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Optimization of rumen anaerobic fungi protein extraction methods to elucidate their CAZyme expression.

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Ås,2024

Lindah Chido Matsapa

Summary

In the past five decades anaerobic rumen fungi (ARF) have been explored more but research and information pertaining to the full extent of their capabilities is still limited compared to rumen bacteria. Anaerobic microbes work in synergy and therefore complement each other in the digestion of plant material for provision of host energy and nutrition. The rhizoidal nature of anaerobic rumen fungi allows them to embed themselves in the substrate cell wall making it difficult to extract them with commonly used extraction methods. The most common extraction method in proteomics is bead beating in the presence of a detergent, and this is effective for protozoa and prokaryotes but might be oblivious of anerobic fungi. Hence the proposal of a pretreatment by freeze grinding (FG) the plant material in liquid nitrogen prior to bead beating (BB). The hypothesis that the pretreatment would extract more fungal proteins was satisfied by the intensity of the proteins recovered. However, for more conclusive research a larger sample size is recommended. In this thesis samples six cannulated cows (from an animal feeding experiment conducted as a part of a research project named Seacow (Funded by the Norwegian Research Council, project number: 302639)) were explored and their biological replicates were used with the two time points (2 hours and 6 hours after feeding)and two diet conditions (control and control+ *Asparagopsis taxiformis (*AT*)*) and for the two extraction methods bead beating versus freeze grinding. The dominating fungal family by protein count was *Neocallimastix,* with *Neocallimastix sp. GF-Ma3-1* as the most detected genus and it had a higher count in freeze grinding (97) than bead beating (81). LFQ intensities showed a clear difference between bead beating and freeze grinding, where the fungal proteins had a significantly higher expression in the freeze grinding samples. The CAZyme annotation resulted in a total of eight protein groups being annotated as CAZymes. Of these, 5 CAZymes being glycoside hydrolases (GH), 2 of them were glycosyl transferases (GT)and 1 being a polysaccharide lyase (PL). These CAZymes were related to *Piromyces MAGS* (1), *Piromyces sp. UH31-1* (2), *Pecoramyces sp. F1* (1), *Neocallimastix sp. WI3-B* (1), and *Neocallimastix sp. GF-Ma3-1* (6).I concluded that a pre-treatment step with freeze-grinding is required for sufficient extraction of anaerobic fungi from rumen samples and a larger sample size will possibly provide satisfactory elucidation of the anaerobic

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Abbreviations

ARF	Anaerobic rumen fungi
AT	Aspragopsis taxiformis
CAZyme	Carbohydrate active enzyme
FG	Freeze grinding
GH	Glycoside hydrolases
GT	Glycosyl transferases
MAGs	Metagenome assembled genomes
PL	Polysaccharide lyase
SAGs	Single amplified genomes
VFAs	Volatile fatty acids

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Introduction

Aims and objectives.

Even though anaerobic eukaryotes play a crucial role in the decomposition of plant and animal feed in the rumen, they are understudied in the rumen ecosystem.(Hess et al., 2020).Their interactions with other microbes complete the efficiency of the rumen ecosystem(Li et al., 2021). Anaerobic rumen fungi (ARF) work in synergy with plant biomass-degrading bacteria, while methanogens take care of the excess hydrogen thereby complementing the fermentation process(Hagen et al., 2020; Hess et al., 2020). The rumen microbes also have a synergetic relationship with their host as they aid in amelioration of fiber digestion and nutrient utilization, production of microbial protein, efficient adaptation to forage diets and bloat reduction (Matthews et al., 2018; Mountfort, 2019). Present protocols favor the extraction of bacteria and protozoa but have been oblivious of the rhizoidal nature of anaerobic rumen fungi (ARF) (Andersen et al., 2023; Hagen et al., 2020), this has led to the procurement of this research thesis. The main objective of this thesis research is to explore the most effective extraction method for anaerobic rumen fungal proteins with the aim of elucidating their CAZyme expression. The optimization of available metaproteomics protocols is essential to aid the study of these microbes. However, the available in-house protocol at NMBU (Norges Miljø og Biovitenskapelige Universitet) for rumen samples is optimized for prokaryotes and it relies on bead beating in detergent to lyse cells directly from the sample (Arntzen.M.Ø., 2014). I hypothesize that this method is not harsh enough to crush all plant and fungal cell walls, leaving more fungal proteins in the cell instead of being extracted and analyzed since fungi typically are buried within the plant material and this is the reason previous methods fails to capture them. Hence the proposal of a second cell lysis method which involves freeze drying the rumen samples and grinding the freeze-dried material to extract more fungal proteins as a pretreatment to the bead beating. Anaerobic fungi are also known to degrade lignocellulosic material by invasive rhizoidal growth hence the need for a harsher cell lysis method than bacteria and protozoa (Hagen, L. H., et.al., 2020)(K.Theodorou, Brookman, & Trinci, 2005). Plant cells have rigid cell walls that contain microfibrils which are responsible for holding the structure of the plant (InglisID et al., 2018). The microfibrils contain cellulose, lignin and hemicellulose and they branch together in order to form that rigidity in the plant cell walls and this

makes it difficult to break them down without the necessary enzymes (Lankiewicz et al., 2013).Fungi are also famous for their sturdy cell wall thus there is need for effective extraction of intracellular proteins during proteomic sample preparation(Williams et al., 2020).

Background

The particular interest for this thesis is the rumen ecosystem due to its immaculate capacity to degrade plant material. The thallus cells of anaerobic rumen fungi (ARF) aid in the plant material degradation by producing cellulases, xylanases and hemicellulases (Akin & Borneman, 1990; Bhardwaj et al., 2021). The cellulases convert cellulose to glucose where endoglucanases, exoglucanases and β-glucosidases break cellulose chains, release cellobiose and convert it to glucose respectively. (Bhardwaj et al., 2021). The presence of rhizoids in fungi is paramount for their efficient lignocellulose degradation as they probe plant cell walls initiating entry of degradation enzymes (Swift et al., 2021). Owing to their rhizoidal nature and CAZyme production ARF play a central role in plant fiber decomposition, yet being understudied (and thus potentially underestimated), this sparked the interest of this thesis and is the basis of the protocol optimization. Significant symbiotic relationships are noted within the rumen microbes and between the rumen and its microbiome. These relationships drive optimal plant fiber decomposition aided by CAZymes to produce VFAs via fermentation (Aschenbach et al., 2010; Hartinger & Zebeli, 2021).The cow as the host then uses the VFAs as essential energy sources for meat and milk production amongst other purposes (Haitjema et al., 2014). Hydrogen gas is produced as a by-product of fermentation and is regulated by methanogens which convert it to methane, which is expelled via burping and flatulence , relieving the cow but exposing the environment to greenhouse gas(Króliczewska, Pecka-Kiełb, & Bujok, 2023; Palangi et al., 2022). To combat methane emissions, several methods have been employed including dietary manipulation like the use of A sparagosis taxiformis (AT) as supplementary feed(Alvarez et al., 2022; Palangi et al., 2022). AT is a red seaweed containing bromoform and other halogenated compounds which are capable of inhibiting methane production by methanogens(Eikanger, 2021).

The Seacow project aims to promote efficient low emitting rumens, through nutritional manipulation of the rumen microbiome by feeding AT to dairy cows to reduce rumen methane production. The samples used in this thesis are from the Seacow project and one of the aims of the Seacow project. Methane possess a significant threat to the environment hence more knowledge is

paramount to engineer strategies to combat enteric methane emission(Króliczewska, Pecka-Kiełb, & Bujok, 2023).As the world evolves from fossil fuels to a greener and circular economy the biorefinery industry is utilizing the efficiency of CAZymes (Palangi et al., 2022). Studying the rumen ecosystem in particular ARF is important in potential discovery of novel CAZymes.

Rumen ecosystem

Ruminants

Ruminants like cattle ingest food, and it goes through the esophagus into the rumen, a pregastric chamber that harbors all the microbes and ferments the feed. The feed is transported to the omasum and abomasum where digestion is aided by glandular secretions (Highfill & Lalman, 2000). In monogastric mammals like humans the feed travels from the mouth to the esophagus then directly into the stomach for breakdown using the glandular secretions without a microbial pre fermentation process. Microbial fermentation sets the ruminants apart and has gained popularity of late in terms of research and analysis, as it can model convenient biotechnological applications in the long run (Kazda1, Langer, & Bengelsdorf, 2014;(Bhardwaj et al., 2021)).

Rumination allows ruminants to swallow copious amounts of food without completely chewing, giving room for regurgitation of food to rechew hence increasing the surface area for enzyme activity and microbial activity (Aschenbach et al., 2010; Highfill & Lalman, 2000). The rumen also harbors billions of microbes essential for feed breakdown, for instance protozoa, bacteria, fungi, and viruses (Highfill & Lalman, 2000). The temperature in the rumen is maintained between 38-42°C for optimal microbial activity and the pH should always be around 6.0 - 7.0 (Yokoyama and Johnson 1993). In the rumen, the feed goes through the three phases of solid, liquid and gas. The solid refers to when the feed has entered the rumen where it accumulates and is digested by microbes into the liquid phase before being transported into the omasum and the phase is represented by the results of fermentation like methane, hydrogen, carbon dioxide and ammonia (Aschenbach et al., 2010).

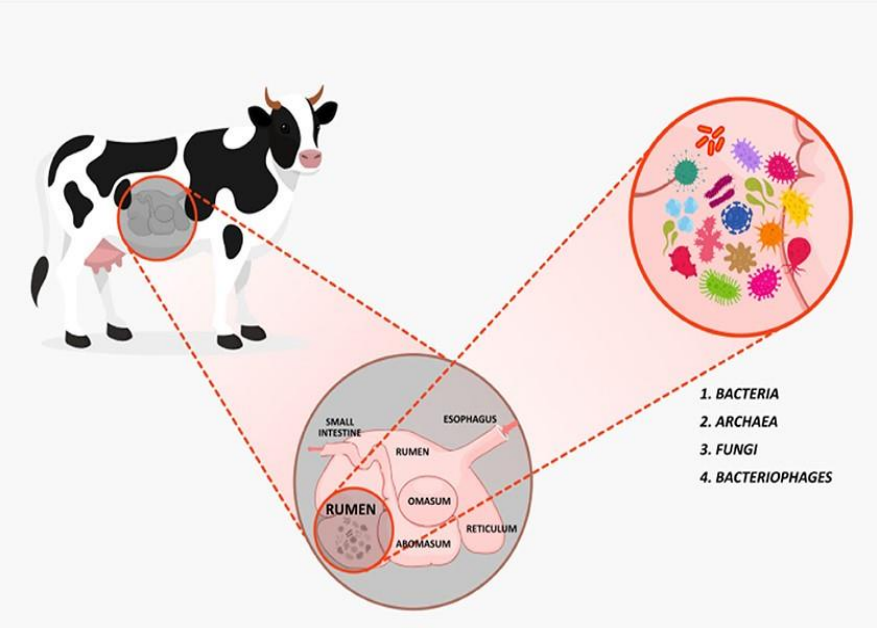


Figure1. Ruminants have 4 compartments in their stomach namely rumen, reticulum, omasum, and abomasum, the rumen is the primary site of digestion and absorption of nutrients and the papillae in the rumen allow increased absorptive capacity. https://www.frontiersin.org/articles/10.3389/fnut.2021.701511/full

Rumen Microbiome

Ruminants ingest feed and masticate it for the microbes and they also provide an optimal environment for microbial growth and in turn the microbes provide energy and nutrients to the rumen.(Highfill & Lalman, 2000). The ruminant is also responsible for removal of the fermentation products that inhibit microbial activity and replication, while the microbes take responsibility for providing the primary energy source for ruminants for instance, they ferment carbohydrates to produce volatile fatty acids (VFAs) (Aschenbach et al., 2011). VFAs influence the milk composition, as they go through the bloodstream and liver, where they are converted to energy source for milk production. Some VFAs result in high milk fat content, others in higher lactose content().

Rumen microbes can utilize non protein nitrogen to make their own proteins and when they die and end up in the small intestines their protein serves to provide almost 75% of the protein required by the ruminant (). Rumens have an impressive microbiome which can be optimized by adding feed additives to optimize target specific microbial groups like adding phytochemicals, probiotics,

fibriolytic enzymes, plant secondary metabolites, methane inhibitors, lipids, essential oils, and algae. (Króliczewska, B.,et.al., 2023) Probiotics are healthy bacteria that stimulate microbiota, they help stimulate an already existing population.

Anaerobic Fungi

History

In the 1900s it was believed that all fungi respired oxygen; hence when found in the anaerobic rumen environment, the *Neocallimastigomycota* were initially identified as flagellated protozoa but in 1975 Collin Orpin figured out these were anaerobic fungal zoospores. So far it has been approximately 5 decades of studying anaerobic fungi and we have only barely scratched the surface of these unique eukaryotes meaning their life cycle, cellular physiology, genetics, and cellulolytic metabolism are not widely understood (Hatijema. C.H et al 2014). Advances in omics research have led to a comprehensive resource for fungal genomics which are available on Mycosom portal (https://mycocosm.jgi.doe.gov/).Early taxonomists focused on morphology, life cycle and enzymatic capabilities (Bauchop, 1979; K.Theodorou, Brookman, & Trinci, 2005). Since genomic technologies were limited researchers focused on culture-based techniques instead . Methods of isolation and extraction of anaerobic fungi were limited to groups who had access to surgically modified, fistulated or recently deceased animals and that limited the study of anaerobic fungi compared to recent studies where they can be isolated from digestive tracts or fresh air-dried feces. (Orpin C, 1975:1976:1977:1988: Davies et al 1993). In this study we use samples collected from the ruminants by canulating directly into the rumen.

Morphology and life cycle

The morphology and lifecycle of AF differentiate them from fellow members of the fungal kingdom as they are conducive to the anaerobic environment. In terms of body structure, they do not possess a true cell wall and they are composed of a multinucleate monocentric thallus(Akin & Borneman, 1990; Griffith et al., 2010). They move via their multi flagellated zoospores which also couple as the reproductive and dispersal machinery. The zoospores are contained in the sporangia where they are formed via mitotic division (Gruninger et al., 2014). Upon maturity the sporangia rapture and release the zoospores into the rumen where the invasive growth of anaerobic fungi

physically disrupts plant structural barriers (Akin & Borneman, 1990). The zoospores swim to acceptable sites of colonization that is the rigid lignocellulosic plant cell walls and attach to the substrate where they encyst and develop a rhizomycellial system that penetrates the plant cell wall and in turn releases CAZymes gainst structural carbohydrates. The formation of the rhizomycellial system into the plant cell wall makes it difficult to extract anaerobic fungi for proteomic analysis. (Akin. D.E and Borneman.W. S ,1990).

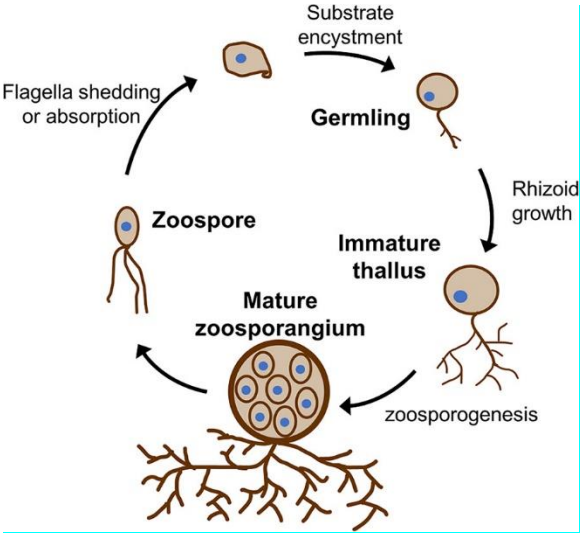


Figure 2. Morphology and lifecycle of the ARF showing the process of germling to formation and release of zoospores highlighting rhizoidal growth and zoospore genesis.

Synergy

Anaerobic fungi have a symbiotic relationship with methanogens where they produce the required hydrogen for the growth of methanogens, (Matthews, C., et al. 2018). Instead of using mitochondria, anaerobic fungi can couple the metabolism of glucose to cellular energy using hydrosomes which do not require oxygen (K.Theodorou, Brookman, & Trinci, 2005). These microbes make up 20 % of the rumen microbiomes in biomass and they increase the rumen fibrous intake by increasing feed digestibility by 7-9 % as they release a wide array of CAZymes (Król et al., 2022). These enzymes are complemented by exoglucanases and proteases from bacteria for efficient degradation of the plant cell wall. (Williams et al., 2020). The rumen bacterial community constitutes a greater part of the rumen ecosystem. Rumen Bacteria aids in fiber degradation to produce acetate ethanol carbon dioxide and hydrogen among other products(Li & Hou, 2007). Methanogens combine hydrogen and carbon dioxide produced to form methane this process helps

in prevention of gaseous buildup in the rumen. However, methane is a toxic greenhouse gas which is also responsible for energy loss in the ruminant(Alvarez et al., 2022). Manipulation of feed with methanogen inhibitors like AT, has been substantial for mitigation of rumen methane production(Eikanger, 2021). Compared to other rumen microbes, protozoa are the largest in size ranging from 20 to 200 micrometers. They help in maintaining the pH of the rumen by engulfing and storing whole starch particles as a way of slowing down immediate fermentation bacteria(Andersen et al., 2023; Matthews et al., 2018).

Proteins and proteomics

Proteins possess a wide range of functions, ranging from transport, catalysts, structure, mobility and communication (LaPelusa & Kaushik, 2022). Liquid rumen samples contain many proteins from different organisms, and they cannot be analyzed simultaneously on MS, hence the need for a separation technique. This separation is paramount to reduce sample complexity and allow identification and quantification of as many protein molecules as possible (Sidoli.S 2016). The 20 amino acids making up proteins have three main ways they associate with water, they can either be polar, nonpolar, or ionic. Molecules like Aspartic acid or Glutamic acid are basic while Lysin and Arginine are acidic hence they have different interactions with water compared amino acids that are hydrophobic which favor other hydrophobic molecules within the protein over water (LaPelusa & Kaushik, 2022).Given this information it is important to note that many proteins are poorly soluble hence they do not stay in solution and can easily precipitate out of the liquid hence cannot be analyzed because most proteins will associate to stabilize their structure then aggregate and precipitate out of solution(LaPelusa & Kaushik, 2022; Novák & cek, 2016) . This process is responsible for the formation of the pellet after centrifugation in proteomics protocols and detergents are then needed to aid protein extraction without compromising the downstream process (Goldman et al., 2019). These detergents are water soluble as they have one end that can be ionized to interact with water and a hydrophobic end that can interact with the hydrophobic ends of the protein, and they work by reducing the surface tension between the proteins and the water (). SDS is the most common surfactant, and we use it in this project because of its strong ionic group which helps solubilize most proteins including membrane proteins(Novák & cek, 2016). To extract, purify and study proteins within a cell, the cell needs to be lysed or broken for the proteins to be

released. There are several cell lysis methods, and they can be categorized into chemical, mechanical and biological. (Novak and Havlicek, 2013(Tan & Yiap, 2009)).

Proteomics

In genomics we have a linear stretch of information essentially four building blocks that we can easily amplify, where as in proteomics we have twenty basic amino acids and a countless number of posttranslational modifications leading to millions of different proteoforms (Sidoli, S 2016), and no way of amplification . One of the main differences in sample prep between a proteome and DNA is that for proteomics there is no one size fits all hence the need to optimize and adjust protocols for different samples (Deracinois et al., 2013). Genomic studies are important when understanding and studying the structure, function, location, and regulation of genes in an environment and the genes in the cell might not accurately portray conditions in the cell due to regulation at the RNA and protein level that cannot be viewed in genomic studies (Deracinois et al., 2013; Xie et al., 2022). Compared to genomics and transcriptomics, proteomics provides additional understanding of the structure and function of an organism and since protein expression is altered according to time and environmental condition, it can become more difficult and complicated than genomics (Safa Al-Amrani et.al, 2021). In this respect proteomics is more beneficial because proteins are functional molecules in cells, and they represent actual conditions. The proteome is dynamic and varies, the set of proteins produced in different tissues varies according to the gene expression while on the other hand the genome is constant, and every cell of an organism essentially has the same set of genes (Aryal.S, 2022).Proteomics is a multistep technique where every step should be carefully controlled to avoid non biological factors interfering with protein expression and interaction. In the traditional bottom-up proteomics intact proteins are digested into peptides before going into the mass spectrometry where they are detected and fragmented (Paulo et al., 2012). The proteins are extracted and digested by a sequence-specific enzyme such as trypsin, which cleaves after the Lys/Arg leaving the C-terminal +charged at low pH meaning the peptides can be analyzed from that end too, this ionization is favored in mass spectrometry (MS) In mass spectrometry, proteins are separated by their charge and mass where the amount of deflection is inversely proportional to the mass over charge ratio and that allows us to identify the different particles. These deflections are plotted on a graph with atomic mass and

relative abundance, giving an abundance calculation which helps us know what type of proteins are present and in what amounts (Aebersold. M 2016)

Metaproteomics

Metaproteomics can be used for characterization of microbial communities and host-associated microbiomes on different levels as it provides deep insights into the biodiversity of microbial communities and the complex functional interplay between microbes and their hosts or environment (Salvato, et.al., 2021). Research and studies involving metaproteomics have brought light to multiple functions and analysis of AF to the microbial community, for instance the synergistic nature between anaerobic bacteria and anaerobic fungi is highlighted through metaproteomic studies of their complementary action in breaking down biomass using CAZymes .Metaproteomics shows that many of these CAZymes are expressed and thus active in the rumen environment hence their significant potential for biomass degradation (Peng, X et.al., 2021). Hagen et.,al in 2020 conducted a genome centric metaproteome study to examine the functional role of AF using data bases from rumen fungal isolates, genomes and metagenome assembled genomes of cultured and uncultured rumen bacteria. Their results also confirmed that AF contribute to CAZymes that complement the anaerobic bacteria that degrade plant cell walls in the rumen. Hence why metaproteomics is paramount for this research analysis.

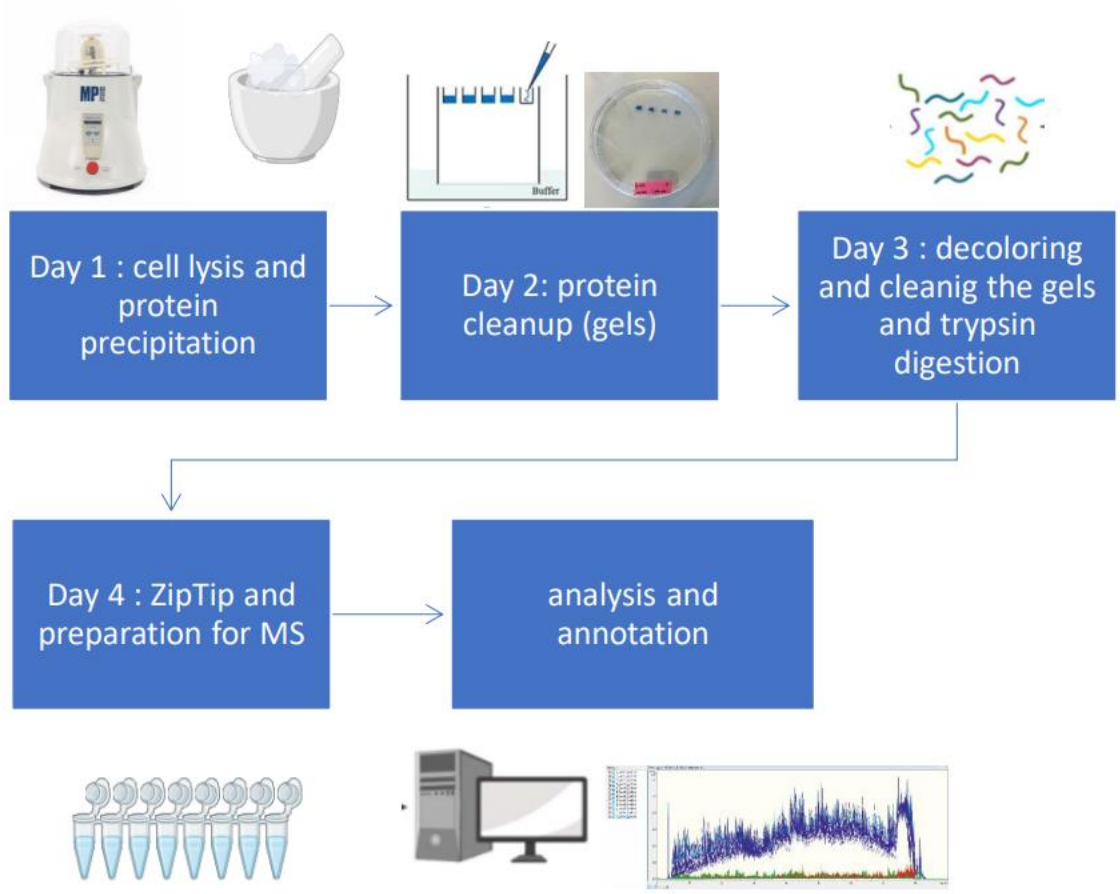


Figure 3 . Proteomics workflow from sample collection to analysis of peptide masses.

Cell lysis methods

As can be seen in Figure 3, proteomics sample preparation starts with cell lysis and protein extraction as it paves way for proteomics research and different techniques have been structured to effectively lyse different cells and produce the most accurate and pure yield (Novák & cek, 2016). The two major categories of cell lysis are reagent based and physical disruption but, in most protocols, they work in combination for efficiency. Physical disruption usually has the potential to produce excess heat which can be countered by chilling the samples on ice in-between rounds as was done during bead beating in this protocol. Detergent based lysis is also used in the form of lysis buffers and salts but if the cell wall is tough then physical disruption is required as well. Each protocol differs from the next and the cell lysis method can be tweaked to accommodate the samples for maximum yield. Bead beating has a record of high efficiency and reproducibility in proteomics hence it is usually the default method in proteomics protocols. The use of the mortar

and pestle in combination with liquid nitrogen is also quite popular and both methods require the aid of lysis buffers for maximum efficiency.

Materials

Laboratory equipment

Laboratory equipment used for the experimental part of this thesis are listed with their respective supplier and catalog number.

PRODUCT	SUPPLIER	CATALOG NUMBER
Blue caps for FastPrep® Tubes	MP Biochemicals, Ohio USA	5065-005
Eppendorf Safe-Lock Tubes (PCR clean) Eppendorf	Hamburg, Germany	0030120094
Eppendorf® epT.I.P.S volume range 0.1-10 µL	Sigma-Aldrich, Missouri, USA	Z640387
Eppendorf® epT.I.P.S volume range 2-200 µL	Sigma-Aldrich, Missouri, USA	Z640336
Eppendorf® epT.I.P.S volume range 50-1000 µL	Sigma-Aldrich, Missouri, USA	Z640433
FastPrep-24™ Classic Grinder	MP Biochemicals, Ohio, USA	SKU116004500
FastPrep® Tubes	MP Biochemicals, Ohio, USA	5076-200
Glass beads, acid washed,	Sigma-Aldrich, Missouri,	G4649-500G

≤ 106 µm, 500g	USA	
Mini-PROTEAN® Tetra Cell	Bio-Rad, California, USA	
Mini-PROTEAN® TGX Stain-Free™ Gels (Any kD, 10 well comb, 30 µl)	Bio-Rad, California, USA	4561023
PowerPac™ Basic Power Supply	Bio-Rad, California, USA	
ThermoMixer® Eppendorf,	Hamburg, Germany	
ZipTips® Pipette Tips Merck-Millipore,	Massachusetts, USA	Z720070
ddH2O, Milli-Q® Reference Water Purification System (0,22 µm filter)	Merch-Millipore, Massachusetts, USA	C79625
Eppendorf® Centrifuge 5418R (4°C) Sigma-Aldrich	Missouri, USA	EP5401000137
Freezer (-20°C)	Bosch, Stuttgart, Germany	
Freezer (-80°C), Innova® C585 Chest Freezer,	MG Scientific, New Brunswick Wisconsin, USA	
MS2 Minishaker IKA® Vortex	Fischer Scientific, New Hampshire, USA	12819435
Nitrile gloves	VWR, Pennsylvania, USA	

Quintix® Weight Santorius,	Göttingen, Germany
Refrigerator (4°C) Bosch,	Stuttgart, Germany
Stainless steel surgical blade	Swann-Morton Limited, Sheffield, UK

Chemicals

Chemicals, premade buffers and reagents, and kits are listed with their respective supplier and catalog number.

CHEMICAL	SUPPLIER	CATALOG NUMBER
2-propanol, 2 L	Honeywell, North Carolina, USA	278475
Acetic acid, 100%, 2,5 L	Merck-Millipore, Massachusetts, USA	1.00063.2500
Acetonitrile (I), CHROMASOLV™ LC-MS grade, 1 L	Honeywell, North Carolina, USA	34967-1L
Ammonium bicarbonate (AmBic), 500 g	Sigma-Aldrich, Missouri, USA	09830-500G
Coomassie Brilliant Blue R-250, 10 g	Bio-Rad, California, USA	161-0400
Dithiothreitol (DTT)	Sigma-Aldrich, Missouri, USA	D0632-25G

EMSUREÒ Hydrochloric acid, 32%, 2,5 L	Merck-Millipore, Massachusetts, USA	1.00319.2500
Ethanol absolute, 5L	VWR, Pennsylvania, USA	20821.365
Iodoacetamine (IAA), 5 g	Sigma-Aldrich, Missouri, USA	I1149
Sodium dodecyl sulfate (SDS), 1 kg	PanReac AppliChem ITW Reagents, Darmstadt, Germany	A2572, 1000
Trifluoroacetic acid (TFA), 100%, HiPerSolv CHROMOANORMÒ, LCMS grade, 1 L	VWR, Pennsylvania, USA	85049.001
TrizmaÒ base, 1 kg	Sigma-Aldrich, Missouri, USA	T1503 2.2.2
99.999% liquid nitrogen instrument grade 5,-196.15°C	Linde	

2.3 Manufactured reagents

REAGENT	SUPPLIER	CATALOG NUMBER

10xTris/Glycine/SDS Buffer (TGS), 5 L	Bio-Rad, California, USA	161-0772
Novex™ NuPAGE™ LDS Sample Buffer (4X), 250 ml	Thermo Fisher Scientific, Massachusetts, USA	NP0008
NuPAGE™ Sample Reducing Agent (10X), 10ml	Thermo Fisher Scientific, Massachusetts, USA	NP0009
TrypsinPorcine, Sequencinggrade modified 20 µg	Promega, Wisconsin, USA	V511A
Trypsin resuspension buffer, 1 ml	Promega, Wisconsin, USA	V542A

Buffers

Buffers used in the protocols are listed below

TRIS-HCl 1M 60 ml

• 7,266g TrizmaÒ base was weighed and dissolved in 20 ml Milli-Q.

• pH was adjusted with 1 M HCl until pH = 8.

• Milli-Q was added to reach total volume (60 ml)

LYSIS BUFFER (3X)

1x	3x

10 mM Dithiothreitol (DTT)	30 mM Dithiothreitol (DTT)
50 mM Tris-HCl (pH=8)	150 mM Tris-HCl (pH=8)
0.1 % Triton X-100	0.3% Triton X-100
4 % SDS	12% SDS

STAIN SOLUTION

25% Isopropanol
10% Acetic acid
0.05% Coomassie Brilliant Blue R-250

DESTAIN SOLUTION

25% Isopropanol
10% Acetic acid

DTT SOLUTION

10 µl 1M Dithiothreitol (DTT)
100 µl 1 Ammonium bicarbonate (AmBic)
890 µl Milli-Q

IAA SOLUTION

10 mg Iodoacetamide (IAA)
100 µl 1M Ammonium bicarbonate (AmBic)
900 µl Milli-Q

TRYPSIN BUFFER

25 µl 1M Ammonium bicarbonate (AmBic)

100µl 100% CAN

875 µl Milli-Q

TRYPSIN SOLUTION

5 µl 500ng/µl Trypsin (frozen at -80°C)

245 µl Trypsin buffer

HPLC Solvents:

• Solvent A: 0.1% formic acid (v/v) (in water

Software tools

- Fragpipe version 19.0
- Perseus version 1.6.15.0
- dbCAN

Methodology

Protocols are constantly optimized to achieve the necessary results for example in this case the original protocol was made for prokaryote extraction, but 3 rounds of pilot experiments were performed to optimize it for anaerobic fungi extraction. The aim was to find out if a freeze-grinding pretreatment method was more efficient and produced a greater yield in anaerobic fungi extraction than the original bead beating strategy. Sample size, protein yield and reproducibility of the methods were fundamental key factors that were considered during evaluation of the efficiency of the protocol.

Sample design

For my thesis project, I received samples collected from an animal feeding experiment conducted as a part of a research project named SeaCow (Funded by the Norwegian Research Council, project number: 302639) These samples originated from six cannulated cows, each providing samples at 2 different time points in respect to feeding. The first samples were collected at 09.00 which was 2 hours after feeding and the second round at 13.00 which was 6 hours after feeding. The cows also belonged to 2 feeding groups; one group given a normal diet (control group) and one fed a diet supplemented with 0.25% Asparagopsis taxiformis on organic matter basis (high AT group). 3 cows were assigned to each feeding group. Since 2 methods are being tested, 24 samples were used with 12 for each method for consistency, compatibility, and reproducibility. The rumen samples were preserved at -80°C before extraction and thawed on ice for 4 hours prior to running the protocol.

Experiment Code	Sample ID	COW ID	Feeding Timepoint	Feeding group
2h after feeding				
D-209	**2021-0641**	6619	09:00h	CONT
D-209	**2021-0643**	6375	09:00h	CONT
D-209	**2021-0645**	6682	09:00h	CONT
D-209	**2021-0652**	6405	09:00h	HIGH AT
D-209	**2021-0654**	6650	09:00h	HIGH AT
D-209	**2021-0655**	6640	09:00h	HIGH AT
6h after feeding				
D-209	**2021-0656**	6619	13:00h	CONT
D-209	**2021-0658**	6375	13:00h	CONT
D-209	**2021-0659**	6682	13:00h	CONT
D-209	**2021-0662**	6405	13:00h	HIGH AT
D-209	**2021-0663**	6650	13:00h	HIGH AT
D-209	**2021-0664**	6640	13:00h	HIGH AT

Figure 4. overview of the sample design.

Protocol optimization.

The rumen samples from the same animal feeding groups were used for troubleshooting, and a comparison between tubing and canulated samples was first suggested. The samples were thawed and aliquoted into the respective required volumes for FastPrepThe first sample (Sample ID: 0243) from a cow given control diet was collected by tubing. The second sample (Sample ID: 0678) was collected by canulation from a cow that had been fed seaweed (AT). The initial test run was done with 4 samples (2 from Sample ID: 0243 and 2 from Sample ID: 0678) and 1mL of sample was used per run with 500µl of lysis buffer. The next day the samples were centrifuged twice at maximum speed 21.000 xg for 15 minutes and the supernatant was removed at each round. 200µL of ice cold 90%ACN/0.01MHCL was added directly to the supernatant since the TCA in the original protocol had been skipped. The samples were further centrifuged at 21.000 xg for 10 minutes and the supernatant was decanted leaving the brownish gluey pellet at the bottom. The pellet was dried and sonicated and checked for protein concentration on nanodrop. The samples showed a high concentration of both protein and contaminants since the cleanup steps had been

skipped, particularly the gels and the reduction and alkylation. The next rounds of trouble shooting were done with the same main samples that is 0243 tubing unfed and 0678 cannulated fed cows. This time 2 aliquots were used from each sample that is, 250µL and 500µL with 125µL and 250µL of lysis buffer respectively to figure out which one would yield the right amount of protein without overload. There was still protein overload with both 250µL and 500µL shown by the gels and the nanodrop, so the sample size was lowered to 200µL with 100µL lysis buffer .It was established that 200µL was the optimal sample size so the full protocol was tested with adjustments favoring 200µL of sample. After adding the ice cold 90%ACN/0.01MHCL the samples were spun down at 15.000 xg at 4 °C for 10 minutes instead of 15 minutes because the pellet from 15 minutes was difficult to dissolve and required extra rounds of heating and loading buffer .The gel injections were adjusted from 20µL to 15µL and finally 10µL to avoid overload and carry over and a well was to be skipped after each load for the same reasons. During trypsin digestion a significant amount of trypsin buffer was added to cover the gel pieces , an extra 20µL of trypsin buffer for bead beating samples and extra 50µL for freeze grinding samples. For the final analysis only canulate samples were used as they contained more biomass material.

The freeze grinding was tested twice before the final run as it was a pre-step and the rest of the protocol was similar to the bead-beating. Liquid nitrogen was poured directly onto the sample in the mortar and the pestle was used to grind the sample into a fine powder. The first attempt required 3 rounds of liquid nitrogen and grinding before the satisfactory powder form was achieved. Due to the excess liquid in the sample the ground particles formed chunks which were difficult to grind to a fine powder and once the powder was formed it immediately turned into a pulp-like paste. Adjustments were made which included squeezing out as much liquid as possible from the samples while in the falcon tube before transferring the sample to the mortar, the squeezing was done by gently pressing the sample against the walls of the falcon tube with a prechilled spatula, this effectively reduced the grinding rounds from 3 to 2 and the powder was stable enough to allow for weighing and preparation for FastPrep. 200mg of fine powder was measured into FastPrep tubes containing 1.5-2mm of glass beads along with 100µL of lysis buffer. The optimized bead beating protocol was followed and protein recovery was lower than expected, hence 150mg of ground sample was used and 50mg of liquid sample was added to compensate for the liquid squeezed out and to reach 200mg before following the bead beating protocol. After trouble shooting I continued with only samples that were collected via canula.

Protocol after optimization

The first set of samples, in this case bead beating (BB) were subjected directly to steps explained in Section () while the second set of samples pretreated with freeze grinding (FG) in liquid nitrogen prior to following the same beadbeating protocol as the first samples.

Freeze-grinding pretreatment

In nucleic acid analysis containing plant material, a common step is to grind the samples after freezing with liquid nitrogen(Tan & Yiap, 2009). This is paramount to ensure effective cell wall breakdown without nucleic acid disruption (Tan and Yiap,2009). Freeze-grinding was used here as a pre-step for breaking through the plant fibers and releasing the anaerobic fungi. Aliquots from the bead beating samples were used, hence 12 samples were analyzed in batches of 4 since freeze grinding is time consuming and the waiting time between samples will allow for activation of enzymes and disruption of protein integrity. 4 samples were taken at a time from -80°C and thawed on ice for 4 hours, prior to freeze grinding and once thawed the samples were mixed by vortexing. Fastprep tubes were labeled, prepared with 1.5-2mm of glass beads and prechilled on ice. The mortar and pestle were thoroughly washed with zalo liquid soap and sterilized with 70% ethanol between samples. The plant material was gently squeezed with a prechilled spatula and transferred to the mortar where liquid nitrogen (99.999% liquid nitrogen instrument grade 5,-196.15°C) was carefully poured into the mortar to cover the samples, chill the mortar and allow manual grinding. The pestle was used to grind the samples by firmly and carefully pressing and twisting in circular motion with downward pressure for the first round. The fragmented pieces were scraped to the center of the mortar and a second round of liquid nitrogen was added, followed by light pressing and twisting while maintaining the circular motion to achieve a fine powder. The 150mg powder was measured into tared prechilled FasPrep tubes and topped up with 50mg of vortexed liquid sample from the falcon tubes to ensure homogenization and avoid sample bias. 100µL of lysis buffer () was added to the FastPrep tubes and vortexed to mix it with the samples which were chilled on ice and followed the optimized bead beating protocol section.

Bead beating method,

Day 1: cell lysis and protein precipitation

Samples from -80 were defrosted on ice to avoid degradation, keep cell activity, and produce more sensitive and consistent results. They were then vortexed to mix them and 200 µL was pipetted into FastPrep® tubes containing 1.5-2 cm of glass beads (≤ 106 µm).In preparation for FastPrep, 100µl of lysis buffer was added to the samples and they were vortexed and placed on ice for 30 minutes to facilitate cell lysis and protein solubilization. Lysis buffer contains SDS, which is a detergent that denatures proteins, ensures complete solubilization, inactivates cellular proteases and aids cell lysis (Scheerlinck, E. et.al,2015). Based on bead beating technology, FastPrep® employs complete and quantitative cell lysis of samples through multidirectional, simultaneous beating of the lysing matrix beads (Jeffy d. whyte 2017). The FastPrep-24™ Classic Grinder with the setting at 6.5m/s for 60 seconds repeated three times was used with 5-minute breaks in between to give the machine a rest from the vigorous shaking. Samples heat up during bead beating hence they need to rest on ice between machine recovery times. Samples were then centrifuged at 21.000 xg for 15 minutes to separate the cell debris and proteins. The cell debris forms a pellet while proteins remain in the supernatant. The supernatant was transferred to the new tubes and centrifuged twice again at maximum speed for 15 minutes to make sure the samples were as clean as possible. 10 % ice cold TCA (Trichloroacetic acid) was added, and the samples were left at 4°C overnight, it is diluted from 80 % to 10% to minimize protein denaturation. TCA disrupts the hydrogen-bonded water molecules (hydration sphere) surrounding a protein causing protein molecules to lose solubility and thus can be recovered during centrifugation and to remove interfering substances such as salts, detergents, and nucleic acids. (Novák, P., & Havlíček, V. 2013, Koontz, L. (2014).).

Day 2: protein clean up (gels)

The gel is run for protein clean-up and as an indication on how much protein was recovered.

Samples were centrifuged for 10 minutes at 15.000 x g at 4 °C to spin down the precipitated proteins which form as a pellet at the bottom. The supernatant was removed and the pellet was air dried for 5 minutes, then washed by adding 300µl of ice cold 0.01M HCl/90% acetone to remove extra contaminants or residual TCA (Di Sanzo,et.al., 2021;). The pellets were dissolved in 30µl of loading gel buffer.LDS (lithium dodecyl sulfate) is comparable to SDS mentioned above, and paramount for protein denaturation as it allows the proteins to unfold into negatively charged polypeptide chains compatible with the gels (D'Silva et.al.,2017) and DTT (dithiothreitol) reduces

the disulphide bonds in proteins and fortifies the uniformity of the negative charge throughout the polypeptide chain making it conducive for gel electrophoresis (Santarino et.al., 2012.).The samples were heated for 10min at 95°C on the thermoblock ,cooled and prepared for running on the gels.(Mini-PROTEAN® TGX Stain-Free™ Gel 30 µL with 10 wells).In the Mini-PROTEAN® Tetra Cell system and the electrode gasket was filled with fresh 1 x TGS buffer(Tris/Glycine/SDS Buffer, Bio-Rad) and the rest of tank was filled with used 1 x TGS buffer up to the 2 gel mark.10µl of sample was loaded into each well skipping a well in between to avoid sample contamination in case of bleeding over. The gel was run for 3 minutes at 270 V with the PowerPac™ Basic Power Supply, to achieve at least a 2cm migration into the gels which is enough for visualization and is efficient enough for MS analysis (Paulo,2016). The gels were carefully transferred to gel staining box and stained with Coomassie Brilliant Blue R-250 solution for 1 hour with slow shaking (30 times/ minute) on the IKA® HS 260 Basic shaker. After cautiously removing the stain solution, the destain solution was added and the gel was destained for 1 hour with slow shaking, the destaining was repeated once more. Then after removing the destain solution, the gel was left overnight in a 50/50 Milli-Q/destain solution with slow shaking.

Day 3: decoloring and cleaning the gels.

Once the gel was destained well and the bands were clean and visible, the 50/50 Milli-Q/destain solution was removed and replaced with Milli-Q water. To increase surface area for trypsin digestion the gels were cut carefully into 1x1mm pieces with a clean sterile scalpel and transferred into marked Eppendorf Safe-Lock tubes. The scalpel was washed in 70% ethanol between incisions to avoid cross contamination between the gels. The gels were then incubated in 200µL Milli-Q at 22°C with 800 rpm shaking on ThermoMixer® for 15 minutes followed by two consecutive incubations of 200µl 50% ACN/25mM AmBic under the same conditions. Liquids were expelled after each incubation. The Milli-Q water is crucial to soften the gel and rehydrate it for downstream processing while the 50% ACN/25mM AmBic solution is requisite for disrupting the gel structure while maintaining slightly alkaline pH which preserves protein stability and avoids deamination during stain removal (Goldman et.al,2019; Paulo,2016). After removing the liquids 100µl of 100% ACN was added, and the samples were incubated for 5 minutes at 22°C on the ThermoMixer® with 800 rpm shaking. The ThermoMixer® was set to 56°C in preparation for reduction and alkylation. ACN is a strong solvent responsible for expelling staining solutions from

gel, removing remaining salts, buffers and other contaminants (Lazarev.et.al., 2009; Paulo,2016). ACN also aids in drying the gels hence the gels were left to air dry for 3 minutes (until they are white and shrunken).

Reduction and alkylation

Dithiothreitol (DTT) is a reducing agent that helps breakdown proteins by dislodging their disulphide bonds to form polypeptide chains and restores all cysteine residues (Alliegro,2000; Borges and Sherma,2014), hence 50µl of DTT solution was added to each sample for reduction and incubated on ThermoMixer® at 56°C for 30 minutes with 800 rpm shaking. The samples were cooled down and the DTT was pipetted out and replaced with 50µl of IAA solution followed by a 30-minute incubation in the dark at room temperature for efficient alkylation. Iodoacetamide (IAA) is an alkylating agent that blocks cystine activity and averts reformation of disulphide bonds by forming covalent bonds with the cystine residues (Sigma 2001;Borges and Sherma,2014). After removing IAA, 200µl of 100% ACN was added and incubated on ThermoMixer® at room temperature 22 °C with 800 rpm shaking for 5minutes and the ACN was removed for the samples to air dry for 3 minutes (until they are white and shrunken) in preparation for trypsin digestion.

Digestion

Once the samples were dry, 30µl of 10ng/µl of trypsin solution and incubated for 30 minutes on ice, then more trypsin buffer was added to cover gel pieces to ensure complete peptide digestion, in this case an extra 20µL of trypsin buffer for bead beating samples and extra 50µL for freeze grinding samples. The samples were incubated overnight at 37°C on ThermoMixer® with 800 rpm shaking. Trypsin is a highly specific protease fundamental for in-gel digestions as it promotes doubly charged peptides and has a high protein yield for LC-MS/MS analysis. (Goldman,et.al, 2019) . This enzyme operates by cleaving at arginine and lysine amino acid residues at the C-terminal (Paulo,2016; Vandermarliere, Mueller, & Martens, 2013).

Day 4: ZipTip and preparation for MS

The samples were removed from the ThermoMixer® and were allowed to cool down for 15 minutes followed by addition of 40µl 1 % TFA to cease the trypsin action (Paulo,2016). This was followed by a 15-minute sonication on Branson 3510 Ultrasonic Cleaner to initiate peptide release from the gels (Cordeiro, et.al., 2007). After the peptides were released, the samples were prepared

for MS on Tims-TOF by ZipTip® using the pipetting method with C-18 material to desalt, purify and concentrate the peptides (Nika, et.al., 2013). Proteomics Eppendorf tubes with 10µl of 70% ACN/0.1 % TFA were prepared and labeled, one for each sample in preparation of drying in the speedvac . To execute effective conditioning and equilibration, 4 Eppendorf tubes were prepared,1 with MeOH, 1 with 70% ACN/0.1 % TFA and 1 with 0.1 % TFA and one for waste. The C-18 material was conditioned by pipetting up 10µl of 100% MeOH and discarding to the waste tube followed by pipetting up 10µl of 70% ACN/0.1 % TFA and discarding it to the waste tube, and the equilibration was done by pipetting up 10µl of 0.1 % TFA and discarding it to the waste tube. Samples binding and eluting was done by pipetting the prepared ZipTip C-18 material up/down in the sample 6 times to ensure that the peptides bind to the ZipTip C18 material. the tip was wiped with a clean tissue to avoid cross contamination of samples then it was washed by pipetting up 10µl of 0.1 % TFA and discarding to the waste tube, to remove any remaining buffers and salts. Peptide elution was executed by pipetting up/down in the 70% ACN/0.1 % TFA 6 times. A new ZipTip was used for each sample and conditioned accordingly. The samples were then prepared for MS by drying them on the speedvac for 28 minutes at 45°C with the Eppendorf tubes open. The peptides were then dissolved in 10µL of 0.1% FA and transferred to HPLC vials. Formic acid is the preferred HPLC solvent as it promotes peptide stability and increases ionization efficiency thereby making it compatible with the TimsTOF and in turn ameliorating the quality and solidity of peptide analysis. The samples were then measured for protein concentration on nanodrop with MilliQ water as the blank and 1.5µL of sample was loaded per measurement.

(section 3.2.1).

HPLC TimsTOF

The samples were analyzed at the NMBU proteomics facility headed by Senior Engineer Morten Skaugen, following the internal protocol adapted below.

•The peptide samples were then analyzed by coupling a nano UPLC (nano Elute, Bruker) to a trapped ion mobility spectrometry/quadrupole time of flight mass spectrometer (TimsTOF Pro, Bruker). The peptides were separated by an Aurora C18 reverse-phase (1.6 µm, 120Å) 25 cm X 75 µm analytical column with an integrated emitter (IonOpticks, Melbourne, Australia). The

temperature of the column was kept at 50°C using the integrated oven. Equilibration of the column was performed before the samples were loaded (equilibration pressure 800 bar). The flow rate was set to 300 nl/min and the samples were separated using a solvent gradient from 5 % to 25 % solvent B over 70 minutes, and to 37 % over 5 minutes. The solvent composition was then increased to 95 % solvent B over 5 min and maintained at that level for an additional 10 min. In total, a run time of 90 min was used for the separation of the peptides. Solvent A is 0.1 % (v/v) formic acid in MilliQ water, while solvent B is 0.1 % (v/v) formic acid in LCMS grade acetonitrile. The TimsTOF Pro was run in positive ion data dependent acquisition PASEF mode with the control software Compass Hystar version 6.0.30.0 (6.2.1.13) and timsControl version 3.1.13.0 (4.1.12.0). The acquisition mass range was set to 100 – 1700 m/z. The TIMS settings were: 1/K0 Start 0.85 V·s/cm2 and 1/K0 End 1.4 V·s/cm2, ramp time 100 ms, ramp rate 9.42 Hz, and duty cycle 100 %. The capillary voltage was set at 1400 V, dry gas at 3.0 l/min, and dry temp at 180 °C. The MS/MS settings were the following: number of PASEF ramps 10, total cycle time 0.53 sec, charge range 0-5, scheduling target intensity 20000, intensity threshold 2500, active exclusion release after 0.4 min, and CID collision energy ranging from 27-45 eV. The raw data were processed using Data Analysis 6.0.313 (6.1.119) and the Bruker processing method “Shotgun PASEF ProteinAnalysis2. 7comp.m”

Analysis software

Fragpipe (powered by MSFragger) was used as the initial software for through proteomic analysis (https://fragpipe.nesvilab.org/).

The data base consisted of 18 protozoa SAGs(Single-Amplified Genomes), an updated list of anaerobic fungi (12 genomes and 1 MAG) and >500 MAGs(Metagenome-Assembled Genomes) from the Seacow metagenome which showed a total of 2,441,974 protein entries (). Data dependent acquisition (DDA) was used since it selects and fragments only specific peptides based on their intensity and abundance, preferably one at a time and gives priority to the most abundant ions. DDA has a near peptide-specific MS2 spectra which is used here for database searches (https://fragpipe.nesvilab.org/). Prior to the Fragpipe analysis parameters were set including filtering contaminants and evaluation of reverse hits. The contaminants are from sample handling and usually keratin from nails while reverse hits are there to estimate the false discovery rate (FDR) meaning when you reverse the sequences there should not be any more matches. The data was then

transferred from Fragpipe to Perseus for further analysis.Perseus (Perseus (maxquant.net)) is commonly used for interpreting proteomics data for visualization and statistical relevance.The data was imported into Perseus v1_6_15_0 and technical filtering was done prior to analysis. The focus on annotations that were relevant for the data, which provided the different relative counts and intensities of the proteins, through normalization of the data and statistical analysis. The data was filtered for contaminants by filtering rows based on categorical values and removing those flagged as contaminating organisms. A log2 transformation of data was done to make the data normal distributed and facilitate statistical test comparisons. Categorical annotations were done hence dividing the data into two groups namely bead beating (BB) and freeze grinding (FG). Rare proteins were filtered out by taking ones that were detected in at least 6/12 samples. Histograms were inspected and checked for the validity of the data, followed imputation and two-sample tests using p value of 0.05. The Student's T-test Difference produced volcano plots which showed the relevant intensities for analysis and interpretation. CAZymes mapped from dbCAN were also annotated in Perseus and were matched according to their relevant fungal proteins of origin.

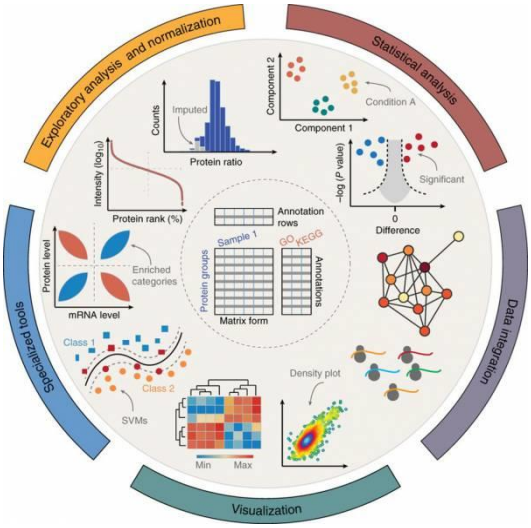


Figure 5. Overview of the Perseus workflow environment https://maxquant.net/perseus/.

Results and discussion

(Akin & Borneman, 1990)recognized ARF as paramount contenders in lignocellulose degradation in the rumen. Throughout the following decades, this led to more research on ARF (Kazda1, Langer, & Bengelsdorf, 2014; Lankiewicz et al., 2023; Ravinder Nagpal et al., 2009) even though up until present day they have not been fully explored, leading to the curiosity of this thesis on whether this could also be attributed to the oblivious nature of available fungal extraction methods. Recently the degradation prowess of ARF when it comes to recalcitrant plant fiber is credited to their rhizoidal nature and their ability to produce relevant CAZymes for effective disintegration of plant fibre into simple sugars (Hagen et al., 2020; Hartinger & Zebeli, 2021). Carbohydrate fermentation then convert these simple sugars into outputs like lactate and pyruvate which are later converted into volatile fatty acids (VFAs) (Ravinder Nagpal et al., 2009). The main focus of this thesis was on ARF extraction with the aim of maximizing their recovery for future further understanding of their significance in plant degradation in the rumen. The experimental focus was on comparison of two lysis methods the first one involving beadbeating (BB) in the presence of a detergent and the second one involving the same beadbeating method but with freeze grinding (FG) in liquid nitrogen as a prestep. Bead beating is the commonly used cell lysis method ()and is lenient to microbes like prokaryotes and protozoa, while freeze grinding is harsher and is capable of crushing tough cell walls like that of plants and fungi. By adding freeze grinding as a pre step, it is expected to provide better access to fungal proteins embedded in the plant fibres. For my research the rumen samples investigated were initially collected via two different methods, that is cannula and tubing. After the initial test run, I settled on only using the rumen samples collected via cannula since they contained more fibrous material. Considering the rhizoidal nature of anaerobic fungi I expected more fungi to be embedded in the plant material in these samples. I hypothesized that I would identify more fungal proteins with the freeze grinding (FG) method than just the beat beating (BB) method due to the ability of (FG) to lyse sturdy cell wall of the substrate (). Even though I was aiming for a method harsh enough for the tough cell walls I had to keep in mind that the method had to still be lenient on protozoa and prokaryotes.

In summary my results suggest that FG as the pretreatment method resulted in slightly higher numbers of detected protein groups originating from the ARF population while a few protozoa

and prokaryote proteins are lost (figure 7). This is also expected since the method is harsh, however the amount that lost is not substantial, suggesting that FG does not compromise with the recovery of prokaryotes and protozoal proteins. Despite only minor differences in the number of protein groups detected the protein intensities (log2- transformed LFQ values)(figure 10) , show that the pretreatment step significantly enriched the abundance of ARF proteins. This suggests that the functional role of ARF could have been previously underestimated specifically fungal families that have a more rhizoidal nature like Piromyces and *Neocallimastix* which were enriched in FG. Bulbous ARF like Caecomyces were generally not affected by FG since they do not form rhizoidal networks to embed in plant cell walls (). In addition to elevating the effect of the lysis strategy I further investigated if sampling time affected ARF proteins, here I found out that the protein abundances (LFQ values) were more enriched in the samples that were collected 6 hours after feeding compared to those collected 2 hours after feeding. This correlates with prior knowledge I had on the significance of dietary changes ( ) and the slow action of ARF. Finally, identification of relevant CAZymes related to filamentous rhizoidal fungal species demonstrated the paramount role of a RF in an effective rumen ecosystem.

Protein counts

The protein count was determined by counting the number of proteins present and trying to map out what our data might be telling us regarding whether BB needed a pre step to extract more fungal proteins. The general view showed that BB produced more proteins (figure 6a), which included fungal, protozoa and prokaryote protein groups. After doing the necessary filtering and categorizing I observed that BB had slightly less recovered fungal proteins than FG (figure 6b). This inspired me to look deeper into the genera of the anaerobic rumen fungi, where I saw that most of the fungal species had slightly higher protein counts in FG compared to BB. This could be due to the effectiveness of FG in breaking not only the fungal cell walls but also plant cell walls, extracting the embedded rhizoidal fungal proteins. The dominating fungal family by count is Neocallimastix (figure 8) with 5 genera (*Neocallimastix sp. GF-Ma3-1, Neocallimastix sp. WI3-B, Neocallimastix constans G3, Neocallimastix lanate, Neocallimastix californiae G1*) and a combined total of 100(BB) and 121(FG).The family *Neocallimastix* was the first to be characterized by Orpin in (), they have multiflagelated zoospores (4-20 flagella thalli) and they form non nucleated filamentous branching rhizoids that penetrate plant material and aid

degradation by CAZyme secretion (Griffith et al., 2010). *Neocallimastix sp. GF-Ma3-1* was the most popular of all fungal species found in the cattle samples in this research, this is in coherence with previous findings since *Neocallimastix sp. GF-Ma3-1* is commonly found in herbivorous ruminants like cattle and giraffes (mycosom).*Piromyces* followed the *Neocallimastix,* (figure 8) with a total count of 62(BB) and 78(FG).This family was represented by 3 genomes and 1 MAG in the database, with the Piromyces MAGs dominating. These monocentric fungi usually possess 1-4 flagella and thallus, and they also possess a filamentous rhizoidal system making them suitable for invasion of the plant cell wall and thus rapidly degrade plant fiber (Theodorou et al., 1996).*Caecomyces* (21(BB) and 22(FG)) and *Anaeromyces* (28(BB) and 28(FG)) have the highest protein counts amongst the families with 1 genus each,and both families have similar values for BB and FG suggesting that FG had limited to no effect as a prestep. *Caecomyces* form monoflagellated zoospores with 1-2 flagella thalli and , they lack a filamentous rhizoidal system but instead have a bulbous system which raptures the plant tissues (K.Theodorou, Brookman, & Trinci, 2005). Anaeromyces form multiple constrictions at regular intervals in its rhizomycellium (K.Theodorou, Brookman, & Trinci, 2005).

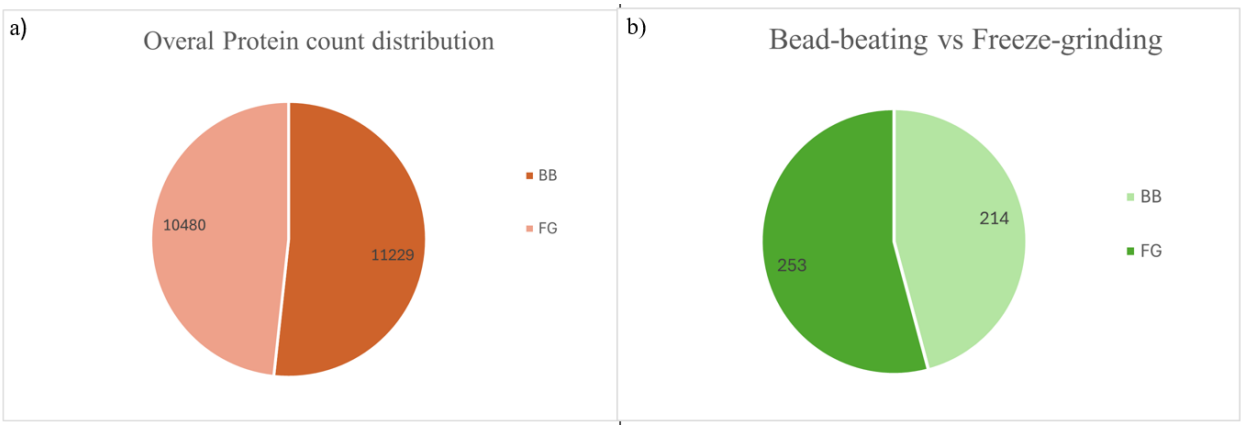


Figure 6 .Protein counts after filtering contaminants showed a slightly higher count in BB than FG.There is a difference of 749 proteins between the two methods which is quite low.Even though I identified approximately the same number of proteins for both methods it can be seen that the number of fungal proteins is slightly higher for FG (253 vs. 214), suggesting that the assumptions regarding the benefit of the FG-method for fungal proteins might be correct.

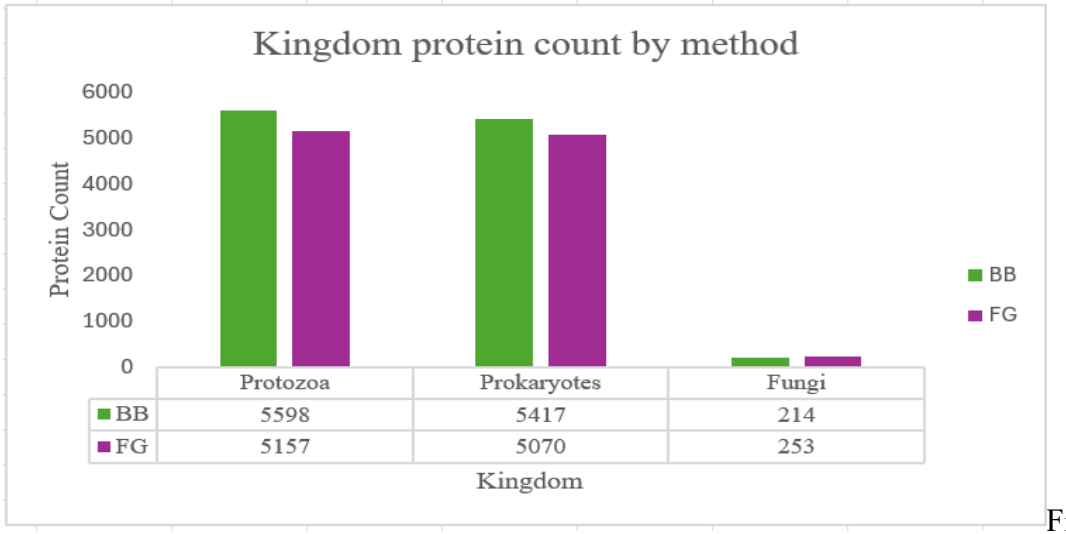


Figure 7. Overall protozoa and prokaryotes have a substaintially higher protein count compared to fungi. However, when considering the lysis methods, protozoa and prokaryotes have lower detection in FG than in BB, this could be attributed to the harsh nature of FG.

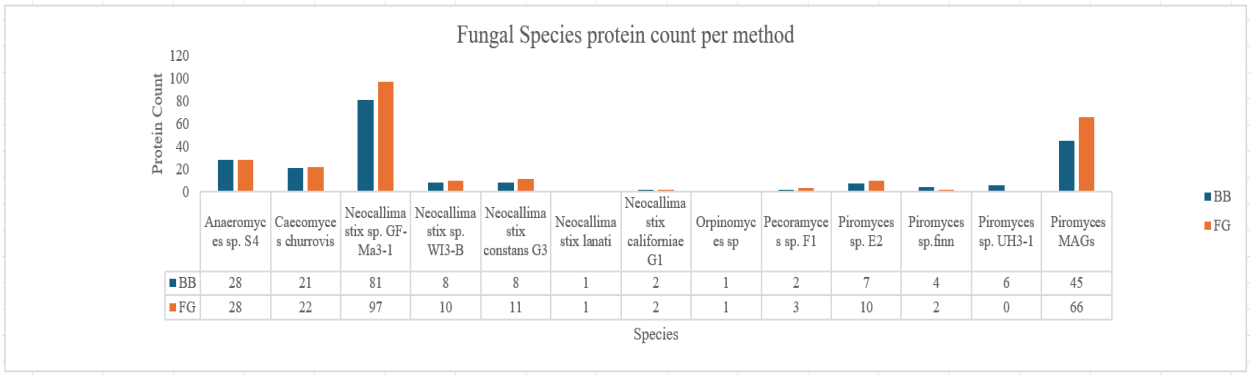


Figure 8. Fungal species show slightly higher protein counts in FG than BB.Some species are higher than others due to presence and absence of a rhizoidal system as in the case of neocallimastix family and caecomyces respectively.

Protein quantification

Quantitative analysis was done based on LFQ; label-free quantification intensities to evaluate differential expression of proteins across the three different categories within my dataset; Methods (BB vs. FG), Diet (control vs. AT) and Time (2h vs. 6h after feeding). Since the samples are from cows and there is a known correlation between the animals a less stringent filtering was used. Therefore, when filtering based on valid values, a minimum of 6 out of 12 samples in at least one

group was used. Values were imputed based on the normal distribution of LFQ scores and the Students test was used to evaluate statistical differences between the means within the groups. When imputing values, I drew randomly from a normal distribution downshifted 2.5 standard deviations from the original distribution of LFQ values.Intensity normalization is usually applied during the proteomics analysis and this visualization helps assessing the technical quality of the samples and whether they can be compared against each other (Fu et al., 2023). I observed that higher LFQ values were dictated more in the samples that had bigger protein pellets during the extraction process.Volcano plots were constructed based on the LFQ intensities in each category with focus on fungal protein intensities and the category of focus was methods. Diet and time were also plotted, although not significant they showed few proteins with statistical difference based on time and , the trend showed more intense fungal proteins in AT compered to control groups (figure 10).

Histograms of identified protein quantifications from Perseus

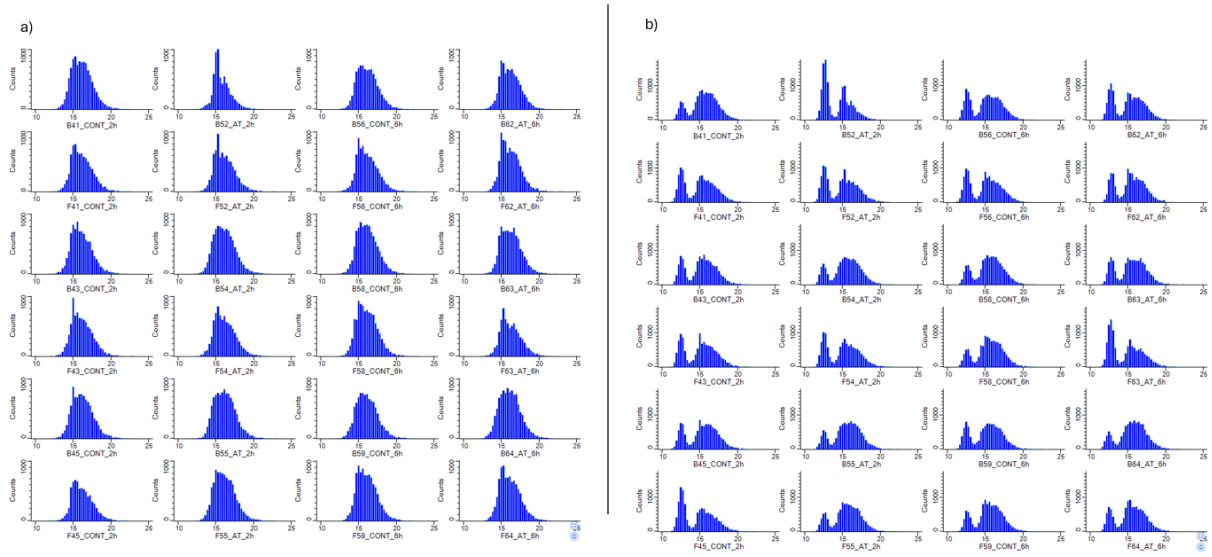


Figure 9 .Histograms were constructed to visualize the distribution of intensity-values (LFQ; label-free quantification in each sample and to identify any outliers among the samples. b) Samples after imputation show new histograms with a clear separation between imputed values (to the left) and true values (to the right).

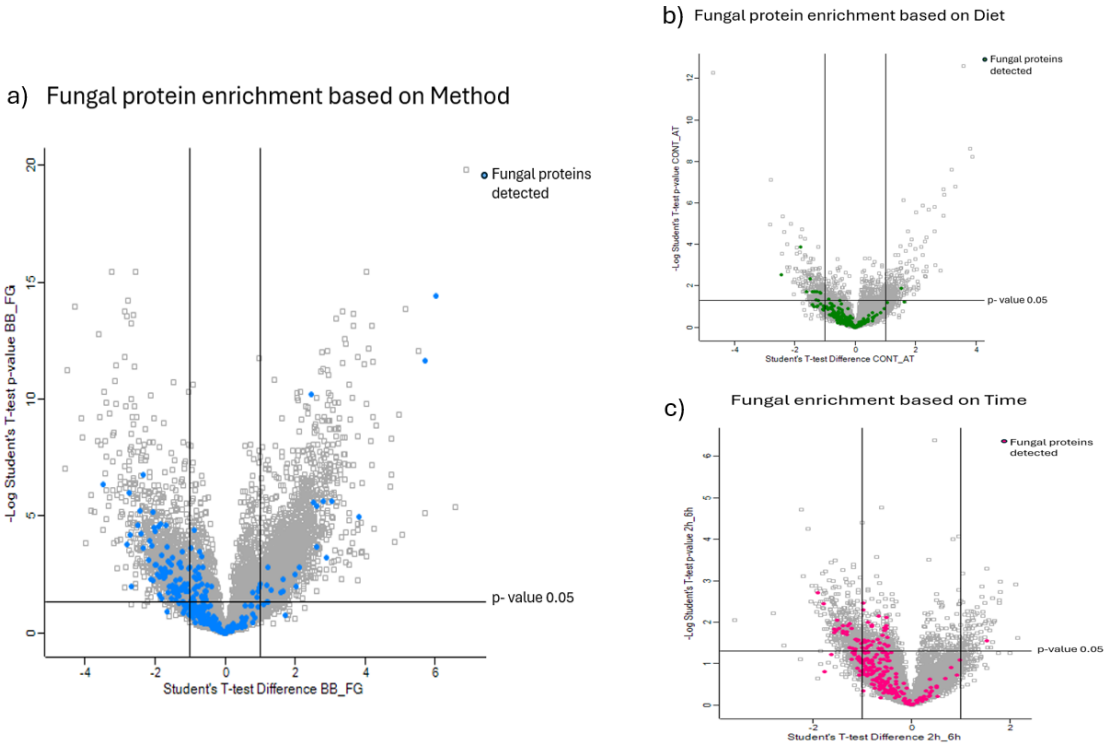


Figure 10 methods a) there is a clear difference between BB and FG , where fungal proteins (in blue) have a significantly higher expression in the samples from the FG method . Diet (green) b) though very few intensities are shown , there is still a higher expression of AT versus control. Time (pink) c) shows very few proteins with statistically different abundance based on time, and 6h is more intense than 2h.

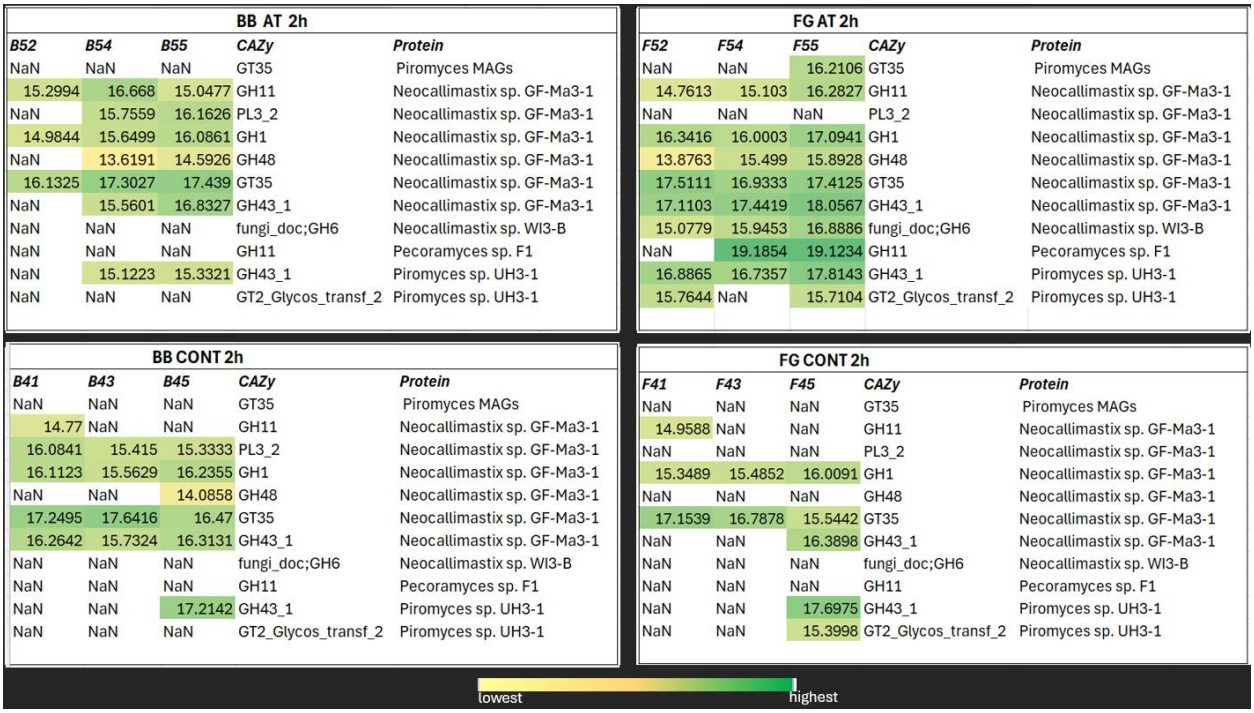
Fungal CAZyme detection

Once the fungal proteins were identified CAZymes were annotated. For this the fungal genomes were functionally annotated using dbCAN. The CAZyme annotation was added to the detected proteins in Perseus which resulted in a total of eight protein groups annotated as CAZymes. Of these, 5 CAZymes being glycoside hydrolases (GH), 2 of them were glycosyl transferases (GT)and 1 being a polysaccharide lyase (PL). These were related to *Piromyces MAGS* (1), *Piromyces sp. UH31-1* (2), *Pecoramyces sp. F1* (1*), Neocallimastix sp. WI3-B (*1), and *Neocallimastix sp. GF-Ma3-1* (6).

CAZymes are of paramount importance in the degradation of plant fiber and are responsible for the cleavage of carbohydrates (Bohra, Dafale, & Purohit, 2019). They are categorized based on function and relevance, where the GHs are responsible for the hydrolytic cleavage of the glycosidic bond (Bernard Henrissat, 1991) while GT catalyzes the synthesis of glycosidic linkages (Lairson et al., 2008). PLs aid in pectin degradation (http://www.cazy.org/). In general my results show detection of more GHs than GTs and PLs indicating possible effective rumen function in fibre degradation. This could be because GHs are known to breakdown polysacharides, while GTs contribute to this degradation by transfering sugar moieties to polysacharides and modifying them to be conducive for GH to break them down (http://www.cazy.org/) (Gruninger et al., 2018). In this thesis identified GH families were GH1, GH6, GH11, GH43_1 and Gh48. GT families identified were GT2 and GT35. The PL family detected was PL3_2. Each CAZyme family had varied abundance based on experimental condition and protein of origin. The high number of GH in the AT condition may suggest efficient fibre digetsion which translates to improved feed efficiency (Bohra, Dafale, & Purohit, 2019; Gruninger et al., 2018). In 2020 Live et al detected an abundance of GH48 and GH46 amongst other ARF CAZymes in relation to degradation of recalcitrant plant cell walls.GH11, GH43 and GH6 amongst others were the most abundant CAZymes related to ARF in terms of cell wall degradation.In the research by (Gruninger et al., 2018)GH6 and GH48 were associated with cellulose digestion while GH11 and GH43 families were related to hemicellulose digestion. In this thesis GH48 seemed to be present in all 6h conditions except FG AT. There was an inconsistent distribution in the 2h condition where in some cases like the control FG it was nonexistent and in control BB there was one hit. GH48 was associated with the fungi *Neocallimastix sp. GF-Ma3-1*, and its presence in all 6h conditions could be attributed to the fact that the fungi are slow to act therefore are more present later after feeding(Akin & Borneman, 1990; Hagen et al., 2020).GH1 a β-glucosidase which hydrolyses terminal, non-reducing glycosyl residues in oligosaccharides and release glucose (Bernard Henrissat, 1991)(http://www.cazy.org/) was detected in all 6h conditions and all 2h conditions but seemed to show a slightly higher abundance in FG compared to BB. Since GH1 is involved in carbohydrate hydrolysis and bond cleavage (http://www.cazy.org/) it is reasonable that it is expressed in all conditions and it's slightly higher in FG due to the efficiency of the pre step. GH6 is responsible for the breakdown of cellulose and β-1,4-glucans (Bohra, Dafale, & Purohit, 2019; Tulsani et al., 2022), and was detected in both FG conditions after 6h but it was either not present

or was low in BB. It was also not detected in all 2h conditions except FG AT for *Neocallimastix sp. WI3-B. GH11* was detected in all 6h conditions, all AT 2h conditions and was low or not present in the control 2h conditions for *Neocallimastix sp. GF-Ma3-1.* For *pecoramyces sp. F1*, GH11 was also present in both FG 6h conditions but either low to not present in the BB 6h conditions. It was absent in all 2h conditions except the FG AT where it had 2 hits, but they have the highest intensity amongst all. GH43_1, which is involved in cleavage of Xylan (Gruninger et al., 2018)(http://www.cazy.org/), was detected in all 6h conditions and in the 2h conditions for FG control and BB control in *Neocallimastix sp. GF-Ma3-1*. It was also found in all 6h conditions except the BB control for Piromyces sp. UH3-1 and had low to no hits in the 2h conditions except the FG AT. *Piromyces sp. UH3-1* also expressed GT2, a glycosyltransferase involved in formation and modification of carbohydrates (Jose et al., 2017). In relation to previos ARF studies (Bohra, Dafale, & Purohit, 2019; Gruninger et al., 2018) GT2 was one of the abundant GTs known to contribute to glycosylation and chitin synthesis. Varsha et al 2019 also recognises GT2 for the same capabilities but in the bacterial genome.In this research GT2 was only enriched in FG samples and had lower values and counts in the 2h condition, which could also be related to the fact that before the freeze grinding the *Piromyces sp. UH3-1* was not being fully extracted and also fungi are more active later hours after initial feeding .GT35 was absent in nearly all samples across lysis method and diet in samples collected two hours after feeding, but it was detected in all freeze grinding samples after six hours. This could be related to the prestep enhancing the cell lysis of the piromyces. GT has been related to the synthesis of glycogen(Park et al., 2021)

GT35 was also highly enriched in all conditions for *Neocallimastix sp. GF-Ma3-1* with slightly higher detection for the 6-hour conditions. This could be since the *Neocallimastix sp. GF-Ma3-1* was the most abundant fungal species detected by protein count, and it was detected in higher amounts after freeze grinding. PL3_2 is not detected in all FG conditions it is only found in BB where It is equally distributed amongst all conditions of diet and time in *Neocallimastix sp. GF-Ma3-1*. It could be argued that the pre step is negatively affecting the presence of the enzyme.



BB AT 2h

B52	B54	B55	CAZy	Protein
NaN	NaN	NaN	GT35	Piromyces MAGs
15.2994	16.668	15.0477	GH11	Neocallimastix sp. GF-Ma3-1
NaN	15.7559	16.1626	PL3_2	Neocallimastix sp. GF-Ma3-1
14.9844	15.6499	16.0861	GH1	Neocallimastix sp. GF-Ma3-1
NaN	13.6191	14.5926	GH48	Neocallimastix sp. GF-Ma3-1
16.1325	17.3027	17.439	GT35	Neocallimastix sp. GF-Ma3-1
NaN	15.5601	16.8327	GH43_1	Neocallimastix sp. GF-Ma3-1
NaN	NaN	NaN	fungi_doc;GH6	Neocallimastix sp. WI3-B
NaN	NaN	NaN	GH11	Pecoramyces sp. F1
NaN	15.1223	15.3321	GH43_1	Piromyces sp. UH3-1
NaN	NaN	NaN	GT2_Glycos_transf_2	Piromyces sp. UH3-1

FG AT 2h

F52	F54	F55	CAZy	Protein
NaN	NaN	16.2106	GT35	Piromyces MAGs
14.7613	15.103	16.2827	GH11	Neocallimastix sp. GF-Ma3-1
NaN	NaN	NaN	PL3_2	Neocallimastix sp. GF-Ma3-1
16.3416	16.0003	17.0941	GH1	Neocallimastix sp. GF-Ma3-1
13.8763	15.499	15.8928	GH48	Neocallimastix sp. GF-Ma3-1
17.5111	16.9333	17.4125	GT35	Neocallimastix sp. GF-Ma3-1
17.1103	17.4419	18.0567	GH43_1	Neocallimastix sp. GF-Ma3-1
15.0779	15.9453	16.8886	fungi_doc;GH6	Neocallimastix sp. WI3-B
NaN	19.1854	19.1234	GH11	Pecoramyces sp. F1
16.8865	16.7357	17.8143	GH43_1	Piromyces sp. UH3-1
15.7644	NaN	15.7104	GT2_Glycos_transf_2	Piromyces sp. UH3-1

BB CONT 2h

B41	B43	B45	CAZy	Protein
NaN	NaN	NaN	GT35	Piromyces MAGs
14.77	NaN	NaN	GH11	Neocallimastix sp. GF-Ma3-1
16.0841	15.415	15.3333	PL3_2	Neocallimastix sp. GF-Ma3-1
16.1123	15.5629	16.2355	GH1	Neocallimastix sp. GF-Ma3-1
NaN	NaN	14.0858	GH48	Neocallimastix sp. GF-Ma3-1
17.2495	17.6416	16.47	GT35	Neocallimastix sp. GF-Ma3-1
16.2642	15.7324	16.3131	GH43_1	Neocallimastix sp. GF-Ma3-1
NaN	NaN	NaN	fungi_doc;GH6	Neocallimastix sp. WI3-B
NaN	NaN	NaN	GH11	Pecoramyces sp. F1
NaN	NaN	17.2142	GH43_1	Piromyces sp. UH3-1
NaN	NaN	NaN	GT2_Glycos_transf_2	Piromyces sp. UH3-1

FG CONT 2h

F41	F43	F45	CAZy	Protein
NaN	NaN	NaN	GT35	Piromyces MAGs
14.9588	NaN	NaN	GH11	Neocallimastix sp. GF-Ma3-1
NaN	NaN	NaN	PL3_2	Neocallimastix sp. GF-Ma3-1
15.3489	15.4852	16.0091	GH1	Neocallimastix sp. GF-Ma3-1
NaN	NaN	NaN	GH48	Neocallimastix sp. GF-Ma3-1
17.1539	16.7878	15.5442	GT35	Neocallimastix sp. GF-Ma3-1
NaN	NaN	16.3898	GH43_1	Neocallimastix sp. GF-Ma3-1
NaN	NaN	NaN	fungi_doc;GH6	Neocallimastix sp. WI3-B
NaN	NaN	NaN	GH11	Pecoramyces sp. F1
NaN	NaN	17.6975	GH43_1	Piromyces sp. UH3-1
NaN	NaN	15.3998	GT2_Glycos_transf_2	Piromyces sp. UH3-1

Figure 11.Heat map of the CAZyme detection from the first 2 hours after feeding including the extraction methods and the diets subjected.The protein detections are based on log 2-transformed LFQ intensities of CAZymes affiliated to anaerobic rumen fungi.Yellow indicates lower intensities, while green indicates higher intensities. All three biological replicates for each diet (Control; CONT and A. taxiformis; AT) are shown. The heatmap indicates that more CAZymes are detected in the groups subjected to FG as a prestep and AT as a feed additive, after 2 hours of feeding. (It should be noted that the sections labled as protein , represent fungal protein of origin)

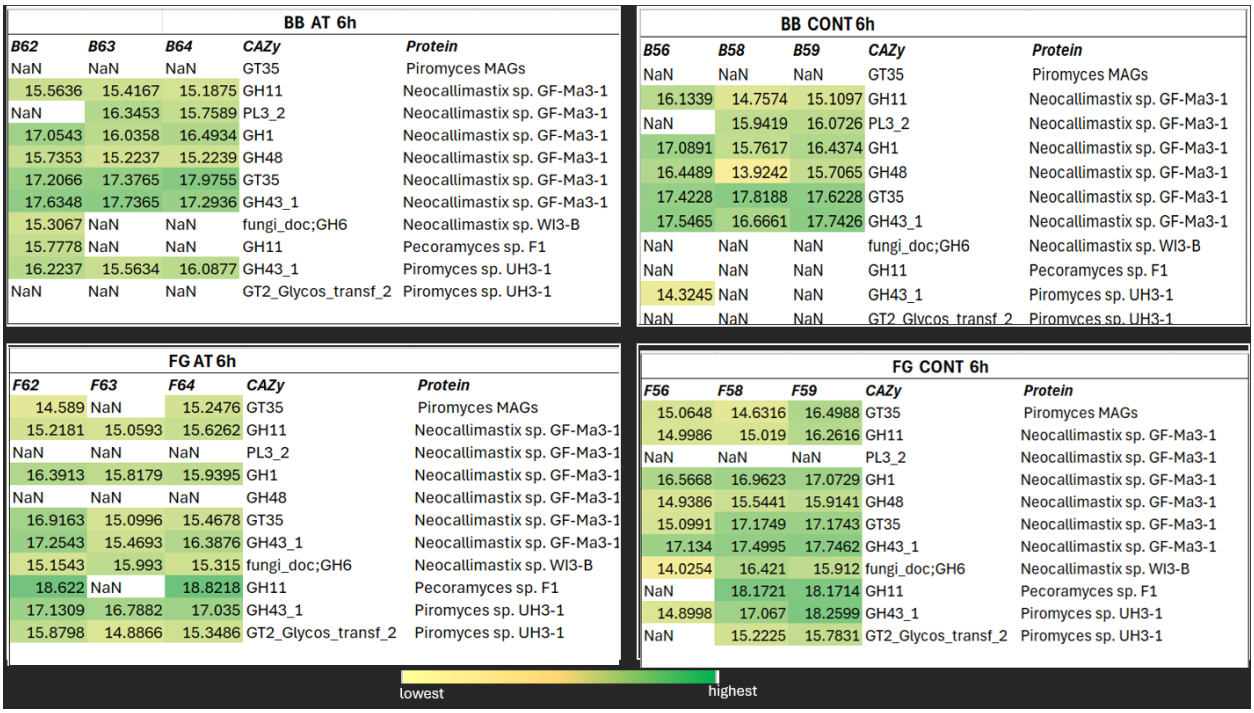


Figure 12

Heat map showing the protein detection (log 2-transformed LFQ intensities) of CAZymes affiliated to anaerobic rumen fungi after six hours from feeding time. Yellow indicates lower intensities, while green indicates higher intensities. All three biological replicates for each diet (Control; CONT and A. taxiformis; AT) are shown. The heatmap indicates that more CAZymes are detected after 6 hours, compared to after 2 hours as seen in (figure 11). (It should be noted that the sections labled as protein , represent fungal protein of origin)

Conclusion

As me(Itned above, ARF are still an understudy compared to other rumen microbes like bacteria but they still play a major role in the degradation of plant fibers in the rumen. The microbes work in synergy with each other and in symbiosis with their host.This study hypothesized that common cell lysis methods are oblivious to some ARF embedded in the cell walls and adding a pretreatment by first freeze grinding the substrates in liquid nitrogen would release more fungal proteins. Even though the sample size was small the hypothesis was still satisfied, since there was significant protein enrichment in the pretreated samples (FG) based on LFQ values. However, the CAZymes detection was a bit lower than expected. The difference in protein counts between kingdoms and

between fungal species were not very significant between bead beating and freeze grinding. This could produce more conclusive results with a larger sample size. The protein intensities in diet and time, though visible, were also very low, hinting the need for a larger sample size. Pretreatment with liquid nitrogen requires care and time and is quite laborious, the feasibility of this method on a larger sample size is food for thought. Overall for this thesis, it can be concluded that a pre-treatment step with freeze-grinding is required for sufficient extraction of anaerobic fungi from rumen samples. However, the sample size used in this thesis is small and more research should be done on larger collections of samples.

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