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Influence of probiotic feed supplements on functional status of rumen

Vliv probiotických krmných doplňků na funkční stav bachoru

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ABSTRACT

The aim of this study was to determine how the administration of probiotic feed supplements affects selected parameters rumen environment of cattle, how it impresses the basic chemical and biological processes in the rumen, and also to check their influence on the total digestibility of feed in the cannulated cattle. For the experiment two adult cows of Aberdeen Angus breed with implanted permanent cannula were used, whom probiotics *Bifidobacterium* sp. were administered daily and subsequently the degradability of the organic matter was determined by the *in sacco* method. From the samples of rumen fluid, the amount of ammonia, volatile fatty acids, ciliates and pH were analyzed. The impact of probiotics has not been demonstrated in testing the influence of probiotics on the different variables with fixed effect of an individual. When testing the influence of probiotics without the effect of an individual, in the linear model, obtained data of acetic and butyric acid were the best. In their dependence, numbers of protozoa were increasing. However, only two experiment individuals were tested, a strong effect of the individual was found. These results indicate that the effect of probiotics *Bifidobacterium* sp. on the functional state of the rumen is low.

Keywords: cannula, cattle, ciliates, food supplements, pH, ruminants, volatile fatty acids

ABSTRAKT

Cílem této studie bylo zjistit, jak podávání probiotických krmných doplňků ovlivňuje vybrané parametry bachorového prostředí, jak působí na základní chemické a biologické procesy v bachoru, a také jejich vliv na celkovou stravitelnost krmiva kanylovaných krav. V pokusu byly použity dvě dospělé krávy plemene Aberdeen-angus se zavedenou permanentní kanylou, kterým byla denně podávána probiotika *Bifidobacterium* sp. a následně stanovena degradovatelnost organické hmoty metodou *in sacco*. Z odebraných vzorku bachorové tekutiny bylo analyzováno množství amoniaku, těkavé mastné kyseliny, nálevníci a pH. Při testování vlivu probiotik na jednotlivé proměnné, s pevným efektem jedince nebyl vliv probiotik prokázán. Při testování bez efektu jedince vyšla v lineárním modelu nejlépe popisujícím má data kyselina octová a máselná. V jejich závislosti se zvyšovaly počty protozoí. Jelikož byli použiti pouze dva pokusní jedinci, je zde silný efekt jedince. Z těchto výsledků vyplývá, že vliv probiotik *Bifidobacterium* sp. na funkční stav bachoru je nízký. Tyto výsledky mohly být ovlivněny nízkým počtem replikací aplikace probiotik a také nízkým počtem zvířat.

Klíčová slova: kanyla, krmné doplňky, nálevníci, pH, přežvýkavci, skot, těkavé mastné kyseliny

INTRODUCTION

The rumen hosts a large number of microorganisms, including bacteria, protozoa and fungi that function on the base of strict anaerobic ambience. These microbes degrade plant fiber to non-fibrous carbohydrates, proteins, volatile fatty acids and ammonia. Ammonia is used by microbes as energy and own source of nitrogen needed for their growth (Flint, 1997; Ozutsumi et al., 2005; Welkie et al., 2010; Fraga et al., 2013; Pinloche et al., 2013; Gillespie and Flanders, 2014). This implies that these microorganisms have an important role in maintaining the stability of rumen ambience and health of the host (Castillo-Gonzalez et al., 2014; Round and Mazmanian, 2009). Bacteria are the most important microbes involved in the digestion of ruminants. 1 ml of rumen fluid contains 10^9 to 10^{12} bacteria (Prescott et al., 2005). The competition of bacteria depends on many factors, such as the preference of substrate, energy requirements and the resistance to certain products which may be toxic (Pitta et al., 2010). *Bifidobacteria* are found in the intestinal tract of animals and humans. *Bifidobacteria* are considered as one of the key genera. Their presence in high numbers is associated with good health of the host. There is a general rule that the presence of this genus in the digestive tract leads to maintaining the balance of microflora, reducing the risk of infection with a pathogen (Biavati and Mattarelli, 2006). Rumen ciliates, anaerobic fermentative organisms contribute significantly to the digestion of ruminants (Ushida, 2011). Ciliates represent 40 – 80% of animal biomass of rumen. Systematically, they are a subclass of *Trichostomatia* and can be divided into two main groups: *Entodiniomorphida* and *Vestibuliferida*. When pH drops below 4.5, in three days the disappearance of fauna in the forestomach occurs. The total number of ciliates varies from 10^4 to 10^7 ml^{-1} of rumen fluid depending on the composition of the feeding ration and the time after feeding (Yañez-Ruiz et al., 2004; Ricard et al., 2006; Firkins et al., 2007). The composition of rumen fluid is affected by the type of feed, the composition of saliva and the absorption of dissolved substances (Jackson and Cockcroft, 2002; Fuller, 2004).

MATERIALS AND METHODS

The experiment was performed in an accredited stable of the school agricultural farm in the period from 25.8.2015 to 29.1.2016. Two dry-cows were used to determine the degradability of organic matter by the *in sacco* method.

Animals and experimental design

For the experiment two adult cows (C – control group, E – experimental group) of breed Aberdeen Angus with implanted permanent rumen cannula (ø 13 cm) to evaluate the impact of administration of probiotics genus *Bifidobacteriunm* sp. (10^7 g^{-1}) were used. Experimental animals were housed loosely in box loges with ad libitum access to the drinking bowls with water and lick. The average body weight during the experiment was in the first animal 799 ± 7.1 kg, in the second animal 594 ± 9 kg. Probiotics *Bifidobacterium* sp. were administered in a lyophilized form of 2 g each, stirred in 100 ml of drinking water and applied through the cannula into the rumen, each day at 9:00 PM during the whole habituating and experimental period.

The experiment was performed in two reruns, which had a consistent pattern of activities. In each of them both cows were included gradually. In the third controlled period animals received the basic feeding ration (BFR). During the whole experiment, stable microclimate was monitored using datalogger.

Feeding

Animals were fed twice a day (at 6:00 and at 15:00). The basic feed ration consisted of hay and water (intake *ad libitum*). The feeding ration of animal **1** was calculated according to the weight of the individual to 7 – 7.5 kg of hay (average 7.25 ± 0.16 kg). For animal **2**, 5 – 5.5 kg (average 5.3 ± 0.15 kg) was set. The intake of water during morning feeding was recorded. Animal 1 drank an average of 31 ± 7.8 l/day, animal 2 drank an average of 33 ± 6.7 l/day.

Organization chart of individual experimental periods:

a) Preparation - 14 days

- during the preparation period animals received only BFR, in order to rumen microflora of both animals reached the same physiological conditions
- during this period samples of rumen fluid and feces were taken and analyzed

b) Habituating period - 14 days

- during these period animals received BFR, and probiotics were administered
- the aim was to get used the rumen microflora to applied feeding supplements
- rumen fluid and feces were taken for laboratory analysis

c) Experimental period - 21 days

- animals received BFR with probiotics simultaneously, rumen fluid and feces were taken for laboratory analysis

Taking of rumen fluid

Rumen fluid samples were taken in all three periods three hours after the morning feeding 3 times a week, every Monday, Wednesday and Friday. Sample collection itself was performed by rumen cannula probe connected to a vacuum hand pump. Rumen fluid was transported to the laboratory immediately. For a subsequent laboratory analysis, the samples were filtered through gauze. Rumen fluid was used to determine the pH, the analysis of nitrogen compounds, VFA and for setting the number of ciliates.

Taking of feces

Feces samples were obtained by manual extracting directly from the rectum, and in plastic sample boxes immediately transported to the laboratory for analysis. Feces were taken at the same time and in the same periods as the samples of rumen fluid.

Sample analysis

Rumen fluid

Altogether 60 samples of rumen fluid from each individual were collected. From the rumen fluid, pH, the amount of VFA (acetic acid, butyric acid, propionic acid), nitrogen compounds (NH_3) and numbers of protozoa were measured.

Determination of crude protein - nitrogen substances (NS) in samples of lyophilized rumen fluid after mineralization, distillation and titration on the device KJELTEC by the method of Kjeldahl (AOAC, 2005) were determined.

Measuring of hydrogen ion concentration (pH) - for measuring the pH of samples by electrometric determination a digital pH meter INOLAB PH LEVEL 2 was used.

Analysis of volatile fatty acids - volatile fatty acids by isotachophoretic method of splitting ions on the basis of different mobility in a DC field on the device Ionosep 2001 according to the application sheets RECMAN were determined.

Determination of content of protozoa - number of protozoa in the rumen fluid by counting in a Bürker chamber (Blau Brand®, Wertheim, Germany) after dyeing and dilution of samples with 0.1% solution of methylene blue in a ratio of 1:10 was determined. To determine the total number of protozoa in 1 ml of rumen fluid, a revealed number was multiplied by a factor of 10,000, comprising the factor of the chamber and an index of dilution (Dohme et al., 1999).

Table 1. Nutritional value of hay in 100% dry matter

Dry matter	Crude protein	Crude fiber	ADF	NDF	Ash	Fat	NEL (MJ/kg)
92,925	7,941	28,072	34,307	59,728	6,355	1,378	4,992

NDF - neutral detergent fiber; ADF - acid detergent fiber; NEL - netto energy lactation.

Feces

Laboratory dry matter - fecal samples were pre-dried at first in petri dishes in a drying cabinet for 48 hours at 55 °C. For determination of laboratory dry matter all samples were subsequently dried in drying bowls in a drying cabinet at 103 °C for 4 hours. Determination of crude protein – it was used pre-dried faecal samples. NS were determined after mineralization, distillation and titration on the device Kjeltec by the method of Kjeldahl (AOAC, 2005). The results were recalculated in laboratory dry matter. Determination of organic matter - dried fecal samples were combusted in a muffle furnace at 550 °C for 6 hours. Organic matter was determined from the difference between the amount of dry matter and ash.

Statistical analysis

At first individual variables of pH, acetic, propionic and butyric acid and ammonia as predictors to probiotics and then towards ciliates using linear regression models (LM) were tested. Further, the influence of probiotics on digestibility of hay and dry matter was also tested. To evaluate the influence of probiotics, pH, acetic, propionic and butyric acids, ammonia, and digestibility of hay on the abundance of ciliates (log-transformed data) linear regression models (LM) with normal dividing was used. In the model at first all explanatory variables were used and the model was then simplified (backward selection) using stepAIC function to the final model that best describes the collected data. To take into account the effect of the individual (since it was used only two animals) in the next LM the variable animal (1 or 2) as a fixed effect was used. To determine the influence of probiotics in each LM always an explanatory variable (pH, VFA, digestibility and amount of protozoa) towards probiotic was tested. Data were analyzed in the R programme, version 3.1.2 (R Development Core Team, 2014). Library Ggplot2 (Wickham, 2009) was used for visualization of graphs and for visualization of the final model results, Library effects (Fox, 2003) and the program Excel (REF) were used.

RESULTS

Analysis of rumen fluid

Measured values of pH (Figure 1) show the minimal difference between groups ($F=0.059$; $P>0.05$). In animal 2, a slight increase in the average pH value occurred. The average pH in both individuals ranged between the values of 6.9-7.

Figure 2 shows the average measured values of acetic acid in rumen fluid. Overall, no changes between the individual groups occurred ($P>0.05$). In animal 2, the average in the experimental period decreased.

Figure 3 shows that the average value of butyric acid quantity do not differ between the individual groups ($P>0.05$). Values differ only between individuals.

Measured values shown in Figure 4 illustrate the average amount of propionic acid in rumen fluid. There is no significant difference between groups ($P>0.05$). It can be seen that the average values in animal 1 increased, whereas the average values in animal 2 decreased during the experimental period.

Figure 5 shows the average amount of NH3 in both individuals. The differences between groups were not proved ($F=1.1943$; $P>0.05$). In animal 1, the average slightly decreased in the experimental group, whereas in animal 2, the average slightly increased.

The amount of ciliates differed significantly only between individuals ($F=26.899$, $P<0.0001$), but not between groups. In Figure 6, a decline of logarithmic amount of ciliates is noticeable in animal 1. In animal 2, the average number of ciliates slightly increased. Statistic evaluation of rumen fluid: At first the influence of variables on the amount of ciliates in rumen without the effect of an individual was tested.

It was used LM (Table 2) from which the values of pH ($F=4.1674$; $P<0.05$) and the amount of acetic acid ($F=6.6834$; $P<0.05$) were significant.

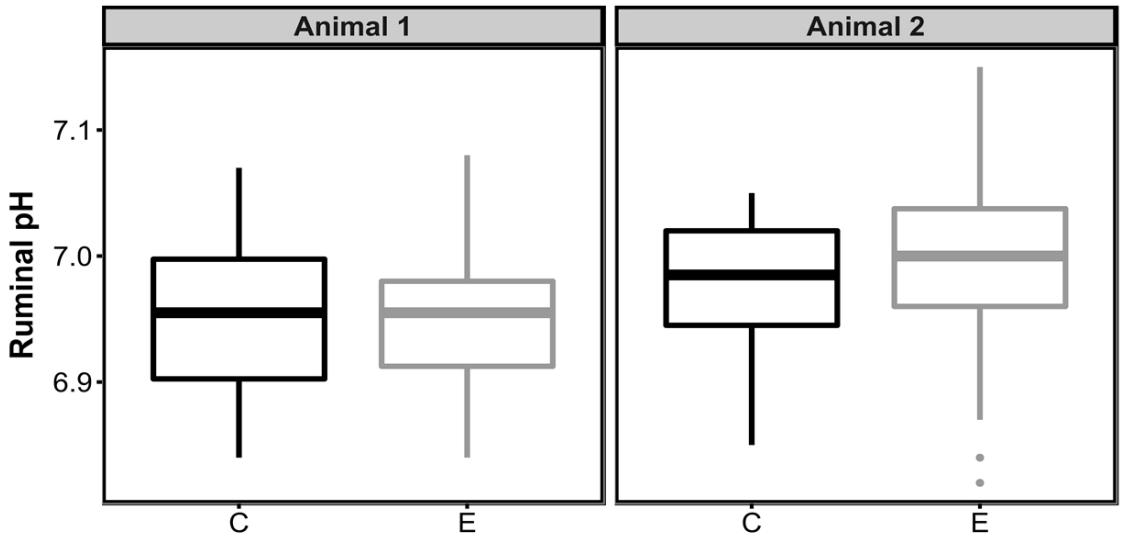

Figure 1. Ruminal pH

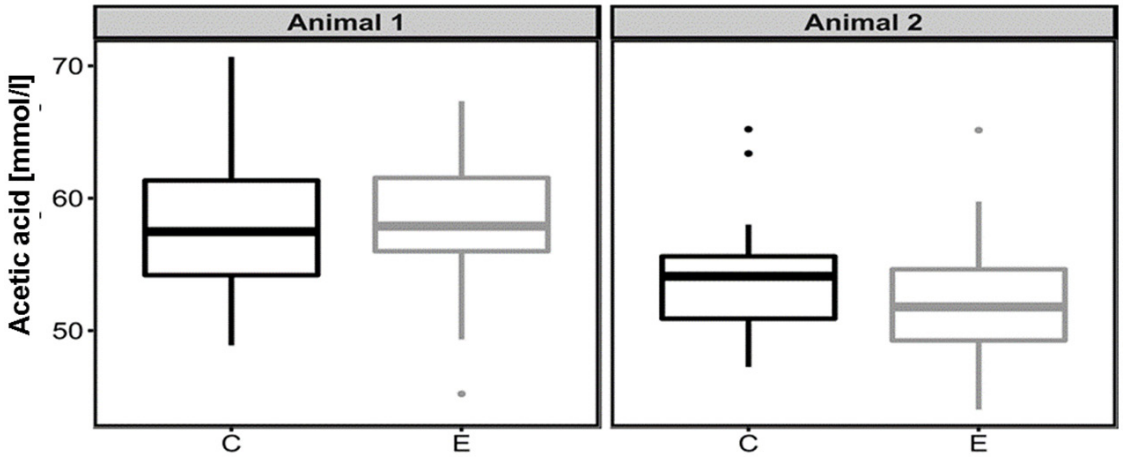

Figure 2. Acetic acid

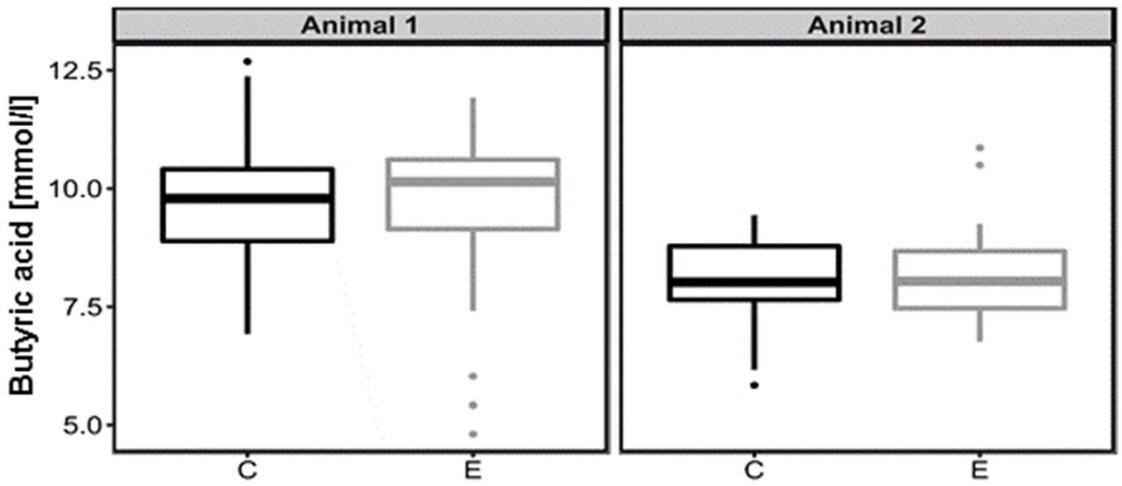

Figure 3. Butyric acid

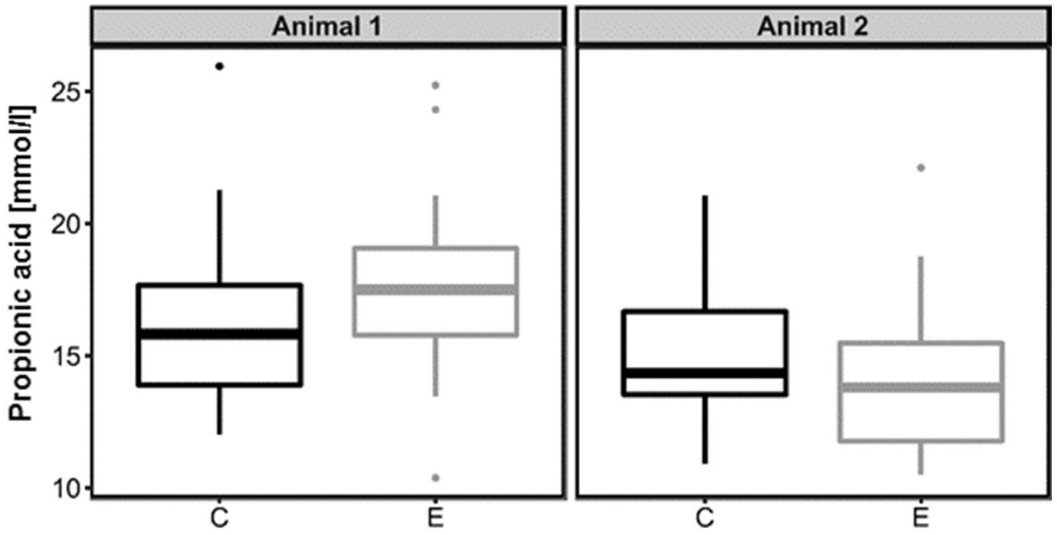

Figure 4. Propionic acid

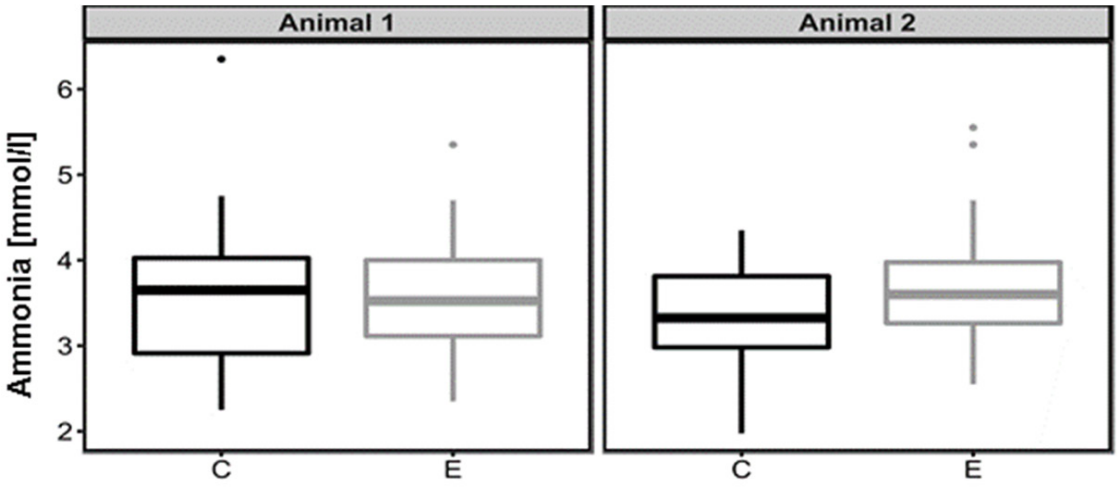

Figure 5. Ammonia

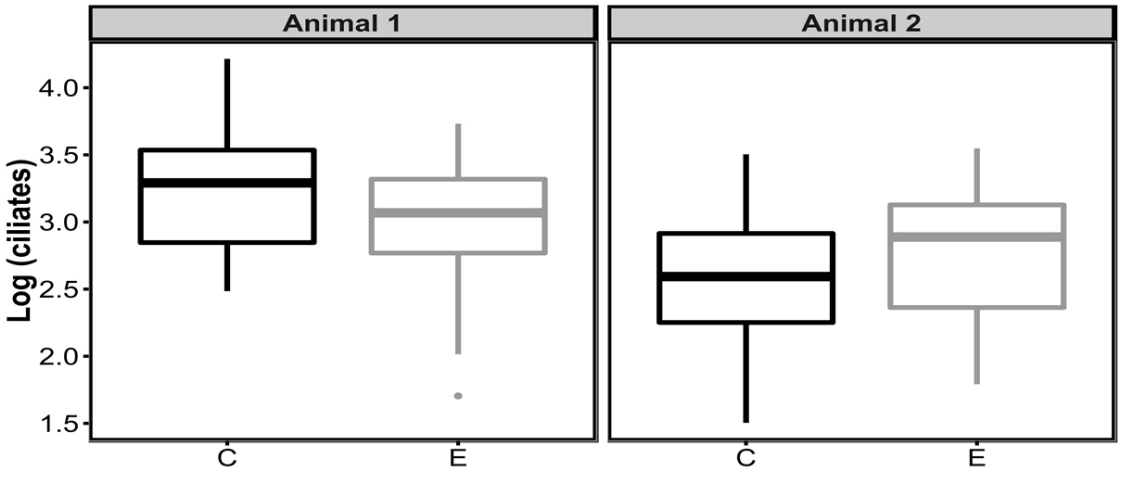

Figure 6. Ciliates

Then LM was simplified into the resulting linear model (Table 3) that best describes the obtained data, where the variable acetic acid was significant (P<0.001) and the butyric acid was inconclusive (P<0.05). Subsequently data were tested towards the influence of a group (experiment, control) with the fixed effect of an individual.

Table 4 shows the overview of the results of each test, where all variables were inconclusive. The resulting linear model best describes influencing on the amount of ciliates by monitored variables, quantity [mM^{-1}] of butyric, propionic and acetic acid, the pH level and amount of ammonia in the rumen fluid in both animals. The overview of the results of each model that tested each variable towards the group (experiment, control) with the fixed effect of an individual was inconclusive in all variables.

Table 2. Basic linear model before simplification without the effect of an individual

	Df	Sum Sq	Mean Sq	F value	P value
pH	1	1.0332	1.03317	4.1674	0.04354*
Acetic acid	1	1.6569	1.65693	6.6834	0.011**
Butyric acid	1	0.6646	0.66457	2.6806	0.10436
Propion acid	1	0.1101	0.11014	0.4442	0.50644
Ammonia	1	0.0937	0.09372	0.378	0.5399

Df - degrees of freedom; Sum Sq - sums of squares; Mean Sq – mean square; *P<0.05; **P<0.01

Table 3. Amount of acetic acid and butyric acid in the rumen fluid in both animals

	Df	Sum Sq	Mean Sq	F value	P value
Acetic acid	1	2.553	2.553	10.56	0.001509***
Butyric acid	1	0.7422	0.7422	3.07	0.082369*

Df - degrees of freedom; Sum Sq - sums of squares; Mean Sq – mean square; *P<0.05; **P<0.01

Table 4. The overview of the results of each model

	Df	Group			
		Sum Sq	Mean Sq	F value	P value
pH	1	0.00027	0.00027	0.0599	0.80714
Acetic acid	1	10.33	10.33	0.4277	0.5144
Butyric acid	1	0	0	0.0001	0.991
Propion acid	1	0.95	0.948	0.1163	0.7337
Ammonia	1	0.585	0.58451	1.1943	0.2767
Ciliates	1	42.6	42.64	0.4995	0.4811

Df - degrees of freedom; Sum Sq - sums of squares; Mean Sq – mean square.

Simultaneously, the effect of the individual was always significant (Table 5).

Table 5. Summary results of testing of individual variables towards a group

	Df	Group			
		Sum Sq	Mean Sq	F value	P value
Lab. dry matter	1	2.813	2.81296	1.6409	0.2027
% ash	1	0.139	0.139	0.1974	0.6576
% NDF	1	16.83	16.834	1.9995	0.16

Df - degrees of freedom; Sum Sq - sums of squares; Mean Sq – mean square; NDF - neutral detergent fiber

Summary results of testing of individual variables towards a group (experiment, control) with a fixed effect of the individual. P value for all variables was inconclusive. The effect of the individual was significant in the share of ash and NDF (Table 6).

Table 6. Average measured values of Ash and NDF

%	Animal 1		Animal 2	
	Control	Experiment	Control	Experiment
Ash	7.9 ± 0.9	8.1 ± 1	8.08 ± 1.1	7.9 ± 1
NDF	58.3 ± 3.4	58.4 ± 2.6	58.18 ± 2.5	58.29 ± 3.4

NDF - neutral detergent fiber

DISCUSSION

The aim of this study was to determine the influence of administration of probiotic feed additive *Bifidobacterium* sp. on the amount of VFA, ammonia and ciliates and pH values in the rumen of cattle and on the total digestibility of feed. It was demonstrated that probiotics have an effect on the stabilization of pH (Table 2 and Figure 1), on the improvement of nutrient intake from the rumen microbiota to the host and on the improvement of ruminal ambience (Beauchemin et al., 2003; Chiquette et al., 2008, 2012).

In this study, the influence of probiotics on the pH was not proved. Only the influence of an individual that notes also Ritz et al. (2014) was found. In both groups the values of pH ranged in diameters between 6.9-7. The optimal pH of rumen fluid is in the range between 6.2-7.2 (Jackson and Cockcroft, 2002). The relatively high pH of the rumen fluid is given by a feed ration. In animals with volume feed pH ranges between 6.2 and 6.8. If the main share of the ration consists of hay, the pH value can increase to above 7 (Beauchemin et al., 2003). Qadis et al. (2014) found pH 6.6–6.8 as a constant pH in the experimental group with the significant effect of probiotics on the pH values. Other significant effect of probiotics on pH value is also claimed by Wang et al. (2016).

It is presumable that the conclusiveness is affected by the species of probiotics. In the study Qadis et al. (2014) probiotics *Lactobacillus plantarum*, *Enterococcus facium* and *Clostridium butyricum* were combined and Wang et al. (2016) used *Bacillus subtilis*. In this study probiotic *Bifidobacterium* sp. was administered, whose influence was already proved (Charteris et al., 1997; Russell et al., 2011), but so far there is no study known where individuals were fed only with hay. PH values are related to the production of volatile fatty acids (Bannink, 2007). Probiotics did not have any effect on the amount of VFA in rumen fluid (Tables 3, 4 and Figures 2-4), which was confirmed also by other studies (Qadis et al., 2014). Wang et al. (2016) reported that probiotics reduced the concentration of propionic and acetic acid, which is the opposite of the Beauchemin et al. (2003) study. In this study only the influence of the individual on all type of VFA was demonstrated (Table 2). In animal 2, VFA had generally lower values, probably due to the lower body frame of the individual. Other VFA occurred only in a trace amount in the ruminal ambience and therefore they are negligible.

To assess rumen fermentation and multiplication of microorganisms themselves, the content of ammonium ions is an important criterion. The level of ammonia in rumen depends on pH of the rumen fluid. When pH is higher than 7.3, the unionized form of NH_3 prevails (Jelínek et al., 2003). Figure 5 shows that the average measured values of ammonia ranged from 2.89 ± 0.7 – 3.89 ± 0.6

mM^{-1}. An optimum for microbial synthesis is 2.9-3.5 mM^{-1} (Firkins et al., 2007; Jallow and Hsia, 2011). In the table is shown that the diameters increased compared to the period before the experiment. However, the increase was also detected in control periods in both individuals, which may be caused by alternating of experimental and control periods in an individual. Li et al. (2009) stated that the concentrations of ammonia are dependent on the area where the rumen fluid was taken.

Values of samples taken from the central rumen area can be up to 10 times lower than the values of samples taken from the cranial parts. Rumen fluid contains approximately 10^6 protozoa (Saleem et al., 2013). These microorganisms are very sensitive to dietary changes and quickly react to the changing conditions, especially pH (Pfeffer and Hristov, 2005). After the application of probiotics, number of ciliates moderately increased in animal 2, nevertheless, statistical conclusiveness was not significant in any of the experimental animals (Figure 6).

According to studies by Gianesella et al. (2012) and Tajima et al. (2007), lower ambient temperature related with higher rumen fermentation, means the higher concentration of volatile fatty acids and ammonia ions. In addition to the temperature of the external environment, also humidity plays an important role in the rumen fermentation. If in the period with a higher temperature, also the humidity of air is higher, fermentative characteristics may be higher in comparison with the period of lower temperatures. Temperature and humidity also have an impact on the quantity and composition of rumen microflora (Tajima et al., 2007).

CONCLUSIONS

Inconclusiveness of this experiment could also be caused by a low number of animals. It was given mainly due to spatial and financial limitations. It is also very complicated to obtain permission for cannulation of animals and the manipulation itself. In studies Qadis et al. (2014) and Lee et al. (2004), twelve cannulated individuals were used for the experiment and Guedes et al. (2008) used only three individuals, which is also a low number for the statistical treatment. In conclusion, the data collected in this experiment and subsequent analysis having used linear models proved neither conclusive results, nor the effect of probiotics *Bifidobacterium* sp. The tests showed the demonstrable effect only of acetic acid and butyric acid, which raised communities of ciliates, but this result did not work significantly in the next test, where the individual as a fixed effect was added to the analysis. Results of the experiment could be influenced by a low number of probiotic replications, and also by a low number of tested animals. The low number of animals is caused mainly as a result of high cost of their operation, by the demandingness of the cannula application itself and by obtaining the permission for the animal manipulation, too. Moreover, the results could have been influenced by the short period of the cannula application, or by the fast alternation of individual groups (experiment and control). Eventually, inconclusiveness of the results could have been affected by the kind of administered probiotics itself (*Bifidobacterium* sp.), and by the amount of the dose. In further research, there would be useful to monitor also the influence of amino acids in the rumen, because their increase would positively affect the digestibility of feed. It would also be appropriate to try other types of probiotics, or to test their various dosages.

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