University of Copenhagen
Faculty of Health and Medical Sciences

Master Thesis
Animal Science

The effect of Animal Biosa on *in vitro* rumen pH, degradability, total gas and methane production with Bolivian dairy cattle feeds

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18th August, 2019

Acknowledgements

First and foremost, we would like to thank our super-advisor Hanne Helene Hansen for her advice and knowledge that helped us tremendously in the process of data collection, statistical analysis and the writing. We would also like to say a big thank you to Biosa Danmark Aps, especially Erik Nielsen, Eli Dollerup Nielsen, and Maja Steensberg for a great collaboration and for giving us an opportunity to conduct an interesting experiment. Furthermore, we are very grateful to Anni Christiansen and Rajan Dhakal for their support in conducting the fermentation trial and teaching us the laboratory procedures.

Abstract

Enteric methane production in cattle is a natural process of anaerobic fermentation, however, it contributes considerably to the world greenhouse gas emissions and global warming. Bacterial probiotics have been proposed to have many beneficial effects on cattle health and productivity, including a reduction in enteric methane emissions. The objective of the current research was to evaluate the ability of a lactic acid based probiotic, Animal Biosa, to affect feed degradability, pH, total gas production, and methane production *in vitro* using rumen fluid from two fistulated Danish research cows and Bolivian dairy cattle feeds. Two consecutive fermentation trials were conducted using an automated vented *in vitro* gas production batch system (ANKOM Technology). Four different rations were formulated using Bolivian feeds, and Animal Biosa was added in four different doses (0, MIN: 0.00125 ml, MED: 0.00250 ml, MAX: 0.00375 ml). Gas was collected and methane percentage was determined using a gas chromatograph. pH was measured before and after incubation. Feed residues post incubation were filtered and organic matter (OM) and fibre degradation were determined. The results showed that, in this study, Animal Biosa did not have a significant effect on pH, OM, hemicellulose or cellulose degradation, total gas production, and methane production. The dose response was also not significant. Despite the lack of significant results in this study, other research showed that bacterial probiotics can be strain, substrate and dose dependent. Therefore, there is a possibility that the effects of Animal Biosa would be different with other feeds, higher doses of the probiotic, or a slight modification of the product with the addition of yeast and/or lactate utilizing bacteria, which could be determined with further research.

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(1) 0.5 g of ration x 50 ml / 20000 g = 0.00125 ml Animal Biosa

(2) 0.5 g of ration x 100 ml / 20000 g = 0.00250 ml Animal Biosa

(3) 0.5 g of ration x 150 ml / 20000 g = 0.00375 ml Animal Biosa

List of Abbreviations

ADF	Acid detergent fibre
ADL	Acids detergent lignin
ADS	Acid detergent solution
AIA	Acid insoluble ash
B1	2 x 10^9 cfu/day of *P. freudenreichii* NP24 + 1 x 10^9 cfu/day of *L. acidophilus* NP51
B2	2 x 10^9 cfu/day of *P. freudenreichii* NP24 + 5 x 10^8 cfu/day *L. acidophilus* NP51 + 5 x 10^8 cfu/day *L. acidophilus* NP45
CH_4	Methane
CO_2	Carbon dioxide
DM	Dry matter
DMI	Dry matter intake
ECM	Energy corrected milk
Exp1	Experiment 1
Exp2	Experiment 2
GC	Gas chromatograph
gDM	grams of dry matter
gOM	grams of organic matter
H	Metabolic hydrogen
H_2	Hydrogen gas
H_2O	Water

IVGPT	*In vitro* gas production technique
LAB	Lactic acid producing bacteria
MAX	Maximum dose of Animal Biosa (0.00375 ml)
MED	Medium dose of Animal Biosa (0.00250 ml)
MIN	Minimum dose of Animal Biosa (0.00125 ml)
NAD	Nicotinamide adenine dinucleotide
NDF	Neutral detergent fibre
NDS	Neutral detergent solution
NH_3	Ammonia gas
O_2	Oxygen gas
OM	Organic matter
P+Lp	*Propionibacterium* P63 and *L. plantarum* 115
P+Lr	*Propionibacterium* P63 and *L. rhamnosus* 32
SEM	Standard error of the mean
SF_6	Sulfur hexafluoride tracer technique
S+La	*S. cerevisiae* and *L. acidophilus*
S+Lp	*S. cerevisiae* and *L. plantarum*
STP	Standard temperature and pressure
TMR	Total mixed ration
VFA	Volatile fatty acids

1. Introduction

Environmental impact of livestock production has received a lot of public and scientific attention in the past few decades. With global annual temperatures rising, it is becoming increasingly important to limit greenhouse gas emissions from their key sources, including the energy production, industry, forestry, land-use and land-use change, as well as agriculture (Victor et al., 2014; Steinfeld et al., 2006). Greenhouse gas emissions from the livestock sector account for 18% of global anthropogenic emissions. Livestock production is responsible for 9%, 37% and 65% of the global anthropogenic carbon dioxide, methane, and nitrous oxide emissions respectively. With global warming potentials 23 and 296 times higher than carbon dioxide, methane and nitrous oxide are the most important emissions from livestock contributing to climate change. The majority of methane emissions in livestock production result from enteric fermentation and manure management. Enteric methane emissions are largely dominated by the cattle industry worldwide (Steinfeld et al., 2006). They do not only contribute to global warming and climate change, but also result in a loss of energy for the animals. This makes enteric fermentation an attractive target to manipulate as decreases in methane emissions are usually accompanied by increased animal productivity (Leng, 1993). With world human population increasing and global beef and milk demand concurrently rising (Gerber et al., 2013), the global enteric methane emissions are expected to rise by more than 20% from 2005 to 2030 (U.S. EPA, 2012). Therefore, it is essential to establish safe, effective and economically feasible strategies to limit methane emissions from cattle industry.

Several methane mitigation strategies have been investigated, such as increasing animal productivity through improved feed quality, farm management, and genetic potential, as well as direct and indirect methane inhibition through defaunation, immunisation, or dietary manipulation (Moss et al., 2000; Shibata & Terada, 2010). Dietary manipulation is the simplest and most practical approach. It can be separated into altering the forage to concentrate ratios of the diet as well as supplementation of different feed additives that have the potential to inhibit rumen methanogens directly or reduce the amount of substrate available for methane production (Haque, 2018). One of the many feed additives with methane mitigation potential in ruminants are probiotics (Nagpal et al., 2015). Bacteria and yeast based probiotics have been widely used in ruminant nutrition in the past few decades

due to their ability to increase animal health and performance (Krehbiel et al., 2002). The effects of probiotics vary with doses, substrates, and strains (Chen et al., 2016; Nocek et al., 2002; Philippeau et al., 2017; Lopez et al., 1999; Alazzeh et al., 2012), and there is also the possibility of interactive effects when the probiotic contains mixed organisms (McAllister et al., 2011). This leads to a considerable variation in probiotic effects between the studies (Rodrigues, 2016), including methane mitigation potential. Consequently, more research into different probiotic strain combinations using a variety of substrates and doses is necessary.

One of the livestock probiotics available on the market in Denmark is Animal Biosa, produced by Biosa Denmark ApS. Animal Biosa consists of eight bacterial strains, including *Bifidobacterium lactis, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus salivarius, Lactococcus lactis,* and *Streptococcus thermophilus,* and 19 organic herbs. An *in vivo* study conducted in Nepal found increased numerical values in milk yield, fat and solids-non-fat percentages in milk in cows supplemented with Animal Biosa compared to control cows (Pant, 2017). Therefore, based on these results as well as the findings of similar studies that used lactic acid producing bacteria (LAB) in their probiotics, Animal Biosa could be expected to affect feed digestibility and enteric methane production in cattle. If the effects were positive, Animal Biosa could become a valuable product that would help to reduce the environmental impact of cattle industry as well as increase animal productivity.

1.1. Research objectives and hypothesis

The objective of this study was to determine the effects of Animal Biosa on total gas and methane production, feed degradability, and pH using the automated *in vitro* gas production batch technique using witch Bolivian dairy cattle feeds as substrate and rumen fluid from Danish research cows as inoculum. Based on the claims by Biosa Denmark ApS and on the findings by Pant (2017), it was hypothesized that Animal Biosa would increase the total gas production, decrease the amount of methane produced, increase feed degradability and increase the pH.

2. Limitations

One of the limitations that were encountered in the present study was the inability to formulate the exact rations that the dairy cows were fed in Bolivia. Some of the feeds were wet and could not be dried properly for transportation, therefore only the feeds fed in a dry form were transported from Bolivia to Denmark and included in the hypothetical rations. Furthermore, although the grass growth in Capinota and Challapata is minimal during the dry season, and most cattle graze only in the wet season, two of the farms from which cattle feeds were collected allowed their cattle to graze during the dry season as well. Estimation of daily forage intake while grazing was outside the scope of this study, therefore it was disregarded in the formulation of the rations. In addition, the farmers provided unrealistic daily feed intake of their animals and were not able to estimate the average daily intake of some of the feeds given to their cows. Some farmers reported measuring the amount of feed given by armfuls, the weight of which could not be measured due to the lack of scales at the time of the visit. As a consequence, the intake of some feeds, such as corn stover and native grass, had to be estimated by weighing an armful of hay and presuming that it would weigh the same as an armful of corn stover or dried native grass. A realistic total daily dry matter intake (DMI) had to be estimated based on literature. The feeds that were not brought back from Bolivia were excluded from the rations. Due to these factors, the formulated rations were not identical to the ones fed in Challapata and Capinota.

Another limitation was the lack of feed material to perform the fibre, dry matter (DM) and organic matter (OM) analyses on both the individual feeds and the formulated rations. No weight of the feed samples was determined before the collection, therefore, some of the collected forage samples did not contain enough weight for both analyses. Consequently, the fibre, DM and OM analyses were carried out on individual feeds and the values for the rations were estimated using the weighted averages of individual feeds.

The volatile fatty analysis analysis (VFA) and varied incubation times have been considered, however, due to the limited resources, they were beyond the scope of this study.

3. Literature review

3.1. Rumen metabolism and carbohydrate digestion

The rumen has a capacity of up to 100 litres, and is, therefore, the largest part of the cow's stomach system (Jouany & Morgavi, 2007). The microbial ecosystem in the rumen is known to be very diverse. The microbes live in a symbiotic relationship with the cow, where they provide energy and protein, while the cow provides nutrients as well as an anaerobic environment that is optimal for microbial fermentation. The microbes, that contribute to the functioning of the rumen and play a major role in feed digestion and fermentation, include bacteria, protozoa, fungi and archaea. Most bacteria can either be found attached to feed particles or free floating in the rumen fluid (Nagaraja, 2016).

The fundamental part of a cow's diet are polymers that include protein and non-protein nitrogenous substances, lipids, lignin, and carbohydrates. The carbohydrates found in feed include polysaccharides, both structural (cellulose, pectin and hemicellulose) and non-structural (starch, simple sugars) (Nagaraja, 2016). Since the diet of ruminants consists of very fibrous and difficult to digest material, these animals regurgite the larger feed particles and chew them again, which acts as an efficient mechanism helping to reduce particle size physically. Once soluble feed particles enter the rumen, they are quickly dissolved and rapidly degraded by the ruminal microbes. The insoluble particles, on the contrary, are colonized by the ruminal microbes, and broken down slowly. Large feed particles tend to move to the top of the rumen, where they are retained and subjected to microbial breakdown, which reduces them to smaller and denser particles. The small particles descend in the rumen fluid and eventually are flushed out. If the passage rate of feed from the rumen increases, the extent of feed digestion is likely to be reduced. This may be an advantage with feeds high in starch or protein, because they are more efficiently digested in the lower gut. On the contrary, it might be a disadvantage with fibrous feeds that require adequate time in the rumen for microbial breakdown (McDonald et al., 2011b).

The metabolic breakdown of carbohydrates in the rumen can be divided into two steps, hydrolysis and fermentation (Jouany & Morgavi, 2007). During hydrolysis, glycosol hydrolases, produced by bacteria, protozoa and fungi, break down polysaccharides to

oligosaccharides (Nagaraja, 2016). Fibre (cellulose and hemicellulose) and starch are broken down by fibrolytic and amylolytic microorganisms respectively. Cellulose is decomposed to cellobiose, which is then converted to di- and monosaccharides. Starch are first converted to maltose or isomaltose, and eventually also to di- and monosaccharides. The final product of hydrolysis is pyruvate, which is considered the key intermediate between hydrolysis and fermentation (McDonald et al., 2011b). The major end-products of ruminal fermentation are VFA, including acetate, butyrate, and propionate, as well as gases, such as CO_2, H_2, NH_3 and CH_4 (Hill et al., 2016). VFA and gas production increases rapidly after feeding, because the increase in available energy leads to a rapid division rate of ruminal microbes. The rapid increase in VFA production also causes the pH to decrease, particularly if the feed consists of a high amount of easily fermentable carbohydrates (Sjaastad et al., 2010). pH has to be maintained above 6 for microbial protein synthesis (Strobel & Russell, 1986) and fibre degradation. The optimal growth of cellulolytic microorganisms occurs at pH 6.7 with normal activity supported at pH 6.7 +/- 0.5 (Van Soest, 1994c). A pH below 6 can cause an inhibition of the activity of cellulolytic bacteria, thus reducing fibre digestibility (McDonald et al., 2011d).

As long as rumen pH remains above 5.7, acetate always constitutes the largest proportion of the VFA produced during fermentation, followed by propionate, and, finally, butyrate. Acetic acid, when produced in the rumen, is rapidly absorbed through the ruminal wall and used as the main energy source by the animal (Nagaraja, 2016). Butyric acid is converted to beta-hydroxybutyrate during absorption through the ruminal wall and used as an energy source by tissues such as skeletal and heart muscle. The majority of propionate produced is absorbed through the ruminal wall, where a small part is converted to lactate, and the rest is carried to the liver, where it is converted to glucose through gluconeogenesis (McDonald et al., 2011c). The types of VFA fermented from pyruvate mostly depend on the composition of the substrate. When the diet is high in fibre, the VFA profile consists of around 70% acetate, 20% propionate and 10% butyrate. When the diet is high in starch, the total amount of VFA produced per feed intake increases, the proportion of acetate decreases to 60%, proportion of propionate increases to 30%, while butyrate is mostly unaffected (Sjaastad et al., 2010).

The production of CH_4 is a major route of H_2 disposal in the rumen. Fermentative bacteria generate adenosine triphosphate for microbial growth via the oxidative reactions, that are

characterized by the removal of an electron from a molecule. In the anaerobic rumen environment, it is hydrogen that is removed from metabolites during catabolism of substrates. The metabolic hydrogen is then used to reduce oxidized co-factors to their reduced forms (Jouany, 2008). One of the most important oxidation-reduction reactions in the rumen fermentation process is $NAD^+ + H^+ + e^- \leftrightarrow NADH$, facilitated by microbial hydrogenases. Nicotinamide adenine dinucleotide (NAD) is an example of a coenzyme that acts as an electron carrier (Bodas et al., 2012). In this reaction, metabolic hydrogen acts as an electron acceptor and combines with an electron to form hydrogen gas: $2H^+ + 2e^- \leftrightarrow H_2$ (Jouany, 2008). Substantial amounts of H_2 are generated in the rumen during this process, which adds to the H_2 produced by protozoa and fungi (Russell & Wallace, 1997). For the fermentation to continue, NADH has to be re-oxidised to NAD^+. This reaction, however, can only occur if the H_2 concentration in the rumen is maintained at a low level (Jouany, 2008), because hydrogenases are inhibited at high H_2 pressures (Bodas et al., 2012). The level of H_2 is efficiently regulated by CH_4 forming bacteria (archaea) that utilize H_2 to reduce CO_2 to CH_4, following the equation $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$. This process generates energy for the growth of methanogens, and at the same time, acts as a sink for the H_2 generated in the rumen. It allows reoxidation of reduced co-factors and, therefore, facilitates the fermentation in the rumen. Alternative routes for H_2 utilization have been recognized, such as reductive acetogenesis, however, the effect of other H_2 sinks compared to methanogenesis, is minimal (Jouany, 2008). The majority of CH_4 generated in the rumen is eructated. Although the process is essential for effective fermentation in the rumen, methanogenesis results in an energy loss for the animal of around 2-12% of digestible energy. It is, therefore, associated with the feed to product conversion ratio, which makes CH_4 an important marker of animal productivity (Hill et al., 2016).

3.2. Fibre analysis

The cell wall surrounding the plant cell consists of lignin, lignin bound protein, and different carbohydrates, including structural (cellulose, hemicellulose and pectin) and non-structural (starch) polysaccharides and simple sugars. Dietary plant fibre can be divided into soluble and insoluble fractions. The soluble cell contents include pectin, starch, lipids and protein, and the insoluble cell wall constituents (the fibre) includes cellulose, hemicellulose and lignin (Van Soest, 1994b; Nagaraja, 2016). Dietary fibre can be analysed using the Van Soest

method (Cherney, 2000), where plant carbohydrates are divided into fibre fractions based on their nutritional accessibility (Sollenberger & Cherney, 1995). First, the feed sample is washed with a neutral detergent solution, which removes the soluble part of the feed. The remaining fibre is referred to as the neutral detergent fibre (NDF) (Figure 1) (Van Soest, 1994b). When feed is high in starch, the neutral detergent solution is unable to remove all the starch. To prevent overestimation of NDF, starch digesting heat-stable alpha-amylase is used (McDonald et al., 2011a). The NDF fraction is then washed with an acid detergent solution, which removes hemicellulose. The remaining cellulose and lignin are referred to as the acid detergent fibre (ADF) (Cherney, 2000). Further, the ADF fraction can be subjected to 72 % sulphuric acid, which dissolves cellulose (McDonald et al., 2011a), and the residues are referred to as acid detergent lignin (ADL) (Van Soest, 1994b).

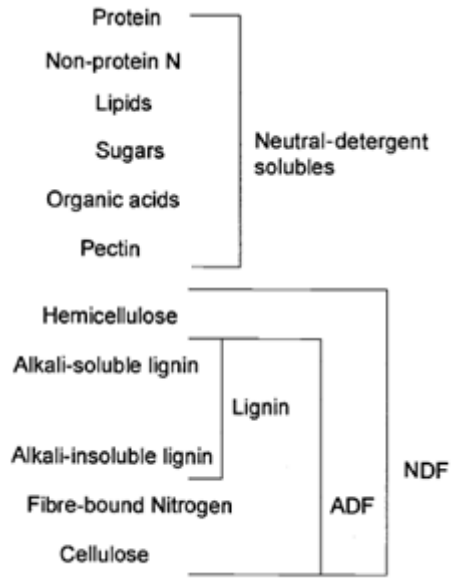

Figure 1. Dietary carbohydrates divided into fractions by the principal of the Van Soest system (NDF: Neutral detergent fibre, ADF: Acid detergent fibre, N: nitrogen) (Adapted from Cherney, 2000).

Lignin is not a carbohydrate, it is an indigestible polymer that consists of phenolic compounds. Lignin is considered the most significant factor in terms of feed digestibility due to its high resistance to chemical degradation. Also, chemical bonds have been found between lignin and different plant polysaccharides, that cause these polysaccharides to become unavailable for digestion. This means that plant fractions that are high in lignin have

low digestibility potential, unless they are treated with a chemical compound that causes the bonds between lignin and other carbohydrates to break (McDonald et al., 2011a).

To be able to compare characteristics of different feeds as well as the findings of different studies, the analytical results of feed evaluation are best described on either DM or OM basis. To determine the DM content, feed samples are dried in an oven with a temperature of 100 °C, which causes the water in the feed to evaporate (Cherney, 2000). When determining the OM, feed samples are placed in an oven overnight at 550 °C. The OM in the sample burns leaving total ash. The OM is calculated by subtracting total ash from the DM. Total ash represents the inorganic component of the feed, and it consists mainly of silica, however, it can also contain traces of organic material such as chloride, sulphur, sodium, phosphorus, and potassium (McDonald et al., 2011a). In addition, total ash represents both the ash of the plant tissue and the acid insoluble ash (AIA), that comes from environmental contamination, such as soil (Rao & Xiang, 2009). If the feed samples have a high level of AIA, it needs to be taken into account when calculating OM to avoid an overestimation of the ash, and thereby an underestimation of OM percentage. AIA is determined by ashing the residue of ADF at 525 °C (Van Soest et al., 1991). AIA is subtracted from the total ash, which leaves the ash that originates from the plant itself.

3.3. *In vitro* gas production technique

In vivo experiments are the most straightforward approach to evaluate the nutritive value of ruminant feeds. A few different techniques exist, including the collection of total urine and faeces, which measures apparent tract digestibility, the use of indigestible markers, which avoids the necessity of labour-intensive collection of total waste, and respiration chambers, that allows the collection of total gases expired and the measurement of total animal metabolism. However, *in vivo* experiments are expensive, time consuming, labour-intensive and logistically complex. They require multiple animals and a large amount of substances that are being tested. Consequently, laboratory methods are often used to predict the results *in vivo* (Owens and Basalan, 2016).

In vitro gas production technique (IVGPT) is one of the most common procedures for *in vitro* ruminant feed evaluation. There are several different methods of IVGPT, including

continuous, semi-continuous, and batch systems that differ in buffer and substrate inflows and outflows (Table 1) (Owens & Basalan, 2016). The *in vitro* batch systems can be either manual or automated. The automated batch system requires minimal labour and can be used to obtain a lot of data from a large number of samples at one time. It is considered to be a simple, yet sensitive, method for determining fermentation kinetics *in vitro* (Rymer et al., 2005). Pre-weighed ground feed samples are incubated with rumen fluid and an anaerobic buffered media into the incubation bottles for a predetermined time period at ~39 °C. Each incubation bottle is connected to a pressure transducer that records the cumulative pressure values in the computer system (Davies et al., 2000). The pressure can then be converted to the volume of gas produced (Rymer et al., 2005). Post incubation the residual feeds can be used to determine substrate degradation (Davies et al., 2000). In closed batch systems, gas produced accumulates in the headspace throughout the fermentation, while in vented batch systems, the gas is periodically released from the bottles and collected in the attached gas bags. It has been suggested that in the closed systems, some of the CO_2 generated can be partially dissolved in the fermentation media due to the gas pressure in the headspace. This can result in underestimation of total gas production and affected methane values as well as disturbed microbial activity. Therefore, for studies of total gas and methane production, it is recommended to use vented systems. The composition of gas can be analyzed by injecting a sample of the collected gas into a gas chromatograph (GC) (Cattani et al., 2014). The technique has been further developed by Muetzel et al. (2014), who directed the fermentation gas straight into the GC, measuring both the CH_4 and H_2 simultaneously, however, to our knowledge, it is not yet available on the market.

Table 1. An overview of continuous, semi-continuous and batch systems of *in vitro* gas production technique (Adapted from Gizzi et al. (1998))

	Continuous system	**Semi-continuous**	**Batch**
Inflow of buffer	continuous	continuous	continuous
Inflow of substrate	continuous	non-continuous (intermittent)	continuous
Outflow	continuous	continuous	none

The IVGPT simulates the fermentation process in the rumen and does not take into account the enzymatic digestion in the abomasum and small intestine, or microbial fermentation in the hindgut (Williams, 2000). It allows the measurement of both fermentation products and

residues. The feed material that is not recovered in the residues after incubation is assumed to be fermented and allows determination of feed degradability. It is generally accepted that the gas produced during IVGPT is proportional to the amount of feed fermented (McDonald et al., 2011d), however, Adesogan (2002) argued that this is not quite true, because the gas produced depends on the composition of the substrate and the type of VFA produced. If the fermentation of the substrate results in a high production of propionate, less gas will be produced than when the fermentation of substrate results in a high production of acetate and butyrate (Beuvink & Spoelstra, 1992). CH_4 and most of the CO_2 produced during fermentation is a result of microbial fermentation of monosaccharides to VFA and is considered a direct gas. Some of the CO_2 originates from the buffering of acids, and is considered an indirect gas (McDonald et al., 2011d). As a consequence, the gas produced indicates microbial activity and is proportional to the amount of VFA produced. If gas is recorded at regular time intervals during the fermentation run, the kinetics of fermentation can also be evaluated. The rate of gas production reflects the accessibility of the degradable feed portion to the microbes and the growth of the microbial population, and in terms of nutritional value of the feeds, it is as important as degradability. IVGPT can be used to predict ruminal OM or DM digestibility and energy content of feeds, as well as to determine fermentation kinetics, changes in microbial populations, feed associative effects and the effects of different feed additives (Getachew et al., 2004).

The extent to which the IVGPT batch system is able to predict fermentation characteristics in live animals has been investigated by a number of studies. Gizzi et al. (1998) studied the ability of IVGPT batch system to simulate the rumen fermentation characteristics of live cows measured by in-situ nylon bag technique. The authors used a slightly modified batch system, where buffer was added in a continuous flow, and compared a number of variables between the *in vivo* and *in vitro* systems, such as feed degradability, microbiome, VFA and gas production. The rumen fluid for the *in vitro* method was collected from the same three cows used in the *in vivo* study. The DM degradability was not significantly different between the two techniques. Also, no significant differences were found in the total microbial population as well as the counts of different bacteria classes (cellulolytic, amylolytic, proteolytic bacteria and archaea) throughout the incubation. The molar percentages of VFA were not significantly different between the two methods neither early in fermentation, nor at 24 hours. The patterns of gas production *in vitro* were in agreement with the DM degradability measured in the nylon bags *in vivo*. The authors concluded that the *in vitro*

system was able to closely simulate the rumen environment and therefore was a valuable alternative to testing *in vivo*.

A few studies have investigated the ability of IVGPT batch system to predict CH_4 production in live cattle. Bhatta et al. (2007) compared the CH_4 values (ml/gDM) obtained by a manual IVGPT batch system to the CH_4 values obtained *in vivo* using sulphur hexafluoride tracer technique (SF_6). The SF_6 technique involves placing a source of SF_6 tracer into the rumen of an animal and collecting the enteric gas into an adjacent canister. The concentration of CH4 and SF_6 can then be determined using GC (McDonald, 2011e). The substrates used in the study were alfalfa hay, corn silage, Italian ryegrass hay, Sudan grass and soybean meal. An average CH_4 production for all diets used in the study was 29.5 and 34.3 ml/gDM measured with SF_6 and batch system respectively. The CH_4 production measured with batch system was significantly higher than that measured with SF_6, however, the authors concluded that it was close enough to provide adequate estimates of CH_4 production in ruminants and was suitable for screening many different feeds and feed additives that could subsequently be tested *in vivo*. A recent study by Danielsson et al. (2017) compared the CH_4 production values (l/day) estimated by an automated *in vitro* batch technique to the values measured in dairy cows in 13 *in vivo* trials. 12 of the *in vivo* trials used respiration chambers and one used the GreenFeed system. The respiration chambers measure the respiratory exchange and CH4 production from animals either by sampling a constant air flow at the entry or the exit of the chamber (open-circuit), or by measuring the amount of O_2 supply and CO_2 absorption and actively taking a sample out for CH_4 determination (closed-circuit) (McDonald et al., 2011e). The GreenFeed is an automated head-chamber system, which consists of an automatic feeder equipped with animal identification, baiting, gas tracer, air handling and measurement systems. The dispensed pelleted feed attracts the cows to the feeder periodically throughout the day, and as the animal's head is inserted all the way into the feeder, the gas emission data is collected (Hristov et al., 2015). For the *in vitro* trial, 49 different diets, similar to those of the *in vivo* studies, were used. The diets differed in forage type and maturity, proportion of concentrate, supplements, and feeding levels. It was found that the correlation between the predicted daily CH_4 production and that measured *in vivo* was high, however, using the *in vitro* system CH_4 production was slightly underestimated (399 l/day *in vitro* and 418 l/day *in vivo*). Nevertheless, the authors concluded that *in vitro* batch technique was useful for initial evaluation of the ability of different feeds and feed additives to affect CH_4 production and fermentation characteristics before testing *in vivo* (Danielsson et al., 2017).

IVGPT have several advantages and limitations compared to techniques *in vivo*. In addition to the slight differences in digestibility and fermentation kinetics between the *in vivo* and *in vitro* experiments, *in vitro* methods lack standardization regarding the diet of the donor animals, the rumen fluid collection time, the handling of inoculum, the medium, the use of blanks, and sample preparation. Considerable differences in results using the same techniques are often found between laboratories and this leads to difficulties in comparing the results of different studies (Williams, 2000; Givens et al., 2000). Furthermore, there is still the necessity of live animals to provide the rumen fluid, which presents a number of animal welfare issues as perceived by the society (McDonald et al., 2011d). Finally, the technique reflects only the production of VFA and measures the proportion of feed that is fermented and does not take into account the proportion of feed that is partitioned to microbial growth and incorporated into microbial biomass (Rymer et al., 2005). On the other hand, *in vitro* methods require a lower number of research animals and less feed, which makes them much cheaper, less labour-intensive and less time-consuming than *in vivo* experiments (Williams, 2000; Adesogan, 2002). With less feed required, it becomes possible to study feeds that are available only in limited amounts. Most importantly, the cumulative gas production measured *in vitro* describes the kinetics of fermentation, reflects the microbial activity, and indicates the amount of substrate available to the microbes in the rumen (Williams, 2000).

3.4. Probiotics

The latest definition of probiotics was accepted by The International Scientific Association for Probiotics and Prebiotics in 2014 as "Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). According to Fuller (1989), to be effective, a probiotic must have no pathogenic or toxic properties, viable cells should be present in large numbers, they should be able to survive and metabolize in the gut, as well as remain stable and viable for adequate periods of time under storage condition. Several possible modes of action of probiotics have been identified. By competing for nutrients and adhesion sites in the digestive tract, producing antibacterial compounds, such as hydrogen peroxide, or lowering the pH in the digestive tract by production of lactic acid, they inhibit the growth of pathogenic bacteria. Probiotics may also neutralize enterotoxins produced by pathogenic bacteria and stimulate the development of an immune system in

young animals by stimulating the production of antibodies and improving the activity of macrophages. They may also increase the production of vitamins, improve nutrient absorption, alter the activity of digestive enzymes, utilize the hydrogen produced during enteric fermentation to produce propionate, thus making less H_2 available for methanogenesis (Fuller, 1989; McDonald et al., 2011f; Alazzeh et al., 2014), as well as increase the activity or the amount of certain microbes (Chen et al., 2016). The probiotics for ruminant animals usually include bacterial and fungal cultures (Rodrigues, 2016), the most common of them being lactobacilli, streptococci, enterococci, bifidobacteria, and propionibacteria and the yeasts *Saccharomyces cerevisiae* (Uyeno et al., 2015; Fuller, 1989) and *Saccharomyces bouldarii* (Poppy et al., 2012).

3.5. Previous results

Several different bacteria, such as acetogens, propionibacteria and lactobacilli, has been investigated for the ability to alter fermentation characteristics in ruminants. Acetogenic bacteria use H_2 and CO_2 to produce acetic acid via the process of reductive acetogenesis (Russell & Wallace, 1997). Lopez et al. (1999) studied the effect of acetogens on CH_4 production, total VFA and pH using a manual *in vitro* batch system with rumen fluid collected from sheep, and a substrate of concentrate and hay. They suggested that acetogens could compete with methanogens for H_2 and, therefore, acetate could be produced at the expense of CH_4. Acetogens had no effects on pH or total VFA. Two out of six acetogenic bacteria strains that were tested, *Eubacterium limosum* and *Ser 5*, showed significant reduction in CH_4 from 374 and 380 µmol/day with autoclaved control to 360 and 369 µmol/day with live culture, respectively. The reduction with both strains, however, was quite low, possibly because acetogens were not able to compete with methanogens effectively due to their lower affinity for H_2. The authors concluded that it was not enough to establish acetogenic population for significant CH_4 reductions in the rumen.

Propionibacteria use either soluble carbohydrates or lactate to produce propionate (Ciani et al., 2008). The process utilizes hydrogen, therefore propionate production acts as a hydrogen sink and provides a pathway of hydrogen utilization in the rumen that is an alternative to methanogenesis. Since hydrogen is the main substrate for methanogens, increased production of propionate leads to decreased production of CH_4 (Jeyanathan et al., 2014). An

accumulation of lactate following the intake of rapidly degradable substrate can severely decrease the ruminal pH. A constant supply of lactate utilizers, such as propionibacteria, presents a potential mechanism to counteract such decreases and reduce the risk of associated diseases (Wiryawan & Brooker, 1995**)**. An *in vivo* study by Vyas et al. (2016) tested the effects of propionibacteria strains *Propionibacterium freudenreichii* T114, *Propionibacterium thoenni* T159 and *P. freudenreichii* T54 on pH, total VFA and CH_4 production in heifers fed a mixed diet of barley silage and barley grain. Total CH_4 production (g/animal/day), measured using respiration chambers for three days, was not affected by any of the strains compared to control, potentially because propionibacteria did not increase the proportion of ruminal propionate. The pH and total VFA were also not affected. Another *in vivo* study by Stein et al. (2006) tested the effect of two doses of *Propionibacterium* strain P169 (low: 6×10^{10} and high: 6×10^{11}) on ruminal pH and VFA levels in Holstein dairy cows fed a total mixed ration (TMR). Although pH was also not affected, the study found a significant decrease (15.4 %) in ruminal acetate to propionate ratio in cows supplemented with the high probiotic dose compared to the control. The increase in propionate proportion could be expected to be accompanied by a reduction in CH_4 production. Significant reductions in CH_4 production were observed by Alazzeh et al. (2012). The study used a manual *in vitro* batch system and a rumen fluid collected from beef cows to test the ability of five species and 16 different strains of propionibacteria to affect CH_4 production with corn and mixed forage diets. Total gas (ml/gDM) and CH_4 (ml/gDM) production were measured at 48 hours. Reductions in CH_4 were observed with six strains using corn and five strains using forage, however, a majority of these reductions were followed by a concurrent decrease in total gas production, which, according to the authors, could indicate that some propionibacteria may produce antimicrobials inhibiting not only methanogens, but also some of the fermentative rumen bacteria. The strains that resulted in decreased CH_4 production without decreasing total gas production were *Propionibacterium propionicus* T83, *P. freudenreichii* T114, *Priopionibacterium jensenii* T121, and *P. thoenii* T159 using the forage diet (8, 9, 4 and 25 % reductions compared to control respectively) as well as *P. freudenreichii* T31 using the corn diet (reduction by 12 % compared to control). These results showed that some strains of propionibacteria were able to reduce CH_4 production *in vitro* with both forage and concentrate diets.

Finally, there has also been some focus on different LAB as probiotics in ruminants. Since LAB ferment feed components to lactate, the most likely effect of a steady supply of LAB to the rumen is stabilization of rumen pH. A slightly lowered, but more stable pH leads to the growth of rumen microorganisms that are adapted to the presence of lactic acid in their environment and more tolerant of lower pH. In addition, LAB stimulate the growth of lactate utilizing bacteria, such as propionibacteria, that prevent large decreases in ruminal pH and reduce production of CH_4 by reducing acetate to propionate ratio (Seo et al., 2010). Santoso et al. (2014) tested the effects of a probiotic containing *L. acidophilus* and *Lactobacillus plantarum* on fermentation characteristics of a grass silage and concentrate substrate, using a manual *in vitro* batch system and rumen fluid collected from cattle. The pH was significantly decreased from 6.87 in control to 6.82 in the treated samples. The decreased pH corresponded well with the total VFA values that were numerically increased from 103.5 mM in control to 112.1 mM with the treatment. Methane production was significantly decreased from 6.8 ml in control to 6.2 ml with the probiotic. The CH_4 values, however, were expressed as ml, therefore it is not clear whether it was correct to compare them directly. DM, OM and NDF degradability were numerically higher with the probiotic treatment compared to control. More pronounced effects on OM degradability were observed in an *in vitro* study by Ellis et al. (2016), who tested the effects of *L. lactis*, *L. plantarum* and a combination of the two strains on OM degradability (g/kgOM) at 72 hours, and the cumulative gas production (ml/gOM), at 6, 12, 24 and 72 hours, with dry grass silage as a substrate. The study used an automated batch system and rumen fluid collected from dairy cows. *L. plantarum* significantly increased OM degradation by approximately 18 g/kgOM compared to control. A combination of *L. lactis* and *L. plantarum* showed a tendency for increased OM degradation (by approximately 12 g/kgOM), while a numerical increase was observed with *L. lactis*. None of the probiotics, however, showed a significant effect on gas production irrespective of time points. The dose response in this study for both degraded OM and total gas production was not significant. These findings were supported by a manual *in vitro* batch study of Baah et al. (2009). Using the rumen fluid collected from steers, the study tested the effects of different doses of a probiotic containing *L. casei* and *L. lactis* (4, 8, 12 and 16 million cfu of LAB/kgDM) on DM degradability (%), total VFA concentration (mmol/gDM) and total gas production (ml/gDM) at 6, 12, 24 and 48 hours of incubation, using a barley silage based and a barley grain based diet. With the barley silage based diet, the probiotic significantly increased total gas production at 12 hours and total VFA at 12 and 24 hours compared to control. With the barley based diet, the addition of probiotic increased the DM degradability

and total VFA at 12 hours, while at the same time the total gas production was decreased. No effects were observed at 48 hours and the dose response was not significant. On the contrary, a study by Chen et al. (2016) observed that LAB effects on total gas and methane production were to some extent dose and substrate dependent. An automated *in vitro* batch system and rumen fluid collected from dairy cows were used to study the effects of four doses of *L. acidophilus* (0, min: 0.25 x 10^7, med: 0.5 x 10^7, max: 0.75 x 10^7) on feed degradability, total gas, CH_4 production and VFA profile with maize stover and rice straw. No effects of *L. acidophilus* on DM and NDF degradability as well as CH_4 production were found. However, numerical increases in CH_4 production (ml/g) were observed with increasing probiotic doses in maize stover. The probiotic was not able to increase the concentration of propionate, which might have resulted in the lack of significant effects on CH_4 production. The min dose of the probiotic significantly increased maximum total gas production (from 76.35 to 80.59 ml/gDM) in maize stover, but not in rice straw. The max dose of the probiotic significantly increased the rate of gas production during early incubation with rice straw, but not maize stover. The influence of substrate on the probiotic effects was also supported by an *in vitro* batch degradability study of Weinberg et al. (2007) that investigated the effects of *L. plantarum*, *Lactobacillus pentosus*, *Enterococcus faecium*, *Lactobacillus buchneri* and a mix of *L. plantarum* and *E. faecium* on DM degradability (g/kg) at 24 hours. They used the rumen fluid from dry Holstein cows and wheat and corn silages as substrate, either on their own, or supplemented with cornstarch at silage to starch ratios of 2:1 (low) and 1:2 (high). When no starch was added, *L. buchneri* increased NDF degradability with corn silage, but not wheat silage, and a mix of *L. plantarum* and *E. faecium* increased NDF degradability with corn silage, while no effect was detected with wheat silage. When low level of starch was added to the diets *L. pentosus*, *L. bucheri* and a mix of *L. plantarum* and *E. faecium* increased NDF degradability with corn silage, but not wheat silage. With high level of starch, *L. plantarum* increased NDF degradability with corn, but not with wheat silage.

Several studies have also been made with a probiotic mix of different LAB and propionibacteria. West & Bernard (2011) tested the effect of two probiotic mixes, containing propionibacteria *P. freudenreichii* and lactobacilli *L. acidophilus* (B1: 2 x 10^9 cfu/day of *P. freudenreichii* NP24 + 1 x 10^9 cfu/day of *L. acidophilus* NP51; and B2: 2 x 10^9 cfu/day of *P. freudenreichii* NP24 + 5 x 10^8 cfu/day *L. acidophilus* NP51 + 5 x 10^8 cfu/day *L. acidophilus* NP45) on DMI and milk yield in dairy cows. The authors suggested that feeding a probiotic mix of lactate producing bacteria in combination with lactate utilizing bacteria would result

in a higher production of propionate, which would possibly lead to higher concentrations of glucose and, therefore, improved animal performance. There were no differences between the two probiotics. The cows that received the probiotic mix showed a tendency for increased milk yield (39.7 and 38.6 kg/day with probiotics B1 and B2 respectively compared to 37.8 kg/day with control), significantly greater energy corrected milk (ECM) yield (37.5 and 37.6 kg/day with B1 and B2 respectively, compared to 35.6 kg/day with control) and significantly higher efficiency of milk production (1.43 and 1.42 ECM/DMI with B1 and B2 respectively compared to 1.39 with control). The DMI (kg/day) was not affected. The authors suggested that the improved milk yield without an increase in DMI could be an indicator of improved feed digestibility or a better yield of microbial protein, both of which are indicators of improved rumen function. The same probiotic strains were used in an *in vivo* study by Ferraretto & Shaver (2015), where mid-lactation dairy cows were fed a total mixed ration supplemented with a probiotic containing *P. freudenreichii* NP24 and *L. acidophilus* NP51. In this study, however, the milk yield (kg/d) and milk components were not affected. There was a tendency for decreased DMI (kg/d) for cows supplemented with the probiotic, however, feed conversion was not affected. The authors concluded that the probiotic had no effect on lactation performance. Another study by Philippeau et al. (2017) investigated the effects of a probiotic mixture of *Propionibacterium* P63 and *L. rhamnosus* 32 (P+Lr) as well as *Propionibacterium* P63 and *L. plantarum* 115 (P+Lp) on milk yield, ruminal pH, OM and fibre digestibility, total VFA and CH_4 production in dairy cows. CH_4 emissions were determined using the SF6 technique. The study divided dairy cows into two groups fed a high-starch diet (38% of total DM) and a low starch diet (2% of total DM). The results on milk yield (kg/day) were in agreement with the aforementioned study by Ferraretto & Shaver (2015) as no effects of the probiotic were observed. Ruminal pH was significantly higher for the treated cows irrespective of the diet, however no significant effect was found on total VFA concentration (mM), OM and fibre digestibility with neither of the diets. There was no significant effect of the probiotic on CH_4 production (gCH_4/kg milk) with the high-starch diet, however, a tendency of decreased CH_4 emission (by 26%) was found with the low-starch diet, reducing the level of CH_4 to that produced by cows fed the high-starch diet. These results suggest that a probiotic, containing a mix of lactate producing and lactate utilizing bacteria, may have a larger potential to decrease CH_4 production when used with a forage based diet compared to concentrates.

Other researchers used LAB and yeast in a probiotic mixture. The direct mechanism connecting *S. cerevisiae* to improved animal performance and CH_4 reduction is yet to be clarified. However, it has been suggested that *S. cerevisiae* can improve fibre digestion, passage rate, and DMI. A possible mode of action involves the respiratory activity of the yeast, which causes a quick removal of oxygen from the rumen, and thereby benefits the fermentative process performed by the anaerobic microorganisms (Nasiri et al., 2019). The rapid removal of oxygen improves growth conditions for the anaerobic cellulolytic bacteria and stimulates their attachment to forage particles (Roger et al., 1990). It also affects the partial pressure of oxygen in the rumen, which plays a substantial role in the regulation of lactate and propionate production. If the partial pressure of oxygen is increased, the formation of propionate is limited, and more lactate accumulates. On the contrary, if the partial pressure of oxygen is decreased, propionate is produced at the expense of lactate (Jouany & Morgavi, 2007), possibly leading to a reduction in CH_4. The previously mentioned study by Santoso et al. (2014) investigated the effects of the yeast *S. cerevisiae* and *L. plantarum* (S+Lp) as well as *S. cerevisiae* and *L. acidophilus* (S+La) on feed degradability, concentration of propionate, CH_4 production, and pH *in vitro*. S+Lp was successful at significantly increasing NDF degradability (56.2 % compared to 51.1 % in control). Both S+Lp and S+La increased the proportion of propionate significantly (20.4 and 19.7, respectively, compared to 15.5 mol/100 mol in control), however, this was followed by only numerical decreases in acetate to propionate ratios as well as CH_4 production. The pH was not affected by the S+Lp treatment, however it was decreased significantly by S+La from 6.87 in control to 6.83 with treatment. An indication of possible probiotic dose response in relation to pH was observed in an *in vivo* study by Nocek et al. (2002), who investigated the effects of a probiotic containing *E. faecium*, *L. plantarum* and *S. cerevisiae* at three different doses (min: 1×10^5 cfu/ml of rumen fluid, med: 1×10^6 cfu/ml, max: 1×10^7 cfu/ml) on rumen pH in dairy cows fed a TMR consisting of 70 % grain. It was found that, compared to control, the min dose of the probiotic numerically increased the mean daily rumen pH and the mean daily lowest pH. The mean daily hours the pH was below 6 were numerically decreased. On the contrary, the med and max doses numerically decreased the mean daily pH and the mean daily lowest pH, while the mean daily hours the pH was below 6 were increased. The authors suggested that LAB supplemented in small doses might produce just enough lactic acid to stimulate lactate utilizers, that can increase the rumen pH. However, it was concluded that there was a threshold for LAB dosage, and with doses that are too high, lactic acid production may exceed the utilization, leading to a decreased rumen pH.

It is clear that the effects of bacterial probiotics in ruminants vary between studies. Several factors may be responsible for this, including the way the probiotic mix is prepared, the age, stage of lactation, parity, health and feeding management of the individual animals used in *in vivo* studies or acting as donors for studies *in vitro* (Xu et al., 2017). As some of the studies showed, the effects of probiotics are also strain (Lopez et al., 1999; Alazzeh et al., 2012) combination of strains (Santoso et al., 2014), dose (Chen et al., 2017), and substrate dependent (Nocek et al., 2002; Philippeau et al., 2017). It is, therefore, of interest to study the effects of new probiotic strains or strain combinations using different doses and substrates *in vitro*, and Animal Biosa presents a combination of bacteria strains that, to our knowledge, has not been tested before.

4. Method and materials

4.1. Collection of feeds and formulation of rations

Feed samples were collected once from selected farms on the 16th and 18th October 2018 in two different areas of Bolivia. The dates correspond to the dry season in Bolivia, therefore the feeds collected represented the diet of the animals in the dry season. The cows in Challapata were grazing for a few hours per day during the dry season, however, it was not possible to determine the amount consumed while grazing. The cows were fed some wet forage, such as fresh alfalfa and corn silage, however it was not possible to dry the wet forage samples properly before transporting them back to Denmark, therefore only dry samples were collected. A 20 kg daily DMI per cow was estimated based on the average DMI used by the Norfor feed evaluation system to develop an equation predicting the intake capacity of large dairy cow breeds (Volden et al., 2011). The average animal weight and daily DMI used by the system was 577 kg and 19.7 kg respectively. The average reported weight of Bolivian dairy cattle was 500 kg however they were fed very fibrous and less nutritious rations compared to the diets of Scandinavian cattle that the Norfor system is mostly used for, therefore, a slightly higher DMI of 20 kg was selected. Using the feeds collected, four different rations were formulated (Table 2).

Table 2. The proportions of feeds (%) on a DM basis in the four rations (A, B, C, D) used for the experiment.

Ration A	Ration B	Ration C	Ration D
Barley straw (44.67 %)	Oat forage (21.19 %)	Maize + barley concentrate (1.85 %)	Corn stover (43.83 %)
Alfalfa 1 (55.35 %)	Barley forage (28.88 %)	Alfalfa 2 (86.71 %)	Concentrate 2 (7.28 %)
	Alfalfa 3 (29.63 %)	Native grass (9.56 %)	Concentrate 3 (7.43 %)
	Native grass (12.30 %)	Concentrate 1 (1.87 %)	Alfalfa 3 (41.46 %)

4.2. Preparation and analyses of individual feed samples

All the feeds were ground with a centrifugal hammer mill through a 2 mm grid. DM and OM of each feed sample were determined. To determine the DM of the feeds, triplicates of each feed sample were oven dried at 101 °C for 2 hours. To determine the OM, the samples were ashed at 530 °C overnight.

Another set of triplicate feed samples was used to determine sequential fibre analyses (NDF, ADF, ADL and AIA). The ANKOM Fibre Analyzer A200 was used for NDF and ADF analyses, and ANKOM Daisy incubator was used for ADL determination. NDF, ADF and ADL analyses were conducted following the protocols by ANKOM (2017a; 2017b; 2017c) except for a few modifications. Sodium sulphite was not added to the nitrogen detergent solution and the ANKOM filter bags were not washed in acetone. Following the ADL analysis, AIA was determined by ashing the samples at 530 °C overnight. The amount of hemicellulose in the rations was calculated by subtracting the amount of ADF (g) from the amount of NDF (g), and cellulose was calculated as the amount of ADL (g) subtracted from the amount of ADF (g). The obvious outliers were excluded from the calculations, and in those cases the averages were calculated using duplicates.

4.3. Experimental design

Biosa Denmark Aps recommended using 1-1.5 ml of Animal Biosa per 5 kg of animal's body weight. The Bolivian dairy cows weigh approximately 500 kg each, therefore the recommended daily dose per animal would be 100-150 ml of Animal Biosa. To investigate the dose response of Animal Biosa, four different doses were selected, including a 0 dose with no probiotic (further referred to as water), minimum dose of 50 ml, a medium dose of 100 ml and a maximum dose of 150 ml per cow per day. Given that the Bolivian cows were expected to consume 20 kg of DM per day, and the amount of ration added to the fermentation bottles was 0.5 g, the Animal Biosa doses were adapted for the fermentation trial by calculations 1, 2, and 3, and they represent the minimum (MIN), medium (MED) and maximum (MAX) doses of Animal Biosa, respectively.

(1) 0.5 g of ration x 50 ml / 20000 g = 0.00125 ml Animal Biosa
(2) 0.5 g of ration x 100 ml / 20000 g = 0.00250 ml Animal Biosa
(3) 0.5 g of ration x 150 ml / 20000 g = 0.00375 ml Animal Biosa

50 bottles in total were used in the first experiment (Exp1) and 54 bottles in the second experiment (Exp2). In both experiments, 11 bottles per ration were used. The bottles included triplicates of water treatment, MIN and MED doses of Animal Biosa as well as duplicates of the MAX dose of Animal Biosa. Regular blanks (buffered rumen fluid, no additives, no feed) and specific blanks (buffered rumen fluid, Animal Biosa, no feed) were used in both experiments. In Exp1, six blanks were used, including three regular blanks, and three specific blanks with MED dose of Animal Biosa. Exp2 included additional two specific blanks with MIN dose of Animal Biosa and two specific blanks with MAX dose of Animal Biosa. The allocation of samples to the incubation bottles was randomized using the randomization function in Microsoft Excel (2010).

In Exp 1, one of the D MED samples (bottle number 38), was considered an outlier in CH_4 production and was not used for further calculations. In Exp2, one of the A MAX samples (bottle number 43) could not be filtered post incubation, therefore, the degradation values and measures based on OM were not available. One of the C MED samples (bottle number 11) was an outlier in total gas production, potentially due to a leak from the gas bag, therefore,

the gas data was excluded from calculations. One of the D MED samples (bottle number 46) was deleted from the experiment due to failed gas measurement. Finally, one of the B MED samples (bottle number 47) and one of the D MIN samples (bottle number 30) were both outliers in CH_4 production and, therefore, were excluded from further calculations. Bottle numbers are provided in Appendix, where outlier values are identified as n/a.

4.4. Preparation of inoculum

The rumen fluid for both experiments was collected from two fistulated research cows owned by the University of Copenhagen and kept at the University Animal Hospital in Taastrup. The research cows were fed a hay diet for maintenance only. The donor diet contained 92.7% DM, 67.9% OM, 591 g/kg DM NDF, 292 g/kg DM crude fibre, and 79 g/kg ash. Rumen fluid was collected before the morning feeding. The water supply was turned off before the rumen fluid collection to prevent dilution. Two litres of rumen fluid from each cow were collected into pre-heated thermo-flasks and transported to the fermentation laboratory at the University of Copenhagen, the Department of Veterinary and Animal Science. The transportation took around one hour. The rumen fluid was filtered through two layers of cheesecloth. The buffer media was prepared according to the four part ingredients (buffer, macro, micro and redox) described by Menke & Steingass (1988) and flushed with CO_2 for three hours before the rumen fluid was added. A reduction agent made from sodium sulphide, sodium hydroxide, and deionised water was added 10 min before the arrival of the rumen fluid. Once the buffered media turned colourless, the filtered rumen fluid was added to the preheated (39 °C) conical flasks with the buffered media and was continuously flushed with CO_2. The inoculum media was dosed into preheated (39 °C) incubation bottles. The pH of the inoculum media was measured before and after the bottling process (Table 3).

Table 3. The pH of the inoculum media before and after the bottling process in experiments 1 and 2 (Exp1 and Exp2).

	pH before bottling	pH after bottling
Exp1	7.06	6.67
Exp2	7.07	6.96

4.5. Incubation conditions

The fermentation trial was conducted using an automated vented *in vitro* gas production batch technique (ANKOM Wireless Gas Production System) according to the protocol by ANKOM (2018). The volume of the bottles used was 132 ml and the headspace volume was 42 ml. 90 ml of inoculant media was added to each bottle containing 0.5 g of ration. Animal Biosa doses were diluted in three volumetric flasks of 50 ml with distilled water for the ease of measurement. The proportions of Animal Biosa doses and distilled water in the dilutions are shown in Table 4. Each incubation bottle received 0.05 ml of Animal Biosa and distilled water solution, which corresponded to a predetermined dose of Animal Biosa (0.00125 ml of Animal Biosa for MIN, 0.0025 ml of Animal Biosa for MED and 0.00375 ml of Animal Biosa for MAX doses). The bottles with no Animal Biosa, received 0.05 ml of distilled water.

Table 4. The proportions of Animal Biosa doses (MIN, MED, MAX) and distilled water in the dilutions used in experiments 1 and 2.

Dose	Animal Biosa	Distilled water
MIN	1.25 ml	48.75 ml
MED	2.5 ml	47.5 ml
MAX	3.75 ml	46.25 ml

Individual gas bags were attached to the fermentation bottles. The system was set to release the gas at 0.75 PSI and the valve opening time was set at 250 ms. Recording interval was set to 10 min. Two fermentation experiments were conducted on the 4-6th and the 19-21st of February 2019. At 48 hours, all the fermentation graphs had reached their asymptote and the incubation was stopped. The fermentation process in the bottles was ended by putting the bottles on ice. The gas bags were detached, and the pH was measured in each bottle. The undegraded feed particles were filtered into preweighed F57 ANKOM filter bags. The filtered residues were first air-dried overnight and then dried for 2 hours in the oven at 101 °C. Afterwards, the NDF and ADF analyses were conducted. A GC (Agilent 7820A GC, Agilent Technologies, Santa Clara, CA, USA) was used to determine the CH_4 percentage in the collected gas. The GC was equipped with a HPPLOT Q column (30 m × 0.53 mm × 40 µmm), with H_2 as the carrier. Column flow was 5 ml/min and the TCD detector was set to

250 ºC with a reference and make up flow of 10 ml/min. A 250 µl gas sample was taken from each gas bag and manually injected into the GC machine. Run time was 3 min at an isothermal oven temperature of 50 ºC. Calibration curves, that were calculated from standards containing 1 %, 2.5 %, 5 %, 10 %, 15 % and 25 % CH_4 in nitrogen, were used to determine the CH_4 concentration in the gas produced (Mikrolab A/S, Aarhus, Denmark).

4.6. Calculations and statistical analysis

The cumulative gas pressure recorded during the fermentation run was corrected for gas produced in the blank bottles. In both experiments, bottles with the water treatment were corrected for the regular blanks. In Exp1, all the bottles with Animal Biosa were corrected for specific blanks with MED dose of the probiotic. In Exp2, the bottles with Animal Biosa were corrected for the specific blanks with respective doses of the probiotic (MIN, MED and MAX). The cumulative pressure was converted to moles of gas produced using the Ideal gas law: $n = \frac{PV}{RT}$, where n is the amount of moles produced, P is the cumulative pressure (PSI), V is the headspace volume (ml), R is the ideal gas constant, and T is the incubation temperature of 312.15 K. Using Avogadro's law, the moles were then converted to the volume of gas produced (ml) at standard temperature and pressure (STP): $V = \frac{nRT}{P}$, where V is the volume of gas produced (ml), n is the amount of moles produced, R is the ideal gas constant, T is a standard temperature of 273.15 K and P is the standard pressure of 1 bar (ANKOM, 2018).

In the fibre analysis, the % increase or decrease in blank bag weight, compared to their original weight, was calculated. In the fibre analysis of individual feed samples, a calculated % increase or decrease was considered a correction factor and was used to correct all samples post neutral detergent solution (NDS) and acid detergent solution (ADS). In the fibre analysis post incubation, the change in blank bag weight was compared between the regular and specific blanks using a t-test in Microsoft Excel (2010). Since no significant differences were found, overall averages of all blanks were calculated and used as a correction factor for samples post incubation, post NDS and post ADS.

The statistical analysis of data was conducted using R (Pinheiro et al., 2016) with significance value set at 0.05. One-way ANOVA tests were used to test for differences in OM, hemicellulose, and cellulose degraded, volume of gas produced and CH_4/gOM between

the two experiments. Two-way ANOVA tests were used to compare the different variables between the rations and the treatments. In order to determine the variation within the different variables, standard error of the mean (SEM) was calculated as follows: $\frac{\sigma}{\sqrt{n}}$, where σ is the standard deviation and n is the sample size.

5. Results

5.1. Chemical composition of the rations

The chemical composition of individual feed samples collected in Bolivia is shown in Table 5. Alfalfa was collected from three different farms, therefore three different alfalfas are referred to as Alfalfa 1, 2 and 3. The composition of three concentrates was unknown, therefore they were named Concentrate 1, 2 and 3. Table 6 presents the chemical composition of the formulated feed rations, calculated by weighted averages of constituent raw feeds.

Table 5. Chemical composition of feeds collected in Bolivia in October 2018.

Feed	% DM	% OM	%NDF /gOM	%ADF /gOM	%ADL /gOM	%Hemi-cellulose /gOM	% Cellulose /gOM	% Ash	% AIA
Alfalfa 1	93.31	90.42	48.53	28.83	5.85	19.70	22.98	9.53	0.52
Alfalfa 2	93.81	91.71	49.13	28.92	6.98	20.21	21.93	8.22	0.86
Alfalfa 3	93.80	89.88	50.76	31.86	11.28	18.91	20.57	9.95	1.40
Oat forage	94.16	95.18	60.17	27.35	2.11	32.82	32.82	4.80	0.45
Barley forage	93.16	92.61	46.77	22.86	1.75	23.91	21.11	7.35	0.55
Barley straw	93.15	92.65	58.60	28.54	2.12	30.06	26.42	7.31	0.55
Native grass	95.17	95.15	74.16	45.43	5.36	28.73	40.07	4.85	0.36
Corn stover	94.76	94.77	84.28	59.14	6.92	25.14	52.22	5.21	0.37
Maize and barley concentrate	92.27	93.95	39.96	12.81	3.54	26.80	9.23	6.04	0.22
Concentrate 1	93.32	94.25	8.87	4.10	0.55	4.77	3.55	5.75	0.17
Concentrate 2	90.65	92.21	18.48	6.66	1.31	11.82	5.35	7.77	0.31
Concentrate 3	92.47	96.26	31.22	7.01	0.80	24.21	6.21	3.72	0.21

Table 6. Chemical composition of the rations (calculated using weighted averages of individual feeds).

Ration	% DM	% OM	%NDF/ gOM	%ADF /gOM	%ADL /gOM	%Hemicellulose /gOM	%Cellulose /gOM	% Ash	% AIA
A	93.24	91.42	53.13	28.70	4.15	24.42	24.55	8.53	0.53
B	93.89	92.86	55.37	29.65	5.06	25.72	24.59	7.07	0.75
C	93.90	92.11	50.57	29.71	6.64	20.86	23.07	7.82	0.79
D	93.89	92.67	61.84	40.26	7.77	21.58	32.49	7.24	0.78

5.2. pH

A one-way ANOVA analysis showed that the pH was significantly different between Exp1 and Exp2. Therefore, the data from the two experiments were treated individually. The pH measured in Exp1 and Exp2 is shown in Tables 7 and 8, respectively. In both experiments, no significant differences in pH were found between the Animal Biosa doses (two-way ANOVA), therefore all the probiotic treatments were pooled together and compared to water. In Exp1 there were no interactions between the rations and the treatments (two-way ANOVA), and no significant differences between pooled Animal Biosa treatments and water (two-way ANOVA). In Exp2, there was a significant interaction between ration B and Animal Biosa treatments (two-way ANOVA), and no significant difference between pooled Animal Biosa treatments and water (two-way ANOVA).

Table 7. Average pH and SEM (standard error of the mean) values for MIN, MED and MAX doses of Animal Biosa doses pooled together and compared to water in rations A, B, C and D (Experiment 1).

	Animal Biosa	**water**
A	6.84	6.83
SEM	0.008	0.012
B	6.81	6.81
SEM	0.012	0.021
C	6.88	6.86
SEM	0.007	0.006
D	6.84	6.83
SEM	0.010	0.009

Table 8. Average pH and SEM (standard error of the mean) values for MIN, MED and MAX doses of Animal Biosa pooled together and compared to water in rations A, B, C and D (Experiment 2).

	Animal Biosa	**water**
A	6.89	6.88
SEM	0.007	0.01
B	6.88	6.91
SEM	0.002	0.025
C	6.93	6.94
SEM	0.005	0.009
D	6.91	6.92
SEM	0.007	0.012

5.3. Organic matter degradation

In both experiments, there were no significant interactions found between the different rations and treatments in OM degradation (two-way ANOVA), indicating that Animal Biosa additives affected all the rations in the same way. There was no significant difference in OM degradation between Exp1 and Exp2 (one-way ANOVA), therefore the data from both experiments were pooled together. The average degraded OM in rations A, B, C and D was

72.6, 73.4, 67.7 and 63.4 %, respectively. No significant difference was found between the rations A and B, however, all the other rations were significantly different from each other (two-way ANOVA) (Figure 2). Within the rations, there were no significant differences in degraded OM between any of the treatments (water, MIN, MED or MAX) (two-way ANOVA).

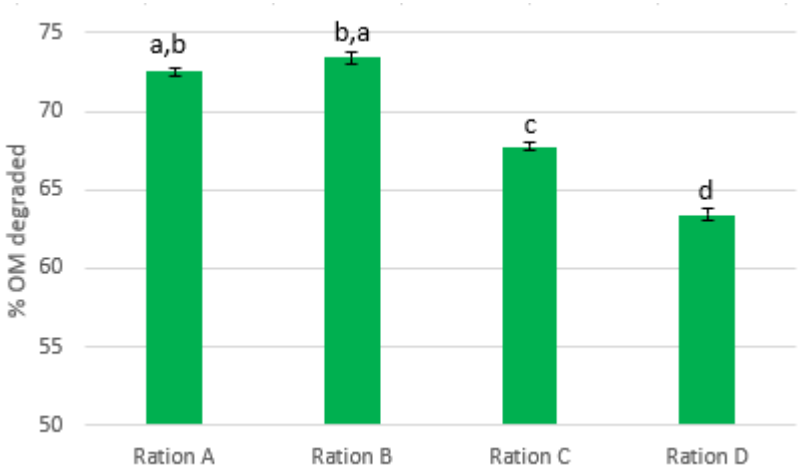

Figure 2. Average % OM degraded in rations A, B, C and D and standard error of the means. The means of degraded OM with different labels (a-d) differ significantly ($p<0.05$).

5.4. Cellulose and hemicellulose degradation

In both experiments, no significant interactions were found between the different rations and treatments in hemicellulose and cellulose degradation (two-way ANOVA), indicating that Animal Biosa additives affected all the rations in the same way. There were no significant differences in % cellulose and hemicellulose degraded between the two experiments (one-way ANOVA), therefore the data from both experiments were pooled together. The average hemicellulose degraded in rations A, B, C and D was 67.6, 66.3, 63.6 and 52.5%, respectively (Figure 3). There was no significant difference in % hemicellulose degraded between the rations A and B, but all the other rations were significantly different from each other (two-way ANOVA). The average cellulose degraded in rations A, B, C and D was 64.1, 72.5, 57.3 and 58.5 %, respectively (Figure 3). There was no significant difference in % cellulose degraded between the rations C and D, but significant differences were found between all the other rations (two-way ANOVA). Within the rations, there were no significant differences in

degraded hemicellulose and cellulose between any of the treatments (water, MIN, MED or MAX) (two-way ANOVA).

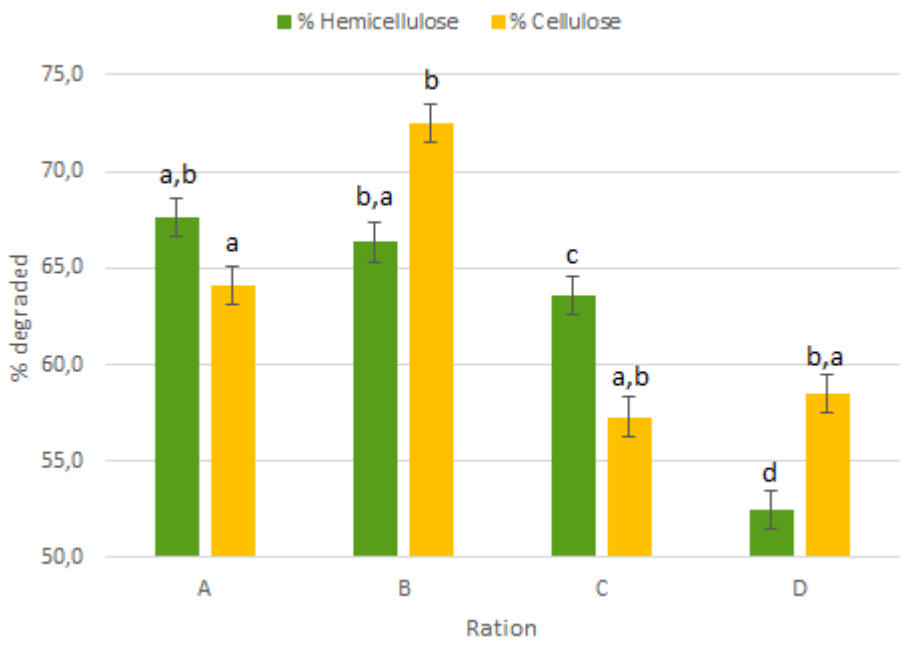

Figure 3. The average hemicellulose (green) and cellulose (yellow) degraded (%) in rations A, B, C and D and standard error of the means. The means of hemicellulose and cellulose degraded with different labels (a-d) differ significantly ($p<0.05$).

5.5. Total gas production

The kinetics of the average total gas produced in different rations during the entire fermentation process in Exp1 and Exp2 are illustrated in Figure 4 and Figure 5 respectively. Compared to ration A and B, ration C had a slightly higher gas production rate from approximately 5 hours (ration A and B produced 34.7 and 36.4 ml gas/gOM respectively and ration C produced 40.0 ml gas/gOM) to 17 hours (ration A and B produced 115.7 and 112.1 ml gas/gOM respectively and ration C produced 117.3 ml gas/gOM) of incubation in Exp1, and 8 hours (ration A and B produced 46.5 and 49.5 ml gas/gOM respectively and ration C produced 53.0 ml gas/gOM) to 16 hours (ration A and B produced 102.8 and 102.0 ml gas/gOM respectively and ration C produced 104.8 ml gas/gOM) of incubation in Exp2. At 21 to 48 hours of incubation, however, the total gas production was lower for ration C compared to ration A and B in both Exp1 (ration A and B produced 131.6 to 161.8 and 128.5

to 167.9 ml gas/gOM respectively and ration C produced 127.3 to 144.3 ml gas/gOM) and Exp2 (ration A and B produced 131.5 to 172.8 and 128.9 to 179.0 ml gas/gOM respectively and ration C produced 125.0 to 153.8 ml gas/gOM). Ration D had a lower gas production rate throughout the incubation, although in Exp1 the difference was to a smaller degree than in Exp2.

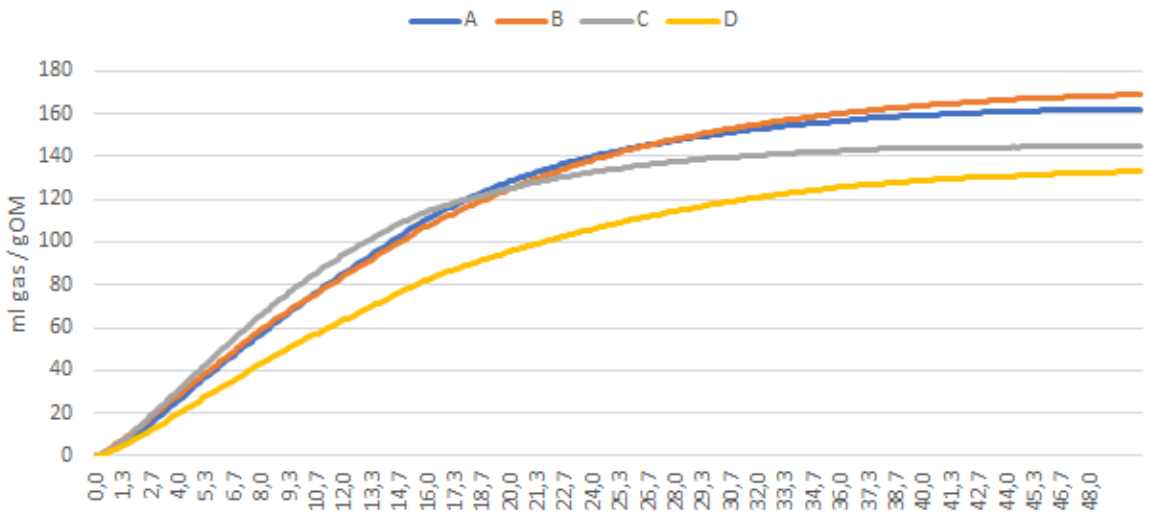

Figure 4. The average total gas produced throughout the incubation period (ml of total gas produced/gOM at standard temperature and pressure) in rations A, B, C and D (Experiment 1).

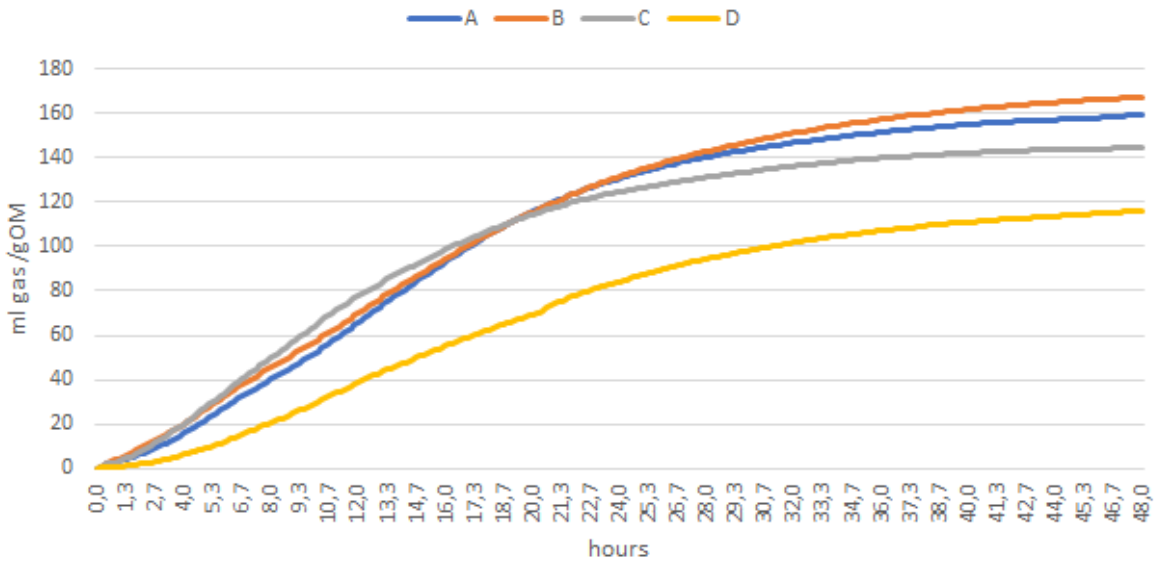

Figure 5. The average total gas produced throughout the incubation period (ml of total gas produced/gOM at standard temperature and pressure) in rations A, B, C and D (Experiment 2).

Seven time points were selected for comparison of total gas production between Exp1 and Exp2 (3, 6, 9, 12, 18, 24, and 48 hours). In both experiments, there were no significant interactions between the different rations and treatments in total gas production (two-way ANOVA), indicating that Animal Biosa had affected the different rations in the same way. There were no significant differences in the volume of gas produced at 12 and 24 hours between the experiments, but there were significant differences at 3, 6, 9, 12 and 18 hours (one-way ANOVA). Due to the differences in gas production early in the fermentation, the data for the two experiments were treated separately. No significant differences in total gas production were found between the Animal Biosa doses (two-way ANOVA), therefore all the probiotic treatments were pooled together and compared to water. In Exp1, at 3, 6, 9, 12 and 18 hours, the samples with water produced 21.3, 44.8, 66.3, 84.8 and 113.0 ml gas/gOM, respectively, and the samples with Animal Biosa produced 18.6, 41.7, 63.4, 82.2 and 111.7 ml gas/gOM, respectively. In Exp2, at the same time points, the samples with water produced 12.6, 31.1, 49.4, 68.0, 101.2 ml gas/gOM, respectively, while the samples with Animal Biosa produced 10.1, 26.3, 42.8, 60.2 and 92.0 ml gas/gOM, respectively. In both experiments, no significant differences were detected between Animal Biosa and water. Figures 6 and 7 show the volume of gas produced at STP per gOM at different time points in Exp1 and Exp2 respectively.

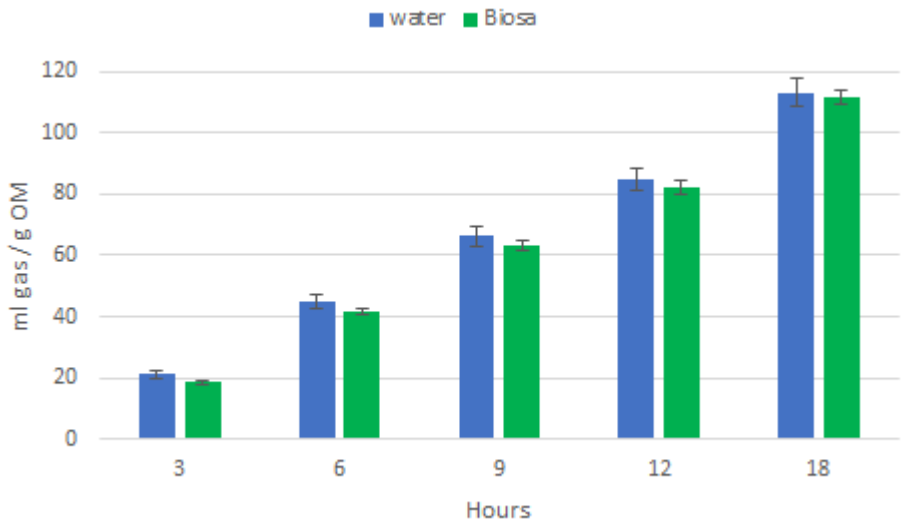

Figure 6. The average of total gas produced (ml total gas produced/gOM) and standard error of the means at 3, 6, 9, 12 and 18 hours in MIN, MED and MAX doses of Animal Biosa combined (green), compared to water (blue) in Experiment 1.

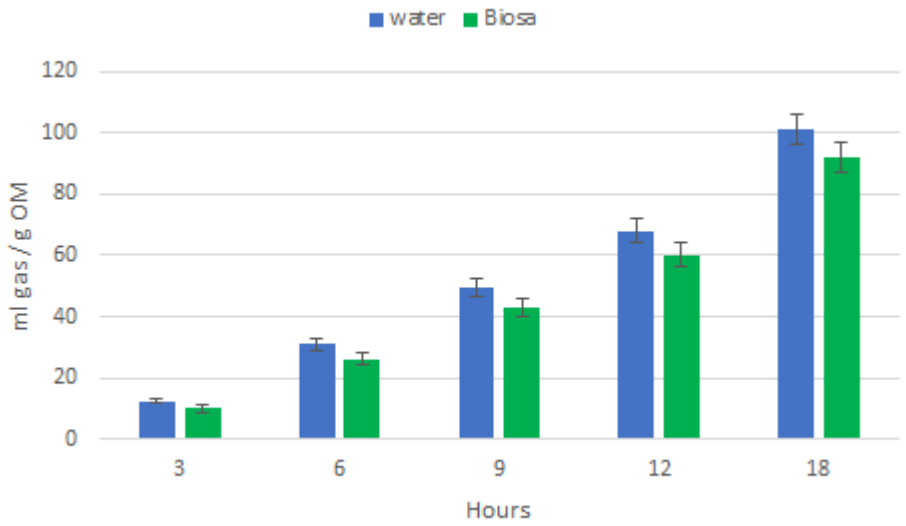

Figure 7. The average total gas produced (ml total gas produced/gOM) and standard error of the means at 3, 6, 9, 12 and 18 hours in MIN, MED and MAX doses of Animal Biosa combined (green), compared to water (blue) in Experiment 2.

At 24 and 48 hours, there was no significant difference in the volume of total gas produced between Exp1 and Exp2, therefore the data from the two experiments were pooled together. The volume of total gas produced at 24 and 48 hours in samples with Animal Biosa was compared to the samples with water (Figure 8). At 24 hours, there was 127.2 and 122.4 ml gas/gOM produced in the samples with water and the samples with Animal Biosa, respectively, while at 48 hours, there was 153.2 and 147.9 ml gas/gOM produced, respectively. The differences, however, were not significant.

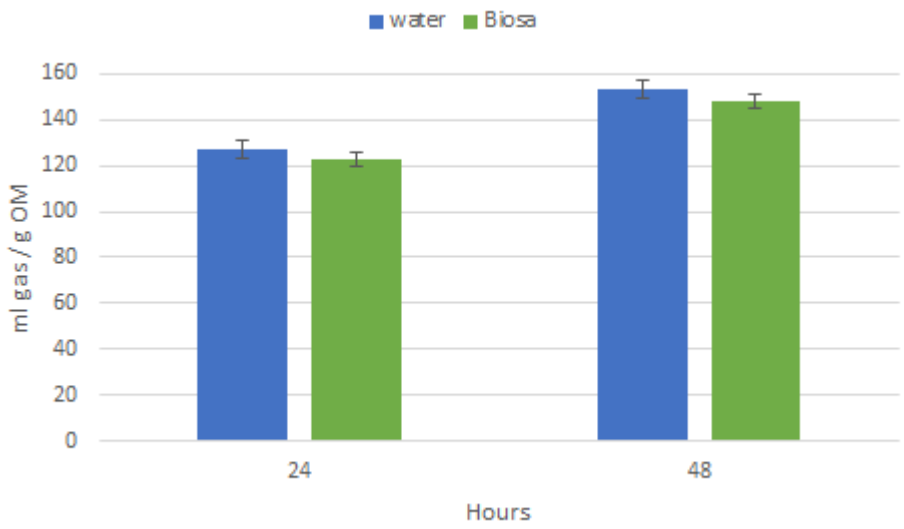

Figure 8. The average total gas produced (ml total gas produced/gOM) and standard error of the means at 24 and 48 hours in MIN, MED and MAX doses of Animal Biosa combined (green), compared to water (blue).

5.6. The relationship between total gas production and organic matter degradation

The relationship between degraded OM and volume of gas produced was tested in order to determine if the gas production was a good predictor of OM degradation (Figure 9). The total gas produced at 48 hours was plotted against % OM degraded in all four rations and both experiments, and all the variables were fitted with linear equations. With these fittings the total gas produced was a poor predictor of degraded OM. The two variables had no correlation in rations A and D (R^2=0.0019 and R^2=0.0038), while correlation was negative in rations B and C (R^2=0.0186 and R^2=0.2218 respectively). However, when the linear equation

was fitted to all the rations combined, the correlation between the volume of total gas produced and the percentage of OM degraded increased (R^2=0.6713).

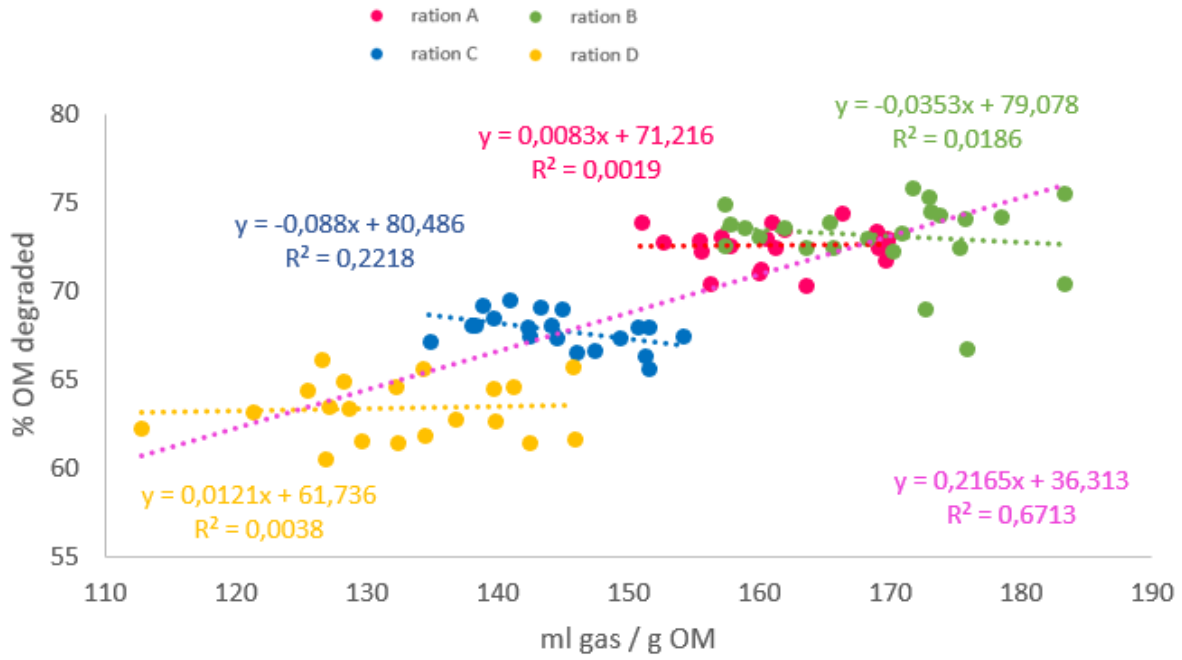

Figure 9. Total gas production as a predictor of % OM degraded in rations A, B, C, D individually (pink, green, blue and yellow lines respectively) and in all the rations combined (purple line).

5.7. Methane production

In both experiments, there were no significant interactions between the different rations and treatments in CH_4 production (two-way ANOVA), indicating that Animal Biosa affected all the rations in the same way. There was a significant difference in CH_4 production (ml CH_4/gOM degraded) between Exp1 and Exp2 (one-way ANOVA), therefore the data for both experiments were treated separately. Figures 10 and 11 show CH_4 production in Exp1 and Exp2, respectively. In Exp1, with ration A, the CH_4 production with water, MIN, MED and MAX doses of Animal Biosa was 15.77, 22.69, 23.86 and 24.13 ml CH_4/gOM degraded, respectively. With ration B, 20.01, 89.90, 21.87 and 22.87 ml CH_4/gOM degraded was produced with water, MIN, MED and MAX doses, respectively. With ration C it was 17.24, 17.79, 19.92 and 19.80 ml CH_4/gOM degraded, while with ration D it was 16.73, 17.67, 18.56 and 21.00 ml CH_4/gOM degraded with the respective treatments. In Exp2, CH_4

production was 19.02, 19.17, 16.77 and 13.71 ml CH_4/gOM degraded with water, MIN, MED and MAX Animal Biosa respectively in ration A and in ration B it was 22.56, 16.48, 20.65 and 18.44 ml CH_4/gOM respectively. In ration C, CH_4 production was 15.46, 14.90, 12.43 and 12.22 ml CH_4/gOM degraded for water, MIN, MED and MAX doses, respectively, while in ration D it was 15.76, 17.33, 14.30 and 15.78 ml CH_4/gOM degraded, respectively. There were no significant differences in CH_4 production between the MIN, MED, MAX and water neither in Exp1 nor Exp2 (two-way ANOVA).

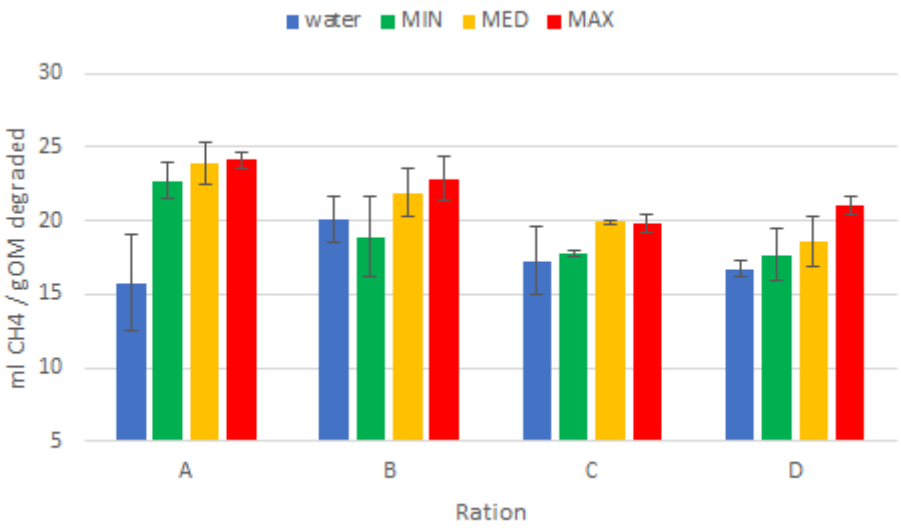

Figure 10. Average methane production (ml CH_4/gOM degraded) in rations A, B, C, D and standard error of the means with water (blue) and MIN (green), MED (yellow) and MAX (red) doses of Animal Biosa in Experiment 1.

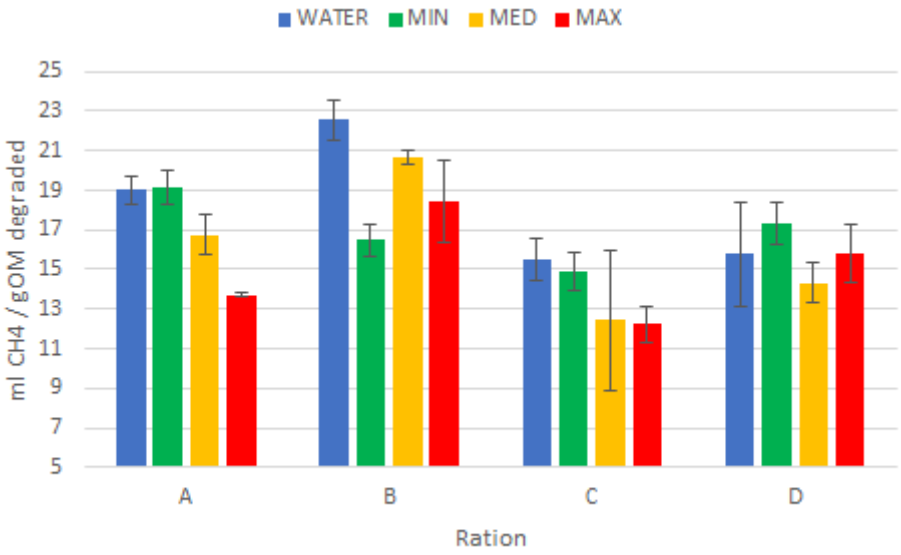

Figure 11. Average methane production (ml CH_4/gOM degraded) in rations A, B, C and D and standard error of the means with water (blue) and MIN (green), MED (yellow) and MAX (red) doses of Animal Biosa in Experiment 2.

6. Discussion

6.1. Discussion of results

6.1.1. Feed analysis

The AIA content in the rations was very low (0.53 - 0.79%), however, for the accuracy of the results, it was still accounted for in the calculation of the OM. For most accurate results, NDF, ADF and ADL fractions should also be accounted for AIA, because it is not part of the plant. In this study, it was only accounted for in the OM calculation. The fibre fraction values would have only been affected to a minimum extent, therefore it was not considered a source of error.

Ration D had the highest fibre content of all the rations, even though 14.71% was formed by concentrates. This was due to a very high fibre content of corn stover, which accounted for 43.83% of the ration. Since corn stover had a markedly higher level of cellulose compared to other feeds, ration D had a cellulose level on average 1.35 times higher than the rest of the

rations. In addition, ration D also had the highest lignin content, due to high lignin in corn stover and alfalfa 3, that together constituted the major part of the ration (85.29%). Although ration C had the lowest fibre content between the rations, it's lignin content was only slightly lower than that of ration D. Considering the high level of fibre, especially cellulose, in ration D as well as the highest amounts of lignin in rations C and D, they were expected to have the lowest OM and fibre degradability and the lowest total gas. Rations A and B, on the other hand, had the lowest lignin content, and, therefore, were expected to have higher degradability and total gas production values.

6.1.2. pH

pH was measured after incubation, and a significant difference between Exp1 and Exp2 was found. Since no significant interaction was found between the two experiments, the pH reacted the same way in both Exp1 and Exp2, however, it was generally higher in Exp2 compared to Exp1. The pH of the incubation media measured before incubation, right after the bottling procedure, was higher in Exp2 compared to Exp1 (6.96 and 6.67 respectively). The difference may have prevailed throughout the fermentation, and the same was observed at the post incubation pH measurement. In the current study, the end pH in both experiments ranged between 6.81 and 6.94, indicating that fermentation was not limited by unfavourable pH.

Since the pH of the incubation media before the bottling procedure was almost identical in both experiments (7.06 and 7.07 for Exp1 and Exp2 respectively), the larger decrease in pH after bottling in Exp1 compared to Exp2 (0.39 and 0.11 respectively) indicated that the media storage conditions during the bottling may have differed between the experiments (such as the duration of the bottling procedure, temperature or stirring fluctuations). Also, there may have been a larger amount of feed residues in the rumen fluid collected for Exp1 compared to Exp2. This could have occurred if the cows in Exp1 were fed later in the evening the day before the rumen fluid collection. As a result, there would have been more feed particles left in the rumen fluid at the collection time in the morning. In addition, if the water supply was turned off earlier for the cows in Exp1 compared to Exp2, the higher concentration of rumen fluid in Exp1 would have resulted in a higher concentration of feed particles in the incubation media. If there were enough feed residues in the rumen fluid to initiate fermentation during

the bottling procedure in Exp1, an increase in VFA in the incubation media could have been the reason for the decreased pH in Exp1. However, it is very unlikely that the resulting VFA production was high enough to induce a relatively large decrease in pH during the bottling process.

In the present study, there was no significant difference in pH between the different rations and different Animal Biosa treatments, as well as no difference between the three Animal Biosa treatments pooled together and water. The findings were similar to those by Chen et al. (2016), where different doses of *L. acidophilus* (0, 0.25 x 10^7, 0.5 x 10^7 and 0.75 x 10^7) tested *in vitro* did not have an effect on pH neither with rice straw nor maize stover. Similar to the present study, the pH was maintained between 6.72 and 6.79 throughout the *in vitro* gas production trial, which is considered to be within the optimal range for microbial growth and fibre degradation.

In Exp1 no significant interactions were found between the different rations or between Animal Biosa and water. In Exp2, however, an interaction between ration B and Animal Biosa treatments was detected. One of the possible explanations could be a higher standard SEM with the water treatment (SEM=0.025) compared to pooled Animal Biosa treatments (SEM=0.002) in ration B. The higher SEM occurred due to a higher pH in one of the three bottles with water in ration B (6.96 compared to 6.89 and 6.88), however the reason for this was not identified. Since no significant interactions were detected within other variables (OM, hemicellulose or cellulose degraded, total gas and CH_4 production), the interaction may have occurred due to random variation.

6.1.3. OM degradability

In the present study, no significant difference in % OM degraded at 48 hours was found between Exp1 and Exp2, as well as between the pooled Animal Biosa treatments and water. These results were supported by the aforementioned *in vitro* studies by Santoso et al. (2014) and Baah et al. (2009) that found no significant effects of LAB based probiotics on DM degradability at 48 hours *in vitro* with grass silage and concentrate, and a barley based diet, respectively. The *in vitro* study by Ellis et al. (2016) measured OM degradability at 72 hours and found a significant effect of *L. plantarum*, which increased OM degradation by

approximately 2.2 % compared to control. A combination of *L. lactis* and *L. plantarum* showed a tendency for increased OM degradation by approximately 1.5 %, while a numerical increase was observed with *L. lactis* by approximately 1 %. It could be discussed whether significant effects of LAB would be observed with the studies mentioned above, including the present study, if the OM degradation was measured at 72 hours. However, the feeds do not normally stay in the rumen for periods longer than 48 hours, therefore, the effects on degradability at 72 hours would not have biological relevance. In this study, no increases in OM degradability were expected with a prolonged incubation time, as the cumulative gas production graphs had reached their asymptote for most of the bottles by 48 hours.

No significant difference in OM degradability was found between rations A and B, however, all the other rations were significantly different. The differences indicated that the effects of Biosa were tested on three rations with different degradability. The potential degradability of a feed is highly dependent on the amount of lignin it contains, since lignin is indigestible. Lignin also tends to bind to cellulose and hemicellulose and, thereby, prevent their degradation, reducing the digestibility of the feed even further (McDonald et al., 2011a). Rations A and B had lower lignin contents (4.15 %/gOM and 5.06 %/gOM respectively) compared to rations C and D (6.64 %/gOM and 7.77 %/gOM), which could account for higher OM degradability in rations A and B.

6.1.4. Fibre degradation

No significant difference in hemicellulose and cellulose degraded (%) was found between Exp1 and Exp2, indicating that the concentration and the activity of fibrolytic bacteria in the inoculum and the incubation conditions were similar in both experiments. No significant differences were found in hemicellulose and cellulose degraded between the Animal Biosa treatments and water. The lack of effects of the probiotic on fibre degradation corresponded well with the studies of Santoso et al. (2014) and Chen et al. (2016), who reported the fibre degradability results on NDF basis. Since NDF includes lignin, cellulose and hemicellulose, and lignin is indigestible, significant results might be less likely to be observed using this expression. Nevertheless, it provides a good indicator of general tendencies in fibre degradation, that can then be compared between the studies. Santoso et al. (2014) found no significant effects of a probiotic containing *L. plantarum* and *L. acidophilus* on NDF

digestibility (%) with a grass silage and concentrate diet *in vitro*. Similar was observed by Chen et al. (2016), whose study showed no effects of different doses of *L. acidophilus* supplementation on NDF disappearance in rice straw and maize stover *in vitro*. The authors suggested that fibre degradability was not affected by the probiotic directly, because common LAB do not have the enzymes required to hydrolyse the structural carbohydrates within the cell wall (Rooke & Hatfield, 2003). The activity of cellulolytic microorganisms might also not have been affected by the addition of LAB either.

The significant differences in hemicellulose degradability between the rations showed that the effects of Animal Biosa were tested using three diets significantly different in their fibre degradability. According to Van Soest (1994b), lignin tends to bind polysaccharides of the plant cell wall, rendering them less available for digestion. All polysaccharides in the plant cell wall can be divided into those that form covalent bonds with a lignified core and are only partly digestible, and those that lack associations with lignin and are more soluble and fully fermentable in the rumen. Hemicellulose is a polysaccharide that is associated with lignin the most because together they form the secondary plant cell wall. The digestibility of hemicellulose in a feed is, therefore, inversely dependent on the level of lignification in the plant. This is reflected in the results of the present study, where increasing level of lignin corresponded to decreasing hemicellulose degradability within the rations (3.71, 4.61, 6.22 and 7.34 % ADL/gOM and 68.0, 66.8, 64.4 and 56.8 % hemicellulose degraded in rations A, B, C and D respectively).

The significant differences in cellulose degradability between the rations showed that the effects of Animal Biosa were tested using three diets significantly different in their fibre degradability. According to Van Soest (1994b), the digestibility of cellulose depends, to a great extent, on the lignin to cellulose ratio in the feed, where high lignin to cellulose ratios are associated with low cellulose digestibility. In this study, rations C and D, with the lowest degradability of 57.3 % and 58.5 % respectively, corresponded well with the highest lignin to cellulose ratios of 0.29 and 0.24 respectively. Here, the slightly higher lignin to cellulose ratio in ration C was reflected by reduced cellulose degradability. Rations B and A with lower lignin to cellulose ratios of 0.21 and 0.17 respectively, also had increased cellulose degradability of 72.5 % and 64.1 % respectively. Here, even though the ratio was higher in ration B, the degradability was also higher. This could happen due to the differences in the

intrinsic properties of cellulose that is free and not associated to lignin. Since the digestibility of this cellulose varies between different plants (Van Soest, 1994b), it is possible that the nutritional availability of free cellulose in the feedstuffs of ration B was higher than in the feedstuffs of ration A.

6.1.5. Total gas production

No significant difference in total gas production (ml/gOM) was found at 24 and 48 hours between Exp1 and Exp2. On the other hand, significant differences were found at 3, 6, 9, 12 and 18 hours. The gas curves, however, followed relatively similar trends, therefore curve fitting was not applied in this study. The gas production in Exp1 was consistently higher at the aforementioned time points regardless of ration, potentially because degradation rate was higher. The degradation of OM and fibre was not measured at intermediate time points, however, since the gas is produced as feed components are being degraded, it would seem like a plausible explanation. The rate of OM degradation could have been higher in Exp1 at 3, 6, 9, 12 and 18 hours due to a possibly higher activity, viability, or concentration of microbes. At 24 hours, the microbial population has potentially stabilized and the gas production between Exp1 and Exp2 equalized.

No significant differences in total gas production were found between any of the Animal Biosa doses (MIN, MED and MAX). As a result, all the Animal Biosa treatments of all the rations were pooled together and compared to water in order to determine if Animal Biosa influenced gas production regardless of dose and ration, however, no significant difference was found. The gas production with water treatment was numerically higher in both experiments at all time points compared to the pooled Animal Biosa doses, indicating that there was a small decrease in gas production with the addition of Animal Biosa, although not significant. Since gas is produced when carbohydrates are being degraded during the fermentation process (Getachew et al., 1998), the lack of probiotic effect on total gas production corresponds well with the lack of effect on OM, hemicellulose and cellulose degradation. The results of the current study are in agreement with the *in vitro* studies by Baah et al. (2009) and Ellis et al. (2016), that did not find any significant effects of LAB based probiotics on the cumulative gas production throughout the fermentation irrespective of time points, substrate, and doses.

6.1.6. The relationship between total gas production and OM degradation

When the gas produced (ml/gOM) at 48 hours was plotted against % OM degraded, logistic and quadratic equations resulted in better fittings than the linear equation. However, these fittings would not have biological relevance, because total gas production increases as more OM is being degraded. Therefore, linear equations were used for all the rations. Within individual rations, the total gas produced had a low accuracy in predicting % OM degraded, however, the accuracy was higher when all the rations were used. The low accuracy within the rations separately could be explained by the heterogeneity of the rations. The rations used in the study consisted of a mixture of feeds. Both rations C and D included a mix of forage and concentrates, while rations A and B consisted of a mix of different forages. All of the constituents individually would most likely have different degradability and different levels of gas produced per gOM, and when these feeds are combined in a heterogenous ration, the correlation is reduced, and the total gas produced is less accurate at predicting % OM degraded.

The gas produced during fermentation is a result of the ruminal microbes converting carbohydrates to VFA. The gas produced from the breakdown of protein is relatively small compared to that from carbohydrates, while the gas produced from the breakdown of fat is imperceptible (Getachew et al., 1998). The total gas production, therefore, is almost proportional to the amount of hemicellulose and cellulose degraded. Ration D had the lowest percentage of hemicellulose and cellulose degraded, followed by ration C. Out of all the rations, ration B had the highest cellulose degradability, and ration A had the highest hemicellulose degradability. The percentage of both hemicellulose and cellulose degraded combined was slightly higher for ration B. This would mean that rations A and B had the highest amount of gas produced, ration B having a slightly higher gas production than ration A. Notably less gas would be produced with ration C while ration D would have the lowest amount of gas produced. This corresponds well with Figure 9, showing the relationship between the volume of gas produced and % OM degraded in both individual rations and all rations combined.

6.1.7. Methane production

There was a significant difference in CH_4 production (ml CH_4/gOM degraded) between Exp1 and Exp2. CH_4 production was consistently higher in Exp1 compared to Exp2. This may have occurred due to a possibly higher concentration of methanogenic archaea, or protozoa that produce hydrogen required for methanogenesis (Cersosimo & Wright, 2015), in the rumen fluid collected for Exp1. The differences in concentration may have appeared due to variations in the period of time between the collection of rumen fluid and inoculation of the bottles. The exact time of transportation was not recorded, however, if the transportation in Exp2 took longer and the temperature was slightly lower, the concentrations of viable rumen microorganisms may have been affected. Also, the experiments were conducted two weeks apart from each other, and there was a prolonged storage period for both Biosa and the feeds. Since the feeds were dried and stored in airtight containers, they were not likely to have altered in their chemical characteristics. The efficacy of Biosa is known to decrease over time, however, a storage period in the fridge of two months is guaranteed by the producer. The concentrations of microorganisms were not tested before the experiments, therefore a possibility of a slight decrease in viability cannot be eliminated. On the other hand, if methanogens and protozoa were affected by the transportation or storage conditions, it is likely that cellulolytic bacteria would have been affected as well, leading to differences in OM, hemicellulose, and cellulose degradation between the experiments. However, such differences were not observed, and the explanation for higher CH_4 production in Exp1 is not clear.

The results showed no significant differences in CH_4 production between the MIN, MED, MAX doses and water neither in Exp1 nor Exp2. These findings are supported by Ellis et al. (2016), who observed no effects of LAB on CH_4 production with rye grass silage *in vitro*. In the present study, there was a numerical increase in CH_4 production with increasing doses of Animal Biosa with rations A and D in Exp1. The same was found by Chen et al. (2016), where increasing doses of *L. acidophilus* increased CH_4 production numerically with maize stover. Another trend was observed with rations B and C in Exp2, where CH_4 production was numerically lower with all of the Animal Biosa doses compared to water. In agreement with this, a reduction in CH_4 production was observed in the *in vitro* study by Santoso et al.

(2014), where a probiotic with *L. plantarum* and *L. acidophilus* was able to significantly reduce CH_4 production with grass silage and concentrate substrate.

To our knowledge, no other *in vitro* studies have been carried out with the same combination of lactic acid bacteria strains as the ones in Animal Biosa, therefore it is not possible to make any direct comparisons and validate the results of the current study. However, the lack of effect of the probiotic on OM, hemicellulose and cellulose degradability, total gas production as well as CH_4 production might be in agreement with the *in vivo* study by Pant (2017), where only numerical increases in animal productivity indicators were found.

6.2. Discussion of methods

6.2.1. Animals

The present study aimed to investigate the effects of Animal Biosa *in vitro* using Bolivian dairy cattle feeds and rumen fluid collected from two non-lactating research cows in Denmark. Although small differences in the rumen microbiota between the Bolivian dairy cows and the Danish research cows could be expected, the results of a study by Henderson et al. (2015) suggest that the core microbiome should be comparable. The authors investigated the composition of rumen microbiome in ruminant animals across continents to determine the effect of animal species, diet and geographical location. The study showed that the dominating bacteria and archaea species were very similar between cattle in South America and Europe. The slight differences that were observed were most likely caused by differences in climate and farm management practices, however, the core microbiome in both continents was the same. However, clear differences were observed in the proportions of different rumen bacteria based on the diet type. The abundance of certain bacteria was different between ruminants fed forage and concentrate based diets. The proportion of Bacteroidales, Ruminococcaceae and Fibrobacter was higher in the microbiome of animals fed forages, while Prevotella was dominating in the animals fed concentrate diets. Butyrivibrio were mostly found in bovines fed a mixed diet of forages and concentrates. Another study by Shanks et al. (2011) studied faecal microbiome in cattle from four different states in USA (Georgia, Colorado, Ohio and Nebraska) fed three different diets: forage based (>80 % forage), processed grains (at least 75 %), and unprocessed grains (>75 %). Faecal microbiome contains microbes from the rumen and the large intestine, therefore it is slightly

different from the actual rumen microbiota but acts as an adequate indicator. In agreement with the aforementioned study, bacteria that were the most sensitive to different feeding practices were of Ruminococcaceae and Prevotella species, with the former higher in forage based diets, and the latter increasing exponentially with diets of unprocessed grains. The results also showed that cattle in the same geographic location had differences in faecal microbiome when subjected to different diets, and faecal microbiota of cattle fed similar diets were associated with one another more than with microbiota of cattle fed different diets. The authors then suggested that the differences in faecal microbiome of cattle were mostly due to variability in diets rather than factors associated with a specific geographic location, such as humidity, water source, or elevation. The findings from the two studies lead to an assumption that the rumen microbiome of Danish research cows, who in the present study were fed a maintenance diet of hay only, should be quite similar to that of Bolivian dairy cows fed forage based diets. Small variations could be expected due to different management practices and a small proportion of concentrates included in the diets of Bolivian dairy cattle.

6.2.2. Blanks

Rumen fluid collected for an IVGPT study might contain a varying amount of residual OM. The gas produced by the breakdown of these residues will be included in the measurement of the total gas production, which can then be overestimated (Rymer et al., 2005), and in order to prevent this, blanks are included to allow correction of gas production values (Araujo et al., 2011). In the current study, three bottles of regular blanks were included in both Exp1 and Exp2, to correct for gas produced by the residual OM in the rumen fluid in the bottles with water. With Animal Biosa treated samples, specific blanks were used instead of regular, because some additives, for example those that include organic acids, can potentially be degraded by rumen microorganisms, contribute to microbial growth (Carro et al., 2005), and, thereby, affect the fermentation of the residual OM in the rumen fluid (Araujo et al., 2011). Carro et al. (2005) advised to include both regular blanks and specific blanks in experiments testing additives to avoid misinterpretation of the results. To prevent overestimation of the total gas produced in the bottles containing any of the Animal Biosa doses, specific blanks were included in the study. Three specific blanks with MED dose of Animal Biosa were included in Exp1, and additional four specific blanks, two with MIN dose and two with MAX dose of Animal Biosa, were included in Exp2.

In the fibre analysis, both regular and specific blanks were included to account for the increase in microbial mass in response to degradation of feed residues in the collected rumen fluid and potentially fermentable substrate in Animal Biosa. In the fibre analysis of individual feeds, three regular blanks were included. In the fibre analysis of rations post incubation, three regular blanks and three specific blanks with MED Biosa were included in Exp1, while three regular blanks, three specific blanks for each probiotic dose were included in Exp2. Since no significant differences in blank bag weight were detected between specific and regular blanks, a total average of all the blanks was used for the blank corrections.

6.2.3. Units of measurement

Animal nutrition studies vary in units of measurement used, however most often the results are expressed on DM or OM basis. In the present study the fibre content, total gas production and level of CH_4 were calculated on an OM basis. The OM of a feedstuff is calculated by subtracting the ash from the DM content. Since ash does not make a significant addition to neither gas nor VFA production, and because the ash content can vary significantly between different feeds or rations, it has been suggested that the results of an *in vitro* analysis are best described on an OM basis (Williams et al., 2000). In the present study, the ash content was 8.53, 7.07, 7.82 and 7.24 % in rations A, B, C and D respectively, and the variation was considered to be too high to base the results on a DM basis.

The total gas production was expressed as gas volume (ml) per gOM, while the amount of CH_4 produced was expressed as CH_4 volume (ml) per gOM degraded. The distinction was made, because CH_4 is produced only during the fermentation of degradable substrate, while total gas also included the indirect gas produced by the reactions between the buffer and VFA. Furthermore, Yáñez-Ruiz et al. (2016) also recommended expressing CH_4 per substrate degraded, rather than incubated. It was emphasized that additives may affect substrate degradability and the effect may be dose dependent, leading to inconsistent CH_4 production measures when it is expressed on an incubated substrate basis.

6.2.4. Fibre analysis

6.2.4.1. Sodium sulphite

The protocol of NDF analysis by ANKOM (2017a) requires the use of sodium sulphite to reduce the level of protein in feed samples. However, Van Soest et al. (1991) suggested that the use of sodium sulphite is optional and is generally not recommended for ruminant feeds, especially if the samples are to be used in sequential fibre analyses, because sulphite also attacks lignin and could lead to lignin underestimation. This was confirmed by the study of Hunt et al. (1995), who found that the addition of sulphite to both the NDS wash and the rinse significantly increased the degradability values in three different types of forage, including alfalfa hay, prairie hay and wheat straw, potentially due to sulphite breaking the lignin bonds. The authors found, however, that when the sulphite was added to the rinse only, the degradability values were not significantly different from those without sodium sulphite. It was suggested that the reason the degradability values decreased with sulphite in NDS wash, but not in the rinse, was the longer exposure time of feed material to sulphite during the wash compared to the rinse. Since adding sulphite to the rinse did not affect the feed degradability, it was suggested that it was the most optimal method for protein separation.

In the current study, both the individual feed samples and the ration samples post incubation were used for sequential fibre analyses, therefore sodium sulphite was not used. The protein levels in the rations used in this study were not expected to be very high due to reported average protein levels of feeds making up the largest part of the rations. Although the protein levels in the added concentrates may have been higher than in the forage, especially because the content of some of the concentrates was unknown, the proportion of these concentrates in the rations was too low to substantially increase the level of protein in rations C and D.

6.2.4.2. Acetone

The protocols for NDF and ADF analyses by ANKOM (2017a; 2017b) suggest that feeds with a fat content of more than 5 % should be subjected to acetone pre-extraction. Although fat content analysis was not performed in the current study, the individual feeds that were used to formulate the rations, were not expected to have ether extract contents lower than 5 % of the DM. As a consequence, the fat extraction with acetone was not performed in the

current study. In addition, the ANKOM protocols require soaking the samples in acetone for three to five minutes after NDF and ADF analyses and rinsing the samples in acetone for three minutes after ADL analysis to remove water. In the current study the acetone was not used due to the finding that acetone affects the weight of the filter bags (Hansen, 2019, personal communication). Instead, the samples were left at room temperature until air-dried and then dried in the oven.

6.2.4.3. Acid insoluble ash

Van Soest (1991) argued, that even though it is possible to determine AIA post ADL analysis, more accurate results are obtained if AIA is determined post ADF, because some of the AIA fractions might be dissolved during the ADL analysis, and this could possibly lead to an underestimation of the AIA. In the present study, AIA was determined post ADL analysis. This, however, was not considered and error, because the AIA in the rations were so low, that if they increased with a small amount, the OM content of the rations would not have been affected.

6.2.5. Total gas production

Apart from indicating the rate of fermentation, gas data from IVGPT alone provides limited direct information about the feeds (Beever & Mould, 2000). Therefore, for a full evaluation of the nutritive value of feeds, gas production data should be complemented with measures of substrate disappearance and VFA profile (Schofield, 2000). The present study measured the disappearance of substrate to determine OM and fibre degradability, as well as assess how well the total gas production was able to predict % OM degraded. The VFA analysis was not performed as it was outside the scope of the study, however, since no significant change in CH_4, total gas production or fibre degradation was detected, the acetate to propionate ratio was not expected to have changed. The VFA analysis would be beneficial if the results showed significant decreases in CH_4 production accompanied by decreases in total gas production. Normally, the decrease in total gas production would be considered not favourable, because it would mean decreased OM degradability, however, the VFA analysis could reveal that the proportion of propionate increased, resulting in the same OM degradation, but decreased total gas production. Since such tendencies were not observed in the current study, there was no necessity for VFA analysis.

6.2.6. *In vitro* vs *in vivo*

The IVGPT is used to simulate the fermentation in the rumen, however, the results obtained *in vitro* are only an indication of the possible effects *in vivo*. Differences between the two methods are common and occur due to several reasons. First of all, by using *in vitro* systems it is not possible to simulate all the digestive processes that occur *in vivo*. In the rumen, the feed particles separate into fractions based on their density and particle size. The fractions differ in their retention time, and this is not simulated by the *in vitro* batch systems, as all the feed particles stay in the incubation bottles for the same period of time. Secondly, mechanical grinding is not able to simulate the distribution of feed particle size that is found in the oesophagus, there is no absorption of VFA, and the microbes do not have sufficient time to adjust to the test substrates (Owens & Basalan, 2016). Furthermore, in the beginning of fermentation, the batch *in vitro* systems are not able to obtain the pH that is typical for the rumen. A high proportion of buffer solution relative to the rumen fluid is necessary to maintain the appropriate pH levels throughout the fermentation period. However, this means that at the start of fermentation the pH levels are considerably higher than would be typically found in the rumen environment (Gizzi et al., 1998), which could lead to differences in fermentation kinetics between *in vitro* and *in vivo*. In addition, certain aspects of *in vitro* gas production batch technique may lead to either overestimation or underestimation of feed degradability compared to that in the rumen. On the one hand, the size of the feed particles is reduced to a great extent during fermentation, and some of the smallest particles may not be recovered in the filter bags during filtration. The material not recovered in the filter bags is considered to be digested. Due to this, and the prolonged retention time of certain substrate fractions, the rumen digestibility might be slightly overestimated. On the other hand, the microbial mass, that is attached to the feed particles or located inside the feed particles, is not always fully removed during filtration. The blanks do not allow to account for the additional weight of retained microbial biomass, because, due to the lack of substrate, the microbes in the blank incubations are likely to die and disintegrate (Owens & Basalan, 2016). As a result, the degradability *in vitro* might be slightly underestimated compared to digestibility *in vivo*. In the current experiment, the rations used were very fibrous and only two of them contained a small proportion of concentrates. Therefore, it is more likely that a considerable amount of microbes remained attached to the undegraded fibrous material during filtration rather than a

lot of small feed particles were lost through the pores of the filter bags. As a result, it can be assumed that degradability was marginally underestimated.

6.3. Sources of error

6.3.1. Animal Biosa

Some of the possible sources of error in the present study could be related to low doses of Animal Biosa as well as dilution with water. The Animal Biosa doses were calculated to be proportional to the dosage that Biosa Denmark ApS recommended for live cows and were based on the average DM intake of a cow weighing 500 kg. Therefore, the amount of Animal Biosa added to the dried feed samples of 0.5 g was directly in proportion to the amount of Animal Biosa recommended for cows consuming 20 kg DM per day. However, it is possible that there is a minimum effective dose of a probiotic for both live animals and *in vitro* studies, and the doses used in this experiment may have been lower than required to have a noticeable effect on gas production and fibre degradation *in vitro*. To date, the information about the minimum effective doses of probiotics is lacking, however, Fuller & Gibson (1998) reported that, in general, a probiotic concentration of 1×10^6 cfu/ml is recommended. Animal Biosa contains at least 5×10^7 cfu/ml, which is considerably above the recommendation. There is also a possibility that the dosage recommended by Biosa Denmark ApS, which is the same for all animals larger than 30 kg in body weight, is too low for it to have significant effects in a ruminant animal. Considering fundamentally different digestive systems and the differences in digestive tract volume to bodyweight ratios, it is quite likely that the dose necessary for the probiotic to induce noticeable effects in cattle is considerably higher than for other animals. However, this is debatable, since the *in vivo* study by Pant (2017) found higher numerical increases in milk production, milk fat and solids-non-fat in cows given 100 ml of Animal Biosa compared to cows given 150 ml of Animal Biosa. These increases were not significant though, and further research is needed to determine whether higher doses would lead to significant results of Animal Biosa.

Since the doses were so low, they would have been very complicated to measure, especially because the automated pipettes of such low calibre were not available. The doses were diluted with deionized water to make the dosing process easier. Both the selection of doses

and the decision to dilute them were in agreement with Biosa Denmark ApS. Since both Animal Biosa itself and the rumen of a cow contains water, and Biosa Denmark ApS recommends administering the probiotic with feed or mixed with water, it was assumed that the dilution would have no negative effect on the viability of the microbes. However, this was not confirmed, and therefore, it remains a possible source of error. It would be beneficial to count the colony forming units in an *in vitro* dose of pure Animal Biosa as well as a corresponding diluted sample. It would help to determine whether dilution affects microbial viability and whether Animal Biosa should be administered together with water.

6.3.2. Fibre analysis

According to ANKOM Technology (2019), during the NDF and ADF analyses with fibre analyser A200 it is important that the fibre analyser is at room temperature (about 20 °C) before the beginning of the first detergent wash. If the temperature of the instrument is above room temperature, the detergent solution will heat up too quickly, and the time of the actual washing procedure will be extended. As a result of this, the fibre content values will be slightly underestimated. In the present study, one of the ADF analyses with 24 post-incubation samples was performed right after an NDF analysis using the same fibre analyser, without allowing it to cool off. This may have been a possible source of error, since the duration of bag exposure to ADS at optimal degradation temperature was slightly extended, and the ADF in these bags may have been slightly underestimated. Even though no particular outliers were identified in the ADF values, the precision may still have been slightly reduced.

6.3.3 Methane determination

Some errors might have occurred in relation to CH_4 determination, which would lead to unrealistic results. Prior to the experiment, all the gas bags were flushed with CO_2 to ensure that there were no traces of CH_4 left in the bags, which could lead to CH_4 overestimation. An error may have occurred if some of the gas bags were not sealed properly after flushing. If air from the surroundings is allowed to enter the gas bags before the fermentation process, the amount of gas in the bag is higher than one that is calculated using the cumulative pressure values recorded by the ANKOM system. CH_4 in the gas samples taken from the gas bags is, therefore, underestimated, because the proportion of CH_4 in the total gas becomes lower with the additional gas that entered prior to fermentation. Some errors might also have occurred in

relation to gas chromatography. The gas bags have to be carefully massaged prior to taking the sample so that the different gasses in the bags are well mixed in order to get a representative sample. If mixing is inadequate, the samples may result in unrealistic CH_4 values.

7. Conclusion

In the current study no significant effects of Animal Biosa on feed degradability and CH_4 production were observed *in vitro*. Using the *in vitro* gas production batch technique with selected feed rations and doses of Animal Biosa, the product did not affect the pH post incubation, the OM and fibre degradability, the total gas production or CH_4 production. The effect of Animal Biosa was the same between the four rations used. There is a possibility that the results would be different with individual feeds or different rations as well as higher doses of Animal Biosa, which could be determined with further research.

8. Perspectives

If the same study using Animal Biosa was to be repeated, certain measures could be considered to improve the accuracy of the results. First of all, individual feeds rather than rations could be used as fermentation substrates, which would allow to determine if Animal Biosa has any effects on specific types of feeds. Otherwise, the rations could be more distinct, with some of the rations high in forage and low in concentrate, some high in concentrate and low in forage, and some rations containing equal parts of forage and concentrates. In this way it could be determined whether Animal Biosa has an effect on any specific forage to concentrate ratio. If there were significant effects on diets high in concentrates, or equal parts of forage and concentrates, the probiotic could be recommended for cattle fed at respective ratio, such as most cattle in beef production systems. If it was discovered that Animal Biosa had significant effects on diets high in forage, the product could be recommended for cattle fed less nutritious, high forage diets, such as those in developing countries like Bolivia.

The doses used in this study were proportional to the dosage recommended for live cattle by the producer, who based it on body weight. The dosage for live cattle, however, was based on

the producer's experience with pigs and horses, where the owners of the animals supplemented with Animal Biosa reported positive results. Since the digestive systems of both pigs and horses are fundamentally different from that of ruminants, it is possible that the dose necessary for noticeable effects is different as well. Therefore, a study with larger doses than the ones used in the current experiment may find different results. There is also a possibility that while the recommended dose would be effective in live cattle, the dose used for the *in vitro* experiment was lower than the minimum effective dose. If the numbers of probiotic bacteria were too low, they may have failed to compete for substrate with established rumen colonies, their growth may have been limited and, therefore, no effects on fermentation characteristics were observed. In this case, it would be worth to conduct an *in vitro* experiment with doses of Animal Biosa (such as 1-5 ml), that would not be directly proportional to live cattle body weight and DM intake, but they would provide a general indication whether the product has a potential to be effective.

To increase the amount of data generated by a single fermentation run, and to get a more comprehensive insight into the effects of Animal Biosa at the onset and throughout the fermentation, it would be interesting to conduct the study with varying incubation times. This would allow the measurement of pH, OM and fibre degradation at intermediate time points throughout the fermentation period. The measures of pH after 48 hours of incubation are not of high relevance, because the amount of buffer mixed with the rumen fluid is able to increase the pH to almost pre-incubation values, even though there is no absorption of VFA. Therefore, it would be beneficial to observe if the probiotic would help stabilize the pH at the intermediate points of the incubation, where the fermentation rate is the highest and the pH is most likely to drop the most. The degradation of OM and fibre would also be interesting at time points earlier in the fermentation, because most of the feed does not stay in the rumen for 48 hours. The effect of Animal Biosa on degradation at intermediate time points would help determine the relevance of its use with feeds having shorter passage rates.

Since no significant effects of Animal Biosa on CH_4 production and fibre degradability were observed in this study, it might be beneficial to slightly alter the microbial composition of the product based on the findings of recent research. Since some studies found positive effects of probiotics when both lactate producing and lactate utilizing bacteria strains were used, it would be worth considering the addition of some lactate utilizers to Animal Biosa. In

addition, several studies have focused on the use of yeast, especially *S. cerevisiae*, in combination with lactate producing and/or lactate utilizing bacteria, and their findings suggest that the addition of yeast to Animal Biosa for cattle might also be beneficial.

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