups effect. In addition to  
399 multivariate analysis, we used the analysis of similarities (ANOSIM) to test for intragroup  
400 dispersion. ANOSIM is a permutation-based test where the null hypothesis states that within-  
401 group distances are not significantly smaller than between-group distances. The test statistic  
402 ( $R$ ) can range from 1 to  $-1$ , with a value of 1 indicating that all samples within groups are  
403 more similar to each other than to any other samples from different groups.  $R$  is  $\approx 0$  when the  
404 null hypothesis is true, that distances within and between groups are the same on average.  
405

406 The Wilcoxon rank-sum test with Benjamini-Hochberg multiple test correction was used to  
407 determine the differentially abundant OTUs, phyla, families, and genera between groups. A  
408  $q \leq 0.25$  was considered significant. This threshold was employed in previous microbiome  
409 studies because allows compensation for the large number of microbial taxa and multiple  
410 comparison adjustment (Lim et al., 2017).  
411

412 This targeted locus study project has been deposited at DDBJ/EMBL/GenBank under the  
413 accession KBTQ01000000. The version described in this paper is the first version,  
414 KBTQ01000000. The bioproject described in this paper belongs to the BioProject  
415 PRJNA413884. The corresponding BioSamples accession numbers were SAMN07773451 to  
416 SAMN07773550.  
417

## 418 **2. 11. Functional metagenomic predictions**

419 The functional prediction for the 16S rRNA marker gene sequences was done using the  
420 phylogenetic investigation of communities by reconstruction of unobserved states  
421 (PICRUST) (Langille et al., 2013). After excluding the unknown OTUs from the GreenGenes  
422 reference database and normalizing by 16S rRNA gene copy number, functional  
423 metagenomes for each sample were predicted from the Kyoto Encyclopedia of Genes and  
424 Genomes (KEGG) catalogue and collapsed to a specified KEGG level. We used Wilcoxon  
425 rank-sum test with Benjamini-Hochberg multiple test correction to evaluate pathway-level  
426 enrichments between groups. A  $q \leq 0.05$  was considered as significant.  
427

## 428 **2.12. Network inference at the genus level**

429 Networks at the genus level were inferred between groups at different time points. In order to  
430 prevent the compositional effects bias typical of the classical correlations methods, we  
431 calculated the correlations among genera using the PCIT method, which identifies significant  
432 co-occurrence patterns through a data-driven methodology based on partial correlation and  
433 information theory as implemented in the PCIT algorithm (Reverter and Chan, 2008). Further  
434 details are depicted in Ramayo-Caldas et al. (2016). The genera with < 0.1% mean relative  
435 abundances were excluded to acquire the results for the taxa that met the statistical conditions  
436 for correlation estimations. Nodes in the network represent the genera and edges that connect  
437 these nodes represent correlations between genera. Based on correlation coefficient and *p*-  
438 values for correlation, we constructed co-occurrence networks. The cutoff of *p*-values was  
439 0.05. The cutoff of correlation coefficients was determined as  $r \geq |0.35|$ . Network properties  
440 were calculated with the NetworkAnalyzer plugin in Cytoscape. We used the “iGraph” R  
441 package to visualize the network. Strong and significant correlation between nodes ( $r \geq |0.60|$ )  
442 were represented with larger edge width in the network.  
443

### 444 **2.13. Real-time quantitative PCR (qPCR) analysis of bacterial, fungal and protozoan** 445 **loads**

446 Loads of protozoa, anaerobic fungi and bacteria in fecal samples were quantified using a  
447 QuantStudio 12K Flex real-time instrument (Thermo Fisher Scientific, Waltham, USA).  
448 Primers for real-time amplification of ciliates, anaerobic fungi and bacteria have already been  
449 described in Mach et al. (2017) and have been purchased from Eurofins Genomics  
450 (Ebersberg, Germany).

451 Amplified fragments of the target genes were used and diluted 10-fold in series to produce  
452 seven standards, ranging from  $2.25 \times 10^7$  to  $2.25 \times 10^{13}$  copies per  $\mu\text{g}$  of DNA for bacteria  
453 and protozoa and ranging from  $3.70 \times 10^6$  to  $3.70 \times 10^{12}$  copies per  $\mu\text{g}$  of DNA for ciliates and  
454 fungi. Each reaction contained, in a final volume of 20  $\mu\text{L}$ , 10  $\mu\text{L}$  of Sybergreen Mix (Power  
455 SYBR Green PCR Master Mix, ThermoFisher, Ullkirch-Graffenstaden, France), 0.6  $\mu\text{M}$  of  
456 each primer to final concentration of 300 mM, and 2  $\mu\text{L}$  of standard or DNA template at 0.5  
457 ng/ $\mu\text{L}$ . The primer concentration of anaerobic fungi was 200 mM and 150 mM for ciliate  
458 protozoa. The DNA template was 0.5 ng/ $\mu\text{L}$ . In all cases, the thermal protocol for qPCR  
459 amplification and detection included an initial step of denaturation of 10 min (95 °C),  
460 followed by 40 amplification cycles [15 s at 95 °C; 60 s at 60 °C]. After each run, melting  
461 curves between 60 and 95 °C were evaluated to confirm the absence of unspecific signals. For  
462 each sample and each gene, qPCR runs were performed in triplicate. The standard curve  
463 obtained the reference genomic fragment was used to calculate the number of copies of  
464 bacteria, protozoa or anaerobic fungi in feces. Taking into account the molecular mass of  
465 nucleotides and fragment length, we calculated the copy number as follows: mass in Daltons  
466 (g/mol) = (size of double-stranded [ds] product in base pairs [bp]) (330 Da  $\times$  2 nucleotides  
467 [nt]/bp). Wilcoxon rank-sum tests were calculated for all possible group combinations. A *p*  
468  $< 0.05$  was considered significant.  
469

### 470 **3. Results**

471 The effects of natural strongyle infection on gut microbiota composition and host phenotypic  
472 variables were determined in ten resistant and ten susceptible grazing ponies over a five-  
473 month grazing season (Figure 1). Metagenomic, parasitological, hematological and  
474 biochemical measures were performed at five time points (Figure S2), hereafter referred to as  
475 days after the onset of the grazing season or grazing days (gd).  
476  
477  
478

479 **3.1. A mixture of abiotic and biotic environmental stressors during the 43-day**  
480 **transitioning period induced shifts in immunological and microbiota profiles**

481 Measured FEC demonstrated a residual egg excretion in one susceptible individual at day 0  
482 and in two susceptible ponies after 24 gd (Figure 2A and 2B). This was due to the known  
483 imperfect efficacy of moxidectin against encysted stages of strongyle. Because we were  
484 interested in studying the effects of strongyle exposure on the gut microbiota, a pyrantel  
485 treatment was administered after 30 gd to reset luminal parasite stages to zero in every pony.  
486 This treatment has a short-lived effect and resulted in negative FECs at 43 gd (Figure 2A and  
487 2B). Therefore, the 0-43 gd period was considered as a transitioning period resulting in both,  
488 mild parasite exposure and changes in environmental conditions.  
489

490 Indeed, a heat wave took place over the first 43 days of the trial resulting in almost no rainfall  
491 (0.2 mm) and high temperatures peaking at 37.7 °C (Figure S3A). This situation ultimately led  
492 to grass senescence and lowered pasture quality (Figure S3B, Table S3). Mild dehydration  
493 also occurred in ponies as supported by a constant rise in measured hematocrits from 32.33 to  
494 39.63 % during the first 43 gd (Figure 2C). To account for this, blood cell counts were  
495 corrected by hematocrit through time. Recorded hematological data showed that ponies were  
496 neither anemic nor thrombocytopenic throughout the experiment (Figure S4).  
497

498 Concomitantly to these challenging conditions, increased levels of some white blood cell  
499 populations were observed, namely eosinophils (2.75-fold increase,  $p = 5.98 \times 10^{-13}$ , Figure  
500 2D), and monocytes (1.64-fold increase,  $p = 2.94 \times 10^{-7}$ ; Figure 2E) during the first 43 gd.  
501 Notably, R ponies had significantly higher levels of these immunological cells from 24 to 43  
502 gd relative to S ponies. Grass samples analysis did not evidence any infective larvae until 43  
503 gd.  
504

505 16S rRNA gene sequencing was used to profile the fecal microbiota of the S and R ponies  
506 across time. Despite the distinctive susceptibility to parasite infection, the overall community  
507 structure showed no statistically significance difference in un-weighted (presence/absence)  
508 Unifrac analysis (PERMANOVA,  $p > 0.05$ ) or abundance-weighted analysis  
509 (PERMANOVA,  $p > 0.05$ ) during the first 43 days of the experiment. Measures of  $\alpha$ -diversity  
510 (Chao1 richness, observed species and Shannon diversity index) were not significantly  
511 different between the two groups (Wilcoxon rank-sum test,  $p > 0.05$ ). Although the overall  
512 gut microbial community of S ponies during the first 43 days of experiment was almost  
513 indistinguishable from those of R ponies, we next evaluated the association between the  
514 abundance of specific gut microbial taxa and the susceptibility to parasite infection. Statistical  
515 differences were not evident at phylum and family levels (Figure 3A). Only seven genera  
516 were statistically significant at a nominal  $p$  value  $< 0.05$  (Wilcoxon rank-sum test; Figure 3B  
517 and 3C, Table S4) at day 0, including *Acetivibrio*, *Clostridium XIVa*, *Ruminococcus*, *Hallella*,  
518 *Syntrophococcus*, unclassified *Lachnospiracea*, and *Lachnospiracea incertae sedis*, whereas a  
519 total of 20 taxa had differential presence at a nominal  $p$  value  $< 0.05$  at day 24 (Wilcoxon  
520 rank-sum test; Figure 3C, Table S4). Specifically, species belonging to the *Blautia* and  
521 *Paraprevotella* genera were relatively more abundant ( $p < 0.05$ ) in the S group compared to  
522 the R group (Table S4) at 24 gd. Conversely, other genera belonging to the Clostridiales order  
523 (e.g. *Clostridium sensu stricto*, *Clostridium IV*, *Clostridium III*, *Syntrophococcus*,  
524 *Oribacterium*, *Dehalobacterium*, *Mogibacterium*, *Acetivibrio*, *Sporobacter* and unclassified  
525 *Ruminococcaceae*) and Bacteroidales (e.g. *Hallella*, *Rikenella*, *Paraprevotella* and  
526 *Bacteroides*) were more abundant in the R group than in the S group at 24 gd (Figure 3B).  
527 These shifts in microbial taxa were not associated with modifications in functional gene  
528 abundances, as predicted from 16S rRNA data analysis ( $q > 0.05$ ; Table S5).

529 **3.2. The predicted levels of resistance matched observed fecal egg counts between**  
530 **resistant and susceptible ponies and shifts in the gut microbiota composition.**

531 By the end of the transition period, *e.g.* after 43gd, ponies were considered adapted to their  
532 new environmental conditions. The resetting of luminal stages with pyrantel resulted in  
533 negative FEC across ponies at 43 days after the onset of grazing (Figure 2A and 2B). After  
534 the patent infection, parasite egg excretion was significantly higher in the susceptible group  
535 after 92 (235 eggs in S and 20 eggs in R ponies on average) and 132 gd (340 eggs in S and  
536 135 eggs in R ponies on average; Figure 2A).

537  
538 The ponies' body weights (Figure S5A) and average daily weight gains (Figure S5B) did not  
539 show significant differences between groups after natural parasite infection, and none of them  
540 displayed clinical symptoms like lethargy or diarrhea. However, strongyle exposure induced  
541 contrasted shifts in white blood cell populations between the two groups of ponies.  
542 Circulating monocyte levels were higher ( $p < 0.05$ ) in R in comparison to S ponies through  
543 the whole period (Figure 2E). But the opposite trend was found for circulating lymphocytes,  
544 which were significantly enriched in the white blood cells population in S ponies during  
545 parasite infection (Figure 2F). Among the white blood cell population, R ponies also  
546 presented higher levels of eosinophils at 132 gd ( $p < 0.05$ , figure 2D). Similarly, serum  
547 biochemical analyses revealed a mild elevation in albumin, cholesterol, the enzyme alkaline  
548 phosphatase and total proteins from 43 gd to the end of the experiment in both groups, as well  
549 as elevated levels of urea, in particular at 92 and 132 gd (Figure S6).

550  
551 Strongyle mediated alterations in microbiota diversity and structure were investigated  
552 between the two groups of ponies. As for the transition period, both groups of ponies  
553 displayed equivalent microbial species richness and alpha-diversity indexes (Wilcoxon rank-  
554 sum test,  $p > 0.05$ , Figure 4A). The diversity Chao1 and Shannon indexes were similar  
555 between the two groups through time (Wilcoxon rank-sum test,  $p > 0.05$ , Figure 4B). The  
556 UniFrac distance followed by PCoA (Figure 4C) showed no distinct clustering between  
557 samples from the S and the R group, which was indicative of, if at all, minor differences in  
558 microbiota composition between the two groups of ponies during parasite infection. Similarly,  
559 the correspondence analysis (Figure 4D) and the Jaccard network (Figure 4E) analyses  
560 suggested that the overall gut microbiota composition was largely similar between S and R  
561 ponies at each time point.

562  
563 However, changes in relative abundance of certain genera concomitantly arose with strongyle  
564 egg excretion at 92 gd. For example, *Paludibacter*, *Campylobacter*, *Bacillus*, *Pseudomonas*,  
565 *Clostridium III*, *Acetivibrio*, and members of the unclassified family *Eubacteriaceae* and  
566 *Ruminococcaceae* increased in S relative to R ponies ( $p < 0.05$  and  $q \leq 0.25$ ; Figure 3C).  
567 Moreover, the relative abundance of *Acetivibrio*, and *Clostridium III* highly correlated with  
568 FEC (Pearson correlation coefficient  $\rho > 0.60$ ). This genera enrichment was concomitant with  
569 depletion in *Ruminococcus*, *Clostridium XIVa* and members of the *Lachnospiraceae* family ( $p$   
570  $< 0.05$  and  $q \leq 0.25$ , Figure 3C). The complete list of differentially expressed genera and  $q$ -  
571 values is presented in Supplementary Table S4. These modifications in the gut bacterial  
572 community structure between S and R also resulted in functional modifications, as inferred  
573 from PICRUSt. Noteworthy, among the well-characterized bacterial functions, S ponies  
574 microbiota tended to show an enrichment ( $q < 0.10$ ) of the mineral absorption, protein  
575 digestion and absorption, as well as of some of the pathways related to cell motility (*e.g.*  
576 bacterial chemotaxis, bacterial motility proteins, flagellar assembly), lipid metabolism  
577 (sphingolipid metabolism), peroxisome, and signal transduction (phosphatidylinositol  
578 signaling system) among others compared to the R group at 92 gd (Table S5).



579 Because the magnitude and direction of changes for *Clostridium XIVa*, *Ruminococcus*,  
580 *Acetivibrio* and unclassified *Lachnospiraceae* observed at 92 gd between the two groups were  
581 already remarked at day 0, we evaluated the host genetics. Interestingly, 80% of the ponies  
582 from group R presented higher genetic relatedness (Figure S1E), whereas there was no  
583 evidence for high genetic relatedness between S individuals.

584  
585 The gut microbiota co-occurrence networks were marginally different between S and R  
586 ponies at 92 gd (Figure S7). The topological properties were calculated to describe the  
587 complex pattern of inter-relationships among nodes, and to distinguish differences in taxa  
588 correlations between these two groups of ponies (Table S6). The structural properties of  
589 the S network were slightly greater than the R network, indicating more connections and  
590 closer relationships of microbial taxa in the S group. Notably, S co-occurrence network  
591 displayed higher levels of betweenness centrality, which measures the number of shortest  
592 paths going through a given node, and higher degree levels, which describes the number of  
593 neighbors relative to R network. Nodes with the highest degree and betweenness centrality  
594 values were identified as key genera in the co-occurrence networks. The keystone genera in S  
595 were related to the Clostridiales order (e.g. *Clostridium IV*, *Roseburia*, *Nakamurella*) or  
596 Spirochaetales (e.g. *Treponema*), whereas R network keystone included members of  
597 Clostridiales order (e.g. *Clostridium XIV*, *Roseburia*) and Bacteroidales (e.g. *Alloprevotella*).

598  
599 To understand the modifications of the gut environment after the natural infection, feces pH  
600 measurements and the fungal, protozoan and bacterial loads in feces were investigated  
601 between groups of ponies. There were no differences in pH between the groups at any time  
602 point, although feces pH significantly decreased after the onset of patent strongyle infection  
603 (Figure S8A). Interestingly, anaerobic fungal loads were higher in the S than in the R group ( $p$   
604  $< 0.05$ ) with more than 0.5 log of difference at day 43 (Figure S8B), while protozoan loads  
605 were lower in S group than in the R group at the same time point (Figure S8C). Bacteria loads  
606 were constant throughout the experiment (Figure S8D).

### 607 608 **3.3. Gut microbiota composition and functions shifted immediately after natural** 609 **parasite infection**

610 Data of both groups were pooled together to assess the influence of natural strongyle infection  
611 (from day 43 to day 132) on gut microbiota composition, irrespective of the ponies' predicted  
612 susceptibility. Analysis of similarities tests demonstrated that microbiota at day 43 were  
613 highly dissimilar and significantly divergent from day 92 and day 132 ( $R=0.197$ ,  $p < 0.001$ ).  
614 Interestingly, species richness increased significantly at day 92 and remained high until the  
615 end of the experiment (Figure 5A). Similarly, PCoA, CA and Jaccard network demonstrated a  
616 high variability in the distribution of microbiota between day 43 and the other points across  
617 the natural parasite infection (Figure 4). Consequently, the most significant alterations at  
618 genus level were found at 92 gd relative to 43 gd, which included a decrease ( $q < 0.05$ ; Figure  
619 5B) in the relative abundances of members of the order Bacteroidales (e.g. *Alloprevotella*,  
620 *Petrimonas*, *Paludibacter*), Clostridiales (e.g. *Clostridium IV*, *Oscillibacter*, *Ethanoligenens*)  
621 and Proteobacteria (e.g. *Desulfovibrio*). On the other hand, the relative abundances of  
622 dominant genera such as *Clostridium XIVa*, *Fibrobacter*, *Ruminococcus*, *Treponema* and of  
623 the members of the as yet unclassified family *Lachnospiraceae* were significantly increased  
624 ( $q < 0.05$ , Figure 6B). The complete list of increased and decreased genera including  
625 direction, coefficient and  $q$ -values is presented in Supplementary Table S7. These temporal  
626 changes on gut microbiota between day 43 and day 132 correlated with an increase in  
627 strongyle egg counts and an improvement in pasture quality with less non-digestible  
628 carbohydrate components and more N and mineral content (Figure S2A).



629

630 These microbiota alterations had an effect on a broad range of biological functions. A total of  
631 94 pathways displayed significantly different abundance (50% related to metabolism)  
632 between 43 and 92 gd. Interestingly, bacterial invasion of epithelial cells was among the top  
633 enriched pathways at day 92 ( $q < 0.0003$ ; Table S8). Conversely, only 34 pathways were  
634 found to be different between 92 and 132 gd. Similarly, 50% of the different metabolic  
635 potentials were related to metabolism, including amino acid, energy, carbohydrate, lipid and  
636 xenobiotic metabolism.

637

#### 638 **4. Discussion**

639 While alternative control strategies are needed for a more sustainable control of horse  
640 strongyle infection, the factors contributing to the over-dispersed distribution of these  
641 parasites in their hosts remain poorly characterized (Debeffe et al., 2016; Kornaš et al., 2015;  
642 Wood et al., 2012). In their preferred niche, strongyles are surrounded by gut microbiota and  
643 reciprocal interactions between them are expected. Our study aimed to identify the  
644 consequences of parasite infection on the gut microbiota and host physiology under natural  
645 conditions and to seek for a metagenomics signature of strongyle infection in resistant and  
646 susceptible ponies. Notably, R and S ponies showed contrasted immune responses toward  
647 natural strongyle infection, and their gut microbiota displayed variations at the genus level.  
648 Additionally, we showed that besides the host susceptibility to strongyle infection, variations  
649 in gut microbiota occurred after the onset of strongyle egg excretion during, expanding earlier  
650 observations on the association between parasites and gut microbiota composition on other  
651 species (Aivelo and Norberg, 2017; Cooper et al., 2013; Ramanan et al., 2016; El-Ashram and  
652 Suo, 2017; Fricke et al., 2015; Houlden et al., 2015; Li et al., 2011, 2012; McKenney et al.,  
653 2015; Newbold et al., 2017; Osborne et al., 2015; Reynolds et al., 2014b; Su et al., 2017;  
654 Walk et al., 2010; Wu et al., 2012; Zaiss et al., 2015; Zaiss and Harris, 2016). Moreover, for  
655 the first time we reported the effect of intestinal parasites have on the gut microbiota in  
656 horses.

657

658 Under our experimental setting, the combined heat wave and the associated reduced pasture  
659 yield and quality induced a mixture of different stresses during the first 43 days of the  
660 experiment. Under these challenging climatic and nutritional conditions, R ponies displayed  
661 higher levels of eosinophils and monocytes from 24 to 43 gd in comparison to the S ponies.  
662 Eosinophilia has been reported in experimentally challenged horses (Murphy and Love,  
663 1997). However, no larvae were recovered from pasture samples in our study suggesting mild,  
664 if any, contamination, in line with the drought conditions that are detrimental to their survival  
665 (Nielsen et al., 2007). In addition, the mild residual egg excretion observed after 24 grazing  
666 days occurred in only two susceptible ponies, which cannot explain the increased eosinophils  
667 in the R group. Therefore, this differential profile in immune cell populations may result from  
668 the stressful environmental conditions as already reported elsewhere (Collier et al., 2008).  
669 Remarkably, the contrast between R and S ponies was also found while comparing their  
670 respective gut microbiota composition. Although the constituent phyla and genera within the  
671 gut microbiota of R and S ponies were congruent with other studies based on horses (Costa et  
672 al., 2012, 2015; Mach et al., 2017; Shepherd et al., 2012; Steelman et al., 2012; Venable et al.,  
673 2017; Weese et al., 2015), *e.g.* members of Firmicutes, Bacteroidetes, Spirochaetes and  
674 Fibrobacteres predominating, R ponies presented an increase of several Clostridiales and  
675 Bacteroides species at day 24, whereas only species related to *Blautia* and *Paraprevotella*  
676 genera were relatively more abundant in the S. Indisputably, individuals with different  
677 susceptibility to parasite infection adapt to environmental stress in different ways. Whether  
678 the gut microbiota differences are ascribed to divergence in the immune response or are due to

679 impaired nutrient availability remains unclear, but micronutrient deficiencies might dictate  
680 microbial-microbial as well as microbial-environmental interactions through the gut (Mach  
681 and Clark, 2017).

682  
683 The most interesting findings brought forward by this work were obtained during the natural  
684 strongyle infection, from day 43 to 132 of the experiment (Figure 6A). Congruent with our  
685 initial hypothesis, the observed FEC matched the predicted resistance levels throughout the  
686 trial, hence supporting the high reproducibility of FEC (Debeffe et al., 2016; Scheuerle et al.,  
687 2016) and the feasibility to select for more resistant individuals (Kornaš et al., 2015).  
688 Observed FEC were associated with differing immune responses characterized by higher  
689 eosinophil and monocyte counts in R ponies and increased levels of circulating  
690 lymphocytes in S ponies during the infection, suggesting a direct functional relationship  
691 between parasite infection and immune response (Howitt et al., 2016). The induction of  
692 lymphocytes in S animals could have been a way to neutralize invading L3 and facilitate  
693 repair and turnover of injury tissue. In R, the eosinophilia, which is a well-recognized  
694 immune response to strongyle infection in horses (Reynolds et al., 2012) and plays an  
695 important role in destroying parasites by acting as a killer cell against larvae (Herbert et al.,  
696 2000), likely played a role in parasitic infection resistance (Lyons et al., 2000). However, the  
697 interpretation of the relationship between strongyle infection and immune profile after  
698 natural parasite infection in our study required accounting for likely confounding effects of  
699 yearly variation in environmental conditions (e.g. extreme heat temperatures and reduction  
700 of pasture quality).

701  
702 Despite the limited infection level monitored throughout the trial, FEC differences between S  
703 and R ponies from day 92 to the end of the experiment were also reflected in the composition  
704 and function of their gut microbiota. Strongyle natural infection in S ponies coincided with an  
705 increase in pathobionts, such as *Pseudomonas*, *Campylobacter*, and *Bacillus*, anaerobic fungi  
706 loads as well as a reduction of commensal genera such as *Clostridium XIVa*, *Ruminococcus*,  
707 and unclassified *Lachnospiraceae* (Figure 6B). Firmicutes belonging to the  
708 families *Ruminococcaceae* (also referred as clostridial cluster IV)  
709 and *Lachnospiraceae* (also referred as clostridial cluster XIVa) comprise most of the  
710 butyrate-producing bacteria in the human gut (Geirnaert et al., 2017). Due to butyrate's  
711 anti-inflammatory properties, it might be suggested that the higher helminth infection in  
712 susceptible ponies alters the abundance of butyrate-producing bacteria which therefore  
713 modulates the gut inflammation (Li et al., 2016). Additionally, the reduced abundance of  
714 *Clostridium XIV* in S ponies could have had functional importance for immune gardening  
715 against the overgrowth of pathobionts such as *Pseudomonas* and *Campylobacter*. *Clostridium*  
716 *XIVa* is one of the main methane-producing bacteria that has been implicated in the  
717 maintenance of mucosal homeostasis and protection against intestinal inflammatory diseases  
718 through the promotion of T<sub>reg</sub> cell accumulation (Atarashi et al., 2011). In addition, it was  
719 notable that a significant number of microbiota functional pathways in S ponies reflected  
720 immunological mechanisms, including pathogen sensing, changes in lipids, and activation of  
721 signal transduction pathways inside of the cell that are critical for regulation of immune  
722 system and maintaining energy homeostasis (Vassart and Costagliola, 2011).

723  
724 Altogether, our data suggest that parasitic infections in S ponies increased the risk of  
725 subsequent pathobionts overgrowth in the gut by reducing butyrate producing bacteria that  
726 may play an important role in mediating interactions between the host immune system and  
727 intestinal parasites (Oliver et al., 2003). Although the mechanisms of the interactions between  
728 these specific gut genera and the host require further elucidation, our findings suggest that

729 specific modulations of the gut microbiota might be an effective strategy for managing  
730 parasite infections in horse.

731  
732 The concomitant increase in fungi abundance in S ponies during the natural parasite infection,  
733 could have had an important pathophysiological consequence, especially in host fiber  
734 metabolism (Dougal et al., 2013) and inflammation (Noverr and Huffnagle, 2004). Fungi have  
735 been found to play a significant role in degrading cellulose and other plant fibers making  
736 them more accessible to bacteria (Güiris et al., 2010; Leng et al., 2011). This results in a  
737 consequent increase in SCFA such as acetate, butyrate and propionate (Ericsson et al., 2016;  
738 Nedjadi et al., 2014) which contribute to 60-70% of energy for horses (Al Jassim and  
739 Andrews, 2009). Consequently, the fungi-mediated increase in SCFA of microbial origin  
740 could have compensated for the decreased abundance of *Ruminococcus* in S ponies. The key  
741 contribution of *Treponema*, a non-pathogenic carbohydrate metabolizer (Han et al.,  
742 2011), to the co-occurrence network of S ponies at day 92 also suggests that compensatory  
743 mechanisms were induced to degrade fiber and supply the host with micro and  
744 macronutrients. More detailed nutritional determinations are needed to resolve this issue.  
745 Fungi can also secrete inflammatory substances that have been shown to reduce T<sub>reg</sub> cell  
746 responses and may elicit a Th17 innate immune responses and pro-inflammatory cytokines  
747 secretion (reviewed by Underhill and Iliev (2014)). In line with the seemingly symbiotic  
748 relationship between *H. polygyrus* and bacteria from the family *Lactobacillaceae* in mice  
749 (Reynolds et al., 2015), it could be speculated that strongyle and fungi may contribute to each  
750 other's success in the ecological niche of the equine intestines.

751  
752 Interestingly, R and S ponies exhibited some contrasting phenotypes from the beginning of  
753 the experiment under worm-free conditions. Indeed, the predicted R ponies displayed higher  
754 levels of circulating monocytes and lower lymphocytes than S ponies at day 0, but they also  
755 exhibited higher *Clostridium XIVa*, *Ruminococcus* and unclassified *Lachnospiracea*  
756 abundances and lower abundance of *Acetivibrio*. Because 80% of the ponies from group R  
757 presented higher genetic relatedness, we could not exclude the possibility that these specific  
758 taxa were influenced by host genetic factors and could sign the intrinsic resistance potential of  
759 ponies to strongyle infection. As explained above, *Clostridium XIVa* has been implicated in  
760 the maintenance of mucosal homeostasis and the protection against intestinal inflammatory  
761 diseases through the promotion of T<sub>reg</sub> cell accumulation (Atarashi et al., 2011). Therefore, it  
762 is tempting to speculate that R and S ponies had an intrinsic different modulation of the  
763 mucosal and systemic immunity as well as the gut bacteria composition and function before  
764 any infection took place.

765  
766 Beyond the effects of susceptibility or resistance to strongyle natural infection on gut  
767 microbiota composition, our findings are consistent with other studies showing that helminths  
768 have the capacity to induce or maintain higher gut microbiota diversity (Giacomin et al.,  
769 2016; Lee et al., 2014; Newbold et al., 2017). In addition, we observed that the patterns of gut  
770 microbial alterations during parasite infection were overall highly consistent with other  
771 studies (Li et al., 2016; McKenney et al., 2015; Reynolds et al., 2014b; Su et al., 2017; Walk  
772 et al., 2010). Specifically, the expansion of Clostridiales has been already reported (Ramanan  
773 et al., 2016; McKenney et al., 2015; Walk et al., 2010; Zaiss et al., 2015) and the reduction of  
774 *Oscillibacter* have been found in pigs infected by *Trichuris suis* (Li et al., 2012; Wu et al.,  
775 2012). Nevertheless, we caution that the late microbiota changes from day 43 to day 92 could  
776 also reflect the effects of pasture quality improvements. Therefore, we hypothesize that the  
777 increased shifts in gut microbiota composition during parasite infection were partially  
778 explained by the nematodes inducing an anti-inflammatory environment and diverting

779 immune responses away from themselves (Cattadori et al., 2016; Fricke et al., 2015; Reynolds  
780 et al., 2014b, 2015; Zaiss and Harris, 2016). This could also be explained by the changes in  
781 the microbial-microbial interactions, the contribution of increased levels of N availability and  
782 the non-fibrous carbohydrates in the pasture, as well as changes in the microbial-  
783 environmental interactions throughout the gut (D'Elia et al., 2009; Midha et al., 2017). In fact,  
784 concomitantly to the parasite infection, pH and fungi loads decreased and protozoa loads  
785 increased. As a result, survival and proliferation of certain microbial species become favored  
786 or depleted.

787  
788 In conclusion, we showed that host parasite susceptibility correlated with parasite burden,  
789 with susceptible ponies having higher egg excretions than resistant animals throughout the  
790 experiment. However, due to the low level of helminth infections observed under natural  
791 conditions, differences in the immune response and gut microbiota composition between  
792 susceptible and resistant animals were modest. Eosinophils and monocytes populations were  
793 more abundant in resistant ponies while lymphocytes were less abundant in their blood, which  
794 may provide a health benefit relative to susceptible animals. Moreover, susceptible ponies  
795 presented a reduction of butyrate-producing bacteria such as *Clostridium XIVa*,  
796 *Ruminococcus*, and unclassified *Lachnospiraceae*, which may induce a disruption of the  
797 maintenance of mucosal homeostasis, intestinal inflammation and dysbiosis. In line with this  
798 hypothesis, an increase in pathobionts such as *Pseudomonas*, *Campylobacter* and fungi loads  
799 were observed in susceptible ponies. Our results therefore suggest that susceptibility to  
800 strongyle infection occurs in the presence of host genetic and other innate and gut  
801 environmental factors that influence immune response and affect individual risk. This  
802 investigation should be followed by experimental work in order to establish the causative  
803 reasons for variation in the microbiota.

804

## 805 **Figures**

### 806 **Figure 1. Experimental design and sampling**

807 A set of twenty female ponies (10 susceptible (S) and 10 resistant (R) to strongylosis) were  
808 selected based on their fecal egg counts history during previous pasture seasons and were kept  
809 inside during the winter. In the spring, they were treated with moxidectin, to ensure that they  
810 were totally free from gastrointestinal nematodes (even from putative encysted larvae) and  
811 were kept indoors for three months. Thereafter, once no more effect of moxidectin treatment  
812 was detected, the ponies were moved to a 7.44 ha pasture to start the study. At day 30 of the  
813 study, a pyrantel treatment was administered to all the animals in order to reset the residual  
814 infections that could interfere in the protocol. A longitudinal monitoring of the parasitism  
815 level in each animal was performed through five time points from June to October. At each  
816 time point, fecal samples were collected from all ponies on 0, 24, 43, 92 and 132 days after  
817 the beginning of the grazing season to carry out fecal egg counts, pH measurements, and  
818 microbiota profiling. Blood samples were taken at the same time points to analyze  
819 biochemical and hematological parameters. This figure was produced using Servier Medical  
820 Art, available from [www.servier.com/Powerpoint-image-bank](http://www.servier.com/Powerpoint-image-bank)

821

### 822 **Figure 2. Fecal egg counts and hematological parameters between susceptible and 823 resistant animals across time**

824 (A) Boxplot of the log parasite fecal egg counts (eggs/g feces) in susceptible (S) and resistant  
825 (R) animals. Purple and green stand for S and R ponies respectively. \*,  $q$  value < 0.05 for  
826 comparison between S and R ponies in each time point; (B) Heatmap of individual egg counts  
827 (eggs/g feces) in S and R animals (each row corresponding to one individual) across time  
828 (column). In the heatmap, egg count values range from 0 (white) and low (blue) to high



829 values (red); (C) Hematocrit (%) between susceptible (S, violet boxes) and resistant (R, green  
830 boxes) animals across time. The quantification of different type of leukocytes: eosinophils  
831 (D), monocytes (E), and lymphocytes (F) were described between susceptible (S, violet  
832 boxes) and resistant (R, green boxes) animals across time. In all cases, boxes show median  
833 and interquartile range, and whiskers indicate 5th to 95th percentile. \*,  $p$  value < 0.05 for  
834 comparison between S and R ponies in each time point.

835

### 836 **Figure 3. Dynamics of microbiota composition between susceptible and resistant animals** 837 **across time**

838 (A) Area plot representation of the phyla detected in feces between susceptible (S) and  
839 resistant (R) animals across time; (B) Area plot representation of the most abundant genera in  
840 feces between susceptible (S) and resistant (R) animals across time; (C) Boxplot graph  
841 representation of genera significantly affected between S and R animals across time. In all  
842 cases, susceptible animals are colored in violet and resistant animals in green. Boxes show  
843 median and interquartile range, and whiskers indicate 5th to 95th percentile. \*,  $p$  value < 0.05  
844 for comparison between S and R ponies in each time point.

845

### 846 **Figure 4. Estimation of the $\alpha$ -diversity indexes and $\beta$ -diversity insusceptible and** 847 **resistant animals during the natural parasite infection (from day 43 to day 132).**

848 (A) Estimation of the  $\alpha$ -diversity indexes in susceptible (S) and resistant (R) during the  
849 natural parasite infection (from day 43 to 132). The box color indicates the time point  
850 analyzed: (pink=43 d, green=92 d, and darkgreen=132 d); (B)  $\alpha$ -diversity indexes between S  
851 and R animals during the natural parasite infection. Susceptible animals were colored in violet  
852 and resistant animals in green; (C) Principal Coordinate analysis of Unifrac distances to  
853 compare fecal communities at the level of genera that differ between S and R animals across  
854 natural parasite infection. Both PC axes 1 and 2 were plotted. Together they explained 58.6%  
855 of whole variation; (D) Correspondence analyses of Unifrac distances to compare fecal  
856 communities at the level of genera that differ between S and R animals across natural parasite  
857 infection. Both CA axes 1 and 2 were plotted; (E) Genus-level network representation  
858 between ponies across natural parasite infection linked within a specified Jaccard distance of  
859 0.85. Two samples were considered “connected” if the distance between them was less than  
860 0.85. In all cases, the relative position of points was optimized for the visual display of  
861 network properties. The point’s shape indicates the susceptibility to strongylosis (triangle:  
862 susceptible (S); round: resistant (R)), the node color indicates the time point analyzed:  
863 (pink=43 d, green=92 d, and darkgreen=132 d).

864

### 865 **Figure 5. Longitudinal dynamics of microbiota composition upon natural parasite** 866 **infection.**

867 (A) Estimation of the  $\alpha$ -diversity indexes across the natural parasite infection: from day 43 to  
868 day 132. The box color indicates the time point analyzed: (pink=43 d, green=92 d, and  
869 darkgreen=132 d); (B) Boxplot graph representation of genera significantly affected ( $q$  <  
870 0.05) at day 92 relative to day 43. In all cases, the box color indicates the time point analyzed:  
871 pink=43 d, green=92 d, and darkgreen=132 d. Boxes show median and interquartile range,  
872 and whiskers indicate 5th to 95th percentile. All genera plotted were statistically significant ( $q$   
873 < 0.05) between day 92 and day 43.

874

### 875 **Figure 6. A model for gut microbiota modifications and their effects on host physiology** 876 **after natural strongyle infection**

877 We hypothesize that natural parasite infection in susceptible ponies increases the release of  
878 lymphocytes but decreases the monocytes and eosinophils cell counts. Concomitantly,



879 parasite infection induced alterations in bacterial-fungal inter-kingdom, increasing the  
880 abundance of *Paludibacter*, *Campylobacter*, *Bacillus*, *Pseudomonas*, *Clostridium III*,  
881 *Acetivibrio* and the overall loads of fungi and parasite egg counts in the feces.  
882 On the other hand, butyrate producing bacteria such as members of *Ruminococcus*,  
883 *Clostridium XIVa* and *Lachnospiraceae* family were found to be depleted in susceptible  
884 ponies, but enriched in resistant animals, suggesting a possible effect of N-butyrate on the  
885 protection of inflammation in resistant animals. Because butyrate is a potent inhibitor of  
886 inflammation, it is suggested that susceptible ponies are prone to the gut inflammation  
887 because of the altered abundance of butyrate-producing bacteria.  
888 The lower N-butyrate bacteria abundance was accompanied by a number of microbiota  
889 functional pathways that reflected immunological mechanisms, including pathogen sensing,  
890 changes in lipids, and activation of signal transduction pathways inside of the cell. This figure  
891 was produced using Servier Medical Art, available from [www.servier.com/Powerpoint-](http://www.servier.com/Powerpoint-image-bank)  
892 [image-bank](http://www.servier.com/Powerpoint-image-bank)

893

#### 894 **Conflict of Interest Statement**

895 The authors declare that the research was conducted in the absence of any commercial or  
896 financial relationships that could be construed as a potential conflict of interest.

897

#### 898 **Authors and Contributors**

899 AB, GS and NM designed the experiment. AC, GS and NM drafted the main manuscript text.  
900 NM designed and carried out the bioinformatics and biostatistical analyses, prepared all the  
901 figures and provided critical feedback on content. VB performed the RT-qPCR analyses. FR  
902 was in charge of pony maintenance and care throughout the experiment and managed  
903 sampling. AM analyzed the chemical composition of the diet. JC and CK performed fecal egg  
904 counts. MR performed the blood analysis. All authors reviewed the manuscript and approved  
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906

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920

#### 921 **List of abbreviations**

922 ADF = acid detergent fiber  
923 ADL = acid detergent lignin  
924 AFNOR = Association Française de Normalisation  
925 ANOSIM = analysis of similarities  
926 ANOVA= Analysis of the variance  
927 CA = correspondence analysis  
928 DM=dry matter

929 FEC= fecal egg counts  
930 KEGG = Kyoto Encyclopedia of Genes and Genomes  
931 NDCS = neutral detergent soluble carbohydrate  
932 NDF = neutral detergent fiber  
933 NFC = Non-fiber carbohydrate  
934 NMDS = non-parametric multidimensional scaling  
935 OTU = operational taxonomic units  
936 PCoA = principal coordinates analysis  
937 PERMANOVA = Permutational Multivariate Analysis of Variance  
938 PICRUST = phylogenetic investigation of communities by reconstruction of unobserved states  
939 QIIME = Quantitative Insights Into Microbial Ecology  
940 qPCR=real time quantitative PCR  
941 RDP = Ribosomal Database Project naïve Bayesian classifier  
942 SCFA=short chain fatty acids  
943

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1278

1279 **Supplementary Material**

1280

1281 **Supporting tables**

1282

1283 **Table S1. Summary of study samples and fecal bacterial 16S rRNA gene amplicon**  
1284 **sequence datasets**

1285

1286 **Table S2. The OTU taxonomical assignments and OTU counts in each pony across time**

1287

1288 **Table S3. Chemical composition of pasture across time.**

1289

1290 **Table S4. Differences in relative abundance of genera between susceptible (S) and**  
1291 **resistant (R) animals across time.**

1292

1293 **Table S5. Differences in KEGG pathway abundance between susceptible (S) and**  
1294 **resistant (R) animals across time.** Pathways values across time are represented as %, where  
1295 normalized counts from a particular pathway is divides by the total number of counts in each  
1296 sample.

1297

1298 **Table S6. Topological properties of co-occurring networks obtained from susceptible**  
1299 **and resistant animals at day 92 of the experiment.**

1300

1301 **Table S7. Differences in relative abundance of genera from day 43 to day 132, regardless**  
1302 **of susceptibility to strongyles.**

1303

1304 **Table S8. Differences in KEGG pathway abundance from day 43 to day 132, regardless**  
1305 **of susceptibility to strongyles.** Pathways values across time are represented as %, where  
1306 normalized counts from a particular pathway is divides by the total number of counts in each  
1307 sample.

1308

1309 **Supporting figures**

1310 **Figure S1. Genetic kinship between the 20 Welsh ponies in the experiment**

1311 (A) Heatmap of the historical egg counts (eggs/g feces) in susceptible (S) and resistant (R)  
1312 animals included in the study. The fecal egg counts values of 4 continuous years were  
1313 included. In the heatmap, rows represent the animals and columns the different time points  
1314 analyzed. Not all animals have information for each time point. In the heatmap, red = high  
1315 values, green = low values of egg counts per g of feces; (B) Violin plot of the historical fecal  
1316 egg counts of the S and R animals included in the study. The median egg counts /g feces were  
1317 800 for S (the mean was 897, the first quartile was 350, and the third quartile was 1,375),  
1318 whereas the median egg counts /g feces were 0 for R (the mean was 246.1864, the first  
1319 quartile was 0, and the third quartile was 150); (C); Density plot of historical fecal egg counts  
1320 values in S and R animals; (D) Pedigree plot. A six-generation pedigree plot is illustrated,  
1321 with different shapes for male (squares) and female (circles). The shapes are black for the 20  
1322 ponies in the study; (E) Heatmap of the kinship coefficient matrix, which assess the genetic  
1323 resemblance between ponies. Each entry in the matrix is the kinship coefficient between two  
1324 subjects. Animals are arranged in the order of their genetic relatedness; genetically similar  
1325 animals are near each other. Note that the diagonal elements did not have values above unity,  
1326 showing no consanguineous mating in the families. The treatment (susceptible (S) or resistant  
1327 (R) is delineated below to the animal name. In the heatmap, red = high values of genetic  
1328 relatedness, white = low values of genetic relatedness.



1329

1330 **Figure S2. Overview of the data analysis in the study**

1331 (A) Effect of parasite susceptibility on gut microbiota composition, parasite egg excretion,  
1332 gut-related parameters and host parameters across time.

1333 Step 1: Measurement of the gut microbiota composition between susceptible (S) and resistant  
1334 (R) ponies across time. This step involved the analysis of the  $\alpha$ -diversity and  $\beta$ -diversity  
1335 between S and R ponies across time, as well as the analysis to assess gut genera whose  
1336 relative abundances changed between groups across time, the determination of the  
1337 corresponding KEGG pathways and the inference of the co-occurrence network.

1338 Step 2: Measurement of the gut related parameters between S and R ponies across time. The  
1339 gut parameters included the pH, the fungal, bacteria and protozoan loads, as well as the  
1340 number of parasite egg in the feces.

1341 Step 3: Measurement of the host parameters between S and R ponies across time. The host  
1342 parameters included the body weight, the daily average gain, hematological and biochemical  
1343 parameters in blood.

1344

1345 **Figure S3. Whether and pasture quality throughout the experiment**

1346 (A) Daily precipitation and temperatures recorded at a meteorological station located 14 km  
1347 from the experimental pasture. Maximum and minimum temperatures are colored in red and  
1348 blue, respectively. Dashed line represents precipitation; (B) Chemical composition (crude  
1349 protein (CP), neutral and acid detergent fiber (NDF and ADF), crude fiber (CF), crude ash,  
1350 and acid detergent lignin (ADL)) of hay at day 0 and pasture at day 24, 43, 92 and 132. From  
1351 day 1 to day 43, the lack of rainfall resulted in significant soil moisture deficits and reduced  
1352 growth rates of pasture. This period coincided with the late-flowering stage of the pasture,  
1353 when stems and leaves are being depleted of nutrients and herbage maturation and  
1354 lignification increases. After day 43, the environmental conditions were eminently favorable  
1355 to start a second pasture cycle, with a quick herbage growth, high protein content and lower  
1356 fiber content. We observed that herbage protein peaked at day 92, after the intense fall rains  
1357 and the increased senescence of green material.

1358

1359 **Figure S4. Hemogram data in susceptible and resistant animals across time.**

1360 The evaluation of the hemogram involved the determination of the hematocrit, total white  
1361 blood cell counts, total erythrocyte count, erythrocyte indices and platelet counts.

1362 The determination of the hematocrit (A) and the total white blood cells (B) was performed  
1363 between susceptible (S, violet boxes) and resistant (R, green boxes) animals across time. The  
1364 quantification of different type of leukocytes: eosinophils (C), monocytes (D), lymphocytes  
1365 (E), neutrophils (F) and basophils (G) were described between S and R animals across time.  
1366 The values corresponding to red blood cell distribution width (H), microcytic (I) and  
1367 macrocytic (J) platelets, as well as microcytic red blood cells (K), and macrocytic red blood  
1368 cells (L) were plotted. The red blood cell distribution width is plotted in (L). In all cases,  
1369 boxes show median and interquartile range, and whiskers indicate 5th to 95th percentile. \*,  $p$   
1370 value < 0.05 for comparison between S and R ponies in each time point.

1371

1372 **Figure S5. Performance between susceptible and resistant animals across time**

1373 (A) Boxplot and violin plot representation of body weight (kg) between susceptible (S) and  
1374 resistant (R) ponies across time; (B) Boxplot and violin plot representation of average daily  
1375 gain (kg) between S and R ponies across time. In all cases, susceptible animals were colored  
1376 in violet and resistant animals in green.

1377

1378 **Figure S6. Biochemical data in susceptible and resistant animals across time.**



1379 Levels of albumin (A), cholesterol (B), globins (C), glucose (D), alkaline phosphatase (E),  
1380 ratio albumin/globins (F), total proteins (G) and urea (H) in susceptible (S, violet boxes) and  
1381 resistant animals (R green boxes) were plotted. Boxes show median and interquartile range,  
1382 and whiskers indicate 5th to 95th percentile. \*,  $p$  value < 0.05 for comparison between S and  
1383 R ponies in each time point.

1384

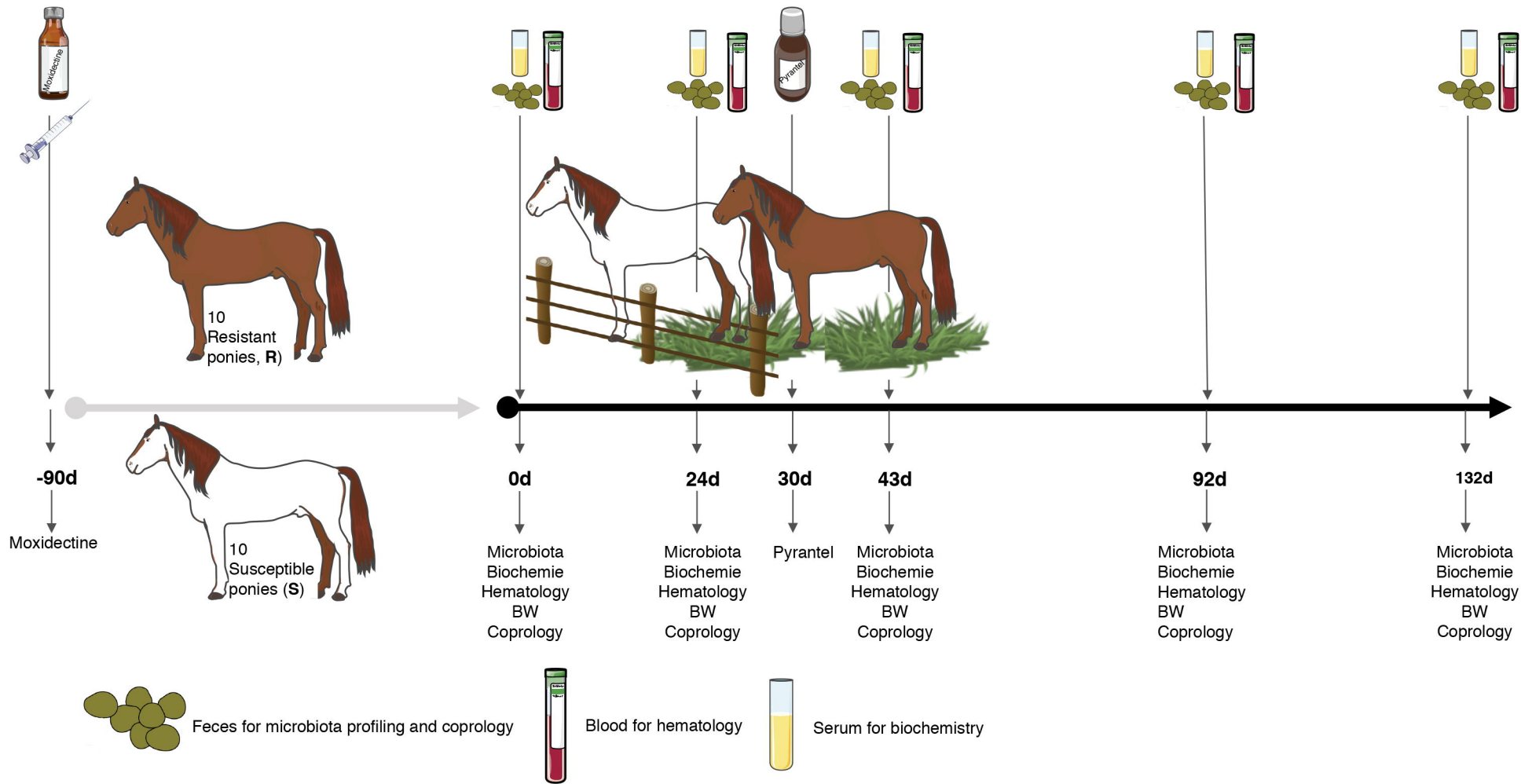
1385 **Figure S7. Co-occurrence network at 92 days after the entry to the pasture for**  
1386 **susceptible and resistant ponies**

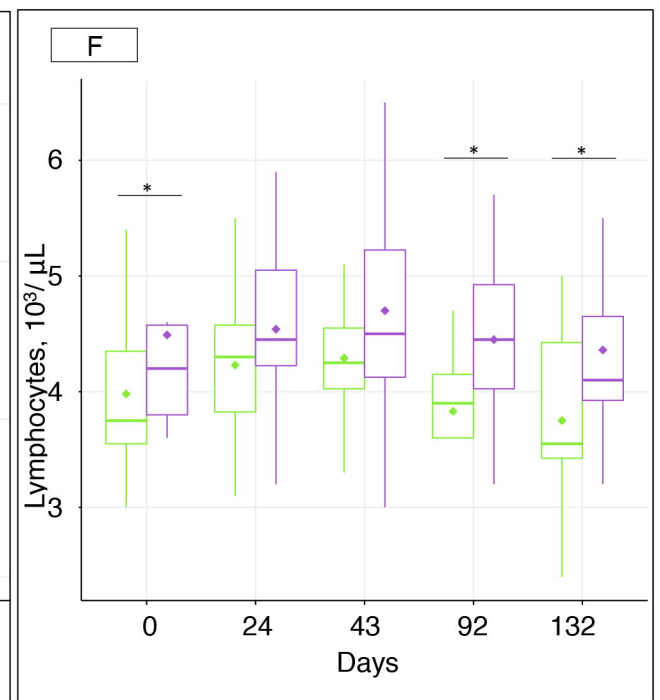
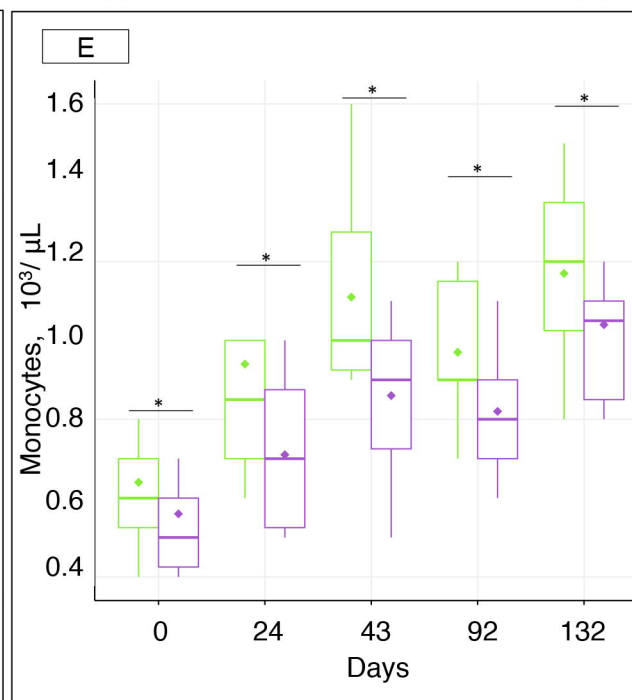
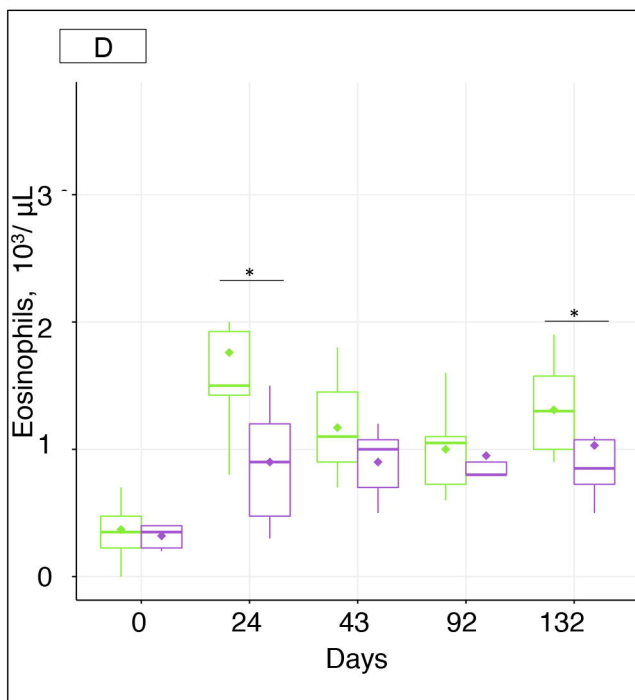
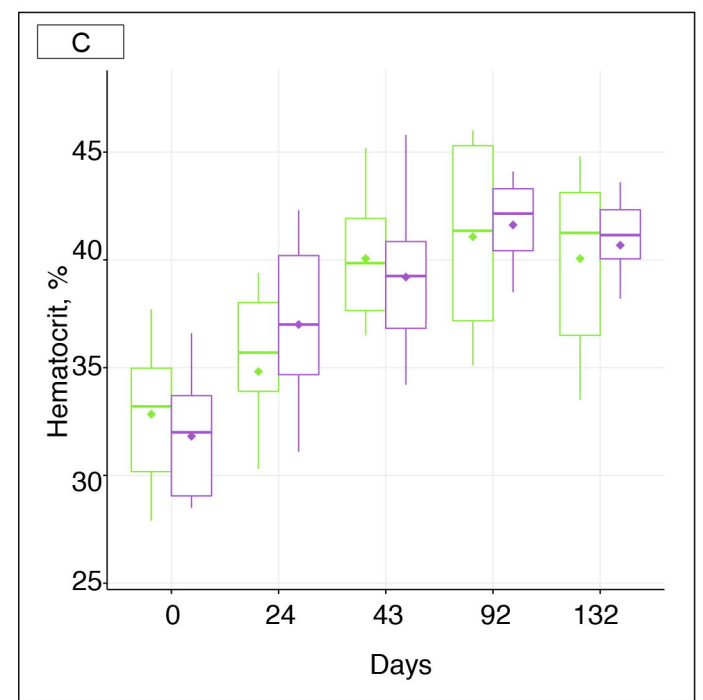
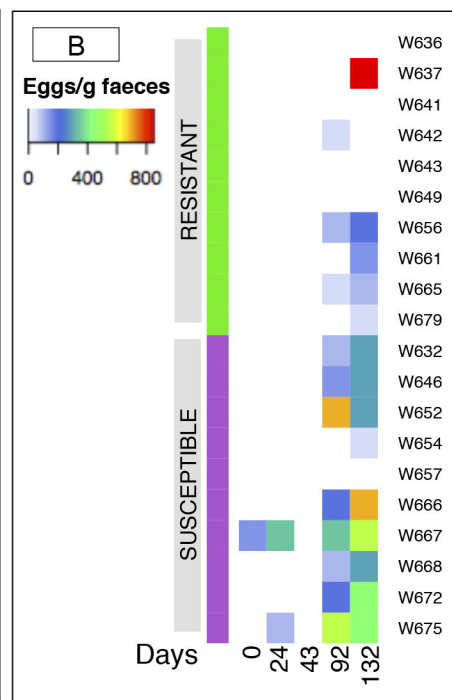
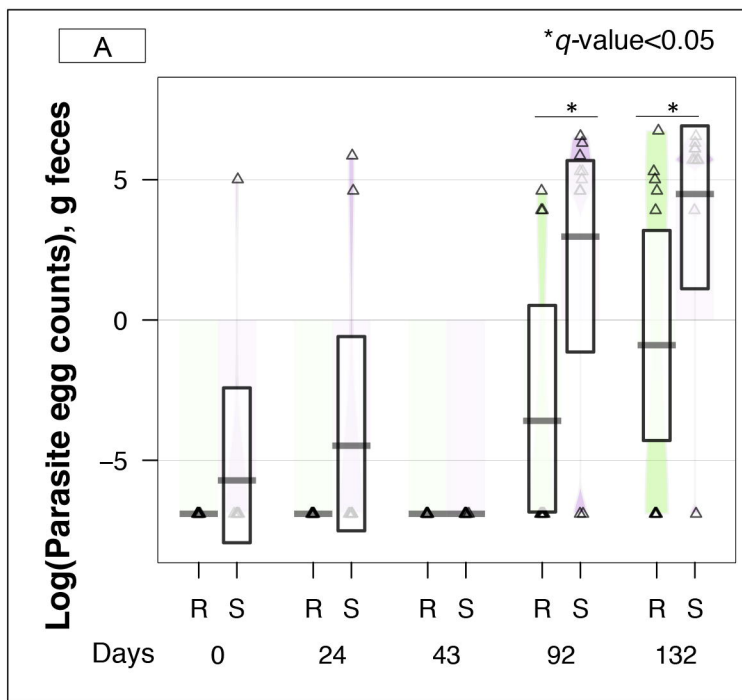
1387 The correlations among genera were calculated using the PCIT method, which identifies  
1388 significant co-occurrence patterns. The size of the node is proportional to genera abundance.  
1389 Node fill color corresponds to phylum taxonomic classification. Edges color represent  
1390 positive (red) and negative (blue) connections, the edge thickness is equivalent to the  
1391 correlation values. Only genera with a relative abundance > 0.10 were included.

1392

1393 **Figure S8. Microbiota functional parameters between susceptible and resistant ponies**  
1394 **across time.**

1395 (A) Boxplot graph representation of pH in feces between susceptible (S) and resistant (R)  
1396 animals at different time points; (B) Boxplot graph representation of loads of anaerobic fungi  
1397 in feces between S and R animals at different time points; (C) Boxplot graph representation of  
1398 loads of protozoan in feces between S and R animals at different time points; (D) Boxplot  
1399 graph representation of loads of bacteria in feces between S and R animals at different time  
1400 points. In all cases, susceptible animals are colored in violet and resistant animals in green. \*,  
1401  $p$  value < 0.05 for comparison between S and R ponies in each time point.





Treatments R S \**P*-value<0.05

