Stapledon Report: Examining the impact of environmental stress on the digestibility of forage grasses.

Awardee: Dr. Divya Kattupalli

Main Supervisor: Prof. Alison Kingston Smith (Aberystwyth University)

Introduction

In a rapidly changing world, we adapt our lifestyles due to the increasing demand for water, food, shelter, and energy (Bruinsma, 2009; Kingston-Smith *et.al*, 2013). The challenge is pressing with 9.1 billion people expected to require a 70% increase in more food by 2050 Animal proteins, such as those found in meat, eggs, and milk, include all of the essential amino acids required by our bodies. Therefore, animal production must play a major role in feeding an expanding human population.

Ruminants possess the microbes-based capacity that degrades the high fibrous plant materials through the process of fermentation that produces volatile fatty acids (VFA's) and microbial proteins (Liang, et.al, 2020). However, efficient production of microbial protein depends on the amount of microbial nitrogen per unit of energy available in the rumen which is known as carbohydrate fermented (Bach and Stern, 2005). Therefore, an imbalance in the availability of protein and digestible carbohydrates for rumen fermentative microbes may lead to an inefficient fermentation process, and as a result high amount of non-environmentally friendly products such as nitrogen, and methane is released into the atmosphere (Kingston-Smith et al 2012). A high volume of ammonia is also released into the environment through the process. The presence of excess waste by-products from the digestive tract typically indicates low feed utilisation. Consequently, there is a need for ongoing scientific research to improve the availability of nutrients from plant-fed feed to ruminants and minimise loss due to digestion defects.

This rumen fermentation process is influenced by the quality of the food. In ruminants, forage grasses are important sources of animal nutrition. Indeed, grassland used for grazing currently represents ~ 67% of the UK's agricultural area. The Institute of Biological, Environmental and Rural Sciences (IBERS) has been involved in forage grass breeding for over 100 years. Its current breeding objectives are the production of varieties combining high nutritive value and

efficient use of inputs like nitrogen. These aim to improve the efficiency of ruminant production and increase the proportion of forage in the ruminant diet. These aim to produce high sugar and high protein grasses but with a high nutrient use efficiency; cutting ammonia and methane from ruminants.

The IBERS forage grass breeding programme is based on field assessments of various grass populations but only recently has it started to systematically consider how environmental stress could impact the nutritional properties of the forage. The biotic and abiotic variables have a significant impact on plant growth as well as crop production and quality features. Weeds, insects, fungi, bacteria, and other biotic and abiotic variables (such as sunshine, temperature, rain, humidity, drought, salinity, and pollution) can all have an impact on plant growth and crop output (Umar, Olayinka Bolaji, et al. 2021).

Hidden stress is represented by attempted “infection” events which fail but against which the plant displays an effective defence. In particular, these defences are based on the recognition of “pathogen” associated molecular patterns (PAMPs) which are in fact, encoded by all microbes. PAMPs are recognised by pattern-recognition receptors such as toll-like receptors, mannose receptors, and nucleotide-binding oligomerization domain-like receptors during the plant immune system response to non-self-molecular bodies. The process can cause systemic resistance by causing defensive pathogenesis-related protein (e.g. PR-1) genes to be expressed in the plant (Bittel and Robatzek, 2007; Boller and Felix, 2009).

Given that PAMPs are encoded by all microbes, it is important to recognise that forage grasses can respond to these as they grow in the field but also as they enter the rumen. Plants ingested by animals are viable and still alive even after being digested, masticated, and driven down into the rumen. Given this, it is likely that extracellular proteins released by rumen bacteria are identified as PAMPs by plant cells. Kingston-Smith and Theodorou (2000) confirmed that continuous stress signals are imposed on plant cells upon entry into the rumen space, with invasion by the rumen microbiome being a contributing factor (Kingston-Smith et al. (2012). It is thus hypothesised that eaten fodder can stimulate the same plant immune response that is activated in aerobic plant-microbial interactions when exposed to rumen bacterial proteins. Plant defence responses may thus contribute to inefficient fodder decomposition by minimising the influence of microbial colonisation and proliferation on eaten leaves (Kingston-Smith et al. 2013). The importance of these host defence responses for animal nutrition compared to the observation that it includes the formation of systematic arrangements of cellulose microfibrils

and multi-level crosslinking of polysaccharides, resulting in a thickened cell wall (Niklas (1989).Wilson (1994). We do not know how plant mechanisms of protection affect nutrient digestion and its influence on excess waste during fermentation in ruminants.

This current study intends to investigate the effects of PAMP stress responses on the nutrient digestibility of ingested plants. To reflect the two possible sources of PAMPs elicitation, the forage grass – AberDart- will be treated with a mutated bacterial pathogen – *Pseudomonas syringe hrpL* – (which only elicits a PAMPs response) and rumen fluid supernatant.

Methods

Plant samples

Ryegrass (*Lolium perenne*) is the most commonly cultivated 'high sugar' forage grass "AberDart" in the UK. These were grown in 20 cm diameter pots in Levington's M2 multipurpose compost under controlled conditions in the glass house (16 h light. 16 ºC) at IBERS, Aberystwyth University. Seeds were sown at high density to mimic sward-like conditions on the pot. At five weeks following sowing the grasses were harvested by cutting the plants ~ 1 cm above the soil layer. The grasses were transferred to beakers with 200 mL of sterile water, a suspension of *Pseudomonas syringe hrpL* (2 x 10^5 cells/mL) or a rumen fluid supernatant to allow uptake through the cut ends for 24 h.

***Pseudomonas syringe hrpL* suspension**

Pseudomonas syringe hrpL were prepared in LB Agar plates and stored in -4 ℃. A single colony of hrp culture was inoculated in LB broth media the day before the experiment. Before use, the culture was centrifuged and the pellet was resuspended in water to give a concentration of 2 x 10^5 cells/mL.

Rumen Fluid Collection and Preparation

For the study, rumen samples (1L) will be required from 3 individual fistulated cows will be used, with approximately 200 ml of rumen fluid taken from each of the four fistulated cows. Each digest sample must be strained through three layers of muslin fabric, divided into aliquots, and used the same day.

Assay

The study involves harvesting perennial ryegrass (AberDart) from controlled conditions and placing 30g of fresh weight forage (FW) in beakers containing A) deionised water, B) *HrpL* suspension (2 x 10^5 cells/mL) and C) rumen fluid supernatant containing PAMPS elicitors (1 mg protein/mL) for 1 hour to allow solutions to enter the transpiration stream. For each treatment (A, B, C) leaves are dried, cut into 1 cm lengths, and placed into washed and autoclaved serum bottles. A further four replicate control bottles contain inoculant but no forage substrate. Rumen fluid is mixed in equal proportions from each sample and used to make a 10 % inoculum with Van Soest solution. The bottles are backfilled with CO_2, capped, and placed at 39 ℃. Total gas is measured at intervals between 0 and 72 h after inoculation using the pressure transducer technique. Gas collected during fermentation is passed through the IRGA for carbon dioxide and NH4 measurement. The end-point analysis is conducted for VFA, CO_2 and NH_4 measurements. Samples are taken from replicate incubations at 0, 2, 4, 6, 12, 24, 48, and 72 h for destructive sampling for VFA, dry matter (DM) and further metabolomics (to assess changes in nutrient quality).

Total bottles = For Gas production (3 treatments x 4 replicates) + 4 controls = 16

For sampling (3 treatments x 4 replicates x 8 timepoints) = 96

Preparing VFA samples

For VFA sample preparation, 200 µl of clear sample was added to 50 µl of external standard (20% Orthophosphoric acid & 20 µM ethylbutyric acid). Mixed well, centrifuged. Inserts were placed inside the glass vials and 100 µl of supernatant was added and stored in -20 ℃ for further analysis.

Results and Discussions

The ruminant's microbiome is dominated by anaerobic microbes such as methanogenic archaea, ciliate and flagella protozoa, fungi, viruses, bacteriophages, and different bacteria that play an important role in bulk digestion in the rumen (Firkins and Yu, 2015). The microbes found in the rumen secrete enzymes that are involved in the digestion and fermentation of the ruminant's swallow; for its involvement in digestion, the rumen is characterised as a fermentation vat (Aschenbach, et al 2011). Rumen microbial fermentation produces CO_2, ammonia and methane (Mackie et al., 2002; Kingston- Smith, *et. al*, 2012).

The data presented represents the outcome of two experimental repeats, both of which yielded similar patterns. The results of a single experiment are shown. *In vitro* fermentation of hrp and rumen treated consequently resulted in lower NH_4 and CO_2 gas emissions compared to water-treated samples (Fig. 1). These observations were consistent with the pre-treatment with the two sources of PAMPs-elicitation suppressing grass digestion.

It would be predicted that this reduced grass digestion would also impact the rate and quantity of substrate fermentation to short-chain fatty acids, carbon dioxide, and methane (Blümmel and Becker 1997). VFA profiling was used to measure the levels of acetic acid, propionic acid, butyric acid and isobutyric acid, which are the building blocks of different organic compounds (Fig. 2A). The PAMPs treatments did not affect the production of the shortest short-chain fatty acid (2C) but reduced production was observed with propanoic (3C) and butanoic (4C) acids. Interestingly, isobutyric acid (a branched fatty acid comprising propanoic acid carrying a methyl branch at the second carbon) was not affected by the PAMP pre-treatments. Longer VFAs were also measured: valeric acid (a C5 straight-chain alkyl carboxylic acid), iso-valeric acid (methyl branched at C2-chain alkyl carboxylic acid), caproic acid (a C6 alkyl carboxylic acid) and iso-caproic acid (methyl branched at C2) (Fig. 2B). The rate of isovaleric acid production was not affected by PAMPs pretreatment of AberDart forage grass. However, the production of valeric acid, caproic acid and iso-caproic acid was reduced if the grass had been pretreated with PAMPs. These data suggest that microbial processes and possibly different components of the rumen microbiome are being differently affected by PAMP pretreatments.

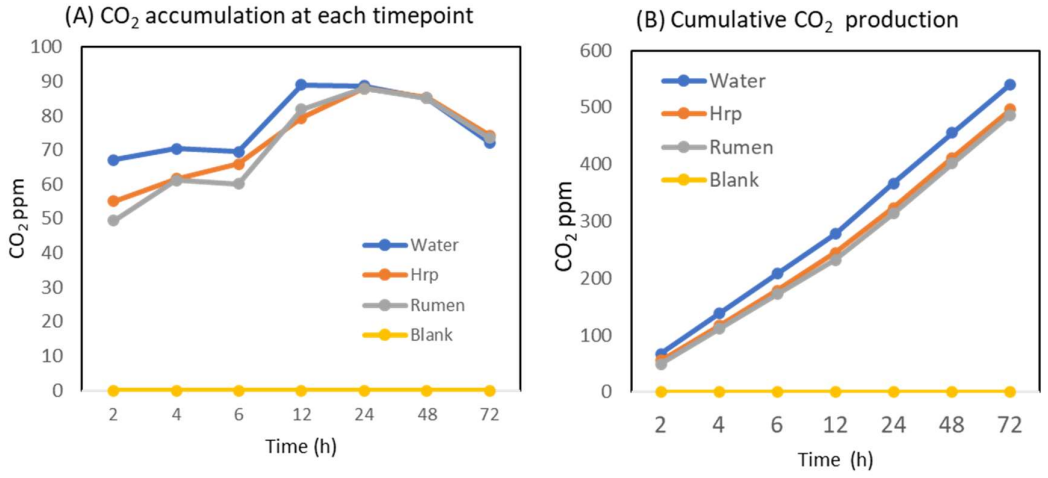

Fig 1A: Estimation of CO_2 at each time point 2, 4, 6, 12, 24, 48, 72 hrs. A) CO_2 & B) Cumulative CO_2 production

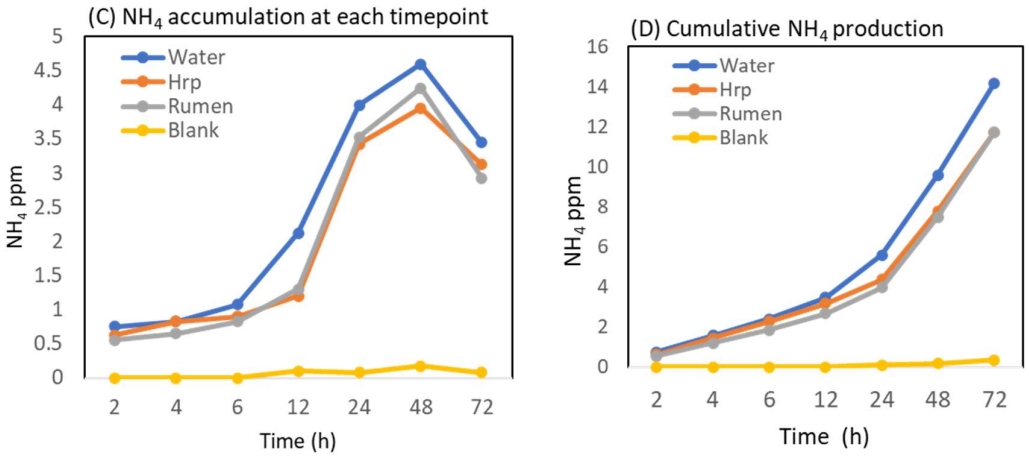

Fig 1B: Estimation of NH_4 at each time point 2, 4, 6, 12, 24, 48, 72 hrs. C) NH_4 & D) Cumulative NH_4 production

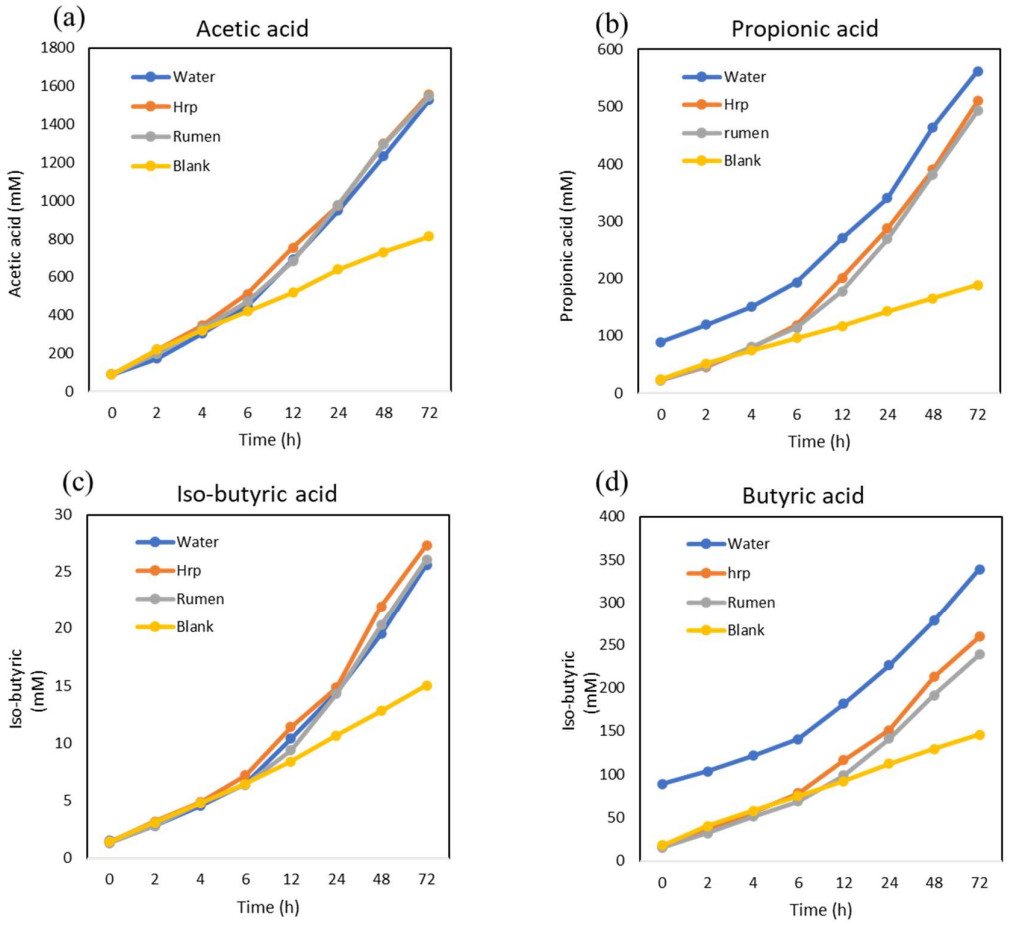

Fig 2A: Detected volatile fatty acids a) Acetic acid, b) Propionic acid, c) Iso-butyric acid, d) Butyric acid.

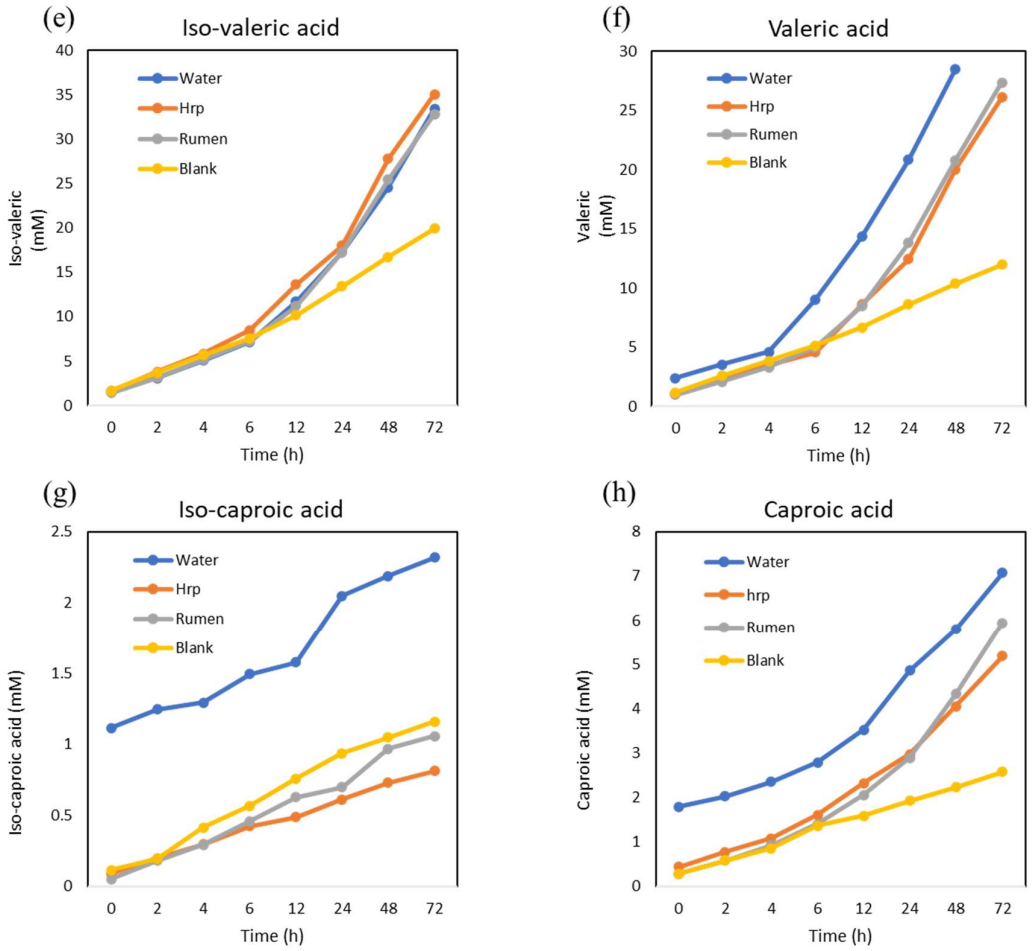

Fig 2B: Detected volatile fatty acids e) Iso-valeric f) Valeric, g) Isp-caproic acid and h) Caproic acid

Conclusions: The observation made in this pilot project indicates that PAMP-elicitation events arising from failed pathogenic attacks and interaction with environmentally microbial and rumen microbes can influence forage grass digestibility. If this is substantiated by follow-up studies, this could influence forage grass breeding targets and strategies.

Future work: IBERS have initiated a PhD programme to attempt to reproduce the observations of this pilot study. It will also use metabolomics to establish how responses to PAMPs can influence the nutritional quality of the forage grass. Microbiomic approaches will be used to define how PAMPs treated forage affect the rumen microbial populations.

I wish to express my profound thanks to the Stapledon Memorial Trust for supporting my project. It has allowed me to develop new skills which I hope will be useful as I develop my career.

References

Bruinsma, J. (2009, June). The resource outlook to 2050. In *Expert meeting on how to feed the world in* (Vol. 2050, pp. 1-33).

Kingston-Smith, A.H., Marshall, A.H. and Moorby, J.M., 2013. Breeding for genetic improvement of forage plants in relation to increasing animal production with reduced environmental footprint. *Animal*, *7*(s1), pp.79-88.

Liang, J., Nabi, M., Zhang, P., Zhang, G., Cai, Y., Wang, Q., Zhou, Z. and Ding, Y., 2020. Promising biological conversion of lignocellulosic biomass to renewable energy with rumen microorganisms: A comprehensive review. *Renewable and Sustainable Energy Reviews*, *134*, p.110335.

Bach, A., Calsamiglia, S. and Stern, M.D., 2005. Nitrogen metabolism in the rumen. *Journal of dairy science*, *88*, pp. E9-E21.

Umar, Olayinka Bolaji, et al. "Stresses in plants: Biotic and abiotic." *Current Trends in Wheat Research* (2021).

Bittel, P. and Robatzek, S., 2007. Microbe-associated molecular patterns (MAMPs) probe plant immunity. *Current opinion in plant biology*, *10*(4), pp.335-341.

Boller, T. and Felix, G., 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual review of plant biology*, *60*, pp.379-406.

Kingston-Smith AH, Theodorou MK. 2000. Post-ingestion metabolism of fresh forage. Tansley Review. New Phytologist 148, 37–55

Kingston-Smith, A.H., Davies, T.E., Edwards, J., Gay, A. and Mur, L.A., 2012. Evidence of a role for foliar salicylic acid in regulating the rate of post-ingestive protein breakdown in ruminants and contributing to landscape pollution. *Journal of experimental botany*, *63*(8), pp.3243-3255

Niklas, K.J., 1989. The cellular mechanics of plants. *American Scientist*, *77*(4), pp.344-349.

Wilson, J.R., 1994. Cell wall characteristics in relation to forage digestion by ruminants. *The Journal of Agricultural Science*, *122*(2), pp.173-182.

Firkins, J. L., & Yu, Z. (2015). Ruminant nutrition symposium: how to use data on the rumen microbiome to improve our understanding of ruminant nutrition. *Journal of animal science*, *93*(4), 1450-1470.

Aschenbach, J. R., Penner, G. B., Stumpff, F., & Gäbel, G. (2011). Ruminant nutrition symposium: Role of fermentation acid absorption in the regulation of ruminal pH. *Journal of animal science*, *89*(4), 1092-1107.

Blümmel M, Becker K (1997) The degradability characteristics of fifty-four roughages and roughage neutral detergentfibre as described by in vitro gas production and their relationship to voluntary feed intake. The British Journal of Nutrition 77, 757–768. doi:10.1079/BJN19970073