Effects of an exogenous fibrolytic enzyme additive on ruminal fermentation, nutrient digestion, performance and health status in early and mid-lactation dairy cows

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ABBREVIATIONS

ADF	Acid detergent fiber
ADG	Average daily gain
AIA	Acid insoluble ash
Al_2O_3	Aluminium oxide
AST	Aspartate-amino transferase
ATP	Adenosine triphosphate
a.p.	Ante partum
BCS	Body condition score
BHB	β-Hydroxybutyrate
BW	Body weight
C2	Acetate
C3	Propionate
C4	Butyrate
$C_6H_{12}O_6$	Glucose
CBM	carbohydrate-binding module
CMCase	Endoglucanase
CH_4	Methane
CP	Crude protein
Cr_2O_3	Chromium(III)oxide
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
EB	Energy balance
ECM	Energy corrected milk
EE	Ether extract
EFE	Exogenous fibrolytic enzymes
EFSA	European Food Safety
FADH	Flavin adenine dinucleotide hydride
FCM	Fat corrected milk
FEQ	fat-protein-quotient
FLI	Friedrich-Loeffler-Institute
FOM	Fermented organic matter
GE	Gross energy
GGT	Gamma glutamyl transferase
GfE	German Society of Nutrition Physiology
GFE	Gross feed efficiency
LSmeans	Least square means

ME	Metabolizable energy
NADH	Nicotinamide adenine dinucleotide hydride
NADPH	Nicotinamide adenine dinucleotide phosphate
NAN	Non-ammonia-N
NDF	Neutral detergent fiber
NEB	Negative energy balance
NEL	Net energy lactation
NIRS	Near infrared spectra
NH_3	Ammonia
nXP	Utilisable crude protein at the duodenum = uCP
OM	Organic matter
p.p.	Post partum
RNB	Ruminal nitrogen balance
SCFA	Short chain fatty acids = VFA
SD	Standard deviation
SEM (SE)	Standard error of the mean
SCC	Somatic cell count
SCS	Somatic cell score
TB	Total bilirubin
TMR	Total mixed ration
uCP	Utilisable crude protein at the duodenum = nXP
UDP	Rumen undegradable crude protein
VFA	Volatile fatty acids = SCFA

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General discussion

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General Introduction

Figure 1. Simplified scheme of potential modes of action of exogenous fibrolytic enzymes (EFE) in ruminants

1 General Introduction

1.1 Background

Given the ever decreasing natural resource base and the increasing demand for animal source food (ASF) in the world challenges in animal nutrition are changing. In the past the most important aim was to balance the digestive physiological needs of farm animals with the properties of available feed stuffs. Animal feeding demands and cost efficient feeding strategies were the main focus, resulting in the use of high quality feeds also useable for human needs, elimination of anti-nutritional factors, and the balancing of nutrient composition through supplementations. Sustainability concerns encompassing environmental externalities and especially societal concerns, such as food security and safety, were imposed more recently and changed the aims and objectives in animal nutrition constantly. With steadily increasing world human population and the dramatic reduction of available arable land, agriculture systems are under increasing pressure to produce more human food with higher efficiency. Under this reality the competition between human food needs, animal feed supply and fuel production from high quality biomass causes a reduction of the available feed base and increases feeding costs.

Ruminants have a specific role in securing global food supply due to their ability to digest fibre-rich and human inedible biomass into human edible food of high nutritive value. This is of relevance in pasture based systems of the world but also in high yielding arable forage production systems, where ruminants are converter of forage based products and their residues and by-products of lower quality. The growing importance of biodiversity, of global warming, and of animal welfare, leads to increasing competition of high-quality forage with more acceptable and economically efficient land use alternatives, especially in temperate regions of Europe, and raises the challenge for optimizing the proportion of forage, reducing the cereal proportion in diets for high yielding dairy cattle.

This shift in paradigm forces the adaptation of digestive physiological properties to the specific features of the forage based ration with the aim of expanding the efficiency of forage biomass utilization. In this context the application of exogenous enzymes, better known for its use to improve the nutritive value of diets for monogastric animals, are also of interest to be explored in ruminant nutrition.

During the past two decades different types of enzymes, such as protease, amylase and cellulases have been tested for their potential ability to improve the digestibility of nutrients and metabolizability of energy (Eun and Beauchemin, 2005; Klingerman et al., 2009; Beauchemin et al., 2004a; Beauchemin and Holthausen, 2010). The main focus was directed towards the application of exogenous fibrolytic enzymes (EFE) increasing the efficiency with which the microbiota of the rumen degrades plant cell wall substrate and digestible energy intake, but responses have been inconsistent (Meale et al., 2014). Given the foremost importance of microbial cell wall degradation this process will briefly be dealt with in the next sub-chapter.

1.2 Microbial degradation of complex carbohydrates

The rumen microbial ecosystem, as a result of evolutionary development, is extremely complex; thus, it is obvious that here we can only deal with selected aspects of cell wall degradation in the rumen to help understand exogenous fibrolytic enzyme effects on rumen fermentation, fibre digestion and animal response.

Almost half of the global carbon fixed annually by photosynthesis is incorporated into plant cell walls (refer to Box 1) making it the largest renewable carbon source on earth (Krause et al., 2003). Cellulose is the most abundant form of fixed carbon, with 10^{11} tons produced in cell walls by plants each year Wilson (2008). The carbon cycle is closed primarily as a result of action of cellulose-utilizing microorganisms present in soil and the fore and hind gut of animals (Lynd et al., 2002). The half-life of cellulose at neutral pH in the absence of enzymes is estimated to be several million years. It requires microbial activity for most of the degradation of carbon into cellulose (Falkowski et al., 2000 in Wilson, 2011).

Box 1: Plant cell walls

Plant cell walls are composed predominantly of the polysaccharides cellulose, hemicellulose, and pectin (Gilbert, 2010). Secondary cell walls contain structural polysaccharides, strengthened further with polymeric lignin covalently cross-linked to hemicellulose (Himmel et al., 2010). The recalcitrance of plant biomass to degradation is a function of how polymers crosslink and aggregate within walls (McCann and Carpita, 2008). Cell walls are chemically characterized as insoluble in neutral detergent and hence are called neutral detergent fibre (NDF). This NDF is considered to consist of cellulose, hemicellulose, lignin and a small amount of nitrogen-containing material (Bannink and Tamminga, 2005).

Cellulose is a linear polymeric chain, consisting solely of ß-(1.4)-linked D-glucose residues that are condensed by hydrogen bonds into crystalline structures, called microfibrils. Their main function is to ensure the rigidity of the plant cell wall (De Vries and Visser, 2001). *Hemicellulose* is a cross-linking glycan that constitutes up to 30% of plant cell walls; the two major hemicelluloses are Xyloglucan and glucuronoarabinoxylan (Flint et al., 2008). Hemicellulose chains are thought to interact with more than one cellulose fibril so that they form non-covalent cross-links between cellulose bundles (Davison et al., 2013). In the bovine rumen approximately 36–79% of the ingested xylan is degraded (Van Soest, 1994). *Pectins* are most abundant in the plant primary cell walls and the middle lamellae, are a class of molecules defined by the presence of galacturonic acid (Mohnen and Caffal, 2009). Pectin is structurally and functionally the most complex polysaccharide in plant cell walls (Mohnen, 2008). There are three major forms of pectin: homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II (for review, see Mohnen, 2008). Pectin making up 35% of primary walls in dicots and non-graminaceous monocots, 2–10 % of grass (Mohnen, 2008). *Lignin* the final main structural polymer, is a complex polyphenolic polymer that partially encases the plant cell wall polysaccharides and cellulose micro fibrils in lignified (i.e., secondary) plant cell walls (Davison et al., 2013). *Aromatic compounds* are thought to play an important role in the structure and function (resistance to pathogens) of the plant cell wall. Ester-linked *p*-coumaric and ferulic acid can be linked to both the hemicellulose and the pectin fractions and is able to cross link these polysaccharides to each other as well as to the aromatic compound lignin (Mathew and Abraham, 2004).

Cellulose hydrolysis can occur under both aerobic and anaerobic conditions. In nature, three dominant microbial paradigms for enzymatic breakdown of plant cell walls have been found. Free enzymes, multifunctional enzymes and multi-enzyme complexes (cellulosomes) are common configurations of microbial cellulase systems (Moraïs et al., 2012). While aerobic microorganisms hydrolyze the cellulose by the secretion of free, soluble enzymes into their surroundings, some anaerobic bacteria have evolved a supramolecular enzymatic complex, termed the cellulosome (Box 2) (Karpol et al. 2013). The division into distinct paradigms is not necessarily strict. Microbes capable of using lignocellulosic substrates and perhaps the bacteria in particular, must employ specific 'intelligent' strategies to survive in often extreme environments (Himmel et al., 2010). The benefits of each strategy have been intensively researched but are still not quantified. Recent studies compared free enzymatic and cellulosomal systems and suggest advantages of cellulosomal system in deconstructing insoluble cellulosic substrates compared to free enzyme systems (Moraïs et al., 2012).

Box 2: Cellulosomes

Cellulosomes were discovered in 1983 from *Clostridium thermocellum* (Lahmed et al., 1983). A cellulosome is a discrete multicomponent, multienzyme complex of anaerobic cellulolytic bacteria and provides enhanced synergistic activity among the different resident enzymes to efficiently hydrolyze intractable cellulosic and hemicellulosic substrates of the plant cell wall (Bayer et al., 2004). The cellulosome consists of a multi-functional integrating subunit (called scaffoldin), responsible for organizing the various cellulolytic subunits (e.g., the enzymes) into the complex. Within a cellulosome, multiple endoglucanases, cellobiohydrolases, xylanases and other degradative enzymes work synergistically to attack heterogeneous, insoluble cellulose substrates. This is accomplished by the interaction of two complementary classes of modules, located on the two separate types of interacting subunits, i.e., a cohesin module on the scaffoldin and a dockerin module on each enzymatic subunit. The high-affinity cohesin-dockerin interaction defines the cellulosome structure. Attachment of the cellulosome to its substrate is mediated by a scaffoldin-borne carbohydrate-binding module (CBM) that comprises part of the scaffoldin subunit (http://wws.weizmann.ac.il/Biological_Chemistry/scientist/Bayer; Dassa et al., 2014).

The cellulases of cellulolytic bacteria and fungi hydrolyze the same type of bond of the cellulose chain (i.e., the b-[1.4]-glucosidic bond), albeit using different cellulase systems. The synergistic actions of three distinct classes of enzymes are involved in the enzymatic degradation of cellulose to glucose (Himmel et al., 2010):

1. The endo-ß-(1.4)-glucanases, which act randomly on soluble and insoluble ß-(1,4)-glucan substrates and are commonly measured by detecting the reducing groups released from carboxymethylcellulose;
2. The exo-ß-(1.4)-d-glucanases, including both the ß-(1,4)-d-glucan glucohydrolases, which liberate d-glucose from ß-(1,4)d-glucans and hydrolyze d-cellobiose slowly, and ß-(1,4)-d-

glucan cellobiohydrolase, which liberates d-cellobiose in a 'processive' manner (successive cleavage of product) from ß-(1,4)-glucans.

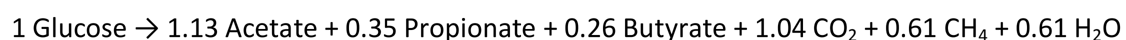
3. The ß-d-glucosidases, which act to release d-glucose units from cellobiose and soluble cellodextrins, as well as an array of glycosides. The above classification scheme is not entirely rigid and a few enzymes have properties that do not fit one of the above definitions.

Products of the hydrolysis are monomers (e.g. hexoses and pentoses) used in the intra-cellular glycolysis, also known as EMP (Embden–Meyerhof–Parnas-pathway) can occur both anaerobically (leading to one or several fermentation pathways) and aerobically through the conversion of pyruvate, but with different ATP forming output. In aerobic systems prokaryotes produce 26 mol ATP/mol glucose metabolized and eukaryotes 38 mol/mol glucose. In anaerobic systems is the energy exploitation drastically reduced to 2 ATP/mol glucose.

1.3 Ruminal cell wall degradation

The ruminal fermentation can be described as an incomplete anaerobic digestion in which plant material is converted to a mixture of C2 to C6 volatile fatty acids (VFAs), of which some are produced via intermediates such as succinic and lactic acids (France and Dijkstra, 2005). Microbial fermentation of fibre comprises several sequential actions: Hydration, adherence of the appropriate microorganisms, release of a mixture of hydrolytic enzymes and finally hydrolysis itself. The resulting release of monomers is followed by their further intracellular degradation into VFA and fermentation gases (Dijkstra et al., 2005). Several factors influence the fermentation characteristics of the NDF (Box 1) in forage, such as stage of maturity, growing season affecting the chemical composition of forages, including extent of lignification of NDF and degradation characteristics (Dijkstra et al., 2005).
The VFAs are used by the host as its primary energy source. The ruminal microflora does also produce other metabolic products such as methane, carbon dioxide and microbial protein, the latter being digested (Suen et al., 2011). Substrate utilized by microbes is partitioned between fermentation end-products (mainly VFA) and microbial biomass formation. Thus an increase in efficiency of microbial protein synthesis (EMPS) usually coincides with a decrease in VFA produced per gram of carbohydrate degraded, which reduces the supply of non-protein energy to the animal (Dijkstra et al., 2007).

The overall fermentation equation for an animal consuming a high roughage diet is according to McSweeney and Mackie (2012):



$$1 \text{ Glucose} \rightarrow 1.13 \text{ Acetate} + 0.35 \text{ Propionate} + 0.26 \text{ Butyrate} + 1.04\ CO_2 + 0.61\ CH_4 + 0.61\ H_2O$$

The molar proportions in which the principal volatile fatty acids are formed in the rumen are (60–70%) acetic, (15-20%) propionic, (10-15%) butyric acid. Higher and branched-chain volatile fatty acids are also produced in small quantities (Breves and Lebzien, 2009).

The actors of the rumen fermentation involve complex microbial communities. Their composition was demonstrated in a recent metagenomics study by Brulc et al. (2009) in which approximately 95% of the coding sequences were bacteria, 0.6-4% archaea, and 1.5% eukaryotic microorganisms (anaerobic fungi and protozoa). The most numerous groups of rumen microorganisms are non-celluloytic bacteria, many of which possess the ability to grow on soluble polysaccharides that are released by the primary degraders (Flint et al., 2012). Bacteria and fungi produce a wide range of highly active plant fiber degrading enzymes. The contribution of protozoa to plant fiber digestion is estimated to be less significant in terms of their contribution to total NDF degradation (Dijkstra and Tamminga, 1995 cited in Koike and Kobayashi, 2009). Although rumen fungi possess superior ability to penetrate the plant cell wall and solubilize lignin, their share in fiber digestion may be low due to their small biomass proportion (Koike and Kobayashi, 2009).

The composition of microbial communities changes dynamically in relation to the dietary composition (Fernando et al., 2010). According to a study by Belanche et al. (2012) cows adapted themselves to fibrous diets by increasing the complexity of the rumen microbial community and the rumen concentrations of protozoa, anaerobic fungi, and methanogens. On the other hand, the reduction in the dietary protein level from 110 to 80% of the N requirements resulted in a substantial decrease in the rumen microbial diversity and the ruminal abundance of total bacteria, anaerobic fungi, methanogens, and most of the cellulolytic bacteria considered.

The Gram-negative *Fibrobacter succinogenes* and the Gram-positve bacteria *Ruminococcus albus* and *Ruminococcus flavefaciens* are the major fibrolytic bacteria, and possess contrasting fibrolytic enzyme systems. *Butyrivbrio fibrisolvens* are a group of highly xylanolic Gram-positive bacteria inhabiting the rumen, which have a central role in fiber digestion (Krause et al., 2003). *Prevotella* species are considered as secondary fibrolytic species, such as *P. ruminicola* and *P. bryantii*. They are not able to breakdown the cellulose polymer but contribute to the breakdown of plant protein and hemicellulose due to their high carboxymethylcellulose-, xylan- and pectin-degrading activities and probably play an important role in overall fibre digestion (Dodd et al., 2011).

The functional importance and the abundance of a species in the rumen are not always correlated (Lin et al., 1997). Specialist cellulolytic bacteria are a clear example. Collectively, major ruminal cellulolytic specialists (*R. albus*, *R. flavefaciens*, and *Fibrobacter succinogenes*) are found 3 h after feeding to represent only 0.3 to 3.9% of the total bacterial RNA, *R. albus* was generally the most abundant of the three species. (Weimer et al., 1999). However, despite their low abundance, the resulting degradation of cellulose is fundamental to ruminal function (Brulc et al., 2011).

Flint et al. (2012) and Dassa et al. (2014) describe *Ruminococcus flavefaciens* as the only gut bacterium so far known to produce a cellulosome-type enzyme complex. *Ruminococcus albus* is a primary cellulose degrader that produces acetate. The mechanism of cellulose degradation by *R. albus* is not well-defined. *R.albus7* does not use classical cellulosomal components to degrade cellulose, but appears to follow an alternative strategy (Christopherson et al., 2014; Dassa et al., 2014).

Fibrobacter strains isolated from the rumen are typically called succinogenes, but there are two recognized species identified through their different 16S rRNA gene sequences, the *F. succinogenes* S85 and *F. intestinalis* NR9 (Russel et al., 2009). *Fibrobacter succinogenes* is an important strict anaerobe member of the rumen microbial community that converts plant biomass into mixture of succinate, acetate and formate as fermentation end products. However, unlike other known cellulolytic microbes, it does not degrade cellulose using a cellulosome or by producing high extracellular titers of cellulase enzymes. Polysaccharide hydrolysis and utilization assays showed that *F. succinogenes* was able to hydrolyze a number of polysaccharides, but could only utilize the hydrolytic products of cellulose (Suen et al., 2011).

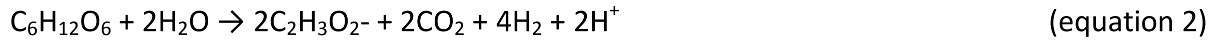
Cross-feeding of fermentation products and of oligomers and monomers is a central feature in anaerobic microbial communities that involves products of fermentation such as hydrogen and lactate as well as partial substrate degradation products (Flint et al., 2012; Koike and Kobayashi, 2009). A relevant example is the interaction between proteolytic and cellulolytic bacteria, the former releasing ammonia, used as preferential nitrogen source for the latter, and the latter releasing soluble sugars from cellulolysis, which are metabolized by proteolytic bacteria (Chaucheyras-Durand et al., 2012). Another example of interactions is the interspecies H_2 transfer between H_2 producing cellulolytics and H_2 utilizing methanogens as H_2 reducer, being fundamental in the functioning of the rumen (Wolin et al., 1997 cited in Morgavi et al., 2010). Without the removal of hydrogen, re-oxidation of reduced cofactors (NADH, NADPH and FADH) is inhibited by the accumulated hydrogen and decreasing the production of VFA. This is the reason why methanogenesis is intimately linked to degradation of plant fibre in the rumen. In this process Carbon dioxide is reduced to methane in the fermentation system (equation 1), and the methane in gaseous form subsequently dissipates from the system shown in equation 1 (Zijderveld 2011).

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \text{(equation 1)}$$

But it is of interest to note, that not all members of the rumen fibrolytic community produce H_2. Increasing the proportion of non-H_2 producing fibrolytic microorganisms (such as *Fibrobacter succinogenes*) might decrease methane production without affecting forage degradability (Morgavi et al., 2010).

Methane production per unit of fermented feed is proportional to the pattern of the VFA produced (acetate, propionate and butyrate). During the production of acetate (equation 2)

and butyrate (equation 4), hydrogen is produced, while the production of propionate (equation 3) leads to a net uptake of hydrogen. Thus, a higher proportion of propionate in the VFA-profile results in reduced methane production (Ellis et al., 2008 cited in Zijderveld 2011).



$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_3O_2- + 2CO_2 + 4H_2 + 2H^+$ (equation 2)

$C_6H_{12}O_6 + 2H_2 \rightarrow 2C_3H_5O_2 + 2H^+ + 2H_2O$ (equation 3)

$C_6H_{12}O_6 \rightarrow C_4H_7O_2 + H^+ + 2H_2 + 2CO_2$ (equation 4)

Another substantial function associated with cell wall degradation is the necessity of adhesion of rumen bacteria to feed particles, which is a common requirement for rapid and efficient cellulose hydrolysis (Lynd et al., 2002). Miron et al. (2001) demonstrated that adhesion-defective mutants of *F. succinogenes* S85 show little or no capacity to degrade cellulose. Polysaccharide-degrading enzymes secreted into the liquid fraction are at risk of inactivation by proteolysis or of being washed out of rumen before they contact their substrates (Wang and McAllister, 2002). Thus, attachments to feed particles are the most efficient way for microbes to prolong their residence in the rumen and to bring their enzymes into contact with substrates. As reported by Miron et al. (2001) microbial populations associated with feed particles are estimated to be responsible for 88 to 91% of ruminal endoglucanase and xylanase, 70 % of the amylase, and 75 % of the protease activity in the rumen, respectively. The study of Brulc et al. (2009) concluded that initial colonization of fiber appears to be a dynamic process, starting by organisms with enzymes that attack the easily available side chains of complex plant polysaccharides which are probably later replaced by another subset of organisms that degrade the main chains of cellulose and xylan. The initial process of colonization including hydration of substrate, removal of digestion inhibitors, or attachment or close association of microorganisms with the substrate, is part of the digestion kinetics and determined as lag phenomena in mathematical modelling of rumen fermentation processes (Huthanen et al., 2006; Mertens, 2005).

Knowledge of fibrolytic mechanisms employed by the specific rumen bacteria is of great importance for manipulation of ruminant’s diet and for improvement of its performance (Dassa et al., 2014).

1.4 Using EFE to promote cell wall digestion in dairy cows

Extensive reviews on the use of exogenous enzymes for ruminants and the potential mode of action are available (Beauchemin et al., 2004a; Beauchemin and Holthausen, 2010; McAllister et al., 2001; Meale et al., 2014; Wang and McAllister, 2002). No attempt will be made to duplicate these efforts in this thesis, but the main outcomes of these reviews are briefly depicted.

Initially, exogenous enzymes were primarily applied as feed additives in diet formulations for poultry and pigs to remove anti-nutritional factors from feeds, to increase the digestibility of existing nutrients, and to supplement the activity of the endogenous enzymes (Adeola and Cowieson, 2011).

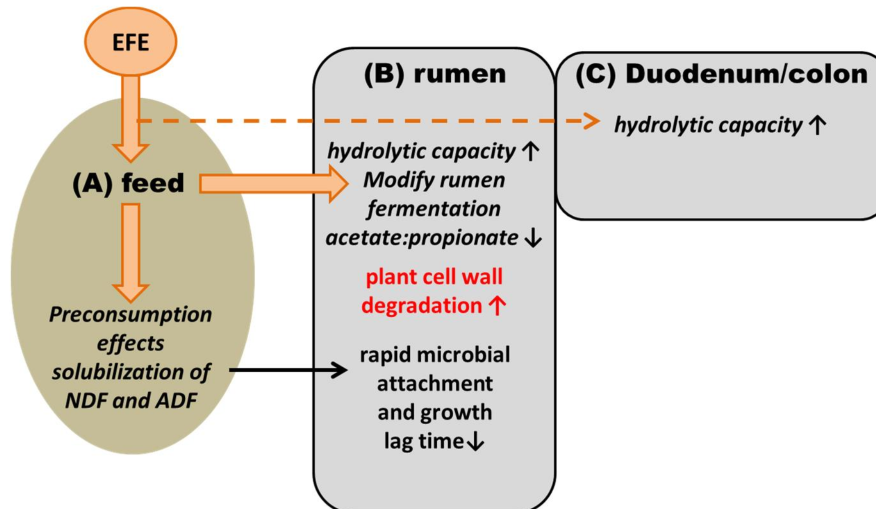


Figure 1. Simplified scheme of potential modes of action of exogenous fibrolytic enzymes (EFE) in ruminants. (A) Effects prior to consumption: Removal of certain plant cell wall polysaccharides by EFE improving the accessibility for the ruminal cellulolytic microorganism; the release of soluble sugars by EFE would provide sufficient additional available carbohydrates to encourage rapid microbial growth, shortening the lag time required for microbial colonization increasing the initial rate but not the extent of microbial cell wall digestion causes in a higher rate of digesta passage and increasing DMI. (B) Direct rumen effects of EFE: provided enzymes are resistant to proteolysis, EFE be able to hydrolyze cell wall polysaccharides directly or act synergistically with rumen microorganism increasing the extent of cell wall digestion; lower acetate:propionate ratio in rumen fluid by EFE indicate a lower availability of metabolic H_2 for methanogenesis. (C) Postruminal effects: Increasing the hindgut fermentation of cell wall polysaccharides. (Morgavi et al., 2000; McAllister et al., 2001; Beauchemin et al., 2004a; Chung et al., 2012; Meale et al., 2014)

The use of exogenous fiber-degrading enzyme additives for ruminants was first examined in the 1960s (Beauchemin et al., 2003) but extensively researched only over the last 15 years. Feed enzymes for ruminants contain mainly cellulases and hemicellulases activities and are of fungal (mostly *Trichoderma longibrachiatum*, *Aspergillus niger*, *A. oryzae*) and bacterial (*Bacillus* spp.) origin (Beauchemin et al., 2004b). The types of cellulases and hemicellulases can differ substantially among commercial enzyme products depending on the source organism and how that organism is grown (Beauchemin and Holtausen, 2010).

Commercial use of EFE in dairy and beef cattle diets is still very limited, although increasing feed costs and declining enzyme costs continue to fuel research efforts to develop and evaluate ruminant enzyme additives. In the EU its commercial use for ruminants is officially not approved. However, the global enzyme market was estimated in 2013 to reach a

monetary value of 4.3 Mrd € (Miloš, 2014) and is dominated by Novozymes, DuPont and DSM accounting for over 70% of the market in 2013.

The ultimate aim of using feed enzymes is to improve feed intake, weight gain, milk yield, and feed efficiency. The inefficiency of nutrient utilization can result in an increase of the diet quantity needed to maintain required levels of animal performance which can subsequently increase the feeding cost and also the environmental pollution due to increased waste.

Potential benefits of using EFE in ruminants are:

1. Improved efficiency of utilization of dietary cell wall components in feedstuffs.
2. Reducing the grain level in the ration decreasing feed costs.
3. The use of feeds with lower digestibility (crop residues, high-fiber byproducts, low quality pastures) without compromising energy intake or animal performance.
4. Higher milk fat concentration due to the increased cell wall degradation.
5. Reduction of metabolic disorders.
6. Minimization of waste, i.e. improving digestion and absorption of nutrients, reducing the amount of manure produced and lowering the nutrition excretion.
7. Reducing greenhouse gas emission.

In the European dairy sector the potential use of EFE is mainly focused on high yielding dairy cows under intensive feeding systems. Major prospects of EFE application are the improvement of feed conversion efficiency and sustainable performance.

Recent studies on the use of EFE in dairy cow feeding have shown improved FCM production efficiency for early lactation dairy cows of 11,3% depending on dosage (Holthausen et al., 2010). Ariola et al (2011) found an increase in efficiency of milk production of 11%, based on 4 cows in a Latin square design, and also concluded that a reduction of concentrate in the diet was overcompensated by EFE supplementation. Other (Bernhard et al., 2010; Dean et al., 2013) found no effects of EFE application prior feeding on intake or performance of dairy cows. Research results on the EFE supplementation are not yet consistent.

1.5 Aim and outline of this thesis

Aim of this Thesis

Improvement of the cell wall digestion in high yielding dairy cows becomes increasingly important, not only from a production economical perspective, but also due to the large interest to increase feed conversion efficiency, to reduce unfavorable condition for animal health (physiological disorders), and to contribute to environmental sustainability. Supplementation of EFE for improving cell wall degradation in the rumen in dairy cows could be a strategy to achieve the above mentions challenges. The current state of research evidence about the prospects of EFE application shows, however, a high degree of variability and a low repeatability. Possible reasons for the divergent results could be related to type of

enzyme product, form of application of EFE, different forage component in the ration, different composition of diet, variable performance level and lactation status of cows, and differences in experimental designs. The available literatures on the use of EFE applications in dairy systems are typical for dairy systems in North America. Under European conditions no experiments have been conducted.

The general aim of this thesis therefore was to identify and quantify the effects of EFE on digestibility and performance of high yielding dairy cows in a typical European dairy feeding system. Furthermore, potential positive or negative effects of EFE on animal health were investigated.

The specific objectives are to answer the following questions:

- Does the selected EFE product lead to an increased digestibility of nutrients of a TMR based on corn silage and grass silage?
- Can we observe a change in the ruminal fermentation pattern due to the enzyme supplementation?
- Does the EFE product influence dry matter intake, performance and animal health of dairy cows in early and mid-lactation?

Thesis Outline

This thesis comprises the results of a literature review, three experiments and a general discussion. In Chapter 2 literature is reviewed regarding the potential mode of action of EFE, the effect of EFE on ruminal fermentation and on *in vivo* digestibility, dry matter intake and milk performance of dairy cows. Chapter 3 presents results on Rumen fermentation, ruminal N metabolism, and ruminal and total digestive tract digestibility in dairy cows. In this study the method of restricted feeding was practiced to avoid feed residues, which prohibits measuring the effect of EFE on DMI and performance. The results of dairy feeding experiments are compiled in Chapter 4. Thirty-three dairy cows in early lactation and 31 cows in mid lactation, fed the similar ration, were exposed to an EFE supplementation, using the same enzyme product and dosage, over a treatment period of 56 days following a 20 days adaptation phase. Data were obtained for Dry matter intake, energy intake based results from the digestibility trials with wethers, milk yield and components, milk energy concentration, calculated energy balance, and selected blood parameter as indicators for metabolic disorders. During second trial rumination activity data were observed to evaluate a possible effect of EFE on the physical feed structure. Finally, chapter 5 discusses findings of this study in the context of the available literature, and reflects on the prospects of EFE application for dairy cows to enhance performance and to mitigate negative effects on the environment (methane).

2 Paper I

Der Einsatz von rationsapplizierten Nicht-Stärke-Polysaccharid-spaltende Enzyme in der Wiederkäuerfütterung unter besonderer Berücksichtigung der Milchkühe

APPLYING NON-STARCH-POLYSACCHARIDE-
HYDROLYSING ENZYMES (NSPHE) TO DIETS IN RUMINANT NUTRITION WITH SPECIAL REGARD TO DAIRY COWS

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ZUSAMMENFASSUNG

Seit Mitte der 90 er Jahre wird der Einsatz von rationsapplizierten NSPsE (Nicht-Stärke-Polysaccharid-spaltende Enzyme) zur Optimierung der ruminalen Abbauprozesse von ß-glykosidisch gebundenen Kohlenhydraten (z.B. Cellulose, Hemicellulose) diskutiert.
In diese Übersicht wird versucht, die Einsatzwürdigkeit von NSPsE bei Wiederkäuern, insbesondere Milchkühen, zu bewerten. Die Ergebnisse der ausgewerteten Studien zu den Wirkungsweisen von NSPsE bei Wiederkäuern sind sehr widersprüchlich. Methodische Probleme der Enzymaktivitätsbestimmung sowie fehlende Kenntnisse über die komplexen Prozesse im Pansen schränken die Forschung zum Enzymeinsatz bei Wiederkäuern ein.
Die T-Aufnahme und die Milchleistung der Kühe in den enzymsupplementierten Gruppen der zugänglichen Literatur wurde im Mittel im Vergleich zu den unbehandelten Kontrollgruppen nur geringfügig erhöht (0,4 ± 0,7 kg/d bzw. 0,5 ± 1,3 kg/d). Die große Variabilität der Versuchsergebnisse ist in Anbetracht der großen Unterschiede in der Rationsgestaltung sowie der Zusammensetzung und den Eigenschaften der geprüften Enzymmischungen verständlich. Aus den derzeitigen Befunden ist für die Anwendung von NSPsE bei Wiederkäuern kein Wirkungsnachweis abzuleiten, wenngleich in einigen Untersuchungen Leistungssteigerungen beschrieben werden. Die Bedingungen, unter denen erzielte Ergebnisse mit hoher Sicherheit reproduziert werden, können gegenwärtig noch nicht ausreichend genau beschrieben werden.

Schlüsselwörter: Wiederkäuer, Enzyme, Faserabbau, Cellulasen, Hemicellulasen, Milchkühe

SUMMARY

Possibilities to improve the degradation of ß-glucosidic bond carbohydrates by applying NSPhE (non-starch-polysaccharide-hydrolysing enzymes) to ruminant diets have been discussed and investigated since the mid 90th. The objective of this review was to analyse the effects of directly fed NSPhE to ruminants, mainly dairy cattle.
Results of those studies included in this analysis are rather divergent and inconsistent. Methodological problems related to the determination of enzyme activity and incomplete understanding of the complex ruminal degradation process are limiting factors in experimental research procedures with NSPhE for ruminants.

DM intake and milk yield of dairy cows were only marginally improved through NSPhE treatment in comparison to controls (0,4 ±0,7 kg/d and 0,5 ± 1,3 kg/d, resp.) The large variation of results and the inconsistency of effects are related to the differences of ration components and type of ration as well as to the composition and properties of enzyme products applied.

Despite some positive experimental results reported in the specific literature, there is no general indication of a positive reaction of NSPhE in rations fed to dairy cows. Conditions for obtaining reproducible results can currently not be described.

Key words: Ruminants, enzymes, fibre digestion, cellulases, hemicellulases, dairy cows

3 Paper II

Effect of exogenous fibrolytic enzymes on ruminal fermentation and nutrient digestion in dairy cows

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Abstract

The aim of the present study was to examine the effects of an exogenous fibrolytic enzyme product applied to a total mixed ration (TMR) prior to feeding on ruminal fermentation, microbial protein synthesis, nutrient digestion, and milk yield and composition. Six multiparous lactating Holstein cows (598+ 29 kg initial live weight and 98+ 30 days in milk) fitted with rumen and duodenal cannulae were allocated to two treatments in a crossover design over three consecutive 28-d periods. The TMR containing 50% concentrates, 30% corn silage and 20% grass silage on dry matter (DM) basis, was mixed once daily and fed twice a day. Treatments were TMR alone (Control) or TMR with an enzyme product containing primarily cellulase and xylanase activities (9000 U endo-1,4-b glucanase, 24000 U endo-1,3(4)-b glucanase and 40000 U 1,4-b xylanase per ml). The enzyme product was applied at a rate of 6.2 ml/kg TMR (DM basis). It was diluted at a rate of 1: 5 with water and applied daily to the TMR. During the control period the cows received a TMR supplemented with 36 ml water/kgTMR on DM basis. Duodenal digesta flow was measured using Cr_2O_3 as flow marker and microbial protein in the duodenal digesta was estimated by near-infrared spectroscopy (NIRS). There were no significant differences in ruminal pH-values, NH_3-N concentrations, total SCFA concentrations and molar proportions of SCFA. No treatment effects on microbial N flow to the duodenum and efficiency ofmicrobial protein synthesis were observed.The apparentruminal digestibilities of DM, organic matter, NDF and ADF, milk yield and composition were also not affected by the enzyme supplementation. In this study the application of exogenous fibrolytic enzymes fed to dairy cows did not show a significant effect on any parameter tested.

Keywords: dairy cows; digestibility; enzymes; feed additives; fermentation; fibre; Rumen

4 Paper III

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

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Abstract

The supplementation of exogenous fibrolytic enzymes (EFE) to dairy cows diets could be a strategy to improve fiber degradation in the rumen which is especially important for the early lactating cow characterized by a high milk energy output and an insufficient energy intake. The objective of this study was to examine the effects of a fibrolytic enzyme product (Roxazyme® G2 Liquid, 3.8 and 3.9 mL/kg TMR DM) supplemented to a total mixed dairy cow ration (TMR) on production performance and blood parameters during early (trial 1) and mid-lactation (trail 2). In addition, rumination activity was measured in trial 2. The nutrient digestibility of the experimental TMR was obtained by using wethers. In the digestibility trial EFE was supplemented at a rate of 4.4 mL/kg Roxazyme® G2 Liquid TMR DM. The TMR contained 60% forage and 40% concentrate (DM basis). Twenty eight (50 ± 16 days in milk; DIM) and 26 (136 ± 26 DIM) Holstein cows were used in two 8-wk, completely randomized trails, stratified by parity and milk yield level. One ml of the enzyme product contained primarily cellulase and xylanase activities (8,000 units endo-1,4-ß glucanase, 18,000 units endo-1,3(4)-ß glucanase and 26,000 units 1,4-ß xylanase). No differences in digestibility of DM, OM, CP, NDF and ADF were observed ($P > 0.05$) between the control and the EFE supplemented TMR. Addition of EFE to the TMR fed to early (trial 1) and mid-lactation cows (trial 2) did not affect daily dry matter intake (DMI), milk yield, 4% fat-corrected milk, energy-corrected milk (ECM), concentration of milk fat, protein, fat-protein-quotients, somatic cell score, energy balance, and gross feed efficiency of early and mid-lactation cows ($P > 0.05$). Mid-lactation cows (trial 2) fed with TMR enzyme showed a tendency of a slightly higher ECM yield ($P = 0.09$). The tested blood parameters were not affected by treatment in trial 1 and 2 ($P > 0.05$). EFE supplementation did not alter daily time spent ruminating in trial 2 ($P = 0.44$). In conclusion, under the conditions of this study no positive effects of enzyme supplementation on dairy performance and health status of dairy cows during early and mid-lactation were observed.

Keywords:
Fibrolytic enzymes, Dairy cows, Milk yield, Feed efficiency

5 General Discussion

5.1 Introduction

The potential usefulness of exogenous fibrolytic enzyme (EFE) preparations to improve nutrient utilization and performance in dairy cows has been subject to various studied during the last two decades and is expected to be a continued research issue. This will be driven by on-going changes in world animal production.

The hypothesis of our study was that the addition of EFE to the diets of dairy cows alters rumen fermentation, increases digestibility of nutrients, improves energy balance (EB), and gross feed efficiency (GFE). We also hypothesized that early lactation cows are more responsive to the EFE supplementation than mid-lactation cows. The literature review (chapter 2) suggested that results of EFE supplementation studies were rather divergent and inconsistent. The high variability of animal response to EFE observed between studies is likely due to factors such as mode of application (i.e., feed versus rumen), time of application before feeding, type and activity of the main enzymes, composition of the basal diet, application level, stability of the enzymes in the rumen, experimental designs, and variable choice of lactation stages. Several studies show that EFE increase the digestibility of DM, NDF and ADF, indicating a possible improvement of milk production and feed conversion. Most of these studies predominantly used diets with a high legume (alfalfa) and concentrate proportion (chapter 2). The effects of EFE on rumen fermentation and digestibility in high yielding dairy cows under a typical European dairy feeding system based on corn silage and grass silage was studied in experiment 1 (chapter 3). There were no effects of EFE supplementation on rumen fermentation and apparent rumen and total tract digestibility of DM; OM, NDF and ADF. These results may have been affected by specific experimental procedures (restricted feeding and lactation stage > 100 DIM). In two feeding trials (experiment 2 and 3) with early and mid-lactation cows, described in chapter 4, we were not able to detect any effects of the EFE addition on milk production and aspects of health status. The results of these experiments were not able to confirm our hypothesis, but did also not indicate negative effects of EFE addition on the tested parameter.

The first part of the following discussion covers methodological aspects of EFE application, EFE resistance to microbial degradation in the rumen and type of enzymes. The second part will give some insight into the effect of EFE supplementation on rumen fermentation and digestibility of nutrients in the context of current literature. Dry matter intake, production performance and feed efficiency are discussed in the third part of this chapter followed by animal health related aspects of EFE supplementation. Finally, the discussion covers questions to which extent EFE can contribute to the mitigation of negative effects on the environment (methane).

5.2 Aspects of EFE application

Dosage, measurement of enzyme activity, key enzymes

Dosage of enzyme products vary considerable between studies cited in the literature (table 4-6), ranging from 2 g to 28 g EFE kg^{-1} diet DM with an average of 6.7 g kg^{-1} DM (SD ± 6.9 g

kg^{-1} DM). The dosages used in the three experiments of this study fall within this range (table 4-6). According to Beauchemin et al. (2004b) and Adesogan et al. (2014) it is possible to under- and over- supplement and an optimal amount of enzyme supplementation may depend on the diet.

It also needs to be considered that extensive pre-ruminal hydrolysis by EFE at a high dosage reduces initial rumen bacterial colonization in fibre rich rations (Wang et al., 2012). It is likely that extensive EFE activity could lead to accumulation of indigestible phenolic compounds-mediated lignin-carbohydrate complexes, which is a key barrier to the microbial colonization and digestion of plant cell walls on the feed surface, thereby inhibiting the attachment of ruminal microbes (Wang et al., 2012). Morgavi et al. (2004) also observed a reduction of *F. succinogenes* attachment to alfalfa and corn silage when EFE was applied at a high dosage.

The dosage of an enzyme product does not reflect their amount of enzyme activity. The enzyme activity is generally determined as substrate converted respectively as product formed per time unit. In the case of enzymes such as xylanase, β-glucanase and other fibrolytic enzymes, a high-molecular-weight (MW) polysaccharide substrate is converted to reaction products consisting of lower-MW oligosaccharides, which are then usually measured by reducing sugar methods such as the DNS (dinitrosalicylic acid) method or the Nelson–Somogyi method (Sheehan, 2010). Most enzyme manufactures declare their enzyme preparations in IU (international unit); 1 IU is defined as the enzyme amount converting 1 µmol substrate (forming the 1 µmol product min^{-1}). But Vahjen et al. (1997) and Eun and Beauchemin (2008) highlight that the comparison of enzyme activities in different studies is not possible because of different assay conditions used (e.g., temperature, pH, ionic strength, substrate, and reaction time), which greatly affect resulting activities, and thus the definition of a unit of enzymatic activity is method dependent and variable among laboratories. This lack of standardization makes it difficult to compare enzyme units across studies. Difficulties to define general standards for enzyme assays are discussed in detail by Bisswanger (2014).

Roxazyme® G2 liquid, as used in our studies, is authorized by the European Food Safety Authority (EFSA 2012) as a feed additive for poultry and piglets. According to the declaration of the manufacturer DSM Nutritional Products, the additive Roxazyme® G2 liquid is a preparation of endo-1,4-beta-xylanase, endo-1,4-beta-glucanase and endo-1,(3)4beta-glucanase produced by a strain of *Trichoderma reesei* (formerly classified as *Trichoderma longibrachiatum*). Enzymatic activities of the three enzymes are expressed in units (U), and one U is the amount of enzyme (xylanase, cellulase or glucanase) that releases 1 µmol of reducing sugar (xylose or glucose equivalent) per minute at 40 °C, pH 5, from wheat arabinoxylan, carboxymethylcellulose or barley beta-glucan, respectively. From these manufacturer declarations it is not possible to determine the enzyme activities of the additive under rumen conditions. If applied in ruminant feeding it seems logical that the pH and temperature used in the assays should resemble the conditions found in the feed or in the rumen, depending upon the site where the enzymes are expected to act (Colombatto and Beauchemin 2003). A methodology to standardize the determination of enzyme activities present in enzyme additives used in ruminant diets was proposed by Colombatto

and Beauchemin (2003) and applied used by Miller et al. (2008a) for Roxazyme® G2 liquid (table 1).

Tab. 1 Enzyme activity of **Roxazyme® G2** using manufacture information and results of Miller et al. (2008a) who using the procedures of Colombatto and Beauchemin (2003) at pH 6.0 and 39°C reflect rumen conditions

Enzyme	EC Number	Enzyme activity	
		Manufacturer information	**According to Miller et al. (2008a)**
Endo-1,4-β-xylanase	3.2.1.8	26000 U/ml	2200
Exo-1,4-ß-glucanase	3.2.1.91	not available	2.6
Endo-1,3(4)-β-glucanase	3.2.1.6	18000 U/ml	not available
Endo-1,4-β-glucanase	3.2.1.4	8000 U/ml	710

μmol reducing sugar equivalents min^{-1} ml^{-1} original product

These results provide clear evidence that an EFE additive used in ruminant feeding studies should be measured under ruminal physiological conditions. Since that has not been done by all cited studies and also our study, the comparison of applied enzyme activities is not possible.

The analysis of enzyme activity in feed samples is important to determine the adequate distribution within the diet. Also for the efficacy assessment of the EFE addition it is necessary to measure enzyme activities in ruminal fluid and digesta. Quantification of enzyme activity after addition to animal feeding stuffs has proven technically challenging (Walsh et al. 2005) since normal methods of assay based upon quantification of reaction products (e.g. reducing sugar measurements) for EFE are not suitable for feed and digesta analysis (Wallace and Hartnell, 2001; König et al., 2002; Sheehan, 2010).

The feed samples of our studies were measured photometrically by the EFE manufacturer DSM. The applied methods, as described in chapter 3, used a relative method in which the Roxazyme product was the standard against which the product concentration (ml product/kg TMR DM) was determined by measuring the enzyme reaction in the feed sample. The analysis method has a bias of 15% and depends on the enzyme product and the feed stuff. The capacity of Roxazyme® G2 liquid to homogenously distribute when added to the TMR was studied in the undermentioned feed samples and was found to be within acceptable values (table 2).

Tab 2. EFE (Roxazyme) dosage and actual product concentrations (mean ± SD) used in the experiments

Experiment	Dosage (ml Roxazyme G2 liquid kg^{-1} TMR DM)	Product concentration[1] (ml Roxazyme G2 liquid kg^{-1} TMR DM)		
		Endo-1,4-β-glucanase	Endo-1,3(4)-β-glucanase	Endo-1,4-β-xylanase
Digestibility trial with wethers*	4.4	3.3	3.5	3.6
Digestion trial	6.2	7.0 ± 2.0	7.9 ± 2.2	9.1 ± 2.1
Feeding trial 1	3.9	3.6 ± 0.3	3.9 ± 0.3	3,8 ± 0.5
Feeding trial 2	3.8	3.5 ± 0.5	3.5 ± 0.5	3.6 ± 0.5

[1] The product concentrations (ml Roxazyme G2 liquid kg^{-1} TMR DM) were measured by DSM Nutritional Products using the assay procedures detailed by chapter 3.
*based on one feed sample.

Increasing the hydrolytic capacity within the rumen

One potential mode of action of EFE for ruminants is the increase of hydrolytic capacity within the rumen. This hypothesis is difficult to verify because of the complexity of measuring the enzyme activity within the rumen (Beauchemin et al., 2004a) and Prauchner et al. (2012) postulated that the methodological procedures for studying the fibrolytic activity of rumen bacteria are not clearly established.

Only few studies have been conducted to quantify the increased enzyme activity through EFE supplementation. Wallace et al. (2001) calculated an increase of xylanase activity of 5% and a CMCase activity (CMC=carboxymethylcellulose) of 15% within the ruminal fluid (table 3) through the supplementation of 1.5 and 2 ml EFE product A or B/kg feed, but argued that both values are within the variation of activities found in the ruminal fluid extracts. According to Wallace et al. (2001), these small quantities of added EFE as recommended by the manufactures are unlikely to stimulate the rate of fermentation directly. It is also relevant to note that many enzyme activities, particularly glycosidase activities, are present at such high concentrations in the feed that added enzymes have no detectable effect on feed-associated enzyme activities.

Tab. 3 Comparison of CMCase and xylanase activities in sonicated ruminal microorganisms and enzyme additives A and B (adopted from Wallace et al. 2001)

Enzyme source	**Enzyme activity in the rumen, mmol/min[1]**	
	CMCase	**Xylanase**
Sonicated ruminal microorganisms	0.33	0.67
Enzyme A	0.05	0.03
Enzyme B	0.06	0.04

[1] Calculated on the basis of a 5-L ruminal volume, an addition rate of enzyme supplement of 1.5 and 2 L per mega gram of feed for enzymes A and B, respectively, and consumption of 0.7 kg of feed per meal.

Inconsequential effects on the polysaccharide-degrading activities of ruminal digesta by EFE were also observed by Hristov et al. (2008), who calculated that adding EFE intraruminal at a 10 g day^{-1} (approx. 0.4 g/kg DMI) application rate would have increased the xylanase activity of ruminal contents by about 1%. Apparently, these exogenous activities were not sufficient to affect indigenous activities of the ruminal microorganisms. Larger doses of EFE, however,

can increase ruminal fibrolytic activities (Hristov et al., 2000). In an experiment of Giraldo et al. (2008a) EFE delivered directly into the rumen of sheep at a rate of 12 g/d significantly increased endoglucanase and xylanase activities and tended to increase numbers of cellulolytic bacteria at 4 h after feeding. Molar proportion of propionate was greater, and acetate:propionate ratio was lower, which however seems not in congruence with the observed stimulated growth of cellulolytic bacteria. Thus, the direct ruminal hydrolytic effect of EFE on improving cell wall digestion is likely minor, at the levels of EFE typically used in feeding studies. Morgavi et al. (2000a) observed a synergistic effect between EFE and ruminal enzymes such that the net combined hydrolytic effect in the rumen was much greater than estimated from the individual activities.

EFE resistance to microbial degradation in the rumen

A precondition of EFE effects after feed intake is the resistance to microbial degradation in the rumen. Only in earlier studies this issue was investigated (Hristov et al., 1998; Morgavi at al. 2000b; Morgavi at al. 2001). The EFE tested by Morgavi et al. 2001 were relatively stable in ruminal fluid and resistant to microbial degradation for a sufficiently long time act in the rumen, which was in accordance with a recent study performed on the resistance of EFE in different biogas reactor set-ups (Hanreich, 2013).

Key enzymes

Due to the complex structure of forages, it has been shown that improved performance can be obtained by appropriate combinations of different enzyme activities. Since the effect of enzyme products depends on the ration composition it seems essential to compose enzyme supplements to the chemical composition of the target forage (Eun and Beauchemin, 2008). Thus, a particular enzyme formulation will not be effective for all diets. In addition the composition of enzyme products to target rations is further complicated by the fact that EFE act synergistically with microbial enzymes in the rumen, and thus the key activities required may vary depending upon the endogenous microflora (Morgavi et al., 2000a). These factors highlight the futility of random addition of EFE to diets as concluded by Adesogan et al. (2014). Given the complex and heterogenic composition of ruminant diets, the need for specific enzyme products to the target ration and need for the optimization of dosage, EFE supplementation requires considerable prior *in vitro* screening to confirm the efficacy at increasing neutral detergent fibre digestion and to find the optimum EFE product and dosage.

From specific *in vitro* experiments of different enzyme products applied to alfalfa hay and corn silage Eun et al. (2007) indicate, that a high application level of xylanase was detrimental to the digestion of corn silage by mixed rumen microbial populations and suggested that an ideal ratio of endoglucanase and xylanase is needed to enhance the effectiveness of EFE (between 0.4:1 and 1:1 for both forages). It further appears that endoglucanase activity is a good indicator for the ability of EFE to stimulate ruminal fermentation of corn silage. The EFE product Roxazyme® G2 liquid used in our studies showed a dominant xylanase activity and an endoglucanase to xylanase ratio of 0.32:1 (assay

condition 39°C and pH 6.0 reflecting ruminal conditions measured by Miller et al., 2008a, shown in table 1). This is below the optimum ratio specified and may be one of the reasons for the lack of animal response in our *in vivo* studies (chapter 3 and 4).

An additional issue to consider is the fact that commercially available EFE products, mostly from fungal origin (e.g. *T. reesei*) do not exhibit optimal enzyme activity under ruminal conditions (Adesogan et al., 2014) due to higher temperature and lower pH optima than provided in the rumen.

Finally, due to the complexity of the fibre fractions in plant cell walls, many fibrolytic activities are required to synergistically degrade forage fiber. Only few of the usually assayed enzymes, typically endoglucanase and xylanase, cannot hydrolyze the recalcitrant phenolic acid-lignin linkages that are the main constraints to ruminal fiber degradation (Adesogan et al., 2014). Lignin degradation is primarily an aerobic process, and in an anaerobic environment lignin can persist for very long periods (Van Soest, 1994). Therefore, most known ligninolytic enzymes such as laccases and peroxidases are expressed by aerobic fungi and oxygen is necessary for catalyzing the oxidation of lignin. Thus, ligninolytic enzyme additives to the anaerobic rumen digestion process seem unsuitable.

5.3 Effect of EFE on ruminal fermentation characteristics and digestibility of nutrients

Dietary carbohydrates, such as cellulose, hemicelluloses, pectin, starch, and soluble sugars, are the main sources of energy and are degraded in the rumen by microorganisms to hexoses and pentoses before being fermented to VFA (Kebreab et al., 2009). In ruminants, VFA can contribute up to 70% (65-75%) of the caloric requirement (Bergman, 1990), while Martin et al. (2001) assume an energy contribution via VFA of 7.8 MJ/kg DM.

The increased total VFA concentration has great potential to increase energy supply in dairy cattle. The main VFAs produced are acetate and butyrate (C2 compounds or lipogenic) and propionate (C3 compound or glucogenic) (Morvay et al., 2011). The acetate-to-propionate ratio (C2:C3-ratio) can be manipulated by ingredients in the diet. Forages (lipogenic dietary ingredients), that stimulate the ruminal production of acetate and butyrate are expected to increase the C2:C3-ratio. Nonfiber carbohydrates (glucogenic dietary ingredients) increase propionate production and lower the C2:C3-ratio. High levels of acetate indicate a high fibre/low starch ration, producing a generally slower, more stable fermentation. High levels of propionate indicate a high starch/low fibre ratio producing a faster rate of fermentation which can lead to reduced rumen pH, depressed fibre digestion and even rumen acidosis.

Effect of EFE on ruminal fermentation characteristics

Several reasons for improvements in rumen fermentation from feeding EFE have been suggested. First, EFE exerts beneficial changes in activity and numbers of the rumen microbes. Wang et al. (2001) suggested that changes in fermentation pattern may reflect a shift in the species profile of colonizing bacteria in response to pre-treatment of feed with EFE. For example, the total rumen anaerobes and cellulolytic bacteria increase with EFE (Wang et al., 2001; Giraldo et al., 2007a and 2007b; Giraldo et al., 2008a; Chung et al., 2012). The opposite is reported by Dong et al. (1999), who could not find changes in total bacterial

or cellulolytic bacteria populations but an overall increase of the methanogenic bacterial population using the RUSITEC technique. This corresponds with the observation of Zeitz et al. (2012) who found no effect of EFE (Roxazyme® G2 liquid) on total or cellulolytic bacterial populations which may be linked to an over-dosage of EFE in their *in vivo* study with non-lactating cows. Second, EFE may also alter the structure of the feed and increase surface area for microbial attachment as stated by Nesereko et al. (2000).

Our hypothesis was that the EFE addition would improve the fiber degradation of the diet and the total VFA production. The higher total VFA with enzyme addition could be a result of higher availability of fermentable soluble carbohydrates due to increased fibrolytic activity in rumen. Consequently, the molar proportion of acetate and butyrate are expected to increase the C2:C3-ratio. Our experimental results (chapter 3) and results of the recent literature (table A) fail to confirm this hypothesis.

On the other hand, the observed (Giraldo et al., 2008a) increased molar proportion of propionate, leading to a decreased C2:C3-ratio might indicate a change in ruminal bacterial populations through EFE addition. This is possibly linked to an increased number of total bacteria and the amount of partial breakdown products, like cellobiose. The promotion of non-cellulolytic, cellobiose-utilizing bacteria (Nesereko et al., 2002) causes other fermentation pattern as expected. This could be a possible explanation for the decreased C2:C3-ratio notable in most recent studies compiled in table 4. In high yielding dairy cows with high glucose requirements a slight reduction of the C2:C3-ratio could even be beneficial since propionate is the main glucose precursor. The optimum C2:C3-ratio should be above 2.2 to avoid SARA (Sub-Acute Ruminal Acidosis) and milk fat depression (Krause and Oezel, 2005; Lounglawan and Suksombat, 2011) which was met in the EFE supplementation studies in table 4. Furthermore it is noteworthy that an EFE over-dosage can decrease the total VFA production as found in a study of Eisenreich (2008). As VFA are the main energy supply in dairy cattle, high doses of EFE may not be beneficial.

The ruminal NH_3-N concentration is a further parameter of fermentation which in our study could be linked to the following action scenarios:

1. Increased NH_3 concentration is related to an increased degradation of crude protein in the rumen. Increased fibre degradation increases indirectly the crude protein degradation (incrusted protein can be made available through EFE supplementation). EFE preparation used in our study could have contained proteolytic enzymes, though not included in the declaration of Roxazyme. In case of EFE being rumen degradable protein NH_3 concentration will be increased as a result of the proteolytic activity.
2. A decreased NH_3 concentration could be a result of improved EFE related fiber degradation, which could lead to an increased supply of fermentable organic matter. This could enhance the efficiency of microbial protein syntheses resulting in a reduced NH_3 concentration.

In our study (chapter 3) the ruminal protein degradation, indicated by ammonia production, was not affected by EFE addition. An increase of the CP content in the ration through EFE addition in our study can be ruled out due the low level of EFE dosage. Other studies found

in the literature (chapter 2 and table 4) report of increased (Gado et al., 2009) as well as reduced NH_3 concentrations (Beauchemin et al., 2000; Dean et al., 2013).
We hypothesized that a stimulation of microbial growth by the use of EFE could result in higher microbial biomass, as would be indicated by enhanced microbial protein synthesis and microbial N flow to the duodenum. Consistent with this hypothesis, Yang et al. (1999) reported that EFE increased feed digestion in the rumen and flow of microbial protein from the rumen. However, in our study (chapter 3) the microbial protein synthesis in the rumen was not affected by EFE supplementation and we were not able to confirm our hypothesis.

Table 4 Effects of EFE on ruminal pH, VFA concentration (mM), molar proportion (mol/100 mmol) and NH_3-N (mg/L)

Enzyme	Animals (n)	Diet (forage:concentrate)	treatment	pH-value	VFA	Molar proportion			C2:C3	NH_3-N	Reference
						Acetate	Pro-pionate	Butyrate			
	dairy cows	CS, GS (71:29)	control	6.66	75.6	50.8	14.1	10.7	3.74	125	Eisenreich
Roxazyme	nonlactating	CS, GS (71:29)	27.7 g EFE kg^{-1} diet	6.70	**59.6†**	**40.3†**	11.2	8.04	3.76	106	2008
Celluclast	(9)	CS, GS (71:29)	6.78 g EFE kg^{-1} diet	6.54	77.5	53.2	14.1	10.1	3.89	128	
	sheep	GH (70:30)	control	6.18	101.1	67.9	18.0	11.8	3.86	88.5	Giraldo
Fibrozyme	(6)	GH (70:30)	12 g EFE d^{-1}	6.07	98.2	**66.2†**	**19.6†**	11.8	**3.41†**	99.0	et al. 2008a
	dairy cows[3]	AH, GH (40:60)	control	5.96	106.3	71.3	22.3	9.7	3.40	153.0	Hristov
Enz A	(4)	AH, GH (40:60)	10 g EFE d^{-1}	5.95	113.9	75.9	23.0	11.4	3.37	175.4	et al. 2008
Enz X		AH, GH (40:60)	10 g EFE d^{-1}	5.92	116.7	78.2	23.5	11.5	3.39	168.6	
Enz AX		AH, GH (40:60)	10 g EFE d^{-1}	5.94	112.2	75.7	22.6	11.6	3.41	172.0	
	steers	Barley (30:70)	control	6.8	71.7	44.7	17.7	7.0		32	Miller
Roxazyme	(16)	Barley (30:70)	5.18 g EFE kg^{-1} diet	6.6	94.4	57.4	21.7	12.0		77	et al. 2008b
		Sorghum (24:76)	control	6.9	70.3	43.4	17.6	6.3		83	
Roxazyme		Sorghum (24:76)	5.18 g EFE kg^{-1} diet	6.8	81.9	65.4	22.5	9.0		87	
	dairy cows[1]	Pasture/Barley	control	7.01	53.0	63.7	16.8	14.7		211	Miller
Roxazyme	(24)	Pasture/Barley	2.52 g EFE kg^{-1} diet	7.02	51.5	63.8	16.8	14.2		193	et al. 2008a
Roxazyme		Pasture/Barley	5.03 g EFE kg^{-1} diet	6.82	71.9	63.4	18.2	13.9		144	
		Pasture/Sorghum	control	6.83	73.9	65.3	16.3	13.9		240	
Roxazyme		Pasture/Sorghum	2.52 g EFE kg^{-1} diet	7.02	60.7	64.7	15.4	15.1		162	
Roxazyme		Pasture/Sorghum	5.03 g EFE kg^{-1} diet	6.99	63.9	64.6	14.9	15.6		183	
	lambs	AH (60:40)	control	6.69	77.6	72.7	14.8	12.5		217	Pinos-Rodríguez
Fibrozyme	(6)	AH (60:40)	2 g EFE kg^{-1} TMR DM	6.65	76.2	72.2	15.3	12.5		155	et al.2008
		AH (50:50)	control	6.58	72.8	73.1	15.0	11.9		182	
Fibrozyme		AH (50:50)	2 g EFE kg^{-1} TMR DM	6.57	71.3	72.8	15.4	11.8		165	
		AH (40:60)	control	6.42	66.5	72.6	15.8	11.6		151	
Fibrozyme		AH (40:60)	2 g EFE kg^{-1} TMR DM	6.28	74.8	72.3	14.5	13.2		174	
	lambs	GGH 35d	control	6.55	73.3	77.2	16.9	5.9		83.0	Avellaneda
Fibrozyme	(4)	GGH 35d	3 g EFE d^{-1}	6.61	72.6	77.6	16.8	5.7		90.3	et al.2009
		GGH 90d	control	6.73	71.8	75.8	18.1	6.1		28.5	
Fibrozyme		GGH 90d	3 g EFE d^{-1}	6.80	66.8	75.8	18.0	6.2		27.2	

Table 4. continued

Enzyme	Animals (n)	Diet (forage:concentrate)	treatment	pH-value	VFA	Molar propotion			C2:C3	NH_3-N	Reference
						Acetate	Pro-pionate	Butyrate			
	dairy cows[1]	CS (70:30)	control	6.1	119.2	60.0	18.3	10.9	3.28	110	Gado
Zado	(20)	CS (70:30)	40 g EFE d^{-1} cow^{-1}	5.9	**128.0†**	**64.0†**	**20.8†**	11.0	**3.08†**	**126†**	et al. 2009
	dairy cows[2]	CS, GS (50:50)	control	6.24	99.7	64.7	16.5	14.3	3.97	231.6	Peters
Roxazyme	(6)	CS, GS (50:50)	7.3 g EFE kg^{-1} TMR DM	6.17	98.8	60.9	19.1	15.1	3.43	185.6	et al. 2010
	dairy cows	CS, AH (67:33)	control	6.26	110.4	60.1	18.6	11.7	3.23	124	Arriola
Econase	nonlactating	CS, AH (67:33)	3.4 g EFE kg^{-1} TMR DM	6.36	118.2	59.5	18.9	12.0	3.15	130	et al. 2011
	(4)	CS, AH (52:48)	control	6.10	118.6	57.7	19.6	12.3	2.94	127	
Econase		CS, AH (52:48)	3.4 g EFE kg^{-1} TMR DM	6.01	133.1	52.4	20.2	12.7	2.59	130	
	dairy	BS, AS, AH (52:48)	Control	6.17	139.0	61.6	23.0	11.4	2.7	98.8	Chung
Econase	(9)	BS, AS, AH (52:48)	0.5 ml EFE kg^{-1} TMR DM	6.12	143.0	60.2	23.3	11.4	2.6	109.0	et al. 2012
Econase		BS, AS, AH (52:48)	1.0 ml EFE kg^{-1} TMR DM	6.13	141.0	61.0	22.7	12.1	2.7	100.5	
	dairy cows[2]	Tifton85 (45:55)	control	6.32	144	58.9	20.8	11.8	2.8	151	Dean
Promote	(30)	Tifton85 (45:55)	EC 4 g EFE d^{-1} cow^{-1}	6.11	125	57.7	20.5	12.7	2.8	136	et al. 2013
Promote		Tifton85 (45:55)	ETMR 4 g EFE d^{-1} cow^{-1}	6.27	**105†**	**56.0†**	**21.7‡**	13.3	**2.6†**	**101†**	
Promote		Tifton85 (45:55)	EF 4 g EFE d^{-1} cow^{-1}	6.26	**115‡**	**55.3†**	20.8	12.6	2.7	170	
Promote		Tifton85 (45:55)	ES 9 g EFE d^{-1} cow-^{1}	6.23	106	59.2	20.2	11.7	**2.9†**	149	

†Differences between control and EFE treatment were significant ($p<0.05$)

‡EFE treatment tend to differ from control ($0.05 \le P < 0.10$)

[1] early lactation; [2] mid-lactation; [3] late-lactation

CS=corn silage; GS=grass silage; GH=grass hay; AH=alfalfa hay; GGH=Guinea grass hay, BS=Barley silage

Eisenreich 2008: 4h post feeding. Giraldo et al. 2008a: 4 h after feeding. Hristov et al. 2008: Enz A: predominantly amylase; Enz X predominantly Xylanase, Enz AX: amylase/xylanase combination; average of 0, 2, 4, and 6 h post feeding. Miller et al. 2008a: 4 h post feeding. Miller et al. 2008b: 6.7 kg concentrate d^{-1} cow^{-1} and pasture; 4 h post feeding. Avellaneda et al. 2009: average of 0, 3, 6, 9 and 12 h post feeding; Guinea grass hay (GGH) cut at 35 and 90 d of growth. Pinos-Rodríguez et al. 2008: average of 3, 6, and 12 h post feeding. Gado et al. 2009: 3 h post feeding. Peters et al. 2010: average of 0, 0.5, 1, 2, 3, 4, and 6 h post feeding. Arriola et al. 2011: average of 0, 2, 4, 6, 8, and 10 h post feeding. Chung et al. 2012: average of 0, 6, and 12 h post feeding. Dean et al. 2013: average of 0, 2, 4, 6, 8 and 10 h after feeding

Mitigate negative effects on the environment (methane)

Methane production by ruminants is a critical issue in the context of greenhouse gas emission contributing to global warming (Opio et al., 2010). Although not measured in our experiments, Beauchemin et al. (2008) proposed that dietary enzyme feed additives may help to mitigate enteric CH_4 emissions.

Only few studies have investigated the effects of exogenous enzymes on methane production in the rumen, and their results are conflicting. Dong et al. (1999) found that the treatment of grass hay with EFE increased cellulose digestibility and methane production in Rusitec fermenters by 15 and 43%, respectively. Giraldo et al. (2007a and 2007b) reported that the treatment of a grass hay and concentrate substrate with EFE increased DM, NDF, and ADF disappearance after 6 and 24 h (and in Giraldo et al., 2007b also at 48 h) of incubation and methane production by 14.3–24.6% and 13.4-14.9% in Rusitec fermenters. Since methane represents a significant loss of energy for the host animal, these results seem to indicate that treating the grass hay and concentrate substrate with EFE would result in the release of more energy (Giraldo et al. 2008b).

The enzyme addition to diets of growing goats did not affect the enteric methane emission and community diversity of ruminal methanogens (Lu et al., 2015) and Zhou et al. (2011) observed an increased CH_4 yield supplementing a dairy cow diet with EFE.

Recently, some EFE products were reported to decrease methane production through improved diet digestibility, a decreased C2:C3 ratio without decreasing total VFA production (Arriola et al., 2011). This finding contradicts that of Johnson and Johnson (1995) who argues, that an improved digestion of cell wall fiber increases methane production, by increasing the total H_2 production and the amount of substrate available for methanogens. However, an indirect effect of EFE on reduction of methane production may occur through the increased feed efficiency (Hristov et al., 2013 and Holtshausen et al., 2011). Increasing forage digestibility and digestible forage intake will generally reduce GHG emissions from rumen fermentation (and stored manure), when scaled per unit of animal product, and are highly-recommended mitigation practices. In general, more specific studies are needed to reach a conclusion on the effects of enzymes in the production of greenhouse gases before EFE can be recommended as an effective mitigation practice (Hristov et al., 2013).

It should also be noted that any assessment of methane emissions of different feeding strategies and feed additives should be based on a live cycle assessment along the whole pathway of a feed chain to avoid assessment biases.

Effect of EFE on digestibility of nutrients

Digestibility of NDF is an important parameter of forage quality. Enhanced NDF digestibility of forage significantly increased dry matter intake (DMI) and milk yield. A one-unit increase in NDF digestibility *in vitro* or *in situ* was reported to be associated with a 0.17 kg increase in DMI and a 0.25 kg increase in 4% fat-corrected milk (Oba and Allen, 1999).

We hypothesized that the addition of EFE to high quality forage based on grass and corn silage would improve the apparent digestibility, especially NDF digestibility.

Table 5 Effect of EFE on apparent total tract digestibilities of DM, OM, NDF, ADF, and CP

Enzyme	Animals (n)	Diet (forage:concentrate)	treatment	DMI (kg/d)	Digestibility (%)					Reference
					DM	OM	NDF	ADF	N	
cellusases	lambs	CS; AH (50:50)	control	1.358	63.5		53.7	52.1	66.5	Reddish and
xylanases		CS; AH (50:50)	10 g EFE d^{-1} lamb^{-1}	1.365	62.7		52.7	51.0	66.3	Kung 2007
	dairy cows	CS, GS (71:29)	control	6.0		72.5	69.0			Eisenreich
Roxazyme	nonlactating	CS, GS (71:29)	27.7 g EFE kg^{-1} diet DM	6.0		75.8	74.5			2008
Celluclast	(9)	CS, GS (71:29)	6.78 g EFE kg^{-1} diet DM	6.0		75.0	73.4			
		CS, GS (66:34)	control	6.1		73.3	61.2			
Roxazyme		CS, GS (66:34)	13.8 g EFE kg^{-1} diet DM	6.1		73.9	62.9			
Roxazyme		CS, GS (66:34)	27.7 g EFE kg^{-1} diet DM	6.1		73.4	61.0			
	sheep	GH (70:30)	control	1.010	67.0	69.4	60.1	54.1	65.2	Giraldo
Fibrozyme	(6)	GH (70:30)	12 g EFE d^{-1} intraruminal	0.993	66.9	69.1	59.6	53.5	64.0	et al. 2008a
	dairy cows[3]	AH, GH (40:60)	control	22.6	68.6	69.9	53.7		68.7	Hristov
Enz A	(4)	AH, GH (40:60)	10 g EFE d^{-1} intraruminal	24.1	70.1	71.6	56.4		70.5	et al. 2008
Enz X		AH, GH (40:60)	10 g EFE d^{-1} intraruminal	24.0	70.7	72.0	56.8		71.6	
Enz AX		AH, GH (40:60)	10 g EFE d^{-1} intraruminal	22.6	64.7	66.1	52.9		65.5	
	steers	Barley (30:70)	control		73.3	74.4	54.3	44.7	68.9	Miller
Roxazyme	(16)	Barley (30:70)	5.18 g EFE kg^{-1} diet DM		74.0	74.8	57.2	46.4	71.7	et al. 2008b
		Sorghum (24:76)	control		56.6	56.2	50.5	45.0	59.2	
Roxazyme		Sorghum (24:76)	5.18 g EFE kg^{-1} diet DM		57.5	57.3	51.4	43.0	57.9	
	steers	High grain (25:75)	control		61.6	61.8	56.9	53.1	58.4	
Roxazyme	(4)	High grain (25:75)	5.18 g EFE kg^{-1} diet DM		61.7	61.6	56.5	49.6	59.6	
		Low grain (49:51)	control		61.9	62.6	63.2	57.4	63.8	
Roxazyme		Low grain (49:51)	5.18 g EFE kg^{-1} diet DM		63.9	64.3	62.6	57.6	64.6	
	lambs	AH (60:40)	control	1.64	66.9		46.0			Pinos-Rodríguez
Fibrozyme	(6)	AH (60:40)	2 g EFE kg^{-1} TMR DM	1.65	67.0		45.9			et al. 2008
		AH (50:50)	control	1.60	69.9		43.3			
Fibrozyme		AH (50:50)	2 g EFE kg^{-1} TMR DM	1.71	70.2		43.7			
		AH (40:60)	control	1.73	73.1		40.4			
Fibrozyme		AH (40:60)	2 g EFE kg^{-1} TMR DM	1.80	73.4		40.1			

Table 5. continued

Enzyme	Animals (n)	Diet (forage:concentrate)	treatment	DMI (kg/d)	Digestibility (%)					Reference
					DM	OM	NDF	ADF	N	
	lambs	GGH 35d	control	1.116	63.4	66.7	76.4	70.8	71.7	Avellaneda
Fibrozyme	(4)	GGH 35d	3 g EFE d^{-1} intraruminal	1.107	63.0	66.4	76.2	69.2	71.0	et al. 2009
		GGH 90d	control	1.121	57.5	61.0	68.6	63.0	56.6	
Fibrozyme		GGH 90d	3 g EFE d^{-1} intraruminal	1.092	57.8	60.9	68.7	64.0	55.0	
	dairy cows[1]	CS (70:30)	control	16.1	66.3	66.7	41.8	40.1		Gado
Zado	(20)	CS (70:30)	40 g EFE d^{-1} cow^{-1}	**18.2†**	**74.3†**	**74.1†**	**58.4†**	**53.2†**		et al. 2009
	dairy cows[2]	CS, GS (50:50)	control	15.1	71.9	74.1	64.7	57.9		Peters
Roxazyme	(6)	CS, GS (50:50)	7.3 g EFE kg^{-1} TMR DM	15.0	72.6	74.6	65.3	57.7		et al. 2010
	dairy cows	CS, AH (67:33)	control	22.1	68.5		52.1	48.6	68.4	Arriola
Econase	nonlactating	CS, AH (67:33)	3.4 g EFE kg^{-1} TMR DM	20.8	**71.2†**		**55.2†**	**52.9†**	71.5†	et al. 2011
	(4)	CS, AH (52:48)	control	25.7	71.1		53.3	52.1	70.0	
Econase		CS, AH (52:48)	3.4 g EFE kg^{-1} TMR DM	23.8	**74.0†**		**57.5†**	**56.6†**	75.1†	
	dairy cows[2]	Tifton85 (45:55)	control	20.9	66.4		50.7		65.6	Dean
Promote	(30)	Tifton85 (45:55)	EC 4 g EFE d^{-1} cow^{-1}	21.6	64.2		51.0		65.7	et al. 2013
Promote		Tifton85 (45:55)	ETMR 4 g EFE d^{-1} cow^{-1}	22.4	66.3		50.4		66.9	
Promote		Tifton85 (45:55)	EF 4 g EFE d^{-1} cow^{-1}	19.9	64.3		51.6		65.7	
Promote		Tifton85 (45:55)	ES 9 g EFE d^{-1} cow^{-1}	21.8	68.3		48.7		67.4	
	sheep	CS, GS (60:40)	control	1.12	76.7	79.8	67.5	64.2	77.2	Peters et al.
Roxazyme	(8)	CS, GS (60:40)	5.2 g EFE kg^{-1} TMR DM	1.11	77.8	80.3	69.7	66.4	75.6	2015

†Differences between control and EFE treatment were significant ($p<0.05$)

‡EFE treatment tend to differ from control ($0.05 \leq P < 0.10$)

[1] early lactation; [2] mid-lactation; [3] late-lactation

CS=corn silage; GS=grass silage; GH=grass hay; AH=alfalfa hay; GGH=Guinea grass hay

Hristov et al. 2008: Enz A: predominantly amylase, Enz X predominantly Xylanase, Enz AX: amylase/xylanase combination. Miller et al. 2008: DMI were restricted to 0.90 of *ad libitum* levels. Avellaneda et al. 2009: Guinea grass hay (GGH) cut at 35 and 90 d of growth. Peters et al. 2010: restricted feed intake. Dean et al. 2013: enzyme applied to bermudagrass Tifton85 at ensiling (ES, 1.3 g/kg DM); enzyme applied at feeding at the rate of 4 g/head/d at mixing to the concentrate (EC), at feeding to the TMR (ETMR) or the forage (EF).

The results of the digestion trail with dairy cows (chapter 3 and table 5) and wethers (chapter 4 and table 5) did not confirm our hypothesis. A reason for the lack of a positive response of EFE on digestibility may be the specific experimental design (restricted feeding and low intake level) that provided optimal conditions for nutrient and specifically NDF digestibility due to a longer rumen retention time associated with a higher digestibility of potentially fermentable NDF (Jung and Allen, 1995).
Further potential reasons for the lack of effect of EFE on ruminal and total-tract nutrient digestibility in our study (chapter 3) include the following: First, low activity of the EFE product in the rumen. The EFE product Roxazyme G2 liquid® used in our studies also show no improvement in other *in situ* and *in vivo* digestibility trials (Dijkstra et al., 2006; Eisenreich, 2008; Miller et al., 2008a). Second, the use of mid-lactation cows (98 DIM) and the use of adult wethers under maintenance conditions in our experiments. Finally, animal-to-animal variation might also indicate that the rumen microbial population of different animals responded differently to the same treatment and prevent clear treatment effects as reported by Zeitz et al. (2012).
The majority of recent *in vivo* studies using different ruminants, as compiled in table 5, did not observe positive effects of EFE on digestibility either. One of two exceptions is the experiment of Gado et al. (2009) in which DMI was 16.1 and 18.2 kg/d (control and treatment, resp.) and the total tract NDF digestibility increased by a striking 40% with EFE treatment without a marked consequence on dairy cow efficiency (0.8 vs. 0.9 kg milk/kg DMI resp.). The other exception is the study of Arriola et al. (2011), who observed an increased apparent total tract digestibility of DM, ADF, NDF and CP resulting in an improved efficiency of milk production (1.4 vs 1.6 kg milk/kg DMI). Biological reasons for this result were not given, but authors related their findings to an increased microbial colonization or by direct cell wall hydrolysis.

5.4 Production and health response to EFE supplementation

The diet used in our experiments for early and mid-lactation cows was a combination of corn silage, grass silage and concentrates fed as TMR (60:40). Our hypotheses were that EFE supplementation to this diet would increase DMI, milk yield and health aspects. We also set the hypotheses that early lactation cows show a higher response to EFE addition because of their increased passage rate, reduced rumen retention time causing a lower fiber digestibility.
The results of our study did not confirm these hypotheses (chapter 4 and table 6) and most recent dairy production studies investigating the effect of EFE, cited in table 6, were also not able to show significant positive production responses. Most of these recent studies found not even a tendency with the exception of the experiment by Holthausen et al. (2011) in which the treatment group had a lower DMI and a slight reduction in milk yield leading to a significant better feed efficiency. In some other publications the effects (table 6, table 3 in chapter 2) were dramatic and as Hristov et al. (2013) stated “not biologically reasonable”.
EFE supplementation did not affect the energy balance of dairy cows in our study, irrespective of stage of lactation. Likewise we did not observe any differences in clinical-

chemical blood parameter related to metabolism and health, thus the health status of the experimental cows was comparable for the treatment groups which did not confirm the hypothesis that EFE supplementation would improve energy balance and the corresponding blood parameters.

In our experiments we did not implement enzyme screening and dose optimization for specific dairy cow rations. This screening strategy includes a large number of variables resulting in an extended set of samples, which can only be covered under *in vitro* conditions. Only with these preliminary tests one can derive at the appropriate choice of enzyme product, dosage and application method to be applied in *in vivo* experiments. Even a positive preliminary *in vitro* screening may not lead to positive *in vivo* results, given the increased complexity of *in vivo* experiments. However, if *in vitro* experiments do not lead to positive effects of the chosen EFE supplementation expensive *in vivo* experiments may not seem sensible.

Table 6 Effect of EFE on DMI (kg/d), milk yield (kg/d), milk fat and milk protein content (%), and feed efficiency

Enzyme	Animals (n)	Diet (forage:concentrate)	treatment	DMI	milk yield	FCM 3.5% *4.0%*	Milk fat	Milk protein	Feed efficiency kg milk/ kg DMI	kg FCM/ kg DMI	kg ECM/ kg DMI	Reference
	dairy	CS; AH (37:63)	control	29.8	44.0		3.54	3.14	1.48			Elwakeel
FP800	cows[2,3]	CS; AH (37:63)	5 g EFE d^{-1} cow^{-1}	29.3	44.3		3.62	3.14	1.52			et al. 2007
FP800	(24)	CS; AH (37:63)	10 g EFE d^{-1} cow^{-1}	28.9	43.4		3.48	3.10	1.50			
FP800		CS; AH (37:63)	15 g EFE d^{-1} cow^{-1}	29.6	44.1		3.43	3.14	1.49			
cellusases	dairy cows[2]	CS; AH (50:50)	control	24.4	40.2	39.3	3.39	2.94				Reddish and
xylanases	(24)	CS; AH (50:50)	10 g EFE d^{-1} cow^{-1}	23.8	40.1	40.4	3.54	2.92				Kung 2007
	dairy cows[1]	Pasture/Barley	control		28.9		3.44	3.11				Miller et al.
Roxazyme	(72)	Pasture/Barley	2.52 g EFE kg^{-1} diet DM		28.6		3.49	3.13				2008a
Roxazyme		Pasture/Barley	5.03 g EFE kg^{-1} diet DM		27.7		3.38	3.11				
		Pasture/Sorghum	control		28.6		3.59	3.07				
Roxazyme		Pasture/Sorghum	2.52 g EFE kg^{-1} diet DM		28.2		3.59	3.09				
Roxazyme		Pasture/Sorghum	5.03 g EFE kg^{-1} diet DM		29.4		3.41	3.07				
	dairy cows[3]	CS, AH (54:46)	control	24.3	41.3		3.76	2.81			1.75	Bernhard
Promote	(44)	CS, AH (54:46)	4 g EFE d^{-1} cow^{-1}	24.2	40.4		3.70	2.81			1.70	et al. 2010
		CS, T85 (50:50)	control	24.9	42.1		3.63	2.75			1.71	
Promote		CS, T85 (50:50)	4 g EFE d^{-1} cow^{-1}	24.6	41.6		3.68	2.81			1.72	
	dairy cows[2]	CS, GS (50:50)	control	15.1	27.6	*30.2*	4.59	2.87				Peters
Roxazyme	(6)	CS, GS (50:50)	7.3 g EFE kg^{-1} TMR DM	15.0	26.3	*26.9*	4.20	2.93				et al. 2010
	dairy cows[1]	CS (70:30)	control	16.1	12.8		3.9	3.5				Gado et al.
Zado	(20)	CS (70:30)	40 g EFE d^{-1} cow^{-1}	18.1†	15.7†		3.8	3.6				2009
	dairy cows[1]	CS, AH (67:33)	control	22.1	31.9	32.5	3.60	2.79		1.46		Arriola et al.
Econase	(60)	CS, AH (67:33)	3.4 g EFE kg^{-1} TMR DM	20.8	32.5	33.9	3.80	2.85		1.69†		2011
		CS, AH (52:48)	control	25.7	33.6	35.9	3.90	2.91		1.42		
Econase		CS, AH (52:48)	3.4 g EFE kg^{-1} TMR DM	23.8	35.8	36.2	3.56	2.89		1.51		
	dairy cows[1]	BS, AS, AH (52:48)	control	24.5	38.1	36.5	3.29	2.95	1.58	1.50		Holthausen
Econase	(60)	BS, AS, AH (52:48)	0.5 ml EFE kg^{-1} TMR DM	**22.9‡**	38.3	36.1	3.19	3.01	1.67	1.58		et al. 2011
Econase		BS, AS, AH (52:48)	1.0 ml EFE kg^{-1} TMR DM	**22.2†**	37.9	36.3	3.26	3.03	**1.75†**	**1.67†**		

Table 6. continued

Enzyme	Animals (n)	Diet (forage:concentrate)	treatment	DMI	milk yield	FCM 3.5% *4.0%*	Milk fat	Milk protein	Feed efficiency kg milk/ kg DMI	kg FCM/ kg DMI	kg ECM/ kg DMI	Reference
	dairy cows[2]	Tifton85 (45:55)	control	20.9	33.1	31.8	3.67	2.91	1.64			Dean et al.
Promote	(30)	Tifton85 (45:55)	EC 4 g EFE d^{-1} cow^{-1}	21.6	30.9	29.9	3.78	**3.07‡**	1.46			2013
Promote		Tifton85 (45:55)	ETMR 4 g EFE d^{-1} cow^{-1}	22.4	32.3	32.4	**3.99‡**	**3.07‡**	**1.42‡**			
Promote		Tifton85 (45:55)	EF 4 g EFE d^{-1} cow^{-1}	19.9	31.2	30.0	3.77	3.03	1.64			
Promote		Tifton85 (45:55)	ES 9 g EFE d^{-1} cow^{-1}	21.8	32.3	30.6	3.72	2.90	1.59			
	dairy cows[1]	CS, GS (60:40)	control	16.6	30.2	*30.8*	4.16	3.14	1.93	*1.99*		Peters et al.
Roxazyme	(28)	CS, GS (60:40)	4.6 g EFE kg^{-1} TMR DM	17.4	30.4	*30.4*	4.04	3.16	1.89	*1.82*		2015
	dairy cows[2]	CS, GS (60:40)	control	18.9	30.7	*31.9*	4.36	3.25	1.64	*1.73*		
Roxazyme	(26)	CS, GS (60:40)	4.5 g EFE kg^{-1} TMR DM	19.5	31.2	*32.5*	4.23	3.28	1.66	*7.72*		

†Differences between control and EFE treatment were significant ($p<0.05$)

‡EFE treatment tend to differ from control ($0.05 \leq P < 0.10$)

[1] early lactation; [2] mid-lactation; [3] late-lactation

CS=corn silage; GS=grass silage; GH=grass hay; AH=alfalfa hay; GGH=Guinea grass hay, BS=Barley silage

Miller et al. 2008: 6.7 kg concentrate d^{-1} cow^{-1} and pasture. Dean et al. 2013: Enzyme applied to bermudagrass Tifton85 at ensiling (ES, 1.3 g/kg DM); enzyme applied at feeding at the rate of 4 g/head/d at mixing to the concentrate (EC), at feeding to the TMR (ETMR) or the forage (EF). 1.3 g EFE kg^{-1} DM

6 Conclusions

Based on the results obtained in this thesis the following conclusion can be drawn:

- The EFE supplementation with Roxazyme G2 liquid (dosage: 3.8 and 6.2 ml/kg TMR DM) to a TMR based on grass and corn silage fed to dairy cows in different stages of lactation had no significant effect on rumen fermentation, microbial protein synthesis, nutrient digestion, energy balance and production performance.
- Furthermore, EFE addition under our experimental conditions did not show the potential to decrease the risk of metabolic disorders in early and mid-lactating dairy cows.
- The results of the current study do not render a general evaluation of the effectiveness of EFE supplementation to dairy cows.

Together with the results of scientific publications on EFE supplementation to dairy cow rations over the past 15 years the following general conclusions can be drawn:

- The large variability in experimental methods applied in EFE studies limit the comparability of EFE effects and impedes a general assessment.
- Considering the substrate and ration specificity of EFE products and the fact that rations for ruminants are generally more heterogeneous than rations for monogastric animals it is obvious that the implementation of substrate-matched EFE supplementation in on-farm ruminant feeding systems is limited.
- EFE supplementation as a possible mitigation strategy to reduce methane emission in dairy cows is not confirmed.
- More investigations are needed to clarify the mode of action and efficacy of EFE supplementation on dairy cows in different feeding systems with a special focus on EFE mixtures specifically adapted to the substrates from different ration types.

7 Summary

Effects of an exogenous fibrolytic enzyme additive on ruminal fermentation, nutrient digestion, performance and health status in early and mid-lactation dairy cows.

The ever increasing challenges on global food security, diminishing natural resources and climate change effects require resource use efficiency improvements also in the dairy industry to reduce negative effects on land use and global warming. One attempt in animal nutrition is the improved feed utilization in dairy cows.

The application of exogenous fibrolytic enzyme (EFE) preparations in different feeding systems of ruminants to improve feed digestibility and nutrient utilization has been investigated and discussed over the last 15 years. The first paper in this study, a literature review, revealed a high variability of experimental results using EFE in dairy cow feeding. However, there are indications that a yield improvement can be expected through the application of EFE especially during early lactation. Cows in this stage of lactation have a higher passage rate with suboptimal rumen conditions for fiber degradation. Since only few studies are available with a focus on the application of EFE in different lactation stages and in intensive dairy feeding systems based on corn silage and grass silage, this study was aimed at investigating the effect of EFE on rumen fermentation, digestibility of nutrients, feed intake, energy balance, milk yield und health status of dairy cows in early and mid-lactation.

The Friedrich-Loeffler-Institute (FLI) applied the enzyme product Roxazyme G2 liquid (RG2 liquid of DSM Nutritional Products, Basel, Switzerland, authorized as feed additive for monogastric animals) in a complex investigation involving 3 experiments with dairy cows and 1 experiment with wethers.

The EFE product RG2 liquid contained 8,000 U/ml endo-1,4-ß glucanase (EC 3.2.1.4), 18,000 U/ml Endo-1,3 (4)-ß glucanase (EC 3.2.1.6) and 26,000 U/ml endo-1,4-ß xylanase (EC 3.2.1.4) specified by the manufacturer. All four experiments used a total mixed ration (TMR) with and without enzyme product (TMR enzyme and TMR control).

The digestibility study involved six lactating German Holstein dairy cows fitted with rumen and duodenal cannulae. The EFE product RG2 liquid was applied with a dosage of 6.2 ml/kg TMR (DM basis) after dilution with water at a rate of 1:5. The ration contained 30 % corn silage, 20 % grass silage and 50 % concentrate, on DM basis and was fed according to the anticipated DM intake of the experimental cows to avoid feed residuals. Data were obtained from 4 cows in each of the three experimental periods extending over four weeks each. The first two weeks of each period were used to adapt the cows to the diets and during the third and fourth week rumen fluid, duodenal chymus and feces were sampled. Rumen fluid was taken on two consecutive days of week three immediately. On the first day at 0, 1, 3, 4, and 6 h and at the second day at 0.5, 2, 3.5, and 5 h after the morning feeding. Duodenal chymus was sampled in two hours intervals over five days in week four. For duodenal flow measurements Cr_2O_3 was used as a marker. Feces samples for the determination of acid-insoluble ash were taken on five days in week four of each period. Milk samples were taken on two days during the third and fourth week of each period. Milk yield was measured based

on the records of the last 12 days of each period. The EFE application did not affect rumen fermentation, apparent ruminal nutrient digestibility, microbial protein synthesis and apparent total tract digestibility.

The digestibility of crude nutrients and energy content in both experimental rations (TMR enzyme and TMR control) was measured using wethers. The content of net energy for lactation (NEL), based on the obtained digestible crude nutrients and the calculated energy content of the rations was 7.2 MJ NEL/kg TMR (DM basis).

Two feeding trials (20 days adaptation and 56 days experimental periods) involved 28 dairy cows during early lactation (< 100 days in milk, feeding trail 1) and 26 dairy cows in mid lactation (> 100 days in milk, feeding trial 2). All cows were randomly allocated to either the TMR enzyme or the TMR control group. Cows were fed with a TMR containing 40% corn silage, 20% grass silage and 40% concentrate, on DM basis. The Enzyme product RG2 liquid with applied with a dosage of 3.9 and 3.8 ml /kg TMR (DM basis). The enzyme product, diluted with water at a rate of 1:10, was applied to the TMR per sprinkler can during the mixing process once per day before the morning feeding.

Dry mater intake, water intake, milk yield parameter, the calculated energy balance, and feed efficiency were sampled or measured daily during the 56 day experimental period. Serum concentration of ß-hydroxybutyrat, total protein, bilirubin, liver enzymes, aspartatamino transferase, and gamma glutamyl transferase were measured on days 28 and 56.

During the second feeding trial rumination frequency during days 14, 28, 42, and 56 over a 24 hour period each was added to the investigation.

The application of EFE did not show significant effects on DM intake, milk yield, energy balance, feed efficiency and metabolic health. In both experiments DM intake was below 20 kg DM / day, which is low for German Holstein. This leads to the expectation that the ruminal retention period must have been sufficient for an optimal NDF digestion and therefore not showing any possible effect of the EFE application.

A specific EFE effect during early lactation of dairy cows was not observable in these experiments in which cows had comparable yields irrespective of lactation stage. The rumination frequencies measured during the second experiment were also not affected by an EFE supplementation which indicates that EFE did not influence the structural effectiveness of fiber negatively.

8 Zusammenfassung

Einfluss einer Zulage exogener fibrolytischer Enzyme auf Pansenfermentation, Nährstoffverdaulichkeit, Leistung und Gesundheitsstatus von Milchkühen im frühen und mittleren Laktationsstadium.

Mit steigenden Anforderungen der Welternährung, knapper werdenden Ressourcen und dem fortschreitendem Klimawandel steht auch die Milcherzeugung unter dem Druck die Ressourceneffizienz zu verbessern und negative Umwelteffekte zu vermeiden. Ein Ansatzpunkt der Tierernährung zur Steigerung der Ressourceneffizienz, ist die Verbesserung der Nährstoffnutzung von Milchkühen. Der Einsatz von Fütterungsadditiven mit exogenen faserspaltenden Enzymmischungen (EFE) in den verschiedenen Fütterungssystemen von Wiederkäuern zur Verbesserung der Futterverdaulichkeit und damit der Nährstoffnutzung wird seit den letzten 15 Jahren untersucht und diskutiert. Das erste Kapitel dieser Arbeit, eine Literaturübersicht, zeigt die hohe Variabilität der Untersuchungsergebnisse von Studien zum Einsatz von EFE in der Milchkuhfütterung. Aus dieser Literaturstudie ergeben sich allerdings Hinweise auf eine mögliche Leistungssteigerung durch eine Zulage von EFE bei Milchkühen insbesondere in der Frühlaktation. Die mögliche besondere Wirksamkeit von EFE in der Frühlaktation erklärt sich aus der hohen Passagerate in diesem Laktationsstadium und den daraus resultierenden ungünstigeren ruminalen Bedingungen für den Faserabbau. Da nur wenige Untersuchungen zum Einsatz von EFE in der intensiven Milchkuhfütterung basierend auf Mais- und Grassilage als Hauptrationskomponente vorliegen, war es Ziel dieser Arbeit den Einfluss von EFE auf Pansenfermentation, Verdaulichkeit der Nährstoffe, Futteraufnahme, Energiebilanz, Milchleistung und Gesundheitsstatus zu untersuchen.
Am Friedrich-Loeffler-Institute (FLI) wurde das Enzympräparat Roxazyme G2 liquid (RG2 liquid) von DSM Nutritional Products, Basel Schweiz, das in der EU für monogastrische Nutztiere als Futterzusatz zugelassen ist, in einem komplexen Untersuchungsansatz mit 4 Einzeluntersuchungen an Milchkühen geprüft. Das verwendete Enzympräparat Roxazyme G2 liquid enthält laut Herstellerangaben 8000 U/ml Endo-1,4-ß Glucanasen (EC 3.2.1.4), 18000 U/ml Endo-1,3(4)-ß Glucanasen (EC 3.2.1.6) and 26000 U/ml Endo-1,4-ß Xylanasen (EC 3.2.1.4). In den 4 Untersuchungen wurde eine totale Mischration (TMR) mit und ohne Enzymzusatz (TMR-Enzym und TMR-Kontrolle) gefüttert.
Für die in vivo Verdaulichkeitsuntersuchung standen 6 laktierende Pansen und Duodenum fistulierte Milchkühe der Rasse Deutsche Holstein zur Verfügung. Das Enzympräparat Roxazyme G2 liquid wurde in einer Dosierung von 6,2 ml /kg TMR (T-Basis) verwendet. Vor der Applikation erfolgte eine Verdünnung der Enzymlösung mit Wasser im Verhältnis 1:5. Die Ration bestand auf T - Basis zu 30 % aus Maissilage, zu 20 % aus Grassilage und zu 50 % aus Kraftfutter. Die gefütterten Mengen orientierten sich an der erwarteten Trockensubstanzaufnahme der Tiere, um Futterreste zu vermeiden. Aus versuchstechnischen Gründen standen in den drei Versuchsperioden jeweils 2 Kühe pro Behandlung zur Verfügung. Zwei weitere Tiere waren in eine parallele Versuchsanstellung involviert. Eine Versuchsperiode ersteckte sich über insgesamt vier Wochen, mit zwei

Wochen zur Adaptation und zwei Wochen für Probenentnahme. Die Pansensaftentnahme fand an zwei aufeinander folgenden Tagen in der dritten Woche statt. Am ersten Tag erfolgte die Pansensaftentnahme zu den Zeitpunkten 0, 1, 3, 4 und 6 h sowie am zweiten Tag um 0,5; 2; 3,5; und 5 h nach der Morgenfütterung. Duodenalchymus wurde in zweistündigen Intervallen über fünf Tage in der vierten Woche gesammelt. Für die Bestimmung des duodenalen Nährstoffflusses kam Cr_2O_3 als Marker zum Einsatz. Kotproben zur Bestimmung der säureunlöslichen Asche wurden über 5 Tage in Woche 4 gesammelt. Die Milchprobennahme erfolgte an zwei Tagen in den Wochen 3 und 4. Die statistische Auswertung der Milchleistungsdaten basierte auf der Milchmengenmessungen der letzten 12 Tage jeder Versuchsperiode. Eine Zugabe von EFE führte zu keiner Veränderung von Pansenfermentation, scheinbarer ruminaler Nährstoffverdaulichkeit, mikrobieller Proteinsynthese, scheinbarer Gesamtverdaulichkeit und Milchleistung.

Die Rohnährstoffverdaulichkeit und Energiekonzentration der beiden Versuchsrationen der Fütterungsversuche (TMR-Enzym und TMR-Kontrolle) wurden in Verdauungsversuchen mit Hammeln gemessen. Die aus den verdaulichen Rohnährstoffen berechneten Energiegehalte betrugen für beide Versuchsrationen 7,2 MJ NEL/kg T-TMR.

In zwei Fütterungsversuchen wurden jeweils eine Gruppe mit 28 Kühen in der Frühlaktation (< 100 Tage post partum, Fütterungsversuch 1) und eine Gruppe von 26 Kühen in der mittleren Laktation (> 100 Tage post partum, Fütterungsversuch 2) randomisiert den beiden Versuchsrationen TMR-Kontrolle und TMR-Enzym zugeteilt. Die Tiere erhielten in beiden Versuchen eine TMR mit 40% Kraftfutter, 40% Mais- und 20% Grassilage auf T-Basis, die ihnen ad libitum zur Verfügung stand. Das Enzympräparat RG2 liquid wurde in den beiden Fütterungsversuchen in einer Dosierung von 3,9 bzw. 3,8 ml /kg TMR T zugesetzt. Die Applikation der Enzyme erfolgte einmal täglich vor der Morgenfütterung. Hierfür wurde die kalkulierte Menge RG2 liquid mit Wasser im Verhältnis 1:10 verdünnt und der TMR mit einer Gießkanne während des Mischvorganges im Futtermischwagen zugegeben. Nach einer Adaptationszeit von jeweils 20 Tagen wurden über eine Versuchsdauer von jeweils 56 Tagen die tägliche Trockensubstanz- und Wasseraufnahme, die Milchleistungsparameter, die kalkulierte Energiebilanz, die Futtereffizienz sowie an den Versuchstagen 28 und 56 die Serumkonzentrationen von ß-Hydroxybutyrat, Gesamtprotein, Bilirubin sowie den Leberenzymaktivitäten Aspartataminotransferase, und Gamma-Glutamyltransferase erfasst. Zusätzlich wurde im zweiten Fütterungsversuch die Wiederkauaktivität an den Versuchstagen 14, 28, 42 und 56 jeweils über 24 h beobachtet.

Die aufgestellte Hypothese, dass die Zulage von EFE bei Milchkühen einen günstigen Effekt auf die Trockensubstanzaufnahme, Leistung, Energiebilanz, Fütterungseffizienz und Stoffwechselgesundheit hat, konnte mit den Ergebnissen, die keinen signifikanten Einfluss der EFE Gabe auf die Untersuchten Parameter zeigten, nicht bestätigt werden. In beiden Fütterungsversuchen lag die Trockensubstanzaufnahme auf einem für die Rasse Deutsche Holstein relativ niedrigem Niveau (< 20 kg T/d), was die Vermutung zulässt, dass die ruminale Retentionszeit für eine optimale NDF Verdaulichkeit ausreichend war, und daher die EFE Gabe zu keiner Verbesserung führte. Die Abhängigkeit der Wirkung der EFE Gabe vom Laktationsstadium konnte ebenfalls durch die Versuchsergebnisse nicht bestätigt

werden, da in beiden Versuchen trotz der unterschiedlichen Laktationsstadien ein vergleichbares Leistungsniveau bestand. Die Ergebnisse der Wiederkaubeobachtungen im zweiten Fütterungsversuch zeigen ebenfalls keinen Einfluss der EFE Gabe. Damit konnte ein Effekt der EFE Gabe auf die Strukturwirksamkeit der Faser ausgeschlossen werden.

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EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation: „Effects of an exogenous fibrolytic enzyme additive on ruminal fermentation, nutrient digestion, performance and health status in early and mid-lactation dairy cows ” selbständig und nur unter der Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe. Die Arbeit lag bisher in gleicher oder ähnlicher Form keiner Prüfungsbehörde vor.

Falkensee, den 11.11.2015 Anja Peters

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