New feed Additives for Ruminants- a European perspective

C.J. Newbold, S.M. Duval, N.R. McEwan, D.R. Yáñez-Ruiz and K.J. Hart[1]
Institute of Rural Sciences, University of Wales,
Llanbadarn Campus, Aberystwyth SY23 3AL UK
[1]Current address: Department of Animal and Poultry Science
University of Saskatchewan, S7N 5A8, Canada

The digestive anatomy and physiology of cattle and other ruminants is markedly different to that of monogastric animals such as man. The ruminant has two additional digestive organs at the anterior end of the tract, namely the rumen and omasum, which allow the host to extract and absorb energy from fibrous plant material not otherwise available to mammalian enzymes. Digestion of food in the rumen occurs by a combination of microbial fermentation and physical breakdown during regurgitation of the food by rumination. Microbial attack is carried out by a mixed population of bacteria and ciliate protozoa, together with a smaller, but possibly mechanically important, population of anaerobic fungi (Dehority, 2004). As a result of the location of the rumen, anterior to the abomasum, feedstuffs consumed by ruminants are exposed to microbial attack prior to gastric and intestinal digestion. The rumen is essentially a fermentation chamber in which microbial attack helps digest the diet. The partly fermented food and the micro-organisms then pass through the omasum, into the abomasum and then into the small intestine. Thus unlike hindgut fermenters the products of microbial fermentation, mainly volatile fatty acids (VFA) and microbial protein, are available for absorption. Indeed VFA formed in the rumen can supply the majority of the animal's energy requirement, while microbial protein leaving the rumen can account for much, if not all, of the protein entering the small intestine. In view of the importance of the rumen in the nutrition of the host, it is perhaps not surprising that a great deal of effort has been devoted to investigating methods for manipulating this complex ecosystem.

Recently legislation has been introduced within the European Union to prohibit the use of growth-promoting antibiotics in animal feeds (EU, 1831/2003). The scientific basis for these restrictions, based around concerns that the use of antibiotics in animal agriculture can give rise to transmissible resistance factors that may compromise therapeutic antibiotic usage for humans, may be questionable (Casewell *et al.*, 2003). Nevertheless, the removal of antibiotic growth-promoters has led to an increased interest in alternative means of manipulating rumen fermentation to increase productivity gain. An enormous variety of secondary compounds are produced by plants to provide protection against microbial and insect attack (Isman, 2000; Kostyukovsky and Rafaeli, 2002; Iason, 2005). Some are toxic to animals, but others may not be, and indeed many have been used to manipulate gut function in both ruminant and non ruminant animals (Greathead, 2003). Here we will describe some of our recent work investigating the effects of one particular sub-class of plant extract; the essential oils on rumen fermentation.

The term ‘essential oil’ is believed to be derived from ‘Quinta essentia’ a name given to the effective component of a drug by Paraclsus von Hobenheim, the Swiss medical pioneer (Guenther, 1948). However, the term essential oil is misleading as they are not as their name

suggests 'essential' for nutrition or metabolism nor are they oils in the sense of being lipids. They are instead volatile, aromatic compounds with an oily appearance, which are obtained from plant materials (Burt, 2004). Hence, they have also been referred to as volatile or ethereal oils (Guenther, 1948).

Essential oils have been used by man as fragrances and flavourings for many years (Guenther, 1948). Typically, essential oils are extracted from plant material by the process of steam distillation, using either water or aqueous alcohol, sometimes with multiple distillates being combined (Losa, 2001). However, small scale extractions using other methods, such as solvent extraction or pressure extraction under liquid carbon dioxide, also occurs (Packiyasothy and Kyle, 2002; Moyler, 1998). Due to the volatile nature of essential oils, those extracted using solvents or at cold temperatures under pressure may have a greater or contrasting bioactivity compared to the corresponding steam distillates (Packiyasothy and Kyle, 2002).

Table 1. Some common essential oil compounds

Hydrocarbon monoterpenes	**Oxygenated monoterpenes**
α-Pinene	Thymol
β-Pinene	Eugenol
Limonene	Citronellol
Terpinene	Terpeneol
Cymene	Geranyl acetate
Myrcene	Linalool
Camphene	Fenchyl alcohol
Terpinolene	Citronellal

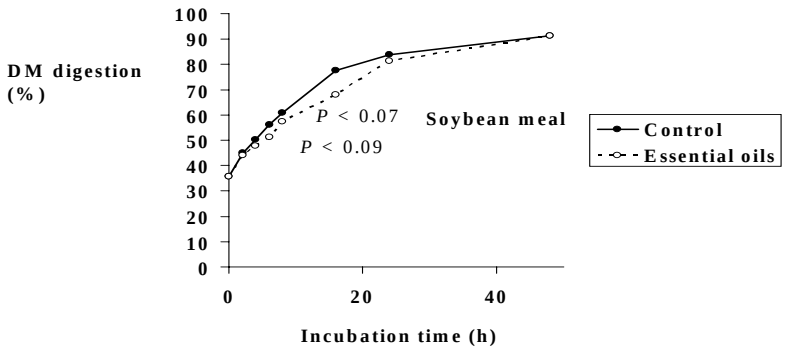
Table 1 list some of the more common essential oil compounds available. Structurally, essential oils can be classified as alcohol, ester or aldehyde derivatives of phenylpropanoids and terpenoids (Greathead, 2003), with the most abundant terpenoid essential oils being the monoterpenes (Dudareva *et al.*, 2004). Essential oils are found throughout the plant, including roots, bark, flowers, petals, leaves, fruit bodies and stems (Hirasa and Takemasa, 1998). As an active component in many spices and preservatives, they have been used to inhibit bacterial growth for many years (Hirasa and Takemasa, 1998). Essential oils appear to be selective in their antibacterial action, with the spectrum of antibacterial activity varying with the compounds tested (Janssen *et al*., 1986; Lis-Balchin and Deans, 1997; Demetzos *et al.*, 1997).

Workers in the 1960's appear to have been the first to investigate the effects of essential oils on rumen fermentation. Oh *et al* (1967) suggested that essential oils from Douglas pine (*Pseudotsuga menziesii*) needles could alter rumen fermentation, whilst Nagy and Tengerdy (1968) found that the addition of essential oils extracted from Sagebush (*Artemisa tridentata*) altered the composition of the bacterial population during a 24 h incubation of rumen fluid *in vitro.* Microbial activity, measured by production of gas during *in vitro* incubations, was either inhibited or promoted depending on the nature of the essential oil added with monoterpene hydrocarbons less toxic and sometimes stimulatory to microbial activity compared to the corresponding oxygenated compounds, the monoterpene alcohols and aldehydes (Oh *et al*.,

1967, 1968). Interest in essential oils as rumen manipulating agents appears to have waned thereafter, possible due to wide spread uptake of ionophoric antibiotics However, as noted above the recent removal of growth-promoting antibiotics, including ionophores from the animal feeds market in the EU has refocused interest on essential oils. Some recent studies have utilised single naturally occurring essential oils (Busquet *et al*., 2005a, 2005b, 2006, Cardozo *et al*., 2004, 2005) but most studies have investigated blends of essential oils. Perhaps the best researched of these, and the compound on which our group has done most of its work, is the product marketed by CRINA SA (Switzerland). Crina is a defined, patented mixture of natural and nature-identical essential oil compounds that includes thymol, eugenol, vanillin, and limonene as its main components on an organic carrier (Rossi, 1995).

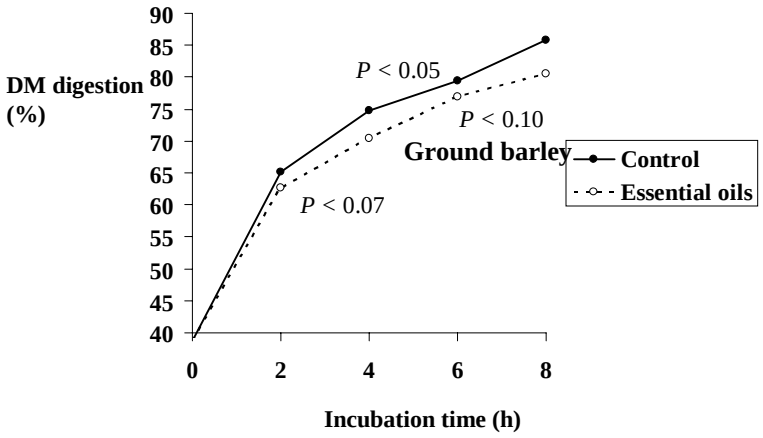
Crina has been shown to affect both volatile fatty acids production plus nitrogen and starch degradation in the rumen. We found that Crina tended to stimulate total volatile fatty acid production 6 h after feeding in sheep Newbold *et al.* (2004), while Castillejos *et al.* (2005, 2006) found a similar effect on total volatile fatty acid production in continuous fermentors and also suggested that essential oil supplementation might selectively stimulate acetate formation. Crina has also been shown to decrease the degradation of both protein- and starch-rich substrates incubated in dacron bags within the rumen (McEwan *et al.*, 2002b; Newbold *et al.*, 2004, Figures 1 and 2). However, no effect on fibre degradation was observed (Newbold *et al.*, 2004).

Figure 1. Influence of dietary essential oils on the loss of soyabean meal barley from nylon bags suspended in the rumen.



The effect on protein and starch degradation seems to be highly selective with Molero *et al.* (2004) reporting that Crina reduced the degradation rate of sunflower meal and green peas but not soyabean meal, fish meal or lupin seeds in the rumen of cattle, whilst Newbold *et al.* (2004) found Crina decreased the rate of soyabean meal degradation but not rapeseed meal in the rumen of sheep.

Figure 2. Influence of dietary essential oils on the loss of ground barley from nylon bags suspended in the rumen.



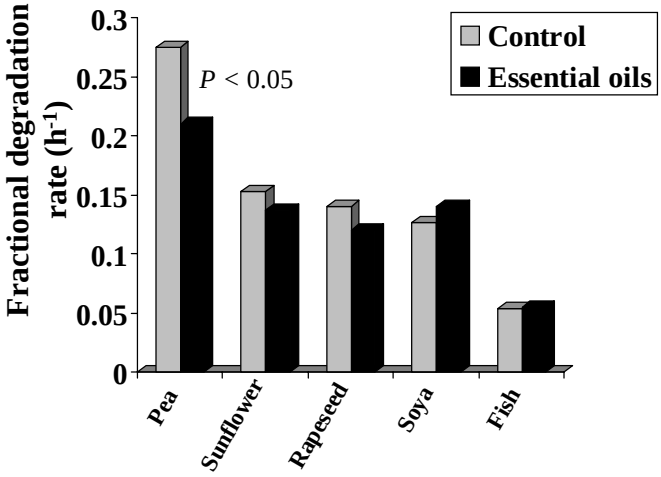
Wallace *et al.* (2002) suggested that the effect of Crina was most obvious with more rapidly degraded protein sources and little to no effect on degradation would be observed with relatively recalcitrant substrates such as fishmeal (Figure 3). Our most recent data (Duval *et al.*, 2006) suggests the same is true with starch-rich substrates, since Crina reduced the rate of degradation of rapidly fermented rolled barley grain but had little effect on the degradation of more resistant cut maize grain.

McIntosh *et al.* (2003) and Newbold *et al.* (2004), using purified substrates, failed to find any effect of Crina on proteolytic and deaminative activity in the rumen of either sheep or cattle. However, Wallace *et al.* (2002) reported that the fractional degradation rate of pea protein measured using the *in vitro* inhibitor method described by Broderick (1978) was decreased in rumen fluid from sheep receiving Crina (Figure 3), whilst Castillejos *et al.* (2006) found a decrease in peptide N accumulation in a rumen simulating fermentor supplemented with Crina consistent with a decrease in proteolysis. The most obvious difference between the trials of McIntosh *et al.* (2003), Newbold *et al.* (2004) and the studies of Wallace *et al.* (2002), and Castillejos *et al.* (2006), is the complexity of the substrate. As noted below one suggested action of Crina is to alter the attachment and colonisation of plant material entering the rumen and this is more likely to affect the breakdown of complex as opposed to soluble protein sources.

Whilst Crina did not effect proteolytic or peptidolytic activity measured with soluble substrates it did cause a 25% decrease in amino acid deamination (Newbold *et al.*, 2004). Subsequently, the decrease in deamination was traced to a specific inhibition of the activity of a

limited group of bacteria within the rumen, the "hyper-ammonia-producing bacteria" (McIntosh *et al.* 2003, see below). This group of bacteria are present in the rumen in low numbers but may be responsible for up to 50% of the deamination therein (Russell *et al.*, 1991). However, the breakdown of particulate protein and starch supplements in dacron bags suspended in the rumen would be unlikely to be affected by a change in amino acid deamination, suggesting that Crina is likely to influence rumen metabolism by more than a single mechanism.

Figure 3. Influence of dietary essential oils on the rate of breakdown of different protein supplements in ruminal fluid *in vitro*



McIntosh *et al.* (2003) found that some, but not all, pure cultures of rumen bacteria could adapt to grow in the presence of Crina when introduced in a stepwise fashion to the presence of increasing concentrations of the oil. However, the extent of the adaptation was small (an average increase, in the concentration of Crina need to inhibit growth by 50%, of 0.8 fold in cultures introduced to the essential oils via stepwise adaptation, and a maximum adaptive response of a 3 fold decrease in sensitivity with *Lactobacillus casei*, McIntosh *et al.*., 2003).

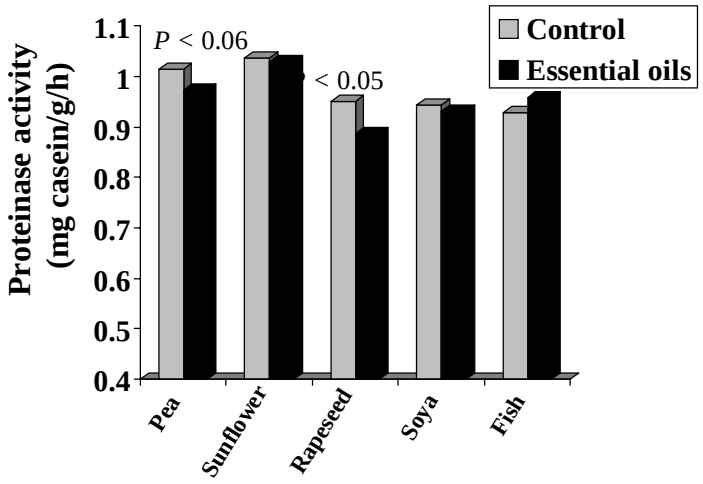
Despite the lack of adaptive response to Crina in the study of McIntosh *et al.* (2003), bacteria did significantly differ in their sensitivity to Crina, with the growth of *Clostridium sticklandii* decreased by 50% at a concentration of 36 mg/l whilst *Streptoccocus bovis* growth was unaffected until Crina concentrations reached 240 mg/l. However, this range in sensitivities is far smaller than had previously been observed with ionophoric antibiotics (the growth of *Eubacterium ruminantium* was decreased by 50% with monensin at 0.03 mg/l whilst *Megaspherea elsdenii* was unaffected by monensin concentrations in excess of 16 mg/l). Indeed, when Crina was added to the rumen in high concentrations (20 times more than that

recommended dose), all fermentation was inhibited suggesting that it is possible to move from a selective effect on the rumen microbial population to a more general inhibition if essential oils are added in excess (Fernandez *et al.,* 1997).

Nevertheless, accumulating information is starting to suggest that Crina has a selective effect on bacterial numbers in vivo. Crina had no effect on total bacterial growth in the rumen (Newbold *et al.*, 2004), however McEwan *et al.* (2002a) found that the numbers of "hyper ammonia producing (HAP) bacteria" determined using selective culture media decreased in sheep receiving Crina; consistent with the metabolic effect reported above. The diversity of HAP bacteria visualised using SSCP to visualise 16 S rDNA amplicons produced from the rumen fluid from the same sheep using primers to amplify the clostridial HAP bacteria also appeared to be reduced by Crina (McEwan *et al.* 2002a).

One of the major inconsistencies in explaining the action of essential oils in the rumen is the effective concentration in the rumen. Newbold *et al.* (2004) reported effects in the rumen of sheep when Crina was fed at 110 mg per day (assuming a 10 l rumen volume and an outflow rate of 10%/ h), peak Crina concentrations would be circa 28 mg/l and the mean concentration over the day would be circa 10 mg/l. Castillejos *et al.* (2006) have reported that in long term continuous culture fermentors 5 mg/l Crina increased VFA production and inhibited deamination. However, McIntosh *et al.* (2003) reported that Crina had it effects on rumen bacteria only at concentrations in the range of 35 to 360 mg/l, higher than that likely to be achieved *in vivo*. Castillejos *et al.* (2006) suggested that the rumen needs to be exposed to essential oils for a period measured in days (6 day for effect on VFA production and up to 28 day for effects on N metabolism to be evident) suggesting that the changes in the microbial population occur relatively slowly consistent with rumen concentrations of the oils being low. Alternatively McIntosh *et al.* (2003) speculated that local concentrations of essential oils, some of which are sparingly soluble, may be higher on the surface of ingested plant material. Indeed, starch granules have been found to bring out of solution and chelate specific components of essential oils (Misharina 2002; 2004; Terenina 2002), an observation that would tend to reinforce the hypothesis of McIntosh *et al.* (2003) and would imply a greater effect of Crina on starch rich substrates as hypothesised by Duval *et al.* (2004).

Figure 4. Influence of essential oils on proteinase activity extracted from protein supplements incubated for 4 h in nylon bags in the rumen of sheep



McEwan *et al.* (2002b, Figure 4) have shown attachment of rumen microbes to starch rich grains and protein is lower in sheep supplemented with Crina. Using 16S rDNA directed PCR-DGGE Duval *et al.* (2004) found that Crina altered the pattern of colonisation of, in particular starch rich, substrates as they entered the rumen suggesting that at least in part the effects of the essential oils might be mediated at the level of bacterial attachment and colonisation of plant material. Wallace (2004) expanded this observation to suggest the effects of essential oils might be regulated through effects on *Ruminobactor amylophilus* an amylolytic and proteolytic organism sensitive to Crina, which he speculated played a pivotal role in initiating attachment and colonisation to protein and starch rich substrates in the rumen. However, recently using real time PCR, we have confirmed that, whilst Crina does indeed effect the colonisation of starch-rich material in the rumen, it has no specific effect on *R. amylophilus* (Duval *et al.*, 2005).

Clearly essential oils are able to manipulate rumen fermentation. The reported effects are likely to be due to selective pressures exerted on different microbial populations, resulting in different bacterial numbers and subsequently different activities, in both the liquid and solid mileu of the rumen. Modern molecular profiling techniques are being employed to better describe the changes in the rumen microbial population and these together with metabolic studies should allow to us to develop new and enhanced rumen manipulators based on essential oils.

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