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

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Abstract

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

Keywords: behavior; diet; microbiota; stress; horses

1. Introduction

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

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

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

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

2. Material and methods

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

2.1. Subjects and facilities

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

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

2.3. Intestinal samples collection and microbiota analyses

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

2.3.1. Bacteria functional group analysis

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

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

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

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

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

Bioinformatic analyses were performed using the FROGS pipeline (Find Rapidly OTU with Galaxy Solution), which associates several tools to work on the file of sequences and obtain both the abundance table of operational taxonomic units (OTUs) and their taxonomic affiliation [28]. OTUs which are not present in at least 2 samples, or whose abundance was less than $5x10^{-5}$, were removed. Remaining OTU (operational taxonomic unit) were then aligned to the silva123 16S data base [29] using BLAST and were affiliated to the highest taxonomic rank possible. The abundance table and the associated multi-hit list were created. Relative abundance of the different families and genera was calculated. Richness (Number of species) and diversity (Shannon) indexes [30] were calculated from the abundance table using FROGS.

2.3.3. pH measurement

The pH of the colon was measured directly after the samples collection using the HI 1053 electrode (Hanna instrument, Lingolsheim, France) connected to an electronic pH-meter (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 m^2) and a test arena (45
202 m^2) 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

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

2.4.2. Umbrella test

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

2.5. Statistical analysis

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

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

3. Results

3.1. Behavioral analysis

During novelty test, the frequency of blowing was significantly higher in HB diet than in H diet (P= 0.04, Table1). The other behavioral variables were not significantly different between H diet and HB diet (P>0.3, Table 1). During umbrella test, behavioral variables were not significantly different between H diet and HB diet (P>0.3, Table 1).

3.2. Intestinal microbiota analysis

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

3.3. Correlations between behavioral and physiological data

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

3.2.1. Novelty test

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

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

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

3.3.2. Umbrella test

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

Behavioral variables and species number were not significantly correlated (|r|<0.6; P>0.3). Behavioral variables and Shannon index were not significantly correlated (|r|<0.6; P>0.3). Relative abundance of Ruminococcaceae (especially genus *Ruminiclostridium* and *Ruminococcaceae UCG-005*) was positively correlated with duration of smelling the floor (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 amylodextrin- 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

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

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

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

5. Conclusion

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

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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.

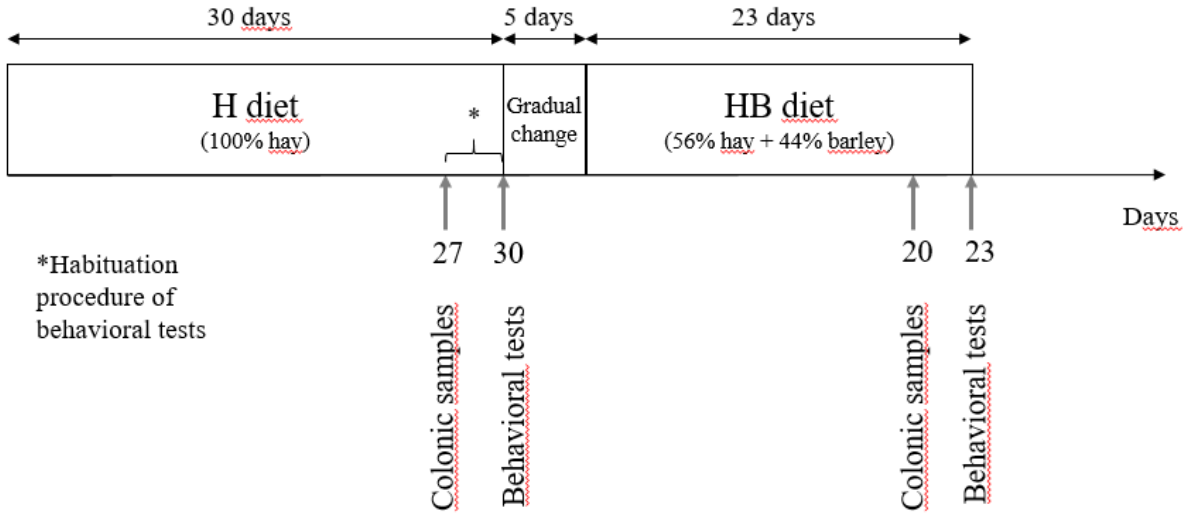
30 days
5 days
23 days
H diet
(100% hay)
*
Gradual change
HB diet
(56% hay + 44% barley)
Days
27
30
20
23
*Habituation procedure of behavioral tests
Colonic samples
Behavioral tests
Colonic samples
Behavioral tests

Fig. 1.

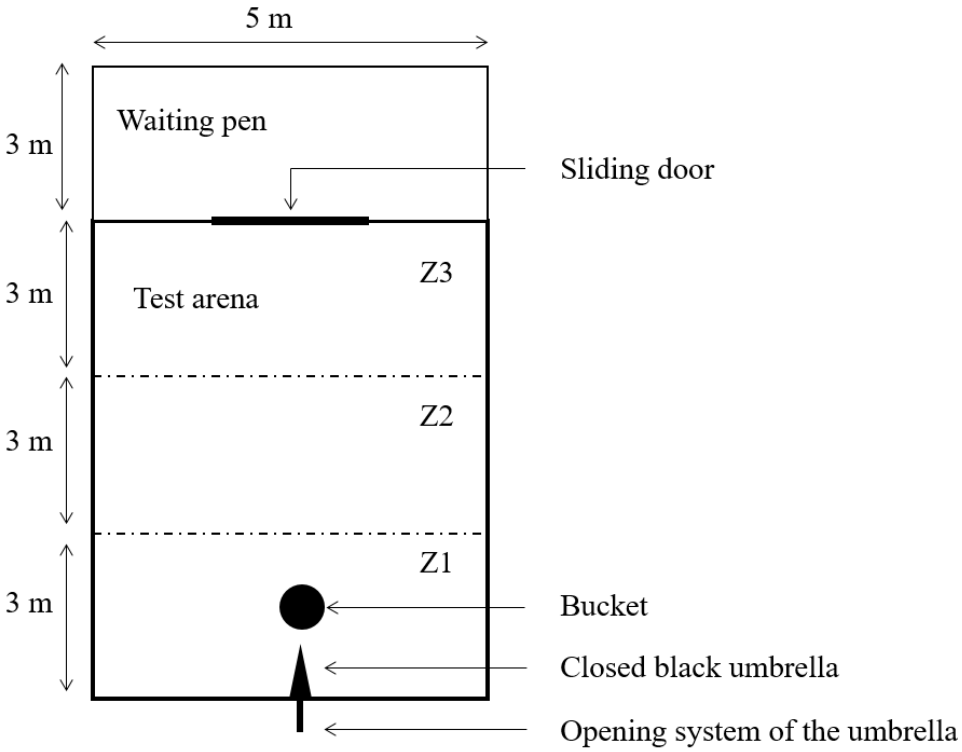
5 m
Waiting pen
3 m
Sliding door
Z3
3 m
Test arena
Z2
3 m
Z1
3 m
Bucket
Closed black umbrella
Opening system of the umbrella

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 ± S.E.	H diet	HB diet	Friedman's test	P-value
Novelty test				
Duration of feeding (s)	130.2 ± 35.8	122.2 ± 35.0	0.03	0.9
Duration of being vigilant (s)	75.7 ± 26.8	85.0 ± 26.0	0.008	0.9
Duration of smelling the floor (s)	29.3 ± 5.6	19.0 ± 8.0	0.8	0.4
Duration of interacting with the novel object (s)	36.0 ± 13.4	49.4 ± 16.3	0.5	0.5
Frequency of blowing	0.8 ± 0.8	3.4 ± 1.5	4.5	0.04
Frequency of moving	8.8 ± 2.9	6.4 ± 1.2	0.08	0.8
Latency to feed (s)	9.2 ± 1.4	12.4 ± 2.6	1.0	0.3
Umbrella test				
Duration of feeding (s)	132.6 ± 38.9	97.4 ± 25.2	0.5	0.5
Duration of being vigilant (s)	116.2 ± 39.5	142.6 ± 25.9	0.5	0.5
Duration of smelling the floor (s)	23.6 ± 9.3	23.0 ± 9.2	0.01	0.9
Frequency of startle response	0.6 ± 0.4	0.4 ± 0.2	0.06	0.8
Frequency of blowing	3.6 ± 2.9	2.0 ± 1.3	0.01	0.9
Frequency of moving	7.2 ± 2.5	7.6 ± 2.4	0.4	0.5
Latency to feed (s)	12.8 ± 2.1	30.8 ± 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 ± 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 ± 0.2	4.2 ± 0.2	3.5	0.09
Concentration (log_{10} CFU/g) of amylolytic bacteria	5.6 ± 0.2	6.5 ± 0.2	13.7	0.005
Concentration (log_{10} CFU/g) of lactate-utilizing bacteria	6.1 ± 0.3	6.9 ± 0.3	2.9	0.1
Concentration (log_{10} CFU/g) of total anaerobic bacteria	6.9 ± 0.3	7.9 ± 0.3	5.3	0.05
pH	6.7 ± 0.1	6.9 ± 0.1	1.4	0.3
Richness and diversity				
Species index	857 ± 50	845 ± 60	0.02	0.9
Shannon index	5.5 ± 0.1	5.4 ± 0.1	0.5	0.5
Family (relative abundance)				
Lachnospiraceae	21.7 ± 1.5	22.8 ± 1.9	0.2	0.7
Ruminococcaceae	16.0 ± 1.0	20.0 ± 3.2	1.4	0.3
Prevotellaceae	16.8 ± 2.5	10.4 ± 2.2	3.6	0.09
Streptococcaceae	0.06 ± 0.02	4.0 ± 2.2	3.4	0.1
Veillonellaceae	0.1 ± 0.05	1.5 ± 1.0	2.0	0.2
Succinivibrionaceae	0.006 ± 0.006	1.3 ± 0.5	6.3	0.04
Lactobacillaceae	0.3 ± 0.09	0.8 ± 0.3	3.2	0.1
Fibrobacteraceae	0.2 ± 0.07	0.3 ± 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