

# Degradation of biogenic amines and *in vitro* evaluation of ruminal parameters of the ruminal fluid of Charolais sheep

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**ABSTRACT** - In this study, we reported the evaluation of ruminal parameters and the rate and degree of degradation of biogenic amines (BA) *in vitro*, using ruminal fluid from sheep fed two different diets (meadow hay and silage of *Medicago sativa* L.). Herein, the Charolais sheep breed with a ruminal cannula was used to gather samples. Multiple rumen parameters were evaluated after 10 days of the feeding period. Samples were processed using fermenters inoculated by BA (histamine, tyramine, putrescine, cadaverine, spermine, and spermidine) during 8-h fermentation. Levels of BA were determined using ion-exchange chromatography, and volatile fatty acids (VFA) were determined using gas chromatography. More rapid BA degradation was noticed in hay than in haylage. Overall, VFA levels increased with duration of the fermentation, while pH lowered. The rapid increase was observed after 4 h of fermentation. Ammonia concentration had a decreasing trend in silage and increasing in the hay. Hypotheses on BA degradation were accepted. Important knowledge of the development of ruminal parameters in time was obtained, and based on gathered information, it is possible to say that other parameters and their impacts on BA in rumen need to be examined further.

**Keywords:** ammonia, hay, haylage, pH, rumen, volatile fatty acids

## Introduction

Biogenic amines (BA) are low-molecular weight bases and products of amino acid decarboxylation by microbial, plant, and animal metabolism. These compounds may be classified as monoamines (tyramine and histamine), diamines (cadaverine and putrescine), and polyamines (spermidine and spermine) (Biji et al., 2016). It has been shown that the concentration of BA in feed depends on the quality of harvested crop and crop processing during feed production. Moreover, BA concentration and their degradation depends on digestive system of an animal (Scherer et al., 2015). Monitoring and measuring levels of BA degradation are fundamental to the safety of both humans and animals (Sirocchi et al., 2013).

Biogenic amines such as tyramine, tryptamine, histamine, cadaverine, and putrescine are widely detected in ruminants. Histamine (HIS) is also one of the minor neurotransmitters in the brain (Passani et al., 2014). It has been shown that low rumen pH plays an important role in the increase of plasma and rumen biogenic amine concentrations (Wang et al., 2013). The occurrence of BA is associated with the presence of intolerance reactions or with a wide variety of physiological syndromes, such as malabsorption syndrome

and ruminal acidosis (Barnes et al., 2001; Nagaraja and Titgemeyer, 2007). High concentrations of HIS and tyramine in the bloodstream cause major toxicological effects, known as histaminosis (Spano et al., 2010). Maciorowski et al. (2007) determined that various production diseases are caused mainly by unbalanced feed rations, higher content of organic acid, or toxic compounds in animal feed.

Volatile fatty acids (VFA) are short-chain (C2–C6) fatty acids produced during anaerobic fermentation by the degradation of compounds such as fiber and polysaccharides (Akiba et al., 2018). The analysis of VFA in the rumen is crucial for animal clinical diagnosis, as they can influence forestomach motility and cause ruminal atony and acidosis. Yohe et al. (2019) demonstrated the innate ability of rumen to absorb VFA, although the extent and mechanism of degradation is a topic for further research. Moreover, the pH level in a sheep rumen plays a significant role and is closely related to the volatile fatty acid level and accumulation (Constable et al., 2017). The following hypotheses have been tested: biogenic amine levels change in time during fermentation and different parameters, such as pH, VFA, and ammonia concentration, affect their concentration in rumen; and the speed of their degradation depends on the biogenic amine and feed type.

The main objective of this study was therefore to investigate the extent of BA degradation by rumen microorganisms. The adaptation of the fermentation process to the diets varying in the amine content and amine-degrading capacity was evaluated. Analysis of VFA in ruminal fluids provides a significant part of the metabolic profile and carries valuable information about the toxicity of feed and clinical condition of animals.

## Material and Methods

Research on animals was conducted according to institutional committee on animal use of the Ministry of Agriculture of the Czech Republic (case number 28766/2018-MZE-17214).

Standards of BA with 99% purity, citric acid, sodium citrate, isopropanol, potassium hydroxide, potassium bromide, trifluoroacetic, ninhydrin, and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). All buffer solutions were prepared with deionized water obtained by using reverse osmosis Aqual 25 (Aqual s.r.o., Brno, Czech Republic).

All analyses of nutrients in feed were carried out in Brno, Czech Republic, according to the methodology for determination of the nutrient content (Horký, 2014). Ash was determined as a weight of material remaining after burning a fixed-weight sample at 550 °C in specified conditions. Dry matter (DM) was determined similarly to ash, except for temperature used in the process (103 °C). Crude fiber (CF) was determined using Soxhlet method. Nitrogenous crude protein substances were measured by Kjeldahl method using a Kjeltac 2300 device (Foss, Hillerød, Denmark). Neutral detergent fiber (NDF) and total fiber were measured on an ANKOM 220 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA).

Charolais sheep were kept separately in the stables and were fed different concentrations of BA. Their feed rations comprised 570 g per animal per day of feed mixture (Tables 1 and 2) and bulk feed accessed *ad libitum*.

In the first stage of the experiment, sheep (as a source of ruminal fluid) were fed meadow hay-based diet with lower concentrations of BA (Table 3). After 10 days, ruminal fluid for *in vitro* analysis was sampled. In the second stage of this experiment sheep were fed silage-based diet with higher BA contents (Table 3). New feed mixture (lucerne silage) was introduced to animals in a three-day changeover. Samples for nutrient analysis of the feed – DM, ash, NDF, CF, crude protein (CP), fat, and BA concentration – were taken at the beginning of each stage. Three samples were taken from hay and silage in each stage.

Ruminal content was divided into liquid and solid parts using 1 mm sieve. The liquid component was sampled for analyses of ruminal environment parameters (pH, ammonia, VFA, and BA concentrations). The mixture of liquid and solid ruminal contents was used in BA degradation analysis. This mixture was prepared in weight ratio 140 g (liquids): 560 g (solids) and added into six fermenters that were kept under controlled conditions (39 °C and CO<sub>2</sub> atmosphere) throughout the incubation. Because

BA was supplied in hydrochloride form, hydrochloric acid was added into the control variants of fermenters. Subsequently, BA (0.232 g of histamine, 0.404 g of tyramine, 0.557 g of putrescine, 0.360 g of cadaverine) and 0.526 M hydrochloric acid were added into fermenters as follows: variant A – 7 mL of BA solution was added into three fermenters and dissolved in 28 mL of distilled water; variant B (control) – 7 mL of 0.526 M-HCl (corresponding quantity of HCl to the variant A solution) were added into three fermenters.

Each fermenter content was mixed thoroughly, and ruminal fluid samples were taken in at this time (time 0). Subsequent sampling and pH measurements using a pH meter (WTW inoLab; Weilheim, Germany) took place after 2, 4, and 8 h after the first sampling. Ruminal fluid was diluted, mixed, and centrifuged. Afterwards, it was filtrated using LUT Syringe Filters Nylon (LABICOM s.r.o., Olomouc, Czech Republic). Samples were kept frozen at  $-20^{\circ}\text{C}$  until subsequent analyses.

Biogenic amines were analyzed by ion-exchange chromatography using AAA 400 apparatus (Ingos, Prague, Czech Republic). This method is based on the affinity of separated molecules of BA to the ion exchanger. The system consisted of a glass filling chromatographic column and steel pre-column, two chromatographic pumps for transport of elution buffers and derivatization reagent, a cooled carousel for 25 Eppendorf tubes, a dosing valve, a heat reactor, a Vis detector, and a cooled chamber for derivatization reagent. The volume of the injected sample was 100  $\mu\text{L}$  with RSD 1%. We used a two-channel Vis detector with a 5  $\mu\text{L}$  flow volume cuvette operated at wavelengths of 440 and 570 nm. A solution of ninhydrin was prepared in 75% methyl cellosolve (v/v) and 25% 4 M acetic buffer (v/v, pH 4.0). Tin chloride was used as a reducing reagent. Prepared solution of ninhydrin was

**Table 1 - Composition of feed mixture**

Constituent	Content (%)
Lucerne pellets	31.2
Unhulled sunflower meal	30.0
Barley	20.0
Malt residual	10.0
Cereal germs	3.0
Limestone	2.5
Monocalcium phosphate	2.0
Molasses	0.5
Feeding salt	0.5
Premix of micronutrients and vitamins	0.3

**Table 2 - Nutrient composition of feed rations (%)**

Feed	DM	Ash	NDF	CF	CP	Fat
Meadow hay	90.93	9.79	50.82	27.88	12.89	2.87
Lucerne silage	32.93	9.30	43.43	28.56	14.08	4.10
Feed mixture, 1st sampling	90.37	9.12	28.90	13.50	16.80	3.41

DM - dry matter; NDF - neutral detergent fiber; CF - crude fiber; CP - crude protein.

**Table 3 - Concentration of biogenic amines in bulk feed (mg/kg)**

	Histamine	Tyramine	Putrescine	Cadaverine	Spermidine	Spermine
Meadow hay	5.9	14.2	50.9	60.4	12.9	22.7
Lucerne silage	97.1	198.8	225.0	406.8	4.2	13.6

stored under an inert atmosphere ( $N_2$ ) with cooling at 4 °C. Flow rate was 0.25 mL·min<sup>-1</sup>. Pressure ranged from 4.5 to 6.0 MPa. Reactor temperature was set to 120 °C. For elution, two buffers were employed: buffer A was composed of 5.5 mM  $C_6H_8O_7$ , 81 mM  $Na_3C_6H_5O_7$ , 257 mM NaCl, 350 mM KBr, and 250 mL of  $C_3H_8O$  per 1 L of Milli-Q water, with a final pH of 5.78. Buffer B was composed of 3 mM  $C_6H_8O_7$ , 130 mM  $Na_3C_6H_5O_7$ , 75 mM NaCl, 20 mM NaOH, and 250 mL of  $C_3H_8O$  per 1 L of Milli-Q water, with a final pH of 5.78. For pH measurements, the WTW inoLab pH meter (Weilheim, Germany) was employed.

Five-milliliter samples of ruminal fluid preserved by 0.1 mL of 50% sulfuric acid were diluted with 95 mL water. After addition of 1 mL of 1 M NaOH, ammonium concentration was determined using gas sensing electrode (Electrochemical detectors, Czech Republic). Samples of ruminal fluid (containing VFA) were kept frozen at -20 °C until subsequent analyses. Prior to analyses, samples were thawed, centrifuged at 3000 *g* for 20 min and 1 mL of 0.3 M oxalic acid to 10 mL of the supernatant was added. Volatile fatty acids in samples were determined according to Křížová et al. (2011) using gas chromatography with CHROM-5 gas chromatograph (Laboratory equipment, Prague, Czech Republic) fitted with glass column, fitted with 80/120 Carbowax B-DA/4% CARBOWAX 20 M.

Data were processed statistically using STATISTICA.CZ, version 12.0 (TIBCO Software, Palo Alto, CA, USA). Results are expressed as mean ± standard deviation (SD). Statistical significance was determined by examining the basic differences between groups and among individual samples using ANOVA and Scheffé's test (two-way analysis) for the parameters: ruminal pH, concentration of ammonia, VFA and BA. Differences with  $P < 0.05$  were considered significant.

## Results

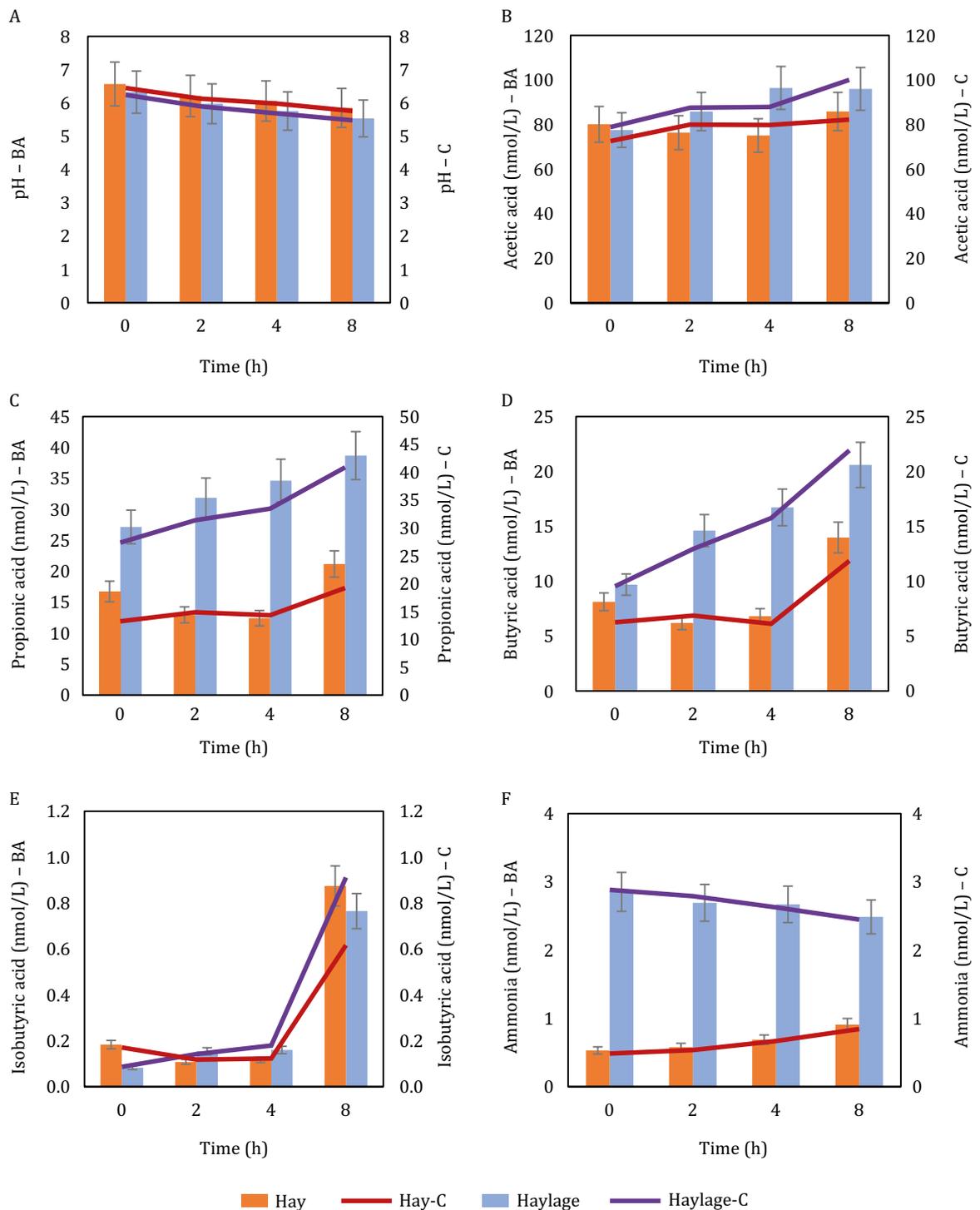
The time-lapse changes for the duration of 8 h in the rumen fluid were graphically expressed. Our data describe levels of digestion indicators (pH, acetic, propionic, isobutyric, and butyric acids, and ammonia) in rumen containing hay and haylage with addition of BA. The control samples did not include the BA addition.

Levels of pH showed a slight decrease from  $6.5 \pm 0.6$  to  $5.5 \pm 0.5$  in both treated and control samples of hay and haylage after 8 h of fermentation (Figure 1A). Concentration of acetic acid showed significant increase up to the concentration 20 nmol/L after 8 h ( $P < 0.05$ ) in the case of silage fermentation. The addition of BA did not cause any change in the acetic acid concentration during the time.

Average concentration of VFA in hay rumen fluid was  $76 \pm 7$  nmol/L (Figure 1B). Approximately two-times higher concentration of propionic acid was determined in haylage rumen fluid in comparison with hay rumen fluid. Moreover, the propionic acid concentration increased from  $27 \pm 3$  to  $38 \pm 3$  nmol/L during 8 h in haylage rumen fluid. In the case of hay rumen fluid, propionic acid concentration was  $13 \pm 2$  nmol/L. The difference between control and samples with added BA was not statistically significant (Figure 1C). There was an increase of butyric acid concentration from  $10 \pm 1$  to  $20 \pm 2$  nmol/L during 8 h in haylage rumen fluid (Figure 1D). On the contrary, the hay rumen fluid showed only a slight increase of butyric acid concentration from  $8.0 \pm 0.7$  to  $14 \pm 1$  nmol/L during 8 h of fermentation. Besides, it can be observed that the control showed similar trend to samples with addition of BA. Isobutyric acid concentration showed a rapid increase from  $0.2 \pm 0.02$  to  $0.8 \pm 0.09$  nmol/L after 8 h of fermentation both in hay and haylage rumen fluid. The addition of BA did not influence isobutyric acid concentration (Figure 1E). Ammonia level in hay rumen fluid was significantly lower ( $0.6 \pm 0.04$  nmol/L) than in haylage rumen fluid ( $2.8 \pm 0.2$  nmol/L) (Figure 1F). From this result, it may be stated that the increase of acid concentration is in correlation with the total pH in the rumen mixture.

The dynamics of BA degradation were studied for 8 h. The level of BA degradation is expressed as slope of the straight line of a least-square regression line. For the simulation of usual contamination, BA were added to the hay/haylage rumen fluid mixture. The BA concentration of the control samples (without BA addition) were subtracted from analyzed levels of BA.

From regression line of both hay and haylage, it is possible to see a decreasing trend of histamine changes throughout the time of fermentation from  $76 \pm 7$  to  $39 \pm 4$  mg/kg (Figure 2A). A steep decrease from  $113 \pm 9$  to  $34 \pm 2$  mg/kg of tyramine in hay rumen fluid was observed, whereas the tyramine content in haylage rumen fluid was  $140 \pm 10$  mg/kg in average (Figure 2B). In the case of putrescine, cadaverine, and spermidine dynamics of degradation, there was an estimated decrease from  $147 \pm 10$  mg/kg to  $59 \pm 5$  mg/kg,  $178 \pm 15$  mg/kg to  $121 \pm 9$  mg/kg, and  $15.0 \pm 0.9$  mg/kg to  $5.2 \pm 0.2$  mg/kg, respectively



BA - biogenic amines.

**Figure 1** - pH and metabolic indicators of digestion of nutrients during 8 h of *in vitro* fermentation.

(Figure 2C-E). Hay rumen fluid showed more rapid degradation of BA compared with haylage rumen fluid. The average contents of BA were  $67 \pm 6$  mg/kg,  $86 \pm 8$  mg/kg, and  $7.3 \pm 0.8$  mg/kg (Figure 2C-E). Rapid decrease of spermine content from  $14 \pm 1$  mg/kg to  $2.2 \pm 0.1$  mg/kg in haylage rumen fluid was also apparent (Figure 2F). Spermine concentration was stable ( $1.8 \pm 0.1$  mg/kg) in time.

## Discussion

Biogenic amines are an important factor to consider in ruminant nutrition and animal health. Until today, there is very limited amount of similar studies observing BA dynamics *in vitro* in real time. Although several authors have observed aspects of BA degradation in cattle, sheep may serve as a

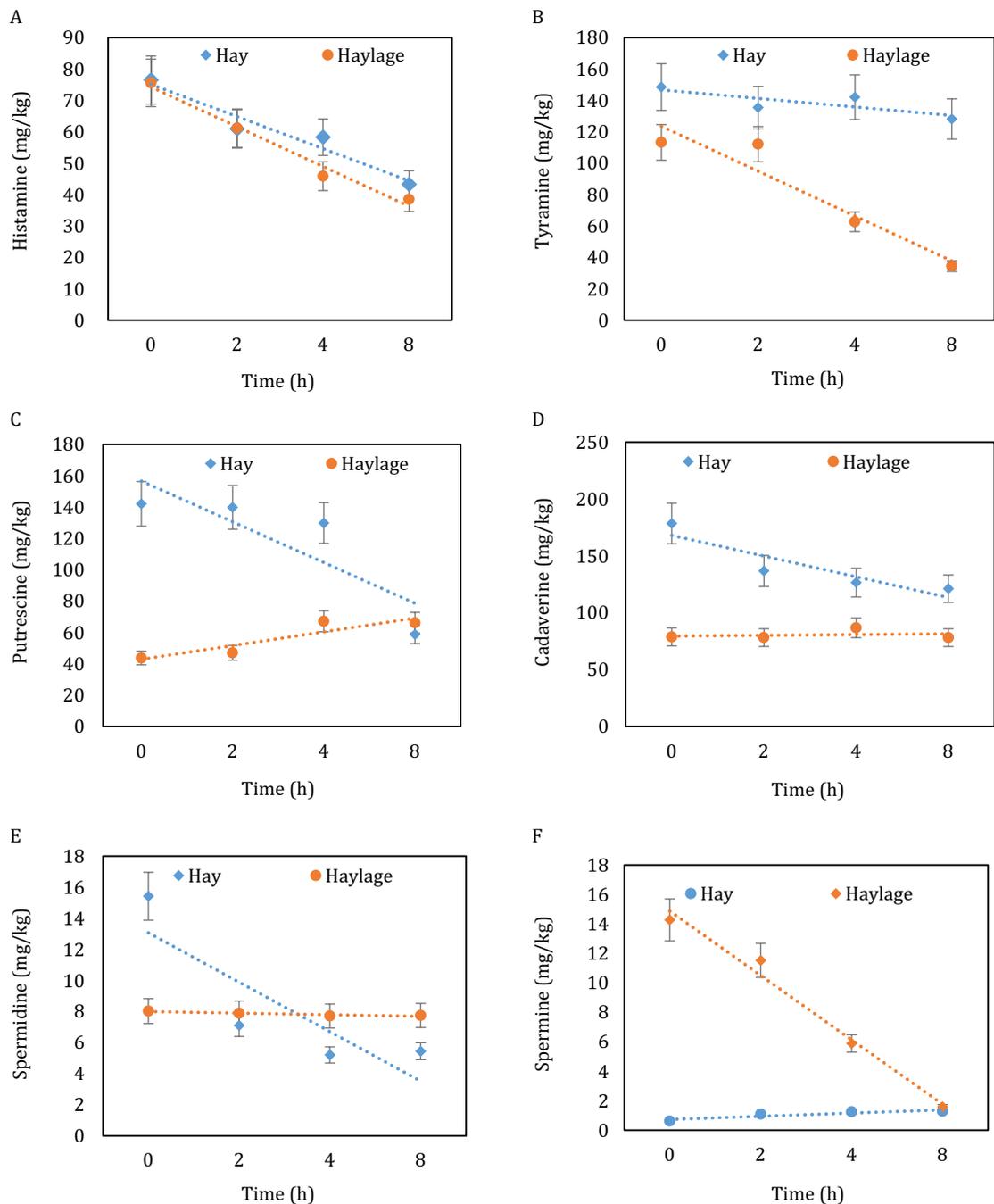


Figure 2 - Analysis of biogenic amines in rumen content during 8 h.

suitable representative of ruminant gastro-intestinal tract as well. However, sheep rearing is less economically encumbering.

Levels of BA in hay or haylage did not seem to change in correlation with VFA levels or ruminal pH throughout the fermentation in our study. Similar association was studied by Wang et al. (2013), who found a negative correlation between pH of rumen fluid and BA concentration. The use of sodium bicarbonate as a buffer increasing ruminal pH was proven to effectively lower BA concentration (Mao et al., 2017) and corroborated this study. Contrariwise, even though pH measured in our study was lower than their experimental measurements, no statistically significant dependence could be concluded in this regard.

Jeong et al. (2015) observed that diet treatments with lower levels of protein resulted in lower concentration of BA and ammonia and lower pH. This partially corresponds with our findings. Hay samples contained lower concentration of spermidine and spermine and lower ammonia after 8 h of fermentation, whereas pH and other BA levels were overall higher than protein-rich haylage samples.

Ammonia concentration increased in haylage, with the highest values obtained after 8 h of fermentation; however, it decreased slowly in hay-fed sheep. This is in contrary to the study of Van Os et al. (1996), in which ammonia levels in silage overall increased in silage rumen fluid during fermentation.

Biogenic amines were degraded by ruminal microflora to some extent in our study. However, sufficiency of degradation levels needs to be broadened and considered in relation to current extensive research done on BA levels in feed. Fusi et al. (2004) determined that only 1.4 g of BA per day reduced body weight and subsequently lowered meat quality of goats. Nevertheless, legislative limits for BA concentration do not currently exist, except for histamine in fish products for human consumption (Ruiz-Capillas and Herrero, 2019). This highlights the importance of our study, since the knowledge of BA degradation process during fermentation may serve as a guideline for future feed safety regulations.

In some studies, it was found that BA could negatively affect DM intake in higher concentrations than 2 g per kg of DM (Scherer et al., 2015; Dawson and Mayne, 1995). However, the combination of BA and formaldehyde was proven to decrease DM intake (Neumark et al., 1964). Therefore, synergistic relationship of BA and other compounds and its influence on ruminal parameters needs to be further studied.

Histamine levels decreased with fermentation time considerably in our study. This may be due to the ability of lactic acid bacteria to degrade BA in feed (Steidlová and Kalač, 2004). Similar findings were observed in the study of Van Os et al. (1995), in which histamine was degraded to the highest extent of all tested BA during fermentation. In this study, a habituation of rumen microflora to BA was also observed after longer periods of BA-contaminated feed intake by the experimental animals.

Slower BA degradation in haylage variant of rumen fluid was observed in comparison with hay variant. Moreover, in the study by Krížek (1993), it was observed that BA concentrations (spermidine, spermine, and histamine) culminated 200 days after ensiling. This suggests that ensiling process increases the risk of silage contamination and thus makes haylage more prone to toxicity. These findings in consideration of our own observation may suggest that higher feed safety standard is needed in ensiled haylage compared with hay.

Concentrations of propionic, butyric, and isobutyric acids increased rapidly after 2 h of fermentation. A similar trend was observed in a study of Shen et al. (2017), in which VFA upregulation by non-fiber carbohydrates occurred in diet 2-5 h after feeding. This suggests that the correlation between ruminal microbiome and VFA production is dependent on the feed nutritional composition, corresponding to the findings of Zhang et al. (2014). On the other hand, Mao et al. (2017) suggested that higher pH led to higher VFA levels in rumen samples. On the contrary, VFA concentrations present in hay rumen fluid changed with fermentation time and the trend shows no dependence on pH levels in our study. However, a negative correlation was observed between pH and VFA in haylage.

## Conclusions

Biogenic amines play an important role in the physiological functions of animals. In this study, degradation of biogenic amines and changes in metabolic indicators during fermentation were observed in Charolais sheep rumen *in vitro*. The degradation of biogenic amines is more rapid in hay, the most noticeable degradation was measured in the case of histamine. Levels of volatile fatty acid increase with duration of the fermentation, while ammonia concentration and pH decrease.

## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

Conceptualization: J. Skladanka and J. Trinacty. Data curation: I. Kolackova, P. Horky and J. Trinacty. Formal analysis: S. Skalickova, N. Cernei, Z. Lackova and V. Adam. Funding acquisition: P. Horky. Investigation: N. Cernei. Methodology: J. Trinacty and V. Adam. Project administration: I. Kolackova and S. Skalickova. Resources: J. Skladanka and P. Horky. Software: S. Skalickova. Supervision: J. Skladanka, P. Horky and V. Adam. Writing-original draft: I. Kolackova. Writing-review & editing: I. Kolackova, S. Skalickova and P. Horky.

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