

5 Conclusions

Screening system antiproteolytics

In the context of the Rumen up project a screening system based on the RPT was developed to monitor the kinetics of ruminal protein degradation *in vitro*. This system had proven to be well suited for the detection of new mechanisms of protein protection other than for example the well-known effect of tannins. This is mainly due to the investigation of various aspects of the protein metabolism, such as measuring the concentrations of soluble and precipitated protein, as well as the end products of proteolysis, the branched short chain fatty acids and ammonium, which helps to distinguish between qualitatively different antiproteolytic activities. By including monensin in the test system, the effects of the plant materials on various aspects of the fermentation could be compared directly to the effects of the GPA, which is to be replaced. Accordingly the system led to the identification of one promising plant material that inhibited protein degradation similar to monensin but by a yet unknown mechanism. Within the framework of the project it could be shown that the active substance is heat stable and soluble in methanol, however, it was not possible to fully identify the active component and to elucidate the mechanism of action.

Effects of Knautia arvensis in vitro

The inclusion of 18% of the plant material *Knautia arvensis* increased the concentration of soluble protein *in vitro* by 64% compared to the control, accounting for 38% of the monensin effect. Unlike monensin, *Knautia arvensis* did not show negative effects on other fermentation parameters such as production of gas and of short chain fatty acids. Thus, the increase in protein concentration without an effect on overall fermentation in the rumen could lead to a higher amount of protein reaching the smaller intestine of the host animal without decreasing the supply of short chain fatty acids. A more detailed investigation revealed a dose dependency for the entire plant material as well as for the methanol extract thereof. Studies of plant material derived from different locations, at various dates, and of related species indicated variability in the efficacy of the material.

Thus, much more research on this promising plant material is needed to elucidate the active component and understand its mechanism of action before it may be applied in animal nutrition. This need is met by a newly proposed research project on *Knautia arvensis* by the University of Hohenheim and a continued cooperation between Hohenheim and the Rowett Research Institute on that topic.

In vivo trials

Knautia arvensis. The inclusion of 10% of *Knautia arvensis* in the feed affected protein metabolism in both *in vivo* trials. The shift in energy retention from fat to protein gain on the diet containing higher crude protein, detected in the Hohenheim trial, may be seen as advantageous, but true benefits would arise only if such an effect was expressed on a lower crude protein diet. The trial performed in Leon, however, which used the diets of lower crude protein content, detected a lower protein content in the carcass and muscle of lambs fed with the additive.

Bellis perennis. The inclusion of 5% of *B. perennis* in the concentrate feed slightly decreased protozoal numbers *in vivo*, however, without any detectable impact on further rumen or production parameters with the exception of a slight decrease in short chain fatty acid concentration in the rumen of the cannulated lambs.

Urtica dioica. The results obtained from *in vitro* experiments and the two *in vivo* trials with the inclusion of 5% of *Urtica dioica* were contradictory, possibly due to the different diets or animals. In particular, the lambs in Spain were very small at slaughter (25 kg)

From the experience gathered within this project it may be learned that the time frame for such a project beginning from the screening of 500 plants to the identification of active compounds and testing them as additives *in vivo* and under production conditions is much too short. Fixed deliverables in the project contract do not allow sufficiently reacting flexibly on needs arising from the actual results. A much deeper investigation of the plant materials and a better evaluation of the experimental conditions *in vitro*, e.g. composition of the feed, would have been beneficial before starting production trials.

From the University of León:

Antimethane agents were effective enough in the *in vitro* tests, and were not tested *in vivo*. It is difficult to say if effects were minor or really important. Effects on methane were certainly smaller than expected, as one would wish to have reductions of 50% or more. However, these effects are comparable to those observed with some organic acids (see our own results) or even with monensin (we have tested monensin in the Rusitec and effects on methane are not much greater of 10-15%).

Effects of plants on methane were not wholly consistent. We confirmed the effects in a number of tests and always observed some decrease in methane production, although to different extent. The main concern is the lack of a clear dose-response trend that compromises the robustness of the results, but as I said before, for *Carduus* and *Rheum*, some decrease was observed in up to 6-8 different incubation runs.

I really like the comment saying that “it was extremely optimistic” to expect the project could be result in a product ready for commercialization. In the discussion I would highlight the fact that we worked with “plant material” rather than with active compounds having a specific activity on any of the fermentation processes. To me this is really important because to be successful active compounds (or at least extracts, oils of any fraction) should have been identified, and the factors affecting the presence and concentration of such active agents in the plant material should have been investigated and characterized. Without this, results are going to be always casual rather than conclusive. We have some indirect information about their composition from literature sources but have no idea at all on which are the compounds responsible for the effect and also on the sources of variation in the amount of those agents present in the plant material. Think that the factors that can affect plant secondary compounds can be countless and we have little ability to control them. If we have a sample of the plant rich in that compound the effect will be positive, if for any reason that compounds is not present the same plant species is going to be not effective at all. Plant secondary compounds can be affected by plant species, variety, part of the plant collected, phenological or growth stage, part of the plant (flower, leaf, stem, root, etc), season, environmental factors (a plant in the shade or at the sun, rainfall or sunlight in that particular year or season), location (altitude, sampling site, etc), harvest or collection procedures, drying, storage conditions and time... I am sure I forget more factors than the ones already enumerated. An example, first daisies were

collected by hand (a few specimens) by your botanist, then 1 kg was cultivated by a company, and the accession used for the in vivo trials was a commercial material bought in Germany. The plant species was the same, the amount of active compound contained in each sample could be completely different.

The dose is an important point. To be really effective you need to establish the optimal dose. If you remember, we expected better results with Crina compounds, but had nothing instead. The problem is that in the screening we could only afford to test a single dose and in the preliminary results we killed everything in the cultures, whereas in the second try we saw almost nothing. I know your work with Crina additives and know that they may be efficient once you know the optimal dose. With some of the compounds effects can be controversial just above or below that dose, with a very narrow range around that dose. So if the dose is not the appropriate, the results are going to be disappointed. Think that the dose may be different for each compound, so an optimal dose for a compound can be insufficient or excessive for a different compound. The question is even more complex when you work with the whole plant material assuming that the active compounds is in there and always in the same amount. You may be adding the same amount of plant material and the amount of compound be drastically different. As a recommendation, I think given the limitations of in vitro procedures, a clear dose-response trend should be an essential requisite, if the response is related to the amount of additive one can expect a real (not just casual) cause-effect relationship. Think that the screening was with 500 plants, and a single dose used for all plants, this clearly may justify some of the weakness of the results observed.

Another important fact is that working with plant material you always have “an inevitable matrix” and we do not know to which point this may have a crossed effect with the intended activity. The active compounds is not administered alone, but contained in the plants that provides a source of digestible organic matter and of other secondary metabolites that may interact with the agent having the specific activity. This has been discussed extensively in the meetings, and I am sure will cause more problems even in monogastric animals (think that plant is 5-10% of the feed) for Replace, I accept that is inevitable, but I am also concerned of the potential influence of this on the results.

As you say, a further chemical knowledge would be required, but this could not be managed by the consortium.

The in vivo trials were subjected to all this questions, together with the fact that an agent can be very effective for some type of animals and under some production conditions. But this was clearly out of the possibility of the project in which only two trials were planned, whereas up to 6 trials were conducted.

Other short thoughts include:

- *Knautia arvensis* may serve as a possible replacement of monensin, as it showed 38% of the efficacy of this GPA on protein degradation without negative side effects on fermentation.
- The active principle of *K. arvensis* is heat stable and soluble in methanol, and its efficacy is dose dependent.

- Based on the *in vitro* studies *K. arvensis* was shown to be a promising antiproteolytic feed additive; although its efficacy could not immediately be confirmed *in vivo*, this plant material is worth further research investigations.
- *Bellis perennis* decreased protozoal activity *in vitro* and protozoal numbers in cannulated sheep, however, without any detectable impact on further rumen or production parameters
- The results obtained from the trials with cannulated sheep show that effects observed *in vitro* need to be validated in a functional study, and optimum experimental conditions need to be evaluated, before carrying out production trials
- Data from a number of *in vitro* screens showed *Urtica dioica* and *Lactuca sativa* to consistently maintained higher pH values and had markedly lower lactic acid contents. This is despite the highly fermentable nature of these two substrates which tends to enhance degradation.
- The *in vitro* screens were unable to generate classic bloat symptoms, a component of which is host animal generated. Due to the difficulty of testing these substrate *in vitro* and the lack of response identified, no candidates were recommended for further work.
- *L. sativa* and *U. dioica* both maintained higher fermentation media pH levels, with *L. sativa* slightly more effective than *U. dioica* at higher doses [50 and 100 mg g⁻¹] while at 10 and 20 mg g⁻¹ the reverse occurred, although the magnitude of effect was also lower.
- with respect to persistency RPT flasks containing wheat inoculated with fluid from RUSITEC vessels that had received either *L. sativa* and *U. dioica* for seven days, showed higher pH levels than control fluid (wheat alone).
- an additive effect was found when perturbed fluid was added to RPT flasks containing *L. sativa* and *U. dioica*
- None of the three solvent (hexane, dichloromethane or methanol) extracted fractions of either *L. sativa* or *U. dioica* showed any activity with respect to fermentation medium pH, when examined at a range of inclusion levels.
- The palatability and tolerance of six plants, *B. perennis*, *L. sativa*, *U. dioica*, *P. pelatum* and *C. pycnocephalus* were examined using short and long term feeding studies and a range of blood parameters, respectively. *L. sativa*, *U. dioica*, *B. perennis*, and *C. pycnocephalus* were readily tolerated [i.e. no changes in blood metabolites and liver enzyme levels were recorded]

Overall, the Rumen-up project must be considered a success. Five hundred collected samples provided a wealth of data, with at least 25 being considered to have potential value as a feed additive to control the target detrimental activities of ruminal fermentation. The data on the rest of the samples, when released into the public domain, will be a useful reference for many producers of plant materials, research nutritionists, agronomists and so on. The fact that none of the targetted samples has reached commercial application as yet is more a function of the short-term nature and limited budget of the consortium than a scientific failure. The EC must be made aware of how much the regulatory framework is a disincentive to the commercial development of products based on natural plant materials. Medium-sized companies would be unable to cope with the expense. For SMEs, such developments are completely out of the question. Thus, the EC's own regulations aggravate the difficulties this sector faces in

copied with the universal ban on growth-promoting antimicrobials in Europe. Novel technologies will continue to be stifled unless solutions are found to the regulatory disincentives.