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The Effects of Cannabidiol on Senior Equine Health

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THE EFFECTS OF CANNABIDIOL ON SENIOR EQUINE HEALTH

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By
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ABSTRACT OF DISSERTATION

THE EFFECTS OF CANNABIDIOL ON SENIOR EQUINE HEALTH

Cannabis sativa L., has been revealed to produce hundreds of phytocannabinoids, of which cannabidiol (CBD) is one of the most desired. It has been revealed that CBD can potentially treat inflammation and act as an analgesic in humans without psychoactive effects of delta-9-tetrahydrocannabinol (THC). Recently, there has been interest in understanding the potential health benefits of CBD for horses. With an increasing senior horse population (< 15 years old), alternatives to the use of non-steroidal anti-inflammatory drugs (NSAIDs) such as phenylbutazone, firocoxib, and flunixin meglumine, are desired as these common anti-inflammatory treatments have negative side effects. Because senior horses may have chronic low-grade inflammation, termed inflamm-aging, they are a natural model to investigate the efficacy of CBD on inflammatory responses as well as other health parameters. Given this interest, and due to the lack of research conducted on CBD in the horse, the overall objective of this dissertation work was to conduct both basic and applied research investigating the effects of CBD on equine immune function and health. The first objective was to determine the *in vitro* effects of CBD as an anti-inflammatory. Peripheral blood mononuclear cells (PBMCs) from senior horses were cultured *in vitro* with increasing concentrations of pure CBD dissolved in dimethyl sulfoxide (DMSO). These cells were then stimulated, cell viability and cytokine production were measured. The concentration of CBD that did not affect cell viability was 4 µg/mL. CBD at 4 µg/mL significantly reduced production of TNFα and IFNγ. RT-PCR results for TNFα and IFNγ at 4 µg/mL showed a reduction compared with the positive control and IL-10 showed a similar reduction at 2 µg/mL and 4 µg/mL. RT-PCR gene expression results showed significance at 10 µg/mL CBD with an increase in gene expression of both CB1 and CB2. CBD reduced *in vitro* production of inflammatory cytokines from senior horses. The second objective was to determine the pharmacokinetics, bioavailability, and pharmacological effects of CBD in senior horses. In a cross-over trial using senior horses, a single oral dose of 2mg/kg CBD in soy oil was compared to a single intravenous (IV) dose of 0.1mg/kg CBD in DMSO. Blood samples were collected at specific time points. Plasma concentrations of CBD and metabolites (7-COH CBD and 7-COOH CBD), were detected in all plasma samples up to 8h post dosing (oral and IV) with 7-COOH CBD being the most predominant metabolite. Pharmacokinetics for CBD oral dosing at 2mg/kg were half-life 7.22 ± 2.86 hours, C_{max} was 18.54± 9.80 and T_{max} was 2.46 ± 1.62 hours. Oral bioavailability for CBD in senior horses was calculated at 7.92%. There were no significant effects of CBD on CBC, serum chemistry or vitals for all horses. Pharmacokinetics and bioavailability of CBD in senior horses was determined, and no adverse effects were found administering either an oral dose or an IV dose of CBD. Lastly, the third objective was to determine the effects of CBD on immune function by measuring inflammatory cytokines and antibody responses to vaccination, as well as various health parameters of body weight, body condition, lameness, and metabolic responses in senior horses. For 90 days two treatment groups of senior horses were orally-dosed once daily with CBD (treatment:

2mg/kg) or soy oil (control). Peripheral blood samples were collected prior to treatments on day 0 and post-treatment on days 30, 60, and 90. On day 90 all horses were vaccinated with a commercial equine influenza vaccine and blood samples were collected post-vaccination. Plasma concentrations of CBD and metabolites, 7-OH CBD, and 7-COOH CBD were measured with 7-COOH CBD the most significant of the two metabolites in the plasma post-treatment. At day 60 in RT-PCR, a significant reduction in the gene production of IFNγ occurred and IL-6 was also significantly reduced at day 60 and 90 for CBD treated horse when compared with control horses. There were no other significant changes in immune function, nor health parameters measured in response to CBD treatment. This body of work provides the foundation for understanding the effects of CBD on the health and well-being of the senior horse.

Key words: Cannabidiol, equine, inflammation, pharmacology

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THE EFFECTS OF CANNABIDIOL ON SENIOR EQUINE HEALTH

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CHAPTER 1. LITERATURE REVIEW

1.1 Senior horses

The equine population in the United States was estimated to consist of over 3 million horses as of a 2015 census by the US Department of Agriculture (USDA) [1]. Within this census it was determined that approximately 11% of the horses were 20 years old or older [1]. This is an increase from a prior census conducted in the U.S. as more horses are living longer with better management and nutrition for the senior horse. Which is similar to the phenomenon in humans of living longer, as our knowledge of care and nutrition increases so does the age of our horses. It has been found that with the right management and care that it is possible that horses can live well into their 30s [2]. Categorizing the horse into what is considered senior should take into consideration both chronological age and biological changes [2]. Biological changes that can occur as the horse ages chronologically can be identified as such things as osteoarthritis that contributes to reduction in range of motion, reduction in weight gain due to multiple factors, laminitis, and metabolic disease or pars pituitary intermedia (PPID) [3, 4]. Through these categories, both chronological and biological, determination of a senior horse can be identified as approximately 15 to 20 years of age [2]. One of the likely causes of some of these biological changes that occur with age are due to chronic inflammation.

1.2 Inflamm-aging

As the horse ages and undergoes physiological changes, an increase in inflammation could be contributing to an increase in the risk for injury or illness.[2, 4]. Chronic inflammation may be a main contributing factor to some of these age-related diseases as it has been shown, both *in vitro* and *in vivo*, [5, 6] that the process of inflamm-aging occurs similar to what happens in a human. The term inflamm-aging is described as a chronic, low-grade inflammation that occurs over time as the individual ages, and can be defined as elevated pro-inflammatory cytokines including; tumor necrosis factor-alpha (TNFα), interleukin 8 (IL-8), or interleukin 6 (IL-6) as well as chemokines [7-9]. As humans age, inflamm-aging is potentially caused by one or more of the following; accumulation of damaged cells (cell self-debris), leaky gut microbiota (where microbiota leaks into the surrounding tissues), accumulation of senescent cells (cells that have responded to damage and stress), the increase in coagulation as the human ages, and changes to the immune system due to age (immunosenescence) [10]. This combination of both inflamm-aging and immunosenescence, a decrease in immune response with age, has been shown to be a determining factor in the health of the senior horse [5, 11, 12]. Depending on the horse, with these age-related maladies, management with nutraceuticals or therapeutics is necessary.

1.3 Therapeutics

In equine soft tissue injury, the effects of inflamm-aging can impact re-injury and contribute to arthritis creating a chronic inflammation situation for the animal [13]. Therefore therapeutics such as non-steroidal anti-inflammatory drugs (NSAIDS) are

used, specifically flunixin meglumine (Banamine), firocoxib (Equiox), and phenylbutazone (Bute) [14]. However, these NSAIDS are strong inhibitors of cyclooxygenase (COX) enzymes which are important for many types of homeostasis of the normal physiologic functions [15]. If administering these NSAIDs that strongly inhibit COX, there are investigations showing that gastrointestinal injury will occur [16]. Thus the use of NSAIDS are slightly less desirable for those animals with chronic inflammation in the event to maintain whole body homeostasis and other options for reducing inflammation for the animal have been of interest. Alternative options include Cannabidiol, derived from *Cannabis sativa L.,* with its potential to modulate the immune system.

1.4 Cannabidiol

Cannabis sativa L. is a multi-faceted plant known by many names (marijuana, hash, hashish) across the world, most commonly called hemp in the United States. It is a dioecious plant meaning it has both male and female flowers but they develop on differing plants [17]. The plant itself has a lot of uses and was one of the oldest sources for textile fiber [17]. It is one of the oldest and most prevalent substance for medicinal purposes, with history that can be traced back to the earliest recorded medical journals [18]. *Cannabis sativa L.* produces hundreds of phytocannabinoids, along with terpenes and other substances within [17].

Cannabidiol (CBD) is a cannabinoid, produced from *Cannabis sativa L.,* which has become desired in human healthcare, now becoming popular as an alternative treatment in companion animal health. It was discovered in 1940 [18] and the structure

was revealed in 1963 [19]. The use of CBD has been previously shown to reduced anxiety, act as an analgesic, reduce inflammation, act as an antioxidant and as an anti-arthritic without psychotropic effects produced by delta 9-tetrahydrocannabinol (THC) [20-23]. The use and investigation of CBD had been severely restricted until recently due to it being a product of *Cannabis sativa L*.

The regulation of *Cannibis* or hemp was enacted with the Marihuana Tax Act of 1937 where it was permitted for doctors, researchers and commercial users to register and obtain the substance by paying a tax fee but outside the medical field its use was restricted [18]. Since then, within the U.S. the federal Controlled Substances Act (CSA), established in 1970, have controlled items that can be psychoactive [24, 25]. There are different classification rules for each category within the CSA which consist of Schedule 1 and Schedule 2 substances [24]. Prior to 2018 CBD was not listed as its own entity and was described as a component of marijuana therefore, it fell under Schedule 1 substances [24].

The medical use of CBD in the United States has been allowed since 1996 but only within 25 states where laws were specifically enacted for this [24]. In 2018 the Agriculture Improvement Act, more commonly referred to as the 2018 Farm Bill, legalized the products derived from the hemp plant by removing it from marijuana in the CSA [25]. Though, even with this legalization the U.S. Food and Drug Administration (FDA) still maintains control over CBD with their own regulations and requirements [25]. Since the 2018 Farm Bill, the market, both human and veterinary, has grown rapidly. In fact, it is forecasted to grow to a 29.9 billion US dollar industry by 2025 [26]. With this industry growing so rapidly more investigations are necessary to understand

CBD's potential health benefits. CBD has the potential to affect many different aspects of health and through initial research an entire internal system, endocannabinoid system, was discovered that is found in all animals and humans.

1.5 Endocannabinoid system

The endocannabinoid system is known to be the internal control for a body to regulate and balance the immune system as well as communicate. The endocannabinoid system (ECS) was originally discovered in 1963 but due to regulations and restrictions involving *Cannabis sativa* it was not extensively studied until the early 1990s [27]. Since its discovery it has been proven that all animals, both vertebrates and invertebrates, contain an endocannabinoid system[27].

The ECS can be simplified into three categories: endogenous ligands, membrane receptors, and deactivating enzymes [27]. Endogenous cannabinoid ligands refers to the endogenous lipids that the body produces [27, 28]. Anandamide and 2-arachidonoyl glycerol (2-AG) are the main and most studied endogenous ligands within the ECS [27, 28]. The endogenous ligands have some similarities in that they contain arachidonic acid however, they are both degraded as well as produced in different ways [28]. Anandamide has a wide range of distribution and influence throughout the central nervous system [29]. The 2-AG ligand is important for lipid synthesis and is a source of arachidonic acid for prostaglandins [28]. The enzymes that degrade these ligands are fatty acid amino hydrolase (FAAH), monoacylglcerol lipase (MAGL), alpha/beta domain hydrolase 6 (ABHD6), and oxidation of cycolooxygenase-2 (COX2) [28, 30, 31]. The main receptors for the ECS are g-protein coupled receptors (GPCR) cannabinoid 1-receptor (CB1) and cannabinoid 2-receptor (CB2) [27, 28]. CB1 receptors are primarily expressed by central

and peripheral neurons and CB2 receptors are found in the immune system [32]. However, it has been demonstrated that CBD indirectly affects CB1 and CB2 [33]. Since CBD has been shown to indirectly affect these receptors other avenues for the mechanism of action has been investigated.

Outside of the ECS, it has been shown that CBD may activate the vanilloid-2 (TRPV2) receptors, which regulate ion channels such as calcium channels. G-protein coupled receptors have also been shown to be affected by CBD treatment including GPR55 for which CBD is an antagonist [17]. CBD has also shown to be an agonist for 5-HT1 serotonin receptors [34]. Additionally, CBD suppresses persistent inflammatory and neuropathic pain by targeting the α3 glycine receptors (GlyRs) in rodents [18]. Since CBD has been shown repeatedly to not affect CB2 receptors the mechanism of how it affects the immune system is still unknown.

1.6 CBD Physiological effects

Analgesic

It has been shown that CBD can act as a pain reliever or analgesic. In an acute pain rat model it was found that CBD can reduce the stimulation of the COX pathway [35]. In this same model they determined that CBD can hinder the increases of the PGE2 pathway in addition to the COX pathway in a dose dependent manner [35]. Further, it has been determined that in addition to acute pain relief, CBD can reduce chronic pain [36]. Therefore, it is possible that CBD has potential as a therapeutic for both chronic and acute pain.

Antioxidant

In the immune response to pathogens monocytes and macrophages are important contributors to anti-inflammation and reactive oxygen species (ROS), some work has shown, *in vitro,* that CBD may have a suppressive effect on these cells [37]. Additionally, some very recent *in vitro* research shows that CBD helps reduce inflammation on oxidative stress [38]. CBD has also been shown to induce apoptosis, which is influenced by oxidative stress, by downregulating and depleting intracellular glutathione (GSH) and by activating the caspase 8 pathway [38, 39]. The pathways which CBD affect oxidative stress are not completely elucidated though, it is thought that CBD downregulates NF-κB and STAT1 activation [38]. The potential for CBD to be an antioxidant is evident but lacking more in depth research.

Anxiolytic effects

CBD has been shown to have reduce anxiety in those that are affected. In a study using mice it was found that the use of CBD can reduce anxiety by reducing expression of fatty acid aminohydrolase (FAAH) which then modulates intracellular signaling further downstream [40]. Further research has supported that CBD promotes neurogenesis thereby reducing anxiety in stressed mice [41]. In humans, research is still lacking with mostly anecdotal evidence of CBD having an anxiolytic effect. However, it has been shown, in humans, that anxiety induced by the thought of public speaking, also known as Generalized Social Anxiety Disorder (SAD), has been reduced with the use of CBD [42]. The investigations into CBD as a potential for anxiety are increasing as popularity increases.

Anti-arthritic

Arthritis is a painful disease that affects humans and animals alike as they age. CBD has been shown to resolve joint inflammation through the deletion of pro-inflammatory immune cells and fibroblasts [43]. This study also demonstrates CBD as anti-arthritic by targeting synovial fibroblasts whilst undergoing inflammation [43]. CBD locally administered in rats with osteoarthritis inhibited pain and peripheral sensitization [44]. Some research has proposed that CBD, has the potential to reduce arthritis induced pain and inflammation through topical application [45]. Regardless of method of administration there is some potential benefit of CBD for reducing arthritis pain.

Anti-inflammatory

Inflammation is important to many biological functions [46], however, it can also be the cause of many health problems [9, 10]. In the early 1990s, CBD was shown to have a discernable suppressive effect on cytokines interleukin 1 alpha (IL-1α), TNFα, and IFNγ in human peripheral blood mononuclear cells [47]. CBD has also been shown to decrease pro-inflammatory cytokines interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and interferon-beta (IFNβ) from lipopolysaccharide-activated microglial cells [48]. Additional *in vivo* and *in vitro* studies, focusing on murine macrophages, determined that CBD can decrease pro-inflammatory cytokines and suggested that this effect may be modulated through interleukin 10 (IL-10) and interleukin 12 (IL-12) [49]. CBD has also been shown to suppress NF-κB activity in both inflammation within the liver of mice and microglial cells activated by lipopolysaccharides (LPS) [50-52]. Studies further indicate that the reduction of inflammatory cytokine production by CBD could be mediated by the

diminished NF-κB activity [52, 53]. MyD88 is an adaptor protein that associates with the cytoplasmic tail of the LPS-recognizing receptor Toll-like receptor 4 (TLR-4) to initiate signal transduction for upregulation of pro-inflammatory cytokine production in response to TLR-4 binding of LPS [46]. MyD88 association with TLR-4 leads to downstream signaling events that activate NF-κB [46], which controls the expression of pro-inflammatory cytokines [54]. In the early 2000's research on the effects of CBD in humans, murine and small animals has increased [22, 48, 55-60]. These studies have shown the potential of CBD to modulate the immune system. Despite the increase in research with CBD, its mechanism of action is complicated and lacking in understanding of how it completely modulates the immune system [2].

1.7 CBD on inflamm-aging

In humans, it has been recently reported that cannabinoids may reduce the inflamm-aging process [7]. CBD has the potential to reduce inflammatory cytokines, specifically TNFα or IL-6, thereby reducing some of the effects of inflamm-aging on the individual [7, 47]. Also, CBD has the potential to reduce transcription factor NF-κB, which has many functions, but can reduce oxidative stress helping to re-integrate the balance of oxidative stress within the body [7, 50, 51]. CBD also interacts with many other receptors, such as TRPV and through these receptors has been shown to down regulate oxidation and reduce apoptosis [7]. Additional interactions of CBD with other receptors such as g-protein coupled receptors (GPR6), serotonin receptors (5-HT_{1A}), and peroxisome proliferator-activated receptor-γ (PPARγ) may influence its ability to restore oxidative balance as well [7]. Combining the potential anti-inflammatory, anti-oxidative,

and anti-apoptosis effects suggests that CBD may provide a protective effect against inflamm-aging for the animal [7]. Therefore, it is important to determine the potential CBD may have in reducing inflamm-aging in senior horses.

1.8 Bioavailability, pharmacology, metabolism

CBD has been shown to be extensively metabolized in the liver which has been shown in other species as well as *in vitro* [61-63]. All metabolic profiles are similar with similar metabolites as well but how they have been metabolized may be slightly different. This difference comes from CBD molecules having multiple sites for hydroxylation and carboxylation, where these reactions are carried out by cytochrome P450 enzymes potentially family CYP2D50 or family CYP3A [64, 65], which break down the structure into its metabolites [66-68]. There have been approximately seventeen different metabolites recorded from the metabolism of CBD [66, 69, 70]. However, the main metabolite 7-CBD COOH (nor-7-carboxy-cannabidiol) resists further degradation for far longer than the parent CBD [71]. Once this metabolism by cytochrome P450 and its related isozymes create secondary metabolites [68], these metabolites proceed where the mechanism by which they are further metabolized is less clear and species-specific [66]. CBD has many different targets within the body therefore, once first pass has occurred it is unknown just how it maneuvers to the necessary targets.

With the first pass in the liver it was proven that CBD has an extremely high extraction ratio which then leads to a very low oral bioavailability [67]. In dogs the oral bioavailability is 13-19% [67]. However, this may improve if the first pass can be

avoided through other possibilities such as encapsulation of CBD or feeding a high-fat diet at the same time of CBD ingestion [72]. In humans it was recently discover that after a fasting episode if CBD is orally administered then the bioavailability is around 6% but can be increased approximately 4-fold if given with a high fat diet [72]. The oral bioavailability of CBD is very low due to the first pass and more methodologies need to be investigated to see if encapsulation could improve this issue.

The pharmacology of CBD has been studied in humans, dogs, and cats, though the most thorough information has come from studying dogs. Oral half-life of CBD is dose dependent and differs slightly in species. In dogs, low doses of CBD give a half-life of approximately 4 hours and high doses of CBD give a half-life of around 7-9 hours [67]. In humans, the dose dependent half-life is anywhere from 10 to 17 hours, this was not affected by a fasted or fed state in humans [72]. There is much that still needs to be determined in the pharmacology and bioavailability of CBD in more companion animal species including equine.

1.9 CBD use in companion animals

The interest in using CBD in companion animal veterinary medicine is high though; there is also a lack of information. An early pharmacokinetic study was completed using dogs which determined the first-pass metabolism, as well as determining that the concentrations of CBD used caused no deleterious effects, neither intravenous or nor oral [67]. Another pharmacology study completed in both dogs and cats revealed that it is safe for use in animals [73]. Several other pharmacology studies have been completed as well in dogs pertaining to delivery method (encapsulation or oil) [63, 74].

Additionally, there has been evidence that CBD can reduce osteoarthritic pain in dogs, though until recently this has been more anecdotal reports. Recently, there have been two different canine study's that have investigated the animal's response to CBD and revealed an improvement in the dog's osteoarthritis [58, 75]. However, more clinical research is necessary to determine if CBD can improve osteoarthritis in dogs.

1.10 CBD use in equine

Prior to 2018 there was a lack of peer reviewed research completed in equine. However, that has slowly changed with the emergence of CBD as one of the most desirable treatments on the market. A single horse case study was one of the first published reports of CBD use in horses [76]. This case study was conducted in Colorado with a single young horse that reported significant improvement in mechanical allodynia after being given pure crystalline CBD (250 mg twice daily) since all other treatments failed [76]. More recently, research has been investigating the pharmacological effects of CBD in the horse.

A pharmacology study was completed using young exercising Thoroughbreds, this study looked at 3 different doses (0.5, 1, and 2mg/kg) of purified CBD in a crossover design as well as the effects on the arachidonic acid metabolism pathway [77]. For 0.5, 1, and 2mg/kg CBD terminal half-life ($t_{1/2}$) was determined to be 10.7 ± 3.61, 10.6 ± 3.84 and 9.88 ± 3.53 hrs, respectively, in young exercising Thoroughbreds [77]. Additionally, this research reported that orally-administered, purified, CBD does not cause changes in behaviors, is well absorbed, nor does it cause any adverse effects on gastrointestinal health after a single dose [77]. As mentioned above this study also looked into the

arachidonic acid metabolism pathway as a means to reduce inflammation and found that CBD did not affect this pathway in the horse after a single dose with the concentrations mentioned [77]. Another pharmacology study completed used a wider age range of horses (6-23 years old) and a commercially available pelletized CBD product at two different doses (0.35mg/kg or 2mg/kg) [78]. This study determined that at 2mg/kg CBD there was a terminal half-life of 10.4 ± 6 hrs and also reported no negative health effects on the horses and good absorption [78].

The most recent research with CBD and equine is a pharmacology study that also worked with synovial fluid and the safety of CBD with single and multiple doses (1 and 3 mg/kg) [79]. It was reported in their study that CBD at 1 and 3mg/kg was detectable in synovial fluid in the majority of the horses, some liver enzymes were elevated and mild hypocalcemia was seen in the majority of the horses but then returned to normal post CBD [79]. Therefore, this study concluded that at the doses provided was a safe concentration to provide to the horse [79]. This research completed in pharmacology with the horse has shown that CBD at these doses is well tolerated by the horse however, more investigations are needed to determine the efficacy of CBD in the horse.

1.11 Conclusion and objectives

Overall there is still a lack of understanding of how CBD functions in large animals, specifically equine. The above pharmacology studies are just the beginning of understanding the effects of CBD on the horse. Our overall hypothesis is that CBD will improve the health and well-being of the horse by acting as an anti-inflammatory, both *in*

vitro and *in vivo* in horses thereby improving health and mobility of senior horses. In order to evaluate the overall hypothesis the following objectives are:

Our **first objective** is to determine the effects of a 99% pure CBD powder, on *in vitro* anti-inflammatory activity using equine peripheral blood mononuclear cells from senior horses as a model of study of the inflammatory response (described in Chapter 2).

Our **second objective** is to measure the bioavailability and pharmacokinetics of CBD, Delta-9-Tetrahydracannabidiol (THC) free distillate oil, in horses to determine detectable dose to use for *in vivo* studies (described in Chapter 3).

Our **third objective** is to determine the *in vivo* effects of CBD, Delta-9-Tetrahydracannabidiol (THC) free distillate oil, in senior horses on inflammation, metabolic responses and joint health (described in Chapter 4).

Our **fourth objective** is to determine the effects of CBD, Delta-9-Tetrahydracannabidiol (THC) free distillate oil, on vaccine response to Equine Influenza vaccine in senior horses (described in Chapter 4).

CHAPTER 2. EFFECTS OF CANNABIDIOL ON THE *IN VITRO* LYMPHOCYTE PRO-INFLAMMATORY CYTOKINE PRODUCTION OF SENIOR HORSES

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2.1 Abstract

Cannabis sativa L. contains cannabidiol (CBD), a compound that has many anti-inflammatory properties. In this study, 99.9% CBD powder was used to determine its *in vitro* efficacy as an anti-inflammatory agent. Heparinized blood was collected *via* jugular venipuncture from senior horses. PBMCs were isolated then incubated for 24 hours with increasing dilutions of CBD dissolved in DMSO. PBMCs were stimulated the last 4 hours of incubation with PMA/IO and Brefeldin A. A Vicell counter was used to evaluate viability after incubation. PBMCs were stained intracellularly for IFNγ and TNFα then analyzed *via* flow cytometry. RT-PCR was used to analyze samples for gene expression. Five equine-specific intron-spanning primers/probes used are: CB1, CB2, TNFα, IFNγ, IL-10, and Beta-glucuronidase. Data was analyzed using RM One-way ANOVA (significance $P< 0.05$). Viability of PBMCs with CBD was completed to determine cytotoxicity. The dilution of CBD that did not affect cell viability was 4 µg/mL ($P<0.05$). CBD at 4 µg/mL significantly reduced production of IFN-γ and TNF-α ($P<0.05$). RT-PCR results for TNFα and IFNγ at 4 µg/mL showed a reduction compared with the positive control and IL-10 showed a similar reduction at 2 µg/mL and 4 µg/mL. RT-PCR gene expression results showed significance for 10 µg/mL CBD in CB1 and CB2. CBD at 4 µg/mL reduced *in vitro* production of inflammatory cytokines from senior horses.

This *in vitro* study supports further investigation of CBD to determine if it may be effective as an anti-inflammatory treatment for chronic inflammation in the horse.

2.2 Introduction

Cannabidiol (CBD), derived from *Cannabis sativa L*., has a long history of being used as an anti-inflammatory for many different ailments [20, 48, 80]. It has become more desired than delta-9 tetrahydrocannabinol (THC) as it has been shown to have no psychoactive components [47]. As it becomes more commonplace to use CBD in human medicine, there is interest to determine if it would help reduce inflammation in horses. Currently, non-steroidal anti-inflammatories (NSAIDs) including phenylbutazone and flunixin meglumine, more commonly referred to as Bute and Banamine, respectively, are administered to horses for inflammation and other ailments. However, long-term use of these NSAIDs can cause serious issues for the horse such as gastric ulcers [81]. CBD may have the potential to reduce inflammation in the horse without negative side effects.

According to the 2015 United States Department of Agriculture National Animal Monitoring System, approximately 11% of the equine population is over 20 years old and over 65% of the population between the ages of 5 and 20 years old [1]. From this survey, while the majority of the horse population in the U.S. is mature, there is still 11% of the population that is considered aged. As horses age, we have shown that the process of inflamm-aging occurs both *in vitro* and *in vivo* [5, 8]. Franceschi et al. introduced the term inflamm-aging in 2000, which is chronic inflammation triggered as macrophages turn on and never turn off thereby, being in a constant state of inflammation [8]. The

previous studies completed by our lab demonstrated that, both *in vitro* and *in vivo,* senior horses when compared to younger horses, exhibit the following inflamm-aging factors; an increase in interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα) by lymphocytes when stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin, and an increase in protein levels of TNFα in serum [5]. Therefore, the senior horse is a useful model to determine if CBD has any impact on inflammation in the horse.

Data demonstrate that CBD has the potential to modulate the immune system. Burstein et al. 2009, have shown CBD decreases pro-inflammatory cytokines interleukin 1 beta (L-1β), interleukin 6 (IL-6), and interferon-beta (IFNβ) from lipopolysaccharide-activated microglial cells. There is evidence that also demonstrates a pro-inflammatory response that may be modulated through CBD-induced interleukin 10 (IL-10) and interleukin 12 (IL-12) both *in vivo* and *in vitro,* in murine macrophages [49]. Yet, there is also a lack of understanding of how CBD, by itself, works on the immune system [20]. In 1991 Watzl et al. demonstrated that CBD has a marked suppressive effect on cytokines interleukin 1 alpha (IL-1α), TNFα, and IFNγ release [47]. Most completed studies are murine models or *in vitro* models, very few studies have been completed in larger animals at this time.

There is limited information in companion animals with CBD, though, a canine and feline single-dose pharmacokinetic and safety study revealed it is safe for animal use [73]. In 1988 Samara et al. used 2 intravenous doses (45 and 90 mg) and 1 oral dose (180 mg) for a pharmacological study using dogs weighing between 16 and 24 kg, which revealed no deleterious effects from any dose, intravenous or oral. This study also determined the half-life in the canine was approximately 4 hours and that the oral dose is

not as bioavailable as the intravenous dosage, a result of first-pass metabolism in the liver [67]. Recently, a canine study utilized the Hudson activity scale and the canine brief pain inventory scoring methods to observe the behavioral response in a variety of dog breeds, a dose of 2 mg/kg administered twice daily revealed improvement in activity and reduced pain [58]. At this time there are no peer-reviewed studies of any CBD research conducted in the horse. Therefore, we analyzed the anti-inflammatory effects of CBD on peripheral blood mononuclear cells (PBMCs) isolated from horses. We hypothesized that CBD will decrease intracellular staining of TNFα and IFNγ in lymphocytes from senior horses and decrease gene expression of TNFα, IFNγ, and IL-10 in a dose-dependent fashion.

2.3 Materials and Methods

All experimental procedures were approved by the University of Kentucky's Institutional Animal Care and Use Committee.

Animals

Six mixed-breed senior horses, 3 geldings, and 3 mares, (mean age 26 years ± 3 yrs) were used in this study. All horses exhibited no clinical abnormalities (e.g. nasal discharge, cough, etc.) at the time of blood collection.

CBD Preparation

The CBD working stock preparation was completed using 99.9%, third-party analyzed for guaranteed analysis, CBD powder (Agtech Scientific, Paris, KY). CBD was diluted into dimethyl sulfoxide (DMSO: Sigma-Aldrich) at concentrations of 2, 4, 6, 8, 10 µg/mL CBD similar to those CBD concentrations used by Jenny et al. 2009, in human mitogen-stimulated peripheral blood mononuclear cells.

Sample Collection, PBMC Isolation, and Culture Preparation.

Heparinized blood was collected *via* jugular venipuncture. Then peripheral blood mononuclear cells (PBMC) were isolated as previously described using ficoll gradient centrifugation [5, 6, 83].

Cell Viability and PMA/Ionomycin stimulation

Peripheral blood mononuclear cells (PBMCs) for each horse were plated in a 24-well plate (4 x 10^6 cells per well) with c-RPMI media [RPMI 1640 (Gibco, Grand Island, NY) supplemented with 2.5% fetal equine serum (FES; Sigma-Aldrich, St. Louis, MO), 55 μM 2-mercaptoethanol (Gibco), and 2 mM l-glutamine, 100 U/ml penicillin, 0.1 mg/mL streptomycin (HyClonePen/Strep/Glutamine solution; Thermo Scientific)] [84] and were incubated with CBD concentrations of 2, 4, 6, 8, 10 μg/mL along with one well of media with DMSO/PMA. Incubation of PBMCs was completed with CBD stimulation for 20 hours at 37 degrees C, 5% CO2. At 20 hours Brefeldin A was added to all wells to ensure protein accumulation intracellularly as previously described [85], in addition to stimulating with phorbol 12-myristate 13-acetate (PMA 25 ng/ml; Sigma) and ionomycin (1μM; Sigma) (PMA/IO) during the last 4 hours of culture [5, 6, 83]. At 24 hours, cells were counted to determine viability using a Vicell Counter-XR (Beckman Coulter, Miami, FL). In addition, PBMC samples were collected for intracellular staining and RT-PCR, described below.

Intracellular Staining and Flow Cytometry of IFNγ and TNFα

Following the 24 hour incubation, aliquots (200 μL each) of cells from each well were placed into duplicate 96-well V-bottom plates. Previously described intracellular staining protocol was used [5, 6, 83]. Following the last incubation, the plates were

washed in saponin buffer and re-suspended in PBS. Using an Attune flow cytometer (Invitrogen) and the Attune Flow software (Invitrogen), samples were analyzed, and lymphocyte populations gated on cell size and granularity to determine percent IFN-γ- and percent TNF-α-positive lymphocytes as well as mean fluorescence intensities (MFI) of lymphocytes [5, 6, 83].

RNA Isolation and RT-PCR

Following the incubation in 24- well plates, samples were aliquoted into microcentrifuge tubes (100 µL) to have RNA isolated then, reverse transcription completed to produce cDNA as previously described [5, 6, 83]. When completed, the cDNA samples were stored at -20 ^{o}C until RT-PCR analysis. To complete RT-PCR, cDNA samples were thawed at room temperature and loaded into the epMotion 5070 (Eppendorf) with 5 equine specific intron-spanning primers and probes (Applied Biosystems), CNR1 (CB1, Ec04949285_s1), CNR2 (CB2; Ec04949475_s1), TNF-α (Ec03467871_m1), IFN-γ (Ec03468606_m1), and IL-10 (Ec03468647_m1) in addition to Beta-glucuronidase (BGUS), as the housekeeping gene. RT-PCR was performed using the 7900HT Fast qPCR System (Applied Biosystems, Foster City, CA), which incubated samples at 95^{o}C for 10 minutes, followed by 40 cycles of 95^{o}C for 15s and 60^{o}C for 60s. The reaction volume for each sample was 10 µL with 5 µL Sensimix II Probe Kit (Bioline), 0.5 µL Taqman assay mix for the gene of interest (inventories equine specific Taqman assays; Applied Biosystems), and 4.5 µL cDNA template. Relative changes in cytokine gene expression are quantified using the ΔΔCT method [86]. The average ΔCT of all the wells treated with cRPMI media alone serves as the calibrator for each cytokine. Data is reported as the natural log of the RQ (relative quantity) values ($RQ = 2^{-\Delta\Delta CT}$)[86].

Statistical Analysis

Statistical analysis was performed by SAS 9.4 (Cary, NC). Linear mixed-effects models (SAS Glimmix procedure), with a random effect for horse, were used to test the effect of CBD dilution on each of the response variables TNFα, IFNγ, IL-10, CNR1 (CB1), and CNR2 (CB2). Pairwise comparisons of dilution effects were tested using the Tukey procedure. All PCR data was log-transformed to improve normality and after parametric models were run, Q-Q plots demonstrated normality. All data were graphed as medians ± interquartile ranges. Significance was defined as $P < 0.05$.

2.4 Results

Cell Viability

The viability of PBMCs incubated with the CBD dilutions, mentioned previously, was compared with PBMCs incubated with the positive control (DMSO and PMA\IO) to determine at what concentration each compound caused cell cytotoxicity (Figure 2.1). The highest dilution of CBD that did not significantly decrease cell viability compared with the positive control was 4 µg/mL (Figure 2.1).

Intracellular staining

At 4 µg/mL dilution, CBD significantly reduced lymphocyte production of TNFα and IFNγ compared to the positive control (Figures 2.2 and 2.3). At 2µg/mL dilution, CBD had no significant effect on both TNFα and IFNγ production compared to the positive control (Figures 2.2 and 2.3). The remaining dilutions of 6, 8, and 10 µg/mL CBD also showed a significant decrease in intracellular staining for both TNFα and IFNγ production when compared with the positive control (Figures 2.2 and 2.3). IFNγ mean

fluorescence intensity (MFI) showed a significant decrease at 4-10μg/mL CBD when compared with the DMSO/PMA control (Figure 2.4). MFI for TNFα also showed a significant decrease at 4-10μg/mL CBD when compared with the DMSO/PMA control (Figure 2.5). At 2μg/mL CBD had no significant effect on TNFα and IFNγ, MFI when compared to the DMSO/PMA control (Figures 2.4 and 2.5).

RT-PCR

For RT-PCR results for gene expression of either CB1 (CNR1) or CB2 (CNR2), there was significance at 10μg/mL CBD dilutions (Figures 2.6 and 2.7) when compared to the DMSO/PMA control. Also, for RT-PCR gene expression of IFNγ and TNFα at 4 μg/mL (Figures 2.8 and 2.9), there was a reduction when compared with the gene expression of the positive control, and gene expression of IL-10 showed a similar reduction at 2 μg/mL and 4 μg/mL (Figure 2.10).

2.5 Discussion

CBD has grown in popularity in human medicine for its anti-inflammatory properties [47, 87, 88]. There have been many CBD *in vitro* and *in vivo* studies conducted in murine models and small animals [22, 44, 48, 56, 57, 88-94]. This is the first *in vitro* experiment using PBMCs from a large animal, the horse, to investigate the effects of CBD on pro and anti-inflammatory cytokine responses. Senior horses were used because they are a unique model of inflamm-aging, characterized by an increase in inflammatory cytokines, which then allows to study the anti-inflammatory effects of CBD [5, 8]. Cytokines used to characterize pro-inflammatory responses were TNFα and IFNγ, both of

which are important to the initial immune response [95]. For the anti-inflammatory response, IL-10 was investigated, as it is a primary cytokine for inhibiting the inflammatory response [95, 96].

Following stimulation with CBD and PMA/IO, the PBMCs showed a concentration-dependent decrease in measurable cell viability. This decrease in viability was detected at the 6-10 μg/mL concentration which is similar to what others have seen when using higher concentrations of CBD [37]. In the current study, these higher concentrations of CBD are likely causing a decrease in cell viability by the process of apoptosis. There have been a few studies that have shown that monocytes and lymphocytes are sensitive to CBD concentrations and have shown that within hours there is a decrease in both cell types due to apoptosis [37, 39, 91]. In lymphocytes, Wu et al. (2008) determined that when stimulated with CBD (1-8μM) apoptotic effects occur that are associated with stress-dependent activation of caspase-8 post-incubation of approximately 1 hour. More specifically, a possible mechanism of action for CBD in apoptosis of monocytes was determined by Wu et al. (2018), where CBD (16μM), *in vitro*, can affect oxidative stress and apoptosis in monocytes 1-2 hours post-incubation by quickly depolarizing the mitochondrial membrane potential and oxidation of cardiolipin. More in-depth studies are needed to determine if apoptosis is the mechanism responsible for the decrease in cell viability in this study.

In the results for both flow cytometry and RT-PCR, a significant decrease in IFNγ was measured. These results were also seen by Kaplan et al. in 2008, specifically that CBD can suppress IFNγ and IL-2 after PMA/IO stimulation. Mechanisms for this decrease are unknown at this time. Additionally, CBD has been shown to decrease IL-10

in human immune cell lines [88]. Often IL-10 is measured following the same pattern of change as inflammatory cytokine production, likely reflective of a regulatory mechanism [49, 84]. If IL-10 is reduced then the inflammatory response will not necessarily be able to turn off as IL-10 is an important cytokine to inhibiting the inflammatory response. The results of this study also show a decrease in the gene expression of IL-10 across concentrations of 4-10 µg/mL with only 2 µg/mL not being significant when compared with the positive control. The results of this study are similar to Sacerdote et al. 2005, in which CBD also decreased IL-10 as well as IL-12 cytokines. The results of this work as well as Srivastava et al. 1998, contradict a study that found CBD is a strong inducer of IL-10 gene expression [98]. With these reductions of IL-10, TNFα and IFNγ, it is thought that at the levels presented in this *in vitro* study, CBD can suppress both anti-inflammatory and pro-inflammatory cytokines in equine peripheral blood mononuclear cells. At higher concentrations of CBD the decreases in cytokines measured could be due to decreases in cell viability however at lower concentrations of CBD, cytokines measured were still decreased but cell viability was not affected. Thus, at a lower or optimal concentration of CBD, the immune response can be modulated however, the mechanisms responsible for these changes remain unknown at this time.

It has been found that CBD can use different receptors to activate many different pathways [87, 99, 100]. Sacerdote et al. 2005, discovered CBD may not work directly through CB1 or CB2 receptors but could be driven through indirect CB1/CB2 receptor channels. In this study, CBD only showed significance at 10 µg/mL for CB1 and CB2 receptors measured through RT-PCR gene expression. The main mechanism is still not

clear, however, that was beyond the scope of this current study and remains to be determined in further experiments.

In summary, the results of this *in vitro* study indicate that CBD has the ability to reduce inflammatory cytokines in cultures of equine PBMCs stimulated with mitogen. This study supports further investigation of CBD as a potential effective anti-inflammatory therapy for chronic inflammation in the horse, *in vivo*.

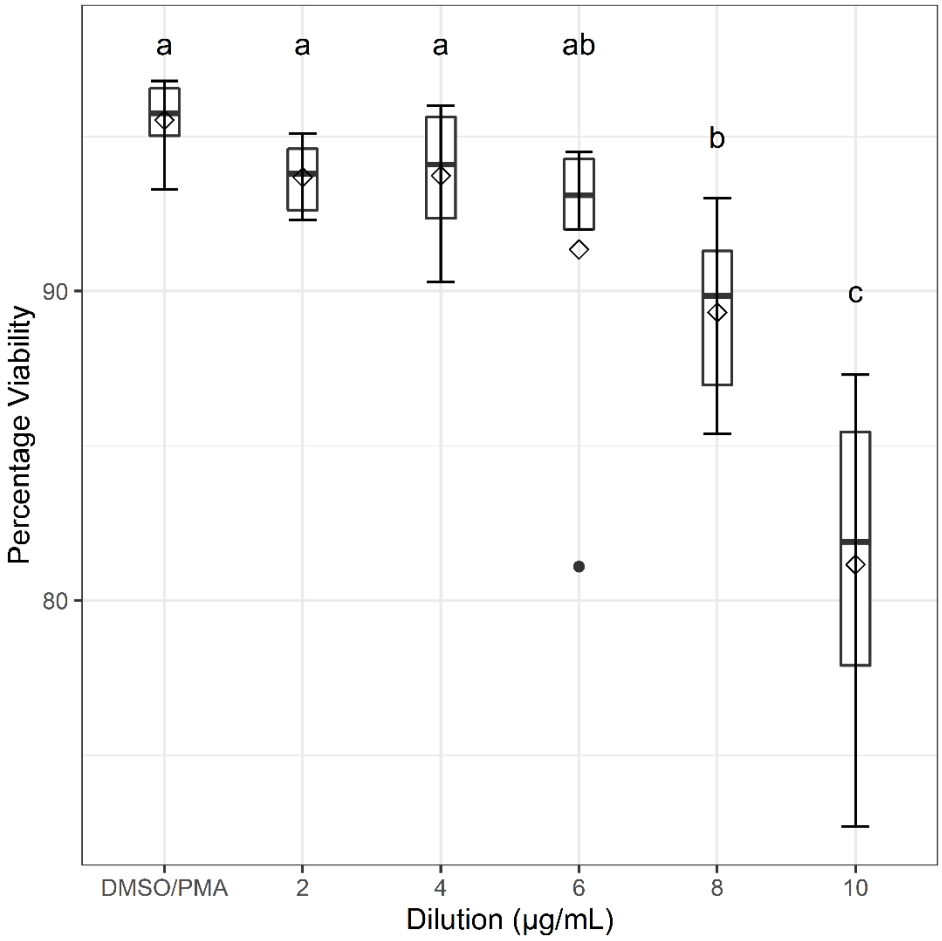

Figure 2.1 Percentage of PBMC Cell Viability

Cell viability across DMSO/PMA (positive control) and CBD dilution range of 2, 4, 6, 8, 10 µg/mL in PBMC cultures from six horses. Significance occurs at dilutions 6-10µg/mL CBD when compared with DMSO/PMA. Different letters above each dilution denote significance of that dilution ($P<0.05$), the same letters above the dilution denote no significance.

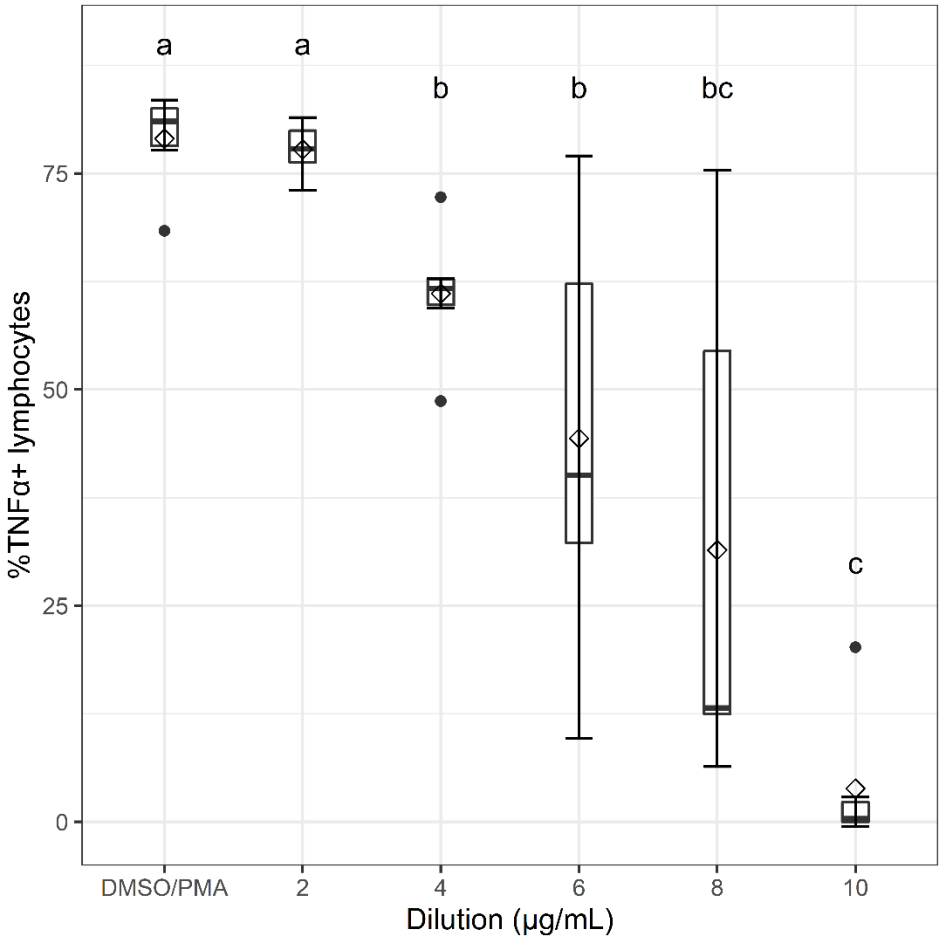

Figure 2.2 Intracellular Staining of TNFα measured by Flow Cytometry.

The percentage of lymphocytes producing TNFα across all dilutions and DMSO/PMA positive control. Significance occurs at dilutions 4-10µg/mL CBD when compared with DMSO/PMA. Different letters above each dilution denote significance of that dilution (P<0.05), the same letters above the dilution denote no significance.

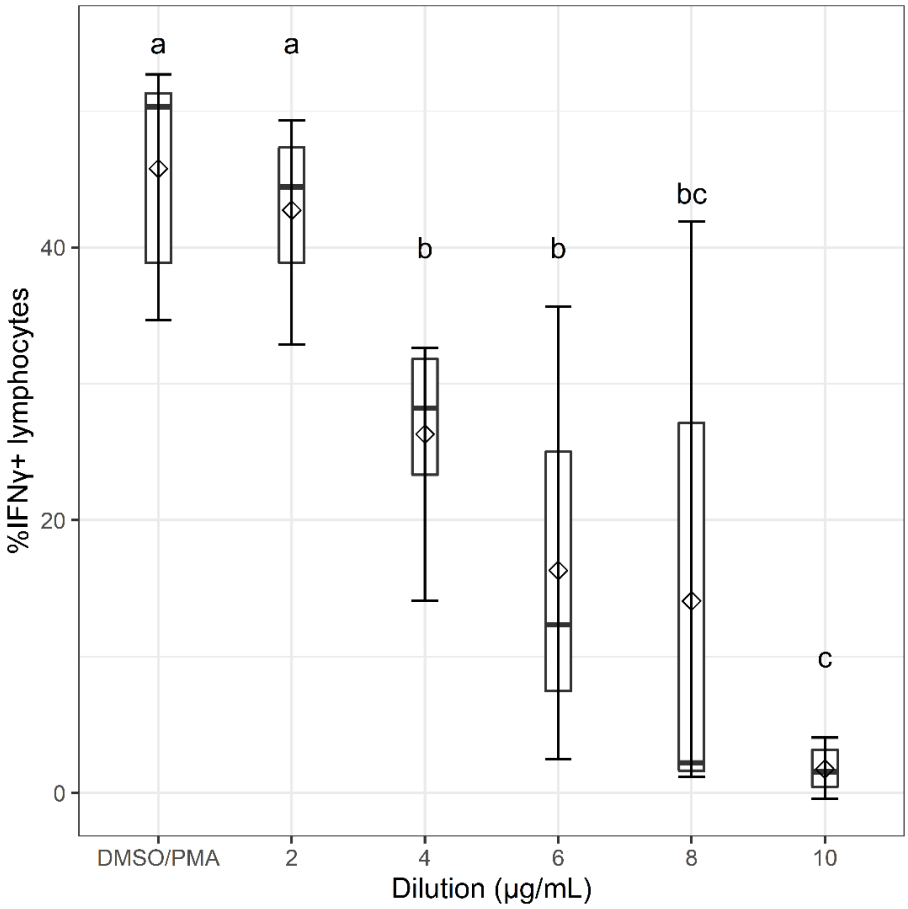

Figure 2.3 Intracellular staining of IFNγ measured by Flow Cytometry.

The percentage of lymphocytes producing IFNγ across all dilutions and DMSO. Significance occurs at dilutions 4-10µg/mL CBD when compared with DMSO/PMA. Different letters above each dilution denote significance of that dilution ($P<0.05$), the same letters above the dilution denote no significance.

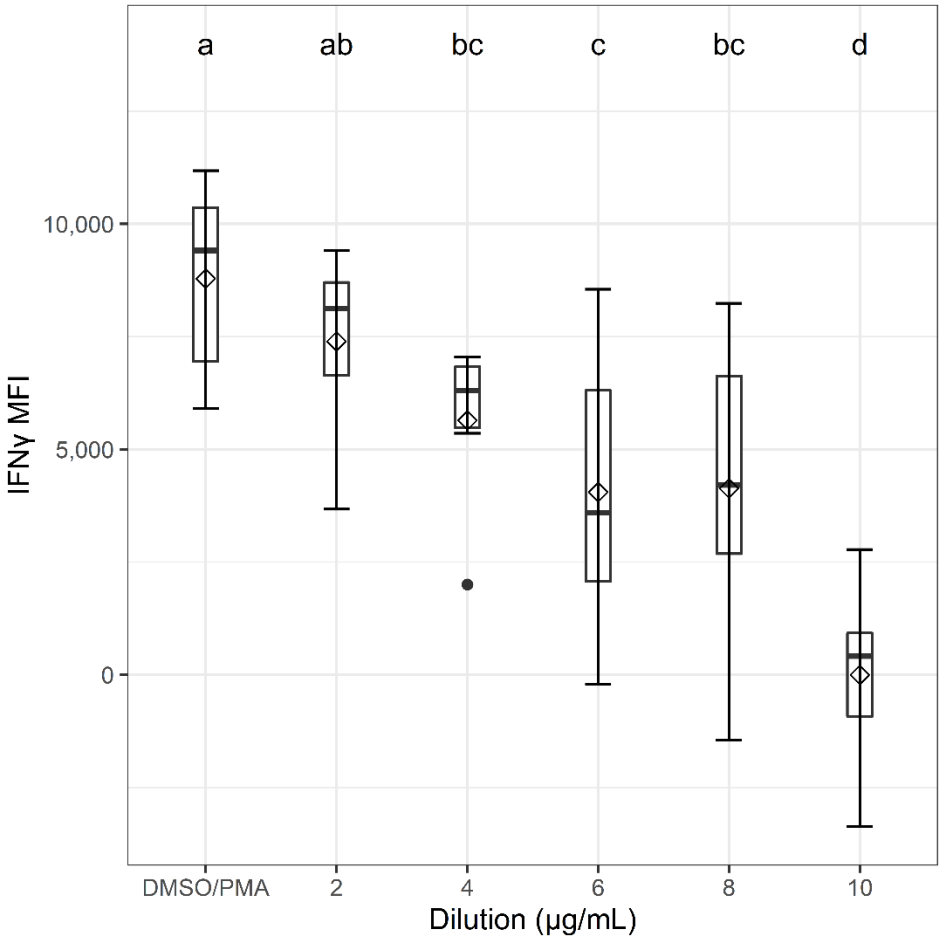

Figure 2.4 MFI of IFNγ

The mean fluorescence intensity (MFI) of IFNγ across all dilutions and DMSO. Significance occurs at dilutions 4-10µg/mL CBD when compared with DMSO/PMA. Different letters above each dilution denote significance of that dilution ($P<0.05$), the same letters above the dilution denote no significance.

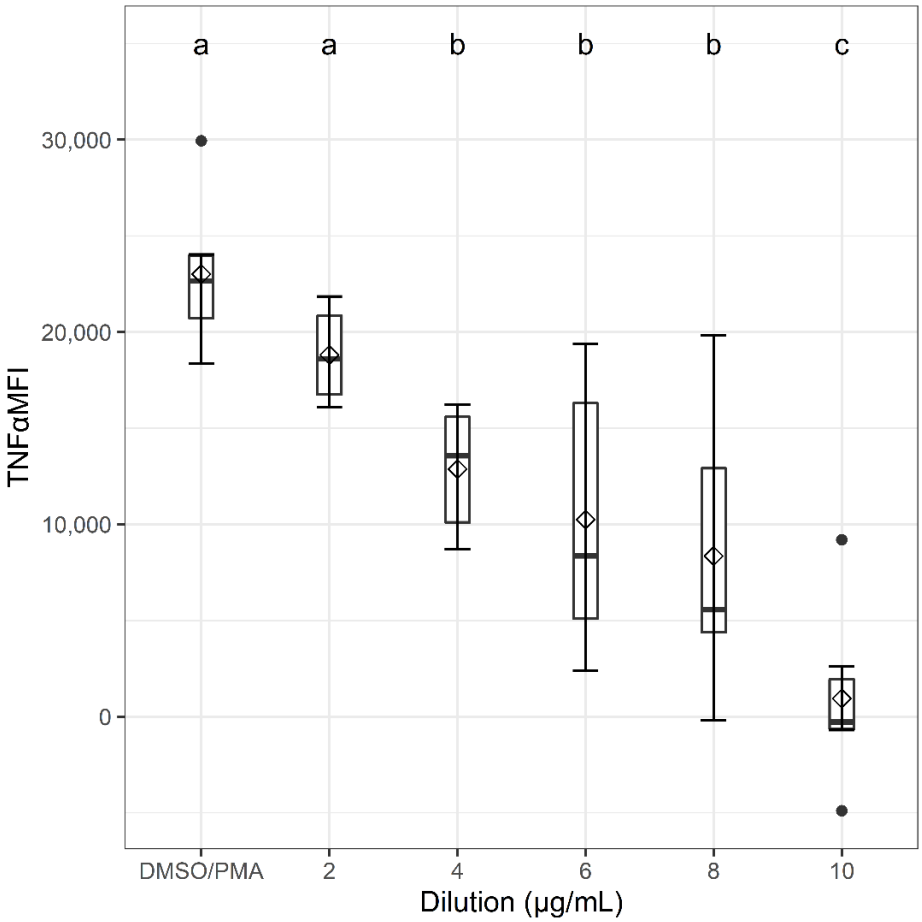

Figure 2.5 MFI of TNFα

The mean fluorescence intensity (MFI) of TNFα across all dilutions and DMSO/PMA. Significance occurs at dilutions 4-10µg/mL CBD when compared with DMSO/PMA. Different letters above each dilution denote significance of that dilution ($P<0.05$), the same letters above the dilution denote no significance.

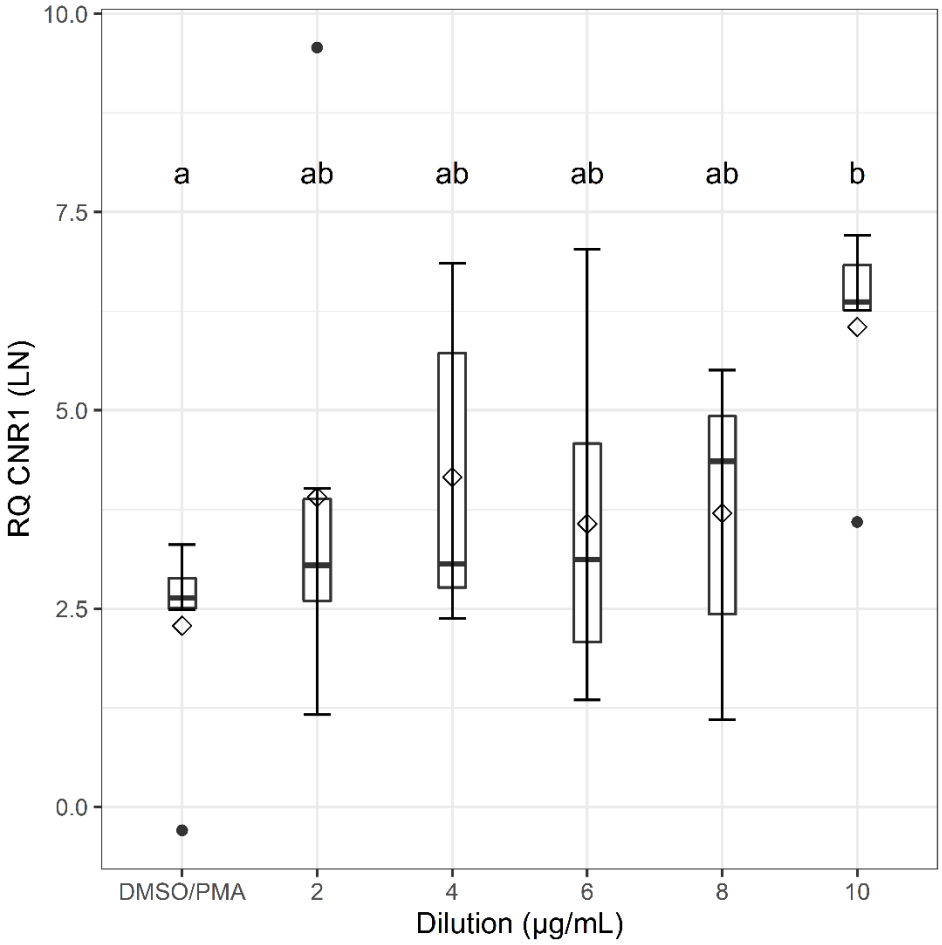

Figure 2.6 RT-PCR CB1 (CNR1).

The natural log of CNR1 across all dilutions and the positive control of DMSO/PMA. Different letters above each dilution denote significance of that dilution ($P<0.05$), the same letters above the dilution denote no significance.

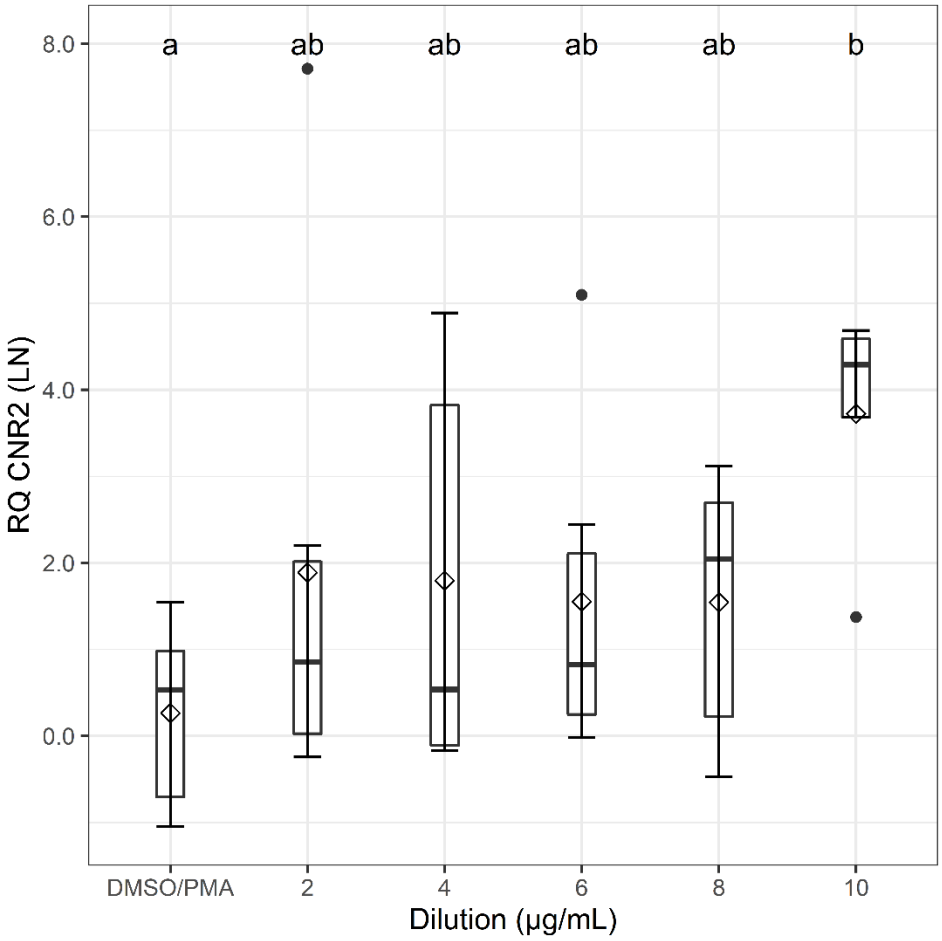

Figure 2.7 RT-PCR CB2 (CNR2).

The natural log of CNR2 across all dilutions and the positive control of DMSO/PMA. Different letters above each dilution denote significance of that dilution ($P<0.05$), the same letters above the dilution denote no significance.

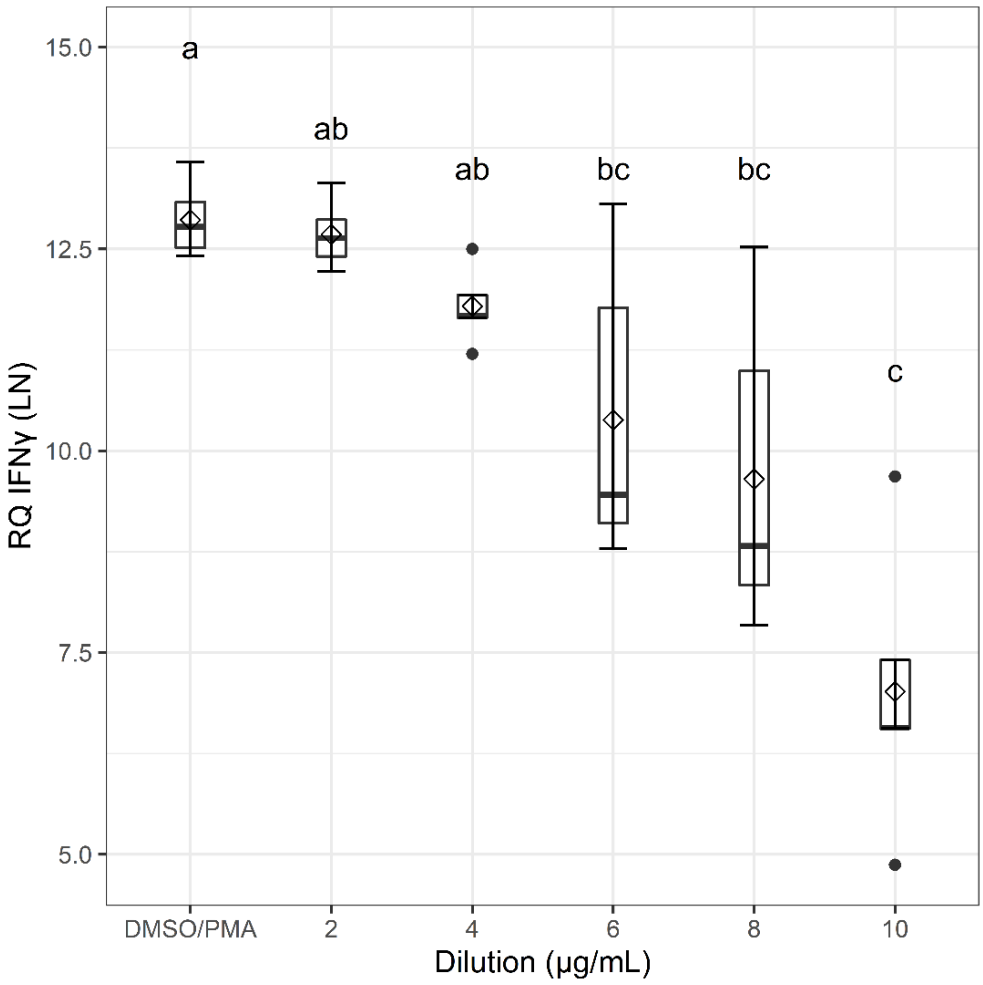

Figure 2.8 RT-PCR IFNγ.

The natural log of IFNγ gene expression across all CBD dilutions and the positive control of DMSO/PMA. Significance occurs at dilutions 6-10µg/mL CBD when compared with DMSO/PMA. Different letters above each dilution denote significance of that dilution ($P<0.05$), the same letters above the dilution denote no significance.

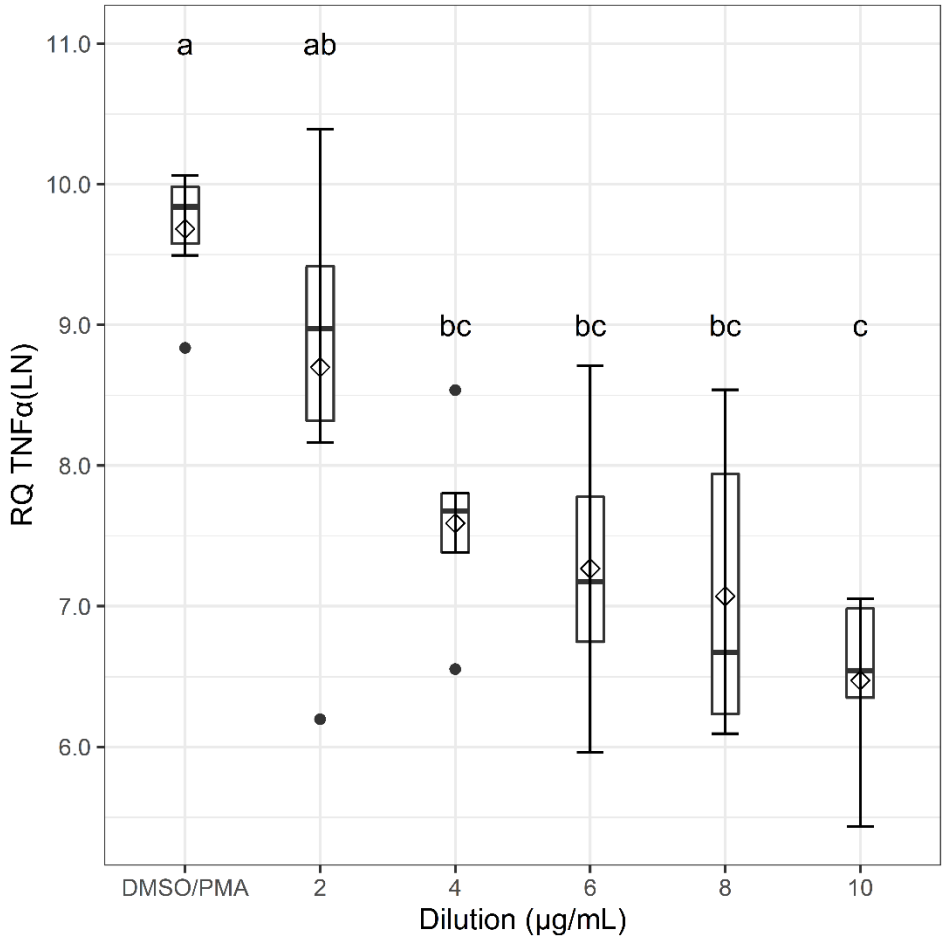

Figure 2.9 RT-PCR TNFα.

The natural log of TNFα gene expression across all dilutions and the positive control of DMSO/PMA. Significance occurs at dilutions 4-10µg/mL CBD when compared with DMSO/PMA. Different letters above each dilution denote significance of that dilution ($P<0.05$), the same letters above the dilution denote no significance.

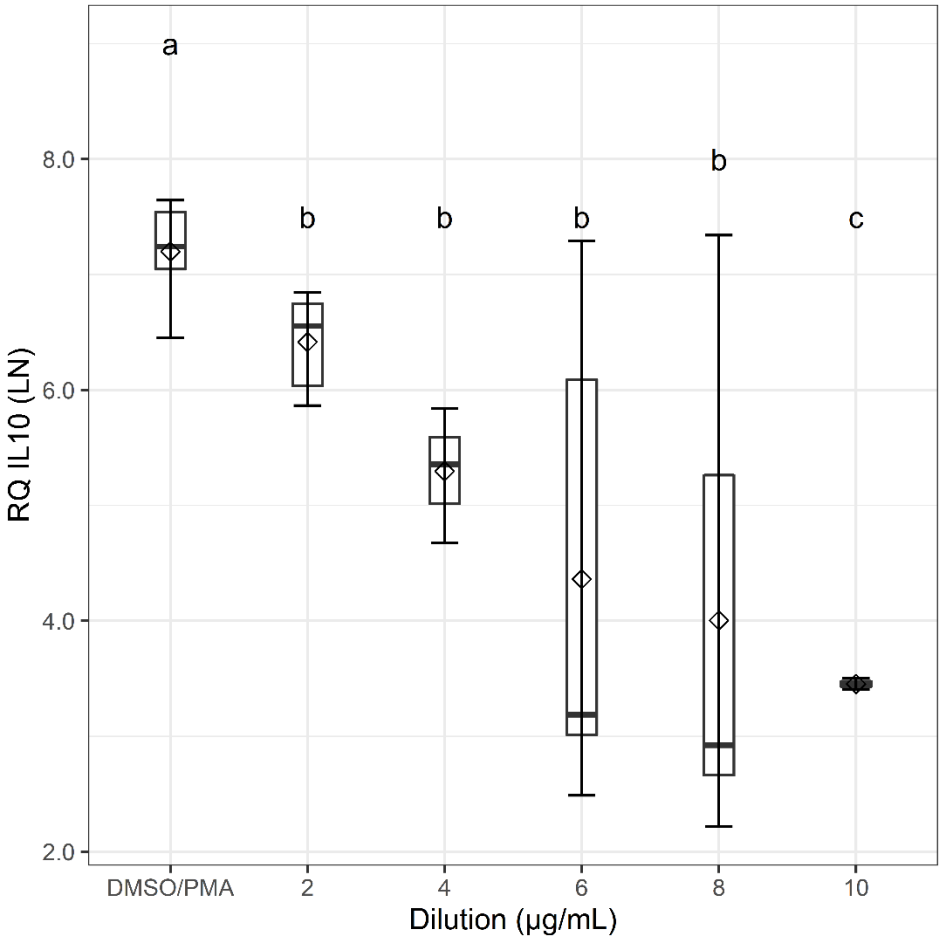

Figure 2.10 RT-PCR of IL-10.

The natural log of IL-10 gene expression across all dilutions and the positive control of DMSO/PMA. It should be noted that for dilution 10 µg/mL there were 3 horses that β-gus did not normalize for and therefore are not included in the statistical analysis. Significance occurs at dilutions 2-8µg/mL CBD when compared with DMSO/PMA. Different letters above each dilution denote significance of that dilution ($P<0.05$), the same letters above the dilution denote no significance.

CHAPTER 3. PHARMACOKINETICS OF A RANDOMIZED CROSS-OVER TRIAL OF CANNABIDIOL IN SENIOR HORSES

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3.1 Abstract

Objective: To determine the pharmacokinetics, bioavailability, and pharmacological effects of cannabidiol (CBD) in senior horses.

Animals: A total of 8 university-owned senior horses (mean age of 24 ± 3 years) were used in this randomized, cross-over study that was conducted from August 10th 2020 to September 4th 2020.

Procedures: Horses received either a single oral dose of 2mg/kg CBD in oil or a single intravenous (IV) dose of 0.1mg/kg CBD in dimethyl sulfoxide (DMSO). Blood samples were collected pre-CBD, 30 mins post-CBD, 1 h, 4, 8, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, and 264 h. Complete blood count (CBC) and serum chemistry profiles were measured. CBD and its metabolites were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results: Plasma concentrations of CBD and metabolites (7-COH CBD and 7-COOH CBD) were detected in all plasma samples up to 8h post dosing (oral and IV) with 7-COOH CBD being the most predominant metabolite. Pharmacokinetics for CBD oral dosing at 2mg/kg were half-life (t1/2λ) 7.22 ± 2.86 hours, Cmax is 18.54± 9.80 and Tmax is 2.46 ± 1.62 hours. For both oral and IV administrations, 7-COOH CBD did not fall below the limit of quantification for the times reported. Oral bioavailability for CBD in

senior horses in this study was calculated at 7.92%. There was no significant effect of CBD on CBC, serum chemistry or vitals for all horses.

Clinical Relevance: This study determined pharmacokinetics and bioavailability of CBD in senior horses, and found no adverse effects of administering either an oral dose or an IV dose of CBD.

3.2 Introduction

The cannabidiol (CBD) market, both human and veterinary, is growing rapidly and has been forecasted to grow to 29.9 billion U.S. dollars by 2025 [26]. CBD has been a focus for human medicine since the early 2000's as a treatment for inflammation, pain, epilepsy, and other diseases [44, 48, 101-104]. More recently, the equine and companion animal industries have shown interest in understanding and investigating the potential health benefits of CBD. However, very little research has been conducted to determine the efficacy of CBD in animals, most reports have been anecdotal. This is evolving, as there have been recent studies [58, 67, 73, 74] conducted with dogs and cats, indicating CBD's safety and pharmacological effects. The equine industry is still in its early stages of investigating the effects of CBD, although this is changing as CBD availability is becoming more widespread.

One of the first published reports of CBD use in horses was a single horse case study [76] conducted in Colorado that reported significant improvement in mechanical allodynia in a young horse when given pure crystalline CBD (250mg twice daily) after all other treatments failed to give a positive response. More recently, a pharmacology study [77] was completed using young exercising Thoroughbreds, comparing 3 different doses

(0.5, 1, and 2mg/kg) of purified CBD in a crossover design. This study [77] found that orally-administered, purified, CBD is well absorbed, does not cause a change in behaviors, nor adversely affects gastrointestinal health after a single dose. An additional pharmacology study [78] was recently completed using a wider age range of horses (6-23 years old) and a commercially available pelletized CBD product at two different doses (0.35 or 2mg/kg). This study [78] also concluded that neither dose caused any adverse health effects and both were readily absorbed. These recent studies have indicated that there is potential use of CBD in the horse, though, more efficacy studies are warranted.

In particular, there is interest in understanding the effects of CBD on health parameters in aging horses. Within the equine industry, horses are considered senior at 15 years and older.[2] Senior horses comprise approximately 11% [1] of the current equine population in the United States. As horses age they become more prone to conditions such as osteoarthritis, laminitis, metabolic disorders, and other musculoskeletal disorders [4]. The main treatments for many of these age-related conditions are non-steroidal anti-inflammatory drugs (NSAIDS) such as flunixin meglumine (Banamine; Merck) and phenylbutazone (Bute; Henry Schein) which have negative side effects such as but not limited to gastric ulcers [14]. Inflammation may be a main contributing factor to some of these age-related diseases. As the horse ages, it has been shown, both *in vitro* and *in vivo*, [5, 6] that the process of inflamm-aging occurs. The term inflamm-aging is described as a chronic, low-grade inflammation that occurs over time as the individual ages, and can be defined as elevated pro-inflammatory cytokines including; tumor necrosis factor-alpha (TNFα), interleukin 8 (IL-8), or interleukin 6 (IL-6) as well as chemokines. [7-9] It has been reported, in humans, [7] that CBD may have the ability to

mitigate the inflamm-aging process by reducing pro-inflammatory cytokines, such as TNFα or interleukin 6 (IL-6), and re-introducing a balance on oxidative stress. Therefore, it is of relevance to determine if CBD may reduce some of the effects of inflamm-aging in senior horses.

In fact, we have recently shown that CBD has an effect on inflamm-aging in vitro [105]. In this study, peripheral blood mononuclear cells collected from senior horses were incubated in vitro with various concentrations of pure CBD. We showed that at increasing concentrations of CBD there was a reduced level of pro-inflammatory cytokines TNFα, and interferon-gamma (IFNγ) [105]. This in vitro study indicated that CBD has the potential to modulate inflammation in the horse, however in vivo studies are warranted. Moreover, before efficacy studies are conducted in the senior horse, pharmacokinetic studies should be undertaken. Thus, the objectives of this study were to measure the pharmacokinetics, bioavailability and pharmacological effects of CBD in senior horses.

3.3 Materials and Methods

All experimental procedures were approved by the University of Kentucky's Animal Care and Use Committee.

Animals

A total of 8 University-owned senior horses, two geldings and six mares of mixed breed, (mean age of 24 ± 3 years) were used in this randomized, unbalanced cross-over design study conducted from August 10th 2020 to September 4th 2020. All horses were housed in their respective paddocks, at the University of Kentucky, C. Oran Little farm.

Before, during, and after all collections, the horses had access to pasture and water, though grain was withheld prior to all dosing. All of the horses exhibited no clinical abnormalities (i.e. nasal discharge, coughing, etc.) at the time of blood collection. All horses were monitored for temperature, heart rate, and respiration prior to receiving CBD and post CBD administration at 30 min, 1hr, and 4hrs.

CBD preparation for oral and IV administration

For each of the randomly chosen 6 horses a single oral dose of 2mg/kg CBD was made by weighing out 2mg CBD, Delta-9-Tetrahydracannabidiol (THC) free distillate oil (Enhanced Pet Sciences, Paris, KY) per 500 kg body weight then dissolved in 15mL of soy oil. The use of 2mg/kg CBD for oral administration was determined with a pilot study using 4 horses. In this pilot study, 2mg/kg was compared with 1mg/kg CBD and it was determined that the 2mg/kg was better detected post-administration (results not shown). For each of the randomly chosen 2 horses, a single intravenous (IV) dose of 0.1mg/kg CBD was made by dissolving the needed amount of CBD powder (Enhanced Pet Sciences, Paris, KY; 99% purity) dissolved in 0.5mL of dimethyl sulfoxide, (DMSO, 99.7% purity; Sigma-Aldrich, USA). Both the CBD distillate oil and CBD powder were analyzed for CBD at a third-party laboratory, confirmed to be THC-free and 98% or 99.9% CBD respectively.

Sample collection

Horses were randomly allocated into either oral dose or IV dose groups. The first oral (n=6) and IV (n=2) dosing both occurred on the same day with a washout period of 14 days between the next administration of CBD. Blood samples were collected via

aseptic jugular venipuncture into ethylene diamine tetra-acetic acid prepared tubes (EDTA; Covetrus, USA). Collection time points for both oral and IV dosing were as follows: pre-CBD administration, 30 mins, 1 hr, 4, 8, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, and 264 hrs post-CBD administration. All blood samples were placed on ice immediately, and within an hour of collection, centrifuged (800 x g for 10 minutes) then plasma was aliquoted into 1.5ml plastic microcentrifuge tubes and stored at -20 C until analyzed. Additional blood samples were collected into 5 mL serum tubes or EDTA tubes for complete blood count (CBC) and basic serum chemistry (Chem) pre-CBD administration and 24 hrs post-administration. All CBC and Chem panels were analyzed at Rood and Riddle Equine Hospital Laboratory (Lexington, KY). CBC and Chem panels were analyzed for hemoglobin, white and red blood cell count, packed cell volume, total protein, as well as blood parameters albumin, serum glutamic-oxaloacetic transaminase (SGOT), aspartate aminotransferase (AST), alkaline phosphate (ALP), and γ-glutamyl transferase (GGT).

CBD and metabolite concentration determination in equine plasma

All plasma samples were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for determination of CBD and the metabolites, 7-Hydroxy Cannabidiol (7-COH CBD), and 7-Carboxy Cannabidiol (7-COOH CBD), as previously described [77].

Pharmacokinetic and bioavailability calculations

Non-compartmental analysis was performed as described previously [77]. Briefly, plasma CBD and metabolite concentrations were analyzed using commercially available software (Phoenix WinNonlin Version 8.1, Certara, Princeton, NJ, USA) [77].

Bioavailability was calculated for the four horses receiving both IV and oral doses using the following equation:

$$\left[\frac{AUC_{0-\infty}\ Oral}{AUC_{0-\infty}\ IV} \times \frac{Dose\ IV}{Dose\ Oral}\right] X\ 100$$

Where AUC is the area under the concentration-versus-time curve from time 0 to infinity and calculated using the linear up log down trapezoidal rule. Dose is the actual oral and IV dose that was used as defined above.

Statistical methods

Statistical analyses were completed using a commercially available statistical software (SAS version 9.4; SAS Institute Inc., Cary, NC). Paired T-Tests with horse as the fixed effect, were used to test the effect of CBD on CBC and Chem results before CBD at time 0 and after CBD at time 24. Normality of data was assessed using Q-Q plots. Non-normally distributed data were log-transformed to adjust for normality and significance for all horses was defined as $P<0.05$.

3.4 Results

All of the horses used in this study had no adverse effects from the doses of CBD either orally or IV administered. Data for all horses dosed with IV CBD (0.1mg/kg) was log transformed to adjust for normality and data from all horses orally dosed (2mg/kg) was normally distributed as reported. For pharmacology analysis, all plasma samples were sent to the same laboratory for analysis by LC-MS/MS. The assay's accuracy and

precision were determined through analyzing the quality control samples in replicates (n=6). Accuracy was stated as a percent nominal concentration and precision as a percent relative standard deviation (Table 3.1). The limit of quantitation (LOQ) was 0.1 ng/mL for all analytes and the limit of detection (LOD) approximately 0.05 ng/mL for all analytes.

The plasma concentrations of CBD and metabolites, 7-COOH CBD and 7-OH CBD over the time points used for this study are shown (Figure 3.1). CBD, 7-COOH CBD, and 7-OH CBD were detected in the plasma post-administration with 7-COOH CBD being the predominant metabolite. Plasma concentration of the most predominant metabolite 7-COOH CBD were detectable throughout all of the collection time-points presented in this study. Concentrations of CBD were non-detectable for 2 of the horses by 24 hours post oral administration and at 48 hours post oral administrations concentration of CBD were non-detectable for half of the horses. By 72 hours post oral administration of CBD all horses were either below the LOQ of the assay or non-detectable. Concentrations of CBD were below LOQ by 24 hours post IV administration for half of the horses. By 48 hours post IV administration, all horses were below LOQ.

Plasma concentrations of 7-OH CBD were much lower overall for all the time points in both oral and IV administration groups. For oral administration, 7-OH CBD concentrations at 24 hours, post dosing, 7 horses were under the LOQ and by 48 hours, post dosing, all horses were under the LOQ. Following IV administration, 7-OH CBD concentrations in plasma were below LOQ for 1/4 horses. By 8 hours post-administration, only 1 of the 4 horses given CBD by the IV route had any detectable

concentrations of 7-OH CBD. Plasma concentrations were of the 7-OH CBD metabolite were too low to calculate the pharmacokinetic parameters.

Pharmacokinetic parameters for oral administration of CBD are shown (Table 3.2). The approximate half-life ($t_{1/2\lambda}$) for this study is 7.22 ± 2.86 (oral 2mg/kg) or 3.15 ± 2.19 (IV 0.1mg/kg) hours. The time to reach maximum concentrations (T_{max}) was 2.46± 1.62 hours. For both the oral administration and IV administration the metabolite 7-COOH CBD did not fall below the LOQ at any of the time points reported. In this study it was calculated that oral bioavailability (F) for CBD administration in senior horses was 7.92% ± 2.85 (mean ± SD).

There were no significant differences in any of the CBD or blood chemistry measures prior to CBD (oral and IV) administration and 24 hours post (oral and IV) administration for all horses in the study (Tables 3.4 and 3.5). There were no significant effects of CBD dosing on any of the vital measures (temperature, pulse, and heart rate) at 30 min, and 1-hour post-administration (data not shown) for all horses.

3.5 Discussion

The objective of this study was to describe the pharmacokinetics and bioavailability of CBD in the senior horse. Post oral and IV CBD administration CBD parent concentrations were shown at low levels 30 minutes post administration with a peak at 4 hours post administration. Similar findings were reported [77] in young exercising Thoroughbreds at the same oral dose (2mg/kg). The $t_{1/2\lambda}$ in the current study was shorter at 7.22 ± 2.86 h for oral dosing of CBD at 2mg/kg than what was previously reported [77] in young horses (10 h). The $t_{1/2\lambda}$ 3.15 ± 2.19 (IV 0.2mg/kg) hours for the IV

dose is much lower than the oral half-life which may be attributed to the formulation of the IV dose versus the oral dose. For IV administration, CBD was formulated in DMSO whereas the oral formulation was in oil. One possible explanation for the differing half-life between routes of administration is the way in which the compound is formulated. Delivery of oral CBD in an oily formulation can lead to a phenomenon termed flip-flop kinetics, whereby the rate of absorption is significantly slower than the rate of elimination due to slow release of the drug from the vehicle. In this case the terminal slope is affected by the process of absorption whereas with the IV formulation, the slope is primarily determined by the rate of elimination. This ultimately leads to differences in the terminal half-life calculation between routes of administration. The carrier for the oral CBD formulation used in the current study was different from previous studies [58, 74, 77] in which sesame or olive oils were used. CBD is a very lipophilic molecule, and when combined with fats or a highly fatty diet absorption is improved in animal and humans [72, 106, 107]. In the presently reported study, a soy oil that was a typical part of the horses daily feeding routine was used as the carrier as it is known to be very palatable for these horses. The horses used here were not fasted during this study as they were in their respective paddocks with grass ad libitum. Feed was withheld immediately following administration of the CBD for thirty minutes. In a recent pharmacology study [78] a pelletized CBD product was used, top dressed over the horses feed and monitored extensively to make sure it was consumed. The delivery method for CBD is still under investigation to determine the best formulation for optimal absorption in horses.

CBD is extensively metabolized in the liver and has been studied extensively in other species as well as *in vitro* [61-63]. Whilst these species all produce metabolic

profiles with similar metabolites they are all slightly different with how they have been metabolized. Cannabinoid molecules have multiple sites for hydroxylation and carboxylation to occur at, however, these reactions are carried out by cytochrome P450 enzymes differently in each species [61, 66]. The major metabolites that were investigated in this study 7-OH CBD and 7-COOH CBD were identified in humans and have a low prevalence in other species [63]. Similar to previous studies, [63, 77] concentrations of 7-OH CBD were extremely low from the initial dosing and were quickly below the lower LOD for most horses, within the first 24 hours following both oral or IV dosing. However, 7-COOH CBD was the predominant metabolite in the horse and in this study was determined to be present in the blood plasma 11 days post-administration of CBD. With little research having been completed at this time with CBD and horses, complete elimination of CBD and its respective metabolites is only partly defined. This study has shown that after 24 hours CBD is below the detection limit in the horse following administration of a 2 mg/kg oral or 0.1 mg/kg IV dose. Furthermore, even after 264 hours post oral administration of a single oral (2mg/kg) or IV (0.1mg/kg) dose, the 7-COOH CBD metabolite is still above detection limits in the senior horse. More research with additional collection time points are needed to define an absolute clearance of CBD and its metabolites in the horse.

This was the first published study to determine absolute oral bioavailability in the horse, in particular the senior horse. The bioavailability (F) of 7.92% ± 2.85 in this study is comparable to other studies [67] in dogs that have reported 13-19% oral bioavailability. The lower bioavailability in the horse is possibly due to the first pass metabolism. CBD has a very high extraction ratio in dogs [67]. If the same is true in horses, low

concentrations of CBD following oral administration may be a result of extensive first pass metabolism. It is also important to note that in the current study, only a small number of horses were administered the IV CBD formulation and therefore the bioavailability reported here represents just four horses. However, using few animals when measuring bioavailability is oftentimes not uncommon [67, 108, 109]. As therapeutic concentrations of CBD in the horse are as of yet unknown, the low bioavailability does not necessarily equate to poor efficacy. Low bioavailability of CBD in the horse could be affected by absorption time due to the amount of forage in the gastrointestinal tract, pH variations in the gastrointestinal tract, absorptive surface areas, protein binding and enzymes specific to the equine digestive tract due to them being hindgut fermenters [110-112]. Another potential explanation to explain the differences in kinetic absorption is horses have different enzymes and protein binding that could be interfering. A protein binding study may be beneficial to be conducted with oral CBD, where it can be determined if the CBD is being bound by the enzymes in the senior horse and maybe the free CBD is the only portion being absorbed. More research with CBD and protein binding in the horse should be investigated in future studies. There may also be an impact of age on bioavailability [113, 114]. More research is warranted to further our understanding of the effects of CBD on health and determine therapeutic concentrations. Studies in human medicine and small animals have investigated alternative dosing routes, [44, 74, 115] and different formulations of CBD,[107] encapsulated by liposomes to help improve bioavailability [74, 75]. Modifying the mode of delivery and CBD formulations could be future areas of research for the horse.

After administering one oral CBD dose of 2mg/kg or one IV dose of 0.1mg/kg there were no observable adverse effects in the horses based on CBC, serum chemistry, and TPR measurements. It has been reported in dogs that ALP, AST, and SGOT enzymes have been slightly elevated [58] possibly due to the first-pass metabolism in the liver [67]. However, monitoring of serum chemistry before and after a single oral dose or IV dose did not show any elevation in prominent liver enzymes such as ALP, AST, and SGOT in the current study.

Overall this study has determined the oral half-life of CBD to be 7.22 ± 2.86 hours and the oral bioavailability of CBD to be 7.92% ± 2.85, following the administration of a 2mg/kg CBD dose in horses. The source of drug was distillate oil CBD, suspended in soy oil and was well tolerated by all horses. Further, there were no adverse effects of administering either an oral dose or an IV dose of CBD to the horses. Future studies are warranted to determine the efficacy of CBD on health parameters in horses.

Table 3.1. Accuracy and precision values for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of cannabidiol (CBD), 7-carboxy cannabidiol (7-COOH CBD), and 7-hydroxy cannabidiol (7-OH CBD) concentrations in horse plasma. Samples tested in replicates (n = 6/concentration).

	Concentration (ng/mL)	Precision (%)	Accuracy (%)
CBD	0.3	8	94
	10	6	99
	250	6	104
7-OH-CBD	0.3	6	99
	10	4	103
	250	9	101
7-COOH-CBD	0.3	4	108
	10	3	100
	250	5	98

Table 3.2. Pharmacokinetic parameters (mean ± SD) for cannabidiol (CBD) and the metabolite 7-carboxy cannabidiol (7-COOH CBD) following a single oral administration of CBD (2 mg/kg; n=12) to university-owned, healthy, senior horses during a randomized cross-over study that had a 2-week washout period between treatments.

All values were generated using noncompartmental analysis.

Parameters	**CBD (n=12)**	**7-COOH CBD (n=12)**
AUC extrap (%)	8.14± 7.52	0.53 ± 1.04
AUC $_{0-\infty}$ (h*ng/mL)	132.44± 64.21	11500 ± 6609
C_{max} (ng/mL)	18.54± 9.80	307 ± 186
$t_{1/2\lambda}$ (h)	7.22± 2.86	43.10 ± 16.10
λ_z (1/h)	0.130 ± 0.10	0.017 ± 0.00
T_{max} (hr)	2.46± 1.62	5.08± 2.31
F_a (%)	7.92 ± 2.85	--

Data are reported as the mean ± SD unless indicated with a superscript letter.

AUC $_{extrap}$ = Percentage of area under the concentration-versus-time curve (AUC) extrapolated

$AUC_{0-\infty}$ = Area under the plasma-concentration curve from time 0 to infinity. C_{max} = Maximum concentration; $t_{1/2\lambda}$ = Terminal half-life. λ_z = Terminal slope. T_{max} = Time to maximum concentration. F = oral bioavailability.

Table 3.3 Pharmacokinetic parameters (mean ± SD) for cannabidiol (CBD) and the metabolite 7-carboxy cannabidiol (7-COOH CBD) following a single IV administration of CBD (0.1 mg/kg) to 4 university owned, healthy, senior horses. All values were generated using noncompartmental analysis.

Parameters	**CBD (n=3)**	**7-COOH CBD (n=4)**
AUC extrap (%)	4.74± 2.47	1.46± 0.77
AUC $_{0-\infty}$ (h*ng/mL)	57.6± 18.19	2000.3± 2422.2
$t_{1/2\lambda}$ (h)	3.15± 2.19	67.5± 10.8
λ_z (1/h)	0.29± 0.15	0.0± 0.0
Vd_{ss}(L/kg)	5481.7 ± 1544.2	--------
Vd_{area}(L/kg)	7569.4 ± 3286.03	---------
C_{max} (ng/ml)	--------	72.6± 98.20
T_{max} (h)	-------	3.5± 3.3

AUC_{extrap}, percent AUC extrapolated; $AUC_{0-\infty}$, area under the plasma-concentration curve from time 0 to infinity; $t_{1/2\lambda}$, terminal half-life; λ_z, terminal slope; Vd_{ss}, volume of distribution at steady state; Vd_{area}, volume of distribution calculated using the AUC method. C_{max}, maximum concentration; T_{max}, time of maximum concentration.

Table 3.4 Mean ± SD serum biochemical results for all 8 horses 24 hours before and after receiving their assigned CBD treatments described in Tables 2 and 3, stratified by treatment group (2 mg/kg, PO [n = 12]; or 0.1 mg/kg, IV [4])

	Oral		**IV**	
	Time 0	Time 24	Time 0	Time 24
Parameter	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Albumin (g/dL)	3.2±0.3	3.3±0.3	3.4±0.2	3.3±0.2
Aspartate aminotransferase (U/L)	239.5±33.3	241.1±27.1	251.3±30.2	266.3±28.7
Alkaline phosphate (U/L)	129.6±33.0	135.5± 34.4	137.8±19.7	140.8±22.5
Total bilirubin (mg/dL)	0.8±0.1	0.9±0.1	0.8±0.1	0.9±0.2
Glucose (mg/dL)	84.7±8.8	94.0±10.9	88.8±13.9	100.0±17.8
γ-Glutamyl transferase (U/L)	17.3±7.6	17.5±7.7	18.3±8.3	17.8±7.5

Table 3.5 Mean ± SD complete blood count results for all 8 horses 24 hours before and after receiving their assigned CBD treatments described in Tables 2 and 3, stratified by treatment group (2 mg/kg, PO [n = 12]; or 0.1 mg/kg, IV [4]).

	Oral		**IV**	
	Time 0	Time 24	Time 0	Time 24
Parameter	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Hemoglobin (g/dL)	11.5±1.2	11.7±1.5	12±1.2	11.8±0.5
Packed Cell Volume (%)	34.6±3.6	35.7±3.9	36.0±3.4	35.7±1.8
Red Blood Cells (10^6)	6.7±0.7	6.8±0.8	6.9±0.2	7.1±0.3
White Blood Cells (10^3)	7.7±1.7	8.4±1.7	7.9±1.7	6.8±1.0
Total Protein (g/dL)	7.43±0.29	7.5±0.6	7.5±0.2	7.6±0.1

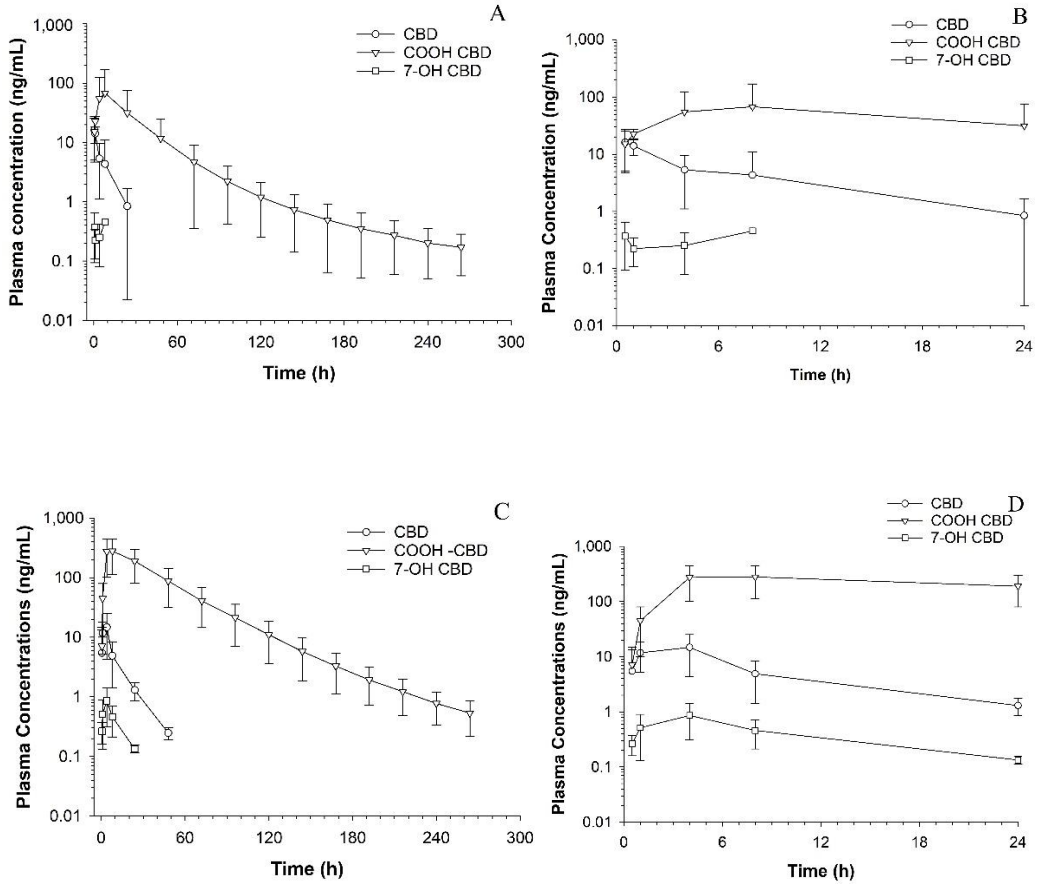

Figure 3.1 Plasma Concentrations

Mean ± SD plasma concentrations of cannabidiol (CBD), 7-carboxy cannabidiol (7-COOH CBD), and 7-hydroxy cannabidiol (7-OH CBD) for 8 university-owned, healthy, senior horses that received a single IV dose of CBD (0.1 mg/kg; n = 4; A and B) or oral dose of CBD (2 mg/kg; 12; C and D) at Time 0 during a randomized cross-over study conducted with a 2-week washout period between treatments. The y-axes are logarithmically scaled. A and C—Results for evaluations at 0.5, 4, 8, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, and 264 hours after treatment. B and D—Expansion of the x-axes allows depictions of the results for just the first 24 hours in greater detail.

CHAPTER 4. THE EFFECTS OF CANNABIDIOL ON IMMUNE FUNCTION AND HEALTH PARAMETERS IN SENIOR HORSES

Submitted to Veterinary Immunology and Immunopathology for publication

4.1 Abstract

Cannabidiol (CBD) has potential to reduce pain and inflammation in humans leading to the interest of use in equine. The purpose of this study was to determine the effects of CBD on immune function by measuring inflammatory cytokines and antibody responses to vaccination, as well as other health parameters in senior horses. Horses were orally-dosed with CBD (2mg/kg: 13 horses) or control (soy oil: 14 horses) daily for 90 days, from July 2021 to November 2021. Peripheral blood samples were collected on days 0, 30, 60, and 90 before administering treatments. On day 90 all horses were kept on treatment and vaccinated with an equine influenza vaccine and blood samples were collected post-vaccination on days 14 and 21. For all time points, plasma samples were analyzed for determination of CBD and metabolites, 7-OH CBD and 7-COOH CBD, using tandem mass spectrometry. For time points 0, 30, 60 and 90, blood samples were analyzed for CBC and chemistry. Additionally, peripheral blood mononuclear cells (PBMC) were isolated, stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, stained intracellularly for interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) then analyzed via flow cytometry. Real time PCR (RT-PCR) analyzed both stimulated PBMCs and whole blood for cytokine gene expression. Inflammatory proteins C-reactive protein, interleukin 1 receptor agonist, and prostaglandin E2 were measured with equine-specific enzyme linked immunosorbent assays. Thyrotropin-releasing hormone stimulation test and oral sugar test were

performed on all horses before and after the study to analyze metabolic function. Hemagglutination inhibition (HI) titers were measured for immune responses pre- and post-vaccination. All data were analyzed using either a paired t-test or a two-way repeated measures analysis of variance (significance $P < 0.05$). Plasma concentrations of CBD and metabolites were determined with 7-COOH CBD, the most significant metabolite, in CBD treated horses compared to control treated horses. A significant decrease was determined for whole blood inflammatory cytokine production of IFN-γ at day 60, and for IL6 at day 60 and 90 for CBD-treated horses when compared to control horses. No other immune measures, HI titers, or health parameters were significantly affected by CBD. This study demonstrated that treatment with CBD reduced some inflammatory cytokine production with no negative side effects as measured by CBC or chemistry profiles. This study reveals the initial understanding of CBD in the horse, however more in-depth research is needed to fully understand its efficacy on the health of the horse.

4.2 Introduction

The plant *Cannabis sativa L.* has been shown to produce hundreds of cannabinoids, of which cannabidiol (CBD) is one of the most prominent [18, 19]. CBD does not stimulate psychoactive effects like Δ-9-Tetrahydrocannabinol (THC) therefore, is more suited to treat pain, inflammation, epilepsy, and other diseases in human medicine [44, 47, 48, 101, 104]. In the past five years, interest in understanding the potential health benefits of CBD in the equine and companion animal industries has garnered research attention. The reports of CBD efficacy within animal health have been anecdotal with very limited scientific research to support these claims. Though recently,

there has been an increase in studies being conducted on the safety and pharmacological effects of CBD in dogs, cats, and horses [58, 67, 73, 74, 77]. With the availability of CBD rapidly becoming more widespread, the equine industry is undertaking more investigations of how CBD impacts the health and well-being of the horse.

The most predominant research available on the study of CBD in the horse are case reports, several pharmacology studies, and one recent safety study [76-79]. These studies are just the beginning of understanding how CBD affects the horse. Understandably, there is a growing interest to understand the effects of CBD on horse health, with a particular interest in senior horses. The senior horse comprises approximately 11% of the current equine population in the United States [1] and horses are often referred to as being senior at 15 years of age and older [2]. As a horse ages, they encounter co-morbidities such as metabolic disorders, laminitis, osteoarthritis, and other musculoskeletal disorders [4]. Non-steroidal anti-inflammatory drugs (NSAIDs) are very common for the treatment of some of these co-morbidities, most frequently used are phenylbutazone, firocoxib, and flunixin meglumine. Inflammatory processes likely contribute to these age-related diseases described above. It has been shown, both *in vitro* and *in vivo*, that as the horse ages a process called inflamm-aging occurs [5, 6]. This chronic, low-grade inflammation occurring over time in an aging individual, referred to as inflamm-aging, is measured as an increase in pro-inflammatory cytokines as well as chemokines with increasing age [7-9]. In humans, CBD may alleviate inflamm-aging through the reduction of pro-inflammatory cytokines and balancing oxidative stress [7]. A recent, *in vitro*, study completed with senior horse peripheral blood mononuclear cells (PBMCs) has shown that CBD can affect inflamm-aging [105]. This study indicated that

CBD may have the potential to reduce inflammation in the horse however, *in vivo* studies are critical to understanding the efficacy of CBD in the horse.

Thus, the objectives of this study were to determine the *in vivo* effects of CBD in senior horses on immune function as measured by inflammatory cytokine production and antibody responses to vaccination, as well as various health parameters of body weight, body condition, lameness, and metabolic responses.

4.3 Materials and Methods

All experimental procedures were approved by the University of Kentucky's Institutional Animal Care and Use Committee. (IACUC: protocol # 2018-2854)

Horses and Study Design

Thirty mixed-breed senior horses 24 mares and 6 geldings (mean age 24 ± 8 yrs) were initially designated for this study which was completed from July to November 2021. Three horses were removed from the study. Accounting for those removed for illness or death a total of 13 horses were used for the CBD treatment group (mean age 24 ± 8 yrs) and a total of 14 horses were used for the control treatment group (mean age 25 ± 7 yrs). The horses were grouped by their respective cohorts on pasture at the University of Kentucky C. Oran Little Research Farm. All of the horses on this study, for routine healthcare, are annually dewormed and vaccinated against core antigens as well as equine influenza. All horses were clinically examined before treatment for overall health status. All horses exhibited no clinical abnormalities (e.g. no nasal discharge, cough, etc.) at the time of blood collection.

All of the horses were weighed and a body condition score (BCS) was assigned, using the Henneke scale of 1-9, 16 days prior to the start of the study and again 2 days post the initial 90 days (Figure 4.1) using a digital floor scale (Tru Test Inc., Mineral Wells, TX) to the nearest kilogram [116]. All horses were randomly blocked to treatment groups (control or CBD) blocked by age, BCS, and inflammatory status as determined by the percentage of lymphocytes producing IFN-γ [6].

Sample Collection

Sample collection and study collection are shown in Figure 4.1. Blood samples were collected via aseptic jugular venipuncture into heparinized blood tubes, ethylene diamine tetra-acetic acid prepared tubes (EDTA: Covetrus, USA), Tempus tubes (Applied Biosystems), and serum tubes (Covetrus, USA) on days 0, 30, 60, and 90 prior to administering treatments (Figure 4.1). Additionally, on day 90 two blood collections occurred. The first blood samples were collected on day 90 (day 90 pre) prior to CBD and control treatment administration and a secondary collection occurred four hours post-CBD and control treatment administration (day 90-4hrs post) for comparing the effect of immediate dosing of CBD on blood parameters. Also on day 90, after blood was collected and treatments administered, all horses were vaccinated with a commercial equine influenza vaccine (FluVac Innovator; Zoetis, USA). Blood samples were collected two weeks (104 days) and again 3 weeks (111 days) post-vaccination while still on designated treatments (Figure 4.1).

Cannabidiol preparation

All CBD oral doses were prepared by using a proprietary broad spectrum hemp extract (98% CBD: 0% THC; Enhanced Pet Science, Paris, KY). CBD was weighed out at 2mg/kg of each horse's body weight and suspended in approximately 15mL of soy oil (Pantry Value Oils, USA) to be administered once per day to treatment horses. All control horses received a daily, approximately 15mL of soy oil at the same time as the treatment horses. Both CBD and control treatments were orally syringed one time per day to ensure all horses received treatments.

Analysis of cannabidiol plasma concentrations

All plasma samples were analyzed for determination of CBD and the metabolites, 7 Hydroxy Cannabidiol (7-OH CBD) and 7-Carboxy Cannabidiol (7-COOH CBD), using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described [77, 117].

Analysis of cannabidiol distillate oil

The CBD distillate oil was sampled on days 0, 30, 60, and 90 and analyzed for CBD content using a previously described liquid chromatography and tandem mass spectrometry (LC-MS/MS) method [77].

Lameness Evaluation

Additionally, all horses underwent a lameness exam before day 0 of the study, specifically on day -12, and again 10 days after the initial 90 days, to determine if CBD had an impact on lameness scores (Figure 4.1). A licensed veterinarian (Rood and Riddle, Lexington, KY), who was blinded to treatments, performed the lameness exams, and the

American Association of Equine Practitioners (AAEP) lameness scale of 1-5 was implemented.

Hematological and biochemical analysis

Peripheral blood samples were taken to Rood and Riddle Equine Hospital Laboratory (Lexington, KY) on days 0, 30, 60, and 90 for all horses for a complete blood count (Beckman Coulter ACT Diff; CBC) and serum chemistry (Beckman Coulter AU480; Chem) analysis. Basic CBC and Chem panels were completed analyzing for hemoglobin, white and red blood cell count, packed cell volume, total protein, as well as serum parameters albumin, serum glutamic-oxaloacetic transaminase, aspartate aminotransferase, alkaline phosphate, and gamma-glutamyl transferase.

PBMC Isolation, and culture preparation

Heparinized blood, collected by jugular venipuncture, was used to isolate peripheral blood mononuclear cells (PBMC) using ficoll gradient centrifugation as previously described [5, 83].

Cell viability and PMA/Ionomycin stimulation

Peripheral blood mononuclear cells (PBMCs) for each horse were counted using a Vicell Counter-XR (Beckman Coulter, Miami, FL), cells were plated in a 24-well plate (4 x 10^6 cells per well) as previously described [5]. Briefly, all cells were suspended in c-RPMI media (RPMI 1640; Gibco, Grand Island, NY) supplemented with 2.5% fetal equine serum (FES; Sigma-Aldrich, St. Louis, MO), 55 μM 2-mercaptoethanol (Gibco), and a combination penicillin, streptomycin, and glutamine solution [HyClone Pen (100 U/ml) /Strep (0.1 mg/mL) /Glutamine (2 mM); Thermo Scientific]. Additionally, phorbol

12-myristate 13-acetate (PMA; 25 ng/mL; Sigma) and ionomycin (IO; 1μM/mL; Sigma) were added to every other well [5]. Lastly, Brefeldin A (2μL per well) was added to all wells as previously described [85] for all plates and then incubated for 4 hours at 37 º C, 5% CO2.

Intracellular staining and flow cytometry

Following the 4-hour incubation period, aliquots (200 μL each) of cells from each well were plated into a duplicate 96-well V-bottom plate, fixed with 2% paraformaldehyde (Sigma), then stored overnight at -4 º C. An intracellular staining protocol that has been described previously was used [5] for the staining of IFN-γ and TNF-α. Briefly, PBMC were stained for IFN-γ using a CC302 mouse anti-bovine antibody IFN-γ FITC (Bio-Rad, Hercules, CA, 0.1mg) and with monoclonal anti-equine TNF-α antibody (antibody HL801; provided by Dr. Robert McKay, University of Florida) as well as secondary antibody FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (Invitrogen, Carlsbad, CA). The use of an Attune^{TM} NxT Flow Cytometer (Thermofischer Scientific, Waltham, MA) was employed, to analyze samples. Results were gated on granularity and cell size determining the percentage of positive lymphocytes for IFN-γ and TNF-α [5].

PBMC culture, RNA Isolation, and RT-PCR

After the 4-hour incubation, aliquots (500 μL each) of the plated PBMCs were placed into microcentrifuge tubes for RNA Stat isolation followed by reverse transcription to produce cDNA using protocols previously described [5]. Until cDNA

samples were analyzed they were stored at -20C until analyzed by real-time PCR (RT-PCR).

Tempus tubes (Applied Biosystems) were used for whole blood collection (approximately 2 mLs) and these samples were frozen at -20C until analyzed. All Tempus tubes were allowed to thaw at room temperature then, RNA was isolated using a KingFisher Flex (Thermo Scientific) and the MagMax Core Kit (Applied Biosystems) per the manufacturer's recommendations, and as previously described [118].

All cytokine gene expression, for both stimulated PBMCs and whole blood, were measured with RT-PCR after samples were allowed to thaw at room temperature. A housekeeping gene, B-glucuronidase (B-Gus) was used as an endogenous control for all samples [119, 120] while using the relative quantitation method previously described [86]. All samples were assayed in duplicate for the following genes using commercially available equine-specific intron-spanning primers and probes (Thermofischer Scientific): βGus (Ec03470630_m1), TNFα (Ec03467871_m1), IFNγ (Ec03468606_m1), IL10 (Ec03468647_m1), IL6 (Ec03468678_m1), IL1B (Ec04260298_s1), PTSG2 (EC03467558_m1). The DCT of the wells treated with cRPMI media alone for each horse serves as the calibrator for each cytokine. Data is reported as the natural log of the RQ (relative quantity) values ($RQ = 2^{-\Delta\Delta CT}$) [86].

Inflammatory protein enzyme-linked immunosorbent assays (ELISA)

C-reactive protein

Serum C-reactive protein (CRP) concentrations were also measured on days 0, 30, 60, and 90. CRP concentrations were determined by using an equine-specific commercially available kit (Kamiya Biomedical Company, Tukwila, WA) according to the manufacturer's instructions. Serum samples were diluted at 1:500, and absorbance was measured at 450 nm in a microplate reader (Agilent Technologies, Santa Clara, CA). The standards were used to create a calibrator curve (R^2=1) which was used was used to obtain CRP concentrations (ng/mL) and were corrected for the dilution factor.

Interleukin-1 receptor agonist

Serum levels of the anti-inflammatory cytokine interleukin-1 receptor agonist (IL-1ra) were also measured for all horses at days 0, 30, 60, and 90. Undiluted serum IL-1ra concentrations were determined by the equine-specific commercially available kit, validated for the serum used (R & D Systems, Minneapolis, MN), and were used per the instructions. Absorbance was measured at 450 nm in a microplate reader (Agilent Technologies, Santa Clara, CA) and then corrected for wavelength at 540nm. Samples and calibrators were background corrected and a calibrator curve (R^2=1) was created using the standards to obtain IL-1ra concentrations (ng/mL).

Prostaglandin E2

Prostaglandin E2 (PGE2) concentrations were also measured on days 0, 30, 60, and 90. PGE2 concentrations were determined by the commercially available kit that has been validated for use in equine (R & D Systems, Minneapolis, MN). Serum samples were diluted to a 3-fold dilution per the instructions. Absorbance was measured at 450 nm in a microplate reader (Agilent Technologies, Santa Clara, CA) and then corrected for

wavelength at 540nm. Samples and calibrators were background corrected and a calibrator curve (R^2=1) was created using the standards to obtain PGE2 concentrations (pg/mL). Results were log-transformed to adjust for normality.

Endocrine Function

In accordance with the Equine Endocrinology Group guidelines, horses underwent testing to determine pituitary pars intermedia dysfunction (PPID) and insulin dysregulation (ID) [121, 122]. To determine pituitary function, the thyrotropin-releasing hormone (TRH) stimulation was performed on all of the horses before the start of the 90 days, specifically at day -40 prior to the start of the study, and after the initial 90 days, specifically 9 days, however, all horses were still on treatment (CBD or control) at this time (Figure 4.1). For the TRH stimulation, peripheral blood was collected into EDTA tubes, then TRH was injected (1mg/mL saline per horse; i.v.: Sigma Aldrich) and at exactly 10 minutes post TRH injection, EDTA blood samples were collected [123]. The oral sugar test (OST) was also used to determine insulin dysregulation by administering light corn syrup (Karo Light, USA; 0.15mL/kg BW, p.o.) and measuring serum insulin concentrations before and at 60 minutes post-administration [124]. The OST testing was conducted -46 days before the start of the 90-day study and again 7 days past the end of the 90-day study with all horses still on treatment (Figure 4.1). EDTA plasma and serum samples were sent to Cornell Animal Health Diagnostic Center for ACTH and insulin analysis respectively as described previously [125]. No other endocrine screening was conducted.

Vaccination

In this study, on day 90, while still on designated treatment, all horses were vaccinated with FluVac Innovator (Zoetis, USA). An additional group of 5 horses (mean age 24 ± 7 yrs) were sampled throughout the vaccination portion of the study to serve as sentinel controls. All horses at this time were monitored for any signs of increased rectal temperature, respiratory distress, or nasal discharge every 7 days. Vaccinated horses were also monitored for any vaccination injection site reactions.

Hemagglutination Inhibition Titer assay

To measure the humoral immune response pre- and post-equine influenza vaccination, serum samples were analyzed for total IgG antibody response by the hemagglutination inhibition (HI) assay with KY/97 equine influenza virus [126]. To remove non-specific inhibitors of hemagglutination all sera were pre-treated with potassium periodate as previously described [127]. A titer of < 1:8 was arbitrarily set to 1:4. For all titers, run in duplicate, the geometric mean and log2 were calculated for each pair.

Statistical Analysis

Statistical analysis was performed by GraphPad Prism 9.0 (San Diego, CA, USA). Paired t-tests with horse as the fixed effect, were used to test the effect of CBD on body weight, and metabolic status before and after CBD treatments. Lameness evaluations were analyzed with a Fischers chi-squared test. Two-way analysis of variance (ANOVA) with repeated measures (RM), was used to determine the effect of CBD on each of the response variables TNF-α and IFN-γ by flow cytometry; cytokine gene expression (TNF-α, IFN-γ, IL10, IL1β, PTSG2, and IL6) in whole blood and stimulated PBMC samples.

Two-way ANOVA with RM was also used to determine the effect of CBD on HI Titers, CRP, IL1ra, and PGE2 concentrations. Any data that did not display normality in a Q-Q plot was log-transformed and then displayed normal distribution (GraphPad Prism, San Diego, USA). All data were graphed as mean ± SD. Significance was defined as $P < 0.05$.

4.4 Results

Body morphometrics and lameness scores

The mean body weight and mean body condition score of all horses were not significantly increased or decreased by CBD treatment (Figure 4.2). Front-end lameness, determined by a licensed veterinarian, of all the horses was also not significantly affected by CBD treatment (Figure 4.3).

Distillate CBD analysis

Additionally, all CBD distillate oil samples were analyzed separately for THC content during days 0, 30, 60, and 90 for verification. The mean of 0.022% and a range of 0.01% THC demonstrated a low amount of THC in the CBD distillate throughout the 90-day study (Results not shown).

CBD Plasma Concentrations

Plasma concentrations of CBD and metabolites, 7-OH CBD, and 7-COOH CBD were detected in the plasma. Metabolite 7-COOH CBD is the most predominant and most significant of the two metabolites in the plasma of the CBD group throughout the 90 days (Figure 4.4).

Time Effect of Collection Sampling

In this study blood samples were collected before CBD administration and 4 hours post-CBD administration when CBD would have been close to its maximum concentration in the plasma of the horses (Data not shown). Therefore, using these sample collection times presented it was determined that there was no effect of this timing on sample collection and the health parameters that were measured in this study.

Hematological and biochemical analysis

Albumin was the only parameter that significantly increase in a time effect in the CBD treatment group from day 0 to day 90 compared to the control group (Table 4.1). There were no further significant treatment effects of CBD on any other complete blood count (CBC) or serum chemistry parameters over time (Table 4.1).

PBMC Intracellular staining for TNF-α and IFN-γ

There was a time effect that was a significant increase from day 0 to day 90 for both TNF-α and IFN-γ. However, there was no treatment effect of CBD on intracellular staining of TNF-α and IFN-γ over time (Figure 4.5).

RT-PCR

Mitogen-stimulated PBMC gene expression

Gene expression was completed using real-time PCR (RT-PCR) from PBMCs that had been stimulated with PMA/IO previously. Expression of the cytokines TNF-α, IFN-γ, IL10, IL1β, PTSG2, and IL6 were not significantly downregulated nor upregulated by the CBD treatment when compared with the control-treated horses at the

same time points (days) (Figure 4.6). However, gene expression of TNF-α, IFN-γ, IL10, IL1β, PTSG2, and IL6 did reveal a significant time increase from day 0 to day 90 for both treatment groups. (Figure 4.6).

Whole blood gene expression

For whole blood gene expression, the expression of IFN-γ at day 60 was significantly reduced for CBD-treated horses when compared with control horses. Expression of IL-6 was also significantly reduced on days 60 and 90 for CBD treatment when compared with the control. There was a significant time increase from day 0 to day 90 for all cytokines measured in both treatment groups (Figure 4.7). No other significant differences were measured when analyzing cytokines TNF-α, IL10, IL1β, and PTSG2 using RT-PCR (Figure 4.7).

ELISAs

Inflammatory proteins CRP, IL-1RA, and PGE2 were not significantly increased nor decreased by CBD treatment at any of the time points collected when compared with the control horses (Figure 4.8). There was a significant time decrease from day 60 to day 90 for the CRP ELISA (Figure 4.8).

Endocrine Function

All horses were metabolically similar before the start of the current study (Figure 4.9). Metabolic responses measured by ACTH to the TRH stimulation test and insulin to the OST before and post the treatment period were not significantly impacted by CBD (Figure 4.9).

Vaccination and HI Titers

There was a significant time increase for both treatment groups from day 0 to day 21 (Figure 4.10). However, in this study, an infectious challenge study with influenza was not conducted to determine if this increase in HI titers post-vaccination was biologically significant (Figure 4.10). Regardless, CBD did not increase nor decrease the antibody response to vaccination. The HI titers of the sentinel horse group were also not increased nor decreased significantly over time (data not shown). In response to the vaccination, some horses developed a reaction at the site of the injection. There were 3 horses in the CBD treatment group that had vaccination site reactions and in the control treatment group, 4 horses had vaccination site reactions. However, reaction sites were monitored by our in-house veterinarian and resolved without the use of NSAIDs.

4.5 Discussion

The awareness of the use of CBD as a therapeutic for a variety of maladies, as well as reducing inflammation is increasing in the equine industry. Given the growing population of senior horses, it is of important to determine the efficacy of CBD in the senior horse. Therefore, the objectives of this study were to determine the effects of CBD on the parameters of body morphometrics, lameness, immune function, endocrine function, and vaccination responses of senior horses. To the authors' knowledge, this is the first research to understand the efficacy of CBD on the health of the horse in an experimentally controlled, 90-day treatment study period.

Senior horses can be susceptible to extreme changes in body weight and condition, thus it was of interest in this study to examine the effects of CBD on these body morphometrics. Also, ideal body condition can be a sign of good health and one of the

goals of this study was to determine if CBD impacted general health of the senior horse. As shown in other CBD studies in dogs [73] during a 12-week study there were no effects of CBD on weight gain. Similarly, in this current study, there was no effect of treatment on body weight or body condition in senior horses treated with CBD over a 90-day period.

A major co-morbidity of aging in senior horses is lameness, caused by osteoarthritis or other musculoskeletal diseases, [2] and there is a desire to identify effective alternatives to NSAIDs for alleviation of inflammation and pain. Thus, another objective of the current study was to determine if CBD had an effect on lameness in senior horses. An observational lameness exam was conducted using the AAEP scale of 1-5, before and post CBD 90-day treatment period and there was no significant effect of CBD on lameness in the horses before or after treatment. More research using non-subjective measures of lameness such as kinetic gait analysis [128] could be useful to investigate the quantifiable effects of CBD on lameness. A recent study investigated the synovial fluid concentrations post CBD administration (.5mg/kg or 1.5mg/kg BID) and determined that CBD was detectable within the synovial fluid after 5 weeks of twice-a-day dosing [79]. Therefore, CBD is found in the synovial fluid of the joint after administration, however, they did not determine how this affected joint mobility [79]. Since CBD can be found in the synovial fluid post-administration, it would be interesting to investigate the effects on lameness using a joint LPS-challenge model in horses [120]. Which may provide further insight into the effects of CBD on joint mobility and osteoarthritis.

Pharmacological research of CBD in equine is on the rise with several studies demonstrating the effects of CBD on the horse [77-79]. The variety of equine CBD doses

ranges from 0.5mg/kg per bw to 3mg/kg per bw administered either once or twice a day [77-79]. The concentration of CBD used in this study was determined based on a previous pharmacology study completed with the senior horse [117]. It was shown that in the first 90 days of the study CBD and its metabolites were detected in the treated horse's plasma. This was determined with the use of a once-a-day oral administration of CBD to the horses. A recent study used a similar concentration and then divided it into two doses daily with a small meal [79] which, may be more beneficial as the half-life of CBD is approximately 7 hours in the horse [117]. The metabolite 7-COOH CBD was the most prominent and significant metabolite present in the plasma post-collection which is consistent with previous studies [117]. While the animals did not demonstrate any adverse effects following CBD administration, the mechanism of action of this particular metabolite is unknown. More in-depth research to determine the effects of this metabolite, 7-COOH CBD, is needed to develop a more cohesive understanding of how it elicits its beneficial actions within the animal. With limited research in the horse, it was unknown if the timing of blood sample collection impacted the ability to detect the effects of CBD on immune parameters of production of inflammatory cytokines and acute phase inflammation markers (CRP, IL-1ra). A sample collection time of prior to CBD administration and 4 hours post CBD administration presented no effect on the health parameters that were investigated in this study. Further research is necessary to investigate the pharmacokinetics of CBD at different dose concentrations and formulations in the horse since effective dose concentrations are unknown.

Another objective of this study was to determine if the administration of CBD caused any adverse effects on the liver of the horse therefore, hematological and

biochemical analyses were conducted. In the current study, there was only a significant effect of CBD treatment on albumin. Albumin can increase in animals that are dehydrated or excited and is influenced by hormonal factors as well, therefore the increase observed in the current study may not be attributed to the treatment of CBD [129]. In previous research describing CBD administration to dogs, an increase in liver enzymes, such as AST and ALK Phosphate was reported [74]. However, in the current study, there were no changes in liver enzymes which is consistent with the CBC and chemistry results in a recent publication investigating the pharmacokinetics of CBD in horses [117]. This data provides evidence that a 2mg/kg by body weight oral dose of CBD administered over an extended period does not cause adverse effects on the liver significant enough to cause an elevation in the liver enzymes monitored in this study.

CBD has been thought to help reduce inflammation in humans and animals since its discovery. Previous research on CBD has been conducted *in vitro,* in rodents, and dogs, and effects on inflammatory responses have been demonstrated [44, 48, 74, 97]. CBD has been shown, *in vitro,* to reduce TNF-α and IFN-γ in equine PBMCs [105] but no research has been conducted to investigate the *in vivo* effects of CBD in the horse. With the potential of CBD to reduce important inflammatory immune responses within the senior horse, the current study measured the effects of CBD administration on levels of the pro-inflammatory TNF-α, INFγ, IL6, and IL1β along with anti-inflammatory IL10 cytokines [96]. No significant effect of CBD treatment on major inflammatory cytokines was observed by intracellular staining and flow cytometry for either TNF-α or IFN-γ in this study. It was discovered that with the equine cells stimulated with PMA/Ionomycin and then analyzed through real-time PCR the expression of TNF-α, IFN-γ, IL1β, IL10,

and IL-6 were not affected by CBD treatment over the entirety of this study. This finding suggests that the strength of PMA/IO mitogen stimulation may overcome any changes in treatment effects.

This study also examined the effects of CBD on whole blood without mitogen stimulation with some significance reported. Specifically, the production of IFN-γ at day 60 was significantly reduced for CBD-treated horses when compared with control horses. Additionally, the expression of IL6 was also significantly reduced on days 60 and 90 for those horses on CBD treatment when compared with the control. No other significant differences were measured during RT-PCR when analyzing cytokines TNF-α, IL10, and IL1β. Research has shown similar results in rodents, with this reduction in IFN-γ and IL6 occurring thereby suggesting the anti-inflammatory effects of CBD [130]. The source of cytokines in whole blood are likely produced by cells other than PBMCs. This could include cells from tissue sources of fat or muscle which could explain some of the differences found between stimulated PBMCs and whole PCR results. A potential reduction of unspecified cell inflammation within whole blood could be occurring compared to a cell specific reduction in inflammation. This finding is further supported by the results of the CRP ELISA which shows a reduction over time from day 60 to day 90. The CRP ELISA is less sensitive than RT-PCR and cannot detect as many changes due to treatments which is evident as both CBD and Control treatments are reduced. Additionally, a major difference between the stimulated PBMCs and whole blood PCR may be a contrast in the situations, *ex vivo* or *in vitro*, and the influences of granulocytes in the whole blood. More research investigating the potential of CBD reducing whole blood inflammation compared to cell specific reduction may be of benefit.

It has been proposed that the anti-inflammatory effects of CBD may be elicited through the inhibition of the arachidonic acid metabolic pathway [131]. Arachidonic acid is converted to prostaglandins with the use of COX enzymes, either COX-1 or COX-2 depending on which prostaglandin is being produced [132]. The inhibition of the COX-2 enzyme as part of the arachidonic acid pathway has been studied with cannabidiolic acid (CBDA), a precursor of CBD [133]. Therefore, CBD, produced by CBDA, may potentially inhibit COX-2 decreasing the production of prostaglandins, thereby reducing inflammation. However, COX-2 was determined not to be affected by CBD in senior horses when compared with the control horses in the current study. Prostaglandin E2, which is downstream of COX-2, was also not inhibited by CBD administration at any of the time points presented in this current study. This is consistent with a recent study completed with young thoroughbreds administered various doses of CBD and no inhibition of the arachidonic acid pathway was reported [77]. In the current study, it was shown that CBD at the current 2mg/kg per body weight, did not affect the arachidonic acid pathway through COX-2 inhibition.

Additionally, an objective of this study was to investigate the effects of CBD on the endocrine system of the senior horse. Animals have an endocannabinoid system that contains a series of cannabinoid receptors, ligands, and enzymes for synthesis and degradation [27]. Effects of cannabinoids have been studied on the endocrine system and investigators have found that many of the endogenous cannabinoids that are interrelated within the endocannabinoid system can inhibit the endocrine system [134]. Though, it has not been determined if CBD alone, can affect these endocrine systems. Further, because a percentage of the senior horse population is affected by endocrine disorders or PPID and

equine metabolic syndrome, an endocrine function response to treatment was investigated. The study horses presented were a mix of PPID, ID, and normal horses before the start of the CBD treatment. Thus, this study examined the effects of CBD on ACTH and insulin before administration of CBD and then after CBD had been administered for 90 days. As the results mentioned there was no effect of CBD treatment on these important endocrine markers. In the current study a purified CBD distillate oil, consisting of 98% CBD and almost 0% THC was utilized and is likely the reason there was not a significant effect of CBD on endocrine function. The endocannabinoid system, which has been shown to affect the endocrine system contains the g-protein receptors, cannabinoid 1 (CB1) and cannabinoid 2 (CB2) [134]. It has been demonstrated that THC interacts with the endocannabinoid system through the CB1 as well as CB2 receptors [27]. A prior *in vitro* study, demonstrated that with equine cells, CBD does not affect these receptors [105]. Therefore, it would be interesting to use a full-spectrum CBD oil that contains THC, which does activate receptor CB2, to determine if the potential is there for affecting the endocrine system in the horse.

Lastly, vaccinations in the horse are routinely administered annually or biannually therefore, this study investigated the effects of CBD on vaccination. It was of interest in this study to measure the immune response to vaccination as a way to determine the effects of CBD on the function of the immune system. Since senior horses undergo inflamm-aging, determining that CBD did not further impact age-associated changes in immune function was of importance. Senior horses have been shown to have a lower immune response to vaccinations than younger horses [135]. Fortunately, for the senior

horse in this current study, it was demonstrated that CBD treatment did not negatively affect the immune response to the equine influenza vaccination.

The current study had some limitations. Horses were dosed once daily with CBD which may have provided some benefits however, with the lack of effect on the majority of inflammatory cytokines measured the current dosing protocol did not achieve the potential benefits that have been suggested. Additionally, only one dose (2mg/kg BW) was administered which may not have been the optimal dose for efficacy. A dose-response study should be conducted to better determine the best dose of CBD for reducing inflammation in the horse. Additionally, additional research is necessary to determine when to dose a horse, as there are multiple factors involved in the absorption of CBD. CBD has been well characterized as a compound that undergoes first-pass metabolism in the liver of both humans and animals [67, 72]. Another potential problem with orally dosing CBD in horses is that they have enzymes within their hindgut that could be affecting the absorption and distribution of CBD within the horse [136]. The CBD was also dosed as a solution, dissolved within a soy oil, which may have affected absorption [117]. Recent pharmacology studies have used different vehicles for administering CBD such as a pelletized CBD [78] or suspension-based CBD for oral administration [77, 79]. As these recent pharmacology studies reveal oral CBD dosage formulation variations, there are lipid encapsulations or other oils that CBD may be suspended in that may improve absorption. Further research should be conducted to determine the optimal route for administering CBD to the horse. Lastly, while not a limitation, it should be noted that the current study did not report any behavioral

responses or changes as that was beyond the focus of this study. Further research investigating the effects of CBD on behavior is of interest and should be considered.

Overall, this study showed that CBD reduces the production of both pro-inflammatory cytokines IFN-γ and IL-6 when compared with control over the entire 90-day study. It was also reported that CBD concentrations were detectable at all time points throughout the study with a one-time per-day administration of 2mg/kg body weight. However, many of the other health parameters were not determined to be significant after horses are treated for 90 days with CBD. More research with CBD is necessary to further determine the effects on equine health as an anti-inflammatory adjunct therapy that could improve lameness or other health parameters, with a particular interest in the senior horse population.

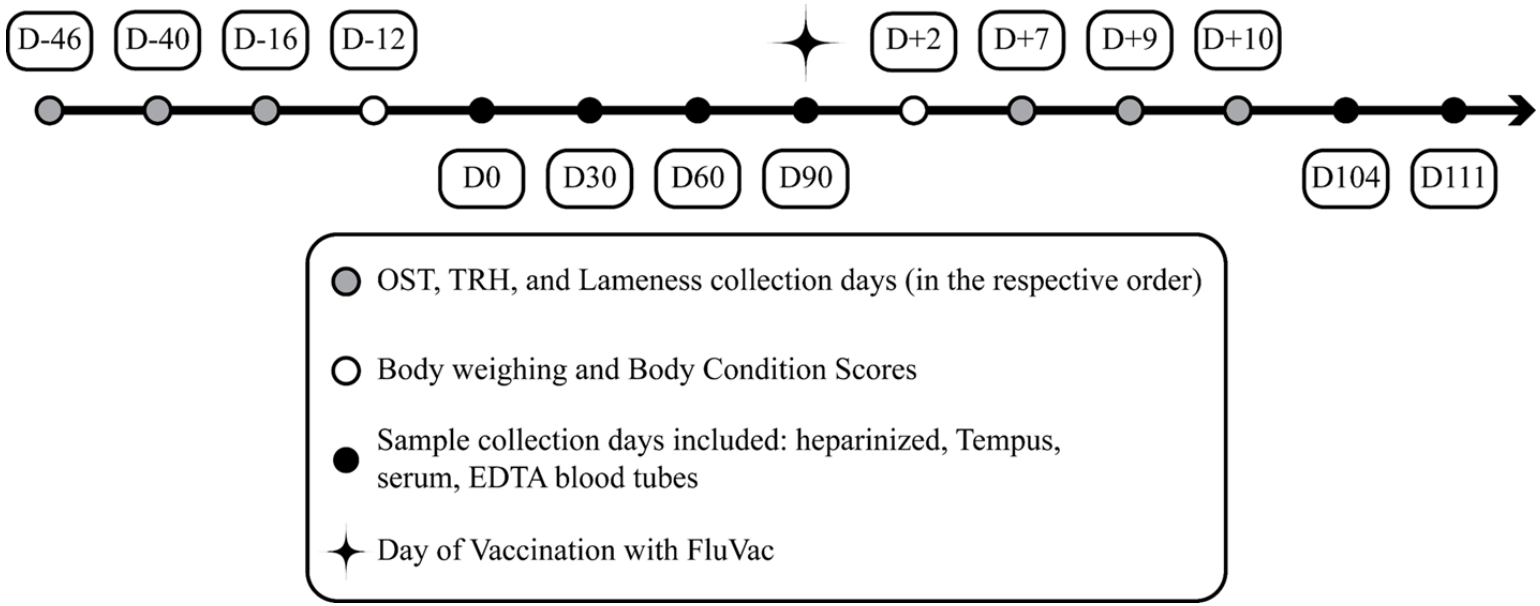

Figure 4.1 Timeline of the sample collections.

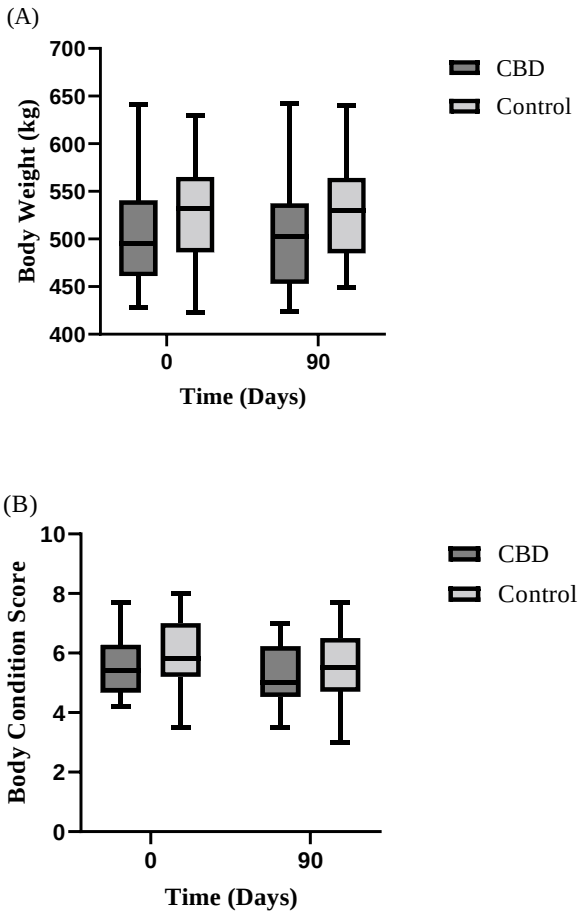

Figure 4.2 Body weight and body condition scores.
(A) Mean ± SD body weight and (B) mean ± SD body condition score for both control (n=14) and CBD (n=13) treated horses on Day 0 and Day 90. No significant time effect or treatment by time effect $P>0.05$.

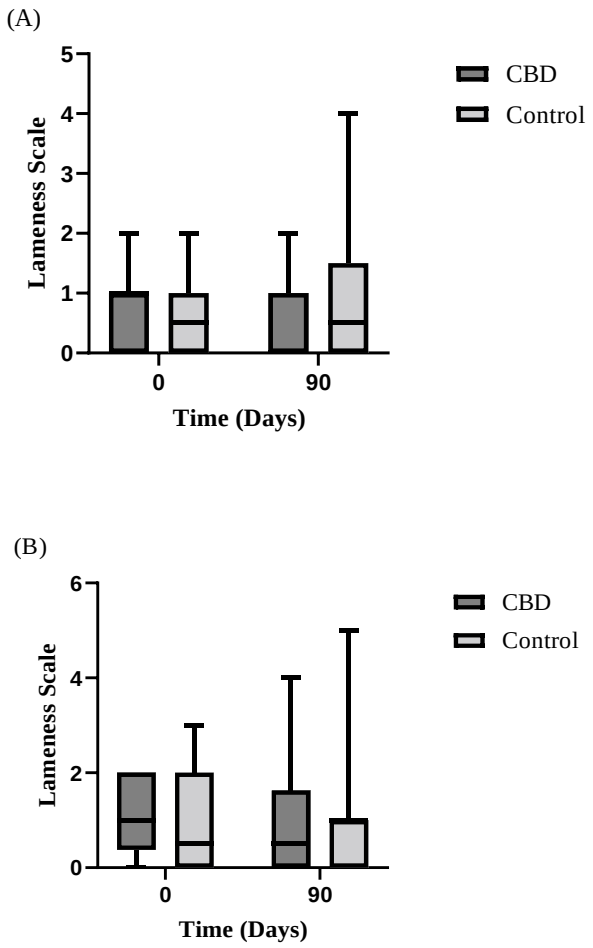

Figure 4.3 Lameness evaluation.

(A) Mean ± SD left front lameness scale and (B) mean ± SD right front lameness scale for both control (n=14) and CBD (n=13) treated horses on Day 0 and Day 90. No significant time effect or treatment by time effect $P>0.05$.

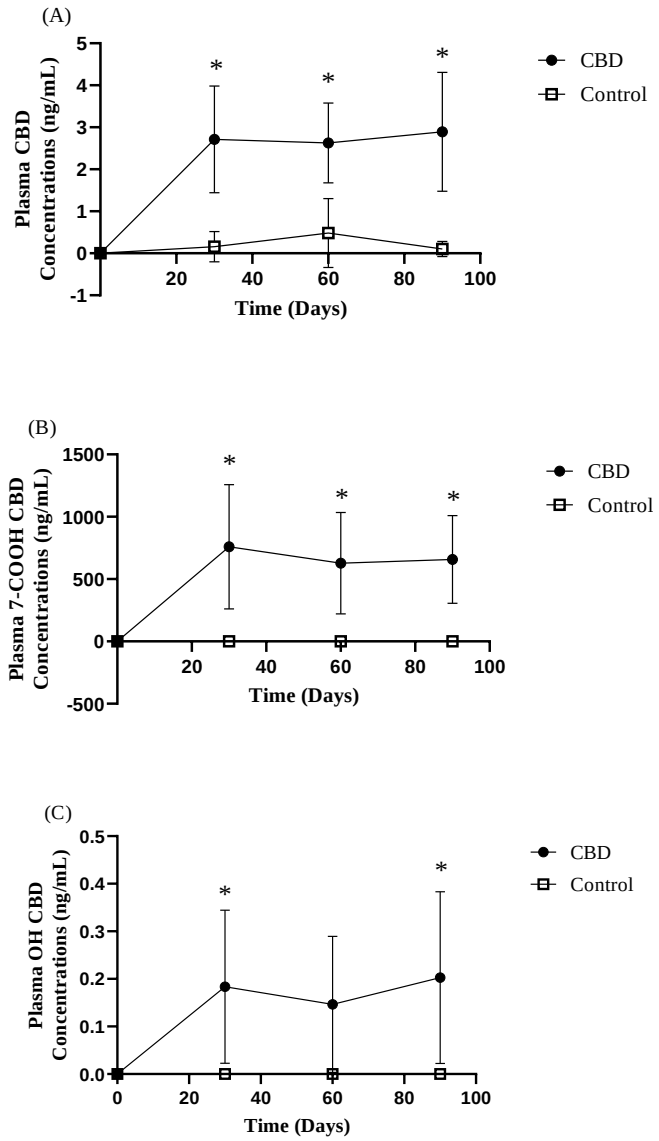

Figure 4.4 Plasma Cannabidiol (CBD) concentrations and metabolites 7-Carboxy Cannabidiol (7-COOH CBD), Hydroxy Cannabidiol (OH CBD)

A) Mean ± SD plasma CBD, B) Mean ± SD Plasma 7-COOH CBD metabolite, and C) Mean ± SD Plasma OH CBD metabolite concentrations with control (n=14) and CBD (n=13). Significance is denoted with the asterisks above the time points for the time by treatment interaction $P<0.05$.

Table 4.1. Complete Blood Count and Serum Chemistry for all horses on days 0, 30, 60, and 90. Presented in mean ± SD. Significant parameters are denoted by superscript letters P<0.05.

	CBD Treatment (n=13)				Control Treatment (n=14)			
	0	30	60	90	0	30	60	90
Total bilirubin (mg/dL)	1.1 ± 0.5	1.4 ± 0.5	1.3± 0.3	1.1± 0.2	1.1 ± 0.4	1.7 ± 0.4	1.4 ± 0.2	1.2 ± 0.2
Albumin (g/dL)	3.0 ± 0.2[a]	3.2 ± 0.2	3.3 ± 0.2[bcd]	3.5 ± 0.1[cd]	3.2 ± 0.3	3.2 ± 0.2	3.2 ± 0.3	3.5 ± 0.3[d]
Total Protein (g/dL)	6.6 ± 0.5	7.2 ± 0.8	7 ± 0.5	7.1 ± 0.5	6.5 ± 0.7	6.8 ± 0.9	7.4 ± 1.4	7.0 ± 1.0
White Blood Cells (10^3)	7.0 ± 0.4	7.3 ± 0.6	7.5 ± 1.0	7.6 ± 1.1	7.2 ± 0.6	7.4 ± 0.8	7.3 ± 0.8	7.4 ± 0.7
Red Blood Cells (10^6)*	7.9 ± 0.7	8.5 ± 1.6	9.2 ±3.8	8.6 ± 1.8	8.4 ± 1.3	8.0 ± 0.9	7.9 ± 1.4	8.1 ± 1.2
Hemoglobin (g/dL)	11.4 ± 2.1	12.0 ±2.4	12.4 ± 2.7	12.4 ± 2.7	11.6 ± 1.5	12.3 ± 2.1	12.7 ± 3.8	13.2 ± 3.4
Packed Cell Volume (%)	18.6 ± 10.4	20.9 ± 11.5	28.0 ± 26.9	24.0 ± 15.4	14.3 ± 2.6	15.8 ± 4.3	17.3 ± 7.1	16.2 ± 4.3
γ-glutamyl transferase (U/L)	34.2 ± 3.8	36.2 ± 5.4	41.5 ± 25.7	40.5 ± 10.6	35.0 ± 4.1	34.9 ± 4.2	36.1 ± 5.4	35.8 ± 4.0
Glucose (mg/dL)	80.9 ±10.9	78.2 ± 8.3	95.6 ± 7.7	89.9 ± 6.7	79.8 ± 11.2	88.9 ± 12.9	95.5 ± 14.3	96.3 ±18.6
Alkaline phosphate (U/L)	106.1 ± 16.9	138.9 ± 41.6	138.9 ±37.1	134.6 ± 36.2	108.3 ± 15.8	123.9 ± 34.1	127.4 ± 29.6	123.4 ±31.9
Aspartate aminotransferase (U/L)	239.6 ± 76.1	258.2 ± 80.3	266.2 ± 98	248.6 ± 86	205.7 ± 36.6	219.4 ± 42.1	220.9 ± 39.3	223.0 ± 46.1

Means with the same letter are not significantly different, those with different letters are significantly different *P*<0.05

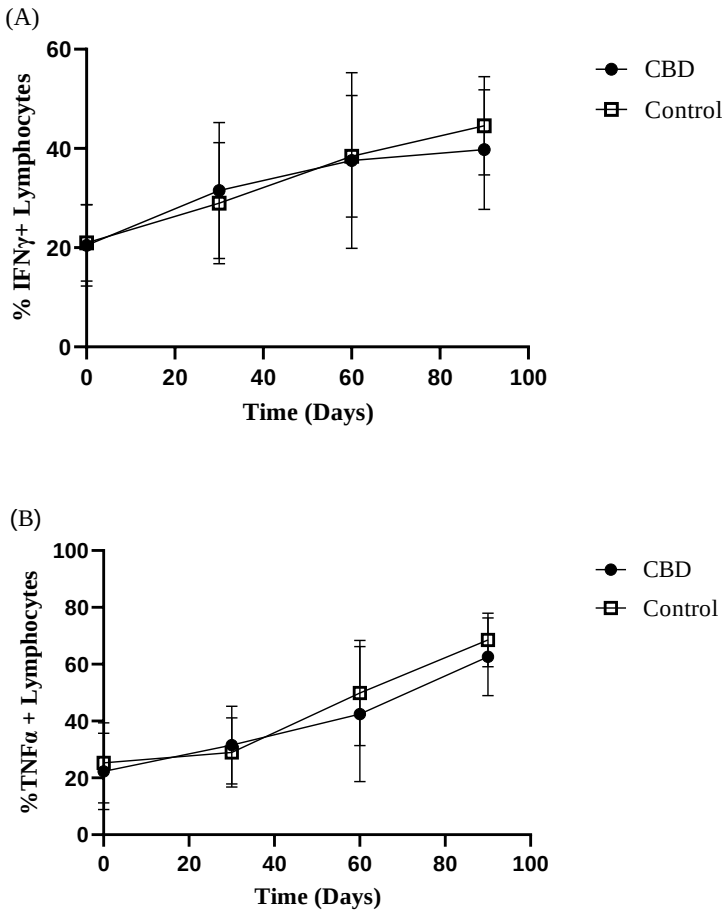

Figure 4.5 Intercellular staining for TNFα and IFNγ.

A.) Mean ± SD % IFNγ –lymphocytes and B). Mean ± SD % TNFα- lymphocytes for both control (n=14) and CBD (n=13) treatment groups. No significant difference of treatment across time points *P>0.05*. There is a significant overall time effect for all cytokines *P<0.05*.

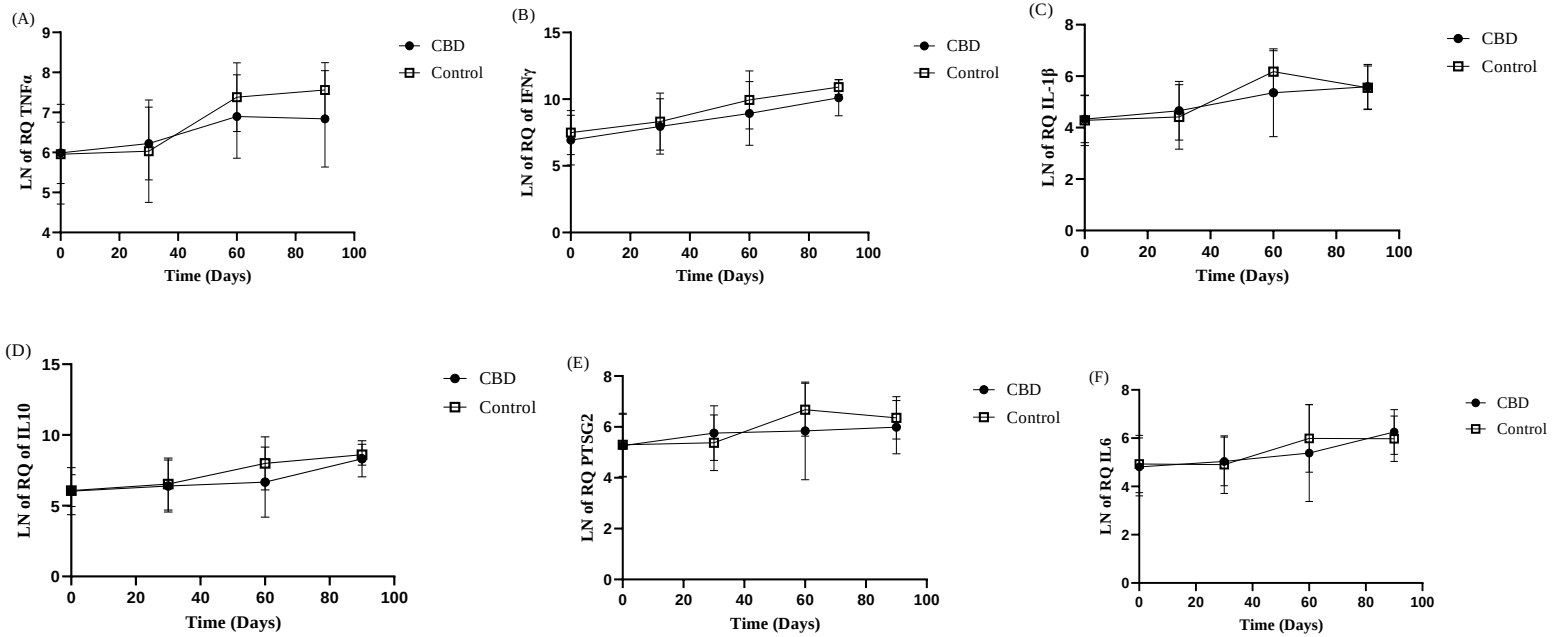

Figure 4.6 PBMC-Stimulated RT-PCR Expression.

A. TNFα, B. IFNγ, C. IL1β, D. IL10, E. PTSG2, F. IL6 Data are presented as means ± SD with control (n=14) and CBD (n=13) treatment groups. No significant treatment by time effect for all cytokines presented $P>0.05$. Though there is a significant overall time effect for all cytokines $P<0.05$.

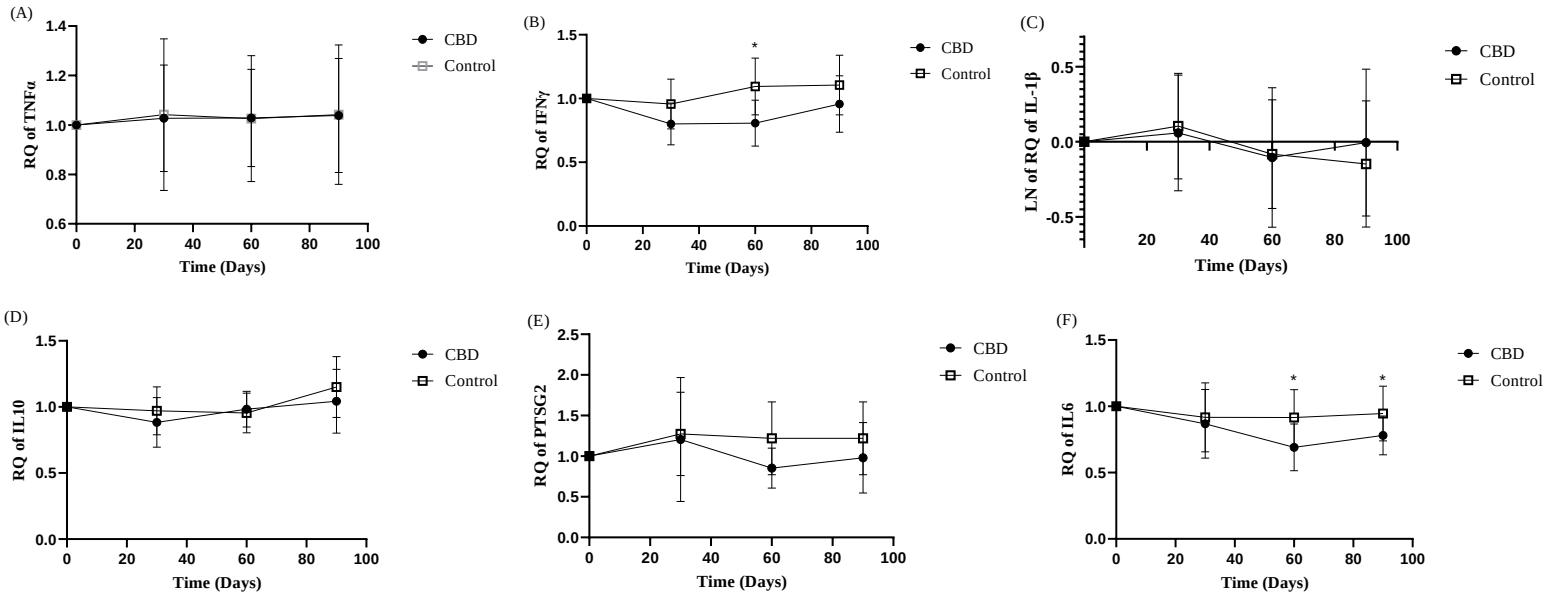

Figure 4.7 Whole blood RT-PCR Expression.

A. TNFα, B. IFNγ, C. IL1β, D. IL10, E. PTSG2, F. IL6 Data are presented as means ± SD with control (n=14) and CBD (n=13) treatment groups. Significant treatment by time effect denoted by asteriks for IFNg and IL-6 $P<0.05$. No significant treatment by time effect for TNFα, IL1B, IL10 and PTSG2 $P>0.05$. Though there is a significant overall time effect for all cytokines $P<0.05$.

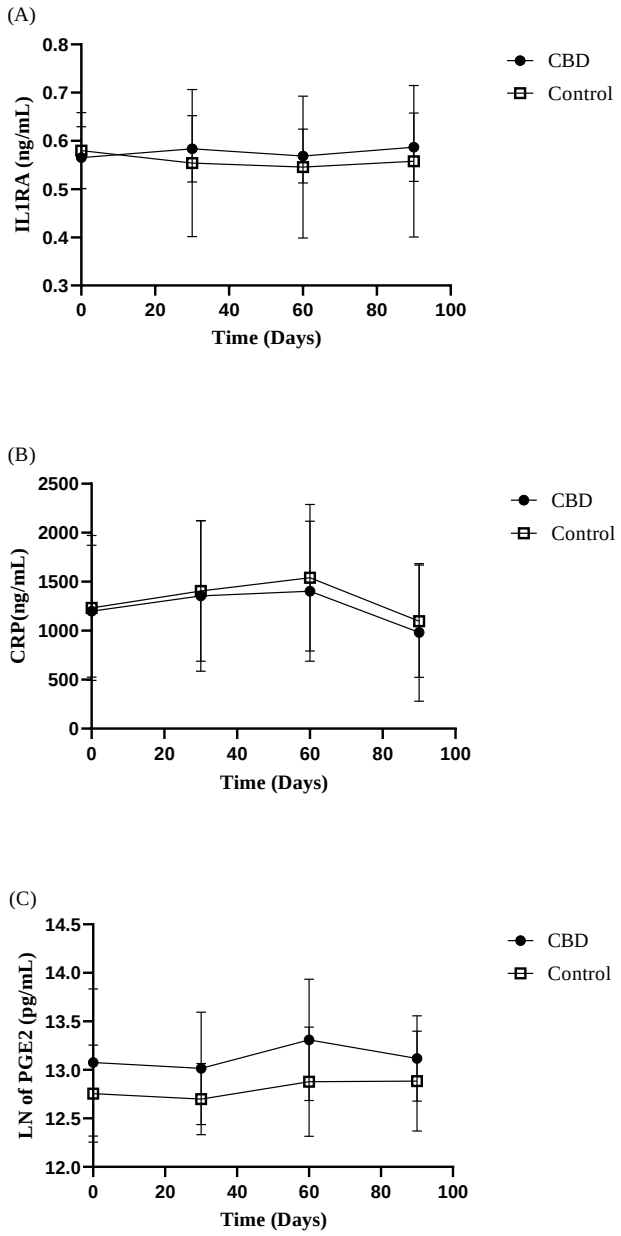

Figure 4.8 Inflammatory Enzyme Linked Immunosorbent Assays (ELISA).
A) Mean ± SD Interleukin-1 receptor agonist (IL-1ra) ELISA serum concentrations, B.) mean ± SD C- Reactive Protein (CRP) ELISA serum concentrations, and C.) Natural log mean ± SD Prostaglandin E2 (PGE2) ELISA serum concentrations for control (n=14) and CBD (n=13) treatment groups. No significant treatment by time effect *P>0.05.* A significant time effect was seen in CRP ELISA from day 60 to day 90 *P<0.05.*

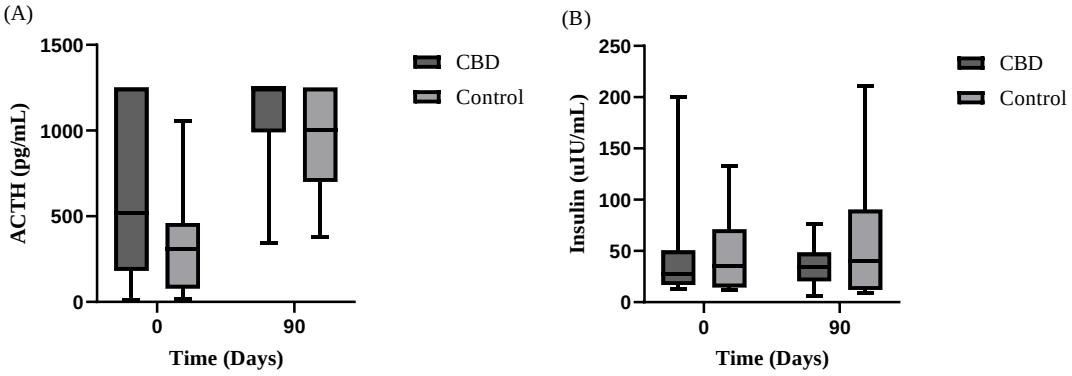

Figure 4.9 Metabolic function.

A) Data are presented as time 10 for ACTH and as mean ± SD and B) Data for insulin are presented at time 60 and as mean ± SD for both control (n=14) and CBD (n=13) treated horses on Day 0 and Day 90. No significant treatment by time effect *P*>0.05.

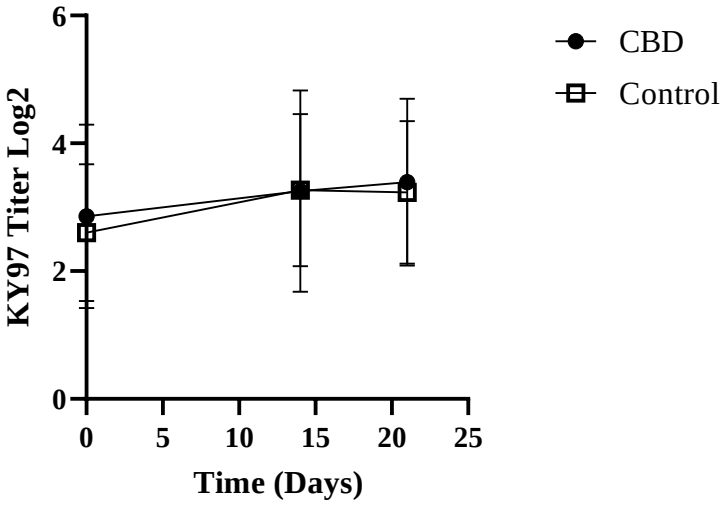

Figure 4.10 HI titers post-vaccination of all horses.
All data are presented as means ± SD with control (n=14) and CBD (n=13) treatment groups. There was a significant overall increase in time effect from day 0 to day 21 $P<0.05$. No significant treatment by time effect $P>0.05$.

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

Popularity of CBD is at an all-time high both in human and veterinary medicine though, this is despite a lack of scientific evidence to support the claims. Most of the claims of CBD's benefits as an anti-inflammatory and anxiolytic treatment are anecdotal at best from centuries of use. In veterinary medicine investigations in the use of CBD for treatment of pain or reducing inflammation has been increasing in dogs, cats, and horses. This dissertation is the first collection of research completed in the senior horse investigating the effects of CBD on immune function and health. The senior horse population in the US is growing and this population of horses is prone to maladies and ailments, such as lameness, metabolic diseases, and other musculoskeletal disorders. Inflamm-aging and immunosenescence may be contributing factors to some of the age-related conditions in the horse. The typical treatments for these co-morbidities of aging have been non-steroidal anti-inflammatories that may have undesirable side effects for the horse. Therefore, it was of interest to see if the CBD potential for anti-inflammation benefits translated to *in vivo* efficacy in the senior horse.

The first study in this body of research was to determine if CBD would affect inflammatory cytokines at a cellular level. No scientific research had been conducted in the horse at the time of this study. Therefore, through extrapolation from other *in vitro* studies a titration amount of CBD was determined and used with peripheral mononuclear cells collected and isolated from the senior horse. Results indicated that at the highest concentrations of the pure CBD powder decreased cell viability. It was also determined that the concentration of CBD that was not affected by viability but that also caused a decrease in cytokines TNFα, IFNγ, and IL-10 was 4ug/mL. This research determined that

CBD does, at a cellular level, decrease inflammatory cytokines, therefore, may potentially decrease inflammation in the senior horse.

This *in vitro* study revealed that high doses of CBD at the cellular level may suppress important cytokines. The study also revealed that at high levels CBD may be cytotoxic. Suppressing cytokines important in inflammation as well as increasing cellular death may translate to negative effects in the animal. Therefore, it is important to consider these effects in the *in vivo* CBD dose concentration formulation.

Following the *in vitro* trial, this next research determined what concentration of CBD should be used as an oral dose as well as determining the oral bioavailability of CBD. After a small trial, it was concluded to use 2mg/kg body weight CBD distillate suspended in soy oil and orally syringed. To determine oral bioavailability an intravenous dose of CBD (0.1mg/mL) dissolved in DMSO was extrapolated from other similar equine drug studies. A cross-over trial was developed using 8 horses; an intravenous dose was administered to 2 and an oral dose was administered to 6 horses on the first day. Following this singular oral or intravenous dose blood collection occurred at predetermined time points. Then twelve days from the original dose the horses were randomized and dosed again with the same blood collections occurring at the pre-distinguished time points as the first collection.

It was determined from this trial that 2mg/kg body weight of CBD was an acceptable oral dose as CBD was detectable in the horse's blood post-dosing. It was also determined that from this one oral dose that no adverse gastrointestinal issues or effects on the animal's livers were measured as reflected in the blood chemistry profiles. CBD is initially degraded in the liver by cytochrome P450 and its related isozymes. This reduces

the amount of CBD to be available for utilization in the body of the animal once it has passed through the liver. In the horse, it was determined from this research that bioavailability was 7%. This is an extremely low bioavailability however, it is unknown how this will translate into *in vivo* efficacy.

The low bioavailability of CBD is proven to be caused by the first past metabolism. In humans, there have been studies that a high-fat diet given at the time of CBD oral administration may improve bioavailability. Future equine studies should consider feeding a small high-fat meal at the time of CBD administration to determine if the diet improves bioavailability in the horse. It has also been demonstrated that an increased dose of CBD causes a proportional increase in bioavailability in the horse. At the time of this pharmacology study, there was a lack of research on the dose concentration in the horse, therefore it was necessary to complete a small titration study to determine what doses to use. Future bioavailability studies may be completed with much higher dose concentration of CBD. Additionally, formulation modifications should also be considered in any future bioavailability studies.

Finally, using the information gathered from the previously described pharmacokinetics trial an *in vivo* trial was devised to determine the efficacy of CBD in the senior horse. A 111-day trial was developed to determine the effect of CBD on lameness, inflammation, endocrine function, and vaccine responses. Before the start of the study as well as after 90 days of the study all of the horses were analyzed for lameness and endocrine function was measured. The twenty-nine horses all were orally syringed daily with either CBD or the control treatment for the entirety of the study. On pre-determined days blood collections were collected on all horses. For each collection

time point, several different health parameters were analyzed for inflammation. Then the horses were vaccinated on day 90 to determine if CBD was going to affect the immune response to routine vaccinations. Blood collections were taken on days 104 and 111 post vaccine while all of the horses were still on CBD or control treatments. Antibody response was measured before vaccination as well as post-vaccination for all horses on CBD or control treatments.

The overall results from this *in vivo* trial with CBD are that CBD orally syringed daily at 2mg/kg of body weight was detected in the blood, CBD decreased the gene production of IFNγ on day 60 and 90 of the trial as well as IL-6 on day 60, and no other health parameters were significantly affected. There was no CBD treatment effect post-vaccination of the horses when compared to the control horses. Overall in the senior horse, orally administered CBD distillate at 2mg/kg body weight was well absorbed, did not cause any symptoms or signs of colic but did significantly reduce the production of anti-inflammatory cytokines on days 60 and 90 of the trial. Also, orally administered CBD distillate (2mg/kg bw) did not affect the endocrine function, and lastly, did not affect routine vaccination.

The lack of anti-inflammatory results from the *in vivo* trial may be the result of having too low of a CBD dose. However, due to a lack of prior research, the dose of 2mg/kg was extrapolated from other species and a small pre-study trial. Further experiments with higher concentrations should be investigated to determine if an increased dose is necessary to achieve the desired anti-inflammatory results. It should also be noted that the methodology of administration may need to be investigated as well. This research used soy oil as the carrier, there may be other lipids that will help the

absorption of CBD in the horse. Encapsulation of CBD may improve the oral bioavailability with the horse as well since they are hindgut fermenters and have enzymes that may have compromised the CBD absorption.

As a whole, this research provides the equine community with the first *in vivo* study of CBD in the horse to determine its immune effects. More research needs to be conducted in the horse to determine what concentration of CBD will improve bioavailability. Additionally, research should build upon the immune health results presented in this body of work to determine if a higher concentration of CBD reduces inflammation. There is potential for CBD to be an effective anti-inflammatory, with more research to be completed.

APPENDIX

Chapter 3 Methods: Pharmacokinetic serum sample analysis

For analysis, working solutions were prepared by diluting each of the stock solutions of Cannabidiol (Cerillant, Round Rock, TX), 7-Hydroxy Cannabidiol (7-OH CBD; BDG Synthesis, Wellington, New Zealand), and 7-Carboxy Cannabidiol (7-COOH CBD; Cerillant, Round Rock, TX) with methanol to concentrations of 0.01, 0.1, and 10 ng/μL. These working solutions were then used to prepare the serum calibrators using drug free equine serum [0.1-50ng/ml CBD and 7-OH CBD; 0.1-1000ng/ml 7-COOHCBD]. A new calibration curve and negative control was prepared for each quantitative assay. Also, for each set of samples a complete set of quality control samples, using drug free equine serum and concentrations within the standard curve, were prepared acting as an additional accuracy check.

An internal standard (CBD-D3; Cerillant, Round Rock, TX) was combined with 0.5mL serum and diluted with 100 μL of water before being analyzed, at 0.1ng/μL. The samples were vortexed to mix. Then use of liquid-liquid extraction (LLE) was employed with the addition of 5 mL of methyl-tert-butyl ether to each sample followed by rotating for 20 min at 40 revolutions/min to mix. All samples were then centrifuged (3300 rpm or 2260g for 5 min at 4º C) and the organic layer was transferred to a new glass tube. The organic layer samples were then dried under nitrogen and dissolved (130 μL of 60% acetonitrile (ACN) in 0.2% ammonium hydroxide). Lastly, each individual sample was injected (30 μL) into the liquid chromatography tandem mass spectrometry (LC-MS/MS) system.

Within the serum, the concentrations of CBD, 7-OH CBD and 7-COOH CBD were measured by LC-MS/MS in ESI negative mode. A TSQ Altis triple quadrapole mass spectrometer coupled with a Vanquish liquid chromatography system (Thermo Scientific, San Jose, CA) was used for quantitative analysis. Spray voltages was set at 2645 V, sheath and auxillary gas were 41 and 24 correspondingly (arbitrary units) with spray voltage temperature set at 350º C. The product masses and collision energies of each sample analyte were optimized by infusing the standards into the mass spectrometer. An ACE 3 C18 10cm x 2.1 mm (Mac-Mod Analytical, Chadds Ford, PA) column and (B) a gradient of neat ACN in 0.2% ammonium hydroxide (A) using of 0.35 mL/min flow rate was used for chromatography. ACN initial concentration was held at 60% for 0.25 min, then ramped to 98% over 3.25min, kept at that concentration for 0.5 min, then re-equilibrating for 2.7 min at the initial concentrations. Specifically the analysis for 7-COOH CBD was altered slightly with the initial ACN concentration was being held at 5% for 0.25 min, then ramped to 98% over 3.45 min, kept at that concentration for 0.2 min, before re-equilibrating for 2.7 min at initial concentrations.

The quantification and detection were measured using selective reaction monitoring (SRM) of initial precursor ion for CBD (mass to charge ratio [m/z] 313.2), 7-OH CBD ([m/z] 329.2), CBD-D3 ([m/z] 316.2) and 7-COOH CBD ([m/z] 343.2). Then using Quanbrowser software (Thermo Scientific) the reactions for the productions for CBD (m/z 179.0 [quan ion], 173.0, 245.1), 7-OH CBD ([m/z] 261.1, 297.1, 299.1), CBD-D3 ([m/z] 182.1, 248.1, 314.0), and 7-COOH CBD ([m/z] 191.1(quan ion), 179.1, 231.1) were plotted and peaks at the proper retention time integrated. Generation of all

calibration curves and the determination of CBD and its metabolites by linear regression was also completed by Quanbrowser software.

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Abstracts

Turner, S., Barker, V.D., Adams, A.A. Effects of Cannabidiol on the *in vitro* lymphocyte pro-inflammatory cytokine production of senior horses. Conference for Research Workers in Animal Disease, Chicago, IL November 2019.

Turner, S, D. R. Ledoux, G. E. Rottinghaus, M. C. Shannon. Effects of dietary treatments containing various concentrations of corn naturally contaminated with fumonisin (FB_1) on performance of nursery pigs. VI Latin American Congress of Mycotoxicology, Mexico, June 27-July 1 2010.

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Turner, S., Knych, H.K., Adams, A.A. (2022). Pharmacokinetics of cannabidiol in a randomized crossover trial in senior horses. *American Journal of Veterinary Research,* doi.org/10.2460/ajvr.22.02.0028

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