Szent István University
Postgraduate School of Veterinary Sciene

Use of fibrolytic enzymes produced by the fungus *Thermomyces lanuginosus* in ruminant nutrition

PhD thesis

by

Dr. Viktor Jurkovich, DVM

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Szent István University
Postgraduate School of Veterinary Science

Supervisor:

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Prof. Dr. **Pál Rafai**, DVM, DSc
Department of Animal Hygiene,
Herd Health and Veterinary Ethology
SzIU Faculty of Veterinary Science

Members of the committee:

Prof. Dr. **Endre Brydl**, DVM, CSc
Head of the Department
of Animal Hygiene, Herd Health
and Veterinary Ethology
SzIU Faculty of Veterinary Science

Prof. Dr. **Ottó Szenci**, DVM, DSc
Head of the Large Animal Clinic,
SzIU Faculty of Veterinary Science

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Dr. Viktor Jurkovich, DVM

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Abbreviations

2h	2 hours after feeding
4h	4 hours after feeding
AA	amino acid
AcAc	aceto-acetate
ADF	acid detergent fibre
ADG	average daily gain
A:P	acetate:propionate ratio
AST	aspartate aminotransferase
BCVFA	branched-chain VFA
BCS	body condition score
BW	body weight
CMC	carboxymethyl cellulose
CO	control period/group
DIM	days in milk
DFM	direct fed microbials
DM	dry matter
DMI	dry matter intake
EC	European Commission
ECM	energy corrected milk
EFE	exogenous fibrolytic enzyme
EX	experimental period/group
FCM	fat corrected milk
FCR	feed conversion rate
FI	final period
FXU	fibre xylan unit
mM	mmol/l
NABE	net acid base excretion
NDF	neutral detergent fibre
NEFA	non esterified fatty acid
NSP	non starch polysaccharide
OM	organic matter
Rusitec	rumen simulation technique
SCAN	Scientific Committee on Animal Nutrition
SE	standard error
T0	before feeding
TVFA	total VFA
VFA	volatile fatty acid

Summary

A strain of *Thermomyces lanuginosus* NCAIM 001288 was used to produce an enzyme preparation. The obtained product (Rumino-Zyme) contains thermally resistant endo-1,4-beta-xylanase with 250 FXU/g activity. Investigations focused on the efficiency of the xylanase product from *T. lanuginosus* in ruminants.

In the first trial we characterised the stability of the xylanase preparation in the rumen of merino wethers. A single dose of 10 g enzyme preparation applied directly into the rumen increased the base-line xylanase activity of the rumen fluid by about 300 % within 5 min after treatment. Total xylanase activity of the rumen decreased to only 63% till the 45th min. After 60 and 90 min the original enzyme activity decreased to 41% and 34%, respectively. Between 90 and 120 min after treatment the enzyme activity settled at a level, slightly over 30% of the original. Three hours after treatment no increment in the enzymatic activity was seen and the activity returned to initial values.

In our second trial the effects of the enzyme product on rumen fermentation characteristics of merino wethers were measured. Adding 2.5 g/sheep/day enzyme preparation to the sheeps' diet had increased the xylanase activity of the rumen fluid in the experimental group compared to the control. There was no change in the pH value of the rumen fluid. Total VFA concentration was higher after feeding in the enzyme supplemented period compared to the control. The molar proportion of acetate was affected by the enzyme supplementation, it was higher in the experimental period than that in the control. The molar ratio of propionate tended to be higher in the enzyme supplemented period but it did not differ significantly from the control. The molar ratio of butyrate was significantly lower then control values in the experimental phase. Ammonia concentration of the rumen fluid was lower in the experimental period than in the control 4 h after feeding.

In our third experiment the effects of the enzyme preparation from *T. lanuginosus* were studied on ruminal VFA concentration, parameters of energy and protein metabolism, milk yield, feed conversion ratio and body condition score of high yielding dairy cows in early lactation (from calving to 110th DIM). The preparation administered in a dose of 30 g/cow/day increased TVFA concentration in the rumen fluid. The milk yield significantly increased in the enzyme supplemented group. There was a better balanced energy metabolism in the experimental cows as indicated by lower incidence rate of hyperketonaemia and lower aceto-acetate and NEFA concentration in the blood and plasma samples. Feed intake and feed conversion rate were also better in the experimental group. Due to the better balanced energy metabolism postparturient body condition loss of the enzyme treated cows was reduced.

In our fourth trial the effects of the direct-fed NSP degrading enzyme preparation were evaluated on the health, milk yield and milk quality of dairy cows in mid-lactation. As a result of this experiment we can conclude that there were no significant differences between the experimental and the control group regarding major blood and urine parameters which are in strong correlation with the health status. Thus enzyme supplementation presumably did not have an adverse effect on the health of the animals though it did not improve metabolic parameters. Our results indicate that feeding NSP enzyme supplement to cows in mid-lactation cows do not improve milk and FCM yield, milk fat and milk protein content significantly. Advantages of oral administration of exogenous enzymes to cows in mid-lactation are uncertain and it is always necessary to calculate the economic benefits.

1. Introduction

Dairy cows of our time are products of genetic selection sustained for many generations, so thus capable of tremendous milk production. The considerable increase in volume and efficiency of ruminant production taken place in the past half century have been beneficial for both producers and consumers. Greater feed consumption and milk yield have demanded higher metabolic capacity of the cow. Consequently, feed components that traditionally were not supplemented must now be added to the diet. Today the ruminal microflora benefits from compounds that had little effect in the past.

Improved feeding standards, in particular, owe much to the growth in the knowledge of ruminant physiology and digestion that has occurred during the past four to five decades. No sooner than it has been established that digestive processes of all animals involve breaking down macro molecules of nutrients by endogenous enzymes, animal nutritionists attempted to enhance these processes by applicating exogenous enzymes. Livestock nutrition has undergone major changes that are, among others, due to the development of microbial feed additives. These feed additives (yeast cultures and enzymes) play an important role in the digestion of nutritional elements in feedstuffs previously not available.

Direct fed microbials or their derivates are mixed to feeds in order to either directly aid digestion in the (fore)stomach and intestines or enzymatically enhance degradation of NSPs. The most commonly used DFM in ruminant diets is the yeast *Saccharomyces cerevisiae.* Microbial preparations are produced in an industrial scale from bacterial and fungal cultures. The main components of the enzyme mixtures are cellulase, xylanase, 1,4-beta-endoglucanase, beta-glucosidase, alfa-amylase and alfa-galactosidase.

Exogenous polysaccharidase enzymes have been most successfully used in the feeding of monogastric animals, specially broiler chickens. Although studies on the use of exogenous proteolytic or fibrolytic enzymes in ruminant diets have been carried out since the 1960s, publications concerning their possible application in the feed of ruminants have significantly grown in number in only the last 15 years. Though results obtained with exogenous fibrolytic enzymes or microbes producing EFEs are far from being unequivocal, use of such enzymes is likely spread.

Adding yeast or enzyme preparations to dairy rations presents us with several challenges. The stage of gestation and lactation influence which additive to choose. Only cows in certain stages of lactation or gestation respond economically. Targeted animal responses are: increase in milk yield, milk components, and dry matter intake; increased rumen microbial synthesis of protein or VFAs through improved fibre degradability; improved weight gain, minimized weight loss after calving, lower incidence of metabolic disorders and overall improvement in general health. Once the additive's role is defined, the cows' response is to be monitored on the farm to assure the additive is needed.

In light of the facts mentioned above, the use of enzymes to improve the quality of ruminant diets seems to be a greater challenge than in case of monogastric animals since the digestive tract of ruminants is far more complex than that of chickens and ruminants digest non-starch polysaccharides with high efficiency. Still the use of enzymes as additives in ruminant nutrition has a great potential. The positive and in some respect controversial data of the relevant literature have prompted us to study the properties of a new polysaccharidase enzyme preparation of the thermophilic fungus *Thermomyces lanuginosus* and its effects on sheep and dairy cows.

2. Review of literature

2.1. Digestion of forages in the rumen

2.1.1. Composition and function of plant cell wall

Cell walls consist of 3 types of layers (Selvendran, 1984; Saupe, 2002; Hangarter, 2003):

- Middle lamella: the first layer formed during cell division. It makes up the outer wall of the cell and is shared by adjacent cells. It is composed of pectic compounds and protein.
- Primary wall: formed after the middle lamella and consists of a rigid skeleton of cellulose microfibrils embedded in a gel-like matrix composed of pectic compounds, hemicellulose, and glycoproteins.
- Secondary wall: formed after cell enlargement is complete. The secondary wall is extremely rigid and provides compression strength. It is made up of cellulose, hemicellulose and lignin. The secondary wall is often layered.

The cell wall has several functions and serves a variety of purposes including (Saupe, 2002; Hangarter, 2003).

- *Maintaining/determining cell shape*. Since protoplasts are invariably rounded, the wall determines the shape of plant cells.
- Providing *support* and *mechanical strength*.
- *Prevent*ing the cell membrane from bursting in a hypotonic medium.
- Controlling the rate and direction of *cell growth* and regulates cell volume.
- Determining plants' *structural design* and controlling plant *morphogenesis* since the wall determines that plants develop by cell addition.
- *Metabolic functions*. Some of the proteins in the wall are enzymes for transport and secretion.
- *Physical barrier* to: (a) pathogens; and (b) water in suberized cells. However, the wall is very porous and allows free passage of small molecules. Pores are about 4 nm in diameter.
- *Carbohydrate storage.* The components of the wall are reused in other metabolic processes (especially in seeds).
- *Signalling*. Fragments of wall, called oligosaccharins, act as hormones. Oligosaccharins, deriving from normal development or defence mechanisms, serve a variety of functions including: (a) stimulating ethylene synthesis; (b) inducing phytoalexin synthesis; (c) inducing chitinase and other enzymes; (d) increasing cytoplasmic calcium levels and (d) participating in the "oxidative burst". This burst produces hydrogen peroxide, superoxide and other active oxygen radicals that attack pathogens directly or cause multiply cross-links in the wall making it harder to penetrate.
- *Recognition responses*. The wall of roots of legumes is important in the nitrogen-fixing bacteria colonizing the root to form nodules. Pollen-style interactions are mediated by wall chemistry.
- *Economic use*. Cell walls are important for products such as paper, wood, energy, shelter, and even fibre in the diet.
  The main components of cell walls are polysaccharides (or complex carbohydrates) which are

synthesized from monosaccharides (or simple carbohydrates). Eleven different sugar monomers are common in these polysaccharides including glucose and galactose. Major wall components are: cellulose, cross-linking glycans, pectic polysaccharides, proteins, lignin, a variety of lipids (like suberin, wax, cutin) and water (Selvendran, 1984; Carpita and Gibeaut, 1993; Saupe, 2002; Hangarter, 2003). In respect of the subject of present thesis hemicellulose compounds are most important. Cross-linking glycans are a diverse group of carbohydrates formerly called hemicellulose. Characteristically they are soluble in strong alkali. A variety of sugars including xylose, arabinose, mannose compose linear, flat molecules with a beta-1,4 backbone and relatively short side chains. Two common types of sugars are xyloglucans and glucuronarabinoxylans. Other less common ones are glucomannans, galactoglucomannans, and galactomannans. The main feature of this group is that they do not form microfibrils. However, they form hydrogen bonds with cellulose and they are therefore called "cross-linking glycans". There may be a fucose sugar at the end of the side chains which may help to keep the molecules planar by interacting with other regions of the chain. Hemicellulose is in abundance in primary walls and also a component of secondary walls.

2.1.2. Microbial strategies for the decomposition of plant cell wall in the rumen

Composition of the rumen flora

The microbial population in the rumen consists of bacteria, protozoa, and fungi. The majority is made up of bacteria, which can number 10^{10}-10^{11} cells/g of rumen content. Bacteria can be classified according to their three main shapes (cocci, rods, and spirilla), according to their size (generally ranging from 0.3 to 50 μm), and according to differences in their structure. They can also be sorted according to the type of substrate and are categorised into eight distinct groups. Bacteria degrade or utilise products such as cellulose, hemicellulose, starch, sugars, intermediate acids, proteins, lipids and certain species produce methane. An extend classification could include pectin utilisers and ammonia producers (Ischler et al., 1996; Dehority, 2003).

Protozoa in the rumen number about 10^5-10^6 cells/g of rumen content and are influenced by feeding practices. Protozoa are generally found in higher numbers when highly digestible diets are fed. The protozoa actively ingest bacteria as a source of protein. They also appear to be a stabilizing factor for end-products of fermentation (Ischler et al., 1996; Dehority, 2003).

Anaerobic fungi are the most recently recognised group of rumen microbes (Orpin, 1975). When animals are fed with a high fibre diet, rumen fungi may contribute to up to 8 percent of the microbial mass. Rumen fungi have been shown to degrade cellulose and xylans, indicating their role in fibre digestion (Ischler et al., 1996). Their contribution to fibre digestion might be low due to the small biomass (Orpin and Joblin, 1997).

Microbes are located in three interconnecting compartments of the rumen. First is the liquid phase, where free-living microbial groups feed on soluble carbohydrates and proteins. This portion makes up 25% of the microbial mass. Next is the solid phase, where the microbial groups associated with or attached to feed particles digest less soluble proteins and insoluble polysaccharides, such as starch and fibre. These populations are numerically predominant and account for up to 70% of the microbial mass. In the last phase, 5% of the microbes are attached to the rumen epithelium or to the protozoa (Ischler et al., 1996; Koike et al., 2003). Micro-organisms attached to feed particles may be retained in the rumen up to three times longer than those free in the liquid phase and are therefore able to maintain their numbers at a lower growth rate (Faichney, 1980).

Enzyme activity of the rumen content

Among the ruminal microbes, bacteria and fungi produce a wide range of highly active plant cell wall degrading enzymes, and their contribution to fibre digestion is estimated to be 80% of total activity (Dijkstra and Tamminga, 1995). Whilst there is a great diversity in the types of NSP found in feedstuffs, there is also a very large number of carbohydrase enzymes produced by the rumen microflora to degrade them. The yet incomplete list of enzymes produced by rumen microorganisms include cellulase, endoglucanase, exoglucanase, beta-glucosidase, xylanase, xylosidase, acetil-xylan-esterase, acetyl-esterase, alpha-L-arabinofuranosidase, ferulic acid esterase, amylase, arabinase, beta-D-glucuronidase, laminarinase, lichenase, pectinase (Hespell and Whitehead, 1990; Annison and Bryden, 1998). Specific activities of polysaccharidase and glycosidase involved in the degradation of structural polysaccarides, were determined referring to total microbial population, solid attached population and solid firmly attached population in the rumen (Michalet-Doreau et al., 2001). Microbial populations associated with feed particles are estimated to be responsible for 80% of total rumen endoglucanase activity (Minato et al., 1966). Cellulase complexes are mainly associated with the cell walls of the bacteria and other microbes. Usually very low free cellulase activity is measured in the rumen fluid (Annison and Bryden, 1998).

Fibre digestion in the rumen

The microbial degradation of complex polysaccharides in the rumen is accomplished by the cooperative efforts of a range of cellulolytic and non-cellulolytic microorganisms (Akin and Borneman, 1990; Annison and Bryden, 1998). Generally, the total digestibility of the hemicellulose and xylan fraction of forages averages about 50% and takes place primarily in the rumen. The two major xylanolytic species in the rumen are *Butyrivibrio fibrisolvens* and *Bacteroides ruminicola*. (Hespell and Whitehead, 1990). Plant matter entering the rumen is rapidly colonised by bacteria and fungi. Koike et al. (2003) showed that after 5 min of incubation, the number of *Fibrobacter succinogenes* and the two ruminococcal species attached to stems were 10^5 and 10^4/g dry matter of stem. At 10 min, the number of all three species attached to stems increased ten fold. Thereafter, attached cell numbers of the three species gradually increased and peaked at 24 h (10^9/g DM for *F. succinogenes* and 10^7/g DM for *Ruminococcus flavefaciens*) or 48 h (10^6/g DM for *Ruminococcus albus*). There are two explanations of the increased cell populations on the incubated stems. One is the attachment of new bacteria from the liquid phase or other particles of rumen content, the other is their proliferation on the stems (Koike et al., 2003).

Krause et al. (2001) showed that the using of recombinant xylanolytic *Butyrivibrio fibriosolvens* does improve the digestibility of fibre compared to the native, but still not reaches the most potent fibre digesters such as ruminococci. Digestion may be improved by genetic manipulation of ruminal bacteria but ecological parameters, such as persistence in vivo or the niche of the organism, must be taken into account.

Barcroft et al. (1944) confirmed the presence of short chain fatty acids or volatile fatty acids in rumen contents and were the first to recognize that the extent of VFA absorption from the rumen was sufficient to supply an appreciable part of the energy requirements of the animal. Dirksen (1970) showed the diminishing tendency in VFA production with decreasing rumen fluid pH. Mould et al. (1983) concluded and this finding was recently reaffirmed by others (Scholz et al., 2001; Yang et al., 2002), that ruminal cellulolysis was inhibited at pH values < 6.0.

2.2. Exogenous non-starch polysaccharidase enzymes

2.2.1. NSP-ase enzymes in animal nutrition

Modern enzyme technology started to develop in 1874, following the first documented production of a refined enzyme that was prepared from the contents of calves' stomachs. That enzyme, rennet, is still used in cheese making. Since then, the technology to identify, extract and produce enzymes on a commercial scale has progressed dramatically and today they are used in many industrial processes. Enzymes are used in detergents, paper production, leather and textile processing, and the food and feed industry. The global market value of enzymes in 1995 was estimated to worth 1 billion US$ and was forecasted to rise to 1.7-2 billion US$ by 2005 (Sheppy, 2001).

Since the early sixties there have been many attempts to enhance the productive preformance of monogastric animals by using enzyme preparations of different (mostly microbial) origin to improve the degradation of NSPs and phytates (Bedford and Schulze, 1998). Trials have proven this method improves the general health status of animals, thus aids management and economical efficiency. Better nutrient utilisation results in environmental benefits. Manure volume reduced by up to 20% and nitrogen excretion by up to 15% in pigs and 20% in poultry. Similarly significant is the ability of enzymes to reduce phosphorus pollution (Sheppy, 2001). A wide variety of EFE products are marketed for livestock, though they are mainly derived from only four bacterial (*Bacillus subtilis*, *Lactobacillus acidophilus*, *L. plantarum* and *Streptococcus faecium* spp.) and three fungal (*Aspergillus oryzae*, *Trichoderma reseii* and *Saccharomyces cerevisiae*) species. Other fungal species including *Humicola insolvens*, *Trichoderma longibrachiatum, Trichoderma viridis* and *Thermomyces* spp. are on the market too (Wang and McAllister, 2002). In the EU, there are numerous enzyme products and DFMs which had undergone the strict registration process and are now authorised zootechnical additives of swine and poultry (European Commission, 2002a, 2002b, 2003). There is a great potential developing the enzyme supplementation of monogastric feeds. By 1996 over 80% of all European broiler diets that contained cereals (barley, wheat, etc.) contained a fibre degrading enzyme also. In a global perspective, current estimates suggest that approximately 65% of all poultry feed containing viscous cereals also contain a fibre degrading enzyme. Penetration into the swine industry is considerably lower, approaching 10%. Despite the impressive upgrade in technology, only approximately 10% of monogastric feeds are supplemented with enzymes giving a total market value of over 100 million US$ in 1999-2000. The phytase market is currently worth up to 50 million US$, of which appr. 8% is contributed by swine and poultry feeds containing phytase (Sheppy, 2001).

Exogenous fibrolytic enzymes have not been traditionally used in feeds of ruminants however the use of EFE has already piqued the interest of ruminant nutritionists over 40 years ago (Bowden and Church, 1959; Burroughs et al., 1960; Rovics and Ely, 1962; Rust et al., 1965). Since the end of the '80s researchers have been re-examining the role of EFE in ruminant production (results described in chapter 2.3) and modern enzyme production technologies significatly contribute to the progress of research. No data are available on the market size and value of enzyme supplements for cattle rations. In 1998, several registered enzyme products were registered in Canada to add to ruminant feeds. Major active ingredients in most of these products were DFMs, microbial extracts – with generally low fibrolytic activity – and vitamin/mineral additives (Rode and Beauchemin, 1998). In the EU, only four DFM products (mainly *S. cerevisiae* strains) are registered in the EU as feed

additives for dairy or beef cattle, and no enzyme product is registered for these animals (European Commission, 2002a, 2002b, 2003, 2004).

2.2.2. EU regulation on the use of direct fed microbials and supplemental enzymes

Since 1970, the use of additives in animal feeds in Europe is regulated by law (Directive 70/524/EEC). This directive was adapted in 1994 to include micro-organisms and enzymes (Directive 94/40/EEC). Directive 94/40/EC was replaced by Directive 2001/79/EC incorporating two parts, one for chemical additives and other substances, and one for micro-organisms and enzymes (Guillot, 2003).

Since 1996, the request for EC authorization of a probiotic is to be accompanied by a dossier drawn up in accordance with the guidelines and the opinion of the SCAN on the safety of the microbiological strains and efficacy of the additive (Guillot, 2003).

An additive is a product intended to improve animal production (animal performance), in particular by affecting the gastro-intestinal microflora or the digestibility of feedingstuffs, therefore, as a part of the assessment process, the amended guidelines (Directive 87/153/EEC) require demonstration of efficacy of microbial products and enzymatic preparations with appropriate data, referring to each target species, in terms of animal production. Improvement in animal performance is expressed in terms of weight gain, feed conversion rate, or in the improvement of quality and yield of animal products, animal welfare or environmentally beneficial effects (European Commission, 2000a, 2000b). Digestion is a complex process influenced by feed intake, passage rate, degradation rate and extent, fluidity of the intestinal contents and finally the rate and extent of absorption of end-products. This confirms the fact that in vitro studies can only provide us indication and can not substitute in vivo trials, therefore the efficacy of enzymes must be determined via the response of the animal (European Commission, 2000a, 2000b). Animal response can be assessed by several experimental methods such as animal performance studies, digestion or balance studies, and in some cases other kinds of studies as it is written in Directive 87/153/EEC. SCAN suggests that each claimed effect in each target animal category should be confirmed with at least three experiments providing significant results ($p<0.05$). Dealing with ruminants, a homogene group of test animals is not easy to obtain, so it is acceptable to use a level of probability of $p<0.10$ (European Commission, 2000a). In addition, producers must confirm that their products are harmless to the animals (with tolerance tests at the level of at least 10 times the maximum proposed dose) and safe to both users and consumers (European Commission, 2002a).

2.2.3. Stability of exogenous fibrolytic enzymes in the ruminal environment

Enzymes have pH and temperature optimals at which they are most effective. For example the cellulase enzyme complex from the fungal species *Trichoderma* has a pH and temperature optimal of 4.5 at 50 ºC (Kung, 2001).

Previous studies suggested that supplemental NSP-enzymes were rapidly decomposed in the rumen due to the protease activity of the microflora. Kopecný et al. (1987) reported that a cellulase enzyme complex from *Trichoderma reseii* was rapidly inactivated by rumen bacterial proteases and adding it to rumen fluid had no effect on in vitro fibre digestion. Some have suggested that feeding

unprotected enzymes may be more useful in immature ruminants where rumen microbial populations are not fully developed. For example Baran and Kmet (1987) reported that a pectinase-cellulase enzyme additive improved ruminal fermentation in newly weaned lambs but not in adult sheep (with evolved rumen microflora).

Recently there has been renewed interest in the use of enzymes in ruminant feeds because some fibrolytic enzymes have been shown to be remain intact when incubated with proteases. Hall et al. (1993) published that the endoglucanase from *Clostridium thermocellum* was completely resistant to inactivation by intestinal proteases in mice. Fontes et al. (1995) incubated NSPase enzymes for 180 min. at 37 ºC in the presence of bovine alpha-chymotrypsin or porcine pancreatin. Cellulase and xylanase enzymes proved to preserve their activity throughout the treatment, while the half-life of endoglucanase decreased to 10 and 70 min, respectively. In their *in vitro* experiments Hristov et al. (1998a) also demonstrated remarkable resistance of carboxymethyl-cellulase, xylanase, beta-glucanase to microbial fermentation, viz. no significant decline in enzyme activity was observed in the first 6 h of incubation. Their *in vivo* experiments showed that peaks in CMC-ase, xylanase, beta-glucanase activities were observed within 1.5 h after treatment with a significant stability thereafter. Morgavi et al. (2001) incubated four commercially available preparations of fibrolytic enzymes (from *Irpex lacteus*, *Trichoderma viridae*, *Aspergillus niger* and their mixture) *in vitro* with rumen digesta of sheep. NSP-enzymes showed different stability. CMC-ase and xylanase from the fungus *Aspergillus niger* was stabile for over 6 h, while beta-glucosidase and beta-xylosidase were much more labile. In an other study (Morgavi et al., 2000b) different commercial products from *Trichoderma longibrachiatum* were tested in rumen fluid. It was concluded that the enzyme additives were relatively stabile in the rumen fluid and resistant to microbial degradation for a time long enough to act in the rumen. However stability of enzymes depended on the kind of preparation and also on the enzymatic activity assayed.

Nsereko et al. (2000a) showed that the application method is an important factor. In their experiment extracts of 14 barley silages inhibited the endo-1,4-beta-xylanase and alpha-amylase of a ruminant feed additive from *Trichoderma longibrachiatum* by 23 to 50 % but had little effect on the cellulase activity. They concluded that barley silages contain low molecular weight thermostable factors that inhibit the enzymes of *T. longibrachiatum*. The results suggest that higher concentrations of feed enzymes are required for silage compared to dry feedstuffs.

In their study, van de Vyver et al. (2004) demonstrated that glycosylation is an important factor in the protection of enzymes from proteolytic degradation. The deglycosylated xylanase lost its activity faster than glycosylated xylanase which was remarkably stable in rumen fluid even up to 6 h of incubation.

2.2.4. Effect of the exogenous fibrolytic enzymes on ruminal fermentation

The results about the stability of EFE in the rumen raises the possibility of using them to manipulate ruminal digestion.

Two main theories exist on the mode of action of the EFEs in monogastric animals (Bedford and Schulze, 1998). One is based on the fact that cell walls of the cereal endosperm composed of xylans, beta-glucans and cellulose, encapsulate starch and protein. Thus the contents of intact endosperm cells escape digestion. Exogenous enzymes which degrade such structures effectively weaken the cell wall thus facilitate digestion. The alternative hypothesis supposes that cell wall components dissolve in the digestive tract and interact to form high molecular weight, viscous aggregates.

Presumably several other effects take place in the rumen as listed below (McAllister et al., 2001).

a) Preconsumptive effects of EFE may be as simple as the release of soluble carbohydrates or as complex as the removal of structural barriers limiting microbial digestion of the feed in the rumen. EFE can release reducing sugars from feed prior to consumption. This is called the indirect use of enzymes (Annison, 1997) and is often used in silages and other conserved forages.

b) Within the rumen EFEs can act directly on the feed particles by the hydrolysis of cell walls. A study of Castañón et al. (1997) on barley and rye proved that enzyme preparations have two concomitant effects on cereal NSPs. Enzymes solubilise insoluble NSP, and hydrolyse them along with the originally soluble sorts. EFE may increase xylanase and cellulase activity in the rumen (Hristov et al., 1998a; 1998b; 2000). Enzyme activity in the fluid usually adds up to less than 30% of the total enzyme activity in the rumen, the remainder is associated with feed particles (Minato et al., 1966; Huhtanen and Khalili, 1992; Michalet-Doreau et al., 2001). However, added EFEs represent a small fraction of the ruminal enzyme activity (may contribute to only 15%, Rode and Beauchemin, 1998), exogenous enzymes can enhance fibre digestion by ruminal microorganisms in vitro (Forwood et al., 1990; Varel et al., 1993; Dong et al., 1999; Wang et al., 2001; Yang et al. 2002) and in vivo (Beauchemin et al., 1995, 1999; Lewis et al., 1996; Yang et al., 1999). Usually (Hristov et al., 2000; Lee et al., 2000; Yang et al, 2002) but not in every case (Kung et al., 2000; Bowman et al., 2002; Sutton et al., 2003). TVFA concentration or molar proportion of individual VFAs increase as a result of enzyme supplementation. The rumen microorganisms are inherently capable of digesting fibre (McAllister et al., 1994), therefore it is difficult to imagine how exogenous enzymes would improve ruminal fibre digestion through direct hydrolysis (McAllister et al., 2001).

c) Enhancement of fibre digestion in the rumen would seem more feasible if these products were working synergistically with ruminal microbes (McAllister et al., 2001). Researchers have shown that extracts from *Aspergillus oryzae* increase the number of ruminal bacteria (Newbold et al., 1992) and work sinergistically with extracts from ruminal microorganisms to enhance the release of soluble sugars from hay (Newbold 1995). In their study Morgavi et al. (2000a) found cooperation in the degradation of CMC, soluble xylan and corn silage between rumen and exogenous fungal enzymes (from *Trichoderma longibrachiatum*), particularly at low pH. This effect is primarily due to the role of cellulosomes. It has been shown that cellulosomes play a role in the adhesion of microbe cells to their substrates (Pell and Schofield, 1993; Beguin et al., 1998, Miron et al., 2001). Adhesion is essential for efficient digestion of forages and cereal grains in the rumen (McAllister, 1994; McAllister and Cheng, 1996). Cellulose-binding domains may be involved in the attachment of rumen bacteria to cellulose (Pell and Schofield, 1993). Morgavi et al (2000c) showed that low levels of enzyme from *T. longibrachiatum* stimulated the adhesion of *Fibrobacter succinogenes* to corn silage and alfalfa hay though this effect was lost at high levels. They concluded that at high levels the fibrolytic enzymes competed with the rumen bacterium for available binding sites on cellulose. Hovewer, it is supposed that exogenous enzymes expose additional microbial adhesion sites on the surface of feeds, probably by modifying feed structure (Morgavi et al., 2000c; Nsereko et al., 2000b; McAllister et al., 2001). Xylanases and esterases are considered to be initiators of the stimulatory effect (Nsereko et al., 2000b). Rezaeian et al. (1999) showed that rumen fungi produce higher xylanase and cellulase activity when the barley straw was treated with sodium-hydroxide (that resulted in structure modifications). Wang et al. (2001) found that ruminal cellulolytic bacteria were more numerous in the presence of EFEs than without and ^{15}N incorporation to bacteria increased as a result of EFE application into Rusitec.

d.) EFEs can reduce the viscosity of the digesta in the gut of poultry and swine thus play an important role in improving feed digestion and thus performance (Bedford and Schulze, 1998).

Increasing viscosity and decreasing avicelase activity was measured in the rumen fluid following barley supplementation of the feed. This may limit the microbial enzyme diffusion or accumulation of inhibitor substances (Martin et al., 2000). Studies have been published in which a decreasing viscosity of rumen fluid was observed due to administration of EFE (Yang et al., 1999; Hristov et al, 2000, Sutton et al., 2003). Viscosity of duodenal digesta in poultry were 3.8 to 16.6 times greater than the values for ruminants in the control group according to the studies of Graham (1996) and Svihus et al. (1997) . Thus, it is supposed that intestinal viscosity may not be a limiting factor of nutrient absorption in cattle (Yang et al., 1999).

e.) Experiments have proven that EFEs not only enhance fibrolytic activity in the rumen, but also increase fibrolytic activity in the small intestine (Hristov et al., 1998a, 2000). In the study of Hristov et al (1998a) it was shown to be particularly characteristical of xylanase activity, as supplementary enzymes increased duodenal xylanase activity by 30%. Cellulase activity increased by only 2-5% in the small intestine as it had been inactivated by the low pH and pepsin in the abomasum (Hristov et al., 1998a). Hydrolysis of complex carbohydrates by EFEs in the small intestine and absorption of released sugars would offer energetic and nitrogen balance benefits to the animal. It is possible that exogenous enzymes work synergistically with the microbes in the large intestine as well. Xylanase activity measured in the faeces increased in correlation with the increasing levels of enzyme addition (Hristov et al., 2000).

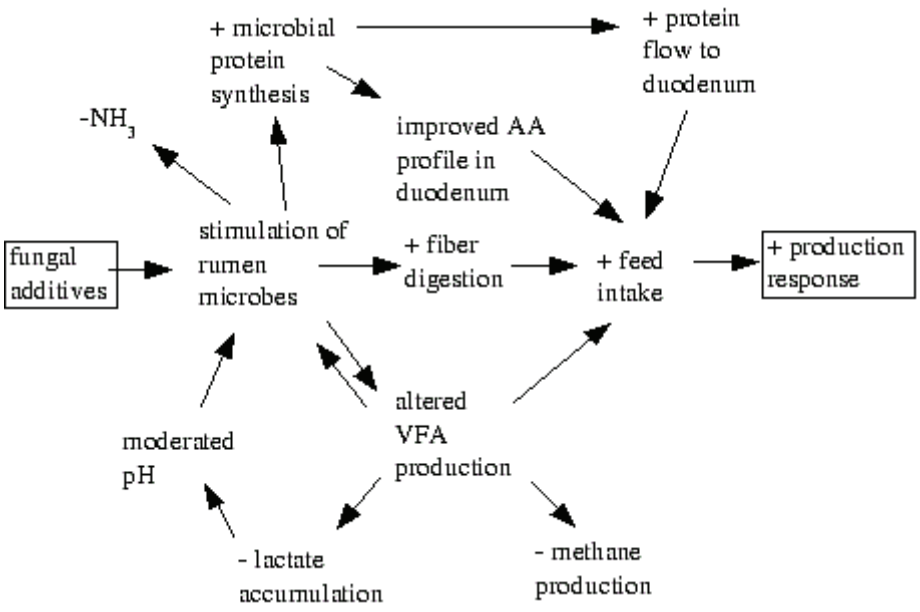

Figure 2.2.1. A possible scheme for the mode of action of exogenous fibrolytic enzymes in the rumen (after Wallace and Newbold, 1992 and Kung, 2001)

2.3. Use of exogenous fibrolytic enzymes to improve performance in ruminants

2.3.1. Indirect use of NSP enzymes

Fibrolytic enzymes isolated from fungal cultures have been shown to enhance fermentation during ensiling of certain forages. These enzymes reduce the amount of unavailable protein and carbohydrate fractions of forages (Gwayumba et al., 1997) by the hydrolysis of structural carbohydrates (McHan, 1986; Stokes, 1992) providing substrate for lactic acid producing microbes (Stokes, 1992). Increased lactic acid production creates a precipitous decline in the pH of the enzyme treated silage (Nakashima et al., 1988; Van Vuuren et al., 1989; Spoelstra et al., 1992), which results in its aerobic stability. Bolsen et al. (1996) inoculated samples of alfalfa silage with 13 different bacterial strains observing a decrease in acetic acid, ethanol and ammonia concentrations compared to control values. The fibrolytic action of enzyme preparations in ensiled forages also improved digestibility when fed to cattle (Stokes, 1992; Shepherd and Kung, 1994) and sheep (Freeden and McQueen, 1993). Using enzymes to improve silage quality holds a great potential and, in my opinion, contrary to Annison (1997), it is more than just a question of feed technology or biochemical engineering and attention needs to be paid to the physiology, biochemistry and nutrition of the ruminants.

2.3.2 Direct use of NSP enzymes in the feeding of calves, lambs and beef cattle

Experiments investigating the effects of enzymes in ruminant feeds have reported on both positive and negative results since the 1960s. Several recent studies however demonstrate positive effects of feed enzymes in diets fed to lambs, calves and beef cattle.

In one of their early studies, Burroughs et al. (1960) demonstrated that adding a dried enzyme mixture of bacterial origin to beef cattle rations (325 cattle were used) improves weight gain, DMI and FCR. Using bacterial and fungal enzyme supplements in their feeding trials with steers, lambs and beef heifers, others (Ward et al., 1960; Rovics and Ely, 1962; Theurer et al., 1963) could support these results. Rust et al. (1963) evaluated the effects of supplementation of calf starter diets with enzymes on the growth of animals. They observed a modest increase in the extent of weight gain when bacterial protease was included in the ration. Supplementation with bacterial amylase and Takamine Cellulase 4000 did not affect the rate of gain, fungal amyloglucosidase depressed the growth rate.

Grainger and Stroud (1960) showed that gumase and amylase, a multiple enzyme preparation and the combination of all three increased apparent dry matter, cellulose and crude protein digestibility in wethers. Others also showed that bacterial supplements (having protease and amylase activity) improved apparent nitrogen digestibility and energy utilization in dairy calves (Rust et al., 1965). The bacterial supplement had no effect on ruminal VFA concentrations neither in total nor proportionally, and there were no significant difference in ruminal ammonia concentrations in the experiments of Ward et al. (1960) and Rust et al. (1965).

Later research focused on bacterial and fungal exogenous enzyme preparations having considerable fibrolytic (cellulase, xylanase etc) activity. Feng et al (1992) showed that as a result of mixing cellulase, hemicellulase and xylanase into the feed of steers total and hay DMI together with

the DM and NDF digestibility were higher compared to control values. Total VFA concentration, acetate:propionate ratio and ammonia concentration in the rumen fluid were not altered by the treatment, though ruminal passage was greater and ruminal retention time was shorter. These changes occured when enzyme preparation was mixed to the hay forage prior to feeding.

Beauchemin et al (1995) published positive effects of fibrolytic enzyme supplements as well. They added incremental levels of xylanase and cellulase enzyme mixtures (from *Trichoderma* spp.) to steers' diets. In case of alfalfa hay, low and moderate levels of enzymes (900 to 4733 U/kg DM) increased weight gain by up to 30%, the improvements were mainly due to increased acid detergent fibre digestibility, resulting in increased DM digestibility. In case of timothy hay, the highest level (12000 IU/kg DM) improved gain significantly by 36%. No response to enzymes was observed in case of barley silage.

Treacher et al. (1997) also pointed out the importance of dose and application method of the enzyme preparations. In their first study they reported that preparations administered intraruminally to wethers reduced the apparent digestibilities of DM and NDF compared to spraying EFEs on the silage. The enzyme preparation did not affect DM and organic matter intake, ruminal pH, endoglucanase or xylanase activity and cellulolytic microbe population in the rumen. In their second study Treacher et al. (1997) found that average daily gain and final weight of steers increased in correlation with enzyme levels. In the third study they showed that ADG was affected while DMI remained unchanged, thus feed conversion rate improved in the enzyme supplemented group. In these studies, treatment of the entire ration was more effective in improving animal performance than was treating only the silage. The positive effect of fibrolytic enzymes on daily weight gain was proved by Gómez-Vázquez et al. (2003) but not affirmed by ZoBell et al. (2000).

Increase of nutrient (ADF, NDF, OM or DM) digestibility after enzyme application was supported by several researchers (Krause et al., 1998; Lee et al., 2000; McAllister et al., 2000; Gómez-Vázquez et al., 2003) but doubted by others (Hristov et al., 2000). Seemingly results are conflicting even in the most recent publications.

Krause et al. (1998) investigated the effects of EFE by spraying PRO-MOTE (a cellulase and xylanase mixture) on barley grain prior to feeding it to steers. The time spent eating was increasing though enzyme treatment had no effect on the number of meals and rumination periods. No significant effect could be observed on the pH of rumen fluid and VFA concentrations, however molar ratio of propionate tended to increase and acetate:propionate ratio tended to decrease.

In contrary to this, in the research of Lee et al. (2000) significantly higher total and proportional VFA concentrations were detected in sheep following application of an enzyme preparation from *Orpinomyces* strain KNGF-2 (showing cellulase and xylanase activity) isolated from Korean native goat. Rumen fluid pH was not affected. Ammonia concentration of the rumen fluid was decreasing and increased nitrogen retention rate was detected in the group receiving enzyme supplementation. Total rumen bacteria and cellulolytic count remained unchanged but the number of rumen fungi increased 1.15 fold due to enzyme administration. The xylanase activity of the rumen fluid was higher in the experimental than in the control group.

Hristov et al. (2000) measured increasing carboxymethyl-cellulase and xylanase activity of the rumen fluid after enzyme application. They added an enzyme product (GNC Bioferm) having CMCase, xylanase, beta-glucanase and amylase activity to the feed of Angus and Shorthorn heifers. Following application researchers found ruminal pH and ammonia concentration decreasing. TVFA and acetate concentrations were elevated while there was no difference in propionate. Protozoa population and the outflow rate of the liquid phase of ruminal contents were not affected by the enzyme treatment, however viscosity of the rumen fluid decreased. Hristov et al. (2000) also

showed that xylanase and beta-glucanase activity of both the rumen fluid and the duodenal digesta increased after enzyme application. Enzyme treatment affected neither urinary excretion of allantoin and uric acid, nor concentrations of glucose and urea in blood.

Using xylanase and endoglucanase preparations (by Finnfeeds Inc) in steers' diets containing forage and concentrate in a ratio of 65:35, ZoBell et al. (2000) did not find average daily gain and DMI changing, however feed digestibility showed a trend for improvement. They found no difference in the mentioned parameters and production or carcass characteristics when feeding finishing steers with a treated diet containing forage and concentrate in a ratio of 20:80.

2.3.3. Direct use of NSP enzymes in dairy rations

Similarly to beef cattle and sheep studies, research in dairy cows recorded inconsistent data about the effects of supplementary enzymes. Investigations in dairy cows started in the mid 1990s, long after beef cattle or sheep studies.

Sanchez et al (1996) established that exogenous fibrolytic (cellulase and xylanase) enzymes improve lactational performance in the early lactation of Holstein-friesian cows. DMI increased in all treated groups (1.25, 2.5 and 5.0 ml enzyme/kg DM forage). Milk, 3.5 % FCM and ECM yield increased significantly in the medium supplemented group. Body weight and body condition score increased in both MED and HIGH groups.

Nussio et al. (1997) did not confirm significancy. They found that milk yield of the group supplemented with a high dose (1.7 l/t of alfalfa hay) of enzymes (cellulase and xylanase) not significantly increased by almost 9 %. Neither of the researchers mentioned any changes in milk fat and protein content.

McGilliard and Stallings (1998) also pointed out the inconsistency of the production response in their wide study with 3417 dairy cows. A microbial supplement (Combo®) containing mainly alpha-amylase and smaller amounts of beta-glucanase, hemicellulase and cellulase was fed to cows. Milk yield increased in 31 herds (17 significantly) and decreased in 15 herds (7 significantly). Average response of milk yield was +0.64 kg/day per cow. Average increase in the milk yield of primiparous cows turned out higher (+0.74 kg/d) than average of all cows. Cows entering the study at fewer than 120 DIM and at 120 to 180 DIM responded similarly to enzyme supplementation. There was no increase in FCM yield, fat and protein content of the produced milk.

Lewis et al. (1999) perceived significant changes after adding enzyme (Cornzyme™: cellulase, xylanase, cellobiase, glucose oxydase) to cow's diet either in early or mid-lactation. They found that cows in mid-lactation produced more milk, 3.5% FCM, and ECM. Milk fat and protein content, DM and NDF digestibility were similar to the control. DMI and BCS were higher in the enzyme supplemented group. The cows in early lactation also yielded more milk, 3.5 % FCM and ECM consuming more DM than cows in the control group. Highest milk yield was observed in the group receiving medium amount of enzyme (2.5 ml/kg forage DM). There was no difference in the milk fat concentration between control and medium groups it was lower however in the groups with low and high enzyme level.

Others, similarly to Sanchez et al. (1996) and Lewis et al. (1999), observed increasing milk yield in early lactation (Schingoethe et al., 1999; Rode et al., 1999; Yang et al., 1999; Zheng et al., 2000) but these findings were not affirmed by others (Dhiman et al., 2002; Knowlton et al., 2002; Sutton et al., 2003) though. Results are also in conflict concerning milk yield in mid-lactation. Similarly to Rode et al. (1999) researchers found milk yiled increasing in the middle of lactation (Kung et al.,

2000) but others did not support this (Schingoethe et al., 1999; Adams et al., 2002; Bowman et al., 2002; Knowlton et al., 2002; Tricarico et al., 2002).

In the study of Rode et al. (1999) the enzyme (Pro-Mote®, with xylanase and cellulase activity) did not increase dry matter intake but total digestibility of nutrients (mainly NDF and ADF except starch) ameliorated. Milk yield was higher but milk composition was unaffected in the enzyme treated group.

Yang et al. (1999) measured the rumen fermentation characteristics beside the production parameters of cows in peak lactation. They found similar DMI among the treatments. Milk production and milk protein content in the higher dose enzyme (Pro-Mote®) treated group were higher. Milk fat content was unaffected by the treatment. Although intakes of OM, NDF and ADF did not differ between groups, the amount of OM fermented in the rumen was 10% higher and digestibility of NDF was 12% higher when fed the high dose enzyme supplemented feed. Microbial protein synthesis was not significantly higher than that in the control. TVFA concentration and propionate ratio of the rumen fluid was higher in the enzyme supplemented groups but this difference was no significant. Viscosity of the rumen fluid was lower in the group consuming a lower dose of enzyme. Kung et al (2000) did not find real difference in the VFA content of the rumen fluid either.

In their study using Natugrain 33-L Beauchemin et al. (2000) established that enzyme supplementation is more useful in cows at the beginning of lactation than those being in positive energy balance. Enzyme supplementation increased proportion of acetate and reduced ammonia concentration in the rumen fluid however there was no difference in the milk yield and milk fat content, although milk protein content was higher than the control values. They found that DMI increased yet there was no difference in the time spent eating and rumination frequency.

2.4. *Thermomyces lanuginosus* as a source of exogenous polysaccharidase enzymes

2.4.1. The fungus

Thermomyces lanuginosus (formerly known as *Humicola lanuginosa*) was discovered over a century ago by Tsiklinskaya, in a potato inoculated with garden soil (Maheshwari et al., 2000). *T. lanuginosus* belongs to the Deuteromycetes class (also called imperfect fungi, mitosporic fungi, asexual fungi or conidial fungi; Guarro et al., 1999) and grows between 30-70 ºC. It is common in all kinds of composts and also in birds' nests and sun-heated soils. It colonises composts during the high-temperature phase of decomposition. It degrades cellulose poorly however and it seems to live as a commensalist with cellulose-decomposing species, sharing some of the sugars released from the plant cell walls due to their cellulolytic activity (Maheshwari et al., 2000; Deacon, 2002). Milieu of composts tends to become anaerobic and CO_2 can reach 10-15% of the gas content of composts. It is likely that CO_2 assimilation plays nutritional and structural roles in the development of thermophilic fungi, which are the primary components of such habitats. Growth of *T. lanuginosus* is severely depressed if gas phase in the culture flasks is devoid of CO_2 (Maheshwari et al., 2000).

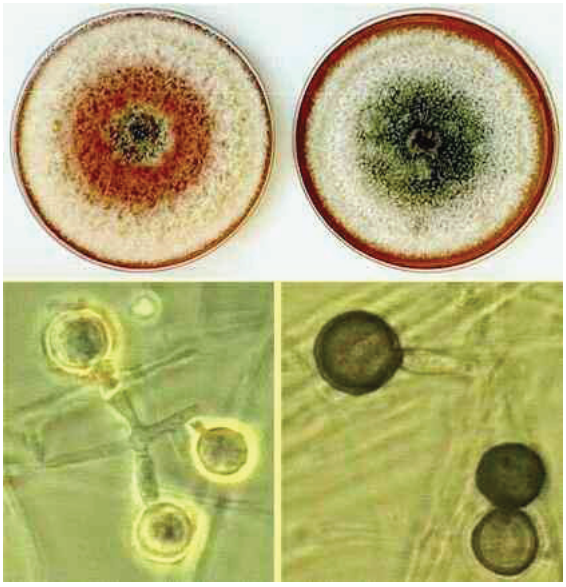

Figure 2.4.1. *Thermomyces lanuginosus*. Colonies growing on potato-dextrose agar (top left) and malt extract agar (top right) at 45 ºC. This fungus produces single spores by a balloon-like swelling process at the tips of short hyphal branches (bottom, left). At maturity (bottom right) the spores have brown, ornamented walls. (Deacon, 2002)

2.4.2. Fibrolytic enzyme production of Thermomyces lanuginosus

As it is written in chapter 2.1.1 xylans are constituents of plant cell walls. The most important enzyme in xylan degradation is beta-xylanase (endo-1,4-beta-D-xylanase, EC 3.2.1.8) and beta-xylosidase (EC 3.2.1.37). It is an inducible extracellular enzyme produced on substrates containing

xylan by several bacteria and fungi, amongst fungi are most potent (Senior et al., 1989). Research has shown that many *Thermomyces lanuginosus* strains produce extracellular proteins having high xylanase activity. Optimisation of xylanase production by *T. lanuginosus* (Gomes et al., 1993a, b), regulation of enzyme prouction (Purkarthofer et al., 1993), purification and physico-chemical characterisation (Cesar and Mrsa, 1996) have been reported previously. Others showed that different *T. lanuginosus* strains have pectinolytic (Bennet et al., 1998), amylolytic (Nguyen et al., 2000), lignocellulolytic (it is able to dissolve the lignin and cellulose units, Deacon, 1985), alpha-galactosidase (Rezessy-Szabó et al., 2003) and beta-D-glucosidase (Lin et al., 1999a) activity as well. Differences in enzyme production exist among *T. lanuginosus* strains of diverse geographical origin (Chadha et al., 1999). The enzyme yield of *T. lanuginosus* strains significantly correlate with the quantity of C and N sources of the medium (Purkarthofer et al., 1993; Purkarthofer and Steiner, 1995).

Structure of the xylanase of *T. lanuginosus*, a polypeptide of 225 amino acids, shows high homology with other xylanases (Maheshwari et al., 2000). The molecular weight of xylanase enzymes produced by *T. lanuginosus* is around 20-30 kDa (Purkarthofer et al., 1993, Hoq and Deckwer, 1995, Lin et al., 1999b; Maheshwari et al., 2000). All *T. lanuginosus* strains studied previously have similar pH (6.0-6.5) and temperature (65-70 ºC) optimums (Gomes et al., 1993a; Lisching et al., 1993; Purkarthofer et al., 1993; Alam et al., 1994; Lin et al 1999b; Maheshwari et al., 2000). These results confirm that basic structures of the catalytic domain of these xylanases are in close relation (Lin et al., 1999b). However, differences in the overall structure of xylanases from various strains might exist, due to differences in specific activities and thermal and pH stabilities (Lin et al., 1999b). Thermostability of *T. lanuginosus* xylanase is ascribed on one hand to the presence of an extra disulfide bridge absent in the majority of mesophilic xylanases, on the other hand to an increased density of charged residues throughout the protein (Maheshwari et al., 2000). As it has been regularly observed, thermostable proteins show increased stability against denaturants as well. Xylanase of *T. lanuginosus* was remarkably resistant to denaturation by 8 M urea (Maheshwari et al., 2000).

2.4.3. Production of an enzyme extract from Thermomyces lanuginosus NCAIM 001288

Two recently published papers (Kutasi et al., 2001; Kutasi et al., 2005) described the production of an enzyme preparation from the fungus *Thermomyces lanuginosus* (deposited in National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary, under the number NCAIM 001288) in the laboratory of Dr. Bata Ltd. Thermal and pH stability and the lignocellulolytic activity of the preparation were measured in vitro. These results are briefly summarised on the pages below.

Examination of the thermal and pH stability of the enzyme preparation (Kutasi et al., 2001)

The enzyme extract preserved more than 70 % of its activity for 2 hours when incubated at 60 ºC, and it decreased below 50 % only after 6 hours of incubation. The extract incubated at 70 ºC retained more than 70% of its activity for an hour showing sharp decline afterwards. Incubation at 80 ºC proved deleterious for the enzyme activity, as the extract lost appr. 50 and 80% of its activity at the end of the 1st and 2nd hour of incubation, respectively and there was no sign of enzymatic activity after hour 4 of the experiment.

Thermal stability examinations indicated that the enzyme preparation has appropriate heat

resistance. It follows, that during manufacturing of the dry fermentation product, the enzyme preparation can be kept at 60 ºC for as long as 2 to 3 hours without considerable loss in the enzyme activity. Owing to this, the moisture content of the end product can be minimized. The enzyme preparation may be dried at 70 and 80 ºC, however, at these temperatures the length of drying should be reduced to 60 and 30 min, respectively. At pH 4.5 the extract retained majority of its enzymatic capacity for 30 min, while the activity was halved by the 60th min of incubation. At this pH the enzymatic activity was practically lost after 120 min of incubation. At pH 6.5, good activity was measured even after 3 hrs of incubation.

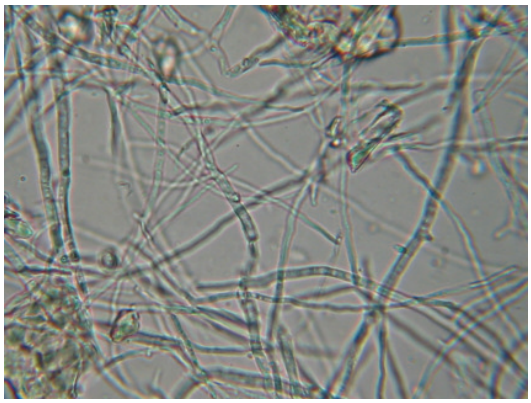

Figure 2.4.2. *Thermomyces lanuginosus* NCAIM 001288 cultured on CCM. Photo was taken by J. Kutasi.

Studies on grain substrates (Kutasi et al., 2001)

More than 50 % of the digestible dry matter content of the rye bran, oat grits, barley groats and wheat bran samples was dissolved within 5 min by the enzyme extracts studied. Degradation was complete after approximately 15 min, though in a few cases it took up to 30 min.

Comparative study of grain samples revealed that the enzyme preparation works similarly to a commercial product studied in these experiments. The reason of the differences might be attributed to the presence of auxiliary enzymes in our preparation and cellulase in the commercial product which were not measured in the present experiments It has been reported (Gomes et al., 1993a; Gomes et al., 1993b; Purkarthofer et al., 1993; Hoq et al., 1994; Alam et al., 1994), that under appropriate culturing conditions, assured in the present studies, *T. lanuginosus* strains produce a considerable amount of xylanase both with and without the presence of amylase and cellulase. The presence of amylase with no or negligible amount of cellulase in preparations rich in xylanase may either decrease or improve the digestive capacity of xylanase in dependence of the substrate quality (Gomes et al., 1993b; Purkarthofer et al., 1993; Alam et al., 1994). This may explain our findings that our enzyme extract had inferior and superior polysaccharidase activity on wheat bran and oat grit samples, respectively in comparison with that of the commercial product.

Experiments with forages and sunflower hulls (Kutasi et al., 2001)

Forage samples (triticale straw, oat straw, alfalfa hay and sunflower hulls) were treated in the same way as the grain samples with the difference that straw samples were incubated for 5 to 60 min, and samples of the sunflower hulls were incubated for 5 to 360 min.

The experimental enzyme extract started to degrade the organic matter of the oat straw, triticale straw, alfalfa hay and sunflower seed hulls after 5 min of digestion. The concentration of the

dissolved dry matter gradually increased until min 30 of incubation and in case of the sunflower, alfalfa, oat and triticale samples it reached 5.6, 5.4, 6.6. and 7.5 %, respectively. Beyond min 30 there was some further increment.

Due to their high lignine content, forages and sunflower seed hulls contain considerably less available xylane than grains (Gomes et al., 1993b; Purkarthofer et al., 1993). This explains the relatively slow and less efficient lignocellulolytic activity. However, applying longer incubation, such as being soaked in aqueous enzyme preparation, polyasaccharides might become available for the enzymes. This was observed in our experiment with the sunflower seed hulls, where the fungal enzyme digested three times more dry matter in 6 hours than in 1 hour of incubation.

Experiments with different T. lanuginosus NCAIM 001288 substrains (Kutasi et al., 2005)

Shaker flask experiments were carried out to determine the enzymatic activity and lignocellulolytic properties of some recently developed fungal substrains cultured under aerobic conditions. During the efficacy test, the five different substrains (A/2000–2004) of the fungus grown on maize stalk reached their maximum xylanase activity at 225–245 FXU/ml in 3 to 6 days. This activity can degrade xylans adequately, as even the xylanase activity of industrial strains used in paper making does not exceed 300 FXU/ml after 7 days of incubation (Cesar and Mrsa, 1996). Cellulase activity was low and did not exceed 3 U/ml, although the cellulolytic ability of the cultures was clearly detectable. At the same time, 30–63% of the maize stalk was decomposed during the 6-day experiment, indicating that the aerated fungal culture had sufficient, comparable to strains used in paper industry, lignocellulolytic activity.

After the shaker flask measurements a one-month lignocellulose degradation experiment was done. The objective of the experiment was to study the enzyme production and the lignocellulolytic activity of the substrain (A/2004) that proved to be the most efficient in the previous experiment, on sterile and on non-sterile solid surface, under microaerophilic conditions. Higher activities and degradation percentages were found in case of maize stalk and wheat straw treated with *T. lanuginosus* fungal culture for 1 month. The quantities of decomposed material clearly showed the high degrading activity in case of sterilised cultures (maize 30.5%, wheat 60%). As a result of decomposition, the wheat straw became liquefied and whitened. This suggests the complete disintegration of the substance and structure of wheat straw, which is a probable indicator of ligninase activity, as confirmed also by data of the literature (Kirk et al., 1976). At the same time, such substantial degrading activity was not found in case of the non-sterile cultures. In case of the latter, fungal cultures appeared also in the control, non-inoculated flasks, which indicates a native fungal biota originally present on the plants and capable of decomposing forage partially. Our fungal culture inoculated in a non-sterile manner can only exert its effect in competition with these fungal cultures (maximum percentage of degraded dry matter: 37.5% for maize and 26% for wheat).

After the one-month study the cellulase activities of the sterile cultures showed the highest activity (24.8 U/g wheat straw), substantially exceeding the activity of the control cultures. The cellulase activity of the non-sterile cultures just slightly, or not at all, exceeded that of the control flasks, indicating the dominance of the already mentioned foreign fungal cultures in terms of cellulolytic activity.

The xylanase activity of the samples was very high after the one-month period had elapsed. Under sterile conditions it reached 1125 U/g on maize stalk, which indicates substantial xylanolytic ability. Although this high value was not achieved under non-sterile conditions, the inoculated cultures displayed an about fourfold increase in xylanase activity over the control (control maize: 100 U/g, inoculated maize: 375 U/g).

The CMC agar experiment confirmed the high cellulolytic activity of sterile cultures; however, in this case, in contrast to the cellulase activity determinations we could surprisingly detect a higher activity on maize stalk (maize st.: cleared zone 2.5 cm in diameter). In case of the non-sterile cultures the difference as compared to the control cultures was smaller (maize: 1.5- to 2.0-cm zone, control maize: 1.2-cm zone).

We also attempted to detect the pectinolytic ability. High pectinase activity was detected in both sterile and non-sterile cultures (wheat st.: 37.1%, maize: 37.1% degraded pectin), while the pectinase activity of the control cultures did not exceed 10.8%.

Among the soluble polysaccharides hemicelluloses, xylans and xyloglucans (arabinoxylans) occur most commonly. They act as connecting agents between the pectin and the cellulose fractions. Maize stalk and wheat straw contains 40-50 % cellulose and 20–30% hemicellulose. Half of the hemicelluloses present in wheat straw is constituted by arabinoxylans and the other half by galactomannans and pectins. The bulk of the hemicellulose fraction of maize stalk is made up of arabinoxylans (Szigethy, 1971).

An important constituent of the cell wall is lignin, which is not a polysaccharide but a polymer of aromatic alcohols deposited primarily during the ageing process of the cell wall. Lignin is linked to the other cell wall constituents by covalent bonds; thus polysaccharides covered by lignin are more resistant to the action of (digestive) enzymes. The decomposition of lignin leads to whitening of the wood material (white rot). During stalk decomposition, all these fibrous materials must be degraded by enzymes (pectinases, ligninases, xylanases, cellulases). Since the decomposition of cellulose requires the synergistic action of multiple enzymes, the group of cellulases is constituted by several different enzymes including cellobiohydrolases, exo- and endoglucanases and glucosidases.

2.4.4. The outcome of product development

The outcome of the product development process detailed above was a preparation under the brand name Rumino-Zyme.

The product is a light-brown granulate (particle size: 400-500 μm, dry matter content 90%), which contains thermally resistant endoxylanase from *T. lanuginosus*. IUB ranking of the enzyme is: endo-1,4-ß-xylanase. It preserves its activity in the range of pH 4.5-8.0 and 30-40 °C. Shelf life at 20 °C is longer than 6 months. Enzyme activity of the product is 250 FXU/g (FXU: one unit of xylanase activity was expressed as μmol of Remazol xylan-degradation products released in one min). The preparation hydrolyses xylans and arabino-xylans into mono-, di-, tri- and oligo-saccharides.

Experiments described in chapter 2.4.3. have clearly demonstrated that this product decomposes different types of plant materials in a different degree. The most active enzyme of the system is xylanase, playing a dominant role in decomposition. Xylanase appeared to be sufficiently active also under non-sterile conditions. After the degradation of arabinoxylans the structure of wheat straw is disintegrated and its fibre components become accessible to the action of less active enzymes such as cellulase, pectinase and ligninase (Grajek, 1986). The high xylan content of maize stalk decelerates this process and, as a result, only partial disintegration of the structure of maize stalk can take place.

Based on data of the relevant literature and data of own experiments described above it is supposed that xylanase from *T. lanuginosus* had a beneficial effect by stimulating ruminal decomposition of the poorly degradable NSP carbohydrates.

The aim of present dissertation is to study the properties of the enzyme preparation, a product developed in collaboration with Dr. Bata Co., in the following aspects:

- activity and stability in the rumen
- detectable effects on ruminal fermentation
- possible benefits on production parameters

The first two aspects were studied in sheep trials (for experimental designs and results see 3.1. and 3.2.). The effect on general condition and production parameters of dairy cows was studied under field conditions on dairy farms, using animals in early (3.3.) and mid (3.4.)-lactation.

3. Own experiments

3.1. Activity and stability of the xylanase preparation in the rumen of sheep*

3.1.1. Aim of the study

The objective of this investigation was to measure the xylanase activity in the rumen and to characterise the stability of the endo-1,4-beta-xylanase of Rumino-Zyme.

3.1.2. Materials and methods

Trial 1

The goal of the experiment was to gain data on the xylanase activity of the rumen fluid of sheep kept on a ration high structural fibre. In this experiment 8 one-year-old, rumen cannulated Merino wethers (average BW=50 kg) were used. The sheep were kept individually in adjacent metabolic cages. The animals were fed with a ration of 800 g meadow hay and 500 g concentrate (7.28 MJ NE_m and 117.4 g crude protein daily) in two equal portions (at 8.00 a.m. and at 4.00 p.m.). Licking salt and water were offered *ad libitum*. In this part of the experiment no enzyme preparation was added to the ration. In the week following the two weeks of preliminary period, 50 ml of rumen fluid samples were taken two times from each sheep just before the morning feeding and 2 and 4 h thereafter. The samples were kept in thawing ice until measuring the xylanase activity. In one occasion a bigger quantity (200 ml) of solid material was taken from the rumen right before the morning feeding in order to measure enzyme activity bound to the solid fibre phase.

Trial 2

The goal of this trial was to collect base line data on the enzyme activity of the rumen fluid in fasting sheep. Four, 3-years-old, rumen cannulated Merino wethers (average BW=70 kg) average weight were used to obtain rumen fluid samples 16, 17.5 and 19 h after the last feeding. In this trial the sheep were fed only once a day (at 4.00 p.m.) with a ration of 700 g alfalfa hay and 400 g concentrate (7.49 MJ NE_m, 125 g crude protein).

Trial 3

With this experiment we attempted to trace the activity change of the exogenous endo-1,4-beta-xylanase enzyme added directly into the rumen at a quantity sufficient to elevate the total extracellular xylanase activity of the rumen fluid. In this experiment the sheep of trial 2 were used with the same accommodation and feeding, with the exception that the ration was fed in two equal portions. At 8.00 a.m. a base-line rumen fluid sample was taken from each sheep. Afterwards a single dose of 10 g enzyme preparation of 250 FXU/g xylanase activity dissolved in 100 ml of water was poured into the rumen via the cannula. Further samples were taken 5, 10, 15, 30, 45, 60, 90, 120 and 180 min after administration of the enzyme preparation.

* Published as: Bata et al., J. Anim. Feed Sci., 2002. 11, 627-635.

Determination of the enzyme activity in the rumen fluid and solid parts

Xylanase activity was measured in the supernatant of rumen fluid samples and also in the solid phase of the rumen samples. Ten ml of rumen fluid was centrifuged for 15 min at 5000 rpm and the supernatant was used for further measurements. The solid fraction of rumen fluid (100 ml) samples were sieved through four layers of gauze. Particles were collected from the gauze and mixed with 500 ml of 0.05 mol phosphate buffer (pH 6.5). The mixture was stirred with a magnetic stirrer for 30 min. The suspension was filtered again through four layers of gauze. The solid part was collected and again mixed in 50 ml phosphate buffer. The suspension was mechanically shaken in a homocentric equipment at 30 °C for 30 min in order to activate the enzyme. The activated suspension was sieved through a filter paper (MN 615 ¼) until a water-clear fluid was obtained. This filtered substance was used for further measurements. The weight of the wet material withheld by the filter paper was measured and then dried until steady weight. Enzyme activity was calculated for the dry matter content.

Measurement of the enzyme activity

Ten mg substrate (4-O-methyl-D-glucorono-D-xylan stained with Remazol blue, Fluka 66960) was put into test-tube and 2.0 ml of test material (rumen fluid supernatant, or filtrate of the rumen solid material) were added. After 120 min incubation at 50 °C, 5 ml of stop reagent (150 ml ethanol + 1 ml HCl) was added to the medium and stirred for 1 min then left standing at room temperature for 15 min then centrifuged for 10 min at 3 000 rpm. The absorbance was measured at 590 nm wavelength against blank that contained phosphate buffer. Colour intensity of the liberated Remazol blue was measured and expressed in FXU units. The standard series consisted of samples of 0.01-0.15 mg *Trichoderma viridis* (Fluka 95595) xylanase with 2.5 FXU/mg activity (Biely et al., 1988).

Statistical analysis

Data were analysed by analysis of variance with the SPSS 8.0 for Windows software.

3.1.2. Results and discussion

Our studies have shown that xylanase activity can be measured with reliable efficiency via the Remazol stained xylane system.

In *Trial 1,* the xylanase activity before the morning feeding was low (Figure 3.1.1.) and increased considerably (from 46.3 to 114.8 and 111.7 FXU/litre; $P<0.001$) following the feed intake. This finding of our experiment is in accordance with the data reported by Huhtanen and Khalili (1992) and Michalet-Doreau et al. (2001). For the time being it is impossible to measure the activity of the internal and exogenous (experimentally added) NSP-enzymes separately. It follows that enzymes produced by the rumen microorganisms may shadow the activity of the external enzymes and may lead to misinterpretation of the data. Therefore, when examining the activity of external NSP-ase enzymes in *in vivo* systems, the precise and reasoned schedule of samplings in relation with feedings is of vital importance.

Fibre-bound enzyme activity was higher than that in the liquid fraction. However, in our experiment enzyme activity of the solid rumen particles is of limited value in characterising the xylanase activity of the ruminal system due to the high standard deviations of the data. Sonication might be better than mechanical stirring to release the xylanase from the crude plant particles. Nevertheless, our analysis of rumen fluid samples can set firm basis for the estimation of the xylanase activity in the rumen.

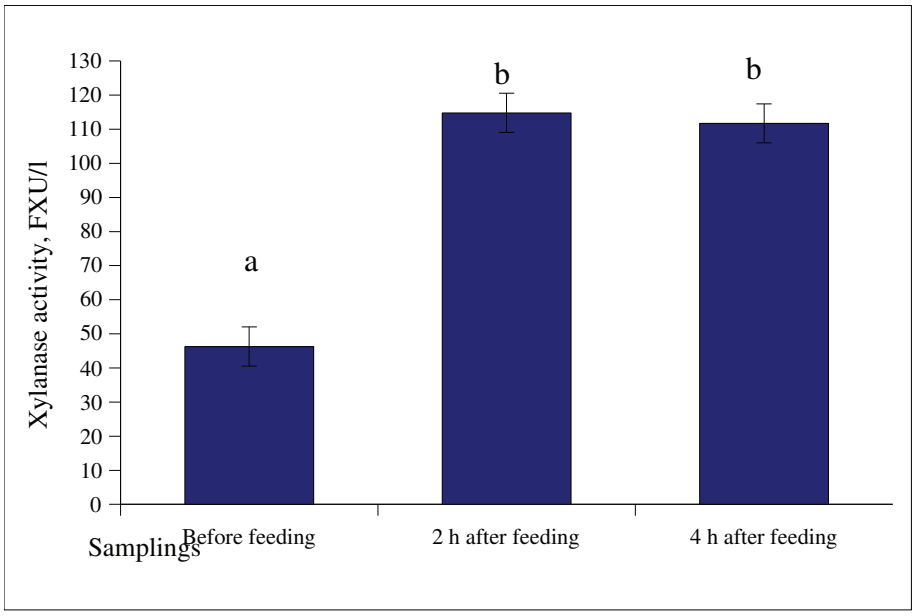

Figure 3.1.1. Xylanase activity in the rumen fluid before and after feeding

a,b: Means with different superscripts are significantly different ($p<0.001$)

In *Trial 2,* the xylanase activity of the rumen fluid was low after 16 h of the last feeding (in a state of "fasting") with low standard deviation and decreased slowly (Figure 3.1.2.). These data indicate that when characterising the base-line xylanase activity of the rumen, the samples should be taken several hours after feeding.

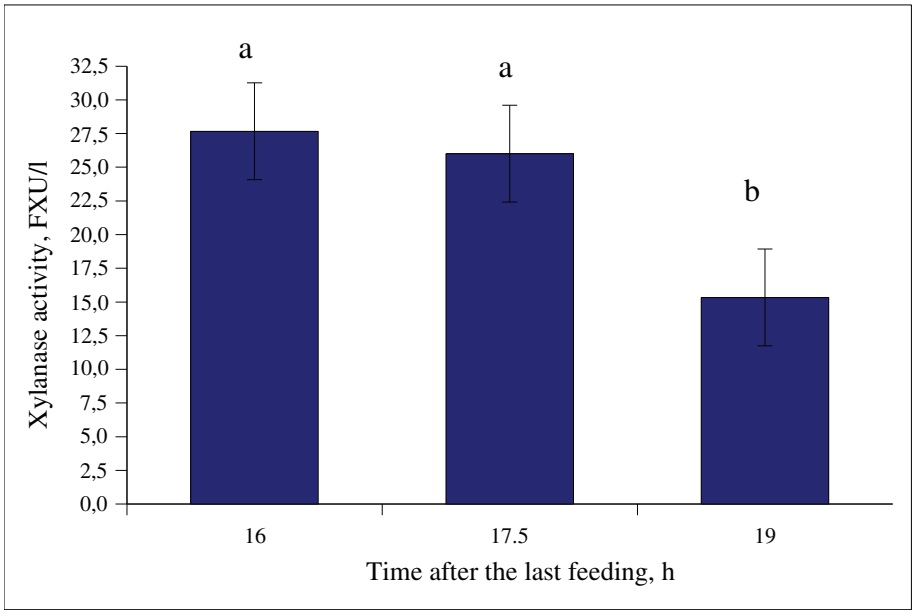

Figure 3.1.2. Xylanase activity in the rumen fluid in fastin sheep

a,b: Means with different superscripts are significantly different ($p<0.05$)

In *Trial 3,* the additional enzyme increased the base-line xylanase activity of the rumen fluid by almost 300 % within 5 min after treatment (Figure 3.1.3.) from 50 FXU/l to 161 FXU/l (P<0.001). The average recovery rate of the original enzyme activity was about 54%. The enzyme activity obtained by the supplementation had been preserved in 63% for 45 min (120 FXU/l). A significant part of the xylanase activity in the rumen is associated with crude plant particles. This may explain incomplete recovery of added xylanase. After 60 and 90 min the original enzyme activity decreased to 41% (96 FXU/litre) and 34% (88 FXU/l), respectively. Between 90 and 120 min after treatment the enzyme activity settled at a level, slightly over 30% of the original activity. Three hours after treatment no increment in the enzymatic activity was seen and the activity returned to initial values (38 FXU/litre).

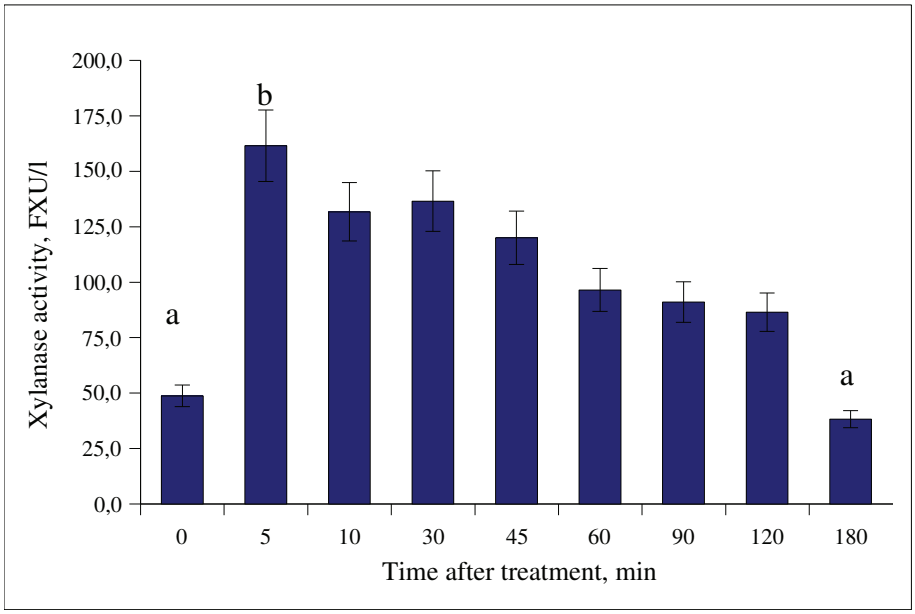

Figure 3.1.3. Xylanase activity in the rumen fluid prior to and after xylanase treatment

a,b: Means with different superscripts are significantly different (p<0.001)

The data (Figure 3.1.3.) indicate a significant increase in the xylanase activity which supports the findings of several authors (Hall et al., 1993; Fontes et al., 1995; Hristov et al. 1998; Morgavi et al., 2001; Wang et al., 2001). In our experiments, however, the increased activity was observed for about two hours only. Hristov et al. (1998b), in their 15 h *in vivo* experiments, found peak enzymatic activity in the rumen 1.5 h after intraruminal application of the enzyme preparation. No data were published about the time when the activity of the external enzyme ebbed away and in their paper no information was found about the origin (microbial or fungal) of the enzyme.

We concluded that dietary (exogenous) endo-1,4-beta-xylanase enzyme is disintegrated in the rumen shortly after treatment (estimated half-life = 45-50 min). It follows that substantial improvement in rumen digestibility of non starch polysaccharides can be expected only from those additives which provide persistent enzymatic activity in the rumen.

3.1.4. Summary

- The base-line xylanase activity of the rumen fluid (samples were taken after several hours of feeding) is low and steady.
- Adding the enzyme preparation from *T. lanuginosus* in one dose intraruminally, increased the xylanase activity of the rumen fluid within 5 min after treatment.
- The supplemental enzyme preserved its maximum activity for 45 min and half of its activity over 2 hours. The enzyme activity of the rumen fluid returned to initial values 3 h after supplementation.

3.2. The effects of the xylanase preparation on the rumen fermentation in sheep*

3.2.1. Aim of the study

The goal of this investigation was to obtain data about the effects of Rumino-Zyme on the rumen fermentation characteristics in sheep.

3.2.2. Materials and methods

Animals and diets

Eight ruminally cannulated yearling Merino wethers (average BW=50 kg) were used in the experiment. The sheep were kept individually in adjacent metabolic cages. The animals were fed with a ration of 800 g meadow hay and 500 g lamb concentrate in two equal portions (at 8.00 a.m. and at 4.00 p.m.). The daily intake was 1.22 kg DM, 7.28 MJ NE_m, 4.27 MJ NE_g, 117.4 g CP, 77.1 g MP, 310.8 g CF, 575.1 g NDF and 357.5 g ADF. Licking salt and water were provided *ad libitum*.

Samplings and laboratory analysis

After 2 weeks of social and feeding acclimatisation there was a baseline data collection period lasting one week. In this control period some parameters (see below) of rumen fermentation were determined. After the control period 2.5 g/day enzyme preparation was administered to the animals, mixed in the concentrate at the morning feeding. This experimental period lasted two weeks, followed by a final washout phase (FI) during which the animals were fed without any enzyme supplementation for two weeks. Rumen fluid samples were taken every day during the control and experimental phase, and every third day in the final phase. Each day samples were taken just before the morning feeding (T0), and at 2h and 4h from different compartments and depth of the rumen. We managed to collect samples from every animal at every sampling, so the number of rumen fluid samples serving as a basis for statistical analysis were the following: 40 in the control phase, 80 in the experimental phase and 40 in the final phase, regarding each of the T0, 2h and 4h samplings.

Rumen fluid pH was measured immediately after the samplings by a digital pH meter (Radelkis OP-211/1, Radelkis Co., Budapest, Hungary). The ruminal fluid samples were sieved through four layers of gauze and then centrifuged (1000 g). Five millilitres of filtrate were added to 1 ml of 25% HPO_3 for volatile fatty acid determination (Supelco Inc., 1975). The acidified fluid was centrifuged and the supernatant was stored at –20 °C until analysed. Ruminal VFAs were separated and quantified by gas chromatography (Shimadzu GC 2010, Japan) using a 30 m (0.25 mm i.d.) fused silica column (Nukol column, Supelco, Bellefonte, PA). The molar proportions of VFAs were calculated by dividing the individual acid concentrations by the total VFA concentration. The ammonia content of ruminal samples was determined by the method of Chaney and Marbach (1962). Xylanase activity in the supernatant of rumen fluid samples was determined by the Remazol blue method as described in previous publications (Biely et al., 1988; Bata et al., 2002).

Statistical analysis

Data were evaluated by univariate analysis of variance and Dunnett's post hoc test using SPSS 8.0 for Windows statistical software. Significance level was established at 5% ($p<0.05$).

* Jurkovich et al.: Acta Vet. Hung., 2006. accepted for publication

3.2.3 Results

Xylanase activity of the rumen fluid increased 2 h after feeding and then slightly decreased 4 h after feeding in all phases of the experiment (Figure 1). Xylanase activity was significantly higher in EX than in CO or FI (n=8; T0: 63.9 vs. 45.7 and 40.7, $p<0.05$; 2h: 103.9 vs. 71.3 and 67.6, $p<0.05$; 4h: 87.8 vs. 63.3 and 53.3, $p<0.05$). There was no significant difference between CO and FI in xylanase activity during the experiment.

The values of pH and total VFA concentration of the rumen fluid and the proportion of the individual VFAs are summarised in Table 1. In the control period the average pH of the rumen fluid was physiological just before the feeding and it fell after feeding. Changes in the pH were similar in the phase when animals were treated with enzyme and in the final phase when enzyme supplementation was terminated. Rumen fluid pH was higher in EX and FI at T0 than in CO but there was no difference between EX, FI and CO at 2h. At 4h the rumen pH was lower in FI than in CO or EX, yet remained in the physiological range.

Total VFA concentration increased after feeding in all groups. It was lower in EX than in CO at T0 but it was higher thereafter. Total VFA concentration in FI differed significantly from that in CO only at 2h.

The molar proportion of acetate decreased 2 h after feeding in all groups and tended to be increased 4 h after feeding. The molar proportion of acetate was higher in EX and also in FI than in CO throughout the experiment. The highest acetate ratio was observed in EX. The molar proportion of propionate increased after feeding in all phases of the experiment. It did not differ in EX and FI from CO at T0 and tended to be higher in EX and FI at 2h. At 4h the molar ratio of propionate was lower in FI than in CO or EX. During the experiment the highest average propionate proportion was measured in EX but it did not differ significantly from CO. Butyrate proportion increased after feeding in CO but was constant in groups EX and FI. The molar proportion of butyrate was significantly lower in EX and FI than in CO ($p<0.05$) during the experiment.

The molar proportion of isobutyrate was similar in all groups before feeding, then it decreased after feeding. Isobutyrate proportion was lower in both EX and FI than in CO after feeding. Valerate proportion increased after feeding in all groups and it was higher in EX and FI during the experiment. The molar proportion of isovalerate decreased after feeding in all phases of the experiment. It was higher in EX and FI than in CO at T0. Subsequently, isovalerate proportion was lower in EX than in CO or FI at 2h and 4h. Acetate:propionate ratio was the highest before feeding in all groups, then it fell at 2h and increased slightly at 4h. A/P ratio was higher in EX and FI than in CO at T0, then it was higher again at 4h.

Ammonia concentration of the rumen fluid increased 2 h after feeding and then it decreased 4 h after feeding in all groups. Ammonia concentration of the rumen fluid did not differ in EX and FI from that in CO (Table 3.2.1.) at T0. A similar trend could be observed at 2h but ammonia concentration was significantly lower in FI. At 4h, ammonia concentration was lower in EX than in CO, and it was lower in FI than in CO and EX.

Table 3.2.1. Rumen fermentation parameters during the experiment

Items	Treatments			
	CO	EX	FI	SE
T0				
pH	6.55[a]	6.72[b]	6.73[b]	0.01
Total VFA, mM	63.09[a]	57.09[b]	59.10[a]	0.83
VFA, mol/100 mol				
Acetate	67.64[a]	69.76[b]	69.41[b]	0.19
Propionate	17.51	16.95	16.76	0.15
Butyrate	12.19[a]	10.20[b]	10.76[b]	0.11
Isobutyrate	1.05	1.02	1.15	0.02
Valerate	0.54[a]	0.69[b]	0.66[b]	0.01
Isovalerate	1.07[a]	1.38[b]	1.26[b]	0.02
A:P	3.86[a]	4.12[b]	4.14[b]	0.05
Ammonia, mM	9.91	9.37	9.10	0.14
2h				
pH	6.00	6.03	6.02	0.01
Total VFA, mM	85.09[a]	96.34[b]	95.27[b]	1.13
VFA, mol/100 mol				
Acetate	63.15[a]	64.85[b]	65.24[b]	0.17
Propionate	21.73	22.44	21.61	0.15
Butyrate	12.94[a]	10.58[b]	10.99[b]	0.11
Isobutyrate	0.71[a]	0.53[b]	0.54[b]	0.01
Valerate	0.96[a]	1.16[b]	1.13[b]	0.02
Isovalerate	0.51[a]	0.44[b]	0.49[a]	0.01
A:P	2.91	2.89	3.02	0.03
Ammonia, mM	12.10[a]	11.48[a]	10.91[b]	0.17
4h				
pH	6.16[a]	6.10[a]	6.05[b]	0.01
Total VFA, mM	85.04[a]	90.90[b]	88.74[a]	1.00
VFA, mol/100 mol				
Acetate	64.06[a]	67.25[b]	66.84[b]	0.21
Propionate	20.99[a]	20.24[a]	19.64[b]	0.17
Butyrate	13.13[a]	10.63[b]	11.66[b]	0.13
Isobutyrate	0.60[a]	0.50[b]	0.51[b]	0.01
Valerate	0.79[a]	1.02[b]	0.94[b]	0.02
Isovalerate	0.43[a]	0.36[b]	0.41[a]	0.01
A:P	3.05[a]	3.32[b]	3.40[b]	0.04
Ammonia, mM	8.99[a]	6.96[b]	5.87[b]	0.23

[a,b]Means with different superscripts within a row are significantly different ($p<0.05$).

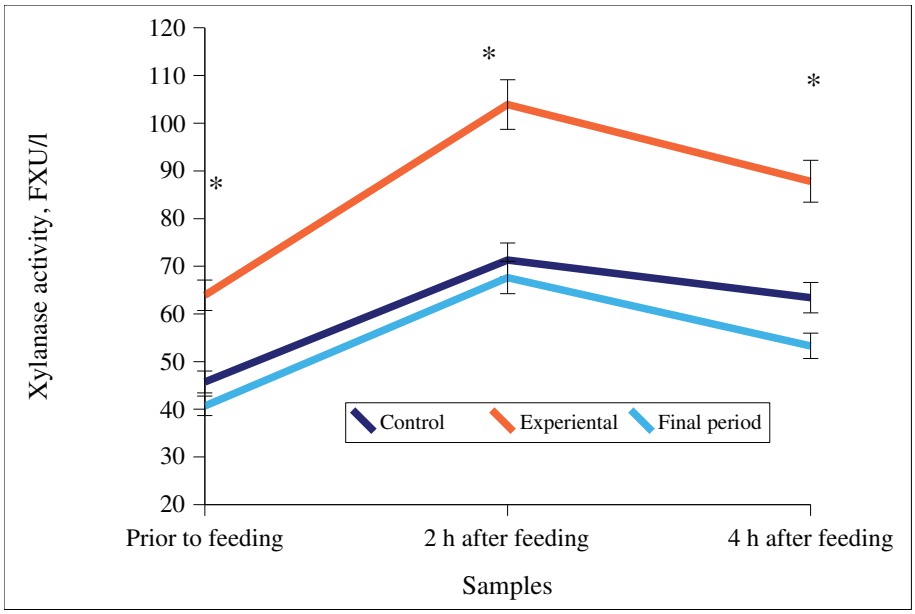

Figure 3.2.1. The xylanase activity in the rumen fluid

* Means are significantly different (P<0.05)

3.2.4. Discussion

In the control phase, xylanase activity increased in the rumen fluid 2 h after feeding as it had been shown by Huhtanen and Khalili (1992) and in our previous study (Chapter 3.1). Fermentation decreased 4 h after feeding as indicated by increasing rumen pH and decreasing xylanase activity. In agreement with our results, Hristov et al (2000) also found that xylanase activity was higher either in the rumen fluid or in the rumen digesta after the application of EFE. In their study the highest xylanase activity was measured 2 h after feeding and a slight decrease was observed 4 h after feeding, but they did not find any difference before feeding. In our study the increased xylanase activity before feeding is possibly due to the high protease resistance of the enzyme preparation, and presumably persist for even 24 hours. Wang et al (2001) showed increased xylanase activity after adding EFE to Rusitec, which is consistent with our findings. Lee et al (2000) also showed that xylanase activity of the rumen fluid was higher in lambs supplemented with EFE after feeding but no difference was found in xylanase activity before feeding. They did not find any increment in xylanase activity after feeding; the highest xylanase activity was measured before feeding and it decreased in the control and was almost constant in the experimental group.

In the control phase, the pH of the rumen fluid decreased 2 h after feeding as expected, indicating a normal digestive process in the experimental animals. Rumen fermentation decreased 4 h after feeding, which was indicated by the rise in ruminal pH. Some researchers (Baran and Kmet, 1987; Lewis et al., 1996; Hristov et al, 2000) found decreased ruminal pH after enzyme application. In contrast to this, in our study the ruminal pH was not affected by the added enzyme preparation (EX) nor the enzyme withdrawal (FI). This finding is supported by the observations of other authors (Krause et al., 1998; Yang et al., 1999 and 2002; Beauchemin et al, 2000; Lee et al, 2000; McAllister et al., 2000; Sutton et al, 2003). Yang et al (2002) have shown that exogenous enzymes are unlikely to improve fibre degradation when low rumen pH limits the fibre degradation process, as exogenous fibrolytic enzymes are often pH sensitive. Our xylanase preparation shows optimal

efficiency between pH 5.5 and 7 (Kutasi et al, 2001). The pH of the rumen fluid did not fall below 6 in the present experiment; therefore, the enzyme preparation could exert its maximum activity and ruminal fibre degradation was presumably not impeded by subclinical rumen acidosis.

The total VFA concentration increased after feeding in the control phase as had been expected and as shown by others (Baran and Kmet, 1987; Lewis et al., 1996; Lee et al, 2000).

Total VFA concentration was found to be lower in EX and FI before feeding than in the control phase. The lower TVFA concentrations observed in the EX and FI periods are presumably a result of increased cellulolysis due to increased hemicellulolysis. The higher TVFA concentration indicates that microbes gain more energy from the degradation of carbohydrates therefore they are able to increase protein synthesis.

Other researchers showed no difference in TVFA concentration before feeding (Baran and Kmet, 1987; Lewis et al., 1996; Lee et al, 2000) between the control and the EFE-supplemented group.

The TVFA concentration was higher after the enzyme administration than in the control 2 and 4 h after feeding. This finding is supported by the observations of other authors (Yang et al, 1999 and 2000; Hristov et al, 2000; Lee et al, 2000) but is inconsistent with other publications where the total VFA concentration was not affected by the enzyme treatment (Beauchemin et al, 1995; Kung et al., 2000; Wang et al, 2001; Yang et al, 2002; Sutton et al, 2003). Inconsistencies might arise from the different properties of administered enzyme products (cellulase or xylanase, with different activities) and different composition of rations.

The molar proportion of acetate slightly decreased after feeding but that of propionate and butyrate increased, which means that the digestion of starch was faster and more intensive just after feeding in the control phase. This finding is supported by the decreasing A/P ratio 2 h after feeding, which ameliorated 4 h after feeding. Four hours after feeding opposite changes are observed. The findings were similar to other observations where the proportion of acetate was higher in the enzyme-supplemented group (Beauchemin et al, 1995; Hristov et al, 2000; Lee et al, 2000; Yang et al, 2002) and the proportion of propionate was unaffected (Beauchemin et al, 1995; Hristov et al, 2000) or reduced (Yang et al, 2002) after enzyme supplementation. Other studies (Krause et al., 1998; Yang et al., 1999; Lee et al, 2000; Wang et al, 2001) showed increasing propionate ratio in the groups supplemented with EFE, whereas, some studies reported no observable changes in the individual VFA ratios (Bowman et al, 2002; Sutton et al, 2003). Since the animals received the same amount of feed during the entire study, the increases in TVFA concentration and molar proportion of acetate are most likely due to the more efficient fibre degradation (though rate of fibre degradation was not measured). This assumption is supported by other authors (Lewis et al., 1996; Yang et al., 1999; Beauchemin et al, 2000; Yang et al, 2002; Sutton et al, 2003) who reported improved fibre digestion under similar conditions.

In our experiment, the molar proportion of branched-chain (BC) VFAs such as isobutyrate and isovalerate decreased as a result of EFE administration. In contrast, there are publications which have showed no effect of EFE on BCVFAs (Baran and Kmet, 1987; Lewis et al., 1996; Hristov et al, 2000; Yang et al, 2002).

In the present study the ammonia concentration was affected by enzyme supplementation. Other authors (Baran and Kmet, 1987; Beauchemin et al, 2000; Lee et al, 2000) also reported that the ammonia concentration of the rumen fluid increased 1 to 3 h after feeding and then decreased 3 to 9 h after feeding. This shows an intensive protein degradation in the rumen fluid and indicates that the ammonia is used by the microbes for their own protein synthesis to a greater extent 4 h after feeding. The ammonia concentration measured in the rumen fluid in this study was above the level (5 mg/100 ml = 2.9 mM) indicated by Satter and Slyter (1974) as being adequate for microbial

growth and microbial protein synthesis.

Lee and others (2000) showed that the ammonia concentration was 10% lower relative to the control after enzyme application. Other investigators also reported a decreasing ammonia concentration in the rumen fluid after EFE administration (Beauchemin et al, 2000; Wang et al, 2001). In contrast, others have reported no difference in ammonia concentration after EFE supplementation (Bowman et al, 2002; Yang et al, 2002; Sutton et al, 2003) or ammonia concentration tended to be higher after EFE forage supplementation 24 h before feeding or infused directly into the rumen just before feeding (Hristov et al, 2000). These results are conflicting. The ammonia concentration of the rumen fluid decreases when the degradability of the consumed protein is lower or when the level of microbial protein synthesis is higher or thirdly, in case of high ruminal pH, due to its increased diffusion through the rumen wall. In the present study the protein content of the feed was the same during the experiment; therefore, it is likely that the decreasing ammonia concentration was due to an increased microbial protein synthesis. However, this response was not measured. This assumption is supported by data from other studies (Yang et al, 1999; Bowman et al, 2002) where increased microbial protein production after EFE administration was reported. Wang and others (2001) showed that EFE treatment increased the incorporation of ^{15}N into feed particle associated and bound bacteria. Lee et al (2000) reported that the total viable bacterial count was not affected by the addition of an enzyme preparation but the number of ruminal fungi increased 1.5 fold. In contrast, in their experiment Yang et al (2002) found that microbial protein synthesis was not affected by EFE, while Hristov et al (2000) showed no effect on the number of protozoa and the urinary excretion of allantoin and urate after EFE supplementation.

It is clear that the literature contains inconsistent results on the supplementation of ruminant feeds with EFE products. These differences may be caused by the different microbe strains used, the different application methods and the dissimilar enzyme activities. It can be concluded that the enzyme product from *Thermomyces lanuginosus* used in the present study has been found to induce favourable changes on rumen fermentation in sheep. It has been suggested that the addition of exogenous fibrolytic enzymes to ruminant feeds improves fibre digestibility by enhancing colonisation of ruminal microorganisms on feed particles and by stimulating the endogenous enzyme activity within the rumen (Beauchemin et al, 2000). Morgavi et al (2000) have shown that there is a synergism between EFE from *Trichoderma longibrachiatum* and ruminal fibrolytic enzymes. Concerning the higher TVFA concentration and acetate proportion, and the lower ammonia concentration measured in the final period of the present experiment, this microbial stimulation seemed to decrease slowly and was maintained after terminating the enzyme supplementation. Further research is needed to better characterise the effects of enzyme preparations from *T. lanuginosus* on fibre digestibility, microbial growth and protein degradation in the rumen.

3.2.5. Summary

- The enzyme product from *T. lanuginosus* significantly increased the xylanase activity of the rumen fluid when added to the feed, both before feeding, 2 and 4 h after feeding.
- The rumen pH remained unchanged after supplementing the feed with the enzyme product from *T. lanuginosus*.
- The total VFA concentration of the rumen fluid significantly increased after adding enzyme product of *T. lanuginosus.*

- The molar ratio of acetate was significantly higher in the rumen fluid of sheep fed with enzyme preparation from *T. lanuginosus* compared to the control group.
- The propionate ratio of the rumen fluid tended to be higher following enzyme supplementation.
- Butyrate ratio was lower in the *T. lanuginosus* enzyme supplemented sheep.
- Ammonia concentration of the rumen fluid was lower in the sheep fed with enzyme preparation from *T. lanuginosus* than that in the controls.

3.3. The effect of the xylanase preparation on dairy cows in early lactation*

3.3.1. Aim of the study

In the present study the effects of Rumino-Zyme were studied on ruminal volatile fatty acid concentration, parameters of energy and protein metabolism, milk yield, feed conversion ratio and body condition score of high yielding dairy cows in early lactation.

3.3.2. Materials and methods

Place and time of the study

The experiment was carried out at a loose housing dairy cattle farm of 2000 Holstein Friesian cows between October 1999 and February 2000.

Animals and diets

By pairing on basis of equal production and parity, two hundred ear tagged Holstein Friesian cows of 2nd and 3rd lactation were assembled into an experimental and a control group of equal size. Housing and feeding regime of the experimental and control cows was identical with the exception that daily ration of the experimental cows contained 30 g enzyme preparation mixed to the concentrate from calving till the 110th day of lactation (see details in Table 3.3.1. and 3.3.2.).

Table 3.3.1. Composition of the daily ration

Feed components kg/day	*Dry cows*		*Early lactation*		*Peak lactation*	
	CO	EX	CO	EX	CO	EX
Maize silage	14.0	14.0	14.5	14.5	15.0	15.0
Meadow hay	6.5	6.5	1.0	1.0	0.0	0.0
Alfalfa hay	0.0	0.0	3.0	3.0	3.0	3.0
Alfalfa haylage	0.0	0.0	2.5	2.5	4.5	4.5
Chopped alfalfa	0.0	0.0	2.5	2.5	4.5	4.5
Wet sugar beet slice	0.0	0.0	1.5	1.5	3.0	3.0
Brewers' grains (wet)	0.0	0.0	1.0	1.0	1.5	1.5
Molasses	0.0	0.0	0.6	0.6	0.6	0.6
Concentrates	1.5	1.5	9.0	9.0	12.1	12.1
Propylenglycol	0.0	0.0	0.1	0.1	0.1	0.1

Data recording and samplings

Of the 100 experimental and control cows, 10 cows in each group were designated for taking ruminal fluid, blood and urine samples by about two weeks intervals from the beginning of the experiment (9±4.6 days before the expected parturition) till its end (107±4.6 days after parturition). Samplings were always carried out 3-5 hours after the morning feeding. Ruminal fluid was obtained via Dirksen-tube. Rumen fluid samples were not contaminated by saliva. Blood and urine samples (20 ml each) were taken from the subcutaneous abdominal vein and via metal catheter from the

* Published as: Jurkovich et al., Acta Vet. Hung., 2002. 50, 395-411.

urinary bladder, respectively. Samples were cooled to 4°C and transported to the laboratory for further analysis. Clotting of the blood was prevented by heparine.

Table 3.3.2. Nutrient content of the daily ration (calculated values)

Nutrients	*Dry cows*		*Early lactation*		*Peak lactation*	
	CO	EX	CO	EX	CO	EX
Dry matter (kg)	12.0	12.0	19.5	19.5	23.4	23.4
NE_l (MJ)	65,9	65,9	141,3	141,3	168,1	168,1
Crude protein (g)	1 448	1 448	3 602	3 602	4 244	4 244
Crude fat (g)	314.5	314.5	1 1202	1 102	1 293	1 293
Crude fibre (g)	3 071	3 071	2 992	2 992	3 509	3 509
Ca (g)	74.9	74.9	165.6	165.6	209.7	209.7
P (g)	72.5	72.5	95.8	95.8	120.7	120.7
Mg (g)	35.8	35.8	69.8	69.8	93.7	93.7
Na (g)	35.4	35.4	39.6	39.6	51.1	51.1
Vitamin A (1 000 IU)	157.5	157.5	149.4	149.4	170.6	170.6
Vitamin D_3 (1 000 IU)	26.2	26.2	39.4	39.4	45.6	45.6
Vitamin E (mg)	1 357	1 357	468.8	468.8	506	506

VFA concentration of the ruminal fluid samples was measured gaschromatographically. Rumen fluid samples were first centrifuged at 10 000 rpm. for 10 min. Five ml of supernatant was blended with 1 ml 0.6 M oxalo-aceticacid. After 2 h rest at room temperature the material was centrifuged again for 10 min at 10 000 rpm then the quantity of VFAs was measured in a Perkin-Elmer Sigma 3B gaschromatograph. Length and diameter of the column was 2 m and 2 mm, respectively. The column was filled with Carbopack B-DA 80/120 4% CW20M. Temperature of the thermostat and the detector was 200 and 260 °C, respectively. Nitrogen gas was used as carrier at a flow rate of 60 ml/min.

Fat- and carbohydrate-metabolism and -balance were monitored by determination of the glucose, and NEFA concentrations in the blood plasma and aceto acetate concentration in whole blood samples. Data of the acid-base balance were also considered. Subclinical fat mobilisation syndrome was studied on basis of the NEFA concentrations and AST activity of blood samples. Occurrence of hyperketonaemia was judged on basis of the AcAc concentration of the blood samples.

Glucose concentration of the blood samples was determined by the method described by Trinder (1969). Urea was measured by an enzymatic method (Tietz, 1987). Activity of AST was estimated by a kinetic method (Bergmeyer et al, 1976) suggested by IFCC (International Federation of Clinical Chemistry). These examinations were carried out by an Autohumalyser 900S Plus clinico-chemical analyser (Human GmbH, Germany). The NEFA and the AcAc concentration of the blood samples was measured by the method of Noma et al. (1973) and Bloom (1958), respectively by using a Unicam Helios Gamma photometer equipped with automatic samplers (Unicam Ltd., UK).

Acid-base balance was studied by determination of the urinary pH and net acid-base excretion (NABE). Urinary pH was measured by a digital instrument (Radelkis OP-211/1, Radelkis Co., Budapest). NABE was estimated by the method of Kutas (1965).

Milk yield of the experimental and control cows was recorded by milkings and computed for daily production by a Pro Vantage™ 2050 Integrated Management System adapted to the Bou-Matic milking system. Milk fat concentration was measured once a month by the laboratory of the Institute of Herd Recording (Gödöllő, Hungary). Feed intake of the cows was measured per feedings by the weighing-instrument of a Seko-Self 500/145 L feed mixer-wagon. Data of feed distribution

was recorded and processed by MC 2 000 (V 1.147) software, interfacing between the scale and the computer.

Body condition of the cows was scored at the time of taking ruminal fluid samples by using a 1 to 5 grade scoring system according to Mulvany (1977).

Statistical analysis

Milk yield of the control and experimental cows was recorded from the time of parturition up to the 110th DIM. The first and consecutive 10-day's milk yield of each cow was averaged and the differences of these ten-day means (± SE) of the experimental and control cows were studied by ANOVA with R statistical software (R Development Core Team, 2004) for significance. This same test was used for analysing the data of ruminal fluid and other biological samples. The significance was established at level 5% ($p<0.05$).

3.3.3 Results

VFA concentration of the ruminal fluid

The total VFA concentration in the experimental group was higher than control values, throughuot the experiment ($p=0.038$).

Table 3.3.3. Average rumen fermentation parameters during the experiment

Parameters	EX	SE	CO	SE	P
Total VFA, mM	109.59	3.76	99.92	3.61	0.038
VFAs, mol/100mol					
Acetate	60.74	0.78	59.29	0.98	0.130
Propionate	29.29	0.72	26.49	0.72	0.006
N-butyrate	11.42	0.52	12.77	0.56	0.063

There was no significant difference in the acetate ratio between the EX and CO groups. On the whole the acetate ratio tended to be higher ($p=0.13$) in the experimental group during the experiment.

Energy and protein metabolism

There was no significant differences in the plasma glucose concentrations between the control and experimental cows.

The blood aceto-acetate concentrations of non-treated cows (Fig. 3.3.1.) tended to be higher ($p=0.069$) than those in the experimental group throughout the experiment. The group average of the control cows on day 22 post partum exceeded 0.1 mM.

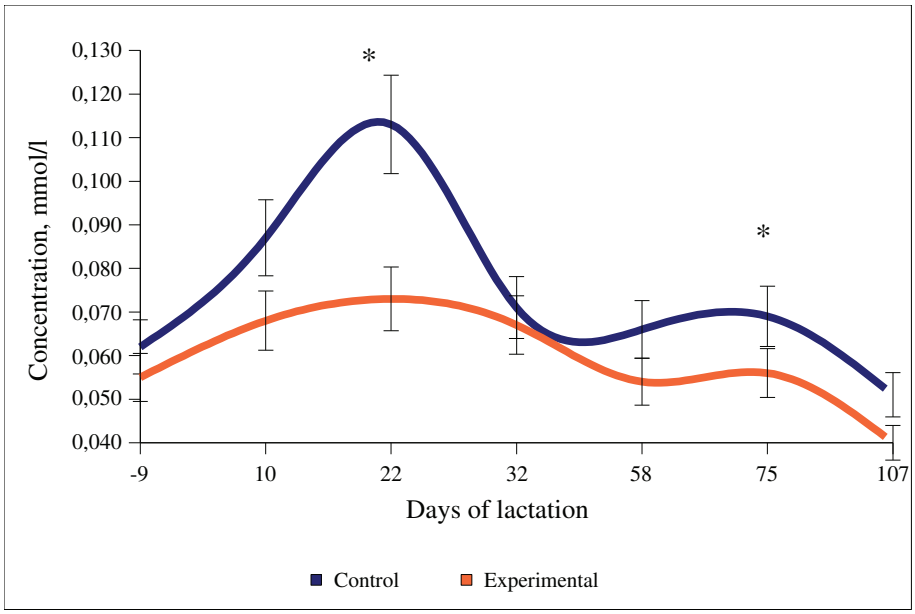

Figure 3.3.1. Aceto-acetate concentration of the blood samples

*Means are different (P<0.05)

Throughout the experiment, plasma NEFA concentrations in the experimental group were higher (p=0.008) compared to control (Figure 3.3.2.). The NEFA concentration in the plasma of the control cows on day 10 after calving exceeded the physiological limit (0.2 mM, Gönye, 1987, Gaál, 1999).

There was no significant difference in AST activity and plasma urea concentration of the control and experimental cows.

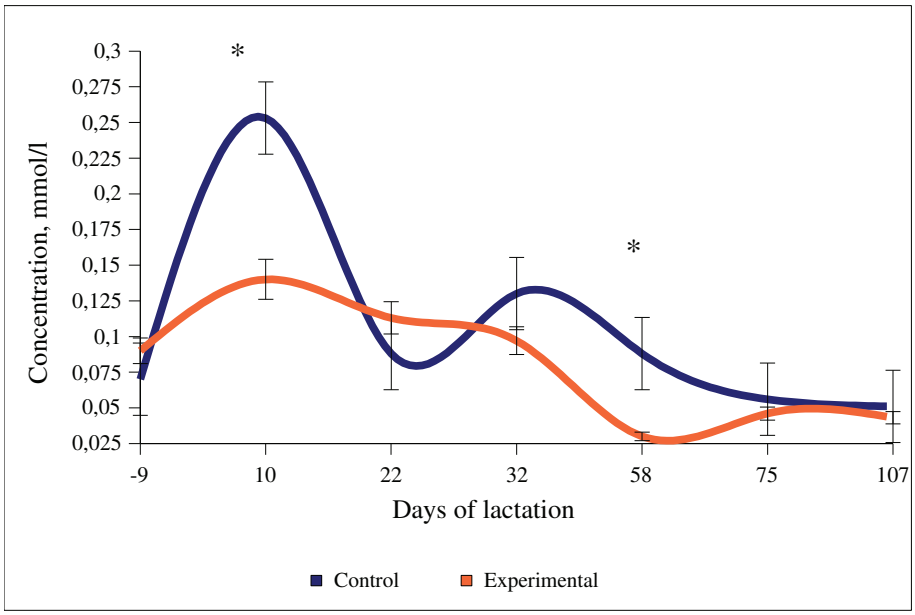

Figure 3.3.2. Non esterified fatty acid concentration of the blood plasma samples

*Means are different (P<0.05)

Laboratory findings concerning the acid-base metabolism of the experimental and control cows reflected balance throughout the experiment. The average net acid-base excretion (NABE) was higher in both groups than the physiological limit value (>100 mM, Kutas, 1965) showing no acid load in the cows.

Table 3.3.4. Average plasma and urine parameters during the experiment

Parameters	EX	SE	CO	SE	P
Glucose, mM	2.7	0.1	2.8	0.1	0.52
AST, U/l	82	2	85	3	0.47
Plasma urea, mM	5.6	0.1	5.7	0.1	0.78
Urine pH	8.5	0.02	8.5	0.03	0.48
NABE, mM	184	6	167	8	0.11

Milk yield

Milk yield of the 100 experimental and 100 control cows is summarised in Fig. 3.3.3. as daily averages of 10 day-periods (l/cow/day). Analysis of the data indicated within group and individual variances, however, in the 1st, 5th, 6th, 7th, 8th 9th and 11th decades the average milk yield of the experimental group was significantly better than that of the control. The experimental cows produced more milk from the very beginning of the lactation. The difference between the groups varied between 0.49 and 3.43 l/day/cow in favour of the experimental groups with an overall surplus of 2.14 l/day/cow. No difference was found with respect to the milk fat and protein content (data are not shown).

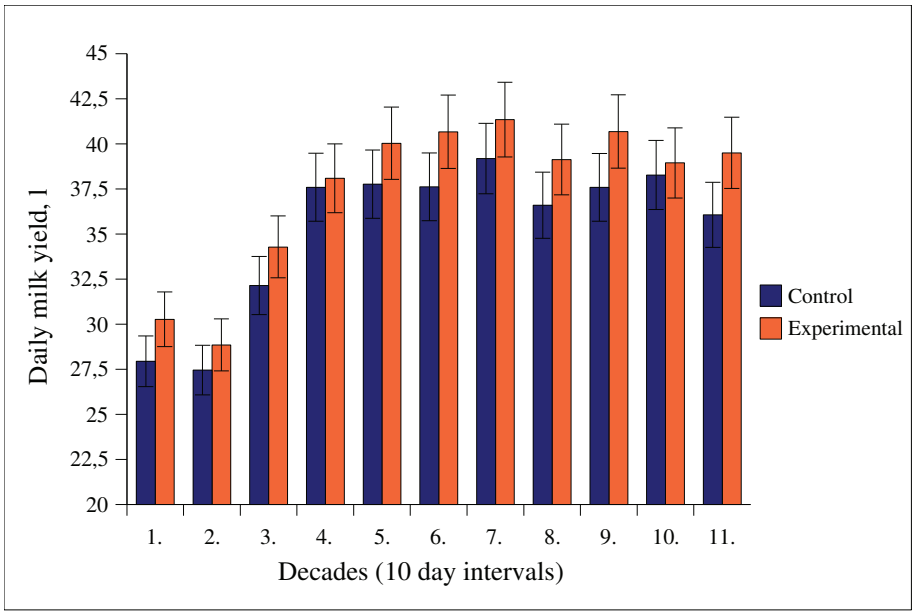

Figure 3.3.3. Daily milk production in the average of 10 day production periods after parturition

Feed intake and conversion

Dry matter intake of experimental and control cows is shown in Figure 3.3.4. as daily averages of 10 day-periods (kg/cow/day). The experimental cows consumed more feed in the first half of

experiment (4 decades). The feed consumption of the experimental cows in this period proved significantly higher ($p<0.001$) than that of the controls. The dry matter intake was numerically higher in the 6th and 7th decades in the experimental cows, and in the 8th decade the difference between the experimental and control cows was statistically significant ($p<0.001$). At the end of the experiment (9th, 10th, 11th decades) the control cows consumed more DM ($p<0,001$). The experimental cows consumed less DM for production of 1 litre of milk.

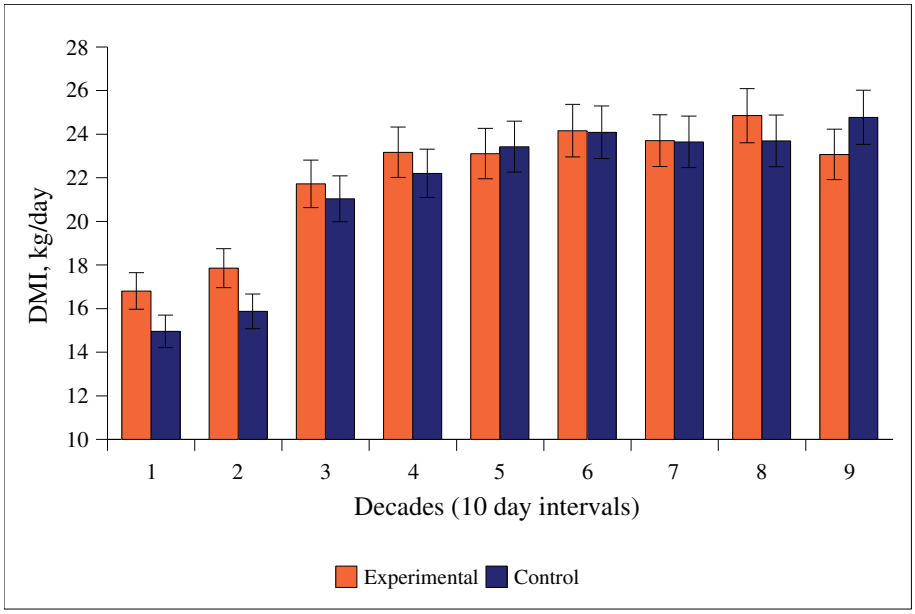

Figure 3.3.4. Dry matter intake in the average of 10 day periods after parturition

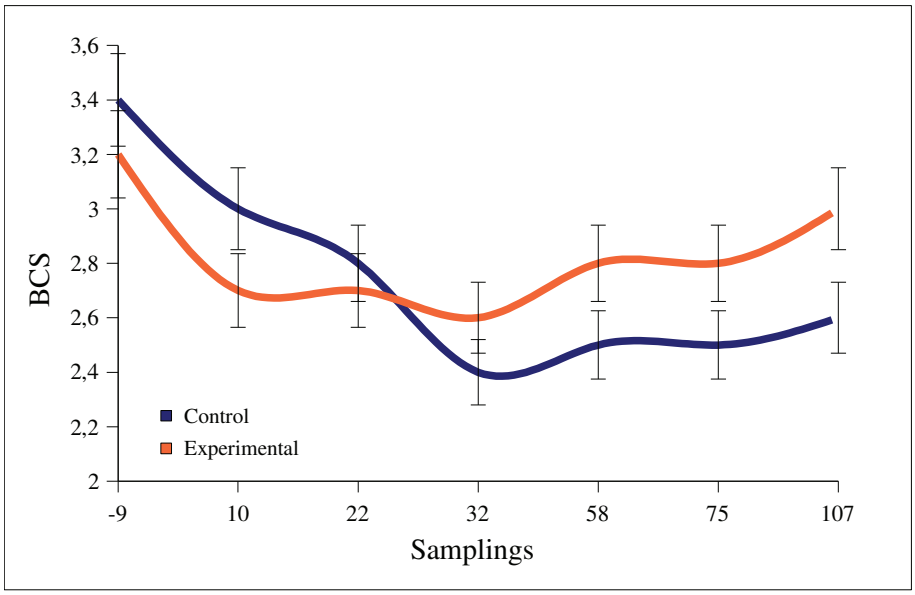

Figure 3.3.5. Body condition scores during the experiment

Body condition of the cows

At the beginning of the experiment there was only 0.2 score difference between the groups in favour of the controls (Figure 3.3.5.), which then decreased and after the third samplings the condition of the experimental cows increased, exceeding the controls, but the 0.2-0.4 score difference was statistically not significant. Body condition of the cows reached nadir at about 32±4.6 days after calving and proved lower throughout the experiment than the score given prior to calving. The condition scores of the experimental cows were never lower then 2.6 (in contrast to the controls), and the decline of body condition in the post-parturient period was about 20% less ($P<0.001$) in the experimental group.

3.3.4. Discussion

Negative energy balance prior to calving and in early lactation is common in high yielding dairy cows entailing consequent decline in milk yield and milk quality and an increased rate of reproduction failures, incidence of clinical diseases and mortality. Subclinical ketosis and subclinical fat mobilisation syndrome occurs most frequently in the first 3-4 weeks post partum (Brydl, 1999; Brydl et al., 2003) .

In cattle, the majority of the energy requirement of the tissues is covered by VFAs produced by the ruminal microflora from dietary carbohydrates. It is suspected that exogenous non-starch polysaccharidase enzymes act synergistically with the endogenous enzymes (Morgavi et al., 2000) and presumably enhancing ruminal digestibility of fibre constituents, resulting in increased protein synthesis (Beauchemin et al., 2000) and in higher VFA production.

Literature provides data on the effect of non-starch polysaccharidase enzymes on ruminal VFA production in dairy cattle. In the comparative study of Beauchemin et al. (2000) the quantity of the ruminal VFAs did not change, but the proportion of acetate in the enzyme treated cows exceeded the control values. In the experiment with growing cattle (Krause et al., 1998) the concentration of VFAs in the ruminal fluid was of the same order in the enzyme treated experimental and control animals, but the proportion of the propionate was higher in the treated cows. Contrary to the findings above, Lewis et al. (1996) found an increase in VFA production in beef cattle when daily forage ration was supplemented with 1.65 ml/kg enzyme preparation of 5800 U/ml xylanase activity.

In our experiment the ruminal concentrations of VFAs were higher than the relevant control values. This finding indicates both the beneficial effect of the treatment on the ruminal VFA production and a considerable time of adaptation. The lower ruminal acetate:propionate ratio of the experimental cows seems to be in accordance with that reported by Yang et al. (1999).

Our results reflected a balanced energy metabolism with a reduced incidence of negative energy balance in the experimental cows in the first few weeks of lactation. This conclusion is proven, among others, by the higher aceto-acetic and NEFA concentrations in the blood of the control cows right after parturition and by the increased incidence rate of hyperketonaemia in this group. It is accepted that aceto-acetic concentration higher than 0.1 mmol/l indicates hyperketonaemia (subclinical ketosis) (Brydl, 1999, Brydl et al., 2000, Radostits et al., 2000). AST activity, indicative of liver cell damage was tendentiously higher in the control group and the proportion of control cows that had AST activity higher than 100 U/l was higher also in the control group. The more intensive lipid mobilisation in the control group was proven also by the expressed decline of the postparturient body condition score in the control group. A significant change could be observed in the NEFA concentration in the control group, between 22-32 DIM. The change is likely to be the

consequence of regrouping at that time (rations have changed), see Table 3.3.1.). Milk yield also increased in both groups at the time referred to (Figure 3.3.3.). Body condition score of the control cows significantly decreased between 22-32 DIM, which was indicated by NEFA concentrations, and presumably due to the increase in milk yield. No similar changes in BCS or NEFA concentrations were observed in the experimental group, suggesting the positive effect of the enzyme supplementation.

Milk yield of the cows correlated with DMI and the increased concentration of VFAs in the rumen. The daily milk yield of the experimental cows was higher than the yield of the control animals. After calving, the milk yield of the treated cows showed correlation with the increased VFA production in the rumen. This experimental result supports those reported by others. Schingoethe et al. (1999) reported increased milk, milk fat and milk protein production as an effect of enzyme treatment and the response was apparent after 2 to 4 weeks. In the experiment of Lewis et al. (1999) enzyme treated cows (2.5 ml enzyme preparation/kg forage; xylanase activity: 5800 IU/ml) maintained higher milk yield throughout the lactation following week 4 of the treatment. Yang et al. (1999) reported earlier response to enzyme treatment. In their experiment the feed of the experimental cows was supplemented with enzyme from the time of calving and the higher milk yield of the treated cows was already seen on the 1st week of the experiment. Others (Sanchez et al., 1996, Nussio et al., 1997) also published positive effect of enzyme treatment on milk yield. In the experiment of Luchini et al. (1997) the milk yield did not increase, but the enzyme treatment caused higher milk-protein and milk fat production.

Contradicting to the findings of Luchini et al. (1997), Rode et al. (1999) and Schingoethe et al. (1999) our data suggest an increase in the appetite of the experimental cows. This finding of the present experiment supports the data published by Sanchez et al. (1996), Nussio et al. (1997), Lewis et al. (1999) and Beauchemin et al. (2000). Besides the increased daily DM intake, our experimental cows consumed less TMR for production of 1 litre of milk indicating better FCR. Sanchez et al (1996) and Luchini et al. (1997) also reported improved FCR of enzyme treated dairy cows. In the first phase of the experiment the daily milk yield shows correlation with the improving appetite of the experimental cows. The improved energy supplementation of the experimental cows resulted in a better balanced body condition in the present experiment.

The mode of action of this preparation in the rumen is not clear in its details. This feed additive is a culture of the fungus *Thermomyces lanuginosus*, which possesses xylanolytic activity. The xylanolytic activity may decrease a few hours after the application, however the effect is persistent as seen in Chapter 3.2. It may explain the increased DMI and higher milk yield after the parturition in the experimental group. The preparation supposedly improves fibre degradation, resulting in a better feed conversion ratio in the experimental cows at the end of the experiment. Further studies are needed to assess the enzyme production of the fungus in the ruminal environment and fibrolytic properties of the enzyme produced.

3.3.5. Summary

The enzyme supplementation had a positive effect on energy balance, milk yield and dry matter intake when fed to cows in early lactation (30 g/cow/day).

- As an effect of the enzyme preparation from *T. lanuginosus* we assessed a better energy balance of cows in early lactation which has been shown by low aceto-acetate and NEFA concentrations in the blood and plasma samples.

- The milk yield was higher in the cows receiving enzyme supplementation from *T. lanuginosus* in the early lactation.
- The cows supplemented with the preparation from *T. lanuginosus* in the beginning of lactation consumed more feed in the first half of the experiment. By the second half, besides no significant difference in DMI between groups, feed conversion rate of the experimental animals was significantly higher as indicated by higher milk yields.
- The body condition of the cows receiving enzyme supplementation was better than that in the control group in the early lactation.

3.4. Effects of the xylanase preparation on dairy cows in mid-lactation*

3.4.1. Aim of the study

The goal of our study was to evaluate the effects of Rumino-Zyme on the clinical condition, milk yield and milk composition of dairy cows in mid-lactation.

3.4.2. Materials and methods

The experiment was carried out at a Hungarian dairy farm between 4th April and 2nd August 2002. 18-18 multiparous, clinically healthy Holstein-Friesian cows were selected in groups A and B of a crossover design. Animals were at 70 DIM on average at start. The feed (Table 3.4.1.) did not change from the regular in the course of the experiment.

Table 3.4.1. Feed components and nutritional values

Feeds	***Group A***	***Group B***
Corn silage (kg)	22	20
Alfalfa silage (kg)	3	3
Alfalfa hay (kg)	4	4
Meadow hay (kg)	2	2
Concentrates (kg)	10	9
Nutrients *(calculated values)*		
DM (kg)	21.7	20.3
NE_l (MJ)	147.8	136.5
NE_l (MJ/kg DM)	6.8	6.7
CP (g)	3405	3266
CP (DM %)	15.8	16.1
MP (g)	2018	1888
Crude fibre (g)	3501	3350
Crude fibre (DM %)	16.1	16.5
Ca (g)	148	146
P (g)	103	99.5

The experimental group was given 30 g Rumino-Zyme daily in the TMR mixed in the concentrate.

During the first treatment period (6 weeks) group A served as experimental, group B as control group. After taking samples and a rest of 2 weeks the second treatment period started (6 weeks) with B as experimental and A as control group according to the crossover design. Milk yield and milk components were measured weekly (Table 3.4.2.) during treatment periods.

* Submitted to the Bulletin of The Veterinary Research Institute in Pulawy

Table 3.4.2. The experimental design, the date and DIM at milk samplings

Group/Sampling	*I.*	*II.*	*III.*	*IV.*	*V.*	*VI.*
Rumino-Zyme (Group A)	04 Apr.	13 Apr	23 Apr	03 May	13 May	23 May
DIM	70	79	89	99	109	119
Control (Group B)	04 Apr.	13 Apr	23 Apr	03 May	13 May	23 May
2 weeks without supplementation						
Control (Group A)	13 June	24 June	04 July	13 July	23 July	02 Aug
DIM	140	151	161	170	180	190
Rumino-Zyme (Group B)	13 June	24 June	04 July	13 July	23 July	02 Aug

Blood and urine samples were taken twice a month (Table 3.4.3.) to monitor clinical condition through biochemical parameters. Blood samples were taken from the subcutaneous abdominal vein into heparinised test tubes 3-5 hours after the morning feeding. Urine samples were taken from the urinary bladder at the same time via a metal catheter. The samples were then cooled to 4°C and transferred to the laboratory for immediate analysis.

Table 3.4.3. The design of blood and urine samplings

Group/Sampling	*I.*	*II.*	*III.*	*IV.*
Rumino-Zyme (Group A)	04 Apr.	23 Apr.	07 May	23 May
DIM	70	89	103	119
Control (Group B)	04 Apr.	23 Apr.	07 May	23 May
2 weeks without supplementation				
Control (Group A)	12 June	02 July	16 July	31 July
DIM	139	159	173	188
Rumino-Zyme (Group B)	12 June	02 July	16 July	31 July

Glucose, NEFA, and urea concentration of blood plasma samples and aceto-acetate concentration of whole blood samples were determined. AST activity of plasma was measured as well. Acid-base balance was studied by measuring the urinal pH and NABE. Methods are detailed in chapter 3.3.2. Body condition score was evaluated at the time of blood samplings by a 1 to 5 grade scoring system according to Mulvany (1977).

Statistical analysis of the data was carried out using SPSS 8.0 for Windows statistical software. Significance of the values was established at 5 % ($p<0.05$) level.

3.4.3 Results

Clinical condition

The parameters discussed below are shown in Table 3.4.4.

The BCS did not change significantly nor were any significant difference between groups. Glucose concentrations of blood plasma were in the physiological range (above 2.3 mM) in both groups, showing no significant difference. Average and individual AcAc and NEFA concentrations remained below physiological limits (0.1 and 0.2 mM). None of the parameters differed significantly between groups. AST activity of the plasma was slightly elevated in both groups, again without any statistically significant difference. Similarly, plasma urea concentrations turned out to be slightly above physiological value (5 mM), while urinal urea concentrations remained in the physiological range (130-300 mM). Experimental and control groups showed no significant difference.

Table 3.4.4. Results of blood, urine and milk samplings

Parameters	EX	SE	CO	SE	P
BCS	2.5	0,1	2.6	0,1	0.66
Glucose, mM	2.5	0.2	2.4	0.2	0.77
AcAc, mM	0.056	0.01	0.063	0.01	0.12
NEFA, mM	0.053	0.01	0.054	0.01	0.38
AST, U/l	98	7	95	5	0.54
Plasma urea, mM	7.2	0.3	6.6	0.3	0.40
Urine pH	8.5	0,1	8.6	0.1	0.38
NABE, mM	115	10	119	11	0.38
4 % FCM, l	31.6	1.5	30.8	1.3	0.25
Milk fat, %	3.72	0.16	3.66	0.17	0.93
Milk protein, %	3.26	0.01	3.22	0.01	0.34

Milk yield

Average milk yields (Figure 3.4.1.) decreased in both experimental and control groups (E: 35.3 – 32.9; C: 34.7 – 31.5; [l]) but at a different degree. The decrease in the experimental group was lower than that of the control group (6.8% vs. 9.3%, $p<0.05$). In spite of this, average milk yield did not differ significantly between groups, though experimental values were at all times higher and a difference of $p=0.085$ could be estimated at the time of the last sampling. The same can be stated regarding 4% FCM. The experimental group produced 0.8 l more 4% FCM on average yet difference between groups was not significant. Average fat- and protein content of milk in the experimental group increased by 0.06% and 1.2%, respectively, still these values showed no statistically significant difference compared to control (Table 3.4.4.).

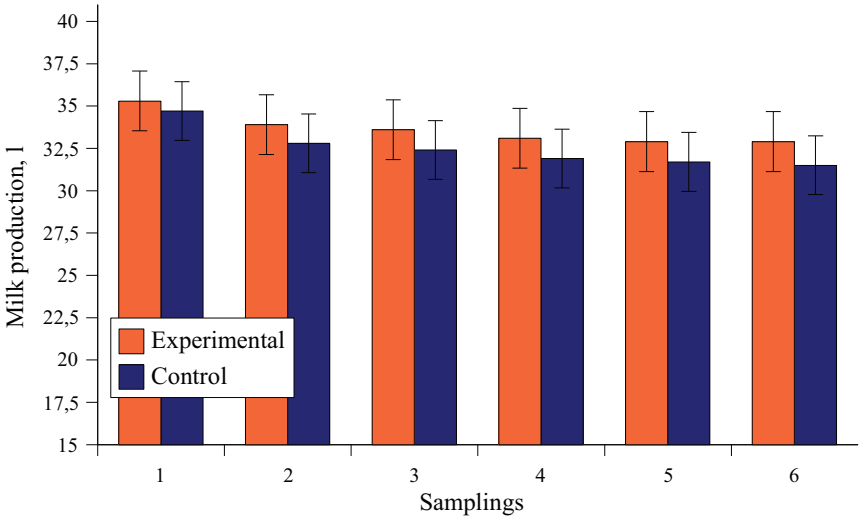

Figure 3.4.1. The average milk production during the experiment

3.4.4 Discussion

According to the results of the present study it can be established that main blood and urine parameters in relation with clinical condition did not differ significantly between experimental and control groups. AST activity of plasma slightly exceeded physiological limit but as no other illnesses (clinical or sub-clinical mastitis, lameness) were present in the course of our investigation, increase is supposedly in relation with elevated blood urea concentrations. Regarding both parameters difference between groups showed no significance therefore the enzyme supplementation had no adverse effect on animal health. Based on the results of our previous study (chapter 3.3.) the same enzyme preparation had a significantly positive effect on the energy-imbalance of the experimental cows in early lactation. Rode et al (1999.) reported similar benefits of oral enzyme supplementation. In the present study cows in mid-lactation already reached positive energy status hence significant differences between treated and untreated groups were not expected to occur.

Scientific research has proved enzyme supplementation to be beneficial for milk yield (Nussio et al., 1997, Lewis et al., 1999; Rode et al., 1999; Schingoethe et al., 1999; Yang et al., 1999) and milk composition (Lewis et al., 1999; Schingoethe et al., 1999; Yang et al., 1999) when fed in the beginning of the lactation period. The first few weeks postpartum is the time of negative energy status when increasing DMI or degradability of feed-components seems obvious to be the way to improve production. Studies mentioned above provided data on increased DMI (Sanchez et al., 1996; Nussio et al., 1997; Lewis et al, 1999) or digestibility (Rode et al., 1999; Yang et al., 1999; Yang et al., 2000) as a result of using enzymes as feed-additives. Knowlton et al. (2002.) experienced an insignificant increase in milk yield of the experimental group correlating with DMI and degradability. Others (Dhiman et al., 2002; Sutton et al., 2003; Higginbotham et al., 2004) could not detect neither increased yield nor significant influence on feed-intake or degradability. In our investigation milk yield decreased with time, which correlates with being in mid-lactation. Our results show insignificantly higher production parameters in the experimental group. The inevitable physiological decrease in daily average milk yield was lower suggesting persistence improved. Other studies confirm the positive but insignificant effect of oral enzyme supplementation on milk production during mid-lactation. Schingoethe et al. (1999), Bowman et al. (2002) reported on increasing yield and milk-parameters, Knowlton et al. (2002.) on the latter. None of them established statistical significance. Bowman et al. (2002.) and Knowlton et al. (2002.) found that though DMI did not change significantly, degradability improved. Contrary to their findings Lewis et al. (1999.) and Kung et al. (2000.) reported on a significant increase in milk yield as an effect of oral enzyme supplementation in mid-lactation. A possible explanation could not be given however, as DMI remained unchanged and degradability or ruminal volatile fatty acid production was not measured. Interestingly, Beauchemin et al. (2000.) experienced increased DMI and degradability while nor yields and composition of milk, neither body condition improved. In our present study DMI was optimised, both groups received the same ration and consumption was 100%. The positive changes in yield are presumably due to enhanced availability of nutrients and/or stimulated ruminal fermentation (confirming measurements could not be carried out). Research suggests that exogenous fibre-degrading enzymes act by improving feed-intake, digestion or both in the stage of pre-top and top lactation and has a beneficial effect on digesting high-fibre rations of post-top and end stages of lactation. Method of application, concentration and nature of feed-components heavily influence efficacy.

3.4.5. Summary

The enzyme product administered (30 g/cow/day) had no significant effect on the production parameters of cows in mid-lactation.

- The biochemical parameters of blood and urine did not differ significantly in the cows fed with and without enzyme preparation from *T. lanuginosus* in the middle of lactation.
- Milk yield, fat and protein content of the milk did not differ between the cows fed with or without enzyme preparation from *T. lanuginosus* in mid-lactation.

4. New scientific results

1. Our research group was the first to test a xylanase enzyme preparation of the fungus *Thermomyces lanuginosus* as feed additive in ruminant nutrition.
2. Adding 10g enzyme preparation from *T. lanuginosus* of 250 FXU/g xylanas activity in one dose intraruminally significantly increased the xylanase activity of the rumen fluid within 5 min after treatment in sheep. The supplemental enzyme preserved its maximum activity for 45 min and half of its activity for over 2 hours. The enzyme activity of the rumen fluid returned back to the initial values 3 h after supplementation.
3. The enzyme product from *T. lanuginosus* significantly elevated the xylanase activity of the rumen fluid when added to the feed in a dose of 2,5 g/animal/day either before feeding or 2 and 4 h after feeding in sheep.
4. Ruminal pH remained unchanged after supplementing the feed of sheep with the enzyme product from *T. lanuginosus*. The total VFA concentration and the molar ratio of acetate of the rumen fluid significantly increased but n-butyrate ratio was significantly lower after adding 2.5 g/day enzyme product of *T. lanuginosus* to the diet of sheep. Ammonia concentration of the rumen fluid was lower in the sheep fed with enzyme preparation from *T. lanuginosus* than that of the controls.
5. As an effect of the enzyme preparation from *T. lanuginosus* fed in a dose of 30 g/animal/day we measured a better energy balance of dairy cows in early lactation which has been shown by reduced AcAc and NEFA concentrations in the blood and plasma samples
6. The milk yield and feed intake was higher in cows in early lactation when fed 30 g/cow/day enzyme preparation from *T. lanuginosus*. The body condition of the cows receiving enzyme supplementation was significantly better than that in the control group in the early lactation.
7. The enzyme supplementation had no significant effect on milk yield, milk components, energy, protein and acid-base metabolism when applied in mid-lactation (30 g/cow/day).

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6. References

1. Adams, S.E., Sniffen, C.J., White, J.H., Knapp, J.R. 2002. Effects of a fibrolytic enzyme supplement on digestion and nutrient utilization by dairy cows fed alfalfa hay and grass silage-based rations. *Journal of Animal Science* 80, Suppl. 1. / *Journal of Dairy Science,* 85, Suppl. 1. (abstract)
2. Akin, D.E., Borneman, W.S. 1990. Role of rumen fungi in fibre digestion. *Journal of Dairy Science,* 73, 3023-3032.
3. Alam, M., Gomes, I., Mohuiddin, G., Hoq, M.M. 1994. Production and characterization of thermostable xylanase by *Thermomyces lanuginosus* and *Thermoascus auranticus* grown on lignocelluloses. *Enzyme Microbiology Technology*, 16, 298-302.
4. Annison, G., 1997. The use of enzymes in ruminant diets. In: *Biotechnology in Feed Industry. Proceedings of Alltech's 13th Annual Symposium*, Nicholasville, Kentucky, pp. 115-129.
5. Annison, E.F., Bryden, W.L. 1998. Perspectives on ruminant nutrition and metabolism. I. Metabolism in the rumen. *Nutrition Research Reviews,* 11, 173-198.
6. Baran, M., Kmet, V. 1987. Effect of pectinase on rumen fermentation in sheep and lambs. *Archives of Animal Nutrition Berlin,* 37, 643-646.
7. Barcroft, J., McAnally, R.A., Phillipson, A.T. 1944. Absorption of volatile fatty acids from the alimentary tract of the sheep and other animals. *Journal of Experimental Biology,* 20, 120-129.
8. Beauchemin, K.A., Rode, L.M., Sewalt, V.J. 1995. Fibrolytic emzymes increase fibre digestibility and growth rate of steers fed dry forages. *Canadian Journal of Animal Science,* 75, 641-644.
9. Beauchemin, K.A., Yang, W.Z., Rode, L.M. 1999. Effects of grain source and enzyme additive or grain source on site and extent of nutrient digestion in dairy cows. *Journal of Dairy Science,* 82, 378-390.
10. Beauchemin, K.A., Rode, L.M., Maekawa, M., Morgavi, D.P., Kampen, R. 2000. Evaluation of nonstarch polysaccharidase feed enzyme in dairy cow diets. *Journal of Dairy Science,* 83, 543-553.
11. Bedford, M.R., Schulze, H. 1998. Exogenous enzymes for pigs and poultry. *Nutrition Research Reviews,* 11, 91-114.
12. Beguin, P., Chavraux, S., Guglielmi, G., Matuschek, M., Leibovitz, E., Chaveroche, M.K., Miras, I., Alzari, P., Gounon, P. 1998. The *Clostridium thermocellum* cellulosome: organization and mode of attachment to the cell. *MIE Bioforum, Genetics, Biochemistry and Ecology of Cellulose Degradation,* Suzuka, Japan, p. 107.
13. Bennet, N. I., Ryen, J., Biely, P., Vrasanska, M. 1998. Biochemical and catalytic properties of an endoxylanase purified from the culture filtrate of *Thermomyces lanuginosus* ATCC 46882. *Carbohydrate Research,* 306, 445-455.
14. Bergmeyer, H.U., Bowers, Jr, G.N., Hörder, M., Moss, D.W. 1976. Provisional recommendations on IFCC Methods for the measurement of catalytic concentrations of enzymes. Part 2. IFCC method for aspartat aminotransferase. *Clinica Chimica Acta*, 70, 19-42.
15. Biely, P., Mislovicova, D., Toman, R. 1988. Remazol brillant blue xylan: a soluble chromogenic substrates for xylanase. *Methods in Enzymology,* 160A, 536.

16. Bloom, W.L.: 1958. The determination of ketone bodies in biologic fluids. *Journal of Laboratory and Clinical Medicine,* 51, 824–828.
17. Bolsen, K.K., Bonilla, D.R., Young, M.A., Hart-Thakur, R.A. 1996. Effect of bacterial inoculants on the fermentation of alfalfa silage. *Journal of Dairy Science,* 79, Suppl 1, 189. (abstract)
18. Bowden, D.W., Church, D.C. 1959. Several enzyme concentrates on cellulose digestion in the artificial rumen. *Journal of Animal Science,* 18, 1523. (Abstr.)
19. Bowman, G.R., Beauchemin, K.A., Shelford, J.A. 2002. The proportion of the diet to which fibrolytic enzymes are added affects nutrient digestion by lactating dairy cows. *Journal of Dairy Science,* 85, 3420-3429.
20. Brydl E. 1999. Occurrence of subclinical metabolic disorders on dairy farms in Hungary between 1991-1997. *Magyar Állatorvosok Lapja,* 121, 82-84 (in Hungarian)
21. Brydl E., Jurkovich V., Könyves L., Tegzes L-né, Kálmán I.: Occurrence of subclinical metabolic disorders on dairy farms in Hungary in 2001. *Magyar Állatorvosok Lapja*, 2003, 125 (7), 393-400 (in Hungarian).
22. Burroughs, W., Woods, W., Ewing, S.A., Greig, J., Theurer, A.B. 1960. Enzyme additions to fattening cattle rations. *Journal of Animal Science,* 19, 458-464.
23. Carpita, N.C., Gibeaut, D.M. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant Journal,* 3, 1-30.
24. Castañón, J.I.R., Flores, M.P., Pettersson, D. 1997. Mode of degradation of non-starch polysaccharides by feed enzyme preparations. *Animal Feed Science and Technology,* 68, 361-365.
25. Chadha, B.S., Jaswinder, K., Rubinder, K., Saini, H.S., Singh, S. 1999. Xylanase production by *Thermomyces lanuginosus* wild and mutant strains. *World Journal of Microbiology and Biotechnology,* 15, 217-221.
26. Chaney, A. L., Marbach, E. P. 1962. Modified reagents for determination of urea and ammonia. *Clinical Biochemistry,* 8, 130-132
27. Cesar, T. and Mrsa, V. 1996. Purification and properties of the xylanase produced by *Thermomyces lanuginosus*. *Enzyme and Microbial Technology*, 19, 289-296.
28. Deacon J.W. 1985. Decomposition of filter paper cellulose by thermophilic fungi acting singly, in combination, and in sequence. *Transactions of British Mycological Society*, 85, 663-669.
29. Deacon, J. 2002. The microbial world, thermophilic microorganisms. Biology teaching organization, University of Edinborough, http://helios.bto.ed.ac.uk/bto/microbes/thermo.htm
30. Dehority, B.A. 2003. *Rumen microbiology*. Nottingham University Press, Nottingham, UK, pp 365.
31. Dhiman, T.R., Zaman, M.S., Gimenez, R.R., Walters, J.L., Treacher, R. 2002. Performance of dairy cows fed forage treated with fibrolytic enzymes prior to feeding. *Animal Feed Science and Technology,* 101, 115-125.
32. Dijkstra, B.J., Tamminga, S. 1995. Simulation of the effects of diet on the contribution of rumen protozoa to degradation of fibre in the rumen. *British Journal of Nutrition,* 74, 617-634.
33. Dirksen, G. 1970. Acidosis. In: *Physiology of digestion and metabolism in the ruminant* (ed. Philipson, A.T.), Oriel press, London
34. Dong, Y., Bae, H.D., McAllister, T.A., Mathison, G.W., Cheng, K.J. 1999. The effect of

exogenous fibrolytic enzymes, alpha-bromoethanesulphonate and monensin on digestibility of grass hay and methane production in the Rusitec. *Canadian Journal of Animal Science,* 79, 491-498.

35. European Commission 2000a. Opinion of the Scientific Committe on Animal Nutrition on the assessment of efficacy of enzymes. www.europa.eu.int/comm/food/fs/sc/scan/out45_en.pdf
36. European Commission 2000b. Report of the Scientific Committe on Animal Nutrition on the assessment under directive 87/153/EEC of the efficacy of micro-organisms used as feed additives. www.europa.eu.int/comm/food/fs/sc/scan/out40_en.pdf
37. European Commission 2002a. Opinion of the Scientific Committe on Animal Nutrition on the use of certain enzymes in animal feedingstuffs. www.europa.eu.int/comm/food/fs/sc/scan/out96_en.pdf
38. European Commission 2002b. List of the authorised additives in feedingstuffs published in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives in feedingstuffs. *Official Journal of the European Communities* C 329, 1-142.
39. European Commission 2003. Opinion of the Scientific Committe on Animal Nutrition on the use of certain micro-organisms as additives in feedingstuffs. www.europa.eu.int/comm/food/fs/sc/scan/out93_en.pdf
40. European Commission 2004. Update (situation as 30 April 2004) of the list of authorized additives in feedingstuffs published in application Article 9t (b) of Council Directive 70/524/EEC concerning additives in feedingstuffs. www.europa.eu.int/comm/food/food/animalnutrition/feedadditives/update.pdf
41. Faichney, G.J. 1980. Measurement in sheep of the quantity and composition of rumen digesta and fractional outflow rates of digesta constituents. *Asian Journal of Agricultural Research,* 31, 1129-1137.
42. Feng, P., Hunt, C.W., Julien, W.E., Haenny, S.C., Pritchard, G.T. 1992. Effect of enzyme additives to cool-season grass forage on voluntary intake and digestive function in mature beef steers. *Journal of Animal Science,* (Suppl. 1) 310. (abstract)
43. Fontes, C.M.G.A,. Hall, J., Hirst, B.H., Hazlewood, G.P., Gilbert, H.J. 1995. The resistance of cellulases and xylanases to proteolytic inactivation. *Applied Microbiology and Biotechnology,* 43, 52-57.
44. Forwood, J.R., Sleper, D.A., Henning, J.A. 1990. Topical cellulase application effects on tall fescue digestibility. *Agronomy Journa*l, 82, 900-913.
45. Freeden, A.H., McQueen, R.E. 1993. Effects of enzyme additives on quality of alfalfa/grass silage and dairy cow performance. *Canadian Journal of Animal Science,* 73, 581-591.
46. Gaál T. (ed) 1999. *Veterinary clinical laboratory diagnostics*. Sík Kiadó, Budapest (in Hungarian)
47. Gomes, J., Gomes, I., Kreiner, W., Esterbauer, H., Sinner, M., Steiner, W. 1993a Production of high level of cellulase-free and thermostable xylanase by a wild strain of *Thermomyces lanuginosus* using beechwood xylan. *Journal of Biotechnology,* 30, 283-297.
48. Gomes, J., Purkarthofer, H., Hayn, M., Kapplmüller, J., Sinner, M., Steiner, W. 1993b. Production of a high level of cellulase-free xylanase by the thermophilic fungus *Thermomyces lanuginosus* in laboratory and pilot scales using lignocellulosic materials. *Applied Microbiology and Biotechnology,* 39, 700-707.
49. Gómez-Vázquez, A., Pérez, J., Mendoza, G.D., Aranda, E., Hernández, A. 2003. Fibrolytic exogenous enzymes improve performance in steers fed sugar cane and stargrass. *Livestock*

Production Science, 82, 249-254.
50. Gönye S. 1987. The disorders of metabolism. In: *Metabolic disorders and poisonings of cattle* (ed. Brydl E.) . Mezőgazdasági Kiadó, Budapest (in Hungarian)
51. Grainger, R.B., Stroud, J.W. 1960. Effect of enzymes on nutrient digestion by wethers. *Journal of Animal Science,* 19, 1263. (abstract)
52. Graham, H. 1996. Feed enzymes influence intestinal microflora. *Zootechnique International,* 19:10, 88-92.
53. Grajek W. 1986. Temperature and pH optima of enzyme activities produced by cellulolytic thermophilic fungi in batch and solid-state cultures. *Biotechnology Letters,* 8, 587-590.
54. Guarro, J., Gené, J., Stchigel, A.M. 1999. Develpoments in fungal taxonomy. *Clinical Microbiology Reviews,* 12, 454-500.
55. Guillot, J.F. 2003. Probiotic feed additives. *Journal of Veterinary Pharmacology and Therapy,* 26, (Suppl. 1) 52-55.
56. Gwayumba, W., Christensen, D.A. 1997. The effect of fibrolytic enzymes on protein and carbohydrate degradation fractions in forages. *Canadian Journal of Animal Science,* 77, 541-542. (abstract)
57. Hall, J., Ali, S., Surani, M.A., Hazlewood, G.P., Clark, A.J., Simons, J.P., Hirst, B.H., Gilbert, H.J. 1993. Manipulation of the repertoire of digestive enzymes into the gastrointestinal tract of transgenic mice. *Bio/Technology,* 11, 376-379.
58. Hangarter, R. 2003. Mechanisms of plant growth and development (Fall Semester 2003), University of Indiana, http://sunflower.bio.indiana.edu/~rhangart/courses/b373/lecturenotes/cellwall/cellwall.html
59. Hespell, R.B., Whitehead, T.R. 1990. Physiology and genetics of xylan degradation by gastrointestinal tract bacteria. *Journal of Dairy Science,* 73, 3013-3022.
60. Higginbotham, G.E., Santos, J.E.P., Juchem, S.O., DePeters, E.J. 2004. Effect of feeding Aspergillus oryzae on milk production and rumen parameters. *Livestock Production Science*, 86, 55-59.
61. Hoq, M. M., Hempel, C., Deckwer W.D. 1994. Cellulase-free xylanase by *Thermomyces lanuginosus* RT9: Effect of agitation, aeration, and medium components on production. *Journal of Biotechnology,* 37, 49-58.
62. Hoq, M. M., Deckwer, W.D. 1995. Cellulase-free xylanase by thermophiloic fungi: a comparison of xylanase production by two *Thermomyces lanuginosus* strains. *Applied Microbiology and Biotechnology,* 43, 604-609.
63. Hristov, A.N., McAllister, T.A., Cheng, K.J. 1998a. Stability of exogenous polysaccharide-degrading enzymes in the rumen. *Animal Feed Science and Technology,* 76, 161-168
64. Hristov, A.N., McAllister, T.A., Cheng, K.J. 1998b. Effect of dietary or abomasal supplementation of exogenous polysaccharide-degrading enzyme supplementation on rumen fermentation and nutrient digestibility. *Journal of Animal Science,* 76, 3146-3156.
65. Hristov, A.N., McAllister, T.A., Cheng, K.J. 2000. Intraruminal supplementation with increasing levels of exogenous polysaccharide-degrading enzymes: effect of nutrient digestion in cattle fed barley grain diets. *Journal of Animal Science,* 78, 477-487.
66. Huhtanen, P., Khalili, H. 1992. The effect of sucrose supplements on particle associated carboxymethylcellulase (EC 3.2.1.4) and xylanase (EC 3.2.1.8) activities in cattle given grass-silage-based diet. *British Journal of Nutrition,* 67, 245-255.
67. Ischler, V., Heinrichs, J., Varga, G. 1996. From feed to milk: understanding rumen function. *Extension circular 422,* The Pennsylvania State University.

68. Knowlton, K.F., McKinney, J.M., Cobb, C. 2002. Effect of a direct-fed fibrolytic enzyme formulation on nutrient intake, partitioning, and excretion in early and late lactation Holstein cows. *Journal of Dairy Science,* 85, 3328-3335.
69. Koike, S., Pan, J., Kobayashi, Y., Tanaka, K. 2003. Kinetics of in sacco fibre attachment of representative ruminal cellulolytic bacteria monitored by competitive PCR. *Journal of Dairy Science,* 86, 1429-1435.
70. Kopecny, J., Marounek, M., Holub, K. 1987. Testing the suitability of the addition of *Trichoderma viridae* cellulases to feed rations for ruminants. *Zivocisna Vyroba*. 32, 587-590.
71. Krause, M., Beauchemin, K.A., Rode, L.M., Farr, B.I., Norgaard, P. 1998. Fibrolytic enzyme treatment of barley grain and source of forage in high-grain diets fed to growing cattle. *Journal of Animal Science,* 76, 2912-2920.
72. Krause, D.O., Bunch, R.J., Dalrympe, B.D., Gobius, K.S., Smith, W.J., Xue, G.P., McSweeney, C.S. 2001. Expression of a modified *Neocallimastix patricarium* xylanase in *Butyrivibrio fibriosolvens* digests more fibre but cannot effectively compete with highly fibrolytic bacteria in the rumen. J*ournal of Applied Microbiology,* 90, 388-396.
73. Kung, L., Treacher, R.J., Nauman, G.A., Smagala, A.M., Endres, K.M., Cohen, M.A. 2000. The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. *Journal of Dairy Science,* 83, 115-122.
74. Kung L. 2001. Enzymes for lactating dairy cows: new theories and applications. In: *Proceedings of the 12th Annual Florida Ruminant Nutrition Symposium*, pp 29-43. www.animal.ufl.edu/dairy/2001ruminantprodconf/Kung2.htm
75. Kutas F. 1965. The measurement of net acid-base excretion in the urine of cattle. (A method for the estimation of acid-base equilibrium). *Magyar Állatorvosok Lapja,* 20, 104-107. (in Hungarian)
76. Kutasi, J., Bata, Á., Brydl, E., Rafai, P., Jurkovich, V. 2001. Characterisation and effects of a xylanase enzyme preparation extracted from *Thermomyces lanuginosus* cultures. *Acta Veterinaria Hungarica,* 49, 175-184.
77. Kutasi J., Jurkovich V., Brydl E., Könyves L., Bata Á. 2005. Degradation of the fibre components of wheat straw and maize stalk by the thermophilic fungus *Thermomyces lanuginosus*. In: Abstracts of 1st Central European Forum for Microbiology (CEFORM), 26-28 October 2005, Keszthely, Hungary, *Acta Microbiologica et Immunologica Hungarica,* 52, Suppl., 89-90. (abstract)
78. Lee, S.S., Ha, J.K., Cheng, K.J. 2000. Influence of an anaerobic fungal culture administration on in vivo ruminal fermentation and nutrient digestion. *Animal Feed Science and Technology,* 88, 201-217.
79. Lewis, G.E., Hunt, C.W., Sanchez, W.K., Treacher, R., Pritchard, G.T., Feng, P. 1996. Effect of direct-fed enzymes on the digestive characteristics of a forage based diet fed to beef steers. *Journal of Animal Science,* 74, 3020-3028.
80. Lewis, G.E., Sanchez, W.K., Hunt, C.W., Guy, M.A., Pritchard, G.T., Swanson, B.I. and Treacher, R.J. 1999. Effect of direct-fed fibrolytic enzymes on the lactational perormance of dairy cows. *Journal of Dairy Science,* 82, 611-617.
81. Lin, J., Pillay, B., Singh, S. 1999a. Purification and biochemical characteristics of beta-D-glucosidase from thermophilic fungus, *Thermomyces lanuginosus*-SSBP. *Biotechnology and Applied Biochemistry,* 30, 81-87.
82. Lin, J., Ndlovu, L.M., Singh, S., Pillay, B. 1999b. Purification and biochemical characteristics of beta-D-xylanase from thermophilic fungus, *Thermomyces lanuginosus*-SSBP.

Biotechnology and Applied Biochemistry, 30, 73-79.
83. Lisching, T., Purkarthofer, H., Steiner, W. 1993. Thermostability of endo-beta-xylanase from the thermophilic fungus *Thermomyces lanuginosus*. *Biotechnology Letters,* 15, 411-414.
84. Lunchini, N.D., Broderick, G.A., Hefner, D.L., Derosa, R., Reynal, S., Treacher, R.J. 1997. Production response to treating forage with fibrolytic enzymes prior to feeding to lactating cows. *Journal of Dairy Science,* 80, (Suppl. 1.) 262. (abstract)
85. Maheshwari, R., Bharadwaj, G., Bhat, M. 2000. Thermophilic fungi: Their physiology and enzymes. *Microbiology and Molecular Biology Reviews,* 64, 461-488.
86. Martin, C., Fernandez, I., Rochette, Y., Michalet-Doreau, B. 2000. Is ruminal viscosity involved in the microbial fibrolytic activity decrease with high cereal diet? In: *Proceedings of 25th Conference on Rumen Function*, Chicago, Illinois (Abstr.) www.msu.edu/user/rumen
87. McAllister, T.A., Bae, H.D., Jones, G.A., Cheng, K.J. 1994. Microbial attachment and feed digestion in the rumen. *Journal of Animal Science,* 72, 3004-3018.
88. McAllister T.A., Cheng K.J. 1996. Microbial strategies in the ruminal digestion of cereal grains. *Animal Feed Science and Technology,* 62, 29-36.
89. McAllister, T.A., Stanford, K., Bae, H.D., Treacher, R.J., Hristov, A.N., Baah, J., Shelford, J.A., Cheng, K.J. 2000. Effect of a surfactant and exogenous enzymes on digestibility of feed and on growth performance and carcass traits of lambs. *Canadian Journal of Animal Science,* 80, 35-44.
90. McAllister, T.A., Hristov, A.N., Beauchemin, K.A., Rode, L.M., Cheng, K.J. 2001. Enzymes in ruminant diets. In: *Enzymes in farm animal nutrition* (eds. Bedford, M.R. and Partridge, G.G.), CAB International. pp. 273-298.
91. McGilliard, M.L., Stallings, C.C. 1998. Increase in milk yield of commercial dairy herds fed a microbial and enzyme supplement. *Journal of Dairy Science,* 81, 1353-1357.
92. McHan, F. 1986. Pretreatment of coastal bermudagrass with sodium hidroxide and cellulase before ensiling. *Journal of Dairy Science,* 69, 1837.
93. Michalet-Doreau, B., Fernandez, I., Peyron, C., Millet, L,. Fonty, G. 2001. Fibrolytic activities and cellulolytic bacterial community structure in the solid and liquid phases of rumen contents. *Reproduction, Nutrition, Development,* 41, 187-194.
94. Minato, H., Endo, A., Ootomo, Y., Uemura, T. 1966. Ecological treatise on the rumen fermentation. II. The amylolytic and cellulolytic activities of fractionated bacterial portions attached to rumen solids. *Journal of General and Applied Microbiology,* 12, 53-69.
95. Miron, J., Ben-Ghadaila, D., Morrison, M. 2001. Adhesion mechanisms of rumen cellulolytic bacteria. *Journal of Dairy Science,* 84, 1294-1309.
96. Morgavi D. P., Beauchemin K. A., Nsereko V. L., Rode L. M., Iwaasa A. D., Yang W. Z., McAllister T. A., Wang Y. 2000a. Synergy between ruminal fibrolytic enzymes and enzymes from *Trichoderma longibrachiatum*. *Journal of Dairy Science,* 83, 1310-1321.
97. Morgavi, D.P., Newbold, C.J., Beever, D.E., Wallace, R.J. 2000b. Stability and stabilization of potential feed additive enzymes in rumen fluid. *Enzyme and Microbial Technology,* 26, 171-177.
98. Morgavi, D.P., Nsereko, V.L., Rode, L.M., Beauchemin, K.A., McAllister, T.A., Wang, Y. 2000c. A *Trichoderma* feed enzyme preparation enhances adhesion of *Fibrobacter succinogenes* to complex substrates but not to pure cellulose. In: *Proceedings of 25th Conference on Rumen Function*, Chicago, Illinois (Abstr.) www.msu.edu/user/rumen
99. Morgavi, D.P., Beauchemin, K.A., Nsereko, V.L., Rode, L.M., McAllister, T.A., Iwaasa, A.D., Wang, Y., Yang, W.Z. 2001. Resistance of feed enzymes to proteolytic inactivation by rumen

microorganisms and gastrointestinal proteases. *Journal of Animal Science,* 79, 1621-1630.
100. Mould, F.L., Ørskov, E.R., Mann, S.O. 1983. Associative effects of mixed feeds. I. Effects of type and level of supplementation and the influence of the rumen fluid pH on cellulolysis in vivo and dry matter digestion of various roughages. *Animal Feed Science and Technology,* 10, 15.
101. Mulvany, P. 1977. Dairy cow condition scoring. *NIRD Paper,* No. 4468.
102. Nakashima, Y., Orskov, E.R., Hotten, P.M., Ambo, K., Takase, Y. 1988. Rumen degradation of straw: effects of polysaccharidase enzymes on degradation characteristics of ensiled rice straw. *Animal Production,* 47, 421.
103. Newbold, C.J., Brock, R., Wallace, R.J. 1992. The effect *Aspergillus oryzae* fermentation extract on the growth of fungi and ciliate protozoa in the rumen. *Letters of Applied Microbiology,* 15, 109-112.
104. Newbold, C.J. 1995. Microbial feed additives in ruminants. In: *Biotechnology in animal feeds and animal feeding* (eds. Wallace, R.J. and Chesson, H.C.) VCH Publishers, New York, pp. 259-278.
105. Nguyen, Q.D., Rezessy-Szabó, J.M., Hoschke, Á. 2000. Optimisation of composition of media for the production of amylolytic enzymes by *Thermomyces lanuginosus* ATCC 34626. *Food Technology and Biotechnology,* 38, 229-234.
106. Noma, A., Okabe, H., Kita, M. 1973. A new colorimetric micro-determinatoin of free fatty acids in serum. *Clin. Chim. Acta.,* 43, 317-320.
107. Nsereko, V.L., Morgavi, D.P., Beauchemin, K.A., Rode, L.M. 2000a. Inhibition of ruminant feed enzyme polysaccharidase activities by extracts from silages. *Canadian Journal of Animal Science,* 80, 523-526.
108. Nsereko, V.L., Morgavi, D.P., Rode, L.M., Beauchemin, K.A., McAllister, T.A. 2000b. Effects of fibrolytic enzymes on degradation of alfalfa hay fibre by mixed rumen microorganisms. In: *Proceedings of 25th Conference on Rumen Function,* Chicago, Illinois (abstract) www.msu.edu/user/rumen
109. Nussio, L.G., Huber, J.T., Theurer, C.B., Nussio, C.B., Santos, J., Tarazon, M., Lima-Filho, R.O., Riggs, B., Lamoreaux, M., Treacher, R.J. 1997. Influence of a cellulase/xylanase complex (C/X) on lactational performance of dairy cows fed alfalfa hay (AH) based diet. *Journal of Dairy Science,* 80, (Suppl. 1) 220. (abstract)
110. Orpin, C.G. 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. *Journal of Genetics and Microbiology,* 91, 249-262.
111. Orpin, C.G., Joblin, K.N. 1997. The rumen anaerobic fungi. In *The rumen microbial ecosystem* (eds. Hobson, P.N. and Stewart, C.S.). Blackie Academic and Professional Publishers, London, pp. 140-195.
112. Pell, A.N., Schofield, P. 1993. Microbial adhesion and degradation of plant cell walls. In: *Forage cell wall structure and digestibility*. (eds. Jung, H.G., Buxton, D.R., Hatfield, R.D., Ralph, J.) American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, Wisconsin, pp. 397-423.
113. Purkarthofer, H., Sinner, M., Steiner, W. 1993. Cellulase-free xylanase from *Thermomyces lanuginosus*: optimization of production in submerged and solid-state culture. *Enzyme Miocrobiology and Technology,* 15, 677-682.
114. Purkarthofer, H., Steiner, W. 1995. Induction of endo-beta-xylanase in the fungus *Thermomyces lanuginosus*. *Enzyme Miocrobiology and Technology,* 17, 114-118.
115. Radostits, O.M., Gay, C.C., Blood, D.C., Hinchdlift, K.W. (eds.) 2000. *Veterinary medicine*. A

textbook of diseases of cattle, sheep, pigs, goats and horses. IX ed. WB Saunders Co. Philadelphia.
116. R Development Core Team. 2004. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, http://www.R-project.org.
117. Rezaeian, M., Parker, D.S., Beakes, G.W. 1999. Cellulase and xylanase activity of the rumen anaerobic fungi grown on untreated and sodium hydroxyde treated barley straw. In: *Proceedings of annual meeting of British Society of Animal Science* www.bsas.org.uk/meetings/annlproc/PDF99/152.pdf
118. Rezessy-Szabó, J.M., Nguyen, Q.D., Bujna, E., Takács, K., Kovács, M., Hoschke, Á. 2003. Thermomyces lanuginosus CBS 395.62/b strain as rich source of alpha-galactosidase enzyme. *Food Technology and Biotechnology,* 41, 55-59.
119. Rode, L.M., Beauchemin, K.A. 1998. Enzymes to enhance utilization of feed in dairy cows. In: *Proceedings of Western Canadian Dairy Seminar*, University of Alberta, www.afns.ualberta.ca/Hosted/WCDS/Proceedings/1998/ch15.htm
120. Rode, L.M., Yang, W.Z., Beauchemin, K.A. 1999. Fibrolytic enzyme suplements for dairy cows in early lactation. *Journal of Dairy Science,* 82, 2121-2126.
121. Rovics, J.J., Ely, C.M. 1962. Response of beef cattle to enzyme supplement. *Journal of Animal Science,* 21, 1012. (abstract)
122. Rust, J.W., Jacobsen, N.L., McGilliard, A.D. 1963. Supplementation of dairy calf diets with enzymes. I. Effect on rate of growth. *Journal of Animal Science,* 22, 1104-1108.
123. Rust, J.W., Jacobsen, N.L., McGilliard, A.D., Hotchkiss, D.K. 1965. Supplementation of dairy calf diets with enzymes. II. Effect of nutrient utilization and on composition of rumen fluid. *Journal of Animal Science,* 24, 156-160.
124. Sanchez, W.K., Hunt, C.W., Guy, M.A., Pritchard, G.T., Swanson, B.I., Warner, T.B., Higgins, J.M., Treacher, R.J. 1996. Effect of fibrolytic enzymes on lactational performance of dairy cows. *Journal of Dairy Science,* 79, (Suppl. 1) 183. (abstract)
125. Satter, L. D., Slyter, L. L. 1974. Effcect of ammonia concentration on rumen microbial protein production in vitro. *British Journal of Nutrition,* 32, 199-208.
126. Saupe, S.G. 2002. Plant physiology. Cell walls – structure and function. College of St. Benedict, St. John's University, Collegeville, MN http://www.employees.csbsju.edu/ssaupe/biol327/cell-wall.htm
127. Schingoethe, D.J., Stegeman, G.A., Treacher, R.J. 1999. Response of lactating dairy cows to a cellulase and xylanase enzyme mixture applied to forages at the time of feeding. *Journal of Dairy Science,* 82, 996-1003.
128. Scholz, H., Hölterschinken, M., Höhling, A. 2001. Cellulase activity in the rumen fluid – a new tool for characterising the ruminal fermentative efficacy? In: *Proceedings of the 3rd Middle-European Buiatrics Congress*, Milovy, Czech Republic, pp. 146-149. (in German)
129. Selvendran, R.R. 1984. The plant cell wall as a source of dietary fibre: chemistry and structure. *The American Journal of Clinical Nutrition,* 39, 320-337.
130. Senior, J.S., Mayers, P.R., Saddler, J.N. 1989. Production and purification of xylanases. In: *Plant cell wall polymers. Biogenesis and biodegradation* (eds. Lewis, N.G. and Paice, M.G.) ACS Symposium Series 399, American Chemical Society, Washington DC, pp. 641-654.
131. Sheperd, A.C., Kung L. 1994. Composition, rumen fermentation, and digestibility of enzyme treated corn silages. *Journal of Animal Science,* 72, (Suppl. 2) 125. (abstract)
132. Sheppy, C. 2001. The current feed enzyme market and likely trends. In: *Enzymes in farm*

animal nutrition (eds. Bedford, M.R. and Partridge, G.G.) CAB International. pp. 1-10.
133. Spoelstra, S.F., Van Wikselaar, P.G., Harder B. 1992. The effects of ensiling whole crop maize with a multi-enzyme preparation on the chemical composition of the resulting silages. *Journal for Science of Food Agriculture,* 60, 223.
134. Stokes, M.R. 1992. Effects of an enzyme mixture, an inoculant and their interaction on silage fermentation and dairy production. *Journal of Dairy Science,* 75, 764-773.
135. Supelco Inc. (1975) G. C. Separation of VFA C2-C5. *Bull. 749* Supelco Inc., Bellefonte, PA.
136. Sutton, J.D., Phipps, R.H., Beever, D.E., Humphries, D.J., Hartnell, G.F., Vicini, J.L., Hard, D.L. 2003. Effect of method of application of a fibrolytic enzyme product on digestive processes and milk production in Holstein-friesian cows. *Journal of Dairy Science,* 86, 546-556.
137. Svihus, B., Herstad, O., Newman, C.W., Newman, R.K. 1997. Comparison of performance and intestinal characteristics of broiler chicken fed on diets containing whole, rolled or ground barley. *British Poultry Science,* 38, 524-529.
138. Szigethy L. 1971. *Use of wheat straw and maize stalk*. Mezőgazdasági Kiadó, Budapest (in Hungarian)
139. Theurer, B., Woods, W., Burroughs, W. 1963, Influence of enzyme supplements in lamb fattening rations. *Journal of Animal Science,* 22, 150-154.
140. Tietz, N.W. 1987. *Fundamentals of clinical chemistry* 3rd edition. W.B. Saunders Company Philadelphia, pp. 676-679.
141. Treacher, R., McAllister, T.A., Popp, J.D., Mir, Z., Mir, P., Cheng, K.J. 1997. Effect of exogenous cellulases and xylanases on feed utilization and growth performance of feedlot cattle. *Canadian Journal of Animal Science,* 77, 541. (abstract)
142. Tricario, J., Johnston, J.D., Dawson, K.A. 2002. The influence of low concentrations of supplemental enzymes on ruminal fermentation and milk production in dairy cows. *Journal of Animal Science,* 80, (Suppl. 1) / *Journal of Dairy Science,* 85, (Suppl. 1) 105. (abstract)
143. Trinder, P. 1969. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Annales of Clinical Biochemistry,* 6, 24.
144. Van Vuuren, A.M., Bergsma, K., Frol-Kramer, F., Van Beers, J.A.C. 1989. Effects of addition of cell-wall degrading enzymes on the chemical composition and the in sacco degradation of grass silage. *Grass Forage Science,* 44, 223.
145. Varel, V.H., Kreikemeier, K.K., Jung, H.C., Hatfield, R.D. 1993. In vitro stimulation of forage fibre degradation by ruminal microorganisms with *Aspergillus oryzae* fermentation extract. *Applied and Environmental Microbiology,* 59, 3171-3176.
146. van de Vyver, W.F.J., Dawson, K.A., Casey, N.H., Tricario, J.M. 2004. Effect of glycolisation on the stability of fungal xylanase exposed to proteases or rumen fluid in vitro. *Animal Feed Science and Technology*, 116, 259-269.
147. Wallace, R.J., Newbold, C.J. 1992. Probiotics for ruminants. In: *Probiotics: the scientific basis* (ed. Fuller, R.) Chapmann Hall, London, pp. 317-353.
148. Wang, Y., McAllister, T.A., Rode, L.M., Beauchemin, K.A., Morgavi, D.P., Nsereko, V.L., Iwaasa, A.D., Yang, W. 2001. Effects of an exogenous enzyme preparation on microbial protein synthesis, enzyme activity and attachment to feed in Rumen Simulation Technique (Rusitec). *British Journal of Nutrition,* 85, 325-332.
149. Wang, Y., McAllister, T.A. 2002. Rumen microbes enzymes and feed digestion. *Asian-Australasian Journal of Animal Science,* 15, 1659-1676.
www.rumen.snu.ac.kr/symposium/lectures/McAllister_TA.pdf

150. Ward, J.K., Richardson, D., Tsien, W.S. 1960. Value of added enzyme preparations in beef cattle rations. *Journal of Animal Science,* 19, 1298. (abstract)
151. Yang, W.Z., Beauchemin, K.A., Rode, L.M. 1999. Effects of enzyme feed additive on extent of digestion and milk production of lactating dairy cows. *Journal of Dairy Science,* 82, 391-403.
152. Yang, W.Z., Beauchemin, K.A., Rode, L.M. 2000. A comparison of methods of adding fibrolytic enzymes to lactating cow diets. *Journal of Dairy Science,* 83, 2512-2520.
153. Yang, W. Z., Beauchemin, K. A., Vedres, D. D. 2002. Effects of pH and fibrolytic enzymes on digestibility, bacterial protein synthesis, and fermentation in continous culture. *Animal Feed Science and Technology,* 102, 137-150.
154. Zheng, W., Schingoethe, D.J., Stegeman, G.A., Hippen, A.R., Treacher, R.J. 2000. Determination of when during the lactation cycle to start feeding a cellulase and xylanase enzyme mixture to dairy cows. *Journal of Dairy Science,* 83. 2319-2325.
155. ZoBell, D.R., Wiedmeier, R.D., Olson, K.C., Treacher, R. 2000. The effect of an exogenous enzyme treatment on production and carcass characteristics of growing and finishing steers. *Animal Feed Science and Technology,* 87, 279-285.

7. The author's papers published concerning the thesis

7.1. Publications used for writing the thesis

Jurkovich V., Kutasi J., Brydl E., Könyves L., Tirián A., Rafai P. 2006. Use of fibrolytic enzymes in ruminant nutrition. *Magyar Állatorvosok Lapja*, submitted for publication (in Hungarian)

Brydl E., Jurkovich V., Könyves L., Kutasi J., Tegzes L. 2006. Effects of a non starch polysaccharidase enzyme preparation from *Themomyces lanuginosus* in dairy cows in the mid-lactation. *Bulletin of The Veterinary Research Institute in Pulawy*, submitted for publication

Jurkovich V., Kutasi J., Fébel H., Reiczigel J., Brydl E., Könyves L., Rafai P. 2006. Rumen fermentation response to a direct-fed xylanase enzyme preparation from *Thermomyces lanuginosus* in sheep. *Acta Veterinaria Hungarica*, accepted for publication

Bata Á., Kutasi J., Jurkovich V., Fébel H., Rafai P. 2002. Activity and stability of a fungal 1,4-beta-endo-xylanase preparation in the rumen of sheep. *Journal of Animal and Feed Sciences*, 11, 627-635.

Jurkovich V., Brydl E., Rafai P., Könyves L., Tegzes L., Kutasi J., Bata Á., Nagy G., Bartyik J., Fülöp A. 2002. Effects of a non-starch polysaccharidase enzyme preparation from Thermomyces lanuginosus on energy and protein metabolism and milk yield of dairy cattle. *Acta Veterinaria Hungarica*, 50, 395-411.

7.2. Other publications related to the subject of the thesis

7.2.1. Scientific journal papers

Kutasi J., Jurkovich V., Brydl E., Könyves L., Bata Á. 2005. Degradation of the fibre components of wheat straw and maize stalk by the thermophilic fungus *Thermomyces lanuginosus*. In: Abstracts of 1st Central European Forum for Microbiology (CEFORM), 26-28. October 2005, Keszthely, Hungary, *Acta Microbiologica et Immunologica Hungarica*, 52, Suppl., 89-90. (abstract)

Kutasi J., Jurkovich V., Brydl E., Könyves L., Tirián A.E., Bata Á. 2004. Influence of different *Saccharomyces cerevisiae* strains on the oxygen concentration in the rumen fluid. In: Proceedings of the 10th International Symposium on Ruminant Physiology, 2-5 September 2004, Copenhagen, Denmark, *Journal of Animal and Feed Sciences,* 13, (Suppl. 1), 131-134.

Kutasi J., Bata Á., Brydl E., Rafai P., Jurkovich V. 2001. Characterisation and effects of a xylanase enzyme preparation extracted from *Thermomyces lanuginosus* cultures. *Acta Veterinaria Hungarica*, 49, 175-184.

Brydl E., Rafai P., Könyves L., Jurkovich V., Mrs Tegzes L., Nagy G., Kutasi J., Bata Á. 2001. NSP-enzymes help rumen microbes. *Feed Mix*, 9, 22-26.

7.2.2. Presentations on scientific congresses

Jurkovich V., Brydl E., Könyves L., Kutasi J., Tegzes L. 2005. Effects of a xylanase enzyme preparation in dairy cows in mid-lactation. In: *Proceedings of 6th Middle European Buiatrics Congress*, Kraków, Poland

Jurkovich V., Kutasi J., Fébel H., Reiczigel J., Brydl E., Könyves L., Rafai P., Bata Á. 2004. The effects of a xylanase enzyme preparation from *Thermomyces lanuginosus* on the rumen fermentation in sheep. In: *Proceedings of 23rd World Buiatrics Congress*, Québec City, Canada, (abstract)

Jurkovich V., Brydl E., Könyves L., Tegzes L., Rafai P., Bata Á., Kutasi J., Bartyik J. 2003. Effects of *Saccharomyces cerevisiae* and non-starch polysaccharidase enzymes on health and milk yield of dairy cows. In: *Proceedings of 4th Central Europan Buiatric Congress*, Lovran, Croatia,

Bata Á., Kutasi J., Fébel H., Jurkovich V. 2003. Characterisation and effect of xylanase enzyme in rumen originated from *Thermomyces lanuginosus* NCAIM 001288. In: *Proceedings of 4th Central Europan Buiatric Congress*, Lovran, Croatia,

Brydl E., Rafai P., Jurkovich V., Könyves L., Tirián A., Tegzes L., Kutasi J., Bata Á., Nagy G., Bartyik J., Fülöp A. 2003. The effects of a Non Starch Polysaccharidase enzyme preparation from *Thermomyces lanuginosus* on the ruminal Volatile Fatty Acid production, energy and protein metabolism and milk yield of dairy cattle. In: *Proceedings of 4th Central Europan Buiatric Congress*, Lovran, Croatia,

Brydl E., Rafai P., Jurkovich V., Könyves L., Tegzes L., Kutasi J., Bata Á., Nagy G., Bartyik J., Fülöp A. 2003. The effects of a Non Starch Polysaccharidase enzyme preparation from *Thermomyces lanuginosus* on the ruminal Volatile Fatty Acid production, energy and protein metabolism and milk yield of dairy cattle. In: *Proceedings of XI International Congress in Animal Hygiene*, Mexico City, Mexico,

Brydl E., Jurkovich V., Könyves L., Mrs Tegzes L., Bata Á., Kutasi J., Alexov M. 2002. The effects of different dosage of an enzyme preparation on health parameters in dairy cows. In: *Book of Abstracts of the XXII World Biuatrics Congress*, Hannover, Germany,

Kutasi J., Jurkovich V. 2002. Degradation of wheat and maize straw with *Thermomyces lanuginosus*. In: *Proceedings of 13th Hungarian Buiatrics Congress*, Hajdúszoboszló, Hungary, pp. 5-11. (in Hungarian)

Brydl E., Jurkovich V., Bata Á., Kutasi J., Mrs. Tegzes L., Nagy G., Bartyik J., Fülöp A., Könyves L. 2000. Effect of polysaccharidase enzymes administered per os on ruminal fermentation and milk production of dairy cows. In: *Proceedings of XXI World Buiatrics Congress*, Punta del Este, Uruguay,

Brydl E., Jurkovich V., Bata Á., Kutasi J., Nagy G., Könyves L. 2000. The effect of polysaccharidase enzyme administered per os on ruminal fermentation of dairy cows. In: *Proceedings of 2nd Middle European Buiatrics Congress,* High Tatras, Slovakia,