Animal Nutriomics

cambridge.org/anr

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

Lena Rippstein[1], Johan S. Sáenz[1], Natascha Titze[1,2], Katharina J. Wild[1], Jana Seifert[1,2] and Markus Rodehutscord[1,2]

[1]Institute of Animal Science, University of Hohenheim, Stuttgart, Germany and [2]HoLMiR – Hohenheim Center for Livestock Microbiome Research, University of Hohenheim, Stuttgart, Germany

Research Article

Cite this article: Rippstein L, Sáenz JS, Titze N, Wild KJ, Seifert J, Rodehutscord M (2025) The technique of sheep faeces preservation affects the microbiome activity and associated gas production kinetics *in vitro*. *Animal Nutriomics* **2**, e23, 1–15. https://doi.org/10.1017/anr.2025.10014

Received: 13 February 2025
Revised: 25 July 2025
Accepted: 26 July 2025

Keywords:
faecal microbiome; gas production technique; *in vitro*; metaproteomics; preservation; storage

Corresponding author:
Markus Rodehutscord;
Email: inst450@uni-hohenheim.de

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

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

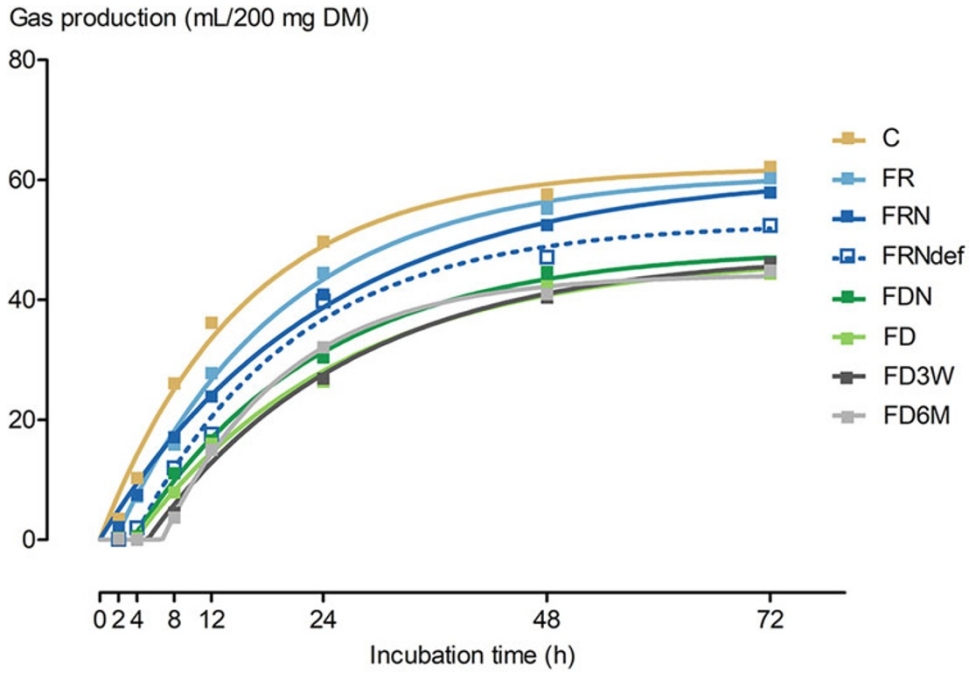

Figure 1. Mean *in vitro* gas production kinetics of faecal inoculum from fresh (C) and differently preserved faeces averaged across nine feed samples incubated for 72 h and fitted with equation [1] or [2]. FR, Frozen faeces; FRN, Shock-frozen faeces with liquid nitrogen; FRNdef, faeces treated like 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					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}	62de	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					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 – $e^{-cgas*t}$) 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 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.

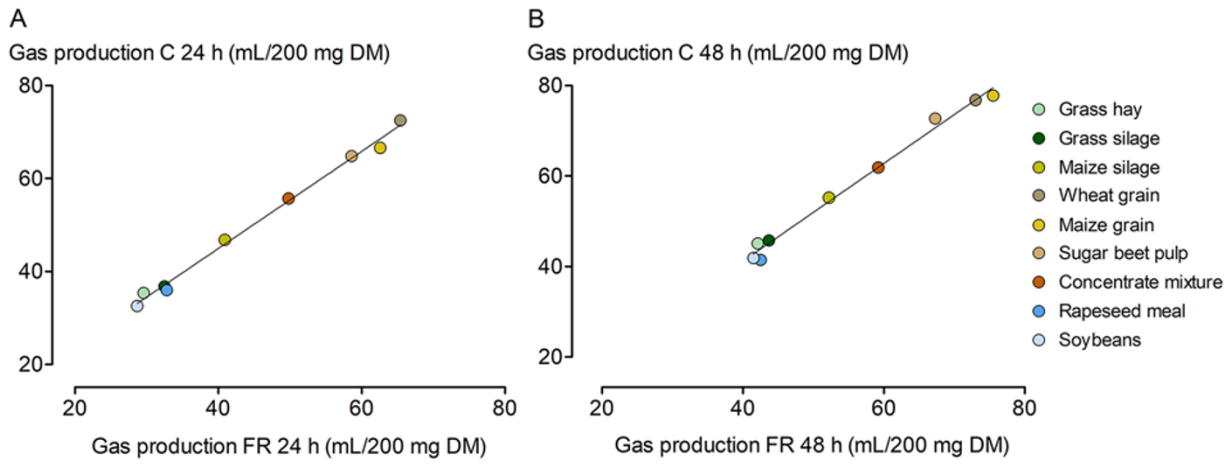

Figure 2. Linear relationships between gas production obtained with faecal inoculum from fresh faeces (C) and frozen faeces (FR) from nine feeds after (A) 24 and (B) 48 h of incubation. y = 1.05 * x + 3.14 (C24h, y; FR24h, x) (R^2 = 0.99; RMSE = 1.21; CV = 2.4%); y = 1.08 * x + 2.02 (C48h, y; FR48h,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

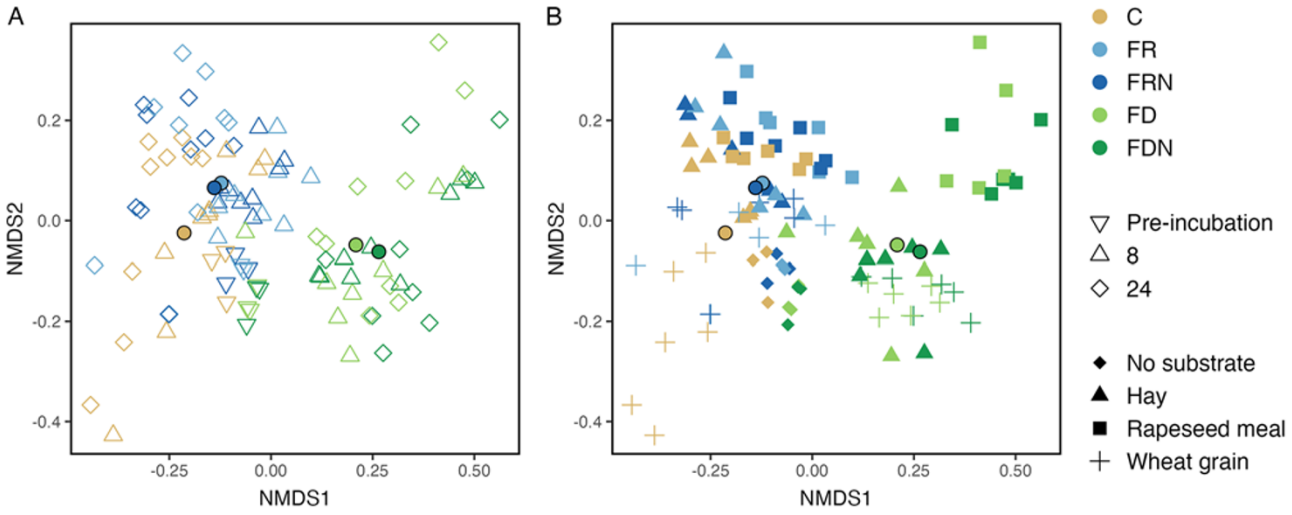

Figure 3. Non-metric multidimensional scaling (NMDS) ordination of faecal sheep samples used as inoculum after preservation with different treatments. Bray–Curtis dissimilarities were calculated based on the abundance of identified protein groups. (A) Clustering by incubation time; (B) clustering by 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.

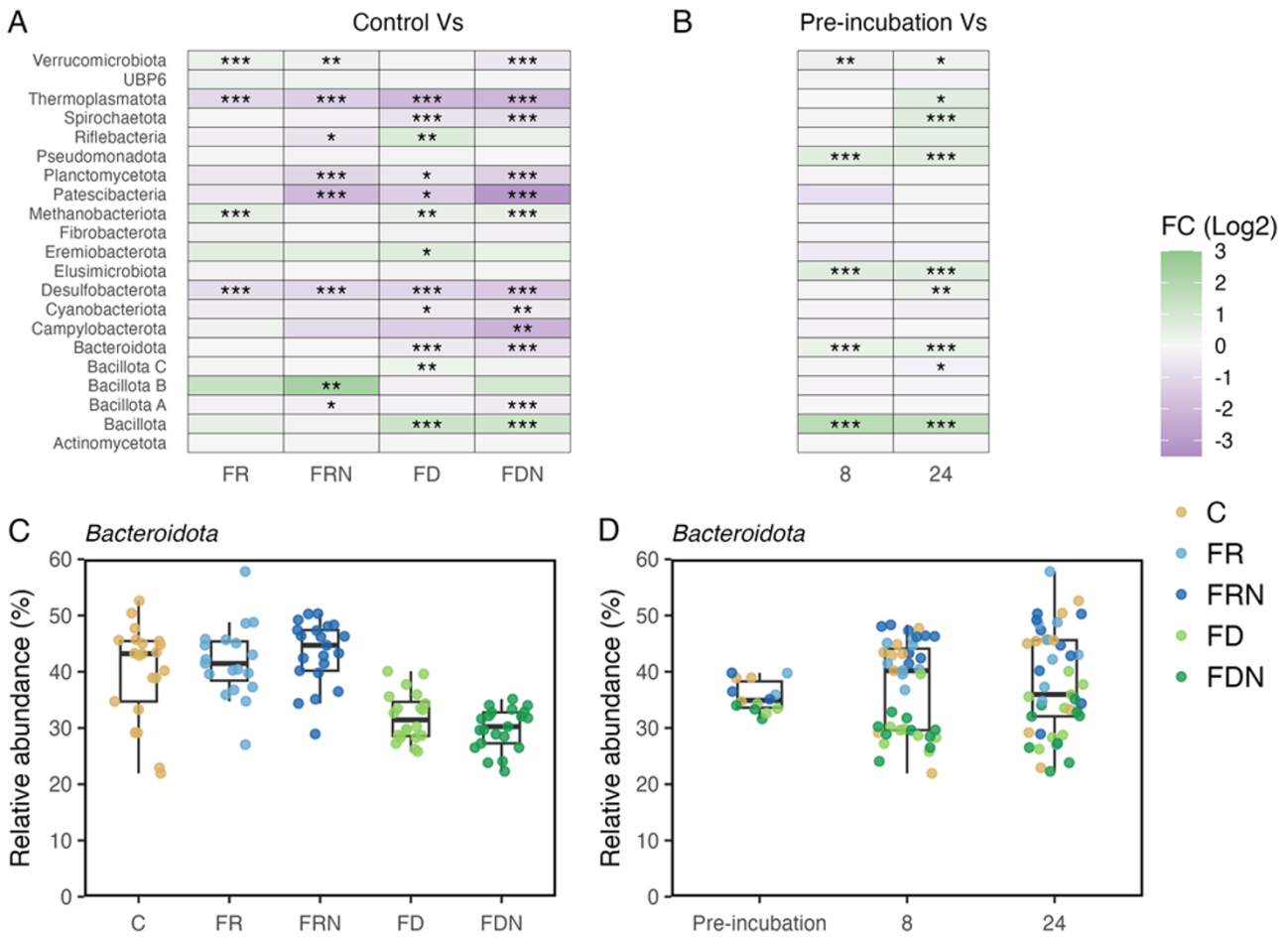

Figure 4. Effect of the (A) preservation methods and (B) incubation time on the relative abundance of different identified phyla. The change of the abundance of the phylum *Bacteroidota* under the different (C) preservation methods and (D) incubation times is shown as an example. The stars indicate the 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.

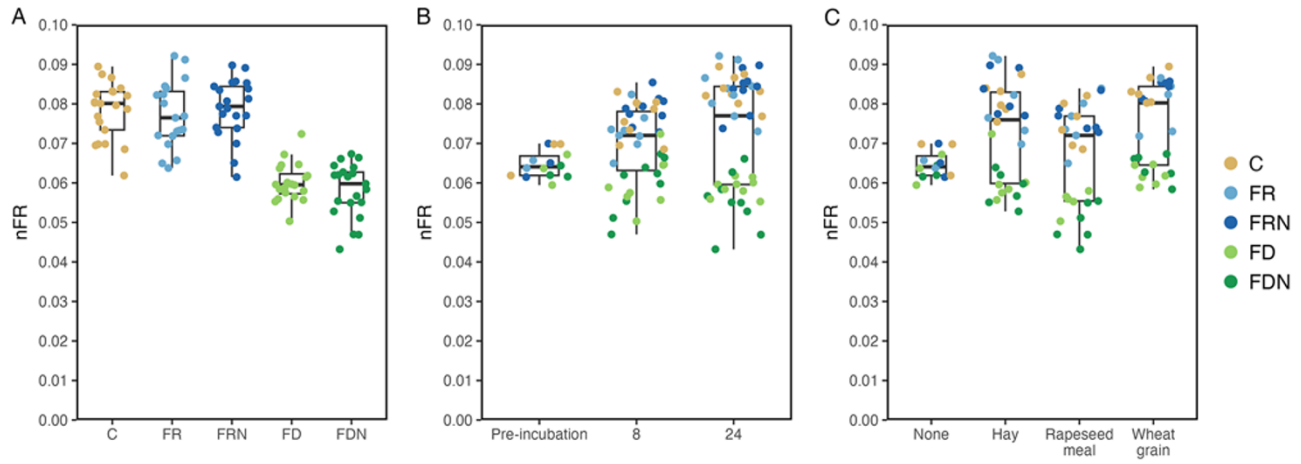

Figure 5. Functional redundancy of the (A) treatment, (B) incubation time, and (C) substrate (feed). High functional redundancy values (nFR) indicate that more different taxa contribute to an ecosystem in similar ways through the expression of redundant functions. C, Fresh faeces; FR, frozen faeces;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

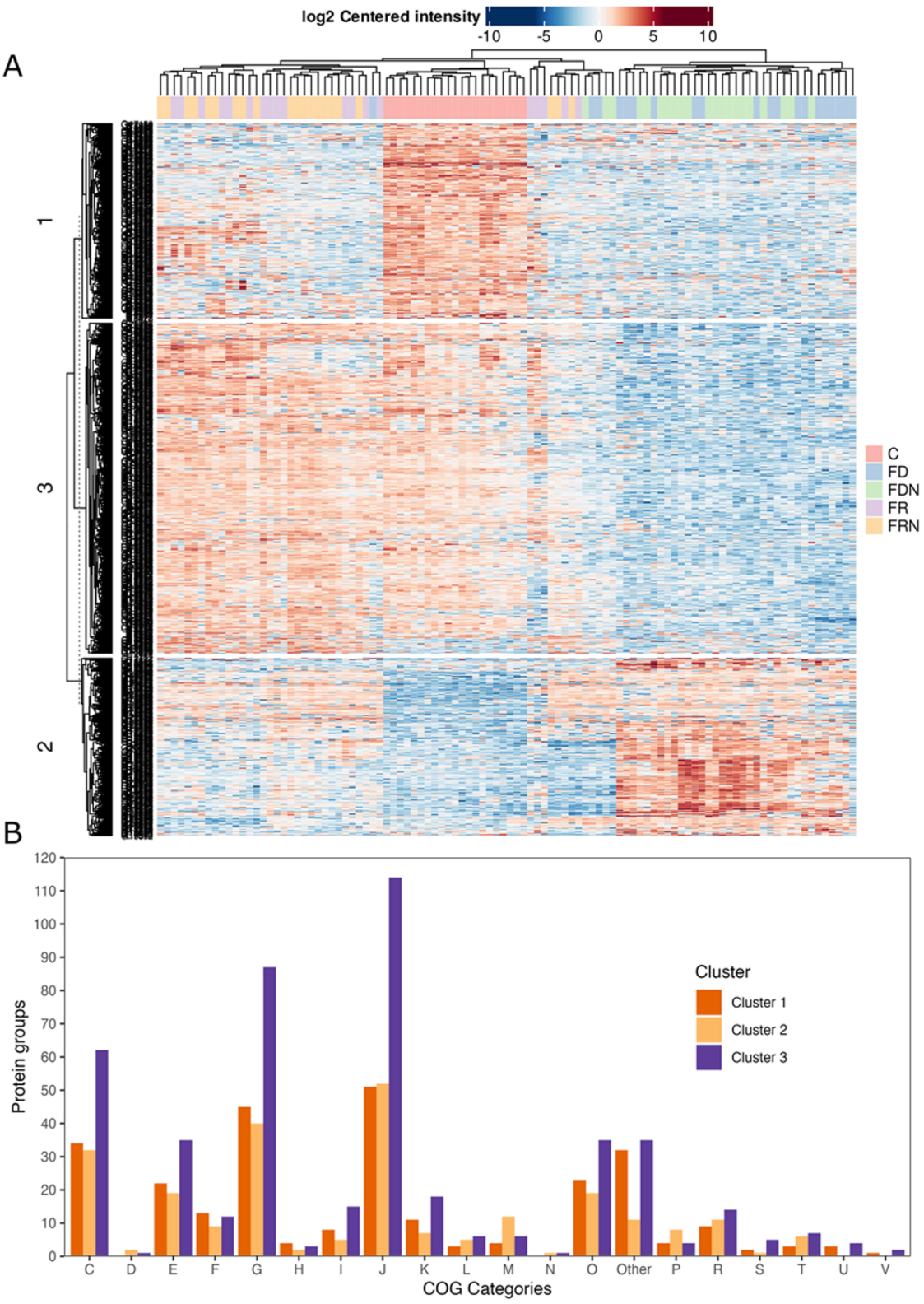

Figure 6. Abundance and clustering of the differentially abundant protein groups (*P*-value $<$ 0.01 and a $>$ 2.5 Log2 fold change) compared between the treatments. (A) Heatmap indicates clustering of proteins based on protein abundances on top tree and protein grouping based on similar abundance patterns 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.

Acknowledgements. This work was supported through a doctoral scholarship for L.R. by the H. Wilhelm Schaumann Foundation (Hamburg, Germany). We also thank Laura Herlein and Yitagele Mekonnen of the Hohenheim Center for Livestock Microbiome Research for their help with the sample preparation, Carolin Bauhof for her help with the investigations, the staff at the Mass Spectrometry Unit at the Core Facility Hohenheim for LC-MS/MS analyses and the Department of Animal Nutrition staff for feed analysis and animal care. The authors acknowledge support by the High Performance and Cloud Computing Group at the Zentrum für Datenverarbeitung of the University of Tübingen, the state of Baden-Württemberg through bwHPC and the German Research Foundation (DFG) through grant INST 37/935-1 FUGG.

Author contributions. L.R.: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – Original Draft. J.S.S.: Formal analysis, Visualization, Writing – Original Draft. N.T.: Conceptualization, Methodology, Writing – Review and Editing. K.J.W.: Conceptualization, Methodology, Formal analysis, Writing – Review and Editing. J.S.: Conceptualization, Methodology, Formal analysis, Funding acquisition, Resources, Project administration, Supervision, Writing – Original Draft. M.R.: Conceptualization, Funding acquisition, Resources, Project administration, Supervision, Writing – Review and Editing.

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.

References

Aiple KP, Steingass H and Menke KH (1992) Suitability of a buffered faecal suspension as the inoculum in the Hohenheim gas test: 1. Modification of the method and its ability in the prediction of organic matter digestibility and metabolizable energy content of ruminant feeds compared with rumen fluid as inoculum. *Journal of Animal Physiology and Animal Nutrition* **67**, 57–66.

Akhter S, Owen E, Fall A, O'Donovan F and Theodorou MK (1994) Use of fresh or frozen cow faeces instead of sheep rumen liquor to provide microorganisms for *in vitro* digestibility assays of forages. *Proceedings of the British Society of Animal Production* **58**, 452A.

Belanche A, Palma-Hidalgo JM, Nejjam I, Serrano R, Jiménez E, Martín-García I and Yáñez-Ruiz DR (2019) *In vitro* assessment of the factors that determine the activity of the rumen microbiota for further applications as inoculum. *Journal of the Science of Food & Agriculture* **99**, 163–172.

Biddle A, Stewart L, Blanchard J and Leschine S (2013) Untangling the Genetic Basis of Fibrolytic Specialization by Lachnospiraceae and Ruminococcaceae in Diverse Gut Communities. *Diversity* **5**, 627–640.

Bircher L, Geirnaert A, Hammes F, Lacroix C and Schwab C (2018) Effect of cryopreservation and lyophilization on viability and growth of strict anaerobic human gut microbes. *Microbial Biotechnology* **11**(4), 721–733.

Chaudry AS and Mohamed R (2012) Fresh or frozen rumen contents from slaughtered cattle to estimate *in vitro* degradation of two contrasting feeds. *Czech Journal of Animal Science* **57**(6), 265–273.

Chaumeil PA, Mussig AJ, Hugenholtz P and Parks DH (2022) GTDB-Tk v2: Memory friendly classification with the genome taxonomy database. *Bioinformatics* **38**(23), 5315–5316.

Cheng K, Ning Z, Li L, Zhang X, Serrana JM, Mayne J and Figeys D (2023) MetaLab-MAG: A Metaproteomic Data Analysis Platform for Genome-Level Characterization of Microbiomes from the Metagenome-Assembled Genomes Database. *Journal of Proteome Research* **22**(2), 387–398.

Costa E, Usall J, Teixidó N, Torres R and Viñas I (2002) Effect of package and storage conditions on viability and efficacy of the freeze-dried biocontrol agent *Pantoea agglomerans* strain CPA-2. *Journal of Applied Microbiology* **92**(5), 873–878.

Fabro C, Sarnataro C and Spanghero M (2020) Impacts of rumen fluid, refrigerated or reconstituted from a refrigerated pellet, on gas production measured at 24h of fermentation. *Animal Feed Science and Technology* **268**, 114585.

Garcia TJ, Cherry NM, Guay KA, Brady JA, Muir JP and Smith WB (2021) Preservation Method of Rumen Fluid Collected From Harvested Cattle Alters *in vitro* Digestibility of Reference Feedstuffs. *Frontiers in Animal Science* **2**, 775345.

Gharechahi J, Vahidi MF, Sharifi G, Ariaeenejad S, Ding XZ, Han JL and Salekdeh GH (2023) Lignocellulose degradation by rumen bacterial communities: New insights from metagenome analyses. *Environmental Research* **229**, 115925.

Hervás G, Frutos P, Giráldez FJ, Mora MJ, Fernández B and Mantecón ÁR (2005) Effect of preservation on fermentative activity of rumen fluid inoculum for in vitro gas production techniques. *Animal Feed Science and Technology* **123-124**(1), 107–118.

Holman DB and Gzyl KE (2019) A meta-analysis of the bovine gastrointestinal tract microbiota. *FEMS Microbiology Ecology* **95**(6), fiz072.

Li C, Zhao K, Ma L, Zhao J and Zhao ZM (2022) Effects of drying strategies on sporulation and titer of microbial ecological agents with *Bacillus subtilis*. *Frontiers in Nutrition* **9**, 1025248.

Li L, Wang T, Ning Z, Zhang X, Butcher J, Serrana JM, Simopoulos CMA, Mayne J, Stintzi A, Mack DR and Liu YY (2023a) Revealing proteome-level functional redundancy in the human gut microbiome using ultra-deep metaproteomics. *Nature Communications* **14**, 3428.

Li XM, Shi X, Yao Y, Shen YC, Wu XL, Cai T, Liang LX and Wang F (2023b) Effects of Stool Sample Preservation Methods on Gut Microbiota Biodiversity: New Original Data and Systematic Review with Meta-Analysis. *Microbiology Spectrum*, **11**(3), e04297–22.

Lin CY, Cross TWL, Doukhanine E and Swanson KS (2020) An ambient temperature collection and stabilization strategy for canine microbiota studies. *Scientific Reports* **10**, 13383.

Malik KA (1991) Cryopreservation of bacteria with special reference to anaerobes. *World Journal of Microbiology & Biotechnology* **7**, 629–632.

Mauricio RM, Owen E, Mould FL Givens I, Theodorou MK, France J, Davies DR and Dhanoa MS (2001) Comparison of bovine rumen liquor and bovine faeces as inoculum for an in vitro gas production technique for evaluating forages. *Animal Feed Science and Technology* **89**(1-2), 33–48.

Megrian D, Taib N, Witwinowski J, Beloin C and Gribaldo S (2020) One or two membranes? Diderm Firmicutes challenge the Gram-positive/Gram-negative divide. *Molecular Microbiology* **113**, 659–671.

Menke KH and Steingass H (1988) Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Animal Research and Development* **28**, 7–55.

Meryman HT (2007) Cryopreservation of living cells: Principles and practice. *Transfusion* **47**(5), 935–945.

Molle V, Fujita M, Jensen ST, Eichenberger P, González-Pastor JE, Liu JS and Losick R (2003) The Spo0A regulon of *Bacillus subtilis*. *Molecular Microbiology* **50**(5), 1683–1701.

Morgan CA, Herman N, White PA and Vesey G (2006) Preservation of micro-organisms by drying; A review. *Journal of Microbiological Methods* **66**(2), 183–193.

Mould FL, Kliem KE, Morgan R and Mauricio RM, (2005) In vitro microbial inoculum: A review of its function and properties. *Animal Feed Science and Technology* **123-124**(1), 31–50.

Neves ALA, Yu J, Suzuki Y, Baez-Magana M, Arutyunova E, O'Hara E, McAllister T, Ominski KH, Lemieux MJ and Guan LL (2021) Accelerated discovery of novel glycoside hydrolases using targeted functional profiling and selective pressure on the rumen microbiome. *Microbiome* **9**, 229.

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P and Stevens MHH (2020) Vegan: Community Ecology Package. Ordination methods, diversity analysis and other functions for community and vegetation ecologists. *R Package Version* 2.5–7.

Perez-Riverol Y, Bandla C, Kundu DJ, Kamatchinathan S, Bai J, Hewapathirana S, John NS, Prakash A, Walzer M, Wang S and Vizcaíno JA (2025) The PRIDE database at 20 years: 2025 update. *Nucleic Acids Research* **53**(D1), D543–D553.

Perry SF (1998) Freeze-Drying and Cryopreservation of Bacteria. *Molecular Biotechnology* **9**(1), 59–64.

Prates A, de Oliveira JA, Abecia L and Fondevila M (2010) Effects of preservation procedures of rumen inoculum on *in vitro* microbial diversity and fermentation. *Animal Feed Science and Technology* **155**(2-4), 186–193.

Richardson L, Allen B, Baldi G, Beracochea M, Bileschi ML, Burdett T, Burgin J, Caballero-Pérez J Cochrane G, Colwell LJ and Curtis T (2023) MGnify: The microbiome sequence data analysis resource in 2023. *Nucleic Acids Research* **51**(D1), D753–D759.

Rippstein L, Titze N, Wild KJ and Rodehutscord M (2024) A systematic study of sheep faeces to be used as an alternative inoculum source in the Hohenheim gas test. *Archives of Animal Nutrition* **78**(3), 298–313.

Rockinger U, Funk M and Winter G (2021) Current Approaches of Preservation of Cells During (freeze-) Drying. *Journal of Pharmaceutical Sciences.* **110**, 2873–2893.

Sáenz JS, Kurz A, Ruczizka U, Bünger M, Dippel M, Nagl V, Grenier B, Ladinig A, Seifert J and Selberherr E (2021) Metaproteomics Reveals Alteration of the Gut Microbiome in Weaned Piglets Due to the Ingestion of the Mycotoxins Deoxynivalenol and Zearalenone. *Toxins* **13**, 583.

Shah AD, Goode RJA, Huang C, Powell DR and Schittenhelm RB (2020) LFQ-Analyst: An Easy-To-Use Interactive Web Platform To Analyze and Visualize Label-Free Proteomics Data Preprocessed with MaxQuant. *Journal of Proteome Research* **19**(1), 204–211.

Song SJ, Amir A, Metcalf JL, Amato KR, Xu ZZ, Humphrey G and Knight R (2016) Preservation Methods Differ in Fecal Microbiome Stability, Affecting Suitability for Field Studies. *mySystems*, **1**(3), e00021–16.

Spanghero M, Chiaravalli M, Colombini S, Fabro C, Froldi F, Mason F, Moschini M, Sarnataro C, Schiavon S and Tagliapietra F (2019) Rumen Inoculum Collected from Cows at Slaughter or from a Continuous Fermenter and Preserved in Warm, Refrigerated, Chilled or Freeze-Dried Environments for In Vitro Tests. *Animals* **9**, 815–830.

Steingass H (2020) Fütterung der Schafe: Ernährung landwirtschaftlicher Nutztiere. Stuttgart, Germany: Eugen Ulmer Verlag.

Tilley JMA and Terry RA (1963) A two-stage technique for the *in vitro* digestion of forage crops. *Grass and Forage Science* **18**, 104–111.

Tunkala BZ, DiGiacomo K, Alvarez Hess PS, Dunshea FR and Leury BJ (2022) Rumen fluid preservation for in vitro gas production systems. *Animal Feed Science and Technology* **292**, 115405.

VDLUFA (2012) Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch): Bd. III Die chemische Untersuchung von Futtermitteln mit 1. – 8. Ergänzungslieferung (1983-2012). VDLUFA-Verlag.

Zeigler LD, Schlegel ML and Edwards MS (2003) Development of a rumen fluid preservation technique and application to an in vitro dry matter digestibility

assay: Proceedings of the Fifth Conference on Zoo and Wildlife Nutrition. Chester, PA, USA: AZA Nutrition Advisory Group.

Zhou H, He K, Chen J and Zhang X (2022) LinDA: linear models for differential abundance analysis of microbiome compositional data. *Genome Biology* **23**, 95.

Zicarelli F, Calabrò S, Cutrignelli MI, Infascelli F, Tudisco R, Bovera F and Piccolo V (2011) *In vitro* fermentation characteristics of diets with different forage/concentrate ratios: comparison of rumen and faecal inocula. *Journal of the Science of Food & Agriculture* **91**, 1213–1221.