Original Research Article

Effect of exogenous fibrolytic enzymes on performance and blood profile in early and mid-lactation Holstein cows

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1. Introduction

The genetic progress in increasing milk yields of dairy cows over the last decades, mainly peak-lactation yields, led to a remarkable increase in energy requirement for milk synthesis. However, the associated improvements in feed intake did not compensate the increased energy demands during early lactation with the consequence of more pronounced negative energy balance and the need to mobilize body reserves. As reported by Ingvartsen and Moyes (2013), this may result in a physiological imbalance, a situation where the regulatory mechanisms are insufficient and the risk for digestive, metabolic and infectious problems is enhanced.

The negative interaction between high milk yield and a prolonged severe negative energy balance has initiated investigations into feeding strategies aimed at improving the energy supply. One of the more recent attempts are directed to improve energy balance by decreasing milk energy output through supplementing conjugated linoleic acids (Pappritz et al., 2011; von Soosten et al.,

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However the most common practical nutritional strategies are aimed at improving the energy balance by raising energy density in the diet. Substituting the forage component with non-fiber carbohydrates in the diet increases the energy intake, but also increases the concentrate induced risk of developing subacute ruminal acidosis (Krause and Oetzel, 2006). A further way of improving the energy balance in early lactation is optimizing the gastro-intestinal degradation of fiber components in the ration (Jung and Allen, 1995). This line of thought led to examine option for improving fiber degradation.

Supplementing dairy cow diets with exogenous fibrolytic enzymes (EFE) has the potential to improve plant cell wall digestibility and therefore, the efficiency of feed utilization (Meale et al., 2014). Most EFE contain mainly xylanases and cellulases of fungal or bacterial origin applied to the ration before consumption with the expectation to improve feed efficiency and animal performance (Beauchemin and Holtshausen, 2010). Several studies with early lactation cows (<100 days in milk [DIM]) reported a significant higher milk performance due to EFE supplementation (Gado et al., 2009; Schingoethe et al., 1999; Yang et al., 1999). Other feeding trials with early lactation cows did not find significant effects of EFE supplementation on milk yield (Arriola et al., 2011; Beauchemin et al., 2000; Bernard et al., 2010; Dhiman et al., 2009; Schingoethe et al., 1999; Yang et al., 1999). This line of thought led to examine option for improving fiber degradation.

Table 1

| Item | Trial 1 | Trial 2 |
|------|--------|--------|
|      | Control | Enzyme | Control | Enzyme |
| **Ingredients, %** | | | | |
| Corn silage | 40.2 | 40.2 | 40.2 | 40.2 |
| Grass silage | 20.1 | 20.1 | 20.1 | 20.1 |
| Concentrate | 39.6 | 39.6 | 39.7 | 39.7 |
| **Chemical composition** | | | | |
| Dry matter, g/kg | 404 | 405 | 363 | 361 |
| **Nutrients, g/kg of DM** | | | | |
| Organic matter | 928 | 931 | 930 | 932 |
| Crude protein | 144 | 142 | 135 | 136 |
| Ether extract | 35 | 36 | 34 | 34 |
| NDF | 408 | 385 | 428 | 420 |
| ADF | 206 | 196 | 226 | 218 |

NDF = neutral detergent fiber; ADF = acid detergent fiber.

1 Composition of concentrate: 25% soybean meal, 20% barley, 27% wheat, 24% sugarbeet pulp dried, 2% soybean oil and 2% mineral vitamin premix. Per kg mineral and vitamin premix: 14.0% Ca; 7.0% P; 12.0% Na; 4.0% Mg; 1,000,000 IU VA; 100,000 IU ID; 1,500 mg VE; 6,000 mg Mn; 5,400 mg Mn; 1,000 mg Cu; 25 mg Co; 100 mg I; 40 mg Se.

2. Materials and methods

2.1. Experimental design and animals

Two experiments were implemented at the Friedrich-Loeffler-Institute in Braunschweig (FLI). The early lactation experiment (trial 1) consisted of twenty-eighth lactating Holstein cows (5 primiparous, 23 multiparous, 50 ± 16 DIM, 33.4 ± 5.9 kg milk yield, 4.24 ± 0.86% fat, 3.07 ± 0.19% protein, 593 ± 45 kg BW), and the mid lactation experiment (trial 2) included twenty-six lactating Holstein cows (6 primiparous, 20 multiparous, 136 ± 26 DIM, 32.7 ± 3.5 kg milk, 4.13 ± 0.56% fat, 3.13 ± 0.16% protein, 625 ± 65 kg BW). Both trials used different cows and were implemented over an experimental period of 56 days. Cows were fed with a total mixed ration (TMR) and blocked by parity and milk yield, and then randomly assigned to 1 of 2 treatments in a completely randomized block design: 1) TMR control (without enzyme supplementation, water only), 2) TMR enzyme (with enzyme supplementation).

2.2. Diet ingredients and chemical composition of trial 1 and trial 2

The diet was formulated to meet the nutritional requirements of the cows as recommended by the German Society of Nutrition Physiology (GfE, 2001). The TMR consisting of 60% forage and 40% concentrate (DM basis). Components and chemical composition of the basal diet are shown in Table 1. All cows were housed together in a free-stall barn and were fed twice a day at 0730 and 1400 h. The TMR was provided in 5 self-feeding stations (TYPE RIC, Insentec, B.V., Marknesse, the Netherlands) per treatment.

2.3. Enzyme product, enzyme level and application method

A commercial enzyme mixture traded as Roxzyme G2 Liquid (RG2, Single lot Nr. 302501, DSM Nutritional Products, Ltd, Basel Switzerland) was used in these experiments. The enzyme mixture was a commercial preparation produced by a strain of *Trichoderma reesei*. One millilitre of the enzyme mixture contained 8,000 units endo-1,4-ß glucanase (EC 3.2.1.4), 18,000 units endo-1,3-(4)-ß glucanase (EC 3.2.1.6) and 26,000 units 1,4-ß xylanase (EC 3.2.1.4), as specified by the manufacturer.

All cows were exposed to a ration-adaptation period of 20 days followed by a 56-day experimental period (supplementation period) on their assigned diet. The RG2 was applied at 3.9 ± 0.14 ml/kg TMR in the trial 1 and 3.8 ± 0.17 ml/kg TMR for trial 2. The RG2 liquid was diluted at a rate of 1:10 with water and added to the TMR using a sprinkler-can while being mixed in a mixer wagon. The daily TMR and the enzyme application were prepared directly before the morning feeding for the TMR.

Details of the measurement of enzyme activities in feed samples are reported by Peters et al. (2010) and results are shown in Table 2.

2.4. Measurement, sampling and analysis

Feed intake was measured daily for individual cows through a transponder assisted automatic feed weighing trough system. Cows were milked twice daily (0530 and 1530 h). Individual milk yields were recording automatically by the milking system at each milking. Milk samples from each cow were collected twice a week (a.m./p.m. composite) and treated with a preservative agent (bronopol) and stored at 8°C until analysis. Milk composition (fat,
protein, lactose, urea concentration, and somatic cell count) was determined using an infrared milk analyser (MilkoScan FT 6000 combined with a Fossomatic 5000, Foss Electric, Hillerød, Denmark). Milk composition data of the two sampling days were averaged and the average values used in the statistical analysis. Body weight was electronically recording after leaving the milking parlor twice daily using automatic walk-through scales. The two measurements were arithmetically averaged to a single value.

Ruminating activities (only in trial 2) were monitored visually for each cow on days 14, 28, 42 and 56 over a 24-h period, according to Kononoff et al. (2002) with modified observation intervals. Rumination activities were observed at 10 min intervals. If the cow was detected to ruminate within a 10 min time span it was noted as a 10-min rumination episode. To estimate the time spent ruminating per kilogram of dry matter intake (DMI) or neutral detergent fiber (NDF) unit, the average intake of the preceding 3 days was used because time spent ruminating was assumed to reflect the DMI of the previous days.

Samples of grass silage and corn silage were taken at alternate days from the mixing wagon after 5 min of mixing. All samples were pooled on a weekly basis and stored at −20 °C until analysis. Feed samples of the TMR enzyme to determine enzyme activities were taken once per week and stored at −18 °C until analysis. Concentrate samples were taken twice per week from the concentrate silo. All samples were pooled fortnightly and directly subjected to Weender Analysis.

Samples of TMR control/TMR enzyme for analyzing DM content were taken daily from each trough directly before feeding. All samples were pooled fortnightly and directly exposed to Weender Analysis.

All methods for analyzing silage, concentrate and TMR samples are in accordance with the book of methods Vol. III of Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (Naumann and Bassler, 2012).

The blood samples were taken on trial days 28 and 56 by puncture of the coccygeal vein/artery. The samples were then centrifuged (2,120 × g for 10 min and 15 °C) within 2 h after collection to obtain serum, which was immediately stored in Eppendorf tubes at −20 °C until analysis.

Serum samples were analyzed at the Clinical Laboratory of the Cattle Clinic Hannover University of Veterinary Medicine, Germany. Haemolysed samples were excluded. The following biochemical blood components were measured by different colorimetric techniques using an automatic multiparameter analyser for clinical chemistry (Cobas-Mira, Hoffmann-La Roche & Co. AG Diagnostika, Basel) and different commercial kits accordance with Deutsche Gesellschaft für Klinische Chemie (DGKC) and International Federation of Clinical Chemistry (IFCC) recommendations.

For total bilirubin (TB), a commercial kit (Jendrassik-Grof colorimetric Diazo method; LT-SYS, Berlin) was used. 8-hydroxybutyрат (BHB) and urea were analyzed with commercial kits (Randox Laboratories Ltd., Wulfrath; LT-SYS, Berlin and Roche Diagnostics GmbH, Mannheim) using an enzymatic UV method. Serum activities of aspartate-amino transferase (AST) and gamma glutamyl transferase (GGT) were determined using an enzymatic assay (ABX Deutschland, Göppingen and Hitado Diagnostic Systems, Möhnesee Deelcke). The spectrophotometric biuret method was used to quantify the concentrations of total protein (Sigma Diagnostics, Deisenhofen).

### 2.5. Digestibility measurements using wethers

The apparent digestibility of the applied TMR control and TMR enzyme was determined through digestibility tests using wethers according to the regulations for the determination of digestibility of crude nutrients with ruminants published by (GfE, 1991). The apparent digestibility of crude nutrients and net energy for lactation (NEL)-content of the offered TMR-Control and the TMR-Enzyme in trials 1 and 2 was measured in two trials with 4 wethers (German Blackhead/SKF) per trial. The daily ration offered contained 1.1 kg TMR (40% corn silage, 20% grass silage, 40% concentrate) plus 44.4 mL water/d (TMR-control) during the first period and an enzyme addition of 4.4 mL/d diluted with 44 mL water/d during the second period, respectively.

Experimental animals were kept in metabolic crates and fed the restrictive ration daily at 0630 and 1430 h. Water was offered ad libitum. Each trial period started with a 12-day adaptation period followed by an 8-day collection period, during which the total faeces were collected after each feeding and stored at −20 °C until further processing.

Before analysis the total faeces samples collected were weighed and thoroughly mixed before several samples were taken. Part of the fresh sample was used for crude protein determination using the Kjeldahl method. Feaces samples were dried at 60 °C and thereafter milled to 2 mm for further chemical determinations. From each daily feed sample 200 g were separated, stored at −20 °C, and at the end of each trial period dried and milled to 2 mm. All feed and faeces samples were subjected to the Weender analyses of crude nutrients and acid detergent fiber (ADF) and NDF determination.

### 2.6. Calculations

The apparent digestibility of nutrients was calculated as follows:

\[
\text{Apparent digestibility} = \left(\frac{\text{nutrient}_{\text{intake}} - \text{nutrient}_{\text{faeces}}}{\text{nutrient}_{\text{intake}}}\right) \times 100.
\]
Based on the equations published by the German Society of Nutrition Physiology (GfE, 2001) the metabolizable energy (ME), gross energy (GE) and NEL were calculated as follows:

\[
\begin{align*}
\text{GE} & \text{ (MJ/kg)} = 0.0239 \times \text{CP} + 0.0398 \times \text{EE} + 0.0201 \times \text{CF} + 0.0175 \times \text{NIE}, \\
\text{ME} & \text{ (MJ/kg)} = 0.0312 \times \text{DEE} + 0.0136 \times \text{DCF} + 0.0147 \times (\text{DOM} - \text{DEE} - \text{DCF}) + 0.00234 \times \text{CP}, \\
\text{NEL} & \text{ (MJ/kg)} = 0.6 \times [1 + 0.004 \times (q - 57)] \times \text{ME} \text{ (MJ/kg)}.
\end{align*}
\]

Where is: CP = crude protein (g/kg); EE = ether extract (g/kg); CF = crude fiber (g/kg); NIE = nitrogen free extract (g/kg); OM = organic matter (g/kg); D = digestible; q = ME/GE \times 100.

Based on the equations published by the German Society of Nutrition Physiology (GfE, 2001) the net energy requirements for maintenance (NE\text{m}) and NEL as well as milk energy concentration and output were calculated as follows:

\[
\text{NE_m} \text{ (MJ NEL/d)} = 0.293 \times \text{BW}^{0.75},
\]

Milk energy concentration (MJ NEL/kg) = 0.38 \times \text{milk fat (\%)} + 0.21 \times \text{milk protein (\%)} + 0.95,

Requirement for milk production (MJ NEL/d) = Energy content of milk (MJ NEL/kg) \times \text{milk yield (kg/d)}.

Fat-corrected milk (FCM) and energy corrected milk (ECM) was calculated as:

\[
\text{FCM} \text{ (kg/d)} = ([\text{milk fat (\%)} \times 0.15] + 0.4) \times \text{daily milk yield (kg/d)},
\]

\[
\text{ECM} \text{ (kg/d)} = \text{milk yield (kg/d)} \times ([0.38 \times (\text{milk fat (\%)} + 0.21 \times (\text{milk protein (\%)} + 1.05)]/3.28).
\]

The net energy balance was calculated with the following equation:

\[
\text{Net energy balance (MJ NEL/d)} = \text{energy intake (MJ NEL/d)} - \text{NE_m} \text{ (MJ NEL/d)} + \text{NEL} \text{ (MJ NEL/d)}.
\]

Gross feed efficiency was determined by dividing the daily 4% FCM yield by DMI of each animal. The changes in body weight (henceforth BW change) were calculated by subtracting the BW at the end from the BW at the start of the trial.

### 2.7. Statistical analysis

The normal distribution of all variables included in the dataset was tested with the PROC UNIVARIATE of SAS (Version 9.3.1, SAS Inst. Inc., Cary, NC, USA) using the Shapiro–Wilk test. Variables with Shapiro–Wilk values (W) ≥ 0.98 were considered normal. The non-normal distributed variables gross feed efficiency, BHH, AST, and GGT were calculated on the log-transformed values (natural logarithm) before being subjected to further statistical evaluation. Milk somatic cell count (SCC) data were converted to somatic cell score (SCS) using a base 2 logarithmic function: SCS = log 2 (SCC/100,000) + 3 to achieve an approximate normal distribution of the test day values for the statistical analysis (Ali and Shook, 1980).

Gaussian and transformed performance (test day information) and blood data were statistically processed by PROC MIXED (Version 9.3.1, SAS Inst. Inc., Cary, NC, USA) using a mixed linear model including a REPEATED statement with subject = cow, to assess the fixed effects of treatment, parity (category 1, 2 and 3 +) and trial day. Differences in milk yield between groups of cows allotted to the treatment groups were corrected using the actual deviation from the common mean obtained during the adaptation period. Cows within trial differed in DIM, thus the day in milk at the first trial day (fDIM) was introduced into the model as a covariate. Ruminating activities data were analyzed with same model, except for fDIM which tested not significant. The variance-covariance matrix structures were evaluated for each response variable using AR (1), SP (Pow), CS and VC covariance structures. Variance-covariance matrix structures were selected for each variable based on the lowest Akaike information criterion (AIC) fit statistic. Significance of the fixed effects was determined using the F-test. Degrees of freedom were calculated using the KENWARDROGER option. Body weight change was analyzed by using the PROC MIXED procedure (Version 9.3.1, SAS Inst. Inc., Cary, NC, USA). The model contained treatment, parity and fDIM as a covariate. Results of all normal distributed traits are shown with their LSmeans and SEM. Log-transformed traits are presented as means with their SD while P-values are taken from the statistical analysis of the log-transformed data. Significance was declared at P ≤ 0.05 and a tendency to significance at 0.05 ≤ P < 0.10, differences were considered to indicate a trend.

### 3. Results

#### 3.1. Digestibility trials

Results of the digestibility trials are compiled in Table 3. The total mixed ration control and TMR enzyme both showed a similar NEL-content of 7.2 MJ NEL/kg DM (P > 0.05). Digestibility values of DM, OM, CP, NDF and ADF did not differ between treatment groups (P > 0.05).

#### 3.2. Production response in lactation trials 1 and 2

Results for performance traits and gross feed efficiency of experimental cows from trials 1 and 2 are presented in Tables 4 and 5, respectively. In general cows in trial 2 had a higher DMI and water intake than those of trial 1 (P > 0.05). Milk yield and milk components were also slightly higher during trial 2 (P > 0.05).

Addition of the fibrolytic enzyme mixture to the TMR had no significant effect on any production parameter in both trials (P > 0.05). The treatment effect on energy balance showed a numerical trend in the early lactation trial (P = 0.11) of a lower negative energy balance in the treatment group during, while no differences were observed during the mid-lactation trial (P = 0.35). Cows fed TMR enzyme showed a tendency of a slightly higher (P = 0.09) ECM yield in trial 2.
Table 3
Nutrient digestibility in sheep fed control or enzyme supplemented total mixed ration (mean ± SD).

| Item                      | Treatment                      | Control (n = 4) | Enzyme (n = 4) |
|---------------------------|-------------------------------|----------------|---------------|
| DMI, g/d                  |                               | 1.117          | 1.100         |
| Apparent digestibility, % |                               |                |               |
| DM                        |                               | 76.7 ± 0.2     | 77.8 ± 2.6    |
| OM                        |                               | 79.8 ± 0.3     | 80.3 ± 2.4    |
| CP                        |                               | 77.2 ± 0.9     | 75.6 ± 2.6    |
| NDF                       |                               | 67.5 ± 0.8     | 69.7 ± 4.4    |
| ADF                       |                               | 64.2 ± 1.7     | 66.4 ± 4.7    |
| Energy content            |                               |                |               |
| MJ ME/kg DM               |                               | 11.7           | 11.7          |
| MJ NEL/kg DM              |                               | 7.2            | 7.2           |

DMI = dry matter intake; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; ME = metabolizable energy; NEL = net energy lactation.

Parity effects were observed for water intake, milk yield, FCM, ECM, Urea and milk energy output in both trials (P < 0.05). The effect of DIM on performance parameter was different in the two trials. In the first trial a significant effect of DIM was observed on FCM, ECM, milk fat, fat-protein-quotient (FPQ), maintenance requirement and milk energy output (P < 0.05) but not on milk yield, DMI and water intake (P > 0.05). During the second trial milk yield, FCM, ECM, milk energy output, energy balance and gross feed efficiency were significantly influenced by fDIM (P < 0.05) but not DMI, water intake and milk components (P > 0.05). Trial day affected all traits significantly (P < 0.05). This is also illustrated in Fig. 1. Significant interactions between treatment and trial day were observed for DMI, water intake, NEL intake and energy balance during trial 1 (P < 0.05), while in trial 2 this interaction also affected milk yield, FCM and ECM and milk energy output (P < 0.05). Gross feed efficiency showed this interaction during both trials (P < 0.05).

Table 4
Performance, milk composition and gross feed efficiency of cows fed the control or the enzyme supplemented total mixed ration in trial 1.

| Item                      | Treatment                      | P-value |
|---------------------------|-------------------------------|---------|
|                           | TMR control (n = 14)          |         |
|                           | TMR enzyme (n = 14)           |         |
|                           | TRT                           | Parity  | fDIM  | Trial day | TRT x Trial day |
| DMI, kg/d                 | 16.6 ± 0.56                   | 17.4 ± 0.62 | 0.36 | 0.29 | 0.17 | < 0.01 | < 0.01 |
| Water intake, L/d         | 63 ± 3.0                      | 63 ± 3.3 | 0.87 | 0.05 | 0.91 | < 0.01 | < 0.01 |
| Yield, kg/d               | 30.2 ± 0.82                   | 30.4 ± 0.90 | 0.84 | < 0.01 | 0.18 | < 0.01 | 0.13 |
| Milk yield                | 30.8 ± 0.70                   | 30.3 ± 0.77 | 0.64 | < 0.01 | < 0.01 | < 0.01 | 0.44 |
| ECM                       | 30.1 ± 0.62                   | 29.3 ± 0.68 | 0.77 | < 0.01 | < 0.01 | < 0.01 | 0.29 |
| Milk components, %        |                               |         |       |         |       |       |       |
| Milk fat                  | 4.16 ± 0.16                   | 4.04 ± 0.18 | 0.60 | 0.21 | 0.01 | < 0.01 | 0.95 |
| Milk protein              | 3.14 ± 0.05                   | 3.16 ± 0.06 | 0.78 | 0.28 | 0.09 | < 0.01 | 0.95 |
| Milk lactose              | 4.73 ± 0.04                   | 4.83 ± 0.04 | 0.08 | 0.02 | 0.19 | < 0.01 | 0.01 |
| FPQ                       | 1.34 ± 0.04                   | 1.28 ± 0.05 | 0.43 | 0.07 | < 0.01 | < 0.01 | 0.86 |
| Urea, mg/L                | 189 ± 6.0                     | 189 ± 6.7 | 0.98 | 0.02 | 0.37 | < 0.01 | 0.03 |
| SCS                       | 2.39 ± 0.33                   | 2.37 ± 0.37 | 0.96 | 0.27 | 0.49 | < 0.01 | 0.99 |
| Energy measure, MJ/d      |                               |         |       |         |       |       |       |
| NEL intake                | 119.1 ± 4.0                   | 125.6 ± 4.5 | 0.28 | 0.29 | 0.19 | < 0.01 | < 0.01 |
| Maintenance requirement   | 35.4 ± 0.4                    | 34.6 ± 0.5 | 0.19 | 0.06 | 0.01 | < 0.01 | 0.08 |
| Milk energy output        | 95.7 ± 2.0                    | 94.9 ± 2.2 | 0.77 | < 0.01 | < 0.01 | < 0.01 | 0.34 |
| Energy balance            | − 12.4 ± 3.7                  | − 32.2 ± 4.1 | 0.11 | 0.59 | 0.97 | < 0.01 | < 0.01 |
| GFE, kg FCM/kg DM         | 1.99 ± 1.09                   | 1.82 ± 0.75 | 0.16 | 0.21 | 0.25 | < 0.01 | < 0.01 |
| BW change, kg/56 d        | 14.0 ± 3.4                    | 15.6 ± 3.8 | 0.75 | 0.16 | 0.40 | nm    | nm    |

DMI = dry matter intake; FCM = fat corrected milk; ECM = energy corrected milk; FPQ = fat-protein-quotient; SCS = somatic cell score; NEL = net energy lactation; GFE = gross feed efficiency; BW = body weight; TMR = total mixed ration; TRT = treatment; fDIM = the day in milk at the first trial day; nm = not included in the model.

1 Least squares means ± SEM (normal distributed data).
2 Means ± SD (log-transformed data).

3.3. Blood parameters

The effects of EFE supplementation on selected blood parameter are shown in Table 6. No treatment effects on blood parameters were observed during both trials (P > 0.05). Trial day significantly influenced all blood parameter (P < 0.05) except total bilirubin in trial 2 (P = 0.13). During the first trial BHB, total bilirubin and AST were significantly affected by parity, while only AST was affected in trial 2 (P < 0.05). The highly significant effect of parity on BHB in trial 1 (P < 0.05) was caused by a markedly higher value in second parity cows (0.48, 0.69 and 0.51 mmol/L for first, second and further parity). The day in milk at the first trial day had an significant influence on BHB and AST in the first trial (P < 0.05) and only on total protein in the second trial (P = 0.05).

3.4. Ruminating activities in trial 2

The effects of EFE supplementation on ruminating are shown in Table 7. Ruminating activity parameters were not affected by treatment and parity (P > 0.05). The trial day was significant (P < 0.05) but no treatment by trial day interaction was observed (P > 0.05).

4. Discussion

4.1. Digestibility

The calculation of nutrient values in the total mixed ration used in the experiments was based on the results of digestibility trials using wethers. The similarity in digestibility values for TMR control and TMR enzyme treatment groups is contrary to our hypothesis that enzyme application improves digestibility of a TMR. Miller et al. (2008a) who used a same enzyme product (Roxzyme G2 Liquid) at a similar dosage fed to lambs also found no significant effects on nutrient digestibility (P > 0.05).
Reddish and Kung (2007). Holtshausen et al. (2011) reported a positive response in DMI (Miller et al., 2008b) for forage silage but not for a barley-based diet, suggesting an EFE effect in diets with a lower OM digestibility. The mode of action of EFE is not completely understood and described because of the complexity of ruminal microbial ecosystem and the process of fiber digestion. One discussed mode of action of EFE is the increased number of cellulolytic bacteria and the population changes in the rumen (Beauchemin and Holtshaussen, 2010). Zeitz et al. (2013) investigated a high dose of Roxazyme G2 Liquid (13.8 and 27.7 mL enzyme product/kg DM) on the specific ruminal bacterial population in non-lactating cows, but did not detect any effect on important rumen bacterial species.

The EFE supplementation did not significantly improve the energy balance (EB) of early and mid-lactation dairy cows. This can be explained by the lack of significant differences in DMI and milk energy output between both diets. The numeric difference of the EB between the TMR control (−12.4 MJ/d) versus TMR enzyme (−3.2 MJ/d) in trial 1, may have been caused by the slightly higher DMI of EFE supplemented cows during early lactation. In the trial 2, we observed a negative energy balance (NEB) in mid-lactation dairy cows (−1.84 and −1.94 MJ/d for TMR control and TMR enzyme, respectively). This is not in agreement with Coffey et al. (2003) who reported a postpartum interval to a positive EB of below 100 DIM. Body weight change of cows was not affected by enzyme treatment. During both trials cows were able to increase BW. These positive BW changes seem not to correspond with our results in EB. This indicates that results of body weight change in our study may not be a valid indicator of energy status in dairy cows.

### 4.2. Dry matter intake and energy balance

This study investigated the supplementation of EFE in corn-grass silage based TMR diets for dairy cows typical for European dairy cow production systems. Exogenous fibrolytic enzymes supplementation for dairy cow diets has been applied with variable success, mainly under North American dairy feeding conditions. Especially in vivo experiments did often fail to show a positive production response by EFE addition (Ortiz-Rodea et al., 2013). In the present study, EFE supplementation did not significantly alter DMI, although EFE supplemented cows had a numerically higher DMI intake in both trials. The result of our study agrees with other studies which observed no effects of EFE supplementation on DMI of dairy cows during different stages of lactation (Arriola et al., 2011; Bernard et al., 2010; Chung et al., 2011; Gado et al., 2009) found increased DMI due to the addition of EFE.

### Table 5

| Item                        | Treatment                  | P-value       |
|-----------------------------|----------------------------|---------------|
|                             | TMR control (n = 13)       | TMR enzyme (n = 13) | TRT | Parity | fdDIM | Trial day | TRT × Trial day |
| DMIm/d                      | 18.9 ± 0.44                | 19.5 ± 0.45 | 0.32 | 0.42 | 0.89 | <0.01 | <0.01 |
| Water intake, L/d          | 75 ± 1.9                   | 74 ± 1.9 | 0.70 | 0.03 | 0.11 | <0.01 | <0.01 |
| Yield, kg/d                 | 30.7 ± 0.32                | 31.2 ± 0.33 | 0.33 | <0.01 | <0.01 | <0.01 | <0.01 |
| Milk yield                  | 4.36 ± 0.16                | 4.23 ± 0.16 | 0.53 | 0.39 | 0.67 | <0.01 | 1.00 |
| Milk fat                   | 3.25 ± 0.04                | 3.28 ± 0.04 | 0.61 | 0.58 | 0.38 | <0.01 | 0.38 |
| Milk lactose               | 4.77 ± 0.03                | 4.81 ± 0.03 | 0.44 | 0.26 | 0.32 | <0.01 | 0.02 |
| FPQ                        | 1.34 ± 0.04                | 1.29 ± 0.04 | 0.36 | 0.36 | 0.90 | <0.01 | 0.96 |
| Urea, mg/L                 | 234 ± 5.2                  | 228 ± 5.3 | 0.35 | <0.01 | 0.95 | <0.01 | 0.01 |
| Energy measure, MJ/d        | 2.90 ± 0.32                | 2.47 ± 0.33 | 0.35 | 0.48 | 0.22 | 0.05 | 0.81 |
| NEL intake                 | 135.3 ± 3.2                | 140.9 ± 3.2 | 0.21 | 0.42 | 0.89 | <0.01 | <0.01 |
| Maintenance requirement    | 37.4 ± 0.78                | 36.7 ± 0.79 | 0.53 | 0.19 | 0.85 | <0.01 | 0.99 |
| Milk energy output         | 99.7 ± 1.0                 | 102.1 ± 1.1 | 0.10 | <0.01 | <0.01 | <0.01 | <0.01 |
| Energy balance             | −1.84 ± 2.9                | −1.94 ± 3.0 | 0.35 | 0.21 | 0.03 | <0.01 | 0.01 |
| GFE, kg FCM/kg DM2         | 1.73 ± 0.45                | 1.72 ± 0.62 | 0.86 | 0.20 | 0.01 | <0.01 | 0.01 |
| BW change, kg/56 d1        | 15.3 ± 3.3                 | 13.3 ± 3.4 | 0.68 | 0.95 | 0.28 | nm | nm |

DMIm = dry matter intake; FCM = fat corrected milk; ECM = energy corrected milk; FPQ = fat-protein-quotient; SCS = somatic cell score; NEL = net energy lactation; GFE = gross feed efficiency; BW = body weight; TMR = total mixed ration; TRT = treatment; fdDIM = the day in milk at the first trial day; nm = not included in the model.

1 least squares means ± SEM (normal distributed data).

2 means ± SD (log-transformed data).

### 4.3. Milk yield, FCM, ECM and milk components

Early and mid-lactation cows of this study showed comparable milk yields. There were no significant effects of EFE on milk yield and FCM in both trials, but EFM tended to increase in mid-lactation cows. These results did not reassure our hypothesis that early lactation cows should be more responsive to an EFE supplementation than mid-lactation cows. Beauchemin and Holtshausen (2010) argued that the stage of lactation appears to be critical for dairy cows in terms of ensuring a response to enzyme additives. While this conclusion is in line with Schingoethe et al. (1999) reporting that cows starting to receive enzyme treated forage during the first 100 days postpartum produced more FCM than cows treated during mid-lactation (> 100 DIM), it is not supported by the results obtained in the current study, where no significant differences were observed for cows in early and mid-lactation.

Increased milk yields or FCM and ECM were published mainly in earlier studies investigating the potential use of EFE (Schingoethe et al., 1999; Yang et al., 1999). In the experiment implemented by Gado et al. (2009) considerable positive effects of EFE are reported (13% greater DMI, 23% greater milk production) in early lactation cows. However, the experimental conditions [milch...
yield 12.8 vs. 15.7 kg/d, protein yield 0.45 to 0.57 kg/d, respectively \((P < 0.05)\) for cows fed the EFE supplemented diet suggest a low yield environment which is not comparable with our study. Dean et al. (2013) observed a decreased milk yield for cows fed with a low EFE supplementation \((1.3 \text{ mL EFE/kg DM})\) applied to concentrate as compared to cows fed the control diet \((P = 0.10)\). These findings indicate a high variability of EFE production responses. However, our results agree with the findings of a recent meta-analysis by Ortiz-Rodea et al. (2013), who evaluated the effect of the addition of EFE in ruminant feeding on milk production and chemical composition \((29\) experiments including \(52\) treatments, \(9\) enzymes, and \(1,187\) animals) and found no increment in milk yield \((P = 0.16)\) due to enzyme addition. Under conditions of a low DMI, as observed in this study, we suggest that the amount of rumen fibrolytic enzymes of the rumen microorganisms and the rumen retention time for NDF should not be limiting factors for fiber digestion which could be an explanation for the lack of production response in both trials. This corresponds with an earlier experiment (Peters et al., 2010), which reported that the EFE (Roxazyme G2 Liquid) supplementation did not affect apparent ruminal and total tract digestibility of DM, OM, NDF and ADF in diets for dairy cows under restricted feeding conditions.

We hypothesized that EFE supplementation improves fiber degradation and energy status, which could influence milk composition. In the present study, no significant effects on milk fat and protein were found. Lack of difference in milk fat content between the treatment groups in both trials is probably related to the absence of improved fiber digestion due to the EFE supplementation. Dietary differences in milk composition are generally reflective of differences in ruminal fermentation patterns. In an earlier study (Peters et al., 2010), we observed no effect of EFE addition on ruminal fermentation using the same EFE product, comparable dosage and the same basal diet. Due to the high energy requirement for protein synthesis, the milk protein yield can be affected by the energy content in the diet (Reynolds et al., 1994). Therefore, the lack of treatment effect on energy intake in both trials of our study is consistent with the absence of differences in milk protein content between cows fed control and enzyme TMR. The results of the our study agree with results of a meta-analysis by Ortiz-Rodea et al. (2013), which showed no effects of EFE addition on milk components \((\text{fat content, } P = 0.88; \text{lactose, } P = 0.39; \text{protein, } P = 0.95)\). In the present study, early lactation cows fed TRM enzyme showed a tendency of a higher milk lactose percentage compared with the TMR control. A significant increase in lactose content was reported by Yang et al. (1999), who explained this effect due to the higher nutrient availability as a result of digestibility improvements caused by EFE application. In our digestibility trial with wethers we did not find any improved digestibility due to EFE treatment. Elwakeel et al. (2007) observed higher lactose content in EFE treated cows, though with a small magnitude of response, while Bowman et al. (2002) found a decrease milk lactose percentage in dependency to a specific EFE application mode. The biological reasons for varying lactose concentrations are not obvious and may relate to specific conditions of the experiment and the statistical analysis since lactose content is rather very constant.

In our study, the fat-protein-quotient (FPQ) of 1.3, with minor differences between trials and treatment, is within the range of reported reference values (H. rle and Sundrum, 2013). During the

Fig. 1. (A,B) Dry matter intake (DMI) and (B,C) milk yield over time by dairy cows fed the control or the enzyme supplemented total mixed ration (TMR) in trials 1 and 2. The values shown are treatment by trial day LSmeans; the SEM for DMI in trials 1 and 2 averaged 0.81 and 0.89 kg, respectively; the SEM for milk yield in trials 1 and 2 averaged 2.26 and 1.04 kg, respectively.
early lactation trial FPQ showed a typical depression while in the mid-lactation trial FPQ values were comparable.

The observed milk urea content found in this study is within the range of 150 and 300 mg/L milk expected in a diet with a balanced energy and protein supply (Kirchgessner et al., 1986). The EFE supplementation did not alter content of milk urea in both trials. In the early lactation cows the milk urea level was around 190 mL/L and in mid-lactation cows around 230 mL/L. Both levels indicate a sufficient ruminal protein and energy supply.

### 4.4. Gross feed efficiency (GFE)

Dairy efficiency defined as yield of milk per unit of dietary DM consumed provides a readily calculated measure of dairy herd productivity. Feed additives of the group digestibility enhancer are expected to increase fiber degradation, leading to increased DMI and milk production. Increased DMI without yield response may indicate a sufficient ruminal protein and energy supply. 

#### 4.5. Ruminating activities

Rumination activities are determined by particle length of forage and are associated with saliva production; coarse particles stimulate chewing activity and increase saliva output which adds to bicarbonate and phosphate buffers required for an optimal rumen function (Beauchemin et al., 2003; Krause et al., 2002). Neutral detergent fiber intake influences ruminating time and EFE supplementation is supposed to reduce ruminating time. Ruminating activities measured in our study did not show treatment effects ruminating time and EFE supplementation did not have a significant effect on rumination time.

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### Table 6

**Blood parameters of cows fed the control or the enzyme supplemented total mixed ration.**

| Item                  | Treatment                  | P-value | Parity | fDIM | Trial day | TRT × Trial day |
|-----------------------|----------------------------|---------|--------|------|-----------|-----------------|
|                       | TMR control (n = 14)       |         |        |      |           |                 |
|                       | TMR enzyme (n = 14)        |         |        |      |           |                 |
| BHB, mmol/L<sup>1</sup> | 0.67 ± 0.417              | 0.56 ± 0.238 | 0.91  | < 0.01 | < 0.01   | < 0.01          |
| Total protein, g/L<sup>1</sup> | 70 ± 73                  | 72 ± 0.81      | 0.10  | 0.07  | 0.20      | < 0.01          |
| TB, μmol/L<sup>1</sup>    | 2.9 ± 0.14                | 2.7 ± 0.16    | 0.37  | 0.03  | 0.09      | < 0.01          |
| AST, U/L<sup>2</sup>      | 61.5 ± 12.1               | 69.8 ± 15.8   | 0.16  | < 0.01 | 0.01      | 0.05            |
| GGT, U/L<sup>2</sup>      | 24.9 ± 7.6                | 29.5 ± 10.6   | 0.24  | 0.96  | 0.74      | < 0.01          |

### Table 7

**Ruminating activity of cows fed the control or the enzyme supplemented total mixed ration trial 2 (LSmeans ± SEM).**

| Ruminating activity | Treatment                  | P-value | Parity | fDIM | Trial day | TRT × Trial day |
|---------------------|----------------------------|---------|--------|------|-----------|-----------------|
|                     | TMR control (n = 13)       |         |        |      |           |                 |
|                     | TMR Enzyme (n = 13)        |         |        |      |           |                 |
| Min/d               | 540 ± 9.7                  | 550 ± 10.0 | 0.44  | 0.42  | < 0.01   | 0.67            |
| Min/kg of DMI       | 29 ± 0.89                  | 28 ± 0.90 | 0.58  | 0.89  | 0.01     | 0.75            |
| Min/kg of NDFI      | 65 ± 2.0                   | 64 ± 2.0  | 0.58  | 0.89  | < 0.01   | 0.55            |

In this study, EFE supplementation did not have a significant effect on feed efficiency (FCE/kg DMI, respectively). In contrast to our results Holtshausen et al. (2011) found a significant improved GFE (P = 0.02; 1.50 vs. 1.67 kg FCM/kg DMI) in EFE supplemented early lactating cows as a result of a significant lower DMI, while Arriola et al. (2011) reported a numeric decrease in DMI and increase in milk yield in early lactation cows causing a significant improved GFE (P = 0.04; 1.44 vs. 1.60 kg FCM/kg DMI). These positive results are based on short term experiments (60 and 70 days) and it would be beneficial to evaluate EFE effects throughout an entire lactation period including health and fertility traits. A negative effect of EFE supplementation on GFE (P = 0.08; 1.64 vs. 1.46 kg milk/kg DMI) was found by Dean et al. (2013), whereas other studies report no differences between diets (Bernard et al., 2010; Elwakeel et al., 2007). Results of available studies do not give a clear indication of EFE effects on GFE, mainly due to high variability in DMI and milk production response. The range in GFE can be quite large. The rather high GFE found in the early lactation trial of our study (TMR-control 1.93 ± 1.08 and TMR-enzyme: 1.89 ± 0.81 kg milk/kg DMI) is presumably mainly due to a low level of DMI. We observed in early lactation cows a NEB without a loss of BW which suggests that any mobilization of body tissue may have rather been associated with the change in the fat: protein ratio of body mass.

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1 Least squares means ± SEM (normal distributed data).
2 Means ± SD (log-transformed data).
differences and fall within the higher end of the range reported by Weigand et al. (1993) and Beauchemin (1991), indicating adequate amounts of dietary physically effective fiber of the TMR. In agreement with our results, EFE supplementation had no effect on rumination activity even when adjusted for DM, NDF or ADF intake in studies by Beauchemin et al. (2000) and Bowman et al. (2003).

4.6. Blood parameters

Blood indicators are used as a tool to diagnose energy balance and hepatic functions. The analysis of EFE effects on blood parameter was restricted due to the rather small number of experimental animals involved. All blood parameter tested were within a normal physiological reference range (Kraft and Dürre, 2005). In this study supplementation of EFE had no significant effect on DMI and energy balance in both trials and; thus, no treatment effects on serum BHB, total protein, total bilirubin and hepatic enzymes (AST, GGT) were observed. These observations are not in agreement with results reported by Holtshausen et al. (2011) and Dean et al. (2013), who observed that the EFE treatment decreased plasma BHB concentration, indicating a positive effect of EFE on body fat mobilization in early and mid-lactation cows. In conclusion one can state, that several blood indicators need to be investigated to detect hepatic disorders as done in this study, but EFE treatments must show marked effects on energy balance to observe differences in blood parameter.

5. Conclusion

The Roxazyme G2 Liquid enzyme applied to a TMR at a rate of 3.9 and 3.8 mL/kg TMR DM prior to feeding of dairy cows in early and mid-lactation did not significantly enhance intake or performance in the current trials. In mid-lactation cows enzyme treatment led to a tendency for a higher ECM yield, but this finding is not sufficient to indicate a beneficial production response to EFE.

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