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USING THE BY-PRODUCT OF INDUSTRIAL THREONINE PRODUCTION IN THE NUTRITION OF DAIRY COWS

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1. RESEARCH BACKGROUND AND OBJECTIVES

Recent years have witnessed an increasing production of amino acids due to progressively diversified amino acid applications in the food, pharmaceutical, chemical industry, and animal nutrition, which has been accompanied by continuously increasing volumes of by-products, as well. In the present dissertation, the potential application of 'mother liquor' (a by-product of industrial threonine production) in the nutrition of ruminants was investigated. Due to the scarcity of relevant literature data, introductory chemical analyses of mother liquor were carried out and followed by nutritional physiology analyses performed on rumen and duodenal cannulated steers as model animals in order to elucidate the mechanisms of action of the by-product (e.g., effects on rumen fermentation, microbial activity, pH and ammonia concentration of rumen fluid, microbial protein ratio in chyme). In addition to cannulated model animals, effects of mother liquor supplementation were examined on late-lactation Holstein-Friesian dairy cows under commercial dairy farm conditions.

The aim of the first model experiment was to investigate the effects of increasing mother liquor supplementation (1 or 2 kg/day) on the following:

- rumen microbial activity,
- short-chain fatty acid (SFCA) content and ratio of rumen fluid,
- to what extent does the ammonia – originating from the microbial decomposition of urea, ammonium sulphate, and free amino acids – increase NH3 content of rumen fluid,
- pH of the rumen fluid.

Based on the results of the first model experiments (i.e., considerably increased ammonia concentration) with rumen canulated Holstein-Friesian steers, the daily dosage of 2 kg mother liquor was decreased to 1.5 kg, furthermore, 1.5 kg mother liquor was also supplemented with 1 kg molasses in the second model trial. The objective of this experiment was to determine the effects of mother liquor and molasses-combined mother liquor supplementation on microbial protein synthesis processes in the rumen. In the second nutritional experiment we sought answers to the following questions:

How do the mother liquor and molasses-combined mother liquor supplementations affect:

- rumen microbial activity during breakdown and synthesis processes?
- ruminal SCFA synthesis?
- ammonia content of the rumen fluid?
- pH value of the rumen fluid?
- amount of microbial protein synthesis in the rumen?

The effect of mother liquor supplementation on milk production was tested in an experiment with a randomised complete block design. The purpose of the experiment was to answer the following questions:

- Does mother liquor supplementation increase milk production? If it does, to what extent?
- Does mother liquor supplementation affect milk composition? If it does, what parameters of the following are affected: protein, fat, urea content?
- Can 1 kg mother liquor replace 0.5 kg sunflower meal? To what extent can mother liquor substitute for sunflower meal?

2. MATERIALS AND METHODS

2.1. Mother liquor

Mother liquor – a by-product of L-threonine production generated in the fermentation plants of the Japanese-Hungarian company Agroferm, Kaba, Hungary – was used in both the model and the farm experiments.

2.2. Methods of the first model experiment

The first model experiment was conducted at the Experimental Animal Farm of Széchenyi István University, Faculty of Agricultural and Food Sciences, with five rumen cannulated Holstein-Friesian steers weighing 600-650 kg, as a periodic experiment with self-controlled design (protocols approved by Győr-Moson-Sopron County Government Office under the reference VIII-I-001/01316-0003/2012). The experiment consisted of one control and two experimental phases. Both the control and the two experimental phases were constructed by a transitional pre-feeding and a sampling period, 10 and 5 days in length, respectively.

After the control phase in the two experimental phases the basal (control) diet of steers was supplemented through rumen cannula with 1 or 2 kg/day mother liquor distributed in two daily feedings as follows: 50% in the morning (7 a.m.) and 50% in the afternoon (3 p.m.).

Rumen fluid samples were collected four times daily from the animals through rumen cannula on each day of the control and the experimental phases. The samplings were done before feeding in the morning, and 1, 2, and 3 hours after feeding. The pH and NH_3 content of rumen fluid was

determined in each sample, whereas microbial activity and SCFA content of the rumen fluid was measured in samples taken before feeding and 3 hours after feeding. In order to maintain actual rumen microbial activity, rumen fluid samples were transferred from the experimental facility to the laboratory in thermos. Analysis of the rumen fluid samples started within 15-20 minutes after sampling.

2.3. Materials and methods of the second model experiment

The second experiment was done with four Holstein-Friesian steers (600-650 kg live weight) in periodic design (control, experiment phase 1, 2 and 3). Each steer was rumen-cannulated, furthermore, two of the experimental animals also received duodenal cannula in order to obtain chyme samples (protocols approved by Győr-Moson-Sopron County Government Office under the reference VIII-I-001/01316-0003/2012).

The experiment consisted of one control and three subsequent treatment phases. Each phase was constructed by a transitional pre-feeding period of 10 days, and a sampling period that lasted 5 days. Experimental animals were fed twice in a day (at 7 a.m. and 3 p.m.), each time receiving 50-50% of the daily dosage. In the experimental phases (experimental phase 1, 2, and 3), animals were fed the same basal diet as in the control phase and in addition received supplementations in various amount and composition (experimental phase 1: 1.0 kg mother liquor; experimental phase 2: 1.5 kg mother liquor; experimental phase 3: 1.5 kg mother liquor + 1.0 kg molasses). Supplements were provided simultaneously with the morning (7 a.m.) and afternoon (3 p.m.) feeding period via the rumen cannula in 50-50% ratio of total daily feed. Feed-

grade liquid molasses (78% dry matter, 50% total sugar content) was provided by Magyar Cukor Co. (Kaposvár, Hungary).

For chemical analyses, rumen fluid samples were collected from 4 rumen cannulated animals four times daily (before feeding, and 1, 2, and 3 hours after feeding) on each day of the sampling period, whereas chyme samples were taken from the duodenum of cannulated steers 3 times daily at 3-hour-intervals after the morning feeding (at 10 a.m., 1 p.m., and 4 p.m).

2.4. Methods of chemical analysis applied in the first and second model experiments

In rumen fluid samples, pH value was determined by OP-211/1 (Radelkis) digital pH meter, whereas ammonia content was measured by means of OP-264-2 (Radelkis) ammonia selective electrode.

Microbial activity of rumen fluid samples was measured by nitrite reduction test that characterizes activity based on the time required by the microbial populations for the reduction of a specific amount of KNO_2 (*Horváth*, 1979).

The SCFA content of rumen fluid was measured by Biotronik-2000 (Biotronik, Maintal, Germany) HPLC instrument with column type Aminex Bio Rad HPX 87H (Bio Red Laboratories Inc., Hercules, USA) sized 300 mm×7.8 mm. Separation was done at 45 ^{o}C with 0.005 M H_2SO_4 eluent. Pump flow rate was 0.85 ml/min, pressure was 77 kg/cm^2. Supelco 10 (Sigma-Aldrich Co.) standard containing a mixture of short-chain fatty acids was applied for the measurement of SCFA content.

Dry matter, crude protein, crude fat, crude fiber, crude ash, Ca and P content of the applied feedstuffs was determined by methods recommended by the *Hungarian Feedstuff Codex Volume II.* (1990). The Kjeldahl method was applied to measure ammonium sulphate content of mother liquor by KjeltecTM 2200 (FOSS, Denmark) instrument. The amino acid content of mother liquor and chyme (collected through duodenal cannula) was determined at the University of Pannonia, Georgikon Faculty of Agriculture, Department of Animal Sciences and Animal Husbandry by the method of column chromatography with INGOS AAA 400 (INGOS Ltd., Prague, Czech Republic) amino acid analyser instrument, Ostion LG ANB ION (INGOS Ltd., Prague, Czech Republic) ion exchange resin column.

2.5. Determination of microbial protein of the total crude protein content of chyme

Microbial protein ratio of chyme crude protein was assessed based on results published by *Csapó et al*. (2008). Their process facilitates the determination of the proportion of microbial protein in chyme crude protein based on DAPA, D-aspartic acid, D-glutamic acid, or crude protein content of chyme. In this experiment, microbial protein ratio in chyme crude protein was calculated by the application of the DAPA-based multiplication factor (165) established by *Csapó et al*. (2008).

2.6. Milk production study under commercial dairy farm conditions

Effects of mother liquor on milk production were investigated under farm conditions on a commercial dairy farm located in the north-west Transdanubia region, Hungary. During the experimental period, the cows were fed a corn silage, alfalfa silage, rye silage, cornmeal, and sunflower meal-based diet (total mixed ration, TMR). TMR preparation was done in a feed mixer facility owned by the dairy farm. Dairy cows in production were fed twice daily.

Multiparous Holstein-Friesian dairy cows in late-lactation participated in the experiment (days in milk (DIM) > 200, average daily milk production/cow < 25 kg). In a randomised complete block design, a total of 40 Holstein-Friesian dairy cows' pairs were used in the control (n=20) and experimental groups (n=20). In the construction of so called 'cow-pairs', the number of completed lactations, last calving date, milk production in the previous lactation, and nutrient content of milk (protein and fat) were taken into consideration. After 2-weeks of pre-feeding, the experimental period lasted two months. Control and experimental animals were kept under identical housing conditions during the experiment (i.e., housing technology, stocking density, feeders and drinkers, etc.).

Compared to the control group, sunflower meal was decreased 0.5 kg and substituted by mother liquor on a crude protein content basis in the TMR of the experimental animals. During the experiment, control and experimental supplementations were mixed into TMR by the feed mixer before feeding.

Milk composition was weekly and individually analysed. The following milk parameters were determined: fat, protein, and urea content. Milk composition analyses were carried out by the Hungarian Dairy Research Institute Ltd. (Mosonmagyaróvár, Hungary) on a Milkoscan FT 120 (Foss Electric, Denmark) system.

2.7. Statistical analysis

The data of the model and the commercial dairy farm experiments were analysed in SPSS 26.0 for Windows (IBM, Armonk, NY).

The Kolmogorov–Smirnov test was applied to assess normal distribution. Regarding the results of the model experiment, normally distributed data were processed by analysis of variances (one-way ANOVA) and means were compared using Bonferroni and Games–Howell tests (range tests), whereas data with non-normal distribution were analysed by Kruskal–Wallis and Mann–Whitney nonparametric tests.

Data from the milk production experiment were analysed by Levene's test, t-test, and nonparametric tests (Kruskal–Wallis, Mann–Whitney).

A p-value less than or equal to 0.05 was considered statistically significant concerning each test applied in the data analysis.

3. RESULTS

3.1. Chemical analysis of mother liquor

According to the introductory chemical analysis, total crude protein of mother liquor consists exclusively of amide compounds. Crude protein primarily contained free amino acids (mainly threonine). Free amino acids made up 63.3% of crude protein; however, this remarkable amino acid content is highly unbalanced due to considerable threonine excess which may result in amino acid imbalance in monogastric animals (e.g. pigs). In ruminants, a part of the free threonine content is deaminated by rumen bacteria, thus resulting in negligible probability of imbalance.

3.2. Results of the first model experiment

In the first model experiment, rumen cannulated Holstein-Friesian steers were supplemented with 1 and 2 kg mother liquor/day in order to evaluate the effects of mother liquor on rumen microbial activity, and on the pH, ammonia and SCFA content of rumen fluid.

Based on the results of nitrite reduction tests, it was concluded that the microbial activity was significantly ($p<0.05$) improved in rumen fluid samples collected 3 hours after feeding in both experimental phases (1 or 2 kg mother liquor/day) due to an enhanced nutrient supply to microbes. Based on the SCFA analysis, both 1 and 2 kg mother liquor supplementation increased SCFA, acetic acid, and propionic acid content of rumen fluid significantly ($p<0.05$) compared to levels before feeding. Regarding fatty acids that are present in a smaller proportion of rumen

fluid, only levels of n-valeric acid and n-butyric acid increased significantly ($p<0.05$) in both the control phase and the experimental phases with 1 or 2 kg mother liquor. The i-valeric acid level increased significantly ($p<0.05$) only in the 1 kg supplementation phase, whereas the increase of n-butyric acid level in rumen fluid was significant ($p<0.05$) only after 2 kg mother liquor supplementation. Based on molar proportions, it can be concluded that mother liquor significantly ($p<0.05$) reduces the proportion of acetic acid in rumen fluid, while significantly ($p<0.05$) increases propionic acid proportion. The increasing SCFA content of rumen fluid after mother liquor supplementation is primarily attributed to the improved N supply to rumen bacteria.

In the first three hours after feeding, levels of rumen fluid NH_3 content were significantly ($p<0.05$) higher in both experimental phases (1 kg or 2 kg mother liquor) compared to that in the control phase. Daily 2 kg supplementation of mother liquor led to a tenfold increase in the NH_3 content of rumen fluid in the first hour after feeding, thereby posing an increased risk of ammonia toxicosis.

3.3. Results of the second model experiment

Similarly to the first experiment, rumen cannulated Holstein-Friesian steers were supplemented with 1, 1.5 kg mother liquor/day, and 1.5 kg mother liquor + 1 kg liquid molasses/day in order to evaluate the effects of mother liquor on rumen microbial activity, and on the pH, ammonia and SCFA content of rumen fluid. Based on the data from this experiment, it was concluded that mother liquor or molasses-combined mother liquor supplementations increased the activity of rumen microbial

populations. The combined supplementation (mother liquor + molasses) reduced the time required for nitrite reduction compared to the experimental phase where animals received only 1.5 kg mother liquor addition to the basal diet. Regarding the rumen fermentation experiment, levels of only two (i- and n-valeric acid) of six analysed SCFA (acetic acid, propionic acid, i- and n-butyric acid, i- and n-valeric acid) differed significantly ($p<0.05$) between the two daily samplings in the control phase. In the experimental phases (1 and 1.5 kg mother liquor, and 1.5 kg mother liquor + 1 kg liquid molasses), levels of only two (i- and n-butyric acid) of six analysed SCFA did not differ significantly ($p>0.05$). When mother liquor supplementation was combined with molasses, the greatest increase was experienced in levels of acetic acid and propionic acid. In the three experimental phases, rumen fluid NH_3 content considerably (30-40 mmol/L) increased as soon as 1 hour after morning feeding; on average, the increasement was 5.9-fold compared to the control phase.

Based on results from model experiments with rumen and duodenal cannulated steers, it was concluded that only the combined mother liquor and molasses supplementation increased the DAPA content of chyme and the microbial protein ratio of chyme crude protein significantly ($p<0.05$). Compared to the control phase, the daily combined supplementation of 1.5 kg mother liquor and 1 kg molasses resulted in a 24.3% increase in the proportion of microbial protein in duodenal chyme, thus contributing to an improved amino acid supply of the experimental animals (*Table 1*).

Table 1. **Effects of mother liquor and molasses-combined mother liquor supplementation on microbial protein synthesis**

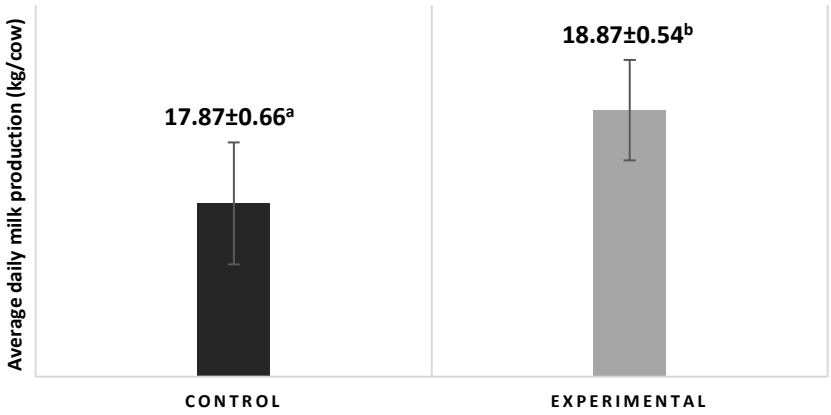
Phase	Control	Experiment 1 (1.0 kg mother liquor)	Experiment 2 (1.5 kg mother liquor)	Experiment 3 (1.5 kg mother liquor + 1 kg molasses)
Chyme crude protein, g/kg	237.33±23.65[b]	266.04±17.97[a]	261.65±17.03[a]	273.98±31.72[a]
DAPA[1] content of chyme, %	0.07±0.011[b]	0.08±0.01[ab]	0.08±0.02[ab]	0.09±0.01[a]
Proportion of microbial protein in chyme crude protein, %	11.71±0.89[b]	13.12±2.13[ab]	13.59±3.63[ab]	14.56±2.03[a]

a,b: Values within lines with different superscripts differ significantly ($p<0,05$)
[1]DAPA: diaminopimelic acid

3.4. Results of the field trial

Based on the results of model experiments and nutritional physiology analyses, effects of mother liquor on milk production, protein, fat, and urea content of milk were evaluated in a preliminary experiment under commercial dairy farm conditions with late lactation cows. After an adaptation pre-feeding period of 14 days, daily milk production was monitored individually throughout a two-month-long experimental phase. Based on results from dairy cows kept under commercial farm conditions, average daily milk production during the experiment significantly ($p<0.05$) increased by 1 kg in the experimental (1 kg mother liquor/day/cow) compared to the control group (without mother liquor supplement) as presented in *Figure 1*. Raw milk samples of the control and the experimental groups did not differ significantly ($p>0.05$) regarding protein, fat, or urea content during the 2-month-long

experiment. Overall, it was concluded – based on the feeding experiment – that mother liquor supplementation of 1 kg/day/cow on a crude protein basis may partially substitute for sunflower meal in the nutrition of late-lactation dairy cows.



Control: without mother liquor supplementation; Experimental: 1 kg/cow/day mother liquor supplementation
a,b: means with different superscripts differ significantly ($p<0.05$)

Figure 1. **Effect of mother liquor supplementation on average daily milk production of dairy cows (kg/cow)**

4. NEW SCIENTIFIC RESULTS

1. The daily supplementation of 1 or 2 kg/steer mother liquor – a by-product of industrial threonine production, rich in free amino acids and ammonium sulphate – significantly ($p<0.05$) enhances microbial activity in the rumen due to an improved N supply to rumen bacteria; furthermore, N-free carbon chains – generated by the deamination of amino acids – provide additional energy for microbial activity improvement, as well.

2. Based on the experiment with rumen cannulated Holstein-Friesian steers, it was concluded that 1-2 kg mother liquor supplemented to a corn silage, grass straw, corn meal-based diet significantly ($p<0.05$) increases after feeding levels of total SCFA, acetic acid, propionic acid, n-butyric acid, and n-valeric acid in rumen fluid compared to levels before feeding. Based on molar proportions, it can be inferred that mother liquor significantly ($p<0.05$) reduces the proportion of acetic acid in rumen fluid, while significantly ($p<0.05$) increases propionic acid proportion; consequently, it can be stated that propionic acid bacteria utilize the nutrients of mother liquor more efficiently compared to acetic acid bacteria.

3. The combined supplementation of mother liquor (1.5 kg/day) and liquid molasses (1 kg/day) significantly ($p<0.05$) increases rumen microbial activity, and total SCFA, acetic acid, propionic acid, i- and n-valeric acid content of rumen fluid.

4. Daily mother liquor supplementations of 1, 1.5, and 2 kg, and the combined supplementation of mother liquor (1.5 kg) and liquid molasses (1 kg) considerably (five to tenfold) increase the NH_3 content of rumen fluid ($p<0.05$) and therefore pose an increased risk of ammonia toxicosis.

5. Based on results from experimental rumen and duodenal cannulated Holstein-Friesian steers, it was concluded that the combined supplementation of mother liquor (1.5 kg) and molasses (1 kg) significantly ($p<0.05$) increases the microbial protein ratio in duodenal chyme compared to the control phase and therefore improves the amino acid supply of animals.

6. Based on results from an experiment conducted under commercial dairy farm conditions, it was inferred that the crude protein-based supplementation of 1 kg mother liquor can partially replace sunflower meal in the total mixed ration (TMR) of late lactation Holstein-Friesian dairy cows.

5. SCIENTIFIC PAPERS ON THE SUBJECT OF THE DISSERTATION

Peer-reviewed papers published in foreign scientific journals

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Peer-reviewed papers published in Hungarian scientific journals

Tóth, T. (2016): A treonin előállítás melléktermékének hatása a bendőfolyadék összetételére és mikrobiális aktivitására (The effect of a threonine production by product on rumen fluid composition and microbial activity. ÁLLATTENYÉSZTÉS ÉS TAKARMÁNYOZÁS. 65:2 pp. 22-34, 13 p.

Tóth, T. – Tempfli, K. (2018): A bendő mikrobás folyamatait befolyásoló tényezők: (Irodalmi áttekintés). ACTA AGRONOMICA ÓVÁRIENSIS 59:1 pp. 141-163, 23 p.

Conference presentations, proceedings

Tóth, T. (2014): A treonin előállítás melléktermékének hatása a bendőben zajló mikrobás fermentációra. In: Anon (szerk.) XXXV. Óvári Tudományos Nap: A magyar és nemzetközi agrár- és élelmiszer-gazdaság lehetőségei [előadások és poszterek teljes anyaga CD]. Mosonmagyaróvár, Magyarország: Nyugat-magyarországi Egyetem Mezőgazdaság- és Élelmiszertudományi Kar, 511-517, 7 p.

Tóth, T. (2016): Anyalúg a kérődzők takarmányozásában. In: Szalka, Éva; Bali, Papp Ágnes (szerk.) XXXVI. Óvári Tudományos Nap: Hagyomány és innováció az agrár- és élelmiszergazdaságban I-II. Mosonmagyaróvár, Magyarország: Széchenyi István Egyetem Mezőgazdaság- és Élelmiszertudományi Kar. 301-309, 9 p.