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Is the rapid fecal blood test safe to asses gastrointestinal health in non-athlete and athlete horses?

Teste rápido para detecção de sangue nas fezes é seguro para avaliação da saúde gastrointestinal em equinos atletas e não-atletas?

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ABSTRACT: Gastrointestinal tract diseases in horses have been reported as frequent health problems. However, there are few rapid tests to screen gastrointestinal health. This research aimed to evaluate biomarkers of equine health in association with the equine fecal blood test (eFBT) in horses. Blood and fecal samples from 160 adult horses, non-athletes and athletes, in different breeding systems for analysis of blood biomarkers and detection of occult blood through eFBT and fecal pH. The non-athlete horses were distributed according to management practices in the G-Mt1, G-Mt2 and G-Mt3, while the athletes were divided according to the type of exercise in the G-Gait, G-Race and G-Vaq groups. The results were analyzed by two-way ANOVA and Tukey's test with p set at 5%. G-Mt1 had the highest frequency of positives (83.3%), while G-Race had the lowest (29.5%). The positive results in non-athletes were associated with a diet rich in soluble carbohydrates in G-Mt1 (83.3%) and crowded housing in G-Mt3 (38.9%). In athletes, it was detected that the age of the animals had an impact on positive results in G-Gait (45.8%), where horses over 6 years old in this group showed more positivity. It was concluded that in horses evaluated, positivity can vary depending on different aspects that stress (housing, diet and, level of competition) the GIT and thus the eFBT can be used as a complementary test and can contribute to the assessment of the health and well-being of horses.

KEYWORDS: equine; colic; ulcer; diagnosis; ELISA.

RESUMO: Doenças do trato gastrointestinal em cavalos têm sido relatadas como problemas de saúde frequentes. No entanto, existem poucos testes rápidos para triagem da saúde gastrointestinal. Esta pesquisa teve como objetivo avaliar biomarcadores da saúde equina em associação com o teste de sangue nas fezes de equinos (eFBT) em cavalos. Amostras de sangue e fezes de 160 cavalos adultos, não-atletas e atletas, em diferentes sistemas de criação foram colhidas para análise de biomarcadores sanguíneos e detecção de sangue oculto por meio de eFBT e pH fecal. Os cavalos não-atletas foram distribuídos de acordo com as práticas de manejo nos grupos G-Mt1, G-Mt2 e G-Mt3, enquanto os atletas foram divididos de acordo com o tipo de exercício nos grupos G-Gait, G-Race e G-Vaq. Os resultados foram analisados por ANOVA bidirecional e teste de Tukey com p definido em 5%. G-Mt1 teve a maior frequência de positivos (83,3%), enquanto G-Race teve a menor (29,5%). Os resultados positivos em não-atletas foram associados a uma dieta rica em carboidratos solúveis no G-Mt1 (83,3%) e alojamento lotado no G-Mt3 (38,9%). Em atletas, foi detectado que a idade dos animais teve impacto nos resultados positivos no G-Gait (45,8%), onde cavalos com mais de 6 anos neste grupo apresentaram mais resultados positivos. Concluiu-se que nos cavalos avaliados, a quantidade de positivos pode variar dependendo de diferentes aspectos que estressam (alojamento, dieta e nível de competição) o TGI e, portanto, o eFBT pode ser usado como teste complementar e pode contribuir para avaliação da saúde e bem-estar dos cavalos.

PALAVRAS-CHAVE: equinos; cólica; disgnóstico; ELISA.

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INTRODUCTION

In recent decades, the emergence of rapid diagnostic tests in equine medicine is notorious. These rapid tests, mostly enzyme-linked serological tests (Enzyme-Linked Immuno Sorbent Assay - ELISA), function as complementary tests in different situations and contribute greatly to establishing the diagnosis. Thus, veterinarians have increasingly used them in different situations, both in the field, for individual assessment, or to predict the health of the herd, and in hospital conditions, since most of the time they have a low cost (Videla & Andrews, 2009; Rebalka & Lindenger, 2021). Among these rapid tests, there is one for detecting fecal occult blood (Equine Fecal Blood Test - eFBT) for horses, which may facilitate the diagnosis and prognosis of certain diseases of the gastrointestinal tract (GIT).

The eFBT can be used by veterinarians to complement initial clinical assessments in a large number of animals or training centers (Pellegrini & Carter, 2012), and in specific individual cases. Albumin and/or hemoglobin are not detected in herbivore feces unless lesions occur. The eFBT, on the other hand, detects the presence of albumin and/or equine hemoglobin in fecal samples and was developed after research with fecal samples obtained during necropsies with healthy horses and those with erosions or gastric and colonic ulcers, in which the two proteins were not present in fecal samples from healthy horses, but its presence was associated with erosions or ulcers in different areas of the GIT (Pellegrini & Carter, 2009; Pellegrini & Carter, 2012). These results can be corroborated by the results of other researchers with other tests, which showed that gastric and/or colonic lesions may be associated with anemia and hypoalbuminemia in horses, due to the leakage of albumin and/or hemoglobin into the ingesta (Galvin, Dillon & McGovern, 2004; Videla & Andrews, 2009).

Recently, a large-scale evaluation of the eFBT showed that the tests had negative and positive predictive values above 75% and can be used as part of the methodologies for the diagnosis and prevention of GIT diseases (Reese & Andrews, 2009; Videla & Andrews, 2009). Injuries, such as equine intestinal erosions and ulcers, do not have a laboratory diagnosis (Reese & Andrews, 2009), making diagnosis difficult and even though these types of injuries can lead to a reduction in the concentration of total plasmatic proteins, mainly albumin, and hemoglobin and/or anemia (Galvin, Dillon & McGovern, 2004; Pellegrini, 2005; Reese & Andrews, 2009), this reduction in these metabolites may be present in different other diseases. Therefore, including eFBT in regular clinical evaluations of horses could be an important test for screening and diagnosis.

There are still few studies on the eFBT test in horses in different breeding systems and sports practices, which is why it is important to disseminate this type of test for a better understanding of the results obtained and better application of the tests. So it was hypothesized that the type of athlete performance or horse's management would influence the frequency of positive cases for the equine fecal blood test (eFBT) in athlete and non-athlete horses, and to test this, a study was developed that aimed to verify the presence of blood (equine albumin and hemoglobin) and pH in fresh fecal samples from healthy athlete and non-athlete horses horses.

MATERIAL AND METHODS

All procedures were approved by the ethics and animal welfare committee of the Committee on Ethics in the Use of Animals with registration number 01200702819/2016-97.

To carry out these studies, 160 clinically healthy adult horses were used and which had not been medicated in the last 30 days prior to collections with anti-inflammatories or antibiotics. All biological samples were collected from horses housed in different locations, during the dry period of the year, between November and February, and under the supervision of veterinarians responsible for training centers or housing farms. These horses were divided into two main groups: non-athlete and athlete horses.

Non-athlete horses were distributed into the following groups:

1. **G-Mt1** (n=6): composed of Arabian horses, housed at the Equine Research Center, at the Federal Rural University of Pernambuco (UFRPE), with ages varying between 5 and 18 years old, kept free in native pasture area (>1.0 hectare/animal), and individually supplemented with concentrate (1x/day with 1.0 Kg/animal, Laminated Equitage, Guabi Nutrição Animal). Tifton hay (Cynodon spp.), salt and water were provided ad libitum. These group of animals were characterized by the free consumption of ripe mangoes, present in abundance on the pasture, during the period of feces and blood collection.
2. **G-Mt2** (n=6): This group was made up of horses described in G-Mt1, but during this period they had not had access to fresh mangoes in the pasture for more than 60 days due to the end of the fruit harvest. The nutritional management was the same as that observed in G-Mt1, with the animals being free on pasture for around 24 hours a day.
3. **G-Mt3** (n=18): the group consisting of mixed-breed animals that were rescued and housed at the Recife-PE Municipal Animal Control Center, with ages varying between 4 and 20yo. They were kept in collective boxes with three or four animals (~6m^2/animal), without individualized diet. They received Tifton hay (Cynodon spp.) 3x a day in a collective trough, with free access to water and mineralized salt. Animals that had been dewormed for more than 30 days and with negative results for EIA and Glanders, and did not have access to exercise areas.

In turn, the athlete horses were distributed into the following groups:

1. **G-Race** (n=24): This group was of racehorses (Thoroughbred), housed in different Studs at the

Jockey-Club Pernambucano, with ages varying between 3 and 6yo, where training and competitions were held. There were housed in an intensive system, in individual boxes (~16m²/animal) with easy visual interaction between the animals. They received 5-6 Kg/animal/day of Tifton grass or hay (Cynodon spp.) divided into two meals. They were supplemented with concentrates from different brands with quantities ranging from 7.5 to 13 kg/animal/day and divided into 3 daily meals. Water and mineralized salt ad libitum. Training took place 4-6 times a week on the racecourse, trotting and galloping for 5-10 minutes, plus walking in the equine village sand roads for 15-25 minutes twice a day every day except race days. They did not have access to free exercise areas.

2. **G-Gait** (n=48): This group was of gait horses (Mangalarga-Marchador and Campolina), which were in training and competition and were housed in gait training centers in different locations in the State of Pernambuco, with ages varying between 4 and 9yo. They were in individual boxes (~12m²/animal) with easy visual interaction between them. They received fresh and chopped elephant grass (Pennisetum purpureum Schum.) and/or Tifton hay (Cynodon spp.). Furthermore, these horses received between 4-6 kg/animal/day of commercial concentrate from different brands, which were divided into 3 daily meals. Water and mineralized salt ad libitum. Training, both walking and walking, lasted 40-50 minutes and 3-5 times a week. All had a paddock (75-100m²) for free exercises where they remained for about 2-3 hours, 2-3 times a week, on days when they didn't train or when they came back from competitions.
3. **G-Vaq** (n=58): This is a group of vaquejada (Quarter Horses) were housed in different locations in the State of Pernambuco, with ages varying between 4 and 14yo. These animals were housed in individual boxes (~12m²/animal), with easy visual interaction between them. They consumed 20-25 Kg/animal/day of chopped elephant grass (Pennisetum purpureum Schum.) or 15 Kg/animal/day of Tifton hay (Cynodon spp.) divided into three meals, in addition to supplementation with 6-8 Kg /animal/day of commercial concentrate, divided into three meals per day. Water and mineralized salt *ad libitum.* The training sessions were carried out four times a week, for 30 minutes, at a trot and gallop, with or without the presence of cattle, and twice in 60-minute walks, but they don't have access to free exercise areas.

Blood samples were obtained after fasting for 12 hours in all groups, in which blood collection was performed through jugular puncture with vacuum tubes containing EDTA. Then, these samples were immediately cooled and sent to the Molecular Biology Laboratory Applied to Animal Production at UFRPE where a blood count was performed using a semi-automatic cell counter (Poch-100iv diff, Roche Diagnostics®), and subsequently centrifuged to obtain plasma. In plasma frozen at -20°C, total plasma protein, albumin, and GGT were subsequently determined in semiautomatic equipment (Doles® D-250, São Paulo-SP, Brazil) using commercial kits (Doles® Reagentes, Goiânia-GO, Brazil). An aliquot of the plasma was used to measure Calpropectin in the samples of the horses in the athlete groups and, for this, an ELISA test was carried out in semiautomatic equipment (Bioclin® Minddray MR-96A, 837 Minas Gerais, Brazil) with a commercial kit (Mouse Calprotectin (Calprotectin) Elisa/Nordic BioSite®).

After fasting, the animals were fed to stimulate intestinal peristalsis, and thus defecation. Then, after natural defecation, stool samples were collected, divided into two aliquots, and immediately analyzed; the first was used for the detection of blood in the feces by eFBT, and the second for the evaluation of the stool type scale and fecal pH determination (Manso Filho *et al*., 2022). The eFBT determination followed the manufacturer's instructions (Succeed® Equine Fecal Blood Test, Freedom Health LLC, USA). Fecal pH was assessed by the direct method with portable equipment (Oakton pH Spear 35634-40, Oakton Instruments, USA) (Trindade *et al*., 2021).

The body score of the animals was defined according to Henneke *et al*. (1983) and the corporal mass was determined using equine weighing tape. For statistical analyses, the horses were subdivided into non-athlete horses which included animals from G-Mt1, G-Mt2, and G-Mt3; and athlete horses, which includes G-Race, G-Gait, and GATVaq animals. The results obtained in the evaluations of the non-athlete group and the athlete group were analyzed separately, due to the great difference in the rearing system and physical activity. Results were submitted to two-way ANOVA (groups and eFBT results), and Tukey's test when indicated, for non-athlete and athlete groups. In both cases, the p-value was set at 5%. The SigmaStat 13.0 program (Systat Software Inc. California, USA) was used for statistical analysis. Results are expressed as mean +/- mean standard error.

RESULTS

The eFBT, indicating the presence of albumin and/or hemoglobin, revealed that of the 160 horses, 39.4% presented positive results (Table 1). However, the groups of non-athlete and athlete horses tested positive differently. The highest percentages of positive eFBT were in G-Mt1 (83.3%) and G-Gait (45.8%), and less frequent in G-Mt2 (33.3%) and G-Race (29.2%), non-athletes and athletes respectively.

The results of the 2-way ANOVA demonstrated that comparisons of results between horses in the non-athlete group showed differences in body mass, fecal pH, total plasma proteins and GGT ($p < 0.05$). G-Mt1 had a higher body score than G-Mt3 and was similar to G-Mt2. Furthermore, G-Mt1 presented lower fecal pH and GGT concentration (Table 2). Analyzing the positive and negative results of eFBT, no significant differences were observed between biomarkers, and there were also no significant interactions between biomarkers and eFBT.

In the group of athletic horses, results of the 2-way ANOVA indicated differences for the following biomarkers according to the groups: age, body mass, body score, fecal scale, fecal pH, total plasma proteins, red blood cells, hematocrit, MVC, leukocytes and lymphocytes ($p < 0.05$) (Table 3). The other biomarkers did not show significant differences ($p > 0.05$).

Still comparing the negative and positive eFBT results, there were no differences between the athlete horses, but

Table 1. The results of eFBT in horses according to Non-Athletes and Athletes Groups.

Groups	eFBT results				Positives*
	Alb+, Hb+	Alb+, Hb-	Alb-, Hb+	Alb-, Hb-	
Non Athletes					
G-Mt1 1 (n=6)	2	2	1	1	83.3%
G-Mt2 (n=6)	1	0	1	4	33.3%
G-Mt3 (n=18)	1	6	0	11	38.9%
Athletes					
G-Race (n=24)	0	7	0	17	29.2%
G-Gait (n=48)	5	10	7	26	45.8%
G-Vaq (n=58)	6	11	3	38	34.5%
Total (n=160)	15	36	12	97	39.4%

Notes: eFBT: equine fecal blood test; ALB: albumin; Hb: hemoglobin; +: positive result; –: negative result. G-Mt1: maintenance 1 in the mango season, G-Mt2: maintenance after the mango season, G-Mt3: animals maintenance at rescue center, G-Race: race horses, G-Gait: four-beat gaited horses, G-Vaq: vaquejada horses; *eFBT-positive animals are horses that are positive for albumin and/or hemoglobin, as indicated by the manufacturer.

Table 2. Results of 2-way ANOVA, independent of the eFBT result, for biometric, fecal and blood biomarkers in the groups of non-athletic horses.

Parameter	Non-Athlete Groups		
	G-Mt1	G-Mt2	G-Mt3
Age, years	12.5±2.6	12.5±2.6	8.6±1.2
Weight, Kg	343.6±31.6	361.0±23.6	302.2±14.0
Body score	5.0±0.4 A	4.8±0.3 AB	4.0±0.2 B
Fecal scale	2.4±0.5	3.7±0.4	3.8±0.2
Fecal pH	5.8±0.3 B	7.2±0.2 A	7.0±0.1 A
TPP, mg/dL	6.0±0.3 B	6.4±0.2 B	7.5±0.1 A
Albumin, mg/dL	2.6±0.3	2.8±0.3	3.0±0.1
GGT, UI/L	21.6±8.3 B	46.8±6.2 A	31.0±3.7 AB
Leukocytes, $x10^3/mm^3$	7.4±1.8	8.4±1.4	11.2±0.8
Lymphocytes, $x10^3/mm^3$	2.6±0.7	2.8±0.5	3.8±0.3
Other cells, $x10^3/mm^3$	4.3±1.8	5.5±1.4	7.5±0.8
Red cells, $x10^6/mm^3$	7.0±0.7	7.6±0.5	7.2±0.3
Hemoglobin, g/dL	10.7±1.1	11.4±0.8	11.0±0.5
Hematocrit, %	31.2±3.0	32.9±2.2	31.3±1.3
RDW-CV, fL	20.1±1.2	20.8±0.5	19.9±0.9
MCV, fL	44.5±2.8	43.4±2.1	44.0±1.2
MCHC, g/dL	34.3±0.5	34.9±0.4	34.7±0.2
Platelets, $x10^3/mm^3$	191.0±33.0	198.0±24.6	193.3±15.00

Notes: Different letters on the same line indicate that p<0.05 by Tukey's test; TPP: total plasma proteins, GGT: γ-glutamyltransferase, RDW-CV: erythrocyte distribution amplitude as coefficient of variation, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration. G-Mt1: maintenance 1 in the mango season, G-Mt2: maintenance after the mango season, G-Mt3: animals maintenance at rescue center.

there were significant interactions between these groups in the eFBT results for age ($p < 0.05$). In G-Gait, the percentage of positive eFBT was higher in older horses in this group ($p < 0.05$). However, no significant differences were observed in G-Race and G-Vaq for the age groups (Table 4).

Comparing the three groups of athletes with each other and according to the eFBT results, regardless of the age of the horses, it was observed that the highest frequency of negative results was in the G-Vaq horses ($p < 0.05$), and positive results were found in the G-Gait followed by G-Vaq ($p < 0.05$) (Table 5).

Table 3. Two-way ANOVA results (athlete groups and eFBT results), according to the athlete groups and independent of the eFBT result.

Parameter	Athlete Groups		
	G-Race	G-Gait	G-Vaq
Age, years	3,3±0,8 B	5,2±0,5 AB	6,6±0,5 A
Weight, kg	430,84±13,62 A	363,08±9,00 B	440,08±9,26 A
Body score	5,00±0,11 A	4,66±0,072 B	5,00±0,07 A
Fecal scale	2,00±0,20 C	3,44±0,13 B	4,13±0,13 A
pH feces	6,71±0,13 B	7,30±0,09 A	7,34±0,09 A
TPP, g/dL	8,49±0,24 A	7,61±0,16 B	6,91±0,16 C
Albumin, g/dL	4,01±0,12	3,68±0,08	3,83±0,08
GGT, UI/L	45,56±8,04	37,51±5,31	34,75±5,47
Calprotectin, ng/dL	41,10±5,30	27,70±3,60	27,82±4,17
Red cells, x10^6/mm^3	9,52±0,32 A	8,41±0,21 B	8,20±0,22 B
Hemoglobin, g/dL	13,58±0,41	12,41±0,27	12,57±0,28
Hematocrit, %	39,2±1,19 A	35,89±0,78 B	35,94±0,81 B
RDW-CV, fL	21,5±0,3	20,7±0,2	21,0±0,2
MCV, fL	42,0±0,6 B	43,2±0,4 AB	43,9±0,4 A
MCHC, g/dL	34,8±0,2	34,7±0,1	35,0±0,1
Leukocytes, x10^3/mm^3	8,58±0,56 AB	9,46±0,37 A	7,56±0,38 B
Lymphocytes, x10^3/mm^3	2,37±0,37 B	4,51±0,25 A	2,95±0,25 B
Platelets, x10^6/mm^3	142,02±11,03	141,35±7,29	126,60±7,50

Notes: Different letters on the same line indicate that p<0.05 by Tukey's test; TPP: total plasma proteins, GGT: γ-glutamyltransferase, RDW-CV: erythrocyte distribution amplitude as coefficient of variation, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration. G-Race: race horses, G-Gait: four-beat gaited horses, G-Vaq: vaquejada horses.

Table 4. Results of significant interactions with two factors (athlete group and eFBT result) dependent on athlete group by age.

eFBT	Athletes					
	G-Race		G-Gait		G-Vaq	
	Negative	Positive	Negative	Positive	Negative	Positive
Age, years old	4.0±0.7	3.0±1.1	5.0±0.6 B	6.4±0.6 A	7.3±0.5	5.9±0.7

Notes: Different letters within the same group of athletes indicate p<0.05, using the Tukey test. G-Race: racing horses; G-Gait: horses with four-beat gait; G-Vaq: bucked horses.

Table 5. Results of significant interactions by two-way ANOVA (athlete group and eFBT result) dependent on eFBT results by age groups.

Athletes	eFBT results					
	Negative			Positive		
	G-Race	G-Gait	G-Vaq	G-Race	G-Gait	G-Vaq
Age, years old	4.0±0.7 b	5.0±0.6 b	7.3±0.5 a	3.0±1.1 B	6.4±0.6 A	5.9±0.7 AB

Notes: Different letters in the same line and in the same results of the eFBT indicate p<0.05, by Tukey's test. G-Race: race horses; G-Gait: four-beat gaited horses; G-Vaq: vaquejada horses.

DISCUSSION

Non-Athlete Horses

In G-Mt1, G-Mt2 and G-Mt3, no significant variations were found in most morphological and blood biomarkers, but some significant differences were observed in particular aspects pertinent to each of the groups. The lowest body score was observed in horses in G-Mt3 when compared to those in groups G-Mt1 and G-Mt2 ($p < 0.05$). The G-Mt3 animals, despite receiving good quality hay ad libitum, were not supplemented with concentrates and were housed in a collective environment with restricted space (less than $2m^2$/horse), which may have compromised the consumption of food necessary to maintain a higher body score.

Unlike the animals in G-Mt1 and G-Mt2, those in G-Mt3 did not receive supplementation with concentrates, although they had 38.9% of positive eFBT and fecal pH above seven, which may be related to the collective husbandry system. As previously mentioned, space was limited and there was competition for food and territory in the stalls, in addition there was a lack of space for free exercise, contributing to chronic stress. This type of stress is associated with the appearance of intestinal lesions, erosions and ulcers, and dysbiosis in horses (Reese & Andrews, 2009; Hepburn, 2011; Mach *et al*., 2020; Garber *et al*., 2020; Rankins *et al*., 2022). Unlike G-Mt3, animals in G-Mt1 and G-Mt2 received hay and concentrate supplementation individually to meet the needs indicated in the NRC for horses (2007), for maintenance animals with body weight around 400 kg. In these last two groups, in addition to having access to pastures with native forage and space for free exercise, they could consume fresh forage without competing with each other, favoring GIT and mental health.

Another parameter that showed significant differences in non-athlete horses was fecal pH, with the lowest value detected in G-Mt1. During the period of the G-Mt1 assessments, they had free access to ripe mangoes in the pasture in large quantities, as regularly occurs at the beginning of the dry summer in the region. It is known that excess carbohydrates, such as starch, can cause a reduction in fecal pH and alter the consistency of equine feces, impacting the microbiota of the GI tract (Santos *et al*., 2009; Hapburn, 2011). Excessive consumption of fresh mangoes, which is rich in soluble carbohydrates, such as what was described in G-Mt1 horses, may be related to dysbiosis and intestinal dysfunction, increasing erosive lesions or even ulcers in G-Mt1 (Al Jassim *et al*., 2008; Silva *et al*., 2022). Unlike G-Mt1, G-Mt2 were only being supplemented with concentrate, hay and native pasture at the end of the dry summer, but without the presence of ripe mangoes or other fruits in the pasture. During this period of the year, G-Mt2 registered an increase in the fecal pH value, suggesting that a diet without excess soluble carbohydrates contributes to the health of the GIT and the well-being of horses in maintenance.

Evaluating the results of the eFBT, it was found that G-Mt1 had a higher frequency of positive results (83.3%), and when the possible triggering factor for the lesions was eliminated, the frequency of positive results decreased, as observed in G-Mt2 animals (33.3%) (Table 1). In this last group, the frequency of positives for eFBT was below the average frequency of positives in the 160 horses in the present trial. Recently, using gastroscopy to diagnose gastric lesions, Silva *et al*. (2022) demonstrated that high consumption of ripe mangoes is associated with the appearance of erosions and gastric ulcers in horses.

The GGT was also biomarker that showed differences between non-athlete horses. This enzyme is related to liver activity, and in groups of non-athlete horses, differences were observed between G-Mt1 and G-Mt2 ($p<0.05$), but with G-Mt3 similar to both. G-Mt1 presented the lowest concentrations among the groups. However, after the end of the heat season, the concentration of GGT increased in G-Mt2 horses. Despite these variations, concentrations in the three groups of non-athlete animals were within the normal range for animals of the equine species. However, it is recommended that new laboratory assessments be carried out to understand the impact of diet, housing and lack of free exercise on variations in this enzyme and its possible relationship with GIT diseases.

The three groups of non-athlete horses were healthy and most biomarkers were within the normal range described in the literature (Perry, 2002), but laboratory tests are not reliable for assessments of various GIT pathologies. According to Reese and Andrews (2009), the presence of erosions and/or ulcers in the GIT does not have a laboratory diagnosis, which makes the inclusion of eFBT an important test in decision-making for carrying out more complex exams to diagnose illnesses in the GIT.

Athlete horses

The three groups of athlete horses, G-Race, G-Gait and G-Vaq, exercised regularly and were fed under different conditions than the horses in the non-athlete horse groups. In addition horses' breeds of these athletes have different morphology and physiological adaptation of the type of exercise. Because that, it was expected that there would be significant variations in several analyzed parameters.

In horses with a profile for speed sports, muscle mass is usually greater than in horses for endurance races, a fact verified in the present trial, in which the horses in the G-Race and G-Vaq were heavier and had a better body score when compared to G-Gait ($p<0.05$). Endurance horses, such as G-Gait, are lighter, have less weight and have a larger body surface when compared to speed horses, facilitating heat loss during medium and long durations exercise (Guthrie & Lund, 1998). This adaptation facilitates thermoregulation and it is important for different resistance equestrian sports and may be related to the performance of horses in different climatic conditions (Jones, 1989; Guthrie & Lund, 1998).

The evaluation of blood biomarkers in athlete horses must take into account both dietary management, training and breed factors. Thoroughbred horses, in training, are the horses that present higher red blood cells concentrations and adaptations in plasma volume (Guthrie & Lund, 1998; Betros *et al*., 2013). In the current experiment, in G-Race, TPP, red blood cells and hematocrit values were higher and significantly different from G-Mar and G-Vaq, which was expected. Variations in MCV were greater in G-Vaq, but all three groups of athlete horses remained within normal limits (Perry, 2002).

Still associated with the training system for athlete horses, G-Race received supplementation with more concentrate and less forage than G-Gait and G-Vaq. This lower forage supplementation may be the factors that impacted the characteristics of racehorse feces. Thus, the lowest score for fecal assessment was observed in G-Race (2.0), followed by G-Gait (3.5), with the highest score being in G-Vaq (4.0). Horses with a fecal score of 2.0 have pasty feces without the formation of syllables, which is a typical characteristic of equids. Pasty stools represent that there is some degree of dysbiosis in the GIT (Manso Filho *et al*., 2022).

These characteristics can also be reflected in the assessment of fecal pH, as the more concentrated the diet, without an adequate supply of forage, the feces tend to become more acidic. In contrast, horses with an adequate diet and good quality forage, which tend to have fecal pH close to neutral (Trindade *et al*., 2021). In G-Race, fecal pH was slightly acidic (6.7) when compared to G-Gait (7.6) and G-Vaq (7.4) ($p<0.05$). Therefore, the veterinarian must seek to relate the fecal pH results with the animal's handling conditions, mainly feeding, to contextualize the fecal score and fecal pH, as they are factors that can alter the results of these tests in clinically healthy horses and suggest new prophylactic measures to maintain GIT health.

When we correlate information about the training system, biometric indices, fecal indices and blood biomarkers to eFBT results, some of them indicate the need for improvements in training and competition practices for these horses. G-Race, even with high supplementation with concentrates to provide the energy necessary for training/competitions, presented the lowest frequency of positive results for eFBT (29.2%). This fact was not expected, due to the more acidic pH and low fecal score, which may be related to the provision of quality forage, including alfalfa hay, and the adaptation of current feeding management conditions.

On the other hand, G-Gait presented a higher frequency of positive tests (45.8%), higher than the average of the 160 animals evaluated, athletes and non-athletes. In G-Gait, these results must be related to food quality, concentrates and forage, as food is often used based on costs. However, the type of training for four-beat gait competitions may also be involved, especially in smaller training centers that have less space for rest after training sections. In G-Vaq, the positive results (34.5%) were below the general average (39.4%), even for animals undergoing intensive training, but it is the animals in the athlete group that are supplemented with higher quality forages and premium concentrates.

The results of the relationships between eFBT and biomarker parameters found a significant interaction between eFBT results and athlete group by animal age. In all horse breeds, age is an important factor in the initiation of training and competition (Jones, 1989; Hodgson, McGowan & McKeever, 2014). For example, racing and vaquejada horses already compete at the age of 3yo, which is not the case for gaited horses. Thus, analyzing the groups of athletes, it was found that in G-Gait the horses with positive eFBT were the oldest (~6.3yo) ($p<0.05$), while in G-Race and G-Vaq these age differences were not observed.

The gaited horses, Campolina and Mangalarga Marchador breeds, begin more intense training at 4yo, but only at 6yo are they in full competition with almost daily training lasting more than 45 minutes and supplemented with an adequate diet for the energy expenditure of the animals that practice this type of exercise. However, older G-Gait horses travel regularly to competitions and are more subject to the chronic stress of training and competitions combined with transport and housing in different locations, which can favor the appearance of intestinal diseases (Venable *et al*., 2016; Connysson *et al*., 2017; Mach *et al*., 2020; Garber *et al*., 2020).

Further analyzing the interactions, it was observed that the positive and negative results of eFBT, regardless of the groups of athletes, demonstrated that old G-Vaq (~7.3yo) was the one with in negative results, while with the positive eFBT results, they were in younger G-Gait (~6.4yo). G-Vaq was similar to the other two groups of athletes ($p>0.05$), reinforcing the indication that G-Gait horses in competition and intensive training should be better monitored during these periods of competition. We must also emphasize that racing and vaquejada horses begin their activities earlier when compared to gaited horses, therefore they must reach the full training and competition phase earlier and, thus, adapt from a metabolic and behavioral point of view to the conditions of competition.

In addition, it should be noted that G-Vaq are transported regularly, but during their competitions they are housed in large collective environments, with free space for exercise, receiving individual supplementation with concentrate and free access to hay, conditions that should reduce chronic stress in this groups of horses. G-Race horses remain housed in Studs for long periods without being transported frequently, thus adapting well to local management conditions. These results may suggest that competitions and its characteristics can interfere with the health of the GIT in equestrian sports throughout the training until the animals reach full adaptation to their modality and that this adaptation may be related to the age of the animals.

Finally, the concentrations of Calprotectin in the athlete group were analyzed because this hormone is known as a biomarker of the degree of intestinal inflammation and can be measured in fecal and blood samples (Jukic *et al*., 2021; Mihelic *et al*., 2022). It is also higher in GIT diseases and has been shown to be a good biomarker for the prognosis of these diseases, even though it may be elevated in different inflammatory processes (Poullis *et al*., 2003). In horses submitted to the ischemia/reperfusion challenge, the concentration of Calprotectin in the blood collected in jugular vein and in colonic vein were 6.3 and 4.9 ng/mL in the pre-test and 4.5 and 5.6 ng/mL after 1 hour of reperfusion , respectively (Grosche *et al*., 2013). In endurance horses the concentration of Calprotectin decreases significantly after competitions, ranging from 30.08 ng/mL in the pre-race to 27.17 ng/mL in the post-race, and there is a positive correlation between this hormone and platelets concentrations (Mihelic *et al*. al., 2022).

In the current experiment, no differences were observed for Calprotectin between athlete horses (p=0.08), including between horses that have positive and negative eFBT. The mean values of Calprotectin showed the lowest concentration in G-Gait (27.70 ng/dL) and the highest in G-Race (41.10 ng/dL), which are similar concentrations to the described by,,, Mihelic *et al*., (2022) but superior to those by Grosche, *et al*., (2013), with samples obtained from the jugular. Thus, even though the G-Race showed a lower frequency of eFBT positives, it had a higher concentration of Calprotectin, stools were more acidic, and had a lower fecal evaluation score, indicating some impairment of GIT health. Therefore, this should be further evaluated to understand the clinical and laboratory findings in horses under different management systems and using other complementary tests, such as gastroscopy and ultrasound, could facilitate the analysis of the impact of Calprotectin on understanding the health of the GIT in this species.

CONCLUSIONS

In non-athlete horses (G-Mt1, G-Mt2, and G-Mt3), the greatest impact of the positive results was related to nutritional management associated with excess soluble carbohydrates and, collective housing. In the groups of athlete horses (G-Race, G-Vaq, and G-Gait), age was the determining factor in positivity, which is related between the beginning and peak of training/competition, but with different impacts on the three groups of athletes. Finally, it is worth highlighting that the eFBT is an exam that can be used as a complementary tool for diagnosing diseases in the GIT, as it is indicative or not of injuries such as erosions and ulcers. And because it is non-invasive and low-cost test, it can be a screening exam to indicate more precise tests such as gastroscopy or intestinal biopsies.

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