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Chicory (*Cichorium intybus*) reduces cyathostomin egg excretion and larval development in grazing horses

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ABSTRACT

Cyathostomins are the most prevalent parasitic nematodes of grazing horses. They are responsible for colic and diarrhea in their hosts. After several decades of exposure to synthetic anthelmintics, they have evolved to become resistant to most compounds. In addition, the drug-associated environmental side-effects question their use in the field. Alternative control strategies, like bioactive forages, are needed to face these challenges. Among these, chicory (*Cichorium intybus,* Puna II cultivar (cv.)) is known to convey anthelmintic compounds and may control cyathostomins in grazing horses. To challenge this hypothesis, we measured fecal egg counts and the rate of larval development in 20 naturally infected young saddle horses (2-year-old) grazing either (i) a pasture sown with chicory (n = 10) or (ii) a mesophile grassland (n = 10) at the same stocking rate (2.4 livestock unit (LU)/ha). The grazing period lasted 45 days to prevent horse reinfection. Horses in the chicory group mostly grazed chicory (89% of the bites), while those of the control group grazed mainly grasses (73%). Cyathostomins egg excretion decreased in both groups throughout the experiment. Accounting for this trajectory, the fecal egg count reduction (FECR) measured in individuals grazing chicory relative to control individuals increased from 72.9% at day 16 to 85.5% at the end of the study. In addition, larval development in feces from horses grazed on chicory was reduced by more than 60% from d31 compared to control individuals. Using a metabarcoding approach, we also evidenced a significant decrease in cyathostomin species abundance in horses grazing chicory. Chicory extract enriched in sesquiterpenes lactones was tested on two cyathostomins isolates. The estimated IC_{50} was high (1 and 3.4 mg/ml) and varied according to the pyrantel sensitivity status of the worm isolate. We conclude that the grazing of chicory (cv. Puna II) by horses is a promising strategy for reducing cyathostomin egg excretion and larval development that may contribute to lower the reliance on synthetic anthelmintics. The underpinning modes of action remain to be explored further.

1. Introduction

Small strongyles of the family Strongylidae are the most prevalent gastrointestinal nematodes in horses (Reinemeyer et al., 1984). Infection-induced clinical signs include colic, diarrhea and weight loss. The massive simultaneous emergence of larval stages encysted in the colonic mucosa (larval cyathostominosis) can lead to the death of the most susceptible hosts, especially young animals (Giles et al., 1985; Love et al., 1999; Love and Duncan, 1992; Peregrine et al., 2014). Therefore, small strongyle control is essential, but the frequent use of synthetic anthelmintics has selected resistant isolates worldwide (Kaplan, 2002, 2004; Traversa et al., 2012; Matthews, 2014). Moreover, the molecules

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remaining the most effective against small strongyles (macrocyclic lactones like ivermectin and moxidectin (Nielsen, 2022)) have the potential to induce toxic effects on coprophagous organisms (Lumaret et al., 2012; Verdú et al., 2018). These accelerate the need to explore alternative solutions for a sustainable control of cyathostomins. In small ruminants facing a comparable challenge, a significant body of research has been published to evaluate the anthelmintic activity of bioactive plants harboring secondary metabolites like alkaloids, tannins, or terpenoids (Santos et al., 2019). To date, *in vitro* tests have evidenced the anthelmintic activity of alkaloids against trichostrongylids (da Silva et al., 2021) but *in vivo* effects were poorly studied or not significant (Dubois et al., 2019). On the other hand, *in vitro* tests have evidenced that condensed tannins could affect larval motility assay or exsheathment (Paolini et al., 2004; Manolaraki et al., 2010). In contrast to alkaloid-containing plant, the anthelmintic efficacy of condensed tannin-rich plants such as sainfoin (*Onobrychis viciifolia*) has been demonstrated *in vivo* in sheep and goat studies (Paolini et al., 2003, 2005; Heckendorn et al., 2006; Manolaraki et al., 2010; Gaudin et al., 2016). However, in horses, two independent trials concluded on the lack of efficacy of the short-term consumption of a sainfoin-rich diet on cyathostomins egg excretion and larval development (Collas et al., 2018; Malsa et al., 2022). In addition, sainfoin pellets did not affect the structure and diversity of the parasite community and interacted negatively with an oral ivermectin treatment, reducing its plasma concentration (Malsa et al., 2022). Finally, terpenes such as sesquiterpene lactones (SLs) (*i.e.* 8-deoxylactucin hereafter referred to as 8-DOL) have demonstrated significant *in vitro* effects against *Ascaris suum* (Valente et al., 2021). Chicory (*Cichorium intybus*), a plant used as animal forage (Rumball, 1986; Niezen et al., 1994; Scales et al., 1995; Street et al., 2013), contains SLs in the leaves (2% DM according to Peña-Espinoza et al. (2015) and Valente et al. (2021)). Significant reductions of L4 and adult counts helminths of the abomasum were observed in infected sheep grazing chicory, but their fecal egg count (FEC) and intestinal worm abundance were not affected (Marley et al., 2003). Moreover, a reduction of abomasal worm numbers of *Ostertagia ostertagi* has been reported in cattle (Peña-Espinoza et al., 2016). To date, all studies evaluating chicory's anthelmintic activity in ruminants have found it to be effective only on abomasum species and for diet including at least 70%DM of chicory (Peña-Espinoza et al., 2018). While a number of studies have explored the potential of chicory against parasitic nematodes in ruminants, none of them assessed its efficacy for the control of equine cyathostomins.

With the aim of reducing the use of chemical antiparasitic, our study investigated the anthelmintic effects of chicory Puna II cultivar (cv.) in grazing horses. A first *in vivo* approach was designed to test if cyathostomin egg excretion and larval development were lower in young horses grazing a chicory pasture during 45 days compared to control individuals grazing a mesophile grassland. To study a potential difference in sensitivity to chicory between cyathostomins species, an ITS-2-based metabarcoding approach was implemented as in (Malsa et al., 2022). Using a molecular "barcode", this approach allows us to quantify cyathostomin species abundances (Mitchell et al., 2019; Poissant et al., 2021; Tombak et al., 2021) and to infer putative species-specific effects of chicory. Finally, an *in vitro* experiment was performed to evaluate the direct activity of chicory leaves extracts enriched in sesquiterpene lactones on the larval development of cyathostomin isolates with varying sensitivity to pyrantel.

2. Materials and methods

The *in vivo* trial was conducted from August 22nd to October 5th, 2022, at the French Horse and Riding Institute experimental farm (IFCE) in Chamberet (45.5820° N, 1.7212° E), France. Each individual fecal sample was taken from the ground directly after its release, taking particular care to collect only the part that has not touched the ground, to avoid contamination from the soil.

2.1. Animal condition

For the *in vivo* experiment, twenty naturally infected saddle horses (Anglo-Arab breed, 2 years old) were used. All horses were born at the IFCE experimental farm and were exposed to the same parasite populations. Infected horses were left undrenched for 264 days before the onset of the trial (last anthelmintic administered on November 30th, 2021 ; moxidectin 400 μg/kg body weight, Equest, Zoetis, France). Two fecal egg counts (FECs, per gram of fresh fecal matter) performed in April and June 2022 on individual feces showed that horses were moderately infested (<380 eggs per gram (EPG)). On August 19th, 2022, three days before the start of the study, animals were assigned to two experimental groups (chicory and control) of ten horses balanced for their last FEC values (chicory group FEC average = 2169 EPG [95% CI = 1676.42; 2661.57] ; control group FEC average = 2169 EPG [95% CI = 1639.97 ; 2698.03]). Horses were weighted (PUEC31, Radwag electronics, Poland) on two consecutive days one week before the start of the study, to ensure that the two groups were also balanced for average body weight (BW) (460.6 ± 20.2 kg and 457.6 ± 18.2 kg in the chicory and control groups, respectively, $P = 0.731$), and sex (eight females and two geldings in each group).

2.2. Parasite material

Two different cyathostomins isolates were used in this study. The first one came from horses of the IFCE experimental farm in Chamberet, France. The *in vivo* effects of grazing chicory were measured on this isolate. The second isolate came from the INRAE experimental station in Nouzilly (47.5434° N, 0.7442° E), France (DOI:10.15454/1.5573896321728955E12). Mock communities were derived from this isolate. Both isolates were used for the *in vitro* larval development assay. Chamberet and INRAE isolates were both benzimidazole-resistant and ivermectin susceptible. While the INRAE isolate was slightly resistant to pyrantel (Boisseau et al., 2023), the sensitivity to pyrantel of the Chamberet isolate was unknown. We therefore performed a fecal egg count reduction test (FECRT) on twenty horses of Chamberet (n = 10 treated and n = 10 not treated) on March 28, 2023, with pyrantel (6.6 mg/kg of BW, Strongid®, Zoetis, France). Fecal samples were collected on the ground at d0 and d14 after treatment and a McMaster assay was performed with a sensitivity of 10 eggs/g (Qualyse lab, Tulle, France). The two isolates showed different pyrantel sensitivity: FECRT of the INRAE isolate (Boisseau et al., 2023) was 91.5%, [95% CI = 78.1; 100] and that of the Chamberet isolate was 99.3% [95% CI = 98.2; 100].

2.3. Experimental design

The trial was conducted over 45 days to evaluate the anthelmintic effect of chicory while preventing horse reinfection. The two groups grazed rotationally at the same stocking rate (2.4 LU/ha, 1 LU (livestock units) = 600 kg BW) either: (i) a pasture sown in April 2022 with pure chicory (cv. Puna II, 9 kg/ha) (45°34′03″N, 1°43′28″E), or (ii) an old mesophile temporary grassland (sown in 2006 and overseeded with 25 kg/ha of perennial ryegrass in October 2020) (control group) (45°34′00″N, 1°43′25″E). Between October 2021 and January 2022, both pastures were fertilized with organic nitrogen and received manure as well as a mixture of calcium and magnesium oxides. Urea was also applied on the mesophile pasture in April 2022. For each treatment, the pasture (3.2 ha) was divided into five subplots (0.64 ha) grazed during three rotation cycles: 1st cycle from day 1 (22/08) to day 15 (05/09), 2nd cycle from day 16 (06/09) to day 30 (20/09) and third cycle from day 31 (21/09) to day 45 (05/10). Horses in both groups grazed each subplot for three days before being moved to the next one. No additives or supplements were given to the horses during the entire experimental period and water was available *ad libitum*.

2.4. Vegetation and dietary choices measurements

In each treatment, vegetation biomass was determined on four occasions along the experiment (d5, d17, d26 and d38), on the second day of the presence of horses in a subplot. For this, six 0.5 m^2 (10 cm × 5 m-long) strips were randomly cut to ground level with manual lawn-mowers and weighted (Sartorius Economy Series EB, Sartorius AG, Germany). Representative samples were dried at 60 °C for 72 h to estimate the dry matter (DM) content. Samples were then ground to 1 mm and analyzed for crude protein (CP ; Dumas method (Jung et al., 2003), N × 6.25) and neutral detergent fiber content (NDF, according to the method of (Van Soest et al., 1991)). The distribution of vegetation heights (100 sample points per diagonal, measured at the first place where a stick contacted the sward surface) was assessed five times during the experiment, on the second day of the presence of horses in a subplot (d5, d11, d23, d32, and d44). At each sample point, we also recorded available bites characterized by the dominant item (chicory, grasses, legumes, or forbs) and the potential presence of plants with anthelmintic properties reported in small ruminants (*i.e.*, *Plantago lanceolata., Rumex obtusifolius* L., and *Chenopodium album* L*)*. On the same day, dietary choices were measured by two observers (one in each group) using a scan-sampling method with activity recorded in both groups every 15 min for each animal (from 7:30 a.m.–11 a.m. and from 4:30 p.m.–8 p.m.). When an individual was grazing, the observer moved as close to the animal as possible (~2 m) without disturbing it to record the composition of one selected bite. Observers decided beforehand which bite to register (*i.e.*, the fifth one) to avoid bias in picking the most visible bites. This required animals to be trained for a week before measurements.

2.5. Fecal sample analysis

Fecal samples were collected individually from the ground, directly after their excretion, at four times during the experiment (d0, d16, d31, and d45). Samples were stored at +4 °C until they were sent to INRAE Centre Val de Loire facilities (Nouzilly, France) where they were processed within 24 h upon collection. Dry matter content of the feces was determined from fecal samples collected at d16 that were dried at 60 °C. As no significant difference in feces DM was observed ($P = 0.06$) between the two groups, we considered FEC values measured on fresh fecal matter. Individual FEC data were determined using a modified McMaster technique (Raynaud et al., 1970) based on the dilution of 5 g of fecal matter in 70 mL of a saturated NaCl solution (density = 1.18). Eggs were counted using an optical microscope (× 150 magnification), the minimum detection limit was set at 50 EPG.

To evaluate the effect of the diet on larval development, the remaining fecal matter (40 g–90 g) was incubated individually for each horse for 12 days at +25 °C and 60% relative humidity. Infective third-stage larvae (L3) of cyathostomins were then collected using a Baermann apparatus after 48 h allowing larval migration for each horse and timepoint. The larval count in each sample was determined from 30 drops of 5 µL of the larval solution under the microscope (× 4 magnification). Based on the method used by Collas et al. (2018), the larval development rate was calculated with individual larval cultures from each horse as follows:

$$\left(\frac{Counted\ L3}{FEC \times g\ of\ cultured\ feces}\right) \times 100$$

2.6. Gastrointestinal nemabiome

To further investigate the antiparasitic activity of chicory, we estimated changes in the cyathostomin community structure during the trial. We used a metabarcoding approach on cyathostomin larval populations harvested from fecal samples collected on d0, d16, d31, and d45 of the experiment. From 80 fecal samples, 13 did not produce any larvae. Ultimately, we extracted the DNA from 67 samples following the DNeasy PowerSoil Pro Kits (Qiagen, Holland). Mock community included 32 worms (14 *Cylicocyclus nassatus*, 12 *Cyathostomum pateratum*, 3 *Cylicostephanus goldi*, 1 *Cyathostomum catinatum*, 1 *Cylicostephanus minutus* and 1 *Cylicocyclus ashworhi*) collected after pyrantel treatment (6.6 mg/kg BW, Strongid®, Zoetis, France) of Welsh ponies from INRAE. The DNA from each adult parasite was extracted using the NucleoSpin Tissue kit (Macherey-Nagel, Germany) and used for genotyping the internal transcribed spacer 2 (ITS-2) and cytochrome *c* oxidase I (COI). DNA concentrations were quantified using the Qubit® double-stranded high-sensitivity assay kit (Life Technologies™, Carlsbad, CA, USA) with a minimum sensitivity of 0.1 ng/µL. Two mock communities were then built with a mix of raw DNA concentrations containing one or all adults of each species (Table S1). For the nemabiome study, the ITS-2 gene region was PCR amplified using the NC1 (5′-ACGTCTGGTTCAGGGTTGTT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) primers (Gasser et al., 1993). Primers also contained between one to three random bases to increase diversity and an Illumina adapter overhang as described previously (Courtot et al., 2023). PCR reactions were realized on 80 µL using 16 µL HF buffer 5X, 1.6 µL dNTPs (10 mM), 4 µL primer mix containing forward and reverse primers, 0.8 µL Phusion High-Fidelity DNA Polymerase (2 U/µL ; Thermo Scientific, Waltham, MA, USA), and 2 µL of a 5 ng/µL genomic DNA solution (Courtot et al., 2023). The libraries were created with 25 µL of each DNA sample concentrated 0.4–74.2 ng/µL in TRis-HCL. Each sample was sequenced on two lanes of NovaSeq 6000 with 250 cycles (IGA sequencing platform, Italy). The adapter trimmed reads were edited to remove the ITS-2 primer sequences using cutadapt v.1.4 (Martin, 2011). Nemabiome data were subsequently analyzed in R v.4.2.1 (R Core Team, 2023) with the dada2 software (Callahan et al., 2016) using the mock community as a standard to choose appropriate bioinformatic parameters as described previously (Courtot et al., 2023). Using this approach, we retained a maximum number of expected errors of 2 and 5 for the forward and reverse reads respectively, a truncation length of 200 bp, and applied the default BAND_SIZE parameter. Downstream analyses and count tables data were analyzed using the phyloseq v.1.42.0 R package (McMurdie and Holmes, 2013) and vegan v.2.6–4 R package (Oksanen et al., 2015). The amplicon sequencing workflow yielded 465,366 sequences on average [820–703,166] for 56 samples. A first filter was applied to remove any amplicon sequence variants (ASVs) with less than 10,000 occurrences. A second filtration was realized to remove the samples that failed, *i.e.* two samples showing less than 10,000 (2 samples removed). Following taxonomy assignment, one false positive ASV (detected in the mock communities while absent) with a summed relative abundance below 5% across the 54 samples was removed. Between filtering and the final analysis, a few samples ($n = 24$) with abnormal behaviors were removed from further analyses. At the end of the pre-treatment, we worked on 30 samples and 14 ASVs, corresponding to 12 species correctly identified. One ASV was attributed only at genus level and another at family level. Due to the low quality of the samples at d0, these were removed from the analysis. The final dataset comprised 30 samples and 14 ASVs, corresponding to 12 species. Species diversity within the two groups was measured at each sampling date, using alpha diversity, an indicator of the number of different species present in a specific condition. We used both Shannon and Simpson indexes, whereby the Shannon index has greater sensitivity to variation in rarer species abundances. We also used β-diversity (Bray Curtis distance) to measure species dissimilarity between the two groups.

2.7. Preparation of sesquiterpenes lactones (SLs) enriched chicory extract

Fresh chicory leaves samples were collected by hand from the entirety of the five subplots at the end of the experiment, when the cumulative effects of chicory consumption on FEC and development of eggs into L3 larvae were maximal (d45; September 05, 2022). After collection, the samples were frozen at −20 °C until extraction. The

isolation and purification of SLs were performed according to a previously published protocol (Peña-Espinoza et al., 2015). First, 11 g of fresh leaves were freeze-dried overnight, then ground to powder and separated in 50 mL tubes (2 g per tube) before 30 mL of methanol/Milli-Q-H_2O (4/1 ; v/v) containing 2% formic acid (v/v) was added. Each tube was vortexed (1 min), sonicated in a water bath (at room temperature) for 10 min, and centrifuged (10 min, 1200 rpm Rotanta 460 R from Hettich®). After centrifugation, the supernatants from each 50 mL tube were pooled in a round-bottomed glass flask. The ground leaves remaining in the tube were extracted three more times, following the protocol described above, and supernatants were pooled in the round-bottomed flask. The supernatant was concentrated under reduced pressure at 35 °C to evaporate methanol and formic acid. To remove Milli-Q-H_2O, the round-bottomed flask was freeze-dried overnight. The crude extract obtained was resuspended in methanol (5 mL) and dissolved with 70 mL of a cellulase enzyme solution (10 mg cellulase from *Aspergillus niger* [Sigma 22,178]/mL Milli-Q-H_2O). The mix was incubated in a water bath for 2 h at 40 °C. After the enzymatic reaction, the extract was distributed into 5 mL tubes and 25 mL of ethyl acetate was added to each tube. The tubes were centrifuged (10 min, 1200 rpm Rotanta 460 R from Hettich®) and the supernatant was pooled in round-bottomed glass flasks. The sedimented material remaining in the tubes were extracted twice, following the protocol described above. The ethyl acetate extracts were evaporated to dryness under reduced pressure (35 °C), redissolved in methanol (8 mL) and transferred to 50 mL tubes. The final purification of SLs was performed by solid-phase extraction (SPE). Dichloromethane (28 mL) was added to each tube and centrifuged (10 min, 1200 rpm Rotanta 460 R from Hettich®). A SPE vacuum manifold (Thermofisher) was equipped with 6 mL SPE tubes (Supelclean® LC-Si SPE tubes, Supelco 505,374). SPE tubes were conditioned with dichloromethane/i-propanol (6 mL, 1/1 ; v/v) and equilibrated with dichloromethane (6 mL). Extract mixed with dichloromethane (6 mL) was loaded in each SPE tube and the collected fraction was dried under reduced pressure (35 °C). Lastly, 590 mg of chicory extract enriched in SLs were obtained and collected in a pillbox. The composition and the concentration of terpenes from the enriched SLs extract were carried out on an ultra-high-performance liquid chromatography-diode array (UHPLC-DAD) adapted from Ferioli and D'Antuono (2012). The extract was dissolved at 10 mg/mL with methanol/water (1/1) solution and filtered at 0.2 µm. SLs titration was performed using UHPLC-DAD protocol adapted from Ferioli and D'Antuono (2012). HPLC analysis were performed on a Dionex UHPLC U3000RS system equipped with a LPG-3400RS quaternary pump, a RSLC WPS-300 T RS automated injector, a TCC-300SD column oven and a UHPLC + DAD-3000 diode array detector (ThermoFisher SA, Voisins le Bretonneux, France). The system was fitted with an Accucore aQ C18 (15 cm × 3 mm i.d., 2.6 µm particle size) column, itself protected by an Accucore aQ C18 10 × 3.0 mm 2.6 µm Defender Guards (ThermoFisher SA, Voisins le Bretonneux, France). The mobile phases were solvent A methanol:water (14:86,v/v), solvent B methanol:water (64:36,v/v). The gradient was set as follow: initial solvent B content was 0%, it was raised to 42% in 7.4 min and maintained for 3.75 min. Then solvent B was raised to 100% in 5.63 min and maintained for 2 min. Post run equilibrium was performed during 5 min. Signal acquisition was performed at 210 and 260 nm and a UV 3D scan was performed between 200 and 400 nm. Lactucopicrin (LACP) titration was performed using a calibration curve of LACP (99%, Sigma PHL84796) in the range of 100–300 ng. In order to build the calibration curve a solution of LACP was prepared at 50 µg/mL and injected volume were set at 2, 3, 4, 5 and 6 µL. Crude extract and enriched SL fraction were injected at 10 mg/mL, with a volume of 5 and 4 µL respectively. Peaks assignment to flavonoid or SL was performed by comparison of the full UV–Visible spectra on each peak, to the reference library.

2.8. In vitro experiment (larval development assay)

To investigate the relative contribution of SLs from chicory leaves on the observed *in vivo* effects, a larval development assay was conducted on the cyathostomin isolate where the trial was conducted (Chamberet) and that from an independent experimental pony herd (INRAE). The eggs were extracted from fecal samples collected from the ground directly after excretion, on 07.03.23 for INRAE samples and on 11.04.23 for Chamberet samples. *Cyathostomum pateratum* and *Cylicocyclus ashworthi* were dominant in both isolates, with *Cylicocyclus nassatus* as an additional species in the INRAE isolate. First, the samples were washed through a colander to remove the largest particles and then through two sieves, a first of 125 µm and a second of 20 µM to retain the eggs. Kaolin (Sigma K7375) was then added to the egg solution before centrifugation. The supernatant was discarded, and the pellet was resuspended in a saturated NaCl solution (density = 1.18) before centrifugation (5 min, 2500 RPM). The supernatant was collected on a 20 µm sieve. After eggs purification, 2.75 eggs/µL (110 eggs) were filled in a 96-well plate with feces mixture made with the residual fecal matter samples (horse feces mixed with water, v/v ; the mixture was crushed through a filter (125 µm) to collect only the liquid and used at an OD_{650nm} of 0.8), bacteria (*Escherichia coli* OP50 strain) to an OD_{650nm} of 1.3 and 500 µg/mL of amphotericin b (Sigma A4888). The plate was incubated at +25 °C for 24 h, letting the eggs develop into L1/L2 larvae. After the incubation, the SLs extract was added to the larvae at the concentrations of interest (between 5000 and 1 µg/mL) and incubated for six days, before the number of undeveloped (L1/L2), dead, or developed (L3) larvae were counted. The six replicates performed were sampled from a single larval batch cultured from 1 INRAE pony and 20 Chamberet horses. The development percentage was calculated as follows:

$$\left(\frac{\text{number of L3}}{\text{number of L1 and L2} + \text{number of L3}}\right) \times 100$$

2.9. Statistical analysis

All data were analyzed using the R software v.4.2.1 (R Core Team, 2023). The difference of BW between the two groups at d45 was tested using an ANOVA (anova_test function from rstatix v.0.7.2 package) considering the BW at d0 (control: 456.6 ± 17.9 kg, chicory: 461.5 ± 20.2 kg) as a covariate. FEC data have been backtransformed (*i.e.* transformed into exp (log data+1) – 1). The effect of chicory on the FEC rate was analyzed with a generalized linear mixed-effects model assuming a negative binomial distribution using the *glmer.nb* function of the lme4 v.1.1–34 package (Bates et al., 2015). We fitted diet, time and diet × time interaction terms as fixed effects, and horse as a random effect. This model estimates the deviation in FEC occurring in the chicory group relative to the control group over time, while accounting for inter-horse variation. The diet effect on the FEC and the larval development rate at each sampling date was analyzed using a Wilcoxon rank sum test (*wilcox.test* function from stats v.4.2.1 package) (R Core Team, 2023). The differences in alpha diversity (Shannon and Simpson index) between the two groups were analyzed using a *t*-test. The efficacy of chicory on reducing FEC was first measured at d16, d31 and d45 based on the Bayesian hierarchical approach as recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Kaplan et al., 2023), with the individual FEC of the control group used as pre-treatment and the individual FEC of the chicory group as post-treatment, including the correction factor for the McMaster technique (50), which resulted in an average fecal egg count reduction (FECR%) and 95% confidence interval (CI). For this, we used the fecr_stan function from eggCount v.2.3–2 package (Wang et al., 2022). In addition, the cumulative FEC of each animal at the end of the trial was calculated according to Vercruysse et al. (1993) and compared between the two groups using the Wilcoxon rank sum test as used in (Peña-Espinoza et al., 2016). Pyrantel efficacy was also measured as

recommended by the WAAVP. The LDA data were used to fit a two-parameter log-logistic regression model on the development ratio to the control as the response variable and the concentration range as the variable, using the *LL.2* function where the lower and upper limits are fixed at 0 and 1 respectively of the drc v.3.0–1 package (Ritz et al., 2015). The inhibitory concentration 50 (IC_{50}) was determined with the *ED* function and statistically compared using the compParm function from the same package. This function compares actual parameter estimates taking into account the model and the parameters used to measure IC_{50}.

The R scripts used and associated datasets are freely available under https://github.com/Joshua-Malsa/Chicory-paper.

3. Results

3.1. Vegetation characteristics and dietary choices

Vegetation structure and quality of chicory and control pastures are presented in Table 1. In the control group, the bites available for horses were dominated by grasses (65.9 ± 7.1% of available bites) and, to a lesser extent, by forbs (plants other than grasses, legumes or chicory) (21.7 ± 8.8% of available bites ; Table S2) including some species with known anthelmintic activity in ruminants (*i.e.*, *Plantago lanceolata, Rumex obtusifolius* L. and *Chenopodium album* L.) (11.8 ± 9.7% of available bites). In the pasture sown with chicory, bites dominated by chicory represented 88.4 ± 5.4% of the total available bites (Table S2) while other plants with known anthelmintic activity in ruminants represented 0.5 ± 0.6% of available bites. Horses in the control group mostly grazed on grasses (72.8 ± 10.3% of the bites consumed by horses) while their counterparts in the other group mostly grazed chicory (88.8 ± 7.6% of their bites were dominated by chicory, and a total of 89.2 ± 7.4% of the bites contained chicory; Table S3). Among forbs with known anthelmintic activity in ruminants, plantain (*Plantago lanceolata*) was the sole consumed by horses in both groups; its presence was observed in 3.7 ± 3.0% and 0.1 ± 0.2% of horses' bites in the control and chicory treatments, respectively. The average BW of horses at the end of the experiment was significantly higher in the control group (471.2 ± 20.4 kg BW and 458.9 ± 17.5 kg BW for the control and chicory groups, respectively; $P < 0.01$).

3.2. Chicory effect on FEC

Mean FEC of the two groups and the corresponding FECR are listed in Table 2. A significant decrease in cyathostomin eggs counts occurred in both groups at the first sampling (on day 16) relative to day 0: from 2169 EPG [95% CI = 1639.97; 2698.03] to 1085 EPG [95% CI = 847.11; 1321.89] in the control group (−50.0%), and from 2169 EPG [95% CI = 1676.42; 2661.57] to 285 EPG [176.04; 393.95] in the chicory group (−86.9% (d16 effect; $P = 0.01$), (Fig. 1). The decrease in FEC between d0 and d16 was higher in the case of the chicory group, which resulted in a lower FEC in this group compared to the control group at d16 (interaction d16 × group, $P < 0.001$). The difference in FEC between groups remained significant on the following occasions ($P < 0.001$ for d31 and d45). The FECR in the chicory group was greater than 70% compared with the control group on days 16, 31 and 45, with a final FECR of 85.5 % [95% CI = 69.5; 99.5]. The cumulative FEC sampled in horses grazing chicory (26929.50 [95% CI = 18047.14; 35811.86]) over the course of the study was also twice lower than the control group (58088.25 [95% CI = 44308.35; 71868.15]) ($P = 0.001$).

Table 1
Vegetation structure and quality (Mean ± sd).

	Control	Chicory
Sward surface height (cm)	6.6 ± 1.3	6.8 ± 2.5
Biomass (g DM/m^2)	53 ± 20	75 ± 42
Dry matter (%)	39.6 ± 9.4	19.3 ± 4.3
Crude protein (DM %)	12.1 ± 1.2	15.9 ± 4.3
Neutral detergent fiber (DM %)	66.8 ± 2.5	52.8 ± 5.9

Table 2
FEC and FECR results.

		FEC (mean EPG [95% CI])	FECR (Efficacy (%) [95% CI])
d0	Control	2169.0 [1639.97–2698.0]	
	Chicory	2169.0 [1676.42–2661.57]	
d16	Control	1085 [847.10–1321.89]	
	Chicory	285 [176.05–393.95]	72.9 [55.9–86.6]
d31	Control	1233 [827.17–1638.85]	
	Chicory	282 [91.13–472.87]	78.5 [55.7–94.7]
d45	Control	864 [667.55–1060.45]	
	Chicory	153 [19.50–289.49]	85.5 [69.5–99.5]

Legends to figures.

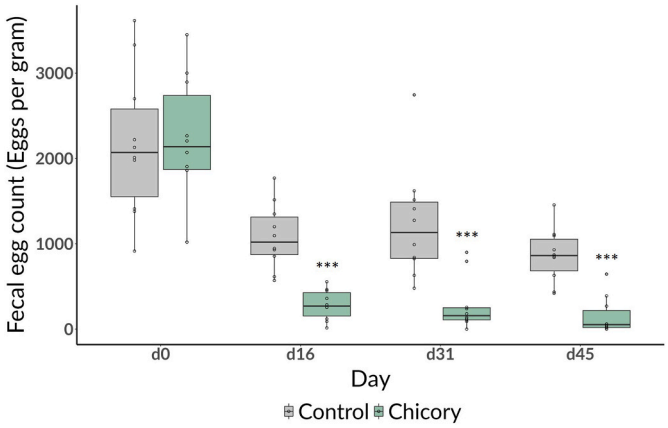

Fig. 1. FEC distribution measured at four time points over the experimental period for control (gray) and chicory (green) groups
Asterisks indicate the statistically significant difference between groups at each time point ***$P < 0.001$.

3.3. Chicory effect on larval development

Despite similar procedures, the average larval development rate at d0 was close to 0% (1.01 ± 2.45%) in both groups. It increased in both groups at d16 (13.2 ± 7.5% and 12.4 ± 6.4% for the control and chicory groups, respectively (mean ± sd)), without any significant difference

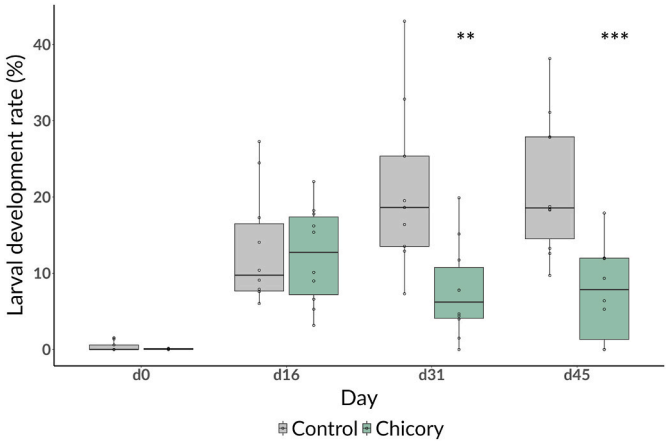

Fig. 2. Larval development rate measured over the experimental period for control (gray) and chicory (green) groups
Asterisks indicate statistically significant differences between the two groups at day 31 (**$P = 0.006$) and day 45 (***$P = 0.001$)

between groups ($P = 0.9$) (Fig. 2). At d31 and d45, the percentage of larval development was significantly lower in the chicory group (2.7 and 2.9 times lower at d31 ($P = 0.006$) and d45 ($P = 0.001$), respectively).

3.4. Chicory effect on equine cyathostomins larval community structure

Following data editing, a significant reduction in alpha-diversity (Shannon's index) was found in the chicory group relative to the control group from day 31 onwards ($P = 0.03$ at day 31 and $P = 0.02$ at day 45 ; Fig. 3A) (Fig. 3A). A slight difference was observed for the Simpson index between the two groups at day 45 ($P = 0.05$). This reduced species richness in the chicory group was concomitant of a significant variation in β-diversity attributable to the experimental group effect (Fig. 3B). The β-diversity shift was underpinned by differential abundances of *Cylicostephanus longibursatus* and *Cylicostephanus minutus* that were less affected by the chicory diet compared to the other species (Fig. 4).

3.5. Sesquiterpenes lactones-enriched extract composition

To test for the putative role played by SLs in the observed *in vivo* activity, the SLs-enriched extract composition was determined using UHPLC-DAD. The obtained chromatogram found a limited subset of SLs. Seven main peaks were observed with retention times of 5.6, 6.8, 10.7, 16.1, 16.4, 17.6, and 18.6 min (Fig. 5). The 16.4 min peak corresponded to the LACP, which was the primary terpene identified with a content of 7.6 μg/mg of extract. By a comparison of the full UV–Visible spectra on each peak, to the reference library we can confirm that peaks with retention times of 5.6, 6.8 and 16.1 min are SLs and those at 17.6, and 18.6 min are flavonoids. However, the peak at 10.7 min remains unknown.

3.6. In vitro anthelmintic activity evaluation of the purified chicory extract enriched of SLs

We next tested the effect of the purified chicory extract on cyathostomin larval development to investigate their role in the inhibitory effect observed *in vivo*, using a pyrantel-sensitive and -resistant isolates (Fig. 6). We noticed, however, that the Chamberet isolate (pyrantel sensitive) displayed a significantly higher sensitivity to the chicory extract enriched of SLs ($IC_{50} = 1.0$ mg/mL [95% CI = 0.6; 1.4]) relative to the INRAE (pyrantel resistant) experimental isolate (IC_{50} of 3.4 mg/mL [95% CI = 3.2 ; 3.5]) ($P < 0.001$).

4. Discussion

This study investigated for the first time the use of chicory as a bioactive forage to control equine cyathostomins. After 16 days of chicory grazing, a significant FEC reduction was observed, reaching an efficacy slightly lower than that of pyrantel treatment at the end of the study (85.5% vs 99.3% FECR for chicory and pyrantel respectively). This diet also reduced the development of eggs into third-stage larvae in feces and affected the cyathostomin community diversity, whereby *Cylicostephanus longibursatus* and *Cylicostephanus minutus,* two of the most prevalent species in the world (Ogbourne, 1978; Bucknell et al., 1995; Lyons et al., 2000; Laugier et al., 2003; Kuzmina, 2012), were the dominant species in the chicory group at the end of the trial. This observation is compatible with a reduced sensitivity of the most abundant species to the chicory regime. On the contrary, minor species would be more prone to disappear following chicory-induced modifications of the fecal microenvironements. The metabarcoding approach would be especially useful to test for species-specific effect of chicory extracts using *in vitro* larval development assay.

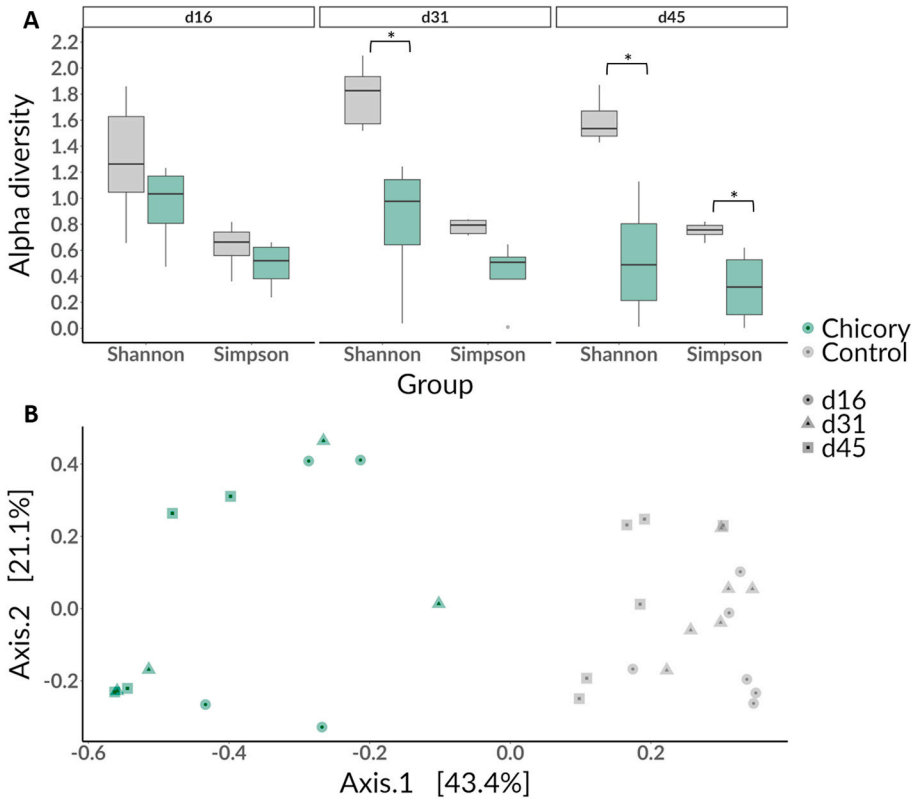

Fig. 3. Effect of chicory on cyathostomin community diversity Upper panel (A) depicts the alpha diversity (Shannon and Simpson index) of cyathostomin species in the control (gray) and chicory-fed horses (green) groups. The bottom panel (B) shows a PCoA plot using Bray Curtis distance (beta diversity) between the group and the day.
Asterisk indicates the statistically significant difference between the two groups at day 31 and day 45 (*$P < 0.05$).

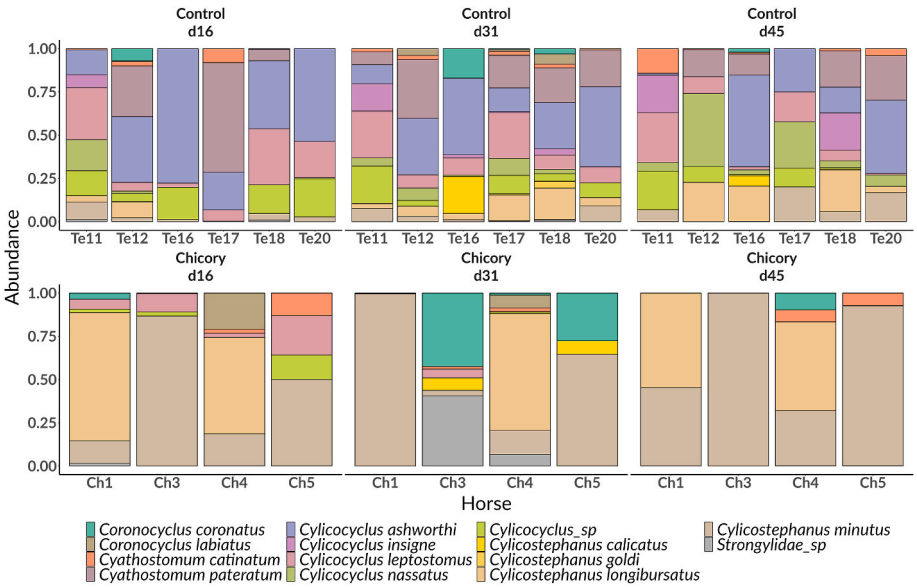

Fig. 4. Cyathostomin larval community abundance estimated using the metabarcoding approach across days and groups.

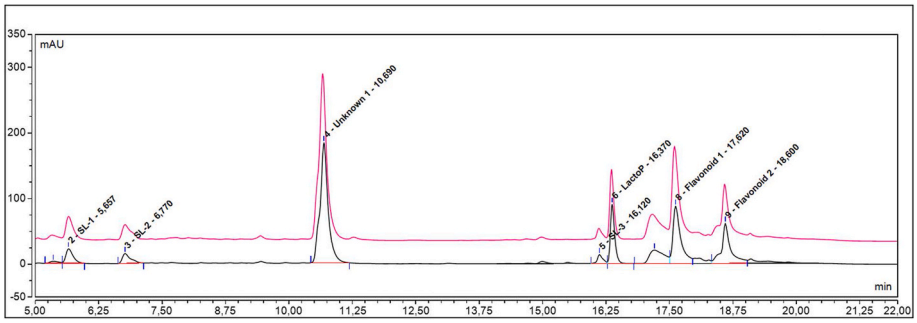

Fig. 5. Chromatogram obtained from chicory (cv. Puna II) extract enriched of SLs used for the *in vitro* LDA
The chromatogram is presented at 215 nm (black) and 280 nm (pink) wavelengths for the chicory extract enriched of SLs. 2 = unknown SL, 3 = unknown SL, 4 = unknown compound, 5 = unknown SL, 6 = lactucopicrin, 8 = flavonoid compound and 9 = flavonoid compound.

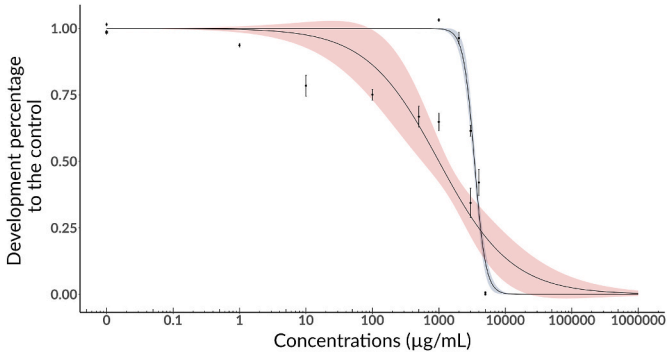

Fig. 6. Dose-response curve of chicory extract enriched of SLs against two cyathostomin populations Development percentage third-stage larvae of cyathostomins isolates from Chamberet (red) and INRAE (blue) in the presence of different concentrations of chicory extract enriched of SLs. A control condition was performed presented by squares found at concentration 0.

While the chicory group showed a significant reduction in FEC overall, this reduction occurred in both groups over the first 16 days of the trial. In the lack of any changes brought neither to horse management or to sample collection and treatment, this might be linked to a seasonal-induced reduction in parasite development. As the chicory efficacy was already high at d16 (*i.e.* 72.9%), we may assume that a 16-day consumption of similar amounts of chicory in the diet (*i.e.* 89% of bites) would control cyathostomins infestations. Nevertheless, further studies over a range of conditions are required to confirm these results.

Knowing that studies conducted in ruminants have reported anthelmintic effects for chicory proportions higher than 70% DM in the diet (Peña-Espinoza et al., 2018), we opted for an almost pure chicory cover. The chicory pasture also contained a low proportion of plantain (*Plantago lanceolata*) (0.05% of the potential bites if horses eat at random) with known antiparasitic activity in small ruminants (Reza et al., 2021). However, as this plant was present in only 0.1% of the bites taken by horses, we consider its potential effect on cyathostomins egg excretion and larval development as negligible. Although vegetation quality was slightly higher in the chicory pasture and both treatments offered close biomasses, the BW of horses grazing chicory remained stable while control horses gained on average 13 kg over the course of the study. It may be that horses grazing chicory achieved lower DM intake compared to control individuals because chicory contained more water and/or was less palatable than graminoids. Characterizing nutrient intake in horses grazing chicory was unfortunately not feasible, as this would first require adaptation of the reference method developed for grasslands (*i.e.* based on total fecal collection and estimation of vegetation digestibility from fecal nitrogen (Mésochina et al., 1998; Penning, 2004),). Whatever the case, it therefore seems unlikely that the lower eggs excretion measured in horses grazing chicory resulted from a

higher nutritional status compared to control individuals. Differences of FEC between groups cannot be explained either by differential ingestion of infective larvae as the duration of our study was lower than the prepatent period of cyathostomins. While the present test was carried out with a substantial number of individuals to support the results, additional trials using chicory across other environments, such as in the face of different parasitic isolates or in the presence of a more diversified plant cover would provide decisive evidence that chicory grazing allows robust cyathostomins control. Moreover, the production of secondary metabolites is influenced by pedoclimatic conditions, the chicory cultivar, the age of the crop and the season of the year (Foster et al., 2006, 2011), which justifies studying the effect of chicory under other conditions.

Studies on small ruminants (Marley et al., 2003; Athanasiadou et al., 2005; Tzamaloukas et al., 2005; Heckendorn et al., 2007; Peña-Espinoza et al., 2016) suggest that chicory has anthelmintic activity against abomasal nematodes, but no effect was observed on small intestinal worms. In horses, our results provide evidence that chicory have an anthelmintic activity against large intestinal cyathostomins. However, the underpinning mechanisms related to the substantial FEC reduction following chicory intake still remains to be elucidated. Chicory harbors sesquiterpenes lactones (SLs) that are known to exert anthelmintic activity against *A. suum, Cooperia oncophora, Oesophagostomum dentatum, O. ostertagi* and *Teladorsagia circumcincta in vitro* (Peña-Espinoza et al., 2015, 2017; Williams et al., 2016; Valente et al., 2021). This is in line with our results of reduced larval development in feces. However, the *in vitro* effects of the chicory extract enriched of SLs against cyathostomin larvae were not able to recapitulate the *in vivo* efficacy in our trial. *In vivo* effects on larval development were observed after 31 days, while *in vitro* larvae were exposed for 6 days. This difference suggests that a longer exposure time may be required for chicory to have an effect on larval development.

Further, chicory SLs may exert indirect *in vivo* effects on adult worms. Indeed, SLs have antimicrobial activity (Mohamed et al., 2017) that may affect the horse gut microbiota. Thus, it can be supposed that chicory grazing would have induced a change in the structure of the gut microbiota, which forms a complex biological system (Rynkiewicz et al., 2015) involving direct interactions with the parasite (Hayes et al., 2010; Reynolds et al., 2014). A longitudinal metagenomic study encompassing the whole range of the horse gut macro- and microbiota, *i.e.* archae, bacteria, fungi, protozoa, helminths and viruses (Mach et al., 2022) would contribute to resolve how the ecosystem is affected by the chicory regime *in vivo* as recently reported for pyrantel treatment (Boisseau et al., 2023). In addition, the observed IC_{50} was also higher than that previously observed (*i.e.* 146 μg/mL measured on *A. suum* L3 (Williams et al., 2016)) suggesting differential sensitivities across nematode clades or different compounds being involved. Moreover, previous studies (Peña-Espinoza et al., 2015, 2017; Williams et al., 2016) have evidenced a reduced sensitivity of larval stage relative to adult worms that may mirror the hereing reported effects on cyathostomins. In addition, some studies (Foster et al., 2011; Peña-Espinoza et al., 2015; Valente et al., 2021) investigated the SLs composition of *Puna cultivar* leaves extract and observed the presence of lactucin (LAC), 11,13-dihydrolactucin (DI-LAC), 8-deoxylactucin (8-DOL), 11,13-dihydro-8-deoxylactucin (DI-8-DOL), lactucopicrin (LACP), and 11,13-dihydro-lactucopicrin (DI-LACP). The activity of these different molecules was evaluated on third-stage larvae *A. suum* and showed a higher activity for the 8-DOL (Valente et al., 2021). Using UHPLC-DAD, we observed the presence of LACP (major compound) and three undetermined SLs. LACP was not the most active SL against *A. suum* (Valente et al., 2021) and its concentration was low in our extract (*i.e.* 7.6 μg/mg compared 19.5 μg/mL (Peña-Espinoza et al., 2015) or 21.5 (Williams et al., 2016)). The low concentration of LACP in our extract is another factor that may explain the difference between *in vitro* and *in vivo* results. Based on these results, we can hypothesize that chicory has an effect on adult fecundity, as mentioned in (Peña-Espinoza et al., 2018). This hypothesis supported the reduction in the number of eggs excreted and a reduction in their development in feces caused by low egg fitness.

5. Conclusion

Our study demonstrated the efficacy of grazing chicory (cv. Puna II) to reduce cyathostomins egg excretion and larval development in horses. The magnitude of this effect was almost as high as expected from synthetic drugs, although we identified differential sensitivity between species. We evidenced a direct but limited, activity of chicory extract enriched of SLs on free living stages. The putative indirect anthelmintic effect of chicory consumption needs to be investigated as it is known that the gut microbiota, gastrointestinal nematodes, and the host immune system are tightly intertwined (Cortés et al., 2020; Williams et al., 2020; Boisseau et al., 2023). The direct activity of chicory also remains to be elucidated and additional studies on the nutritive values of this plant in horses are also needed to adjust recommendations in the field.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2024.100523.

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