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

PhD thesis (brief sumary)

by

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Abbreviations

2h	2 hours after feeding	EFE	exogenous fibrolytic enzyme
4h	4 hours after feeding	EX	experimental period
AcAc	aceto acetate	FCM	fat corrected milk
ADF	acid detergent fibre	FCR	feed conversion rate
A:P	acetate:propionate ratio	FI	final period
AST	aspartate aminotransferase	FXU	fibre xylan unit
BCS	body condition score	mM	mmol/l
BW	body weight	NABE	net acid base excretion
CO	control period	NDF	neutral detergent fibre
DIM	days in milk	NEFA	non-esterified fatty acid
DFM	direct fed microbials	NSP	non starch polysaccharide
DM	dry matter	T0	before feeding
DMI	dry matter intake	VFA	volatile fatty acid

1. Introduction

Dairy cows of our time are products of genetic selection sustained for many generations, thus capable of tremendous milk production. 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.

The microbial feed additives (yeast cultures and enzymes) play an important role in the digestion of nutritional elements in feedstuffs. DFMs are mixed to feeds in order to either directly aid digestion in the (fore-) stomach and intestines, or enhance degradation of non-starch polysaccharides (enzymatic pre-treatment of cereals or forages). 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.

EFEs have most succesfully been used in feeding monogastric animals, especially broiler chickens. Though results obtained with exogenous fibrolytic enzymes or microbes producing EFE are far from being unequivocal in ruminants, use of such enzymes is likely to spread.

Adding yeast or enzyme preparations to dairy rations presents us with several challenges. Only the 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 and lower incidence of metabolic disorders. 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 complex digestive tract of ruminants can digest non-starch polysaccharides with high efficiency. 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. Materials and methods

2.1. Aims of the study

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 see 2.3. and 2.4.). The effect on general condition and production parameters of dairy cows was studied under field conditions on dairy farms, using animals in early (2.5.) and mid (2.6.)-lactation.

2.2. The material tested

The product (Rumino-Zyme) is a light-brown granulate (particle size: 400-500 μm) with 90% DM content. It contains thermal resistant endoxylanase from *T. lanuginosus* NCAIM 001288. IUB ranking of the enzyme is endo-1,4-beta-xylanase, which preserves its activity within the range of pH 4.5-8.0 and 30-40 ºC. Shelf life at 20 ºC is longer than 6 months. Enzyme activity of the product is 250 FXU/g. The preparation hydrolyses xylans and arabino-xylans to mono-, di-, tri- and oligo-saccharides.

2.3. Trial 1

This experiment aimed at tracing the changes in the activity of the exogenous endo-1,4-beta-xylanase enzyme added directly into the rumen at a dosis sufficient to elevate the total extracellular xylanase activity of the rumen fluid. Four, 3-year-old, rumen cannulated Merino wethers of (average initial BW = 70 kg) were used to obtain the rumen fluid samples. In this trial the sheep were fed with a ration of 700 g alfalfa hay and 400 g concentrate (7.49 MJ NE_m, 125 g crude protein) a day in two equal portions, at 8.00 am and 16.00 pm. At 8.00 a.m. a base-line rumen fluid sample was taken from each animal. 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 the application of the enzyme preparation.

2.4. Trial 2

Eight rumen cannulated yearling Merino wethers (average initial BW = 50±6 kg) were used in the experiment. The animals were fed with a ration of 800 g meadow hay and 500 g lamb concentrate a day in two equal portions. 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*.

After 2 weeks of social and feeding acclimatisation there was a baseline data collection period lasting one week. In this control period some parameters of rumen fermentation (see below) 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. Rumen fluid pH was measured immediately after the samplings. Ruminal VFA were separated and quantified by gas chromatography. The molar proportions of VFA were calculated by dividing the individual acid concentrations by the total VFA concentration. The ammonia content and xylanase activity of ruminal samples were determined as well.

2.5. Trial 3

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 110 DIM.

Of the 100 experimental and control cows, 10 cows in each group were designated for taking rumen 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. Rumen fluid was obtained via Dirksen-tube. Blood and urine samples (20 ml each) were taken from the subcutaneous abdominal vein and via metal catheter from the 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.

VFA concentration of the rumen fluid samples was measured gaschromatographically. Fat- and carbohydrate-metabolism and -balance were monitored by determination of the glucose, and NEFA concentrations in the blood plasma and AcAc concentration in whole blood samples. Urea concentration and AST activity of plasma samples were measured as well. Acid-base balance was studied by determination of the urinary pH and NABE.

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 rumen fluid samples by using a 1 to 5 grade scoring system.

2.6. Trial 4

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 70 DIM on average at start. The feed did not change from the regular in the course of the experiment. 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 production and milk components were measured weekly during treatment periods.

Blood and urine samples were taken twice a month to monitor clinical condition through biochemical parameters. Blood an urine samples were taken 3-5 hours after the morning feeding. Glucose, NEFA, and urea concentration

of blood plasma samples and AcAc 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. BCS was evaluated at the time of blood samplings by a 1 to 5 grade scoring system.

3. Results

3.1. Trial 1

The additional enzyme increased the base-line xylanase activity of the rumen fluid by about 300 % within 5 min after treatment 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 why the recovery of the added xylanase was incomplete. 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 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/l).

3.2. Trial 2

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. 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.

In the control period the average pH of the rumen fluid was physiological just before the feeding and it decreased 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 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.

3.3. Trial 3

TVFA concentration and molar proportion of propionate were higher in the EX than that in the control group ($P=0.038$ and $P=0.006$).

The blood aceto-acetate concentrations of non-treated cows tended to be higher ($P=0.069$) than those in the experimental cows throughout the experiment. The group average of the control cows on day 22 post partum was higher than 0.1 mM. During the study higher plasma NEFA concentrations ($P=0.008$) were found in the control group. The NEFA concentration in the plasma of the control cows on day 10 after calving exceeded the physiological level. There was no significant difference in AST activity of the control and experimental cows. There was no significant difference in plasma urea

concentration between the control and experimental cows. Laboratory findings concerning the acid-base metabolism of the experimental and control cows indicated the presence of balance throughout the experiment. The average NABE was in both groups higher than the physiological limit value (>100 mM) showing no acid load in the cows.

Analysis of the milk production data indicated that the average milk yield of the experimental group was significantly higher 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 for 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.

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

At the beginning of the experiment there was only 0.2 body condition score difference between the groups in favour of the controls, which then decreased and after about the third samplings the condition of the experimental cows increased exceeding the controls, though 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 to be lower throughout the experiment than the score taken prior to calving. The condition scores of the experimental cows were never lower than 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.

3.4. Trial 4

The BCS did not change significantly nor were any significant difference between groups. Blood plasma glucose concentrations were above physiologic− al level in both groups, showing no significant difference. Average and indi− vidual AcAc and NEFA concentrations remained below the physiological limit. 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, while urinal urea concentrations remained in the physiological range. Experimental and control groups showed no significant difference. Average milk yield 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 the control.

4. New scientific results

1. Our research group was the first to test an 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 2,5g/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 30g/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 (30g/cow/day).

5. The author's papers published concerning the thesis

5.1. Publications used for writing the thesis

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5.2. Other publications related to the subject of the thesis

5.2.1. Scientific journal papers

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