Effects of hops (Humulus lupulus L.) extract on volatile fatty acid production by rumen bacteria

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Abstract

Aims: To determine the effects of hops extract on in vitro volatile fatty acid (VFA) production by bovine rumen micro-organisms.

Methods and Results: When mixed rumen microbes were suspended in media containing carbohydrates, the initial rates of VFA production were suppressed by \( \beta \)-acid-rich hops extract. The rates of VFA production increased over extended incubations (24 h), and hops extract caused an increase in the propionate to acetate ratio. Hops extract inhibited the growth and metabolism of Streptococcus bovis, but Selenomonas ruminantium and Megasphaera elsdenii were not affected. Likewise, the propionate production of M. elsdenii/S. bovis co-cultures, but not M. elsdenii/S. ruminantium co-cultures, was decreased in the presence of hops extract.

Conclusions: These results are consistent with the hypothesis that the hops inhibit Gram-positive lactic acid bacteria (S. bovis), and the rumen microbial community requires a period of adaptation before normal VFA production resumes. Selenomonas bovis and S. ruminantium both produce lactate, which is the substrate for propionate production by M. elsdenii. However, S. ruminantium has an outer membrane, while S. bovis does not.

Significance and Impact of Study: The enhanced production of the gluconeogenesis precursor, propionic acid, provides further evidence that plant secondary metabolites from hops could be used to improve rumen fermentation.

Introduction

Ruminants are unique in that the first stage of digestion is carried out by symbiotic microbes that colonize the rumen (Russell 2002). When a ruminant consumes herbage, bacteria ferment the plant tissues and generate many products, including bacterial cells and volatile fatty acids (VFA). The microbial cells are an important nitrogen source because much of the plant protein and amino acids are degraded in the rumen. Microbial protein requires digestion in the gastric stomach and intestines, but VFA are absorbed directly through the rumen epithelium. The VFA are transported into the blood stream and undergo gluconeogenesis. In this way, the animal is rapidly provided with blood sugar, and the microbes are free of end-product inhibition.

A principle theory in rumen microbiology is that the activities of the microbes can be optimized just like any other fermentation (Weimer et al. 2009). This concept has been applied in a number of ways, and antimicrobial feed additives are one of the most successful (Van Nevel and Demeyer 1977). Ionophores (e.g. monensin, lasalocid) are a membrane-active class of antimicrobials that promote the growth of ruminants when they are included in the diet (Callaway et al. 2003). These compounds inhibit the hyperammonia-producing bacteria (HAB) that degrade amino acids (Chen and Russell 1989). They also mitigate rumen acidosis (Barley et al. 1983) and promote lower acetate to propionate ratios (Richardson et al. 1976).

Ionophores are made by bacteria, but plants also produce antimicrobial compounds (Nicholson and Hammerschmidt 1992). Commercial interest in alternatives
to antibiotics is increasing, and plant-based products, such as essential oils, are among the alternatives (Benchaa et al. 2008). A number of essential oils have been shown to decrease ruminal ammonia production (McIntosh et al. 2003; Newbold et al. 2004; Benchaa et al. 2006). It should be pointed out that both bacteria and protozoa conduct deamination, but it appears that the efficacy of essential oils is because of antibacterial rather than antiprotozoal activity (Newbold et al. 2004; Benchaa et al. 2007). When inhibitors are used to decrease ammonia production, the effects of VFA production must also be considered. In this case, various essential oil components were shown to have differing effects. For instance, cinnamaldehyde and garlic oil decreased the acetate to propionate ratio in vitro (Busquet et al. 2005). However, eugenol had the opposite effect under similar conditions (Castillejos et al. 2006).

Hops (Humulus lupulus L.) have been used in brewing for centuries, with the original purpose of this bitter herb being to preserve the beer (Sakamoto and Konings 2003). The flowers, which are typically called ‘cones’, contain several prenylated phloroglucinol derivatives that have antimicrobial properties (Zuurbier et al. 1995). These compounds are typically divided into the heat-labile α-acids (humulone, cohumulone, adhumulone) and the more stable β-acids (lupulone, colupulone, adlupulone). It was recently demonstrated that both hops cones and lupulone-rich, hops β-acid extract inhibited the growth and ammonia production of ruminal HAB (Flythe 2009). However, the effects of hops on VFA production were not determined. The following experiments were conducted to evaluate the effects of hops on VFA production from carbohydrates.

Materials and Methods

Animals and diets

Four Holstein steers (approx. 2 year, 275 kg ± SD) fitted with ruminal cannulae were maintained at the University of Kentucky Animal Research Center. The diet was composed of 85% corn silage, 12% cracked corn and 3% soybean meal. A mineral mix and fresh water were provided free choice. The University of Kentucky animal care and use committee approved all animal procedures (protocol 00674A2004).

Medium composition and culture conditions

Basal medium contained (per litre) 240 mg K₂HPO₄, 240 mg KH₂PO₄, 480 mg (NH₄)₂SO₄, 480 mg NaCl, 100 mg MgSO₄·7H₂O, 64 mg CaCl₂·2H₂O and 600 mg cysteine hydrochloride. The initial pH was adjusted to 6.7 by adding NaOH. The medium was autoclaved to make anaerobic and cooled under O₂-free CO₂, and 4.0 g Na₂CO₃ was added. The medium was anaerobically transferred to Hungate tubes (Bellco Biotecnology, Vineland, NJ, USA) or serum bottles (160 ml) and capped with butyl rubber stoppers before sterilizing in an autoclave (Russell et al. 1988). Glucose or sodium lactate were prepared as anaerobic solutions and aseptically added to basal media with tuberculin syringes as indicated. Cultures were routinely transferred in basal medium. All incubations were performed at 39°C in an incubator or shaking water bath as indicated. The propylene glycol-based hops extract contained 30% (w/w) β-acids as determined by the manufacturer (S.S. Steiner, Inc., Yakima, WA, USA).

Rumen microbe suspensions

Rumen contents were removed and squeezed through four layers of cheesecloth. The fluid was collected into an Erlenmeyer flask (4 l) and incubated (39°C) until three phases separated (45 min). Anaerobic conditions were maintained during separation by endogenous gas production. Subsequently, all samples were handled under a stream of O₂-free CO₂, and all vessels were purged with air with CO₂. The centre phase was removed with a pipette and subjected to low-speed centrifugation (100 g, 10 min) to remove plant particles and protists. The supernatants were transferred to new bottles and subjected to centrifugation (25 600 g, 10 min). The pellets were resuspended in basal medium, and low-speed centrifugation was repeated (100 g, 10 min). The supernatants were pooled into a water-jacketed (39°C) glass vessel (500 ml) that was sparged with CO₂. The cell suspension (~20.0 OD, 200 mg cell protein OD⁻¹ L⁻¹, pH 6.7) contained no visible plant fibre and few protozoa. The cell suspension (50 ml) was dispensed into serum bottles that contained glucose, cellobiose, soluble starch, crystalline cellulose (200 mg each) and a CO₂ headspace. Hops extract was added as indicated and the starting concentrations based on previous work (Flythe 2009). The bottles were incubated in a shaking water bath (39°C, 180 rpm). Samples were clarified by centrifugation and frozen (~20°C). Initial metabolic rates (Fig. 1) were determined in the first 30 min (5 min between samples), and the values reported in Table 1 were obtained after 24-h incubation.

Pure strains

Megasphaera elsdénii B159 and Selenomonas ruminantium HD4 were obtained from the culture collection of Herbert J. Strobel, University of Kentucky. Streptococcus bovis JB1 was obtained from the culture collection of James B. Russell, Cornell University.
Growth experiments

Growth experiments were conducted with basal medium (10 ml, pH 6.7) in Hungate tubes. Hops extract was added to the tubes with a Hamilton syringe. The minimum concentration that achieved the maximum observed effect during mixed rumen microbe experiments was used in all pure culture experiments (30 ppm β-acid). Each tube was inoculated (1%/v) from stationary phase 16 h cultures and incubated (39°C). Optical density was determined by spectrophotometry (600 nm). Samples for VFA analysis were clarified by centrifugation and frozen immediately. The hops extract was not sterilized, but when sterile media were amended with hops extract, growth was not observed even if incubation periods were greater than 96 h. The propylene glycol base affected neither growth nor product formation.

Table 1  in vitro volatile fatty acid production by mixed rumen microbes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acetate (mmol L⁻¹)</th>
<th>Propionate (mmol L⁻¹)</th>
<th>Lactate (mmol L⁻¹)</th>
<th>Butyrate (mmol L⁻¹)</th>
<th>A : P ratio</th>
<th>Total VFA (mmol L⁻¹)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>52 (5)</td>
<td>56 (8)</td>
<td>0 (0.3)</td>
<td>16 (10)</td>
<td>0.95 (0.15)</td>
<td>119 (16)</td>
<td>5.2 (0.2)</td>
</tr>
<tr>
<td>Hops extract (30 ppm β-acid)</td>
<td>38 (3)**</td>
<td>67 (10)</td>
<td>0</td>
<td>11 (8)</td>
<td>0.58 (0.07)**</td>
<td>116 (9)</td>
<td>5.4 (0.1)</td>
</tr>
<tr>
<td>Hops extract (60 ppm β-acid)</td>
<td>36 (3)**</td>
<td>74 (6)*</td>
<td>0</td>
<td>11 (10)</td>
<td>0.48 (0.02)**</td>
<td>121 (15)</td>
<td>5.4 (2)</td>
</tr>
</tbody>
</table>

Acid concentrations in 24-h cell suspension are expressed in mmol L⁻¹ and indicate the means of four replicates (n = 4 steers). Standard deviations are shown in parentheses, and asterisks indicate values are statistically different from the control (**p < 0.005).
catabolism, acetate production and propionate production decreased when hops extract was added to the cell suspensions. The maximum response was observed at approximately 30 ppm β-acid, and even concentrations as great as 300 ppm failed to decrease metabolism further (data not shown). There was little effect on butyrate production in any case (Fig. 1). Longer incubations (24 h) revealed similar total VFA production for the control or hops extract treatments (Table 1). However, lower concentrations of acetate were observed when hops extract was used. Furthermore, propionate production was stimulated when hops extract was added to a final concentration of 60 ppm β-acid. Consequently, the ratio of acetate to propionate was lower in the hops treatments.

When the basal medium was inoculated with pure cultures of Selenomonas ruminantium or Megasphaera elsdenii, neither the growth rates nor the cell yields were affected by the addition of hops extract (Fig. 2a). However, the growth of Streptococcus bovis was inhibited in the presence of hops extract (Fig. 2b). Additionally, hops extract decreased the glucose catabolism and lactate production of S. bovis even in the absence of growth (Fig. 3). When the basal medium was inoculated with both S. ruminantium and M. elsdenii, the co-culture grew and both lactate and propionate were produced (Fig. 4a). The addition of hops extract (30 ppm β-acid) had little effect on the metabolism of the co-culture (Fig. 4b). Megasphaera elsdenii was also co-cultured with S. bovis. In this latter co-culture, S. bovis produced lactate more rapidly than S. ruminantium, and the lactate was gradually converted to propionate by M. elsdenii (Fig. 5a). However, the production of the fermentation acids was inhibited by hops extract (Fig. 5b).

**Discussion**

Antimicrobial compounds act on some aspect of the sensitive organism’s physiology. When an exogenous chemical comes into contact with a cell, the first structure encountered is the outermost component of the cell envelope and this is often the site of antimicrobial action (Bryskier 2005). When the cell envelope loses integrity, osmotic and pH homoeostasis are compromised and the
concentration gradients that drive metabolism dissipate (Harold 1986). Ionopores, polymyxins, bacteriocins and β-lactams are well-known categories of antimicrobials that act on either bacterial cell walls or cell membranes (Bryskier 2005). Plant essential oils normally contain a number of antibacterial components; many of which also act on the cell membrane (Burt 2004).

The Reinheitsgebot, or German Purity Law, of 1516 identified hops (Humulus lupulus L.) as a principal ingredient of beer (Sakamoto and Konings 2003). The herb was originally added as a preservative, and the antimicrobial components are commonly called α-acids (humulone and related compounds) and β-acids (lupulone and related compounds). Like the aforementioned antimicrobial compounds, lupulone is membrane active (Teuber and Schmalreck 1973). When lupulone comes into contact with the cell membranes of Gram-positive bacteria, integrity is lost and metabolism is inhibited. Most of the lactic acid bacteria (LAB) that contaminate beer are Gram positive, but Megasperha cerveisae is an exception (Engelmann and Weiss 1985). Like these LAB, M. cerevisae belongs to Phylum Firmicutes, which is often called the ‘up G+C Gram-positive bacteria’ (Doyle et al. 1995). However, M. cerevisae is a member of the Family Veillonellaceae. It, like many species in this family, is
protected by an outer membrane, which prevents uptake of the crystal violet stain and some inhibitors, such as the hops β-acids.

The rumen bacterium *Megasphaera elsdii* is a close relative of *M. cerevisiae* (Eladen et al. 1951; Rogosa 1971; Engelmann and Weiss 1985; Doyle et al. 1995). *Megasphaera elsdii* also stains Gram negative and has an outer membrane that decreases sensitivity to ionophores (Dennis et al. 1981). The current results also indicate that *M. elsdii* was less sensitive to β-acids in the concentrations used. This latter observation is important because *M. elsdii* is responsible for as much as half of the propionate production in the rumen (Russell 2002). Unlike many fermentative bacteria, *M. elsdii* does not occupy a carbohydrate-fermenting niche (Russell and Baldwin 1978). Instead, ruminal LAB first ferment the carbohydrate to lactate. Then, *M. elsdii* converts the lactate to propionate via the acylate pathway (Gottschalk 1986).

When *M. elsdii* was co-cultured with *Streptococcus bovis*, the fast-growing *S. bovis* rapidly converted all of the glucose to lactate. As *M. elsdii* grew, the lactate was gradually converted to propionate. However, both lactate and propionate productions were inhibited by hops extract. *Streptococcus bovis* has a Gram-positive cell envelope (Dennis et al. 1981), so the hypothesis that it was sensitive to hops β-acid was reasonable. The experiments that employed pure cultures of *S. bovis* revealed that the growth and metabolism of the streptococcus were inhibited by hops extract. These observations were consistent with previous studies, which reported that nonruminant Gram-positive bacteria, e.g. *Listeria monocytogenes* (Larson et al. 1996) and *Clostridium perfringens* (Siragusa et al. 2008), are sensitive to hops β-acid. In this way, hops could have a negative impact on ruminal propionate even if propionate producers, like *M. elsdii*, are not sensitive.

Fortunately, not all ruminal LAB are Gram positive. *Selenomonas ruminantium* is also a member of Family Veillonellaceae and has an outer membrane (Kingsley and Hoeniger 1973). *Selenomonas ruminantium* produces lactate and primarily occupies a carbohydrate-fermenting niche (Russell and Baldwin 1978). Our results indicate that *S. ruminantium* was not sensitive to hops β-acids, and when it was co-cultured with *M. elsdii*, there was no effect on propionate production. These results support the hypothesis that a Gram-negative cell envelope protects rumen bacteria from the antimicrobial activity of β-acids.

The composition of the rumen flora is still a topic of debate, but it is clear that the members of this community are phylogenetically and physiologically diverse (Edwards, et al. 2004; Kobayashi 2006; Stevenson and Weimer 2007). The unequal sensitivities of Gram-positive and Gram-negative cell envelopes offer a plausible explanation for the effect of hops extract on mixed rumen microbes. When the mixed rumen microbes were incubated in the presence of hops extract, the rates of fermentation initially decreased by almost half. It is well known that *S. bovis* flourishes in carbohydrate-excess conditions (Russell and Hino 1985), and it seems likely that hops extract decreased the metabolism of *S. bovis* and similar organisms. Furthermore, it appears that over a longer period of time (24 h), *S. ruminantium* and similar bacteria took advantage of the decreased competition and utilized a greater proportion of the substrate. The result was a greater proportion of the carbohydrates were converted to propionate rather than acetate. If hops have a similar effect in vivo, more of this valuable three-carbon acid would be available to the animal.

As the European Union disapproved the use of antibiotics to promote animal growth, there has been considerable interest in finding alternatives to ionophores (Russell and Houlihan 2003; Wallace 2004). Ionophores have several positive aspects that are exploited in ruminant production: (i) kill intestinal parasites (Croft 1997), (ii) mitigate bloat and acidosis (Dennis et al. 1981; Bartley et al. 1983), (iii) promote a favourable acetate to propionate ratio (Richardson et al. 1976) and (iv) inhibit hyperammonia-producing bacteria, which increases 'bypass' amino acids (Russell and Strobil 1989). However, ionophores are produced by bacteria; thus, they fit the traditional definition of antibiotic. While there is little evidence that ionophores contribute to clinical antibiotic resistance (Russell and Houlihan 2003; Edrington et al. 2006), the broad toxicity of these compounds remains an issue (Oehmke and Pickrell 1999).

Hops cones are nontoxic, and humans have consumed them for centuries. In fact, clinical researchers are interested in the plant for properties ranging from tumour suppression and anti-inflammatory activity to mild sedative effects (Magalhaes et al. 2009). It was previously shown that hops inhibited the growth and ammonia production of ruminal HAB (Flythe 2009), and the current study indicates a favourable effect on propionate production. These results suggest that hops warrant evaluation as a feed additive. In vivo studies with ruminants are needed to determine whole-animal response and possible economic benefit to producers. Animal studies are particularly important in this case because in vivo studies with essential oils have demonstrated that effective concentrations can be difficult to achieve in production situations (Benchaar et al. 2007). Moreover, most of what we know about rumen microbiology focuses on energetically important primary metabolites (e.g. carbohydrates). This study illustrates the impact that a plant secondary metabolite can have on the microbial physiology and ecology in the rumen.
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