EVALUATION OF AN ORAL SUPPLEMENTAL CANNABIDIOL PRODUCT FOR ACCEPTABILITY AND PERFORMANCE IN MATURE HORSES

A Thesis

by

JULIA MARIE LEISE

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Chair of Committee,	Jessica L. Leatherwood
Committee Members,	Tryon A. Wickersham
	Kati P. Glass
Head of Department,	Cliff Lamb

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ABSTRACT

Thirty mature geldings (mean ± SD; 14.83 ± 2.61 yr; 556 ± 62.77 kg BW) were used in a randomized complete design for a 28 d trial to determine the influence of CBD oil supplementation on the health of mature horses by conducting a blood chemistry panel and evaluating the presence of banned substances. Horses were balanced by body weight (BW) and age, and randomly assigned to 1 of 3 treatments: canola oil (CON; n=10), canola oil with 0.13 mg CBD/kg BW (TRT1; n=10) or 0.12 mg CBD/kg BW (TRT2; n=10). Treatments were top-dressed and mixed onto concentrate that was delivered twice daily at 12 h intervals using attachable feed bags. Diets were formulated to meet or slightly exceed nutritional requirements for mature horses at maintenance. Between meals, horses were maintained in adjacent dry lots and received coastal bermudagrass hay *ad libitum*. Body weight was obtained, and body condition scores (BCS) were assigned every 14 d. Blood was collected via jugular venipuncture and a blood chemistry panel was performed as well as banned substance testing at the Texas Veterinary Medical Diagnostic Laboratory (College Station, TX). Data were analyzed using PROC MIXED of SAS (v9.4). Model contained effect for treatment, time, and treatment × time interaction for BW and BCS. A covariate was used to account for differences in blood chemistry on d 0 and main effect tested was treatment. Dietary treatments did not affect concentrate intake, BW, or BCS; however, BCS varied over time ($P \leq 0.01$) across treatments. Supplementation of CBD did not reveal the presence of banned substances, including tetrahydrocannabinol (THC). A linear dose response

was observed in concentrations of serum Ca (P = 0.01). Creatinine levels tended (P = 0.07) to be lower in TRT1 compared to CON while gamma-glutamyl transferase levels (P = 0.03) demonstrated a quadratic response by dietary treatments with CON being lower than TRT2, but values remained within normal physiological limits. Canola based CBD oil appeared to be well-accepted by mature horses, banned substances were not detectible in blood, and blood chemistry parameters were not adversely impacted as a result of supplementation.

DEDICATION

Dedicated to my parents whose unwavering support has taught me perseverance.

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NOMENCLATURE

A2A	Adenosine A2A Receptor
ADF	Acid Detergent Fiber
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AMPK	Adenosine Monophosphate-activated Protein Kinase
AST	Aspartate Transaminase
BCS	Body Condition Score
BUN	Blood Urea Nitrogen
BW	Body Weight
Ca	Calcium
CB1R	Cannabinoid Type 1 Receptor
CB2R	Cannabinoid Type 2 Receptor
CBC	Cannabichromene
CBCA	Cannabichromenic acid
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBG	Cannabigerol
CBGA	Cannabigerolic Acid
CBPI	Canine Brief Pain Inventory
CON	Control Group
CP	Crude Protein

d	day
DEA	Drug Enforcement Agency
dL	deciliter
ENT	Equilibrative Nucleoside Transporter
FDA	Food and Drug Administration
FFA	Free Fatty Acids
g	gram
Gαi	Heterometric Gi protein subunit
GGT	Gamma-glutamyl Transferase
H	Hydrogen
h	hour
IL-1β	Interleukin-1β
INFγ	Interferon-γ
kg	kilogram
LLC	Limited Liability Corporation
LPS	Lipopolysaccharide
LSD	Lysergic acid Diethylamide
MAG	Monoacylglycerol
mg	milligram
NDF	Neutral Detergent Fiber
NRC	National Research Council
O	Oxygen

OA	Osteoarthritis
P	P-value (the lower the p-value, the greater the statistical significance of the observed difference)
QoL	Quality of Life index
R	Radical
SAS	Statistical Analysis System
SD	Standard Deviation
SEM	Standard Error of the Mean
THC	Tetrahydrocannabinol
TNFα	Tumor Necrosis Factor-α
TRT1	Treatment Group 1
TRT2	Treatment Group 2
TVMDL	Texas Veterinary Medical Diagnostic Laboratory
U/L	Units per Liter
U/mL	Units per milliliter
US	United States
USDA	United States Department of Agriculture

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CHAPTER I

INTRODUCTION

The use of cannabidiol (CBD), one of the non-psychoactive compounds found in hemp, is gaining in popularity as a potential alternative to conventional pharmaceutical treatments for a variety of conditions including but not limited to arthritis, anxiety, and seizures (Brighenti et al., 2021). Studies conducted in mice and horses have shown that CBD reduces production of inflammatory cytokines such as tumor necrosis factor-α (TNFα) via competitive inhibition of equilibrative nucleoside transporter 1 (ENT1), leading to an increase in endogenous adenosine availability. As a result, the adenosine (A2A) receptor is upregulated and reduces the amount of TNFα produced, which may help reduce inflammation in various body tissues (Ribeiro et al., 2012a; Turner et al., 2021). Furthermore, studies on dogs and people with epilepsy have demonstrated that CBD is effective in reducing seizure frequency by reducing the nerve activity associated with seizures (Devinsky et al., 2018; McGrath et al., 2019).

Despite the potential advantages of CBD supplementation, the introduction and level of dietary CBD may have a negative impact on equine health and performance. Averse responses reported in mice provided a dose of 615 mg CBD/kg BW were lethargy, loss of appetite, and body weight loss (Ewing et al., 2019). It has also been identified in multiple studies that CBD may induce the cytochrome p450 mediated oxidative metabolism in the liver and cause an increase in alkaline phosphatase (ALP) and alanine aminotransferase (ALT) activity, which suggests that liver injury is present.

The increase in serum ALP levels were seen in dogs at 2 mg CBD/kg BW and at 2.5 mg CBD/kg BW (Gamble et al., 2018; McGrath et al., 2019). Increases in ALT serum levels were reported in mice at 615 mg CBD/kg BW and in cats at 2 mg CBD/kg BW (Deabold et al., 2019; Ewing et al., 2019). These studies indicate that CBD may have the potential for dose-dependent liver injury.

Gamma-glutamyltransferase (GGT) is another useful indicator of long-term liver damage in ruminants and horses due to its association with hepatocytes responsible for bile production (Kahn and Line, 2010; McAtee and Lidbury, 2017). In general, a rise of liver enzymes is concerning due to the many metabolic functions involving the liver. Observation of liver enzymes requires some level of elevated pattern recognition. Alanine aminotransferase and AST will increase together if there is hepatocellular damage. Alkaline phosphatase and GGT will increase together if there is cholestasis. Some liver diseases such as cholangitis or phenobarbital hepatopathy can display a mixed pattern (McAtee and Lidbury, 2017). In addition to the potential of liver injury, CBD may have a positive or negative effect on kidney function depending on the dosing level. Cannabinoid Type 1 (CBR1) and Type 2 (CBR2) receptors are found in the kidneys and interact with G-proteins, which produces several effects including an increase in calcium levels (Park et al., 2017).

Elevated blood urea nitrogen (BUN), calcium, and creatinine are additional indicators of kidney function and signify if the kidneys are under stress (Yazdi, 2021). Studies in horses have indicated similar concerns to health as those in rodents and companion animals. A pilot pharmacokinetics study evaluating three different dosage

levels of a pelleted CBD supplement given to horses was performed by Draeger et al. (2021a). Although serum chemistry were within normal ranges, BUN levels increased with CBD supplementation level and creatinine decreased then increased as supplementation level increased. Previous work involving dietary supplementation levels has focused primarily on companion animals, including dogs and cats, with limited information available regarding appropriate CBD oil supplementation levels in mature horses. Therefore, the objectives of this study were to (1) determine the influence of CBD oil supplementation levels on the health of mature horses by conducting a blood chemistry profile to measure BUN, creatinine, total protein, albumin, Ca, P, glucose, and liver enzymes as well as (2) evaluate the presence of banned substances in dietary CBD oil through the detection of amphetamines, barbiturates, opiates, marijuana, and other drug types that have implication to competitive performance.

CHAPTER II

LITERATURE REVIEW

Introduction

Hemp has been extracted from the stem of the cannabis plant and used historically as a fiber source for making textiles, rope, paper, and other products in the United States (US), but it was banned in 1937 after the Marijuana Tax Act was passed by Congress. Interest in hemp and its derivatives resurfaced in the US in the mid 1990's due to Europe and Canada legalizing hemp production (Fike, 2016). Under the 2014 Farm Bill, hemp was permitted to be grown by institutions of higher education and the state Department of Agriculture for research purposes if state laws allowed hemp production (Smith, 2020). This legal exception has allowed for new research opportunities that were previously limited by the US Drug Enforcement Agency (DEA) due to the DEA's classification of hemp as a Schedule I controlled substance. The DEA defines a Schedule I controlled substance as one that has no current accepted medical use, a lack of accepted safety for use under medical supervision, and has a high potential for abuse (Controlled Substances Act, 1970). Examples of Schedule I substances include heroin and lysergic acid diethylamide (LSD).

The 2018 Farm Bill legalized hemp, declaring it a Schedule V controlled substance. A Schedule V substance has a currently accepted medical use in the United States, may lead to limited physical dependence or psychological dependence, and a low potential for abuse relative to the drugs or other substances in Schedule IV (Controlled Substances Act, 1970). Examples of Schedule V substances include cough preparations

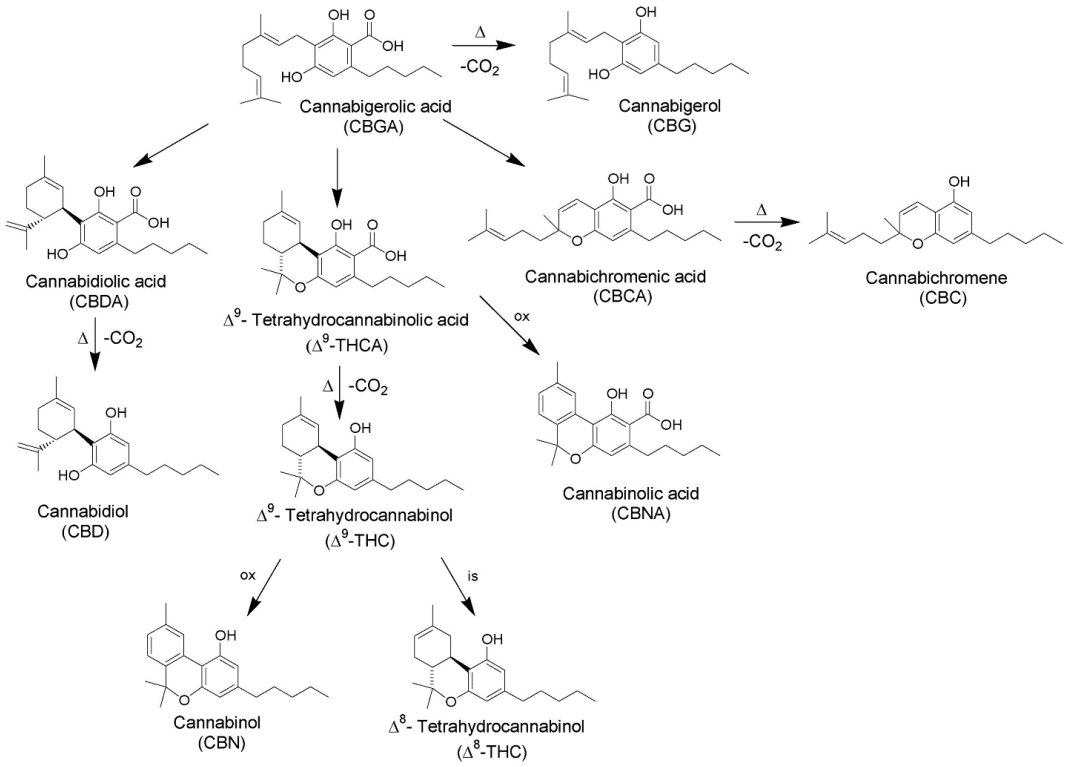
containing not more than 200 mg of codeine per 100 ml or per 100 g. This change in classification allowed for commercial hemp production and defined industrial hemp as cannabis with less than 0.3% tetrahydrocannabinol (THC) (Smith, 2020). The bill not only removed industrial hemp from the Schedule I controlled substance category, but gave regulatory authority to the US Department of Agriculture (USDA). Following this legislation, there was a sharp increase in the amount of acreage licensed for hemp production. A report on the hemp market estimated that the total acreage in the top 10 hemp-growing states increased nearly tenfold (37,122 to 310,721 acres) by July 2019 (Sterns, 2019). At the end of 2020, it was reported that the US hemp industry was valued at $4.7 billion (Gomez, 2021).

Cannabidiol

Cannabidiol (CBD) is one of several non-psychoactive compounds found in hemp and contains low levels of Δ9-THC, unlike medicinal and recreational Cannabis strains (Brighenti et al., 2021). Several methods of extraction are used to extract CBD oil from hemp including cold press, accelerated solvent, rapid solid-liquid, pressurized liquid, and supercritical-fluid extraction (Fathordoobady et al., 2019; Brighenti et al., 2021). The most commonly used method of CBD extraction is by mechanically breaking down the plant, then using organic solvents (e.g., methanol, ethanol, etc.) to extract the CBD from the solids and distill the CBD out of the solvent (Brighenti et al., 2021). By using hemp plants bred for low THC levels and organic solvents that have high specificity for non-psychoactive cannabinoids, the amount of THC in the oil extract is extremely low.

Cannabigerolic acid (CBGA) is the starting compound for CBD and several other main cannabinoids present in hemp (Figure 1). Cannabidiol and its precursor cannabidiolic acid (CBDA) are the main cannabinoids found in hemp, but others including CBGA, cannabigerol (CBG), cannabichromene (CBC), and cannabichromenic acid (CBCA) can be found in lower amounts (Brighenti et al., 2021).

Figure 1. Chemical structures of cannabinoids in hemp. Reprinted with permission from Brighenti et al., 2021.



These derivatives have potential biological significance due to the effects on the endocannabinoid receptor system, which play a role in regulating pain and mitigating inflammation by modulating cytokines associated with an inflammatory response.

Mechanisms of Inflammatory Cytokine Modulation

Recent in vitro and in vivo studies on the effects of CBD on inflammatory responses identified several possible mechanisms of action. The primary proposed mechanism of action is thought to be the upregulation of A2A receptors. The A2A receptors are a type of G-protein coupled receptor that are primarily located in the spleen, thymus, leukocytes, blood platelets, neurons, and the olfactory bulb and also present in the heart, lungs, blood vessels, and other brain tissues. The A2A receptors are important mediators of vasodilation, supporting the formation of new blood vessels, and protecting tissues from collateral inflammatory damage (Ruiz et al., 2014).

The upregulation of the adenosine A2A receptors reduces the amount of inflammatory cytokines present such as tumor necrosis factor-α (TNFα) and interferon-γ (INFγ) which reduces secondary tissue damage. Tumor necrosis factor-α is a pro-inflammatory cytokine found in macrophages, natural killer cells, lymphocytes, and adipocytes that encourages cytokine production, cell proliferation, apoptosis, and fights off infection (Chen et al., 2018). Interferon-γ is another pro-inflammatory cytokine produced by T cells, natural killer cells, and natural killer T cells that triggers innate and adaptive immunity and is anti-viral (Chen et al., 2018). These cytokines are important in the modulation of the immune system response to inflammation and infection. However, it is important to control the amount of TNFα and INFγ due to their ability to inflict

further tissue damage by encouraging the immune cells to continue attacking healthy tissue (Chen et al., 2018).

In a study of lipopolysaccharide (LPS)-treated mice, administration of CBD before the LPS injection resulted in a significant decrease of TNFα. Cannabidiol is a competitive inhibitor for equilibrative nucleoside transporter 1 (ENT1), which led to an increase in availability of endogenous adenosine. The ENT1 is responsible for transporting adenosine to A2A receptors. The inhibitive effect of CBD on TNFα was eliminated when an A2A receptor antagonist was given and absent in mice with no A2A receptors (Carrier et al., 2006). Another study in mice with induced acute lung injury confirmed the findings in the LPS study and also found that CBD inhibits the leukocyte migration into lung tissue via the A2A receptor (Ribeiro et al., 2012).

Another proposed mechanism is the induction of autophagy via the cannabinoid type 2 receptor (CB2R). Cannabidiol activates the adenosine monophosphate-activated protein kinase (AMPK), which then induces the autophagy of inflammasome. Inflammasomes are multiprotein complexes that serve as platforms for activation of caspase-1, which leads to processing and secretion of major pro-inflammatory cytokines such as interleukin-1β (IL-1β). Interleukin-1β is found in macrophages and monocytes and is responsible for cell proliferation, apoptosis, and differentiation of T helper cells (Chen et al., 2018; Shao et al., 2014). Mice with an experimentally-induced autoimmune encephalomyelitis demonstrated reduced inflammation after being treated with CBD. Further analysis investigating the co-localization of inflammasome components revealed that the levels were lower, indicating that the activation of CB2R may decrease

inflammation by stopping the assembly of inflammasome components (Shao et al., 2014). Escalation of the inflammatory response and secondary necrosis is prevented by autophagy due to the important role it has in the phagocytosis and clearing cells undergoing programmed cell death (Van Niekerk et al., 2019). Cannabidiol-induced autophagy may assist in the clearing of TNFα, INFγ, and IL-1β which may ultimately reduce the levels of these inflammatory cytokines in the blood serum that would have negative implications on a host of various tissues.

Dietary Supplementation of CBD Products

Cannabidiol is commonly supplemented to domestic animals in a variety of forms such as biscuits, edibles, capsules, and liquid. Multiple studies have indicated that an oil-based form of CBD has the highest bioavailability due to its lipophilicity in the carrier oil and within a biological system (Zgair et al., 2016; Gamble et al., 2018; Fathordoobady et al., 2019; Brighenti et al., 2021). The lipophilicity of CBD allows it to redistribute rapidly into adipose tissue and be retained longer in the body. In the small intestine, lipid-based substances must be broken down into small droplets that are primarily made up of monoacylglycerol (MAG) and free fatty acids (FFA) before being absorbed by the enterocyte. Upon absorption, FFA either goes directly into the bloodstream, to the liver, or undergoes further processing within the enterocyte depending on the length of the fatty acid chain. Monoacylglycerol is broken down further in the enterocyte into a chylomicron before entering the lymphatic system for transport to tissues other than the liver (Cheeke and Dierenfeld, 2010). Storage of lipid-based CBD in the adipose tissue may maintain higher levels of CBD in the body for

longer periods of time (Zgair et al., 2016). Studies involving supplementation of CBD oil to dogs indicated that top-dressing CBD oil on feed may have greater absorption since the feed can act as a transport vehicle, but there are others that seem to contraindicate this due to first-pass metabolism in the liver (Gamble et al., 2018; Deabold et al., 2019; Brioschi et al., 2020; Wakshlag et al., 2020; Brighenti et al., 2021).

Historically, the most commonly studied properties of CBD oil are its antiepileptic properties. In dogs and humans being treated for epilepsy, CBD reduces seizure frequency by binding to specific transient receptor potential channels, which leads to decreased release of glutamate, activation of 5-hydroxytryptophan 1A receptors, and inhibition of adenosine reuptake (Devinsky et al., 2018; McGrath et al., 2019). The changes in the activity cause a reduction in the nerve activity associated with seizures. The reduced seizure frequency may improve the overall quality of life for both humans and dogs. This has led to CBD oil being approved by the Food and Drug Administration (FDA) for use in treatment-resistant epilepsy (Devinsky et al., 2018; McGrath et al., 2019). The FDA approved the drug Epidiolex in June 2018 for use in humans as an oral solution for the treatment of seizures associated with Lennox-Gastaut syndrome and Dravet syndrome (in patients two years of age and older). This is the first FDA-approved drug that contains a substance derived from marijuana (Felberbaum, 2018). Although the FDA did not approve the drug for use in canines, veterinarians can prescribe it off label as the FDA did not restrict such use (McReynolds, 2018).

A growing number of pet owners are beginning to use CBD oil-based products as an alternative to conventional pharmaceutical treatments for a variety of conditions

including but not limited to arthritis, anxiety, and seizures, due to the increasing availability of these types of products for pets and humans alike. In an online survey conducted by Colorado State University, it was reported that some of the most common reasons for using CBD products were a positive view of the fact that these products came from natural sources and that they could be used as an adjunct to other therapies (Kogan et al., 2016). The respondents of the survey noted that the products provided pain relief and reduced inflammation in their pets. In other studies, it has been shown that CBD oil relieves pain in dogs suffering from osteoarthritis (OA), reduces seizure frequency in dogs with intractable idiopathic epilepsy, and reduces inflammation (Ribeiro et al., 2012b; Deabold et al., 2019; McGrath et al., 2019; Brioschi et al., 2020). These studies indicate that a potential exists for CBD products to be used for pain relief.

Rate of Supplementation

Antiepileptic, immunomodulatory, anti-hyperalgesic, anti-inflammatory, antinociceptive, and antibacterial properties are multiple targets of CBD that have been documented (Devinsky et al., 2018; Gamble et al., 2018; Fathordoobady et al., 2019; McGrath et al., 2019; Brioschi et al., 2020; Mlost et al., 2020; Brighenti et al., 2021). Cannabidiol targets G-protein coupled receptors, ionotropic receptors, intracellular endocannabinoid transporters, and cytochrome p450 liver enzymes (Mlost et al., 2020). Ionotropic receptors are protein complexes that span the cell membrane in neural cells and are responsible for converting neurotransmitter signals into electrical information (Sakimura, 2009). G-protein coupled receptors are found in all cells and are transmembrane proteins that bind to an agonist on the outside of the cell, which then

induces a change on the cytoplasmic side (Voet et. al., 2016). There are some G-protein coupled receptors that are associated with opioids and CBD may be a potential alternative to opioids in managing severe pain.

In several pharmacokinetic studies performed in dogs, the time to max concentration was reported to be 1 to 2 hours and the half-life of elimination median for CBD oil was found to be between 1 and 4.4 hours for a 2 mg/kg dose of CBD oil (Gamble et al., 2018; Deabold et al., 2019; Wakshlag et al., 2020). The max CBD serum concentration levels varied from 102 ng/mL to 347 ng/mL (Gamble et al., 2018; Deabold et al., 2019; Vaughn et al., 2020; Wakshlag et al., 2020). It is not fully understood why there is a wide variance in the elimination and max concentration of CBD oil, but it may be due to studies using different methods to administer the CBD oil, product formulations, and the co-administration of other medications or dietary products including nonsteroidal anti-inflammatory drugs, fish oil, and/or glucosamine/chondroitin sulfate.

In a study of efficacy on OA, pharmacokinetics was performed using four dogs at the 2 mg/kg dose and an 8 mg/kg dose. The half-life of elimination median was measured as 4.2 h with 2 mg/kg and the same with 8 mg/kg. Median maximum concentration of CBD oil was 102.3 ng/mL and 590.8 ng/mL for the 2 mg/kg and 8 mg/kg doses, respectively. Dosing level was chosen at 2 mg/kg for the study due to vendor recommendations and perception of prohibitive costs as a practical treatment regimen at the higher levels. The results of the study showed a significant decrease in pain and increase in activity as observed at weeks 2 and 4 compared to week 0.

Important to note however, 9 of the dogs had an increase in alkaline phosphatase (ALP) activity (Gamble et al, 2018).

There is limited information concerning the appropriate supplementation rates for companion animals in order to elicit beneficial effects. One such study was performed using sixteen dogs with osteoarthritis (OA). Each dog had radiographic evidence of OA, detectable pain in their joints on palpitation, and visible lameness on gait evaluation. Each dog was studied with 2 mg/kg CBD oil for 4 weeks and a placebo for 4 weeks. Both treatments were given every 12 hours. A 2 week washout period was used between studies. Both owner and veterinarian were blinded to the treatment being given. Feeding was given 2 hours after dosing with observations made at dosing, and at 4, 8, and 24 hours post dosing. Blood samples were collected at dosing, and at intervals of 0.5, 1, 2, 4, 8, 12, and 24 hours after dosing. Subjective evaluation of the dogs' attitude, behavior, proprioception and gait were made while running, walking and weaving around traffic cones (Gamble et al., 2018).

Another study evaluated the use of CBD oil in combination with a multimodal pharmacological protocol of gabapentin and amitriptyline to treat OA. Two groups were established, 9 dogs receiving CBD oil with the pharmacological protocol and 12 dogs receiving only the pharmacological protocol. Canine Brief Pain Inventory (CBPI) and Quality of Life Index (QoL) evaluations were used to assess the comfort level of the dogs over the 12 week program. The dogs receiving CBD oil were given 2 mg/kg every 12 hours. The CBD oil treated group scored significantly lower in Pain Severity and Pain Interference than the other group (CBPI specific measures). The CBD oil treated group

scored significantly higher in QoL than the other group. The study did not measure liver enzyme values (Brioschi et al., 2020).

A safety study of CBD oil in cats and dogs used a dosage of 2 mg/kg during their assessment and reported that there were no significant changes in the blood chemistry in dogs, but the blood chemistry for one of the cats demonstrated elevated alanine aminotransferase (ALT) levels for the duration of the study. It was not known whether the elevated ALT level was due to an unknown disease or the CBD-infused oil (Deabold et al., 2019). In a study of escalating cannabinoid doses in healthy dogs, one group was given a CBD-predominant oil in ten escalating doses containing 2 up to 62 mg/kg CBD and reported only mild adverse events, which was described as gastrointestinal upset (Vaughn et al., 2020). This safety study of escalating CBD doses in dogs may indicate that CBD can be administered in increasing doses without serious short-term side effects, but further research is needed to determine the max safe dose and if there are any effects from long-term administration of CBD.

Manufacturing Process and Testing

As use of CBD is becoming popular, use of it in the CBD oil mode of delivery is also standing out as one of the easiest and more popular to administer. It touts the benefits of being easily digestible in large doses without risk of intoxication due to its processing out of THC products. There are currently dozens if not hundreds of producers ranging from individuals to compounding pharmacies, to pharmaceutical companies. Unfortunately, the industry is still struggling to deliver consistent concentrations of CBD within CBD oil, or even agree on analysis methods (Hazekamp, 2018).

CBD oil is extracted from cannabis flowers or leaves and dissolved in an edible oil such as sunflower, hemp, or olive. Solvents are used to perform the extraction and can be organic (such as ethanol or isopropyl alcohol) or petroleum based (such as naphtha). The type of solvent used and conditions will impact taste, color and viscosity of the final product. Other components which are co-extracted with the CBD must be removed. Often this is accomplished through a process called “winterization”. This process places the extract in a freezer where components with a higher melting temperature (e.g. waxes, triglycerides, chlorophyll) are precipitated and removed through filtration or centrifuge. All of these processes can cause variability in the final concentration. The cannabis variety used will affect concentration. Finally, regulatory differences between countries has added to inconsistency in concentrations as entrepreneurs now spread activities of cultivation, processing, extraction, and final lab testing across multiple countries. Such diversification of process steps challenges traceability and responsibility for consistent results (Hazekamp, 2018).

Handling and storage of CBD can also cause degradation with time. Sensitivity to elevated temperatures and photo-instability has been reported in CBD-infused oils. One study found an average degradation in concentration of 20% for 30 days storage at 37ºC (98.6ºF). Storage at 4 ºC (39.2ºF) and room temperature averaged around 5% for the same 30 day period. It was also found that storage in sunlight for the same 30 day period caused 15% degradation in concentration. This same study also found product label concentrations differed significantly from actual measured concentrations. Thirteen samples were measured from nine different companies. Concentrations higher than

labeled were as much as 17% high. Concentrations lower than labeled were as much as 92% low (Mazzetti et al, 2020).

A study of consistency between testing labs and consistency in popular strains of flowers used to produce CBD oil in Washington state revealed significant differences in concentrations of CBD reported by the six largest state-licensed laboratories (by volume). Systemic discrepancies between laboratories were observed with certain labs "always high" and other labs "always low". These issues were attributed to systemic differences in their testing methods. Heterogeneity within the strains of flowers used was not present nor credited with the disparate results. The study emphasizes the need for adoption of testing standards by industry, states, or even the federal government (Jikomes and Zoorob, 2018).

A 12 week study in dogs with intractable idiopathic epilepsy (performed by the Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University) was the only study that tested CBD oil products by a third party laboratory. In this study, CBD concentrations were verified by the pharmacology lab at Colorado State University. Two groups of canines were evaluated with and without CBD supplementation. The CBD group received CBD infused oil at 2.5 mg/kg body weight (BW). The study did not cite discrepancies in the CBD infused oil. The control group received cold-pressed hemp oil. The control oil was not verified by the independent lab. Both groups had blood plasma tested every 4 weeks. One dog in the control group tested positive for a trace amount of CBD (at week 8 only). This level was 6.4 ng/mL. The average for the CBD group was 450.1 ng/mL. Three other dogs in

the control group had received the same placebo batch and did not test positive (McGrath et al., 2019).

Liver Health

Liver enzymes are proteins that act as catalysts for chemical reactions in the body. They contribute to breaking down food, removing toxins, fighting infections, producing blood clotting factors, and producing bile. When the liver is damaged or injured, it releases these enzymes into the blood. Liver enzymes are not direct markers of liver damage, however they provide clues as to the presence of liver damage when elevated levels are present in blood serum.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are markers of hepatocellular damage. Alanine aminotransferase, a cytoplasmic enzyme found in hepatocytes, is released when there is hepatocellular injury. Although ALT is liver specific, it can be released due to muscle damage or ex vivo hemolysis. Creatine kinase can be used as a co-indicator as it increases with muscle damage. Aspartate aminotransferase is a cytoplasmic and mitochondrial enzyme found in hepatocytes. Both forms are released from liver damage, but only irreversible damage will release mitochondrial AST. They are indistinguishable in blood serum and additional testing is required to identify root cause.

Similar to ALT, AST can also be caused by muscle damage or ex vivo hemolysis. Alanine aminotransferase is the preferred marker of liver damage between the two as its half-life is longer. In dogs, the half-life of ALT is 60 hours where AST is 22 hours (McAtee and Lidbury, 2017). In normal metabolism, ALT is responsible for

mediating the transamination of alanine into pyruvate. Alanine aminotransferase is a catalyst taking amino groups from alanine to alpha-ketoglutarate, glutamate, and pyruvate. Pyruvate is a very important metabolite in the liver as it is a precursor molecule used in gluconeogenesis as well as other metabolic pathways (Voet et al., 2016 and Aulbach, 2017).

Alkaline phosphatase (ALP) is found in hepatocytes that line the bile canaliculi. Intra- or extrahepatic cholestasis will cause its release. It can increase due to bone and glucocorticoid-induced isoenzymes. Alkaline phosphatase will be higher in young animals due to bone growth. It also can be increased due to osteomyelitis or osteosarcoma. High levels of ALP have been reported with cholestasis, steroid hepatopathy, chronic hepatitis, and hepatic necrosis. Alkaline phosphatase levels can be hard to interpret due to multiple potential tissue sources. The half-life of ALP in dogs is 70 hours (McAtee and Lidbury, 2017).

Gamma-glutamyltransferase (GGT) is a marker of intrahepatic or extrahepatic cholestasis. It is associated with the cell membranes of hepatocytes of the bile canaliculi and ducts. It is also associated with periportal hepatocytes. Alkaline phosphatase and GGT have been observed to decrease at a similar rate in dogs after liver injury indicating similar half-life (McAtee and Lidbury, 2017). Gamma-glutamyltransferase is particularly useful for horses and ruminants because it is indicative of long-term liver damage (Kahn and Line, 2010). Observation of liver enzymes requires some level of elevated pattern recognition. Alanine aminotransferase and AST will increase together if there is hepatocellular damage. Alkaline phosphatase and GGT will also increase

together if there is cholestasis. Some liver diseases such as cholangitis or phenobarbital hepatopathy can display a mixed pattern (McAtee and Lidbury, 2017).

Cytochrome p450 are a family of enzymes with over 30 distinguishable enzymes. Besides being found in the liver, there are some levels in the small intestinal mucosal enterocytes and other organs. These enzymes can metabolize drugs, toxins, and carcinogens as well as normal endogenous compounds (Gregg, 2004). The cytochromes P450 is a superfamily of heme-containing enzymes present in nearly all living organisms. These enzymes catalyze the following reaction: $RH + O_2 + 2\,H^+ + 2e^- \rightarrow ROH + H_2O$. Different cytochromes P450 has specificity to a broad range of lipophilic compounds, which must be converted into a water soluble form to be excreted by the kidneys (Voet et al., 2016).

The expansive range of cytochrome P450 specialties has been speculated to be an evolutionary response to numerous toxins produced by plants, but the specificities tend to be somewhat generalized and overlap frequently (Voet et al., 2016). In addition to performing oxidative metabolism, the cytochromes P450 can mediate reactions that convert harmful compounds into safe forms and vice versa. Depending on the compound, various metabolic pathways may be overwhelmed if the dosage is high enough and can lead to fatal hepatotoxicity.

It has been identified in multiple studies that CBD may induce the cytochrome p450 mediated oxidative metabolism in the liver and cause an increase in ALP and ALT activity (Gamble et al., 2018; Deabold et al., 2019; Ewing et al., 2019; McGrath et al., 2019). Previously reported data involving elevated liver enzymes and CBD

supplementation strongly indicate that CBD oil may have a negative impact on liver function across multiple species. Normal ALP levels in blood serum of horses (.094 U/mL), calves (0.137 U/mL), sheep (0.101 U/mL), and goats (0.09 U/mL) are similar. Poultry are almost double to these grazing animals (chickens 0.284 U/mL and turkey 0.213 U/mL) (McComb et al, 1979). Normal levels of ALP in canines run from 0.005 to 0.160 U/mL with a mean of 0.083 U/mL similar to the levels for grazing animals (Hershey, 2019). The similarity of mean ALP levels between grazing animals and canines allows extrapolation of results between species in planning studies and dosage levels. Furthermore, ALT is not a useful indicator in horses and other large animals because of low enzyme activities in the liver of these animals; however, it is used in dogs and cats as an indicator of hepatocellular injury (eClinPath.com, Cornell University, https://eclinpath.com/chemistry/liver/liver-injury/alanine-aminotransferase/, 2022).

A hepatotoxicity study conducted in mice reported moderately elevated ALT and AST serum levels 24 hours after mice received a dose of 738 mg/kg or 2460 mg/kg CBD in their acute toxicity study (Ewing et al., 2019). In addition to the elevated ALT and AST levels 24 hours after dosing, mice that were given the 2460 mg/kg dose also had increased total bilirubin levels. The authors also did a sub-acute toxicity study for a period of 10 days and found that ALT and AST levels increased in mice given a dose of 615 mg/kg. A study of dogs being treated with CBD oil for pain relief from osteoarthritis reported a marked increase in ALP levels after 4 weeks of treatment and a slight increase in creatinine levels after 2 and 4 weeks (Gamble et al., 2018). In another study, dogs

with intractable idiopathic epilepsy had elevated ALP levels after being treated with CBD oil for 12 weeks (McGrath et al., 2019). A preliminary safety assessment of CBD oil supplementation in healthy dogs and cats showed an increase in ALP levels in dogs, but this was not considered statistically significant by the authors (Deabold et al., 2019).

To date, there are a small number of studies done on CBD in horses. A pilot pharmacokinetics study evaluating three different dosage levels (50 mg, 100 mg, and 250 mg) of a pelleted CBD supplement given to horses in a single dose found that there were dose-dependent differences in BUN and creatinine serum levels (see "Kidney Function" below). Alkaline Phosphatase was measured but did not show a statistical difference or trend (Draeger et al., 2021a). A follow-up pharmacokinetic study by the same author looked at the effects of CBD on the reactivity and heart rate (HR) of horses exposed to novel stimulus. The study fed 40 g of a pelleted CBD supplement containing 100 mg of CBD top dressed onto a concentrate and maintained control horses on their standard diet for 6 weeks. After 6 weeks, the horses were exposed to a novel object and responses were scored on a scale of 1 to 5, with 1 being no reaction and 5 being a complete loss of control. Heart rates were monitored during the test using a wireless HR monitor. No differences in HR were seen, but the treated horses had a lower reactivity score compared to the control group. Blood serum was not tested (Draeger et al., 2021b). This indicates that CBD may help calm horses suffering from anxiety or those easily overstimulated. Many situations stressing horses are only during certain handling procedures or environmental conditions (e.g., show anxiety). Although this study shows a potential for anxiety reduction, horse owners may desire a treatment that provides

benefits within hours or less. Further studies are needed to determine dosing levels and verify that this benefit can be obtained without negative impacts on the liver and kidneys.

Another pharmacokinetics study in exercising thoroughbreds administered a single oral dose of 0.5, 1, or 2 mg/kg of CBD suspended in sesame oil. Urine and blood samples were collected prior to the dosing and 72 h post administration. The study reported a terminal half-life of 10.7 ± 3.61 h, 10.6 ± 3.84 h, and 9.88 ± 3.53 h for 0.5, 1, and 2 mg/kg, respectively. In addition to pharmacokinetics, the study also evaluated the effects of CBD on inflammatory biomarkers using an ex vivo model. For this portion of the study, additional blood samples were collected at time 15, 30, and 45 min, and 1, 2, 3, 4, 5, 6, 8, 12, 18, 24, 30, 36, and 48 h following CBD administration. Reduction in inflammatory markers were reported shortly after stimulation of the CBD infused blood serum, however they increased with increasing time then fluctuated over time. Liver and kidney markers were not reported (Ryan et al., 2021). Another recent study evaluating the effects of CBD on in vitro lymphocyte pro-inflammatory cytokine production in senior horses reported that there was a significant reduction in cytokine production for a CBD-dimethyl sulfoxide solution at CBD concentration levels of 4, 6, 8, and 10 μg/mL. Liver and kidney markers were not reported (Turner et al., 2021). The results of the recent study and previous studies suggest that CBD has potential as an anti-inflammatory drug.

Kidney Function

Cannabinoid Type 1 Receptor (CB1R) and Cannabinoid Type 2 Receptor (CB2R) are found in numerous tissues including the kidneys as a result of CBD supplementation. Studies suggest stimulation of these receptors in the kidneys could have positive or negative effects. Dependencies on receptor distribution, type and level of renal injury, and timing of the stimulation relative to the state of the injury are not fully known. Stimulation of these receptors has impact on numerous physiological processes including memory, mood, pain sensation, sleep patterns, energy metabolism, and immune function. As a result, a large number of research areas are gaining interest covering effects on anxiety, cachexia, obesity, metabolic syndrome, atherosclerosis, depression, nausea, epilepsy, hypertension, multiple sclerosis spasms, rheumatoid arthritis, and others. Since CB1R and CB2R are expressed in the kidneys, there is potential for both positive and negative effects.

Cannabinoid Type 2 Receptors interact with heterotrimeric G-proteins consisting of Gαi subunits. The range of CB1R and CB2R response on cell signaling pathways is not fully known. Cannabinoid Type 1 Receptors have been shown to associate with other Gα subunits to produce a variety of effects including increase of intracellular calcium (Ca) levels (Park et al, 2017). Normal calcium levels in blood serum are 2.9 to 3.9 mmol/L or (converting using 0.2495 mmol-dL/mg-L) 11.62 to 15.63 mg/dL (Huntington, 2012). Blood Urea Nitrogen (BUN) and creatinine levels in the blood provide insight into kidney function. Urea is removed by the kidneys at a rate based on physiological need. Depending on factors such as hydration and blood pressure, the

kidneys will return different levels of urea to the blood. Elevated BUN levels are an important marker of kidney dysfunction. Creatinine is a waste output produced by the muscles. The muscles use creatine to generate energy for contractions. Creatinine levels are a reflection of muscle mass and therefore are largely constant from day to day. The kidneys filter creatinine from the blood into the urine. As the muscles are producing creatinine at a fairly constant rate, the removal process is also fairly constant. An increase in creatinine level in the blood is a good indicator of kidney stress (Yazdi, 2021).

A pilot pharmacokinetics study evaluating three different dosage levels (group TXT1 received 50 mg, group TXT2 received 100 mg, and group TXT3 received 250 mg) of a pelleted CBD supplement given to horses was performed by Murray State University (Murray, KY). The study evaluated pharmokinetics and short-term safety for the three dosage levels using 18 quarter horse geldings randomly assigned to the three treatment groups. Blood was collected within a 30 minute window prior to the treatment and at 0.5, 1, 2, 4 and 12 hours after. Blood serum and plasma were measured and statistics completed on serum chemistry. Serum chemistry and CBC results were within normal ranges. Normal ranges of BUN in horses are 10 to 25 mg/dL. Normal levels of creatinine in horses are 0.45 to 1.8 mg/dL. Differences were observed for BUN and creatinine. Pre-treatment levels were averaged across all three groups yielding BUN=15.89 mg/dL and creatinine=1.39 mg/dL. Increasing levels of BUN indicate kidney stress. Initial decrease in creatinine levels between TXT1 and TXT2 shows

potential benefit; however, the increase in creatinine between TXT2 and TXT3 suggests a point of diminishing return and even kidney stress (Draeger et al., 2021).

Summary

Safety and efficacy of CBD oil is currently being explored in dogs and cats, and clinical trials have shown a reduction in osteoarthritic pain and epileptic seizure frequency in dogs. Given the clinical success with dogs and cats, there is potential that CBD oil may elicit similar benefits to horses. Despite the increase of research in dogs and cats, there is limited information available regarding the acceptability, efficacy, and safety of dietary CBD oil products in horses. The cited research indicated a calming effect with no risk to the heart (i.e., no changes to heart rate), reduction in inflammation potential based on changes in inflammatory biomarkers, and potential kidney treatment based on reduction of creatinine. One reported a risk to kidney function based on elevated markers but did not see an increase in liver markers. Therefore, a lack of information is available concerning dietary CBD supplementation to mature horses.

CHAPTER III

MATERIALS AND METHODS

To evaluate the use of CBD oil in mature horses, 30 Quarter horse geldings (mean ± SD; 14.83 ± 2.61 y; initial body weight (BW) 556 ± 62.77 kg) were selected from an established herd at Texas A&M University (College Station, TX) and utilized in a completely randomized design. Horses were evenly stratified and balanced based on BW and age, and randomly assigned to one of three dietary treatment groups (n = 10 horses/treatment) for a 28-day trial. All horses were placed on a similar nutritional background for 30 d prior to the start of the trial, and blood samples were obtained via jugular venipuncture into a 7.5 mL sterile non-additive collection tube (BD Vacutainer, BD#367987, Fisher Scientific, Pittsburgh, PA). A blood chemistry profile panel was performed (Texas Veterinary Medical Diagnostic Laboratory, TVMDL, College Station, TX) and evaluated by a board-certified veterinarian to ensure values were within normal physiological limits prior to enrolling horses into the study.

Horses meeting the inclusion criteria received dietary treatments consisting of canola oil supplementation (CON) and two levels of CBD containing oil (canola base; Arrowhead Laboratories, Costa Mesa, CA) that provided 0.13 mg CBD/kg BW (TRT1) or 0.12 mg CBD/kg BW (TRT2) of CBD. Treatments were divided evenly between the two daily concentrate meals, top-dressed and thoroughly mixed, immediately prior to feeding. Levels of CBD supplementation were determined based on previous literature in horses (Ryan et al., 2021) and other species using up to 1.5 mg CBD/kg BW (Gamble et al., 2018; Deabold et al., 2019; Wakshlag et al., 2020; Brioschi et al., 2020) to avoid

negative implications on liver health. An oil-based formulation was chosen due to ease of administration and higher bioavailability when compared to solid-based formulations (Zgair et al., 2016; Gamble et al., 2018; Fathordoobady et al., 2019; Brighenti et al., 2021).

All horses were offered 0.75% BW (as-fed) of a commercially available concentrate (Producer's Golden Years, Producer's COOP, Bryan, TX) daily to meet or slightly exceed the nutritional requirements for mature horses at maintenance (NRC, 2007). Concentrate was divided evenly between two meals offered at 12-h intervals using attachable feed bags (Derby Originals LLC; North Canton, OH). Horses were allowed 60 min to consume concentrate meals. Intakes and orts were recorded daily.

Horses also had ad libitum access to water and round bales of coastal bermudagrass (Cynodon dactylon) hay in a group-housed setting and all horses grazed 2 h/d on the same coastal bermudagrass pasture. Every 14 days, BW was obtained using a calibrated livestock scale and BCS were assigned by three independent observers using the Henneke et al. (1983) system. Composited samples of concentrate, hay, and pasture were analyzed for nutrient content (Table 1) using a commercial laboratory (Cargill Inc., Elk River Forage Lab, Elk River, MN). Composited samples of each oil were also collected, and CBD levels were determined by an independent laboratory (TVMDL, College Station, TX).

Table 1. Nutrient composition of a commercially available pelleted concentrate, Coastal bermudagrass (Cynodon dactylon) hay, and Coastal bermudagrass pasture fed to mature horses

Item[1]	Concentrate[2]	Hay	Pasture
Nutrient, % DM			
CP, %	18.55	6.18	12.87
Starch, %	17.99	2.16	1.49
Crude Fat, %	6.09	1.60	2.33
ADF, %	23.23	35.47	37.84
NDF, %	32.34	62.15	62.70
Ca, %	0.82	0.27	0.31
P, %	0.60	0.17	0.29

[1]Elk River Forage Lab (Elk River, MN)
[2]Concentrate consisted of 0.75% BW (as-fed) daily of a commercially formulated concentrate (Producer's Golden Years, Producer's Cooperative Association, Bryan, TX)

CBD Oil Extraction

In brief, 20 µL of sample was pipetted into a screw-top tube. After adding 5 mL of 0.1 % acetic acid acetonitrile to the extract, the tube was capped, and roto-racked for 10 min and centrifuged for 5 min at 2700 x g. The supernatant was then transferred to another tube and evaporated to dryness under a stream of nitrogen at approximately 40°C. The residue was reconstituted with 1ml of 20% methanol before loading onto the CEREX® WWP cartridge, previously conditioned with 1 mL of methanol, followed by 1 mL of deionized water. The cartridge was then washed with 1 mL of deionized water, 1 mL of 1N acetic acid and 1 mL of 20% methanol. The target analyte was eluted into two fractions using 1 mL of Hexan and 1 mL of ethyl acetate/Hexan (1:1, v/v). The two

fractions were then combined and dried under nitrogen at 40°C. The extract was reconstituted with 80 µL 50% acetonitrile for instrumental analysis.

All LC–MS/MS analyses were performed using a Thermo Altis (Thermo, San Jose, CA) triple quadrupole mass spectrometer with an electrospray ionization (ESI) source. LC separations were carried out on an Ascentis® Express C18 column (100×2.1 mm ID, 2.7 µm) with a C18 guard column (5×2.1 mm ID, 2.7 µm) (St. Louis, MO) maintained at 40°C. Mobile phase A (water/formic acid, 100:0.1, v/v) and B (ACN/formic acid, 100:0.1, v/v) were employed for elution of the analyte from the column. The following gradient elution was used at a flow rate of 0.3 mL/min: 0.00–0.50 min: 50% B; 0.50–3.00 min: 50% B → 95% B; 3.00–4.50 min: 95% B; 4.50–5.00 min: 95% B → 50% B, and 5.00–7.00 min: 50% B. All MS/MS data were collected in positive ion mode by selected reaction monitoring (SRM) of the transition m/z 315.1500→ m/z 123.0540 and 259.1250 for cannabidiol (CBD) and m/z 318.2500→ m/z 196.1370 for CBD-d3 (internal standard). The optimized parameter settings for ESI were: positive ion voltage, 4.5 kV; ion transfer tube temperature, 325°C; vaporizer temperature, 400°C. A 10 µL of extracted analyte was injected for LC– MS/MS analysis.

Sample Collection and Analysis

Blood samples were collected prior to the start of the trial (d 0) and on d 28 following the onset of supplementation. Serum was collected into non-additive sterile blood collection tubes and remained at room temperature for approximately 1 h prior to centrifugation. Samples were centrifuged at 2700 x g at 10°C for 20 min (ALC, PM140R, Thermo Fisher Sci., Waltham, MA), and then harvested and transported to

TVMDL on ice to evaluate blood parameters and liver enzymes. Serum samples were also tested for drugs of abuse using liquid chromatography/mass spectrometry for the detection of amphetamines, benzodiazepines, promazines, barbiturates, opiates, marijuana, and other drug types (TVMDL, College Station, TX).

Drugs of abuse sample extraction included 0.5 ml of 1N acetic acid that was added to 1 ml serum sample. Before loading onto the CEREX® MD SCX-60 cartridge, the cartridge was conditioned with 1 mL of methanol, followed by 1 mL of deionized water and 0.5 ml 1N acetic acid. The cartridge was then washed with 1 mL of deionized water, 0.4 mL of 1N acetic acid, 1 mL of 20% methanol and 1ml Hexan. The target analyte was eluted with into two fractions using 1 mL of ethyl acetate/methanol (1:1, v/v) and 1 mL of ethyl acetate/methanol/ammonium hydroxide (70:30:4, v/v/v). The two fractions were then combined followed by adding 1 drop of 1N methanol HCl and dried under nitrogen at 40°C. The extract was reconstituted with 80 µL 10% acetonitrile for instrumental analysis.

All drugs of abuse were performed using a Q Exactive–Orbitrap (Thermo, San Jose, CA USA) with heated electrosprayionisation (HESI-II) with full scan. The HESI source was heated to 350°C and equipped with a high-flow metal needle insert. The sheath gas and auxiliary gas pressure were set at 50 and 15 arbitrary Q Exactive units respectively. The sweep gas was set to 3 arbitrary units. The ion spray voltage was + 3.2 kV and the capillary temperature was set at 350°C. The S-Lens RF level was 50%. Full scan mass spectra were acquired using a mass resolution of 70,000. The acquired mass range was from m/z 120–1200. LC separations were carried out on an Ascentis®

Express C18 column (100×2.1 mm ID, 2.7 μm) with a C18 guard column (5×2.1 mm ID, 2.7 μm) (St. Louis, MO, USA) maintained at 40 °C. Mobile phase A (water/formic acid, 100:0.1, v/v) and B (ACN/formic acid, 100:0.1, v/v) were employed for elution of the analyte from the column. The following gradient elution was used at a flow rate of 0.3 mL/min: 0.00–1.00 min: 5% B; 1.00–9.00 min: 5% B → 95% B; 9.00–10.00 min: 95% B; 10.00–10.20 min: 95% B → 5% B, and 10.2–12.20 min: 5% B. A 10 μL of extracted analyte was injected for LC– MS/MS analysis.

Statistical Analysis

Investigators were blinded to dietary treatments until data analysis, with CON and the two levels of CBD alphabetically coded (A, B, C) in pre-weighed bottles provided by the manufacturer. Data were analyzed using the mixed procedure in SAS (v9.4). Data were tested for normality and outliers were identified using box plots of the residuals and removed if greater than two standard deviations from the mean.

Body weight and BCS were analyzed separately from values obtained from the blood chemistry panel. For performance variables, the model contained fixed effects for treatment, time and an interaction for treatment × time. Both RANDOM and REPEATED statements were also utilized in the model to account for variability between animals. A covariate was used to account for differences in blood chemistry on d 0, and main effect tested was treatment. Linear and quadratic effects were tested in the form of orthogonal contrasts. Contrast values were determined using PROC IML in SAS. The effects were considered significant if $P \leq 0.05$ with a trend towards significance if $P \leq 0.10$.

CHAPTER IV

RESULTS AND DISCUSSION

Cannabidiol Concentrations

Levels of CBD supplementation were formulated by the manufacturer to achieve a targeted intake of 0.75 mg CBD/kg BW and 1.5 mg CBD/kg BW per day. The required concentrations to achieve these targets were 23 and 46 mg/mL, respectively. However, composited samples of each treatment that were obtained during the trial revealed supplemented products contained 3.7 and 4.0 mg/mL for the TRT2 and TRT1 groups when tested by an independent laboratory (TVMDL). Therefore, horses in TRT2 and TRT1 received an average of 0.12 mg CBD/kg BW and 0.13 mg CBD/kg BW, respectively. This represents an 84% and 91.3% reduction from targeted to actual intake of CBD to mature horses in the current study.

Literature indicates inconsistencies across industry in concentrations due to plant type and processing (Hazekamp, 2018). This would not explain the present study results as differences were seen in measured concentration of the same product. Temperature and photo-instability have a role (Mazzetti et al., 2020), but would not account for the magnitude of differences seen here. Supplements were maintained in a room temperature environment until needed and only kept in the barn (ambient outdoor temperatures) for 1 to 3 days in a shaded location.

Literature indicates that a lack of standardized test methods is the most significant issue, emphasizing the need for adoption of testing standards by industry, states, or even the federal government (Jikomes and Zoorob, 2018). Note, the producer

measured concentrations using High Performance Liquid Chromatography (HPLC) and the verification lab (TVMDL) measured concentrations using Liquid Chromatography/Mass Spectrometry (LC/MS). It cannot be concluded that these different methods resulted in the discrepancy in concentration. To fully reconcile the differences would require a comparison of test methods between the manufacturer and TVMDL, production of new samples, strict control of handling including temperature and sunlight exposure, and then testing comparisons between the two facilities.

Although the manufacturer tested the product prior to shipment, this study must rely on the independent verification as the levels actually studied for two reasons: 1) the independent verification was on samples taken at the time of the study, and 2) the authors have intimate understanding of the test methods used by TVMDL.

Intake and Performance Characteristics

The current study examined the effects of cannabidiol oil on horse performance and blood chemistry parameters in mature horses. Top-dressing the canola-based oil onto the concentrate, did not affect ($P = 0.97$) the intake of concentrate, as horses prefer vegetable oils such as canola oil over animal-based oils (Warren, 2011). Similarly, there was no effect of CBD oil supplementation on BW ($P = 0.97$) or BCS ($P = 0.44$); however, BCS increased ($P \leq 0.01$) in all horses (mean ± SEM; 6.04 ± 0.14 to 6.33 ± 0.14) to d 14 then decreased from d 14 to 28 (mean ± SEM; 6.13 ± 0.14) across treatments (Figure 2). While differences were observed in this study over time, BCS remained within normal ranges of 4 to 6 for mature horses at maintenance (Camargo et al., 2019; Henneke et al., 1983). No other studies have reported effects of CBD

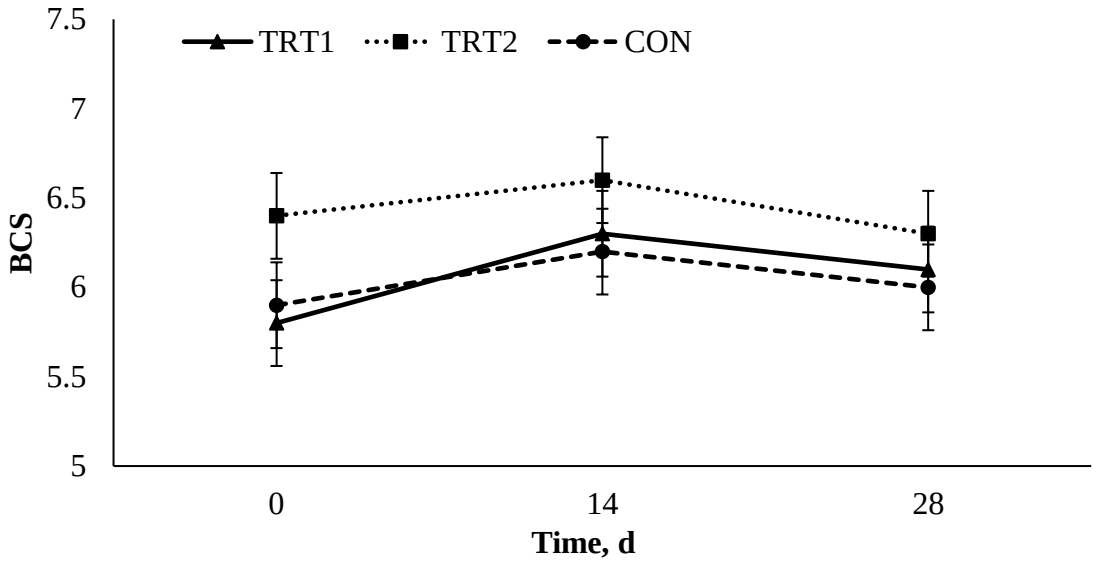
supplementation on BW and BCS in horses. A study of CBD supplementation in dogs and cats did not indicate a change in BW after 12 weeks using a dose of 2 mg/kg twice daily (Deabold et al., 2019). Similarly, Vaughn et al. (2020) provided increasing dosages from 1.75 mg/kg to 61.75 mg/kg over a 30-d period without a change in BW and McGrath et al. (2019) administered 2.5 mg/kg twice daily for 12 weeks without a change in BW. Limited information is available concerning the effects of CBD supplementation on performance variables in dogs, cats, and horses and should continue to be investigated further in future studies.

No drugs of abuse (amphetamines, benzodiazepines, promazines, barbiturates, opiates, or marijuana) were detected in blood on d 0 and following 28 d of supplementation. This is important in that most competition rulebooks prohibit these substances, and their presence would result in disqualification of the horse and competitor. However, this is not sufficient for use in the competitive horse industry as CBD itself is banned. For example, the current National Cutting Horse Association (NCHA) bans any substances considered a Class I or II drug as defined by the Uniform Classification Guidelines for Foreign Substances provided by the Association of Racing Commissioners International (ARCI) (National Cutting Horse Association Rules and Regulations, 2022). The ARCI define a Class I substance as those listed by the US Drug Enforcement Agency (DEA) as a Schedule I controlled substance and some of the Schedule II substances (Association of Racing Commissioners International, 2020; Controlled Substances Act, 1970). ARCI Class II substances are those with high potential to affect the outcome of a race, and CBD is currently on that list. The

Federation Equestre Internationale (FEI) has specifically listed CBD and cannabidiolic acid (CBDA) as controlled substances, which may not be used in competition (2022 Equine Prohibited Substances List, 2021). Finally, the United States Equestrian Federation (USEF) does not allow CBD or any other cannabinoid compounds (USEF Guidelines and Rules for Drugs and Medications, 2022).

Many of the attributes of CBD (e.g. treatment of inflammation) are of particular value to competition horses. In order to use CBD outside of competition (i.e. during training), attention to withdrawal times is needed to allow clearing from the blood prior to an event. With the inconsistencies in concentrations of CBD products, this is a challenge that may outweigh benefit. This is a significant market segment for CBD based products, especially with the ease of access to these products (Ryan et al., 2021; Williams et al., 2021). Therefore, horse owners will be discouraged to use CBD in competition horses if disqualification concerns arise from its use.

Figure 2. Body condition score (BCS) over time (d) for mature horses receiving a supplement formulated to provide 0.13 mg/kg BW cannabidiol oil (TRT1; n=10), 0.12 mg/kg BW cannabidiol oil (TRT2; n=10) or canola oil (CON; n=10) on d 0, 14, and 28. [a-c]Different superscripts denote the main effect of time ($P \leq 0.01$).



Blood Chemistry Parameters

A linear dose response was observed ($P \leq 0.05$) for blood Ca levels, with Ca concentrations increasing from 11.37 ± 0.07 mg/dL in CON to 11.67 ± 0.07 mg/dL in TRT1 horses. Levels of serum Ca, following 28 d of supplementation, were below the physiologically normal range (11.62 to 15.63 mg/dL) for both CON and TRT2 horses, while TRT1 remained within limits (Huntington, 2012).

Cannabinoid Type 1 Receptor (CB1R) and Cannabinoid Type 2 Receptor (CB2R) are found in numerous tissues including the kidneys as a result of CBD supplementation. Studies suggest stimulation of these receptors in the kidneys could

have positive or negative effects. Dependencies on receptor distribution, type and level of renal injury, and timing of the stimulation relative to the state of the injury are not fully known. Cannabinoid Type 2 Receptors interact with heterotrimeric G-proteins consisting of Gαi subunits. The range of CB1R and CB2R response on cell signaling pathways is not fully known.

Cannabinoid Type 1 Receptors have been shown to associate with other Gα subunits to produce a variety of effects including increase of intracellular calcium (Ca) levels (Park et al, 2017). Increased calcium levels beyond normal ranges may indicate chronic renal failure, primary hyperthyroidism, cancer, or other disorders in dogs, cats, and horses (Kahn and Line, 2010). This study did not see Ca levels in blood serum exceed normal levels with CBD supplementation, which is similar to previous studies in which values remained within normal limits (Deabold et al., 2019; Gamble et al., 2018; Vaughn et al., 2020; Draeger et al, 2021a)

In contrast to Ca, creatinine levels tended to decrease (P = 0.06) with increasing levels of CBD supplementation (CON = 1.41 mg/dL, TRT2 = 1.34 mg/dL, TRT1 = 1.31 mg/dL). Creatinine is a waste output produced by the muscle (Yazdi, 2021). The muscle uses a protein known as creatine to generate energy for contractions. Creatinine levels are a reflection of muscle mass and therefore are largely constant from day to day. Normal levels of creatinine in horses are 0.45 to 1.8 mg/dL. The kidneys filter creatinine from the blood into the urine. As the muscles are producing creatinine at a fairly constant rate, the removal process is also fairly constant. An increase in creatinine level in the blood is a good indicator of kidney stress. A reduction is indicative of improved

kidney function (Yazdi, 2021). The current study observed a reduction in creatinine levels, with increasing CBD treatment level. A reduction of creatinine can be produced by improving kidney health or function. Increasing fiber in the diet is an example of another means of reducing creatinine (Yazdi, 2021).

A similar study saw the same reduction in creatinine with CBD supplementation levels of 0.09 mg/kg BW and 0.18 mg/kg BW yielding 1.41 mg/dL and 1.22 mg/dL, respectively. (Draeger et al., 2021a). The remaining blood chemistry parameters including blood urea nitrogen, total protein, albumin, glucose, phosphorous, sodium, potassium, Na:K, and Cl were not influenced ($P \geq 0.13$) by dietary treatment. Draeger et. al. (2021a) was the only study in horses that reported a significant decrease in albumin when comparing the 50 mg group to the 250 mg group. The current study did not observe a similar decrease in albumin levels. Furthermore, previous work done in dogs and cats has demonstrated similar findings in which no other changes were observed in blood chemistry values (Brioschi et al., 2020; Vaughn et al., 2020; Deabold et al., 2019; McGrath et al., 2019; Gamble et al., 2018).

Table 2. Least square treatment means of blood chemistry parameters in serum of mature horses receiving 0.13 mg/kg BW cannabidiol oil (TRT1; n=10), 0.12 mg/kg BW cannabidiol oil (TRT2; n=10) or canola oil (CON; n=10) top-dressed onto a pelleted concentrate

	Dietary Treatments				*P*-values[1]	
Parameter	**TRT1**	**TRT2**	**CON**	**SEM**	**Trt**	**Contrast**
Blood Urea Nitrogen mg/dL	18.16	18.61	18.91	0.61	0.67	0.42
Creatinine mg/dL	1.31*	1.34*,ǂ	1.41ǂ	0.03	0.07	0.06
Total Protein g/dL	6.38	6.46	6.48	0.08	0.67	0.39
Albumin g/dL	3.37	3.35	3.32	0.03	0.56	0.32
Glucose mg/dL	77.14	79.04	80.05	2.10	0.61	0.34
Calcium mg/dL	11.67[a]	11.42[b]	11.37[b]	0.06	0.01	0.01
Phosphorous mg/dL	3.05	3.26	3.27	0.11	0.28	0.12
Sodium mEq/L	137.19	137.09	137.16	0.32	0.97	0.89
Potassium mEg/L	3.78	3.87	3.67	0.07	0.13	0.13
Na:K	36.24	35.54	37.08	0.56	0.21	0.82
Chloride mEq/L	100.02	99.81	99.86	0.39	0.93	0.72

[1] Trt = main effect of treatment for the 28-d trial; Contrast = linear contrast
[a,b] Superscripts denote a difference in dietary treatment ($P < 0.05$)
*,ǂ Superscripts denote a tendency in dietary treatment ($P < 0.10$)

Enzyme Activity

Gamma-glutamyl transferase (GGT) demonstrated a quadratic dosage response, as TRT2 was greater than CON (P = 0.03). The values were within normal levels of 8 to 33 U/L and did not indicate any negative effects on the liver in the current study, but it

has been demonstrated in other studies done in dogs, cats, and horses that elevated liver values indicates potential harmful effects to the liver. Gamma-glutamyl transferase is a marker of intrahepatic or extrahepatic cholestasis, and it is associated with the cell membranes of hepatocytes of the bile canaliculi and ducts. It is also associated with periportal hepatocytes. Cannabidiol can cause hepatotoxicity resulting in elevated GGT (McAtee and Lidbury, 2017; Ewing et. al., 2019). In general, gamma-glutamyl transferase is a particularly useful indicator for horses and ruminants for long term liver damage (Kahn and Line, 2010). The remaining liver enzymes (alkaline phosphatase, creatine kinase, aspartate aminotransferase, total bilirubin, bilirubin direct, albumin:globulin, globulins, glutamate dehydrogenase) were not influenced by dietary treatment ($P \geq 0.16$). One study by Draeger et al. (2021a) examined alkaline phosphatase and did not see a difference. Further studies should investigate the effects of CBD on liver function in horses at higher dosages.

Table 3. Least square treatment means of liver enzyme analytes in serum of mature horses receiving 0.13 mg/kg BW cannabidiol oil (TRT1; n=10), 0.12 mg/kg BW cannabidiol oil (TRT2; n=10) or canola oil (CON; n=10) top-dressed onto a pelleted concentrate

	Dietary Treatments				*P*-values[1]	
Parameter	**TRT1**	**TRT2**	**CON**	**SEM**	**TRT**	**Contrast**
Alkaline phosphatase U/L	160.77	165.07	156.40	6.17	0.60	0.32
Creatine kinase U/L	274.22	249.35	223.58	21.52	0.23	0.40
Aspartate aminotransferase	273.56	273.34	280.25	6.37	0.66	0.44
Total bilirubin mg/dL	1.02	1.06	1.19	0.07	0.16	0.53
Bilirubin direct mg/dL	0.28	0.27	0.33	0.02	0.17	0.09
Albumin:Globulin	1.09	1.05	1.08	0.02	0.43	0.30
Globulins g/dL	3.06	3.14	3.08	0.07	0.74	0.53
Glutamate Dehydrogenase U/L	4.48	4.93	4.09	0.63	0.66	0.37
Gamma-glutamyl transferase U/L	16.58[b]	17.38[b]	14.86[a]	0.77	0.08	0.03

[1] Trt = main effect of treatment for the 28-d trial; Contrast = quadratic contrast
[a,b] Superscripts denote a tendency in dietary treatment ($P < 0.10$)

CHAPTER V

CONCLUSIONS

The present study supports the use of top-dressed CBD oil supplementation to feed concentrate as a convenient and reliable means of administration. Horses appear to willingly accept the product on feed. Banned substances were not found in blood serum which would present potential legal problems in developing CBD as a therapeutic and be a detraction to the competitive horse market. Cannabidiol is currently banned in competition and its use complicated by concentration inconsistencies. Management of withdrawal times is problematic with a lack of consistent products and standardized testing.

In the current study, markers related to the health of both the liver and the kidneys, did not exceed normal limits. Despite remaining in normal ranges, serum calcium increased with CBD treatment level. Since CBD receptors interact with G-proteins in the kidneys, this is a parameter that future studies and therapeutic development will need to monitor. This study saw a reduction in creatinine level indicating potential benefit to kidneys. Other studies in mice, dogs, and horses suggest that CBD has potential positive and negative impact to the kidneys (Draeger et al., 2021a; Gamble et al., 2018; Park et al., 2017). Findings herein would agree with a positive impact, suggesting potential as a therapeutic for kidney disease or injury. Levels of GGT demonstrated a quadratic dosage response, and it is the only liver marker change with statistical significance observed in this study. Dosage levels in this study were lower when compared to other studies, where a greater number of liver markers were

impacted. Overall, presented data herein can be combined with results from other studies to develop plans for future studies to establish more comprehensive dosing level guidelines.

REFERENCES

2022 Equine Prohibited Substances List. 2021. In: FEI Equine Anti-Doping and Controlled Medication Regulations. 3rd ed.

AQHA Official Handbook of Rules and Regulations. 2022. 70th ed.

Brighenti, V., M. Protti, L. Anceschi, C. Zanardi, L. Mercolini, and F. Pellati. 2021. Emerging challenges in the extraction, analysis and bioanalysis of cannabidiol and related compounds. J. Pharm. Biomed. Anal. 192. doi:10.1016/j.jpba.2020.113633.

Brioschi, F. A., F. Di Cesare, D. Gioeni, V. Rabbogliatti, F. Ferrari, E. S. D'urso, M. Amari, and G. Ravasio. 2020. Oral transmucosal cannabidiol oil formulation as part of a multimodal analgesic regimen: Effects on pain relief and quality of life improvement in dogs affected by spontaneous osteoarthritis. Animals. 10:1–14. doi:10.3390/ani10091505.

Camargo, F., L. Lawrence, and B. Coleman. 2019. Body Condition Scoring Horses: Step-by-Step. Univ. Kentucky Dep. Anim. Food Sci. 1-9. Available from: https://thehorse.com/164978/body-condition-scoring-horses-step-by-step/

Carrier, E. J., J. A. Auchampach, and C. J. Hillard. 2006. Inhibition of an equilibrative nucleoside transporter by cannabidiol: A mechanism of cannabinoid immunosuppression. Proc. Natl. Acad. Sci. 103:7895–7900. Available from: www.pnas.orgcgidoi10.1073pnas.0511232103

Cheeke, P. R., and E. S. Dierenfeld. 2010. Comparative Animal Nutrition and Metabolism. First. (S. Hulbert and K. Hill, editors.). CABI.

Chen, L., H. Deng, H. Cui, J. Fang, Z. Zuo, J. Deng, Y. Li, X. Wang, and L. Zhao. 2018. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 9:7204–7218.

Controlled Substances Act. 1970. Congress. Available from: https://uscode.house.gov/view.xhtml?req=(title:21 section:812 edition:prelim)OR (granuleid:USC-prelim-title21section812)&f=treesort&num=0&edition=prelim

Deabold, K. A., W. S. Schwark, L. Wolf, and J. J. Wakshlag. 2019. Single-Dose Pharmacokinetics and Preliminary Safety Assessment with Use of CBD-Rich Hemp Nutraceutical in Healthy Dogs and Cats. Animals. 9. doi:10.3390/ani9100832.

Devinsky, O., A. D. Patel, J. H. Cross, V. Villanueva, E. C. Wirrell, M. Privitera, S. M. Greenwood, C. Roberts, D. Checketts, K. E. VanLandingham, and S. M. Zuberi. 2018. Effect of Cannabidiol on Drop Seizures in the Lennox–Gastaut Syndrome. N. Engl. J. Med. 378:1888–1897. doi:10.1056/nejmoa1714631.

Draeger, Anna L, L. K. Hoffman, P. R. Godwin, and A. J. Davis. 2021a. Pharmacokinetics of a Single Feeding of Pelleted Cannabidiol in Horses. Steeplechase An ORCA Student J. 4.

Draeger, Anna L., E. P. Thomas, K. A. Jones, A. J. Davis, and C. A. S. Porr. 2021b. The effects of pelleted cannabidiol supplementation on heart rate and reaction scores in horses. J. Vet. Behav. 46:97–100. doi:10.1016/J.JVEB.2021.09.003.

Ewing, L. E., C. M. Skinner, C. M. Quick, S. Kennon-McGill, M. R. McGill, L. A. Walker, M. A. ElSohly, B. J. Gurley, and I. Koturbash. 2019. Hepatotoxicity of a Cannabidiol-rich cannabis extract in the mouse model. Molecules. 24:1–18. doi:10.3390/molecules24091694.

Fathordoobady, F., A. Singh, D. D. Kitts, and A. Pratap Singh. 2019. Hemp (Cannabis Sativa L.) Extract: Anti-Microbial Properties, Methods of Extraction, and Potential Oral Delivery. Food Rev. Int. 35:664–684. doi:10.1080/87559129.2019.1600539.

Gamble, L.-J., J. M. Boesch, C. W. Frye, W. S. Schwark, S. Mann, L. Wolfe, H. Brown, E. S. Berthelsen, and J. J. Wakshlag. 2018. Pharmacokinetics, Safety, and Clinical Efficacy of Cannabidiol Treatment in Osteoarthritic Dogs. Front. Vet. Sci. 5:1–9. doi:10.3389/fvets.2018.00165.

Gomez, B. 2021. Hemp CBD Market to Reach $16 Billion By 2025. Available from: https://blog.brightfieldgroup.com/hemp-cbd-market-size

International, R. C. 2017. The Association of Racing Commissioners International ’ s Model Rules of Racing.

Kahn, C. M., and S. Line. 2010. The Merck Veterinary Manual. Tenth. Gary Zelko.

Kogan, L. R., P. W. Hellyer, and N. G. Robinson. 2016. CONSUMERS' PERCEPTIONS OF HEMP PRODUCTS FOR ANIMALS Scientific Report. AHVMA J. •. 42. Available from: http://www.drugabuse.gov/

McAtee, B. B., and J. A. Lidbury. 2017. Liver Enzyme Interpretation and Liver Function Tests. Today's Vet. Pract. 7:29–38.

McGrath, S., L. R. Bartner, S. Rao, R. A. Packer, and D. L. Gustafson. 2019. Randomized blinded controlled clinical trial to assess the effect of oral cannabidiol administration in addition to conventional antiepileptic treatment on seizure frequency in dogs with intractable idiopathic epilepsy. J. Am. Vet. Med. Assoc. 254:1301–1308. doi:10.2460/javma.254.11.1301.

Mlost, J., M. Bryk, and K. Starowicz. 2020. Cannabidiol for pain treatment: Focus on pharmacology and mechanism of action. Int. J. Mol. Sci. 21:1–22. doi:10.3390/ijms21228870.

Park, F., P. K. Potukuchi, H. Moradi, and C. P. Kovesdy. 2017. Cannabinoids and the kidney: Effects in health and disease. Am. J. Physiol. - Ren. Physiol. 313:F1124–F1132. doi:10.1152/ajprenal.00290.2017.

Ribeiro, A., V. Ferraz-De-Paula, M. L. Pinheiro, L. B. Vitoretti, D. P. Mariano-Souza, W. M. Quinteiro-Filho, A. T. Akamine, V. I. Almeida, J. Quevedo, F. Dal-Pizzol, J. E. Hallak, A. W. Zuardi, J. A. Crippa, and J. Palermo-Neto. 2012. Cannabidiol, a non-psychotropic plant-derived cannabinoid, decreases inflammation in a murine model of acute lung injury: Role for the adenosine A 2A receptor. Eur. J.

Pharmacol. 678:78–85. doi:10.1016/j.ejphar.2011.12.043. Available from: http://dx.doi.org/10.1016/j.ejphar.2011.12.043

Ruiz, M. D. L., Y. Lim, and J. Zheng. 2014. Adenosine A2A Receptor as a Drug Discovery Target. J. Med. Chem. 57:3623–3650.

Ryan, D., D. S. McKemie, P. H. Kass, B. Puschner, and H. K. Knych. 2021. Pharmacokinetics and effects on arachidonic acid metabolism of low doses of cannabidiol following oral administration to horses. Drug Test. Anal. 13:1305–1317. doi:10.1002/dta.3028.

Sakimura, K. 2009. Ionotropic Receptor. In: M. D. Binder, N. Hirokawa, and U. Windhorst, editors. Encyclopedia of Neuroscience. Springer Berlin Heidelberg, Berlin, Heidelberg. p. 2056–2060. Available from: https://doi.org/10.1007/978-3-540-29678-2_2596

Shao, B.-Z., W. Wei, P. Ke, Z.-Q. Xu, J.-X. Zhou, and C. Liu. 2014. Activating Cannabinoid Receptor 2 Alleviates Pathogenesis of Experimental Autoimmune Encephalomyelitis Via Activation of Autophagy and Inhibiting NLRP3 Inflammasome. CNS Neurosci. Ther. 20:1021–1028. doi:10.1111/cns.12349.

Smith, S. 2020. Hemp on the Horizon : The 2018 Farm Bill and the Future of CBD. North Carol. Law Rev. 98:35–58.

Sterns, J. A. 2019. Is the Emerging U . S . Hemp Industry Yet Another Boom – Bust Market for U. S. Farmers? Choices. 34:1–8.

Turner, S., V. D. Barker, and A. A. Adams. 2021. Effects of Cannabidiol on the In Vitro Lymphocyte Pro-Inflammatory Cytokine Production of Senior Horses. J. Equine Vet. Sci. 103. doi:10.1016/j.jevs.2021.103668.

USEF Guidelines and Rules for Drugs and Medications. 2022. United States Equestrian Federation.

Van Niekerk, G., · T Mabin, and A.-M. Engelbrecht. 2019. Anti-inflammatory mechanisms of cannabinoids: an immunometabolic perspective. Inflammopharmacology. 27:39–46. doi:10.1007/s10787-018-00560-7. Available from: https://doi.org/10.1007/s10787-018-00560-7

Vaughn, D., J. Kulpa, and L. Paulionis. 2020. Preliminary Investigation of the Safety of Escalating Cannabinoid Doses in Healthy Dogs. Front. Vet. Sci. 7:1–13. doi:10.3389/fvets.2020.00051.

Voet, D., J. G. Voet, and C. W. Pratt. 2016. Fundamentals of Biochemistry: Life at the Molecular Level. 5th ed. (J. Kalkut, E. Swain, and B. Ray, editors.). John Wiley & Sons, Inc.

Wakshlag, J. J., W. S. Schwark, K. A. Deabold, B. N. Talsma, S. Cital, A. Lyubimov, A. Iqbal, and A. Zakharov. 2020. Pharmacokinetics of Cannabidiol, Cannabidiolic Acid, Δ9-Tetrahydrocannabinol, Tetrahydrocannabinolic Acid and Related Metabolites in Canine Serum After Dosing With Three Oral Forms of Hemp Extract. Front. Vet. Sci. 7:1–12. doi:10.3389/fvets.2020.00505.

Warren, L. K. 2011. The Skinny on Feeding Fat to Horses. Florida Equine Inst. Allied Trade Show. 8:19–24.

Yazdi, P. 2021. BUN_Creatinine Ratio_ High & Low Levels + Normal Range - SelfDecode Labs. SelfDecode Labs.

Zgair, A., J. C. M. Wong, J. B. Lee, J. Mistry, O. Sivak, K. M. Wasan, I. M. Hennig, D. A. Barrett, C. S. Constantinescu, P. M. Fischer, and P. Gershkovich. 2016. Dietary fats and pharmaceutical lipid excipients increase systemic exposure to orally administered cannabis and cannabis-based medicines. Am. J. Transl. Res. 8:3448–3459.