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*Brief Report*

# Activity of Cannabidiol on Ex Vivo Amino Acid Fermentation by Bovine Rumen Microbiota

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**Abstract:** Amino-acid-fermenting bacteria are wasteful organisms within the rumens of beef cattle that remove dietary amino nitrogen by producing ammonia, which is then excreted renally. There are currently no on-label uses for the control of this microbial guild, but off-label use of broad-spectrum antimicrobials has shown efficacy, which contributes to antimicrobial resistance. Plant-derived antimicrobials supplemented into the diets of cattle may offer worthwhile alternatives. This study sought to investigate the role of cannabidiol (CBD) as a terpenophenolic antimicrobial. Ex vivo cell suspensions were harvested from the rumen fluid of Angus × Holstein steers in non-selective media with amino acid substrates. The suspensions were treated with five concentrations of CBD (860 μg $mL^{-1}$–0.086 μg $mL^{-1}$) and incubated (24 h), after which ammonia production and viable number of cells per substrate and treatment were measured. The data demonstrated a ~10–15 mM reduction in ammonia produced at the highest concentration of CBD and negligible changes in the viable number of amino-acid-fermenting bacteria. CBD does not appear to be a biologically or economically viable terpenophenolic candidate for the control of amino acid fermentation in beef cattle.

**Keywords:** amino acid fermentation; cannabidiol; ex vivo cell suspensions; hyper-ammonia-producing bacteria; bovine rumen fluid



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## 1. Introduction

The innate commensal microflora within ruminants allows these animals to amass nutrients from a great range of carbohydrates, i.e., fiber, which enables them to productively utilize a wide variety of forages [1]. However, some rumen organisms produce metabolic end-products that cannot be directly utilized by the animal, such as ammonia. A portion of the ammonia produced by these microorganisms is assimilated into microbial protein and, therefore, available to the animal, but the vast majority is converted to urea and excreted with the urine [2,3]. This process necessitates the supplementation of protein in feed, often an expensive part of the diet, to subvert this loss by rumen microorganisms and to maintain animal performance.

Ammonia production in the rumen proceeds in two distinct steps: proteolysis and deamination of amino acids. A variety of microorganisms within the rumen utilize proteolysis to obtain amino acids for anabolic synthesis of needed compounds, while few catabolically ferment amino acids to ammonia (hyper-ammonia-producing bacteria and generalists) [2,4]. Generalist bacteria occupy several niches and preferentially utilize certain substrates (e.g., glucose) but also ferment amino acids. In contrast, hyper-ammonia-producing bacteria (HAB) are specialists that ferment amino acids at an increased rate compared to generalists, making them a consistent source of reduced weight gain in cattle and a rather wasteful group of organisms [5–7].

Antibiotics are beneficial for the near-immediate treatment of microbial infection as well as facilitating weight gain in feed animals via off-label uses. For example, the ionophore monensin can be used to treat bacterial, fungal, and parasitic infections, and it promotes growth via the inhibition of HAB [8]. However, growth promotion is an off-label use that is counter-indicated by veterinary feed directives in the United States and the European Union. Monensin, however, is a broad-spectrum antimicrobial affecting far more of the bacterial community in the rumen than amino-acid-fermenting organisms. The continuous use of such compounds with the mutation rate of affected species often results in the rapid development of antibiotic resistance [9]. Thus, there is a definitive motivation to develop sustainable, growth-promoting technologies and control the subsequent spread of antibiotic resistance. Plants are a source of novel antimicrobials as they accumulate secondary metabolites, compounds not essential for growth, which are often bioactive [10].

Previous work explored the efficacy of plant secondary metabolites as a mechanism to bypass the need for antibiotic administration. For example, data indicates that isoflavones (e.g., biochanin A) found in red clover and other legumes inhibit ammonia production by HAB [11–14]. Hemp (*Cannabis sativa* L.) accumulates a wide range of phenolic, terpenoid, and cannabinoid compounds, the latter having both phenolic and terpenoid moieties, thus garnering the designation terpenophenolics [15]. Some phenolic compounds are antimicrobial, such as biochanin A and beta acids from hops, which make these promising candidate compounds [11,16]. Previous works with cannabidiol (CBD) have demonstrated its antimicrobial capabilities in vitro against common pathogens, some of which are known to metabolize amino acids, e.g., *Clostridioides difficile* [17]. Additionally, unpublished works from our group have demonstrated the activity of CBD against four representative ruminal HAB and *C. difficile* [18], thus demonstrating CBD as a potentially efficacious antimicrobial compound for the control of amino-acid-fermenting bacteria. This study aimed to elucidate the effectiveness of CBD on the inhibition of ammonia production via amino-acid-fermenting bacteria ex vivo.

## 2. Materials and Methods

### *2.1. Culture Medium*

Basal medium (BM) was prepared as described previously [5] and contained (per 1 L) 600 mg cysteine HCl, 240 mg $KH_2PO_4$, 100 mg $MgSO_4 \cdot 7H_2O$, 1 mg $FeSO_4 \cdot 7H_2O$, 0.05 mg $ZnSO_4 \cdot 7H_2O$, 240 mg $K_2HPO_4$, 2 mg CaD pantothenate, 480 mg NaCl, 516 mg $Na_2SO_4$, 0.05 mg folic acid, 64 mg $CaCl_2 \cdot 2H_2O$, 0.05 mg cobalamin, 2 mg thiamine HCl, 2 mg riboflavin, 0.1 mg p-aminobenzoic acid, 1 mg pyridoxal HCl, 0.05 mg biotin, 0.1 mg $CoCl_2 \cdot 6H_2O$, 1 mg lipoic acid, 0.015 mg $NaMoO_4 \cdot 2H_2O$, 1 mg pyridoxamine 2HCl, 2 mg nicotinamide, 2.5 mg $Na_4EDTA$, 1 mg pyridoxine, 0.1 mg $H_3BO_3$, 5 µg $CuCl_2 \cdot 2H_2O$, 0.01 mg $NiCl_2 \cdot 6H_2O$, and 1 mg $MnCl_2 \cdot 4H_2O$. The medium was adjusted to pH 6.5 via NaOH solution addition, autoclaved to remove $O_2$, and finally cooled under $CO_2$. After cooling, 4.0 g $Na_2CO_3$ was added as a buffer, and the broth was then dispensed into sealed glass vessels and autoclaved for sterility.

### *2.2. Animals and Rumen Fluid Microbial Cell Suspensions*

All procedures and animal use in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee. Housing and animal care were consistent with the Guide to Care and Use of Agricultural Animals in Research and Teaching [19]. Three rumen-fistulated, mature Angus × Holstein steers (BW = 470.9 ± 23.9 kg) were used as rumen digesta donors. Steers were fed corn silage and dried distiller's grain with solubles to meet protein and energy requirements. Additionally, steers had ad libitum access to conventional loose minerals and water. When rumen digesta were needed for experiments, samples (~0.25 kg) were collected using direct access to the rumen via cannula from each steer individually and transported to the laboratory in a sealed, insulated container within 1 h of collection.

Microbial cell suspensions from rumen fluid (RF) were prepared as described [20] using a series of centrifugation steps. Briefly, the collected rumen material from each animal was first passed through a fine-mesh cheesecloth to filter out large organic material, such as forage. The liquid RF (~500 mL per steer) was then subjected to low-speed centrifugation (500× *g*, 5 min) to remove finer particulate and protists. The supernatants were transferred to clean vessels and then subjected to high-speed centrifugation (10,000× *g*, 5 min) to pellet the prokaryotic fraction of the RF. Supernatants were then discarded, and pellets were resuspended in anaerobic BM and then pooled (per animal) into $CO_2$-sparged glass vessels. Optical density (600 nm; OD) per vessel (animal) was adjusted via the addition of BM to a final OD of 4.0–4.5 prior to any experimentation.

### *2.3. Ex Vivo Cannabidiol Experiment, Viable Number, and Ammonia Production*

The effect of cannabidiol (CBD) on metabolism and growth of microbial RF cell suspensions were determined in 10 mL of BM supplemented with either trypticase tryptone (15 mg $mL^{-1}$ final concentration; T) (BD Difco™, Franklin Lakes, NJ, USA), or casamino acids (15 mg $mL^{-1}$ final concentration; CAA) (BD Difco™, Franklin Lakes, NJ, USA) in Hungate tubes. The tubes were amended (1% *v*/*v*) from an 86 mg $mL^{-1}$ stock solution of CBD and serially diluted (10% *v*/*v*) to obtain five working concentrations of CBD: 860 µg $mL^{-1}$–0.086 µg $mL^{-1}$. Post dilution series, the tubes were inoculated (80% *v*/*v*) with RF cell suspensions prepared as described above, then incubated at 39 °C for ~24 h. An unamended growth control for each replicate (animal) was prepared in 10 mL of BM in the same manner as the experimental tubes above, without the inclusion of the CBD for each substrate type (CAA or T).

A sample (1 mL) was taken from each replicate vessel (0 h; as described in the cell suspension section above) and inoculated into 9 mL of BM supplemented with either T or CAA, serially diluted 9× (10 total tubes per dilution series) and incubated for ~24 h at 39 °C to determine an initial viable number of amino-acid-fermenting organisms. Following the ~24 h incubation of the CBD treated and control tubes for each substrate type (CAA or T) described above, viable number dilution series were prepared in the same manner as for the 0 h replicate vessels.

Supernatant samples were taken for each experimental (CBD) and control tube (for all replicates) at 0 h and 24 h, clarified by centrifugation (21,100× *g*, 1 min), and stored at −20 °C for later ammonia analysis. Additionally, ammonia samples were taken for several tubes in sequence for each viable number dilution series (Day 0 and Day 1, marked by turbid versus light growth) to determine the viable number of hyper-ammonia-producing bacteria versus generalists per CBD concentration and substrate type. Ammonia concentration was determined using the phenolic/hypochlorite methods, as previously described [21,22]. Significant results ($p < 0.05$) were determined using a 1-way ANOVA and Tukey's post hoc test utilizing OriginPro statistical software (version 9.5) (OriginLab Corporation, Northampton, MA, USA).

## 3. Results

### *3.1. CBD Affects Ex Vivo Ammonia Production at High Concentration*

RF cell suspensions in BM supplemented with either CAA or T produced similar concentrations of ammonia after 24 h of incubation at 39 °C (~45 mM; Tables 1 and 2). Regardless of growth substrate (CAA or T), RF cell suspensions had decreased ammonia production after 24 h when incubated under the highest concentration of CBD, 860 µg $mL^{-1}$ (~30 and 35 mM ammonia, respectively; $p < 0.05$). All other concentrations of CBD tested, regardless of substrate type, had no measurable effect on ammonia production of RF microbial cell suspensions when compared with control or each other (Tables 1 and 2).

**Table 1.** Ammonia production (mM) by amino-acid-fermenting bacteria (ex vivo) after 24 h in casamino acids supplemented media.

| Cannabidiol Concentration (μg mL$^{-1}$) | Average | Maximum | Minimum | SD | *p*-Value * |
|---|---|---|---|---|---|
| 0 (control) | 43.88 | 47.30 | 40.41 | 3.44 | - |
| 0.086 | 43.31 | 46.53 | 37.85 | 4.76 | 0.999 |
| 0.86 | 46.70 | 48.30 | 44.12 | 2.26 | 0.911 |
| 8.6 | 45.78 | 49.61 | 43.34 | 3.36 | 0.982 |
| 86 | 44.98 | 45.84 | 43.68 | 1.14 | 0.999 |
| 860 | 30.25 | 35.43 | 27.08 | 4.53 | 0.005 |

* *p*-values indicate the difference in ammonia (average) produced between the cannabidiol-treated groups and the control group. Significance is defined as $p < 0.05$.

**Table 2.** Ammonia production (mM) by amino-acid-fermenting bacteria (ex vivo) after 24 h in tryptone peptone supplemented media.

| Cannabidiol Concentration (μg mL$^{-1}$) | Average | Maximum | Minimum | SD | *p*-Value * |
|---|---|---|---|---|---|
| 0 (control) | 46.17 | 46.65 | 45.25 | 0.80 | - |
| 0.086 | 50.87 | 53.84 | 48.52 | 2.71 | 0.752 |
| 0.86 | 52.95 | 56.74 | 49.31 | 3.72 | 0.416 |
| 8.6 | 54.67 | 63.37 | 48.09 | 7.86 | 0.212 |
| 86 | 50.70 | 52.84 | 48.94 | 1.98 | 0.777 |
| 860 | 35.06 | 38.56 | 29.89 | 4.57 | 0.049 |

* *p*-values indicate the difference in ammonia (average) produced between the cannabidiol-treated groups and the control group. Significance is defined as $p < 0.05$.

### *3.2. Ex Vivo Cell Suspension Growth Is Unaffected by CBD*

The viable number of RF cell suspensions grown in BM supplemented with CAA or T yielded no differences, nor were there discernable differences between CBD treatments. Specifically, average growth with either substrate (CAA or T) unamended or amended with CBD of any concentration used was between $10^7$ and $10^8$ cells. Within the guild of amino-acid-fermenting bacteria preferentially selected by BM and substrate (including HAB and generalists), differences in total number were indistinct.

## 4. Discussion

Amino-acid-fermenting bacteria are generally considered to be wasteful organisms in the rumens of beef cattle as they directly contribute to lower weight gain in animals as the available nitrogen sources are removed and excreted in the urine [2,3]. Previous research identified CBD as a viable antimicrobial against common pathogens, which include the amino acid fermenter *Clostridioides difficile* [17]. This study aimed to determine the effectiveness of CBD in inhibiting ammonia production of ruminal amino-acid-fermenting organisms ex vivo.

The experimental design employed here utilized a reduced medium specifically supporting the growth of bacteria capable of utilizing only free amino acids (casamino acids; CAA) or oligopeptides (trypticase tryptone; T). This type of culture medium allowed for the preferential selection of amino-acid-fermenting organisms (HAB and generalists) from ex vivo bovine rumen fluid samples. The results indicated that CBD at the highest concentration (860 μg mL$^{-1}$; 2.73 mM) decreased ammonia production of amino-acid-fermenting bacteria cultured ex vivo by ~10–15 mM (Tables 1 and 2). The reductions in ammonia production by ruminal microorganisms exposed to CBD are comparable to those previously reported with biochanin A in a study utilizing caprine ruminal microbes (~15–20 mM reductions) [11]. However, the suppression of ammonia was attained using a much lower concentration of biochanin A than what was determined effective with CBD in this study (70 μM versus 2.73 mM). Additionally, the use of CBD as an antimicrobial

agent in livestock would be costly and impractical due to the current legislative hurdles and cultivation/isolation costs [23].

Additionally, while the viable number data indicate that high concentrations of CBD exerted a metabolic effect on the bacteria (reduced amino acid fermentation), there was no discernable bactericidal effect on the guild of amino-acid-fermenting bacteria ex vivo. While the inhibitory action of CBD against certain Clostridia in vitro is known [17,18], other studies with amino-acid-fermenting bacteria have yet to be published. However, it is likely that CBD is active against other amino-acid-fermenting organisms within the class Clostridia, among other taxonomic designations [17,24]. Therefore, while CBD might have inhibitory activity against some amino-acid-fermenting organisms at the species level, the rumen microbial community appears to have enough redundant function in the amino-acid-fermenting guild that there is little net effect on overall viable number. In other words, there are species within the guild unaffected by CBD under the conditions used in this study that are capable of increasing their population to occupy the spaces left within the niche by those species that were susceptible. Reduction in ammonia without a concomitant reduction in viable number of total amino-acid-fermenting organisms may be a function of high-ammonia-producing species, such as *Acetoanaerobium sticklandii*, succumbing to high concentrations of CBD ex vivo and more robust species, such as Gram (−) generalists, persisting. Alternatively, one of the suspected mechanisms of action of CBD lies in the depolarization of the bacterial membrane [25]. Depolarization results in the loss of ion motive force, which directly impacts cells' ability to transport substrates across the membrane [26–28], thereby inhibiting metabolism and the release of metabolic byproducts such as ammonia. Regardless, the inhibition of select species in an environment may prove useful for certain indications as microfloral populations are fluid and competitive by nature. For example, co-culture experiments with *Campylobacter jejuni* and *Clostridium aminophilum* (an HAB) revealed a competitive relationship for amino acid substrates where *C. aminophilum* was more fit and ammonia accumulation was high as a result [29]. However, when the culture medium was treated with monensin, the population of *C. aminophilum* was greatly reduced, while the population of *C. jejuni* was unaffected, but importantly, ammonia accumulation was greatly reduced. Importantly, while CBD may find use for species-specific inhibitory action, further exploration of effective application techniques and antimicrobial specificity of the compound is needed. Current research indicates that CBD has strong activity against Gram (+) bacteria with some activity against Gram (−) bacteria [17,18]. Therefore, CBD has the potential to act as a broad-spectrum antimicrobial compound, and the impact on commensal organisms would need to be assessed prior to any in vivo commercial applications.

The work presented here indicates that CBD is likely not a reasonable antimicrobial terpenophenolic (as there are other more effective plant-derived options) for suppression of the amino-acid-fermenting guild within ruminants. This study demonstrates a suppressive effect on ammonia production, albeit at high concentrations (2.73 mM) of CBD, though importantly lacks evidence of a guild-wide bactericidal effect. Thus, CBD would likely be ineffective in controlling the community of amino-acid-fermenting organisms and ammonia production within the rumens of livestock. In addition, the inclusion of CBD as a feedstuff or antimicrobial agent would be costly and impractical; however, it may be an effective choice for the control of specific species pending further research.

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