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1 Combining *in vivo* and *in vitro* approaches to investigate the effect of  
2 sainfoin on strongyle infection, immunity and large intestine ecosystem  
3 of horses

4

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## LAY SUMMARY

14 The use of bioactive plants in the diet of herbivores represents an innovative approach to  
15 controlling gastrointestinal parasites. Sainfoin, a forage legume rich in polyphenols, has been  
16 studied for its anti-parasitic properties. Sainfoin may act directly or indirectly on parasites, by  
17 modulating the intestinal microbiota and/or host immunity. In horses, the anti-parasitic effect  
18 of sainfoin on strongyles may vary depending on various factors, including the concentration  
19 and composition of polyphenols and the type of basal diet. This study was designed to  
20 investigate the effect of long-term inclusion of dehydrated sainfoin pellets in horses *in vivo*.  
21 Parasitic, microbial and immune parameters were measured to evaluate the direct and indirect  
22 anti-parasitic effects of sainfoin on strongyles. The anti-parasitic effect of two different sainfoin  
23 pellets was also compared *in vitro*, using Egg Hatch Tests and Larval Migration Inhibition Tests.  
24 The *in vivo* results showed no anti-parasitic effect of sainfoin on strongyles. The *in vitro* results  
25 showed that different sainfoins have different anti-parasitic efficacy. Overall, these results  
26 suggest that the polyphenolic composition of different sainfoin should be taken into account  
27 when studying its potential anti-parasitic effect *in vivo*.

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## TEASER TEXT

The anti-parasitic effect of the legume sainfoin is likely to depend on its polyphenolic concentration. The use of sainfoins with different polyphenolic concentrations may explain the inconsistency of *in vivo* results on the anti-parasitic effect of this plant.

## ABSTRACT

Sainfoin (*Onobrychis viciifolia*), a polyphenol-rich plant, has shown promising anti-parasitic properties in ruminants, but results in horses are fewer and inconsistent. The mechanisms of action involved are not fully understood and different factors may influence its anti-parasitic properties. Recently, it has been shown that the effect of sainfoin depends on the horse's diet. Indeed, the inclusion of dehydrated sainfoin pellets in a high starch diet limited the rate of increase in strongyle egg shedding over a short period of time (21 days). The objective of this study was to evaluate, *in vivo*, the effect of long-term inclusion of sainfoin in a high starch diet on strongyle infection and intestinal health in horses and to compare *in vitro* anti-parasitic activity of two different dehydrated sainfoin pellets. Horses known to have a past history of strongyle egg excretion (n=16) were allocated to two groups and fed with a high starch diet containing either sainfoin pellets (SF) or control pellets (sunflower and hay) (CONT) for 84 days. *In vitro* tests including Egg Hatch Test (EHT) and Larval Migration Inhibition Test (LMIT) were performed with different concentrations of aqueous extracts of the sainfoin pellets. No effect of the inclusion of sainfoin in the high starch diet was observed on the number of strongyle eggs excreted in the feces ( $p=0.671$ ). At the different sampling dates, including sainfoin in a high starch diet induced some changes in the relative abundance of bacterial taxa (e.g., *Oscillibacter*,  $p<0.05$ ), but not on bacterial diversity (all  $p's>0.05$ ), function ( $p>0.05$ ) or activity ( $p>0.05$ ). Adding sainfoin to a high starch diet increased plasma acetate concentration ( $p=0.032$ ) but no statistically significant differences were observed on other markers of

54 intestinal integrity (plasma lipopolysaccharides) and health (complete blood count) (all  
55  $p$ 's>0.05). Both sainfoin pellets showed anti-parasitic activity in the two *in vitro* tests (all  
56  $p$ 's<0.05), and one sainfoin had a stronger anti-parasitic effect than the other (EHT, all  $p$ 's<0.05;  
57 LMIT,  $p=0.008$ ). Overall, these results suggest that the anti-parasitic effect of sainfoin may  
58 depend on its polyphenolic concentration. Thus, preliminary *in vitro* testing may help to identify  
59 sainfoin best suited for *in vivo* use.

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61 Keywords: Bioactive plant, egg hatch test, hindgut microbiota, immunity, larval migration  
62 inhibition test, strongyles

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## LIST OF ABBREVIATIONS

- 70 ASV: Amplicon sequence variant
- 71 BW: Body weight
- 72 C2: Acetate
- 73 C3: Propionate
- 74 C4: Butyrate
- 75 C5: Valerate
- 76 CBC: Complete blood count
- 77 CFU: Colony forming unit
- 78 DM: Dry matter
- 79 DMI: Dry matter intake
- 80 EHT: Egg hatch test
- 81 FEC: Fecal egg count
- 82 FROGS: Find rapidly OTU with galaxy solution
- 83 HCT: Hematocrit
- 84 iC4: Isobutyrate
- 85 iC5: Isovalerate
- 86 L3: Infective larvae
- 87 LA: Lactic acid
- 88 LDA: Linear discriminant analysis
- 89 LEfSe: Linear discriminant analysis effect size
- 90 LMIT: Larval migration inhibition test
- 91 LPS: Lipopolysaccharides
- 92 rDNA: ribosomal deoxyribonucleic acid
- 93 VFA: Volatile fatty acids

## INTRODUCTION

95 Strongyles and particularly cyathostomins are the most common gastrointestinal parasites  
96 infecting equids worldwide (Kaur et al. 2019). Excessive infections with these helminths can  
97 cause severe health issues such as diarrhea, weight loss, colic, or even death in severe cases  
98 (Corning, 2009). Deworming is the most common strategy used to treat gastrointestinal  
99 helminth infections, but the inappropriate use of anthelmintics has led to the emergence of  
100 drug resistance. In horses, resistance to benzimidazole and pyrimidine is well established, and  
101 resistance to macrocyclic lactone drugs is currently increasing (Nielsen, 2022; Merlin et al.,  
102 2024). Furthermore, dewormers might interfere with the gastrointestinal microbiota (Kunz et  
103 al., 2019) and its fibrolytic function (Daniels et al., 2020). Finally, once released in the  
104 environment, anthelmintic drugs can be toxic for terrestrial and aquatic organisms (Lumaret et  
105 al., 2012). For these reasons, it is necessary to seek more sustainable strategies for the control  
106 of gastrointestinal parasites.

107 Among the possible strategies, dietary interventions including supplementation with the  
108 polyphenol-rich legume sainfoin (*Onobrychis viciifolia*) represent an innovative approach to  
109 control strongyle infection. The principal hypothesis put forward to explain the anti-parasitic  
110 properties of this plant is its high concentration of secondary metabolites, especially condensed  
111 tannins and other polyphenols (Hoste et al., 2006). The potential anti-parasitic properties of  
112 sainfoin *in vivo* have been first studied in ruminants. Both *in vitro* and *in vivo* experiments have  
113 provided evidence showing that sainfoin can impair parasite performance, alleviating infection  
114 burden and reducing the transmission potential of parasites (Hoste et al., 2015), although this  
115 might also depend on the species of parasites involved. In sheep and goats infected with the  
116 strongyles *Haemonchus contortus*, and *Trichostrongylus colubriformis*, sainfoin consumption  
117 has been associated with a decrease in infection burden and parasite fecundity (Heckendorn et  
118 al., 2006; Valderrábano et al., 2010). A reduction in parasite fecundity was also observed in

119 calves infected with *Ostertagia ostertagi* but the parasite burden was not reduced. However, no  
120 anti-parasitic effect of sainfoin was observed when the calves were infected with *Cooperia*  
121 *oncophora* (Desrues et al., 2016). A few studies in ruminants have also investigated the possible  
122 effect of sainfoin on host immunity and microbiota, which may suggest indirect mechanisms of  
123 action of this plant. Helminth-infected sheep fed sainfoin hay for 42 days had higher numbers  
124 of Th2-associated mucosal eosinophils and mast cells than sheep fed grass hay (Ríos-De  
125 Álvarez et al., 2008). In addition, lambs fed sainfoin pellets for 14 days showed changes in the  
126 relative abundance of specific rumen microbiota compared to lambs fed meadow hay (Petrič et  
127 al., 2022), but the targeted analysis of bacterial taxa using qPCR did not allow an assessment  
128 of the effect of sainfoin on the ruminal microbiota as a whole.

129 An important consideration for the potential anti-parasitic effect of sainfoin and the composition  
130 of its secondary metabolites include the inherent plant variability in nutrient profile influenced  
131 by environmental conditions (e.g. seasons/weather) and plant genetic variability (Manolaraki,  
132 2011; Hoste et al., 2015). The type of tannins (hydrolysable or condensed) can also have an  
133 effect on the ruminal microbiota, as lambs fed with different tannins for 75 days showed  
134 different changes in the composition of the ruminal microbiota (Salami et al., 2018). These  
135 results on the anti-parasitic effect of sainfoin in ruminants prompted further research in horses,  
136 to apprise the potential for its use as a feed supplement. To the best of our knowledge, four  
137 studies have investigated the potential of sainfoin as an anti-parasitic feed in horses, with  
138 somewhat inconsistent results. The first study reported an *in vitro* effect of sainfoin, with a  
139 reduction in strongyle egg hatching rate and larval development rate (Collas et al., 2018).  
140 However, adding sainfoin to the diet did not affect the number of strongyle eggs excreted in the  
141 feces. These results were corroborated by a subsequent study, which again reported no effect of  
142 sainfoin on egg excretion *in vivo* (Malsa et al., 2022). On the contrary, two recent studies  
143 reported a reduction in the number of eggs excreted and a decrease in the motility of infective

144 larvae in horses fed a sainfoin-supplemented diet (Grimm et al., 2022, Laroche et al., 2024). In  
145 the most recent study, it was shown that the effect of the sainfoin supplementation on egg  
146 excretion depended on the type of diet offered to the horses (high fiber or high starch) (Laroche  
147 et al., 2024). Horses fed the high starch diet excreted more strongyle eggs than horses fed the  
148 high fiber diet, possibly due to the alteration of the large intestinal microbial ecosystem with  
149 the high starch diet, and the inclusion of dehydrated sainfoin pellets in the high starch diet  
150 limited the rate of increase in egg excretion. Laroche et al., (2024) also assessed markers of  
151 immunity, and hindgut microbiota diversity, composition and function to investigate the  
152 potential indirect anti-parasitic effect of sainfoin and its impact on digestive health. The results  
153 showed minor changes in these parameters, suggesting that dehydrated sainfoin pellets exert  
154 their potential anti-parasitic properties mainly through direct effects on strongyles. Finally,  
155 these rather inconsistent results are based on short-term dietary supplementations [maximum  
156 28 days in Grimm et al. (2022)], and long-term studies are needed to complete the current  
157 knowledge on the possible effects of sainfoin on the intestinal health of horses.

158 The current work investigated the direct and indirect effects of a long-term sainfoin-  
159 supplementation in horses receiving a high starch diet (84 days). Specifically, we examined if  
160 sainfoin-supplementation affected i) the excretion of strongyle eggs, ii) the diversity,  
161 composition, function, and activity of the large intestine microbiota, iii) the integrity of the  
162 intestinal mucosa, and host health. Then, we compared *in vitro*, the anti-parasitic effect of two  
163 different dehydrated sainfoin pellets [the pellets used in a previous study (Laroche et al. 2024)  
164 and the pellets used in the current study]. To this purpose, an Egg Hatch Tests (EHT) and a  
165 Larval Migration Inhibition Tests (LMIT) were carried out.

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## MATERIALS AND METHODS

### 170 *IN VIVO EXPERIMENT*

171 The study was approved by the Committee on the Ethics of Animal Experiments of the  
172 University of Burgundy, and authorized by the French Ministry of Research with the permit  
173 number #36339-2022040417102331

174

### 175 *ANIMALS AND MANAGEMENT*

176 This study was conducted in the experimental stables of Lab To Field (Créancey, France) from  
177 May 2022 to October 2022, during the period of highest strongyle egg shedding (Wood et al.,  
178 2013). Sixteen adults ( $\geq 5$  years) « Trotteurs Français » geldings with a known past history of  
179 strongyle egg excretion (Mean $\pm$ SD: 366 $\pm$ 406 at D-28 and 925 $\pm$ 716 at D0) were included in this  
180 trial. All horses were up to date with their vaccinations according to the protocol established by  
181 the in-house veterinarian. Five horses were last dewormed 8 months prior to the start of the  
182 study with moxidectin (Equest, Zoetis, 0.4 mg/kg) and seven horses were last dewormed with  
183 ivermectin (Alverin, Zoetis, 0.2 mg/kg) 3 months prior to the start of the trial. The date and the  
184 type of the last deworming of four horses were unknown. Horses were housed individually in  
185 13.3 m<sup>2</sup> boxes. Every day, they were exercised 1 hour in an automatic walker (6 km/h) and spent  
186 4.5 hours in overgrazed paddocks in groups of 2 within the same dietary treatment. Horses had  
187 free access to water and salt lick blocks (Solsel, K+S Minerals and Agriculture GmbH, Kassel,  
188 Germany). The health of the horses was monitored daily.

189

### 190 *EXPERIMENTAL DESIGN AND DIETARY TREATMENTS*

191 Horses were included in a longitudinal study of 113 days (D<sub>-28</sub> to D<sub>84</sub>) (Fig 1). Prior to the study,  
192 the horses were grazed in groups and fed with hay *ad libitum*. At the start of the trial, each horse  
193 was fed individually, and an acclimatization period was conducted from D-28 to D0, during

194 which all horses were fed a high starch diet (1.4g starch/kg of bodyweight/meal - 14.61% of  
195 DMI). This diet contained hay (61.1% of DMI), barley (23.3% of DMI) and a mixture (15.3%  
196 of DMI) of sunflower and hay pellets (Table 1). During the first 5 days of this period, barley  
197 was gradually introduced into the horses' diet to reduce the risk of gastrointestinal disease. Fecal  
198 egg count of horses was monitored every week during this period (Fig S1). At D<sub>0</sub>, the horses  
199 were divided into two homogeneous experimental groups (n = 8 individuals in each group)  
200 according to their Fecal Egg Count (FEC), bodyweight (BW), age, and fecal pH (Table S1). An  
201 experimental period was then carried out from D<sub>1</sub> to D<sub>84</sub> during which one group of horses was  
202 kept on the same diet (CONT), while the other group of horses was fed a sainfoin diet (SF). The  
203 SF diet consisted of the same high-starch diet but with 15.3% DMI of dehydrated sainfoin  
204 pellets (Perly cultivar, Equifolia, Multifolia, Viapres Le Petit, France) instead of the mixture of  
205 sunflower and hay pellets (Table 1). Dehydrated sainfoin pellets contained 1.15% DM of  
206 condensed tannins and 2.16% DM of total phenols (Folin method N-AOEN/M/119, Inovalys  
207 Laboratory, Nantes, France); tannins and phenols were considered negligible in the mix of  
208 sunflower meal and hay pellets. Analysis of the biochemical composition of the feed ingredients  
209 was performed on one grab sample by DairyOne (Equine Complete analysis; Ithaca, USA). The  
210 digestible energy of the feed ingredients was calculated using the National Research Council  
211 (2007) equations. The two diets were formulated to be iso-caloric and iso-nitrogenous (Table  
212 1) and to meet the energy requirements for horses moderately exercised (National Research  
213 Council, 2007). Digestible Energy of each diet was calculated by using the following formula:

$$214 \quad DE \text{ (kcal/kg DM)} = \sum \left( \frac{\% \text{ DMI ingredient} \times DE \text{ ingredient}}{100} \right)$$

215 Ration was given at 08:00 and 16:45 in two equal portions per day. There was no feed refusal.

216

217 ***STRONGYLE FECAL EGG COUNT***

218 Fecal samples were collected for each horse at day 0 (D<sub>0</sub>) and then every two weeks (from D<sub>14</sub>  
219 to D<sub>84</sub>) by rectal grab and were stored at 4°C until fecal egg counts were carried out on the same  
220 day. To assess the number of strongyle eggs excreted in the feces, 42 ml of NaCl-saturated water  
221 (300g/L) were added to three grams of feces and mixed. The suspension was then filtered  
222 through a sieve (500µm pore size) and the filtrate was inserted into the two chambers of a  
223 McMaster slide with a detection limit of 50 eggs per gram of feces. After ten minutes, the  
224 number of eggs was counted under an optical microscope (magnification ×10) in each chamber.  
225

226 Fecal egg count reduction tests (FECRT) were carried out using the eggCounts package for R  
227 (version 4.1.1). The confidence interval was estimated using the fecrtCI function.  
228

### 229 ***ANALYSIS OF FUNCTIONAL BACTERIAL GROUPS***

230 At D<sub>0</sub>, D<sub>42</sub> and D<sub>84</sub> the conventional anaerobic culture technique in roll tubes (Hungate and  
231 Macy, 1973) was used to enumerate total anaerobic, cellulose-utilizing, starch-utilizing and  
232 lactate-utilizing bacteria contained in each fecal sample. For each horse and immediately after  
233 collection by rectal grab, feces were sampled in a container filled to capacity to avoid the  
234 presence of oxygen. One gram of fresh feces was directly isolated and mixed with a mineral  
235 solution and serially diluted to a decimal concentration. Dilutions were then introduced under  
236 a continuous flow of CO<sub>2</sub> (Bryant and Burkey, 1953) onto a non-selective medium for the  
237 cultivation of total anaerobic bacteria (Leedle and Hespell, 1980; Julliand et al., 1999).  
238 Additionally, selective media containing starch (Lowe et al., 1985) or lactic acid (Mackie and  
239 Heath, 1979) were inoculated to cultivate amylolytic and lactic acid-utilizing bacteria,  
240 respectively. After 72 hours of incubation at 38°C, bacterial concentrations were quantified  
241 through colony enumeration, expressed as Colony Forming Units (CFU) per gram of feces.  
242 Cellulolytic bacteria were cultivated in a complex liquid medium with a cellulose source

243 provided by a filter paper strip, maintained for 14 days at 38°C (Halliwell and Bryant, 1963).  
244 Concentrations were determined using the McGrady method, based on the most probable  
245 number (MPN) per gram of feces. All bacterial concentrations were log<sub>10</sub>-transformed.

246

#### 247 ***BACTERIAL 16S RIBOSOMAL RNA GENE SEQUENCING ANALYSIS***

248 At D<sub>0</sub>, D<sub>42</sub> and D<sub>84</sub>, an aliquot of fresh feces was sampled sterilely, and stored at -80°C for  
249 molecular biology analysis. The method described by Yu and Morrison (Yu and Morrison, 2004)  
250 was used to extract total DNA from 0.25 g of feces. The quantity and purity of DNA obtained  
251 were assessed using a spectrophotometer (SpectraMx ID3, Molecular devices, San Jose, USA).  
252 The V3-V4 hypervariable region of the bacterial 16S DNA was amplified, sequenced and  
253 analyzed as described by Grimm et al. (2020). Briefly, a first round of polymerase chain reaction  
254 (PCR) was run to obtain amplicons of the V3-V4 region and checked by electrophoresis on a  
255 2% agarose gel. Amplicons were submitted to a second round of PCR after purification and the  
256 resulting products were sequenced using an Illumina MiSeq run of 250 base paired ends  
257 (Illumina Inc., San Diego, CA, USA) at the Genotoul bioinformatics platform (Toulouse,  
258 France). FROGS pipeline (Find Rapidly OTU with Galaxy Solution) (Escudié et al., 2018) was  
259 used to perform the bioinformatic analyses. First, sequences were assembled and cleaned.  
260 Swarm method was used to carry out the clustering. Chimeras were eliminated and the resulting  
261 Amplicon Sequence Variants (ASVs) that were not present in at least four samples or whose  
262 abundance was  $< 5 \times 10^{-5}$  were removed. Remaining ASVs were then aligned to the SILVA 16S  
263 database (version 138.13) using BLAST. At the end of this step, the abundance table and the  
264 associated multi-hits list were generated. The affiliations were then filtered to eliminate ASVs  
265 with a percentage of coverage below 99% and a percentage of identity below 90%. Finally, a  
266 normalization of ASVs was carried out by reducing all samples to the same number of  
267 sequences based on the sample with the lowest number of sequences. Richness (number of

268 ASVs and Chao1), diversity indices (Shannon and inverse Simpson), and Bray-Curtis distance  
269 were calculated from the abundance table.

270

### 271 ***ANALYSIS OF FECAL DRY MATTER, pH AND FERMENTATION END PRODUCTS***

272 Before the start of the experimental period (D<sub>0</sub>) and then every two weeks (from D<sub>14</sub> to D<sub>84</sub>),  
273 100 grams of feces were weighed, oven-dried at 70°C for 72h and reweighed to measure fecal  
274 dry matter (DM).

275 Approximately 100 grams of feces were pressed through a 100 µm filter. The filtrates were used  
276 to measure pH with an electronic pH meter (CyberScan pH 510; Eutech Instrument Europe  
277 B.V., Landsmeer, The Netherlands). The filtrates were subsequently frozen (-20°C) with a  
278 preservative solution composed of 4.25% H<sub>3</sub>PO<sub>4</sub> and 1.0% HgCl<sub>2</sub> for later analyses of  
279 Volatiles Fatty Acids (VFAs), and without the preservative solution for lactic acid analysis.

280 The concentration of total VFAs, acetic (C<sub>2</sub>), propionic (C<sub>3</sub>), isobutyric (iC<sub>4</sub>), butyric (C<sub>4</sub>),  
281 isovaleric (iC<sub>5</sub>) and valeric (C<sub>5</sub>) acids were analyzed by gas-liquid chromatography (Clarus  
282 500; PerkinElmer, Courtaboeuf, France), as described by Jouany (Jouany, 1982). Concentration  
283 of total VFAs and each VFA was computed as mmol/L of fecal filtrate and as the proportion of  
284 the total VFAs. To determine D-lactic and L-lactic acid concentrations, a spectrophotometric  
285 method at 340nm with an enzymatic colorimetric kit (Megazyme, D-/L-lactic acid (rapid) Assay  
286 Kit, Megazyme International Ireland Ltd., Wicklow, Ireland) was used following the procedure  
287 described by Grimm et al. (2017). Lactic acid concentration (mmol/L of fecal filtrate) was  
288 expressed as a proportion of the total lactic acid concentration.

289

### 290 ***ANALYSIS OF MARKERS OF HEALTH AND INTESTINAL INTEGRITY***

291 Before the start of the experimental period (D<sub>0</sub>) and then every two weeks (from D<sub>14</sub> to D<sub>84</sub>),  
292 20 ml of blood were collected by venipuncture of the jugular vein of each horse, in EDTA and

293 heparin tubes. One tube was used immediately to performed complete blood count (CBC). The  
294 other tubes were centrifuged immediately (1500g for 20 min at 4°C), aliquots were stored at -  
295 20°C and used for lipopolysaccharides (LPS) and acetate (C2) analyses.

296 Complete blood count was used as a marker of health and carried out with the analyzer Scil Vet  
297 abc plus+ to determine the hematocrit (HCT) as the percentage of packed red blood cells,  
298 hemoglobin (g/dL) and total peripheral white blood cells, lymphocytes, granulocytes, and  
299 eosinophils concentrations ( $10^3/\text{mm}$ ).

300 Plasma concentration of total esterified LPS was used as a marker of mucosal permeability and  
301 was determined by direct quantification of 3-OH C10, C12, C14, C16 and C18 using HPLC  
302 coupled with tandem MS (HPLC-MS/MS, Agilent QQQ 6460 device) as described in Pais de  
303 Barros et al., (2015). Plasma acetate concentration was used as a marker of mucosal absorption  
304 and was measured spectrophotometrically at 450 nm in a 96-well microplate (AMR 100) using  
305 an enzymatic colorimetric kit (Acetate Colorimetric Assay Kit, Merk, Darmstadt, Germany),  
306 following the procedure described by Laroche et al. (2024).

307

## 308 ***IN VITRO EXPERIMENT***

### 309 ***STRONGYLE EGG COLLECTION***

310 The strongyle eggs used for the *in vitro* experiment were collected from the fresh feces of three  
311 naturally infected horses (FEC > 1000 eggs/g of feces), which were immediately brought to the  
312 laboratory. The egg suspension was prepared by passing the feces through a series of four sieves  
313 (500 $\mu\text{m}$ , 250 $\mu\text{m}$ , 150 $\mu\text{m}$  and 38 $\mu\text{m}$ ). Eggs were retained on the last sieve (38 $\mu\text{m}$ ) and collected  
314 with 100ml of distilled water. The suspension was then centrifuged 3 min at 240g and the  
315 supernatant was removed to obtain a pellet containing the eggs. To obtain a clear strongyle egg  
316 suspension, another centrifugation for 3min at 240g with saccharose (6.8g/mL) was carried out.

317 The supernatant was transferred on a 38 $\mu$ m sieve to wash eggs with distilled water. The eggs  
318 were finally diluted in distilled water at a concentration of approximately 10 eggs per 10 $\mu$ L.

319

### 320 ***INFECTIVE LARVAE (L3) CULTURE***

321 The cultures of infective larvae were carried out in 48-well plates and 100  $\mu$ l of the suspension  
322 containing approximately 100 eggs were added to each well. The plate was then incubated 24h  
323 at 25°C to allow egg hatching. Culture medium was then added in each well and the plate was  
324 again incubated for 5 days at 25°C to allow for the development of infective larvae. Culture  
325 medium was prepared according to the protocol described by Hubert and Kerboeuf (1984) with  
326 9 mL of yeast extract solution (1g yeast extract + 90mL 0.85% solution of NaCl; autoclave for  
327 20 min) mixed with 1 mL of 10  $\times$  concentrated Earle's solution. To finish, *Escherichia coli*  
328 suspension at 1.5mg/mL was added in proportion 2:1. After the complete incubation period,  
329 each well content was collected and washed with 200 $\mu$ L of ultra-pure water and pooled in a  
330 50mL tube. The number of larvae in 4 drops of 10 $\mu$ L solution was counted under a microscope  
331 (obj  $\times$ 10) and the average was calculated to determine the concentration of larvae. The  
332 concentration was then adjusted to obtain approximately 5-10 larvae per 10 $\mu$ L. Infective larvae  
333 were stored at 4°C and used within eight weeks post-harvesting.

334

### 335 ***PREPARATION OF SAINFOIN AQUEOUS EXTRACTS***

336 Two different aqueous extracts of Equifolia pellets provided by Multifolia (Viâpres-le-Petit,  
337 France) were tested *in vitro*. The first (SF1) refers to the Equifolia pellets used in a previous *in*  
338 *vivo* study (Laroche *et al.*, 2024) and the second (SF2) to the Equifolia® pellets used in the  
339 present *in vivo* study. Sainfoin pellets were grounded and passed through a sieve of 1mm. For  
340 the extraction of the water-soluble components, ultra-pure water was added to the resulting  
341 sainfoin powder and heated at 80°C for 25 min to obtain an initial concentration at 265mg/ml.

342 The aqueous extracts were then centrifuged at 4100g for 15 min and the supernatant was  
343 removed and sterilized through a 0.2µm filter.

344

#### 345 ***EGG HATCH TEST (EHT)***

346 For EHT, 48-well plates were used, and 100µL of the egg suspension were added to each well.  
347 The sainfoin aqueous extract and ultra-pure water were then added to obtain the different final  
348 concentrations detailed below, with a final volume of 265µL in each well. Each plate was  
349 incubated 48h at 25°C and the test terminated by the addition of 10µL Lugol iodine solution  
350 (Sigma Aldrich, UK). The number of eggs and L1 larvae were counted in each well using an  
351 inverted microscope (Optika IM-3). The percentage of hatched eggs at each sainfoin  
352 concentration was calculated as follows:

$$353 \left( \frac{\text{Number of L1}}{\text{Number of L1} + \text{Number of eggs}} \right) \times 100$$

354

#### 355 ***LARVAL MIGRATION INHIBITION TEST (LMIT)***

356 For each LMIT performed, approximately 100 L3 were incubated for 12 hours in a 48-well  
357 plate with different concentrations of each sainfoin aqueous extracts detailed below, with a final  
358 volume of 265 µL in each well. For the migration, the content of each well was then transferred  
359 on a 20µm mesh sieve (pluriStrainer, pluriSelect) disposed on another 48-well plate containing  
360 200µL of ultra-pure water in each well for 2 hours at 25°C in the dark. Sieves were then lifted  
361 out and ultra-pure water used to wash the outside of the sieves into the corresponding well.  
362 Afterwards, the sieves were inverted and the L3 that did not migrate were washed into the  
363 corresponding well in the next column. The L3 were fixed with 100% molecular grade ethanol  
364 and migrated and non-migrated L3 counted using an inverted microscope (Optika IM-3). The  
365 percentage of migrating L3 at each sainfoin concentration was calculated as follow:

366 
$$\left( \frac{\text{Number of migrated L3}}{\text{Number of migrated L3} + \text{Number of non - migrated L3}} \right) \times 100$$

367

368 ***EXPERIMENTAL DESIGN***

369 Two EHT were performed using two different strongyle egg suspensions and two different  
370 aqueous extracts from each Equifolia pellets used. For the first series of tests (EHT 1), nine  
371 concentrations of sainfoin were used (100, 80, 60, 40, 20, 10, 15, 5 and 0 mg/mL) and tested in  
372 six replicates. For the second series (EHT 2), again nine concentrations of sainfoin were used  
373 covering a smaller range (15, 12, 10, 9, 8, 7, 6, 5 and 0 mg/mL) in six replicates.

374 One LMIT was performed using a mix of two different L3 suspensions (one that was stored in  
375 the fridge for 2 weeks and the other for 1 week) to obtain a larval concentration of L3 > 1000  
376 L3/mL. One aqueous extract from each Equifolia pellets was used for this test, with eight  
377 concentrations 100, 60, 40, 20, 15, 10, 5 and 0 mg/ml, in six replicates.

378

379

380 ***STATISTICAL ANALYSES***

381 For the *in vivo* experiment, General Linear Mixed Models (GLMM) were run using PROC  
382 MIXED (SAS) to assess the effect of diet, time, and their interaction on FEC, functional  
383 bacterial groups, microbiota diversity and richness, microbial fermentation parameters and the  
384 markers of health and intestinal integrity. Logarithmic transformation was performed on the  
385 FEC and concentrations of each blood cell type to reduce skewness. For dependent variables  
386 that were measured every two weeks (FEC, fecal dry matter, pH, fermentation end products,  
387 and marker of health and intestinal integrity), time was considered as a continuous variable.

388 A principal coordinates analysis was used to analyze the beta diversity of the fecal microbiota  
389 with the vegan and ggordiplots packages in R, based on Bray Curtis distances. Differences

390 between dietary treatments at each day were assessed using a permutational multivariate  
391 analysis of variance (PERMANOVA) with 9999 permutations, employing the adonis function  
392 of the vegan package in R.

393 To analyze the abundance of bacterial taxa between the dietary treatments at each day, we ran  
394 a linear discriminant analysis effect size (LEfSe) with the Galaxy software package of the  
395 Huttenhower Laboratory (Segata et al., 2011). This model employs the non-parametric factorial  
396 Kruskal-Wallis (KW) sum-rank test to identify taxa with significant differential abundances  
397 between groups (using one-against-all comparisons). Finally, we performed linear discriminant  
398 analysis (LDA) to estimate the effect size using a threshold of 3 log LDA scores. We used  
399 bacterial taxa with varying abundances to generate cladograms that illustrate the effect of diet.  
400 For the results of the *in vitro* experiment, a logistic regression model was run using PROC  
401 PROBIT (SAS). The model allowed us to assess the dose-dependent effect of each sainfoin on  
402 egg hatching rate and larval migration and to compare the anti-parasitic efficacy of the two  
403 sainfoins.

404

## 405 **RESULTS**

### 406 ***IN VIVO EXPERIMENT***

#### 407 ***EFFECT OF SAINFOIN ON STRONGYLE FECAL EGG COUNT***

408 We did not find any difference in the fecal egg count (FEC) between the dietary treatments  
409 during the experimental period of 84 days (Fig 2, Table 2). The FECRT also showed that the  
410 two groups did not differ at either D14 or D84, as indicated by the 95% CI overlapping zero  
411 (S2 Table). However, the number of eggs excreted in the feces increased over time ( $p < 0.001$ ),  
412 possibly reflecting a seasonal effect.

413

414 ***EFFECT OF SAINFOIN ON THE DIVERSITY AND COMPOSITION OF THE FECAL***  
415 ***MICROBIOTA***

416 The initial 1,419,659 sequences of 16S rDNA V3-V4 regions obtained from the 48 fecal  
417 samples were reduced to 496,024 after quality filtering during bioinformatic processes, with a  
418 mean ( $\pm$  SD) of 10,334 ( $\pm$  1,848) reads per sample. These sequences were clustered into 2,520  
419 ASVs and assigned to 11 phyla, 18 classes, 31 orders, 53 families and 95 genera. The lowest  
420 number of sequences per sample was 7,073 and was used to normalize the data prior to the  
421 analysis of microbial richness,  $\alpha$ - and  $\beta$ -diversity, and relative abundances.

422 The dietary treatment had no effect on bacterial richness (the number of ASVs, Chao1 index)  
423 nor on the indices of  $\alpha$ -diversity (Shannon and inverse Simpson) (Fig 3, S3 Table). The principal  
424 coordinate analysis (PcoA) and PERMANOVA model showed that  $\beta$ -diversity did not differ  
425 between dietary treatments over time (Fig 4A, 4B and 4C).

426 Given the high number of unknown (1603/1768 ASVs; 90.7%) or multi-affiliated (127/1768  
427 ASVs; 7.2%) species, the analysis of relative abundances was conducted at the genus level as  
428 the lowest taxonomic level. The LEfSe analysis showed differences between relative  
429 abundances according to the experimental diet at the different sampling date (Fig 5, S4 Table).

430 When all horses were fed the control diet prior to the start of the experimental period (D<sub>0</sub>), the  
431 relative abundances of 5 genera differed between the two groups of horses (Fig 5A, S4 Table).

432 After 42 days of the start of experimental diets, the relative abundance of *Phoenicibacter*, an  
433 unknown genus from the *Bacteroidales RFI6 group* family and another unknown genus from  
434 the *UCG\_011* family was higher in the fecal microbiota of SF fed horses. On the other hand,  
435 *Succinivibrionaceae UCG\_002*, an unknown genus from *Barnesiellaceae* family and another  
436 unknown genus from *gir\_aah93h0* family were more abundant in the fecal microbiota of CONT  
437 fed horses (Fig 5B, S4 Table). At D84, the unknown genus from *gir\_aah93h0* family and  
438 *Succinivibrionaceae UCG\_002* were still more abundant in the fecal microbiota of CONT fed

439 horses and another unknown genus from the *Bacteroidales BS11 gut group* family was also  
440 more abundant in this group at this day. At the same day, the fecal microbiota of horses fed the  
441 SF diet was enriched in *Oscillibacter* (Fig 5C, S4 Table). The LEfSe analysis also showed  
442 differences between relative abundances within a same diet (CONT or SF) over time (Fig 6, S5  
443 table). In the fecal microbiota of CONT fed horses, the relative abundances of 3 genera  
444 including *Phoenicibacter*, *Acetitomaculum*, *Lachnospirillum*, and an unknown genus from  
445 *M2PB4\_65 termite group* family, were higher at D0. At D42, the relative abundance of  
446 *Candidatus Soleaferrea*, *Oscillibacter*, *Rikenellaceae RC9 gut group* and an unknown genus  
447 from the *Barnesiellaceae family* were higher and at D84 the genus *Papillibacter* was enriched  
448 (Fig 6A, S5 table). The fecal microbiota of SF fed horses was enriched in *Lachnospirillum*  
449 and an unknown genus from *gir\_aah93h0 family* at D0, in an unknown genus from *UCG-011*  
450 family at D42 and in *Faecalibacterium* at D84 (Fig 6B, S5 table).

451

#### 452 **EFFECT OF SAINFOIN ON FUNCTIONS AND ACTIVITY OF THE FECAL** 453 **MICROBIOTA**

454 No differences were found in the concentration of total anaerobic, cellulolytic, amylolytic and  
455 lactate utilizing bacteria between the two dietary treatments over time (Fig 7, S6 Table).  
456 However, the concentration of cellulolytic bacteria was lower at D<sub>84</sub> compared to D<sub>42</sub> (p=0.035)  
457 and the concentration of amylolytic bacteria was lower at D<sub>84</sub> compared to D<sub>0</sub> (p<0.001),  
458 irrespective of the experimental group (Fig 7, S6 Table), possibly reflecting an alteration in  
459 fibrolytic and amylolytic functions due to the high starch diet fed over a long period of time.  
460 There were no significant differences in fecal dry matter, fecal pH, fecal concentrations and  
461 proportions of fermentation end products (VFAs and lactate) between the two diets over time  
462 (Fig 8, S6 Table).

463

464

465 ***EFFECT OF SAINFOIN ON HEALTH PARAMETERS AND LARGE INTESTINE***  
466 ***MUCOSA INTEGRITY OF HORSES***

467 Complete blood counts (CBC) were carried out to assess different health parameters (Fig 9, S7  
468 Table). Hematocrit, hemoglobin and the amount of total white blood cells, lymphocytes,  
469 granulocytes, and eosinophils did not differ between diets over time. Horses in SF group had  
470 higher number of granulocytes than horses in CONT group independently of the sampling day  
471 ( $p=0.010$ , Fig 9E). A decrease in hematocrit ( $p<0.001$ , Fig 9A) and lymphocyte count ( $p=0.012$ ,  
472 Fig 9D) and an increase in eosinophil count ( $p=0.006$ , Fig 8F) were also observed over time,  
473 independently of diet. Including the FEC in the model showed a marginally non-significant  
474 negative correlation between this parameter and the hematocrit of horses ( $p=0.051$ ) and no  
475 correlation with lymphocytes and eosinophils count (Fig S2, S7 Table).

476 Concentrations of blood LPS, blood acetate and fecal acetate were used to assess the effect of  
477 the diet on the integrity of the large intestine mucosa of horses. We did not find any difference  
478 in blood LPS and fecal acetate between the diets over time (Fig 10, S8 Table). However, the  
479 concentration of blood acetate increased in SF fed horses over time (Mean $\pm$ SD =  $1.29\pm 0.52$   
480 mmol/L at D0 to  $2.16\pm 0.54$  mmol/L at D84) compared to CONT fed horses (Mean $\pm$ SD =  
481  $1.32\pm 0.56$  mmol/L at D0 to  $1.32\pm 0.50$  mmol/L at D84) ( $p=0.032$ ) (Fig 10C, S8 Table).

482

483 ***IN VITRO EXPERIMENT***

484 ***EFFECT OF TWO DIFFERENT SAINFOIN PELLETS ON STRONGYLE EGG***  
485 ***HATCHING***

486 To compare the effect of two dehydrated sainfoin pellets on strongyle egg hatching and inter-  
487 assay repeatability, two EHT were carried out and the 50% lethal dose (LD50) of each sainfoin  
488 pellets was assessed for each test. In both EHTs, we found an inhibitory effect of sainfoin on

489 strongyle egg hatching. We also found that SF1 was significantly more effective at inhibiting  
490 egg hatching than SF2 in EHT 1 ( $p<0.001$ ) and EHT 2 ( $p=0.038$ ) (Fig 11 A and B). In both  
491 EHTs, the LD50 of SF1 was lower compared to the LD50 of SF2 (EHT 1: Mean - CI95% for  
492 SF1 = 5.78 – [5.51;6.04] mg/ml and Mean – CI95% for SF2 = 8.95 – [9.56;9.35] mg/ml; EHT  
493 2: Mean - CI95% for SF1 = 10.64 – [11.41;11.89] mg/ml and Mean – CI95% for SF2 = 13.40  
494 – [13.04;13.82] mg/ml) (Fig 11C and S3).

495

#### 496 ***EFFECT OF TWO DIFFERENT SAINFOIN PELLETS ON MIGRATION OF*** 497 ***STRONGYLE INFECTIVE LARVAE***

498 We performed a LMIT to compare the effect of two dehydrated sainfoin pellets on the migration  
499 of strongyle infective larvae. An inhibitory effect of sainfoin on the migration capacity of  
500 infective larvae was found, with a better inhibition effect for SF1 compared to SF2 ( $p<0.008$ )  
501 (Fig 12).

502

### 503 **DISCUSSION**

504 The objectives of this study were to investigate the effect of a long-term inclusion of dehydrated  
505 sainfoin pellets in a high starch diet on the strongyle infection, the large intestine microbiota  
506 and health of horses and to compare, *in vitro*, the anti-parasitic effect of two different dehydrated  
507 sainfoin pellets.

508 Contrary to our expectations, we did not find an effect of supplementation with the tested  
509 sainfoin on strongyle egg excretion. Similarly, we found no effects on the  $\alpha$  and  $\beta$  diversity of  
510 the fecal microbiota. The relative abundance of a few bacterial taxa changed during the  
511 experimental period when horses were fed the SF diet. However, we did not find any changes  
512 in the bacterial functions and activity of the large intestine microbiota. Finally, supplementation  
513 with the sainfoin used in this study improved the concentration of blood acetate but had no

514 effect on the other markers of intestinal mucosal integrity and health. The *in vitro* results showed  
515 that aqueous extracts of dehydrated sainfoin pellets inhibited the hatching of strongyle eggs and  
516 the migration of infective larvae. The two sainfoins were not equally effective at inhibiting egg  
517 hatching and migration of infective larvae.

518 Supplementing horses with sainfoin over 84 days had no effect on the number of strongyle eggs  
519 excreted in the feces. These results are not in agreement with those reported in a previous study  
520 where horses were fed a high starch diet and supplemented with sainfoin over a period of 21  
521 days (Laroche et al. 2024). Indeed, a slower rate of increase in egg excretion over time was  
522 reported in sainfoin supplemented horses. Several reasons might have caused the discrepancy  
523 between the two studies. First, Laroche et al. (2024) used a Latin square experimental design in  
524 which each individual moved between different experimental groups over time, thus limiting  
525 the weight of within-group differences between individuals. Individual variation in the efficacy  
526 of sainfoin to reduce strongyle egg excretion has already been reported (Grimm et al. 2022),  
527 therefore, experimental design that do not fully get rid of individual effects might be less  
528 powerful to appraise the effect of dietary treatments. Second, the two studies also differed in  
529 the amount of starch provided in the diet and the sainfoin pellets used for the supplementation  
530 were not the same. In particular, given that horses received a high starch diet for a longer period  
531 (113 days vs 21 days), the amount of starch fed in this study was deliberately lower (14.61% of  
532 DMI – 1.4 g/kg of BW/meal) compared to the previous study (21.5% of DMI – 1.9 g/kg of  
533 BW/meal in Laroche et al., 2024) to avoid jeopardizing the health of the horses (Julliand et al.,  
534 2006). While the amount of sainfoin fed daily to horses in this study was the same, the  
535 proportion of condensed tannins and total phenols were lower in the sainfoin used in this study  
536 (1.15% DM and 2.16% DM, respectively) compared to the previous one (1.52% DM of  
537 condensed tannins and 2.76% DM of total phenols) (Laroche et al., 2024). Therefore, it is  
538 possible that the amount of sainfoin pellets fed in the present study did not provide a sufficient

539 amount of polyphenols to have an anti-parasitic effect *in vivo*. This finding is consistent with  
540 results reported in ruminants, where it has been shown that the higher the concentration of  
541 condensed tannins, the stronger the anti-parasitic effect (Gaudin, 2017).

542

543 As reported in the study of Laroche et al. (2024), we found no effects of sainfoin on the  
544 diversity, functions, and activity of the large intestinal microbiota despite a longer  
545 supplementation period. However, compared to the previous results, horses that were fed  
546 sainfoin in a high starch diet over a long term had a higher number of bacterial taxa whose  
547 abundance was altered, although this number remained small. After 42 days, two unknown  
548 genera from two families known to be fiber degraders (*Bacteroidales RF16 group* and  
549 *UCG\_011*) were higher in the fecal microbiota of SF-fed horses compared to CONT-fed horses.  
550 Among the most notable changes observed at both 42 and 84 days in the fecal microbiota of SF  
551 fed horses in comparison to CONT fed horses, we observed a sustained reduction in the  
552 abundance of an unknown genus belonging to the *gir-aah93h0* family within the Bacteroidota  
553 phylum, and in the abundance of *Succinivibrionaceae UCG-002* belonging to the  
554 Succinivibrionaceae family within the Proteobacteria phylum. The unknown genus of *gir-*  
555 *aah93h0* was already found to be less abundant in horses receiving sainfoin in a high fiber diet  
556 in our previous study (Laroche et al., 2024), but its role in the large intestine ecosystem is  
557 undetermined. The *Succinivibrionaceae UCG-002* genus and Succinivibrionaceae family were  
558 found to be associated with high starch dysbiosis (Grimm et al., 2020, Laroche et al., 2024),  
559 probably as several members of this family are involved in the digestion of starch (Dworkin et  
560 al., 2006). Based on this result, we can therefore hypothesize that starch-associated dysbiosis  
561 was reduced when horses were fed a starch-rich diet including sainfoin. The alteration in starch  
562 fermentation was not reflected in the concentration of amylolytic bacteria or in the faecal  
563 microbial fermentation parameters. However, these findings must be interpreted with caution,

564 as these changes in microbiota composition cannot be attributed solely to the effect of the diet,  
565 given the differences in fecal microbiota composition observed at D<sub>0</sub> when all horses were still  
566 fed with the control diet. The changes observed in the composition of the large intestine  
567 microbiota of horses may also be related to the high concentration of phenolic compounds and  
568 tannins in sainfoin. Similar results were reported in lambs fed a diet containing different vegetal  
569 sources of tannins for 75 days (Salami et al., 2018). This study showed that a 4% tannin  
570 inclusion modified the bacterial composition of the ruminal microbiota without affecting its  
571 diversity, structure, and fermentative activity. The changes induced by tannins varied according  
572 to their type (hydrolysable or condensed) and plant of origin (chestnut, tara, mimosa or gambier)  
573 and were also not comparable to those we found in the equine fecal microbiota, indicating that  
574 tannins do not modify the microbiota in a consistent way. However, regardless of the tannin  
575 source, Salami et al. (2018) found a general reduction in the abundance of Fibrobacter in  
576 tannins-supplemented lambs, confirming the previously reported potential inhibitory effect of  
577 tannins on fibrolytic bacteria (Bae et al., 1993). Fibrobacter is also a major fibrolytic bacterium  
578 of the hindgut microbiota of horses and our data showed no change in the abundance of this  
579 phylum nor in the concentration of cellulolytic bacteria in the sainfoin supplemented horses.  
580 This suggests that the fibrolytic function was maintained when sainfoin was added at the dose  
581 tested.

582

583 In horses, both a high-starch diet and a high intensity of strongyle infection can cause  
584 inflammation and damage to the large intestine mucosa (Stewart et al., 2017; Walshe et al.,  
585 2019). This may be reflected by an increase in mucosal permeability with the leakage of some  
586 microbial fragments such as LPS, a decrease in the absorption of nutrients, or an increase in the  
587 amount of circulating white blood cells. No changes in the concentration of different types of  
588 circulating white blood cells and in the amount of circulating LPS were observed after long-

589 term inclusion of sainfoin in the high starch diet of horses naturally infected with strongyles.  
590 However, we observed that the horses fed the SF diet had a higher concentration of blood  
591 acetate over time in parallel to no change in the fecal acetate concentration, suggesting a higher  
592 absorption through the intestinal mucosa or a higher production of acetate by the microbiota.  
593 The addition of a polyphenol-rich plant to the diet can lead to a strengthening of the integrity  
594 of the intestinal mucosa through an increased immune response (Andersen-Civil et al., 2021).  
595 This hypothesis cannot be confirmed since our results do not show any change on the health  
596 and immunological parameters measured when sainfoin was included in the diet. To better  
597 understand these results, measurements of local markers of immunity would be required.  
598 Alternatively, the amount of starch used, or the intensity of strongyles infection may not have  
599 been sufficient to cause serious damage and inflammation of the intestinal mucosa. As discussed  
600 above, we fed the horses with a level of starch lower (1.4 g/kg BW/meal) than the level  
601 identified as the threshold for hindgut dysbiosis and risk of digestive disease occurrence (2 g/kg  
602 BW/meal) (Julliand et al., 2006), to ensure their good health throughout this long-term trial.  
603 Regarding intensity of strongyle infections, our results showed that fecal egg count increased  
604 progressively over the 84 days of follow up, together with a decrease in haematocrit, both in SF  
605 and CONT group of horses. Moreover, strongyle eggs excretion and haematocrit of horses tend  
606 to be negatively correlated. These results might reflect changes in worm burden, *per capita*  
607 fecundity or both.

608 *In vitro* experiments showed an anti-parasitic effect of sainfoin, with a strong inhibitory effect  
609 on the hatching of strongyle eggs and less pronounced inhibitory effect on the migration of  
610 infective larvae. These findings are consistent with those previously reported in horses (Collas  
611 et al., 2018). In all tests, we reported a better inhibition of egg hatching and migration of  
612 infective larvae by SF1 than by SF2. The concentration of total phenols in SF1 was higher  
613 (2.76%) than in SF2 (2.16%), which may explain these differences. However, the polyphenolic

614 composition might also play a role as shown by the effect of different flavan-3-ols on the  
615 inhibition of egg hatching and larval migration of *Trichostrongylus colubriformis* (Molan et al.,  
616 2003). In ruminants, *in vitro* studies of the anti-parasitic properties of different sainfoin have  
617 also shown a better efficacy against *Ostertagia ostertagi* and *Cooperia oncophora*, depending  
618 on the polyphenolic concentration (Novobilský et al., 2011, 2013). The better efficacy of SF1  
619 than SF2 *in vitro* could contribute to explain the lack of anti-parasitic effect of SF2 *in vivo* and  
620 suggests that the concentration of total phenols could be another reason which might cause the  
621 discrepancy between the present study and the previous study of Laroche et al. (2024). Another  
622 interesting point that could explain the differences between horses and ruminants is that in  
623 ruminants, strongyles are located in the foregut, and thus sainfoin has not been digested prior  
624 to come into contact with them, unlike in horses. However, preliminary *in vitro* testing of  
625 sainfoin could help identify sainfoin with biochemical properties best suited for an *in vivo* use.  
626 However, a detailed metabolomic analysis of sainfoin may be necessary to understand which  
627 polyphenolic molecules are involved in the anti-parasitic effect of this plant on equine  
628 strongyles.

629

630

## LIMITATIONS

631 There are several limitations that deserve to be acknowledged and discussed. First, as already  
632 mentioned, the amount of starch might not have been enough to produce the expected effect on  
633 the intestinal ecosystem, but this was done to preserve horse health. Second, we did not perform  
634 experimental infections of naïve animals which would have allowed to assess any potential  
635 effect of diet on the infection dynamics, independently of the previous history of  
636 infection/exposure of each individual. However, when studying large mammals, it is often  
637 impossible to perform experimental infections for ethical reasons, and the number of available  
638 individuals is often limited. This point brings us to the last limitation which concerns the

639 statistical power associated with small sample sizes. We cannot discard the possibility that with  
640 a larger number of individuals per group, some of the differences would have reached the  
641 threshold for statistical significance; however, it is important to remind that the effect sizes of  
642 the *in vivo* experiment appear to be small, and only with an unrealistic sample size these  
643 differences would have reached the significance threshold.

644

645

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647

## CONCLUSION AND PERSPECTIVES

648 In this study, the combination of *in vivo* and *in vitro* approaches suggests that the variability in  
649 anti-parasitic activity of dehydrated sainfoin pellets observed in *in vivo* studies may be related  
650 to their phenol's concentration and possibly to their metabolomic profile. The characterisation  
651 of a polyphenolic composition of interest of the used plants would therefore make it possible to  
652 standardise the expected anti-parasitic effects under *in vivo* conditions and ensure the efficacy  
653 of the final products. To achieve this, it may be necessary to first select the products of interest  
654 using *in vitro* tests before testing them under *in vivo* conditions. This study was also the first to  
655 evaluate the effect of long-term administration of dehydrated sainfoin pellets on the integrity of  
656 the intestinal mucosa. Interesting results suggesting an improvement in the absorptive capacity  
657 of the intestinal mucosa were observed, indicating that further studies may be required to  
658 investigate the health benefits of sainfoin on the intestinal mucosa, which plays a crucial role  
659 to preserve digestive homeostasis in animals.

660

661

## DISCLOSURES

662 The authors declare no conflict of interest.

663

664

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670

671

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809 **Table 1.** Composition of diets. (CONT: Control diet; SF: Sainfoin diet)

	CONT	SF
<b>Ingredients (% DMI)</b>		
Hay	61.1	61.1
Rolled barley	23.3	23.3
Sunflower meal	5.3	0.0
Hay pellets	10.3	0.0
Dehydrated sainfoin pellets (Equifolia®)	0.0	15.6
<b>Chemical composition</b>		
Dry matter intake (DMI, % BW)	1.96	1.96
Digestible Energy (DE, kcal/kg DM)	2371	2403
Crude protein (% DM)	9.94	9.77
Neutral Detergent Fiber (NDF, % DM)	52.9	52.0
Acid Detergent Fiber (ADF, % DM)	31.2	31.3
Lignin (ADL, % DM)	4.73	5.15
Starch (% DM)	14.6	14.6
Sugars (Ethanol Soluble Carbohydrates) (% DM)	3.48	2.98
Crude fat (% DM)	1.96	2.22
Ash (% DM)	6.52	6.44
Calcium (% DM)	0.46	0.56
Phosphorus (% DM)	0.29	0.25

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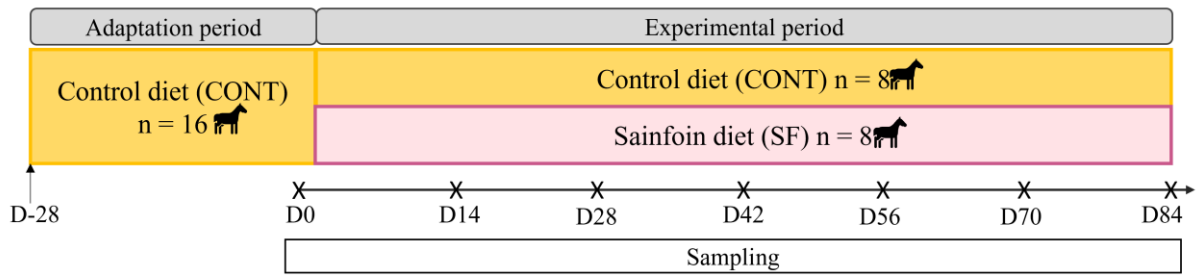
813 **Table 2.** Mixed model exploring the effect of diet (CONT vs SF) fecal egg count over the 84  
814 days experimental period. We report the fixed effects but the model also included the day, horse  
815 ID. We report the degrees of freedom, F and *p*-value.

<b>Effect</b>	<b>DF</b>	<b>F value</b>	<b><i>p</i>-value</b>
Diet	1.21	0.31	0.584
Day	1.94	20.21	<b>&lt;0.001</b>
Diet*Day	1.94	1.05	0.308

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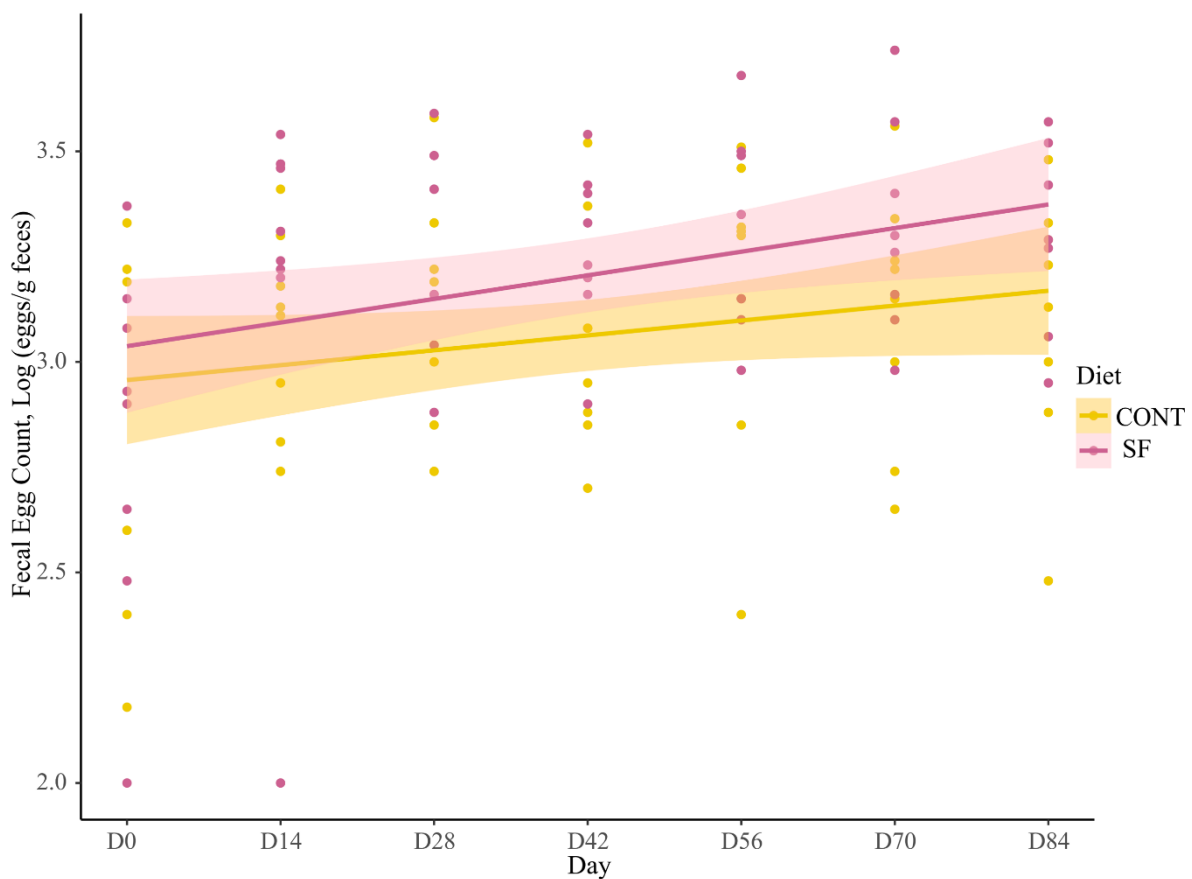


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820 **Fig 1.** Experimental design of the study. Longitudinal design with an adaptation period of 2  
821 days and an experimental period of 84 days. (CONT : high starch diet considered as control ;  
822 SF : high starch diet including sainfoin pellets)

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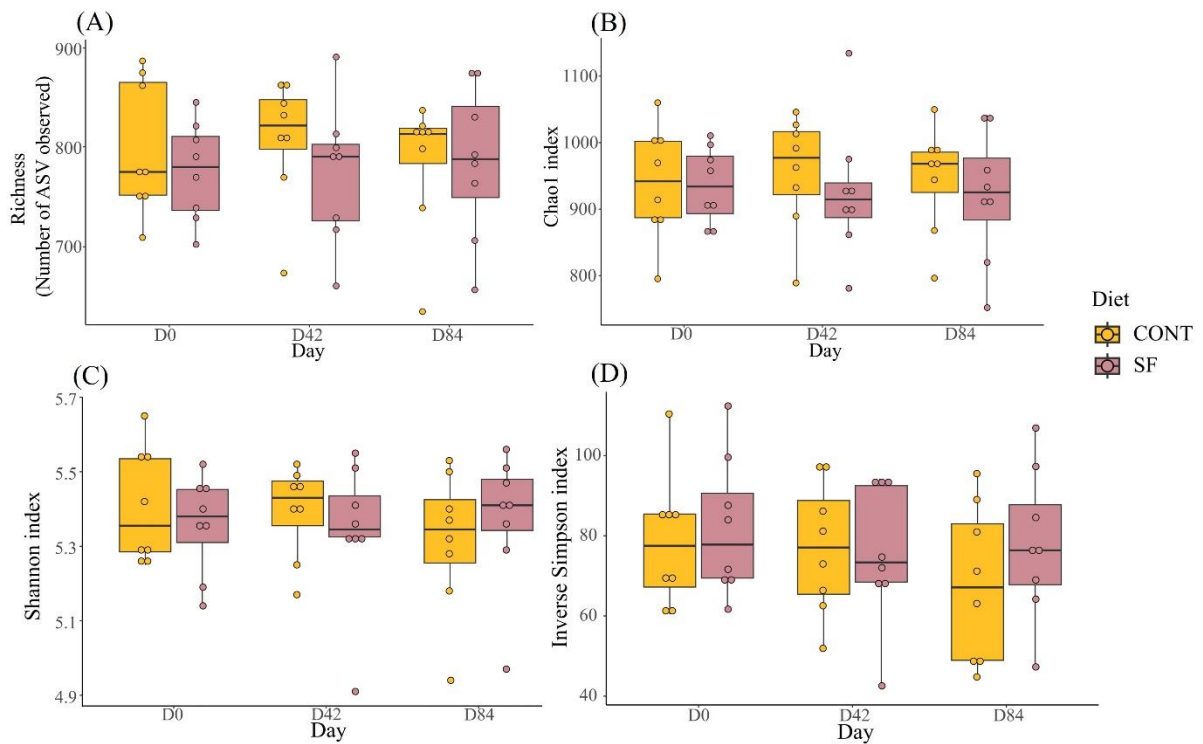
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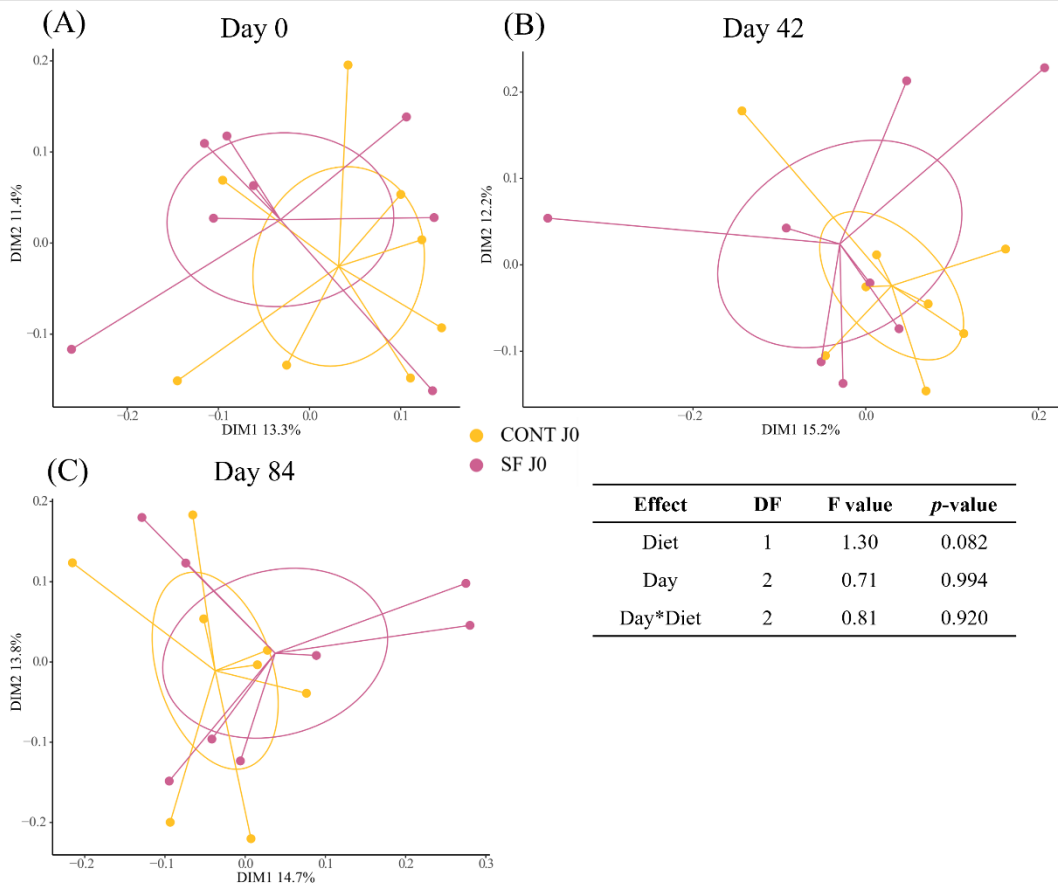
826 **Fig 2.** Changes in fecal egg count (FEC) over the 84 days of the experimental period for  
827 horses fed control (yellow points) and sainfoin (pink points) diets. We report the trend line  
828 and the 95% confidence intervals (light colors).

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832 **Fig 3.** Fecal bacterial richness (A: number of ASVs ; B: Chao 1 index) and  $\alpha$ -diversity (C:  
 833 Shannon index; D: Inverse Simpson index) at D0, D42 and D84 in horses fed control diet  
 834 (CONT) or sainfoin diet (SF). We report the median and the interquartile range.



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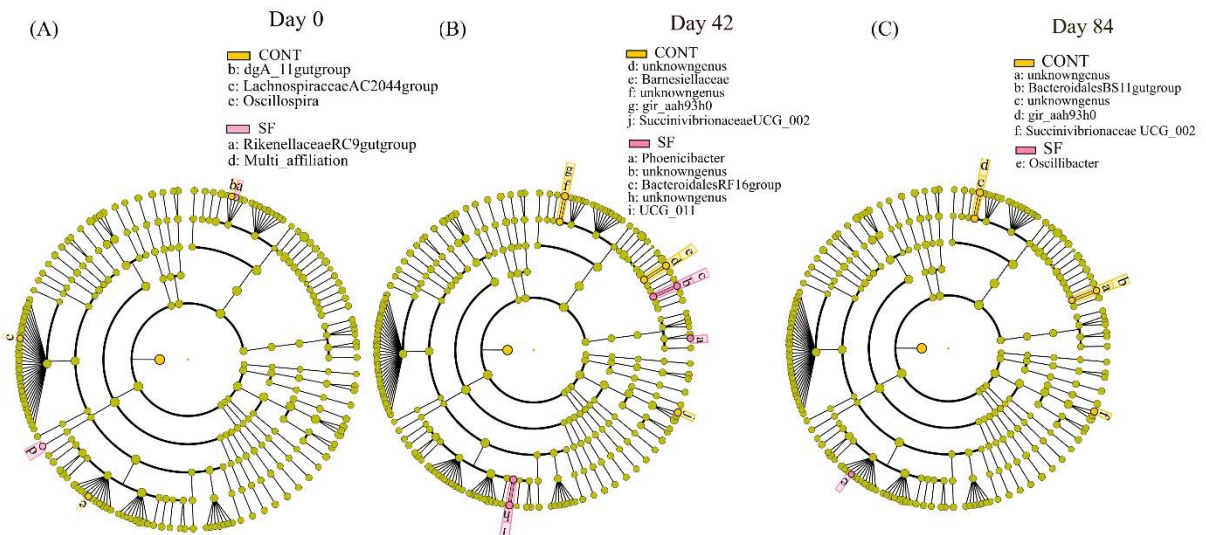
836 **Fig 4.** Principal coordinates analysis (PCoA) at amplicon sequence variants level showing the  
 837  $\beta$ -diversity (Bray-Curtis distance) in horses fed control diet (CONT) or sainfoin diet (SF) at  
 838 each sampling day. Ellipses represent the 95% confidence intervals.

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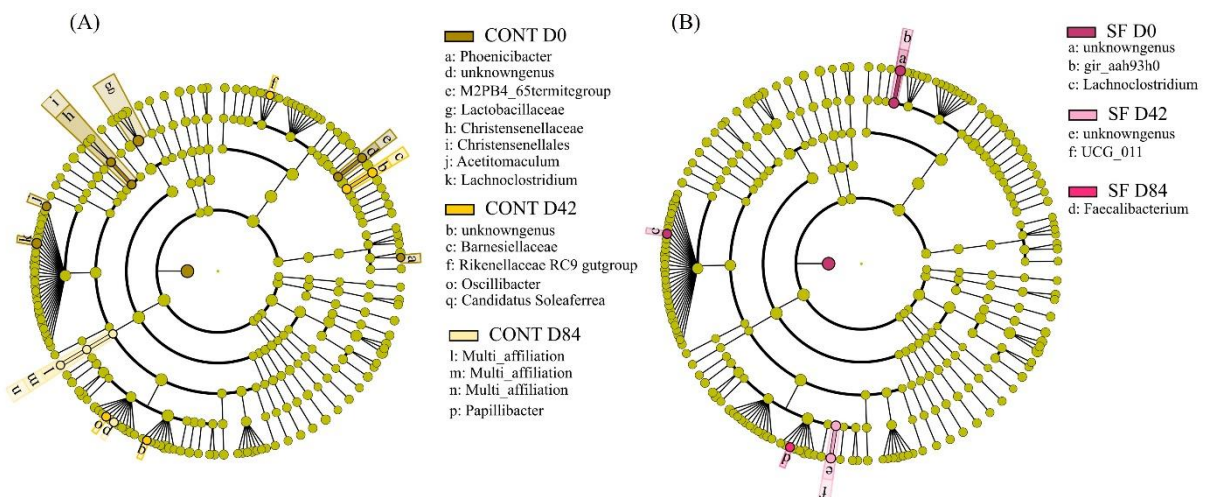


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844 **Fig 5.** LDA effect size (LEfSe) cladograms showing the taxa most differentially associated with  
 845 CONT or SF diet at D0 (A), at D42 (B) and at D84 (C) of the experimental period. The circles  
 846 represent, going from the inner to outer circle: phylum, class, order, family and genus. Pink  
 847 circles represent taxa overrepresented in SF-fed horses and yellow circles taxa overrepresented  
 848 in CONT-fed horses.

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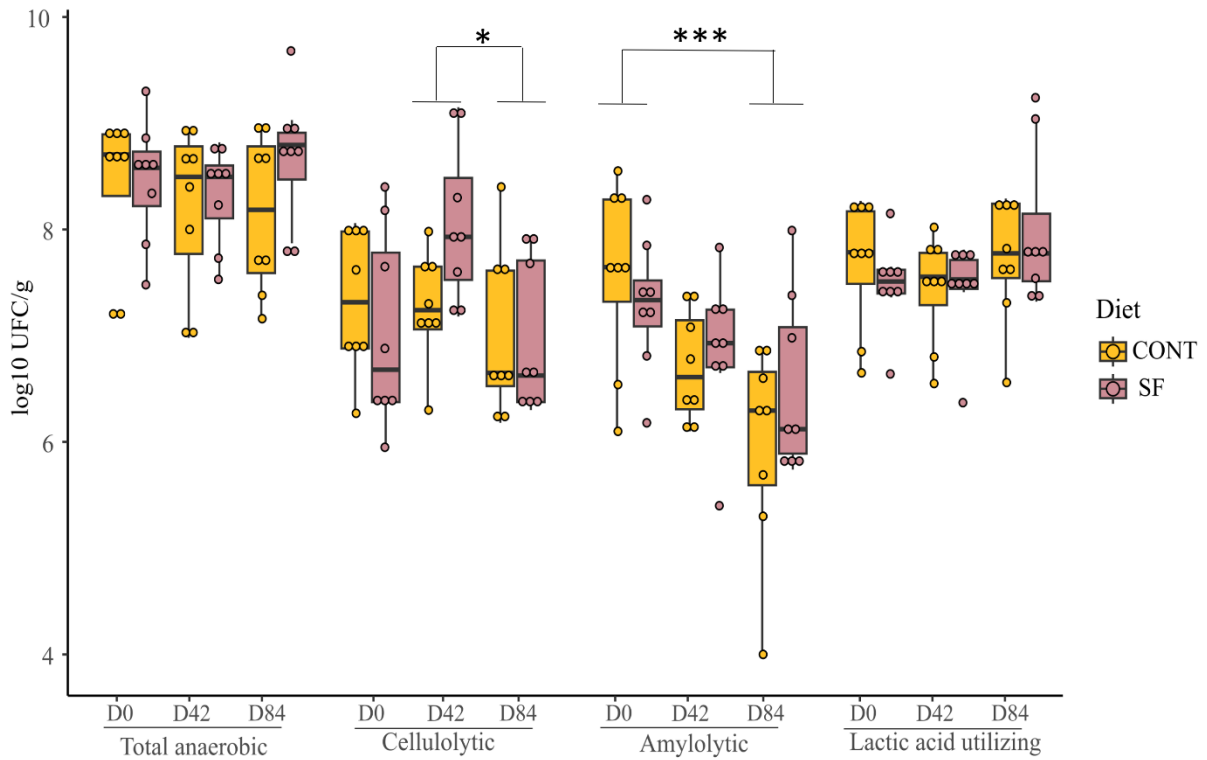


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852 **Fig 6.** LDA effect size (LEfSe) cladograms showing the taxa most differentially associated with  
 853 D0, D42 or D84 for horses fed CONT diet (A) or SF diet (B). The circles represent, going from  
 854 the inner to outer circle: phylum, class, order, family and genus.

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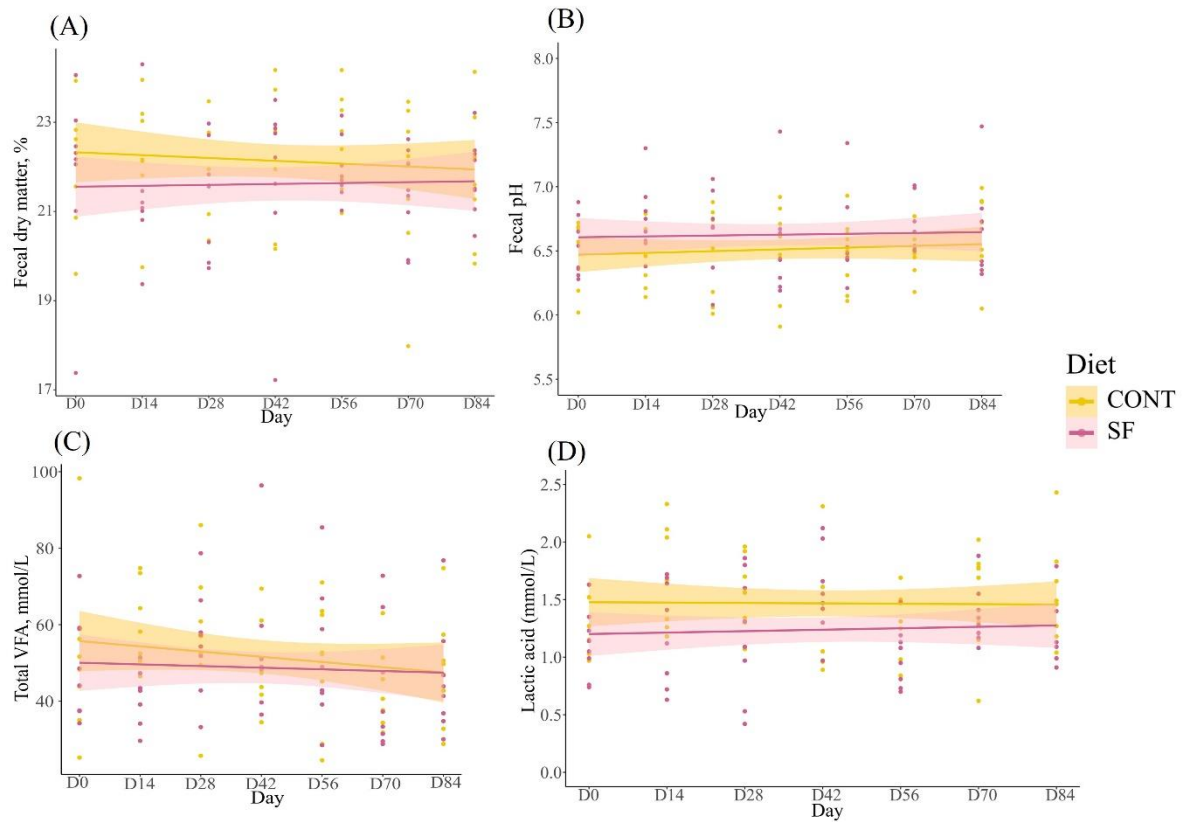


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858 **Fig 7.** Total anaerobic, cellulolytic, amylolytic and lactate utilizing bacteria concentrations at  
 859 D0, D42 and D84 in fecal samples of horses fed high starch control diet (CONT) or sainfoin  
 860 diet (SF). We report median values and interquartile range.

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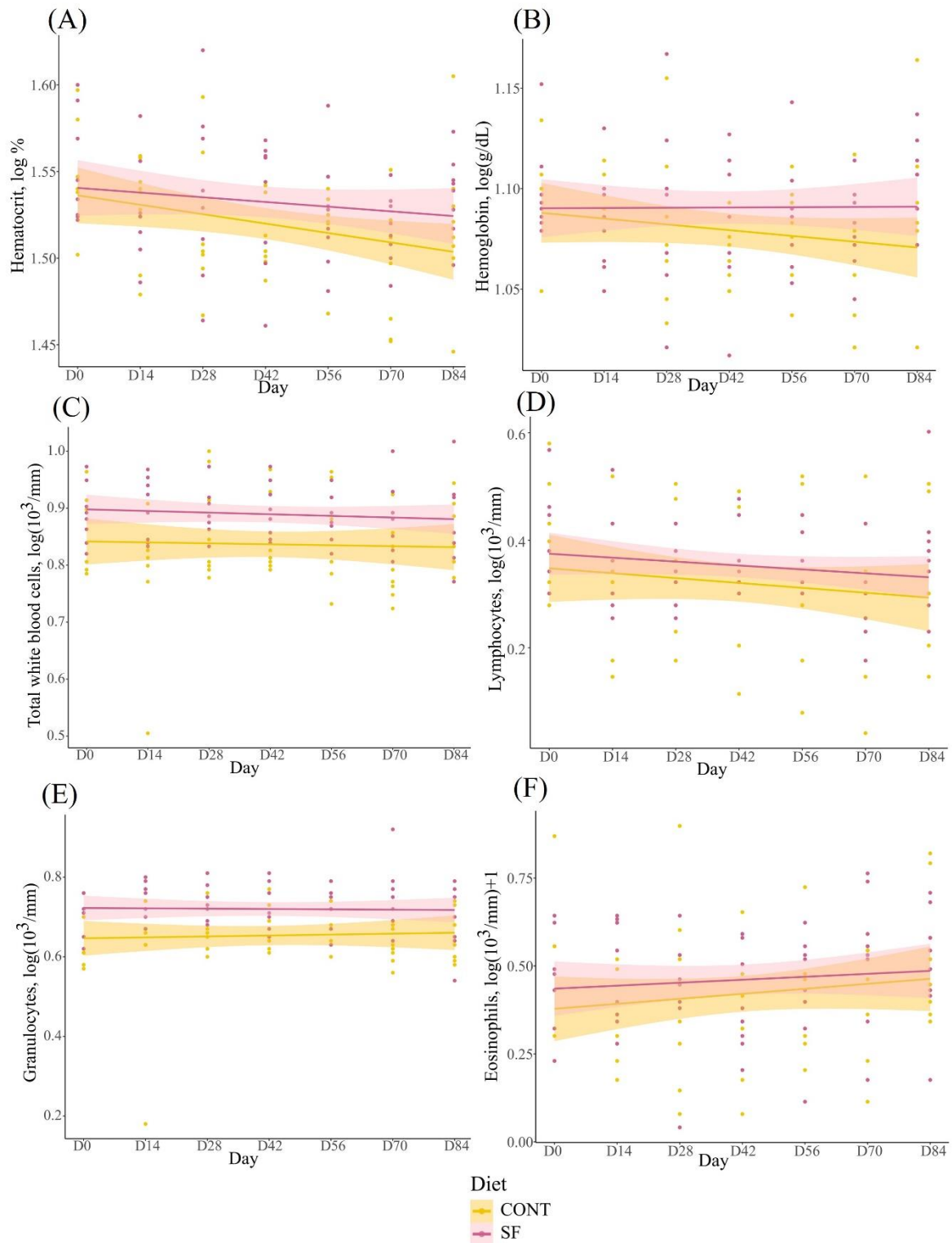


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864 **Fig 8.** Changes in fecal dry matter (A), fecal pH (B), fecal concentration of total volatile fatty  
 865 acids (C) and fecal concentration of lactic acid (D) over the 84 days of the experimental period  
 866 for horses fed control (yellow points) and sainfoin (pink points) diets. We report the trend line  
 867 and the 95% confidence intervals.

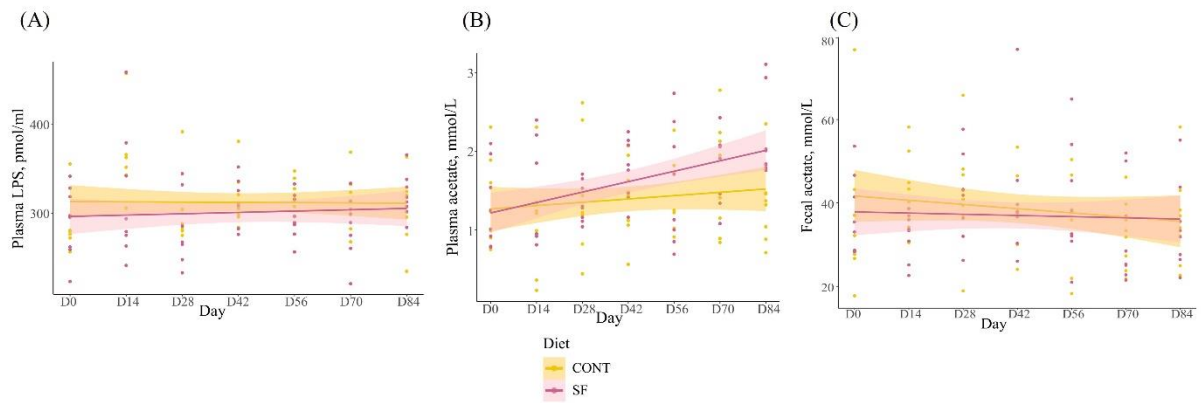
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871 **Fig 9.** Hematocrit (A), hemoglobin (B), concentration of total white blood cells (C), blood  
 872 concentration of lymphocytes (D), blood concentration of granulocytes (E) and blood  
 873 concentration of eosinophils over the 84 day of the experimental period for horses fed control  
 874 (yellow points) and sainfoin (pink points) diets. We report the trend line and the 95% confidence  
 875 intervals.

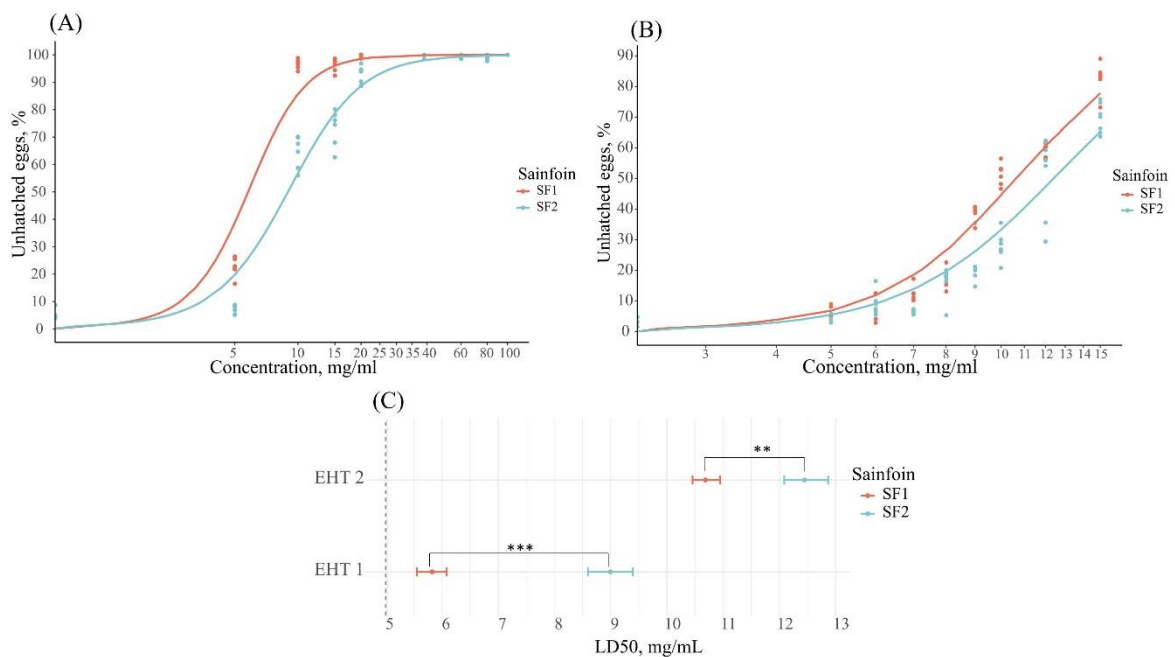


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877 **Fig 10.** LPS concentration in plasma (A), acetate concentration in plasma (B) and fecal acetate  
 878 concentration (C) over the 84 days of the experimental period for horses fed control (yellow  
 879 points) and sainfoin (pink points) diets. We report the trend line and the 95% confidence  
 880 intervals.

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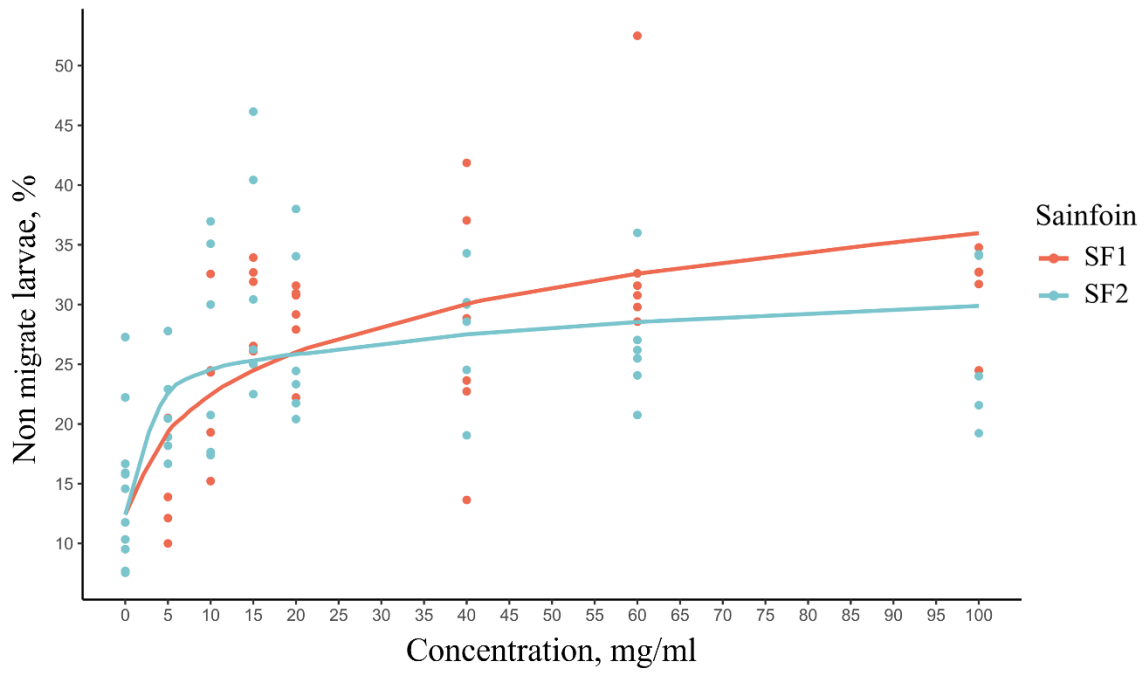
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884 **Fig 11.** Proportion of unhatched strongyle eggs in the first hatching test (EHT 1) (A) and the  
 885 second (EHT 2) (B) for sainfoin 1 (SF1, red dots) and sainfoin 2 (SF2, blue dots) at each  
 886 concentration tested. We report the prediction curve for each sainfoin (solid lines). Lethal doses  
 887 to obtain 50% of unhatched eggs (LD50) and the 95% confidence intervals predicted by the  
 888 probit model in each EHT (C).

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890

891 **Fig 12.** Proportion of non-migrate infective larvae for sainfoin 1 (SF1, red dots) and sainfoin 2  
 892 (SF2, blue dots) at each concentration tested.

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