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IMPACT OF INULIN ON PRODUCTION OF METHANE, CARBON DIOXIDE AND GASTROINTESTINAL CANAL FUNCTIONALITY IN CALVES

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Abstract
Ruminants produce a large amount of methane (CH_4) and carbon dioxide (CO_2) in their foregut. These gases cause greenhouse effect. There are a lot of studies about different feed additives which can reduce the production of greenhouse gases in ruminants. Prebiotics can also change the amount of bacteria in animal gastrointestinal tract and reduce the occurrence of diarrhoea. The aim of this study was to test whether the prebiotic inulin affects the production of CH_4 and CO_2 in calves' rumen and whether it affects the bacteria count in the rumen fluid and bacterial overgrowth in intestines. We used the flour of Jerusalem artichoke (*Helianthus tuberosus* L.) containing 50% of inulin. Approximately fifty days old, Holstein Friesian crossbreed calves were used in this study. Eight were in the control group, 8 received 12 g of flour and 8 received 24 g per day. On the 28th and 56th day of the research, we measured the amount of CH_4 and CO_2 in calves' rumen, took rumen fluid samples for bacterial analysis and urine to measure the level of phenol and indican. We concluded that adding the flour of Jerusalem artichoke at doses 12 g and 24 g did not significantly impact the production of CH_4 and CO_2 in calves' rumen, the prebiotic inulin may suppress the growth of anaerobic microorganisms in the rumen at concentration 12 g of inulin reaching 56th day of experiment. The amount of phenol and indican in calves' morning urine did not correlate with the faecal consistency of calves.
Key words: calves, inulin, methane, carbon dioxide, phenol, indicant.

Introduction

Greenhouse gas effect negatively affects ecosystem in the whole world and can cause tremendous climate changes. Unfortunately, the development of the economic activity, including agricultural sector, negatively affects the emission of gases, which causes the greenhouse effect. Many countries invest enormous resources to decrease the greenhouse effect (Jordaan *et al*., 2017). After regaining independence, Latvia took active part in reducing the greenhouse effect by finding different solutions to reduce these emissions in different sectors of economic activity (Lenerts, Popluga, & Rivza, 2017).

In Latvia, greenhouse gas (GHG) emission is actual in dairy cattle and beef cattle farms where the emission of nitrous oxide (N_2O) and methane (CH_4) can reach 83–94%, but carbon dioxide (CO_2) 6 – 17% of the total gas amount (Kreišmane, 2011).

Bērziņa with her colleagues found out that, in 2012, emissions produced in agriculture sector contributed approximately 20% of the total emissions in Latvia and it was the second largest source of GHG emissions (Bērziņa *et al*., 2014). There were 393.1 thousand cattle in Latvia (2012); dairy cows were 164.6 thousand from them (Centrālā…, 2013). The most recent data show that, in 2016, the number of cattle in Latvia rose to 412 084, dairy cows – 153 927 from them (Latvijas…, 2017), therefore, it may be concluded that emissions have risen.

Ruminants have a stomach consisting of four compartments; the largest is rumen with enormous number of microorganisms providing biological digestion processes of feed. Due to these processes, two major gases CO_2 and CH_4 are produced in large quantities making 65.5% and 26.8% respectively (Chianese, Rotz, & Richard, 2009). Some of the CO_2 producing microorganisms in the rumen are *Ruminococcus albus*, *Butyrivibrio fibrisolvens* and *Lachospira multiparus*. *Methanobacterium ruminatium* and *Methanobacterium mobilis* and other methanogens produce CH_4 by using hydrogen (H_2) and CO_2 produced by other fermentative members of the rumen microbiome (Rother & Krzycki, 2010). It is known that CO_2 and H_2 are major precursors of CH_4 (Asanuma, Iwamoto, & Hino, 1999).

Although agriculture is not considered to be the main source of CO_2 emissions, still CO_2 emissions occur on farms, mainly due to animal respiration and decomposition of soil organic matter (Chianese, Rotz, & Richard, 2009). The same authors made conclusions that CO_2 emission from animal respiration make up to 90% of the total CO_2 emissions on a dairy farm. Although CH_4 is 23 times more potent than CO_2, it still should be considered when talking about GHG (Loh *et al*., 2008).

Methane production is considered an energy loss for the host (Kristensen *et al*., 2011). There are a lot of factors impacting the production of CH_4 in rumen, such as animal size, growth rate, level of intake, feed quality, genetics, and environmental temperature (Shibata & Terada, 2010). It is impossible to completely stop methanogenesis in rumen as it is the integral component of rumen fermentation (Cengic-Dzomba, Dzomba, & Musanovic, 2012). GHG emission may be decreased either by increasing the productivity of the animal through improved nutrition to produce less

CH_4 per unit of meat or milk or by altering the rumen fermentation process (Iqbal *et al.,* 2008).

The promising results of prebiotics on human health have encouraged researchers to explore its potential on different livestock species like cattle, sheep and other ruminants (Fraser *et al.*, 1998; Nabuurs, 1998). Feed additives have the potential to improve nutrient utilization in farm animals by modifying ruminal microbial population and, consequently, ruminal fermentation and digestion (McGuffey, Richardson, & Wilkinson, 2001). Many researchers have found that prebiotics can increase the daily weight gain in calves and have potential to reduce CH_4 production (Mwenya *et al*., 2004; Hasunuma *et al*., 2011; Ilgaza *et al*., 2016).

The prebiotic inulin was used in the present research. It is a polydisperse non-starch polysaccharide naturally occurring as a storage carbohydrate in some 36 000 species of plants. The main sources of inulin are chicory (*Cichorium intybus* L.) and Jerusalem artichoke (*Helianthus tuberosus* L.). Inulin is used successfully in monogastric animals, however, there is not enough evidence of its use in ruminants. The process of fermentation that occurs in the large intestines of monogastric animals is identical to that occurring in the forestomach of ruminants (Öztürk, 2008). Several studies have revealed that adding inulin to the milk replacer of pre-ruminant calves leads to significantly higher live-weight gains and better faecal consistency (Kaufhold, Hammon, & Blum, 2000; Verdonk & Van Leeuwen, 2004). There is no information about the impact of inulin on the production of CH_4 and CO_2 in the rumen of calves.

Mul (1997) found that adding oligofructose to the milk replacer of calves resulted in improved body weight gains, feed conversion efficiency, firmer faeces and reduction of diarrhoea. The same results were recorded in the study where prebiotic inulin was used (Ārne & Ilgaža, 2016). Faecal consistency may also be affected by bacterial overgrowth in intestines. In human medicine, the quantitative evidence of phenols in night and morning urine with increased values is a reliable indicator of bacterial overgrowth in intestines. With the help of special analysis of phenol and indican, it is furthermore possible to locate a growth in the intestine. Phenol mainly is resorbed in the small intestine, indican is a bacterial metabolic product, which is resorbed in colon (Toskes, 1999; Lord & Bralley, 2008). Acknowledging it, we asked whether inulin supplement changes the amount of phenol and indican in calves' morning urine and whether these changes are related to faecal consistency.

Phenol is formed in the bowel from amino acid tyrosine and is eliminated to a considerable extent in the faeces. Part of it is absorbed and excreted largely unchanged in urine (Smith & Macfarlane, 1997).

Indican is an end-product of indole produced when bacteria in the intestine act on the amino acid, tryptophan. Most indoles are excreted in faeces. The rest is absorbed, metabolized by the liver, and excreted as indican in urine (Greenberger, Saegh, & Ruppert, 1968; Jackson, Riordan, & Neathery, 2000). Indican is an indicator of overgrowth of anaerobic bacteria (Greenberger, Saegh, & Ruppert, 1968).

The aim of this study was to find out how the prebiotic inulin affects the production of CH_4 and CO_2 in calves' rumen on 1 kg body weight and how it affects the bacteria count in rumen fluid and the bacterial overgrowth in intestines. Previously it has been found that inulin supplement positively affects calves' live weight gain and faecal consistency (Jonova, Ilgaza, & Grinfelde, 2017).

Materials and Methods

The research was conducted in a dairy farm of intensive production from July until the end of September, 2016. The farm is located in Jelgava district (latitude: N 56° 27', longitude: E 23° 37') Latvia. Clinically healthy different sex Holstein Friesian (*Bos taurus* L.) crossbreed calves were included in our research. Their average age was 49 ± 10 days and average weight was 79.6 ± 12.82 kg. Calves were kept in groups in a partly closed space with natural ventilation through windows.

Eight calves were in the control group (CoG), 8 were fed with additional 12 g of flour of Jerusalem artichoke containing 6 g of prebiotic inulin (Pre12) and 8 received 24 g of this flour containing 12 g of inulin (Pre24). These dosages were chosen to compare the results with the results from the previous research in a different farm. Jerusalem artichoke contains just around 10% of inulin, but by using special technologies, the content of inulin can be increased up to 48.5 – 50% (Fleming & Groot, 1979). Inulin supplement, which is used in this study, is produced in Latvia. We added this prebiotic to barley flour once a day. All calves had free access to water and hay and they also got whole milk (2.5 – 3 L per calf twice a day) and a calf starter meal (0.5 kg per calf once a day) (Ilgaza *et al*., 2016; Jonova, Ilgaza, & Grinfelde, 2017).

The research lasted for two months (56 days). Methane and carbon dioxide were measured by using cavity ring down spectroscopy device Picarro G2508 connected in the closed circulation system with 1 L chamber and external zero leaching vacuum pump. The air samples from calves' rumens were collected on the 28th and 56th day of the research. Each sample of calves' rumen gas was taken in syringes with 20 mL volume by puncturing the rumen at the point where the gas accumulation was visually noticed. 10 mL of calf's rumen gas were injected in the chamber with air and measured for 180 s.

The rumen fluid was obtained by puncturing the rumen at a lower point and aspirating the fluid in the syringe 2 h after feeding. Then samples were securely closed to maintain an anaerobic environment and frozen at –80 °C. The count of anaerobic bacteria in rumen fluid was determined by growing viable anaerobic bacteria from rumen fluid samples at 10^{-6} dilution on plate count agar. Petri dishes were incubated in anaerobic conditions at +37 °C for 3 days. Bacteria colonies were counted, and bacteria count in one millilitre of rumen fluid was mathematically calculated and converted to logarithmic scale. Testing was performed in Latvia University of Life Sciences and Technologies, Scientific Laboratory of Biotechnology, Department of Molecular Biology and Microbiology.

The urine was collected early in the morning before feeding by catching urine flow in the middle. The samples were stored in special urine vacutainers with preservative to keep it stable and kept in a dark place. Samples were sent to Laboklin laboratory in Germany for testing for phenol and indican (indicators for bacterial overgrowth in small intestines) by using standardized chemical photometric method.

On the 28th and 56th day of the research faecal consistency was evaluated. Faeces were scored for physical shape as 1: firm; 2: slightly loose; 3: loose; 4: watery (Larson *et al*., 1977).

The assumption of normal data distribution was assessed by Shapiro-Wilk's test and visual inspection of their histograms, normal Q-Q plots and box plots. The assumption of homogeneity of variances was tested by Levene's test. Box plots detected presence of outliers. To determine whether there were any statistically significant differences among three or more independent groups, one-way ANOVA (with Tukey post hoc test) (if the data were normally distributed, homogeneous and without outliers) was used. Otherwise, the data were analysed using the Kruskal – Wallis H test with pairwise comparisons using Dunn's (1964) procedure with a Bonferroni adjustment. To determine if there was a statistically significant monotonic trend between an ordinal independent variable and a continuous variable the Jonckheere-Terpstra test was used, however, it does not inform how strong this trend might be. Therefore, Kendall's tau-b (τb) was used as a measure of effect size (Kraska-Miller, 2014). Those tests were carried out using SPSS Statistics version 22 (IBM Corporation, Chicago, Illinois). All statistical analyses were performed at the significance level of α=0.05.

Results and Discussion

In this study it was found out that the production of CH_4 on 1 kg body weight on the 28th day of the research was the highest in Pre12 but the lowest in Pre24 (11.9 ± 2.94 mg m^{-3} and 9.2 ± 1.06 mg m^{-3}, respectively), but in CoG – 10.1 ± 2.46 mg m^{-3}, the situation changed at the end of the research, the lowest production of CH_4 on 1 kg body weight now was in CoG, but the highest in Pre24 (7.2 ± 1.68 mg m^{-3} and 9.1 ± 2.45 mg m^{-3}, respectively), but in Pre12 – 8.0 ± 1.53 mg m^{-3}). Similar tendency was observed about CO_2. The highest CO_2 production on 1 kg body weight on the 28th day of the research was in Pre12, but it was the lowest in Pre24 (49.1 ± 10.54 mg m^{-3} and 39.0 ± 8.81 mg m^{-3}, respectively), but in CoG – 42.9 ± 6.98 mg m^{-3}. On the 56th day of the research the highest CO_2 production on 1 kg body weight was observed in groups fed with additional inulin supplement, but the lowest in CoG (Pre12 – 46.1 ± 8.52 mg m^{-3}, Pre24 – 43.4 ± 11.12 mg m^{-3} and CoG – 39.1 ± 10.93 mg m^{-3}). Numerous studies show that prebiotics can reduce the production of CH_4. For example, galacto-oligosaccharides can reduce the production of CH_4 up to 11% (Mwenya *et al*., 2004). Our results are contrary, the analysis of production of CH_4 and CO_2 on 1 kg body weight showed that there were no significant outliers, data were normally distributed for each group, and there was homogeneity of variances. The one-way ANOVA showed that there was no significant difference between experimental and control groups ($p>0.05$). It means that adding the flour of Jerusalem artichoke at doses 12 g and 24 g does not significantly influence the production of CH_4 and CO_2 in calves' rumen. We suggest that other dosages of inulin or other feed additives should be chosen for mitigation of GHG.

A lot of microorganisms living in the rumen use prebiotics as the source of energy (Samanta *et al*., 2013). Bunce, Howard & Kerley (1995) found that the inclusion of prebiotic oligosaccharides into the diet of calves reduces the population of total anaerobes in the gut.

Data of the present study showed that the count of anaerobic bacteria in rumen fluid was not normally distributed, however, conversion to logarithmic scale has made data normally distributed. A Welch t-test was run to determine if there were differences in log of anaerobic bacteria between experimental and control groups due to the assumption of homogeneity of variances being violated, as assessed by Levene's test for equality of variances ($F(5.42) = 7.224$, $p<0.001$).

The Tukey post hoc test showed that the log of anaerobic bacteria was significantly lower in experimental group Pre24 on 56th day comparing to CoG on the 28th and 56th day and Pre24 on the 28th day and Pre12 on the 28th day ($p<0.05$) (Fig 1.).

The rumen only starts to grow at 2–3 weeks of age and its growth will continue until about 6 months. Calves can be called full-ruminants at 12 weeks of age when their rumen has fully developed and calves are

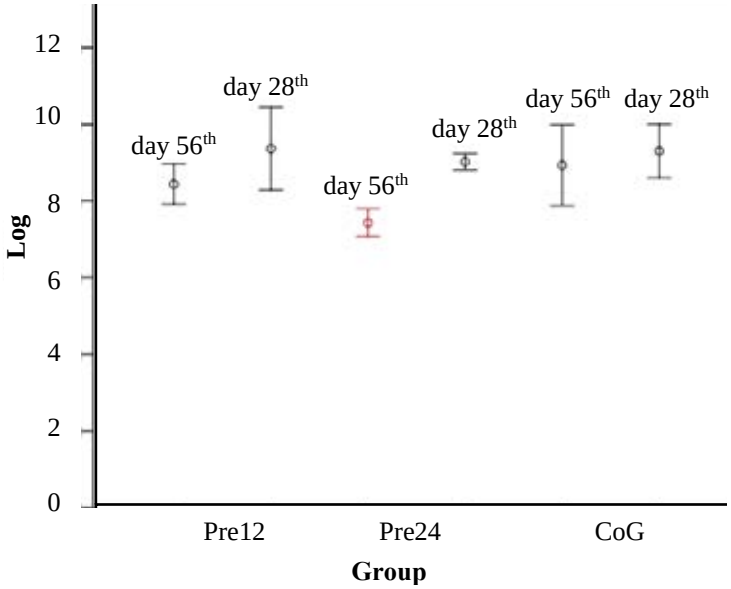

Figure 1. The amount of viable anaerobic microorganisms in the rumen fluid on different sampling days.

able to eat and digest dry food at the level of adult ruminants (Govil *et al*., 2017). Ruminant animals harbor a complex microbial community consisting of a diverse array of anaerobic microbes in the rumen (Fathallh Eida *et al*., 2012). These microorganisms interact with one another and take part in the systematic digestion of fibrous plant material, which they anaerobically ferment into end products later used as energy sources by the host (Martin, Morgavi, & Doreau, 2010). Our results suggest that the prebiotic inulin may suppress the growth of anaerobic microorganisms in the rumen at concentration 12 g of inulin reaching the 56th day of experiment.

Median of phenol in calves' morning urine in three study groups was 681.1 mg dL^{-1} for CoG (n=9), 675.0 mg dL^{-1} for Pre12 (n=7) and 384.5 mg dL^{-1} for Pre24 (n=8). A Jonckheere-Terpstra test determined that there was a statistically significant decreasing monotonic trend in median of phenol in calves' morning urine, p= 0.032. Kendall's τ_b between study groups and phenol level was -0.353 (moderate effect size). Median of indican in calves' morning urine in three study groups was 40.0 mg dL^{-1} for CoG (n=9), 20.0 mg dL^{-1} for Pre12 (n=7) and 10 mg dL^{-1} for Pre24 (n=8). A Jonckheere-Terpstra test determined that there was a statistically significant decreasing monotonic trend in median of indican in calves' morning urine, p= 0.002. Kendall's τ_b between study groups and indican level was -0.534 (strong effect size). It means that there is moderate negative association between dosage of inulin and phenol in calves' morning urine, as well as strong negative association between dosage of inulin and indican in calves' morning urine. The level of indican and phenol in the urine decreases with a higher dosage of inulin.

Hidaka *et al.* (1986) supplemented rat diets with short-chain fructooligosaccharides and observed that concentrations of phenols in urine samples decreased, whereas the concentration of indican did not change. Those data only partly coincide with our results, because we recorded decrease of both phenol and indican.

These two substances – indican and phenol are considered to be the indicators of the bacterial overgrowth in the intestines and can affect the faecal consistency in humans and monogastric animals (Toskes, 1999; Lord & Bralley, 2008). Although during the implementation of this study, changes in the amount of phenol and indican were observed, these changes were not related to the faecal consistency ($p>0.05$). On the 28th day recorded results were as follows in Pre12 Me = 2 (IQR 1.25 – 2.75), Pre24 Me =1 (IQR 1 – 2), CoG Me = 2 (IQR 1 – 2) and on the 56th day: in Pre12 Me = 2 (IQR 1 – 2), Pre24 Me = 2 (IQR 1 – 2), CoG Me = 1 (IQR 1 – 2). Faecal consistency shows that faeces were quite firm in all groups on sampling days and the difference between scores was not significant. However, we cannot confirm that the bacterial overgrowth in the intestines has any connection with the level of phenol and indican in the calves' urine.

Conclusions

1. The flour of Jerusalem artichoke at doses 12 g and 24 g does not reduce the production of CH_4 and CO_2 in calves' rumen at age 49 ± 10 days.
2. The prebiotic inulin may suppress the growth of anaerobic microorganisms in the rumen at concentration 12 g of inulin reaching the 56th day of experiment.

3. The amount of phenol and indican in calves' morning urine does not correlate with the faecal consistency of calves, however, it negatively correlates with inulin's concentration in feed.

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