Evaluation of fresh and preserved sheep faeces as an inoculum source in *in vitro* gas production assays

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Evaluation of fresh and preserved sheep faeces as an inoculum source in *in vitro* gas production assays

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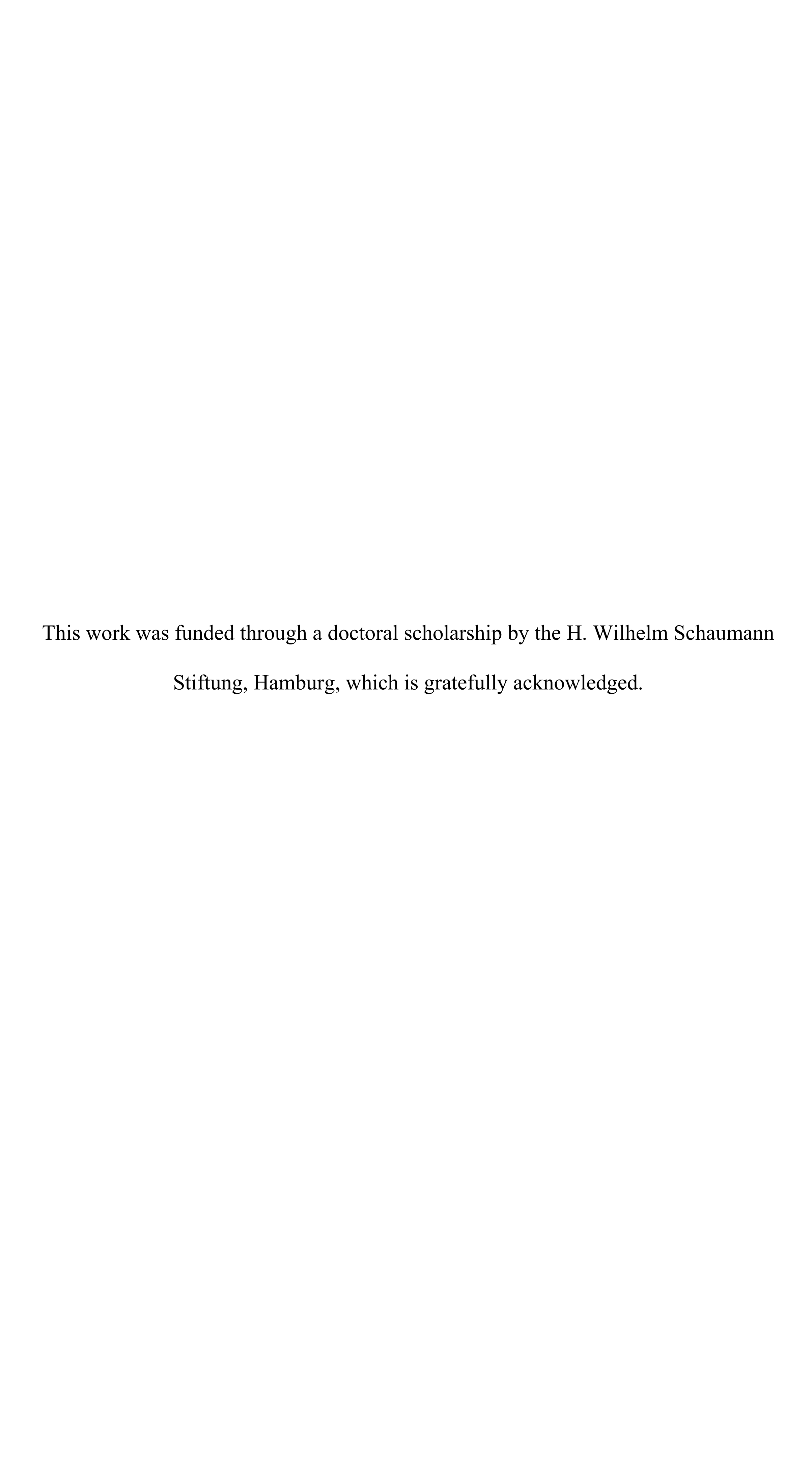

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LIST OF ABBREVIATIONS

ADFom	Acid detergent fibre expressed exclusive of residual ash
aNDFom	Neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash
C	Fresh faeces as control to preserved faeces
CHO	Carbohydrate
CP	Crude protein
CV	Coefficient of variation
DM	Dry matter
EE	Ether extract
FAN	Feed intake level
FD	Freeze-dried faeces
FDN	Shock-frozen faeces with liquid nitrogen and freeze-dried prior to incubation
FD3W	Freeze-dried faeces stored 3 weeks at 23 °C prior to incubation
FD6M	Freeze-dried faeces stored 6 months at 23 °C prior to incubation
FI	Faecal inoculum
FI-GP	Faecal inoculum-gas production
FI-mN	Faecal inoculum-microbially bound nitrogen
FI-NH_3-N	Faecal inoculum-ammonia-nitrogen
FR	Frozen faeces stored 8 days at -20 °C prior to incubation
FRN	Shock-frozen faeces with liquid nitrogen stored 8 days at -20 °C prior to incubation
FRNdef	FRN followed by defrosting for 12 h at 4 °C prior to incubation
FR6M	Frozen faeces stored 6 months at -20 °C prior to incubation
GP	Gas production
HGT	Hohenheim gas test
MCP	Microbial crude protein
mN	Microbially bound nitrogen
N	Nitrogen
nFR	Functional redundancy
NH_3-N	Ammonia-nitrogen
OM	Organic matter
OMD	Organic matter digestibility
RI	Rumen fluid inoculum
RI-GP	Rumen fluid inoculum-gas production
RI-mN	Rumen fluid inoculum-microbially bound nitrogen

RI-NH_3-N	Rumen fluid inoculum-ammonia nitrogen
RUP	Ruminally undegradable crude protein
SD	Standard deviation
TMR	Total mixed ration

1 GENERAL INTRODUCTION

Trends and levels of per capita consumption of animal source foods in many regions of the world increase (Qaim et al., 2024) and world's population is expected to continue growing (United Nations, 2024). Global consumption of animal source foods can, therefore, be expected to continue to increase in the future. Ruminants, in particular, play a key role in this context because of their ability to utilise low-quality fibrous biomass that is inedible for humans. Rumen microbes convert fibre-rich feeds into high-quality protein to produce valuable human food without directly competing with the human population. Natural resources such as land, water, and energy will be limited, and emissions and environmental changes are expected to increase in the future (NRC, 2015). From ecological and economic perspectives, it is therefore becoming increasingly important to use ruminant livestock to produce high-quality human food and to ensure that animals are fed according to their requirements. This will save resources, avoid nutrient losses in manure, and reduce feed costs.

To meet the animals' requirements, it is essential to characterise the nutritional value of feeds. This will be necessary on an ongoing basis because the basis for animal feed is continuously changing. This is due to various factors, such as changes in plant breeding and cultivation, advances in processing technology, shifts in demand, such as for alternative products, and particularly changes in environmental conditions (GfE, 2017). The gold standard for assessing the nutritional value of feeds, which is mainly determined by digestibility, energy and protein content, is an *in vivo* digestibility trial. When determining the digestibility of nutrients as a basis for energetic feed evaluation, a number of methodological requirements must be met in order to obtain reliable results (GfE, 1991). These involve large numbers of animals and repetitions, which is expensive, laborious, and often impractical. Hence, *in vivo* digestibility trials are not suitable for analysing large numbers of feed samples or for routine testing of feeds. In addition, digestibility experiments severely restrict the animals' freedom of movement and opportunities for contact and thus impair animal welfare.

In order to formulate rations for ruminants that meet their nutritional requirements, information on ruminal degradation and fermentation is crucial, which can be provided by *in situ* and *in vitro* assays. *In situ* methods have been developed, in which synthetic fibre bags containing the test feeds are incubated in the rumen (Mehrez and Ørskov, 1977; Ørskov and McDonald, 1979). These methods are useful for assessing dry matter (DM) and nitrogen (N) degradation, as well as particle outflow. However, its reproducibility across laboratories is low, partly due to variations in the animals' proteolytic activity caused by different diets and physiology. Additionally, *in situ* methods require rumen-cannulated animals, raising ethical concerns, increasing costs, and limiting sample sizes (Mohamed and Chaudry, 2008). Therefore, interest has grown in *in vitro* techniques for predicting the organic matter digestibility (OMD), as well as the energy and protein values of feeds for ruminants, as these methods reduce the need for *in vivo* or *in situ* approaches and offer cost-effective, rapid, and standardisable analyses. *In vitro* methods using enzymes have been applied, including pepsin-cellulase techniques for estimating DM

digestibility (McLeod and Minson, 1978), OMD (Aufrere and Michalet-Doreau, 1988; McLeod and Minson, 1978), enzyme soluble organic substance (ELOS) for estimating OMD and metabolisable energy (VDLUFA, 2018) or *Streptomyces griseus*-protease for determining ruminal crude protein (CP) degradation (Chaudry, 2007, 2005; Licitra et al., 1999; Licitra et al., 1998). However, using single enzymes may not be appropriate to represent the total activity of the rumen microbiome, and results obtained using enzyme mixtures did not accurately reflect rumen microbial activity either (Mohamed and Chaudry, 2008). Therefore, a comprehensive estimation of the nutritional value of various ruminant feeds by using enzymes seems not possible to date. In contrast, techniques such as the two-stage *in vitro* digestion method by Tilley and Terry (1963) and the *in vitro* gas production (GP) technique by Menke et al. (1979) "Hohenheim gas test" (HGT) include rumen fluid as a complex microbial inoculum. These techniques have been established for the estimation of OMD and the energy value of feeds for ruminants. The newly revised recommendation for the energy and nutrient supply of dairy cows in Germany (GfE, 2023) introduced novel approaches for determining the energy value of diets and for supplying dairy cows with protein and amino acids. *In vitro* and *in situ* methods are particularly important in these approaches, as the OMD is a key parameter for energy and protein evaluation. Furthermore, the extended HGT (eHGT), a modified version of the standard HGT method, allows the estimation of the protein value of ruminant feeds (Steingass and Südekum, 2013). It is therefore crucial to improve the established *in vitro* systems, such as the HGT, which relies on a microbial inoculum source, in terms of animal welfare and accessibility.

The aim of scientific animal welfare is based on the 3 R principles: replacing animal testing, reducing the number of animals used, and refining methods to minimise suffering. Although the above-mentioned established *in vitro* techniques already reduce the need for *in vivo* testing, these approaches still depend on using rumen fluid, commonly obtained from rumen-cannulated animals. The surgical procedure and ongoing care required to prevent infection in cannulated animals demands skilled surgery, specialised research organisations, and ethical committee approval in most countries. For ethical and practical reasons, it is therefore desirable to replace surgically modified animals as donors of microbial inoculum sources for *in vitro* testing in the HGT. In this context, previous studies have already demonstrated the potential of faeces as an alternative inoculum source to rumen fluid (Aiple et al., 1992; Akhter et al., 1999; El-Meadaway et al., 1998; Mauricio et al., 2001). However, to date, no such approach has been established for routine ruminant feed analysis.

Hence, the objective of the doctoral thesis was to systematically evaluate the suitability of sheep faeces to be used as an alternative inoculum source to rumen fluid regarding *in vitro* GP in the HGT, as well as the potential suitability of faeces for use in the eHGT. Moreover, to reduce animal numbers and enhance standardisability, the possibility of faeces preservation was investigated by examining effects on the GP and the microbiome.

2 LITERATURE OVERVIEW AND RESEARCH QUESTIONS

2.1 Rumen cannulation

The rumen content is a complex mixture of feed, water, saliva, fermentation products and living organisms, including bacteria, fungi, and protozoa, that changes over time and in response to different feeds (Durmic et al., 2015). To date, it has not been possible to reproduce this complex system in the laboratory, making surgical rumen cannulation a valuable tool for studying the physiology and biochemistry of the ruminant digestive system to obtain information such as ruminal nutrient degradability (Castillo and Hernández, 2021). Rumen cannulation is a form of rumenostomy in which a cannula connects the rumen to the body surface, allowing continuous access for research purposes. Historically, Schalk and Amadon (1928) described some of the first rumen cannulation procedures in cattle, later modified for sheep by Hecker (1969). Since then, new variations of this technique have been developed (Durmic et al., 2015; Lopes Muzzi et al., 2009) and rumen-cannulated animals are widely used by research organisations around the world. Despite the important benefits, the cannulation of the rumen represents an invasive intervention for animals (Lozier and Niehaus, 2016). Research into the effects of permanent rumenostomy on health and rumen function in cattle has provided no indication of compromised welfare or health, even at low-grade pain level (Hartinger et al., 2025). Therefore, with appropriate management, experimental rumenostomy for research purposes in early lactating dairy cows appears to be ethically acceptable (Hartinger et al., 2025), as previously demonstrated in small male ruminants (Schramm et al., 2021).

However, rumen cannulation continues to be a subject of public controversy, with animal welfare groups claiming it as cruel. Animal welfare has emerged as an important scientific and social issue in recent decades, and there is a drive in the scientific community to continuously improve and replace animal testing according to the 3 R principles. In the case of rumen cannulation to obtain rumen fluid for research questions, such as feed evaluation, the aim is to collect rumen fluid or other suitable inoculum sources by alternative and less invasive methods, thereby improving animal welfare considerations.

2.2 Alternative rumen-associated inoculum sources

Alternatively, ruminal contents can be obtained by oesophageal intubation, but this is an uncomfortable procedure for the animal and samples are often highly contaminated with saliva (Shen et al., 2012). In addition, the microbial composition and metabolites vary across different regions of the rumen (Cheng and McAllister, 1997; Shen et al., 2012), and by using oesophageal intubation only free rumen liquid can be collected, the composition of which is quite different from particle-associated liquid and solid digesta (Cheng and McAllister, 1997). Such samples may therefore not fully represent the rumen ecosystem (Hartinger et al., 2025).

Other options for obtaining ruminal microbial communities are the collection of regurgitated digesta (bolus) or buccal fluid. Ruminants routinely regurgitate ruminal contents to chew partially digested plant material (Kennedy, 1985). After thorough chewing, the bolus is swallowed again for continued microbial breakdown. Given this process, it is reasonable to assume that the oral samples may therefore contain appropriate representations of the ruminal microbiome (Tapio et al., 2016). Confirming this, Kittelmann et al. (2015) found that buccal swab samples of sheep contained rumen bacterial taxa with similar diversity to the corresponding samples collected via a rumen cannula. In line with this result, Tapio et al. (2016) found that the taxa present in buccal and bolus samples from dairy cows were similar to rumen samples, but relative abundances varied. Bolus samples exhibited a bacterial community more closely resembling ruminal digesta than buccal swab samples, which the authors suggested is likely because the gingival microbiota mixes with ruminal microbes from regurgitated feed. However, collection from the oral cavity probably poses a stress factor for the animals. Additionally, since the bolus primarily consists of feed components, it would need to be separated from the liquid before being used as an inoculum source. Obtaining a sufficient quantity would require interrupting several chewing cycles, making the process time-consuming and potentially comprising the quality of the inoculum source. Moreover, the representativeness of the rumen content of such samples is questionable.

Using rumen fluid from slaughtered animals could be another alternative to rumen cannulation. Some studies investigated the suitability of rumen fluid from slaughtered animals, demonstrating its potential for predicting *in vivo* DM digestibility (Denek et al., 2006) and reporting no significant differences in *in vitro* DM digestibility and OMD compared to rumen fluid obtained from rumen-cannulated cattle (Beyihayo et al., 2015). Reducing the variability of results is an important prerequisite for ensuring the repeatability and comparability between studies and is highly dependent on sampling and storage procedures from the slaughterhouse to the laboratory (Fortina et al., 2022). When properly collected and stored, rumen fluid from slaughter animals retained its fermentative activity for up to 300 minutes without significantly affecting feed digestibility (Fortina et al., 2022). However, the inoculum can be substantially affected by the diet provided to the animals, as the diet can distinctly influence the rumen microbial populations (Saro et al., 2014; Tassone et al., 2020). In this context, two variables, *in vitro* neutral detergent fibre digestibility and acetate production, were found to account for the greatest degree of variability between rumen fluid obtained from rumen-cannulated and slaughter animals (Alba et al., 2023). In the experiment of Alba et al. (2023), the cannulated cattle received diets containing concentrate feed, while the slaughtered cattle diet was unknown, but probably consisted of pasture without concentrate supplementation. The authors concluded that ruminal inoculum from slaughtered cattle is viable for the *in vitro* analysis of concentrate-based feeds only if the donor animals are adapted to such diets. However, information on the diet of slaughtered cattle is often lacking, and pasture-based feeding predominates in some regions. Furthermore, not all research organisations have

a slaughterhouse in close proximity, and access to such facilities is not always permitted. This restricts the feasibility of using rumen fluid from slaughtered animals for routine feed evaluation.

2.3 Faecal inoculum sources – Host species

Although the above-mentioned alternative procedures could partially replace rumen-cannulation, the limitations of these methods for applying to feed evaluation have been demonstrated. The use of faeces could represent a viable source of inoculum, eliminating the need for surgically modified animals and providing a non-invasive, flexible and easily accessible method for routine feed evaluation.

Equine faeces

Since *in vitro* techniques have been established as valuable methods for ruminant nutrition research and feed evaluation, they have also been adopted to assess hindgut fermentation in monogastric animals such as horses (Bush et al., 2001; Jansen et al., 2007; Lowman et al., 1996). In this regard, methods that have been applied in equine studies are the *in vitro* GP technique, as documented by Murray et al. (2009, 2014) or the ANKOM Daisy II Incubator (Earing et al., 2010; Lowman et al., 1999). Kujawa et al. (2020) recently evaluated the use of equine rectal content in the *in vitro* GP technique. Their results indicated that, while the use of equine faeces may lead to an overestimate of total tract digestibility in forage-based feeds, it could be suitable for evaluating grain-based substrates. Apart from the *in vitro* estimation of equine hindgut fermentation activities, equine faeces may also have the potential to be used in the research of ruminant feed evaluation; particularly as equids are hindgut fermenters and thus when using equine faeces, microbial cells undergo little or no post-fermentative digestion and absorption (Lowman et al., 1999). In contrast, when using ruminant faeces, the predominant microbial fermentation occurs in the rumen, followed by substantial digestion and absorption in the small intestine. Consequently, equine faeces may be used to replace rumen fluid as inoculum source (Can et al., 2009). Strong relationships were found between horse faecal suspension and rumen fluid suspension as inoculum sources for *in vivo* DM digestibility ($R^2 = 0.91$; $r = 0.83$) (Denek and Can, 2007) and between 24 h rumen fluid inoculum-gas production (RI-GP) and 48 h faecal inoculum-gas production (FI-GP) ($R^2 = 0.86$) in the HGT (Can et al., 2009).

However, ideally, the inoculum should be obtained from an appropriate host animal, fed a diet comparable to the experimental feeds (Mould et al., 2005). In addition, the availability and husbandry of ruminant animals on farms or in research organisations, and in some regions of the world, may be more accessible and therefore have practical advantages.

Bovine and ovine faeces

Given that a considerable proportion of rumen bacterial species persist in the hindgut and their microbial residues are eventually excreted with the faeces (van Soest, 1994), faecal material of ruminants can also constitute a viable alternative source of microbial inoculum for *in vitro* assays. Several researchers have

investigated the use of ruminant faecal material as an inoculum source, either by comparing rumen fluid with bovine faeces (Akhter and Hossain, 1998; Cone et al., 2002; Mauricio et al., 2001) or with ovine faeces (Aiple et al., 1992; Borba et al., 2001; El Shaer et al., 1987; Váradyová et al., 2005). Using the two-stage technique of Tilley and Terry (1963), El Shaer et al. (1987) observed a close correlation between *in vivo* apparent and *in vitro* predicted digestibility with faecal inoculum (FI) ($r = 0.98$) and Akhter and Hossain (1998) found the relationship between estimated OMD using rumen fluid inoculum (RI) and FI to be highly significant ($p < 0.001$; $R^2 = 0.97$). Faecal material has also been applied as an inoculum source for use in GP techniques, as carried out by Mauricio et al. (2001) who followed the method of Theodorou et al. (1994). The authors reported a strong correlation between *in vivo* OMD and *in vitro* OMD estimated from FI ($r = 0.77$), as well as between *in vitro* OMD derived from FI and RI ($r = 0.81$). In addition, Aiple (1993) applied the use of FI in the HGT to predict *in vivo* OMD by including FI-GP and chemical composition of the feeds in multiple linear regression models, resulting in a highly accurate prediction ($R^2 = 0.93$, RSD = 5.1%). According to these results, both bovine and ovine faeces appear to have the potential to be used as inoculum sources to replace rumen fluid in the *in vitro* evaluation of ruminant feeds. However, even under identical dietary and environmental conditions, the host species remains an important factor influencing the microbial composition of the ruminant gastrointestinal tract (Henderson et al., 2015; Szeligowska et al., 2021). Accordingly, the selection of bovine or ovine faeces as the inoculum source is likely to impact the results of *in vitro* analyses. Aiple (1993) investigated the effect of donor animal species on GP and observed that fermentation activity in sheep faeces was clearly superior to that in cow faeces, as across all tested feeds, the GP measured after 48 h of incubation was significantly higher when sheep faeces were used as the inoculum source. The author hypothesised that this difference could be attributed to the pellet-like structure of sheep faeces, which contrasts with the mushy consistency of cow faeces. It is possible that the pellet-like form may offer more favourable conditions for microbial survival, potentially by providing enhanced protection against oxygen exposure.

2.4 Objectives of the included studies

As described above, the idea of using faeces as an alternative inoculum source to replace rumen fluid for *in vitro* evaluation of feeds dates back several decades. The basis of this doctoral thesis was the work of Aiple et al. (1992) and Aiple (1993), who examined a large number of ruminant feeds in *in vivo* digestibility trials and *in vitro* in the HGT by using rumen fluid and sheep faeces as inoculum sources. From this, linear multiple regression equations were derived, including RI-GP or FI-GP, to estimate the OMD and energy value of ruminant feeds. A comparison of the regression analyses demonstrated that using FI provided a level of estimation accuracy comparable to that achieved with RI. For compound feeds and individual ingredients of compound feeds, the equations showed almost identical goodness of fit measures. Thus, Aiple et al. (1992) and Aiple (1993) have already demonstrated in their studies that sheep faeces have the potential to be used as an inoculum source for estimating the OMD and energy

value of ruminant feeds. However, in 1995, the reference method for calculating the metabolisable energy from digestible nutrients (GfE, 1995) was changed. Since then, the estimation equations developed by Aiple (1993) for the use of FI in the HGT were no longer applicable. Given that the feeds and the comprehensive associated data from the *in vivo* studies conducted by Aiple (1993) are no longer accessible, a direct continuation or further development of the equations is not feasible. The overall objective of the present thesis was, therefore, to use the protocol developed by Aiple (1993) in order to finally verify and establish the applicability of faeces as an inoculum source in the HGT.

In the *Manuscript 1* experiment, a wide range of currently relevant feeds for ruminants were used to systematically compare GP kinetics between sheep faeces and cow rumen fluid as inoculum sources *in vitro* in the HGT. The aim was to determine whether the GP of RI and FI follows a generally similar progression over time and to identify possible feed-specific differences between the two inocula. In addition, the objective was to examine the relationship between the GP of the two inocula at different time points to predict RI-GP of different feeds based on FI-GP. If this estimation could be achieved with high accuracy, the existing equations of the HGT using RI (calibrated against *in vivo* measurements) could continue to be used to estimate OMD and metabolisable energy of ruminant feeds, without the need to develop new equations based on additional *in vivo* experiments.

Since the results of the first experiment showed similar GP progressions and strong linear relationships between FI-GP and RI-GP, it was concluded that FI prepared with fresh faeces can be used as an alternative inoculum source in the HGT. The proven methodological approach with fresh faeces was the basis for further investigations with FI.

The ability to store faecal samples as an inoculum source for use in the HGT would enable stockpiling, which would help to standardise results and provide greater flexibility, while reducing the overall number of donor animals required. It was known from the literature that fresh rumen fluid could, in principle, be replaced by preserved rumen fluid, but preservation suitability strongly depends on the technique used, and microbial activity might be depressed. However, such data was not available for faeces. The *Manuscript 2* experiment, therefore, focused on preserving faeces prior to *in vitro* use in the HGT. Thus, several preservation methods of sheep faeces, including different freezing and freeze-drying processes and storage times, were studied for their effects on GP and the microbiome.

Besides applying FI in the standard GP technique of the HGT, the aim of the *Manuscript 3* experiment was to evaluate the potential of FI for estimating the protein value of ruminant feeds *in vitro*. Using RI, the eHGT method has been established, enabling the estimation of ruminally undegradable crude protein (RUP) and microbial crude protein (MCP) synthesis. These estimates are based on the measurement of NH_3-N after incubation of the feed samples in the eHGT. NH_3-N is a metabolic intermediate of feed CP degradation and serves as a source of N for microbial growth. Its concentration reflects the balance between CP degradation and microbially bound N (mN). Therefore, the aim was to measure and

compare this key parameter using FI and RI for different feeds *in vitro*. In addition, the calculated mN and the response to an extra source of energy, provided alongside the feed sample, were to be compared between the two inocula to better assess the suitability of sheep faeces as an alternative inoculum source for protein value estimation of ruminant feeds.

3 GENERAL DISCUSSION

3.1 Methodological considerations

Several factors relating to the animals and their feeding, faeces collection, inoculum preparation, and incubation severely influence the quality of the inoculum and thus the results of the *in vitro* assays. For this reason, these factors have been carefully considered in the planning of the experiments and will be discussed in the following. As mentioned above, the methodological approach to using FI was based on Aiple et al. (1992) and Aiple (1993), so reference is made to these studies where appropriate in the methodological considerations.

It should be noted that the methodological procedure for incubations using the RI source was based on the standard technique described by Menke and Steingass (1988) and all steps were conducted according to the established protocol of the standard HGT method (VDLUFA, 2012). This methodological approach is therefore not discussed below.

Sheep as donor animals – Housing and feeding

To reduce individual variation in faecal microbial composition between animals (Mamun et al., 2019; Szeligowska et al., 2021), inocula samples should be collected from different animals and pooled prior to use (Rymer et al., 2005). Therefore, in all experiments conducted within the present thesis, faeces were collected from at least three adult wether sheep. For the *Manuscript 1 and 3* experiments, three sheep were used, whereas for the *Manuscript 2* experiment, six sheep were used simultaneously to obtain the required amount of faeces for all treatments.

To standardise the conditions between the three experiments (*Manuscripts 1, 2, 3)*, the factors of housing and feeding of the sheep were handled equally. Changes in rumen population dynamics (van Glyswyk et al., 1992; Warner, 1966a, 1966b) and, consequently, in *in vitro* GP are known to occur as a result of differences in feed intake (Payne et al., 2002). To reduce variation in the composition and activity of the inoculum, Menke and Steingass (1988) suggested sampling rumen contents just before feeding and Mould et al. (2005) emphasised that donor animals should be offered the feed frequently to ensure *ad libitum* access. As faeces may be less directly affected by variations in digestive processes and passage rates than rumen fluid, faeces may also be less susceptible to diurnal variations in response to feeding. Supporting this assumption, Corbett and Pickering (1983) found that abomasal flow rates of organic matter (OM) and N within 24 h varied greatly depending on sampling time, while the variation in estimates of faecal OM and N excretion was small and essentially random. However, for animal welfare reasons, the aim was to avoid individual housing or separation, even during the feeding period, and to ensure permanent group-housing throughout the experimental periods. For this reason, a total mixed ration (TMR) in dry form was prepared for *ad libitum* access of the sheep in the present work.

Concerning the composition of the donor animal diet, a considerable effect on *in vitro* fermentation using rumen fluid has been observed, and this effect was even greater than that of the donor animal

species (Boguhn et al., 2013). The addition of concentrates to ruminant diets resulted in higher GP rates, lower lag times, and a higher extent of degradation in the rumen (Hervás et al., 2005), as well as higher total and viable bacterial counts in rumen fluid (Furchtenicht and Broderick, 1987) compared to pure roughage diets. It is known from studies in cattle that the diet composition also has a strong influence on the faecal microbiota (Kim et al., 2014; Shanks et al., 2011). Although little is known about the nutrient requirements and supply of hindgut microbes, their demand is likely similar to that of ruminal bacteria (Frey et al., 2010). Thus, to favour a dense and active microbial population in the hindgut and consequently in the faeces, the diet of the donor animals should provide some fermentable substrates for the lower digestive tract (Aiple et al., 1992). In addition, the diet should be compositionally diverse to avoid the influence of individual feed components on GP, so that the inoculum can be used to test roughages, single concentrate feeds, and concentrate mixtures *in vitro* (Aiple et al., 1992). Therefore, maize grain, rapeseed meal, grass hay, and straw were included in the TMR of the thesis experiments. Although not all ruminally undegraded substrate reaches the hindgut due to digestion in the small intestine, the TMR was formulated to contain ruminally undegradable starch and RUP, with the aim of providing nutrients likely accessible to hindgut microbes. Unlike Aiple (1993), who used wheat grain, barley grain, and oat grain as starch sources, maize grain was chosen for the experiments of the present thesis because maize starch is degraded more slowly and to a lesser extent in the rumen (Krieg et al., 2017; Seifried et al., 2016). Rather than using soybean meal as a protein source, as done by Aiple (1993), rapeseed meal was chosen in the present experiments due to its lower ruminal *in situ* CP degradation rate, reduced effective CP degradation, and higher proportion of RUP (Maxin et al., 2013). Molasses was added to bind the concentrate components to the chopped roughages. The animals were kept on sawdust to ensure that their nutrient intake came exclusively from the TMR.

Since dietary changes alter the microbiota and fermentation processes in the rumen, and the time required to re-stabilise differs (Hackmann, 2015), the donor animals must be given a sufficient time to adapt to the experimental diets. Reports on the time required for the ruminal environment to adapt to a dietary change vary, ranging from 3 to 6 days (Annison et al., 1959) or 7 days (Storry and Sutton, 1969). Machado et al. (2016) found that rumen fermentation patterns and rumen microbiota stabilised within 4 to 11 days and 3 to 9 days, respectively, after cows were subjected to a diet change. The authors concluded that it is logical to assume that faecal content is affected by the quantity and composition of digesta leaving the rumen. They observed that faecal mass and composition stabilised about 0.7 days after ruminal digesta composition had stabilised following the diet change. Therefore, a period of 14 days for adaptational feeding was considered appropriate for the present experiments to stabilise the conditions and the microbial composition of the sheep faeces.

Faeces collection, inoculum preparation, and* in vitro *incubation

A standardised procedure of faeces collection and inoculum preparation is necessary to minimise variability of the inoculum and to ensure reproducibility. Figure 1 illustrates the approach applied in

Manuscripts 1 and 3, including the collection of faeces in the stable, transport to the laboratory, pooling of faeces, combination with the medium solution, and conditions up to starting the incubation.

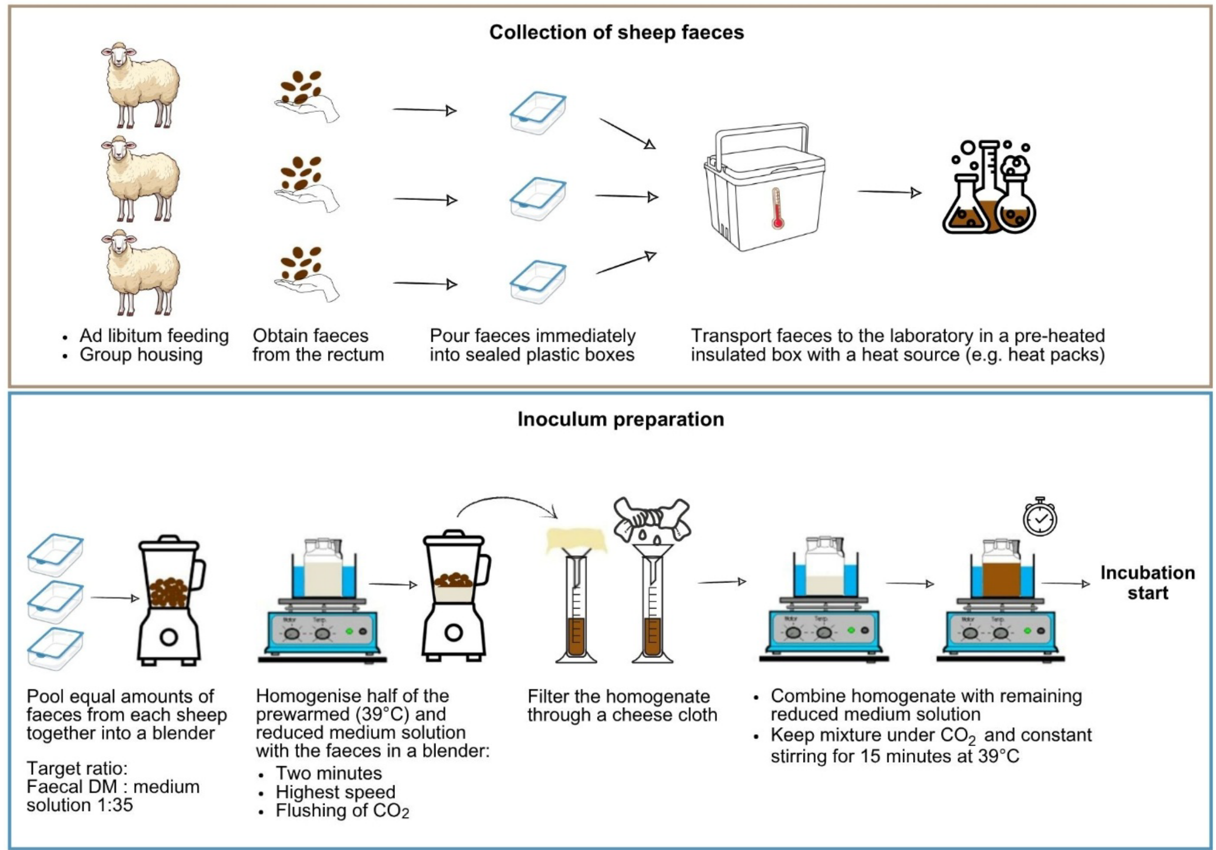


Figure 1. Sheep faeces collection and inoculum preparation procedures for *in vitro* incubation in the Hohenheim gas test.

The rumen environment is essentially anaerobic (Hobson, 1969) and the microbial community, consisting of anaerobic bacteria and ciliate protozoa, is specifically adapted to these conditions (Dehority, 2002). Many of the obligate anaerobic bacteria of the rumen are also present in the hindgut (Demeyer, 1991). Some of the faecal microbes are highly sensitive to oxygen, which has been observed in the study of Aiple et al. (1992). The authors reported that fresh faeces stored aerobically at room temperature rapidly lost microbial activity, resulting in decreased GP within one hour for incubated hay and within 12 h for concentrate feeds. This highlights the sensitivity of bacteria, especially cellulolytic bacteria, to unfavourable conditions, which is why the authors suggested that faeces should be collected rectally to maintain high microbial activity. Therefore, the faeces of the *Manuscript 1*, *2*, *and 3* experiments were obtained from the rectum of the sheep.

To achieve a high microbial activity of the FI, Aiple (1993) investigated different dilutions of faeces with the medium solution. The dilution was defined as the ratio of faeces in g DM to the volume of medium solution in mL. Dilutions ranging from 1:10 to 1:90 were tested, and the results showed that those between 1:20 and 1:50 yielded similar GP values. Dilutions above or below this range resulted in

lower GP. To remain within this suggested dilution ratio despite individual and daily variations in faecal DM of the animals, a ratio of 1:35 was aimed for in all experiments of this thesis.

As the consistency of the sheep faeces is very compact, the pooled faeces from the three sheep (*Manuscripts 1 and 3*) or six sheep (*Manuscript 2*) were mixed with half of the prewarmed medium solution in a blender. This step was intended to detach microbes from particulate matter, thereby supporting a high microbial density in the liquid faecal suspension after filtration.

Variations may also occur between incubation runs due to the incubation environment, such as the incubation oven. Therefore, in order to perform a direct comparison of FI and RI *in vitro* in the HGT, it would have been preferred to incubate the feeds with the two inocula simultaneously in the same run and oven. This was difficult to implement due to the large number of feeds to be tested and the resulting capacity constraints. In addition, parallel or staggered preparation of both inocula would have carried the risk of a higher level of error because the laboratory workflow would have been far more complex. Therefore, after careful consideration, it was decided to conduct the FI and RI incubations consecutively in separate blocks (*Manuscripts 1 and 3*). The replicates were generated over the number of incubations, thus balancing fluctuations in individual incubation runs. This procedure was considered appropriate, as each run included blank values containing only the buffered inoculum to provide information on possible interferences during incubations. As an additional control, standard hay and concentrate feed samples with known GP values of RI were included in each run.

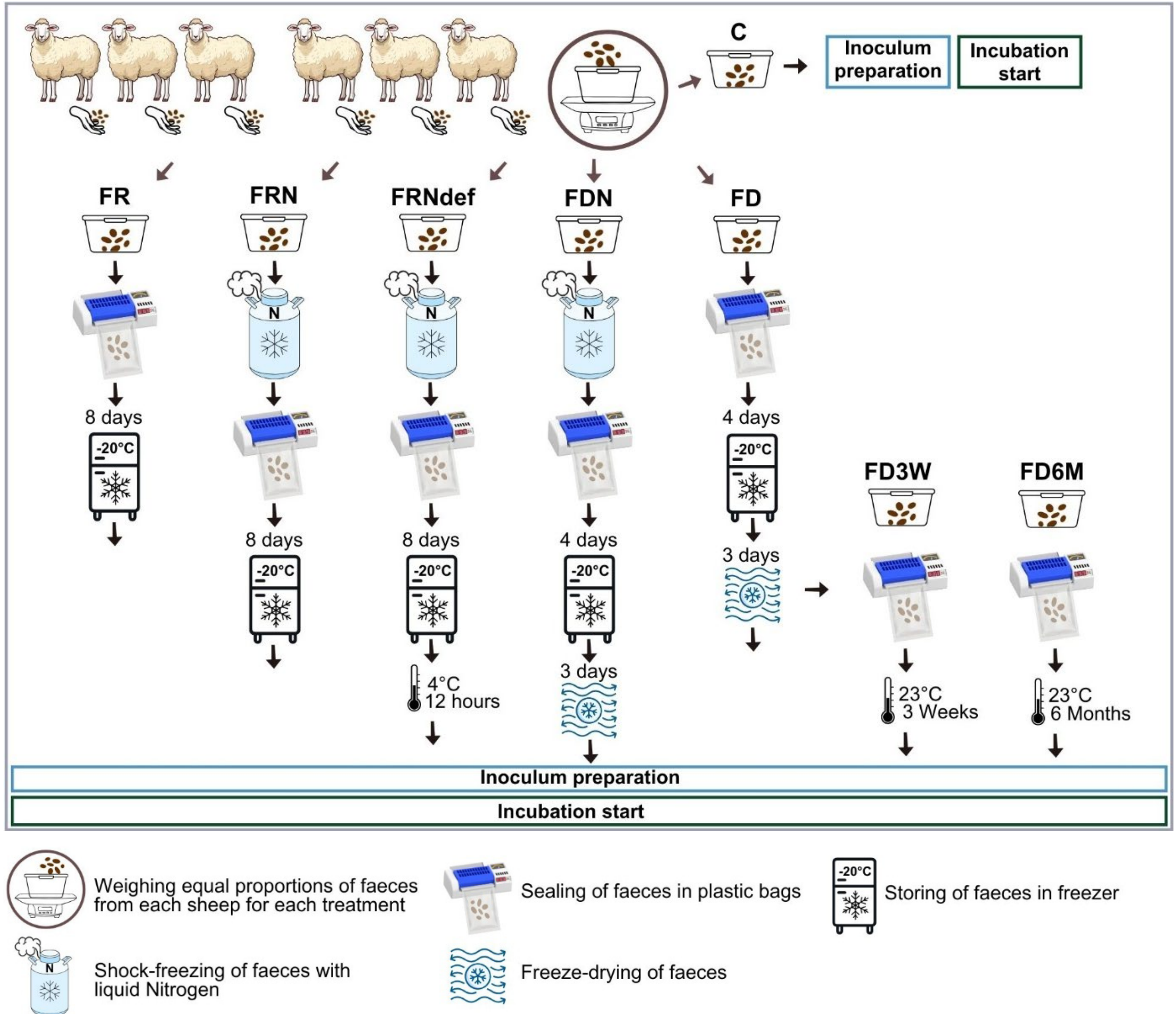
Faeces preservation

Several methodological considerations were taken into account, aiming to maintain a high microbial activity in FI after preservation of faeces in the *Manuscript 2* experiment. In addition, factors of practicality and, therefore, the future suitability to be used as a standardised technique for the HGT also played a role. Due to the lack of reports on preserving faeces before use as an inoculum source for *in vitro* analysis of ruminant feeds, previous studies on rumen fluid preservation for this purpose were used as guidance.

Refrigeration is a preservation method that has been applied to rumen fluid and suggested as a possible alternative to fresh rumen fluid as an inoculum source for short storage periods. Several researchers found that GP (Hervás et al., 2005) or other fermentation traits (Spanghero et al., 2019) were not depressed up to 6 h of refrigerated storage. Fabro et al. (2020) observed no negative effect on GP after 72 h of refrigeration, but found lower values after 96 h. Hervás et al. (2005) reported a reduced fermentation rate after 24 h of storage. However, despite the potential of short-term storage of rumen fluid, this method was not included in the present experimentation, as the focus was on achieving longer storage durations for the inoculum source. Another method considered was the use of cryoprotectants such as glycerol or dimethyl sulfoxide. These substances protect cells during freezing by promoting vitrification, thus preventing ice crystal formation and mechanical damage (Denek et al., 2010; Prates et al., 2010). Glycerol can also serve as an energy source for the bacteria after thawing, but this can lead

to an increase in propionate and butyrate proportions, while acetate levels decrease (Prates et al., 2010; Rémond et al., 1993). Prates et al. (2010) also found a higher GP *in vitro* and overall concluded that, while glycerol is beneficial for preserving bacterial diversity, it is not suitable for use in fermentation studies. Dimethyl sulfoxide can be cell toxic, especially at high temperatures (Wolfe and Bryant, 2001). For the present experiment, it was therefore decided not to use cryoprotectants for the *in vitro* analysis of feeds.

Figure 2 provides an overview of the faeces collection and subsequent processing steps for all chosen treatments and preservation techniques conducted to study GP kinetics in the *Manuscript 2* experiment.



C, Fresh faeces; FR, Frozen faeces; FRN: Shock-frozen faeces with liquid nitrogen; FRNdef, Faeces treated like FRN followed by defrosting prior to incubation; FDN, Shock-frozen faeces with liquid nitrogen and freeze-dried prior to incubation; FD, Freeze-dried faeces; FD3W, Freeze-dried faeces stored for 3 weeks; FD6M, Freeze-dried faeces stored for 6 months.

Figure 2. Faeces collection and preparation of the fresh control treatment and preservation treatments.

Direct freezing of the inoculum source would be a practical and easy-to-implement method of preservation. In contrast to liquid rumen fluid, where a high surface-to-volume ratio is necessary for

rapid freezing and thus successful preservation (Prates et al., 2010), the loose, pellet-like sheep faeces provide a decisive advantage. Because of this favourable physical property, the simple technique of direct freezing was included (FR). FR was frozen at -20°C, as this temperature provides a lower maintenance cost compared to -80 °C and has been shown to have no significant adverse effect on the pH value, NH_3-N, MCP, or volatile fatty acid concentration compared to -80 °C in a study that used rumen fluid (Fu et al., 2024). Since liquid N considerably accelerates the freezing process due to its extremely low temperature of approximately -196 °C, it was also tested in the experiment. One purpose was to assess whether the treatment with liquid N could improve the preservation effect of frozen faeces (FRN). In both frozen treatments (FR, FRN), the faeces were directly mixed with the prewarmed medium solution, as described for fresh faeces in Figure 1, since rapid thawing of rumen fluid, similar to rapid freezing, has been shown to be beneficial (Prates et al., 2010). However, as the microbiome was exposed to a severe temperature shock during this process, a potentially gentler method of thawing faeces for 12 h at 4 °C before use in the HGT was also tested (FRNdef). The method of freeze-drying has been previously examined in rumen fluid studies (Belanche et al., 2019; Spanghero et al., 2019) and could provide advantages in terms of storage and transport of faeces. For these practical and logistical reasons, it was included in the present experiment. Freeze-drying was carried out on faeces that had previously been either shock-frozen with liquid N (FDN) or frozen at -20 °C (FD). The latter was additionally stored at room temperature for 3 weeks (FD3W) or 6 months (FD6M). These two stored treatments were of particular importance, as the practical benefit of this method depends on preserving microbial activity over an extended period.

Ultimately, the suitability of using preserved faeces in the HGT depends on the observed GP, which indirectly provides information on fermentation processes and thus microbial activity present in the inoculum. However, the microbiome was also analysed in this experiment to understand better the effect of each preservation treatment on the microbial community and to assess better which treatments maintain or reduce microbial diversity. Aiming to identify the microbial cells contributing to fermentation directly and thus to GP, a metaproteomic workflow was used to detect proteins recently translated in actively growing microbial cells, as opposed to DNA, which may be artefacts from remaining inactive cells. For the microbiome assay, only treatments C, FR, FRN, FD, and FDN were examined for capacity reasons, following the same methodological approach as shown in Figure 2.

A critical reflection on the methodological approaches presented here is provided in Chapter 3.5.

3.2 Comparison of fresh faeces and rumen fluid as inoculum sources

The experiments comparing FI and RI in the HGT revealed differences in GP kinetic parameters, NH_3-N values, and mN values between the two inocula, particularly in the first hours of incubation. The following section intends to outline factors that influence the characteristics of the two inoculum sources, likely affecting the microbial activity and, consequently, may have influenced the study results.

3.2.1 Microbial community differences between faeces and rumen fluid

The differences observed between incubations with FI and RI (*Manuscripts 1 and 3*) are probably attributable to the different microbial communities in rumen fluid and faeces, which show the greatest differences in diversity, relative to other gastrointestinal tract sections (Oliveira et al., 2013). The rumen microbiota is predominantly composed of bacteria, with smaller populations of archaea, anaerobic fungi, and ciliate protozoa (Tapio et al., 2016). The rumen bacterial community is dominated by the phyla Firmicutes and Bacteroidota (Ozbayram et al., 2018; Tapio et al., 2016). At the genus level, *Prevotella*, *Ruminococcus*, *Succiniclasticum, Saccharofermentans* (Holman and Gzyl, 2019; Li et al., 2012; Liu et al., 2016), and *Fibrobacter* (Holman and Gzyl, 2019) were most abundant in rumen contents. In faeces, Firmicutes is much more prominent (Oliveira et al., 2013; Ozbayram et al., 2018) and Bacteroidota is less abundant (Oliveira et al., 2013; Tapio et al., 2016). Genera that are strongly associated with faeces include *Clostridium*, *Turicibacter,* unclassified *Peptostreptococcaceae, Treponema* (Liu et al., 2016), *Alistipes*, *Bacteroides*, *Faecalibacterium*, and *Escherichia-Shigella* (Holman and Gzyl, 2019). The anaerobic fungal species identified in faeces were similar to those in the rumen, with minor differences in abundance (Tapio et al., 2016). Studies have found protozoa to be low in abundance (Tapio et al., 2016) or absent (Hobson, 1971; Kern et al., 1974) in faeces. The faecal archaeal population was dominated by *Methanocorpusculum* rather than *Methanobrevibacter* (Tapio et al., 2016), with the latter being an important member in the rumen (Oliveira et al., 2013). Rumen and large intestine microbiota should therefore be regarded as different populations that happen to share many common species (Mould et al., 2005). Thus, differences in the microbial activity of the two inocula FI and RI, and hence differences in the traits studied in the *Manuscript 1 and 3* experiments, were to be expected.

3.2.2 Microbial activity differences in the hindgut and rumen

The *Manuscript 1* experiment showed a reduced potential GP and GP rate on average across all examined feeds and regardless of the feed categories. The *Manuscript 3* experiment demonstrated overall lower NH_3-N and mN values with FI compared to RI across the incubated feeds. In addition, FI incubations showed a prolonged lag phase in GP (*Manuscript 1*) and an apparent delay in N binding by microorganisms (*Manuscript 3*) compared to RI. These findings may be attributed to a smaller microbial population and lower microbial density in the hindgut than in the rumen, likely resulting from a less degradable substrate in the large intestine and colon (Tanca et al., 2017) and a relatively high passage rate in the hindgut (Fon and Nsahlai, 2012). Information on the microbial density of ruminant faeces is scarce in the literature. When the approach of Nagadi et al. (1999) for estimating rumen microbial population densities by absorbance measurements was applied to FI, inconsistent effects were reported (Rymer and Givens, 2000). The authors therefore concluded that absorbance measurements may not be appropriate for analysing faeces. Posada et al. (2012) studied the microbial density in FI and RI obtained from cattle faeces and rumen fluid by counting bacteria, fungi, and protozoa after cultivation. FI thereby showed considerably lower total bacterial counts and about half the fungal counts than RI, and no

protozoa were present. As the microbial density was examined in the two inocula rather than the inoculum sources faeces and rumen fluid in the study of Posada et al. (2012), the inoculum preparation procedure may have influenced the microbiome, preventing direct information about initial microbial densities of faeces and rumen fluid. However, since inoculum preparation was carried out with both inoculum sources prior to analysis, and the differences in microbial counts were pronounced between FI and RI, it is reasonable to assume that faeces initially had a lower microbial density in that study. In contrast, Mao et al. (2015) found no significant differences in total bacterial density between rumen and rectal digesta in cattle by conducting 16S rRNA gene sequencing, a DNA based method. DNA is rapidly degraded in the bovine ileum (Smith and McAllan, 1971) and PCR results are therefore ought to represent cell-bound DNA (Frey et al., 2010). However, PCR results are associated with dead or damaged cell artefacts (Codony et al., 2020). The most commonly used DNA-based methods are unable to distinguish between viable and non-viable community members, which limits knowledge of basic microbiome functions (Wang et al., 2023). The passage of digesta and its associated microbes trough the abomasum, characterised by a low pH, may contribute to the absence of sensitive microorganisms such as protozoa in faeces (Posada et al., 2012). These microorganisms may transiently appear in an inactive state, potentially leading to an overestimation of total bacterial density in DNA-based analyses of faeces. A lower microbial density in the faeces could therefore have contributed to a generally lower microbial activity in FI and a longer time to colonise the substrate after the incubation started.

Alternatively, the lower GP and lower N metabolism of the microbes observed with FI incubations could be due to a lower enzymatic activity of the faecal microbiome compared to the rumen microbiome. This was suggested from results of Michalet-Doreau et al. (2002), who investigated RNA and enzymatic activity in the rumen and caecum to compare the cellulolytic microbial ecosystems. The authors reported that the sum of the three main cellulolytic bacterial species (*Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus*), expressed relative to the total bacterial signal, was equal between the two digestive compartments. On the other hand, enzyme activities (polysaccharidase, glycosylase) were higher in the rumen than in the caecum, possibly due to different physiochemical conditions and the amount of potentially degradable substrate in the rumen *vs.* caecum (Michalet-Doreau et al., 2002).

Ultimately, these published findings point to a reduced microbial activity in ruminant faeces compared to rumen fluid. It is likely, though, that a lower microbial density and a lower enzymatic activity contributed in combination. Accordingly, degradation and fermentation of the feeds incubated with FI were slower and less extensive than with RI in the *Manuscript 1 and 3* experiments. This is further supported by several studies reporting lower total volatile fatty acids production with FI compared to RI (Cutrignelli et al., 2007; Váradyová et al., 2005; Zicarelli et al., 2011).

Another metabolic pathway may also have contributed to the results of *Manuscript 1 and 3* experiments. In addition to the production of fermentation end products (e.g. gases, volatile fatty acids), microbial biomass is synthesised, a process which can significantly influence the fermentation balance

(Zhang et al., 2024). Blümmel et al. (1997) demonstrated an inverse relationship between microbial biomass and *in vitro* GP per unit of degraded substrate using RI in the HGT. Similarly, Zhang et al. (2024) found that net microbial yield, defined as the difference between microbial mass at the end and start of *in vitro* fermentation, was negatively correlated with total GP using RI in the HGT. The authors also reported that a higher initial inoculum microbial mass was related to a lower net microbial yield. It is therefore possible that an initially lower microbial biomass in FI, as suggested by the above discussion, resulted in a greater proportion of degraded substrate being partitioned to microbial synthesis rather than to the production of gases and acids. This could explain the lower GP observed in the *Manuscript 1* experiment and the lower volatile fatty acids production reported in the literature using FI than RI *in vitro*. Furthermore, it could also account for the similar response of FI and RI to an additional energy source, i.e. the incorporation of N into MCP per mL of GP, which was found after 24 and 48 h of incubation in the *Manuscript 3* experiment. This similar response was notable, as the lower mN and NH_3-N values obtained with FI than RI indicated a more inert microbial activity. However, an initial lower microbial biomass in FI could have shifted the metabolic pathways towards a higher microbial synthesis per unit of degraded substrate in FI, possibly compensating for the generally lower activity compared to RI.

Overall, despite the delayed start of fermentation and the apparently lower microbial activity in FI compared to RI, similar GP progressions were observed with increasing incubation time (*Manuscript 1*), suggesting that the two inoculum sources undergo a similar type of fermentation. This finding highlights the potential of FI to be applied in the HGT, since a similar GP progression and a constant ratio between FI-GP and RI-GP are crucial prerequisites for mathematical approaches to estimating RI-GP from FI-GP, which will be discussed in Chapter 3.4.1. Furthermore, the comparable responses of FI and RI to an additional energy source in terms of microbial N incorporation suggests the potential for using FI in the eHGT, which will be further addressed in Chapter 3.4.3.

3.3 Comparison of fresh and preserved faeces as inoculum sources

3.3.1 Preservation impact

In the *Manuscript 2* experiment, all faeces preservation treatments (Chapter 3.1, Figure 2) affected the actively growing microbial cells and GP kinetic parameters *in vitro* compared to the fresh control treatment C. The magnitude of the preservation effect on GP and the microbiome varied between treatments. Lower potential GP and GP rates of the frozen treatments FR, FRN, and FRNdef compared to C were likely a result of some freezing stressors. Intra- and extracellular ice crystal formation, as well as an increased osmotic pressure, can lead to bacterial membrane disruption (Malik, 1991; Meryman, 2007) and thus may represent such stress for the microbes. However, across all feeds, the potential GP in FR and FRN incubations decreased by less than 2% compared to treatment C incubations and showed GP curves close to C. This was consistent with the common grouping of metaproteomic datasets in these three treatments, based on the relative abundance of core proteins (Figure 3, *Manuscript 2*), the

abundance of the phylum Bacteroidota (Figure 4C and D, *Manuscript 2*), and functional redundancy values (nFR) (Figure 5, *Manuscript 2*). In contrast to these results, some studies that investigated frozen rumen fluid as an inoculum source *in vitro* found severely lower GP parameters compared to the use of fresh rumen fluid (Hervás et al., 2005; Prates et al., 2010; Tunkala et al., 2022). The more adverse results of frozen rumen fluid reported in the literature compared to frozen faeces in the *Manuscript 2* experiment were likely related to the physical properties of the sheep faeces. Cold denaturation occurs in water because lower temperatures increase the hydration, and therefore the solubility, of nonpolar groups, which weakens solvophobic forces and reduces protein stability (Wang, 2000). This process could have affected the rumen fluid as a liquid more than the pellet-like sheep faeces with a rather low moisture content. Additionally, the sheep faeces presumably freeze more rapidly than the liquid rumen fluid. A faster freezing rate produces smaller ice crystals due to the rapid crystallisation of supercooled water, thus preventing protein denaturation caused by extensive crystal growth and reducing loss of microbial activity (Wang, 2000). Regarding fast freezing, liquid N was used to preserve faeces in the treatment FRN in the *Manuscript 2* experiment. Contrary to expectations, shock-freezing with liquid N prior to freezing at -20 °C in FRN was not beneficial compared to FR, possibly because direct freezing at -20 °C was a gentler process compared to that caused by the extremely low temperatures of liquid N. Also, the additional defrosting process included in the FRNdef treatment did not improve GP parameters compared to FR and FRN. Bacterial groups adapt to low temperatures through a complex cold shock response, which involves various metabolic processes, including the synthesis of proteins that favour sugar metabolism (Phadtare and Inouye, 2008). An increase in sugar metabolism may have resulted in a faster consumption of the available energy resources by the faecal microbes already during storage at 4 °C for 12 h with FRNdef. This could have potentially led to an earlier onset of microbial starvation and lysis once energy became limited. As a consequence, this may have reduced the fermentation activity of the FRNdef inoculum from the beginning of the incubation, which could explain the extended lag phase and the reduced overall GP.

In contrast to C and the frozen treatments, the freeze-dried treatments FD and FDN revealed a strong decrease in potential GP and a distinct lag phase across all feeds. This was supported by a clear separation of these treatments from C and the frozen treatments in the metaproteomic dataset across the different analyses included in *Manuscript 2*. Furthermore, proteins associated with potential spore formation were identified and assigned to Negativicutes in higher abundance in FD and FDN samples. This demonstrates the strong impact of freeze-drying on the microbiome, which may be due to this process causing both freezing and drying stresses. As the water content of a freeze-dried product is less than 10%, a part of the hydration shell is removed during freeze-drying, which can disrupt the natural state of a protein and lead to denaturation (Wang, 2000). Additionally, water molecules can also be an integral part of protein active sites, thus removal of these water molecules due to dehydration can readily cause inactivation of proteins (Wang, 2000). When comparing FD and FDN, a common grouping of these two treatments was found based on a low variance among the relative abundance of core protein

groups (Figure 3, *Manuscript 2*). However, compared to C, treatment FDN more frequently caused significant negative effects on the abundance of proteins assigned to different phyla than FD (Figure 4A, *Manuscript 2*). Although a fast freezing process can help to protect the protein structure, as mentioned above in frozen treatments, a slower freezing process that possibly generates larger ice crystals may be beneficial during subsequent drying. Larger ice crystals increase water sublimation during the primary drying stage of the freeze-drying process, whereas small ice crystals, possibly generated by liquid N freezing, result in slower sublimation rates (Hottot et al., 2004). This may have optimised the drying process in FD and produced a more stable freeze-dried product than FDN.

Across the feeds examined in the *Manuscript 2* experiment, FR and FRN treatments revealed no significant differences in potential GP compared to C. In contrast, FD and FDN were associated with a significantly lower potential GP and a significantly prolonged lag phase for all feeds compared to treatment C. Moreover, a pronounced decrease in potential GP and an extended lag phase or the impossibility of estimating GP parameters were found for roughages rather than concentrate feeds with FD and FDN. This was consistent with several reports in the literature that used preserved rumen fluid as an inoculum source and observed a more pronounced negative effect of preservation on *in vitro* fermentation of fibrous feeds than high-starch feeds (Garcia et al., 2021; Hervás et al., 2005; Tunkala et al., 2022; Zeigler et al., 2003). Two terms – resilience and redundancy – are likely to have contributed to the overall and feed-associated differences observed and are therefore outlined in the following. Microbial resilience describes the ability to withstand and recover from perturbations, while the microbial response depends on the intensity, frequency, and duration of the perturbation (Weimer, 2015). Redundancy in terms of the rumen microbial community can be defined as the overlapping distribution of physiological abilities among multiple microbial taxa (Weimer, 2015). The high number of different rumen microbial species relative to the degradation sites suggests that several species contribute to the degradation of each feed substrate or linkage, i.e. the specific chemical bonds within these substrates, making the rumen microbial community highly redundant (Weimer, 2015). This agrees with studies reporting similar basic ruminal fermentation parameters such as pH, volatile fatty acid concentrations, or molar volatile fatty acid proportions despite substantial changes in microbial community composition (Sandri et al., 2014; Welkie et al., 2010). It is most likely that microbial resilience and redundancy in other ecosystems, such as the rumen, are also present in the hindgut and thus in faeces. In the *Manuscript 2* experiment, significant adverse effects on the abundance of proteins assigned to the different phyla were observed for the FR and FRN treatments. Still, on the other hand, there was also a significant increase in the proteins of some other phyla compared to C. It is therefore possible that, due to a high microbial redundancy, all feeds were fermented to a similar extent compared to the fresh faecal treatment C, although freezing altered the faecal microbial composition. This assumption is supported by the nFR analysis (Figure 5, *Manuscript 2*), which showed no significant differences in nFR values between C, FR, and FRN. In contrast, significantly lower nFR values were observed for FD and FDN. Regarding these treatments, the adverse effects on relative abundance of

phyla occurred considerably more often compared to FR and FRN. Although for some phyla the abundance was also increased in the freeze-dried treatments, the significantly negatively affected phyla predominated in these treatments. It is therefore possible that the microbiome of the faeces was highly resilient to freezing. The physical perturbation caused by the freeze-drying process, on the other hand, could have appeared to be too intense to ensure a high recovery rate of the microbial community, especially of the microbes involved in fibre degradation, which are apparently more susceptible to preservation. As a result, microbial redundancy in FD and FDN did not appear to maintain the fermentation activity of C compared to FR and FRN.

3.3.2 Storage impact

Freeze-dried storage

The adverse effects of the freeze-drying process on the GP kinetic parameters became more pronounced with storage in the *Manuscript 2* experiment, particularly after 6 months. Wang (2000) stated that even when the freeze-drying process was successful, long-term storage stability can be limited due to various chemical degradation processes, such as protein aggregation, oxidation, or hydrolysis. Common reactives that affect the stability of freeze-dried products are moisture, oxygen, or temperature (Costa et al., 2002; Lievense and van’t Riet, 1994; Morgan et al., 2006; Wang, 2000). Despite the typically low moisture contents in freeze-dried products, hydrolysis can still occur during storage (Wang, 2000). Furthermore, it has been reported that oxidation in freeze-dried samples in air-filled vials occurred at a rate comparable to that in a solution sample at temperatures between 25 °C and 30 °C (Fransson et al., 1996). As small amounts of oxygen can already cause severe microbial damage (Lievense and van’t Riet, 1994), this may have contributed to the poor GP results obtained with FD3W and FD6M. The sealing of faeces from these treatments in low barrier plastic bags could have facilitated possible oxygen ingress (Costa et al., 2002). In addition, increasing temperatures enhance the mobility of protein molecules, which promotes protein interactions and thus accelerates the physical aggregation of proteins in a solid state, which is one of the greatest instabilities for freeze-dried proteins (Wang, 2000). Lievense and van’t Riet (1994) also pointed out that storage stability increases with decreasing temperature, and that storing bacterial cultures above 10 °C for several months does not appear feasible without a significant loss of activity. This supports the findings observed for FD3W and particularly for the prolonged storage of FD6M, as both treatments were stored at 23 °C. This demonstrates that, in addition to the unavoidable stresses on the microbiome associated with the freeze-drying process, factors during subsequent storage also appeared to affect the stability of microbiome activity. However, Granja-Salcedo et al. (2017) stored freeze-dried pellets prepared from rumen fluid at -20 °C for 3, 6, and 12 months and found that the yield of extracted DNA decreased with increasing storage time. Although the experimental conditions differed from those of the faecal treatments used in the *Manuscript 2* experiment, these results suggest that even frozen storage of freeze-dried samples is not sufficient to preserve microbial activity over an extended period. Furthermore, the storage condition of freezing

would be of limited practical value as it would negate the clear advantages of freeze-dried faeces in terms of logistical requirements for storage and transport.

Frozen storage

If storage at lower temperatures appears to be beneficial for maintaining microbial activity as mentioned above, it would be more appropriate to store the frozen instead of the freeze-dried faeces under freezing conditions. In addition to the aforementioned freeze-dried stored samples, Granja-Salcedo et al. (2017) also investigated the effect of storage at -20 °C for 3, 6, and 12 months on rumen fluid pellets that were frozen prior to storage. Although a decrease in the yield of extracted metagenomic DNA was also observed in the frozen pellets, it was considerably less pronounced compared to the difference between the freeze-dried samples and the control treatment.

The *Manuscript 2* experiment did not include any frozen storage treatment due to capacity reasons. However, a further trial was conducted to study the effect of frozen storage on *in vitro* GP parameters. For this purpose, faeces were collected as described in *Manuscript 2*, but from three wether sheep. The faeces were either used immediately as an inoculum source in the HGT as the fresh control, or treated as FR, but stored for 6 months instead of 8 days at -20 °C prior to *in vitro* use (FR6M). These inocula were used to incubate the feed sample material from the *Manuscript 2* experiment. For the sake of clarity, this trial will be referred to as *FR6M experiment* in the following. In contrast to the *ad libitum* TMR in dry form used in the *Manuscript 1, 2, and 3* experiments, grass hay and straw were offered *ad libitum* in the *FR6M experiment*, but the concentrates were fed to each sheep in two equal portions in the morning and evening. As in the TMR, the concentrates consisted of crushed maize grain and rapeseed meal, of which each sheep received 0.34 kg and 0.14 kg per day, respectively. The daily amount of concentrates was based on the average amount of concentrates consumed per sheep and day with the TMR used in the *Manuscript 1, 2 and 3* experiments. In the *FR6M experiment*, the GP kinetic parameters were calculated by using the approach described in *Manuscript 2* for each feed and averaged across the nine feeds. The FR6M treatment was then compared with the treatments FR and C of the *Manuscript 2* experiment, as it should be interpreted in the context of the *Manuscript 2* results. Treatment C of the *FR6M experiment* was also included in the GP kinetic comparison on average of all feeds, to provide a direct reference to the initial fresh faeces, from which the FR6M treatment was prepared. All mean GP kinetic curves are presented in Figure 3. The mean *in vitro* GP curves of the treatments C from both experiments were almost identical and showed no lag phase. This high level of agreement enabled the FR6M treatment to be compared with the results of the *Manuscript 2* treatments, despite the differences in sheep feeding. The GP curve of the FR6M treatment was at a considerably lower level than that of the C treatments and that of FR. Compared to FR, FR6M prolonged the lag phase by 1.7 h and reduced the potential GP by 16 mL/200 mg DM.

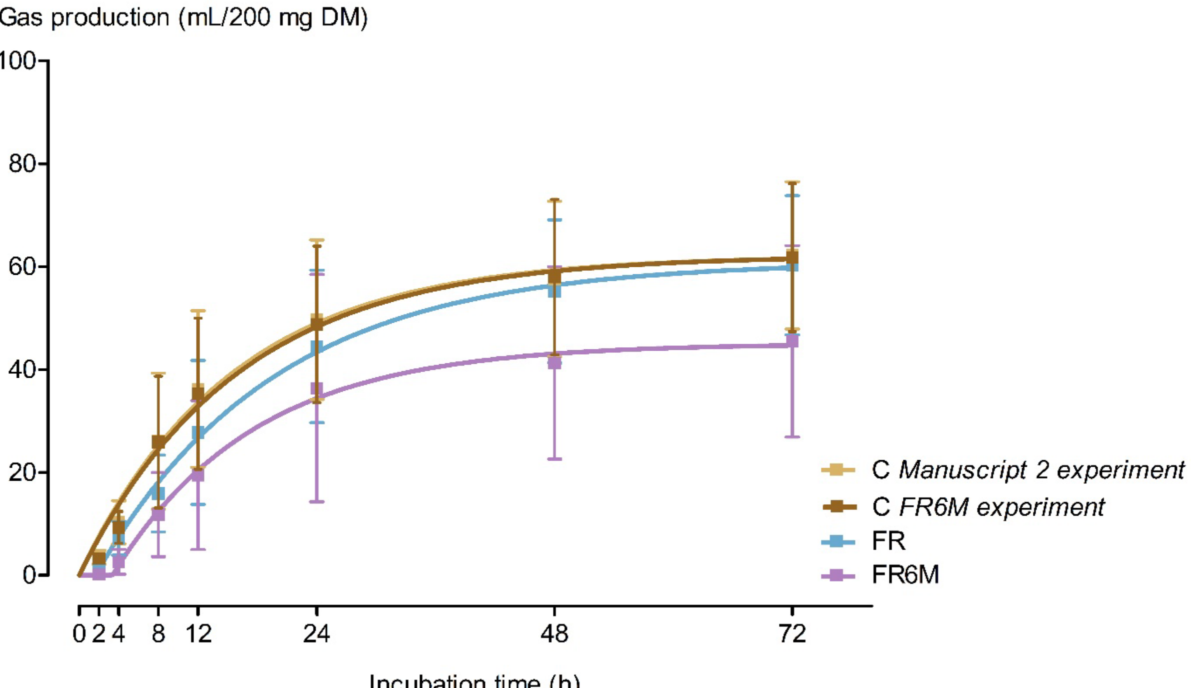


Figure 3. Mean *in vitro* gas production kinetics of faecal inoculum from fresh faeces of the *Manuscript 2* experiment (C *Manuscript 2 experiment*) and the *FR6M experiment* (C *FR6M experiment*), frozen faeces (FR), and frozen faeces stored for 6 months (FR6M) across nine feeds. Equation for C *Manuscript 2 experiment*: $Y = 62.09*(1 - e^{-0.06486*X})$, equation for C *FR6M experiment*: $Y = 62.22*(1 - e^{-0.06251*X})$, equation for FR: $Y = 61.03*(1 - e^{-0.05558*(X - 1.642)})$, equation for FR6M: $Y = 45.08*(1 - e^{-0.06983*(X - 3.359)})$. Bars represent standard deviation.

The GP kinetic parameters of the treatments C of *Manuscript 2* experiment, FR, and FR6M were analysed in a two-way ANOVA as described in *Manuscript 2* and visualised using bar charts. The two-way interaction of feeds and treatments was significant for potential GP and GP rate ($p < 0.001$) and for the lag phase ($p < 0.05$) (Figure 4). FR6M was associated with a lower potential GP for all feeds except wheat grain and resulted in a prolonged lag phase for all feeds except rapeseed meal, for which none of the treatments showed a lag phase. High GP rates were observed with FR6M, which were probably related to the simultaneously occurring prolonged lag phase. Differences between FR6M and the treatments C and FR were more pronounced for roughages than for concentrates.

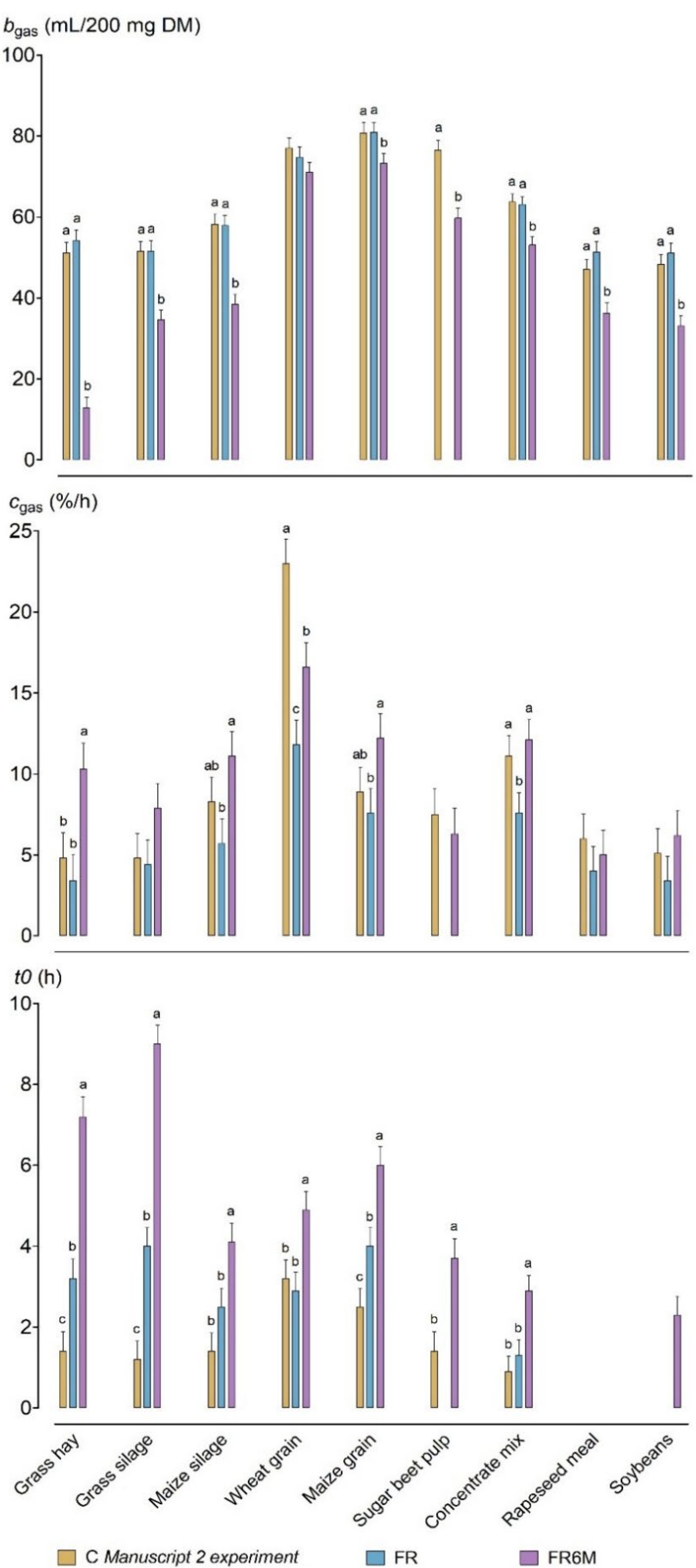


Figure 4. Effect of feed and preservation treatment on gas production kinetic parameters. Columns within statistical comparison not sharing the same letter are significantly different ($p \leq 0.050$). $t0$, b_{gas} and c_{gas} calculated from the fitted equation either without lag phase: GP = $b_{gas}*(1 - e^{-cgas*t})$ or with lag phase: GP = $b_{gas}*(1 - e^{-cgas*(t - t0)})$, with GP = gas production after t hours; b_{gas} = potential GP, c_{gas} = rate of GP of b; $t0$ = time of lag phase (h). C, Fresh faeces; FR, Frozen faeces; FR6M, Frozen faeces stored for 6 months. Bars represent standard deviation.

Overall, except for a shorter lag phase, the FR6M treatment closely resembled the freeze-dried stored FD3W and FD6M treatments of the *Manuscript 2* experiment, averaged over the feeds and concerning the individual feeds incubated. These findings indicate that, based on the applied approach, the storage duration had a detrimental impact on the activity of the faecal microbiome, even when the faeces were frozen stored at -20 °C. In this context, Fu et al. (2024) stored rumen fluid for up to 240 days and observed significant alterations in the concentration and proportion of volatile fatty acids, changes in NH_3-N concentrations, and significant changes in the pH value and MCP after 30, 14, and 7 days of storage, respectively. The authors therefore suggested that the assessment of rumen fermentation parameters should be carried out within 7 days post-collection. This supports the findings of the *FR6M-* and *Manuscript 2* experiments, as FR, stored for 8 days, showed low differences in the GP and metaproteomic datasets compared to C, which is in clear contrast to the impaired GP parameters observed with FR6M. The storage period of 6 months in the *FR6M experiment* seems rather long, considering the adverse effects after at least 7 days of frozen storage in the study by Fu et al. (2024). However, from reports on the storage of rumen fluid in the literature, no direct conclusion can be drawn regarding the storage impact on sheep faeces. Nevertheless, findings in the literature have demonstrated the significant impact of several stresses that appear to occur during the preservation and storage process. All reports indicate that storage duration influences microbiome stability, suggesting limitations in feasible storage time.

3.4 Relationships of sheep faeces and rumen fluid as inoculum sources - implications and recommendations for future application of faecal inoculum

3.4.1 Estimation of gas production using fresh faeces

Although the GP kinetic parameters were generally lower with FI than with RI in the *Manuscript 1* experiment and in other reports (Aiple et al., 1992; Cone et al., 2002; Mauricio et al., 2001; Zicarelli et al., 2011), FI incubations provide endpoint measures such as GP after 24 or 48 h that correlate well with RI measures (Rymer et al., 2005). This is of great relevance, given that the common application of the HGT relies on such endpoint measurements, and a close relationship between FI and RI in GP is a prerequisite for establishing the use of FI in the HGT. FI-GP and RI-GP were highly related in the *Manuscript 1* experiment. This was demonstrated by significant positive correlations between measured RI-GP at 24 h and estimated FI-GP throughout the 24 to 60 h of incubation period analysed. Furthermore, close linear relationships were observed for predicting RI-GP at 24 and 48 h from FI-GP at 24 and 48 h, respectively, when all 90 feed samples were included in the analysis. As the *Manuscript 1* experiment contained 90 different ruminant feed samples, a wide range of different-quality feeds was covered, and a large database was provided to obtain a high level of statistical confidence for the estimation of RI-GP from FI-GP. This was confirmed by the high accuracy obtained by validating the linear regression equations by splitting the dataset into a training and validation dataset (Table 5, *Manuscript 1*).

An additional and independent dataset was created from unpublished FI-GP and RI-GP data from previous investigations of different ruminant feeds at the Institute of Animal Science at the University of Hohenheim, the dataset of Aiple (1993), and the *Manuscript 1* experiment dataset. The aim was to combine these datasets to increase statistical power. This analysis comprised GP data from a total of 423 *in vitro*-studied feeds. The unpublished data was derived from three experiments conducted between 2015 and 2020, all of which applied a similar approach as the one described in Chapter 3.1, using adult wether sheep as faecal donor animals. In two of these experiments, the sheep were fed with hay *ad libitum* and additionally received a diet consisting of 400 g chopped hay, 105 g grass cobs, and 280 g of a mixed concentrate feed for sheep (ingredients: wheat bran, maize grain, palm kernel cake, sugar beet pulp, malt sprouts, dried distillers grains, rapeseed meal, barley grain, calcium carbonate, molasses, wheat grain, sodium chloride) in the morning and evening, respectively. In the third experiment, the sheep were fed a TMR in dry form for *ad libitum* access, consisting of 50% hay, 15.5% straw, 12.5% maize grain, 12.5% wheat grain, 8% molasses and 1.5% mineral feed. Annex 1 provides a list of the feeds that were tested *in vitro* in the three previous experiments, along with their chemical composition and GP data.

The following flowchart (Figure 5) provides an overview of the key steps involved in creating the dataset. The complete procedure is described in detail in the subsequent text.

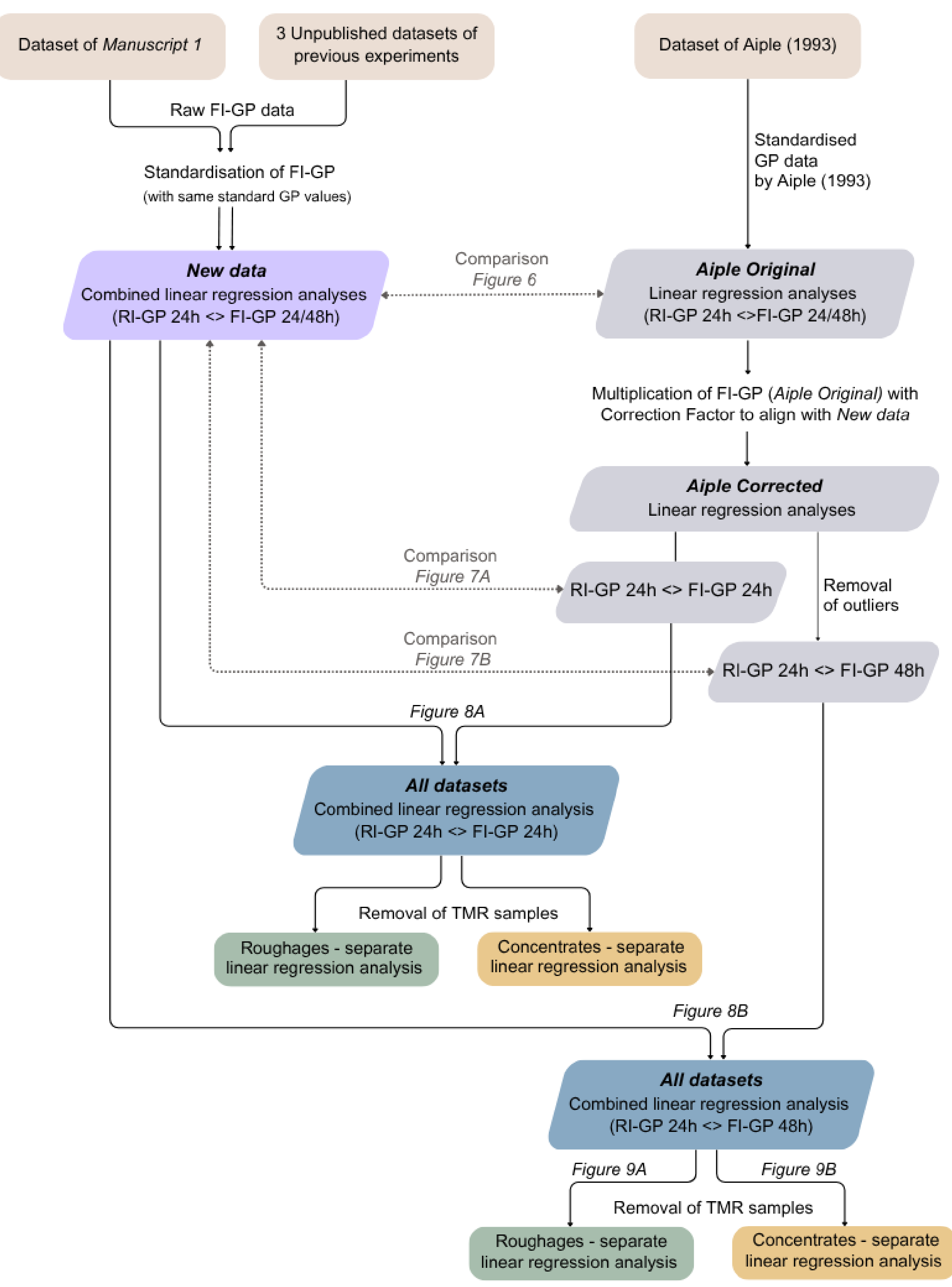


Figure 5. Flowchart illustrating the key steps involved in creating the independent dataset, based on the *Manuscript 1* dataset, three unpublished datasets conducted at the Institute of Animal Science, University of Hohenheim, and the dataset published by Aiple (1993). Abbreviations: FI-GP, faecal inoculum-gas production; RI-GP, rumen fluid inoculum-gas production; GP, gas production; TMR, total mixed ration.

It is important to note that, for FI-GP of the *Manuscript 1* experiment, the hay and concentrate standard GP were calculated and averaged after 48 h of incubation from the data of these three previous experiments and the *Manuscript 1* experiment to standardise GP data of all incubation times (hay: 44.7, concentrate: 61.4 mL/200 mg DM). It was possible to use the raw GP data of the three previous experiments and standardise them by the same averaged standard GP values used in the *Manuscript 1* experiment. After standardisation, the datasets of the three previous experiments were analysed together with that from *Manuscript 1* in a combined regression analysis, which is referred to as *New data* in the following. No raw GP data of the study by Aiple (1993) were available. The author averaged GP from hay and concentrate standards in his experiment after 24 h (hay: 37.1, concentrate: 57.6 mL/200 mg DM) and 48 h (hay: 44.4, concentrate: 65.8 mL/200 mg DM) to standardise FI-GP of the incubated feeds at the respective incubation times. Therefore, two separate linear regression equations were calculated for the *New data* and the Aiple (1993) dataset (hereafter termed as *Aiple Original*) to estimate RI-GP at 24 h from FI-GP at 24 and 48 h, following the statistical approach used in the *Manuscript 1* experiment (Figure 6, Table 1).

In these datasets, the HGT using rumen fluid as the inoculum source was conducted according to the standard HGT assay (Menke and Steingass, 1988). The GP data of RI incubations had already been standardised in all original datasets, and those values were used for the present analysis. The standard GP of RI at 24 h used in the *New data* was 61.6 and 61.1 mL/200 mg DM (in two datasets each) for concentrate, and 45.9 mL/200 mg DM (in all datasets) for the hay. In the dataset of Aiple (1993), the values were 62.6 mL/200 mg DM for hay and 44.2 mL/200 mg DM for concentrate.

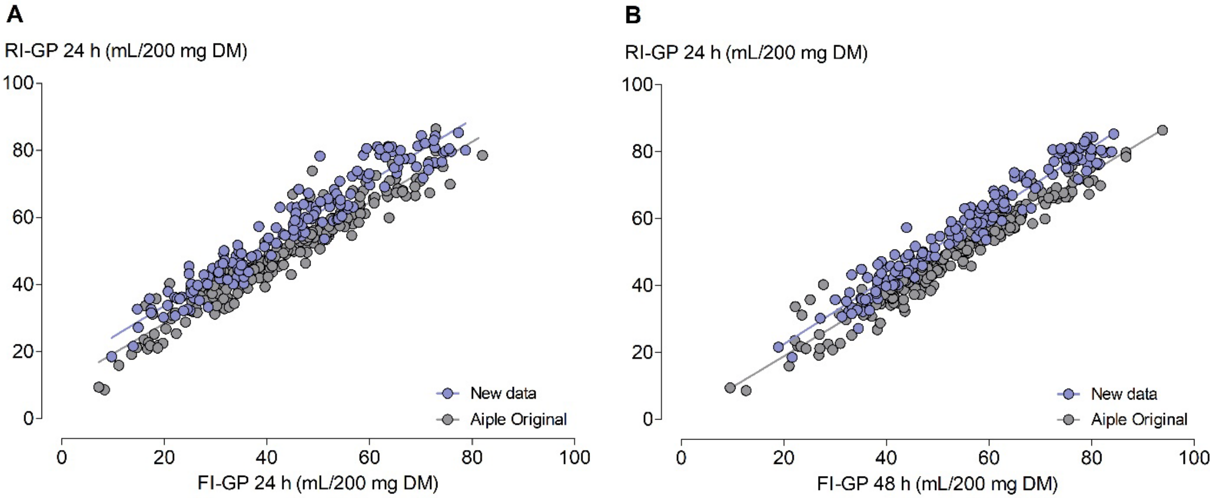


Figure 6. Linear relationships between rumen fluid inoculum-gas production (RI-GP) after 24 h and faecal inoculum-gas production (FI-GP) after (A) 24 h and (B) 48 h from 161 feeds of the *New data* (three unpublished datasets and *Manuscript 1* dataset) and 262 feeds of the *Aiple Original* dataset (Aiple, 1993). The estimated parameters of the linear regressions are shown in Table 1.

Table 1. Linear regression equation parameters to predict rumen fluid inoculum-gas production (RI-GP) at 24 h from faecal inoculum-gas production (FI-GP) at 24 and 48 h for all feeds and separately for roughages and concentrates, using different datasets and data modifications.

Feeds	n	Dataset	Incubation time		Slope	Intercept (mL/200 mg DM)	R^2	RMSE	CV (%)
			RI-GP	FI-GP					
All feeds	262	Aiple Original[b]	24	24	0.90	10.41	0.92	3.72	7.5
	161	New data[c]			0.93	15.13	0.93	4.40	7.6
	262	Aiple Corrected[b]			1.04	10.41	0.92	3.72	7.5
	423	All datasets[d]			0.98	12.60	0.93	4.06	7.7
Roughages	170	All datasets[d]	24	24	1.02	11.46	0.89	3.51	8.4
Concentrates	247				0.96	13.35	0.89	4.43	7.4
All feeds	262	Aiple Original[b]	24	48	0.92	0.53	0.94	3.15	6.4
	161	New data[c]			0.98	2.87	0.95	3.64	6.3
	262	Aiple Corrected[b]			1.03	0.53	0.94	3.15	6.4
	423	All datasets[d]			1.00	1.71	0.95	3.35	6.4
All feeds[a]	257	Aiple Corrected[b]	24	48	1.06	-1.14	0.96	2.74	5.5
	160	New data[c]			0.98	2.62	0.95	3.54	6.1
	417	All datasets[d]			1.02	0.85	0.96	3.11	5.9
Roughages	169	All datasets[d]	24	48	1.02	-0.03	0.91	3.18	7.6
Concentrates	242				0.97	4.16	0.95	2.94	4.9

DM, dry matter; RMSE, root mean squared error; CV, coefficient of variation; Total mixed ration samples were excluded from the separate analyses of roughages and concentrates.
[a] Outliers removed; [b] Dataset originally published by Aiple (1993); [c] Includes three unpublished datasets conducted at the Institute of Animal Science at the University of Hohenheim and the dataset of *Manuscript 1*; [d] Includes the datasets of footnote b and c.

The regression equations of the *New data* and *Aiple Original* datasets showed close linear relationships between RI-GP at 24 h and FI-GP at 24 and 48 h. The dependency between FI-GP and RI-GP differed between the two datasets for both incubation times, with FI-GP of the *New data* having a greater influence on the estimated RI-GP at both incubation times, as demonstrated by the higher slopes. With simultaneously higher intercepts of the *New data*, the corresponding regression equation lines were above those of the *Aiple Original* data for both incubation times. This suggests a systemic difference between the two datasets, which is not surprising given the large time span of 20 to 30 years between the studies. Differences in the *in vitro* fermentation in the HGT and thus the resulting GP may be attributed to the variability of the donor animals and feeds. Breeding has altered animal genetics, which may have affected factors such as metabolic traits and the microbiome. In terms of feed, changes driven by breeding, such as improved stress tolerance and enhanced nutrient profiles, may have influenced the feed quality, potentially resulting in changes of the *in vitro* incubated feeds.

Aiming to combine the *New data* and the dataset of Aiple (1993) into one linear regression equation, a mathematical approach was applied to adjust for the observed differences in the level of the two regression equation lines. As the *New data* contained currently relevant feeds for ruminants and the included *Manuscript 1* experimental approach has proven to be suitable for predicting RI-GP from FI-GP with high accuracy, it was considered to adjust the *Aiple Original* data to align with the *New data*. For this purpose, the average FI-GP at 24 and 48 h was calculated based on both datasets, incorporating all feeds that were incubated. These averaged FI-GP values of 24 and 48 h were used as the independent variable in the respective linear regression equations of the *New data* and *the Aiple Original* datasets for predicting the dependent variable RI-GP at 24 h. The predicted RI-GP values were higher by using the *New data* regression equations and the difference was 5.8 mL/200 mg DM for both 24 and 48 h compared to the RI-GP estimated from the *Aiple Original* equations. This value was subtracted from the GP values of the standard feed samples of Aiple (1993) in order to calculate a Correction Factor, as shown in the following equation:

$$Correction\ Factor = \frac{\left(\frac{GP\ hay\ standard - 5.8}{GP\ hay\ standard}\right) + \left(\frac{GP\ concentrate\ standard - 5.8}{GP\ concentrate\ standard}\right)}{2}$$

This Correction Factor was calculated for 24 and 48 h. The *Aiple Original* FI-GP values at 24 and 48 h were then multiplied by the respective Correction Factor. To ascertain whether the level of the *Aiple Original* regression data could be adjusted to the level of the *New data* through the correction step, new linear regression analyses were carried out using the corrected FI-GP values, termed as *Aiple Corrected.* These linear regression equations are shown in comparison with those of the *New data* in Figure 7. For the 24 h regression data of *Aiple Original* (Figure 7A), the overall data spread was greater compared to 48 h (Figure 7B), thus all GP values were retained for the 24 h *Aiple Corrected* regression analysis. In contrast, for the 48 h regression, the data spread was considerably lower but five feeds in the *Aiple Corrected* and one in the *New data* regression were clearly separated from the rest of the data. These were identified as molasses residues, more specifically as concentrated, desugared, fermented sugar beet (*Aiple Corrected*) and African moringa leaves (*New data*), which were removed from the regression datasets (Figure 7B) in view of their high specificity and low relevance in ruminant feeding. The regression parameters, with and without these removed outliers, are provided in Table 1.

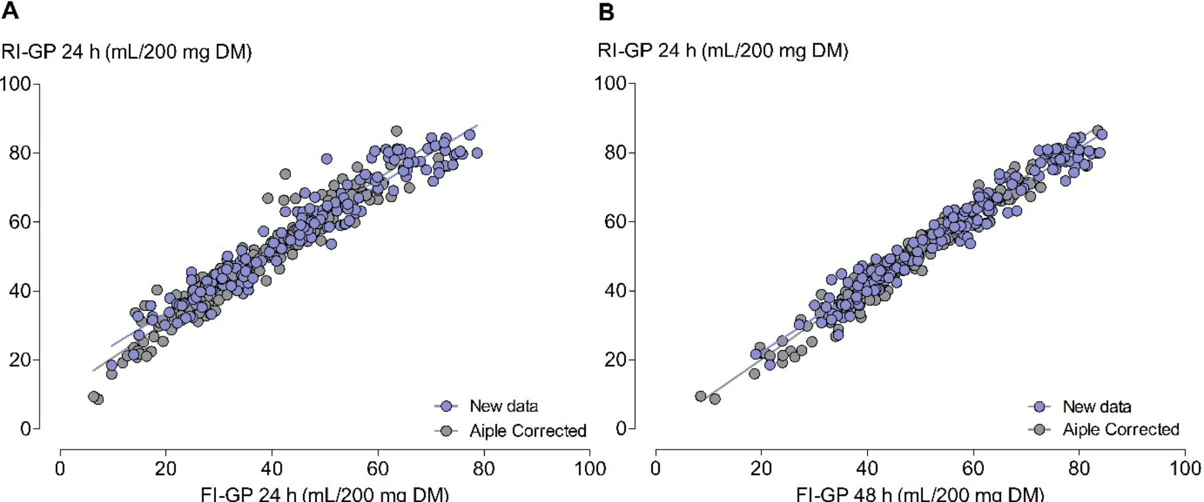


Figure 7. Linear relationships between rumen fluid inoculum-gas production (RI-GP) after 24 h and faecal inoculum-gas production (FI-GP) after (A) 24 h (n = 161) and (B) 48 h (n = 160) from the *New data* values (three unpublished datasets and *Manuscript 1* dataset), and after 24 h (n = 262) and 48 h (n = 257) from the *Aiple Corrected* values (Aiple, 1993). Data in panel B are without removed outliers. The estimated parameters of the linear functions are shown in Table 1.

The applied correction step appeared to have successfully aligned the *Aiple Corrected* regression line with the level of the *New data* by increased slopes and unchanged intercept values compared to the *Aiple Original* regression equations. This facilitated the integration of the *New data* and the *Aiple Corrected* data into a single regression analysis (*All datasets*), the results of which are presented in Figure 8 and Table 1.

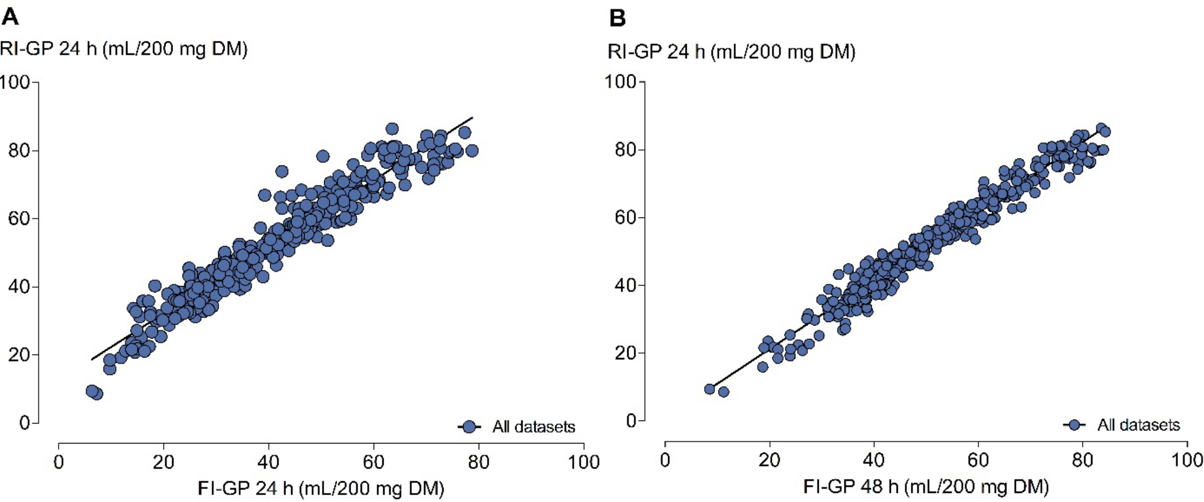


Figure 8. Linear relationships between rumen fluid inoculum-gas production (RI-GP) after 24 h and faecal inoculum-gas production (FI-GP) from *All datasets* values (including three unpublished datasets, the *Manuscript 1* dataset, and the dataset of Aiple (1993)) after (A) 24 h (n = 423) and after (B) 48 h (n = 417). The estimated parameters of the linear functions are shown in Table 1.

The regression analyses for predicting RI-GP at 24 h from FI-GP of the combined *All datasets* indicated close linear relationships at 24 and 48 h of incubation. The RI-GP values at 24 h could be predicted with higher accuracy from FI-GP after 48 h (R^2 = 0.96; root mean squared error, RMSE = 3.11; coefficient of variation, CV = 5.9) than after 24 h (R^2 = 0.93, RMSE = 4.06: CV = 7.7), as also demonstrated by the greater data spread after 24 h (Figure 8A). In contrast, in the *Manuscript 1* experiment, the prediction of RI-GP after 24 h from FI-GP after 24 and 48 h was similarly accurate, with only slightly lower error parameter values for FI-GP at 48 h. This difference in the estimation accuracy by using FI-GP after 24 h between the *Manuscript 1* experiment and the *All datasets* is probably attributed to the included sample size. While the *Manuscript 1* experiment involved 90 different feeds, the combined analysis included a total of 423 distinct feeds. This comprised additional samples of the feed types used in the *Manuscript 1* experiment, as well as a considerable number of newly added diverse feeds. It is reasonable to assume that a constant ratio between RI-GP and FI-GP provides a high estimation accuracy of the linear regression equation. It is likely that this ratio was less constant with FI after 24 than after 48 h. This is probably due to the fact that, as explained in Chapter 3.2, fermentation with FI is associated with an initial lag phase and a slower GP rate. As shown in the estimated GP curves in *Manuscript 1*, the discrepancy between RI-GP and FI-GP diminished as the incubation time progressed. It is therefore possible that fermentation activity in FI stabilised with increasing incubation time and fluctuations were more frequent at 24 than 48 h of incubation. This is supported by the analysis of variation among replicated GP, which decreased with increasing incubation time and was lower on average after 48 than 24 h (*Manuscript 1*). A greater variation in the fermentation of feeds at 24 compared to 48 h may have become more apparent with a larger sample size of feeds across a wide range of nutrient compositions in the *All datasets*.

Using the approach described in the *Manuscript 1* experiment, a validation of the obtained regression parameters for predicting RI-GP at 24 h from FI-GP at 24 and 48 h of the combined *All datasets* was carried out. The validation parameters are given in Table 2.

Table 2. Validation parameters of linear regression equations to estimate rumen fluid inoculum-gas production (RI-GP) after 24 h from faecal inoculum-gas production (FI-GP) after 24 h from 423 feed samples and 48 h from 417 feed samples with a training dataset of 80% and a validation dataset of 20% of the feed samples.

Incubation time (h)		**n**		**Slope**	**Intercept**	**R^2**	**RMSE**	**CV**
RI-GP	**FI-GP**	**Training dataset**	**Validation dataset**		(mL/200 mg DM)			(%)
24	24	338	85	1.00	-0.12	0.93	4.07	7.7
	48	334	83	1.00	-0.04	0.95	3.27	6.2

DM, dry matter; RMSE, root mean squared error; CV, coefficient of variation.
Includes a dataset originally published by Aiple (1993), three unpublished datasets conducted at the Institute of Animal Science at the University of Hohenheim, and the dataset of *Manuscript 1.*

Both linear regression equations for predicting RI-GP at 24 h from FI-GP at 24 and 48 h using the *All datasets* were validated with high accuracy. This was demonstrated by the similarity in the values of R^2, RMSE, and CV between the linear regression equations for RI-GP predictions and the corresponding validation equations. A slightly higher level of agreement was observed in the validation with FI-GP at 48 h. This reflects the higher estimation accuracy obtained between RI-GP at 24 h and FI-GP at 48 h than FI-GP at 24 h.

In the *Manuscript 1* experiment, data subsets for roughage and concentrate feeds were evaluated in separate linear regression analyses to assess whether this may lead to higher estimation accuracies. This approach followed the conclusion of Menke and Steingass (1988), who suggested that using different equations for different feed categories is advantageous to using a single equation for all feeds, based on their estimates of metabolisable energy and OMD from GP. Therefore, the *All datasets* was also divided into roughage and concentrate subsets to calculate separate linear regression equations for predicting RI-GP at 24 h from FI-GP at 24 and 48 h (Table 1). The latter is also shown in Figure 9. As in the *Manuscript 1* experiment, the six TMR samples were not included in this analysis, as they consisted of roughage and concentrate feeds.

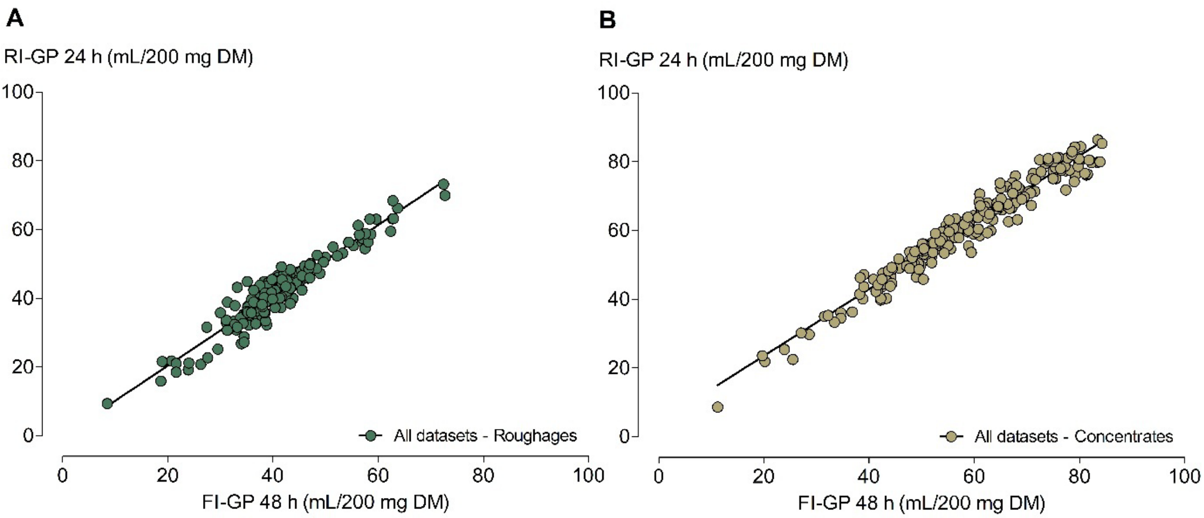


Figure 9. Linear relationships between rumen fluid inoculum-gas production (RI-GP) after 24 h and faecal inoculum-gas production (FI-GP) after 48 h from the *All datasets* values (including three unpublished datasets, the *Manuscript 1* dataset, and the dataset of Aiple (1993)), covering 169 roughages (A) and 242 concentrates (B). Total mixed ration samples were excluded from these analyses. The estimated parameters of the linear functions are shown in Table 1.

Using only roughages in separate regression analyses in both the *Manuscript 1* experiment and the combined *All datasets* did not improve or even reduced the estimation accuracy compared to the common regression equation when predicting RI-GP at 24 h from FI-GP at 24 and 48 h. For the separate analysis of concentrates, both datasets led to slightly higher estimation accuracies for predicting RI-GP at 24 h from FI-GP at 48 h, but no distinct improvement was observed with FI-GP at 24 h. Given that a constant ratio between RI-GP and FI-GP is the basis for a high estimation accuracy, this ratio appeared

to be poorer for roughages compared to the dataset of all feeds and concentrate feeds. Although the microbial population in the large intestine is adapted to poorly degradable substrates, such as those contained in high-roughage feeds, and is therefore able to ferment them efficiently, the microbes have limited time for fermentation and growth due to the high passage rate in this section of the digestive tract (Fon and Nsahlai, 2012). This contributes to the lower microbial activity of FI as discussed in Chapter 3.2.2, thereby reducing the presumed advantage of the faecal microbiome over the rumen microbiome in terms of adaptation to high-roughage feeds. This may have been particularly evident for low-degradable feed samples such as straw. In *Manuscript 1*, an extended lag phase was estimated for all straw samples with FI, whereas with RI, only two straw samples were associated with a lag phase that was approximately half as long as that observed with FI. Although incubations using FI generally resulted in a lower fermentation activity than using RI, this difference appeared to remain highly consistent between RI after 24 h and FI after 48 h for concentrate feeds. In contrast, some roughages showed more pronounced deviations between FI and RI than others, apparently resulting in a less homogeneous ratio of RI to FI among the roughage dataset and hence reduced estimation accuracy in the separate roughages regression analyses, even after 48 h of incubation with FI.

Overall, in the *Manuscript 1* experiment and the combined *All datasets*, splitting the dataset into the feed categories roughages and concentrates to calculate separate linear regression equations did not provide a clear advantage over using a single equation for all feeds. Therefore, a single regression equation incorporating various feeds is considered more robust and is therefore recommended for future applications. The regression equations of the *Manuscript 1* experiment, which were based on 90 different ruminant feeds, showed a superior goodness of fit compared to those derived from the combined *All datasets*, which were based on either 423 or 417 different ruminant feeds. Nevertheless, a close linear relationship between RI-GP and FI-GP was also observed with the latter dataset, allowing RI-GP to be predicted with high accuracy. It is important to consider which equation should be preferred. Although the equations derived from the *Manuscript 1* experiment are associated with a supposedly higher estimation accuracy, this advantage could be diminished when evaluating feeds not covered by this dataset. Equations derived from the *All datasets*, comprising over 400 different feeds, would cover a broader range of nutrient compositions, thus considerably improving the predictive capacity, which is why the application of these equations is recommended. The *Manuscript 1* experiment demonstrated that an incubation time of 24 h with FI is sufficient to reliably predict RI-GP at 24 h, which would offer great practical advantages regarding laboratory practice and analysis costs. However, this could not be confirmed by the regression analyses based on the *All datasets*, as estimation accuracy was higher with FI-GP at 48 h. This appears plausible, given the slower and more delayed microbial fermentation activity found with FI (Chapter 3.2.2), and rather suggests that an incubation time of 24 h with FI may not be sufficient for all feeds. Therefore, an incubation time of 48 h with FI seems more appropriate for predicting RI-GP after 24 h.

3.4.2 Estimation of gas production using preserved faeces

Simple linear regression analyses between the fresh control treatment C and the preserved faecal treatments in the *Manuscript 2* experiment revealed strong relationships for some treatments, despite variations in microbial composition and lower GP values of the preserved treatments, as discussed in Chapter 3.3. This is not surprising, considering the strong linear relationships outlined in the previous Chapter between FI-GP with fresh faeces and RI-GP, although there appear to be notable differences in the microbial community and activity between FI and RI and a reduced GP with FI (Chapter 3.2.). The strongest relationship was observed between C-GP and FR-GP at both incubation times of 24 and 48 h, but also between C-GP and FD-GP after 48 h. FR-GP showed a highly constant ratio to C-GP across the nine feeds studied. Similarly, despite the considerably lower GP observed for FD, a relatively strong consistency with C-GP was evident across the different feeds. In contrast, the ratios of FRNdef-GP, FD3W-GP, and FD6M-GP to C-GP demonstrated a higher variability across the different feeds, which is reflected in the reduced estimation accuracy of the regression equations, even after 48 h of incubation (Table S5, *Manuscript 2*).

These regression analyses were useful for evaluating the potential of replacing FI prepared from fresh faeces with FI prepared from preserved faeces in the HGT. However, a crucial objective of the present thesis was to evaluate whether RI-GP could be predicted from FI-GP with high accuracy by using preserved rather than fresh faeces as inoculum source. Accordingly, linear regression analyses were performed to examine the relationship between the FI-GP values of preserved faeces and the RI-GP values, based on the nine feeds examined in *Manuscript 2*, for which corresponding RI-GP data had been obtained in *Manuscript 1*. The simple linear regression equations to estimate RI-GP at 24 h from GP of the preserved treatments at 24 and 48 h were calculated according to the statistical approach used in *Manuscript 2* and are presented in Table 3. As the chemical composition of these nine feeds was available, additional multiple regression analyses were carried out. These were conducted using PROC REG in SAS (version 9.4 for Windows, SAS Institute, Cary, NC, USA), with backward elimination applied for variable selection. Variables were removed sequentially based on a significance level of $p > 0.1$. The final model, which contains significant predictors, can be expressed as:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 + \beta_6 x_6$$

where y (mL/200 mg DM) was the predicted RI-GP after 24 h, $ß_0$ was the intercept (mL/200 mg DM), x_1 (mL/200 mg DM) was the GP of the faeces preservation treatment after 24 or 48 h, x_2 was the ash (g/kg DM), x_3 was the ether extract (EE, g/kg DM), x_4 was the CP (g/kg DM), x_5 was the neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash (aNDFom, g/kg DM), x_6 was the acid detergent fibre expressed exclusive of residual ash (ADFom, g/kg DM), $ß_1$, $ß_2$, $ß_3$, $ß_4$, $ß_5$, and $ß_6$, represented the regression coefficients for the independent variables GP, ash, EE, CP, aNDFom, ADFom. The results of the multiple linear regression equations are shown in Table 3.

Table 3. Simple and multiple linear regression equations to predict rumen fluid inoculum-gas production (RI-GP, mL/200 mg dry matter) of *Manuscript 1* experiment from gas production (GP, mL/200 mg dry matter) of differently preserved faeces after 24 and 48 h of incubation and chemical crude nutrient and fibre fractions (g/kg dry matter) of nine incubated feeds of *Manuscript 2* experiment.

Regression equation	R^2	RMSE	CV (%)
24 h Incubation time			
Y = 7.06 + 1.19 FR-GP	0.96	4.01	6.7
Y = -26.14 + 1.61 FR-GP + 0.09 EE + 0.06 ADFom	0.99	2.77	4.6
Y = 18.65 + 1.01 FRN-GP	0.95	4.19	7.0
Y = 29.37 + 0.77 FRNdef-GP	0.92	5.44	9.1
Y = 30.30 + 0.98 FDN-GP	0.88	6.79	11.3
Y = 38.38 + 0.82 FD-GP	0.73	9.93	16.6
Y = 41.58 + 0.68 FD3W-GP	0.61	12.01	20.0
Y = 39.02 + 0.65 FD6M-GP	0.62	11.84	19.7
Y = 31.57 + 0.78 FR6M-GP	0.92	5.55	9.3
Y = 17.89 + 0.94 FR6M-GP + 0.05 ADFom	0.95	4.61	7.7
48 h Incubation time			
Y = -10.09 + 1.27 FR-GP	0.96	3.62	6.0
Y = -50.22 + 1.72 FR-GP + 0.08 EE + 0.07 ADFom	0.99	1.90	3.2
Y = -60.66 + 1.84 FR-GP + 0.08 EE + 0.02 CP + 0.08 ADFom	1.00	1.35	2.2
Y = -2.36 + 1.19 FRN-GP	0.97	3.39	5.6
Y = -27.94 + 1.48 FRN-GP + 0.06 EE + 0.05 ADFom	0.99	2.79	4.7
Y = 18.06 + 0.89 FRNdef-GP	0.93	5.21	8.7
Y = 14.53 + 1.02 FDN-GP	0.95	4.31	7.2
Y = -16.80 + 1.35 FDN-GP + 0.08 EE + 0.04 aNDFom	0.98	2.94	4.9
Y = 10.04 + 1.18 FD-GP	0.96	3.97	6.6
Y = -20.49 + 1.57 FD-GP + 0.08 EE + 0.06 ADFom	0.98	3.05	5.1
Y = 25.32 + 0.86 FD3W-GP	0.87	6.82	11.4
Y = 30.92 + 0.71 FD6M-GP	0.83	7.95	13.2
Y = -54.23 + 1.64 FD6M-GP + 0.20 EE + 0.24 ADFom	0.93	5.86	9.8
Y = 21.98 + 0.92 FR6M-GP	0.92	5.55	9.2

RMSE, root mean squared error; CV, coefficient of variation; FR, Frozen faeces; FRN, Shock-frozen faeces with liquid nitrogen; FRNdef, Faeces treated like FRN followed by defrosting prior to incubation; FDN, Shock-frozen faeces with liquid nitrogen and freeze-dried prior to incubation; FD, Freeze-dried faeces; FD3W, Freeze-dried faeces stored for 3 weeks; FD6M, Freeze-dried faeces stored for 6 months; FR6M, Frozen faeces stored for 6 months; EE, ether extract; CP, crude protein; ADFom, acid detergent fibre expressed exclusive of residual ash; aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash.

Including chemical fractions as additional regressors in multiple linear regression equations alongside the GP of the respective preservation treatment did not improve the prediction of RI-GP at 24 h for all treatments. For some treatments, a simple linear regression equation performed better. Overall, EE and

ADFom were the primary contributors to improving the prediction. There may be a connection between EE and ADFom, as both share the characteristic of negatively affecting the fermentation potential and thus having an immediate influence on GP. Dietary fat has a negative effect on different rumen bacterial groups, including fibrolytic bacteria, and on the activity of fibre-degrading enzymes (Patra, 2014). This leads to a reduced metabolic activity and decreases the amount of feed fermented in the rumen (Patra, 2013). An increase in cell wall content and its lignification, reflected in a rising ADFom concentration, is considered to inhibit microbial activity by reducing the availability of rapidly fermentable carbohydrates (Wilson and Hatfield, 1997). Consequently, there is a negative correlation between this fraction and *in vitro* GP (Kafilzadeh and Heidary, 2013; Larbi et al., 1998). Therefore, it is likely that the EE and ADFom concentrations of the incubated feeds had a similar relative effect on RI-GP and FI-GP of preserved faeces. Consequently, including these two fractions may have strengthened the relationships, leading to improved predictions. The crude nutrients and fibre fractions were more frequently identified as significant predictors after 48 than after 24 h of incubation of the preserved treatments. This is likely to be related to the initially slower and delayed GP found with the preservation treatments, which probably led to a higher degree of unexplained variability and thus a lower accuracy of the regression equations after 24 than after 48 h. Consequently, additional variables may have had little predictive value at 24 h. In contrast, after 48 h, the ratio of GP between RI and the preserved treatments may have become more constant across the feeds, resulting in a higher proportion of the variation in RI-GP that could be explained by the GP of the preserved treatments. This could have allowed more predictors to become significant and improve the overall prediction.

In comparison to the simple linear relationships found in the *Manuscript 1* experiment between RI-GP at 24 h and FI-GP using fresh faeces at 24 h (R^2 = 0.97, RMSE = 2.85, CV = 5.1%) and 48 h (R^2 = 0.97, RMSE = 2.77, CV = 4.9%), the predictions of RI-GP from GP of all preserved treatments resulted in lower R^2 values and estimation accuracies. The best equation at 24 h was achieved with FR-GP, whereas at 48 h, both FR-GP and FRN-GP demonstrated similar estimation accuracy, with FRN-GP performing slightly better. By including significant regressors of crude nutrient and fibre fractions, 99 to 100% of the variation in RI-GP at 24 h of the nine feeds was explained by FR-GP at 24 and 48 h, and 99% by FRN-GP at 48 h. In addition, the estimation accuracies of these multiple linear regression equations based on FR-GP and FRN-GP were higher, and those based on FD-GP and FDN-GP were similar, compared to the predictions from fresh FI-GP using the simple linear regression equations. However, the linear regression analyses conducted with fresh faeces in the *Manuscript 1* experiment were based on 90 different-quality feeds, whereas in the *Manuscript 2*, the linear regression analyses with preserved faeces were based on only nine feeds. Although these nine feeds differed in their chemical composition and were assigned to different feed categories, the variety of chemical composition was considerably lower compared to the *Manuscript 1* experiment. Hence, the statistical power of the regression analyses is lower in the *Manuscript 2* experiment and consequently, a direct comparison with the regression equations of the *Manuscript 1* experiment is not possible. However, the

results of the regression equations provided valuable information on predicting RI-GP from GP of the preserved faeces and demonstrated the potential of FR-GP for this purpose. Nevertheless, FR6M-GP showed slightly stronger relationships with RI-GP than the freeze-dried stored treatments FD3W and FD6M, but its estimation accuracy was considerably lower than that obtained with FR-GP, even when using a multiple regression model. This is in line with the lower GP and the more pronounced differences for individual feeds with FR6M compared to the C or FR treatments, as reported in Chapter 3.3.2.

Overall, the FR treatment has proven to be most suitable for predicting RI-GP at 24 h. Due to the higher estimation accuracy with FR after 48 h, this incubation period is recommended. By including EE, ADFom, and CP as regressors, estimation accuracy could be enhanced compared to the simple linear regression equations derived from the fresh FI source (Chapter 3.4.1). However, even the simple linear regression equation for predicting RI-GP at 24 h from FR-GP at 48 h was comparable to the proposed equation based on the *All datasets* (Chapter 3.4.1). Therefore, it is advisable to use the multiple regression equation that includes the nutrient and fibre fractions when feasible. Otherwise, the simple equation could be used, accepting a slightly higher estimation error. In conclusion, it appears that there is a great potential for the future application of frozen faeces instead of fresh faeces as inoculum source to estimate RI-GP in the HGT. However, the FR treatment would have limited practical value if it could not be stored. The findings of the impaired relationships between FR6M-GP and RI-GP highlight the necessity of further investigations on frozen storage of faeces.

The following overview summarises the recommended simple and multiple linear regression equations of Table 3 for predicting RI-GP at 24 h from FI-GP at 48 h using preserved faeces as inoculum source.

Recommended linear regression equations	**R^2**	**RMSE**	**CV** (%)
48 h Incubation time			
Y = -10.09 + 1.27 FR-GP	0.96	3.62	6.0
Y = -50.22 + 1.72 FR-GP + 0.08 EE + 0.07 ADFom	0.99	1.90	3.2
Y = -60.66 + 1.84 FR-GP + 0.08 EE + 0.02 CP + 0.08 ADFom	1.00	1.35	2.2

3.4.3 Estimation of protein value using fresh faeces

Similar to the results of the GP (Chapters 3.2 and 3.4.1), despite the generally lower NH_3-N levels across the different incubated feeds by using FI as the inoculum instead of RI, close linear relationships were found between faecal inoculum-ammonia-nitrogen (FI-NH_3-N) and rumen fluid inoculum-ammonia nitrogen (RI-NH_3-N) (*Manuscript 3*). The regression analyses first of all aimed to better characterise the relationship between FI and RI regarding NH_3-N amounts at different incubation times. The strongest linear relationships were found after 24 h across the six different feeds and feeds plus carbohydrate (CHO) source, while the weakest were observed after 8 h. This closely reflected the results of the NH_3-N values, as these were higher for some feeds and lower for others after 8 h with FI than with RI, possibly contributing to an inconsistent relationship between RI and FI at that time point. However, the close

linear relationships found thereafter may reflect comparable microbial mechanisms in faeces and rumen fluid. Close relationships were observed between FI-GP and RI-GP and between FI-NH_3-N and RI-NH_3-N. Since both parameters are included in the calculation of mN according to Raab et al. (1983) (*Manuscript 3*), it could be assumed that there may also exist a close relationship between faecal inoculum-microbially bound nitrogen (FI-mN) and rumen fluid inoculum-microbially bound nitrogen (RI-mN). This could subsequently allow a prediction of RI-mN from FI-mN, and thereby of MCP. Contrary to this assumption, however, the ratio between FI-mN and RI-mN values was inconsistent across the different feeds and incubation times, which prevented a reliable application of linear regression analysis. These observed differences in the ratio of FI to RI for NH_3-N values *vs.* mN values are plausible, as both traits result from different underlying microbial metabolic processes, each following different kinetics. NH_3-N is released during the rapid hydrolysis of feed CP by rumen microbes (McDonald, 1952; Russell et al., 1992) as well as faecal microbes (Hecker, 1971), while microbial N binding depends on energy availability and decreases as energy becomes limiting (Bach et al., 2005), which is likely to occur at some point in the batch culture system. The apparent delay of this time point with FI is likely the result of a slower and delayed fermentation activity (Chapter 3.2.2), which varies depending on the incubated feed. This could explain the non-uniform pattern observed between FI-mN and RI-mN across different feeds.

Besides the information on the amount of MCP, the quantity of RUP is essential for assessing the protein value of ruminant feeds. Therefore, the calculation of RUP for both inocula RI and FI was also a subject of the present thesis and followed the calculation described by Steingass and Südekum (2013). Although calculating RUP using FI seems illogical because it is based on faecal microbial activity, this calculation was done to investigate differences and the ratio to RUP values calculated with RI. Using the GP and the NH_3-N values of the feed and the feed plus CHO source, linear regressions were calculated according to the equation [2] described in *Manuscript 3*. The intercept of this regression equation represented a theoretical value of NH_3-N that would result if no energy was available. In the next step, the NH_3-N content of the blanks, containing only buffered inoculum, was subtracted from this intercept to determine the degraded N originating from the feed only (rumen-degradable nitrogen, RDN). The RUP of the feed then represents the difference between the total N content of the feed sample and the RDN. Table 4 presents RUP values for all feed samples and both inocula for comparison.

Table 4. Comparison of ruminally undegradable crude protein (RUP) of six different feeds calculated from *in vitro* incubations of 8, 24, and 48 h.

	RUP (g/kg dry matter)					
	8 h		**24 h**		**48 h**	
Feed	**RI**	**FI**	**RI**	**FI**	**RI**	**FI**
Rapeseed meal	230	282	144	218	87	190
Soybean meal	238	377	119	232	77	170
Sunflower meal	143	236	99	146	73	123
Pea grain	41	144	17	41	-2	15
Maize grain	46	78	-51	57	-18	45
Rye grain	18	59	3	10	-19	14

RI, rumen fluid inoculum; FI, faecal inoculum.

The estimated RUP was higher for all feeds and incubation times when FI was used instead of RI. This finding is reasonable when considered in the context of the lower fermentation activity in faeces and the lower NH_3-N levels observed with FI than RI, indicating a slower and less extensive degradation of feed CP with FI. For pea grain, maize grain, and rye grain, negative values were obtained after 24 and 48 h of incubation with RI. The estimation of RUP in the eHGT is only reliable when the CP/ME ratio is above 20 g/MJ (Steingass and Südekum, 2013), which probably explains the negative values observed with RI after 24 and 48 h for maize grain, rye grain, and pea grain as their ratios were below this threshold. There was an inconsistent ratio between RUP values calculated from RI and FI incubations across the different feeds, whereas the three oilseed meals showed less variation in this ratio. Given the overall high variability across all feeds and the fact that the oilseed meal group comprised only three feeds, no reliable regression analysis could be performed to accurately predict RUP of RI from RUP of FI.

As direct predictions of RI-mN from FI-mN and RUP of RI from RUP of FI were not appropriate with the present dataset, another consideration was the prediction of RI values from FI values based on the input variables used in the calculation of mN and RUP. Close linear relationships between FI and RI were found for NH_3-N values (*Manuscript 3*) and GP values (*Manuscript 1*). Therefore, in principle, RI-NH_3-N and RI-GP could be predicted from FI-NH_3-N and FI-GP, respectively, and the predicted values could then be used to calculate mN and RUP. However, this approach currently faces several obstacles. Firstly, the calculation of RUP includes blank values of NH_3-N that could not be estimated for RI from FI using the regression equations derived from the feed samples. Furthermore, the linear regression equations for predicting RI-GP from FI-GP are based on the incubated feeds only and not on the feeds plus CHO source. Although FI and RI responded similarly to the extra energy source in terms of the amount of N incorporation per mL GP after 24 and 48 h (*Manuscript 3*), no conclusion can be drawn about the extent of fermentation and the resulting GP of such extra energy between FI and RI. Predictions inherently contain estimation errors that propagate through the calculation. When multiple

predictions are applied, these errors can accumulate, potentially increasing the overall uncertainty of the final result, such as mN or RUP. If RI-mN and RUP of RI were calculated based on FI-derived predictions of the calculation input variables, up to five estimation errors could accumulate in the final results.

To better assess the approach used regarding incubation times, the corresponding ruminal passage rates for retention times of 8, 24, and 48 h were calculated using the following equation:

$$k_p = \frac{100}{T_{retention}}$$

where k_p is the passage rate (%/h) and $T_{retention}$ is the retention time (hours). By applying this calculation, the retention times 8, 24, and 48 h corresponded to passage rates of 13, 4, and 2%/h, respectively. Additionally, these passage rates were categorised within the context of the newly revised recommendation for energy and nutrient supply of dairy cows in Germany (GfE, 2023). This recommendation introduces a new measure: the feed intake level (FAN). FAN 1 corresponds to the maintenance requirement, FAN 2 to a low milk yield in late lactation, FAN 3 to a medium milk yield in mid-lactation, and FAN 4 to a very high milk yield in early to mid-lactation. For concentrate feeds, FAN 1, 2, 3, and 4 are associated with ruminal passage rates of 3.5, 4.7, 6.0, and 7.2%/h, respectively. This demonstrates that the calculated passage rate based on an 8 h retention time is considerably higher than what the real retention time of concentrate feeds in the rumen would be. In contrast, the calculated passage rates for 24 and 48 h are at the lower end of, or considerably lower than, the realistic values, respectively. Therefore, the chosen incubation times do not correspond well to the actual ruminal retention times of concentrate feeds.

Taken altogether, the prediction of the RI input variables from FI for the calculations of mN and RUP does not appear to be appropriate for obtaining reliable results. Conversely, it could be possible to directly predict the RUP of RI from the RUP of FI, based on the consistent ratio between FI and RI regarding the RUP values of the three oilseed meals. A direct prediction of RI-mN from FI-mN may also be feasible, given a high consistency of the ratio after 8 h of incubation. For that purpose, it would be necessary to include a larger amount of feeds in the analysis. It would be most appropriate to initially focus on a broader range of protein feeds with a fairly homogenous chemical composition to examine whether a systematic relationship between FI and RI exists with respect to mN and RUP. Furthermore, it would be useful to investigate incubation times that better reflect physiological conditions, such as durations between 14 and 21 h, based on the passage rates of 7.2 and 4.7%/h, which correspond to FAN 4 and FAN 2, respectively.

3.5 Critical reflection on the applied methods and recommendations for future research and application

This section critically reflects on the methods applied in the present thesis, evaluating the strengths and limitations of donor sheep feeding, faeces collection, inoculum preparation, reproducibility, and preservation treatments. The aim of this section is to provide guidance on establishing a standard protocol to achieve reproducible results when using sheep FI in the HGT.

Donor sheep feeding

The composition of the TMR in the present thesis aimed to supply the hindgut microbiome with sufficient nutrients to favour microbial growth and thus the fermentative capacity of the faecal microbiome. Furthermore, a diverse feed composition was intended to enable the FI application across a wide range of feeds *in vitro*. Whether these objectives were met cannot be directly assessed, as the present thesis did not involve the comparison of different diet compositions. However, similar GP progressions between FI and RI (*Manuscript 1*) and a considerable microbial growth (*Manuscript 3*) have been demonstrated. Additionally, no systematic under- or over-evaluation of individual feed categories was indicated in the linear regression analyses between FI-GP and RI-GP (*Manuscript 1*). The findings of the thesis, therefore, suggest that the dietary composition of the donor sheep, consisting of grass hay, straw, maize grain, rapeseed meal, and molasses, was overall appropriate for achieving the stated objectives.

Regarding the feeding schedule, the *ad libitum* access to the TMR resulted in a high daily feed intake, averaging 2.5 kg per sheep across the three experiments (*Manuscript 1, 2, and 3*). Also, it is likely that the maize grain and molasses have contributed to a high palatability of the TMR. Increasing feed intake levels accelerate the passage rate, which in turn reduces retention time and feed digestibility. In this context, Chiaravalli et al. (2019) reported lower total tract OMD at higher DM intake levels of cows, along with increased NDF digestibility in *in vitro* long-time incubations when using a FI prepared from faeces of those cows. The authors concluded that a higher quantity of undigested OM may have promoted microbial growth in FI. Thus, a high feed intake, together with the diet composition, could have increased the amount of fermentable substrate in the hindgut, thereby enhancing microbial proliferation and fermentation activity of the faecal microbiome.

As outlined in Chapter 3.3.2, a further experiment was conducted to study the effect of a frozen storage of faeces. As explained in that Chapter, a control treatment with fresh faeces as inoculum source was included. In that experiment, faeces were collected from sheep fed the same dietary components as in the TMR (excluding molasses), with roughages provided *ad libitum* and concentrates offered in two equal portions in the morning and evening. The FI, prepared from that experiment, is therefore referred to as $FI_{restrictive\ concentrate}$ in the following. The aim was to compare $FI_{restrictive\ concentrate}$ with FI from the *Manuscript 1* experiment, termed as $FI_{\textit{ad libitum}}$ hereafter, to assess whether these different feeding

schedules influence *in vitro* GP of different feeds. To this end, grass silage, maize silage, maize grain, wheat grain, rapeseed meal, soybeans, and sugar beet pulp samples were incubated in the HGT according to the approach of *Manuscript 1*, using $FI_{restrictive\ concentrate}$. For $FI_{ad\ libitum}$, the GP of these feeds has already been studied, as the same feeds were included in the *Manuscript 1* experiment. The GP at 24 and 48 h of both assays was corrected for the blank values and standardised using hay and concentrate standard values according to *Manuscript 1*. The GP at 24 and 48 h for both inocula was analysed using a two-way ANOVA, as described in *Manuscript 2*, and visualised using bar charts. The two-way interaction of feeds and inocula was significant for both incubation times ($p < 0.001$) (Figure 10). The GP between the two inocula was very similar across the different feeds at both incubation times, with significant differences observed only for a few individual feeds. These differences did not indicate a systematic effect, as both inocula showed either higher or lower GP compared to one another, without a consistent pattern related to specific feed categories. Based on the above considerations, it could have been conceivable that $FI_{ad\ libitum}$, due to TMR feeding, might have led to a higher feed intake and subsequently to a higher GP. This assumption was not confirmed. However, the sheep of the $FI_{restrictive\ concentrate}$ also had *ad libitum* access to roughage, but no data on feed intake was recorded. It is therefore possible, that total daily intake was comparable between the sheep of the two feeding schedules. This hypothesis is supported by the findings of Fan et al. (2002), who compared three feeding regimes for dairy cows: TMR feeding, and roughage offered *ad libitum* with concentrates fed either twice or four times daily, and found no significant difference in roughage or total DM intake. At most, in the present analysis, a slight tendency toward higher GP values with $FI_{restrictive\ concentrate}$ was observed for concentrates. It could be speculated that the intake of a large portion of concentrate at once increased the passage rate slightly, allowing more undegraded substrate to reach the hindgut if not intestinally digested. Nevertheless, the overall differences were so small that a substantial effect cannot be assumed. On average of the 12 feeds, the mean GP (mL/200 mg DM) ± standard deviation (SD) for $FI_{restrictive\ concentrate}$ and $FI_{ad\ libitum}$ were almost identical, with 47 ± 16.3 and 46 ± 15.2 at 24 h, and 56 ± 16.3 and 56 ± 15.8 at 48 h. This similarity was also evident in the almost identical mean *in vitro* GP curves for treatment C from the *FR6M experiment* ($FI_{restrictive\ concentrate}$) and treatment C from the *Manuscript 2* experiment ($FI_{ad\ libitum}$) (Chapter 3.3.2).

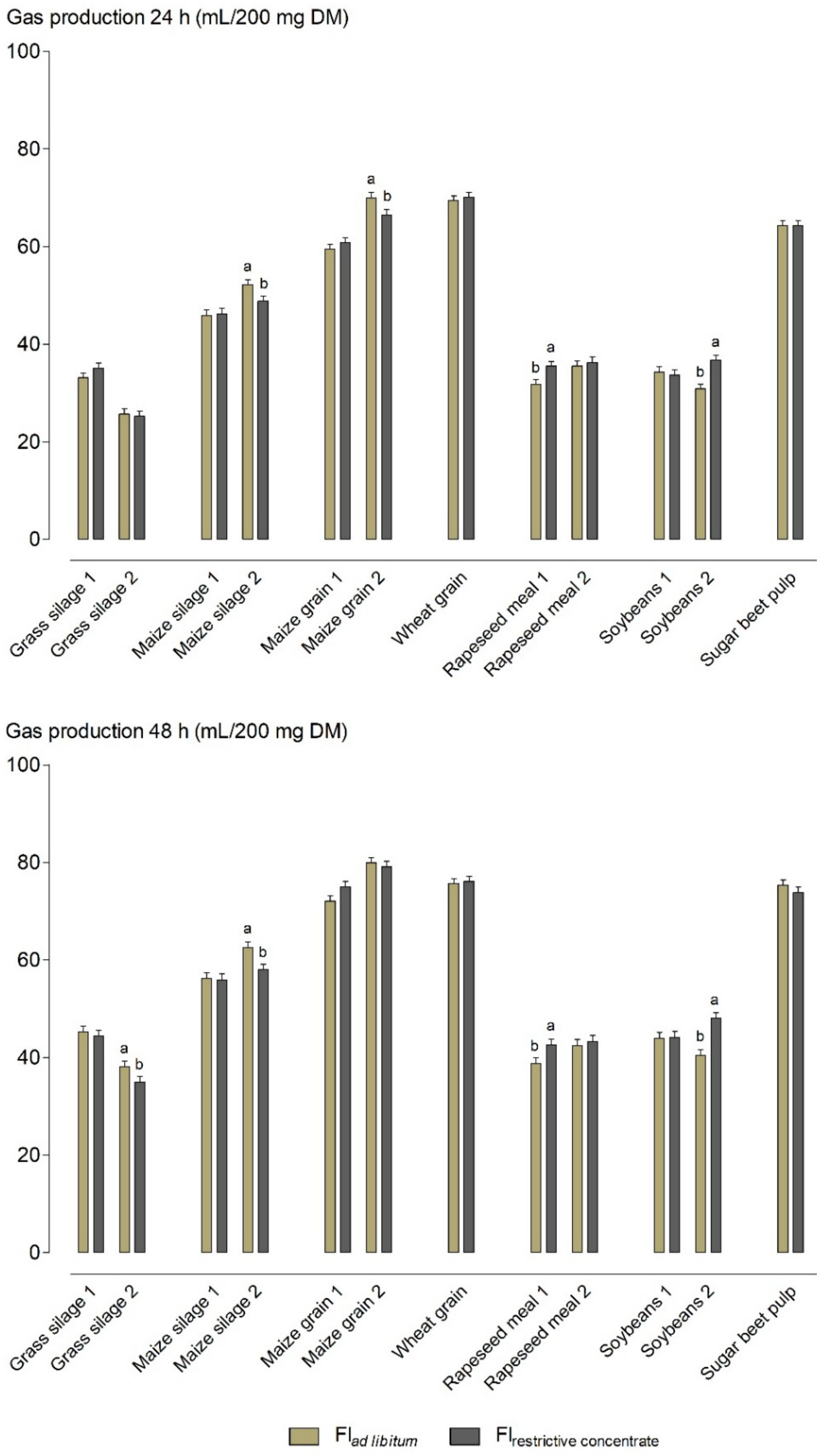


Figure 10. Effect of feed and inoculum on *in vitro* gas production after 24 and 48 h of incubation. Columns within statistical comparison not sharing the same letter are significantly different ($p \leq 0.050$). $FI_{ad\ libitum}$, faecal inoculum prepared from sheep fed a total mixed ration for *ad libitum* access; $FI_{restrictive\ concentrate}$, faecal inoculum prepared from sheep fed roughage *ad libitum* and concentrate twice daily. Bars represent standard deviation.

In conclusion, providing the donor animals with roughages *ad libitum* and concentrates twice a day can be a suitable alternative feeding schedule, when providing the same feed components as included in the TMR. This is an important finding, as it allows for some flexibility and could be applied according to the practical circumstances.

Faeces collection

The present thesis was driven by the overarching aim of improving animal welfare by attempting to replace the use of rumen-cannulated animals for *in vitro* feed evaluation in the HGT. Conversely, when using an alternative inoculum source, such as sheep faeces in the present thesis, it is equally important to ensure the welfare of these donor animals is not impaired in accordance with the 3R principles. Therefore, the following section critically reflects on the sheep husbandry and faeces collection procedures applied in the present thesis, identifying potential future improvements.

When working with animals, it is important to understand the behavioural characteristics of the particular animal species. The four key traits of sheep are their flocking instinct, following behaviour, visual perception, and intelligence, which form the basis of all behavioural principles of sheep handling (Hutson and Grandin, 2024). Based on these characteristics, several stressors experienced by sheep can be derived, including isolation, particularly visual isolation, and isolation for extended periods, as well as the approach of a human (Hutson and Grandin, 2024). Taking these aspects into account, the three sheep were group-housed throughout all experiments conducted in the present thesis, with visual contact to the rest of the herd most of the time. Furthermore, all persons involved in handling the animals spent time familiarising the animals with their presence to avoid aversive behaviour during the subsequent rectal faeces collection. Faeces collection was consistently performed with two people, one holding the sheep and the other collecting the faeces. Most of the sheep involved in the experiments remained calm during holding and faeces collection. However, some sheep became slightly agitated during faeces collection and a few individuals were restless throughout both holding and faeces collection. These individuals appeared noticeably stressed and reacted with resistance during the handling. Hargreaves and Hutson (1990) investigated the effect of gentling on adult sheep to reduce their aversion to following handling. In line with the behaviour of the sheep in the thesis experiments, the authors reported that gentling reduced the sheep’s flight distance and heart rate in response to humans, but did not diminish their aversion to handling.

Apart from animal welfare considerations, an increased stress response may also affect the experimental results, as it might influence factors such as feed intake, and consequently the passage rate and overall digestive processes. It has been shown that cortisol concentrations affect feed intake and glucose metabolism in sheep (Bassett, 1963). Additionally, psychological stressors can stimulate a neuroendocrine response that may directly or indirectly affect the microflora of the digestive system (Cholewińska et al., 2021).

A possible alternative to avoid stress associated with the procedure of rectal collection could be the use of faeces collection bags. For this, the sheep could be fitted with a harness to which plastic collection bags are attached. These could be removed after a defined period of time, necessary to obtain the required amount of faeces per sheep. Akhter et al. (1996) investigated the effect of different time periods under aerobic conditions between the collection of cattle faeces and their use as an inoculum source in *in vitro* digestibility evaluation of forages. The authors observed that a reduction in temperature to 30 °C or a time period of 2 h did not affect digestibility, whereas a reduction in temperature to 22 °C or an extension of the duration to 5 h led to a decrease in digestibility. However, Aiple et al. (1992) reported a significantly reduced GP of hay incubations even after one hour of aerobically stored sheep faeces at room temperature. Therefore, the period of collection time could represent a critical point. It should, therefore, be studied how long it takes to collect the required amount of faeces using collection bags. It is conceivable that a specific time span can be identified during which the sheep excrete more faeces, or that defaecation could be stimulated by specific stimuli, such as movement or providing fresh feed. The faeces collected in bags must then be compared with rectally obtained faeces in terms of *in vitro* GP for different feeds in the HGT. It should be noted, however, that the fitting and wearing of a harness also involves a handling to which the sheep must be accustomed, and which may also induce stress reactions in some sheep.

An alternative approach might be to systematically prepare the sheep for rectal faeces collection through targeted training. Different learning mechanisms, including habituation and conditioning, have been shown to reduce the animal's fear responses and improve their handling (Fonsêca et al., 2019; Pluchot et al., 2024; Schiøler et al., 2025). For example, Fonsêca et al. (2019) successfully trained sheep to voluntarily enter a squeeze chute and tolerate the use of a facial mask for up to 50 minutes for indirect calorimetry trials, without observable changes in behavioural or physiological responses. Therefore, the authors applied the principles of repetition, regularity, and connection with positive stimuli. In another study, sheep could be trained to participate voluntarily in magnetic resonance imaging sessions, without the need for anaesthesia or restraint (Pluchot et al., 2024). The approach of Pluchot et al. (2024) was based on positive reinforcement, a positive human-animal relationship, and voluntary cooperation of the sheep. The authors developed a step-by-step animal welfare-friendly training protocol in which each correctly performed behaviour was followed by a social or a feed reward, and any form of physical restraint was avoided. Likely, implementing such habituation and conditioning processes through training protocols prior to the experiments of the present thesis would have enabled a less stressful handling of individual sheep and an increased overall voluntary cooperation of the sheep for rectal faeces collection. Hypothetically, conditioning such as clicker training (Skinner, 1951) might eventually allow for the stimulation of defaecation. Therefore, it is highly recommended to develop such a training protocol for the specific situation of rectal faeces collection to limit fearful reactions and improve animal welfare. This would be of particular relevance if the collection of faeces were centralised in specialised institutions, in which the sheep would be kept for this purpose and potentially used over an extended

time period. Nevertheless, it is also likely that there will always be individual sheep that, despite habituating and training, do not cooperate voluntarily and react with fear responses. Pluchot et al. (2024) initially selected the sheep that displayed the most positive reactions with the trainers and the introduced objectives in their experiment. The authors also reported that some sheep failed to progress to the subsequent higher training level and that a certain proportion of sheep will possibly never fulfil the specific behavioural criteria required. Such animals that do not meet the necessary behavioural criteria could then be permanently excluded from rectal faeces collection, as it is likely that the handling and this procedure would always cause stress in these individuals.

Faecal inoculum preparation

A substantial amount of GP (*Manuscript 1 and 2*) and microbial growth (*Manuscript 3*) was observed in the FI incubations using fresh faeces in the HGT, and similar GP progressions between FI and RI incubations were demonstrated (*Manuscript 1*). It can, therefore, be concluded that the approach chosen for FI preparation, including the mixing and filtering processes, as well as the dilution rates with the medium solution, was appropriate. With a mean ratio of DM (g) : medium solution (mL) of 1:33 (SD = 3) across all 60 incubation runs conducted for the present thesis, the actual ratio remained within the range of 1:20 to 1:50 recommended by Aiple et al. (1992). Overall, the GP of FI was generally at a lower level compared to RI incubations (*Manuscript 1*), which was consistent with findings in the literature (Aiple et al., 1992; Cone et al., 2002; Mauricio et al., 2001; Zicarelli et al., 2011). Posada et al. (2012) concluded that FI is not suitable in replacing RI for evaluating DM degradability kinetics, as differences in GP reflect a longer time to colonise the feed and lower microbial concentrations in FI. They emphasised that using FI is not feasible without prior increase of microbial density. Likewise, Cone et al. (2000) concluded that faeces are not appropriate as an alternative inoculum source to rumen fluid for *in vitro* GP evaluations based on their findings of a prolonged lag phase, lower GP rates, and lower total GP with FI compared to RI, regardless of different faecal dilutions with the buffer solution. However, it does not appear feasible to increase the concentration of faeces in the FI arbitrarily in order to achieve a microbial density comparable to that in RI, given that Aiple (1993) reported that dilutions below 1:20 resulted in a highly viscous faecal suspension that was difficult to handle and reduced GP. Nevertheless, in the view of the present thesis's overall objective to predict RI parameters, such as GP or NH_3-N, from FI, it is not necessary to achieve comparable microbial densities and fermentation activities between faeces and rumen fluid. Instead, maintaining a consistent ratio that enables strong relationships between FI and RI is of great importance and was successfully achieved, as demonstrated in the *Manuscript 1 and 3* experiments. The targeted ratio of 1:35, therefore, appears to have been appropriate and is recommended for future application.

Potential for standardisation and reproducibility of fresh faeces as an inoculum source

The inoculum is a primary source of variation in GP measurements, with factors such as the donor animal diet, the sampling method, and the dilution with the medium solution all contributing to this variation (Rymer et al., 2005). As described in the previous sections, the methods applied concerning these factors have proven to ensure sufficient volumes of GP using fresh sheep faeces, reflecting a high level of fermentation activity in the faeces. This forms the basis for establishing the methods applied in this thesis for routine analysis of ruminant feeds in the HGT. The high accuracy observed in the statistical approach of validation of the linear regression equations (*Manuscript 1*, Chapter 3.4.1) already demonstrated their potential for application to other datasets. However, the next crucial step in the context of standardisation is to ensure the repeatability of results across different experimental series and reproducibility across different facilities.

In the *Manuscript 1* experiment, a comparative analysis of the CV among replicated GP values was conducted for different feed categories and incubation times, based on FI and RI incubations. The results showed that GP variability was considerably higher in the first hours of incubation when using FI, but variation was comparable between both inocula at 24 and 48 h, which represent the relevant time points regarding feed evaluation in the HGT using FI. This highlights that a certain degree of variability is inevitable due to the biological nature of the inoculum sources, even when rumen fluid is used according to the standard HGT method (Menke and Steingass, 1988). At the same time, the results indicated comparable repeatability between the FI and RI approaches at the 24 and 48 h incubation times. However, these results are based on comparisons within a single experiment conducted over a short period of a few weeks. Therefore, it should also be examined whether FI prepared from fresh sheep faeces according to the approach described in this thesis are related to each other across different experimental series conducted at different time points. To this end, correlations between FI-GP of the *Manuscript 1* experiment and C-GP (fresh faeces) of the *Manuscript 2* experiment were calculated using PROC CORR of SAS (version 9.4 for Windows, SAS Institute, Cary, NC, USA). The same nine feeds were included in both experiments, allowing for this analysis, whereby the feed GP values were corrected by the respective blank GP values. The FI-GP and C-GP were highly correlated, with correlation coefficients increasing over time from 2 h ($r = 0.671$, $p < 0.05$), 4 h ($r = 0.935$, $p < 0.001$), 8 h ($r = 0.994$, $p < 0.001$), 12 h ($r = 0.974$, $p < 0.001$), 24 h ($r = 0.986$, $p < 0.001$), 48 h ($r = 0.995$, $p < 0.001$), and 72 h ($r = 0.993$, $p < 0.001$). Furthermore, the mean *in vitro* GP curves of the treatments C from the different experiments of *Manuscript 2* and the *FR6M experiment* (Chapter 3.3.2, Figure 3) were almost identical. Although all of these experiments were conducted in the same institution using the identical laboratory equipment by the same persons, they took place over extended periods of time and always involved different sheep. In total, 17 sheep were used for the *Manuscript 1, 2, and 3* experiments, as well as the *FR6M experiment*, with six sheep being used twice.

The findings presented so far already indicate a high potential for obtaining reproducible results with FI, when applying the methodological approach described in this thesis. The next essential step in

establishing a standardised protocol based on this approach is to perform experiments under reproducibility conditions. This means to obtain independent measures using the same approach and research question in different facilities with different persons using different equipment (ISO – International Organization for Standardization, 2017). For this purpose, inter-laboratory ring tests should ideally be conducted to comprehensively assess and validate both the methodological approach of the modified HGT using fresh faeces and the linear regression equations established in this thesis, with particular focus on the equation based on the *All datasets* and the suggested incubation time of 48 h (Chapter 3.4.1).

Faeces preservation techniques

Among all preservation treatments, freeze-drying of the sheep faeces with additional storage caused the most pronounced negative effects on the GP kinetic parameters, particularly after 6 months of storage (*Manuscript 2*). As stated in Chapter 3.3.2, the storage conditions of the freeze-dried stored faeces could have been improved by sealing in high-barrier plastic bags at lower temperatures, which may have increased the subsequent recovery rate of faecal microbial cells during incubation. Nevertheless, the freeze-dried treatments without storage also resulted in distinct differences in terms of GP and metaproteome analyses compared to C. The literature also provides indications of possible improvements in cell recovery after freeze-drying during subsequent incubation. One such suggestion concerns the reconstitution medium. A slower rehydration process (7-16 days) may enhance the recovery of freeze-dried microorganisms (Morgan et al., 2006), as rapid reconstitution may prevent proteins from refolding into their native conformation, leading to denaturation and/or aggregation (Wang, 2000). Furthermore, Morgan et al. (2006) concluded that the rehydration temperature could also affect the recovery following freeze-drying, given that Ray et al. (1971) found lower numbers of recovered cells at 35-45 °C than at 15-25 °C. Additionally, the use of a complex rehydration medium enriched with additional nutrients may promote the repair of damaged cells (Morgan et al., 2006); however, this appears to be inappropriate in the context of fermentation studies. One consideration that emerged regarding the freeze-dried treatments was whether it might be possible in future studies to compensate for the strong negative impact on microbial activity by targeted adding of probiotic strains to the incubation. Weimer (2025) identified several critical challenges that may impair the successful establishment of a probiotic strain in the rumen. These include withstanding the stresses related to delivery and inoculation (culture storage, exposure to air), identifying and utilising the nutrient sources within the rumen, successfully competing with the established microbial community, avoiding predation and antagonistic agents, establishing beneficial mutualistic interactions with other microbes, and growing at a rate that exceeds the dilution rate of ruminal contents. Similar challenges are expected when establishing probiotic strains during *in vitro* incubations with a microbial inoculum source. Furthermore, the results of the *Manuscript 2* experiment indicated that the preservation treatments did not deplete bacterial or archaeal taxa. Consequently, the observed differences in GP between the

treatments could be attributed primarily to the respective differences in the relative abundances of the active bacterial and archaeal taxa. Therefore, besides the aforementioned obstacles of establishing probiotic strains during incubations, it appears to be highly complex and impractical to specifically balance the relative abundances of individual taxa in the precise proportions required. Overall, while the discussed factors can potentially improve the recovery of freeze-dried microbial cells during incubation, they appear to be either unsuitable for GP evaluation or would considerably prolong the inoculum preparation time, making them impractical and difficult to implement for routine application. Considering these aspects and the detrimental effects of the freeze-drying process on GP and the microbiome observed in the *Manuscript 2* experiment, it is currently not recommended to prioritise this treatment and to further optimise its approach.

In contrast, the FR treatment overall showed GP kinetic parameters most comparable to those of treatment C and a high degree of similarity in the metaproteomic datasets (*Manuscript 2*). These findings support the methodological approach applied in the FR treatment. However, the FR6M treatment resulted in poor GP kinetic parameters compared to FR and C (Chapter 3.3.2). As the FR treatment would have limited practical value without the possibility of storage due to logistical constraints, further investigations on the frozen storage conditions and storage duration are required. The sealing of frozen faeces in low-barrier plastic bags may also have had a negative effect during frozen storage, which could potentially be improved by using high-barrier plastic bags. Furthermore, the two experiments on preservation and storage of frozen faeces conducted in this thesis (*Manuscript 2*, Chapter 3.3.2) do not provide sufficient data to determine the time period for which frozen sheep faeces can be stored without a significant reduction in *in vitro* fermentation activity. Further studies should, therefore, investigate narrow storage time intervals with frozen faeces. Given the high potential demonstrated by the FR treatment, such further investigations are strongly recommended to identify the minimum feasible storage duration under freezing conditions. Based on this, in the next step, investigations on the reproducibility of the GP of such frozen stored faeces as inoculum source should be conducted. At the same time, this would represent a potential future scenario, in which faeces could be collected in specialised centres and distributed to different facilities to be used as inoculum source in the HGT, thereby enabling an even higher level of standardisation.

The following points summarise the main research questions that should be addressed in future studies to improve and validate the approach applied in this thesis:

- Faeces collection:
  - Is faeces collection using collection bags a suitable alternative to rectal faeces collection?
    Investigation of the collection duration and comparison of in vitro *GP in the HGT using rectally collected faeces vs. faeces collected via bags*

- Could targeted training prepare the donor sheep for rectal faeces collection to reduce or avoid stress during faeces collection?
  Implementing habituation and conditioning processes by developing a specific training protocol for the situation of rectal faeces collection

- Prediction of RI-GP from FI-GP using fresh faeces:
  Can the methodological approach and the derived linear regression equations generate reproducible data across different laboratories?
  Conducting inter-laboratory ring tests to validate the methodological approach of the modified HGT using fresh faeces and the regression equations derived in this thesis

- Prediction of RI-GP from FI-GP using preserved faeces:
  Can microbial fermentation activity be maintained in frozen faeces for more than 8 days of storage, and if so, can reproducible data be generated using these samples?
  Investigation of narrow storage time intervals with frozen faeces and sealing in high-barrier plastic bags; Evaluation of reproducibility concerning GP data of frozen stored faeces

- Estimation of protein value using fresh faeces:
  Is it possible to accurately predict RUP of RI and RI-mN from RUP of FI and FI-mN, respectively?
  Assessing these predictions by using a larger number of feeds, initially focusing on a broader range of protein feeds with fairly homogenous chemical composition; Investigation of incubation times between 14 and 21 h to better reflect ruminal retention times

3.6 Conclusions

In conclusion, using sheep faeces as an inoculum source has proven suitable in replacing rumen fluid and thus rumen-cannulated animals for evaluating ruminant feeds *in vitro* in the HGT. In general, by using fresh faeces, FI incubations were associated with consistently lower GP kinetic parameters and a prolonged lag phase compared to RI incubations. This points to a slower and less extensive fermentation of feeds *in vitro* when FI is used as the inoculum source, apparently reflecting a lower microbial activity in FI than in RI. However, strong correlations and linear relationships between FI-GP and RI-GP were demonstrated across a large number of different-quality ruminant feeds, allowing for precise predictions of RI-GP from FI-GP. The methodological approach applied in this thesis, therefore, appears suitable for achieving an appropriate level of FI-GP that remains proportional to RI-GP. These findings support the application of the described approach and regression equations for establishing a standardised protocol for routine feed analysis in the HGT using fresh faeces as an inoculum source.

The preservation treatments had a clear impact on both the microbiome and GP during incubations in the HGT. Among the preserved faeces, frozen treatments showed GP kinetic parameters and metaproteomic profiles most similar to C, while freeze-drying had the strongest negative impact on both

traits, and storage of freeze-dried faeces even pronounced the negative effects on GP. This appears reasonable in light of the various stress factors associated with the freeze-drying and storage processes. The potential to predict RI-GP from FI-GP using preserved faeces closely reflects the findings of the GP kinetic and metaproteomic data, with the strongest relationship and highest accuracy observed with FR-GP. Contrary to expectations, the frozen storage in the treatment FR6M had a considerable negative effect on GP and weakened the relationship with RI-GP. Overall, FR demonstrated great potential to be used as an alternative inoculum source to both rumen fluid and fresh faeces, enabling centralisation of donor animal husbandry and overall reduction of animal numbers. However, this is only feasible if frozen stored faeces provide results comparable to those obtained with non-stored frozen faeces, which requires further research.

Although FI incubations of different feeds resulted in lower NH_3-N contents compared to RI incubations, strong linear relationships were observed between the two inocula for this trait. This provides a valuable basis for further estimations of the protein value of ruminant feeds, since NH_3-N is the key parameter in estimating MCP and RUP in the eHGT. However, due to an inconsistent ratio regarding mN and RUP contents between FI and RI across the six different incubated feeds, RI values could not be reliably predicted from FI values for these parameters based on the available data in this thesis. Nevertheless, a higher consistency regarding these ratios was found within the group of oilseed meals, indicating a greater potential for reliable estimation when using more homogenous feed groups, which should be addressed in future studies.

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4 INCLUDED MANUSCRIPTS

4.1 Manuscript 1: A systematic study of sheep faeces to be used as an alternative inoculum source in the Hohenheim gas test

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A systematic study of sheep faeces to be used as an alternative inoculum source in the Hohenheim gas test

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ABSTRACT

In vitro studies of gas production (GP) using rumen fluid obtained from rumen-cannulated animals are common in feed evaluation for ruminants. The objective was to compare sheep faeces with rumen fluid from dairy cows as inocula in the Hohenheim gas test (HGT) using a large number of different-quality feeds. It then was evaluated whether GP obtained by using rumen inoculum (RI-GP) can be estimated from GP using faecal inoculum (FI-GP). Ninety feeds for ruminants including roughages ($n = 27$), total mixed rations ($n = 6$), commercial compound feeds for dairy cows ($n = 18$), cereal grains ($n = 10$), energy-rich by-products ($n = 5$), legume grains ($n = 6$), oilseed meals ($n = 6$), and other protein-rich feeds ($n = 12$) were incubated with either rumen inoculum (RI) or faecal inoculum (FI), and GP was recorded at 2, 4, 8, 12, 24, 48, and 72 h. Nonlinear equations were fitted to the GP data to determine GP kinetic parameters. Correlations between RI-GP and FI-GP were calculated for different time points. Linear regressions were calculated to estimate RI-GP from FI-GP using the entire data set or subsets of roughages and concentrates. GP kinetic parameters were lower for all feed categories when incubated with FI instead of RI. On average of all feed samples, the potential GP was 9 mL/200 mg dry matter (DM) lower and the rate of GP was 3.1%/h lower with FI than RI. The estimation of kinetic data for FI included a lag phase of 1.51 h on average, whereas no lag phase was estimated for RI. Estimated parameters indicated an overall lower fermentation activity of FI than RI. However, there was a very similar progression of GP curves of RI-GP and FI-GP. RI-GP after 24 h was significantly correlated with FI-GP in the time period of 24 to 60 h ($r = 0.973 - 0.982$, including all feed samples). Linear regression analysis showed that RI-GP after 24 h can be estimated from FI-GP after 24 h (Slope = 1.02, $R^2 = 0.97$) and 48 h (Slope = 1.1, $R^2 = 0.97$) including the complete data set. Splitting the data set into roughages and concentrate feeds did not lead to distinctly higher estimation accuracy. In conclusion, sheep faeces can be used as an alternative inoculum for studying a wide range of different-quality feedstuffs. The standard gas test may be modified without the need for rumen-cannulated animals.

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1. Introduction

To contribute to a sustainable livestock sector that meets the challenges of global food security while reducing environmental impact, animal nutrition concepts, including feed evaluation, are very important. Several methods, such as the two-stage *in vitro* digestion method (Tilley and Terry 1963) and the *in vitro* gas production (GP) technique (Menke et al. 1979; "Hohenheim gas test" (HGT)) have been established as standard procedures for simulating fermentation in the forestomach of ruminant animals. Data obtained with these assays are widely used to determine the digestibility and energy value of feeds for ruminants. The standard HGT assay uses rumen inoculum to obtain GP (RI-GP) after 24 h of incubation (Menke and Steingass 1988). These assays depend on using rumen fluid as inoculum, commonly obtained from rumen-cannulated animals. The surgical procedure and need for continued care of cannulated animals are associated with practical and ethical obstacles that may impair the implementation of the assays by research organisations. In tropical regions, in particular, cannulation of the rumen may not be feasible due to the increased risk of infections (Pandian et al. 2016).

For ethical reasons, the aim is to improve animal welfare according to the 3 R principles (reduce, replace, refine) by reducing the use of cannulated animals. Earlier studies indicated that freshly voided faeces have the potential to be used as an alternative to rumen fluid (Aiple 1992; El-Meadaway et al. 1998; Akhter et al. 1999; Mauricio et al. 2001; Hughes et al. 2012). Aiple (1992) compared sheep and cattle faeces and found sheep faeces to be superior for use in the HGT, assuming that the compact form of sheep faeces may provide more stable conditions for the microorganisms until the assay is started. Although it has been frequently recommended that faeces be considered as a potential inoculum source for *in vitro* studies, it has not been done in routine feed evaluation to date. Cutrignelli et al. (2005) pointed out that further research on a wide range of feeds is necessary to better assess the suitability of faeces as an inoculum.

Therefore, this study used a wide range of currently relevant feedstuffs for ruminants aiming at a systematic evaluation of sheep faeces as an inoculum used for *in vitro* testing. The second aim was to quantify the relationship between GP data obtained by using the different inocula. The overall objective was to study whether the prediction of RI-GP from GP using faecal inoculum (FI-GP) is possible and the duration of the incubation period is a relevant factor.

2. Materials and methods

2.1. Feed samples

A total of 90 samples from different feeds for ruminants were evaluated in the HGT including roughages ($n = 27$), total mixed rations (TMR) ($n = 6$), commercial compound feeds for dairy cows ($n = 18$), cereal grains ($n = 10$), energy-rich by-products ($n = 5$), legume grains ($n = 6$), oilseed meals ($n = 6$), and other protein-rich feeds ($n = 12$) (Table S1). Data on the nutrient composition of the 90 feeds were not available. Each sample was incubated *in vitro* in the HGT using two inocula substrates: rumen fluid from lactating dairy cows and faeces from sheep. For technical reasons, the incubations using rumen inoculum (RI) and faecal inoculum (FI) were conducted in two separate incubation blocks.

2.2. Animals and diets

Two ruminally-fistulated lactating Jersey cows were used to obtain rumen fluid for the RI incubation block. The cows were offered water and a TMR for *ad libitum* access. The TMR consisted of 33% concentrate mixture, 22% maize silage, 18% grass silage, 15% grass hay, 7% rapeseed meal, 2% barley straw, 1% soybean cake, 1% mineral mixture, and 1% limestone. The concentrate mixture was composed of 22% maize grain, 22% rapeseed cake, 20% barley grain, 19% field beans, 14% peas, and 3% linseed cake.

Adult wether sheep aged about three years were used as donor animals to obtain fresh faeces for the FI incubation block. Since, apart from environmental conditions, individual variation in faecal microbiota composition exists among sheep (Mamun et al. 2019; Szeligowska et al. 2021), the faeces from three sheep were pooled before producing the FI. The three sheep were group-housed on sawdust and offered water and a TMR in dry form for *ad libitum* intake during adaption and sampling periods. The TMR was calculated to meet the requirements of adult wethers according to Steingass (2020) and was composed as shown in Table 1. The adaptation period lasted two weeks and was followed by three weeks of faecal sampling. After three weeks of sampling, one group of sheep was replaced by three other sheep, which were treated and fed in the same way. Animal care and all procedures were in accordance with the German Animal Welfare rules and ethical approval was obtained from the Regierungspräsidium Stuttgart with protocol numbers 35-9185-99/299.

2.3. Substrate collection, inoculum preparation, and in vitro incubation

For the block of the RI incubations, rumen fluid was collected from the two cows before the morning feeding and RI preparation followed the method of Menke and Steingass (1988), which is used as the standard HGT assay by VDLUFA (2012). In

Table 1. Ingredients and nutrient composition [g/kg dry matter (DM), unless otherwise stated] of dry total mixed ration fed to wether sheep.

Ingredients	
Grass hay, chopped	430
Straw, chopped	310
Grain maize, crushed	130
Rapeseed meal	60
Molasses	70
Chemical composition[a]	
DM [g/kg]	914
Ash	56
Ether extract	16
CP	81
aNDFom	592
ADFom	366

CP, crude protein; aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; ADFom, acid detergent fibre expressed exclusive of residual ash.
[a]Analysed chemical composition is averaged from six samples during the experimental period.
[b]Minerals were supplemented as a lick stone placed in the pen (SALEC® Mineral-Leckstein eco,Südwestdeutsche Salzwerke AG, Heilbronn).

brief, a buffer solution (Table S2) was prepared and kept in a water bath at 39°C under constant stirring and flushing with CO_2. The rumen fluid from both cows was mixed and filtered through four layers of cheesecloth. The filtered rumen fluid was added to the reduced buffer solution and kept under CO_2 and constant stirring until the incubation started.

The block of FI incubations used fresh faeces, which were obtained from the rectum of each sheep with two fingers and immediately poured into a sealed plastic box. The three plastic boxes of faeces were immediately transported in a polystyrene box with heat packs to the laboratory. To start the FI preparation, equal amounts of faeces from each sheep were pooled together. Aiple (1992) suggested a ratio of dry matter (DM) [g] : medium solution [mL] of 1 : 20–1 : 50 is adequate for high microbial activity of the FI. Thus, a ratio of 1: 35 was intended in the present study. The required amount of fresh faeces was calculated by this ratio and a measurement of faecal DM averaged of the three sheep taken during the adaptation period. Calculation of the realised faecal DM : medium solution ratio of each faecal sampling for one incubation start was based on measurement of faecal DM after each faeces collection. On average of the 12 performed incubations, the faecal DM concentration was 27% (standard deviation (SD) = 1.8). The average amount of fresh faeces used for one incubation in the HGT with an oven capacity of 57 syringes was 207 g (SD = 10.2) and the realised faecal DM : medium solution ratio was on average 1 : 36 (SD = 3.6). The FI was prepared using a modification of the HGT method using rumen fluid as described by Aiple (1992) with minor modifications. In brief, a buffer solution with the same reagent composition as for the HGT with rumen fluid (Table S2) was prepared and kept in a water bath at 39°C under constant stirring and flushing with CO_2. Half of the reduced medium solution was mixed with the faeces and homogenised in a blender (Robert Bosch GmbH) for two minutes at the highest speed under flushing with CO_2. The homogenate was filtered through four layers of cheesecloth before being combined with the remaining reduced buffer solution. The mixture was kept under CO_2 and constant stirring for 15 minutes until the incubation started.

All incubations were conducted according to the standard method of the HGT (VDLUFA 2012). The feed was ground through a 1-mm sieve (ultracentrifuge mill; type ZM1; Retsch GmbH) and precisely weight at approximately 200 mg into 100-mL glass syringes. After pre-warming the syringes in an oven, 30 mL of either buffered RI or FI was dispensed into each of the syringes, which were then placed into a rotating disk in an oven maintained at 39°C. Due to the oven capacity of 57 syringes, one run for all 90 feed samples was carried out in two ovens on two consecutive days. One run contained one syringe per feed, randomly allocated to the two ovens. Five runs were carried out to achieve five replicates per feed and inoculum. Additionally, in each run, three syringes with hay and three syringes with a concentrate standard with known RI-GP as well as three syringes containing only buffered inoculum, termed as blanks, were included. The GP was recorded after 2, 4, 8, 12, 24, 48, and 72 h of incubation for each syringe. After completion of the five runs with either FI or RI, outliers were detected using the Grubb´s test and removed. The corresponding feed samples were incubated again in two replicates as described before. Eventually, a minimum number of four replicated syringes was used to calculate GP kinetic parameters for each feed and inoculum combination.

2.4. Calculations and statistical analyses

The GP values recorded at each incubation time were corrected for the respective GP values of the blanks. In addition, GP data were standardised by using the correction factor of the standard samples according to the method of VDLUFA (2012) for RI after 24 h. For FI, hay and concentrate standard GP were calculated and averaged after 48 h from previous studies of our institute and the present experiment and were used to standardise GP data. For individual feeds, feed sample categories, and all feed samples together, an exponential equation was fitted to the resulting standardised GP data either without lag phase:

$$GP = b_{gas} * (1 - \exp^{-c_{gas}*t})$$

or with lag phase:

$$GP = b_{gas} * \left(1 - \exp^{-c_{gas}*(t-t0)}\right),$$

where GP [mL/200 mg DM] is the GP after t hours, b_{gas} is the potential (asymptotic) GP [mL/200 mg DM], c_{gas} [%/h] is the rate constant of GP, t is the time [h], and $t0$ is the time of the lag phase [h]. Parameters were estimated by an iterative least-square procedure using the software GRAPHPAD PRISM (GraphPad Software, San Diego, CA, USA). For the choice of the model, a preliminary calculation was made: first, the slope of the GP [mL/200 mg DM/h] was calculated using the difference of the GP between two adjunct individual reading times starting at $t = 0$ h. If this slope was ≥ 2 between 0 and 2 h after incubation, only the model without lag phase was used. If this slope was < 2, the AICc was used to investigate whether the data could be better fitted to an equation with or without lag phase. The time period [h] in which the slope was < 2 was defined as the setting for the model. Differences between the average GP parameters of FI and RI were analysed for various feed categories using PROC TTEST of SAS. (version 9.4 for Windows, SAS Insititute, Cary, NC, USA)

Since the RI-GP after an incubation period of 24 h is generally used for estimating the metabolisable energy concentration and organic matter digestibility of feedstuffs (Menke and Steingass 1988), this incubation time was focused on for comparisons with FI-GP. Therefore, RI-GP after 24 h was used to investigate correlations with data obtained from FI. By using the estimated parameters of the exponential equation for FI-GP from all feed samples, the FI-GP after t hours was estimated for every desired time point. Thus, the period from 24 to 60 h incubation time was evaluated at one-hour intervals. Correlations between the estimated FI-GP values within this period and the measured RI-GP values at 24 h were calculated using PROC CORR of SAS to determine a time period for FI-GP with the highest correlation with RI-GP after 24 h of incubation. As some researchers also use the RI-GP at 48 h, this relationship was also examined.

The effect of inoculum on the variation among replicates within a feed sample was investigated by calculating the coefficient of variation (CV) for each feed sample and both inoculum sources to average the CV for different feed categories using MS-Excel.

The relationships between RI-GP and FI-GP after 24 and 48 h, respectively, were assayed by linear regression analyses for the complete data. Additionally, data subsets for roughages and concentrate feeds were evaluated according to the equation:

$$y = \text{slope} * x + \text{intercept},$$

where y [mL/200 mg DM] was the predicted RI-GP after 24 or 48 h, and x [mL/200 mg DM] was the measured FI-GP after 24 or 48 h. The parameters were estimated using PROC REG in SAS. For validation of the obtained parameters, the data set was randomly divided 100 times into two data subsets, a training data set of $n = 72$ feed samples and a model validation data set of $n = 18$ feed samples by using PROC GLM in SAS.

3. Results

The estimated GP kinetic parameters are presented in Table 2. The potential GP ranged from 48 to 82 mL/200 mg DM for FI and from 54 to 91 mL/200 mg DM for RI, respectively. Significant differences ($p \leq 0.01$) in potential GP were found for roughages, TMR, compound feeds, and cereal grains. The differences between FI and RI for the potential GP were similar among the feed categories and ranged from 8 to 14%. The rates of GP were significantly lower for all feed categories when incubated with FI (range 3.3 to 7.4%/h) compared to RI (range 5.6 to 12.9%/h). The largest differences between the two inocula were found for energy-rich by-products, oilseed meals, and other protein-rich feeds. Averaged across all feeds, the GP curve of FI was consistently below that of RI (Figure 1). The GP kinetic equation for FI had an average potential GP value of 57 mL/200 mg DM and a GP rate of 6.5%/h, whereas the values for RI were 66 mL/200 mg DM and 9.6%/h, respectively. At the beginning of incubation, the GP kinetic curve of FI was associated with a lag phase of 1.51 h, whereas no lag phase was estimated for RI.

When the feed samples were incubated with FI instead of RI, consistently higher CV values in the different feed categories were observed at each incubation time (Table 3). As the incubation period progressed, differences in the CV between FI-GP and RI-GP decreased.

The RI-GP at 24 h was highly positively correlated with the estimated FI-GP over the entire period examined between 24 h ($r = 0.973$, $p < 0.001$) and 60 h ($r = 0.983$, $p < 0.001$)

Table 2. Gas production kinetic parameters for faecal inoculum-gas production (FI-GP) and rumen inoculum-gas production (RI-GP) of different feed categories.

		b_{gas}[a] [mL/200 mg DM]					c_{gas}[a] [%/h]				
		FI-GP		RI-GP			FI-GP		RI-GP		
Feed category	*n*	Mean	*SD*	Mean	*SD*	*p*-value	Mean	*SD*	Mean	*SD*	*p*-value
Roughages	27	55	*7.7*	62	*10.2*	0.01	3.3	*1.35*	5.6	*2.33*	<0.001
TMR	6	56	*3.4*	62	*3.1*	0.01	5.0	*1.28*	8.8	*1.58*	<0.01
Compound feeds	18	64	*6.7*	74	*7.8*	<0.001	7.2	*0.78*	12.1	*1.27*	<0.001
Cereal grains	10	82	*3.8*	91	*3.2*	<0.001	7.4	*1.55*	11.3	*2.24*	<0.001
EBP	5	72	*16.1*	78	*12.4*	0.50	5.6	*1.58*	12.5	*0.96*	<0.001
Legume grains	6	70	*7.9*	78	*6.6*	0.09	6.5	*0.66*	11.5	*1.30*	<0.001
Oilseed meals	6	48	*7.4*	54	*8.4*	0.21	4.7	*0.70*	11.6	*1.46*	<0.001
PRF	12	49	*5.6*	54	*7.2*	0.11	4.9	*1.08*	12.9	*3.81*	<0.001

DM, dry matter; TMR, total mixed rations; Compound feeds for dairy cows; EBP, energy-rich by-products; PRF, other protein-rich feeds; SD, standard deviation of n feed samples for each feed category.

P-values indicate differences between FI-GP and RI-GP ($p < 0.05$).

[a] b_{gas} and c_{gas} calculated from the fitted equation either without lag phase: $GP = b_{gas}*(1 - e^{-c_{gas}*t})$ or with lag phase: $GP = b_{gas}*(1 - e^{-c_{gas}*(t - t0)})$, with GP = gas production after t hours; b_{gas} = potential GP; c_{gas} = rate of GP of b; $t0$ = time of lag phase [h]; lag phases were not averaged for feed categories, due to different underlying models with and without lag phase; lag phases of individual feeds are presented in Supplementary table 1.

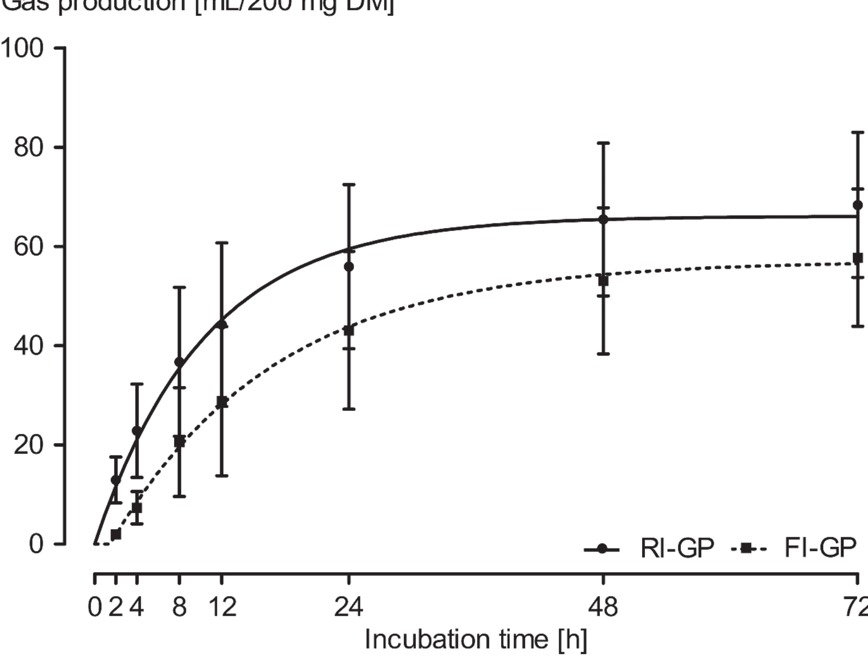

Figure 1. Comparison of mean *in vitro* gas production kinetics between rumen inoculum-gas production (RI-GP) and faecal inoculum-gas production (FI-GP) from 90 feed samples for ruminants. Equation for RI-GP $Y = 66.08*(1 - e^{0.09604*X})$, equation for FI-GP $Y = 57.13*(1 - e^{0.06466*(X - 1.511)})$. Bars represent standard deviations (SD).

Table 3. Comparison of the variation among replicated gas production values for different feed categories and incubation times between faecal inoculum (FI) and rumen fluid inoculum (RI).

		Averaged CV [%]													
		2 h		4 h		8 h		12 h		24 h		48 h		72 h	
Feed category	n	FI	RI	FI	RI	FI	RI	FI	RI	FI	RI	FI	RI	FI	RI
Roughages	27	109	17	38	12	12	8	41	7	13	4	6	3	5	3
TMR	6	71	9	48	6	16	5	9	4	4	4	4	4	6	4
Compound feeds	18	62	8	25	5	14	3	7	3	4	3	4	3	4	3
Cereal grains	10	58	13	36	6	23	4	8	3	4	3	5	3	4	3
EBP	5	76	7	22	6	17	3	18	2	7	2	7	2	6	2
Legume grains	6	85	12	33	7	25	3	11	3	6	2	3	2	3	2
Oilseed meals	6	68	11	30	9	18	5	17	4	6	4	4	4	5	6
PRF	12	70	11	30	7	17	5	15	4	8	4	6	4	6	4

CV, coefficient of variation; TMR, total mixed rations; Compound feeds for dairy cows; EBP, energy-rich by-products; PRF, other protein-rich feeds.
For gas production measurements n counted a minimum number of four replicated syringes.

of incubation (data not shown). The highest correlation was determined at 41 to 52 h of incubation with FI ($r = 0.987$, $p < 0.001$).

Regression analyses to predict RI-GP after 24 and 48 h from FI-GP after 24 and 48 h, respectively, indicated close linear relationships between the two inocula when all 90 feed samples were evaluated together (Figure 2, Table 4). The prediction of RI-GP after 24 h resulted in slope values ranging from 1.02 to 1.10 and high R^2 values ($R^2 = 0.97$ for both,

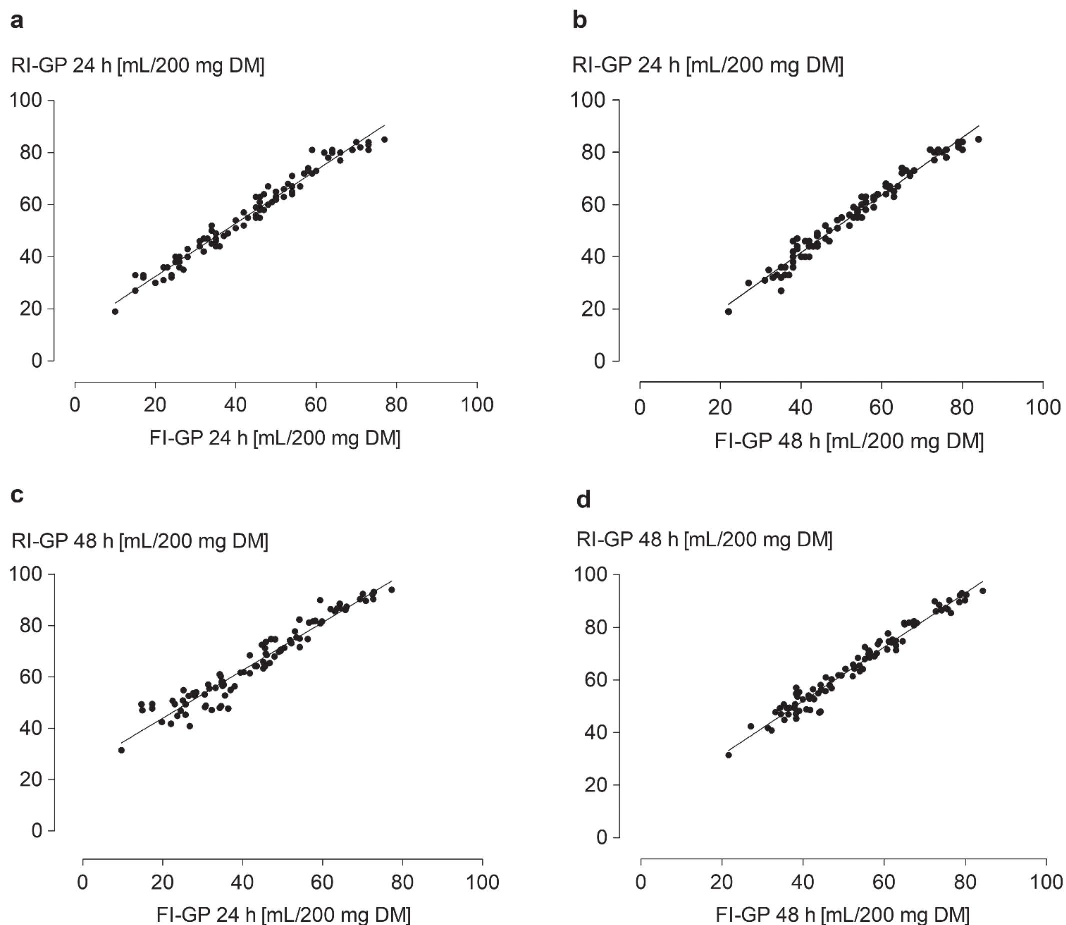

Figure 2. Linear relationships between rumen inoculum-gas production (RI-GP) after 24 and 48 h and faecal inoculum-gas production (FI-GP) after 24 and 48 h from 90 feed samples. The estimated parameters of the linear functions are shown in Table 4.

FI-GP after 24 and 48 h) (Figure 2a,b). There were small differences in the root mean squared error (RMSE) and CV values, with 2.85 and 5.1% for FI-GP after 24 h (Figure 2a) and 2.77 and 4.9% after 48 h (Figure 2b), respectively. The RI-GP values after 48 h could be predicted with greater accuracy from FI-GP after 48 h ($R^2 = 0.97$; RMSE = 2.81; CV = 4.3%) (Figure 2d) than after 24 h ($R^2 = 0.93$; RMSE = 4.12; CV = 6.3%) (Figure 2c). Separate consideration of the feed categories in the regression analysis between RI-GP after 24 h from FI-GP after 48 h (Figure S1) showed that the individual feeds were evenly distributed around the trendline of the regression equation with slightly positive and negative residual values within each category (data of residual values not shown). Therefore, for separate regression analyses, concentrate feeds were summarised in one category in addition to roughages as data subsets. The separate linear regressions for roughages and concentrate feeds resulted in a slightly more precise prediction of RI-GP after 24 h from FI-GP after 48 h for concentrate feeds when compared to the entire data set. For the prediction of RI-GP after 48 h, compared to the entire data set, the separate analysis of roughages resulted in a more accurate prediction and for concentrate feeds in a slightly better prediction by using FI-GP values after 24 h (Table 4). All linear

Table 4. Linear regression equation parameters to estimate rumen inoculum-gas production (RI-GP) after 24 and 48 h from faecal inoculum-gas production (FI-GP) after 24 and 48 h for all feeds and separately for roughages and concentrates.

		Incubation time [h]						
Feed category	n	RI-GP	FI-GP	Slope	Intercept [mL/200 mg DM]	R^2	RMSE	CV [%]
All feeds	90	24	24	1.02	11.84	0.97	2.85	5.1
			48	1.10	−2.70	0.97	2.77	4.9
		48	24	0.93	25.25	0.93	4.12	6.3
			48	1.03	10.98	0.97	2.81	4.3
Roughages	27	24	24	1.03	11.79	0.95	2.57	6.2
			48	1.17	−7.62	0.97	2.07	4.9
		48	24	1.02	11.84	0.97	2.85	5.1
			48	1.00	11.86	0.95	2.33	4.3
Concentrates	57	24	24	1.03	11.84	0.96	3.06	4.8
			48	1.01	4.04	0.98	2.07	3.3
		48	24	1.05	18.68	0.94	3.85	5.4
			48	1.03	10.71	0.96	3.10	4.4

DM, dry matter; RMSE, root mean squared error; CV, coefficient of variation.

Table 5. Validation parameters of linear regression equations to estimate rumen inoculum-gas production (RI-GP) after 24 h from faecal inoculum-gas production (FI-GP) after 24 and 48 h from 90 feed samples with a training data set of *n* = 72 feed samples and a validation data set of *n* = 18 feed samples.

Incubation time [h]						
RI-GP	FI-GP	Slope	Intercept [mL/200 mg DM]	R^2	RMSE	CV [%]
24	24	0.99	0.26	0.97	2.92	5.24
	48	0.99	0.33	0.97	2.81	5.04
48	24	0.99	0.30	0.92	4.27	6.55
	48	1.00	0.22	0.96	4.43	2.90

DM, dry matter; RMSE, root mean squared error; CV, coefficient of variation.

regression equations for predicting the RI-GP after 24 and 48 h from FI-GP after 24 and 48 h, respectively, using the complete data set of the 90 feeds, could be validated with high accuracy (Table 5).

4. Discussion

4.1. Potential GP and GP rate

We aimed to compare bovine rumen fluid and sheep faeces as inocula in the HGT in order to better assess the possibilities of using FI instead of RI in *in vitro* feed evaluation assays. Consistent with previous studies that compared rumen fluid with cow faeces (Aiple 1992; Mauricio et al. 2001; Cone et al. 2002) or sheep faeces (Aiple 1992; Borba et al. 2001; Váradyová et al. 2005; Zicarelli et al. 2011) as inoculum for *in vitro* incubation assays, the total GP was lower with FI than RI on average of all feeds and regardless of the feed categories. The consistently lower GP rate in the present study was also detected in earlier studies (Aiple 1992; Mauricio et al. 2001; Cone et al. 2002; Váradyová et al. 2005; Zicarelli et al. 2011). Different microbial communities in RI and FI probably caused these differences in the GP kinetic parameters. A meta-analysis of the bovine gastrointestinal

tract microbiota found that, despite some shared traits, considerable differences in the groups of microorganisms exist between the rumen content and faeces of cattle at the genus level (Holman and Gzyl 2019). No protozoa were found in the caecum and colon (Hungate 1966; Hobson 1971), whereas they have high cellulolytic activity in the rumen (Dehority 2003). The differences in the bacterial communities between rumen fluid and faeces follow the available substrates and physiochemical conditions which shift in the digestive tract of ruminants (Mao et al. 2015). Precaecally undigested organic matter enters the large intestine and thus represents a less degradable substrate, including some starch and fibre, for the microbial population in the large intestine and colon compared to that in the rumen (Tanca et al. 2017). It is assumed that the microbes in the hindgut are able to ferment less degradable substrates, such as high roughage feeds, very effectively (Fon and Nsahlai 2012). However, the passage rate in the hindgut might be relatively high, giving the microbes limited time to ferment and grow, which is why the initial microbial count may be lower when using faeces compared to rumen fluid for *in vitro* incubations (Fon and Nsahlai 2012). Overall, due to the less nutritive value of the substrate, shorter retention time of the digesta, and a smaller bacterial population in the caecum and colon than in the rumen, FI has a lower fermentation activity than RI (Mauricio et al. 2001).

4.2. Lag phase and GP progression

In the present study, FI but not RI incubations were associated with a lag phase of GP. This is consistent with the results of El-Meadaway et al. (1998) and Mauricio et al. (2001), who reported longer lag phases from FI. The lag phase may reflect a longer time to colonise the substrate with FI due to an overall lower microbial density of faeces (Posada et al. 2012). Furthermore, the microorganisms in faeces are probably naturally in a "state of suspended animation" from which they have to grow, divide and thus develop, which takes longer with faeces than rumen fluid (Rasouli and Amiri 2016). The initially delayed and slower FI-GP approached the RI-GP over time, as described before (Aiple 1992; Fon and Nsahlai 2012), revealing the faecal microbial growth with time. Despite the described differences in the microbiota between rumen fluid and faeces, Liu et al. (2016) and De Graeve and Demeyer (1988) observed no significant differences in the relative proportions of volatile fatty acids between rumen contents and faeces, as did Ramin et al. (2015) between *in vitro* incubations with RI and FI. These authors suggested that both microbial communities appear to have a similar type of fermentation. However, the study by Ramin et al. (2015) also showed that less CH_4 per unit of fermented substrate and less total gas per mmol of volatile fatty acids were produced from incubations with FI compared to RI. They concluded that more carbon was used to produce volatile fatty acids instead of gas using FI than RI, possibly through acetogenesis from CO_2. In addition, they found lower blank volatile fatty acid concentrations in FI compared to RI, indicating a generally lower fermentation activity level in faeces. Accordingly, although the averaged curve of FI-GP was at a lower level, the close progression of the two GP curves (Figure 1) after initial delayed activation of the faecal microorganisms indicates a similar fermentation function in the rumen and faecal microbial communities.

4.3. Variation of GP between RI and FI

Faeces may represent a more constant inoculum in the HGT compared to RI, as the compact form of sheep faeces could protect the microorganisms for a short time from external influences, such as oxygen or temperature changes. Also, faeces may be less affected by fluctuations of the pH value, digestive activity, and passage rate than rumen fluid, which is directly influenced by feed and water intake, rumination, and saliva flow. However, this hypothesis was not supported by the results of the present study because a greater variation among the replicates of individual feeds was found for incubations with FI compared to RI (Table 3). This result is similar to that of Mauricio et al. (2001), who concluded that the more active and larger microbial population of the rumen fluid may be less sensitive to external influences than faeces. This could have affected the collection, preparation and incubation processes in the present study. Adherence to a strict standardised protocol, therefore, seems to be particularly important in order to produce an inoculum with faeces with which reproducible results can be achieved.

4.4. Relationship between RI and FI

The analysis of the relationship between RI-GP and FI-GP was focused on RI-GP after 24 h because this is the incubation time used to estimate metabolisable energy and organic matter digestibility in the standard HGT method (Menke and Steingass 1988). Ideally, new estimation equations for metabolisable energy and organic matter digestibility would be derived using *in vivo* data for the same feeds studied *in vitro*. However, such *in vivo* data were impossible to determine for the present data set and samples from previous *in vivo* studies were unavailable for the present *in vitro* study. Therefore, our approach was to estimate 24 h RI-GP by using FI-GP and then continue using established equations based on RI-GP in estimating digestibility.

The highest correlation of 24 h RI-GP was found within the time period of 41 to 52 h for FI-GP in the present study. Other authors found that GP, volatile fatty acid production, degraded organic matter, and fermentation rate were correlated (Zicarelli et al. 2011) or potential GP and organic matter digestibility were highly correlated (Mauricio et al. 2001) between RI and FI. Nsahlai and Umunna (1996) reported a high correlation ($R^2 = 0.85$) between the GP of sheep rumen fluid and faeces after 48 h but considerably lower correlations for GP after 24 h. This was not consistent with the results of the present study, where RI-GP after 24 h was highly correlated overall with FI-GP in the period of 24 to 60 h. A reason for this discrepancy could be related to the sampling method. While Nsahlai and Umunna (1996) collected the faeces within 2 h after defaecation, we obtained them from the rectum. Although they stored the faeces in a CO_2-flushed thermal flask, this procedure may have harmed the microbial activity, leading to delayed and reduced microbial activation *in vitro*. Váradyová et al. (2005) investigated potential GP, GP rates, *in vitro* DM digestibility, and total volatile fatty acid production with RI and FI from sheep. The estimated parameters were poorly related overall between the two inocula and the authors speculated that the concentration of fresh faeces might have been too low. The ratio of faecal DM to buffer medium should be within a range of 1 : 20 to 1 : 50, with deviations above or below reducing the GP (Aiple 1992). Such reduction of GP was more pronounced for feeds with a high structural carbohydrate content, whereby the feeds

examined in the study of Váradyová et al. (2005) consisted of meadow hay, wheat straw, amorphous cellulose, and barley grain. This highlights again that a standardised protocol, which includes consistent inoculum preparation, is required to achieve comparable results.

Some studies have conducted regression analyses between the GP obtained with rumen fluid and faeces as inocula. RI-GP after 48 h of incubation was accurately predicted from FI-GP after 48 h by linear regression analysis (Slope = 0.97, R^2 = 0.86) (Nsahlai and Umunna 1996). Cone et al. (2002) found a similar relationship for that time period (Slope = 0.95, R^2 = 0.88), whereas an incubation time of 24 h for both inocula resulted in a less precise prediction of RI-GP from FI-GP (Slope = 0.75, R^2 = 0.61). In the present study, an even higher estimation accuracy was found for predicting RI-GP after 24 and 48 h from 24 and 48 h incubations with FI-GP, respectively, considering all feeds. Differences in the results between the present and previous studies may be related to the diet of the donor animals. In general, hay feeding provides sufficient amounts of fermentable substrate for the lower digestive tract microbial community, as high FI-GP values were obtained at 100% hay feeding of the donor animals (Aiple 1992). Nevertheless, the ingredient composition of the donor animal diet should be manifold when the inoculum is used for testing different feeds (Aiple 1992). Cone et al. (2002) fed the cows, from which they collected rumen fluid and faeces 2 h after the morning feeding, with hay for *ad libitum* intake and 1 kg of a standard concentrate with low CP concentration once in a day. The low CP concentration in that concentrate may have been particularly relevant, as many of their incubated feed samples were high in CP concentration (Cone et al. 2002). Can et al. (2009) conducted an *in vitro* GP assay by using the method according to Aiple (1992) with a dilution (faecal DM : buffer medium) of 1 : 27 for preparation of the sheep FI. In contrast to the good relationship between RI-GP and FI-GP of the aforementioned and the present study, Can et al. (2009) found poorer relationships using linear regression analyses for GP after 24 h (Slope = 0.64, R^2 = 0.39) and 48 h (Slope = 0.70, R^2 = 0.56). The sheep from which rumen fluid and faeces were collected had a diet containing 600 g grass hay and 600 g concentrate mixture per day. Although this diet contained diverse ingredients, the concentrate proportion was very high. FI-GP after 48 h of incubation was reduced with faeces from sheep fed 30–40% concentrates compared to a pure hay diet (Aiple 1992). To provide the microbial population in the hindgut of the donor animal with various nutrients and, at the same time, extend the retention time of the feed, the TMR of the sheep in the present study contained straw in addition to grass hay, and the concentrate proportion was only 26%. Maize grain provided some ruminally undegraded starch, and rapeseed meal provided some ruminally undegraded protein likely available for the hindgut microbes. Divergent results found in literature and the present study conducted with varying diets and feeding schedules, therefore, highlight the necessity of standardised donor animal feeding when the FI method is used.

4.5. Potential of the regression equations

Including nutrient concentrations as regressors in the linear regression analysis might increase prediction accuracy, but the nutrient composition data of the tested samples were not available. Nevertheless, it was possible to assess whether the prediction of RI-GP from FI-GP for specific feed categories leads to different estimation accuracy than a common equation. This is important because systematic under- or over-

evaluation of individual feed categories should be avoided (Menke and Steingass 1988). Such deviations were not found for any feed category in the present study (Figure S1), indicating that the estimation of RI-GP by FI-GP is suitable for each feed category investigated. Menke and Steingass (1988) concluded from their estimates of metabolisable energy and organic matter digestibility from GP that different equations for different feed categories are advantageous above a single equation for all feeds. By using feed categories of the present study, RI-GP after 24 h was estimated from FI-GP after 48 h with slightly higher accuracy when concentrate feeds were considered separately. However, this slightly higher accuracy did not occur in a separate analysis of roughages. The application of feed category-specific equations requires that the particular feeds be correctly identified, which is not a simple task in all laboratories. However, the choice of a wide range of nutrient composition leads to more robust equations that can cover possible variations occurring in unknown feed samples (Aiple 1992). Thus, the use of one single linear regression equation for all feeds of different categories may be preferred, as very high estimation accuracy has been shown. Furthermore, the high accuracy achieved by validating the linear regression equations for all feed samples showed the potential for applying the equations to other data sets.

4.6. Standardisation of the methodology

Overall, divergent results in the literature and the present study indicated that the procedure regarding the collection, processing, and incubation with FI, as well as the feeding of the donor animals, have a major impact on the *in vitro* GP data. The approach of the present study has proven to be suitable for generating a high-quality FI. Nevertheless, methodological improvements would still be desirable, such as the collection of faeces. Since Aiple (1992) found a significant decrease in GP when incubating hay after one hour of aerobic storage at room temperature, we chose to collect the faeces in the present study from the rectum. For reasons of animal welfare, however, the collection of dispensed faeces should be comprehensively examined in further studies.

Consensus on a standard protocol and application of such protocol is suggested for obtaining reproducible results in GP studies with a FI.

5. Conclusions

The present results showed consistently lower GP kinetic parameters and a lag phase when faeces instead of rumen fluid were used as the source of inoculum. Similar GP progression with both inocula, high correlations, and strong linear relationships between RI-GP and FI-GP were observed. One overall regression equation for diverse feedstuffs may be adequate to estimate RI-GP from faecal data. In conclusion, FI can be used as an alternative inoculum for the *in vitro* GP technique of the HGT to predict RI-GP for various feedstuffs for ruminants. The development and application of a standard protocol for obtaining and processing faeces is suggested. Further studies should examine the faecal microbiome and how it is influenced by the preparation and incubation processes to better assess faeces as an inoculum source.

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Disclosure statement

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Data availability statement

Data that support the findings of this study will be provided upon justified request by the authors.

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4.2 Manuscript 2: The technique of sheep faeces preservation affects the microbiome activity and associated gas production kinetics *in vitro*

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The technique of sheep faeces preservation affects the microbiome activity and associated gas production kinetics *in vitro*

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Abstract

In vitro systems involving microbial fermentation typically require freshly obtained inocula, such as rumen fluid or faeces. The objective of this study was to test whether preserved faeces can be used instead of fresh faeces in the Hohenheim gas test (HGT). Fresh faeces from sheep (control, C) was compared with seven differently preserved faeces by using nine different feeds and studying *in vitro* gas production (GP) ($n = 6$–9 per treatment). Preservation involved freezing at −20°C (FR), shock-freezing with liquid nitrogen (N) and additional freezing at −20°C (FRN), FRN followed by defrosting (FRNdef), shock-freezing with liquid N and freeze-drying (FDN), freeze-drying (FD) and freeze-drying with storage for 3 weeks (FD3W) or 6 months (FD6M). Metaproteomics was used to analyse microbiome composition and function in treatments C, FR, FRN, FD, and FDN (n = 3 per treatment). On average across all feeds, the potential GP with FR and FRN (61 mL/200 mg DM) was comparable to that of C (62 mL/200 mg DM), whereas values for FRNdef, FDN, FD, FD3W, and FD6M were 85, 78, 76, 78 and 71% of C, respectively. All estimated GP kinetic parameters were affected by feed and preservation interactions ($P<0.001$). Microbiomes from C, FR, and FRN differed from those of FD and FDN based on the relative abundance of the core proteins ($P<0.001$). FD and FDN showed a significant decline of Bacteroidota, functional redundancy values, and specific proteins such as carbohydrate esterases (CE) ($P<0.05$) and glycoside hydrolases (GH) ($P<0.01$). Overall, frozen faeces closely resembled fresh faeces and can serve as a viable alternative inoculum source in the HGT. This may reduce animal numbers used for scientific purposes, but preservation and storage must be strictly standardised to maintain an active microbiome for GP-based *in vitro* tests.

Introduction

In vitro gas production (GP) techniques have become increasingly relevant for evaluating the nutritive value of feeds for ruminants, such as the organic matter digestibility, or the metabolisable energy. Standard *in vitro* methods use rumen fluid as an inoculum source, usually obtained from rumen-cannulated animals (Menke and Steingass 1988; Tilley and Terry 1963). To avoid the use of rumen fluid for ethical and practical reasons, the suitability of freshly obtained faeces from ruminants as an inoculum source has been studied (Aiple et al 1992; Mauricio et al 2001; Zicarelli et al 2011). Rippstein et al (2024) have recently shown that freshly voided sheep faeces can be used as an alternative inoculum source to rumen fluid for assaying various feeds for ruminants, including roughages, total mixed rations, commercial compound feeds for dairy cows, cereal grains, energy-rich by-products, legume grains, oilseed meals, and other protein-rich feeds.

The need for fresh faeces requires access to donor animals. Research organisations maintain a donor animal herd or obtain faeces from livestock farms, which causes practical and organisational challenges. Loss of microbial activity of the inoculum source due to storage and transport of the faeces, or variation caused by host animal management, including feeding and sampling techniques, can affect the inoculum quality (Mould et al 2005). Preservation of the inoculum source for subsequent *in vitro* assays would help in minimising inoculum variability and better comparability of data by restricting the collection of faeces to only a few specialised centres (Spanghero et al 2019). To improve animal welfare following the 3R principles (replace, reduce, refine), such stockpiling would also reduce the overall number of donor animals required. In this regard, several previous studies have investigated different methods of preserving rumen fluid as a source of inoculum for *in vitro* feed evaluation (Belanche et al 2019; Chaudry and Mohamed 2012; Hervás et al 2005; Spanghero et al 2019; Tunkala et al 2022). These studies have shown that

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fresh rumen fluid could, in principle, be replaced by preserved rumen fluid. However, preservation suitability was highly dependent on the preservation technique, and microbial activity may be reduced. Since microbial communities in bovine rumen content and faeces are different (Holman and Gzyl 2019) and the physical properties of these two inoculum sources differ, the preservation processes may affect microbial activity in rumen fluid and faeces differently. Information on the effects of preserving sheep faeces as an inoculum source for *in vitro* studies is not available. Furthermore, to the best of our knowledge, the active part of the microbial community in a preserved inoculum source has not been studied in ruminant *in vitro* assays. However, studies conducted with faeces from humans (Li et al 2023b; Song et al 2016) and dogs (Lin et al 2020; Song et al 2016) using 16S rRNA have shown that different preservation methods can alter the microbial community in different ways.

This study investigated different techniques of sheep faeces preservation by examining effects on the GP and microbiome, intending to maintain high microbial activity during *in vitro* incubations. Using a metaproteomics workflow, proteins assigned to actively growing microbial cells were detected, as only these cells contribute to fermentation and GP. The overall objectives of the study were to test whether the associated data on the microbiome could explain the GP of the preserved sheep faeces as an inoculum source and whether a technique could be described that would allow the use of preserved faeces instead of fresh faeces.

Materials and methods

Study organisation

The study included a GP kinetic assay and a microbiome assay. For capacity reasons, the two assays could not be examined simultaneously. Therefore, the assays were conducted in two consecutive blocks, with each block comprising all incubation runs for the respective assay.

Animals and diet

In total, 12 adult wether sheep aged approximately four years were used as donor animals to obtain faeces to be used for *in vitro* incubations. The animals were kept on sawdust and were offered water and a total mixed ration (TMR) in dry form for *ad libitum* intake during adaptation and sampling periods. The TMR was formulated to meet the requirements of adult wethers according to Steingass (2020) and was composed as presented in Table S1. The sheep were housed in groups of three at the same time and replaced with six other sheep during the experiment according to the ethical protocol. For all sheep, the adaptation period lasted 2 weeks and was followed by 2 weeks of faecal sampling. Animal care and all procedures were in accordance with the German animal welfare regulations and approved by the Regierungspräsidium Stuttgart with protocol numbers 35-9185-99/408.

Feed samples and their processing for the *in vitro* assays

Grass silage, maize silage, wheat grain, maize grain, sugar beet pulp, rapeseed meal and soybeans, standard concentrate mix, and standard grass hay were used as feeds in the GP kinetics assay. The standard concentrate and hay samples are references with known GP and used as controls in the standardised HGT assay using rumen fluid (VDLUFA 2012). For these samples, the GP after 48 h was known from a previous study that used fresh faecal inoculum (Rippstein et al 2024). For capacity reasons, only the grass hay, wheat grain, and rapeseed meal were used for the microbiome assay. All feed samples were ground with a centrifugal mill (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan, Germany) through a 1-mm sieve before being used. Each sample was incubated *in vitro* in the HGT using differently preserved sheep faeces as a faecal inoculum source as described later. All incubations were carried out according to the standard method of the HGT (VDLUFA 2012) using prewarmed 100-mL glass syringes that contained approximately 200 mg of the ground feed sample.

Faeces collection and preservation treatments

For both assays, fresh faeces were obtained from the rectum of six sheep and mixed. Immediately afterwards, equal proportions of the faeces were weighed in random order for the different preservation treatments. The amount of faeces required per treatment was calculated to achieve a ratio of faecal dry matter (DM) (g): medium solution (mL) of 1: 35 (Rippstein et al 2024) for incubations in the HGT. Two measurements of faecal DM were taken during the adaptation period and averaged for the six sheep to calculate the required amount of fresh faeces. Calculation of the actual faecal DM: medium solution ratio of each faecal sampling was based on the measurement of faecal DM after each faeces collection. In the GP kinetic and microbiome assays, on average across all incubation runs, the actual ratio of faecal DM: medium solution was 1: 31 (SD = 1.3) and 1: 30 (SD = 0.8), respectively. Each incubation run comprised 30 syringes for the GP kinetic assay and 18 syringes for the microbiome assay, using 131 g (SD = 3.7) and 76 g (SD = 0.6) of fresh faeces, respectively.

A description of the preservation treatments is summarised in Table 1. For the GP kinetic assay, fresh faeces were immediately transported in a polystyrene box with heat packs to the laboratory to prepare non-preserved, fresh faecal inoculum as the control treatment (C) in the HGT. For the other treatments, faeces were handled in one of the following ways before being used as inoculum source for the *in vitro* incubations to measure GP in the HGT (n = 6–9 incubations per treatment). In the **FR** treatment, faeces were stored frozen at −20°C for 8 days. In the **FRN** treatment, faeces were shock-frozen with liquid nitrogen (N) and then stored at −20°C for 8 days. Faeces of the **FRNdef** treatment were treated like FRN and defrosted for 12 h at 4°C. In the **FDN** treatment, faeces were shock-frozen with liquid N, stored frozen at −20°C for 4 days, and then freeze-dried for 3 days. The faeces of **FD** were stored frozen at −20°C for 4 days and then freeze-dried for 3 days. The **FD3W** and **FD6M** faeces were treated like FD, but were additionally stored at room temperature for 3 weeks or 6 months.

Treatments C, FR, FRN, FD, and FDN were used to study the microbiome in the *in vitro* incubations of the HGT (n = 3 incubations per treatment). FRNdef was not analysed for the microbiome, as this treatment revealed poorer GP parameter estimates than FR and FRN. For capacity reasons, the FD3W and FD6M treatments were also excluded from microbiome analysis.

The faeces from all treatments except C were stored vacuum-packed in plastic bags until use in the laboratory for both assays.

Inoculum preparation and *in vitro* incubation

Faecal inoculum was prepared using the same procedure for all treatments by following a modification of the HGT assay according to Aiple et al (1992) with minor adjustments. In brief, a buffer solution (Table S2) was prepared and kept in a water bath at 39°C under constant stirring and flushing with CO_2. Half of the reduced medium solution was mixed with the fresh or preserved faeces and homogenised in a blender (Robert Bosch GmbH)

Table 1. Overview of the control (C) and faeces preservation treatments

Code	Treatment
C	Fresh faeces
FR	Frozen faeces stored for 8 days at −20°C
FRN	Shock-frozen faeces with liquid nitrogen stored for 8 days at −20°C
FRNdef	Faeces treated like FRN followed by defrosting for 12 h at 4°C
FDN	Shock-frozen faeces with liquid nitrogen and freeze-dried
FD	Freeze-dried faeces
FD3W	Freeze-dried faeces stored for 3 weeks at 23°C
FD6M	Freeze-dried faeces stored for 6 months at 23°C

for 2 min at the highest speed under flushing with CO_2. The homogenate from faeces and medium solution was filtered through four layers of cheesecloth before being mixed with the remaining reduced buffer solution. The mixture was kept under CO_2 and constant stirring for 30 min until the incubation started. Thirty millilitres of the buffered faecal inoculum from the respective treatment were dispensed into each of the feed-containing syringes, which were then placed into a rotating disk in an oven maintained at 39°C.

For the GP kinetic assay, three runs per treatment were conducted, each containing two repeated syringes per feed sample, three syringes each with the standard hay and concentrate mix samples, and four syringes containing only buffered inoculum, termed as blanks. Hence, the minimum number of replicates was $n = 6$. All syringes were allocated randomly in the oven. The GP was recorded after 2, 4, 8, 12, 24, 48, and 72 h of incubation.

Three runs per treatment were also carried out in the microbiome assay. After inoculum preparation and 30 min of CO_2 flushing, two blanks containing only buffered inoculum were taken and pooled (Pre-incubation). Each run contained four replicated syringes per feed sample, randomly allocated in the oven, of which two syringes were pooled after 8 h and two syringes after 24 h of incubation. Thus, for each treatment, feed and blank samples, and incubation time, three replicates resulted for microbiome analysis. All samples from the HGT in microbiome assay were centrifuged after sampling (10,000 g, 30 min, 4°C), and the residual pellet was stored at −80°C until microbiome analyses after the end of the study.

Analyses

Chemical analysis of sheep diet and feed samples

Samples of the sheep diet and the nine feed samples were ground through a 0.5-mm screen for chemical analysis. Dry matter (method no. 3.1), N (method no. 4.1.1), ash (method no. 8.1), ether extract (method no. 5.1.1) and fibre fractions (method no. 6.5.1 and 6.5.2) were analysed according to the standard methods used in Germany (VDLUFA 2012). The chemical composition of the sheep diet and feed samples is shown in Table S1 and Table S3.

Gas production calculations and statistical analyses

The GP at each incubation time was corrected for the respective GP of the blanks according to the method of VDLUFA (2012). Mean GP, SD, and coefficients of variation (CV) per treatment and incubation time, calculated across the nine feeds, are shown in Table S4.

An exponential equation for all feed samples together and for each single syringe per feed sample was fitted to the corrected GP values following incubation with C, FR, FRN, FRNdef, FDN, FD, FD3W, and FD6M either without a lag phase:

$$GP = b_{gas} * \left(1 - exp^{-c_{gas}*t}\right) \quad [1]$$

or with a lag phase:

$$GP = b_{gas} * \left(1 - exp^{-c_{gas}*(t-t0)}\right) \quad [2]$$

where GP (mL/200 mg DM) is the GP after t hours, b_{gas} is the potential (asymptotic) GP (mL/200 mg DM), c_{gas} (%/h) is the rate constant of GP, t is the incubation time (h) and $t0$ is the time of the lag phase (h). Parameters were estimated by an iterative least-square procedure using the software GRAPHPAD PRISM (version 5.00 for Windows, GraphPad Software, San Diego, CA, USA). Both equations were applied to the dataset and the equation with the lowest Akaike Information Criterion (AICc) was used. The GP parameters estimated for each syringe were subjected to a two-way ANOVA using PROC MIXED of SAS (Version 9.4 for Windows, SAS Institute, Cary, NC, USA). Implausible values were eliminated by calculating the standard error (SE) in relation to the estimated value of the parameter b_{gas} for each syringe; if this value was > 25%, the respective syringe was not included in the two-way ANOVA. If less than three syringes remained for the analysis after outlier detection, the corresponding combination of feed and treatment was not included in the ANOVA. In this case, a dot was placed in the results table. For the two-way ANOVA, the following model was used:

$$y_{ij} = a_i + b_j + (ab)_{ij} + e_{ij} \quad [3]$$

where y_{ij} is the observation of the estimated parameter, a is the fixed effect of the treatment (C, FR, FRN, FRNdef, FRNdef, FDN, FD, FD3W, and FD6M), b is the fixed effect of the feed sample (grass hay, grass silage, maize silage, wheat grain, maize grain, sugar beet pulp, concentrate mixture, rapeseed meal, and soybeans), $(ab)_{ij}$ is the interaction between the i-th treatment and the j-th feed sample, and e_{ij} is the residual error. Variance homogeneity and normal distribution were verified on the residuals for each trait. The results are presented as least squares means and standard error of the mean (SEM). Statistical significance was set at $P \leq 0.050$.

The relationships between GP of C (C-GP) and GP of all preserved treatments at 24 and 48 h were assayed by linear regression analyses, including all nine feed samples according to the equation:

$$y = slope * x + intercept \quad [4]$$

where y (mL/200 mg DM) is the predicted C-GP after 24 or 48 h, and x (mL/200 mg DM) is the measured GP of the preserved treatments after 24 or 48 h. The parameters were estimated using PROC REG in SAS.

Metaproteomics analyses

The effect of different preservation techniques on the microbiome was analysed by a metaproteomics workflow, which allows the identification of proteins recently translated in active microbial cells in contrast to DNA which might be artefacts of residual inactive cells. Sample preparation was done using 1 g of still slightly frozen faecal pellet which was mixed intensively with 8.75 mL ice-cold MC-buffer (0.2 M NaCl; 50 mM Tris-HCl pH 8; 0.1%

methylcellulose 400cP) and rotated for 2 h at 4°C. Samples were sonicated for 1 min in a sonication bath, vortexed, and stored on ice. The suspension was pressed through a two-layered, sterile cheesecloth into a 50 mL Falcon tube. Residues were rinsed with 6.25 mL Rinse-buffer (2 M NaCl; 50 mM Tris-HCl pH 8) and pressed again through the cheesecloth. The filtrate was centrifuged at 200 *g* for 10 min at 4°C. The supernatant was filtered through glass microfibre filters (grade GF/A, 25 mm, Whatman™) in sterile plastic filter holders into fresh 50 mL Falcon tubes. The filtrate was centrifuged at 10,000 *g* for 15 min at 4°C. The pellets were resolved in 1.25 mL wash buffer (50 mM Tris-HCl pH 7.5; 0.1 mg/mL chloramphenicol; 1 mM phenylmethylsulfonyl fluoride) on ice and subsequently centrifuged at 10,000 *g* for 15 min at 4°C. Washing was repeated with 625 µL wash buffer and the suspension was transferred to a fresh 2 mL tube to reveal a 100 mg biomass pellet after centrifugation at 10,000 *g* for 20 min at 4°C. Cell pellets were stored at −20°C until protein extraction followed by protein digestion and peptide purification as described by Sáenz et al (2021).

Peptide mixtures were measured using an Exploris 480 mass spectrometer (Thermo Fisher Scientific) faced with an Ultimate 3000 nano-RSLC (Thermo Fisher Scientific) at the Mass Spectrometry Unit of the Core Facility Hohenheim. Peptides were concentrated and desalted on a trap column (5 mm × 30 µm, Thermo Fisher Scientific). Separation of peptides was performed on a 25 cm × 75 µm nanoEase MZ HSS T3 reversed-phase column (100 Å pore size, 1.8 µm particle size, Waters, USA). Peptides were loaded onto the column in solvent A (0.1% formic acid) at a flow rate of 300 nL/min and subsequently eluted with increasing levels of solvent B (80% ACN in 0.1% formic acid) using a gradient with the following profile: 2–10% solvent B in 2 min, 10–17% solvent B in 16 min, 17–32% solvent B in 17 min, 32–50% solvent B in 8 min, 50– 96% solvent B in 5 min, isocratic 96% solvent B for 2 min, 96–2% solvent B in 3 min and isocratic 2% solvent B for 7 min. The MS/MS instrument was set to positive ion mode. Full scans were acquired in the mass range from m/z 300 to 1600 in the Orbitrap mass analyser at a resolution of 120,000 followed by fragmentation (HCD, normalized collision energy of 30) of the 25 most intense precursor ions (Minimum Intensity for Intensity Threshold = 3.0e5). High-resolution MS/MS spectra were acquired with a resolution of 15 000. For MS/MS analysis, only precursor charge states from 2 to 4 were considered, and the isolation width was set to 1.6 Da. The target values were 1e6 charges for the MS scans and 9e4 charges for the MS/MS scans with a maximum fill time of 50 ms and 40 ms, respectively. Fragmented masses were excluded for 30 s after MS/MS.

A total of 116 raw files were analysed using the software MetaLab-MAG (Cheng et al 2023). Microbial and host peptides/proteins were identified and quantified using the default parameters. The MGnify Cow Rumen metagenome-assembled genomes (MAG) catalogue v1.0 (Richardson et al 2023) was used as a reference for microbial proteins, while the *Ovis aries* (sheep) proteome (Uniprot ID: UP000002356) was used for host proteins. The catalogue contains 5,578 prokaryotic genomes from the cow rumen microbiome, clustered into 2,729 species representatives. To obtain an updated taxonomy, all MAGs were downloaded from the European Nucleotide Archive (ENA) and their taxonomy was reannotated using GTDB-Tk v2.3.0 (Chaumeil et al 2022) and the Genome Taxonomy Database (GTDB) r214.1 (https://gtdb.ecogenomic.org/). Unless otherwise mentioned, the comparison between groups was done using the relative abundances of the label-free quantification (LFQ) intensities from the protein and taxonomic groups using the tool LFQ-Analyst (Shah et al 2020). The relative abundance of the different phyla was compared between treatments using a linear model for differential abundance analysis (LinDA) (Zhou et al 2022). The C treatment and the pre-incubation were used as reference groups. Ordination analyses were based on Bray–Curtis dissimilarities and the permutational multivariate analysis of variance using distance matrices was done with the function *Adonis2(*permutations = 1000*)*. Ordination based on protein groups was done using the core proteins. Core protein groups were defined as the proteins that were present in at least 81% of the total samples (71 samples). Data were imputed following the Perseus approach. Changes between the proteins were considered significant if $P<0.05$, Log2 fold change > 2 and > 1 identified peptides. *P*-values were corrected by the Benjamini–Hochberg procedure. Normalised functional redundancy (nFR) was calculated at the proteome level across all samples as described in Li et al (2023a). The workflow and scripts were adapted for use of the Metalab-MAG outputs. The means of nFR were compared using ANOVA and Tukey test. Data wrangling and statistical tests were performed in R base v.4.0.2 (Libraries: "tidyverse," "patchwork," "car" and "vegan") (Oksanen et al 2020). R and python scripts for data wrangling and plotting are available in (https://github.com/SebasSaenz/Papers_wf/tree/main/conservation_protocol/code).

Results

Effects of preservation methods on GP

On average across all feeds, the GP curve of C was above all preservation treatments, without a lag phase (Fig. 1). The progression of the FR and FRN GP curves was closest to that of C with low or no estimated lag phase, and comparable potential GP (Table 2). The GP curve of FRNdef was on a lower level than FR and FRN and associated with a longer lag phase and a lower potential GP. The GP curves of the freeze-dried treatments FDN, FD, FD3W, and FD6M were at an even lower level and were all associated with a lag phase. The GP rate was highest with FD6M and C, and it was reduced for all other treatments compared to C.

The two-way interaction of feed and treatment was significant ($P<0.001$) for potential GP, GP rate, and the lag phase (Table 3). The potential GP did not differ for any feed between C, FR and FRN. The potential GP accounted for 97% (wheat grain) to 109% (rapeseed meal) with FR and 93% (wheat grain) to 109% (soybeans) with FRN of the potential GP with C as inoculum. FDN, FD, and FD3W were associated with a lower potential GP for all feeds, except for rapeseed meal when using the FD3W inoculum. While the FRNdef, FDN, FD, FD3W, and FD6M treatments prolonged the lag phase for all feeds compared to C, the lag phase of FRN and FR was not different when compared to C for most of the feeds. The highest GP rate was estimated for wheat grain when being incubated with C. GP rates of maize grain using FD and FD3W as inoculum and of hay, maize silage and soybeans using FD6M were higher compared to C. Overall, FDN, FD, FD3W, and FD6M had an adverse effect on potential GP compared to C. This effect was more pronounced with roughages than concentrate feeds. In addition, for some preservation treatments, GP parameters could not be estimated for grass hay, grass silage, and molassed sugar beet pulp due to atypical GP progression.

Regression analyses for predicting C-GP after 24 and 48 h from GP of the preservation treatments showed close linear relationships between C and some preserved treatments when the nine

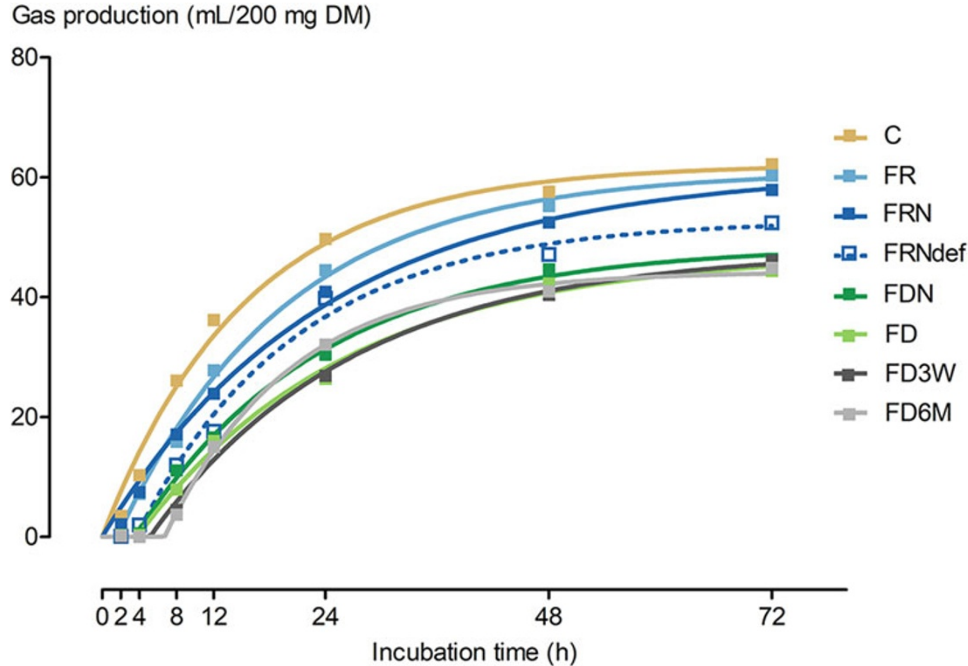

Figure 1. Mean *in vitro* gas production kinetics of faecal inoculum from fresh (C) and differently preserved faeces averaged across nine feed samples incubated for 72 h and fitted with equation [1] or [2]. FR, Frozen faeces; FRN, Shock-frozen faeces with liquid nitrogen; FRNdef, faeces treated like FRN followed by defrosting prior to incubation; FDN, Shock-frozen faeces with liquid nitrogen and freeze-dried prior to incubation; FD, Freeze-dried faeces; FD3W, Freeze-dried faeces stored for 3 weeks; FD6M, Freeze-dried faeces stored for 6 months.

Table 2. Gas production kinetic parameters of faecal inoculum from non-preserved control (C) and differently preserved faeces averaged across nine feed samples incubated for 72 h

Treatment	*t0*	*SE*	b_{gas}	*SE*	c_{gas}	*SE*	R^2
	(h)		(mL/200 mg DM)		(%/h)		
C	.		62	*3.4*	6.5	*0.01*	0.75
FR	1.6	*0.87*	61	*3.7*	5.6	*0.01*	0.81
FRN	.		61	*4.9*	4.2	*0.01*	0.75
FRNdef	3.6	*1.40*	53	*4.7*	5.8	*0.02*	0.69
FDN	3.5	*1.41*	49	*4.4*	5.1	*0.02*	0.73
FD	3.7	*1.54*	47	*5.2*	4.4	*0.01*	0.71
FD3W	5.1	*2.57*	48	*6.4*	4.6	*0.02*	0.67
FD6M	6.7	*1.77*	44	*4.5*	7.4	*0.03*	0.61

t0, b_{gas} and c_{gas} calculated from the fitted equation either without lag phase: GP = $b_{gas}*(1 - e^{-cgas*t})$ or with lag phase: GP = $b_{gas}*(1 - e^{-cgas*t - t0})$, with GP = Gas production after *t* hours; b_{gas} = potential GP, c_{gas} = rate of GP of *b*; *t0* = time of lag phase (h), a dot indicates that the model without lag phase was used.
SE, standard error of *t0*, b_{gas} and c_{gas}.
FR, Frozen faeces; FRN: Shock-frozen faeces with liquid nitrogen; FRNdef, faeces treated like FRN followed by defrosting prior to incubation; FDN, Shock-frozen faeces with liquid nitrogen and freeze-dried prior to incubation; FD, Freeze-dried faeces; FD3W, Freeze-dried faeces stored for 3 weeks; FD6M, Freeze-dried faeces stored for 6 months.

feed samples were included (Table S5). The best equations to predict C-GP after 24 and 48 h were obtained with FR-GP (Fig. 2) and the estimate at 24 h (Slope = 1.05; R^2 = 0.99; RMSE = 1.21; CV = 2.4%) was slightly better than that at 48 h (Slope = 1.08; R^2 = 0.99; RMSE = 1.58; CV = 2.7%).

Effects of preservation methods on the active microbiome

In total, 685,686 peptide sequences were identified from the MS/MS spectra of 116 samples. Identified peptides were clustered in 24,654 protein groups and about 98% of them were classified as bacterial or archaeal proteins using the cow-rumen catalogue as a protein sequence database. The variances among the samples were determined in non-metric multidimensional scaling (NMDS) analyses using the relative abundance of core protein groups (Fig. 3), which were defined as the proteins that were present in at least 81% (71 samples) of the total samples. This set of proteins (8962) were less prone to measurement bias and represented 36% of the total proteins. The effect of the experimental treatments became apparent from two separate groups in each ordination analysis (C, FR, FRN *vs.* FD, FDN) defined by differences in the relative abundances of the core proteins among these groups. In addition, a clear separation according to incubation time was observed in both groups (Fig. 3A). These results were confirmed by a permutational

Table 3. Effect of feed and preservation treatment of faeces on gas production kinetic parameters

	Feed								
	Grass hay	Grass silage	Maize silage	Wheat grain	Maize grain	Sugar beet	Concentrate	Rapeseed meal	Soybeans
Treatment					$\boldsymbol{b_{gas}}$ (mL/200 mg DM)				
C	51^{a}	51^{ab}	58^{a}	77^{ab}	80^{a}	76^{a}	64^{a}	47^{ab}	48^{a}
FR	54^{a}	51^{ab}	58^{a}	74^{ab}	81^{a}	.	63^{a}	51^{a}	51^{a}
FRN	.	55^{a}	54^{a}	71^{bc}	77^{a}	74^{a}	62^{ab}	51^{a}	52^{a}
FRNdef	.	49^{b}	48^{b}	79^{a}	79^{a}	63^{b}	58^{bc}	41^{c}	37^{b}
FDN	.	.	43^{bc}	67^{cd}	71^{bc}	62^{b}	50^{d}	41^{c}	34^{b}
FD	33^{b}	.	46^{b}	59^{e}	64^{d}	.	48^{d}	40^{c}	38^{b}
FD3W	.	.	40^{cd}	62^{de}	68^{cd}	.	56^{c}	41^{bc}	36^{b}
FD6M	26^{b}	.	37^{d}	73^{b}	76^{ab}	73^{a}	59^{ac}	36^{c}	25^{c}
Pooled SEM	*2.1*	*2.0*	*2.1*	*2.0*	*2.0*	*2.1*	*1.6*	*2.0*	*2.0*
P-values (ANOVA) Feed: < 0.001 Treatment: < 0.001 Feed *Treatment: < 0.001									
Treatment					$\boldsymbol{c_{gas}}$ (%/h)				
C	4.8^{bc}	4.9^{a}	8.4^{b}	23.1^{a}	9.0^{cd}	7.6^{ab}	11.1^{ab}	6.2^{a}	5.2^{b}
FR	3.4^{c}	4.6^{a}	5.8^{bc}	11.9^{d}	7.7^{d}	.	7.6^{c}	4.1^{a}	3.6^{b}
FRN	.	3.4^{a}	4.9^{bc}	12.5^{d}	8.8^{cd}	5.7^{ab}	7.2^{c}	3.3^{a}	2.9^{b}
FRNdef	.	3.0^{a}	6.3^{bc}	11.6^{d}	8.6^{cd}	7.8^{a}	8.5^{bc}	3.5^{a}	4.7^{b}
FDN	.	.	5.7^{bc}	13.4^{cd}	7.1^{d}	5.9^{ab}	9.3^{bc}	3.7^{a}	4.1^{b}
FD	8.3^{ab}	.	3.9^{c}	18.0^{b}	15.7^{a}	.	9.4^{bc}	2.5^{a}	2.7^{b}
FD3W	.	.	5.9^{bc}	16.7^{bc}	12.9^{ab}	.	10.6^{ab}	2.5^{a}	3.2^{b}
FD6M	9.9^{a}	.	15.2^{a}	18.2^{b}	11.4^{bc}	3.6^{b}	13.5^{a}	2.9^{a}	19.7^{a}
Pooled SEM	*0.01*	*0.01*	*0.01*	*0.01*	*0.02*	*0.01*	*0.01*	*0.01*	*0.01*
P-values (ANOVA) Feed: < 0.001 Treatment: < 0.001 Feed *Treatment: < 0.001									

Table 3. (*Continued.*)

	Feed								
	Grass hay	Grass silage	Maize silage	Wheat grain	Maize grain	Sugar beet	Concentrate	Rapeseed meal	Soybeans
Treatment	***t0* (h)**								
C	1.4^{d}	1.2^{c}	1.4^{d}	3.2^{e}	2.5^{d}	1.4^{b}	0.9^{d}	0.0^{c}	0.0^{d}
FR	3.2^{c}	4.0^{b}	2.6^{cd}	2.9^{e}	4.0^{c}	.	1.3^{d}	0.0^{c}	0.0^{d}
FRN	.	4.6^{b}	1.7^{d}	2.5^{e}	2.9^{cd}	1.3^{b}	0.7^{d}	0.0^{c}	0.0^{d}
FRNdef	.	6.4^{a}	3.5^{c}	5.5^{cd}	5.9^{b}	5.4^{a}	3.1^{c}	2.6^{b}	2.5^{c}
FDN	.	.	3.2^{c}	5.2^{d}	5.8^{b}	5.2^{a}	3.6^{bc}	2.6^{b}	2.0^{c}
FD	21.8^{a}	.	3.8^{c}	6.6^{bc}	8.2^{a}	.	4.1^{bc}	3.0^{b}	2.8^{bc}
FD3W	.	.	5.8^{b}	7.6^{b}	8.6^{a}	.	4.6^{b}	4.9^{a}	3.9^{b}
FD6M	6.6^{b}	.	9.3^{a}	9.3^{a}	8.7^{a}	6.0^{a}	5.9^{a}	2.8^{b}	7.5^{a}
Pooled SEM	*0.49*	*0.47*	*0.49*	*0.47*	*0.47*	*0.49*	*0.39*	*0.48*	*0.47*
P-values (ANOVA) Feed: < *0.001* Treatment: < *0.001* Feed *Treatment: < *0.001*									

a–d Values in the same column within one gas production parameter not sharing the same superscript letter are significantly different ($P \leq 0.050$).
t0, b_{gas} and c_{gas} calculated from the fitted equation either without lag phase: $GP = b_{gas} * (1 - e^{-c_{gas} * t})$ or with lag phase: $GP = b_{gas} * (1 - e^{-c_{gas} * (t - t0)})$, with GP = Gas production after *t* hours; b_{gas} = potential GP, c_{gas} = rate of GP of *b*; *t0* = time of lag phase (h); a dot indicates that less than three syringes remained for ANOVA, so that the combination was not included.
C, Fresh faeces; FR, Frozen faeces; FRN: Shock-frozen faeces with liquid nitrogen; FRNdef, Faeces treated like FRN followed by defrosting prior to incubation; FDN, Shock-frozen faeces with liquid nitrogen and freeze-dried prior to incubation; FD, Freeze-dried faeces; FD3W, Freeze-dried faeces stored for 3 weeks; FD6M, Freeze-dried faeces stored for 6 months.

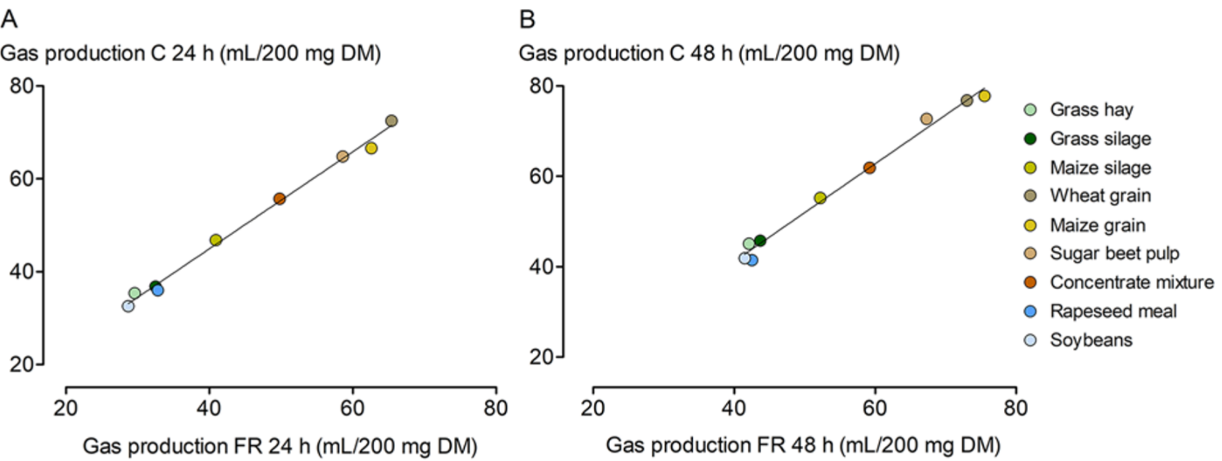

Figure 2. Linear relationships between gas production obtained with faecal inoculum from fresh faeces (C) and frozen faeces (FR) from nine feeds after (A) 24 and (B) 48 h of incubation. y = 1.05 * x + 3.14 (C24h, y; FR24h, x) (R^2 = 0.99; RMSE = 1.21; CV = 2.4%); y = 1.08 * x + 2.02 (C48h, y; FR48h, x) (R^2 = 0.99; RMSE = 1.58; CV = 2.7%)

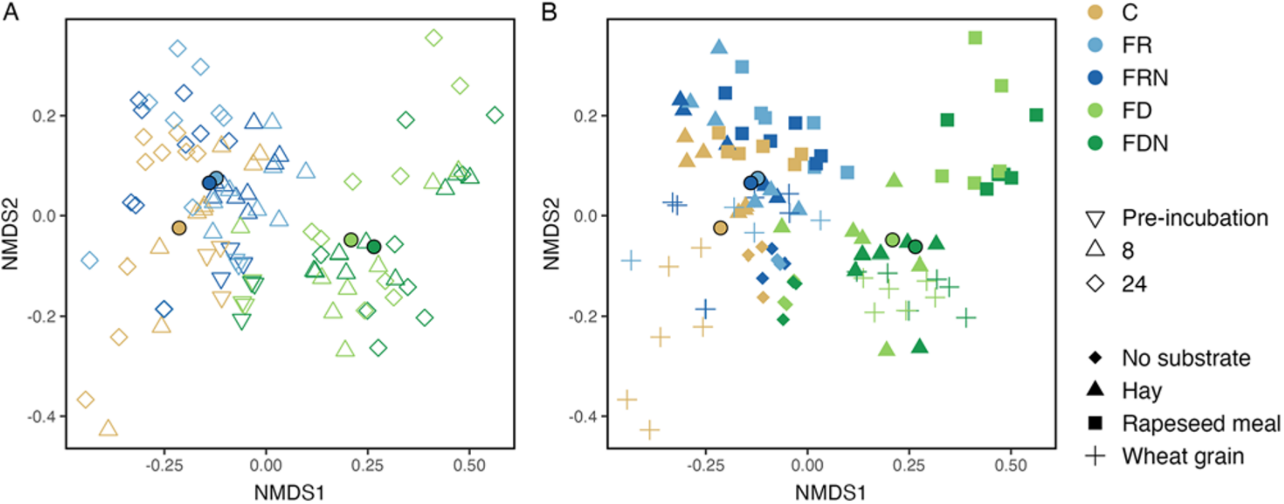

Figure 3. Non-metric multidimensional scaling (NMDS) ordination of faecal sheep samples used as inoculum after preservation with different treatments. Bray–Curtis dissimilarities were calculated based on the abundance of identified protein groups. (A) Clustering by incubation time; (B) clustering by substrate (feed). Circle shapes with black edge represent the centroid per group. C, Fresh faeces; FR, Frozen faeces; FRN, Shock-frozen faeces with liquid nitrogen; FD, Freeze-dried faeces; FDN, Shock-frozen faeces with liquid nitrogen and freeze-dried prior to incubation.

multivariate analysis of variance (Adonis) test indicating that treatment and interaction between treatment and incubation time had a significant effect on the clustering of the samples (Table S6). Additionally, the treatment was the variable that explained most of the variance in both ordinations. The effect of the feed used was not significant but was apparent in the ordination plot (Fig. 3B).

Changes in relative abundance of specific microbial taxa

The significant changes of the core metaproteome among the preservation methods and the incubation time was also detectable in the taxonomic composition of the total metaproteome. The absolute frequency of all assigned proteins showed that 86% of the phyla, 91% of the families, and 94% of the genera were shared among all samples (Figure S1, Table S7). Proteins with the highest relative abundances from bacteria were assigned to Bacteroidota (25–60%), Bacillota and Bacillota A (10–55%) and Spirochaetota (2–10%) as well as to Methanobacteriota (up to 1%) as representative of the archaea. The relative abundance of the different phyla was compared using LinDA and the treatment C and the pre-incubation samples were used as references for the comparison. In general, the preservation methods tended to significantly negatively affect the abundance of proteins assigned to the different phyla, while the incubation time had a positive effect (Fig. 4A, B). The feed was not included in the model due to the lack of repetitions. Significantly reduced abundances were identified for proteins assigned to Bacteroidota in FD and FDN compared to C, FR, and FRN (Fig. 4C). This difference specifically developed during the incubation period where proteins assigned to Bacteroidota were increased after 8 and 24 h in treatments C, FR, and FRN compared to FD and FDN (Fig. 4D). In contrast, proteins assigned to Bacillota also increased in relative abundance after 8 and 24 h but with a concomitant increase in FD and FDN (Fig. 4A, B). Archaeal proteins were assigned to Methanobacteriota and Thermoplasmatota, with low abundances of the last one (<0.05%). Proteins of Methanobacteriota were significantly higher in FR, FD, and FDN than in control, with insignificant changes over time.

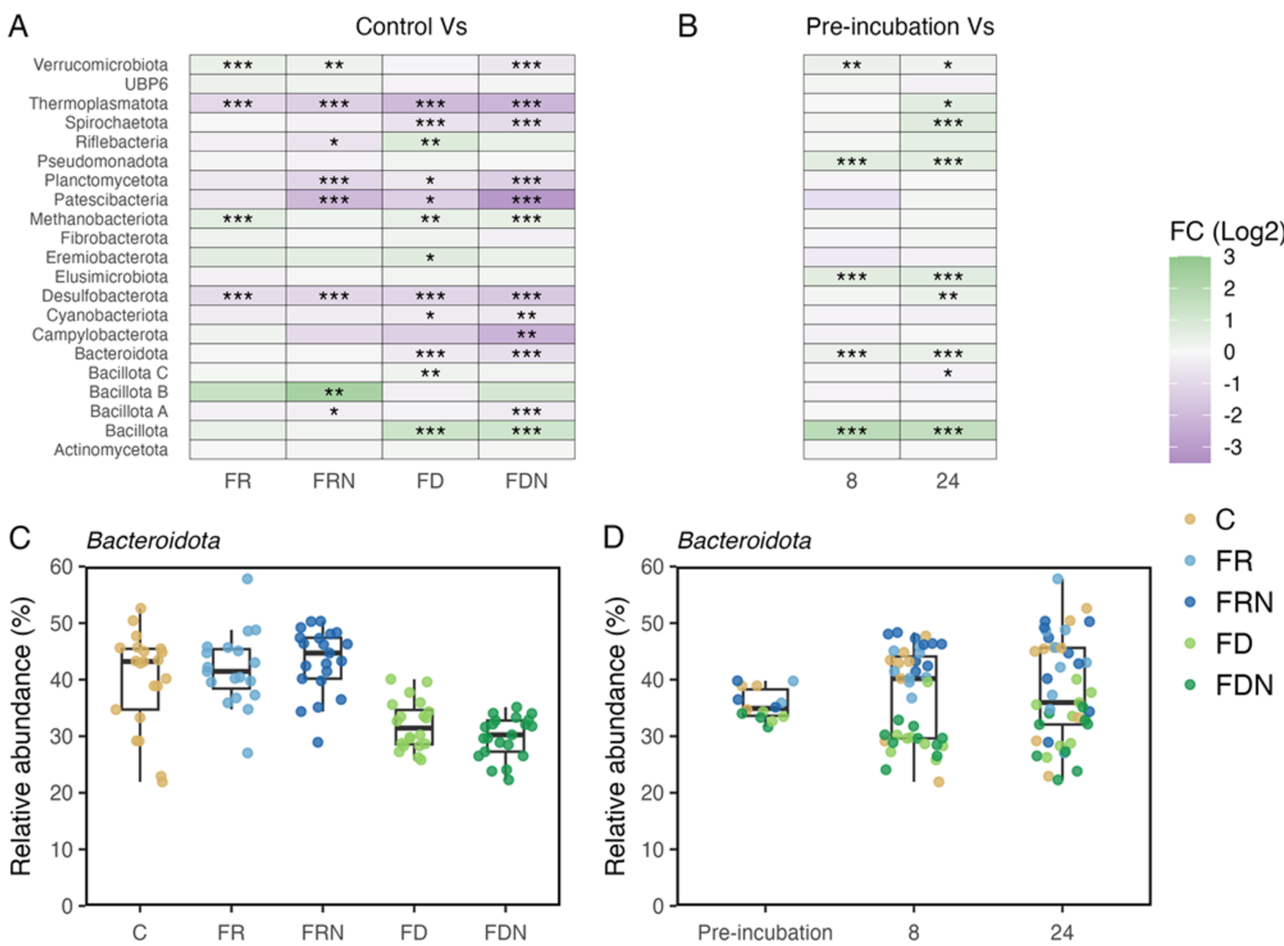

Figure 4. Effect of the (A) preservation methods and (B) incubation time on the relative abundance of different identified phyla. The change of the abundance of the phylum *Bacteroidota* under the different (C) preservation methods and (D) incubation times is shown as an example. The stars indicate the level of significance: * < 0.05, ** < 0.01 and *** < 0.001. C, Fresh faeces; FR, Frozen faeces; FRN, Shock-frozen faeces with liquid nitrogen; FD, Freeze-dried faeces; FDN, Shock-frozen faeces with liquid nitrogen and freeze-dried prior to incubation.

Effects on the functional redundancy and abundance of proteins involved in carbohydrate metabolism

The diversity of the taxa was linked with the annotated functions of the respective proteins by calculating the functional redundancy across all samples. This metric incorporates the presence/absence of each function, the protein abundance of each function and the biomass of each taxon, and appears to outperform diversity metrics in detecting significant microbiome responses to environmental factors (Li et al 2023a). High functional redundancy values (nFR) indicate that more different taxa contribute to an ecosystem similarly through the expression of redundant functions. The effect of the preservation method, incubation time, and substrate (feed) was compared using a linear model "nFR ~ treatment*incubation*substrate." The test indicated that the three factors influenced the functional redundancy and interactions existed between the treatment and the incubation time (Table S7). Differences in nFR were significant between C, FR, and FRN on the one hand and FD and FDN on the other. The lower nFR values of FD and FDN appeared also in the analyses of the incubation and substrate effect (Fig. 5).

A global analysis of the differentially abundant proteins revealed that out of a total of 12,331 protein groups that passed pre-filtering quality by LFQ-Analyst, only 996 were differentially abundant across all conditions. When comparing proteins between treatments, protein groups with an adjusted P-value < 0.01 (Benjamini–Hochberg test) and a 2.5 Log2 fold change were considered to be differentially abundant. The clustering tree at the top of the heatmap (Fig. 6A) shows that, based on the abundance of the differentially abundant proteins, the preservation methods FR and FRN are more similar to C than FD and FDN.

Proteins from cluster 3, which were highly abundant in C, FR, and FRN, were mostly associated with translation (J), carbohydrate metabolism and transport (G), and energy production and conversion (C) (Fig. 6B). The influence of the preservation method was also present in the abundance of Carbohydrate-Active enZYmes (CAZy). Significant differences between C, FR, FRN *vs.* FD, FDN were identified in carbohydrate esterases (CE) and glycoside hydrolases (GH) (Figure S2).

In cluster 2, proteins affiliated with cell wall/membrane/envelope biogenesis (M) were predominant in FD and FDN samples (Fig. 6B). An impressive example of this dominance is the average relative abundance of S-layer protein (MGYG000291655_02036) and outer membrane porin (MGYG000291447_00915), which were 50% higher in FD and FDN (Ø 0.9%, Ø 0.42%) than in FR and FRN (Ø 0.49%, Ø 0.21%). Both proteins are assigned to Negativicutes.

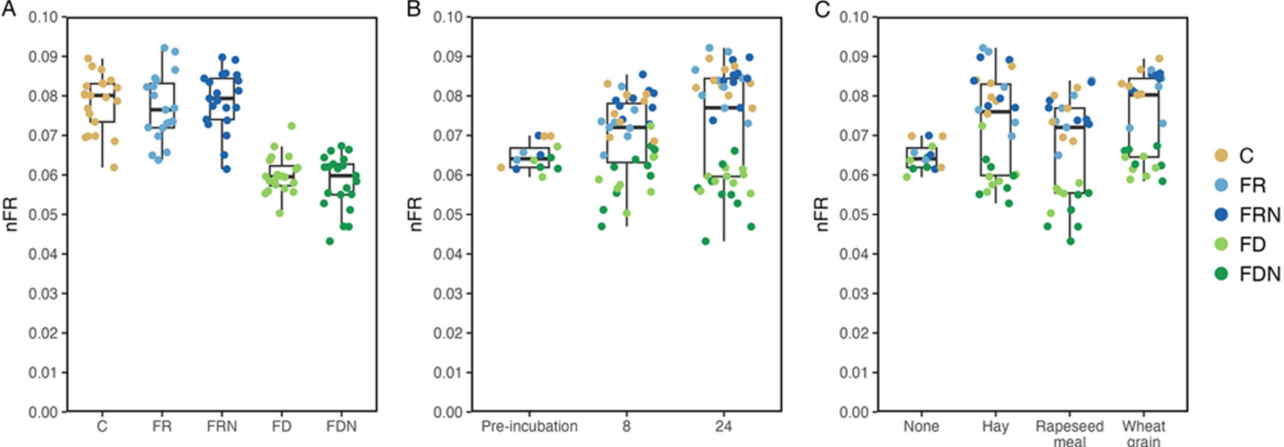

Figure 5. Functional redundancy of the (A) treatment, (B) incubation time, and (C) substrate (feed). High functional redundancy values (nFR) indicate that more different taxa contribute to an ecosystem in similar ways through the expression of redundant functions. C, Fresh faeces; FR, frozen faeces; FRN, shock-frozen faeces with liquid nitrogen; FD, freeze-dried faeces; FDN, shock-frozen faeces with liquid nitrogen and freeze-dried prior to incubation.

Discussion

Effects of preservation treatments

All variants of faeces preservation affected the actively growing microbial cells and GP kinetic parameters in the HGT when the faeces were used as inoculum, to varying degrees. The high number of shared taxa indicates that the preservation variants did not deplete bacterial or archaeal taxa (Fig. S1). The observed differences in GP parameters were, therefore, primarily due to the respective differences in the relative protein abundances of the active bacterial and archaeal taxa.

The freezing process exposes bacterial cells to stress through intra- and extracellular ice crystal formation. In addition, the osmotic pressure increases due to dissolved substances in the remaining unfrozen fraction, which may lead to bacterial membrane disruptions and, thus, to lethal damage (Malik 1991; Meryman 2007). Despite these potentially damaging processes, the least impairment of GP was caused by freezing. The treatments FR and FRN showed GP curves and potential GP values close to treatment C, averaged across all feed samples. This was consistent with the common grouping of metaproteomic datasets in treatments C, FR, and FRN, as indicated by their low variance in protein composition of the actively growing cells.

Effects of preservation of ruminant faeces for *in vitro* applications have rarely been studied. Therefore, results from studies with rumen fluid are used for comparisons here. Consistent with a similar potential GP of FR, FRN, and C in the present study, Hervás et al (2005) found a considerable potential GP *in vitro* after freezing rumen fluid at −18°C for 24 h, but the GP rate was significantly reduced. As freezing in liquid N is considered to maintain high cellular viability (Malik 1991; Perry 1998), we included this method for comparison with FR. FR and FRN showed similar GP parameters averaged across all feed samples but a higher CV at almost every incubation time when FRN was used, suggesting a greater impact on the microbial community when liquid N was used. Consistently, significant differences in the relative abundance of proteins assigned to different phyla occurred twice as often between FRN and C compared to FR and C. Some studies that used rumen fluid as inoculum in *in vitro* GP assays have also compared frozen and liquid N frozen preservation and found lower cumulative GP with both preserved inocula compared to fresh inocula (Prates et al 2010; Tunkala et al 2022). In contrast to the present study, these authors reported the smallest differences in GP and ammonia-N (Tunkala et al 2022) or fewer differences in the first hours of incubation and a lower variability of GP (Prates et al 2010) compared to fresh rumen fluid by using liquid N frozen rumen fluid instead of rumen fluid frozen at −20°C. They concluded that freezing rumen fluid with liquid N is more appropriate for preservation. There is a consensus in the literature that rapid freezing preserves a high microbial activity of the inoculum (Prates et al 2010; Spanghero et al 2019; Tunkala et al 2022). In this respect, the physical properties and the surface-to-volume ratio of the inoculum source appear to play a decisive role. While freezing rumen fluid as a liquid with a high surface area can be achieved more quickly with liquid N, sheep faeces freezes quickly at −20°C in its loose and compact form with a small surface area. Concerning sheep faeces, freezing at −20°C without liquid N may, therefore, be considered a gentler process for the microbial cells, as it causes a less severe temperature shock.

The supposedly gentle thawing process of the FRNdef treatment, caused by a lower temperature difference when mixing with the pre-warmed buffer solution, resulted in a longer lag phase and lower potential GP compared to C, FR, and FRN. Fabro et al (2020) preserved rumen fluid at 4°C and found increased ammonia-N concentrations compared to fresh rumen fluid. Based on this finding, Tunkala et al (2022) concluded an increased proliferation and activity of some microbial groups when the inoculum source is stored at 4°C. As a result of increased fermentation during storage, individual species may become dominant and thus reduce the degradative activity of the inoculum (Mould et al 2005). The findings of the present study may, therefore, be explained by an increase in microbial activity during the 12 h thawing process at 4°C. Unfortunately, this cannot be verified for FRNdef in the present study, as the microbiome was not analysed for this treatment.

A substantial decrease in potential GP and a pronounced lag phase occurred across all feed samples with all freeze-dried treatments compared to C, FR, and FRN. This is consistent with the clear separation of the freeze-dried treatments from C and the frozen treatments in the metaproteomic dataset. Approaches that used rumen fluid also reported that freeze-dried treatments showed the greatest differences from the fresh inoculum source

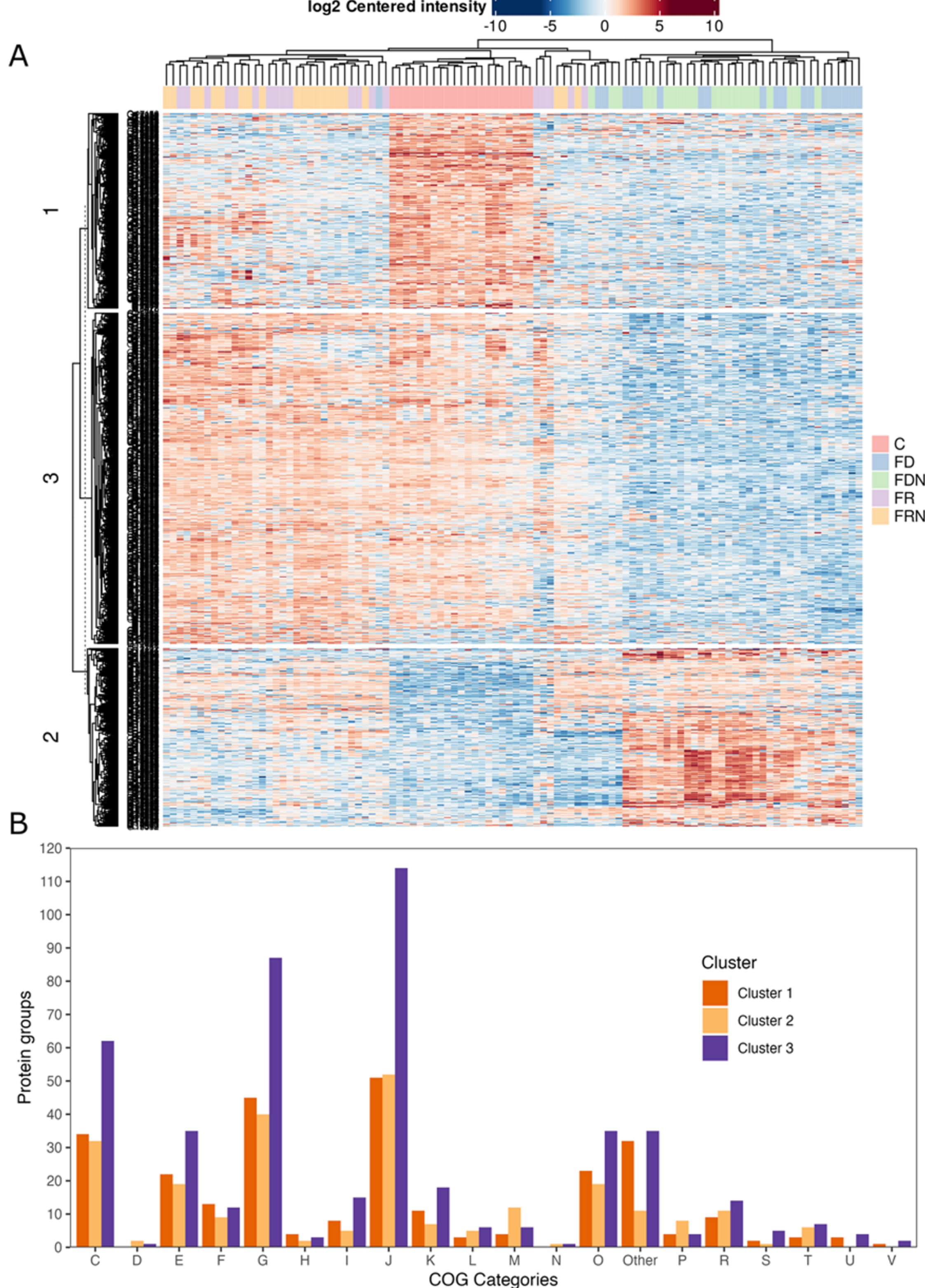

Figure 6. Abundance and clustering of the differentially abundant protein groups (*P*-value < 0.01 and a > 2.5 Log2 fold change) compared between the treatments. (A) Heatmap indicates clustering of proteins based on protein abundances on top tree and protein grouping based on similar abundance patterns in the left tree (three clusters). Red indicates high abundant and blue low abundant proteins. (B) Number of protein groups per cluster and cluster of orthologous groups (COG) category. C, Fresh faeces; FD, Freeze-dried faeces; FDN, Shock-frozen faeces with liquid nitrogen and freeze-dried prior to incubation; FR, Frozen faeces; FRN, Shock-frozen faeces with liquid nitrogen.

(Belanche et al 2019; Spanghero et al 2019). These authors observed the lowest GP and volatile fatty acid concentrations with freeze-dried treatments, indicating remarkable adverse effects of this preservation process on the microbial activity of the inoculum. In the present study, this adverse effect was also indicated by a greater number of proteins assigned to different phyla being negatively affected by freeze-drying compared to C than by freezing. In contrast, *Streptococcus* spp., seemed to benefit from the freeze-drying process as their proteins increased in abundance in FD and FDN with all feed substrates. In addition, proteins related to possible spore formation, such as S-layer proteins, were identified and assigned to Negativicutes in higher abundance in FD and FDN samples. These bacteria were recently described to be diderm Firmicutes (Megrian et al 2020) and seem to be susceptible during the freeze-drying procedure, which might induce the formation of spores or at least the precursor membranes of spores. This substantial impact of freeze-drying is likely attributable to the combined effects of the stress caused by freezing and drying. As reviewed by Rockinger et al (2021), several studies have shown that freeze-drying can severely affect cell membrane integrity, particularly during the drying phase. Water removal disrupts the hydration shell of phospholipid head groups, leading to tighter lipid packing, increased van der Waals interactions, and a shift from a liquid crystalline to a gel-like membrane phase. These structural changes promote membrane fusion and leakage, contributing to cell damage. Rockinger et al (2021) also summarised that intracellular structures and proteins are affected during drying, as removing intracellular water disrupts hydrogen bonds and forces formerly separated molecules to interact, leading to aggregation processes. Such dehydration stress induced by freeze-drying decreased the water fraction amplitudes and distribution in the biomass medium, which in turn influenced the sporulation of *Bacillus subtilis* in the study of Li et al (2022). The authors observed an upregulation of the Spo0A gene, the primary regulator gene of sporulation initiation (Molle et al 2003), demonstrating the intensified sporulation process associated with freeze-drying. The impact of freeze-drying on various rumen community members was also reported by Belanche et al (2019) using qPCR. They found lower values of methanogens (−1.71 logs), anaerobic fungi (−0.18 logs) and an almost complete eradication of protozoa (−2.66 logs) due to freeze-drying of rumen fluid, while the freezing process caused a less pronounced decrease of anaerobic fungi (−0.14 logs) and protozoa cells (−0.70 logs) and no adverse effect on methanogens. In the present study, the number of proteins assigned to methanogens was even enhanced under the tested preservation conditions compared to fresh faecal samples indicating a stable or even enhanced activity of these archaea. This is an important finding because studies testing feed additives to inhibit methanogenesis are initially tested in *in vitro* experiments, which are gaining increasing attention to replace animal experiments.

The adverse effects on GP parameters that occurred through the freeze-drying process in the present study became greater by prolonged storage, especially after 6 months. In contrast, Bircher et al (2018) reported that the viability of freeze-dried cultures was maintained during storage at 4°C for 3 months. Morgan et al (2006) also indicated that a storage temperature of 4°C compared to 25°C and storage in high-barrier plastic bags compared to low-barrier plastic bags could help to improve the recovery rate of freeze-dried and stored cells. It seems likely that these factors had an adverse effect on treatments FD3W and FD6M, as the freeze-dried faeces of these treatments were stored at 23°C sealed in low-barrier plastic bags. Potential oxygen ingress (Costa et al 2002) and subsequent oxygen exposure during storage may have affected microbial survival and temperatures may have increased the metabolic activity of the microbiome during storage. Both could have contributed to the prolonged lag phase compared to FD and FDN after both storage times and the lower potential GP after 6 months of storage. These effects of storage conditions should, therefore, be considered and improved in future studies.

Effects of incubated feeds

No significant differences in potential GP were found among the examined feeds when being incubated with FR or FRN compared to C. Again, this was in accordance with the metaproteomic data, as nFR values did not significantly differ between C, FR, and FRN when hay, rapeseed meal, and wheat grain were used. Also, the data on differentially abundant protein groups, where FR and FRN were more similar to C than FD and FDN, highlight the altered microbial metabolism and, hence, fermentation activity of the FD and FDN inocula. This consistency across feeds with various nutrient compositions with FR and FRN is notable, as several studies have shown a greater negative impact on the *in vitro* fermentation of fibrous feeds compared to feeds with high starch contents by using frozen rumen fluid as an inoculum source (Garcia et al 2021; Hervás et al 2005; Tunkala et al 2022; Zeigler et al 2003). Such a pronounced decrease in fermentation activity in incubations with fibrous feed compared to starch-rich or protein feeds was also detected in the present study, but only when freeze-dried faeces were used as the inoculum source. This was demonstrated by a lower potential GP and a longer lag phase when grass hay was used and the impossibility of estimating GP parameters for grass silage with all freeze-dried treatments, probably caused by an accumulation of atypical GP progressions. Therefore, it is reasonable to assume that microbes involved in fibre degradation are particularly susceptible to preservation processes. The adverse effects of freezing on GP found in previous studies with rumen fluid were, therefore, only caused by freeze-drying and not by freezing with faeces as the inoculum source. This highlights the advantage of sheep faeces' properties during the freezing process in protecting microbial activity. Freeze-drying, on the other hand, appears to impair microbial activity to such an extent that it cannot be compensated for by the favourable conditions that the sheep faeces apparently provide. A clear impact of freeze-drying was detected for *Fibrobacter* spp. as their proteins were twice as abundant in hay incubations in C, FR, and FRN compared to FD and FDN. Proteins of starch-utilising bacteria assigned to Lachnospiraceae (Biddle et al 2013) were reduced by half in abundance in FD and FDN in wheat incubations compared to C, whereas protein abundances of *Ruminococcus* spp. were only different in FD and FDN wheat samples with a doubled value compared to C, FR, and FRN. The analysed data on CAZymes (Fig. S2) support the general assumption of a declined fibre degrading potential, as CAZymes are required for the enzymatic digestion of lignocellulose (Gharechahi et al 2023; Neves et al 2021). GHs include different hydrolase families, all of which are involved in the degradation of lignocellulosic substrates (xylose and cellulose) by hydrolytic processes (Neves et al 2021). The abundance of GHs was slightly lower in FD and FDN than C, FR, and FRN, indicating reduced digestion of plant cell walls due to the freeze-drying process. The observation of the treatments FD and FD6M also showed that the less potential GP was estimated for hay (no GP parameters could be estimated for grass silage), the more potential GP was estimated for the high-starch feeds wheat grain and maize grain. Such an effect could result from reduced competition

with other, damaged microbial groups during *in vitro* incubation (Belanche et al 2019) or from releasing intracellular enzymes to compensate for the loss of microbial activity (Hervás et al 2005). Likewise, lysed cells and other damaged microbes could be used by active microbes as an additional fermentation substrate (Tunkala et al 2022). The inconsistent values for different feeds and the generally lower potential GP for all feeds when incubated with FDN, FD, and FD3W, and the longer lag phase with these treatments and with FRNdef and FD6M emphasise the detrimental effects of these preservation techniques on the microbes and their functionality, meaning their capacity to hydrolyse various feed components, to ferment the respective substrates efficiently, and sustain key enzymatic processes. In addition, FD, FD3W, and FD6M showed high GP rates for some feeds, which is probably related to the simultaneously occurring prolonged lag phase of these treatment and feed combinations. These high GP rates, as well as non-estimable GP parameters for several freeze-dried treatments, confirm that GP progressions of these treatments differ greatly from C and frozen treatments.

Relationship between fresh and preserved faeces as inoculum source

Linear regressions between GP of some preserved treatments and C-GP (Table S5) showed that close relationships exist despite differences in microbial composition and some lower GP parameters of the preserved treatments. The strongest relationship was found between FR-GP and C-GP for both considered incubation times of 24 h and 48 h across all feeds, reflecting the similarity of the microbiome and GP data between C and FR. The consistently similar GP values across all feeds with the FR treatment compared to C, as described before, likely contributed to this close relationship. In this context, an even ratio to C also seems to be relevant. Despite the consistently significantly lower values for FD regarding potential GP, the relative proportion of FD to C was relatively constant across the different feeds. This could explain the strong relationship observed after 48 h between FD-GP and C-GP. Spanghero et al (2019) also found a satisfactory relationship ($r = 0.797$) among GP of different feeds between frozen and fresh rumen fluid inoculum, despite a lower GP level with frozen rumen fluid. In addition, they determined an even higher correlation with freeze-dried rumen fluid ($r = 0.850$), although this inoculum source demonstrated the lowest fermentation activity. Similarly, Chaudry and Mohamed (2012) found that, despite lower degradation of feeds with frozen rumen fluid inoculum, high correlations ($R^2 = 0.97$–0.99) between fresh and differently frozen and thawed rumen fluid for DM and crude protein degradability existed. Akhter et al (1994) also found highly significant correlations of *in vitro* organic matter digestibility of different feeds between the use of fresh and frozen rumen fluid ($R^2 = 0.996$), but also between fresh rumen fluid and frozen cow faeces ($R^2 = 0.974$). Therefore, it seems possible to predict the *in vitro* digestibility of feeds using a frozen inoculum source (Akhter et al 1994; Chaudry and Mohamed 2012). In the present study, the highest estimation accuracy of the regression equation between FR-GP and C-GP also suggests that FR-GP can predict C-GP *in vitro* in the HGT for different feeds. However, the effect of storing faeces in the frozen state FR for a more extended period was not tested in the present study. This would be a further necessary investigation concerning the possibility of stockpiling faeces for use as an inoculum source.

Conclusion

Different preservation methods alter the microbiome and the GP in the HGT. A distinct difference was found between FD and FDN *vs*. FR, FRN, and C regarding GP. This was consistent with a common grouping of the respective active fraction of the microbiome, including taxonomic and functional differences for these treatments. The supposedly more favourable preservation of microbial cells by using liquid N or defrosting at 4°C did not improve microbial cell recovery. Overall, frozen faeces showed the least differences and the best relationship with fresh faeces. In conclusion, frozen sheep faeces can be used as a preserved inoculum source to replace fresh faeces in the *in vitro* GP technique of the HGT to analyse ruminant feeds with different nutrient compositions.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/anr.2025.10014

Data availability statement. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al 2025) partner repository with the dataset identifier PXD060439 (Data will be publicly available after acceptance, currently use reviewer access details: Log in to the PRIDE website using the following details: Project accession: PXD060439, Token: OPo3SMNUZbM4). Other data that support the findings of this study will be provided upon justified request by the authors.

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Conflicts of interest. The authors declare that they have no competing interests.

Ethical standards. The animal study was in accordance with the German animal welfare regulations and approved by the Regierungspräsidium Stuttgart with protocol numbers 35-9185-99/408.

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4.3 Manuscript 3: Evaluation of sheep faeces as an alternative inoculum source to cow rumen fluid for estimation of ruminal ammonia-nitrogen release and microbially bound nitrogen *in vitro*

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Evaluation of sheep faeces as an alternative inoculum source to cow rumen fluid for estimation of ruminal ammonia-nitrogen release and microbially bound nitrogen *in vitro*

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ABSTRACT

In vitro gas production (GP) techniques, such as the extended Hohenheim gas test (eHGT), are being used to estimate the protein values of ruminant feeds and commonly apply rumen fluid obtained from rumen-cannulated animals as inoculum. This study aimed to compare rumen fluid inoculum (RI) with faecal inoculum (FI) concerning *in vitro* ammonia-nitrogen (NH_3-N) release and microbially bound nitrogen (mN). Rumen fluid was obtained from lactating dairy cows and faeces from adult wether sheep. Six feeds for ruminants, including rapeseed meal, soybean meal, sunflower meal, pea grain, maize grain, and rye grain, were incubated for 8, 24, and 48 h with and without an additional carbohydrate (CHO) source. NH_3-N increased with the incubation time for all feeds and both inocula. For NH_3-N values after 8, 24 and 48 h of incubation, the two-way interaction between feed and inoculum source was significant ($p < 0.001$). Across the six feeds, the NH_3-N values did not differ between RI and FI after 8 h, whereas RI-NH_3-N was 17% and 23% higher than FI-NH_3-N after 24 and 48 h, respectively. For most feeds, the mN values decreased with RI as incubation time increased. In contrast, mN in FI remained constant or increased between 8 and 24 h before decreasing at 48 h. Overall, the NH_3-N and mN values were lower with FI than RI. After 24 and 48 h, the two inocula did not significantly differ in the response to the CHO supply, studied as the ratio of mg NH_3-N decrease per 1 mL of GP. Linear regression analysis showed that FI-NH_3-N can be used to predict RI-NH_3-N using the six feeds or the CHO-supplemented feeds at all incubation times. The highest estimation accuracy was found at 24 h for the feeds alone (slope = 1.39, R^2 = 0.98, CV = 3.9%) and the feeds plus CHO source (slope = 1.55, R^2 = 0.99, CV = 4.1%). In conclusion, FI has the potential to be applied as an inoculum to assess the protein values of ruminant feeds *in vitro*.

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1. Introduction

Providing adequate amounts of crude protein (CP) in the feed to meet the animals' requirement is crucial, as protein is an essential nutrient for animal health, growth, and productivity. CP oversupply increases nitrogen (N) excretion of animals and contributes to environmental pollution, which is why an accurate characterisation of the protein requirement on one hand and the protein value of feeds on the other is important. For ruminant feeds, the *in situ* technique (Ørskov and McDonald 1979) is widely used for estimating CP degradation in the rumen, but its application needs strict standardisation (GfE 2022) and its high effort and limited sample throughput prevent its use in routine feed protein evaluation practice. *In vitro* techniques reduce the need for *in vivo* or *in situ* approaches and could provide inexpensive, rapid, and standardisable analysis of rumen CP degradation.

The Hohenheim gas test (HGT; Menke and Steingass 1988) is a widely used *in vitro* gas production (GP) assay for estimating organic matter digestibility and energy value of ruminant feeds. Already at an early stage of assay development, its application has been extended to the estimation of ruminally undegradable CP (RUP) and microbial crude protein (MCP) production in the "extended HGT" (eHGT; Steingass et al. 2001; Edmunds et al. 2012; Steingass and Südekum 2013). This extension is based on results by Raab et al. (1983), who described an approach to determine ruminal CP degradation by measuring GP (Menke et al. 1979) and ammonia concentrations in incubation residues. To assess the estimation accuracy of the eHGT, in a comparative study, a high proportion of the variation of *in vivo* omasal CP flow in cattle was explained by the sum of *in vitro* RUP and MCP ($R^2 = 0.87$; Gidlund et al. 2018).

Conducting the eHGT requires rumen fluid, which is usually obtained from rumen-cannulated animals. Regarding the estimation of organic matter digestibility and energy value of feeds, the potential of sheep faeces as an alternative inoculum source for *in vitro* GP has been shown (Aiple et al. 1992; Rippstein et al. 2024). To the best of our knowledge, only one study has assessed the protein value of ruminant feeds *in vitro* using faeces as the inoculum source (Zhao and Chen 2004). These authors incubated ruminant feeds with faecal inoculum (FI) and rumen fluid inoculum (RI) according to the method described by Zhao and Lebzien (2000). This method is based on the first step of the technique described by Tilley and Terry (1963) and follows the approach of directly estimating the CP that reaches the duodenum and is therefore intended to represent the sum of RUP and MCP. In contrast to the eHGT assay, the approach of Zhao and Lebzien (2000) does not allow for a distinction between RUP and MCP and does not include GP measurements. As GP reflects the fermented organic matter, it is an indicator of the energy available for MCP synthesis (Raab et al. 1983). This information is used in the eHGT approach.

The aim of this study was to use FI in comparison with RI in the eHGT assay. The two inocula were compared based on ammonia-nitrogen (NH_3-N) and the calculated microbially bound N (mN), based on considerations of Raab et al. (1983). The objective was to determine whether and to what extent the two inocula cause differences in these *in vitro* traits, how they respond to an extra source of fermentable energy, and whether they are related to each other regarding the amount of NH_3-N in incubation residues. It was

hypothesised that a close relationship might exist between FI and RI in terms of NH_3-N, and that the results of this study could indicate a similar functional characteristic of the two inocula. These aspects should provide information on the suitability of FI as an alternative inoculum to RI for estimating the protein value of ruminant feeds *in vitro*.

2. Materials and methods

2.1. Examined feeds

Six feeds, including rapeseed meal, soybean meal, sunflower meal, pea grain, maize grain, and rye grain were used for *in vitro* incubations. All feeds were ground with a centrifugal mill (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan, Germany) through a 1-mm sieve before use in the eHGT. Feeds were additionally ground through a 0.5-mm sieve for analysis. Dry matter (DM) (method no. 3.1) and total N (method no. 4.1.1) were analysed according to VDLUFA (2012) (Table 1).

2.2. Animals and diets

All feeds were incubated *in vitro* using two inoculum sources: rumen fluid from dairy cows and faeces from sheep. Rumen fluid was obtained from two ruminally fistulated lactating Jersey cows offered water and a total mixed ration (TMR) for *ad libitum* intake. The TMR consisted of (on a DM basis) 37% concentrate mixture, 21% maize silage, 13% grass silage, 17% grass hay, 4% lucerne silage, 4% rapeseed meal, 2% barley straw, 1% mineral mixture, and 1% limestone. The concentrate mixture was composed of 22% maize grain, 22% rapeseed cake, 20% barley grain, 19% field beans, 14% pea grains, and 3% linseed cake.

Adult wether sheep were used as donor animals to obtain fresh faeces. Three sheep aged about 3 years were group-housed and kept on sawdust. They were offered water and a TMR in dry form for *ad libitum* intake during adaptation and sampling periods. The TMR was calculated to meet the requirements of adult wethers according to Steingass (2020) and was composed as shown in Table 2. The adaptation to this TMR lasted 2 weeks and was followed by 3 weeks during which faecal samples were obtained on assay days.

Animal care and all procedures were in accordance with the German Animal Welfare rules and ethical approval was obtained from the Regierungspräsidium Stuttgart with protocol numbers 35–9185–99/299 for sheep and 35–9185.82/0365 for cows.

Table 1. Dry matter (DM) and nitrogen (N) concentrations of the tested feeds.

Feed	DM [g/kg]	N [g/kg DM]
Rapeseed meal	898	59
Soybean meal	901	83
Sunflower meal	906	51
Pea grain	924	36
Maize grain	897	13
Rye grain	877	14

Table 2. Ingredients and nutrient composition (g/kg dry matter (DM), unless otherwise stated) of the dry total mixed ration fed to wether sheep.

Ingredients	
Grass hay, chopped	430
Straw, chopped	310
Maize grain, crushed	130
Rapeseed meal	60
Molasses	70
Chemical composition[a]	
DM [g/kg]	914
Ash	56
Ether extract	16
CP	81
aNDFom	592
ADFom	366

CP, crude protein; aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; ADFom, acid detergent fibre expressed exclusive of residual ash.
[a]Analysed chemical composition is averaged from six samples during the experimental period.
Minerals were supplemented as a lick stone placed in the pen (SALEC® Mineral-Leckstein eco,Südwestdeutsche Salzwerke AG, Heilbronn)

2.3. Substrate collection and inoculum preparation

For technical reasons, the incubations using RI and FI were conducted in two separate incubation blocks, carried out sequentially, each lasting 4 weeks. For the RI incubation block, rumen fluid was collected from the two cows before the morning feeding and RI was prepared according to the method of Menke and Steingass (1988), with minor modifications of the buffer solution composition (Table 3) according to the eHGT protocol (Steingass et al. 2001). In brief, the buffer solution was prepared and kept in a water bath at 39°C under constant stirring and flushing with CO_2. Four layers of cheesecloth were used to filter the rumen fluid from both cows. The filtered rumen fluid was added to the reduced buffer solution and kept under CO_2 with constant stirring for 15 minutes until incubations began.

The block of FI incubations used fresh faeces collected from the rectum of each sheep and immediately poured into sealed plastic boxes. In a polystyrene box with heat packs, the three plastic boxes with faeces were immediately transported to the laboratory. Equal amounts of faeces from each sheep were pooled to begin FI preparation. The amount of faeces required for HGT incubation was calculated as described before (Rippstein et al. 2024). On average of all incubation runs, the ratio of faecal DM: medium solution was 1: 34 (standard deviation (SD) = 1.4) and the total amount of fresh faeces used in each incubation run with 57 syringes was 219 g. The FI preparation followed a modification of the HGT method according to Aiple et al. (1992) with minor adjustments. Briefly, a buffer solution with the same reagent composition as for the rumen fluid HGT (Table 3) was prepared and maintained in a water bath at 39°C with constant stirring and flushing with CO_2. Half of the reduced medium solution was mixed with the faeces and homogenised in a blender (MMB11B1/07, Robert Bosch Hausgeräte GmbH, Munich, Germany) for 2 minutes at the highest speed under CO_2 flushing. The homogenised mixture was filtered

Table 3. Reagent composition of buffer solutions used for *in vitro* incubations in the Hohenheim gas test for rumen inoculum (RI) and faecal inoculum (FI).

Buffer solution[a]		RI[f]	FI[g]
Composition Medium solution			
Aqua dest.	mL	620.00	950.00
Trace element solution[b]	mL	0.16	0.24
Buffer solution[c]	mL	310.00	475.00
Main element solution[d]	mL	310.00	475.00
Resazurin solution[e]	mL	1.60	2.40
Composition Reduction solution			
Aqua dest.	mL	62.0	95.0
NaOH [1 mol/L]	mL	2.6	4.0
Na_2S x 7 H_20	mg	373.0	575.0

[a]Calculated for one oven with 57 syringes.
[b]Trace element solution: 13.2 g $CaCl_2$ x 2 H_2O, 10.0 g $MnCl_2$ x 4 H_2O, 1.0 g $CoCl_2$ x 6 H_2O, 8.0 g $FeCl_3$ x 6 H_2O, 100 mL Aqua dest.
[c]Buffer solution: 33.0 g $NaHCO_3$, 6.0 g NH_4HCO_3, 1000 mL Aqua dest.; [d] Main element solution: 5.7 g Na_2HPO_4, 6.2 g KH_2PO_4, 0.6 g $MgSO_4$ x 7 H_2O, 1000 mL Aqua dest.; [e] Resazurin solution: 100.0 mg Resazurin, 100 mL Aqua dest.
650 mL of rumen fluid was added to the buffer medium for RI.
[f]Prepared according to Menke and Steingass (1988) with minor modifications of the buffer solution composition according to Steingass et al. (2001); [g] Prepared according to Aiple et al. (1992).

through four layers of cheesecloth before combining with the remaining reduced buffer solution. The mixture was kept under CO_2 with constant stirring for 15 minutes until incubations began.

2.4. In vitro incubation and NH_3-N determination

All incubations were performed according to the standard method of the HGT (VDLUFA 2012) using prewarmed 100-mL glass syringes, but containing either approximately 130 mg of the ground feed or 130 mg of the ground feed and additional 130 mg of a carbohydrate (CHO) source (50% maize starch, 30% cellulose, 20% sucrose) according to the eHGT method (Steingass et al. 2001; Steingass and Südekum 2013). Thirty millilitres of either buffered RI or FI were dispensed into each of the prepared syringes, which were then placed in a rotating disk in an oven maintained at 39°C. Eight runs per incubation block were conducted, each run containing three replicate syringes per feed, three replicate syringes containing the feed and the CHO source, and 12 syringes containing buffered inoculum only, referred to as blanks. All syringes were allocated randomly to the disc positions in the oven. After 8, 24, and 48 h of incubation, GP was recorded from one syringe containing the feed only, one syringe containing the feed and CHO source, and four blanks. All syringes were immediately placed on ice to minimise microbial fermentation. Within 24 h after the placement on ice, the syringe contents were quantitatively transferred to distillation syringes and the NH_3-N contained in each syringe was measured by distillation (Vapodest 50; C. Gerhardt GmbH & Co. KG, Königswinter, Germany).

2.5. Calculations and statistical analyses

After completion of the eight runs with either FI or RI, outliers of NH_3-N values of feeds and feeds plus CHO source were detected using the Grubb´s test and removed. Finally, a minimum of six replicate syringes per incubation time, feed or feed plus CHO source, and inoculum source were the experimental unit for calculations and statistical analyses.

The NH_3-N amount in each syringe with the feed only was subjected to a two-way ANOVA with PROC MIXED from SAS using the following model:

$$y_{ij} = a_i + b_j + (ab)_{ij} + e_{ij} \quad [1]$$

where y_{ij} is the observed value of the estimated parameter, a is the fixed effect of the inoculum (FI and RI), b is the fixed effect of the feed (rapeseed meal, soybean meal, sunflower meal, pea grain, maize grain, rye grain), $(ab)_{ij}$ is the interaction between the $_i$th inoculum and the $_j$th feed, and e_{ij} is the residual error. Variance homogeneity and normal distribution were checked on the residuals for the estimated parameter. Results are presented as least-squares means per feed, inoculum, and incubation time and standard errors of the means (SEM). Statistical significance was set at $p \leq 0.050$. The mean, minimum, and maximum amount of NH_3-N (mg) determined with FI (FI-NH_3-N) and NH_3 -N determined with RI (RI-NH_3-N) of the blanks were calculated over all incubation runs and replicate syringes, and the mean and SD were calculated per incubation run at 8, 24, and 48 h.

The GP values recorded at 8, 24, and 48 h were corrected for the GP values of the corresponding blanks. GP is assumed to be a measure of the energy available for MCP synthesis (Raab et al. 1983). Linear relationships of NH_3-N (mg) and GP (mL) based on data from both the feeds only (130 mg DM) and the feeds plus CHO source (260 mg DM) were calculated to analyse the ratio of GP: incorporation of NH_3-N into MCP at each point in time. The slopes of the regression equations indicate how much energy was expended per mg of MCP synthesised, i.e. how much NH_3-N was incorporated into MCP, namely protein and non-protein N compounds such as nucleic acids, per mL of GP. This relationship was calculated for all feeds, incubation times, and both inoculum sources by linear regression analyses according to the equation:

$$y = slope * x + intercept \quad [2]$$

where y (mg) was the measured amount of NH_3-N and x (mL) was the measured GP after 8, 24 or 48 h for each feed and both inoculum sources. Significant differences between slopes (mg NH_3-N/mL GP) of FI and RI were tested by calculation of linear regression equation parameters and p-values (two-tailed) using GRAPHPAD PRISM software (version 5.00 for Windows, GraphPad Software, San Diego, CA, USA).

A schematic representation between CP degradation and CP synthesis *in vitro* from Raab et al. (1983) was used to calculate mN (mg) by applying the following equation:

$$mN = H - A \quad [3]$$

where H was the estimated intercept of the linear regression equation of NH_3-N (y, mg) and GP (x, mL) between feeds only (130 mg DM) and feeds plus CHO source (260 mg DM) according to equation [2]. A was the NH_3-N content (mg) determined for the feed. The results of the calculated amounts of mN are presented graphically as mean value with

standard deviation (SD) together with the NH_3-N content of the syringe. GRAPHPAD PRISM software was used to create the graphic illustrations.

The relationships between RI-NH_3-N and FI-NH_3-N at 8, 24 and 48 h were investigated by linear regression analyses, including the six feeds (130 mg DM) or the six feeds plus CHO source (260 mg DM) according to the equation [2]. In the equation, y (mg/130 or 260 mg DM) was the predicted RI-NH_3-N after 8, 24 or 48 h and x (mg/130 or 260 mg DM) was the measured FI-NH_3-N after 8, 24 or 48 h, respectively. The parameters were estimated using PROC REG in SAS.

3. Results

The two-way interaction of feed and inoculum was significant ($p< 0.001$) for NH_3-N after 8, 24, and 48 h of incubation (Table 4). Significant differences between amounts of FI-NH_3-N and RI-NH_3-N were observed for each feed and incubation time, except for pea grain after 8 h. After 8 h of incubation, differences between FI and RI were the lowest and FI-NH_3-N was higher than RI-NH_3-N with rapeseed meal, maize grain, and rye grain. However, after 24 and 48 h of incubation, RI-NH_3-N was higher than FI-NH_3-N for all feeds, as the NH_3-N values increased more over time with RI than with FI. Across the eight incubation runs and four replicate syringes per run, mean amounts per 30 mL of FI-NH_3-N of the blanks at 8, 24, and 48 h were 7.9 mg (min. – max. 7.6–8.3), 8.6 mg (8.3–9.1), and 9.2 mg (8.8–9.6), respectively. Amounts of RI-NH_3-N for the blanks at the same incubation times were 7.2 mg/30 mL (6.6–7.6), 10.1 mg/30 mL (9.4–10.7), and 11.5 mg/30 mL (10.8–12.3). The means and SD of NH_3-N values of the blanks per run are shown in Table S1.

The response in the NH_3-N decrease to the CHO addition was similar for the three oilseed meals within an inoculum source and also when comparing the two inoculum sources, despite different levels of NH_3-N, as exemplarily shown for 24 h in Figure 1. The slopes of all feeds and feeds plus CHO source differed significantly between FI and RI

Table 4. Effect of feed and inoculum source on amount of ammonia-nitrogen (NH_3-N, mg/130 mg DM) in the incubation residue.

		Feeds					
Incubation time [h]	Inoculum source	Rapeseed meal	Soybean meal	Sunflower meal	Pea grain	Maize grain	Rye grain
8	FI	8.5^a	9.1^b	8.6^b	7.8	6.0^a	5.7^a
	RI	8.2^b	10.0^a	9.1^a	7.8	5.2^b	5.4^b
	Pooled SEM	*0.10*	*0.10*	*0.10*	*0.10*	*0.11*	*0.11*
	p-values (ANOVA)	*Feed: <0.001 Inoculum: 0.962 Feed*Inoculum: <0.001*					
24	FI	10.4^b	12.6^b	10.9^b	9.9^b	7.4^b	7.9^b
	RI	12.9^a	15.9^a	13.3^a	11.4^a	9.0^a	8.8^a
	Pooled SEM	*0.18*	*0.18*	*0.18*	*0.19*	*0.18*	*0.18*
	p-values (ANOVA)	*Feed: <0.001 Inoculum: <0.001 Feed*Inoculum: <0.001*					
48	FI	11.9^b	14.9^b	12.3^b	11.6^b	8.7^b	9.4^b
	RI	16.3^a	19.2^a	15.8^a	14.5^a	11.7^a	11.9^a
	Pooled SEM	*0.20*	*0.19*	*0.20*	*0.19*	*0.20*	*0.20*
	p-values (ANOVA)	*Feed: <0.001 Inoculum: <0.001 Feed*Inoculum: <0.001*					

a and *b* Values in the same column within a statistical comparison not sharing the same superscript letter are significantly different ($p \leq 0.050$).
A minimum of $n = 6$ replicate syringes per incubation time, inoculum source, and feed were included in the analysis.
FI, faecal inoculum; RI, rumen fluid inoculum.

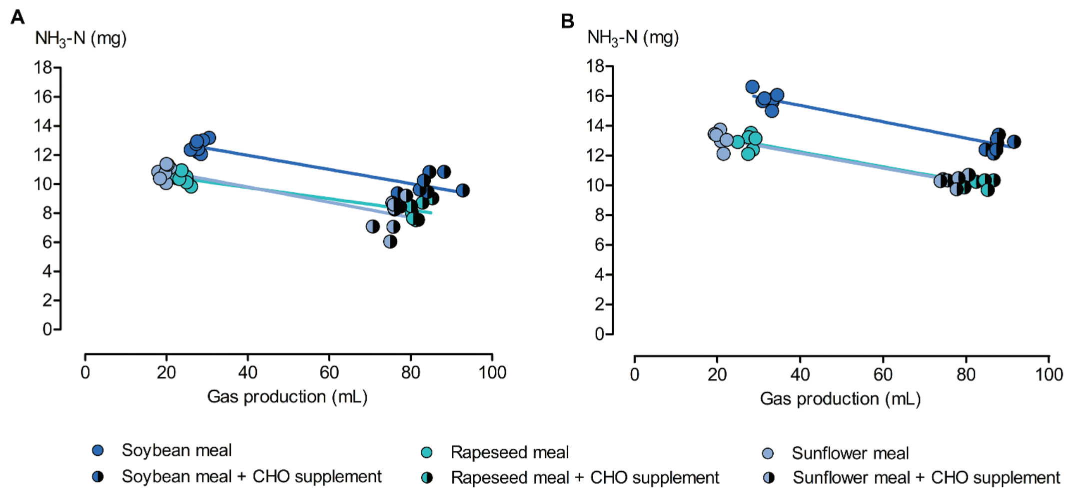

Figure 1. Amount of ammonia-nitrogen (NH_3-N) as a function of the gas production after 24 h incubations with feeds only (130 mg DM) and feeds with additional carbohydrate (CHO) source (260 mg DM) with faecal inoculum (A) and rumen fluid inoculum (B). Predicted linear regression parameters are shown in Table 5.

Table 5. Linear regression equation parameters of the relationship between the ammonia-nitrogen (y, NH_3-N) content (mg) and the gas production (x, mL) of feeds only (130 mg dry matter) and feeds plus carbohydrate supplement (260 mg dry matter).

		Slope			Intercept			R^2	
Incubation time [h]	Feeds	FI	RI	*p*-value	FI	RI	*p*-value	FI	RI
8	Rapeseed meal	−0.07	−0.08	0.015	9.4	9.7	.	0.97	0.97
	Soybean meal	−0.08	−0.10	0.036	10.4	12.4	.	0.88	0.96
	Sunflower meal	−0.07	−0.09	0.006	9.4	10.5	.	0.95	0.98
	Pea grain	−0.07	−0.10	<0.001	9.4	10.7	.	0.93	0.97
	Maize grain	−0.07	−0.09	0.001	7.8	7.8	.	0.93	0.97
	Rye grain	−0.07	−0.09	0.052	8.5	8.4	<0.001	0.95	0.93
24	Rapeseed meal	−0.04	−0.05	0.096	11.3	14.3	<0.001	0.85	0.92
	Soybean meal	−0.05	−0.05	0.448	13.9	17.6	<0.001	0.79	0.92
	Sunflower meal	−0.05	−0.05	0.796	11.9	14.2	<0.001	0.76	0.92
	Pea grain	−0.05	−0.06	0.473	12.0	13.9	<0.001	0.77	0.94
	Maize grain	−0.04	−0.07	0.033	9.3	12.3	.	0.66	0.90
	Rye grain	−0.04	−0.06	0.183	10.0	11.5	0.094	0.72	0.95
48	Rapeseed meal	−0.02	−0.02	0.899	12.5	16.8	<0.001	0.55	0.54
	Soybean meal	−0.02	−0.02	0.895	15.5	19.8	<0.001	0.64	0.64
	Sunflower meal	−0.02	−0.02	0.574	12.8	16.2	<0.001	0.80	0.53
	Pea grain	−0.02	−0.03	0.107	12.5	15.9	<0.001	0.50	0.73
	Maize grain	−0.02	−0.03	0.325	9.6	13.4	<0.001	0.31	0.76
	Rye grain	−0.02	−0.03	0.203	10.6	13.3	<0.001	0.70	0.78

p-values refer to comparisons between FI and RI.
Dots indicate that the slopes differ so much that it was not possible to test whether the intercepts differ significantly.
A minimum of $n = 6$ replicate syringes per incubation time, feed, and inoculum source were included in the analysis.
FI, faecal inoculum; RI, rumen fluid inoculum.

after 8 h, but not after 24 and 48 h of incubation (Table 5). The intercepts differed significantly between FI and RI for each feed and incubation time.

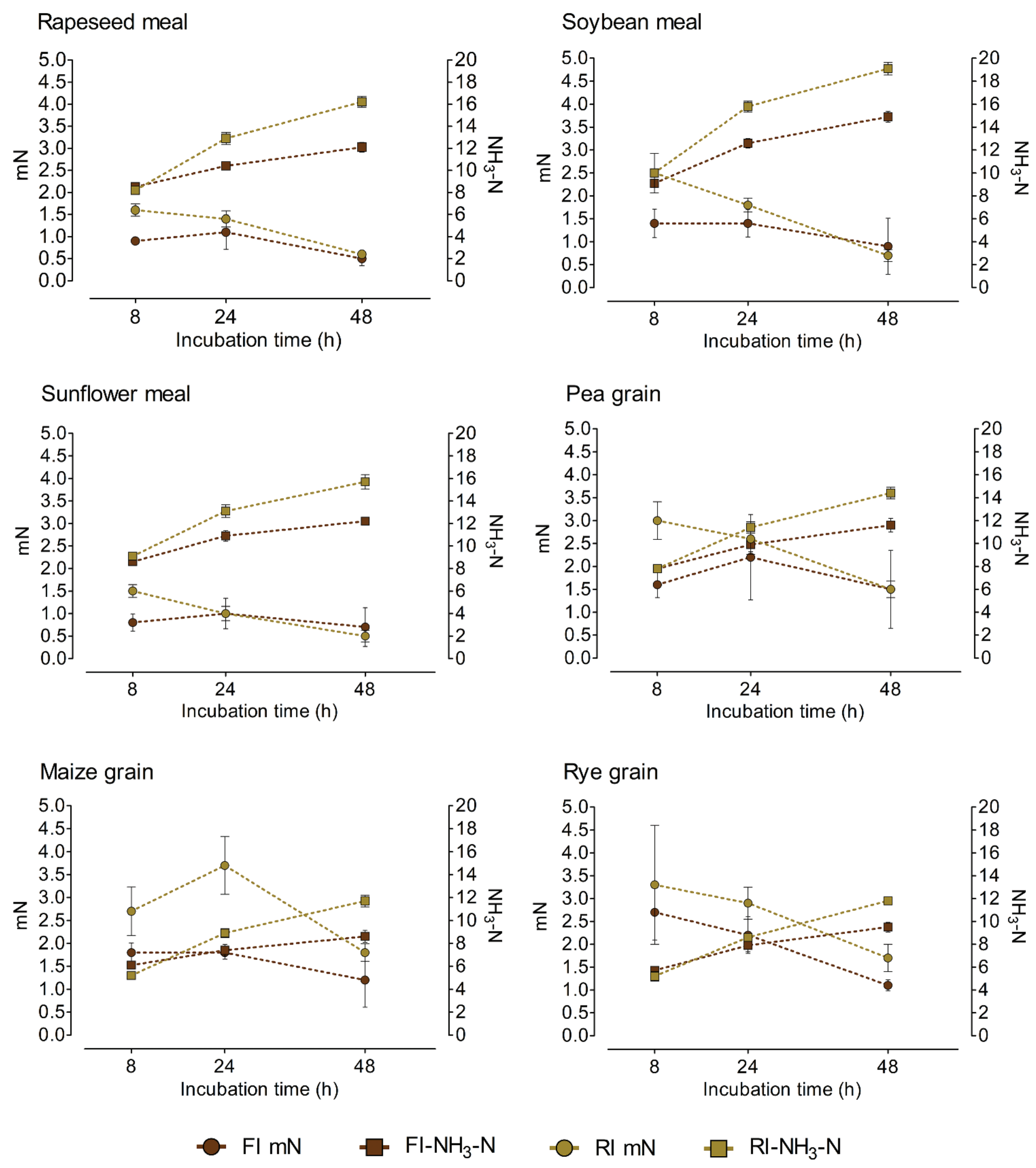

Figure 2. Amount of microbial nitrogen (mN mg/130 mg DM) and ammonia-nitrogen (NH_3-N, mg/130 mg DM, from Table 4) of feeds after 8, 24, and 48 h of incubation with faecal inoculum (FI) and rumen fluid inoculum (RI). The lines connecting the three points are shown for illustration purposes only.

Figure 2 shows that, compared to 8 h, calculated mN values were consistently lower and NH_3-N values were consistently higher at 48 h for all feeds incubated with FI and RI. Between 8 and 24 h and using RI, the amount of mN increased for maize grain. Using FI, it remained constant for soybean meal and maize grain, but increased for rapeseed meal, sunflower meal, and pea grain. For both inoculum sources, NH_3-N increased with each increase in incubation time. Overall, NH_3-N and mN values were at a lower level with FI than RI, but showed a similar trend with incubation time.

Table 6. Linear regression equation parameters to estimate ammonia-nitrogen contents (mg) of rumen inoculum (RI) incubations after 8, 24, and 48 h from ammonia-nitrogen contents (mg) of faecal inoculum (FI) incubations after 8, 24, and 48 h for feeds and feeds plus carbohydrate (CHO) source.

Feeds	n	Incubation time [h]		Slope	Intercept	R^2	RMSE	CV [%]
		RI	FI					
Feeds[a]	6	8	8	1.39	−3.05	0.96	0.42	5.6
		24	24	1.39	−1.93	0.98	0.46	3.9
		48	48	1.25	0.43	0.97	0.59	4.0
		24	48	1.21	−2.10	0.96	0.63	5.3
Feeds plus CHO source[b]	6	8	8	1.43	−2.87	0.96	0.44	10.3
		24	24	1.55	−2.44	0.99	0.36	4.1
		48	48	1.31	−0.14	0.95	0.76	5.7
		24	48	1.22	−3.80	0.95	0.74	8.5

[a]For feeds, the amount per feed was 130 mg dry matter.
[b]For feeds plus CHO source, the amount per feed and CHO source was 130 mg dry matter each, for a total of 260 mg dry matter.
RMSE, root mean squared error; CV, coefficient of variation.

Regression analyses to predict RI-NH_3-N at 8, 24 and 48 h from FI-NH_3-N using all six feeds or the feeds with CHO source showed close linear relationships at all incubation times (Table 6). The highest estimation accuracy was found at 24 h for both the feeds and the feeds plus CHO source.

4. Discussion

4.1. Comparison of NH_3-N and mN between FI and RI

The analysed NH_3-N values at 8 h of incubation were on a similar level for all feeds and higher for the blanks when FI instead of RI was used, whereas at 24 h and 48 h, all NH_3-N values were higher with RI. Differences in NH_3-N between the two inoculum sources are likewise related to differences in the microbial composition between rumen content and faeces, as summarised by Rippstein et al. (2024). In brief, FI appears to have a lower fermentation activity than RI due to less fermentable substrates, shorter retention time of the digesta, and a smaller bacterial population in the caecum and colon than in the rumen (Mauricio et al. 2001). The predominantly higher NH_3-N values with FI at 8 h may be related to differences in the proteolytic activity of the inoculum sources. Hecker (1971) found a significantly higher proteolytic activity in FI (0.60 units) compared to RI (0.022 units). According to this author, soluble proteolytic enzymes found in caecal and faecal, but not in ruminal inoculum, may contribute to this difference. Consistent with the higher NH_3-N values with FI after 8 h in the present study, Hecker (1971) found a higher deaminase activity, expressed as mg NH_3-N production of casein hydrolysate per 100 mg digesta sample, for faecal (34.4) compared to rumen contents (26.5) after a 120 min incubation period. Therefore, it would be interesting to know the development of NH_3-N during the first hours of incubation. The first measurement of NH_3-N in the present study was after 8 h and shorter incubation times should be considered in future studies. However, based on similar relative proportions of volatile fatty acids between FI and RI (Ramin et al. 2015) and an overall similar GP progression (Aiple et al. 1992; Rippstein et al. 2024) in *in vitro* incubations, it could be assumed that the microbes contained in

these two inoculum sources have similar functional characteristics. Consistent with this assumption, N binding by microorganisms occurs in the large intestine (Ørskov et al. 1970; Nolan et al. 1976), but to a much lesser extent than in the rumen (Mason 1969). Accordingly, a considerable amount of mN could be calculated for FI incubations in the present study, but at almost all incubation times, it was lower than that calculated from RI incubations. Although not all ruminally undegraded substrate reaches the hindgut owing to small intestinal digestion, the TMR was formulated to contain ruminally undegradable starch and RUP by including maize grain and rapeseed meal, respectively, aiming to supply nutrients likely accessible to microbes in the hindgut. Therefore, the diet fed to the sheep may have contributed positively to mN with FI, as MCP synthesis has been enhanced by an increase in the amount of sugar or starch present in the hindgut (Ørskov et al. 1970).

In the present study, the diets fed to cows and sheep differed, and it is known that the diet affects the microbial community in the rumen (Newbold and Ramos-Morales 2020) and accordingly, the *in vitro* fermentation using rumen fluid as inoculum source (Boguhn et al. 2013). Such an effect of dietary composition also applies to the microbial community in the hindgut (Kim et al. 2014). Providing identical diets to all donor animals could, therefore, be assumed as a crucial prerequisite for ensuring a reliable comparison between the two inocula. However, the substrate reaching the hindgut inherently differs from that in the rumen, even under identical feeding conditions. It was, therefore, not intended in the present study to apply identical feeding of both donor animal species, but rather to provide high levels of fermentable substrate reaching the hindgut to promote a high microbial activity in the sheep faeces.

For RI and all feeds except maize grain, mN values decreased consistently with increasing incubation time, while the NH_3-N values increased simultaneously. Raab et al. (1983) registered a high GP of starch incubations with RI up to 12 h and for cellulose up to 24 h, with a rather low GP thereafter. The authors concluded that the decreasing GP indicates a period of microbial starvation and lysis, and, therefore, the extent of microbial lysis must be higher for rapidly fermentable carbohydrates if the incubation time is longer than required for a given amount of substrate. As the mN values with FI increased or remained at about the same level for all feeds except rye grain between 8 and 24 h, it is likely that the incubation time required for fermentation of the same amount of substrate is longer with FI compared to RI. A slower but prolonged supply of energy for the microbes may, therefore, account for the postponed initiation of microbial lysis with FI. As mentioned before, overall fermentation activity is reduced with FI compared to RI. Furthermore, FI incubations are often associated with a lag phase (El-Meadaway et al. 1998; Mauricio et al. 2001; Rippstein et al. 2024), which may reflect a longer time for colonisation of the substrate with FI due to the overall lower microbial density of faeces (Posada et al. 2012). This is supported by the GP kinetic parameters of Rippstein et al. (2024), who used the same feeds as in the present study. Apart from maize grain, which showed no differences in GP rates between RI and FI, all feeds had lower GP rates when incubated with FI instead of RI. This further indicates that microbial lysis is likely to start faster with RI than with FI, leading to a faster decrease in mN and a concomitant faster increase in NH_3-N, resulting in the observed higher amounts of NH_3-N at 24 and 48 h when RI was used instead of FI. The exceptions between 8 and 24 h of maize grain, for which mN increased with RI, and of rye grain, for

which mN decreased with FI, are probably related to the availability of energy required for microbial synthesis. Starch-rich feeds differ considerably in the rate and extent of ruminal starch degradation. In general, maize starch has a slow rate and low extent of ruminal degradation, whereas rye starch has a fast rate and high extent of degradation (Krieg et al. 2017; Seifried et al. 2016). As a result, the maximum amounts of mN during *in vitro* incubations with RI were obtained later for maize grain compared to the other feeds. The microbes in FI, on the other hand, had a readily available energy source during incubations with rye grain, which apparently favoured microbial growth and thus, as in RI, led to an early decrease in mN between 8 and 24 h.

The observed differences in NH_3-N and mN levels between the different feeds can be attributed to variations in their chemical composition. The N concentrations and also the energy contents varied between the feeds and thus did the respective amounts available to the microbes at the standardised amount of feed used. This influences the extent to which N is microbially bound and ultimately the amount of NH_3-N released.

4.2. Comparison of CHO utilisation between FI and RI

The regression between GP and NH_3-N values showed a negative linear relationship at 8 h, as indicated by the high R^2 values for all feeds, but R^2 decreased for both inocula as incubation proceeded. This trend was probably related to changes in fermentative and synthetic activities with increasing incubation time. Decreasing fermentation activity after several hours of incubation was apparent with both RI and FI from the GP kinetic curves of Rippstein et al. (2024). While a steep rise in the curves could be observed at 8 h, the curves flattened out at 24 h and approached a plateau at about 48 h, probably because the energy available to the microbes in the batch culture becomes limiting at some point, thus restricting microbial growth. At a low growth rate of the microbes, their efficiency decreases and a large proportion of the available energy is used for maintenance and cell composition, and energy expenditure for transport processes changes (Hespell and Bryant 1979). After long incubation periods, such changes may explain the lower slopes representing less incorporated NH_3-N per mL GP. Another possible explanation is the lysis of microbial cells as incubation time increases. The NH_3-N from microbial lysis then enters the NH_3-N pool and can thereby reduce the ratio of NH_3-N decrease (mg) per mL GP (Raab et al. 1983).

The slopes of the linear regression equations were significantly different between FI and RI after 8 h, but not after 24 h, excluding maize grain, and 48 h (Table 5). This indicated that, after a certain incubation time, the amount of NH_3-N incorporation per mL GP was similar between the two inocula. This similarity may be surprising, as the overall more inert microbial activity with FI has been discussed before. A possible explanation could be the difference in microbial composition between rumen fluid and faeces, particularly due to protozoa that are not present in the colon (Hungate 1966; Hobson 1971). Rumen microbes are inefficient in producing cellular protein, partly because they do not use all adenosine triphosphate (ATP) for growth (Hackmann and Firkins 2015). They expend ATP by alternating between post-feeding accumulation and subsequent mobilisation of reserve carbohydrate, as some of the ATP is irreversibly expended during this process (Hackmann and Firkins 2015). In a batch culture study, Hackmann and Firkins (2015) observed that protozoa, rather than bacteria, were

responsible for most glycogen accumulation, so protozoa appear to be the predominant group accumulating reserve carbohydrate. Another factor that reduces growth efficiency is the recycling of MCP and most recycling was thought to occur through predation by protozoa, based on lysis of pure bacterial cultures in the presence of rumen fluid containing protozoa (Wallace and McPherson 1987). As discussed before, NH_3-N released by microbial lysis adds to the NH_3-N pool, potentially lowering the ratio of NH_3-N decrease (mg) per mL GP (Raab et al. 1983). Therefore, the protozoa-free microbial community in the hindgut may have a less wasteful N metabolism than that in the rumen. In consequence, microbial population growth with FI may be less restricted than with RI, possibly compensating for the generally lower fermentation activity of faeces as discussed before. Eventually, this might have caused the close relationship between the two inocula regarding NH_3-N utilisation per unit of GP.

4.3. Relationship of NH_3-N between FI and RI

Close linear relationships between FI-NH_3-N and RI-NH_3-N were found at all incubation times for both feed alone and feed with CHO source, with the highest estimation accuracy at 24 h (Table 6). Zhao and Chen (2004) studied the protein value of feeds by using sheep faeces compared to rumen fluid for estimating the CP entering the duodenum, i.e. the sum of RUP and MCP. The authors found a significant relationship between the CP reaching the duodenum estimated from incubations with FI after 48 h and RI after 24 h, but the R^2 value of 0.75 was low compared to the linear regressions of the present study. Apart from the fact that the author's regression analysis was based on the estimated sum of RUP and MCP instead of the NH_3-N contents as in the present study, the differences could be due to differences in assay details. For instance, Zhao and Chen (2004) used only one sheep for inoculum preparation, which was fed restrictively in the morning and evening. They collected the faeces in bags and used the *in vitro* incubation method based on Tilley and Terry (1963). Since *in vitro* GP data appear to be strongly influenced by factors such as the feeding of the donor animals, the collection and processing of faeces, and the incubation procedures (Rippstein et al. 2024), it can be assumed that these factors also affected *in vitro* NH_3-N data. In addition, in the study of Zhao and Chen (2004), 35 different feeds were used in the regression analysis, while only six feeds were included in the present study. This highlights the need for further research using FI in the HGT concerning the estimation of protein values of feeds. This includes the investigation of a larger number of feeds with diverse nutrient compositions to enhance the reliability of the regression equations. However, some studies have also found strong linear regression equations between FI and RI for GP (Nsahlai and Umunna 1996; Cone et al. 2002; Rippstein et al. 2024), or highly correlated GP and organic matter digestibility between the two inocula (Mauricio et al. 2001). These close relationships are consistent with the results of the present study and confirm the suitability of using FI instead of RI, also for estimating NH_3-N, due to apparently similar mechanisms of the microbiome present.

5. Conclusion

There is a high potential for the use of FI to estimate the protein value of feeds for ruminants in the eHGT. CP degradation and N binding by microorganisms appear to be faster and

more extensive in RI incubations, leading to higher NH_3-N contents at 24 and 48 h when RI was used as the inoculum instead of FI. However, at these incubation times, both inocula respond similarly to an extra energy source and exert strong linear relationships regarding the measured amounts of NH_3-N. These close relationships may reflect comparable microbial mechanisms in faeces and rumen fluid, and provide the basis for future mathematical approaches to estimating the protein value of feeds *in vitro* using FI instead of RI.

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Data availability statement

Data that support the findings of this study will be provided upon justified request by the authors.

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5 SUMMARY

In order to meet the animals' requirements of energy and nutrients, knowledge of the feed value of individual feed components is essential. In this context, information on ruminal degradability of feeds is crucial for formulating rations for ruminants. Information of this kind can be obtained using *in vitro* methods, such as the Hohenheim gas test (HGT). This method allows for the estimation of the organic matter digestibility and the energy value, as well as the protein value of ruminant feeds when applying the extended HGT (eHGT). *In vitro* methods provide a cost-efficient, rapid, and standardisable alternative to *in situ* and *in vivo* approaches, while contributing to reducing animal burden and the number of experimental animals. However, the HGT currently depends on using rumen fluid, which is commonly obtained from rumen-cannulated animals. Due to ethical concerns related to animal welfare and practical considerations, there is growing interest in replacing rumen-cannulated animals for the *in vitro* feed evaluation in the HGT and eHGT systems. However, the use of individual enzymes or enzyme mixtures for *in vitro* evaluation of feeds has not yet proven suitable for adequately representing the complex microbial activity of a rumen fluid inoculum (RI). In contrast, several findings in the literature indicate the potential of faeces as an alternative inoculum source to rumen fluid. However, to date, this approach has not been established in the routine analysis of ruminant feeds.

The overarching aim of the present thesis was to systematically evaluate the suitability of sheep faeces as an alternative inoculum source to rumen fluid in the HGT. To this end, *in vitro* gas production (GP) was compared between faecal inoculum (FI) and RI. Additionally, the potential of using preserved faeces as an alternative inoculum source to fresh faeces, as well as the application of FI in the eHGT system, was evaluated. To compare faeces and rumen fluid as inoculum sources in the HGT and eHGT, RI was prepared according to the standard procedure using rumen fluid obtained from two rumen-cannulated lactating dairy cows and FI was prepared from rectally collected faeces of three adult wether sheep.

The objective of *Manuscript 1* was to examine whether FI and RI generally follow similar GP kinetics and to assess whether feed-specific variation could be observed. Furthermore, there was considerable interest in determining whether FI-GP and RI-GP are related to each other, as this could provide the basis for the future applicability of FI. A total of 90 currently relevant ruminant feeds from various categories and differing nutrient compositions were incubated *in vitro* with both FI and RI for 72 h, with multiple readings in the HGT. By using FI, lower GP kinetics were observed across all feed categories compared to RI. On average of all feeds, the potential GP was 9 mL/200 mg dry matter (DM) lower and the GP rate was 3.1%/h lower with FI than RI. Additionally, a lag phase of 1.51 h was estimated with FI, whereas no lag phase was observed for RI. The results indicate an overall lower fermentation activity of FI compared to RI. Despite these differences, the GP kinetic curves of the two inocula exhibited a very similar progression. Moreover, strong linear relationships were found between RI-GP at 24 h, the

common incubation time of RI in the HGT, and FI-GP at both 24 h (Slope = 1.02, $R^2 = 0.97$) and 48 h (Slope = 1.1, $R^2 = 0.97$). Additionally, within the scope of this thesis, linear regression analyses were conducted based on a combined dataset from *Manuscript 1* and previous studies. By using data from more than 400 different feeds in these analyses, the strong linear relationships between RI-GP at 24 h and FI-GP at 24 h (Slope = 0.98, $R^2 = 0.93$) as well as 48 h (Slope = 1.02, $R^2 = 0.96$) were confirmed. Dividing the dataset into the feed categories roughages and concentrates for the calculation of separate regression equations did not provide a clear advantage over using a single equation for all feeds. Despite the lower GP observed with FI, a consistent relationship was evident between the GP of both inocula across the different feeds, enabling a reliable estimation of RI-GP from FI-GP in the HGT.

The use of preserved instead of fresh faeces would allow for a centralised housing of donor sheep, thereby reducing the number of animals required and improving standardisation. The aim of *Manuscript 2* was to investigate the effect of differently preserved sheep faeces on the *in vitro* GP of nine different feeds and the microbiome in the HGT, intending to maintain a high level of microbial activity during incubations. Seven different freezing and freeze-drying treatments were applied. On average across all feeds, the potential GP of the frozen treatments (61 mL/200 mg DM) was comparable to that of the fresh faeces (62 mL/200 mg DM), whereas the freeze-dried treatments accounted for only 71-85% of the fresh faecal value. The results were confirmed by metaproteome analyses, as the microbiomes of the fresh and frozen treatments were significantly different from that of the freeze-dried treatments based on the relative abundance of the core proteins ($p < 0.001$). This demonstrated that stress factors associated with the freeze-drying process significantly impaired the microbiome, consequently affecting fermentation activity and GP. By contrast, the freezing process appeared more gentle on the microbiome, preserving a high microbial activity. Furthermore, strong relationships were found between RI-GP at 24 h and GP of the frozen treatments at 48 h of incubation (Slope = 1.27, $R^2 = 0.96$). Additionally, the effect of storage on freeze-dried and frozen treatments was investigated, revealing a considerable negative impact on GP and its relationship with RI-GP for both treatments. This limits the high potential for estimating RI-GP, which was particularly demonstrated with frozen faeces, and therefore requires further research.

Manuscript 3 aimed to investigate the potential suitability of FI in the eHGT for estimating the protein value of ruminant feeds. The eHGT is used to estimate ruminally undegradable crude protein (RUP) and microbial crude protein. Ammonia-nitrogen (NH_3-N) is a key parameter in this context, as it is released during microbial crude protein degradation and provides a nitrogen source for the microbes. FI and RI were therefore compared based on NH_3-N and calculated microbially bound nitrogen (mN) following *in vitro* incubation of six different feeds for 8, 24, and 48 h. The NH_3-N content was 17 and 23% lower with FI than with RI after 24 and 48 h, respectively. With RI, mN values decreased over the incubation time for most feeds, whereas with FI, mN initially increased before declining at later incubation times. This suggests that crude protein degradation and microbial binding of nitrogen occur more slowly and

to a lesser extent with FI. However, both inocula demonstrated a comparable response to an additional energy source and showed strong linear relationships for NH_3-N, particularly after 24 h (Slope = 1.39, $R^2 = 0.98$), indicating similar microbial mechanisms in faeces and rumen fluid. The RUP was also estimated for both inocula in this thesis, and the results showed an inconsistent ratio between FI and RI incubations across the six feeds. Similarly, an inconsistent ratio between the two inocula was observed for the mN data. Therefore, further studies involving a larger number of feeds, as well as the testing of mathematical approaches, are necessary to better evaluate the suitability of FI for estimating the protein value of ruminant feeds with the eHGT.

In conclusion, the use of sheep faeces as an inoculum source for the *in vitro* analysis of ruminant feeds can be considered suitable for replacing rumen fluid and thus rumen-cannulated animals in the HGT. By reliably predicting the RI-GP from FI-GP, the predicted RI-GP can be used in the official and validated equations to estimate organic matter digestibility and metabolisable energy of ruminant feeds. The methodological approach applied in this thesis, including sheep feeding, faeces collection, and inoculum preparation, appeared appropriate in achieving a high and consistent microbial activity in the FI. Furthermore, a high potential was demonstrated for using preserved, particularly frozen, sheep faeces for application in the HGT, as well as the use of FI for estimating the protein value of feeds in the eHGT. However, further investigations are required for the two application fields to assess the suitability of FI comprehensively.

6 ZUSAMMENFASSUNG

Um eine bedarfsgerechte Versorgung von Tieren mit Energie und Nährstoffen zu gewährleisten, ist die Kenntnis des Futterwerts der einzelnen Futterkomponenten essenziell. Für die Formulierung von Rationen für Wiederkäuer sind in diesem Zusammenhang Informationen zur Abbaubarkeit von Futtermitteln im Pansen von entscheidender Bedeutung. Einen wichtigen Beitrag dazu können *in vitro* Verfahren wie der Hohenheimer Futterwerttest (HFT) leisten. Hierdurch kann die Schätzung der Verdaulichkeit der Organischen Masse und des energetischen Wertes sowie im erweiterten HFT (eHFT) die Schätzung des Proteinwertes von Futtermitteln für Wiederkäuer erfolgen. *In vitro* Verfahren bieten eine vergleichsweise kostengünstige, schnelle und gut standardisierbare Alternative zu *in situ* oder *in vivo* Verfahren und können gleichzeitig zur Reduktion der Tierbelastung und der Anzahl von Versuchstieren beitragen. Der HFT basiert derzeit jedoch auf der Verwendung von Pansenflüssigkeit, welche üblicherweise von pansenfistulierten Tieren gewonnen wird. Aus ethischen Gründen in Bezug auf das Wohlergehen der Tiere und praktischen Erwägungen besteht ein großes Bestreben darin, pansenfistulierte Spendertiere für die *in vitro* Untersuchung von Futtermitteln im HFT und eHFT zu ersetzen. Ein dahingehender Einsatz einzelner Enzyme oder Enzymmischungen, für die *in vitro*-Bewertung von Futtermitteln, kann jedoch gegenwärtig nicht adäquat die komplexe mikrobielle Aktivität des Pansenflüssigkeits-Inokulums (RI) abbilden. Dahingegen weisen einige Ergebnisse in der Literatur auf das Potential von Kot als alternative Inokulumquelle zu Pansenflüssigkeit hin, jedoch fand dieser Ansatz bislang keine Anwendung in der Routineuntersuchung von Futtermitteln für Wiederkäuer.

Das übergeordnete Ziel der vorliegenden Arbeit war deshalb, die Eignung von Schafkot als alternative Inokulumquelle zu Pansenflüssigkeit im HFT systematisch zu evaluieren, um dessen Anwendbarkeit zu forcieren. Hierzu wurde die *in vitro* Gasbildung (GB) zwischen Kot-Inokulum (FI) und RI verglichen. Zudem wurde das Potential der Verwendung von konserviertem Kot als alternative Inokulumquelle zu frischem Kot sowie die Anwendung von FI im eHFT geprüft. Um Kot und Pansenflüssigkeit als Inokulumquellen im HFT und eHFT zu vergleichen, wurde das RI gemäß der Standardmethoden unter Verwendung von Pansenflüssigkeit zweier pansenfistulierter Milchkühe hergestellt, während für die Herstellung des FI rektal gewonnener Kot von drei ausgewachsenen Hammeln verwendet wurde.

Das Ziel von *Manuskript 1* bestand darin, zu prüfen, ob FI und RI grundsätzlich einer ähnlichen GB-Kinetik folgen und ob hierbei futtermittelspezifische Unterschiede vorliegen. Zudem bestand ein großes Interesse an der Frage, ob zwischen der FI-GB und RI-GB ein Zusammenhang besteht, was die Grundlage für die künftige Anwendbarkeit von FI darstellen könnte. Dazu wurden 90 aktuell relevante Futtermittel für Wiederkäuer aus verschiedenen Kategorien und unterschiedlicher Nährstoff-zusammensetzung jeweils mit FI und RI für insgesamt 72 h mit mehreren Ablesezeiten *in vitro* im HFT inkubiert. Dabei zeigte sich mit FI für alle Futtermittelkategorien eine niedrigere GB-Kinetik als mit RI. Im Durchschnitt aller Futtermittel lag die potentielle GB von FI um 9 mL/200 mg Trockenmasse (TM)

unterhalb der von RI und wies eine um 3.1 %/h niedrigere GB-Rate als RI auf. Zudem wurde für FI eine anfängliche Verzögerungszeit von 1.51 h geschätzt, die für RI nicht beobachtet wurde. Die Ergebnisse deuten auf eine insgesamt geringere Fermentationsaktivität von FI im Vergleich zu RI hin. Trotz dieser Unterschiede zeigten die GB-Kinetikkurven der beiden Inokula einen sehr ähnlichen Verlauf. Außerdem wurden starke lineare Zusammenhänge zwischen RI-GB nach 24 h, der üblichen Inkubationszeit mit RI im HFT, und FI-GB nach 24 h (Steigung = 1.02, $R^2 = 0.97$) sowie nach 48 h (Steigung = 1.1, $R^2 = 0.97$) beobachtet. Ergänzend wurden im Rahmen dieser Arbeit lineare Regressionsanalysen auf Basis eines zusammengeführten Datensatzes aus *Manuskript 1* und früheren Untersuchungen durchgeführt. Unter Einbezug von über 400 verschiedenen Futtermitteln bestätigten sich dabei die starken lineare Zusammenhänge zwischen der RI-GB nach 24 h und der FI-GB nach 24 h (Steigung = 0.98, $R^2 = 0.93$) sowie nach 48 h (Steigung = 1.02, $R^2 = 0.96$). Eine Aufteilung des Datensatzes in die Kategorien Grobfuttermittel und Konzentratfuttermittel zur Berechnung separater Regressionsgleichungen zeigte insgesamt keinen deutlichen Vorteil gegenüber einer Gesamtgleichung für alle Futtermittel. Trotz der niedrigeren FI-GB bestand demnach über die verschiedenen Futtermittel hinweg ein konstantes Verhältnis zwischen der GB der beiden Inokula, welches eine zuverlässige Schätzung der RI-GP aus FI-GP im HFT ermöglicht.

Die Verwendung von konserviertem anstelle von frischem Kot würde eine zentralisierte Haltung der Spenderschafe ermöglichen, wodurch sich die Anzahl der hierfür eingesetzten Tiere reduzieren und die Standardisierbarkeit verbessern ließe. Das Ziel von *Manuskript 2* bestand deshalb darin, den Einfluss von unterschiedlich konserviertem Kot auf die *in vitro* GB von neun verschiedenen Futtermitteln sowie auf das Mikrobiom im HFT zu untersuchen. Dabei wurde der Erhalt einer hohen mikrobiellen Aktivität angestrebt. Hierfür wurden sieben verschiedene Gefrier- und Gefriertrocknungsbehandlungen durchgeführt. Im Durchschnitt aller Futtermittel war die potentielle GB der gefrorenen Behandlungen (61 mL/200 mg TM) vergleichbar mit derjenigen des frischen Kots (62 mL/200 mg TM), während die gefriergetrockneten Behandlungen lediglich 71-85% des Frischkotwertes erreichten. Die Ergebnisse der GB wurden durch Metaproteomanalysen gestützt, da sich das Mikrobiom der frischen und gefrorenen Behandlungen, basierend auf der relativen Häufigkeit der Coreproteine, signifikant von dem der gefriergetrockneten Behandlungen unterschied ($p < 0.001$). Dies verdeutlicht, dass die mit dem Gefriertrocknungsprozesses verbundenen Stressfaktoren das Mikrobiom und in der Folge die Fermentationsaktivität und GB deutlich beeinträchtigen. Der Gefrierprozess schien hingegen schonender für das Mikrobiom gewesen zu sein und eine hohe mikrobielle Aktivität bewahrt zu haben. Darüber hinaus konnten in dieser Arbeit starke lineare Zusammenhänge zwischen RI-GB nach 24 h und der GB der gefrorenen Kotbehandlung nach 48 h Inkubation (Steigung = 1.27, $R^2 = 0.96$) festgestellt werden. Weiterhin wurde auch der Einfluss einer Lagerung der gefriergetrockneten und gefrorenen Behandlungen geprüft, welche sich bei beiden Behandlungen deutlich negativ auf die GB und die Beziehung zu RI-GB auswirkte. Dies begrenzt das hohe Potential zur Schätzung der RI-GB, welches insbesondere für gefrorenen Kot gezeigte wurde, und erfordert daher zusätzliche Forschungsarbeit.

Manuskript 3 verfolgte das Ziel, FI hinsichtlich der potentiellen Eignung im eHFT zur Proteinwertschätzung von Wiederkäuerfuttermitteln zu untersuchen. Mithilfe des eHFT kann eine Schätzung von im Pansen nicht abgebautem Futter-Rohprotein (UDP) und mikrobiellem Rohprotein erfolgen. Dabei ist Ammoniak-Stickstoff (NH_3-N) ein zentraler Messwert, da er während des mikrobiellen Rohproteinabbaus freigesetzt wird und den Mikroben als Stickstoffquelle dient. FI und RI wurden daher auf Basis des NH_3-N sowie des berechneten mikrobiell gebundenen Stickstoffs (mN) durch die Inkubation von sechs verschiedenen Futtermitteln nach 8, 24 und 48 h Inkubation miteinander verglichen. Der NH_3-N Gehalt war mit FI nach 24 und 48 h jeweils um 17 und 23% niedriger als mit RI und der mN Gehalt sank für die meisten Futtermittel mit RI mit zunehmender Inkubationszeit, während er mit FI zunächst anstieg und erst zu einem späteren Zeitpunkt abnahm. Dies deutet darauf hin, dass der Rohproteinabbau und die mikrobielle Bindung von Stickstoff mit FI langsamer und insgesamt in geringerem Umfang erfolgen. Die beiden Inokula reagierten jedoch ähnlich auf eine zusätzliche Energiequelle und zeigten starke lineare Zusammenhänge bezüglich des NH_3-N, insbesondere nach 24 h (Steigung = 1.39, R^2 = 0.98), was auf ähnliche mikrobielle Mechanismen in Kot und Pansenflüssigkeit hindeutet. Im Rahmen dieser Arbeit wurde jedoch zudem das UDP für beide Inokula geschätzt, wobei sich ein uneinheitliches Verhältnis zwischen FI und RI für die sechs untersuchten Futtermittel zeigte, was auch für den Parameter mN beobachtet wurde. Daher sind weitere Untersuchungen mit einer größeren Futteranzahl und die Prüfung mathematischer Ansätze nötig, um die Eignung von FI für die Proteinwertschätzung von Futtermitteln für Wiederkäuer im eHFT besser bewerten zu können.

Zusammenfassend kann die Verwendung von Schafkot als Inokulumquelle zur *in vitro*-Untersuchung von Wiederkäuerfuttermitteln als geeignet bewertet werden, um Pansenflüssigkeit und somit pansenfistulierte Tiere im HFT zu ersetzen. Durch die zuverlässige Schätzung der RI-GB aus FI-GB kann perspektivisch die geschätzte RI-GB in den offiziellen und validierten Gleichungen zur Schätzung der Verdaulichkeit der Organischen Masse und Umsetzbaren Energie für Wiederkäuerfuttermittel verwendet werden. Der in dieser Arbeit angewandte methodische Ansatz bezüglich der Fütterung der Schafe, der Kotentnahme und der Herstellung des Inokulums schien daher erfolgreich gewesen zu sein, um eine hohe und konstante mikrobielle Aktivität des FI zu erzielen. Zudem zeigte sich ein hohes Potential für die Verwendung von konserviertem, insbesondere gefrorenem, Schafkot für die Anwendung im HFT sowie für die Verwendung von FI zur Proteinwertschätzung im eHFT. Für diese beiden Anwendungsbereiche sind jedoch weitere Untersuchungen erforderlich, um die Eignung von FI umfassend beurteilen zu können.

7 ANNEX

Annex 1. Chemical composition of tested feeds (g/kg dry matter (DM), unless otherwise stated) and rumen fluid inoculum-gas production (RI-GP) at 24 h and faecal inoculum-gas production (FI-GP) at 24 h and 48 h of incubation of the three unpublished datasets from previous investigations at the Institute of Animal Science at the University of Hohenheim.

Feed sample	DM (g/kg)	Ash	EE	CP	aNDF$_{om}$	ADF$_{om}$	RI-GP 24 h	FI-GP 24 h	FI-GP 48h
Dataset 1									
Oat feed	934	133	31	226	484	326	49	32	42
Rhodes grass feed	943	131	13	135	651	370	46	25	40
Guinea grass feed	949	140	13	140	566	344	50	32	47
Col. Guinea grass feed	945	102	21	70	633	322	36	17	30
Para grass feed	950	167	12	159	504	277	38	21	33
Common vetch feed	941	147	17	346	404	336	42	29	36
Alfalfa feed	952	151	13	257	364	302	43	25	33
Pigeon pea leaves	948	86	44	200	413	276	22	14	19
Sesban leaves	917	127	63	227	136	122	45	29	35
African moringa leaves	926	119	40	296	182	148	57	38	44
Wheat straw	939	100	3	29	762	576	34	21	36
Barley straw	934	126	4	53	678	532	36	22	35
Wheat bran	914	42	27	163	309	114	63	56	63
Papaya fruit	904	121	8	128	178	208	73	65	72
Banana fruit	943	187	40	51	299	244	54	47	58
Maize (Grosso) [a]	924	35	18	62	484	277	56	46	58
Maize (Grosso) [a]	917	34	26	63	417	231	63	43	60
Maize (Grosso) [b]	932	50	10	54	626	374	49	35	48
Maize (Bm3) [b]	925	58	7	72	578	347	42	35	45
Maize (Ayrro) [a]	923	39	24	69	445	244	60	49	62
Maize (Ayrro) [a]	916	34	27	76	369	198	68	46	63
Maize (Ayrro) [b]	931	57	9	48	617	371	53	40	53
Maize (Ayrro) [b]	929	65	9	44	661	396	49	34	47
Pea grain	866	28	23	230	138	74	77	67	77
Pea grain	891	28	21	222	160	86	70	60	70
Pea grain	884	30	20	248	142	81	75	66	75
Lupin grain	902	47	57	440	261	210	56	47	59
Lupin grain	907	45	114	380	190	214	54	51	59
Lupin grain	885	39	79	311	249	209	63	56	66
Distillers grains	929	.	.	.	.	.	36	23	37
Distillers grains	955	.	.	.	.	.	42	34	42
Distillers grains	928	.	.	.	.	.	40	33	39
Wheat grain	877	16	20	140	111	29	76	73	81
Wheat grain	876	16	24	132	132	33	76	73	82
Wheat grain	872	17	21	162	134	34	76	71	79

Table continued on next page

Annex 1. Continuation

Barley grain	879	24	25	122	180	53	72	70	77
Barley grain	881	25	31	123	186	50	78	68	78
Barley grain	884	25	31	128	152	46	77	66	75
Rye grain	879	17	20	112	172	33	77	74	81
Rye grain	879	17	19	113	139	27	79	74	80
Triticale grain	881	19	21	131	163	31	74	71	79
Triticale grain	882	18	22	113	130	29	80	75	83
Oat grain	893	29	51	130	268	123	59	53	60
Oat grain	893	27	48	125	267	120	65	54	61
Oat grain	890	27	52	121	261	117	59	54	62
Maize grain	911	16	123	99	96	27	63	48	68
Maize grain	907	13	43	80	110	29	79	59	80
Maize grain	901	14	45	95	81	30	78	50	75
Dataset 2									
Maize grain 1	869	14	51	87	109	37	79	62	77
Maize grain 2	874	14	41	87	98	29	81	64	80
Maize grain 3	864	13	44	83	91	15	81	62	78
Wheat grain 1	847	15	25	133	103	25	80	76	82
Wheat grain 2	878	18	21	126	198	23	80	79	84
Rye grain	876	17	21	106	150	24	81	76	82
Maize germ meal	858	34	6	275	424	160	60	54	60
Maize gluten feed	877	74	26	192	331	71	58	45	57
Wheat distillers grains	912	63	73	339	298	125	40	35	42
Dataset 3									
Maize grain	897	14	43	84	84	30	78	67	77
Wheat grain	887	19	31	141	86	36	75	69	75
Barley grain	894	28	33	126	162	76	69	63	69
Rapeseed meal	898	92	38	367	304	223	42	36	41
Soybean meal	901	74	26	517	111	66	49	41	49
Sunflower meal	906	78	30	319	402	307	33	29	33
Lupin grain	891	38	65	331	243	222	60	47	59
Pea grain	885	30	19	228	118	75	75	65	72
Field bean grain	893	35	16	299	157	131	60	55	61
Compound feed 18% CP	901	49	41	177	232	105	63	57	63
Compound feed 22% CP	901	36	72	219	145	76	64	56	62
Grass hay	956	92	12	96	609	365	32	25	33
Maize silage	930	30	.	76	444	246	59	51	59
Grass silage	925	83	24	127	575	353	42	31	40

EE, ether extract; CP, crude protein; aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; ADFom, acid detergent fibre expressed exclusive of residual ash; Col. Guinea grass feed, Coloured guinea grass feed.

[a]whole plant; [b]residual plant.

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