



HAL
open science

Dietary-induced modulation of the hindgut microbiota is related to behavioral responses during stressful events in horses

Alexandra Destrez, Pauline Grimm, Véronique Julliand

► To cite this version:

Alexandra Destrez, Pauline Grimm, Véronique Julliand. Dietary-induced modulation of the hindgut microbiota is related to behavioral responses during stressful events in horses. *Physiology and Behavior*, Elsevier, 2019, 202, pp.94-100. 10.1016/j.physbeh.2019.02.003 . hal-02067208

HAL Id: hal-02067208

<https://hal-agrosup-dijon.archives-ouvertes.fr/hal-02067208>

Submitted on 21 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial | 4.0 International License

1 **Dietary-induced modulation of the hindgut microbiota is related to behavioral responses**
2 **during stressful events in horses**

3

4 **Authors**

5 Alexandra Destrez¹, Pauline Grimm², Véronique Julliand³

6

7 **Affiliations**

8 ¹ AgroSup Dijon, Univ. Bourgogne Franche-Comté, INRA, CNRS, UMR6265 CSGA -
9 Centre des Sciences du Goût et de l'Alimentation, Dijon, F-21000, France

10 ² Lab To Field

11 ³ Univ. Bourgogne Franche-Comté, AgroSup Dijon, PAM UMRA 02.102, F-21000 Dijon,
12 France

13

14 **Corresponding author**

15 alexandra.destrez@agrosupdijon.fr

16 +33 (0)3 80 77 25 57

17 **Abstract**

18 The bidirectional communication between the central and the enteric nervous system named
19 the gut-brain axis has been widely recognized. The gut microbiota has been implicated in a
20 variety of stress-related conditions including anxiety, depression and irritable bowel syndrome
21 based on rodent studies or correlative analysis in human patients. The aim of the present study
22 was to investigate to what extent changes in behavior during stressful events and in the
23 microbial composition of the colonic ecosystem were associated in horses. The microbiota
24 alterations were induced by a change from a high-fiber diet (100% hay, H diet) to a
25 progressive low-fiber and high-starch diet (56% hay and 44% barley, HB diet) on six
26 fistulated horses. Colonic total anaerobic, cellulolytic, amylolytic and lactate-utilizing bacteria
27 were enumerated once on H diet and once on HB diet. Bacterial richness, diversity and
28 structure at family and genus level were also determined. The behavior of horses was assessed
29 through two standardized stressful tests: a novelty test and an umbrella test. The different
30 alterations measured in the colonic microbiota demonstrated a lower fibrolytic capacity and a
31 higher amylolytic capacity of the ecosystem when horses received HB compared to H diet.
32 During the novelty test, the frequency of blowing was significantly higher in HB than in H
33 diet and was positively correlated with the concentration of amylolytic bacteria and the
34 Succinivibrionaceae relative abundance. During the umbrella test, behavioral variables were
35 not significantly different between the diets but the colonic content pH was negatively
36 correlated with the frequency of startle response. Behavioral responses of anxiety were related
37 to hindgut microbiota indicators of a high-starch diet. Dietary-induced modulation of the gut
38 microbiota composition may have changed the horses' behavioral reactions in stressful
39 situations.

40

41 *Keywords: behavior; diet; microbiota; stress; horses*

42 **1. Introduction**

43 The bidirectional communication between the central and the enteric nervous system named
44 the gut-brain axis has been widely recognized [1-3]. Over the past decade, fundamental
45 studies emphasized the importance of the gut microbiota in influencing this communication,
46 revealing a microbiota-gut-brain axis with two-way interactions between the microbiota and
47 stress behavior [2, 4]. The gut microbiota has been implicated in a variety of stress-related
48 conditions including anxiety, depression and irritable bowel syndrome based on animal
49 studies or correlative analysis in patient populations [4]. For instance, human patients with
50 depression exhibited modified and less diverse fecal microbiota signatures than controls [5].
51 In lab animals altered gut microbiota profiles have been associated with changes in stress-
52 related behavior and exacerbation of the hypothalamic-pituitary-adrenal axis reactivity [6]. As
53 an example, stress hormones (plasma ACTH and corticosterone) elevation in response to
54 restraint stress was higher in germfree mice than in conventional mice [7]. In open-field test
55 (i.e. test of spontaneous activity), germfree rodents were more anxious and active than
56 conventional rodents [mice: 8, rats: 9].

57 Bearing in mind this influence of the gut microbiota on the central nervous system, there is
58 great temptation for changing behavior by modulating gut microbiota structure and function.
59 It is largely recognized that dietary modulation is a major and easy way for inducing changes
60 in the composition of the gut microbiota [10]. Furthermore there is scientific evidence
61 supporting a causal relationship between overall diet quality and occurrence of depression and
62 anxiety [11]. For example, mice on high-sucrose diet displayed less anxiety than mice on
63 high-fat diet in three stressful tests [12]. However, biological pathways and targets that
64 mediate the associations between diet, gut microbiota and anxiety are not clearly identified.
65 This is why we investigated in a earlier study to what extent behavioral changes were
66 associated with changes of the microbiota through an alimentary change in horses [13]. Far

67 from their natural living, most horses are submitted to stressful conditions in terms of
68 housing, feeding or training that have been associated with the occurrence of altered
69 behavioral responses or even of diseases such as gastric ulcers, colic or laminitis [14].

70 Our earlier study indicated that the gut microflora and the horse's brain might have a
71 bidirectional relationship because the animal's response in a novelty and a social test varied
72 with diet [13]. More research is needed to evaluate the impact of the dietary stress factor on
73 particular bacterial taxa and their interactions with behavioral changes. Hence, the present
74 study aimed to investigate the link between changes in behavior during stressful events and
75 changes in microbial composition and activity of the colonic ecosystem in horses. In view of
76 our previous study, another behavioral test known to be more stressful for animals than a
77 novelty test was used. In addition to bacteria enumeration, we determined bacterial richness,
78 diversity and structure at family and genus level as well as pH.

79

80 **2. Material and methods**

81 The protocol was approved by the Committee on the Ethics of Animal Experiments of Grand
82 Campus Dijon (registration number: 105; April 05, 2013).

83

84 2.1. Subjects and facilities

85 Six adult crossbred geldings fitted with cannulas in the right ventral (RV) colon were used.
86 The barrel of each fistula (in the cecum and in the right-ventral colon) was 15 cm long and
87 2.25 cm internal diameter. Surgery procedure used to set fistulas was the same as that
88 described by [15], and was performed at least five years ago. Hence, the effect of fistula on
89 behavior was assumed to be negligible. Horses were aged 13–21 years and weighing 474 to
90 517 kg. They were housed in 3.3 × 4 m stalls which contained an automatic waterer (water
91 provided free choice), a plastic feed bucket for hay, a plastic feed bucket for barley with a

92 trace mineral and NaCl block, and wood shavings (Copeaux Classic, Thierwhol, Retteinmaier,
93 France) as bedding over rubber stall mats. Horses were fed at 08:00 and 17:00; stalls were
94 cleaned and bedding replaced each morning. The horses were released daily in a small dry
95 paddock (10 × 10 m) for 2h. Horses were exercised six times a week (except days of digestive
96 collection or behavioral tests) in an automatic walker 1h per day at 4-6 km/h. Their
97 vaccinations against tetanus and influenza (ProteqFlu TE, Merial) and their de-worming
98 (Equest Oral Gel (moxidectin), Fort Dodge Animal Health) were updated before the start of
99 the experiment.

100

101 2.2. Experimental design and diets

102 The horses were submitted to a longitudinal experiment composed of 8 weeks and separated
103 in 2 periods, each associated with a diet. During H diet (30 days), horses were fed a high-fiber
104 diet composed of 100% of hay (H diet; 2.1 kg of Dry Matter (DM)/day/100 kg of Body
105 Weight (BW)). Then, during a period of gradual transition (over five days), they were
106 submitted to a modification of diet from the H diet to a progressive low-fiber and high-starch
107 diet (from 90% hay and 10% barley to 60% hay and 40% barley in 4 days). During HB diet
108 (23 days), horses were fed a diet composed of 56% of hay and 44% of barley (HB diet; 1.4 kg
109 of DM/day /100 kg of BW with 0.8 kg of DM/day/100kg of BW hay). All the diets were
110 formulated to be iso-energetics and to meet 105% of the energy requirements for horse
111 subjected to a very light work [16]. Both diets were offered in two equal meals per day. Every
112 period, the body weight of each horse was assessed visually through a body condition score
113 and horses were weighed.

114 Recent data (not published yet) in horses showed that 6-weeks after a change from a high-
115 starch diet to a hay diet the hindgut microbial ecosystem was still statistically different from
116 its status under the hay diet (Grimm & Julliand, personal data). Thus, we hypothesized that

117 the modulation of the hindgut microbiota after a high-starch diet would last more than 4
118 weeks and we chose to observe all the 6-fistulated horses during hay diet (their usual diet)
119 then during the high-starch diet. The timing of the experimental procedures is described in
120 Fig. 1.

121

122 2.3. Intestinal samples collection and microbiota analyses

123 Colonic contents were collected 4h after the morning meal the 27th day of the H diet and the
124 20th of the HB diet (Fig.1). The colonic contents were obtained by gravity via the cannulas.
125 An aliquot of the unfiltered contents (solid and liquid phase) was sampled for molecular
126 biology analysis (1 mL, storage at -80 °C until analysis). Another aliquot of unfiltered content
127 was also sampled in a container filled to capacity (to avoid the presence of oxygen) for
128 microbiological analyses performed immediately after the sample collection.

129

130 2.3.1. Bacteria functional group analysis

131 Total anaerobic bacteria and cellulolytic, amyolytic and lactate-utilizing bacteria were
132 enumerated in the colon using conventional anaerobic culture techniques. The content of
133 colon (1mL) was serially diluted in a mineral solution [17] and then inoculated in roll tubes
134 on specific media under continuous flow of CO₂ [18]. Concentrations of total anaerobic
135 bacteria (dilutions representing 10⁻⁵, 10⁻⁶, or 10⁻⁷ mL of intestinal contents) were determined
136 on a non-selective modified complete agar medium [19, 20] after 48 h of incubation at 38°C.
137 Concentrations of lactate-utilising bacteria (dilutions representing 10⁻³, 10⁻⁴, or 10⁻⁵ mL of
138 intestinal contents) were determined on a selective medium [21] after 48h of incubation at
139 38°C. Concentrations of amyolytic bacteria (dilutions representing 10⁻⁴, 10⁻⁵ and 10⁻⁶, mL of
140 intestinal contents) were determined on a modified selective medium containing 1% (w/v)
141 soluble starch as the main energy source [22], after 72 h of incubation at 38°C. Most probable

142 number [23] of cellulolytic bacteria (dilutions representing 10^{-5} , 10^{-6} , and 10^{-7} mL of
143 intestinal contents) were determined using a modified broth medium [20, 24] after 15 d of
144 incubation at 38°C. Finally, all the bacteria concentrations were turned into decimal
145 logarithms (\log_{10}).

146

147 2.3.2. Bacterial 16S rDNA extraction, sequencing and bioinformatical analysis

148 Total DNA was extracted from 0.25 g RV colonic content as described by Yu and Morrison
149 [25]. The quantity of obtained DNA was assessed on a spectrophotometer (Eppendorf,
150 Hamburg, Germany) and its purity was evaluated by calculating A260/280 ratios to verify
151 contamination by proteins (ratio values between 1.6 and 2 were acceptable for nucleic acid
152 extractions from digestive samples). DNA samples were then frozen at -80°C. The V3-V4
153 hypervariable region (459 bp in E. Coli) of the bacterial 16S ribosomal RNA gene (16S
154 rRNA, 1542 bp in E. Coli) was amplified by PCR using the forward and reverse primers F343
155 (CTTTCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG) (adapted from [26])
156 and R784 (GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT)
157 (adapted from [27]). The PCR mix was constituted of 10 ng of DNA, 5 μ L of buffer (MP
158 Biomedicals, Illkirch-Graffenstaden, France), 1 μ L of dNTP mix, 0.5 μ L of Taq polymerase (5
159 U/ μ L MTP TM Taq DNA Polymerase, Sigma, Saint-Louis, Missouri, USA), 1.25 μ L (20
160 μ M) of forward and reverse primer and was completed to 50 μ L with sterile water. The
161 conditions of PCR were: one cycle at 94 °C during 1 min, followed by 30 x (94 °C during 1
162 min, 65 °C during 1 min and 72 °C during 1 min). The amplification was followed by a
163 melting program (72°C during 10 min). To verify the correct amplification of the V3-V4
164 region, the DNA samples were electrophoresed on a 2% agarose gel. The PCR products (4 μ L)
165 were applied per well and electrophoresed for 1h15 at 45°C using a fixed voltage of 70 V on a
166 Mupid One system. After purification with magnetic beads amplicons were submitted to a

167 second PCR aiming at ligate Illumina adapters and an index allowing the identification of the
168 sample. The PCR mix was the same than for the first PCR with a forward primer
169 (AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC) and a reverse
170 primer(CAAGCAGAAGACGGCATACGAGAT-Index-GTGACTGGAGTTCAGACGTGT).
171 The conditions of PCR were similar to the previous ones except 12 cycles of elongation were
172 performed. PCR products were again purified using magnetic beads. The resulting PCR
173 products were sequenced using an Illumina MiSeq run of 250 base paired-ends according to
174 the manufacturer instructions (Illumina Inc., San Diego, CA). The quality of the run was
175 checked using control libraries generated from the PhiX virus (Illumina PhiX control;
176 Illumina Inc., San Diego, CA).

177 Bioinformatic analyses were performed using the FROGS pipeline (Find Rapidly OTU with
178 Galaxy Solution), which associates several tools to work on the file of sequences and obtain
179 both the abundance table of operational taxonomic units (OTUs) and their taxonomic
180 affiliation [28]. OTUs which are not present in at least 2 samples, or whose abundance was
181 less than 5×10^{-5} , were removed. Remaining OTU (operational taxonomic unit) were then
182 aligned to the silva123 16S data base [29] using BLAST and were affiliated to the highest
183 taxonomic rank possible. The abundance table and the associated multi-hit list were created.
184 Relative abundance of the different families and genera was calculated. Richness (Number of
185 species) and diversity (Shannon) indexes [30] were calculated from the abundance table using
186 FROGS.

187

188 2.3.3. pH measurement

189 The pH of the colon was measured directly after the samples collection using the HI 1053
190 electrode (Hanna instrument, Lingolsheim, France) connected to an electronic pH-meter
191 (CyberScan pH 510, Eutech Instrument, Strasbourg, France).

192

193 2.4. Behavioral analysis in the stressful tests

194 In order to examine the reactions specific to the stimulus studied, we attempted to test the
195 horses' reaction in a context as neutral as possible: novelty and umbrella tests were thus
196 performed in a barn adjacent to the stall where horses were living. Both tests were realized the
197 same day, the 30th days of the H diet and the 23rd days of the HB diet (Fig 1.). Novelty tests
198 were realized on mornings and umbrella tests on afternoons.

199

200 2.4.1. Novelty test

201 The testing area (Fig. 2) consisted of a pre-test pen (waiting pen, 15 m²) and a test arena (45
202 m²) made of wooden walls covered with white sheets (2 m high). A sliding door allowed
203 passage from waiting pen to the area [31]. At the opposite side of the entrance, the test arena
204 contained a bucket containing pellets (75 g of concentrate) and a closed black umbrella fixed
205 on the wall (1.5 m high, the opening system of the umbrella was on the other side of the wall
206 thanks to a hole in the wall). Three zones were determined in the test arena to measure the
207 horse activity (Z1, Z2 and Z3). Between the 27th day and the 30th of the H diet, horses were
208 submitted to a habituation procedure. On day one, the animals were individually allowed to
209 freely explore the test arena for 5 min. On day two, each horse was led by a stockperson to the
210 bucket containing the pellets and ate them. On day three, animals were allowed to eat pellets
211 on their own. After the habituation procedure, horses were submitted to the novelty test on
212 two occasions (Fig 1.). Each horse entered the test arena where a novel object (H diet: a red
213 fire extinguisher, 1 m high; HB diet: a gold cardboard display box, 1.5 m high) had been
214 placed 20 cm in front of the food-filled bucket. The test lasted for 5 min. The behavior of the
215 horse was recorded (using a SONY Handycam HDV Camera) and analyzed using the
216 following behavioral categories: feeding (taking food into the mouth, chewing food), being

217 vigilant (being immobile with head in an upright position, ears immobile and tail slightly
218 raised), smelling the floor, interacting with the novel object (head and ears oriented towards
219 the object whereas animal's nose is less than 10 cm from the object), blowing (high pitched
220 'whoosh' sound produced as the horse exhales through the nose) and moving (sum of the
221 frequencies of being of the different zones Z1, Z2 and Z3). The order that animals entered in
222 the testing area was randomly made.

223

224 2.4.2. Umbrella test

225 The day of the novelty test (in the same test arena), horses were submitted to the umbrella test
226 on two occasions (Fig 1.). Each horse entered the test arena and started to feed. After 30 s
227 feeding, the sudden event occurred i.e. the black umbrella was open by an experimenter
228 placed outside the pen. As soon as the umbrella was open, the test lasted for 5 min. The
229 behavior of the horse was recorded (using a SONY Handycam HDV Camera) and analyzed
230 using the following behavioral categories: feeding, being vigilant, smelling the floor, having a
231 startle response (having a visible transient contraction of the shoulder and/or hindquarter of
232 the animal occurring with a flexion of the legs or with the legs moving away from each other),
233 blowing and moving. Times spent in the different zones of the test arena (Z1, Z2 and Z3) and
234 latencies to feed after the sudden event were also recorded. The order that animals entered in
235 testing area was randomly made.

236

237 2.5. Statistical analysis

238 Data were analyzed using XLSTAT software (version 2015.3.01.19097, Addinsoft, Paris,
239 France). Since the animals were physiologically stable (adult, non-productive), restricted fed,
240 and maintained in a controlled environment, time effect was assumed to be negligible [32].
241 Diet effect on microbial parameters (concentration of bacteria functional group, relative

242 abundance of bacterial families and genera, richness and diversity indexes) was analyzed
243 using ANOVAs. Friedman test was used to analyze diet effect on horses' behaviors during
244 novelty and umbrella tests. Spearman correlations (with Holm's method adjustment) were
245 used to correlate microbial parameters and horses' behaviors during novelty and suddenness
246 tests. The limit of significance was set at $P < 0.05$.

247

248 **3. Results**

249

250 3.1. Behavioral analysis

251 During novelty test, the frequency of blowing was significantly higher in HB diet than in H
252 diet ($P = 0.04$, Table 1). The other behavioral variables were not significantly different between
253 H diet and HB diet ($P > 0.3$, Table 1). During umbrella test, behavioral variables were not
254 significantly different between H diet and HB diet ($P > 0.3$, Table 1).

255

256 3.2. Intestinal microbiota analysis

257 Concentrations of amylolytic bacteria and of total anaerobic bacteria were significantly higher
258 on HB diet than in H diet (respectively $P = 0.005$ and $P = 0.05$, Table 2). pH and the other
259 concentrations of bacteria were not significantly different between the two diets (Table 2).
260 The number of species and Shannon index were not significantly different between diets
261 (Table 2). The relative abundance of Succinivibrionaceae family was significantly higher in
262 HB diet than in H diet ($P = 0.04$, Table 2). The relative abundance of the other bacteria
263 families were not significantly different between the two diets ($P > 0.1$, Table 3).

264

265 3.3. Correlations between behavioral and physiological data

266 The adjusted P-values with Holm's method showed that behavioral and physiological data
267 were not significantly correlated (adjusted P-values>0.5). In the following results, P-values of
268 Spearman correlations without Holm's method adjustment are shown.

269

270 3.2.1. Novelty test

271 Concentration of amylolytic bacteria and frequency of blowing were positively correlated (P=
272 0.009, Table 3). The other behavioral variables and concentrations of bacteria were not
273 significantly correlated (P>0.1, Table 3).

274 Behavioral variables and Species index were not significantly correlated ($|r|<0.5$; P>0.1).
275 Shannon index and duration of smelling the floor; Shannon index and frequency of moving
276 were positively correlated ($|r|>0.6$; P<0.04). Shannon index and duration of feeding were
277 negatively correlated ($r=-0.8$; P=0.01). The other behavioral variables and Shannon index
278 were not significantly correlated ($|r|<0.5$; P>0.1).

279 Relative abundance of Succinivibrionaceae (genus: *Succinivibrio*) and frequency of blowing
280 were positively correlated (P=0.02, Table 4). The other behavioral variables were not
281 significantly correlated to colonic bacteria families relative abundance (P>0.1, Table 4).

282

283 3.3.2. Umbrella test

284 Behavioral variables and bacterial concentrations were not significantly correlated (P>0.1,
285 Table 5). pH was negatively correlated with frequency of startle response (P=0.02, Table 5).

286 Behavioral variables and species number were not significantly correlated ($|r|<0.6$; P>0.3).

287 Behavioral variables and Shannon index were not significantly correlated ($|r|<0.6$; P>0.3).

288 Relative abundance of Ruminococcaceae (especially genus *Ruminiclostridium* and
289 *Ruminococcaceae* UCG-005) was positively correlated with duration of smelling the floor
290 (P=0.05, Table 6). Relative abundance of Ruminococcaceae (especially genus

291 *Ruminiclostridium 5*, *Ruminococcaceae UCG-002* and *Ruminococcaceae UCG-003*) was
292 negatively correlated with latency to feed (P=0.006, Table 6). Relative abundance of
293 Prevotellaceae was positively correlated with latency to feed (P=0.04, Table 6). The other
294 behavioral variables and bacteria family abundances were not significantly correlated
295 (P>0.05, Table 6).

296

297 **4. Discussion**

298 In this study, we examined and found data supporting the association between the fluctuations
299 of the colonic microbial ecosystem due to dietary changes and changes in behavior reflecting
300 anxiety-like disorders in horses. One strength of our study included the use of live horses as
301 model for their own species. Indeed interactions between the intestinal microbiota and the
302 host are host specific [33]. There is a marked inter-individual variability of the colonic
303 bacterial composition, which could be a confounding factor. Using fistulated animals, this
304 limitation could be resolved as we worked with the same individuals along the study. To
305 increase the relevance of the findings, horses should be tested after different lengths of time
306 eating each diet. Another study should be conducted to test the diets short- and long-term
307 effects on the microbiota.

308 To study the variations of the colonic microbial ecosystem, we combined the analysis of
309 bacterial composition (family) and microbial functionality of the colonic ecosystem. Recent
310 data brought out the fact that studying the microbial metabolism could be as important and
311 relevant as examining the gut microbiota composition in order to better understand the
312 complexity of the dynamic relationship of microbiota, diet, and mental health [34]. Under
313 dietary changes, the capability of bacteria to modify their metabolism could indeed be faster
314 than their capacity to alter their composition. The two diets that we distributed to the horses
315 modulated significantly the colonic microbiota in accordance with data reported in the

316 literature. As expected [35], the different alterations demonstrated a lower fibrolytic capacity
317 and a higher amylolytic capacity of the ecosystem when horses received **higher** starch intake.
318 This change was reflected by the significant increase of amylolytic bacteria concentration in
319 the colonic content of horses fed the high-starch diet compared the high-fiber diet.
320 Furthermore, the relative abundance of Succinivibrionaceae family increased with starch.
321 Bacterial species of *Succinovibrio* are included among the predominant amylose-,
322 amyloextrin- and maltose-utilizing bacteria [36-38]. And it was even reported that
323 *Succinivibrio dextrinosolvens* constitutes a high proportion of the cecal microbiome before the
324 onset of laminitis [39]. Cellulolytic and amylolytic bacteria hydrolyse plant structural
325 cellulose and starches respectively into simple sugars (cellobiose, glucose) which are further
326 fermented by other intestinal microorganisms into pyruvate and then short chain fatty acids,
327 lactate, and gases (CO₂ and CH₄). The types of fermentation metabolites produced from
328 pyruvate in the hindgut of horses depend on the dietary environment [35] and can impact the
329 fecal pH. Although we found no significant differences in the colonic content pH of horses
330 fed high-starch or high-fiber diet, pH was negatively correlated with the frequency of startle
331 response in umbrella test. Hence, when the colonic content pH decreased which can induce
332 acidosis [40], the frequency of startle response increased which can reveal more anxiety in
333 horses. Our data support the fact that a dietary factor can induce changes in behavior during
334 stressful events. Likewise, during the novelty test our horses demonstrated a **significantly**
335 higher frequency of blowing when they were fed the high starch compared to the hay diet.
336 This frequency of blowing was positively correlated with the concentration of amylolytic
337 bacteria and the relative abundance of Succinivibrionaceae family. Blowing in horses may be
338 considered as an alert or alarm type of behavior and may be associated with anxiety [41]. In
339 our study, the horses seemed to be **more** over reactive in the novelty test **when fed the high**
340 **starch** diet than the hay diet. However, they did not show significant behavioral differences

341 during the umbrella test. During this test, all the horses stopped eating few seconds and some
342 of them showed a startle response in reaction to the sudden event, with no difference between
343 the diets. This lack of difference may be due to a ceiling effect, because all the horses
344 exhibited very strong responses to the sudden opening of the umbrella. This hypothesis was
345 also suggested in calves [42] and in lambs [31]. In addition, according to the appraisal
346 theories of cognitive psychology of Scherer to assess emotions [43], the evaluation of
347 suddenness is the most automatic evaluative process. It can be argued that suddenness caused
348 specific reflex responses, making it difficult to discriminate the variation in sentience and
349 decision making between individuals.

350 A recurring criticism in studies examining the link between colonic microbiota and anxiety-
351 like disorders is that data do not permit the elucidation of whether an altered microbiota are
352 the cause or consequence of behavioral changes. As each individual in our study was
353 submitted to the same controlled management parameters in terms of housing, bedding,
354 exercise, etc. except for the diet, we could hypothetically assign the observed behavioral
355 changes during stressful tests to the alterations of the microbial ecosystem due to dietary
356 modifications. We also found positive correlations between the duration of smelling the floor
357 or the frequency of moving during novelty test and the diversity of colonic bacteria; and
358 between the duration of smelling the floor during umbrella test and the relative abundance of
359 Ruminococcaceae. Oppositely, bacterial diversity was negatively correlated with the duration
360 of feeding during novelty test. Smelling the floor and moving may reflect an exploratory
361 behavior in horses in a calm situation [44]. Therefore individuals who were more reactive in
362 our study spent less time smelling the floor but more time before eating and for eating, and
363 concomitantly had a lower bacterial diversity within the colon. Ruminococcaceae is a family
364 of fibrolytic and acid-intolerant bacteria that may decrease in abundance in response to dietary
365 change (concentrate diet) and intestinal disease in horses [45]. The decrease in bacterial

366 richness and diversity potentially reduces the resilience of the microbial ecosystem and can
367 result in a higher susceptibility to dysbiosis. Epidemiologic data have suggested that colic
368 could result from dysbiosis in the hindgut ecosystem as a result of dietary factors [35]. Other
369 epidemiological data have cited the temperament, and especially the irritability and
370 excitability of the horse as a risk factor for colic [46].

371

372 **5. Conclusion**

373 Modulation of the gut microbiota by changing the diet seemed to change the horses'
374 behavioral reactions in stressful situations. As in our previous study [13], behavioral
375 responses of anxiety were related to hindgut microbiota indicators of a high-starch diet. The
376 ability of diet to modulate the microbial structure and function of the gut shows the gut
377 microbiota to be not only a sensitive organ but also one with great potential to modulate
378 anxiety. More studies are needed to better understand the effect of diets on anxiety disorders
379 in mammals.

380

381 **Acknowledgments**

382 The authors wish to thank staffs of the horse facility for technical assistance.

383

384 **References**

385 [1] Collins, S. M., Surette, M., Bercik, P. The interplay between the intestinal microbiota and
386 the brain. *Nature reviews. Microbiology*. 2012,10:735.

387 [2] Cryan, J. F., Dinan, T. G. Mind-altering microorganisms: the impact of the gut microbiota
388 on brain and behaviour. *Nat. Rev. Neurosci*. 2012,13:701-12.

389 [3] Tengeler, A. C., Kozicz, T., Kiliaan, A. J. Relationship between diet, the gut microbiota,
390 and brain function. *Nutrition Reviews*. 2018,76:603-17.

- 391 [4] Foster, J. A., Rinaman, L., Cryan, J. F. Stress & the gut-brain axis: regulation by the
392 microbiome. *Neurobiology of Stress*. 2017.
- 393 [5] Liu, Y., Zhang, L., Wang, X., Wang, Z., Zhang, J., Jiang, R., et al. Similar fecal
394 microbiota signatures in patients with diarrhea-predominant irritable Bowel syndrome and
395 patients with depression. *Clinical Gastroenterology and Hepatology*. 2016,14:1602-11.e5.
- 396 [6] Vuong, H. E., Yano, J. M., Fung, T. C., Hsiao, E. Y. The microbiome and host behavior.
397 *Annu. Rev. Neurosci.* 2017,40:21-49.
- 398 [7] Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X. N., et al. Postnatal microbial
399 colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice.
400 *The Journal of physiology*. 2004,558:263-75.
- 401 [8] Nishino, R., Mikami, K., Takahashi, H., Tomonaga, S., Furuse, M., Hiramoto, T., et al.
402 Commensal microbiota modulate murine behaviors in a strictly contamination-free
403 environment confirmed by culture-based methods. *Neurogastroenterology & Motility*.
404 2013,25:521-371.
- 405 [9] Crumeyrolle-Arias, M., Jaglin, M., Bruneau, A., Vancassel, S., Cardona, A., Daugé, V., et
406 al. Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine
407 response to acute stress in rats. *Psychoneuroendocrinology*. 2014,42:207-17.
- 408 [10] Scott, K. P., Gratz, S. W., Sheridan, P. O., Flint, H. J., Duncan, S. H. The influence of
409 diet on the gut microbiota. *Pharmacol. Res. Rés. Pharmacol Res*. 2013,69:52-60.
- 410 [11] Jacka, F. N. Nutritional psychiatry: where to next? *EBioMedicine*. 2017,17:24-9.
- 411 [12] Jørgensen, B. P., Hansen, J. T., Krych, L., Larsen, C., Klein, A. B., Nielsen, D. S., et al.
412 A possible link between food and mood: dietary impact on gut microbiota and behavior in
413 BALB/c mice. *PloS one*. 2014,9:103398.

- 414 [13] Destrez, A., Grimm, P., Cézilly, F., Julliand, V. Changes of the hindgut microbiota due
415 to high-starch diet can be associated with behavioral stress response in horses. *Physiol.*
416 *Behav.* 2015,149:159-64.
- 417 [14] Bell, R. J. W., Mogg, T. D., Kingston, J. K. Equine gastric ulcer syndrome in adult
418 horses: a review. *New Zealand Veterinary Journal.* 2007,55:1-12.
- 419 [15] Drogoul, C., Poncet, C., Tisserand, J. L. Feeding ground and pelleted hay rather than
420 chopped hay to ponies: 1. Consequences for in vivo digestibility and rate of passage of
421 digesta. *Anim. Feed Sci. Technol.* 2000,87:117-30.
- 422 [16] INRA. *Alimentation des chevaux.* Versailles: INRA editions; 1990.
- 423 [17] Bryant, M. P., Burkey, L. A. Cultural methods and some characteristics of some of the
424 more numerous groups of bacteria in the bovine rumen. *J. Dairy Sci.* 1953,36:205-17.
- 425 [18] Hungate, R. E. Chapter IV A roll tube method for cultivation of strict anaerobes. In:
426 Norris JR, Ribbons DW, eds. *Methods Microbiol.:* Academic Press; 1969. p. 117-32.
- 427 [19] Leedle, J., Hespell, R. B. Differential carbohydrate media and anaerobic replica plating
428 techniques in delineating carbohydrate-utilizing subgroups in rumen bacterial populations.
429 *Appl. Environ. Microbiol.* 1980,39:709-19.
- 430 [20] Julliand, V., de Vaux, A., Millet, L., Fonty, G. Identification of ruminococcus
431 flavefaciens as the predominant cellulolytic bacterial species of the equine cecum. *Appl.*
432 *Environ. Microbiol.* 1999,65:3738-41.
- 433 [21] Mackie, R., Heath, S. Enumeration and isolation of lactate-utilizing bacteria from the
434 rumen of sheep. *Appl. Environ. Microbiol.* 1979,38:416-21.
- 435 [22] Lowe, S. E., Theodorou, M. K., Trinci, A. P., Hespell, R. B. Growth of anaerobic rumen
436 fungi on defined and semi-defined media lacking rumen fluid. *Microbiology.* 1985,131:2225-
437 9.

438 [23] Clarke, K. R., Owens, N. J. P. A simple and versatile micro-computer program for the
439 determination of 'most probable number'. *J. Microbiol. Methods.* 1983,1:133-7.

440 [24] Halliwell, G., Bryant, M. P. The cellulolytic activity of pure strains of bacteria from the
441 rumen of cattle. *J. Gen. Microbio.* 1963,32:441-8.

442 [25] Yu, Z., Morrison, M. Improved extraction of PCR-quality community DNA from digesta
443 and fecal samples. *BioTechniques.* 2004,36:808-13.

444 [26] Liu, Z., Lozupone, C., Hamady, M., Bushman, F. D., Knight, R. Short pyrosequencing
445 reads suffice for accurate microbial community analysis. *Nucleic Acids Res.* 2007,35:e120.

446 [27] Andersson, A. F., Lindberg, M., Jakobsson, H., Bäckhed, F., Nyrén, P., Engstrand, L.
447 Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PloS one.*
448 2008,3:e2836.

449 [28] Escudie, F., Auer, L., Bernard, M., Mariadassou, M., Cauquil, L., Vidal, K., et al.
450 FROGS: find, rapidly, OTUs with galaxy solution. *Bioinformatics.* 2017,7.

451 [29] Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., et al. SILVA: a
452 comprehensive online resource for quality checked and aligned ribosomal RNA sequence data
453 compatible with ARB. *Nucleic Acids Res.* 2007,35:7188-96.

454 [30] Peet, R. K. The measurement of species diversity. *Annu. Rev. Ecol. Syst.* 1974,5:285-
455 307.

456 [31] Destrez, A., Deiss, V., Leterrier, C., Boivin, X., Boissy, A. Long-term exposure to
457 unpredictable and uncontrollable aversive events alters fearfulness in sheep. *Animal.*
458 2013,7:476-84.

459 [32] Brossard, L., Martin, C., Michalet-Doreau, B. Ruminal fermentative parameters and
460 blood acido-basic balance changes during the onset and recovery of induced latent acidosis in
461 sheep. *Anim. Res.* 2003,52:513-30.

- 462 [33] Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., et al. Host-
463 gut microbiota metabolic interactions. *Science*. 2012,336:1262-7.
- 464 [34] Pizarro, N., de la Torre, R. Inter-relationship of the intestinal microbiome, diet, and
465 mental health. *Current Behavioral Neuroscience Reports*. 2018,5:1-12.
- 466 [35] Julliand, V., Grimm, P. The impact of diet on the hindgut microbiome. *J. of Equine Vet.*
467 *Sci*. 2017,52:23-8.
- 468 [36] Kotarski, S. F., Waniska, R. D., Thurn, K. K. Starch hydrolysis by the ruminal
469 microflora. *The Journal of Nutrition*. 1992,122:178-90.
- 470 [37] Chesson, A., Forsberg, C. Polysaccharide degradation by rumen microorganisms. *The*
471 *rumen microbial ecosystem*: Springer; 1997. p. 329-81.
- 472 [38] Stewart, C., Flint, H., Bryant, M. The rumen bacteria. *The rumen microbial ecosystem*:
473 Springer; 1997. p. 10-72.
- 474 [39] Milinovich, G. J., Klieve, A. V., Pollitt, C. C., Trott, D. J. Microbial events in the hindgut
475 during carbohydrate-induced equine laminitis. *Vet Clin North Am Equine Pract*. 2010,26:79-
476 94.
- 477 [40] Julliand, V., de Fombelle, A., Drogoul, C., Jacotot, E. Feeding and microbial disorders in
478 horses: Part 3—Effects of three hay:grain ratios on microbial profile and activities. *J. of*
479 *Equine Vet. Sci*. 2001,21:543-6.
- 480 [41] Bagshaw, C., Ralston, S., Fisher, H. Behavioral and physiological effect of orally
481 administered tryptophan on horses subjected to acute isolation stress. *Appl. Anim. Behav. Sci*.
482 1994,40:1-12.
- 483 [42] Boissy, A., Veissier, I., Roussel, S. Behavioural reactivity affected by chronic stress: An
484 experimental approach in calves submitted to environmental instability. *Anim. Welf*.
485 2001,10:S175-S85.

486 [43] Scherer, K. R. On the sequential nature of appraisal processes: indirect evidence from a
487 recognition task. *Cognition & Emotion*. 1999,13:763-93.

488 [44] Wolff, A., Hausberger, M., Le Scolan, N. Experimental tests to assess emotionality in
489 horses. *Behav. Process*. 1997,40:209-21.

490 [45] Daly, K., Proudman, C. J., Duncan, S. H., Flint, H. J., Dyer, J., Shirazi-Beechey, S. P.
491 Alterations in microbiota and fermentation products in equine large intestine in response to
492 dietary variation and intestinal disease. *Br. J. Nutr*. 2012,107:989-95.

493 [46] Gonçalves, S., Julliand, V., Leblond, A. Risk factors associated with colic in horses. *Vet.*
494 *Res*. 2002,33:641-52.

495

496

497

Figure captions

Fig 1. Diagram depicting the timing of the diets (**H** diet: high-fiber diet composed of 100% of **H**ay; **HB** diet: very low-fiber diet composed of 56% of **H**ay and 44% of **B**arley), the sampling collection and the behavioral tests.

Fig 2. The testing area for novelty and umbrella tests. Three zones were defined: Z1, Z2 and Z3.

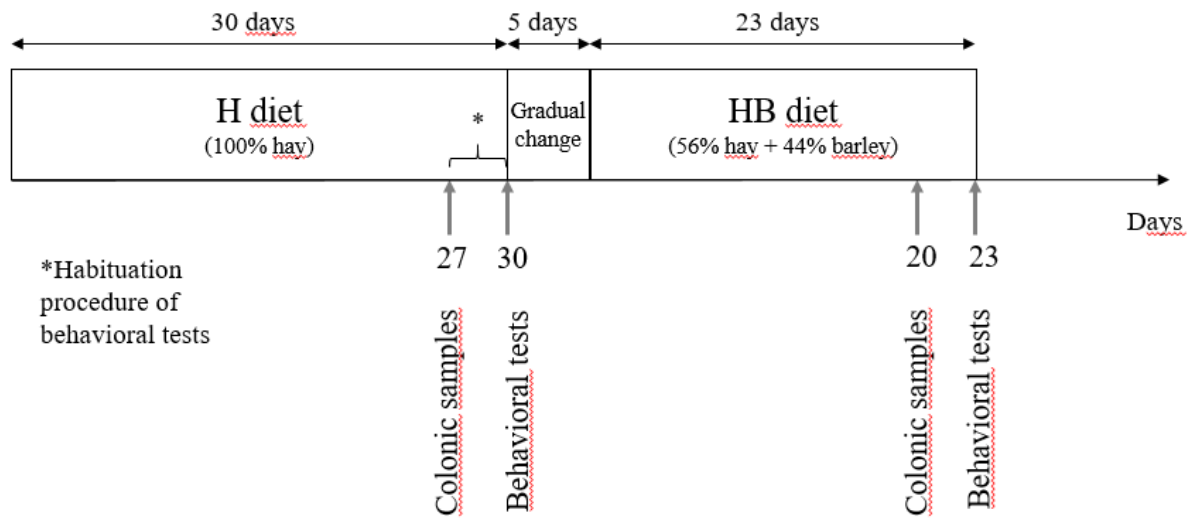


Fig. 1.

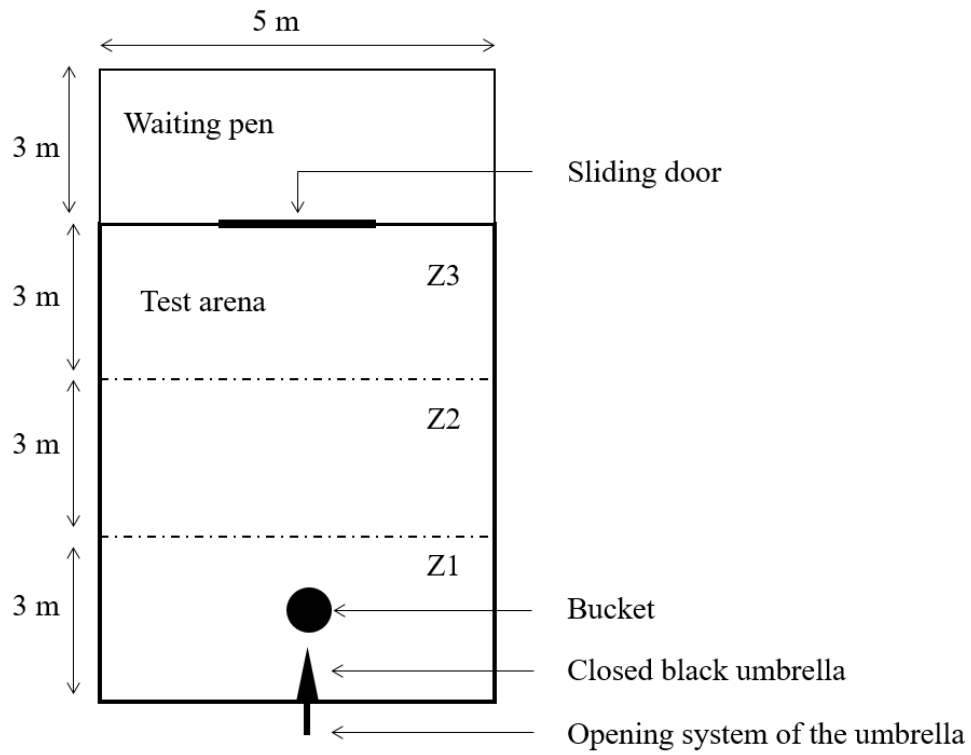


Fig. 2.

Table 1. Observed behaviors (n= 6 horses) during H diet (100% hay) and HB diet (56% hay and 44% barley) in both standardized stressful tests (novelty and umbrella tests)

Mean \pm S.E.	H diet	HB diet	Friedman's test	P-value
Novelty test				
Duration of feeding (s)	130.2 \pm 35.8	122.2 \pm 35.0	0.03	0.9
Duration of being vigilant (s)	75.7 \pm 26.8	85.0 \pm 26.0	0.008	0.9
Duration of smelling the floor (s)	29.3 \pm 5.6	19.0 \pm 8.0	0.8	0.4
Duration of interacting with the novel object (s)	36.0 \pm 13.4	49.4 \pm 16.3	0.5	0.5
Frequency of blowing	0.8 \pm 0.8	3.4 \pm 1.5	4.5	0.04
Frequency of moving	8.8 \pm 2.9	6.4 \pm 1.2	0.08	0.8
Latency to feed (s)	9.2 \pm 1.4	12.4 \pm 2.6	1.0	0.3
Umbrella test				
Duration of feeding (s)	132.6 \pm 38.9	97.4 \pm 25.2	0.5	0.5
Duration of being vigilant (s)	116.2 \pm 39.5	142.6 \pm 25.9	0.5	0.5
Duration of smelling the floor (s)	23.6 \pm 9.3	23.0 \pm 9.2	0.01	0.9
Frequency of startle response	0.6 \pm 0.4	0.4 \pm 0.2	0.06	0.8
Frequency of blowing	3.6 \pm 2.9	2.0 \pm 1.3	0.01	0.9
Frequency of moving	7.2 \pm 2.5	7.6 \pm 2.4	0.4	0.5
Latency to feed (s)	12.8 \pm 2.1	30.8 \pm 22.6	1.1	0.3

Table 2. Bacteria functional group, biochemical parameters and bacteria family (n= 6 horses) during H diet (100% hay) and HB diet (56% hay and 44% barley)

Mean \pm S.E.	H diet	HB diet	F	P-value
Bacteria enumeration				
(CFU:Colony-forming unit)				
Concentration (\log_{10} CFU/g) of cellulolytic bacteria	4.7 \pm 0.2	4.2 \pm 0.2	3.5	0.09
Concentration (\log_{10} CFU/g) of amylolytic bacteria	5.6 \pm 0.2	6.5 \pm 0.2	13.7	0.005
Concentration (\log_{10} CFU/g) of lactate-utilizing bacteria	6.1 \pm 0.3	6.9 \pm 0.3	2.9	0.1
Concentration (\log_{10} CFU/g) of total anaerobic bacteria	6.9 \pm 0.3	7.9 \pm 0.3	5.3	0.05
pH	6.7 \pm 0.1	6.9 \pm 0.1	1.4	0.3
Richness and diversity				
Species index	857 \pm 50	845 \pm 60	0.02	0.9
Shannon index	5.5 \pm 0.1	5.4 \pm 0.1	0.5	0.5
Family (relative abundance)				
Lachnospiraceae	21.7 \pm 1.5	22.8 \pm 1.9	0.2	0.7
Ruminococcaceae	16.0 \pm 1.0	20.0 \pm 3.2	1.4	0.3
Prevotellaceae	16.8 \pm 2.5	10.4 \pm 2.2	3.6	0.09
Streptococcaceae	0.06 \pm 0.02	4.0 \pm 2.2	3.4	0.1
Veillonellaceae	0.1 \pm 0.05	1.5 \pm 1.0	2.0	0.2
Succinivibrionaceae	0.006 \pm 0.006	1.3 \pm 0.5	6.3	0.04
Lactobacillaceae	0.3 \pm 0.09	0.8 \pm 0.3	3.2	0.1
Fibrobacteraceae	0.2 \pm 0.07	0.3 \pm 0.2	0.1	0.8

Table 3. Correlations between behavioral data (frequencies or duration in s) during novelty test and bacteria enumeration and pH

	Concentration of cellulolytic bacteria	Concentration of amylolytic bacteria	Concentration of lactate-utilizing bacteria	Concentration of total anaerobic bacteria	pH
Duration of feeding	-0.2	0.005	0.04	0.1	0.3
Duration of being vigilant	0.04	0.02	-0.2	-0.3	-0.3
Duration of smelling the floor	0.4	-0.5	-0.1	-0.3	0.1
Duration of interacting with the novel object	0.03	0.3	0.3	0.1	0.08
Frequency of blowing	-0.3	0.8	0.4	0.4	-0.2
Frequency of moving	0.4	-0.4	-0.4	-0.2	0.2
Latency to feed	-0.2	-0.1	-0.4	-0.2	0.3

Table 4. Correlations between behavioral data (frequencies or duration in s) during novelty test and bacteria family data (relative abundance)

	Lachnospiraceae	Ruminococcaceae	Prevotellaceae	Streptococcaceae	Veillonellaceae	Succinivibrionaceae	Lactobacillaceae	Fibrobacteraceae
Duration of feeding	-0.1	-0.4	-0.1	-0.03	-0.04	-0.1	-0.09	-0.5
Duration of being vigilant	0.3	0.4	0.2	0.07	0.3	0.2	-0.3	0.4
Duration of smelling the floor	-0.4	-0.02	0.3	-0.3	-0.03	-0.08	-0.2	0.6
Duration of interacting with the novel object	-0.04	0.6	-0.5	0.6	-0.5	0.4	0.6	0.4
Frequency of blowing	0.2	0.2	-0.1	0.5	0.3	0.7	0.1	0.01
Frequency of moving	-0.3	0.4	-0.07	-0.3	0.09	-0.09	0.01	0.5
Latency to feed	0.1	0.4	-0.2	0.1	0.4	0.3	0.009	0.4

Table 5. Correlations between behavioral data (frequencies or duration in s) during umbrella test and bacteria enumeration and pH

	Concentration of cellulolytic bacteria	Concentration of amylolytic bacteria	Concentration of lactate-utilizing bacteria	Concentration of total anaerobic bacteria	pH
Duration of feeding	0.02	-0.2	-0.2	-0.2	0.4
Duration of being vigilant	-0.08	0.3	0.3	0.3	-0.3
Duration of smelling the floor	-0.2	0.07	0.2	-0.1	-0.4
Frequency of startle response	0.03	0.2	0.4	0.4	-0.7
Frequency of blowing	0.03	0.2	0.1	0.1	-0.4
Frequency of moving	0.04	0.3	0.1	0.01	0.09
Latency to feed	-0.1	-0.2	-0.1	-0.08	-0.04

Table 6. Correlations between behavioral data (frequencies or duration in s) during umbrella test and bacteria family data (relative abundance)

	Lachnospiraceae	Ruminococcaceae	Prevotellaceae	Streptococcaceae	Veillonellaceae	Succinivibrionaceae	Lactobacillaceae	Fibrobacteraceae
Duration of feeding	-0.3	-0.2	-0.2	-0.2	-0.3	-0.3	-0.2	-0.4
Duration of being vigilant	-0.03	-0.1	0.4	0.3	0.4	0.4	0.07	0.5
Duration of smelling the floor	0.3	0.6	-0.5	0.2	-0.6	-0.04	0.4	-0.4
Frequency of startle response	-0.2	0.2	0.1	0.06	-0.3	-0.2	0.4	0.01
Frequency of blowing	0.2	0.3	-0.1	-0.03	-0.3	-0.08	0.2	0.03
Frequency of moving	0.6	0.1	-0.4	0.1	-0.09	0.2	0.09	-0.04
Latency to feed	0.2	-0.8	0.7	-0.3	0.4	-0.3	-0.4	-0.2