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Hops (*Humulus lupulus*) ß-Acid as an Inhibitor of Caprine Rumen Hyper-Ammonia-Producing Bacteria *In Vitro*

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ABSTRACT

Antimicrobial plant secondary metabolites increase rumen efficiency and decrease waste products (i.e. ammonia, methane) in some cases. A promising source of bioactive secondary metabolites is the hops plant (*Humulus lupulus* L.), which produces ß-acid, a suite of structurally similar, potent antibacterial compounds. The efficacy of hops has been shown in bovines. Additionally, the ß-acid mechanism of antimicrobial action was determined on rumen bacteria of bovine origin. The objective of the current study was to determine the effect of hops ß-acid on amino acid degradation and ammonia production by goat rumen bacteria. The growth of two rumen hyper-ammonia-producing bacteria of caprine origin (*Peptostreptococcus* spp. BG1 and BG2) was inhibited by ß-acid (≥ 45 ppm and ≥ 4.5 ppm, respectively) when either amino acids or peptides were the growth substrate. Uncultivated, mixed rumen microorganisms harvested from goats produced approximately 35 mM ammonia from amino acids and 50 mM ammonia from peptides during 24 h incubation. The addition of ß-acid reduced the final ammonia concentration, and there was a dose-response relationship between the ß-acid concentration and the ammonia concentration. Peptide catabolism was more sensitive to ß-acid inhibition than free amino acid catabolism to ß-acid inhibition because as little as 3 ppm resulted in less ammonia production than the control ($P < 0.05$). These results demonstrate that hops ß-acid is effective against caprine HAB and suggest that hops could be useful in controlling wasteful metabolic processes in the caprine rumen.

Keywords: antibiotic alternative, ammonia, beta-acid, bypass protein, feed efficiency, hops, goat, hyper ammonia producing bacteria, inhibition, ionophore, lupulone, phytochemical, phytoprotectant, plant secondary metabolite, rumen

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INTRODUCTION

A symbiotic relationship with the rumen microflora is the adaptation that gives ruminants metabolic access to fibrous plant tissues (Russell and Rychlik, 2001). This dense community of microorganisms includes bacteria, archaea, fungi and protists, which collectively degrade fiber and convert the resulting sugars to fermentation acids and gases. The fermentation acids can be transported across the rumen epithelium and utilized by the ruminant host, and the gases, which are essential for reducing equivalent cycling in the microorganisms, are eructated (Russell and Rychlik, 2001).

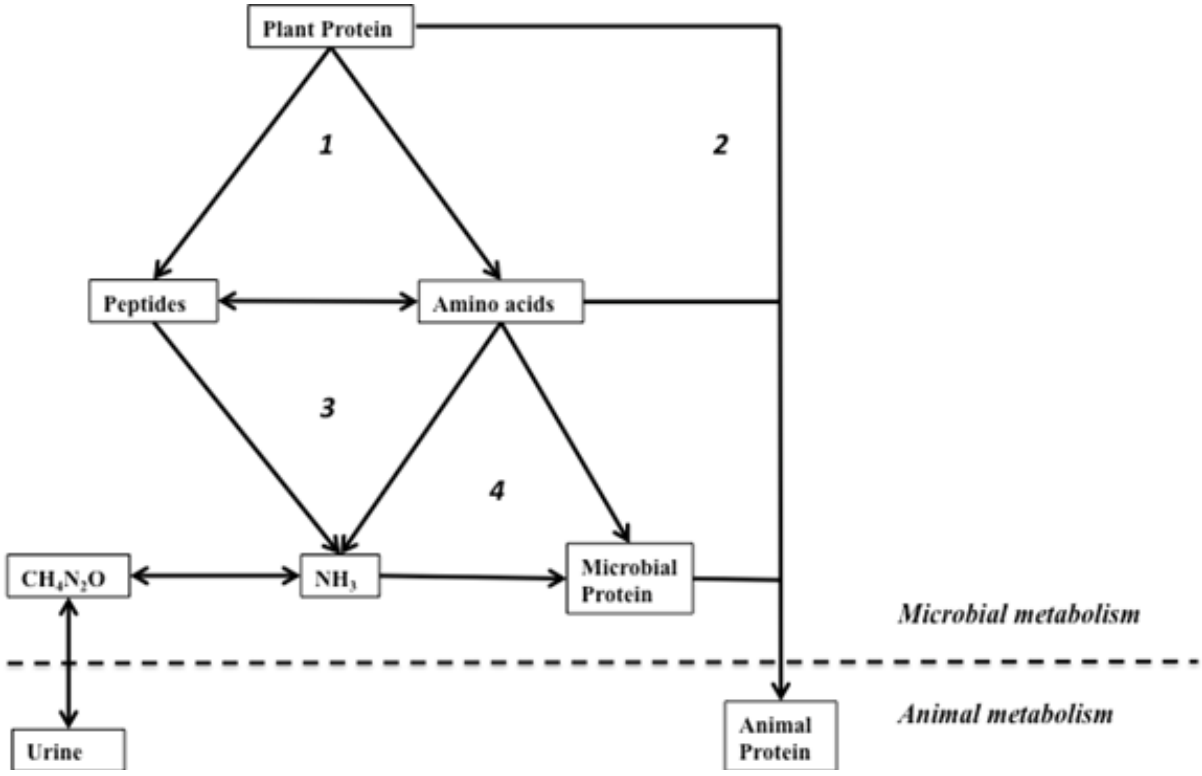
In addition to carbohydrates, plant proteins, peptides and amino acids are also catabolized. Rumen proteolysis and deamination (diagrammed in Figure 1) are not inherently harmful to ruminants. In fact, some cellulolytic bacteria, such as *Ruminococcus albus*, require the aromatic- and branched-acids produced by deamination of particular amino acids (Caldwell and Bryant, 1966). Bacteria can assimilate some of the ammonia allowing the ruminant host to utilize the resulting microbial protein (Satter and Slyter, 1974). The ruminant converts excess ammonia to urea, some of which can be recycled into the rumen, and goats are exceptionally good at this process (Kohn *et al*., 2005). Ruminants have lower nitrogen clearance rates than non-ruminants and goats tend to be lower than other ruminants (Kohn *et al*., 2005). The urea transport rate through the rumen epithelum and kidney increases as nitrogen in the diet decreases (Muscher *et al*., 2010; Starke *et al*., 2014). When urea is not recycled it is lost in the urine. The loss of feed amino-nitrogen in the urine is an economic loss to the rancher or dairyman, and a source of environmental pollution (Tedeschi *et al*., 2003). Additionally, amino acid fermentation results in the production of indole and related compounds, which can cause flavor notes in milk that are undesirable to some consumers (Attwood *et al*., 2006).

Proteolysis is carried out by a variety of rumen microorganisms, but most of the ammonia production from the resulting peptides and amino acids is attributable to ciliate protozoa (Abou Akkada and El-Shazly, 1964) and to a guild of bacteria termed Hyper Ammonia-producing Bacteria, HAP or HAB (Russell *et al*., 1988). The contribution of each type of microorganism to rumen ammonia depends on host, diet and other factors. However, ammonia production in defaunated ruminants supports the hypothesis that bacteria are sufficient for amino acid degradation (Abou Akkada and El-Shazly, 1964). Fortunately, both HAB and ciliates are susceptible to ionophores, which are included in many ruminant diets (Chen and Russell, 1989; Dennis *et al*., 1986). Ionophores (e.g. monensin, lasalocid) inhibit ammonia production, increase feed efficiency, promote growth and decrease amino-nitrogen release into the environment (Tedeschi *et al*., 2003; Callaway *et al*., 2008).

In spite of the production and environmental advantages and limited clinical utility of ionophores, the fact that they are antibiotics has led to some resistance to their use in industrialized nations (Russell and Houlihan, 2003). For more than a decade, researchers have looked for phytochemical alternatives to control ammonia production and other aspects of rumen fermentation (Wallace, 2004). Some phytochemicals, like those derived from the hops plant (*Humulus lupulus*) even have an ionophore-like mechanism of action on HAB (Flythe 2009). Antimicrobial phytochemicals have the added advantage of production from local botanical sources. For example, hops might be an appropriate choice in northern Europe or northern North America where this plant is cultivated. Spearmint might be a better local source of phytochemicals in the Middle East, and spearmint essential oil has been shown to have antimicrobial activity on HAB from Mehraban sheep (Taghavi-Nezhad *et al*., 2013).

The use of regionally available antimicrobial phytochemicals in ruminant production raises the question of which animals and microorganisms should be used to test these compounds. Most rumen microbiology research has been conducted on dairy cows in North America and Western Europe, but goats are the predominant domestic ruminants in many parts of the world (FAO, 2011). Goat production increased more than other species, except poultry, in both developed and developing countries (FAO,

Figure 1. Simplified schematic of amino-nitrogen cycling the rumen. Processes are labeled: 1- Proteolysis by proteolytic microorganisms, 2- "By-pass" protein that is not deconstructed in the rumen, 3- Deamination by HAB and other microorganisms, 4- Assimilation of ammonia and amino acids by microorganisms for anabolic purposes.



2011). The goat industry in the US increased by 23% between 1997 and 2007, this increase was 116% for the 12 Southeastern states (USDA 2004 and 2009; USDA-APHIS 2005), which is where the current work was conducted. The size of the industry steadied between 2002 and 2012 goat numbers increased by just over 1% in these same states (USDA 2004 and 2014). The purpose of the study was to test the effect of a model antimicrobial phytochemical on: 1) the growth of caprine HAB, and 2) ammonia production by uncultivated rumen microorganisms from goats. Hops ß-acid is a suite of structurally similar compounds (lupulone, colupulone, adlupulone) that are potently inhibitory to Gram-positive bacteria. It was selected as the model antimicrobial phytochemical because inhibition and the mechanism of action have already been determined on bovine HAB (Flythe 2009).

MATERIALS AND METHODS

Animals and diet

All animal husbandry and procedures were approved by the University of Kentucky care and use committee. Rumen fistulated Kiko goat wethers (n=4; 2 y, 40-50 kg) were used as rumen digesta donors. They were maintained on pasture in a herd of Kiko goats at the University of Kentucky's Research Farm, Lexington, Kentucky, USA. The botanical composition of the pasture is shown in Table 1. The herd was supplemented with 1.0 kg $head^{-1}$ d^{-1} orchard grass hay (*Dactylis glomerata*; 18% crude protein), and 0.25 kg $head^{-1}d^{-1}$ soya-based supplement (16% crude protein; Southern States Cooperative, Richmond, Virginia, USA). Water and a mineral mixture (Southern States Cooperative, Richmond, Virginia, USA) were provided free choice.

Table 1. The botanical composition of the pasture

	Pasture Component	Percent
Forage		
Tall Fescue	*Lolium arundinaceum* (Schreb.) Darbysh.	44.4
Orchardgrass	*Dactylis glomerata* L.	14.9
White Clover	*Trifolium repens* L.	14.5
Kentucky Bluegrass	*Poa pratensis* L.	8.9
Red Clover	*Trifolium pratense* L.	3.3
Bermudagrass	*Cynodon dactylon* (L.) Pers.	1.4
Weeds*		12.6

* Weed species include: *Rumex crispus* L., *Taraxacum officinale* F.H. Wigg., *Plantago coronopus* L., *Solanum carolinense* L., *Convolvulus arvensis* L., *Digitaria* sp., *Ambrosia artemisiifolia* L.

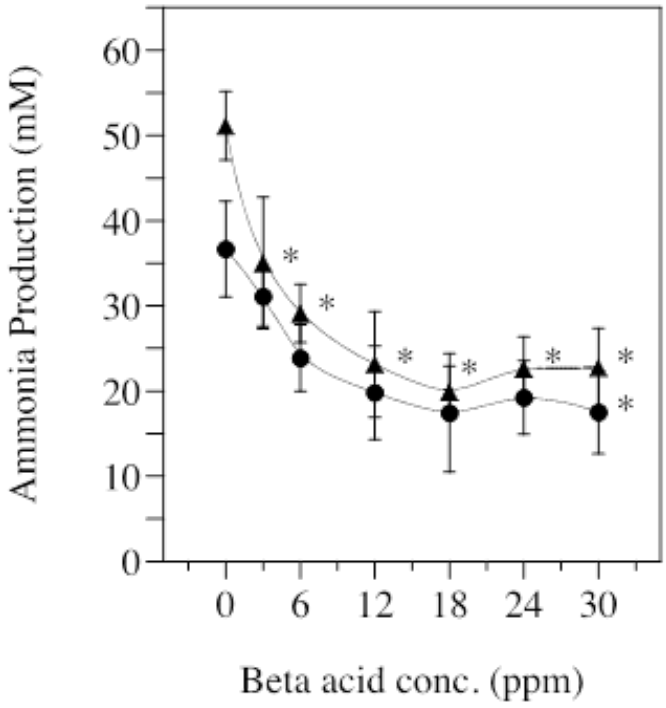
Bacterial strains and media composition

The isolation and characterization of the HAB cultures (*Peptostreptococcus* spp. BG1 and BG2) used in these experiments were previously reported (Flythe and Andries, 2009). The HAB medium contained (per liter): 240 mg K_2HPO_4, 240 mg KH_2PO_4, 480 mg NaCl, 480 mg Na_2SO_4, 64 mg $CaCl_2{\cdot}2H_2O$, 100 mg $MgSO_4{\cdot}7H_2O$, 600 mg cysteine hydrochloride, mineral and vitamin solutions as previously described (Russell *et al*., 1988). The initial pH was adjusted to 6.5 with 20% NaOH. The liquid was autoclaved to remove O_2 and cooled under O_2 -free CO_2. The buffer (4.0 g Na_2CO_3) was added before dispensing anaerobically and autoclaving again for sterility. Casamino acids and Trypticase (Fisher BioReagents, Fair Lawn, New Jersey, USA) were prepared separately using anaerobic technique, as described above. They were aseptically added when indicated (15 mg ml^{-1} final concentration).

Pure culture growth experiments

The HAB medium was amended with Casamino acids or Trypticase (15 mg ml^{-1}). Overnight *Peptostreptococcus* spp. BG1 and BG2 cultures were used as the inocula (10% v/v). "Beta-Bio" hops extract was added to the media, vigorously mixed and serially diluted with to achieve the concentration indicated. The propylene glycol-based extract contained 45% ß-acid and no detectable ß-acid, as reported by the manufacturer (S.S. Steiner, Inc., Yakima, Washington, USA). The concentrations were verified by HPLC (Dionex; Sunny Vale, California). The Phenomineex 250×4.6 mm Luna 5u C18 column (Torrance, California) was 30˚C. The mobile phase was 80% methanol, 20% water, pH 2.5. Eluting compounds were detected by UV absorbance (314 nm). The standard curve was generated using hops standards obtained from the American Society of Brewing Chemists (St. Paul, Minnesota). The limits of detection were 3.3 ppm and 4.1 ppm for colupulone and adlupulone, respectively. The extract was not autoclaved, but was added to uninoculated media and incubated as a control. Controls for the effect of the propylene glycol carrier were also performed. The incubations were conducted in a shaking incubator (150 rpm, 39˚C). Growth was determined by optical density at 24 and 48 h (600 nm).

Figure 2. Effect of hops extract on ammonia production by uncultivated microorganisms from the goat rumen. Ammonia concentrations from peptides (squares) or amino acids (circles) after 24 h incubation (39 ° C) are shown. Error bars indicate standard error of the mean. Asterisks indicate treatments that are different than the 0 ppm control ($P < 0.05$).



Ammonia production by uncultivated rumen microorganisms

The rumen digesta (approx. 0.5 kg) was collected from each goat individually (n=4) and transported back to the laboratory in an insulated, airtight container within 30 min. Rumen contents were removed and filtered through four layers of muslin cheesecloth into centrifuge bottles. The fluid was subjected to low-speed centrifugation (100 x g, 10 min) to remove feed particles. The supernatants were transferred to new bottles and subjected to high-speed centrifugation (25,600 x g, 10 min) to harvest the microorganisms. The pellets were resuspended in HAB medium without a growth substrate, and low-speed centrifugation was repeated to wash the cells. The pellets were resuspended in HAB medium, pooled into a glass vessel (500 ml), and sparged with O_2-free CO_2. Microscopic examination revealed that the cell suspension (approximately 15.0 OD, pH 6.7) contained no visible plant fiber and few protozoa. The cell suspension was dispensed into serum bottles that contained CO_2. Growth substrates (Trypticase or casamino acids, 15 mg ml^{-1}) and hops extract were added as indicted. The bottles were incubated in a shaking water bath (39°C 150 rpm). Samples were clarified by centrifugation and frozen (-20°C) until ammonia analysis. Ammonia concentrations were determined with a colorimetric method (Chaney and Marbach, 1962).

Replication and statistics

The pure culture growth experiments were repeated three times with identical results. The *in vitro* ammonia production experiments were repeated four times using cell suspensions from a different goat each time. The data were subjected to an analysis of variance with Tukey's test *post hoc*. Results were considered significant when $P < 0.05$.

RESULTS

Two caprine HAB pure cultures (*Peptostreptococcus* spp. BG1 and BG2) grew when either free amino acids (Casamino acids) or peptides (Trypticase) were the sole growth substrate, and produced ammonia at rates as great as 600 nmol mg cell protein^{-1}min^{-1} (data not shown). Hops extract inhibited growth of the two HAB cultures when the ß-acid concentrations were ≥ 45 ppm and ≥ 4.5 ppm, respectively. The inhibitory concentration was not changed if amino acids or peptides were used as the growth substrate.

Cell suspensions of uncultivated rumen microorganisms from goats produced approximately 35 mM ammonia from free amino acids (Casamino acids) and 50 mM ammonia from peptides (Trypticase) in 24 h (Fig. 2). The addition of hops extract decreased the total ammonia produced during the incubations. Ammonia from amino acids was not significantly less than the control unless the ß-acid concentration was 30 ppm or greater. When the ß-acid concentration was 30 ppm, ammonia production from amino acids was 30% less than the control ($P < 0.05$). When peptides were the growth substrate as little as 3 ppm ß-acid caused a decrease in ammonia production ($P < 0.05$). Ammonia production from peptides was decreased by 50% relative to the control when the ß-acid concentration was 12 ppm or greater ($P < 0.05$).

DISCUSSION

Several other experiments have been performed to assess the usefulness of hops as a feed additive, and all of these employed cows or microorganisms of bovine origin. Previous *in vitro* experiments indicated that hops ß-acid did not inhibit bovine ruminal protozoa (Schmidt *et al.*, 2006). However, hops ß-acid did inhibit *Streptococcus bovis* and decreased the acetate: propionate ratio during *in vitro* experiments (Flythe and Aiken, 2010). *S. bovis* is proteolytic (Russell *et al.*, 1981) thus, the results of the latter study is consistent with recent work by Lavrenčič and colleagues (2013), who determined that two varieties of hops decreased proteolysis by bovine rumen microorganisms. Wang and colleagues (2010) showed the *in vivo* efficacy of hops in a feeding trial. In that experiment, the average daily gain of steers was improved by the inclusion of hops cones in the diet.

The results of the current study indicate that hops, specifically the ß-acid component, can decrease ammonia production by microorganisms from the goat rumen. Inhibition of caprine HAB in pure culture demonstrated that the decrease in ammonia could be accomplished by antimicrobial of the ß-acid on HAB. These results are consistent with previous work that showed antimicrobial action on three bovine HAB species (Flythe, 2009).

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