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Gastric content technique collection for *in vitro* degradation and gas production in horses supplemented with live yeast and protected yeast

[Técnica de coleta de conteúdo gástrico para degradação e produção de gases in vitro *de equinos suplementados com levedura viva e levedura viva protegida]*

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ABSTRACT

Due to the difficulty in acessing certain sites of fermentation and possible starch digestion, studies show the need for equine gastric digestive fisiology evaluation. The aim of this experiment was to assess horses supplemented with live yeast and protected live yeast (S*accharomyces cerevisae* NCYC Sc 47) to validate a new procedure of gastric content collection for determination of pH, latic acid, short chain fatty acids and gas production and degradation to be used in further *in vitro* fermentative studies. The experimental design used was a the latin square (4x4) for 4 periods and 1 day of gastric content collection, with 15 days of rest between it. The groups were divided in control (CTRL), non protected live yeast (NPYEA), protected live yeast (PYEA) and a combination of live yeast + protected yeast (COMB). Treatment means were compared using orthogonal contrasts (C1: CTRL *versus* NPYEA, PYEA and COMB; C2: COMB *versus* NPYEA and PYEA; C3: NPYEA *versus* PYEA) and Tukey´s test was used at a 5% significance level. The procedure was highly tolerated and provided great accuracy of the collection site. The live yeast supplementation increased the concentration of acetic acid at the horses' stomachs. When gastric content was used as inoculum for *in vitro* fermentation, the protected live yeast produced less gas.

Keywords: degradation, equine, fermentation, nutraceutical, stomach

RESUMO

*Com dificuldade de fazer abordagens a locais de processos fermentativos com uma possível digestão de amido, estudos vêm demonstrando a importância da avaliação da fisiologia digestiva gástrica de equinos. O objetivo desta pesquisa foi avaliar equinos suplementados com levedura viva e levedura protegida (*Saccharomyces cerevisae *NCYC Sc 47) para validar um novo procedimento de coleta de digesta gástrica, avaliando-se os parâmetros de pH, ácido lático, ácidos graxos de cadeia curta, degradação e produção de gases para estudos do processo fermentativo* in vitro. *O delineamento utilizado foi o quadrado latino duplo (4x4) por 4 períodos, com um dia para coleta de conteúdo gástrico e 15 dias de descanso. Os grupos foram divididos em: controle (CTRL), levedura viva (NPYEA), levedura viva protegida (PYEA) e a combinação de viva e protegida (COMB). Os tratamentos foram comparados por contrastes ortogonais (C1: CTRL* versus *NPYEA, PYEA e COMB; C2: COMB* versus *NPYEA e PYEA; C3: NPYEA* versus *PYEA), e foi utilizado o teste de Tukey a 5% de significância. O procedimento apresentou alta tolerância por parte dos animais e proporcionou precisão do local de coleta. O uso de*

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levedura viva aumentou a concentração de ácido acético no estômago dos cavalos. Quando utilizado conteúdo gástrico como inóculo para fermentação in vitro*, a levedura viva protegida apresentou menor produção de gases.*

Palavras-chave: degradação, estômago, equinos, fermentação, nutracêuticos

INTRODUCTION

Studies on gastric secretion in the equine specie have been often performed in fasted horses, therefore most of the postprandial characteristics of the gastric content remain unknown. This is related to technical difficulties in obtaining postprandial gastric content for analysis. To investigate equine gastric ulcer syndrome (EGUS) or normal gastric physiology, different procedures have been developed for the study of gastric contents in horses (Varloud *et al*., 2007), both for fasted and fed horses.

The stomach content pH is a good parameter to determine the collection site. Therefore, it has been studied for *in situ* evaluation of live horses, by means of nasogastric tube (Murray and Schusser, 1993). Once the probe can move within the organ and lead to data variation, a fluoroscopy (Baker *et al.*, 1993) or a radiography (Sanchez *et al.*, 1998) are required to monitor it's position. To avoid this complication, the probe can also be introduced through a cannula (Clark *et al*., 1996; Merritt *et al*., 2003) surgically inserted into the horse's stomach (Campbell-Thompson and Merritt, 1987).

Some other biochemical parameters such as lactate or short chain fatty acids (SCFA) concentrations can only be determined after the sample collection. Currently, another three procedures have been used to achieve it in horses: eutanasia, gastric puncture and aspiration of gastric contents through a nasogastric tube (NG). The technique based on blind aspiration through an NG tube was previously tested by Varloud *et al*. (2007) but did not provide adequate samples, since only small volumes (approximately 10 mL in 20 minutes) could be aspired.

In vitro gas production (GP) techniques were developed to predict the fermentation of ruminant feeds. The food is incubated with inoculum and the gases produced are measured as indirect indicators of fermentation kinetic. This technique allows assessing the rate and the extent of feed degradation. The latter is important for horses due to the faster total transit time of their digestive tract compared to ruminants (Mclean *et al*., 1995; Drogoul *et al*., 2001).

The inoculum is the main source of variation in gas production profiles. The fermentative activity of the inoculum can change due to several factors, such as sampling time, storage, diet, concentration, preparation, etc (Desrousseaux *et al*., 2012).

Variable techniques with different results were already performed to minimize misfortunes of gastric content collection, but all of them could only obtain small samples. The aim of this study was to validate a new gastric content collection technique in horses supplemented with live yeast and protected live yeast (S*accharomyces cerevisae* NCYC Sc 47), evaluating parameters such as pH, lactic acid and short chain fatty acids degradation and gas prodution.

MATERIAL AND METHODS

The experiment was carried out in Pirassununga (SP), Brazil, Latitude 21° 36'00"S; Longitude: 47° 18'00"W. Eight Arabian gelding (60 ± 5 mouths and 457 ± 28kg of body weight) were used. Two years before the trial the horses received routine deworming, vaccinations, and dental floating, During the study, the horses were individually housed in 4 x 4m stalls bedded with wood shavings and had access to a sand paddock for 2 hours a day. During the collection period, the horses were in maintenance and did not perform physical exercise. Wasted hay was removed, and stalls were cleaned daily before each morning feeding. At the beginning and end of each experimental phase, a complete blood cell count in all horses was performed using a Hematoclin 2.8 Vet (Quibasa Bioclin, Minas Gerais, Brazil) to ensure animals were healthy. All animal protocols were approved by the Institutional Animal Care and Use Committee of the School of Veterinary Medicine and Animal

Science of the University of São Paulo, (#6006030315).

Dietary treatments (yeast supplementation strategies) were evaluated in a replicated 4 x 4 latin square design experiment. Horses were weighed using a livestock platform scale (MGR 4000, Toledo do Brasil, São Paulo, Brazil) before starting the study and were separated into two groups (squares) with similar body weight (BW). Within each square, horses were randomly assigned to one of the four supplementation strategies. Each experimental period lasted 21 days and consisted of a 15-day dietary adaptation (day 1 to 15), followed by a 5 day total feces collection (TFC) (day 16 to 20), and a 1 day gastric liquor sampling (day 21). After each experimental period, horses were fed the basal diet for 15 days.

The basal diet was comprised of bermudagrass hay (*Cynodon dactylon* L. 'Tifton 85') and a pelleted concentrate. The chemical composition of the diet and the ingredients used to formulate the pelleted concentrate are shown in Tab. 1. The pelleted concentrate was manufactured at 70ºC maximum temperature and 1 m^3 pressure at the Feed Mill at the University of São Paulo (Pirassununga, Brazil). Diet was formulated to meet the daily nutritional requirements of maintenance horses (Martin-Rosset, 2012) fed at 1.75%BW on a dry matter (DM) basis with a forage-to-concentrate ratio of 57:43. Meals were offered twice a day (7 am and 4 pm) representing 50% of the daily DM intake each. The hay was offered to the horses in nylon-roping hay nets with 10-cm diamond-shape openings, while the concentrate was offered in concrete bunk feeders. Horses had unlimited access to fresh, clean water and mineral supplements (Kromium, Tortuga) throughout the study.

Dietary treatments consisted of a basal diet associated with one of the following yeast supplementation strategies: CTRL: without yeast supplementation ; NPYEA: 15g/d of non-protected live yeast (*Saccharomyces cerevisiae* 1.5×10^{10}cfu/g; Procreatin-7; LeSaffre Feed Additives, Marquette-Lez-Lille, France); PYEA: 20g/d of protected live yeast (*Saccharomyces cerevisiae* 1.0×10^{10}cfu/g; Actisaf HR Plus Sc 47; Phileo LeSaffre Feed Additives, Marquette-Lez-Lille, France), and COMB: a combination of the daily doses of NPYEA and PYEA (15 and 20g/d, respectively) totalizing a daily dose of 35g/d. The NPYEA daily dose was top-dressed onto the pelleted concentrate, divided into 7.5g along the meals while the PYEA was incorporated in the pelleted concentrate during the concentrate manufacturing process.

Table 1. Chemical composition (%) of the diet ingredientes based on the dry matter (%)

Nutrients	Hay	Control Concentrate	Protected live yeast concentrate
Dry Matter (DM)	91.12	89.00	89.00
Crude Protein (CP)	20.60	15.00	14.50
Ether Extract (EE)	2.26	7.30	7.00
Neutral Detergent Fiber (NDF)	70.20	25.00	26.00
Acid Detergent Fiber (ADF)	48.30	9.30	12.40
Energy digestive (ED: Mcal/kg)	2.20	3.55	3.38

The collection of gastric contents occurred two hours after the first meal (concentrate and hay) according to a Varloud *et al*., (2007) adapted methodology One hour and fifty minutes after the meal, the horses were sedated with detomidine intravenously (Detomidin®, Syntec, Votuparim, Brasil) at with a dose of 0.02mg/kg. Ten minutes later, a nasogastric (NG) tube (PVC with a 2.85m lenght, 15mm of internal diameter and 19mm of external diameter) previously warmed in hot water and lubricated with gel was inserted through one of the nostrils into the esophagus (Delfine, 1999). A flexible vídeo-endoscope (3 meters) with 10.8 mm diameter, previously lubricated with gel was introduced into the contralateral nostril and pushed together with the NG tube into the stomach. The nasogastric tube was positioned with its end in contact with the mass of the digesta visualized through the vídeo-endoscope in the *marcus plicatus* region. The probe was immersed in the digesta and confirmed by the image through the vídeo-endoscope. The appropriate collection site was determined, and a vacum pump was used to aspirate the content into a glass container. NG intubation was paired with a video

gastroendoscope and five trained people were required for each sampling session. One person was responsible for restricting the horse's head and another one for collecting the material. Three other people oversaw positioning the endoscope and NG tube for content aspiration.

Samples of gastric contents were immediately distributed in their respective containers for later analysis of latic acid and put the meaning of the SCFA (short chain fatty acids). The pH of the content was measured by a digital pH meter (Quimis®) right after the collection. The second measurement was taken further, along with other analysis including microbiology.

For SCFA analysis (acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate), a separated 10g subsample was immediately diluted and homogenized with 20mL of distilled water manually compressed through a double layer of cheesecloth. A 4mL aliquot of filtered fecal liquor was transferred to a 12 × 75 mm glass tubes (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA) containing 1mL of formic acid. Tubes containing the fecal fluid/formic acid mixture were then immediately centrifuged for 12 minutes at 10,000×g. A 2mL aliquot of the supernatant was further transferred to a 2mL microtube (Axygen Scientific Inc., Union, CA) and stored at -20ºC for posterior analysis.

The gas chromatograph was equipped with capillary column (Stabilwax; Restek Corp., Bellefonte, PA) at 145ºC, a split-splitless injector and a dual flame ionization detector at 250ºC. Short chain fatty acid analysis was carried out according to Erwin *et al*. (1961) and adapted by Getachew *et al*., (2002). Helium was used as the carrier gas, synthetic air was used as the oxidizing gas and hydrogen was used as the fuel gas. Samples stored in the microtubes were thawed at room temperature and centrifuged for 10 minutes at 14,500×g. The supernatant (800µl) was transferred to a tube with 100µL of internal standard (ethyl-butyric acid 100 mM; Chem Service Inc., West Chester, PA). The external standard was prepared with acetate, propionate, isobutyrate and butyrate (Chem Service Inc., West Chester, PA). The gas peaks were identified and performed using the GCSolution software (Shimadzu Corp., Kyoto, Japan). For analysis of lactic acid, a 10mL subsample was stored in conical tubes (Corning Falcon; Fisher Scientific Inc., Fair Lawn, NJ) at -20ºC. The lactic acid concentration was determined by an automatic biochemical analyzer (Randox RX; Randox Laboratories Ltd., Antrim, UK) through an enzymatic ultra-violet method using lactate dehydrogenase (Pryce, 1969).

To the author's knowledge, this was the first time an in vitro fermentation assay using gastric contents was performed using a semi-automatic gas production technique (Mauricio *et al*., 1999). Horses were submitted to different treatments: control, non protected live yeast, protected live yeast and combinantion, and its gastric contents were used as inoculum in different moments. Immediately after collecting 160mL the content (Theodorou *et al.*, 1994) were filled with 100mL of gastric contents and constantly sprayed with CO_2. For each treatment, 3 replicates were incubated, totalizing 24 flasks. No susbtrate was used. Only the gastric contesnts of each animal were incubated. After incubating, the flasks, they were closed and all residual pressure was removed, so it could return to the forced ventilation oven at 39ºC. Gas productin readings were taken at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 hours post inoculation. The accumulated gas was also verified (Fig. 1). Readings were all performed with a pressure transducer (LOGGER AG100, Datalogger Universal) coupled to a three-way and a syringe.

Cumulative gas production was submitted to variance analysis and adjusted to the biocompartmental model described by France *et al*. (1993).

$$A = b\ x\ [1 - e^{-c(t-L)}]$$

A: accumulated volume of gases produced until time t; b: asymptotic gas production; c: gas production rate/hour; L: time (hours) of colonization before the gases production

Data were analyzed using a latins square design and SAS (Statistical Analysis System, version 9.1). The residue normality was previously verified by the Shapiro-Wilk test (Proc Univariate) and the homogeneity of variance by the Hartley test.

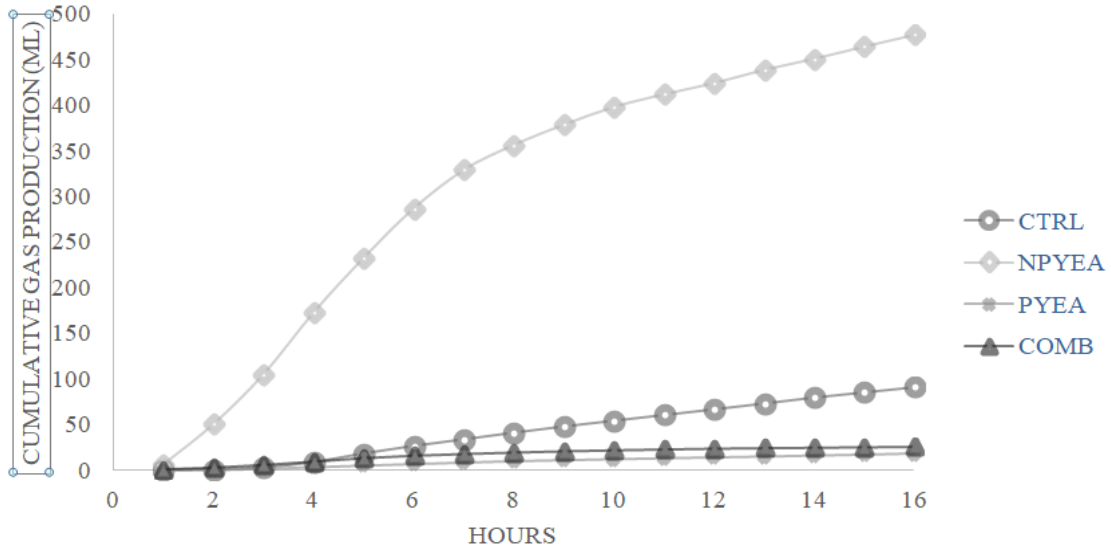

Figure 1. Gas content cumulative gas production (ml/g MS) for the diets CTRL: without yeast supplementation; NPYEA: 15g/d of non-protected live yeast (*Saccharomyces cerevisiae* 1.5 × 10^{10}cfu/g; PYEA: 20g/d of protected live yeast (*Saccharomyces cerevisiae* 1.0 × 10^{10}cfu/g; and COMB: a combination of the daily doses of NPYEA and PYEA (15 and 20g/d, respectively).

The gases production was analyzed with the same variation causes and he fator repeated measurements over time was added referring to the different moments of collection, by the PROC MIXED procedure of Statistical Analysis System (SAS Institute Inc., 2010). The model included the treatment effect as a fixed fator and animal effects within square, square, and period as random factors. The significance level of the Tukey's test was 5%.

RESULTS AND DISCUSSION

No significant difference in pH values of gastric contents was observed between treatments ($P<0.05$). The pH in different treatments showed averages: 6.2; 6.4; 6.1; 6.3 of CTRL, NPYEA, PYEA and COMB respectively.

It was possible to observe an increase of acetic acid concentrations with the use of yeast when evaluating he meaning of the SCFA (short chain fatty acids) of the stomach content. The control group had the lowest concentration followed by the NPYEA and PYEA groups. The highest concentration was in the COMB group (Table 2).

The mathematical model showed significant diferences in constant *b* of the asymptotic gas production. Group COMB had a higher rate of gas production, while the control and protected group had lower rates (Table 3). Incubation period of treatments showed significant differences of gas production at 4, 5 and 6 hours ($P<0.05$).

The NG tube insertion has a potential stimulatory effect, while the sedation impacts gastric emptying, which can complicate the content collection. To reduce the risk of accidents for both the animals and the veterinary team, horses were sedated with Detomidine before the procedure.

The endoscope associated technique allowed better control of the NG tube and minimized the collection time when compared to Varloud *et al*. (2007), that performed a blind post prandial aspiration.

Table 2. Means and standard error of the mean (SEM) of short chain fatty acids concentrations in gastric contents

Itens (mmol/L)	Treatments				SEM	P- value			
	CTRL	NPYEA	PYEA	COMB		Treat	C1	C2	C3
Acetic. Ac.	3.15^{c}	4.37^{bc}	5.27^{ab}	6.36^{a}	0.3538	0.0082*	0.0062	0.044	0.2899
Ac. Pr.	1.59	1.55	1.60	1.66	0.0546	0.8712	0.4831	0.4904	0.7013
Ac. Isop.	-	-	-	-	-	-	-	-	-
Ac. But	-	-	-	-	-	-	-	-	-
Ac. Isob.	-	-	-	-	-	-	-	-	-
Ac. Val.	-	-	-	-	-	-	-	-	-
Ac. Lat.	0.30	0.90	0.73	0.22	0.6029	0.5953			
pH	6.2	6.4	6.1	6.3	0.2409	0.8420			

(-) No detectable values; Acetic Aci. (acetic acid); Ac. Pr (propionic acid); Ac. Isop (Isopropionic acid); Ac. But (butyric acid); Ac. Isob (isobutyric acid); Ac. Val (valeric acid); Ac Lat. (latic acid); (CTRL: control group; NPYEA: non protected live yeast; PYEA: protected live yeast; COMB: combination NPYEA + PYEA; SEM: stand error mean; C1: CTR *versus* NPYEA, PYEA and COMB; C2: COMB *versus* NPYEA and PYEA; C3: NPYEA *versus* PYEA.

Table 3. Cumulative gas production from gastric contents after 16 hours of incubation

Item	Treatments				SEM	P-value
	CTRL	NPYEA	PYEA	COMB		
A (mL. g^{-1} MS)	5685	1309	12571	1015	258.96	0.4916
b (h^{-1})	0.1467^{b}	0.2385^{ab}	0.1216^{b}	0.3227^{a}	0.0216	0.0484*
c (h$^{-1/2}$)	-0.3582	-0.3779	-0.2637	-0.6171	0.1226	0.2587
L (h)	1.606	1.819	0.608	1.921	6.4236	0.7480
GP 1h	0.87	10.05	7.16	22.45	3.33	0.1447
GP 2h	1.13	52.33	24.11	71.70	11.08	0.1309
GP 3h	14.69	110.15	53.76	149.95	19.08	0.0755
GP 4h	42.08	184.29	90.13	243.29	27.31	0.0499*
GP 5h	81.83	250.87	116.29	344.51	35.40	0.0456*
GP 6h	118.53	318.83	151.14	412.57	40.81	0.0483*
GP 7h	154.97	366.62	180.62	463.03	40.81	0.0605
GP 8h	187.46	407.55	208.96	507.66	49.39	0.0776
GP 9h	215.29	445.41	231.47	536.39	51.61	0.0878
GP 10h	246.82	484.90	268.86	563.71	55.60	0.1312
GP 11h	276.96	511.67	304.41	587.56	59.33	0.1903
GP 12h	302.52	543.10	335.27	609.86	62.18	0.2312
GP 13h	333.40	565.78	364.99	631.27	65.86	0.3033
GP 14h	360.53	596.52	395.24	652.14	69.35	0.3609
GP 15h	390.61	617.89	422.78	674.69	72.93	0.4287
GP 16h	419.15	634.37	454.33	695.40	76.71	0.5090

^{ab} means with different superscripts on the same column are different ($P \leq 0.05$); A = final volume of gases; b = asymptotic gas production; c = gas production rate/hour; L = colonization time; CTRL: control group; NPYEA: non protected live yeast; PYEA: protectedlive yeast; COMB: combination NPYEA + PYEA; SEM: stand error mean; IVDDM: *In vitro* degradability of dry matter; IVDNDF: *In vitro* degradability of neutral detergente fiber.

Paul *et al.* (2021) was to characterize the glandular mucosal and gastric fluid microbiomes of horses with and without equine glandular gastric disease. The samples were aspirate with gastroscopy, but with blind, and just 20mL was collected the fluid gastric (EGGD).

Contrary to the blind technique, that allowed sampling from the ventral part of the stomach, the use of the endoscope allowed a guided collection from a specific gastric portion In the present study, it was possible to observe different digesta between animals. Solid and concentrated contents with hay probably belonged to animals with little water intake, as well as liquid digestas probably belonged to horses who ingested water after feeding (± 300-500mL).

According to Frape (2008), carbohydrates are fermented into lactic acid and the pH of the digesta decreases approximately 2.6 in the stomach. In the study performed by Varloud *et al*. (2007), the gastric content pH values decreased from 5.23 to 5.02 in horses after feeding. Although the blind collection, the authors believe the samples were collected below the *marcus plicatus*, where some studies point the starch digestion and intense microbial activity occur. Procedure and handling conditions allowed the satisfactory conservation of the collected samples and that the treatments did not interfer with the pH of stomach. Our technique and handling conditions allowed proper conservation and analysis of the samples, and manipulation did not interfer in the gastric pH.

More recent, Bachmann *et al*. (2020) was study if digesta batch cultures taken from horses adapted to Fructooligosaccharides (FOS) and inulin show different fermentation compared with such taken from nonsupplemented horses. With the purpose of incubating gastric content for 48 hours, to evaluate gas production, the authors needed to euthanize the horses. There is a need, even in the most recent studies, for a less invasive method.

Nadeau *et al*. (2003) studied the reasons for gastric alcers by comparing the injury to the non-glandular gastric mucosa caused by hydrochloric acid (HCl) or SCFA in horses fed hay or hay and grains. Acetic acid was found in most concentration, followed by butyric and propionic acids. Furthermore, another study carried out by the same group revealed that gastric pH and concentrations of SCFA are important factors that contribute to the severity of gastric ulcers in horses.

Acetic, propionic, and butyric acids are the predominant SCFA and are produced mainly in the fermentation of carbohydrates from plants, such as cellulose, hemicellulose, pectin, starch, and sugars (Bergman, 1990). Acetic acid concentrations showed higher values when compared to those in literature, in the treatment used COMB in stomach samples. Probably, the higher SC concentration values in this treatment provided a better substrate for the fermentative parameters, since COMB also showed greater gas production.

According to Varloud *et al*. (2007) starch enriched diets lead to an increase of anaerobic bacteria, especially *Lactobacillus spp*., which results in increase of lactic acid and SCFA production in the stomach. During the present research, diet digestibility analysis was carried out.

A relation between acetic acid concentrations and initial fiber digestion process was found. The use of live yeast or protected live yeast seemed to increase gram-negative bacteria without interfering in the pH, what could influence fermentative patterns in the first compartment of the gastrointestinal tract. The study is part of a line of research where the work is pioneering evidence of the use of live and protected yeast to obtain parameters of the gastric ecosystem, proving that the use of probiotics can model important gastric parameters.

Al Jassim and Andrews (2009) evaluated lactic acid concentrations in different compartments of the gastrointestinal tract of horses to monitor lactic acidosis and laminitis. Cecum and rectum had values between 2-3.5mmol. The stomach pointed to undetectable values until 4.3mmol. Julliand and Grimm (2017) worked with horses in diferent forage diets and detected 0.75 to 1.51mmol concentrations of lactic acid.

Differences observed, in the mathematical model, with gastric contents inoculum was already expected. Since protected live yeast treatment was performed with protected dry yeast, any alteration was observed in aqueous medium. PYEA and CTRL had a lower rate of constant *b*. PYEA probably remained stable to remove oxygen and favor fiber consumed by bacteria. CRTL probably needed more time for hydration, fixation and microorganism

colonization of the substrate. According to Jouany *et al*. (2009) a positive mechanism of SC yeast is the improvement of cellulolytic bacteria enzymatic activity without directly acting on the microbial population. Live yeast present in NPYEA and COMB in contact with aqueous medium, was consumed faster by microorganisms what can explain higher gas rates in asymptotic phase.

CONCLUSION

With the experimental design performed in this research, it was possible to work with a great number of live animals, submitted to little or non disconfort, and still collect samples for evaluation of different physiological parameters. Live yeast *Saccharomyces cerevisiae* increased acetic acid concentrations in the horses' stomachs. When gastric contents are used as inoculum for in vitro fermentation, protected live yeast results in less gas production.

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