Impact of a Diet Supplemented with Exoenzymes and Yeast on Predicted Circadian Rhythm of Reticulorumen pH

Ignas Šilinskas[1], Ingrida Monkevičienė[1], Gintarė Dovydaitienė[2], Ilma Tapio[3], Kristina Musayeva[1], Sigitas Japertas[4], Antanas Sederevičius[1] and Rasa Želvytė[1]

[1]The Research Center of Digestive Physiology and Pathology, Veterinary Academy, Lithuanian University of Health Sciences, Lithuania

[2]Departament of Animal Nutrition, Veterinary Academy, Lithuanian University of Health Sciences, Lithuania

[3]Natural Resources Institute Finland, Jokioinen, Finland

[4]Public Institution Practical training and trial center of Lithuanian University of Health Sciences, Lithuania

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Abstract. *Reticulorumen pH is affected by rumen fermentation processes and, therefore, is a good indicator of subclinical disease in dairy cattle. The aim of this study was to estimate the effect of exoenzymes and yeast supplementation on the reticulorumen pH circadian rhythm and predict the reticulorumen pH diurnal fluctuation. The study was performed in a commercial dairy farm. Thirty clinically healthy Lithuanian Red cows were assigned into outdoor (OD) and indoor (ID) trials (15 cows each). In each trial, the cows were divided into three homologues groups (5 cows each): control (C) – diet with no supplementation; enzyme (E) – diet supplemented with exoenzymes; enzyme and yeast (EY) – diet supplemented with exoenzymes and active yeast. The reticulorumen pH values were recorded for each cow over a 30-day period using "SmaXtec Premium Bolus" (SmaXtec animal care GmbH. Graz. Austria). The diurnal reticulorumen pH fluctuation was analyzed using the Z-probability equation. In group OD–C, the greatest probability for the reticulorumen pH values to reach maximum was at 06:45 ± 00:20 h. It was by 09 h 14 min ($p < 0.001$) and 09 h 32 min ($p < 0.001$) earlier in the day as compared with groups OD–E and OD–EY, respectively. The probability for the reticulorumen pH to reach maximum occurred earlier in group OD–E by 8 h 34 min ($p < 0.001$) as compared with group ID–E. No significant differences were observed between the groups in the probability for the minimum reticulorumen pH. The time for reticulorumen pH to stay at its maximum value was significantly longer by 06:30 h in trial ID as compared with trial OD. The total amount of time per day of the probability for minimum reticulorumen pH was by 03:34 h ($p < 0.05$) longer as compared with the probability for maximum reticulorumen pH. According to our results, the Z-probability equation can be used to predict the reticulorumen pH diurnal fluctuation. Total mixed ration supplementation with exoenzymes and active yeast positively affected the time and the length of maximum pH values occurrence.*

Introduction

Rumen plays the most important role of feedstuff digestion in dairy cows. The rumen ecosystem is a sensitive microbiological niche with well-established interactions between different types of microorganisms that are responsible for optimal feed digestion and energy extraction for the host animal (Deng et al., 2008). For efficient forage degradation, not only the quality and the composition of material itself are important, but also physical conditions of the environment where fermentation will be performed. One of the physical parameters is rumen fluid pH. Rumen pH is dependent on rumen microbial fermentation end-products. Optimal rumen pH is close to neutral, pH below 6.0 reduces activity of fiber degrading bacteria, and pH below 5.8 could promote sub-acute ruminal acidosis (SARA), or lead to animal health problems (Kleen at al., 2003; Zebeli et al., 2007; Plaizier et al., 2008). One of the feeding management priorities is to increase fiber digestion and at the same time keep ruminal pH in the normal range (Zebeli et al., 2012). Biological diet supplements, such as active yeast or exoenzymes, can induce non-starch polysaccharide degradation. Exoenzymes are capable of direct hydrolysis of cellulose and hemicellulose matrix, and they also stimulate bacterial attachment to plant cell walls (Adesogan et al., 2019). Active yeast supports anaerobic conditions by reducing the oxygen level in rumen and by increasing ruminal pH through stimulation of lactic acid utilizing bacteria, thus improving conditions for fiber degradation (Fonty and Chaucheyras-Durand, 2006; Adesogan et al., 2019). Both of them affect ruminal pH, but there is a lack of information about an effect of such diet supplements on diurnal pH cycles.

On a commercial dairy farm, rumen pH could be measures by taking ruminal fluid samples using esophagus–rumen probes, or performing rumenocentesis (AlZahal et al. 2007). Both of them are stressful for animals, samples could be contaminated with saliva or blood and have limitations in sampling frequencies. A modern and safe method for animals is to use wireless-indwelling reticulorumen pH measurement systems (Antanaitis et al., 2016). Such systems continuously measure reticulorumen pH and provide large volumes of data, which could be used to calculate average reticulorumen pH at a certain period

Correspondence to Ignas Šilinskas, The Research Center of Digestive Physiology and Pathology, Faculty of Veterinary Medicine, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės 18, LT-47181 Kaunas, Lithuania
E-mail: ignas.silinskas@lsmuni.lt

or amount of time spent below the given threshold. Denwood et al. (2018) suggested that such data could be used to define and diagnose abnormal reticuloruminal pH by identifying deviations from normal pH. A similar study was performed by Josson et al. (2019). They calculated the probability of the reticulorumen pH value to occur below a given threshold in farms with different feeding strategies. But statistical analysis suggested by both studies does not reflect the circadian rhythm of reticulorumen pH.

By predicting the reticulorumen pH diurnal fluctuation, the ruminal digestion and nutrient supply to dairy cows on a given diet can be evaluated. Besides, it is beneficial to avoid ruminal and metabolic diseases, such as acidosis or laminitis. For this reason, AlZahal et al. (2007) used four equations which were growth functions used to describe the growth of animals and plants. But the applied equations only predict reticulorumen pH accumulated time under a given threshold and do not predict the reticulorumen pH diurnal fluctuation. To solve this issue, we considered to use the Z-probability (Z-score) equation (Whitlock, 2005).

We hypothesized that the use of exoenzymes and active yeast supplements with different feeding strategies would affect the reticulorumen pH diurnal fluctuation. The aim of this study was to predict the reticulorumen pH diurnal fluctuation by using Z-probability and evaluate the effect of exoenzymes and yeast supplementation on the reticulorumen pH circadian rhythm.

Materials and Methods

This study was performed on a commercial organic dairy farm (700 cows) in Panevėžys region, Lithuania. Thirty clinically healthy Lithuanian Red cows (an average rectal temperature of 38.7 ± 0.1 °C, rumen motility 5–6 times per 3 min, without signs of mastitis, metritis, or lameness) were used in the experiment. The cows were on average 50 ±7 days in milk and produced on average 31.3 ± 3.0 kg/d of milk. They were assigned to two trials (15 cows each): outdoor (OD), which was performed in the grazing period, and indoor (ID), which was performed in the winter time. During the OD trial, the cows were grazing *ad libitum* on pasture between 11 am and 5 pm, and spent the rest of the time in a free style barn. During the ID trial, the cows were continuously kept in a free stall type barn. In both trials, all cows were offered a total mixed ration (TMR) at 7 am and 5 pm. Diets of both trials were balanced to meet maintenance and production needs (Table 1). The accessibility to water was *ad libitum*. Milking occurred twice daily at 6:00 am and 6:00 pm.

In each trial, the cows were divided into three groups (5 cows each): control (C) – diet with no supplementation; enzyme (E) – diet supplemented with exoenzymes mixture of endo-β-xylanase 37 × 10^4 U/cow/day, endocellulase 45 × 10^4 U/cow/day, and endo-β-glucanase 12 × 10^4 U/cow/day (Vilzim®NSP, UAB Biorro, Lithuania); enzyme and yeast (EY) – diet supplemented with exoenzymes and active dry yeast *Saccharomyces cerevisiae* CNCM-1077, 10 x 10^9

Table 1. The ingredients and chemical composition of farm diet

Item	Value	
	% of diet DM	
	Outdoor trial	Indoor trial
Ingredient		
Grass silage[1]	26	42.8
Corn silage[1]	9	12.3
Straw[1]	3	5.1
High-moisture corn[1]	5	9.1
Ground concentrate mixture[1,2]	29	30.7
Pasture grass[3]	28	0
Diet chemical composition		
DM, %	40.5	44.1
OM, % of DM	71.1	74.6
CP, % of DM	15.08	13.6
NDF, % of DM	43.11	37.1
ADF, % of DM	27.46	22.2

DM – dry matter, OM – organic matter, CP – crude protein, NDF – neutral detergent fiber, ADF – acid detergent fiber.
[1] Ingredients of TMR.
[2] Composition of the concentrate mixture. Dry grains: wheat, triticale, oats; dry ground peas and ground beans, dry rapeseed, soybean meal, sodium bicarbonate, feed chalk, salt, vitamins and trace mineral premix.
[3] Pasture grass: ryegrass, red clover, white clover, alfalfa grass.

CFU/cow/day (Levucell SC, Lallemand Animal Nutrition, France). The corresponding treatment was administered by top-dressing forages and was mixed by using a TMR mixer – feeder "Kuhn Euromix I" (Bucher Industries AG. Switzerland).

The cows were adapted to the diet supplement for 21 days, before each experimental period. The reticulorumen pH values were recorded for 30 days in each trial. All cows were equipped with intrareticuloruminal telemetric sensor device "SmaXtec Premium Bolus" (SmaXtec animal care GmbH. Graz. Austria). The bolus specifications and guidelines for application to animals were described by Antanaitis et al. (2016).

Probabilities for the reticulorumen pH to reach the highest or the lowest values during the diurnal cycle were calculated using the Z-probability table. The Z-probability test takes advantage of the one-to-one mapping of the standard normal curve to the p value of a one-tailed test (Whitlock, 2005). By Z, we mean a standard normal deviation, which would be a daily reticulorumen pH distribution with mean and standard deviation. As Z goes from negative infinity to infinity, P will go from 0 to 1, and any value of P will uniquely be matched with a value of Z and vice versa. The Z values of −1.96 and 1.96 were selected as points in data set to describe 2.5% of the lowest and the highest measurement values in the daily data range, respectively. An equation to find the necessary reticulorumen pH value was used: .

All measured reticulorumen pH values which were equal, above (for maximum) or below (for minimum) the estimated value were selected and marked as −1 or 1, respectively. Time values were also recorded. A probability for pH to reach the maximum or the minimum value at a certain point of time was calculated using the equation:

$$P(A) = \frac{n(E)}{n(S)}$$

where E is positive observations (a sum of −1 or 1) and S is all possible observations (30 days).

A probability in separate trial groups was calculated as means and expressed in percent. For regression analysis, the Pearson correlation coefficient (R) was used. Statistical analysis was performed by ANOVA with the Fisher's least significant difference test. Statistical calculations were carried out using IBM Statistic SPSS version 15 (license No. 9900457; IBM. USA). The results were considered statistically significant when $p \leqslant 0.05$.

Results

In general, the diurnal reticulorumen pH fluctuation of each trial group had two periods when the probability was higher for maximum diurnal pH values and two periods when the probability was higher for the minimum diurnal pH values (Fig. 1). The difference between the treatment groups was in the time when the maximum or the minimum was reached, in the size of probability and the length of periods.

In the first half of the day, the probability for maximum pH values started to increase before the morning feeding. The earliest probability started to occur in group ID–EY at 05:24 ± 00:32 am , and the latest probability occurred in group OD–EY at 06:05 ± 00:41 am . The highest probability was registered in group OD–C (22.67 ± 4.87%) and the lowest probability in group OD–E (11.33 ± 1.8%). The longest period in the first half of the day was recorded in group ID–EY for 05:34 ± 00:21 h, and the shortest was recorded in group OD–E for 01:38 ± 00:17 h. In the first half of the day, the probability for maximum pH values increased fastest in group OD–C (18.46%/h, R = 0.92) and slowest in group ID–EY (4.8%/h, R = 0.67). As group OD–C was compared with group OD–E and group OD–EY, better probability for maximum pH values occurred 09:14 h ($p < 0.001$) and 09:32 h ($p < 0.001$) later in the day, respectively.

After the morning feeding, the probability for maximum pH started to decrease and the probability for minimum pH started to increase. The earliest probability for minimum pH was registered in group OD–E (09:10 ± 00:29 am) and the latest was registered in group OD–EY (11:08 ± 00:34 am). The highest probability was registered in group OD–C (6.67 ± 1.4%) and the lowest probability was in group ID–E (3.33 ± 0.7%). The longest period in the first half of the day of the probability for minimum pH values was recorded in group ID–E for 05:50 ± 00:34 h and the shortest was in group OD–C for 02:16 ± 00:20 h.

In the second half of the day, the reticulorumen pH fluctuation was similar to pH fluctuation in the first half of the day: the probability for maximum pH values had the greatest values within evening feeding and then started to decrease followed by increased probability for minimum pH values. The earliest probability for maximum pH values occurred in group OD–EY at 1:17 ± 00:13 pm and the latest in group ID–E at 3:58 ± 00:47 pm . The probability for maximum pH values ranged from 7.33 ± 1.3% at 3:18 ± 00:38 pm (group OD–C) to 28.89 ± 3.05% at 4:30 pm (± 00:22) (group OD–EY), while the probability increased fastest in group OD–E (10.2%/h, R = 0.87) and slowest in group OD–C (3.67%/h, R = 0.71). The shortest period in the second half of the day of the probability for maximum pH values was recorded in group OD–C for 01:58 ± 00:24 h and the longest was in group ID–EY for 04:50 ± 00:26 h. No significant differences in the length of the periods between treatments were observed.

The probability for minimum reticulorumen pH values was observed during the nighttime in all groups. Group OD–C was the first group when the probability for minimum pH started to increase (at 5:00 ± 00:19 pm). This group had the longest period (10:00 ± 00:20 h) as well. The latest (started to increase at 8:20 ± 00:17 pm) with the shortest time (08:02 ± 00:14 h) was group ID–EY. The highest probability values of minimum pH was registered in group OD–EY (14.14 ± 2.08%), while the lowest occurred in group ID–E (9.98 ± 0.1%).

The overall probability time length of maximum reticulorumen pH during the day varied from 06:10 h (group OD–E) to 10:20 h (group ID–EY). The probability of minimum reticulorumen pH varied from 8:50 h (group OD–C) to 13:30 h (group OD–E). The probability of minimum reticulorumen pH was by 03:34 h ($p < 0.05$) longer than the probability of maximum reticulorumen pH. As trial ID was compared with trial OD, the period of probability for maximum reticulorumen pH values was longer for trial ID by 06:30 h ($p < 0.05$).

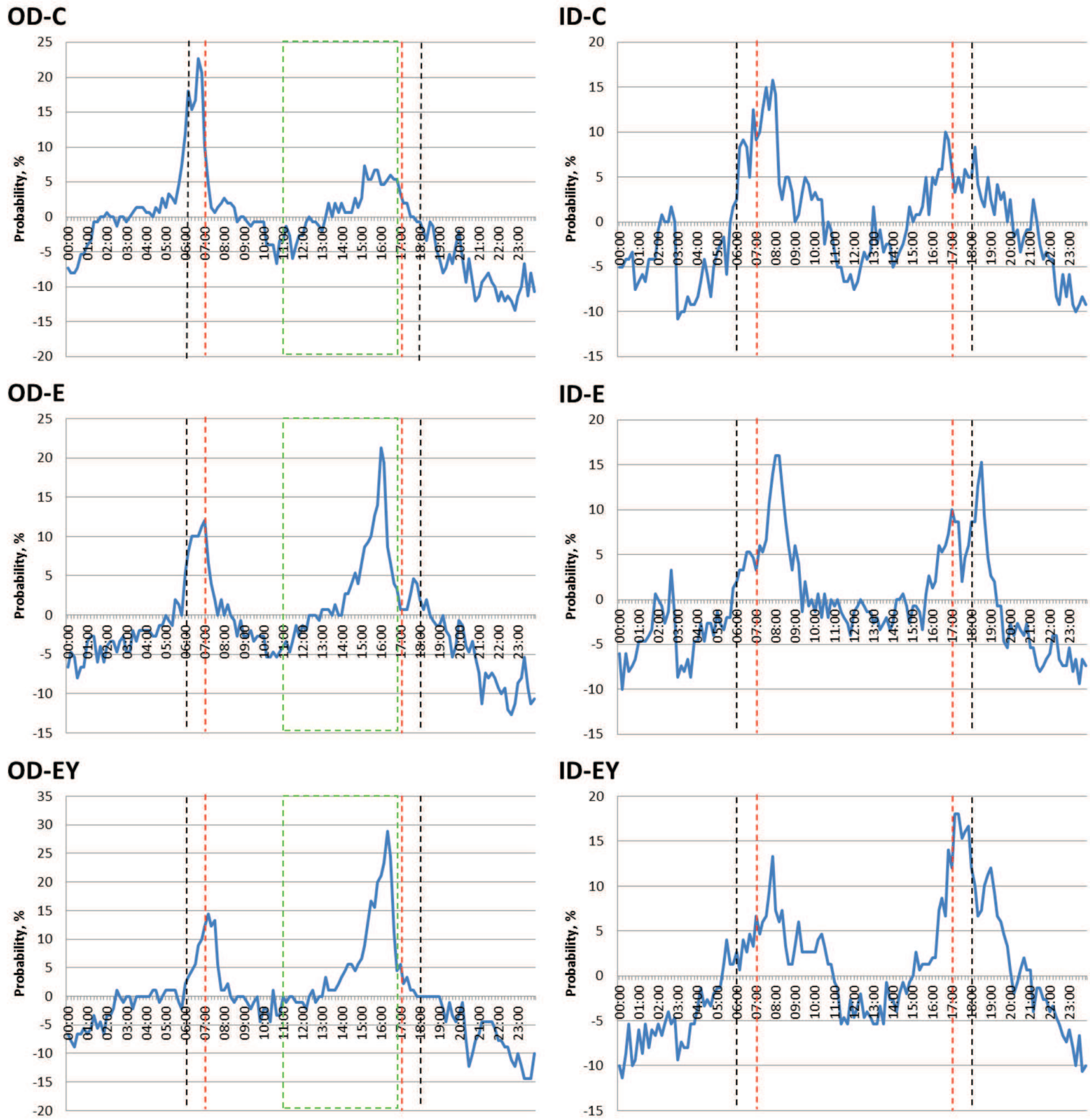

Fig. 1. Graphically visualized reticulorumen pH diurnal probability patterns
Trials and groups are represented in the upper left corner of the separate graph. Positive Y-axis values represent maximum pH values and negative values represent minimum values. The X-axis represents the time of the day in a 24 h (hh:mm) format with a smaller tick of 10 min. Black dotted lines represent the milking time, red dotted lines represent feeding with TMR, and the green dotted square is grazing time.

Discussion

To our knowledge, this is the first study that has used the Z-probability equation to predict the reticulorumen pH diurnal fluctuation. The results suggested that the probability for reticulorumen pH to be at maximum or minimum at a certain time had only minor differences across the treatment groups. The probability for maximum or minimum pH was more dependent on the feeding time rather than on feed supplements or the feeding strategy. In all treatment groups, the probability for maximum pH tended to be greatest within the morning and evening feeding, when the probability for minimum pH on average occurred at 5:33 ± 1:30 h after feeding. Our study results of ruminal pH diurnal fluctuation are consistent with those of Palmonari et al. (2010) and Salfer et al. (2018), where ruminal pH was highest just before feeding and declined for approximately 5 to 7 h thereafter, before gradually increasing again. Henk van Lingen et al. (2017) also reported that rumen fluid had pH 7.0 at feeding, decreased to pH 6.3 two hours after feeding, remained relatively constant for 5 h, and then increased to pH 6.7 at 10 h after feeding. Nordlund and Garrett (1994) suggested that the best time to take samples by rumenocentesis for SARA diagnostic is 5 to 8 h post-feeding in TMR-fed herds. According to the results from independent trials, we can conclude that cows' ruminal fermentation cycles are adapted to the feeding time.

Ruminal fermentation is performed by rumen microorganisms where the most important role belongs to rumen bacteria. Rumen bacteria consist of a vast amount of species where each species plays an important role in feed degradation and conversation into volatile fatty acids (VFA), an acceptable form of energy for a host animal. According to Kolver and De Veth (2002), the most abundant rumen bacteria, detected a few hours after feeding, were *Pseudobutyrivibrio spp.*, *Lactobacillus spp.*, *Selenomonas spp*, *Streptococcus spp.*, and *Prevotella spp.,* which rapidly convert carbohydrates into volatile fatty acids (VFA) and lactic acid. VFA and lactic acid strongly negatively correlate with rumen pH, which is responsible for increased probability for minimum reticulorumen pH. Kolver and Veth (2002) reported that in their study VFA concentration reached its maximum 3 h after feeding. The molar proportion of propionate reached a maximum of 22% in total VFA at 1.5 h after feeding. The proportion of butyrate steadily increased to a peak of 15% at 6 h after feeding and the molar proportion of acetate decreased from 68% to 62% over the first 1.5 h and then recovered after 3 h after feeding. Lactic acid was detected from 0.5 h to 4 h after feeding. The inflation of VFA and lactic acid in the first hours after feeding and their strong influence on rumen pH suggest that the Z-probability model of reticulorumen pH could be used to predict the diurnal fluctuation of VFA and lactic acid but the additional measurement is necessary.

Different feeding strategies could not necessarily result in different patterns of the diurnal reticulorumen pH fluctuation. The main difference between trials was that in trial OD along with TMR feeding a fresh pasture grass was included, while in trial OD the cows were only fed TMR. Fresh grass consists of soluble sugars and easily fermentable fiber, but according to the results, no significant differences in reticulorumen pH probabilities were observed between the treatments in general, or in the period when the cows were grazing. These observations could suggest that, while grazing, lactic acid is not produced in necessary quantities to induce a significant rumen pH decrease. However fresh grass is a good source of VFA production, whereas VFA are relatively weak acids and do not reduce the activity of fiber degrading bacteria (De Veth and Kolver, 2001). Besides, by comparing trial OD to trial ID, cow feeding behavior should be taken into account. The study of Pitt and Pell (1997) suggested that cow feed intake was not dependent on a feeding frequency. Cows fed 2 times and 12 times per day consumed the same amount of DM during the day. During our trials, the cows were always fed at the same time 2 times per day. The cows in trial OD instead of grazing fresh grass tended to wait for the main TMR meal, but further investigation of DM intake is necessary.

Forage supplementation with exoenzymes and active yeast did not give expected significant results to reticulorumen pH. It is possible that neither exoenzymes nor active east may have abilities to affect reticulorumen pH, but more likely that the z-probability equation and its adaptation to the diurnal reticulorumen pH fluctuation are not suitable to find meaningful differences between treatments. Only the small positive effect of yeast was observed in group ID–EY when the time length of probability for maximum reticulorumen pH was longest. A meta-analysis prepared by Desnoyers et al. (2009) revealed that yeast supplementation increased rumen pH (+0.03 on average) and rumen volatile fatty acid concentration (+2.17 mM on average), tended to decrease rumen lactic acid concentration (–0.9 mM on average) and increased total-tract organic matter digestibility (+0.8% on average).

By comparing the results with other studies where the circadian rhythm of reticulorumen pH was analyzed, the results of both trials in group C were similar to those of Denwood et al. (2018) and Josson et al. (2019) where the reticulorumen pH was highest in the first half of the day. Our results contribute to the research where reticulorumen pH values started increasing after 22:00 h (Antanaitis et al., 2016), explaining a pH increase due to saliva excretion during rumination at the nighttime (Josson et al., 2019).

Conclusions

According to our results, we can conclude that the Z-probability equation is acceptable for the prediction of reticulorumen pH diurnal fluctuation. The diurnal fluctuation of reticulorumen pH had high chances to be at maximum two times per day: in the morning and in the evening; minimum pH values have the greatest probability to occur at midnight. TMR supplementation with exoenzymes and active yeast could affect the daily time of the maximum pH values to occur, but has no effect on time for the minimum pH values. This research also has practical benefits. Using reticulorumen pH probability patterns, cow feeding strategies and rumen sampling strategies could be improved.

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