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# The diurnal patterns of ruminal enzymatic activity and in vitro digestibility of starch, neutral detergent fiber, and protein

**E. Raffrenato,[1,2]* M. J. Badenhorst,[1] K. J. Harvatine,[3] M. N. T. Shipandeni,[4] L. du Plessis,[5] G. Esposito,[1] and W. H. van Zyl[5]**
[1]Department of Animal Sciences, Faculty of AgriSciences, Stellenbosch University, Private Bag X1, Matieland, 7602, South Africa
[2]R&D RUM&N Sas, Reggio Emilia, Italy 42123
[3]Department of Animal Science, Pennsylvania State University, University Park 16802
[4]Department of Animal Sciences, Faculty of Agriculture and Natural Resources, University of Namibia, Private Bag 13301, Windhoek, Namibia
[5]Department of Microbiology, Faculty of AgriSciences, Stellenbosch University, Private Bag X1, Matieland, 7602, South Africa

## ABSTRACT

The objective of this study was to determine whether diurnal patterns in starch, neutral detergent fiber (NDF) and protein digestibilities and amylolytic, fibrolytic, and proteolytic activities exist in dairy cows. Rumen fluid was collected from 4 ruminally cannulated Holstein dairy cows before the morning feeding and subsequently every 4 h for a 24-h period. Two of the cows were restricted from feed for 8 h overnight, and the other 2 continued to receive their feed ad libitum, to isolate and quantify the effects of changes in feeding behavior at night. After 2 runs the cows were crossed over between night feeding treatments. Rumen fluid was analyzed for enzymatic activity and in vitro starch, NDF, and nitrogen digestibility. Circadian rhythm analyses of enzymatic activity and in vitro digestibility were conducted by fitting the linear form of a cosine function with a 24-h period. Patterns were observed in activity for amylase, lichenase, endoglucanase, and xylanase, with the highest activities observed at the time points subsequent to milking and feed delivery. Protease activity was unaffected by either feeding treatment or possible feeding behavior. When fitted to a cosine function, all the parameters tested followed a daily pattern that was sensitive to the overnight availability of feed, although the parameters responded differently to the feeding treatment. The patterns displayed by in vitro digestibility results of starch, NDF, and nitrogen, across the various fluid collection time points, were highly variable. The time at peak (acrophase) observed in the enzymatic analysis did not correspond to those observed in the in vitro analysis. These results suggest that different interpretations should be given to enzymatic activities and in vitro digestibility values, and the time of rumen fluid collection relative to feeding time should be considered and reported when rumen fluid is used for research or commercial purposes. Maximum digestibility appears in fact to be reached around 4 to 5 h after the main ration delivery for NDF and starch and around ration delivery for protein.
**Key words:** diurnal variation, enzymatic activity, digestibility, rumen fluid, dairy cows

## INTRODUCTION

Circadian rhythms have been observed in most organisms, including animals, plants, and bacteria, and allow them to adapt their physiology pre-emptively across the day (Brown and Schibler, 1999). A circadian rhythm can be defined as any internal process that displays an entrainable oscillation with a 24-h period. Endogenous clocks allow organisms to synchronize their physiological and behavioral activities with changes in the exogenous environment (Harvatine, 2012). In animals, the wake and sleep pattern is the most apparent activity regulated by circadian rhythms (Takahashi et al., 2008). Circadian patterns will continue in the absence of environmental signals, as they are controlled by timekeeping mechanisms in the peripheral tissues and central nervous system (Niu et al., 2014). In mammals, the brain houses the master timekeeper in the hypothalamic suprachiasmatic nucleus (Brown and Schibler, 1999; Takahashi et al., 2008). The circadian clock located in the suprachiasmatic nucleus is entrainable by light (Brown and Schibler, 1999) and is reset daily by the photoperiod when light signals perceived by the retinohypothalamic tract are transmitted to the suprachiasmatic nucleus (Balsalobre et al., 2000). Pacemakers in peripheral tissues are also entrainable by environmental stimulants, including temperature cycles, hormonal signals, and the timing of feed availability (Balsalobre et al., 2000; Damiola et al., 2000; Harvatine, 2012). Harvatine (2012) suggested that certain

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*Corresponding author: emiliano@sun.ac.za

management strategies employed by dairy farmers can desynchronize the master timekeeper and mammary timekeepers in cows and possibly desynchronize absorption of nutrients and milk production, consequently decreasing milk yield.

Numerous physiological parameters in cows have been reported to follow a circadian pattern, including glucose, urea, nonesterified fatty acids, total lipids, total cholesterol, hemoglobin, creatinine, magnesium, phosphorus, respiratory rate, and rectal temperature (Lefcourt et al., 1999; Piccione et al., 2007; Giannetto and Piccione, 2009). Milk production in dairy cows has also been shown to follow a circadian pattern that is sensitive to the timing of feed intake (Rottman et al., 2014). Under natural grazing conditions, cows display a diurnal feeding pattern where most of the feeding activity occurs during the day, especially at sunset and sunrise (DeVries, 2013). However, the diurnal feeding pattern of dairy cows in a freestall housing setup tends to be influenced by milking, feed push-up, and time of feed delivery (DeVries et al., 2003). The feed delivery time of dairy cows has the ability to retrain the daily rhythm of feeding, core body temperature, fecal NDF and indigestible NDF concentrations, lying behavior, and plasma blood urea nitrogen, insulin, and glucose concentrations (Niu et al., 2014). Robinson et al. (1997, 2002) showed that the time of feeding protein supplements to dairy cows affected the diurnal patterns of ruminal fermentation, VFA, and amino acids. Blackburn and Hobson (1960) also reported that VFA were affected by the time of feeding. Additionally, Russell et al. (1981) showed that the most significant increase in ammonia was observed 1 to 3 h after feeding.

In addition to protein metabolites, Cone et al. (1989) and Fickett and Allen (2002) showed that the extent of in vitro starch digestibility was also affected by time relative to feeding. Fickett and Allen (2002) attributed the variation in digestibility rates to the difference in enzymatic activity of rumen fluid collected before and after feeding. Therefore, we have reason to speculate that diurnal patterns for enzymatic activity also exist, as bacterial numbers have already been shown to be influenced by time of feeding (Bryant and Robinson, 1968). Furthermore, when performing ruminal in vitro studies, little attention is given to the time of day when rumen fluid is sampled, thereby not controlling variation caused by diurnal patterns and the effect it might have on starch, NDF, and protein digestibility. The results of these studies are used to characterize feeds, to determine the amount of feed needed in the diet of the cow to meet its requirements, and especially for the determination of starch, NDF, and protein requirements. Additionally, often results are compared between in vitro studies without considering time of rumen fluid collection or feeding time. We therefore hypothesize that rumen enzymatic activities in dairy cows are characterized by diurnal patterns. Thus, the objective of this study was to determine whether diurnal patterns in starch, NDF, and protein digestibility and amylolytic, fibrolytic, and proteolytic activity exist in rumen fluid of dairy cows.

## MATERIALS AND METHODS

All the procedures used in this study were approved by the Research Ethics Committee for Animal Care and Use of Stellenbosch University (SU-ACUD14-00052; Stellenbosch, South Africa). Rumen fluid was collected by hand from 5 different rumen sites (cranial dorsal, cranial ventral, central, caudal dorsal, and caudal ventral rumen) of 4 ruminally cannulated lactating Holstein cows and treated separately at the Welgevallen Experimental Farm of Stellenbosch University. Cows were 516 ± 89 d in milk, 736 ± 88 kg of BW, and 5.4 ± 2.3 yr old, with 17.1 L ± 10.4 daily milk production (mean ± SD). Rumen fluid was collected every 4 h over a 24-h period (0730, 1130, 1530, 1930, 2330, and 0330 h). The first collection took place before the morning feeding and immediately after cows were milked. The cows were milked at 0600 and 1600 h. The cows were fed a TMR (Table 1) consisting of corn as the main source of starch and alfalfa hay and wheat straw as the main sources of NDF, fed twice a day to achieve 10% orts, at 0730 and 1600 h. The other ingredients of the TMR consisted of (in decreasing amount) corn gluten, sugarcane molasses, soybean meal, barley malt, potato by-product meal, dry molasses, feather meal, limestone, blood meal, salt, urea, and monocalcium phosphate. The TMR did not include any additive or supplement that could have interfered with the microbial normal activity (e.g., essential oils, monensin). The diet was formulated and evaluated using NDS Professional Version 3.9 (RUM&N), based on the Cornell Net Carbohydrate and Protein System version 6.5.5 (Van Amburgh et al., 2015), using the mentioned animal inputs and DMI of 19 kg. The intake level was decided based on DMI records of the previous 20 d. Two of the cows were restricted from feed for 8 h overnight (i.e., from 2200 h), and the other two continued to have access to the TMR, to determine the effect of feeding behavior at night. After 2 runs the cows were crossed over and were allowed 5 d of adaptation as suggested by Grant et al. (2015) and Van Soest (1994).

### *Determination of Enzymatic Activities*

Rumen fluid was placed on ice at sampling to slow down microbial activity and then filtered through 4 lay-

**Table 1.** Phase and amplitude of a cosine function with a 24-h period fit to enzymatic activity and in vitro digestibility of cows fed ad libitum over the overnight period, or cows fasted

| Parameter | Treatment[1] | Phase[2] (h) | Amplitude[3] | *P*-value[4] |
|---|---|---|---|---|
| Enzymatic activity | | | | |
| Amylase, μmol/min | Ad lib | 8.74 | 1.93 | <0.0001 |
| | Fasted | 17.18* | 1.28* | <0.0001 |
| Lichenase, μmol/min | Ad lib | 16.30 | 0.64 | <0.0001 |
| | Fasted | 16.78* | 0.68* | <0.0001 |
| Endoglucanase, μmol/min | Ad lib | 15.79 | 0.27 | <0.0001 |
| | Fasted | 15.20* | 0.25* | <0.0001 |
| Xylanase, μmol/min | Ad lib | 7.32 | 0.95 | <0.0001 |
| | Fasted | 15.47* | 0.83* | <0.0001 |
| Protease, optical density/min | Ad lib | 9.32 | 0.01 | 0.0028 |
| | Fasted | 6.30* | 0.00* | 0.0002 |
| In vitro digestibility | | | | |
| Starch, % of starch | Ad lib | 17.14 | 2.25 | <0.0001 |
| | Fasted | 14.46* | 3.93* | <0.0001 |
| NDF, % of NDF | Ad lib | 12.50 | 1.29 | 0.0005 |
| | Fasted | 11.84* | 2.16* | <0.0001 |
| Nitrogen, % of N | Ad lib | 7.13 | 2.88 | <0.0001 |
| | Fasted | 10.69* | 5.32* | <0.0001 |

[1]Treatments were cows fed ad libitum (ad lib) or cows fasted for approximately 8 h over the night period.

[2]Phase refers to the time (h) where the highest value in the 24-h cycle occurs. Values are decimal representations of hours and minutes, where 12.5 = 12 h, 30 min.

[3]Amplitude refers to the halfway distance between the peak and the trough of a cosine function with a 24-h period. Units are shown next to the parameter.

[4]Zero-amplitude test for a cosine function with a 24-h period for each treatment.

*Ad libitum fed differed from fasted ($P < 0.05$).

ers of cheesecloth, glass wool, and 2 layers of 200-μm porosity mesh into a 1-L pre-cooled Erlenmeyer flask. The samples were centrifuged at 16,000 × *g* for 15 min at 4°C, and only the supernatant was used for enzymatic essays, which followed immediately.

Enzymatic activity of amylase, endoglucanase, lichenase, and xylanase were assessed colorimetrically by measuring the amount of reducing sugars released using dinitrosalicylic acid (Miller, 1959; Raffrenato et al., 2021). Amylolytic activity was determined using 0.2% (wt/vol) cooked maize starch (S4126, Sigma-Aldrich) resuspended in 0.05 *M* citrate buffer (pH 6.0). Endoglucanase activity was determined using 0.1% (wt/vol) lichenan (L6133, Sigma-Aldrich) and 1% (wt/vol) carboxymethyl cellulose (C5678, Sigma-Aldrich). Activity measured using carboxymethyl cellulose and lichenan as substrates will be referred to as endoglucanase and lichenase activity, respectively. Endoxylanase activity was determined using 1% (wt/vol) Beechwood xylan (X4252, Sigma-Aldrich) resuspended in the same buffer. Enzymatic activities were determined by measuring the amount of reducing sugars released from substrates during 15 min at 39°C with 450 μL of substrate and 50 μL of enzyme sample. The mixture was boiled with 750 μL of dinitrosalicylic acid for 15 min to stop the reaction. Spectrophotometry was used to quantify the reducing sugars at 540 nm. One unit was determined as 1 μmol of reducing sugar liberated per minute using glucose or xylose as standards. Protease activity was assessed colorimetrically by measuring the amount of azo dye released from 2% azocasein (A-2765, Sigma-Aldrich) in a 0.1 *M* sodium phosphate buffer (pH 6.8) as per the procedure described by van de Vyver et al. (2004). Protease activity was defined as optical density per minute (OD/min) and was determined by subtracting the optical density of the blank from the optical density of the test sample and dividing that value by the total incubation time (in minutes).

### *In Vitro Fermentations*

Rumen fluid for the in vitro digestibility trial was placed in a pre-warmed insulated flask at collection and then filtered into a 1-L prewarmed Erlenmeyer flask through 4 layers of cheesecloth, glass wool, and 2 layers of 200-μm porosity mesh. In vitro starch digestibility (**ivSd**; 7 h of incubation), NDF digestibility (**NDFd**; 12 h), and nitrogen digestibility (**Nd**; 8 h) were determined using maize, oat hay, and soy oilcake as substrates, respectively. All the substrate samples were milled through a 1-mm screen using a Wiley mill (Thomas Scientific) and analyzed for DM (method 934.01; AOAC International, 2005). Before incubations, substrates were weighed into 125-mL Erlenmeyer flasks, and 40 mL of in vitro buffer medium (adapted from Goering and Van Soest, 1970) was added to each flask. The flasks were then placed in a water bath (39°C), closed with rubber stoppers, and flushed with

$CO_2$ to reduce the medium pending incubation with rumen fluid. Rumen fluid (10 mL) was injected into each flask using a warmed automatic syringe (Dosys, Socorex). All samples were incubated in duplicate, and all fermentations were completed across 4 runs.

Residual starch was analyzed as described by Hall (2000). Residual NDF was analyzed as described by Raffrenato and Van Amburgh (2011), with the addition of amylase and sodium sulfite anhydrous. Nitrogen digestibility was quantified by measuring N disappearance from residual N, as per the rumen digestibility procedure of Ross et al. (2013), and crude protein, used for the determination of Nd, was determined using a Nitrogen Gas Analyzer FP528 (LECO Africa Pty Ltd.).

### Statistical Analysis

Enzymatic activity and in vitro digestibility data were analyzed as response variables by the GLIMMIX procedure of SAS (version 9.3; SAS Institute Inc.) using a factorial arrangement of ad libitum versus fasting (treatment), collection time, and their interactions. Cow was treated as a random factor. Differences were declared significant at $P \leq 0.05$ using the least squares means and the Tukey adjustment. Statistical differences resulting in $0.05 < P \leq 0.10$ were considered tendencies. The results of treatments are reported as least squares means.

Circadian rhythm analysis of enzymatic activity and in vitro digestibility were conducted by fitting the linear form of a cosine function with a 24-h period according to Bourdon et al. (1995) by random regression in SAS (Seltman, 1997). A zero-amplitude *F*-test was performed to compare the cosine fit against a linear fit to determine the significance of the 24-h cosine fit for each of the treatments. Phase refers to the time at which the peak of the cycle occurs and is reported as acrophase. The 95% confidence limits were determined for both phase and amplitude (Bourdon et al., 1995). Treatment differences were considered significant when the difference of phase or amplitude were 1.96 times the square root of the sum of squares of the standard error values ($P < 0.05$; Knezevic, 2008). All response variables were checked for outliers and removed if respective Studentized residuals were outside of ± 3 (Niu et al., 2014). No more than 2 data points were removed per response variable.

## RESULTS

### Enzymatic Activity

We found no significant differences between the amylolytic ($P = 0.78$), lichenase ($P = 0.26$), endoglucanase ($P = 0.86$), xylanase ($P = 0.72$), and protease ($P = 0.51$) activities of the cows fasted the night before and the cows fed ad libitum. The time of rumen fluid collection did, however, affect the amylolytic, lichenase, endoglucanase, and xylanase activity ($P < 0.0001$) but not the protease activity ($P = 0.49$), as presented in Figure 1.

The highest amylolytic activity was at 1930 h and was higher than the activity at all the other time points when pooling the main treatment levels (fasting vs. ad lib), namely 0330 h ($P < 0.0001$), 0730 ($P < 0.0001$), 1130 ($P = 0.02$), 1530 ($P < 0.0001$), and 2330 ($P < 0.0001$). The second highest amylolytic activity was at 1130 h, and the lowest activities were at 0730 and 1530 h. The interaction between treatment (ad libitum vs. fasting overnight) and the time of collection tended to influence amylolytic activity ($P = 0.06$). We observed no difference between the amylolytic activity for the fasted and ad libitum-fed cows at 0330 h ($P = 0.63$), 0730 ($P = 0.61$), 1130 ($P = 0.35$), 1530 ($P = 0.98$), or 1930 ($P = 0.59$). However, at 2330 h the amylolytic activity of the cows fasted overnight tended to be lower than the activity of ad libitum-fed cows ($P = 0.07$).

Lichenase activity was highest at 1130 and 1930 h, and no difference was detectable between the 2 time points ($P = 0.78$). Lichenase activity for the rumen fluid collected at 1930 h was higher than for the rumen fluid collected at 0330 ($P < 0.0001$), 0730 ($P < 0.0001$), 1530 ($P < 0.0001$), and 2330 ($P < 0.0001$). We found no difference in lichenase activity between rumen fluid collected at 0730 and 1530 h ($P = 0.67$), when lichenase activity was the lowest. No interaction occurred between overnight fasting and the time of rumen fluid collection for lichenase activity ($P = 0.40$).

The highest endoglucanase activities were observed at 1130 and 1930 h, with no difference between these 2 time points ($P = 0.51$). The lowest endoglucanase activities were observed at 0330, 0730, 1530, and 2330 h. No difference was detectable in activity for the rumen fluid collected at 0330, 0730, 1530, and 2330 h. Additionally, we found no interaction between the treatment and the time of collection on endoglucanase activity ($P = 0.29$).

The activity of xylanase was highest at 1130 and 1930 h, and we observed a tendency for the xylanase activity for the rumen fluid collected at 1930 h to be higher than the activity observed at 1130 ($P = 0.10$). The xylanase activity of the rumen fluid collected at 1930 h was higher than 0330 ($P < 0.0001$), 0730 ($P < 0.0001$), 1530 ($P < 0.0001$), and 2330 ($P < 0.0001$). Similarly, the xylanase activity of the rumen fluid collected at 1130 h was higher than the activities at 0330 ($P < 0.0001$), 0730 ($P < 0.0001$), 1530 ($P < 0.0001$), and 2330 ($P < 0.0001$). The xylanase activity of rumen

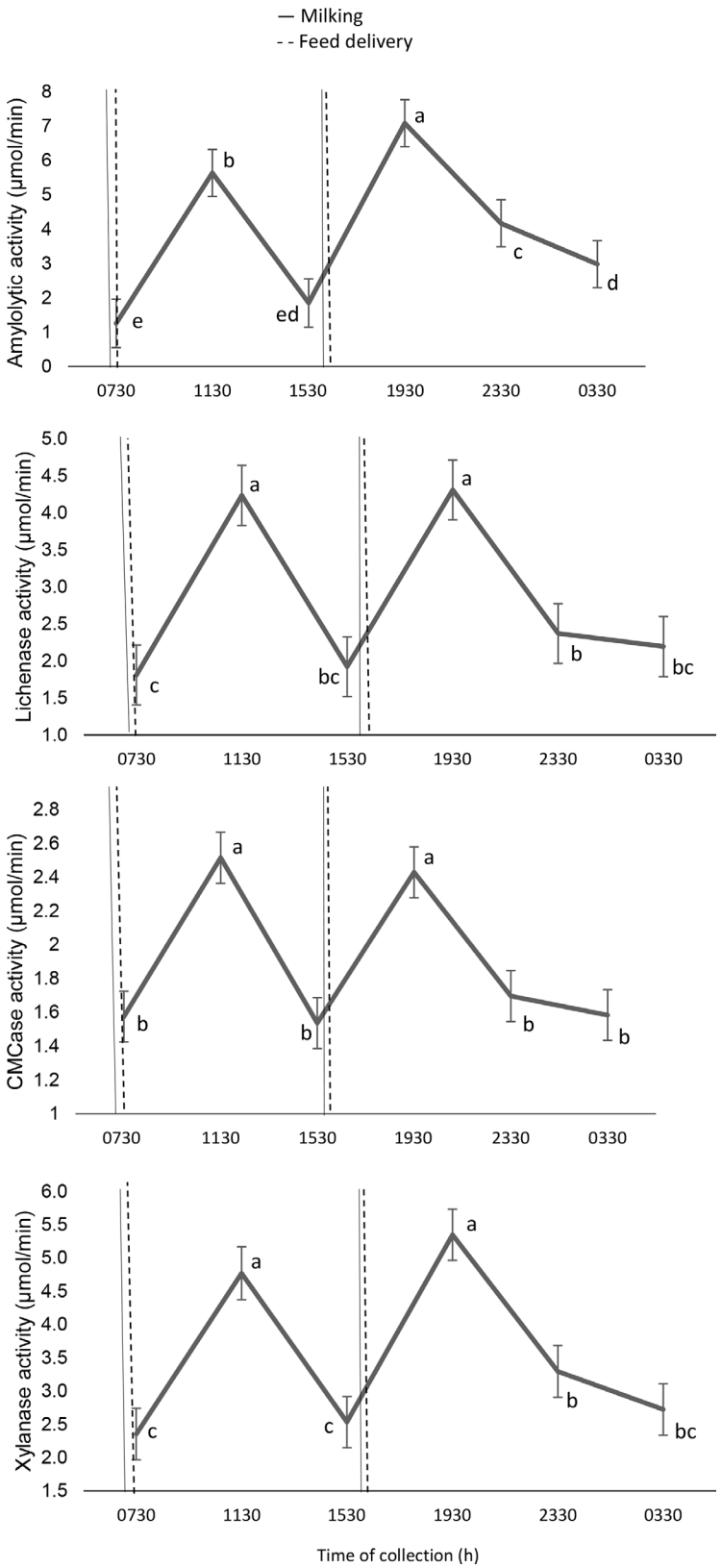


**Figure 1.** Effects of time of collection on enzymatic activities of amylase, lichenase, carboxymethyl cellulose (CMC)-ase, and xylanase. Vertical lines indicate milking time and feed delivery time. Different letters (a–e) within each panel and at each respective time point value represent significant differences among the time points ($P < 0.05$). Error bars represent SE.

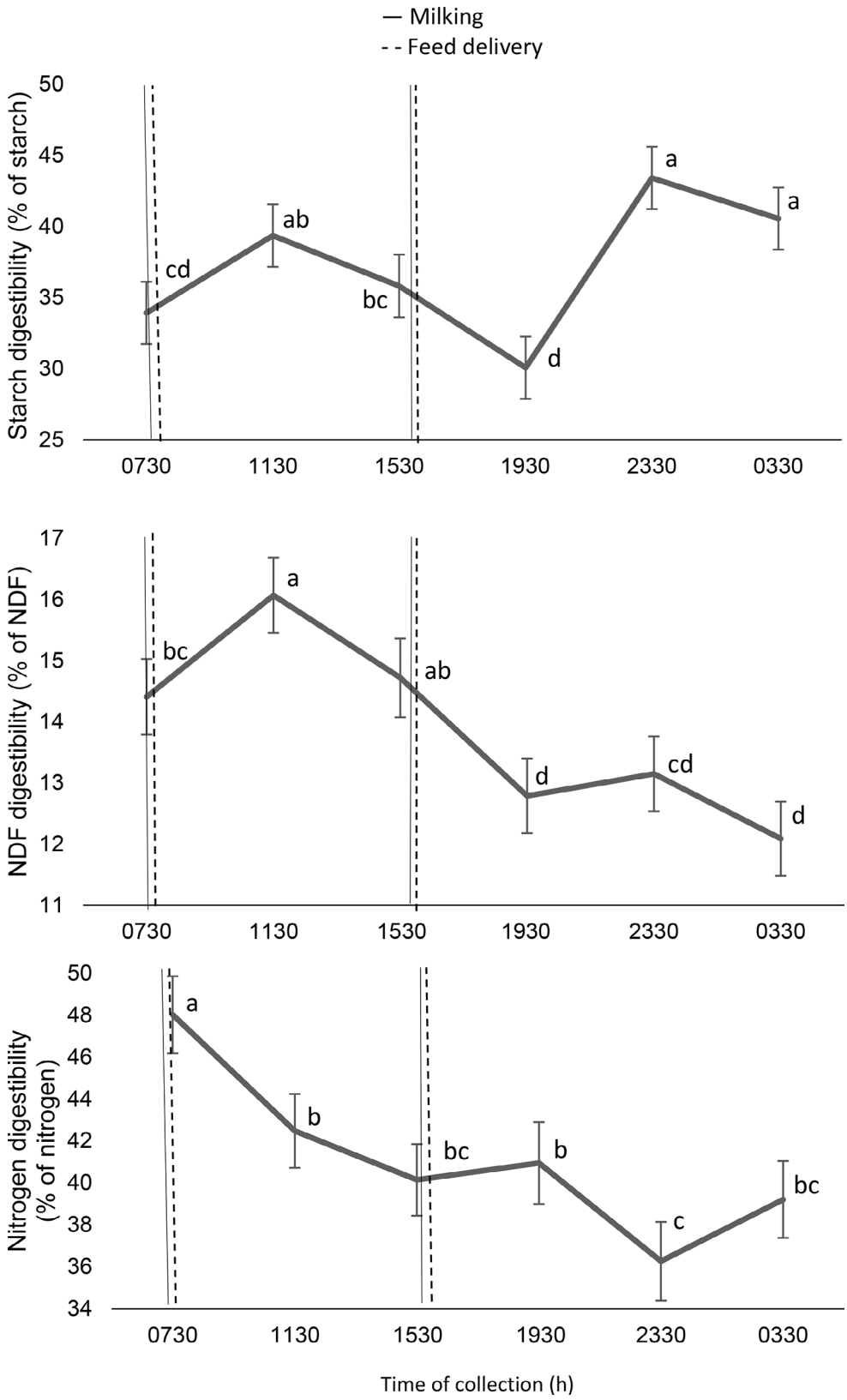


**Figure 2.** Effects of time of collection on in vitro starch, NDF, and nitrogen digestibility. Vertical lines indicate time of feeding and time of milking. Different letters (a–d) within each figure and at each respective time point value represent significant differences among the time points ($P < 0.05$). Error bars represent SE.

fluid collected at 2330 h was higher than the activities at 0730 ($P = 0.0055$) and 1530 ($P = 0.0238$) and tended to be higher than the activity observed at 0330 ($P = 0.09$). The xylanase activity of rumen fluid collected at 2330 did not differ from that collected at 0330 ($P = 0.27$) and 1530 ($P = 0.57$). Similarly, we observed no difference in xylanase activity between the rumen fluid collected at 0730 and 1530 h ($P = 0.58$). An interaction occurred between the hour of rumen fluid collection and treatment ($P = 0.0034$). Although we found was no difference between ad libitum and fasting overnight at 0330 ($P = 0.63$), 0730 ($P = 0.95$), 1530 ($P = 0.32$), 1930 ($P = 0.12$), and 2330 h ($P = 0.56$), a difference was observed at 1130 h ($P = 0.03$). In addition, we detected no effect of the interaction between the treatment and the time of collection on protease activity ($P = 0.49$).

### In Vitro Digestibility

No differences were detectable in ivSd ($P = 0.98$), NDFd ($P = 0.91$), and Nd ($P = 0.48$) between feeding ad libitum overnight compared with fasted, indicating that fasting overnight did not affect digestibility for the subsequent 24 h. There was, however, an effect of the time of rumen fluid collection on starch, NDF, and nitrogen digestibility ($P < 0.0001$). The effect of time of collection on in vitro digestibility is presented in Figure 2.

The highest ivSd values were observed at 0330, 1130, and 2330 h. Starch digestibility at 2330 did not differ from ivSd at 0330 ($P = 0.23$), but the rumen fluid collected at 2330 tended to have a higher ivSd than the rumen fluid collected at 1130 ($P = 0.09$). Starch digestibility from rumen fluid collected at 2330 was higher than ivSd when using rumen fluid collected at 0730 ($P < 0.0001$), 1530 ($P = 0.0017$), and 1930 h ($P < 0.0001$). The lowest ivSd values were observed when using fluid collected at 0730 and 1930 h. We detected no interaction of treatment and the time of collection on ivSd ($P = 0.81$).

We observed a peak in NDF digestibility at 1130 h. Cell wall digestion when using rumen fluid collected at 1130 was higher than NDFd from rumen fluid collected at 0330 ($P < 0.0001$), 0730 ($P = 0.02$), 1930 ($P < 0.0001$), and 2330 h ($P < 0.0001$), and tended to be higher than the rumen fluid collected at 1530 ($P = 0.073$). The lowest NDFd values were observed from rumen fluid collect at 0330, 1930, and 2330. No interaction of treatment and time of collection occurred for the extent of NDFd ($P = 0.69$).

Nitrogen digestibility peaked at 0730 h, when it was higher than that of fluid collected at 0330 h ($P < 0.0001$), 1130 h ($P = 0.0063$), 1530 h ($P = 0.0001$), 1930 h ($P = 0.0017$), and 2330 h ($P < 0.0001$). The lowest Nd values were observed at 0330 and 2330 h, and no difference in Nd occurred between these 2 time points ($P = 0.15$). There also was no interaction between treatment and time of rumen fluid collection on the extent of Nd ($P = 0.20$).

### Circadian Patterns

Amylase, lichenase, endoglucanase, xylanase, and protease activity, and in vitro starch, NDF, and nitrogen digestibility all fit a cosine function with a 24-h period for both treatments (Table 1). The cosine function was modified by treatment and therefore was sensitive to the availability of feed at night.

Amylolytic activity was phase advanced by 8.4 h in the ad libitum compared with the fasting treatment. For amylolytic activity, the amplitude of the ad libitum-fed cows were more than 50% greater than for the fasted cows. Lichenase activity was phase advanced by 0.48 h for the ad libitum cows compared with the fasted cows. For lichenase activity, the amplitude of the fasted cows was 7.4% greater than the ad libitum cows. Endoglucanase activity was phase delayed by 0.59 h for the cows fed ad libitum compared with the fasted cows. For endoglucanase activity, the amplitude for the ad libitum-fed cows were almost 10% greater than the fasted cows. Xylanase activity was phase advanced by 8.15 h for the cows fed ad libitum compared with the fasted cows. For xylanase activity, the amplitude of the ad libitum fed cows was more than 15% greater than the fasted cows. Protease activity of the ad libitum-fed cows was phase delayed by 3.0 h compared with the fasted cows. For protease activity, the amplitude of the ad libitum-fed cows was 32.5 times greater than the fasted cows.

Starch digestibility was phase delayed by 2.7 h for the ad libitum cows compared with the fasted cows. For ivSd the amplitude of the fasted cows was almost 75% greater than the ad libitum-fed cows. Neutral detergent fiber digestibility was phase delayed by 0.66 h for the ad libitum-fed cows compared with the fasted cows. For NDFd, the amplitude for the fasted cows was more than 67% greater for the ad libitum-fed cows. Nitrogen digestibility was phase advanced by 3.6 h when fed ad libitum compared with the fasted cows. For Nd, the amplitude of the fasted cows was almost 85% greater than the ad libitum-fed cows.

## DISCUSSION

The lack of differences between the treatments (ad libitum vs. fasted) indicates that fasting overnight did not affect amylase, lichenase, endoglucanase, xylanase,

and protease activities or the in vitro digestibility of starch, NDF, or nitrogen over the subsequent 24 h. However, except for protease, all the parameters displayed an effect of the time of rumen fluid collection on enzymatic activity and in vitro digestibility. Both peaks observed in amylase, lichenase, endoglucanase, and xylanase activities were for the sampling times subsequent to feed delivery and milking. As previously mentioned, under natural grazing conditions, cows display a diurnal feeding pattern, where most of the feeding activity occurs during the day, especially at sunset and sunrise (DeVries, 2013). However, the diurnal feeding pattern of dairy cows in freestall housing tends to be influenced by milking, feed push-up, and the time of feed delivery (DeVries et al., 2003). It could be that the feeding behavior of the cows were affected by feed delivery and milking, as described by DeVries et al. (2003).

The lack of difference in protease activity by both overnight feeding treatment and time of rumen fluid collection could indicate that protease activity is constant across the day and is not responsive to timing of feed intake. Alternatively, the assay we used (van de Vyver et al., 2004) was only able to detect a very low protease activity and was also highly variable (ranging from 0.000008 to 0.000708 OD/min at 275 nm in the samples analyzed), resulting in high standard errors and limiting the ability to detect differences. Extracting enzymes from the particle and cell-bound fractions by sonication could have increased the activity detected. However, the number of parameters tested throughout this trial did not allow for sonication, as it would have taken at least 30 min to sonicate the samples individually for protease analysis.

The amylase activity assays displayed 2 peaks subsequent to milking and feed delivery, but the ivSd assay showed a delayed peak after the second milking and feed delivery. This difference could be attributed to the fact that enzymatic assays give an instant result of the enzymatic activity at that time point. For in vitro assays, rumen fluid is handled for longer, as it needs to be injected into all the flasks and results are only obtained after the incubation period, indicating the abilities of the microorganisms sampled at the given time point to adjust to the new microenvironment and to digest a substrate. In the case of ivSd, the values obtained were measured 7 h after the enzymatic activity. If the cows adapted their feeding behavior according to the time of feed delivery and milking, it could be suggested that after the second feed delivery there was a lag in microbial activity that only reached a maximum 7.5 h after feed delivery. Coincidentally, 7 h is also the most common time point used for starch digestibility, because 7 h is believed to be the average retention time for starchy materials. A similar lag time was observed by Cone et al. (1989) and showed that cows fed once a day only reached their maximum ivSd 12 and 16 h after feeding for a low- and high-level diet. Starch and NDF digestibility values, across the various fluid collection time points, were in general more variable than expected. The most distant ivSd values (1930 vs. 2330 h) had a difference of 0.13 starch$^{-1}$ and NDFd values (1130 vs. 0330 h) had a difference of 0.04 NDF$^{-1}$ ($P < 0.0001$). Such differences could result in important consequences when computing rates of digestion (Raffrenato and Erasmus; 2013), especially for starch (Hall, 2000), to be used for rationing software systems such as the Cornell Net Carbohydrate and Protein System (Van Amburgh et al., 2015), depending on how rates are estimated and especially when using few time points to calculate a fractional rate of digestion in case of NDF (Raffrenato and Van Amburgh, 2011). A 12-h NDFd incubation was used in this study to attempt to highlight the differences caused by the moment of rumen fluid collection across the 24 h. Logistical limitations did not allow us to have multiple time points from the same rumen fluid. However, we do believe that the differences would be smaller for farther time points (e.g., 24 or 30 h), reducing the consequences of collecting rumen fluid at different time points. However, this speculation needs to be investigated further.

The decrease in digestibility observed in in vitro starch and NDF digestibility after the second feed delivery and for N after the first feed delivery could be a result of a decrease in bacterial numbers. Leedle et al. (1982) reported a decrease in viable bacterial numbers after feeding. Additionally, Bryant and Robinson (1968) found that bacterial numbers were at their lowest 1 h after feeding, attributed to the dilution of rumen contents by water, feed, and saliva. However, Leedle et al. (1982) attributed the loss in viable bacterial numbers to a rapid change in the ruminal environment after feeding. Leedle et al. (1982) suggested that some ruminal bacteria could be adapting to the changed ruminal environment after feeding by participating in substrate-accelerated death, as described by Postgate and Hunter (1964). Moreover, Leedle et al. (1982) suggested that osmotic shock caused by water intake and oxygen shock caused by oxygen intake with feeding should also be considered as factors that could cause a loss in viable bacteria numbers and subsequently a decrease in digestibility after feeding. Therefore, it is possible that the feeding behavior of the cows was affected by feed delivery and milking, as shown by DeVries et al. (2003), resulting in a decrease in bacterial and ciliate numbers after feed delivery, producing the lag times observed in the ivSd, NDFd, and Nd peaks. However, monitoring feeding activity could confirm this speculation.

No interactions occurred between the feeding treatment and the time of rumen fluid collection on the activity of lichenase, endoglucanase, and protease, and digestibility of starch, NDF, or nitrogen. The tendency of lower amylolytic activity at 2330 h for the fasted cows could be attributed to the absence of substrates for the amylolytic microorganisms. However, this would be expected after a few hours into the overnight fasting. Such a difference at 2330 h is therefore difficult to explain, and it is more likely related to different feeding patterns among the cows that were not monitored. A higher xylanase activity at 1130 h for the fasted cows might have been caused by the different feeding behaviors or a change in the rumen microenvironment pertaining to amylase and xylanase activity.

When fitted to a cosine function, all the parameters tested followed a daily pattern that was sensitive to the overnight availability of feed, although the parameters responded differently to the feeding treatment. Lactating dairy cows naturally display highest feeding activity during the day, with a marked decrease in activity at night (DeVries and von Keyserlingk, 2009). The effect of overnight fasting on amylase and xylanase activities produced a daily pattern with a delayed acrophase and reduced amplitude. Overnight fasting also delayed the acrophase of lichenase activity but increased the amplitude. The effect of overnight fasting on endoglucanase and protease activity produced a daily pattern with an advanced acrophase and a reduced amplitude. The reason for the phase delay in endoglucanase activity observed in the ad libitum-fed cows could be attributed to higher levels of glucose in the rumen compared with the fasted cows, as Mountfort and Asher (1985) demonstrated that glucose could play a role in the regulation of endoglucanase. Fasting the night before could have caused a change in the rumen microenvironment in relation to the ad libitum-fed cows, producing an advanced acrophase for endoglucanase activity. The effect of fasting on enzymatic activity varied between the substrates. Both lichenase and carboxymethyl cellulose were used to measure for endoglucanase activity. However, fasting had the opposite effect on their daily patterns.

The effect of overnight feeding on ivSd and NDFd produced a daily pattern with an advanced acrophase and increased amplitude. The fact that the effect of fasting on Nd produced a delayed acrophase and a higher amplitude corresponds with the results of a study by Ørskov and McDonald (1979) showing that restricted feeding reduced the passage rate with a subsequent increase in ruminal protein degradability compared with unrestricted feeding in sheep. The reason for the variability in enzymatic activity observed for amylase, lichenase, xylanase, and protease activity, and in vitro starch and nitrogen digestibility is not clear but might have been caused by different feeding behaviors displayed by the cows, resulting from the treatment the night before.

## CONCLUSIONS

The data confirm that different interpretations should be given to enzymatic activities and in vitro digestibility values. Daily variation in in vitro digestibility would result in important consequences when computing rates of digestion, especially for starch (estimated changes can be approximately ±20% from the calculated rates). When fitted to a cosine function, all the parameters tested followed a daily pattern that was sensitive to the overnight availability of feed. Analyzing for bacterial diversity using modern techniques, such as 16S rDNA sequencing, should also be considered to characterize the bacterial population dynamic across a 24-h period in relation to enzymatic activity and in vitro digestibility assays. Although no robust conclusions can be inferred relative to the day and night feed regimen, if the objective is to maximize NDF or starch digestibility in vitro, rumen fluid should not be harvested immediately after cows are fed but, rather, after 4 to 5 h. For protein digestibility, sensitivity will be lower and rumen fluid harvest should occur before the cows are fed, when the bacteria and protozoa seem to be N deficient.

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## ORCIDS

E. Raffrenato https://orcid.org/0000-0002-1789-662X
M. J. Badenhorst https://orcid.org/0000-0001-5177-4696
K. J. Harvatine https://orcid.org/0000-0001-6422-2647
G. Esposito https://orcid.org/0000-0003-4884-6999
W. H. van Zyl https://orcid.org/0000-0001-8195-353X