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THERAPEUTIC AND SAFETY EVALUATION OF CURCUMIN'S ANTIMICROBIAL AND ANTI-INFLAMMATORY PROPERTIES IN CANINE AND EQUINE

by

Stephanie D. Bland

B.S., Murray State University, 2012
M.S., Murray State University, 2014

A Dissertation
Submitted in Partial Fulfillment of the Requirements for the
Doctoral Degree

Department of Agriculture Sciences
in the Graduate School
Southern Illinois University Carbondale
August 2016

DISSERTATION APPROVAL

THERAPEUTIC AND SAFETY EVALUATION OF CURCUMIN'S ANTIMICROBIAL AND ANTI-INFLAMMATORY PROPERTIES IN CANINE AND EQUINE

By

Stephanie D. Bland

A Dissertation Submitted in Partial

Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

in the field of Animal Science, Food & Nutrition

Approved by:

Dr. Rebecca Atkinson, Chair

Dr. Erin Venable

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Southern Illinois University Carbondale
March 2, 2016

AN ABSTRACT OF THE DISSERTATION OF

Stephanie D. Bland, for the Doctor of Philosophy degree in Agriculture Sciences, presented on March 2, 2016, at Southern Illinois University Carbondale.

TITLE: THERAPEUTIC AND SAFETY EVALUATION OF CURCUMIN'S ANTIMICROBIAL AND ANTI-INFLAMMATORY PROPERTIES IN CANINE AND EQUINE

MAJOR PROFESSOR: Dr. Rebecca Atkinson

In total, four experiments were conducted to determine the therapeutic and safety effects of the nutraceutical, turmeric, and its active ingredient curcumin on canines and equines. Two studies were conducted on client-owned, moderately arthritic canines, studying the therapeutic and safety effect of curcumin's anti-inflammatory properties. In Exp. 1, two different dosages, 500 mg, SID of 95% curcumin and 250 mg, BID of 95% liposomal-curcumin, were evaluated in ten moderately arthritic dogs over five months. The dogs in the 95% curcumin group, overall, had a greater reduction in pain by Day 60. Exp. 2, was a follow-up experiment to Exp. 1. In Exp. 2, two different dosages, 500 mg, SID or 100 mg, SID of 95% curcumin, were evaluated in ten moderately arthritic dogs over five months. We observed that dogs in the 500 mg, SID group had an overall greater significance in pain reduction by Day 60. Experiment 3 and 4 were conducted as a two-part project looking at the antimicrobial and anti-inflammatory properties of turmeric, curcumin, and liposomal-curcumin. The purpose of these studies were to investigate both form and dose of turmeric and its active ingredient, curcumin, on reducing opportunistic bacteria found in the equine hindgut. The bacterial strains of interest included *Streptococcus bovis/equinus* complex (SBEC), *Escherichia coli* K-12, *Escherichia coli* general, *Clostridium difficile*, and *Clostridium perfringens*. Exp. 3, was a two-part *in vitro* study; the first part looked at the antimicrobial effects of turmeric, curcumin, and liposomal-curcumin (LIPC) on reducing

opportunistic bacteria found in the equine hindgut, including SBEC ($P = 0.006$), *E. coli* K-12 ($P = 0.50$), *E. coli* general ($P = 0.11$), *C. difficile* ($P < 0.0001$), and *C. perfringens* ($P = 0.24$). The follow-up *in vitro* 24 h batch culture examined four different dosages (15 g, 20 g, 25 g, and 30 g) of 500 mg/g of LIPC, at reducing the concentration of opportunistic bacteria. These results were utilized to determine the dosing rate *in vivo*. Exp. 3, *in vitro,* evaluated the efficacy of antimicrobial and anti-inflammatory properties of LIPC dosed at 15, 20, 25, and 35 g. These results were utilized to determine the dosing rate *in vivo*. Exp. 4, *in vivo,* evaluated the efficacy of antimicrobial and anti-inflammatory properties of LIPC dosed at 15, 25, and 35 g compared to a control. *In vivo*, LIPC's antimicrobial properties, at 15 g, significantly decreased ($P = 0.02$) SBEC compared to other treatments. In addition, *C. perfringens* tended ($P = 0.12$) to decrease as LIPC dose increased. Non-significant results in digestion, blood parameters, and range of motion suggest there were no adverse side effects from oral dosing increasing doses of curcumin. Valerate decreased ($P = 0.005$) linearly as LIPC dose increased. As LIPC dose increased, butyrate and iso-valerate decreased ($P \leq 0.03$) linearly. However, acetate tended ($P = 0.10$) to increase linearly as the dose of LIPC increased. Treatment did not affect ($P \geq 0.19$) any of the other individual VFAs measured, but increasing doses of LIPC tended ($P = 0.10$) to increase total VFA concentrations. Additionally, LIPC tended ($P = 0.11$) to increase total VFA concentrations when compared to control. In the future, further work should be conducted examining liposomal-curcumin's antimicrobial properties in canine and anti-inflammatory properties in equine over a longer period of time

ACKNOWLEDGMENTS

Accomplishing this great achievement has been nothing short of a life-changing journey. I would not be standing where I am today without everyone's support, sacrifices, and words of encouragement and inspiration.

I would first like to thank my advisor, Dr. Rebecca Atkinson, for her constant words of wisdom, encouragement, and being an aspiring role model. Dr. Atkinson is not only an outstanding scientist, I achieve to be one day, but also she is a great motivator and has constantly cheered me on through my Ph.D. journey. I know my questions seemed never ending, but thank you for never giving up on me and constantly staying patient with all my inquires. Thank you for your mentorship and support.

I would also like to thank the rest of my committee members, Dr. Amer AbuGhazaleh, Dr. Erin Venable, Dr. Clay Nielsen, and Dr. Buffy Ellsworth, for their support and guidance through my education and research projects.

This incredible journey would have been impossible without the help and support from my fellow graduate and undergraduate students. I would like to give a big thank you to Brandy Strohl, Jennifer McPherson, and Victoria Braner for their unwavering friendship and help during sampling, especially in -6° F weather at 3 A.M. I would also like to thank Darcie Hastings for her never-ending lab knowledge and always been a great friend when lab analysis never went the way it should have. You ladies have made my time her much more enjoyable and for that I will never be able to thank you enough.

Lastly, I would like to give a heart-felt thank you to my entire family, who has supported my nomadic lifestyle over the past years while I was in graduate school and made countless sacrifices for my success. I would like to thank my Dad for being my "words of wisdom" when

things got tough and my Mom for constantly giving me strength and compassion. I would also like to thank my fiancé, Jonathan, all the while serving our country in the United States Air Force. He supported my dreams, constantly provided words of encouragement, and never gave up on me. Thank you for allowing me to fulfill my dreams; for that I will always be grateful.

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CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Currently, the topic of alternative care, specifically dietary supplements and nutraceuticals, is extremely controversial. Dietary supplements are defined as an herb or other phytochemical, amino acid, vitamins, and minerals added into the diet (Mechanick, 2003). Nutraceuticals are defined as dietary supplements that contain a concentrated form of a bioactive substance, which is derived from food (Mechanick, 2003). In 1994, the United States Congress passed the Dietary Supplement Health and Education Act (DSHEA). The DSHEA promotes the use of dietary supplements and nutraceuticals based on their presumed safety and medicinal properties. However, there are no federal regulations and little scientific evidence on the health benefits and potential side effects of nutraceuticals. Due to an increasing push for new, safer alternative care, nutraceuticals are becoming a more popular choice, resulting in a need for safety and efficiency research to be conducted.

NUTRACEUTICALS

Pharmaceuticals have a high risk of toxicity and adverse side effects; because of this, there is push for alternative treatments in the form of food supplements. A nutraceutical, typically plant based, food source, which provides medical or health benefits including the prevention and treatment of a disease (Rajat et al., 2012). Stephen DeFelice, MD, the founder and chairman of the Foundation for Innovation in Medicine, coined the word “nutraceutical” in 1989, from the words “nutrition” and “pharmaceutical” (Rajat et al., 2012). However, the use of

food supplements to treat diseases dates back to Hippocrates, the father of medicine, (460-377 BC) when he predicted the health benefits of foods (Singh et al., 2011). Nutraceuticals are gaining popularity with health professionals and the public, since certain foods play an important role in maintaining normal functions in the human body without the risk of adverse side effects. Currently, there are over 470 nutraceuticals with documented health benefits (Singh et al., 2011; Rajat et al., 2012). Nutraceuticals are classified into two types, traditional foods and non-traditional foods. Traditional food is defined as natural, whole food with new information about potential health qualities. For example, omega-3 fatty acids, in salmon and other seafood, help reduce undesirable cholesterols. Non-traditional foods result from agriculture, crop and animal breeding or adding nutrients and ingredients to boost traditional food's nutritional value. Examples include orange juice that is fortified with calcium; milk fortified with vitamins; and crops fortified with vitamins, minerals, and omega-3 fatty acids. However, to date few focus directly on osteoarthritis.

Unlike pharmaceuticals, there are no FDA regulations for the health claims of nutraceuticals or non-traditional foods (Rajat et al., 2012). Even though there are few regulations on the health claims of nutraceuticals, safety must be assured in advance. Therefore, extensive, independent, testing must be conducted on a nutraceutical before health professionals recommend it to their patients. During the research process, nutraceuticals can be classified as potential or established nutraceuticals. Potential nutraceuticals provide a promising approach toward a particular health or medical benefit, while established nutraceuticals have multiple, independent, peer-reviewed, research reports backing up their claimed benefits (Sanghi et al., 2008; Singh et al., 2011).

Herbal medicine is increasing its popularity in veterinary medicine. Popularity may be due to low cost and a belief that there are minimal to no side effects. Herbal medicine is becoming a common treatment for mastitis occurrences, foot-and-mouth disease outbreaks, skin allergies, food poisonings, tympany, and expulsion of placentae. In the past, nutraceuticals were a common therapy for livestock in treating a variety of diseases including, hepatitis, chronic heart disease, skin disorders, wounds, and arthritis (Sanghi et al., 2008; Mahima et al., 2013). Some nutraceuticals affect the progression of arthritis by preventing degradation and enhancing the repair of joint cartilage (Sanghi et al., 2008).

TURMERIC

Turmeric is a rhizomatous herbaceous perennial plant, *Curcuma longa* Linn, belonging to the ginger family, *Zingiberaceae* (Chan et al., 2009). Turmeric is native to Southeast India and grows in temperatures between 20-30° C, with high amounts of rainfall. Once picked, typically in August, the rhizomes are boiled, dehydrated, and then ground into orange-yellow powder, which is used for curries, dyeing, and mustard condiments (Prasad et al., 2011). Turmeric, also known as *haldi,* is one of the oldest sources of spice, coloring pigments, and medicine, dating back to 1900 B.C. (Hassaninasab et al., 2010). In culinary, turmeric is used in many South and Southeast Asian dishes, typically in the powder form. Turmeric has also been a major part of Siddha medicine for over a thousand years as a remedy for stomach and liver ailments, healing sores, and has antimicrobial properties. Turmeric is said to help with a range of diseases and conditions including, skin, pulmonary, gastrointestinal, aches, pains, wounds, sprains, liver disorders, and cancer (Prasad et al., 2011). Turmeric's anti-inflammatory properties are said to come from an antioxidant, curcumin, specifically diferuloylmethane. In *in vitro* studies, curcumin was able to inhibit the production of cyclooxygenase-II enzymes, lipoxygenases,

prostaglandins, and nuclear factor-kappaß (NF-Kß), which are involved in the cascade of inflammation (Rosenbaum et al., 2010). According to the National Center for Complementary and Alternative Medicine, turmeric has little reliable evidence to support these claims due to the few studies that have been conducted (Esatbeyogula et al., 2012). Even though there have been over 3,000 curcumin related studies to date, most are *in vitro* and due to the poor bioavailability it is hard to extrapolate the results in an animal model (Belcaro et al., 2010).

Chemical Composition of Turmeric

Turmeric is comprised of protein (6.3%), fat (5.1%), minerals (3.5%), carbohydrates (69.4%), and moisture (13.1%) (Chattopadhyay et al., 2004). Essential oils can be collected from turmeric by steam distillation of the rhizomes in the amounts of: alpha-phellandrene (1%), sabinene (0.6%), cineol (1%), borneol (0.5%), zingiberene (25%), and sesquiterpines (53%) (Chattopadhyay et al., 2004). Curcumin, the active ingredient of turmeric (3-4%), was first isolated in 1815, by Roughley and again by Whiting in 1973. It was noted that turmeric's melting point is at 176° C and forms a reddish-brown salt with alkali, which is soluble in ethanol, alkali, ketone, acetic acid, and chloroform (Chattopadhyay et al., 2004).

Curcuminoids

The most important chemical component of turmeric is the group of active ingredients, curcuminoids. Curcuminoids consist of diferyloymethane (curcumin I), demthoxycurcumin (curcumin II), and bis-demthoxycurcumin (curcumin III), which are mostly seen in commercial supplements, structures listed below. In addition to this vital, active ingredient, turmeric also contains volatile oils, including tuermerone, atlantone, and zingiberene. Curcuminoids are natural phenols and give turmeric its yellow coloring. This group makes up roughly 2-6% of the spice, with curcumin, belonging to the diarylheptanoid group, as the main compound (Jagetia

and Aggarwal, 2007; Wynn and Fougere, 2008). Typical commercial products contain 77% curcumin, 17% demethoxycurcumin, and 3% bis-demethoxycurcumin. These curcuminoids are said to work synergistically and have a greater effect than if used alone (Wynn & Fougere, 2008). Commercial curcumin is often 95% curcumin, instead of 100%, because there is not an increase of bioavailability from 95% to 100%. However, the cost to manufacture 95% curcumin is less than 100% curcumin (Jagetia and Aggarwal, 2007; Wynn and Fougere, 2008).

Curcumin is known for its wide range of medicinal benefits, including anti-inflammatory, antioxidant, antimicrobial, wound healing, and anti-tumor properties (Zhu et al., 2014). In *in vivo* studies, it has been suggested that, despite the poor bioavailability, curcumin can cross the blood brain barrier, making it a potential treatment for neuro-inflammatory and neurodegenerative conditions in the central nervous system (Zhu et al., 2014). These properties are due to curcumin's chemical features and its ability to interact with signaling molecules (Zhu et al., 2014). In Ayurvedic medicine, turmeric is used as an anti-inflammatory and in Chinese medicine; it is used for stimulant, aspirant, carminative, astringent, detergent, and as a diuretic (Li et al., 2011). Curcumin has been used for thousands of years in Eastern medicine; however, the biological actions have been recently studied (Li et al., 2011). Throughout multiple studies on a variety of species, curcumin has potential for being a therapeutic agent for inflammatory diseases, including inflammatory bowel disease, pancreatitis, and arthritis. In clinical trials, it has been reported that curcumin may have an anti-cancer effect, as a chemoprevention agent (Li et al., 2011). Overall, curcumin and turmeric are considered relatively safe under daily consumption. However, in a human study, high dosages of curcumin over a period of time caused mild side effects, including nausea and diarrhea. In more recent studies, curcumin was

found to affect iron metabolism by chelating iron and suppressing the protein hepcidin, resulting in iron deficiency.

Numerous studies have been conducted on the medicinal properties of turmeric and its active ingredient, curcumin. However, little has been studied in regards to metabolic pathways. In 2010, Hassaninasab et al. identified a curcumin-converting enzyme in the cecum, CurA, a sub-strain of *Escherichia coli.* The bioavailability of curcumin is noted to be minimal due to it being hydrophobic, with low intrinsic activity, poor absorption, and a high rate of metabolism and elimination from the body (Anard et al., 2009). However, curcumin can be encapsulated into liposomes, liposomal-curcumin, to increase bioavailability (Li et al., 2007; Li et al., 2011). Liposomes can carry both hydrophobic and hydrophilic molecules, which makes them ideal for drug delivery (Anard et al., 2009). Turmeric and curcumin have been suggested to have numerous medicinal benefits and seem to have a relatively low risk of adverse side effects; however, it is vital to conduct research to identify to what degree of anti-inflammatory and antimicrobial properties they have, how safe they are to use, and the proper dosage for mammals, specifically canine and equine.

OSTEOARTHRITIS

Osteoarthritis is a disease that has been described for over a hundred years (Nelson et al., 2011). Currently there are about 27 million Americans diagnosed, but numbers are expected to reach 67 million by 2030 (Lawley et al., 2013). Osteoarthritis is the most common form of arthritis in humans, dogs, and horses. In almost every form of arthritis there is a loss of bone or cartilage that results in changes in the shape of joints (Lawley et al., 2013). Osteoarthritis, also known as degenerative joint disease (DJD), is a chronic inflammatory joint disease, which causes pain, soreness, stiffness, swelling, and lameness; due to the diminished cushion and changes in

the synovial fluid (Vaughn-Scott et al., 1997; Pasquini et al., 2007). Osteoarthritis affects the entire synovial joint, including cartilage, synovial fluid, and bone. This disease is characterized by degeneration of the cartilage and soft tissues, hypertrophy of bone at the margins, and changes in the synovial membrane (Vaughn-Scott et al., 1997; Pasquini et al., 2007). Mechanical stress is thought to induce changes in biochemical factors within affected joints, leading to articular cartilage degradation (Renberg, 2005). The disease process limits the amount of protein, released from the cartilage's cells, to repair cartilage in the joints; this is referred to as pitting and fraying of cartilage (Gupta et al., 2009; Gupta et al., 2011; Fleck et al., 2013; Lawley et al., 2013). Pitting and fraying results in the cartilage losing its elasticity and protective surface due to enzymatic cleavage of proteoglycans (Reid and Miller, 2008). As the cartilage continues to break down and deteriorate completely, it causes friction between the bones, which leads to inflammation, thickening of soft tissues, and loss of mobility of the joint (Reid and Miller, 2008). Trying to maintain its normal balance of injury and repair, as the cartilage wears away, the joint begin to lose its normal shape and the space between the joint narrow. Osteophytes (spurs) formation begins where the ligaments and joint capsule attach to the bone. In addition, fluid filled cysts form, and fragments of bone and cartilage can be found floating in the joint space (Gupta et al., 2009; Gupta et al., 2011; Fleck et al., 2013; Lawley et al., 2013). All of the changes to the joints and bones can cause pain, swelling, and the joint may even appear enlarged.

JOINT ANATOMY

Osteoarthritis has multiple causes and risk factors, however once the cartilage is lost, the joint fails (Erye et al., 2006). There are three different types of joints, fibrous, cartilaginous, and synovial. Fibrous and cartilaginous joints consist of fibrous tissue or hyaline cartilage, which allow little or no movement. Synovial joints are made up of synovial fluid and dense irregular

connective tissue, which creates a synovial joint capsule allowing the joints to freely move (Pasquini et al., 2007). The main focus will be on the synovial joint, especially the ball and socket (hip and shoulder) and hinge joints (elbow), because these types of joints are most commonly affected by osteoarthritis (Pasquini et al., 2007). The synovial fluid in the synovial joint capsule provides nutrients, lubrication, and a cushion for articular cartilage (Vaughn-Scott et al., 1997; Pasquini et al., 2007). Articular cartilage, which is composed of hyaline cartilage, is avascular tissue consisting of chondrocytes embedded within an extracellular matrix of collagens, proteoglycans, and non-collagenous proteins. Articular cartilage reduces friction and makes movement of the synovial joints less painful (Bos et al., 2010). The cartilage is 75% water and divided into four zones; superficial, middle, deep, and calcified zones (Tomiosso et al., 2005). Articular cartilage consists of three zones (I through III), which are delineated from the calcified cartilage (Zone IV) (Renberg, 2005). The tissue's material strength depends on the cross-linking of collagen and the zoning changes within tissue depth. Hyaline cartilage (50% cartilage, 35% proteoglycan, 10% other glycoproteins, and 5% other lipids and minerals) covers the subchondral bone and forms the articulating surface in the joint (Pasquini et al., 2007; Bos et al., 2010). The hyaline cartilage, which has a high content of collagen type II, serves as a shock absorber by distributing pressure from the load over the subchondral bone. In healthy joints, there is a fine balance between injury and repair amongst chondroblasts and chondroclasts (Gupta et al., 2009; Gupta et al., 2011; Fleck et al., 2013; Lawley et al., 2013). However, in osteoarthritis this balance is disrupted by an overproduction of osteoblasts that can cause pain and swelling. Early diagnosis of osteoarthritis is key to help prevent further damage and try to repair the damage already done.

Measuring Joint Mobility

Osteoarthritis patients struggle with limited range of motion (ROM), a reduction in the ability to move one's joints. Pain, stiffness, and swelling, all symptoms of osteoarthritis, can hinder mobility. Measuring the ROM can help identify what condition the articular surface, joint capsule, ligaments, and muscles, are in (Lin et al., 2013). Assessing the ROM is widely used in human medicine and is becoming more popular in canine and equine veterinary medicine, as more patients are being diagnosed with arthritis. Universal goniometry is a commonly preferred way to measure ROM in humans and other species (Ates et al., 2011). A goniometer is an affordable, reliable, commonly used, non-invasive tool used to measure flexion and extension degrees of joint mobility in the forelimbs and hind limbs in animals, as well as humans during physical therapy sessions. When using a goniometer, place the tool over the fulcrum of the joint, aligning the stationary arm with the stationary line of the body. Move the desired joint, either flexed or extend, and follow the moving line of the body with the moving arm of the goniometer; look at the readings on the goniometer for the degree of range of motion.

Erythrocyte Sedimentation Rate

The erythrocyte sedimentation rate (ESR) test, also known as the sed rate, sedimentation rate, and Autozero Westergren sedimentation rate, is a quick and simple test that has been used for many years to detect inflammation associated with infections, autoimmune diseases, and arthritis. A Polish pathologist, Edmund Biernacki, invented the ESR test in 1897. In 1918, two Swedish pathologists, Robert Sanno Fahraeus and Alf Vilhelm Albertsson Westergren used sodium citrate-anticoagulant specimens. This method of the test is widely used today and known as the Westergren method (Provet, 2014).

Due to the ESR test not being specific, it is used in addition to other blood tests, including C-reactive protein, antinuclear antibody (ANA), and rheumatoid factor. Typically,

ESR tests are ordered when a condition or disease is suspected to cause some form of chronic inflammation in the body. For example, people who suffer from arthritis may have an ESR test run to detect the amount of inflammation in the joints. ESR measures the rate at which red blood cells settle out over one hour. The test is performed with anti-coagulated blood, typically in an ethylenediaminetetraacetic acid (EDTA) tube that is mixed with a tube containing sodium citrate and then is placed in an upright 150 mm tube, also known as a Westergren tube. After an hour, the rate at which the red blood cells have fallen is reported in millimeters of plasma per hour (Blair Street Vet Hospital, 2014). The ESR test works by a precise balance of pro-sedimentation factors, specifically fibrinogen, and resisting sedimentation factors, such as the negative charge of erythrocytes. During a state of inflammation, the fibrinogen increases, causing the red blood cells to stick together in a stacked pattern known as rouleaux. The stacked erythrocytes are denser and cause the cells to settle faster than normal (Provet, 2014).

STAGES OF OSTEOARTHRITIS

Osteoarthritis is a progressive disease that consists of four stages. In stage one of osteoarthritis, minor bone spurs begin to develop. The cartilage matrix begins to break down due to chondrocyte's metabolism being affected and increasing the production of matrix destroying enzymes, matrix metalloproteinases (MMP). The severity of cartilage lesions can be correlated with the levels of collagenase present (MMP-1) (Reid and Miller, 2008). Cartilage lesions disrupt the function of cartilage, increasing friction and inflammation in the joints, resulting in pain. Stage two of osteoarthritis is considered the "mild" stage. This stage involves erosion of the bone due to the cartilage lesions. This can cause new bone growth, osteophytes, also called bone spurs, which affect normal joint movement. In this stage, proteoglycan and collagen fragments are released into the synovial fluid (Lawley et al., 2013). In the adult dog, proteoglycan turnover

is quicker (300 days) than estimated collagen turnover (120 years). Marked proteoglycan loss of articular cartilage is irreversible and results in joint degeneration (Renberg, 2005). Stage three is considered "moderate" osteoarthritis. The cartilage, in-between the bones, thins out and loses cushion. The space between the bones is also narrowing, causing grinding between the adjacent subchondral bones (Renberg, 2005). During stage three, symptoms are more severe and inflammation begins to occur. Production of synovial macrophage occurs, including MMP, cytokines (interleukin-1), and tumor necrosis factor-alpha (Renberg, 2005). Once the synovial macrophages are produced they can destroy tissues by diffusing back into the cartilage and can also stimulate chondrocytes. The fourth and final stage of osteoarthritis is considered "severe" osteoarthritis. In this stage the joint space is dramatically reduced, the cartilage is almost gone, and joint mobility is reduced greatly (Renberg, 2005).

TYPES OF OSTEOARTHRITIS

There are two types of osteoarthritis, primary and secondary. Primary osteoarthritis, also known as "wear and tear" is characterized by aging or normal wearing of the cartilage in the joint. This form of osteoarthritis is more commonly diagnosed. Secondary osteoarthritis is characterized by a specific cause, such as an injury, secondary issue from obesity, genetics, inactivity, or other diseases. An injury to a bone can cause an earlier onset of osteoarthritis. Obesity, and inactivity, which leads to obesity, can cause the joint to wear away faster due to extra pressure that is exerted on a joint (Vaughn-Scott et al., 1997). According to the Arthritis Foundation, for every pound gained, three pounds of pressure are added to the knees and six pounds of pressure are added to the hips (Vaughn-Scott et al., 1997). Despite the type of osteoarthritis, the treatment for both primary and secondary are the same (Vaughn-Scott et al., 1997).

TREATMENTS OF OSTEOARTHRITIS

When treating osteoarthritis, the main goals are to reduce pain and inflammation, improve joint function, eliminate or control the cause of arthritis, and even halt the process via surgery. Treatment can either occur through therapy or medication. Osteoarthritis is more common in overweight dogs; therefore, putting the dog on a strict diet to promote weight loss may result in a decrease in mechanical stress on the joints. By incorporating a weight loss program into the treatment plan, this can lower the amount of medication required. Along with strict dieting, a modified exercise plan should also be established for the dog. An exercise program can help in reducing weight while maintaining range of motion and muscle mass. Modified, low-impact exercises, such as walking or swimming, can also strengthen joint supporting structures, muscles, ligaments, tendons, and joint capsules (Vaughn-Scott et al., 1997). These forms of treatment can also be applied to other animal species and humans.

Non-Steroidal Anti-Inflammatory Agents (NSAIDs)

Pharmacological management of osteoarthritis includes steroidal or non-steroidal anti-inflammatory drugs (NSAIDs). These drugs do not address the underlying issue; they just control pain and inflammation. NSAIDs work against prostaglandins, a family of chemicals that are produced by cells and promote inflammation. During inflammation, proliferation of prostaglandins can result in pain, fever, and increased platelet clumping (Vaughn-Scott, et al., 1997). The cells that produce prostaglandins are called cyclooxygenase (COX). There are two forms of COX enzymes; COX-I enzymes produce prostaglandins that support platelet clumping and protect the stomach lining, and COX-II enzymes produce prostaglandins that are responsible for pain and inflammation. Since NSAIDs inhibit both forms of COX enzymes, NSAID usage

can result in gastrointestinal side effects, including ulceration, vomiting, anorexia, melena, and abdominal pain (Vaughn-Scott, et al., 1997).

Aspirin (acetylsalicylic acid) was the first NSAID to be used in modern medicine and still is widely used. Aspirin, despite its side effects, is commonly recommended in veterinary medicine for dogs that suffer from osteoarthritis, due to it being relatively inexpensive. However, studies have shown that aspirin can decrease chondrocyte production of collagen and proteoglycans and can enhance cartilage degradation over time (Vaughn-Scott, et al., 1997). Aspirin is also a unique NSAID, in the fact that it prolongs blood clotting for 4-7 days post-consumption. This makes in an ideal drug for preventing blood clots that can cause heart attacks and strokes (Vaughn-Scott, et al., 1997). However, excessive use can cause internal bleeding and decrease surgical recovery prognosis. Since there are many problems associated specifically with taking aspirin for osteoarthritis treatment, other NSAIDs are becoming more popular. The six most commonly used NSAIDs, prescribed by veterinarians, other than aspirin, for osteoarthritis patients, include Rimadyl™, Deramaxx™, Etogesic™, Metacam™, Zubrin™, and Previcox™ (Vaughn-Scott, et al., 1997).

Corticosteroids

Corticosteroids and glucocorticosteroids, often referred to as steroids, can be considered lifesaving and increase the quality of life (McDonald and Langston, 1995). Cortisone is a hormone that naturally occurs in the cortex of the adrenal gland. This is where the “cortico” prefix comes from. Corticosteroids are produced from the same chemical base that produces sex hormones (Jones and Dohetry, 1996). Cortisol is naturally produced when an animal gets stressed; however, man-made cortisol is 5-6 times stronger than naturally produced cortisol. Any production, natural or drug induced, of cortisol has a negative feedback and slows or stops

natural production. Suppression of naturally produced cortisol typically occurs within 12-48 h and takes a few days to start the process back up (Jones and Dohetry, 1996). Stopping the use of steroids quickly can result in a withdrawal syndrome, which includes fatigue, joint pain, stiffness, tenderness, and fever (, 2009).

Corticosteroids are the most used and misused, pharmaceutical in veterinary medicine (McDonald and Langston, 1995). Steroids, generally in an oral tablet, are used for stress response, immune system issues, inflammation, nutrient metabolism, and maintaining electrolyte levels in the blood (McDonald and Langston, 1995). Corticosteroids are a popular treatment plan for patients suffering from arthritis because they are extremely effective in relieving pain and inflammation (Fields, 2009). Steroids inhibit the production of arachidonic acid, which can stop the inflammation and stop the production of prostaglandins, similar to NSAIDs (Jones and Dohetry, 1996). However, when using steroids the body cannot separate the anti-inflammatory properties from the immunosuppressant properties (Jones and Dohetry, 1996). Therefore, low doses of steroids are used to suppress inflammation and high doses of steroids are used as immune-suppressants (McDonald and Langston, 1995). Since steroids affect nearly all cells of the body, their benefits are widespread; however, their side effects can be long lasting and devastating (Jones and Dohetry, 1996). The side effects, which vary depending on the dose and duration of steroid use, include sore mouth, weight gain, osteoporosis, high blood sugar levels (diabetes), cataracts, insomnia, gastrointestinal bleeding and ulcers, suppressed immune system, fluid retention, atherosclerosis resulting in increased risk of heart disease, and aseptic necrosis. To reduce the probability of side effects from steroid use, one must avoid using steroids on a daily basis and no longer than 3-4 months without re-evaluating organ functions. Due to the devastating side effects of steroid use, alternative medicine such as acupuncture, nutraceuticals,

and physical therapy are becoming more popular as treatments for osteoarthritis, especially in veterinary medicine.

Glucosamine

As the body ages, the production of glucosamine slows down; therefore, it is important to supplement glucosamine to avoid joint issues (Narvy et al., 2010). Glucosamine (2-amino-2-deoxy-D glucose), the most abundant monosaccharide, is a naturally occurring compound composed of sugar and amino acids. Glucosamine has been used for nearly 40 years in human medicine (Narvy et al., 2010). Glucosamine supplements are extracted from crustacean exoskeletons or from fermentation of grains such as corn or wheat (Narvy et al., 2010). It is strictly used as a dietary supplement in the United States, but is a regulated pharmaceutical throughout Europe (Simoens and Laekeman, 2010). There are three different types of glucosamine; glucosamine sulfate, glucosamine hydrochloride, and N-acetyl-glucosamine. However, glucosamine sulfate may be more effective for arthritis treatment because sulfate is needed to produce cartilage and the other two forms of glucosamine do not contain sulfates (Narvy et al., 2010). Glucosamine supplements are often combined with chondroitin sulfate. Chondroitin sulfate addresses the disease process of arthritis by aiding in the repair of damaged connective tissue. It is also beneficial to stress injuries, by keeping joints hydrated and helps protect existing cartilage breakdown (Irsay et al., 2010).

Glucosamine is one of the most commonly used nutraceuticals, especially for arthritic patients, due to it being involved in the body's production of joint lubrication, shock absorption, and maintaining healthy cartilage and joint function (Narvy et al., 2010). Glucosamine is the precursor in the biochemical synthesis of glycosylated proteins and lipids, glycosaminoglycans. Glycosaminoglycans are a major component of joint cartilage and the extracellular matrix of

articular cartilage (Narvy et al., 2010). Glucosamine also aids in the rebuilding of damaged cartilage and is a building block for articular cartilage (Narvy et al., 2010). Glucosamine has anti-inflammatory properties by inhibiting synthesis of degradation enzymes, increasing synthesis of extracellular matrix, and reduces apoptosis of articular chondrocytes (Narvy et al., 2010). Glucosamine is also good for nail growth, tendons, skin, eyes, synovial fluid, ligaments, heart valves, and mucous secretions of the digestive, respiratory, and urinary tract (Irsay et al., 2010). Glucosamine supplements have little to no side effects when used at the recommended dose; however, if taken above the recommended dose, it can cause damage to pancreatic cells and increase the risk of diabetes. Short-term side effects of glucosamine include stomach upset, constipation, diarrhea, headaches, and rashes (Simoens and Laekeman, 2010). In recent years, in a series of preliminary experiments, researchers have evaluated several nutraceuticals, individually and in combination, with several other supplements, and found that they are significantly effective in ameliorating arthritic pain (Gupta et al., 2009; Gupta et al., 2011).

OSTEOARTHRITIS IN DOGS

Osteoarthritis is the most common type of arthritis in dogs and is the most common source of chronic pain in older dogs (Vaughn-Scott, et al., 1997). This is due to the constant wearing away of the cartilage from dogs running, jumping, and other strenuous exercise. Arthritis commonly affects large breed dogs, i.e. German Shepherds, Labradors Retrievers, Siberian Huskies, and Rottweilers, more than small breed dogs. Prevalence of osteoarthritis can be as high as 20% in dogs more than a year old, with middle-aged and older dogs being at higher risk. Dogs that are diagnosed with arthritis tend to be lethargic, have difficulty moving from a sitting or lying position, cracking joints, stiffness, muscle wastage, and visible pain (Gupta et al., 2009; Gupta et al., 2011). Diagnosing osteoarthritis in dogs begins with owners observing the

pain and stiffness while the animal is running, walking, jumping, or rising from a lying or sitting position. Radiographic evidence, patient symptoms, and osteoarthritis risk factors, such as age, gender, and body mass index, can all aid in predicting the risk of rapid, highly predictable joint degradation (Gupta et al., 2011). During physical examinations, the patient may show signs of pain, including whining, biting, or trying to move away. Radiographs can show the breaking down of cartilage between bones and narrowing joint space. Domestic species including cats and dogs can be diagnosed for arthritis via ultrasounds. In addition, a multitude of blood tests can be used to determine the degree of inflammation in the joints from arthritis, aiding in the diagnosis. One test used to assess inflammation is the erythrocyte sedimentation rate test along with complete blood counts and chemistry panels. By properly diagnosing patients with osteoarthritis, this will help establish a future plan to help ease pain, prevent further damage, and overall increase the quality of life.

Along with osteoarthritis, dogs may also suffer from hip dysplasia, a form of osteoarthritis present in the ball and socket joints. Hip dysplasia is an inherited condition from improperly formed hip joints typically seen in large breed dogs (WebMD, 2013). Dogs that suffer from inherited hip dysplasia, show signs within the first year and should be spayed or neutered to avoid passing this genetic tendency of malformation to offspring. Bulldogs, St. Bernard's, Blood Hounds, and Boykin Spaniels are a few examples of breeds that are at a higher risk factor for developing hip dysplasia. Dogs can also be at risk for hip dysplasia if there is excessive weight gain during the early stages of growth, typically 3-8 months of age, and from putting excessive pressure on the hip joint from strenuous exercise. Hip dysplasia is caused from an abnormal development of the hip joint, leading to excess laxity in the hip joint. Laxity in the hip joint can cause stretching of the supporting ligaments, joint capsules, and surrounding

muscles, leading to permanent damage to the anatomy of the hip joint. The permanent damage to the anatomy causes the poorly developed head of the femur to loosely fit into a shallow acetabulum (WebMD, 2013). OFA, Orthopedic Foundation for Animals, radiographs can also be performed to diagnose hip dysplasia. According to the Orthopedic Foundation for Animals, OFA radiographs must be performed with the animal in dorsal recumbancy with rear limbs extended parallel. The stifles are rotated inward and the pelvis is symmetric. This type of radiograph allows veterinarians to assess how the femoral head fits into the acetabulum, which is the diagnosis of hip dysplasia (WebMD, 2013).

OSTEOARTHRITIS IN HORSES

Musculoskeletal diseases, including osteoarthritis, affect horses the same way it affects humans and dogs. Osteoarthritis is a degenerative, career-compromising disease in horses and is responsible for 60% of lameness in performance and pleasure horses (Frisbie et al., 2002). Osteoarthritis can be emotionally and financially draining for the horse's owner. Therefore, finding a safe, effective, and economically sound treatment is vital for horse owners and their horses.

Horses can get osteoarthritis by two means, normal forces on damaged cartilage or damaging force on normal cartilage (Farinacci et al., 2009). Horses need to keep up with physical demand, which can lead to abnormal forces, including heavy athletic activity resulting in loss of joint or limb stability. The most common joints susceptible for osteoarthritis are the knee, fetlock, coffin, pastern, and hock (Todhunter and Lust, 1990). If a horse has osteoarthritis, their symptoms are commonly very subtle and non-specific. These symptoms, include spending more time laying down, difficulties getting up, lethargy, behavior changes, slow or stiffness, abnormal gait, swollen joints, decreased appetite, and unexplained muscle wastage (Todhunter

and Lust, 1990). When diagnosing osteoarthritis in a horse, a veterinarian must perform a clinical examination to evaluate the horse's lameness and locate which joint/s are affected. Radiographs are important when diagnosing osteoarthritis because they can help eliminate the possible presence of fractures and bony prominences. However, radiographs are limiting in the fact that they cannot identify early stages of osteoarthritis (McIlwraith, 2003). Nuclear imaging is becoming more popular in diagnosing osteoarthritis because it is very sensitive, but has poor specificity. In addition to nuclear imaging, computed tomography (CT) and MRIs are being used to show early stages of osteoarthritis by looking at subchondral bone changes (McIlwraith, 2003). Synovial fluid samples can also be tested to evaluate the amount of inflammation in a joint. In addition to these diagnostic tools, recent studies have identified biomarkers that can detect early degradation of proteoglycans and collagen (the early stages of osteoarthritis) (McIlwraith, 2003). Currently, biomarkers are up to 90% accurate.

Once a horse has been diagnosed osteoarthritic, there are two different approaches for managing the disease; (1) return the joint to its normal healthy state as quickly as possible (2) prevent the recurrence or reduce the severity of the arthritis (Todhunter and Lust, 1990). The principle factor at the top of the inflammatory cascade in horses is interleukin-1 (IL-1), a deleterious cytokine, or inflammatory mediator (Farinacci et al., 2009). To slow down the inflammation process non-steroidal anti-inflammatory agents, such as phenylbutazone (Bute) and flunixin meglumine are commonly used. However, due to the gastrointestinal side effects, firocoxib (Equioxx), a specific COX-II inhibitor, is gaining favor (Farinacci et al., 2009). Horses can also receive intra-articular corticosteroids, such as methylprednisolone acetate (Depo-Medrol), triamcinolone acetonide (Vetalog), and betamethasone esters (Celestone). These drugs are the most potent in treating osteoarthritis, but due to adverse side effects, such as

cardiovascular disease, allergies, dermatitis, gastrointestinal upset, and musculoskeletal weakening, they are less preferred. There are also other treatments for acute inflammation and joint injuries, which can be used on horses suffering from osteoarthritis, including autologous conditioned serum (interleukin-1 receptor antagonist protein- IRAP), and platelet-right plasma (Farinacci et al., 2009). Oral nutraceuticals are gaining popularity, although still controversial due to lack of evidence. Many *in vitro* studies have been conducted on glucosamine and chondroitin sulfate, but no studies have looked at the safety and therapeutic effects from oral joint supplements (McIlwraith, 2003). Due to the lack of scientific evidence and FDA approval for oral nutraceuticals, there is little information on dosage, side effects, and health benefits.

EQUINE GASTROINTESTINAL HEALTH

In addition to owners commonly using supplements and switching from pharmaceuticals to nutraceuticals in canines, it is also becoming popular in the equine industry. Horse owners are beginning to use supplements for arthritis in riding horses and performance horses as well as supplementing to help avoid gastrointestinal diseases and colic. Gastrointestinal disorders, including diarrhea, lesions, enterocolitis, bloat, and colic are very common and can cause physiological consequences including death in horses. By supplementing a nutraceutical that can suppress proliferation of opportunistic and pathogenic bacteria and control inflammation, equine caretakers and owners can help their horses with a majority of disorders that horses commonly suffer from.

Horse Hindgut Overview

Horses are monogastric animals with a relatively small stomach. From the horse's mouth to their large intestine their gastrointestinal tract is similar to that of a human's. However, past the cecum, a horse's gastrointestinal tract has more similarities to a cow's (Hansen et al., 2014).

A horse's gastrointestinal tract can be divided into three segments: foregut, midgut, and hindgut (Fraga et al., 2012). The foregut consists of the esophagus and stomach. Once food has passed through the stomach, it enters the small intestine (midgut), duodenum, jejunum, and ileum, which join the hindgut, cecum, colon, and rectum, at the ilocecal junction. The small intestine and stomach can receive a continuous flow of food (Hansen et al., 2014). The cecum is a large fermentation vat located on the right side of the animal. Carbohydrates fermented by fibrolytic bacteria produce volatile fatty acids (VFAs), which account for 60-70% of the their energy. The volatile fatty acids that are yielded during fermentation include acetate, a source of energy for tissues, propionate, a precursor for gluconeogenesis, and butyrate, a source of energy for colonocytes and helps regulate differentiation of gut epithelia (Hoffman, 2009; Milinovich et al., 2010; Costa et al., 2012).

In modern management practices, horse owners and caretakers do not let horses graze like they naturally should. In result, caretakers and owners substitute the horse's diet with grains and fats, which the horse cannot properly digest. This unbalanced feeding regimen causes numerous digestive disturbances (Hansen et al., 2014).

Horses are classified as hindgut fermenters, a balance of beneficial and harmful bacteria aid in the digestion of foodstuff in the cecum and large intestine (Costa et al., 2012). The hindgut is not only a fermentation vat, but it also stimulates immune responses, protects against pathogens, production and neutralization of toxins, and gene expression in host epithelial tissues (Milinovich et al., 2010). The cecal microbiome is extremely sensitive and can be affected by factors like gastrointestinal disease and dietary changes, which can lead to systemic consequences and even death (Costa et al., 2012). Therefore, a healthy and balanced microbiota is vital for the overall wellbeing of the animal. By understanding external factors and how they

affect the gut microbiota, this could help in diagnosing medical conditions and provide better treatment and prognosis of gastrointestinal diseases resulting in colic.

EQUINE HINDGUT MICROBIOME

Gastrointestinal microbiota play an essential role not only in digestion, but also in colonic disease (Marteau et al., 2001). Gut microbiome is one of the densest, most dynamic, and complex microorganism populations located in the body (Costa et al., 2012). Gut microbes act against transient pathogens, aid in digestion and absorption, stimulate the immune system, and support enterocytes (Suchodoiski et al., 2012). Gut microbiome population differs between species, individuals, and organs (Fraga et al., 2011). It is noted that there are one billion microbes within one drop of cecal fluid, consisting of anaerobic microorganisms such as bacteria, fungi, protozoa, and archaea (Fraga et al., 2012). If these microbes are changed, this could result in gastrointestinal disease and even death. *Clostridium perfringens, Clostridium difficile, Escherichia coli* general and K-12, and *Streptococcus bovis/equinus* complex (SBEC) are common bacteria found in the microbiome of the hindgut. These strains are considered opportunistic bacteria, and if the immune system becomes compromised by changes to the hindgut microbiome, this will trigger proliferation of harmful and opportunistic bacteria that can cause numerous gastrointestinal diseases.

The role of the microbes during digestion is fermenting carbohydrates and turning them into VFAs (two carbons: acetic, three carbons: propionic, four carbons: butyric, and five carbons: valeric). Microbes can digest alpha glucose in the form of starch and beta glucose from crude fiber from plant cell walls. When starch is digested by amylase, it is broken down to dextrin, then maltose, which is made up of two glucose molecules. Crude fiber is made up of cellulose, hemicellulose, and lignin. Cellulose is digested by the microbes, and broken down to insoluble

fiber, and produce volatile fatty acids, as a byproduct. Hindgut microbes in the horse only produce energy and vitamin B12, while microbes in cattle manufacture B-complex vitamins and vitamin K. In general, there are five major types of microbes; cellulolytic bacteria that digest fiber, proteolytic bacteria that break down protein, lactic acid-producing bacteria that digest starch, protozoa that produce volatile fatty acids, fungi/yeast that breaks down fiber, and other bacteria that produce B12-vitamin (Hussein et al., 2004). These five types of microbes are found throughout the gastrointestinal tract, but prefer the neutral pH environment of the cecum and colon; with cellulolytic bacteria being most abundant in the cecum and colon because that is the primary location of fiber digestion (Hussein et al., 2004).

In literature, gastrointestinal microbiota in general has been the most studied microbiota; however, equine microbiota has not been extensively studied (Fraga et al., 2012). In the past, equine studies have looked at the microbiota changes during laminitis, identifying and detecting lactic acid bacteria (LAB), and bacterial changes during equine grass sickness and foal heat diarrhea (Daly et al., 2001). A majority of these studies used fecal samples due to the ease of sampling. However, no studies have been conducted on identifying the relationship, short-term and long-term, between the cecal and the fecal microbiome in living horses (Daly et al., 2001). Even though similar studies have been conducted on humans, dogs, and cattle, data cannot be extrapolated to horses due to differences in anatomy, functions, and microbiome composition (Schoster et al., 2013). Therefore, only superficial knowledge exists on the equine hindgut microbiome due to culturing limitations when identifying bacteria. However, with new technological advances and next generation sequencing, scientists can now achieve great strides in identifying bacteria down to the genus and species level.

Clostridium perfringens

Clostridium perfringens, formerly known as *C. welchii* or *Bacillus welchii*, is a gram-positive, rod-shaped, anaerobic, spore forming bacterium. The first association *C. perfringens* had with gastrointestinal disease was in the 1920s (Songer, 1996). The next case was post-World War 1, in Germany, in the 1940s, when it caused gangrene of the bowel, enteritis necroticans. Since then, *C. perfringens* has been the most commonly associated with gas gangrene (Lawrence et al., 1997). In 1950, there was a confirmed food poisoning case that linked back to *C. perfringens* (McDonel, 1986). It was not until the late 1970s that there was a correlation made between equine enteric disease and *C. perfringens*. However, it was not extensively studied until 1977, when a connection was made between high levels of *C. perfringens* type A in the feces of racehorses suffering from colitis in comparison to the lower levels detected in healthy horses (Borriello, 1995). Currently, *C. perfringens* is associated with causing severe colitis in horses, yet can sometimes be ingested without causing any harm. Therefore, it is vital to understand what type of strain and toxins are causing gastrointestinal diseases and how to control and prevent them.

C. perfringens, Bacteria (Domain), Firmicutes (Phylum), Clostridia (Class), Clostridiales (Order), Clostridiaceae (Family), Clostridium (Genus), *C. perfringens* (Species), is found in the intestinal tract as well as decaying vegetation, marine sediment, and soil (Herholz et al., 1999). This bacterium is a mesophile with optimum growing temperatures at 37° C. It is non-motile, but has the ability to produce endospores in a short generation time of 6.3 min. *C. perfringens* has a protective thick cell wall made up of peptidoglycans and is a single circular chromosome, containing 10 rRNA genes and 96 tRNA genes, made up of approximately 3.6 million base pairs, with a guanine-cytosine content ranging from 24-55% (CDC, 2014). Similar to *Mycoplasma spp.* and *Bacillus subtills*, *C. perfringens*' genes are arranged in a specific way that their

transcriptional process orients the same directions as their replication direction (CDC, 2014). *C. perfringens* requires essential amino acids from the environment due to its inability to perform its own amino acid biosynthesis. However, it can perform anaerobic respiration using nitrates, allowing an increased yield of energy. *C. perfringens,* thrives in little to no oxygen in the environment; therefore, it can perform anaerobic fermentation to produce gases such as carbon dioxide, to create an anaerobic environment that is optimal for the bacterium to grow and survive. The bacterium can carry out glycolysis and glycogenolysis, utilizing simple sugars such as glucose. The primary end products of *C. perfringens'* metabolisms are ethanol, lactate, acetate, butyrate, and carbon dioxide.

C. perfringens is the most common cause of food borne illness in the United States, with a million cases each year (CDC, 2014). *C. perfringens* is able to produce up to 15 different toxins, making it versatile. These toxins are used to isolate the five different types of *C. perfringens*: type A, B, C, D, and E. The four toxins that are primarily used to isolate the different types include alpha, beta, epsilon, and iota-toxins. Type A is the most common and most variable, and subdivided into enterotoxigenic and non-enterotoxigenic strains (Herholz et al., 1999). Enterotoxigenic type A and C are associated with equine enterocolitis, gas gangrene, infections, avian and canine necrotic enteritis, colitis in horses, and diarrhea in pigs (Divers and Ball, 1996). Types B, C, D, and E can cause severe enteritis, dysentery, toxemia, and high mortality rates in young lambs, calves, pigs, and foals. Types B, C, D, and E have been intermittently associated with foal enterocolitis, and equine antibiotic associated diarrhea (Divers and Ball, 1996). Even though the alpha toxin is noted to be relatively nonpathogenic, the beta2 toxin plays a significant role in digestive disease, specifically, enterocolitis in equine (Herholz et al., 1999). This is mainly due to the *C. perfringens* enterotoxin (CPE); the main virulence factor

that initiates many critical gastrointestinal diseases across species (Herholz et al. 1999). CPE works in a four-step mechanism against membrane action (CDC, 2014). First, CPE binds to the target receptors on plasma membrane protein or claudin protein, which leads to the formation of a small complex. This changes the anatomical structure of the intestinal tissue due to binding to claudins, proteins that maintain tight junction integrity and establishment of paracellular barriers. These barriers control the flow of molecules between the cells of the gastric epithelium (Herholz et al. 1999; CDC, 2014). Secondly, the complex undergoes physical changes when it binds to other membrane proteins, forming a larger complex in the membrane. Thirdly, this results in the disruption of the membrane's permeability, leading to cell death due to the osmotic equilibrium not being maintained (CDC, 2014). As the CPE in the intestinal lumen increases, more deaths of pathways are triggered (Chakrabarti et al., 2003). Lastly, the CPE is capable of forming a larger complex in the membrane and its toxic levels are enhanced when the first 45-N terminal amino acids are eliminated (Herholz et al., 1999). This contributes to intestinal fluid and electrolytes being lost through diarrhea (McClane, 2000). It has also been noted that high levels of CPE can have a pro-inflammatory effect, which can worsen the diarrhea symptoms (Chakrabarti et al., 2003). In a recent study, *C. perfringens* enterotoxins were detected in 19% of adult horses and 28.6% of foals with diarrhea symptoms, in contrast to not being detected in healthy horses (Herholz et al., 1999). Due to the increased interest of horse enterocolitis, CPE, and beta2 toxins, many studies have looked at *C. perfringens* enterotoxin in horses with diarrhea; yet it needs to be further investigated to identify types, strains, toxins, and how to prevent related gastrointestinal diseases.

Clostridium difficile

Clostridium difficile, formerly named *Bacillus difficilis*, due to how difficult it was to isolate and cultivate was first isolated from newborn infants in 1935 (Hall and O'Toole, 1935). It was not until 1970, that a correlation was identified between *C. difficile* and humans with colitis (Ehrich et al., 1984). The first time *C. difficile* was identified in mature horses, located in the Potomac River area, with diarrhea was in 1984. Cases of *C. difficile* colitis in horses treated with antimicrobials increased in 1993. Since then, many studies have examined horses with diarrhea associated with the presence of *C. difficile* (Baverud et al., 1997).

C. difficile, Bacteria (Domain), Firmicutes (Phylum), Clostridia (Class), Clostridiales (Order), Clostridiaceae (Family), Clostridium (Genus), *C. difficile* (Species), is a large gram-positive, anaerobic, spore-forming, motile, rod bacteria. *C. difficile* is associated with colitis and diarrhea, especially in horses (Divers and Ball, 1996). This bacterium requires five amino acids for energy metabolism, (leucine, lysine, proline, tryptophan, and valine) and an addition of glycine has been shown to increase growth. To generate energy in the form of ATP, *C. difficile* utilizes amino acid fermentation and simple sugars such as glucose (Kim et al., 1981). The primary fermentation end product of *C. difficile* is acetic, iso-butyric, iso-valeric, valeric, and iso-carproic acid.

C. difficile is one of the top three most common bacteria linked to diarrhea. In the United States it contributes to 14,000 human deaths each year and contributes to 20-30% of acute diarrhea cases in equine (CDC, 2014). *C. difficile* is also directly linked to equine gastrointestinal inflammatory diseases, such as enterocolitis. *C. difficile* produces protein toxins A, B, and/or binary toxin CDT in the intestine (Divers and Ball, 1996). Protein toxin A is an enterotoxin that causes hyper section of the fluid into the intestinal lumen and can cause tissue damage. Protein toxin B is a potent cytotoxin that induces inflammation and necrosis. Lastly, protein toxin CDT

is still relatively unstudied and little is known about it (Divers and Ball, 1996). Typically, *C. difficile* has a low transient level in healthy horses. Due to the opportunistic properties of *C. difficile*, when the healthy microflora are compromised, this allows the *C. difficile* spores to travel down the gastrointestinal tract without being affected by the gastric acid barrier; resulting in rapid multiplying of the bacterium in the colon. This overgrow of bacteria in the intestines is a precursor for many gastrointestinal diseases (Divers and Ball, 1996). Compromised immune systems can from antineoplastic, immunosuppressive, and antimicrobial treatments or stress. In horses, stress can be caused by dietary changes, including the addition of a new supplement, environmental changes, transportation, starvation, surgery, and other medical treatments (Divers and Ball, 1996). In humans, the pathogenesis is fecal-oral route; however, in horses it has still yet to be identified. Clinical symptoms of *C. difficile* can vary, but may include abdominal pain, diarrhea with or without blood, abdominal distention, dehydration, toxemia, and shock (Divers and Ball, 1996). If an outbreak occurs, proper isolation and disinfecting with sporicidal disinfectants are ideal.

Identifying and culturing *C. difficile* is difficult because the toxin type must also be identified. In 1979, a medium called cycloserine-cefoxitin-fructose agar (CCFA)-selective was developed. Once fecal samples were collected, they were streaked onto selective and differential medium to help identify toxin types. Also, different additives were tested for culturing *C. difficile*, including horse serum, sodium taurocholate, and media with mannitol replacing fructose (Dezfulian et al., 1981). Further modifications have been made for cycloserine and cefoxitin for an ideal media. *C. difficile* can also be tested by cytotoxicity assays, ELISA tests, and microwell enzyme immunoassays (EIA) that are toxin specific, for fecal samples (CDC, 2014). Lastly, anaerobic cultures can be tested by PCR to determine if it is a toxigenic or a non-toxigenic strain

(Divers and Ball, 1996). In horses, it is important to test for the presence, but also the type of toxin. Due to the numerous tests for identifying toxins along with the variation in degree to which toxins are produced between equine isolates, it is important to take multiple samples over time to avoid false negatives in horses (Bårerud et al., 2003). Overall, horses have some level of *C. difficile* in their hindgut (0%-7.59%), depending on the study, and are considered carriers; therefore, it is important to be mindful of these percentages when analyzing a microbial sample (Bårerud et al., 2003).

Escherichia coli

Escherichia coli is the most prevalent infecting organism in the family of gram-negative bacteria known as enterobacteriaceae. *E. coli* was first discovered in the human colon in 1885, by German bacteriologist, Theodor Escherich. Escherich also showed that certain strains of *E. coli* were associated with infant diarrhea and gastroenteritis. *E. coli* was initially named *bacterium coli*, but was later changed to *Escherichia coli* in honor of its discoverer (CDC, 2014; EPA, 2014). In the 1960s and 1970s, mass amounts of information were discovered about *E. coli.* The need for information about this bacterium came from the affordable and quick methods that became available to identify enteric bacteria and the major shift in nosocomial infections from gram-positive to gram-negative (EPA, 2014). *E.coli* is referred to as the best and most-studied, free-living organism and is noted to have over 700 serotypes identified (Bertone et al., 1990). By studying the "O" and "H" antigens on the bacteria and the flagella, scientists can help distinguish between the different serotypes (Bertone et al., 1990).

E. coli, Bacteria (Domain), Proteobacteria (Phylum), Gammaproteobacteria (Class), Enterobacteriales (Order), Enterobacteriaceae (Family), Escherichia (Genus), coli (Species), is a gram-negative, facultative anaerobic, rod-shaped bacterium, with optimum growing temperatures

at 37° C. This bacterium is commonly found in the lower intestines of warm-blooded animals. *E. coli* makes up about 0.1% of gut microbes and most strains are harmless. Some strains are part of the normal gut microbiome, produce vitamin K_2, and prevent colonization of the intestine with pathogenic bacteria (CDC, 2014). *E. coli* makes ATP by aerobic respiration, if oxygen is present, but can switch to fermentation or anaerobic respiration if oxygen is limited or absent. The end product of fermentation is lactate, succinate, ethanol, acetate, and carbon dioxide (CDC, 2014; EPA, 2014).

Even though *E. coli* normally lives in the intestines, and most strains are harmless, some strains can cause diarrhea. This bacterium is also responsible for numerous reports of contaminated food and beverages (Bertone et al., 1990). The most widely known strain, *E. coli* 0157:H7, produces a toxin called shiga toxin, which is identical to the *shigella dysenteria* type 1 bacteria. *E. coli* 0157:H7 is known for causing over 100,000 illnesses, 3,000 hospitalizations, and 90 deaths, annually, in the United States (CDC, 2014). The incubation period is, typically, 3-4 days, but can range anywhere from one to ten days. Once inoculated with the bacterium, it rapidly multiplies in the large intestine and then binds tightly to cells in the intestinal lining. From there, it attaches to receptors on white blood cells and is transferred all over, resulting in inflammation due to hemorrhagic colitis with abdominal pains, severe cramps, and diarrhea (Bertone et al., 1990). Rarely, *E. coli* can cause bowel necrosis and perforation without progressing to hemolytic uremic syndrome (HUS). In humans, the pathogenesis of *E. coli* is fecal-oral route (CDC, 2014).

One of the many serotypes of *E. coli*, is *E.coli* K-12. *E. coli* K-12 is a descendant isolate used commonly in molecular biology as a model organism and in broths. *E. coli* K-12 was first isolated in 1920, by the Lister Institute in London. In 1922, at Stanford University, the strain was

isolated from a stool sample from a patient with diphtheria (CDC, 2014). Charles E. Clifton, in the 1940s, used *E. coli* K-12 to study nitrogen metabolism, which then deposited it in the ATCC and lent it to Edward Tatum for his study in tryptophan biosynthesis (CDC, 2014). Different strains of K-12 have developed by treating *E. coli* K-12 with agents such as nitrogen, mustard, ultra-violet radiation, and x-rays (Bertone et al., 1990). Currently, a study showed that curcumin-converting microorganisms were isolated from human feces and had a high activity level to *E. coli*, specifically *E. coli* K-12, substrain DH10B (Hassaniansab et al., 2010). In the study, researchers observed that *E. coli* was able to act on curcumin by using a two-step reduction process. Curcumin was being converted, NADPH-dependently, into an intermediate product, dihydrocurcumin, and then the end product, tetrahydrocurcumin (Hassaniansab et al., 2010). The "NADPH-dependent curcumin/dihydrocurcumin reductase" was called CurA (Hassaniansab et al., 2010). Due to its recent discovery in humans, little is known about CurA, as a whole, and in other species.

Streptococcus bovis/equinus complex

Streptoccocus bovis/equinus complex (SBEC) is a heterogeneous group within the Lancefield group D streptococci. The genus, *Streptococcus*, is gram-positive, aerobic cocci, lactic acid bacterium (LAB) that belongs to the phylum Firmicutes (Hastie et al., 2008). Most *Streptococcus* genomes are 1.8-2.3 megabase pairs in sizes and can encode 1,700 to 2,300 proteins. In 1984, *Streptococcus* was split into two genera, *Enterococcus and Lactococcus* and can be found in the microbiomes of the mouth, skin, intestine, and upper respiratory tract. Different species of *Streptococcus* can be classified by their hemolytic properties on blood agar: alpha-hemolytic, green hemolysis zones; beta-hemolytic, clear hemolysis zones; gamma-hemolytic, no hemolysis zones (Hastie et al., 2008). Alpha-hemolytic species such as *S.*

pneuomniae and *S. virdans*, cause oxidation of iron within red blood cells. Beta-hemolytic species completely rupture red blood cells. Lastly, gamma-hemolytic species cause no hemolysis. SBEC is classified as a Lancefield group D (*enterococci)* beta-hemolytic species, which can cause many infections in species such as cattle and horses.

Streptococci have been divided into six groups based on their 16S rDNA sequence, which is why *Streptococcus bovis* and *Streptococcus equinus* are considered a complex; with only 15 base pairs different, most labs will report them as a group, while other labs will classify them down to their subspecies level by conducting a follow-up fermentation study (Jans et al., 2011). However, the subspecies in this complex differ in their microbiology, pathogenesis, and epidemiology (Jans et al., 2011). Some of the species in this complex are pathogenic and can have detrimental tolls on the fermentation process of the equine hindgut and cause lesions in the colon (Jans et al., 2011). SBEC is also known for diseases, such as meningitis, neonatal sepsis, peritonitis, ruminal acidosis, feedlot bloat, septic arthritis, and vertebral osteomyelitis (Jans et al., 2011). The diseases caused by *Streptococci* are heightened by their virulence factors, including streptolysin, DNAases, and hyaluronidase (Hastie et al., 2008). Some strains also release exotoxins that activate T-cells, which trigger the release of cytokines. The released cytokines activate detrimental physiological processes, such as coagulation, inflammation, shock, organ failure, and death (Hastie et al., 2008).

In hindgut fermenters, such as horses, a diet high in starch or sugar can promote proliferation of SBEC. As a lactic acid bacterium, the fermentation of these carbohydrates to lactic acid can cause a decrease in pH, which can lead to acidosis, bloat, starch-induced colic, and other gastrointestinal conditions. Therefore, it is important to manage a horse's diet, but to also control the concentration of SBEC in the hindgut.

CONCLUSION

In summary, nutraceuticals, especially turmeric and its active ingredient, curcumin, are increasing in popularity in veterinary medicine due to it being relatively inexpensive and minimal to no side effects. Turmeric is used as an anti-inflammatory, stimulant, aspirant, carminative, astringent, detergent, and diuretic (Li et al., 2011). Curcumin, the major component and active ingredient of turmeric, has been used for thousands of years in Eastern medicine. However, only recently have the biological actions of curcumin been examined (Jagetia and Aggarwal, 2007; Wynn and Fougere, 2008). In clinical trials, it has been reported that curcumin may have an anti-cancer effect, in the form of a chemoprevention agent (Li et al., 2011). Throughout multiple studies on a variety of species, curcumin has potential for being a therapeutic agent in inflammatory diseases, including inflammatory bowel disease, pancreatitis, and arthritis (Jagetia and Aggarwal, 2007; Wynn and Fougere, 2008; Li et al., 2011). Curcumin is also known to have antimicrobial properties. Turmeric and curcumin, with their anti-inflammatory and antimicrobial properties, have potential to alleviate arthritic symptoms in canines and equines as well as controlling colic and gastrointestinal upset by reducing opportunistic and harmful bacteria proliferation.

In a four-part study, the first part will evaluate the therapeutic efficacy and safety of 95% curcumin (500 mg, SID) and 95% liposomal-curcumin (250 mg, BID) in ten moderately arthritic dogs. The objective of this study was to compare the two groups, given either curcumin or liposomal-curcumin, to determine which form of the nutraceutical alleviates symptoms better in moderately arthritic dogs. As a follow up study, the second study evaluated the therapeutic efficacy and safety of 95% curcumin, 100 mg or 500 mg, SID, in ten moderately arthritic dogs. The objective of this study was to compare the two groups, given either 100 mg or 500 mg of

95% curcumin, to determine which dosage of the nutraceutical improves the symptoms and ROM in moderately arthritic canines more.

In project three, the species will transition to equine. Project three consists of a two *in vitro*, closed-system, batch culture studies looked at the effects of 95% turmeric, 95% curcumin, and 95% liposomal-curcumin on five opportunistic strains of bacteria found in the equine hindgut. The objective of this study was to assess the effects of the different forms of the nutraceutical, used in the previous studies, on bacteria in the equine hindgut. A follow-up *in vitro* study was conducted looking at different dosages of 95% liposomal-curcumin, the nutraceutical that had the greatest reduction in the five opportunistic bacteria in the first *in vitro* study. The results from the third study were used for the fourth and final study, based on which form of the nutraceutical, 95% turmeric, 95% curcumin, or 95% liposomal-curcumin, had the greatest overall effect on the hindgut bacteria. The fourth study was a repeat of the second *in vitro* study except taking *in vivo* with dosages of 15 g, 25 g, and 35 g, and looking at the anti-inflammatory properties in addition to the antimicrobial properties of liposomal-curcumin. This study looked at therapeutic and safety effects of liposomal-curcumin at three different dosages because there has yet to be an approved dose of orally administered curcumin in equines. By conducting these studies, we hope to gain information about turmeric, curcumin, and liposomal-curcumin in relationship to its dosage, therapeutic efficacy, safety, and effects on gut microbes and inflammation conditions.

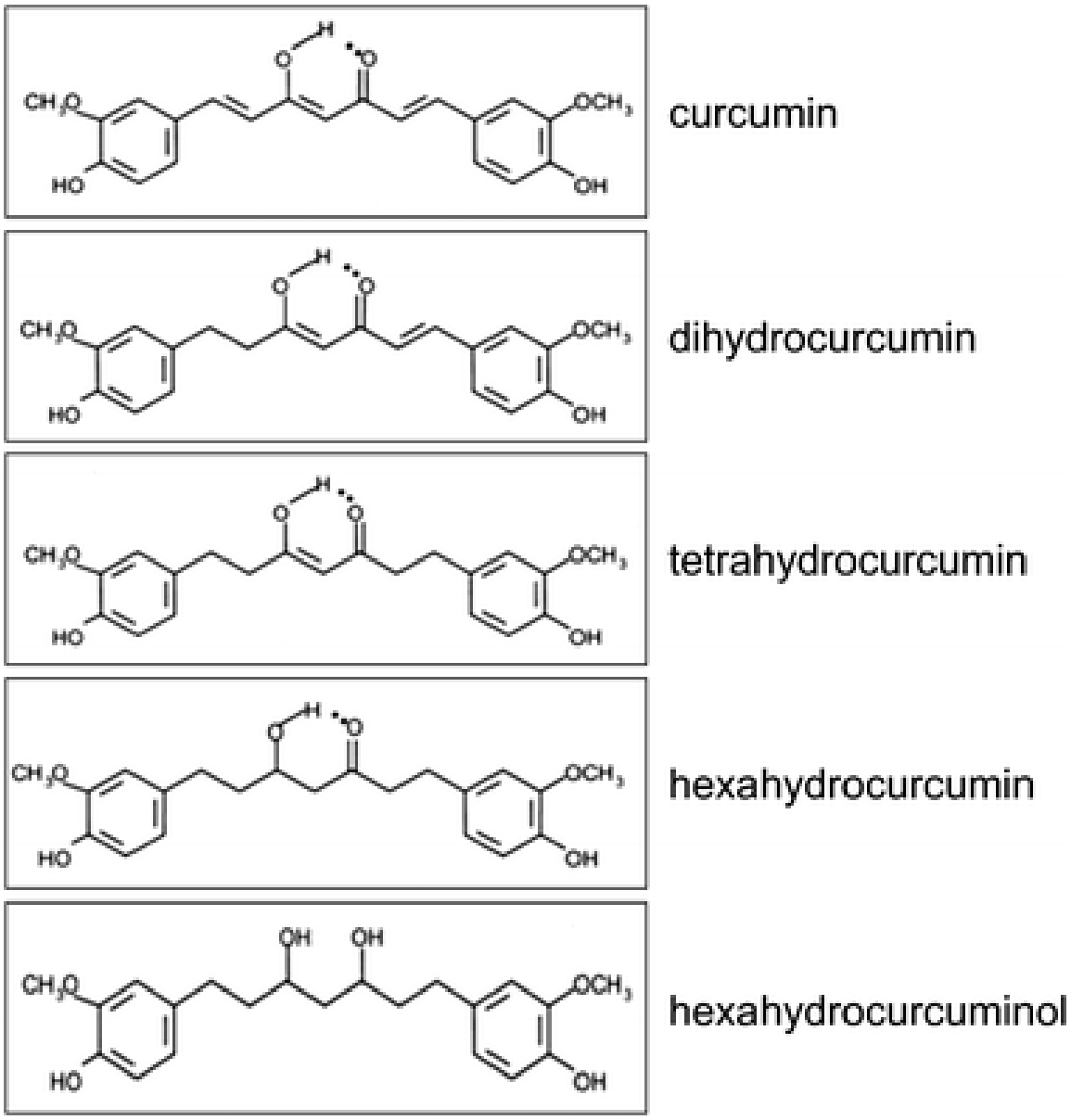

Figure 1.1. Chemical structures of turmeric, curcumin, and its derivatives

Table 1.1. Maximum joint motion in canine (Millis, 2004, p. 536)

Joint	Extension	Flexion
Shoulder	142 degrees-ground	125 degrees-ground
Elbow	124 degrees-ground	98 degrees-ground
Carpus	124 degrees-ground	97 degrees-ground
Hip	141 degrees-ground	115 degrees-ground
Stifle	141 degrees-ground	109 degrees-ground
Hock	135 degrees-ground	115 degrees-ground

Table 1.2. Erythrocyte sedimentation rate for small domestic animals, mm/hr (Provet, 2014)

Species	Normal Range
Canine	0-5
Feline	0-12

Table 1.3. Erythrocyte sedimentation rate for equine, mm/hr (Blair Street Vet Hospital, 2014)

Packed Cell Volume	Normal Range
35	13-43
37	8-28
39	3-9
40	0-8
45	0-3

CHAPTER 2

THERAPEUTIC AND SAFETY EVALUATION OF CURCUMIN AND LIPOSOMAL-CURCUMIN IN MODERATELY ARTHRITIC DOGS

ABSTRACT:

The objective of this investigation was to evaluate the efficacy and safety of 95% curcumin and 95% liposomal-curcumin in moderately arthritic dogs. Ten client-owned dogs in a randomized, double-blinded study received either 95% curcumin (500 mg) once a day (SID) or 95% curcumin (250 mg) twice a day (BID) for a period of five months. Dogs were evaluated each month for physical condition (body weight, body temperature, heart rate, and respiratory rate), pain associated with arthritis (overall pain, pain from limb manipulation, and pain after physical exertion), and range of motion was measured using a goniometer on the stifle, shoulder, and elbow joints. Serum samples collected from these dogs were examined each month for biomarkers of liver (total bilirubin, ALT, and AST), kidney (BUN and creatinine), heart and muscle (creatine kinase) functions. The findings of this study revealed that dogs receiving 95% curcumin (Group-I) and 95% liposomal-curcumin had a significant ($P < 0.05$) reduction in pain from limb manipulation by day 150. Group-1 had a significant reduction in overall pain by day 60 and Group-II had a significant reduction in overall pain by day 90. Group-I had a significant reduction in pain after physical exertion by day 90 and Group-II had significant reduction in pain after physical exertion on day 150. Dogs in either group showed no significant changes ($P > 0.05$) in physical parameters or serum markers, suggesting that both 95% curcumin and 95% liposomal-curcumin were well tolerated by moderately arthritic dogs. It was concluded that both

95% curcumin and 95% liposomal-curcumin significantly ($P < 0.05$) reduced pain in osteoarthritic dogs and markedly improved their daily life activity without any side effects.

INTRODUCTION

Arthritis is a commonly occurring chronic illness in human and animals (Gupta et al., 2009). Among all domestic and pet animal species, dogs and horses suffer from arthritis more often because of excessive running or exercise, injury, and/or genetic predisposition. Presently, one in four of 77.2 million pet dogs in the United States are diagnosed with some form of arthritis (Lawley et al., 2013). In dogs, osteoarthritis is more common than rheumatoid arthritis and pain is the number one observation. Osteoarthritis, also known as degenerative joint disease (DJD), is a slowly progressive inflammatory disease. Osteoarthritis is characterized by degeneration of the cartilage, hypertrophy of bone at the margins, and changes in the synovial membrane, and that eventually results in pain and stiffness of joints (Reid and Miller, 2008). Alterations in joint structures can decrease flexibility, and lead to severe pain due to lack of hydration and inflammation. Cells within the damaged joints release pro-inflammatory cytokines, which further the inflammatory process (Reid and Miller, 2008). This causes more breakdown of the cartilage collagen type II and proteoglycans. This perpetuating destructive cycle ultimately results in cartilage destruction, subchondral bone thickening, and synovial membrane inflammation (Renberg, 2005).

Currently, osteoarthritis is treated or managed by invasive as well as noninvasive means. In the recent past, the treatment options for arthritis were typically non-steroidal anti-inflammatory drugs (NSAIDs) given alone or in combination with other disease-modifying agents. NSAIDs (COX enzymes inhibitors) eliminate pain, but do not eliminate the signs and symptoms of active disease nor do they repair cartilage (Vaughn-Scott, et al., 1997). In recent years, chronic use of

NSAIDs has been linked to numerous side effects, including gastrointestinal (GI) bleeding, and renal and hepatic dysfunction. Anti-inflammatory drugs such as aspirin and ibuprofen are non-specific inhibitors of COX enzymes (COX-I and COX-II) (Vaughn-Scott, et al., 1997). They inhibit the production of inflammatory prostaglandins, resulting in their therapeutic effect, but also inhibit the production of constitutive prostaglandins, resulting in side effects, such as GI bleeding. Therefore, under these circumstances, a safe therapy is warranted for arthritic dogs.

Herbal medicine is increasing its popularity in veterinary medicine due to it being relatively inexpensive and minimal to no side effects. Herbal medicine is becoming a common treatment for mastitis, foot-and-mouth disease, skin allergies, food poisoning, tympany, and expulsion of placenta (Chan et al., 2009). In the past, nutraceuticals were a common therapy for livestock in treating a variety of diseases including hepatitis, chronic heart disease, skin disorders, wounds, and arthritis (Mahima et al., 2013). According to past studies, particular nutraceuticals can possibly affect the progression of arthritis by preventing degradation and enhancing the repair of joint cartilage (Sanghi et al., 2008).

Turmeric is a rhizomatous herbaceous perennial plant, *Curcuma longa* Linn, belonging to the ginger family, *Zingiberaceae* (Chan et al., 2009). Turmeric is native to southeast India and grows in temperatures between 20-30° C, with high amounts of rainfall. Once picked, the rhizomes are boiled, dehydrated, and then ground into orange-yellow powder, which is used for curries, dyeing, and mustard condiments (Prasad et al., 2011). Turmeric is one of the oldest sources of spice, coloring pigments, and medicine, dating back to 1900 B.C. (Hassaninasab et al., 2010).

Out of all *Curcuma longa* Linn species, *Curcuma longa* is the most chemically investigated (Li et al., 2011). Curcuminoids, belonging to the diarylheptanoid group, are the most

important chemical components of turmeric and are the main active ingredient in turmeric. This group makes up roughly 2-6% of the spice, with curcumin as the main compound (Wynn and Fougere, 2008). Three main curcuminoids observed in commercial supplements are curcumin (curcumin I), demethoxycurcumin (curcumin II), and bis-demethoxycurcumin (curcumin III). Typical commercial products contain 77% curcumin, 17% demethoxycurcumin, and 3% bis-demethoxycurcumin. These curcuminoids are said to work synergistically (Jagetia and Aggarwal, 2007; Wynn and Fougere, 2008). Commercial curcumin, it is often 95% curcumin instead of 100% because there is not an increase of bioavailability from 95% to 100% and it costs less to manufacture (Wynn and Fougere, 2008). The bioavailability of curcumin is noted to be minimal due to its hydrophobic and low intrinsic activity, poor absorption, and high rate of metabolism and elimination from the body (Anard et al., 2009). However, curcumin can be encapsulated into liposomes, liposomal curcumin, to increase bioavailability (Li et al., 2007; Li et al., 2011). Liposomes can carry both hydrophobic and hydrophilic molecules, which make them ideal for drug delivery (Anard et al., 2009).

In Ayurvedic medicine, turmeric is used as an anti-inflammatory, and in Chinese medicine, used as stimulant, aspirant, carminative, astringent, detergent, and diuretic (Li et al., 2011). Curcumin has been used for thousands of years in Eastern medicine. However, only in recent studies has the biological action of curcumin have been examined (Jagetia and Aggarwal, 2007; Wynn and Fougere, 2008). Throughout multiple studies on a variety of mammalian species, curcumin has potential for being a therapeutic agent in inflammatory diseases, including inflammatory bowel disease, pancreatitis, and arthritis (Li et al., 2011). Therefore, this is important to note that dogs suffering from osteoarthritis could potential be given curcumin over NSAIDs to help alleviate symptoms, without the negative side effects. In the present

investigation, curcumin was evaluated for its therapeutic and safety evaluation in osteoarthritic dogs.

MATERIALS AND METHODS

Animals

Ten client-owned moderately arthritic dogs, weighing between 40-65 pounds, 10 ± 2 years, were used in this study. These dogs, based on signs of joint stiffness, lameness, degree of range of motion, had pain at the level of moderate arthritis (>2 on a 4-point scale). My inclusion criteria of dogs in this study excluded those having any concurrent diseases (liver, kidney, or heart disease, neoplasia, cancer or any other major disease) and were heartworm negative. Institutional Animal Care and Use Committee (IACUC) approval and owner's consent were obtained prior to the initiation of this study.

Experimental Design

In a randomized double-blind study, ten client-owned dogs, divided into two groups (n = 5), received 95% curcumin (500 mg) once a day (Group-I) or 95% liposomal-curcumin (250 mg) twice a day (Group-II). The study was carried out for a period of five months at Murray State University. None of the dogs received any treatment or supplements for four weeks prior to the study or during the study period.

Pain Measurement

At pre-determined intervals (i.e. 30 days), each dog was evaluated for overall pain, pain upon manipulation, and pain after physical exertion, for a period of five months. Overall pain, on a scale of 0-10, was graded as: 0, no pain: 2.5, mild pain: 5, moderate pain: 7.5, severe pain: 10, severe and constant pain. Pain after manipulation, on a scale of 0-4, was evaluated as: 0, no pain: 1, mild pain: 2, moderate pain: 3, severe pain: 4, severe and constant pain. Pain after physical

exertion, on a scale of 0-4, was evaluated as: no pain: 0, no pain: 1, mild pain: 2, moderate pain: 3, severe pain: 4, severe and constant pain. Range of motion was evaluated with a goniometer and the degree of motion during flexion was noted. The physical examination of each limb started with the forelimbs and ended with the hind limbs. The evaluation focused on manipulation of the limbs in a forward, backward, and circular motion. Three main joints in each dog were evaluated, including shoulder joint, elbow joint, and stifle joint. Popping and cracking of the joints as well as vocal pain were noted for each canine. Detailed criteria of the measurement of pain are provided in our earlier publications (Deparle et al., 2005; D'Altilio et al., 2007; Peal et al., 2007; Gupta et al., 2009; Gupta et al., 2011; Fleck et al., 2013; Lawley et al., 2013). The present investigation was carried out on moderately arthritic dogs. A moderately arthritic dog exhibits overall pain of about a 5 on a scale of 0-10; pain upon limb manipulation about a 2 on a scale of 0-4; and pain after physical exertion about a 2 on a scale of 0-4.

Physical Examination

On a monthly basis, dogs were given a full physical examination for body weight, body temperature, heart rate, and respiratory rate (Table 2.1).

Serum Biomarkers Assays

Blood samples were collected from the cephalic vein using a 3 mL syringe with a 22-gauge, 1-inch needle and were stored in a marble top tube and lavender top tube. Samples in the marble top tubes were then spun to collect serum and transferred to a red top tube for evaluation. Serum samples were collected each month and analyzed for liver (total bilirubin, ALT, and AST), kidney (BUN and creatinine), heart and muscle (CK) functions, using a Beckman AU 480 serum analyzer. The lavender top tubes were analyzed for a complete blood count including a five-part differential (neutrophils, lymphocytes, monocytes, eosinophils, and basophils). Whole

blood stored in lavender top tubes was collected each month to analyze effects on red blood cells and white blood cells. The serum and whole blood sample assays indicated that neither 95% curcumin nor 95% liposomal-curcumin produced adverse effects in vital organs of arthritic dogs.

Statistical Analysis

The data presented are means ± SEM. Statistical significance of difference comparing each month against baseline (day 0) was determined by analysis of variance (ANOVA) coupled with Tukey-Kramer *post-hoc* test ($P < 0.05$) using the Statistical Analysis and Graphics Software for Windows (NCSS9).

RESULTS

On a monthly basis, each dog was examined for pain level (overall pain (Figure 2.2), pain after limb manipulation (Figure 2.3), and pain after physical exertion (Figure 2.4) While evaluating overall pain, the key points were to observe the dog's gait, joint range of motion, ability to sit or lie down, ability to rise from a sitting position and from a lying position. Group-I dogs receiving 95% curcumin (500 mg, SID), showed significant ($P = 0.01$) reduction in overall pain by day 60 (4.8 ± 0.34) compared to day 0 (6.6 ± 0.51). The maximum reduction in overall pain was noted on day 150 (1.9 ± 0.18). Group II dogs receiving 95% liposomal-curcumin (250 mg, BID), showed significant ($P = 0.02$) reduction in overall pain by day 90 (4.1 ± 0.24) compared to day 0 (7.2 ± 0.66). The maximum reduction in overall pain was noted on day 150 (2.9 ± 0.6).

Pain after limb manipulation was measured in each limb of the dog for flexibility, joint integrity, and vocalization. The pain level was significantly reduced by day 150 in both groups, Group-I (0.4 ± 0.3) and Group-II (0.6 ± 0.29). The canines were evaluated for pain after two minutes of jogging. After jogging, pain level was assessed based on the dog's body position,

limping, flexibility, and vocalization. Group-I had noted significant reduction in pain after physical exertion on day 90 (0.2 ± 0.2) and day 150 (0.2 ± 0.2). Group-II had significant reduction in pain after physical exertion only on day 150 (0.5 ± 0.5).

Data of physical parameters (body weight, body temperature, heart rate, and respiratory rate) were not significantly different (Table 2.1). Dogs receiving 95% curcumin or 95% liposomal-curcumin had no significant change in any physical parameters. Dogs receiving 95% curcumin or 95% liposomal-curcumin had no significant change in serum biomarkers during the study of 150 days (Table 2.4).

DISCUSSION

In the present paper, we report that 95% curcumin or 95% liposomal curcumin at a dose of 500 mg is effective in reducing arthritic pain and enhancing the daily activity of dogs without exerting any side effects. Curcumin and liposomal-curcumin administration ameliorated arthritic pain in all three categories (overall pain, pain after limb manipulation, and pain after physical exertion) with maximum effect noted on day 150. Curcumin, the active ingredient in turmeric, is known for its medicinal properties, including anti-inflammatory, antioxidant, antimicrobial, wound healing, and anti-tumor properties (Zhu et al., 2014). Curcumin, has been used both for preventative health and for treating many diseases such as bowel disease, pancreatitis, skin, pulmonary, gastrointestinal, and aches, pains, wounds, sprains, liver disorders, and cancer for thousands of years. Since curcumin has multiple medicinal benefits, it is highly likely that it reduced the arthritic pain due to a variety of pharmacological mechanisms, including anti-inflammatory and antioxidant properties.

In conclusion, curcumin is an all-natural supplement, which offers significant anti-arthritic properties including reduction of pain and inflammation and increasing joint range of

motion. On average, dogs experienced significant increase in ROM and decrease in pain 60-90 days after beginning the treatment. All dogs responded well to curcumin administration without exhibiting any adverse effects, thereby giving this supplement an edge over many other anti-arthritic nutraceuticals and pharmaceuticals. Further work needs to be conducted examining curcumin's anti-inflammatory properties in dogs on the same diet and exercise regime.

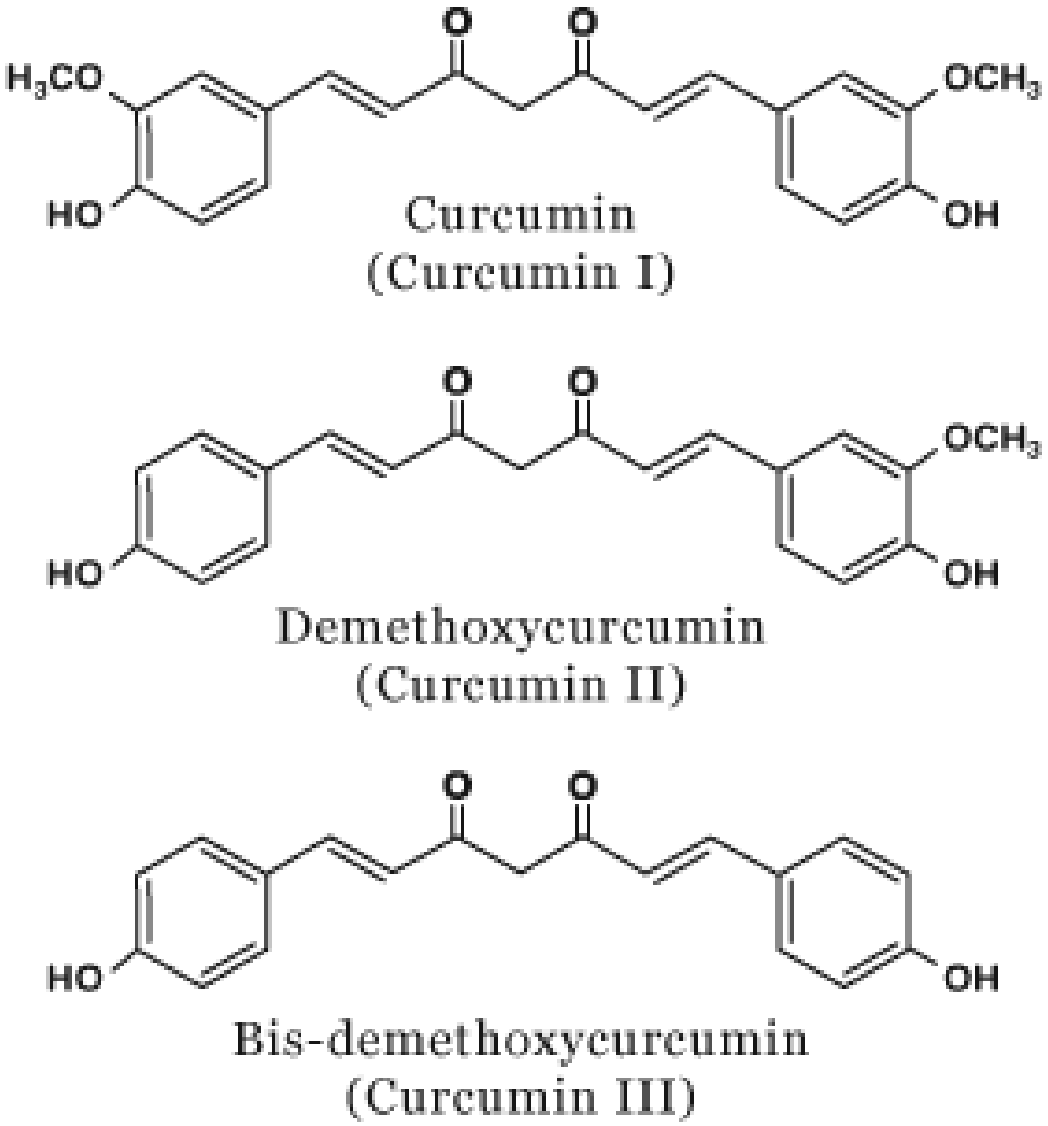

Figure 2.1. Chemical structures of curcumin I, II, and III

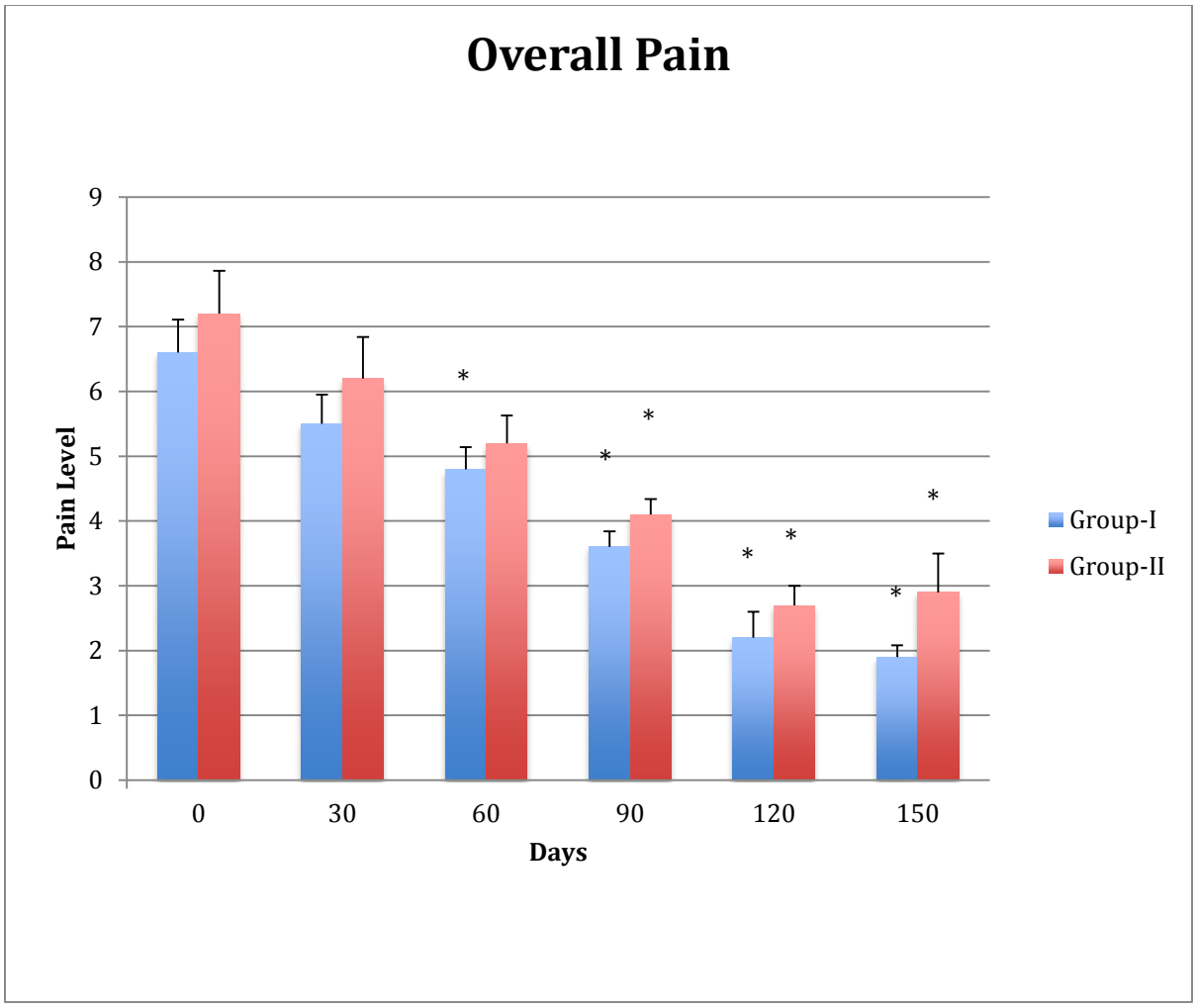

Figure 2.2. Effects of (Group I) 95% curcumin (500 mg, SID) or (Group II) 95% liposomal-curcumin (250 mg, BID) on overall pain in moderately arthritic dogs.

Overall pain was graded on a scale of 0-10 (0, no pain: 2.5, mild pain: 5, moderate pain: 7.5, severe pain: 10, severe and constant pain).

*Significantly different compared to Day 0 ($P < 0.05$)

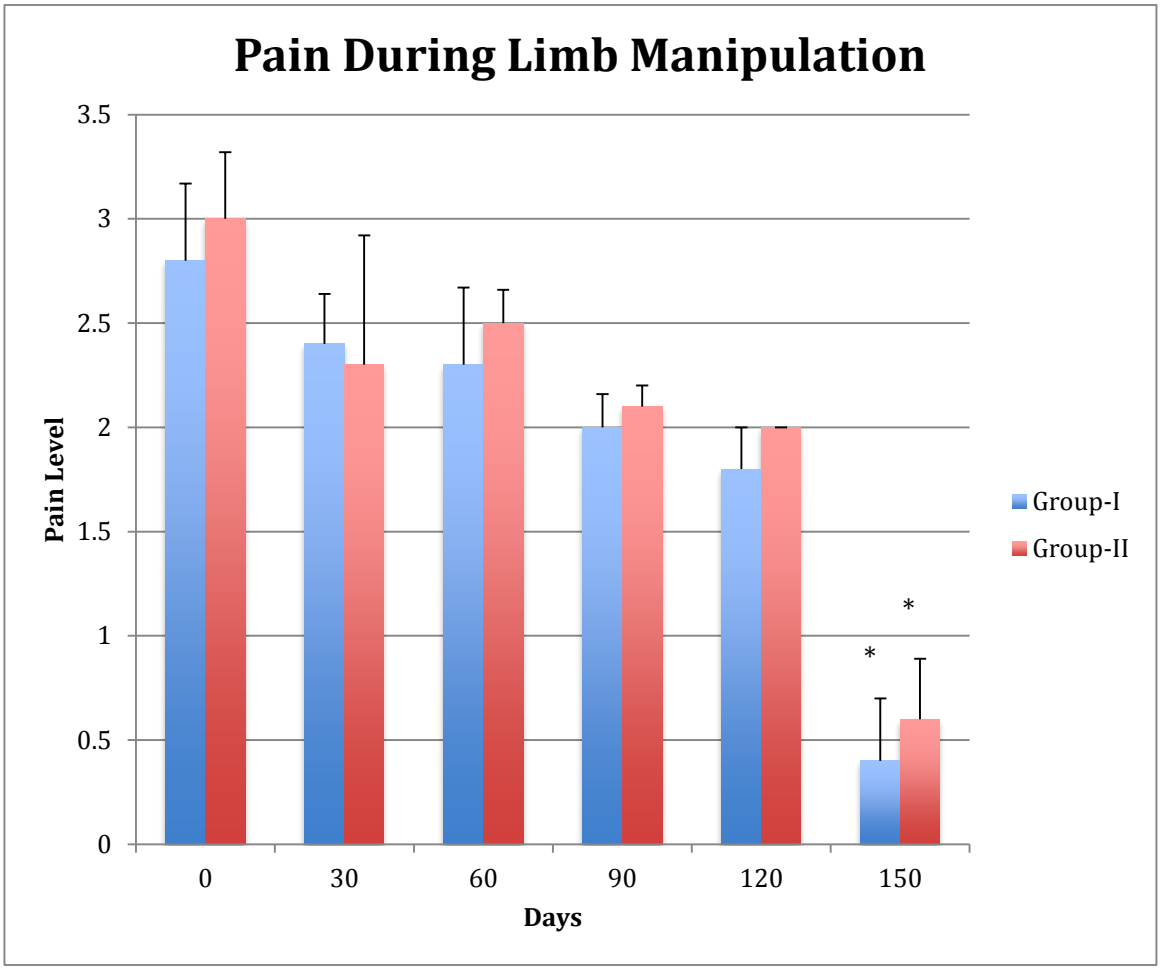

Figure 2.3. Effects of (Group I) 95% curcumin (500 mg, SID) or (Group II) 95% liposomal-curcumin (250 mg, BID) on pain from limb manipulation in moderately arthritic dogs.

Pain from limb manipulation was graded on a scale of 0-4 (0, no pain: 1, mild pain: 2, moderate pain: 3, severe pain: 4, severe and constant pain).

*Significantly different compared to Day 0 ($P < 0.05$)

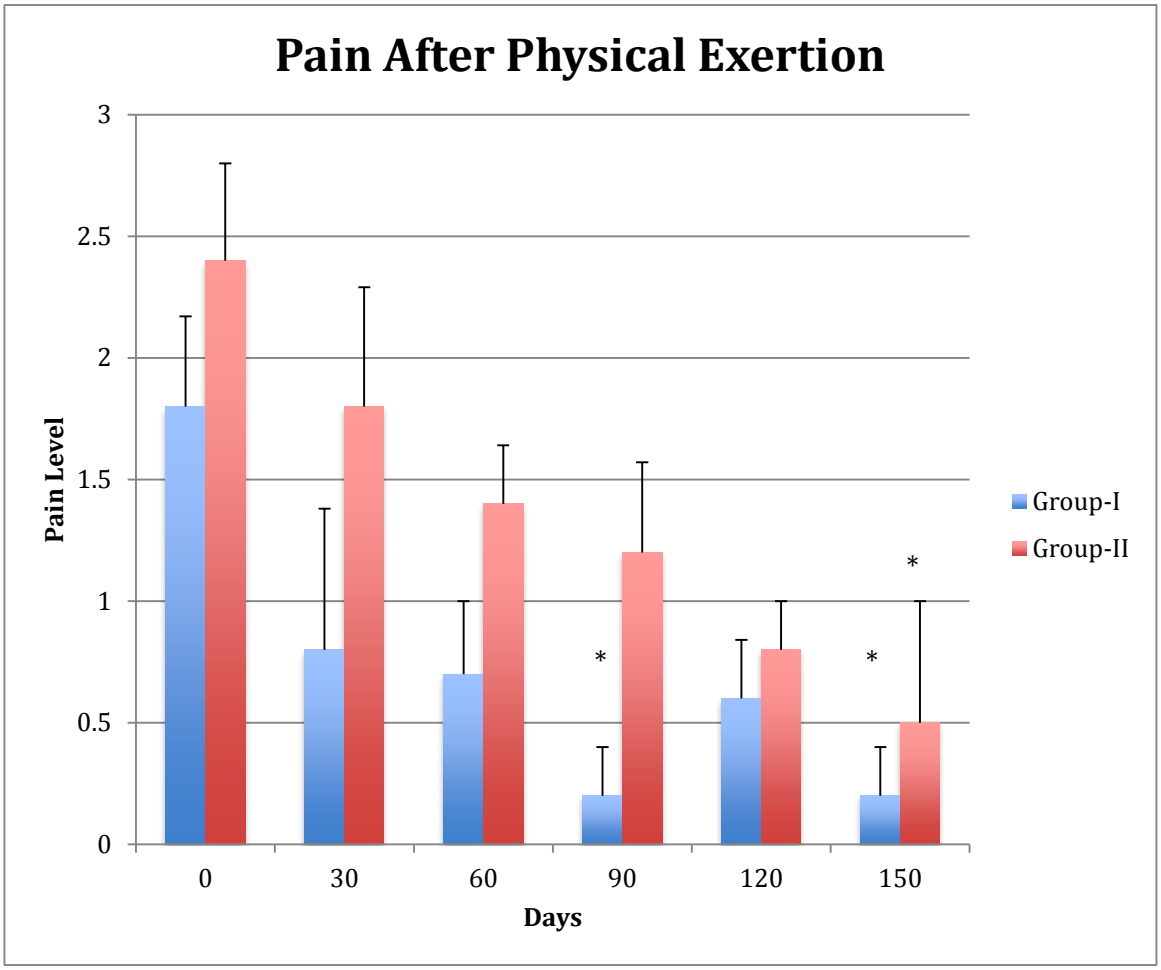

Figure 2.4. Effects of (Group I) 95% curcumin (500 mg, SID) or (Group II) 95% liposomal-curcumin (250 mg, BID) on pain after physical exertion in moderately arthritic dogs.

Pain from limb manipulation was graded on a scale of 0-4 (0, no pain: 1, mild pain: 2, moderate pain: 3, severe pain: 4, severe and constant pain).

*Significantly different compared to Day 0 ($P < 0.05$)

Table 2.1. Effects of curcumin on physical parameters in osteoarthritic dogs

*Significantly different compared to Day 0 ($P < 0.05$)

Parameters	Group	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150
Body Weight (lbs)	I	52.50 ± 5.82	49.70± 5.48	51.20± 5.41	50.16± 5.51	50.64± 4.62	49.04± 5.44
	II	57.80 ± 10.05	58.90± 9.93	58.50± 10.53	53.92± 9.05	56.04± 9.38	57.68± 7.86
Temperature (°F) Normal range: 101-102.5°F	I	100.00± 0.49	101.70± 0.29	101.02± 0.18	100.86± 0.23	100.28± 0.37	100.12± 0.15
	II	99.86± 0.53	101.12± 0.18	101.16± 0.20	100.86± 0.42	100.40± 0.39	100.90± 0.15
Heart Rate (bpm) Normal range: 70-160 bpm	I	118.80± 12.21	118.80± 6.68	116.40± 14.89	118.40± 16.40	133.20± 13.47	108.80± 13.96
	II	9.63± 0.87	9.53± 1.16	9.86± 1.05	10.23± 0.85	10.97± 1.33	10.30± 1.09
Respiratory Rate (bpm) Normal range: 10-35 bpm	I	18.67± 0.67	24.67± 2.91	24.50± 2.06	22.00± 2.28	24.60± 2.94	23.20± 2.06
	II	20.50± 1.26	20.67± 1.76	20.66± 1.76	21.50± 1.50	20.20± 2.58	20.00± 2.02

Table 2.2. Effects of curcumin on arthritis associated pain level in osteoarthritic dogs

*Significantly different compared to Day 0 ($P < 0.05$)

Parameters	Group	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150
Overall Pain Score (0-10)	I	6.60± 0.51	5.50 ± 0.45	4.80± 0.34*	3.60± 0.24*	2.20± 0.40*	1.90± 0.18*
	II	7.20± 0.66	6.20± 0.64	5.20± 0.43	4.10± 0.24*	2.70± 0.30*	2.90± 0.60*
Pain Severity Score (0-4)	I	2.90± 0.15	2.05± 0.23	1.85± 0.34	1.85± 0.16	1.87± 0.18	1.70± 0.27*
	II	2.60± 0.31	2.26± 0.34	2.05± 0.37	1.87± 0.32	1.62± 0.31	1.62± 0.44
Pain Interference Score (0-10)	I	7.00± 0.64	6.00± 0.71	4.20± 0.39*	2.73± 0.21*	1.80± 0.37*	1.30± 0.30*
	II	6.72± 0.66	6.32± 0.66	4.37± 0.88	4.02± 0.57	2.10± 0.33*	1.70± 0.41*
Pain from Limb Manipulation (0-4)	I	2.80± 0.37	2.40± 0.24	2.30± 0.37	2.00± 0.16	1.80± 0.20	0.40± 0.30*
	II	3.00± 0.32	2.30± 0.62	2.50± 0.16	2.10± 0.10	2.00± 0.00	0.60± 0.29*
Pain After Physical Exertion (0-4)	I	1.80± 0.37	0.80± 0.58	0.70± 0.30	0.20± 0.20*	0.60± 0.24	0.20± 0.20*
	II	2.40± 0.40	1.80± 0.49	1.40± 0.24	1.20± 0.37	0.80± 0.20	0.50± 0.50*

Table 2.3. Effects of curcumin on joint flexibility measured by goniometer in osteoarthritic dogs

*Significantly different compared to Day 0 ($P < 0.05$)

Parameters	Group	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150
Right Shoulder	I	56.20± 9.08	93.80± 7.21*	80.00± 5.00	91.20± 1.88*	86.00± 2.53*	96.40± 2.50*
	II	53.20± 5.90	80.20± 7.66*	82.60± 2.71*	86.20± 4.64*	89.20± 3.71*	93.00± 4.83*
Right Elbow	I	79.00± 14.4	99.40± 4.43	96.20± 3.53	98.80± 2.76	96.20± 3.69	103.40± 2.31
	II	77.00± 11.19	97.00± 3.33	94.00± 1.87	94.60± 2.48	93.60± 2.73	95.80± 1.15
Right Stifle	I	59.20± 11.49	80.80± 3.53	81.60± 4.23	85.40± 5.35	73.40± 1.88	92.40± 4.50
	II	50.20± 9.49	86.80± 7.29*	84.60± 4.27*	80.60± 3.92*	92.20± 2.51*	92.20± 2.08*
Left Shoulder	I	60.00± 6.89	86.80± 2.13*	78.80± 2.92*	89.60± 3.67*	83.40± 3.05*	92.40± 3.58*
	II	42.60± 5.07	88.20± 5.90*	73.20± 8.56*	82.40± 8.20*	88.80± 4.80*	91.60± 2.46*
Left Elbow	I	85.8± 6.81	95.60± 3.31	93.60± 2.40	97.00± 3.00	94.60± 4.31	95.20± 5.47
	II	75.00± 10.72	92.60± 6.51	94.00± 1.51	96.00± 3.67	89.60± 0.40	93.80± 3.80
Left Stifle	I	67.60± 8.15	87.40± 5.53	81.00± 4.55	87.00± 5.26	84.00± 4.30	97.80± 3.00*
	II	62.20± 6.44	95.60± 6.99*	81.60± 2.04	83.20± 5.51	84.80± 2.92*	92.60± 3.91*

Table 2.4. Effects of curcumin on serum biomarkers of liver, kidney, and heart functions in osteoarthritic dogs *Significantly different compared to Day 0 ($P < 0.05$)

Parameters	Group	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150
Total Bilirubin (mg/dl)	I	0.16± 0.02	0.24± 0.07	0.22± 0.04	0.16± 0.02	0.16± 0.02	0.22± 0.02
Normal range: 0.1-0.6 mg/dl	II	0.22± 0.05	0.20± 0.03	0.18± 0.02	0.14± 0.02	0.14± 0.02	0.18± 0.02
ALT (IU/L)	I	90.40± 30.10	71.00± 22.78	90.60± 31.45	81.00± 30.03	82.80± 30.96	72.00± 29.80
Normal range: 10-120 IU/L	II	72.60± 22.70	89.80± 47.70	46.00± 13.09	43.40± 10.89	38.20± 11.29	40.20± 10.28
AST (IU/L)	I	25.40± 1.60	26.20± 2.29	27.00± 2.12	23.80± 2.22	21.60± 1.24	25.20± 2.88
Normal range: 15-65 IU/L	II	27.20± 0.92	26.20± 2.58	21.40± 1.57	21.60± 2.04	19.60± 2.25	24.40± 1.50
BUN (mg/dl)	I	17.96± 8.33	11.60± 1.17	12.20± 1.07	13.80± 1.24	14.20± 1.31	15.00± 3.56
Normal range: 7-26 mg/dl	II	15.00± 2.39	13.00± 1.48	16.60± 1.43	11.60± 1.86	14.00± 1.30	14.40± 1.43
Creatinine (mg/dl)	I	0.79± 0.08	0.86± 0.09	0.83± 0.10	0.89± 0.09	0.93± 0.11	0.96± 0.12
Normal range: 0.0-1.35 mg/dl	II	0.86± 0.11	0.88± 0.10	0.86± 0.11	0.93± 0.14	0.92± 0.12	0.95± 0.12
CK (IU/L)	I	109.40± 22.13	82.20± 9.87	101.40± 22.02	75.00± 3.48	82.80± 13.37	89.00± 17.50
Normal range: 60-450 IU/L	II	134.40± 35.11	106.20± 30.0	88.40± 16.79	66.40± 6.50	69.00± 10.37	88.20± 13.76

Table 2.5. Effects of curcumin on complete blood count in osteoarthritic dogs

*Significantly different compared to Day 0 ($P < 0.05$)

Parameters	Group	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150
White Blood Cells (/µL)	I	9.48± 1.11	10.30± 1.31	10.47± 1.64	9.26± 1.28	9.91± 1.45	9.60± 1.22
Normal range: 6-17x10^3/µL	II	8.63± 0.63	8.46± 0.49	8.89± 0.32	8.26± 0.87	8.88± 1.36	8.64± 0.84
Red Blood Cells (/µL)	I	6.56± 0.28	6.97± 0.34	6.57± 0.23	6.78± 0.29	6.3± 0.25	6.41± 0.21
Normal range: 5.5-8.5x10^6/ µL	II	8.63± 0.63	7.10± 0.42	6.78± 0.29*	7.10± 0.36	6.70± 0.34*	6.85± 0.32*
Hemoglobin (g/dL)	I	15.98± 0.95	17.02± 0.35	16.22± 0.87	16.72± 1.03	15.64± 0.86	15.86± 0.76
Normal range: 12-18 g/dL	II	16.34± 1.31	16.48± 1.28	15.98± 0.89	16.86± 1.21	16.16± 1.11	16.42± 0.81
Hematocrit (%)	I	48.86± 2.18	51.38± 2.99	49.12± 1.87	50.34± 2.43	45.30± 1.91	47.54± 2.22
Normal range: 37-55%	II	48.62± 4.21	50.36± 3.39	48.78± 2.42	50.94± 3.21	46.56± 2.79	49.62± 2.20
MCV (fL)	I	74.48± 0.68	73.58± 0.79	74.68± 0.67	74.12± 0.59	71.9± 0.53	74.02± 1.07
Normal range: 60-77 fL	II	69.18± 2.19	70.34± 1.65	71.92± 1.90	71.66± 2.03	69.5± 1.44	72.5± 1.38
MCH (pg)	I	24.28± 0.42	24.38± 0.42	24.60± 0.46	24.56± 0.45	24.78± 0.38	24.72± 0.45
Normal range: 19.5-24.5 pg	II	23.32± 0.62	22.98± 0.68	23.54± 0.71	23.70± 0.76	24.06± 0.64	23.96± 0.46

Table 2.5. (Continued)

MCHC (g/dL) Normal range: 32-36 g/dL	I	32.62± 0.56	33.12± 0.43	32.92± 0.55	33.12± 0.56	34.46± 0.51	33.36± 0.46
	II	33.74± 0.66	32.64± 0.37	32.72± 0.25	33.06± 0.37	34.60± 0.37	33.04± 0.18
Number of Neutrophils (/µL) Normal range: 3-11.5x10^3/ µL	I	6.50± 0.77	6.99± 1.03	7.30± 1.06	6.18± 0.77	6.29± 0.57	6.33± 0.74
	II	65.44± 2.24	65.20± 3.98	66.68± 4.24	63.34± 4.01	63.44± 3.25	64.30± 3.45
Percentage of Neutrophils (%) Normal range: 60-77%	I	68.72± 2.13	67.40± 2.48	70.28± 1.12	67.38± 2.98	65.54± 3.95	66.54± 3.54
	II	65.44± 2.24	65.20± 3.98	66.68± 4.24	63.34± 4.01	63.44± 3.25	64.30± 3.45
Number of Lymphocytes (/µL) Normal range: 1-4.8x10^3/ µL	I	1.87± 0.22	2.10± 0.24	1.97± 0.35	1.91± 0.37	2.16± 0.49	2.08± 0.39
	II	2.14± 0.25	2.02± 0.27	2.12± 0.25	2.16± 0.36	2.16± 0.18	2.16± 0.27
Percentage of Lymphocytes (%) Normal range: 12-30%	I	20.12± 1.76	21.18± 2.18	19.40± 2.35	21.08± 2.93	21.42± 2.13	21.90± 2.67
	II	24.68± 1.57	23.76± 2.41	24.82± 3.06	26.36± 3.32	25.44± 2.23	25.42± 3.10
Number of Monocytes (/µL) Normal range: 0.1-1.4x10^3/ µL	I	0.37± 0.06	0.40± 0.05	0.37± 0.06	0.38± 0.08	0.45± 0.09	0.36± 0.06
	II	0.38± 0.05	0.40± 0.07	0.37± 0.06	0.33± 0.07	0.40± 0.08	0.34± 0.05

Table 2.5. (Continued)

Percentage of Monocytes (%)	I	3.90± 0.54	3.92± 0.39	3.60± 0.52	4.02± 0.62	4.50± 0.58	3.78± 0.65
Normal range: 3-10%	II	4.38± 0.45	4.74± 0.88	4.16± 0.66	3.88± 0.55	4.40± 0.49	3.92± 0.44
Number of Eosinophils (/µL)	I	0.72± 0.26	0.79± 0.20	0.82± 0.39	0.78± 0.32	0.98± 0.47	0.81± 0.29
Normal range: 0.1-1.2x10^3/ µL	II	0.47± 0.09	0.52± 0.14	0.37± 0.10	0.52± 0.08	0.54± 0.11	0.53± 0.10
Percentage of Eosinophils (%)	I	7.12± 1.83	7.46± 1.42	6.66± 2.86	7.44± 2.38	8.46± 3.09	7.70± 1.86
Normal range: 2-10%	II	5.32± 0.78	6.22± 1.55	4.28± 1.25	6.36± 1.20	6.62± 1.74	6.28± 1.21
Number of Basophils (/µL)	I	0.01 ± 0.00	0.00± 0.00	0.00± 0.00	0.01± 0.00	0.01± 0.00	0.00± 0.00
Normal range: 0-0.05x10^3/ µL	II	0.01± 0.00	0.01± 0.00	0.01± 0.00	0.01± 0.01	0.01± 0.00	0.01± 0.00
Percentage of Basophils (%)	I	0.12± 0.02	0.04± 0.02	0.06± 0.02	0.08± 0.02	0.08± 0.04	0.08± 0.02
Normal range: 0-0.5%	II	0.18± 0.03	0.08± 0.04	0.06± 0.02	0.06± 0.05	0.10± 0.03	0.08± 0.03

CHAPTER 3

THERAPEUTIC AND SAFETY EVALUATION OF CURCUMIN AND LIPOSOMAL-CURCUMIN IN MODERATELY ARTHRITIC DOGS: PHASE 2

ABSTRACT:

The objective of this investigation was to evaluate the efficacy and safety of two different dosages of 95% curcumin (100 mg and 500 mg, once daily) in moderately arthritic dogs. Ten client-owned dogs, in a randomized, double-blinded study, received either 500 mg of 95% curcumin once a day (SID) or 100 mg of 95% curcumin once a day, for a period of five months. Dogs were evaluated each month for physical condition (body weight, body temperature, heart rate, and respiratory rate), pain associated with arthritis (overall pain, pain during limb manipulation, and pain after two minutes of physical exertion), and range of motion was measured using a goniometer on the shoulder, elbow, and stifle joints. Serum samples collected from these dogs were examined each month for biomarkers of the liver (total bilirubin, ALT, and AST), kidney (BUN and creatinine), heart and muscle (creatine kinase) functions. Whole blood samples were also analyzed to detect inflammation biomarkers using an Autozero Westergren erythrocyte sedimentation rate test. The findings of this study revealed that dogs receiving 95% curcumin, 500 mg, SID (Group-III) and 95% curcumin, 100 mg, SID (Group-IV) had a significant ($P < 0.0001$) reduction in pain of overall pain, pain of limb manipulation, and pain after physical exertion by day 150. Group-III had a significant reduction in overall pain by day 60 and Group-IV showed significant reduction in overall pain by day 90. Both groups had a significant reduction in pain during limb manipulation and after physical exertion on day 90. Dogs in either group had no significant changes ($P > 0.05$) in physical parameters or serum

markers, suggesting that both treatments, 500 mg and 100 mg of 95% curcumin, were well tolerated by moderately arthritic dogs. It was concluded that both, 500 mg and 100 mg of 95% curcumin, significantly ($P < 0.05$) reduced pain in osteoarthritic dogs and markedly improved their daily life activity without any side effects.

INTRODUCTION

Osteoarthritis is the most common type of arthritis in dogs and is the most common source of chronic pain in older dogs (Gupta et al., 2009; Gupta et al., 2011). Osteoarthritis is a chronic inflammatory joint disease, which causes pain/soreness, stiffness, swelling, and lameness, due to the diminished cushion and changes in the synovial fluid (Vaughn-Scott et al., 1997; Pasquini et al., 2007). Osteoarthritis affects the entire synovial joint, including the cartilage, synovial fluid, and bone. Mechanical stress is thought to induce changes in biochemical factors within affected joints, leading to articular cartilage degradation (Renberg, 2005). All of these changes in the joints and bones can cause pain, swelling, and enlargement of the joints, which can affect the quality of life. Arthritis mainly affects large breed dogs, i.e. German Shepherds, Labradors Retrievers, Siberian Huskies, and Rottweilers, more than small breed dogs. However, presently, one in four dogs are being diagnosed with osteoarthritis in the United States. Dogs that are diagnosed with arthritis tend to display signs of lethargy, have difficulty moving from a sitting or lying position, cracking joints, stiffness, muscle wastage, and visible pain (Gupta et al., 2009; Gupta et al., 2011). Diagnosing osteoarthritis in dogs begins with owners observing the pain and stiffness while the animal is running, walking, jumping, or rising from a lying or sitting position. Properly diagnosing patients with osteoarthritis will help establish a future treatment plan to help ease the pain.

Pharmacological management of osteoarthritis includes steroidal or non-steroidal anti-inflammatory (NSAID) agents. However, these drugs just control pain and inflammation and do not address the underlying issue. NSAIDs work against prostaglandins, which are a family of chemicals that are produced by cells and promote inflammation. NSAIDs have a high risk of toxicity and multiple adverse side effects, due to this; there is a push for alternative treatments in the form of food supplements such as nutraceuticals.

A nutraceutical is defined as a food, typically plant based, which provides medicinal or health benefits, including the prevention and treatment of diseases (Rajat et al., 2012). Currently, there are over 470 nutraceuticals with documented health benefits (Rajet et al., 2012). Curcumin, the active ingredient of turmeric, is isolated from the plant *Curcuma longa*. Curcumin is a member of the curcuminoid family and is closely related to ginger. Curcumin, diarylheptanoid, has been extensively studied for over 30 years, and past studies have shown that curcumin plays a vital role in preventing and treating a wide range of pro-inflammatory chronic diseases such as cardiovascular, pulmonary, autoimmune, and neurodegenerative diseases (Prasad et al., 2014). In addition, curcumin is also known for other medicinal benefits, including anti-inflammatory, anti-oxidant, wound healing, and antimicrobial properties (Prasad et al., 2014). Three main curcuminoids that are seen in commercial supplements are curcumin (curcumin I), demethoxycurcumin (curcumin II), and bis-demethoxycurcumin (curcumin III) (Figure 3.1). These curcuminoids are said to work synergistically and have a greater effect compared to if used alone (Wynn and Fougere, 2008).

According to past studies, curcumin can possibly affect the progression of arthritis by preventing degradation and enhancing the repair of joint cartilage (Sanghi et al., 2008). Curcumin can also inhibit pro-inflammatory transcription factors, nuclear factor-kappaβ, as well

as inhibit inflammatory cytokines, including TNF and cyclooxygenases-2 (Prasad et al., 2014). Overall, curcumin seems to be an ideal alternative treatment due to significant evidence pointing towards it as a potent agent against chronic diseases without being toxic to any metabolic pathways (Prasad et al., 2014).

Despite curcumin's medicinal benefits, the downfalls of curcumin are noted to be its poor aqueous solubility, low bioavailability, and its staining properties (Anard et al., 2009). Curcumin's low bioavailability is due to its poor absorption, bio-distribution, and quick rate of metabolism. Multiple studies have tried increasing curcumin's bioavailability, longer circulation, and resistance to metabolic processes by changing the preparation of the formula to include nanoparticles, micelles, liposomes, and phospholipids (Li et al., 2007; Anard et al., 2009; Li et al., 2011; Prasad et al., 2014). Due to curcumin being a nutraceutical and only having recommended dosages available, it is vital to identify the therapeutic dosage of curcumin anti-inflammatory properties in dogs. In the present investigation, 95% curcumin was evaluated for its therapeutic and safety evaluation in osteoarthritic dogs.

MATERIALS AND METHODS

Animals

Ten client-owned moderately arthritic dogs, weighing between 40-65 pounds, 8 ± 3 years, were used in this study. These dogs, based on signs of joint stiffness, lameness, and degree of range of motion, had pain at the level of moderate arthritis. My inclusion criteria of dogs in this study excluded those having any concurrent diseases (liver, kidney, or heart disease, neoplasia, cancer or any other major disease). All dogs were tested and were heartworm negative for the entire duration of the study. Institutional Animal Care and Use Committee (IACUC) approval and owners' consent were obtained prior to the initiation of this study.

Experimental Design

In a randomized double-blind study, ten client-owned dogs, divided into two groups (n = 5), received 95% curcumin, 500 mg, once a day (Group-III) or 95% curcumin, 100 mg, once a day (Group IV). The study was carried out for a period of five months at Murray State University. None of the dogs received any treatment or supplements four weeks prior to the study or during the study period.

Pain Measurement

At pre-determined intervals (i.e. 30 days), each dog was evaluated for overall pain, pain upon limb manipulation, and pain after physical exertion, for a period of five months. Overall pain, on a scale of 0-10, was graded as: 0, no pain: 2.5, mild pain: 5, moderate pain: 7.5, severe pain: 10, severe and constant pain. Pain after manipulation, on a scale of 0-4, was evaluated as: 0, no pain: 1, mild pain: 2, moderate pain: 3, severe pain: 4, severe and constant pain. Pain after physical exertion, on a scale of 0-4, was evaluated as: no pain: 0, no pain: 1, mild pain: 2, moderate pain: 3, severe pain: 4, severe and constant pain. Range of motion was evaluated with a goniometer and the degree of motion during flexion was noted. The physical examination of each limb started with the forelimbs and ended with the hind limbs. The evaluation focused on manipulation of the limbs in a forward, backward, and circular motion. Three main joints in each dog were evaluated, including the shoulder joint, elbow joint, and stifle joint, which are the top three joints that are affected by osteoarthritis in dogs. Popping and cracking of the joints as well as vocal pain were noted for each canine during examination. Detailed criteria of the measurement of pain are provided in our earlier publications (Deparle et al., 2005; Peal et al., 2007; D'Altilio et al., 2007; Gupta et al., 2009; Gupta et al., 2011; Fleck et al., 2013; Lawley et al., 2013). The present investigation was carried out on moderately arthritic dogs. A moderately

arthritic dog exhibits overall pain of about a 5 on a scale of 0-10, pain upon limb manipulation about a 2 on a scale of 0-4, and pain after physical exertion about a 2 on a scale of 0-4.

Physical Examination

On a monthly basis, dogs were given a full physical examination for body weight, body temperature, heart rate, and respiratory rate (Table 3.1).

Serum Biomarkers Assays

Blood samples were collected from the cephalic vein using a 3 mL syringe with a 22-gauge, 1-inch needle and were stored in a marble top tube (serum separator tubes) and lavender top tube (EDTA tubes). Samples in the marble top tubes were then spun to collect serum and transferred to a red top tube for evaluation. Serum samples were collected each month and analyzed for liver (total bilirubin, ALT, and AST), kidney (BUN and creatinine), heart and muscle (CK) functions, using a Beckman AU 480 serum analyzer. The lavender top tubes were analyzed for a complete blood count, including a five-part differential (neutrophils, lymphocytes, monocytes, eosinophils, and basophils). Whole blood, stored in lavender top tubes, was collected each month to analyze the effects of curcumin on red blood cells and white blood cells (Table 3.4). Whole blood was also analyzed for the presences of inflammation biomarkers by performing an erythrocyte sedimentation rate test (Table 3.5) using the Autozero Westergren erythrocyte sedimentation rate (ESR) system (Globe Scientific Inc.).

Statistical Analysis

The data presented are means ± SEM (n = 5). Statistical significance of difference between each month compared to baseline (day 0) was determined by analysis of variance (ANOVA) coupled with Tukey-Kramer *post-hoc* test ($P < 0.05$) using the Statistical Analysis and Graphics Software for Windows (NCSS9).

RESULTS

On a monthly basis, each dog was examined for pain level (overall pain, pain during limb manipulation, and pain after physical exertion), shown in Figures 3.2-3.4. Overall pain was assessed by the dog's gait, joint range of motion, ability to sit or lie down, and ability to rise from a seated or lying position. Group-III dogs, receiving 500 mg of 95% curcumin, had a significant ($P < 0.0001$) reduction in overall pain by day 60 (4.40 ± 0.29) compared to day 0 (5.90 ± 0.24). The maximum reduction in overall pain was noted on day 150 (2.90 ± 0.29). Group-IV dogs, receiving 100 mg of 95% curcumin, had a significant ($P = 0.002$) reduction in overall pain on day 90 (3.80 ± 0.25) compared to day 0 (6.30 ± 0.43). The maximum reduction in overall pain was noted on day 150 (3.00 ± 0.71).

Pain during limb manipulation was measured in each limb of the dog for flexibility, joint integrity, and vocalization. The pain level during limb manipulation for Group-III was significantly ($P = 0.02$) reduced by day 90 (1.30 ± 0.12), compared to day 0 (2.60 ± 0.18). The pain level during limb manipulation for Group-IV was significantly ($P = 0.04$) reduced by day 60 (1.95 ± 0.16) compared to day 0 (3.00 ± 0.00). The canines were also evaluated for pain after two minutes of jogging. After jogging, pain level was assessed based on the dog's body position, signs of pain or limping, flexibility, and vocalization. Group-III and Group-IV had a notably significant reduction in pain after physical exertion on day 60 (1.60 ± 0.10) and (1.80 ± 0.12), respectively.

Data of physical parameters (body weight, body temperature, heart rate, and respiratory rate) are shown in Table 3.1, and were within normal range. Dogs in both groups did not have any significant changes in any of the physical parameters. Dogs in both groups also did not have any significant changes in the serum biomarkers during the duration of the study (Table 3.4).

Although the erythrocyte sedimentation rate test showed a decreasing trend for both groups, it was not significantly different compared to day 0 (Table 3.5).

DISCUSSION

In the present paper, we report that 95% curcumin at a dose of 500 mg or 100 mg, SID, is effective in reducing arthritic pain and enhancing the daily activity and quality of the dog's life without exerting any side effects. Curcumin administration can aid in alleviating pain in all three categories (overall pain, pain during limb manipulation, and pain after physical exertion) with a maximum reduction noted on day 150. Curcumin, the active ingredient in turmeric, is widely known for its anti-oxidant and anti-inflammatory properties, which makes it a promising nutraceutical for arthritic dogs. In addition to reducing arthritic pain, curcumin has been used as a preventative treatment for bowel disease, pancreatitis, skin conditions, pulmonary and gastrointestinal issues, wound healing, sprains, liver disorders, and cancers. While testing therapeutic dosages, further research needs to be conducted examining the same dosages with a controlled diet and exercise plan.

In conclusion, curcumin offers significant anti-arthritic properties, including reduction of overall pain and inflammation, increasing range of motion, especially in the stifle joint, and help reduce pain during limb manipulation and after physical exertion. All dogs responded well to curcumin administration without experiencing any adverse side effects; therefore, giving the supplement a competitive edge over many other anti-arthritic pharmaceuticals.

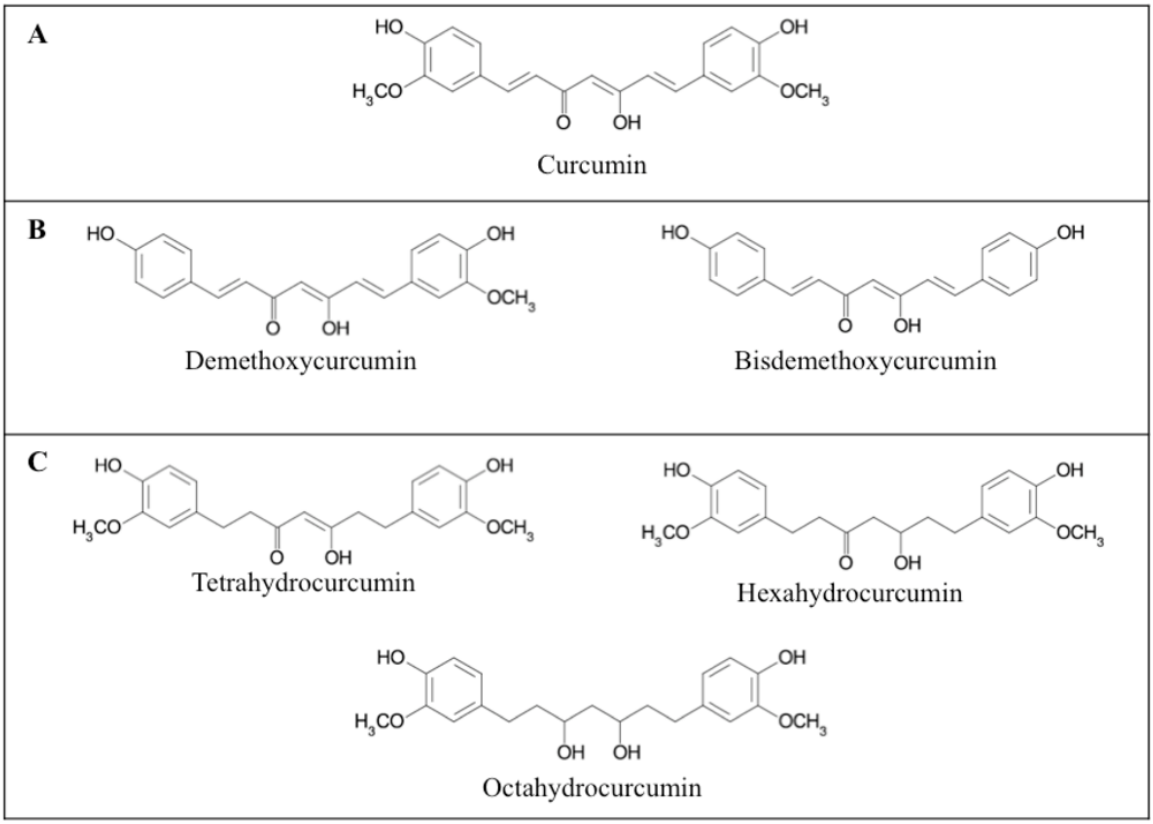

Figure 3.1. Chemical structures of curcumin I, II, and III and derivatives

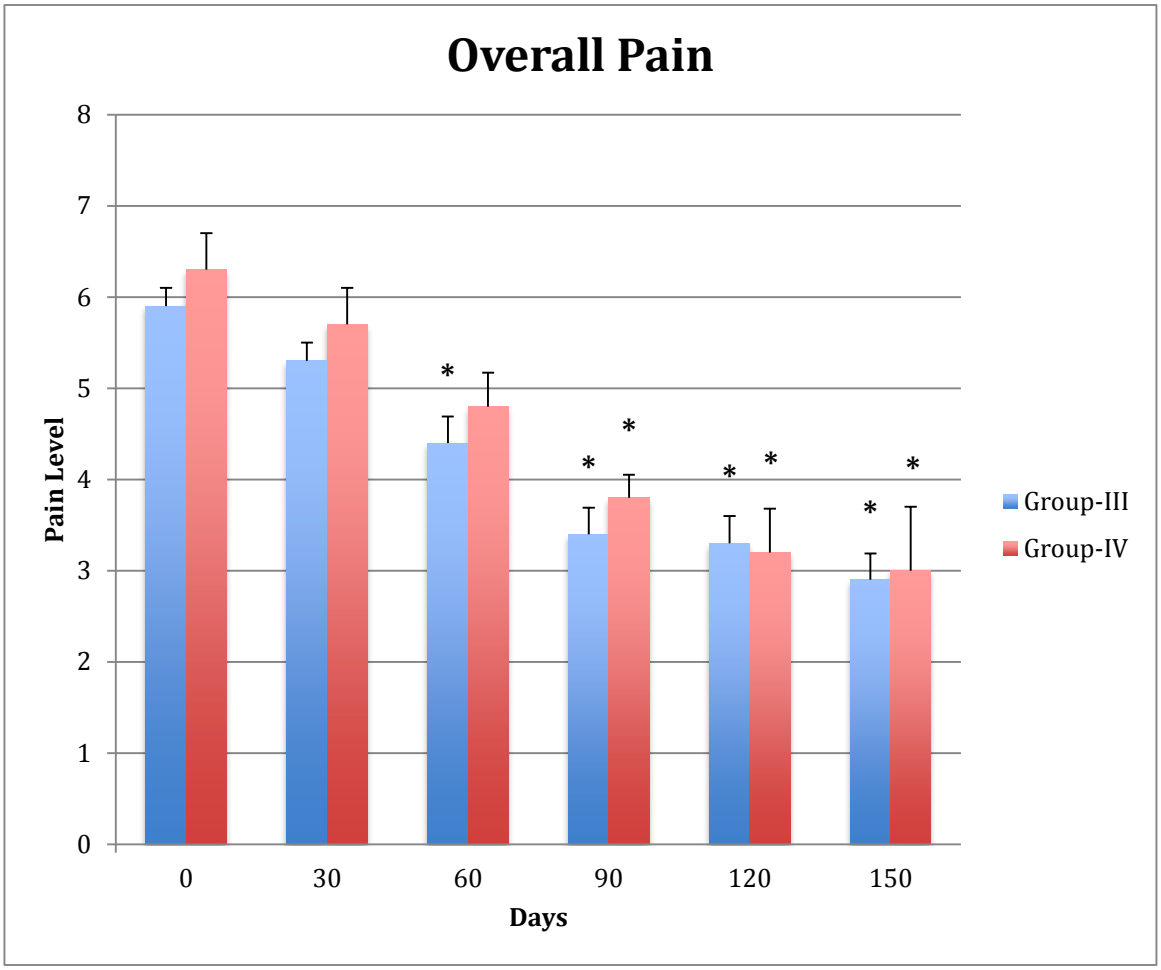

Figure 3.2. Effects of 500 mg of 95% curcumin (Group-III) or 100 mg of 95% curcumin (Group-IV) on overall pain in moderately arthritic dogs.

Overall pain was graded on a scale of 0-10 (0, no pain: 2.5, mild pain: 5, moderate pain: 7.5, severe pain: 10, severe and constant pain).

*Significantly different compared to Day 0 ($P < 0.05$)

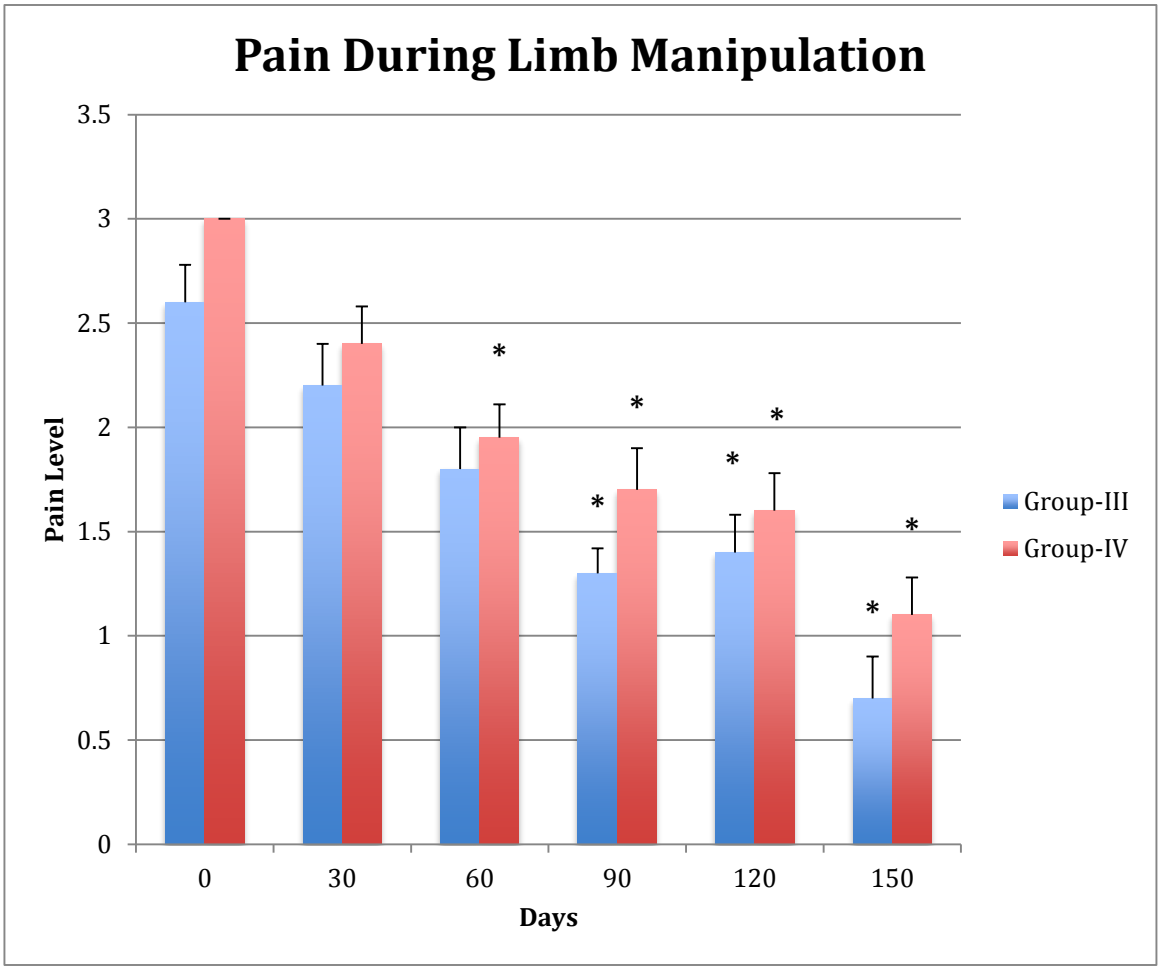

Figure 3.3. Effects of 500 mg of 95% curcumin (Group-III) or 100 mg of 95% curcumin (Group-IV) on pain during limb manipulation in moderately arthritic dogs.

Pain from limb manipulation was graded on a scale of 0-4 (0, no pain: 1, mild pain: 2, moderate pain: 3, severe pain: 4, severe and constant pain).

*Significantly different compared to Day 0 ($P < 0.05$)

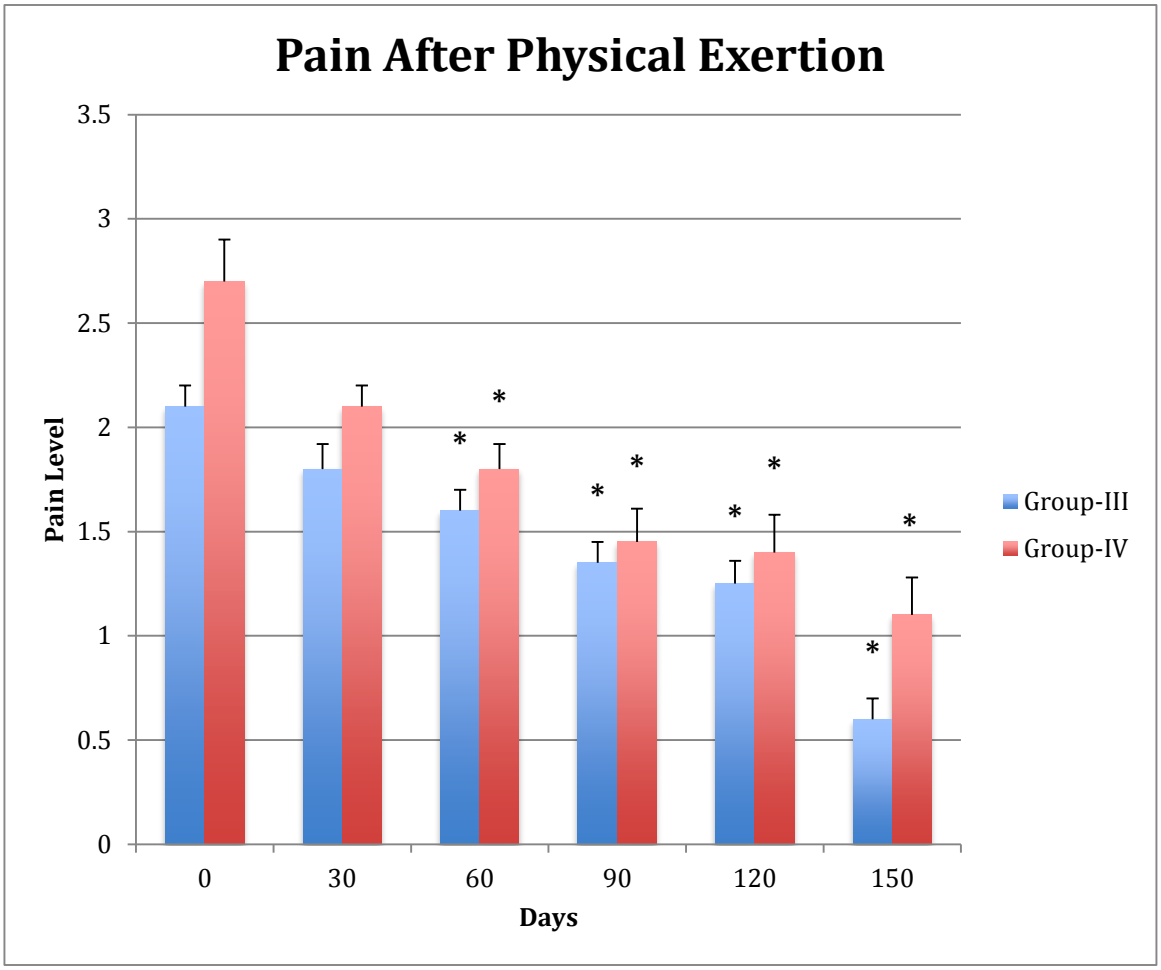

Figure 3.4. Effects of 500 mg of 95% curcumin (Group-III) or 100 mg of 95% curcumin (Group-IV) on pain after physical exertion in moderately arthritic dogs.

Pain from limb manipulation was graded on a scale of 0-4 (0, no pain: 1, mild pain: 2, moderate pain: 3, severe pain: 4, severe and constant pain).

*Significantly different compared to Day 0 ($P < 0.05$)

Table 3.1. Effects of curcumin on physical parameters in osteoarthritic dogs

*Significantly different compared to Day 0 ($P < 0.05$)

Parameters	Group	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150
Body Weight (lbs)	III	58.04± 5.14	58.04± 5.14	57.28± 5.34	57.20± 4.36	57.00± 5.15	55.92± 5.15
	IV	59.56± 6.71	58.40± 6.62	59.60± 6.53	58.48± 5.81	57.84± 5.75	59.20± 5.96
Temperature (°F)	III	101.46± 0.53	100.8± 0.28	100.20± 0.22	101.10± 0.15	100.08± 1.03	100.92± 0.18
Normal range: 101-102.5°F	IV	101.66± 0.25	101.62± 0.19	100.26± 0.66	100.00± 0.12	99.78± 0.34	100.18± 0.20
Heart Rate (bpm)	III	109.20± 14.72	198.80± 7.84	96.40± 14.78	99.60± 9.21	106.80± 5.04	93.60± 12.05
Normal range: 70-160 bpm	IV	123.20± 11.67	135.50± 12.78	120.00± 6.12	110.40± 11.49	108.40± 12.10	102.40± 14.45
Respiratory Rate (bpm)	III	19.66± 2.49	21.60± 2.40	22.00± 2.00	20.60± 1.66	21.60± 2.48	23.60± 2.40
Normal range: 10-35 bpm	IV	23.80± 2.90	24.00± 2.45	22.80± 1.20	23.60± 1.83	24.20± 3.35	27.20± 2.73

Table 3.2. Effects of curcumin on arthritis associated pain level in osteoarthritic dogs

*Significantly different compared to Day 0 ($P < 0.05$)

Parameters	Group	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150
Overall Pain Score (0-10)	III	5.90± 0.24	5.30± 0.20	4.40± 0.29*	3.40± 0.29*	3.30± 0.30*	2.90± 0.29*
	IV	6.30± 0.43	5.70± 0.46	4.80± 0.37	3.80± 0.25*	3.20± 0.49*	3.00± 0.71*
Pain Severity Score (0-10)	III	4.80± 0.49	5.00± 0.42	4.90± 0.85	2.85± 0.34	3.60± 0.92	2.90± 0.29
	IV	5.30± 0.66	4.70± 0.71	4.90± 0.90	3.77± 0.66	3.90± 1.31	3.40± 0.96
Pain Interference Score (0-10)	III	5.66± 0.36	4.73± 0.58	4.29± 0.49	2.82± 0.22*	3.25± 0.95	3.06± 0.60*
	IV	6.36± 0.62	5.58± 0.71	4.69± 0.69	3.96± 0.76	3.87± 1.21	3.70± 1.00
Pain from Limb Manipulation (0-4)	III	2.60± 0.18	2.20± 0.20	1.80± 0.20	1.30± 0.12*	1.40± 0.18*	0.70± 0.20*
	IV	3.00± 0.00	2.40± 0.18	1.95± 0.16*	1.70± 0.20*	1.60± 0.18*	1.10± 0.18*
Pain After Physical Exertion (0-4)	III	2.10± 0.10	1.80± 0.12	1.60± 0.10*	1.35± 0.10*	1.25± 0.11*	0.60± 0.10*
	IV	2.70± 0.20	2.10± 0.10	1.80± 0.12*	1.45± 0.16*	1.40± 1.87*	1.10± 0.18*

Table 3.3. Effects of curcumin on joint flexibility measured by goniometer in osteoarthritic dogs

*Significantly different compared to Day 0 ($P < 0.05$)

Parameters	Group	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150
Right Shoulder	III	69.00± 4.84	55.00± 4.30	57.00± 12.51	70.00± 1.58	74.00± 1.58	82.00± 3.74
	IV	66.00± 10.77	64.00± 4.30	73.00± 3.39	69.00± 3.31	76.80± 4.33	85.00± 3.53
Right Elbow	III	94.00± 4.30	86.00± 1.58	97.00± 7.17	99.00± 8.86	100.00± 6.51	109.00± 4.58
	IV	85.00± 6.70	79.00± 5.78	93.00± 3.74	91.00± 7.81	96.00± 4.00	104.00± 6.96
Right Stifle	III	74.00± 4.00	82.00± 5.15	94.00± 1.87	89.00± 7.64	96.00± 5.56*	110.00± 1.58*
	IV	63.00± 5.14	69.00± 4.30	84.00± 5.09	79.00± 6.20	78.00± 5.14	98.00± 3.39*
Left Shoulder	III	81.00± 4.00	70.00± 2.74	76.00± 4.30	75.00± 2.23	75.00± 3.16	91.00± 1.87
	IV	60.00± 5.70	68.00± 2.00	75.00± 5.70	76.00± 1.86	72.00± 3.74	82.00± 3.39
Left Elbow	III	96.00± 1.00	95.00± 2.74	96.00± 8.12	102.00± 3.39	96.00± 2.45	100.00± 7.41
	IV	87.00± 5.38	87.00± 3.39	88.00± 6.81	96.00± 4.58	98.00± 3.74	103.00± 6.44
Left Stifle	III	57.00± 6.63	66.00± 5.09	78.00± 5.83	93.00± 7.00	94.00± 3.31	103.00± 6.63
	IV	68.00± 5.83	61.00± 8.28	72.00± 2.00	79.00± 4.30*	82.00± 5.83*	97.00± 1.22*

Table 3.4. Effects of curcumin on serum biomarkers of liver, kidney, heart, and muscle functions in osteoarthritic dogs *significantly different compared to Day 0 ($P < 0.05$)

Parameters	Group	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150
Total Bilirubin (mg/dl)	III	0.30± 0.05	0.18± 0.02	0.24± 0.05	0.18± 0.03	0.20± 0.03	0.10± 0.00
Normal range: 0.1-0.6 mg/dl	IV	0.20± 0.05	0.32± 0.10	0.26± 0.04	0.18± 0.04	0.16± 0.02	0.14± 0.02
ALT (IU/L)	III	55.80± 24.44	67.20± 25.18	63.80± 24.58	70.40± 24.50	56.00± 20.53	55.60± 22.32
Normal range: 10-120 IU/L	IV	45.00± 5.30	38.60± 2.91	41.40± 2.01	46.20± 5.80	45.00± 2.05	49.20± 5.75
AST (IU/L)	III	29.40± 2.62	25.40± 1.91	25.20± 2.22	27.80± 3.31	26.2± 1.83	25.60± 2.69
Normal range: 15-65 IU/L	IV	25.40± 2.33	29.00± 4.83	25.20± 0.80	25.60± 1.63	26.80± 1.98	25.80± 2.31
BUN (mg/dl)	III	17.80± 1.71	17.60± 1.21	17.00± 1.58	16.60± 1.60	16.60± 1.77	18.8± 1.39
Normal range: 7-26 mg/dl	IV	15.00± 2.07	15.20± 2.20	13.6± 1.56	13.60± 1.43	12.80± 1.56	14.40± 1.74
Creatinine (mg/dl)	III	1.09± 0.11	0.95± 0.09	1.02± 0.07	0.95± 0.08	0.97± 0.11	0.95± 0.08
Normal range: 0.0-1.35 mg/dl	IV	0.95± 0.09	0.90± 0.10	0.95± 0.10	0.96± 0.09	0.87± 0.08	0.89± 0.08
CK (IU/L)	III	97.80± 16.09	106.00± 28.37	88.00± 19.21	113.20± 22.27	100.60± 13.12	94.40± 14.00
Normal range: 60-450 IU/L	IV	127.6± 45.52	159.00± 41.57	114.00± 20.17	111.20± 21.47	140.80± 21.09	116.80± 19.54

Table 3.5. Effects of curcumin on complete blood count in osteoarthritic dogs

*Significantly different compared to Day 0 ($P < 0.05$)

Parameters	Group	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150
White Blood Cells (/µL)	III	10.46± 0.91	9.29± 1.00	10.00± 1.57	10.08± 1.06	8.64± 0.86	10.00± 1.57
Normal range: 6-17x10^3/µL	IV	8.83± 0.64	8.88± 0.85	8.19± 0.97	8.30± 0.84	7.46± 0.69	8.11± 1.02
Red Blood Cells (/µL)	III	7.37± 0.35	7.20± 0.25	7.35± 0.31	7.29± 0.24	7.55± 0.38	7.35± 0.31
Normal range: 5.5-8.5x10^6/ µL	IV	7.63± 0.24	7.42± 0.34	7.71± 0.31	7.74± 0.33	7.67± 0.24	7.75± 0.38
Hemoglobin (g/dL)	III	17.56± 0.64	17.02± 0.55	17.40± 0.48	17.26± 0.48	17.94± 0.76	17.40± 0.48
Normal range: 12-18 g/dL	IV	18.10± 0.63	17.50± 0.84	18.28± 0.73	18.38± 0.72	18.14± 0.24	18.25± 0.92
Hematocrit (%)	III	50.46± 1.82	52.58± 2.05	51.52± 1.73	51.62± 1.87	53.98± 2.44	51.52± 1.73
Normal range: 37-55%	IV	53.84± 1.52	55.24± 2.10	56.36± 1.13	57.06± 1.43	57.22± 1.77	55.70± 2.19
MCV (fL)	III	68.68± 1.72	73.04± 1.65	70.26± 1.76	70.92± 1.70	71.62± 1.77	70.26± 1.76
Normal range: 60-77 fL	IV	70.74± 2.06	74.62± 1.49	73.38± 2.00	73.94± 2.39	74.66± 1.82	72.12± 2.25
MCH (pg)	III	23.86± 0.41	23.66± 0.39	23.72± 0.46	23.70± 0.51	23.78± 0.35	23.72± 0.46
Normal range: 19.5-24.5 pg	IV	23.72± 0.31	23.58± 0.31	23.74± 0.34	23.78± 0.55	23.64± 0.32	23.57± 0.34

Table 3.5. (Continued)

MCHC (g/dL) Normal range: 32-36 g/dL	III	34.76± 0.36	32.38± 0.29	33.80± 0.35	33.44± 0.38	33.26± 0.46	33.80± 0.35
	IV	33.62± 0.71	31.64± 0.61	32.40± 0.74	32.20± 0.62	31.68± 0.56	32.77± 0.69
Number of Neutrophils (/µL) Normal range: 3-11.5x10^3/ µL	III	6.11± 0.44	5.76± 0.64	6.00± 0.93	6.44± 0.79	5.23± 0.75	6.00± 0.93
	IV	6.01± 0.41	6.29± 0.69	5.70± 0.78	5.58± 0.68	5.04± 0.45	5.22± 0.65
Percentage of Neutrophils (%) Normal range: 60-77%	III	59.30± 4.11	62.28± 4.70	60.02± 3.70	63.52± 3.55	59.82± 4.77	60.02± 3.70
	IV	68.34± 1.70	70.62± 2.14	68.94± 1.49	67.02± 3.04	67.70± 1.73	64.82± 4.46
Number of Lymphocytes (/µL) Normal range: 1-4.8x10^3/ µL	III	3.10± 0.45	2.42± 0.38	2.81± 0.47	2.51± 0.26	2.35± 0.25	2.81± 0.47
	IV	1.79± 0.13	1.58± 0.10	1.64± 0.10	1.65± 0.07	1.34± 0.03	1.71± 2.32
Percentage of Lymphocytes (%) Normal range: 12-30%	III	29.34± 2.73	26.58± 4.13	28.62± 3.03	25.62± 2.97	28.10± 3.44	28.62± 3.03
	IV	20.64± 1.99	18.3± 1.76	20.72± 1.49	20.30± 1.45	18.62± 1.82	21.80± 2.32
Number of Monocytes (/µL) Normal range: 0.1-1.4x10^3/ µL	III	0.42± 0.09	0.43± 0.12	0.43± 0.10	0.50± 0.15	0.37± 0.85	0.43± 0.10
	IV	0.37± 0.05	0.37± 0.04	0.40± 0.07	0.36± 0.06	0.31± 0.04	0.36± 0.07

Table 3.5. (Continued)

Percentage of Monocytes (%) Normal range: 3-10%	III	3.98± 0.69	4.44± 0.70	4.26± 0.60	4.74± 0.95	4.16± 0.59	4.26± 0.60
	IV	4.22± 0.55	4.24± 0.35	4.88± 0.64	4.38± 0.56	4.24± 0.68	4.32± 0.53
Number of Eosinophils (/µL) Normal range: 0.1-1.2x10^3/ µL	III	0.79± 0.25	0.66± 0.27	0.75± 0.29	0.60± 0.21	0.67± 0.18	0.75± 0.29
	IV	0.63± 0.23	0.63± 0.16	0.43± 0.64	0.69± 0.27	0.76± 0.27	0.81± 0.46
Percentage of Eosinophils (%) Normal range: 2-10%	III	7.24± 1.86	6.58± 2.43	6.94± 2.25	5.94± 1.75	7.78± 1.93	6.94± 2.25
	IV	6.74± 2.03	6.76± 1.41	5.32± 0.36	8.22± 2.66	9.36± 2.45	9.02± 3.96
Number of Basophils (/µL) Normal range: 0-0.05x10^3/ µL	III	0.02± 0.01	0.01± 0.00	0.01± 0.00	0.02± 0.00	0.01± 0.00	0.01± 0.00
	IV	0.01± 0.00	0.00± 0.00	0.01± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
Percentage of Basophils (%) Normal range: 0-0.5%	III	0.14± 0.05	0.12± 0.04	0.16± 0.06	0.18± 0.05	0.14± 0.50	0.16± 0.06
	IV	0.06± 0.02	0.08± 0.02	0.14± 0.02	0.08± 0.02	0.08± 0.37	0.02± 0.02
Erythrocyte Sedimentation Rate (mm/hr) Normal range: 0-5mm/hr	III	3.20± 1.20	3.60± 0.51	3.40± 0.81	2.20± 0.37	3.60± 0.51	2.20± 0.20
	IV	2.80± 0.49	4.8± 1.24	2.40± 0.60	2.60± 0.51	2.00± 0.31	2.30± 0.43

CHAPTER 4

EFFECTS OF TURMERIC, CURCUMIN, AND LIPSOMAL-CURCUMIN ON BACTERIA FOUND IN THE EQUINE HINDGUT- AN *IN VITRO* STUDY

ABSTRACT:

The purpose of this study was to investigate both form and dose of turmeric and its active ingredient, curcumin, on reducing opportunistic bacteria found in the equine hindgut. The bacterial strains of interest included *Streptococcus bovis/equinus* complex (SBEC), *Escherichia coli* K-12, *Escherichia coli* general, *Clostridium difficile*, and *Clostridium perfringens*. The first *in vitro*, 24 h batch culture, consisted of the following treatments; 1) control, no nutraceutical (CON); or 500 mg/g of turmeric as 2) 95% turmeric (TUR); 3) 95% curcumin (CUR); or 4) 95% liposomal-curcumin (LIPC). All turmeric treatments significantly decreased ($P = 0.006$) SBEC compared to CON. Both CON and TUR had significantly lower ($P = 0.0001$) concentrations of *C. difficile*. These results, along with the numerical decreases in bacterial concentrations, when compared to CON were the criteria used to select LIPC for the second batch culture. The follow-up *in vitro* 24 h batch culture examined four different dosages (15 g, 20 g, 25 g, and 30 g) of 500 mg/g of LIPC, at reducing the concentration of opportunistic bacteria. These results were utilized to determine the dosing rate in the follow-up study, *in vivo*.

INTRODUCTION

Gut microbiota are one of the densest, most dynamic, and complex microorganism populations located in the body (Costa et al., 2012). Gut flora act against pathogens, aid in digestion and absorption, and stimulate the immune system (Suchodoiski et al., 2012). If the

microbiome is altered, this could result in gastrointestinal diseases such as enterocolitis, diarrhea, colic, and even death. *C. perfringens, C. difficile, E. coli* general and K-12, and *S. bovis/equinus* complex (SBEC) are common bacteria found in the microbiome. These five strains are considered to be opportunistic bacteria, and if the immune system becomes compromised or changes occur to the normal gut flora, this could trigger an increase of opportunistic bacteria that may result in numerous gastrointestinal diseases such as diarrhea and enterocolitis (Suchodoiski et al., 2012).

To help aid in preventing gastrointestinal diseases associated with inflammation, such as enterocolitis, many horse owners supplement their horses with turmeric, at a suggested dosage of 15 g, once daily (Kellon, 2012). Turmeric is a rhizomatous herbaceous perennial plant, *Curcuma longa* Linn, belonging to the ginger family, *Zingiberaceae,* and has been used for thousands of years in Ayurvedic medicine (Chan et al., 2009). Curcumin, the active ingredient in turmeric, has been suggested to have numerous medicinal benefits, including anti-inflammatory, antioxidant, antimicrobial, and wound healing properties, with a relatively low risk of adverse side effects (Zhu et al., 2014). However, due to its low bioavailability, curcumin can be encapsulated in liposomes in hopes to increase bioavailability. Although turmeric and curcumin, are considered relatively safe, little is known about their anti-microbial effects in the equine hindgut and at what dosage rate is it effective. The objective of this first batch culture *in vitro* study was to determine what form of 500 mg/g of turmeric, 95% turmeric, 95% curcumin, or 95% liposomal-curcumin, had the greatest effect on opportunistic bacteria in the equine hindgut microbiome. The follow-up batch culture *in vitro* study was to determine what dosage of 500 mg/g of 95% liposomal-curcumin had the greatest effect on reducing the opportunistic bacteria in the equine hindgut microbiome.

MATERIALS AND METHODS

Animals

Four cecally-cannulated horses (Beard et al., 2011) weighing 522.95 ± 16.59 kg and having a BCS of 5.5 ± 0.5, were used for the two *in vitro* batch culture experiments and in the *in vivo* study. Southern Illinois University Animal Care and Use Committee (Protocol 14-048) approved care and handling of animals used in this study. Cannulated horses utilized in this study had not received any medical treatment one month prior to the start of this study and there were no concurrent medical issues between experiments nor during any of the studies.

Batch Culture

Treatments and Sample Collection

Two, 24 h, *in vitro* batch cultures were conducted to determine the form and dose of turmeric to be utilized *in vivo*. The first *in vitro* batch culture examined which form of the nutraceutical, turmeric, at 500 mg/g, 95% turmeric, 95% curcumin, or 95% liposomal-curcumin (with the other 5% comprising of cellulose, magnesium stearate, vegetable source, and silicon dioxide) (Life Xtend Labs, Las Vegas, NV), had the greatest effect on reducing opportunistic bacteria, *E. coli* general and K-12, *C. difficile*, *C. perfringens*, and SBEC, found in the hindgut of equine. Erlenmeyer flasks (125 mL) were randomly assigned to one of the following treatments, in quadruplicate: 1) control, no nutraceutical (CON); or 500 mg/g of turmeric as 2) 95% turmeric (TUR); 3) 95% curcuma (CUR); or 4) 95% liposomal-curcumin (LIPC). Dosages (0.025 g, 0.033 g, 0.042 g, and 0.05 g) were based off recommended dosage of 500 mg/g of turmeric at 15 g per 454.54 kg horse (Farinacci et al., 2009; Casie, 2014). Erlenmeyer flasks also contained 0.50 g (Bailey et al., 2003) of ground alfalfa hay.

Cecal fluid was collected from four cecally-cannulated horses (2.5 L/horse) and composited to eliminate animal variation. Cecal samples were filtered through eight layers of cheesecloth to remove fibrous debris. Prior to incubation in the water bath, a 0 hour sample was collected, stored in a 15 mL conical tube, and frozen at -80° C for later analysis. Composited cecal fluid was mixed with McDougall's buffer at a 1:4 ratio (Bailey et al., 2003). Then 50 mL of cecal fluid-buffer mix was poured into 16-125 mL Erlenmeyer flasks, degassed with CO_2, and placed in a water bath at 39° C. Flasks were manually shaken every two hours for 24 h. At 24 h, the flasks were pulled from the water bath and total contents were aliquoted into 15 mL conical tubes and frozen at -80° C for subsequent laboratory analysis.

The second *in vitro* 24 h batch culture examined the effect of dose on bacteria concentrations when supplementing LIPC. The LIPC treatment was selected based on results from the first *in vitro* batch culture. Erlenmeyer flasks (125 mL) were randomly assigned to one of the following treatments, in quadruplicate: 500 mg/g of LIPC at 1) recommended dose, (15); 2) 1.33 times the recommended dose, (20); 3) 1.66 times the recommended dose (25); or 4) two times the recommended dose, (30). Dosages were based off recommended dosage of 500 mg/g of turmeric at 15 g per 454.54 kg horse (Farinacci et al., 2009; Casie, 2014) and were increased in 5 g increments up to two times the recommended dose. The 125 mL Erlenmeyer flasks also contained 0.50 g (Bailey et al., 2003) of ground alfalfa hay. The *in vitro* protocol was the same as previously discussed except for pH measurements taken at 0 and 24 h with an Oakton pH 110 Advanced Portable Meter (Vernon Hills, IL).

Growth of Bacteria

Pure cultures of selected opportunistic bacteria were grown and used as standards for qPCR. Luria-Bertani medium was made for *E. coli* general and K-12, and Trypticase soy yeast

extract medium was made for SBEC, according to Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Germany) media recipes. *Clostridium* medium was made for both *C. difficile* and *C. perfringens*, according to Difco™ (Becton, Dickson and Company, Sparks, MD). Ten mL of broth was pipetted into glass Hungate tubes and deoxygenated with nitrogen. Rubber stoppers and metal caps were crimped on the tubes and then autoclaved at 121° C, 15 psi, for 15 min. Hungate tubes were inoculated with pelleted strains of *E. coli*, *C. difficile*, *C. perfringens*, and SBEC. Dense bacterial samples were transferred to a new Hungate tube every three days for 10 d to ensure pure cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Germany)).

DNA Extraction

DNA was extracted from the cecal fluid samples using PowerSoil Mo Bio DNA Extraction Kits (Mo Bio Laboratories, Carlsbad, CA). The pure cultures that were used as standards for qPCR (Bio-Rad MyiQ Optical System Software 2.0) were extracted using PowerSoil Mo Bio DNA Extraction Kits (Mo Bio Laboratories, Carlsbad, CA) and then purified using UltraClean 15 DNA Purification Kits (Mo Bio Laboratories, Carlsbad, CA). All DNA extractions were assessed for concentration and quality using a Nano Drop ND-1000 Spectrophotometer (Wilmington, DW).

Real Time PCR

All real-time PCR runs were performed in triplicate, and each reaction mixture was prepared using Maxima SYBR Green/ROX qPCR (Thermo Scientific, Waltham, MA). The total volume of the reaction mixture consisted of 216 ng of sample, 12.5 µL 1X SYBER Green master mix, and 2 uL of forward and reverse primers (Table 4.1). Standards were set to 10-fold dilution at 35, 3.5, 0.35, 0.035, 0.0035, 0.00035, 0.000035, and 0.0000035 ng, in duplicates. The thermal

cycling protocol for *E. coli* general and K-12 were as follows: initial denaturation for 10 min at 95° C, followed by 40 cycles of 5 s at 95° C, 5 s at 60° C, and 5 s at 72° C. After amplification, the melting peak was cooled down over 15 s to 65° C (Lee et al., 2007). The thermal cycling protocol for *C. difficile* was as follows: initial denaturation for 10 min at 95° C, followed by 45 cycles of 15 s at 95° C and one min at 60° C. After amplification, the reaction mixture was heated over 15 s to 65° C, for the melt curve (Avbersek et al., 2011). The thermal cycling protocol for *C. perfringens* was as follows: initial denaturation for 10 min at 95° C, followed by 45 cycles of 15 s at 95° C, 20 s at 56° C, and 20 s at 72° C. After amplification, the reaction mixture was heated over 10 s to 65° C, for the melt curve (Karpowicz et al., 2009). The thermal cycling protocol for SBEC was as follows: initial denaturation for 10 min at 95° C, followed by 40 cycles of 15 s at 95° C, 30 s, annealing at 60° C, and a 10 s melting curve at 65° C (Hastie et al., 2008).

Statistical Analysis

The *in vitro* experiments were analyzed as a completely randomized design using the MIXED procedure of SAS (SAS 9.4 Inst., Inc., Cary, NC). Flask was the experimental unit and the model included the effect of treatment. The significance level was set at ($P \leq 0.05$).

RESULTS AND DISCUSSION

All nutraceutical treatments significantly decreased ($P = 0.006$) SBEC concentrations compared to CON, but CUR and LIPC significantly increased ($P = 0.001$) *C. difficile* compared to CON (Table 4.2). It is possible that the treatments did not decrease all the opportunistic bacteria for two reasons. First, this was only a 24 h *in vitro* batch culture using cecal fluid and may not have been long enough to see significant decreases (Bailey et al., 2003). Second, it may be possible that the dose used, was not enough (Farinacci et al., 2009; Casie, 2014). Curcumin,

the active ingredient of turmeric, is known and widely used for its medical benefits, including anti-inflammatory, antioxidant, antimicrobial, wound healing, and anti-tumor properties (Robson, 1959). However, curcumin has a low bioavailability due to its hydrophobic properties, low intrinsic activity, poor absorption, and high rate of metabolism and elimination from the body (Anard et al., 2009). To improve this, when encapsulated in liposomes, which are highly hydrophilic, curcumin may potentially have increased bioavailability, which increases its beneficial potency. Based on the literature and the results of this study, LIPC was utilized in the second *in vitro* batch culture to examine the effects of increasing doses on opportunistic bacteria concentrations.

Follow-up In Vitro

In the follow-up *in vitro* study, every flask had a pH within the normal equine cecum pH range of 6.5-7.1 (data not shown) and was not significantly different ($P = 0.54$) among treatments (Willard et al., 1977). The recommended dose (15) significantly decreased ($P < 0.0001$) SBEC concentrations compared to increasing doses of LIPC (Table 4.3). *E. coli* substrain K-12 concentrations were decreased ($P = 0.01$) with 15 and 20 treatments compared to 25 and 30 treatments. Concentrations of *E. coli* general were significantly less ($P = 0.03$) for 15, 20, and 30 compared to the 25 treatment. Doses of LIPC had no effect ($P \geq 0.42$) on *C. difficile* and *C. perfringens*, but numerically, 30 had the lowest concentration of *C. difficile*, and 25 had the lowest concentration of *C. perfringens* out of the four treatments. Previous work, with human subjects, showed *E. coli* substrain K-12 possesses curcumin-converting activity, which is responsible for curcumin transformation and slowing down the degradation and metabolic process of curcumin (Hassaninasab et al., 2011). Thus, increasing the dosage of LIPC may have increased the growth activity of *E. coli* general and K-12. In addition, due to curcumin being

broken down in the cecum of humans, this could have had a direct effect on these strains, and may be the reason for the observed responses (Hassaninasab et al., 2011). A follow-up study needs to be conducted examining the therapeutic effects of liposomal-curcumin and determine if oral dosing at different dosages elicits negative side effects.

CONCLUSION

In conclusion, based on the literature and previous work on turmeric and its active ingredient, curcumin, and their medical properties, this study supports the theory that encapsulating curcumin in liposomes is associated with increased bioavailability, potentially resulting in heightened medicinal benefits, specifically, antimicrobial properties compared to non-encapsulated forms. In the second *in vitro* batch culture, there was an unexplainable concentration response that may or may not have been related to curcumin-converting enzyme activity. Liposomal-curcumin demonstrated antimicrobial properties in reducing opportunistic bacteria, including *C. perfringens, C. difficile, E. coli* general and K-12, and SBEC, which are documented for causing foal-heat diarrhea, enterocolitis, and colic. Future *in vivo* studies are required to determine the causes of the concentration responses seen in these *in vitro* 24 h batch cultures.

Table 4.1. Forward and reverse primers used for qPCR in five opportunistic strains of bacteria found in equine cecal fluid

Strains	Forward Primers (5'-3')	MW (g/mol)	Conc (nmol)	Reverse Primers (5'-3')	MW (g/mol)	Conc (nmol)
SBEC[1]	GCCTACATGAAGTCGGAATCG	6455.3	59.2	CAAGTTGAGCGATTTACTTCGGTAA	6455.3	61.3
E. coli K-12[2]	TACAAGGCCGGGAACGTA	6119.0	60.4	CTAATCAGACGCGGGTCCAT	6188.1	62.0
E. coli general[3]	GCTACAATGGCGCATACAAA	6386.3	52.6	AAATGTAACAGCAGGGGCA	6396.3	54.5
C. difficile[4]	TTCATGGAGTCGAGTTGCAG	7696.1	64.5	TGAAATTGCAGCAACTCTAGC	6102.0	59.3
C. perfringens[5]	GTTAATACCTTTGCTCATTGA	5894.9	58.3	ACCAGGGTATCTAATCCTGTT	6414.3	32.4

[1]*Streptococcus bovis/equinus* complex (Hastie et al., 2008)

[2]*Escherichia coli* general (Lee et al., 2007)

[3]*Escherichia coli* substrain K12 (Lee et al., 2007)

[4]*Clostridium difficile* (Magdesian and Leutenegger, 2010)

[5]*Clostridium perfringens* (Karpowicz et al., 2009)

Table 4.2. Effects of 500 mg/g of 95% turmeric, 95% curcumin, and 95% liposomal-curcumin, on opportunistic bacteria (ng/μL) found in equine cecal fluid

	Treatment[1]					
Strains	CON	TUR	CUR	LIPC	SEM	*P*-value
SBEC[2]	9.53E+04[a]	3.30E+04[b]	3.12E+04[b]	1.66E+04[b]	1.31E+04	0.006
E. coli K-12	1.96E+01	1.08E+02	1.58E+01	3.80E+01	4.70E+01	0.51
E. coli general	1.67E+01	2.20E+01	8.06E+01	2.62E+01	1.87E+01	0.11
C. difficile	5.80E-01[a]	1.06[ab]	4.23[c]	2.07[b]	3.77E-01	0.0001
C. perfringens	5.20E-01	1.26	2.10E-01	1.40E-01	4.09E-01	0.24

[a-c]Means ± SEM within a row with different superscripts differ ($P < 0.05$).

[1]Treatments: CON = control (no nutraceutical); TUR = 0.025 g of 500 mg/g 95% turmeric; CUR = 0.025 g of 500 mg/g 95% curcumin; LIPC = 0.025 g of 500 mg/g 95% liposomal-curcumin.

[2]*Streptococcus bovis/equinus* complex

Table 4.3. Effects of different dosages of 500 mg/g of 95% liposomal-curcumin on opportunistic bacteria (ng/μL) found in equine cecal fluid

	Treatment[1]					
Strains	15	20	25	30	SEM	*P*-value
SBEC[2]	5.49E+09[a]	1.79E+11[b]	5.07E+13[c]	2.60E+12[d]	2.73E+07	<0.0001
E. coli K-12	7.93E+03[a]	1.30E+04[a]	2.86E+06[b]	3.39E+06[b]	7.67E+05	0.01
E. coli general	1.30E+02[a]	9.60E+01[a]	2.08E+04[b]	4.81E+03[a]	4.85E+01	0.03
C. difficile	2.14E+03	1.74E+03	2.15E+01	1.07	1.33E+03	0.56
C. perfringens	5.20E-01	1.74E-02	2.06E-01	6.56E+01	3.20E+01	0.42

[a-d]Means ± SEM within a row with different superscripts differ ($P < 0.05$).

[1]Treatments: 15 = 0.025 g of 500 mg/g 95% liposomal-curcumin; 20 = 0.033 g of 500 mg/g 95% liposomal-curcumin; 25 = 0.042 g of 500 mg/g 95% liposomal-curcumin; 30 = 0.05 g of 500 mg/g 95% liposomal-curcumin.

[2]*Streptococcus bovis/equinus* complex

CHAPTER 5

EFFECTS OF LIPSOMAL-CURCUMIN ON BACTERIA FOUND IN THE EQUINE HINDGUT- AN *IN VIVO* STUDY

ABSTRACT:

The purpose of this study was to investigate the dose of turmeric and its active ingredient, curcumin, on reducing opportunistic bacteria found in the equine hindgut. The bacterial strains of interest included, *Streptococcus bovis/equinus* complex (SBEC), *Escherichia coli* K-12, *Escherichia coli* general, *Clostridium difficile*, and *Clostridium perfringens*. This study utilized four cecally-cannulated horses to determine the efficacy of antimicrobial and anti-inflammatory properties when oral dosing, increasing levels of liposomal-curcumin dosed at 15, 25, and 35 g compared to a control. *In vivo*, liposomal-curcumin's antimicrobial properties, at 15 g, significantly decreased ($P = 0.02$) SBEC compared to other treatments. In addition, *C. perfringens* tended ($P = 0.12$) to decrease as liposomal-curcumin doses increased. Acetate tended to increase linearly ($P = 0.10$), as the dose of liposomal-curcumin increased. Valerate was greatest ($P = 0.02$) in control horses compared to liposomal-curcumin treated horses. Butyrate tended ($P = 0.01$) to increase in the control group and horses dosed liposomal-curcumin at 15 g. Treatment did not affect any of the other individual VFAs measured ($P \geq 0.54$), but increasing doses of liposomal-curcumin tended ($P = 0.10$) to increase total VFA concentrations. Lastly, no adverse side effects were observed, suggesting these dosages are relatively safe. In regard to these findings, further projects need to be conducted to examine oral administration of 95% liposomal-curcumin with a longer acclimation period to study the potential therapeutic anti-inflammatory properties and anti-microbial properties on the cecal microbiome of horses.

INTRODUCTION

Horses may suffer from inflammation issues in their gastrointestinal (GI) tract, colic, enterocolitis, diarrhea, and inflammatory bowel disease, and joints, arthritis, osteochondritis, and bursitis. Additionally, due to the magnitude of stress applied to the joints, cartilage begins to break down, resulting in pain, inflammation, and osteoarthritis (Todhunter and Lust, 1990). Inflammation in horse's GI tract can cause enterocolitis and colic due to physiological changes that can affect the balance of the microbiome in the cecum (Todhunter and Lust, 1990). If the microbiome is changed, this could result in gastrointestinal disease and even death. *C. perfringens, C. difficile, E. coli* general and K-12, and SBEC are common bacteria found in the microbiome of the hindgut. These strains are considered opportunistic bacteria, and inflammation, stress, and pain along with a compromised immune system changes the hindgut microbiome which can trigger proliferation of harmful and opportunistic bacteria that can cause numerous gastrointestinal diseases. Both *C. perfringens* and C. *difficile* can cause colitis and diarrhea in horses, *E. coli* can lead to enterocolitis, and SBEC can affect the lactic acid bacteria concentration in the hindgut and can trigger inflammatory cytokines (Mackie and Wilkins, 1988; Todhunter and Lust, 1990).

Commonly, osteoarthritis and GI diseases caused from dysbiosis or inflammation can be managed with non-steroidal anti-inflammatory drugs (NSAIDs) to reduce pain (Ricciotti and FitzGerald, 2011). NSAIDs are used to alleviate pain and inflammation by suppressing eicosanoids and inhibiting cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Since NSAIDs inhibit COX-1, common side effects are GI bleeding, ulcers, colitis, and colic (Farinacci et al., 2009). Due to the many adverse side effects, there is a high demand for alternative treatments, such as nutraceuticals.

Curcumin is the active ingredient in turmeric, *Curcuma longa*. Over the past 30 years, studies in horses, humans, and mice, have shown that curcumin has properties that can manage and prevent a magnitude of inflammatory diseases while being nontoxic to metabolic pathways (Prasad et al., 2014). Curcumin also possesses antimicrobial properties, which have been studied in humans, chickens, and horses (Mackie and Wilkins, 1988; Prasad et al., 2014; El-Bahy and Bazh, 2015). The antimicrobial properties could aid in preventing pathogenic and opportunistic bacteria found in the hindgut from proliferating and causing GI issues. However, while curcumin seems to be the ideal alternative treatment for a wide variety of diseases, it has poor bioavailability (Prasad et al., 2014). Curcumin's poor bioavailability, observed in humans, dogs, and mice, is related to its hydrophobic properties and quick elimination from the body (Prasad et al., 2014). However, past studies have speculated that encapsulating curcumin in liposomes could increase its bioavailability (Prasad et al., 2014). The primary objective of this research was to evaluate the antimicrobial properties of liposomal-curcumin (LIPC) in an *in vivo* study. The secondary objective was to determine if increasing doses would elicit a negative effect on cecal characteristics and blood parameters. And lastly to examine range of motion and therapeutic effects of orally administered liposomal-curcumin in cecally-cannulated horses.

MATERIALS AND METHODS

Animals

Four Southern Illinois University-owned, cecally-cannulated horses (Beard et al., 2011), weighing 522.95 ± 16.59 kg and having a BCS of 5.5 ± 0.5, were used for the two *in vitro* batch culture experiments and in the *in vivo* test. Southern Illinois University Animal Care and Use Committee (Protocol 14-048) approved care and handling of animals used in this study. None of the horses utilized in this study were given any medication one month prior to the start of this

study or between experiments and they had no concurrent medical issues at the initiation of, nor during the study.

Treatment and Sample Collection

Four cecally-cannulated horses were utilized in a 4 x 4 Latin square to evaluate increasing doses of LIPC on the same opportunistic bacteria stated in Chapter 4, and to evaluate cecal characteristics as well as inflammation. Horses were randomly assigned to one of four treatments: 1) no LIPC, (0); 2) 15 g of 500 mg/g of LIPC, recommended dose (15); 3) 25 g of 500 mg/g of LIPC (25); or 4) 35 g of 500 mg/g of LIPC (35). Horses were fed 2-3 lbs of Strategy® (Purina Mills, St. Louis, MO), once a day at 0600 and the treatments were top-dressed on the grain. Strategy was used for treatment delivery and to maintain a BCS of 5-6. Once grain and treatment was consumed horses were then turned out to pasture (predominantly K31 Tall Fescue) and allowed to graze until 1600, at which time they were stalled and fed hay that was cut from the same pasture the previous hay season. This was the daily procedure with the exception of d 9 for each period, during which they were stalled all day and had *ab libitum* access to hay and water after complete consumption of Strategy® and treatment.

Each period was 14 days with a 9 d acclimation period and a 5 d withdrawal period (Weese et al., 2003; Farinacci et al., 2009). Cecal fluid was collected at 0 h on d 0 and 8, and again on d 9 at 0, 3, 6, 9, 12, 15, 18, and 21 h. Whole cecal contents (100 mL) were collected, pH recorded (Oakton pH 110 Advanced Portable Meter (Vernon Hills, IL), subsampled (15 mL), and immediately frozen for later analysis of opportunistic bacteria. On d 9, after pH was recorded, contents were filtered through eight layers of cheesecloth into a 15 mL collection tube and immediately frozen for later analysis of VFA and ammonia concentrations. Blood was also collected via jugular venipuncture on d 0 and 8 into a serum separator tube and a 7.5%

Ethylenediaminetetraacetic acid (EDTA) tube (Coviden, Mansfield, MA) for chemistry panel analysis, complete blood count analysis, and erythrocyte sedimentation rate assessment. On d 0 and 8, range of motion was measured for the knee and hock joints, using a universal goniometer (Valley Vet, Marysville, KS) (McGann et al., 2013).

Growth of Bacteria

Pure cultures of selected opportunistic bacteria were grown and used as standards for qPCR. Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) was made for *E. coli* general and K-12, and Trypticase soy broth (30 g/L) and yeast extract (3 g/L) medium was made for SBEC, according to Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Germany) media recipes. *Clostridium* medium (17 g/L digest casein, 3 g/L digest soy, 5 g/L NaCl, 2.5 g/L K_2Pho4 and dextrose) was made for both *C. difficile* and *C. perfringens*, according to Difco™ (Becton, Dickson and Company, Sparks, MD). Ten mL of broth was pipetted into glass Hungate tubes and deoxygenated with nitrogen. Rubber stoppers and metal caps were crimped on the tubes and then were autoclaved at 121° C, 15 psi, for 15 min. Hungate tubes were inoculated with pelleted strains of bacteria, *E. coli*, *C. difficile*, *C. perfringens*, and SBEC. Dense bacterial samples were transferred to a new Hungate tube every 3 d for 10 d to ensure pure cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Germany)).

DNA Extraction

DNA was extracted from the cecal fluid samples using PowerSoil Mo Bio DNA Extraction Kits (Mo Bio Laboratories, Carlsbad, CA). The pure cultures that were used as standards for qPCR (Bio-Rad MyiQ Optical System Software 2.0) were extracted using PowerSoil Mo Bio DNA Extraction Kits (Mo Bio Laboratories, Carlsbad, CA) and then purified

using UltraClean 15 DNA Purification Kits (Mo Bio Laboratories, Carlsbad, CA). All DNA extractions were assessed for concentration and quality using a Nano Drop ND-1000 Spectrophotometer (Wilmington, DW).

Real Time PCR

All real-time PCR runs were performed in triplicate, and each reaction mixture was prepared using Maxima SYBR Green/ROX qPCR (Thermo Scientific, Waltham, MA). The total volume of the reaction mixture consisted of 216 ng of sample, 12.5 μL 1X SYBER Green master mix, and 2 uL of forward and reverse primers (Table 4.1). Standards were set to 10-fold dilution at 35, 3.5, 0.35, 0.035, 0.0035, 0.00035, 0.000035, and 0.0000035 ng, in duplicates. The thermal cycling protocol for *E. coli* general and K-12 were as follows: initial denaturation for 10 min at 95° C, followed by 40 cycles of 5 s at 95° C, 5 s at 60° C, and 5 s at 72° C. After amplification, the melting peak was cooled down over 15 s to 65° C (Lee et al., 2007). The thermal cycling protocol for *C. difficile* was as follows: initial denaturation for 10 min at 95° C, followed by 45 cycles of 15 s at 95° C and one min at 60° C. After amplification, the reaction mixture was heated over 15 s to 65° C, for the melt curve (Avbersek et al., 2011). The thermal cycling protocol for *C. perfringens* was as follows: initial denaturation for 10 min at 95° C, followed by 45 cycles of 15 s at 95° C, 20 s at 56° C, and 20 s at 72° C. After amplification, the reaction mixture was heated over 10 s to 65° C s for the melt curve (Karpowicz et al., 2009). The thermal cycling protocol for SBEC was as follows: initial denaturation for 10 min at 95° C, followed by 40 cycles of 15 s at 95° C, 30 s, annealing at 60° C, and a 10 s melting curve at 65° C (Hastie et al., 2008).

Serum Chemistry Panel and Complete Blood Count Assays

Blood samples were separated via centrifugation to collect serum and transferred to a red top tube for evaluation (Coviden, Mansfield, MA). Serum was analyzed for liver (total protein, ALT, and ALP), kidney (BUN and creatinine), and blood glucose level with an Abaxis VetScan V2 (Union City, CA). Blood collected into tubes containing 7.5% EDTA, was analyzed for complete blood count, including a five-part differential (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) with an Abaxis VetScan HM5 (Union City, CA). Whole blood was also analyzed for inflammation by performing an erythrocyte sedimentation rate test using the Autozero Westergren Erythrocyte Sedimentation Rate (ESR) system (Globe Scientific Inc., Paramus, NJ).

Ammonia and Volatile Fatty Acid Analysis

Cecal NH_3 concentrations were determined by the phenol-hypochlorite procedure (Broderick and Kang, 1980). Cecal fluid was analyzed for VFA concentrations (Goetsch and Galyean, 1983) using a Shimadzu GC-2010 gas chromatograph (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with a flame-ionization detector and 30-m SP-2560 fused silica capillary column (Restek Stabil WAX DA column, Bellefonte, PA). The internal standard 2-ethyl butyrate was used for VFA analysis (Goetsch and Galyean, 1983). The helium carrier gas was maintained at a linear velocity of 23 cm/s. The oven temperature was programmed to 65º C for 3 min, increased at 12º C/min to a final temperature of 225º C, which was held for 9 min. The column temperature was maintained at 65º C and the flame ionization detector temperature at 225º C.

Statistical Analysis

Bacterial concentrations, ESR, chemistry panel data, complete blood count data, and goniometer range of motion data was analyzed using the MIXED procedure of SAS (SAS 9.4

Inst., Inc., Cary, NC) using the model for a Latin square design with a Tukey *post-hoc* adjustment. The model included treatment and period with animal specified in the RANDOM statement of SAS. Cecal fermentation data (NH_3, pH, and VFA) were analyzed using the MIXED procedure of SAS for repeated measures. The model included period, treatment, and time as well as treatment × time interactions. The RANDOM statement of SAS included the interaction of period × time within subject. An autoregressive covariance structure (AR1 of the MIXED procedure of SAS) was determined to be most appropriate based on Akaike's Information Criterion. There were no treatment x time interactions; therefore, only treatment means are reported. Comparisons of main effects were determined using least square means and Fisher's protected LSD. Calculation of coefficients for linear orthogonal polynomials with unequal spacing was done using IML of SAS (Robson, 1959). Significance was set at ($P = 0.05$) and tendency was set at ($P \leq 0.15$).

RESULTS AND DISCUSSION

Based on the results of the batch cultures, in the previous chapter, the authors decided to investigate 15 g, 25 g, and 35 g of 500 mg/g of 95% LIPC for the *in vivo* study. Day 0 samples confirmed that opportunistic bacteria were present. SBEC bacterial concentrations increased linearly ($P = 0.008$) as LIPC dose increased, but concentrations were similar for 0 and 15 (Table 5.1). As the dose of LIPC increased the concentration of *C. perfringens* decreased linearly ($P = 0.03$). The remaining three opportunistic bacteria strains, *C. difficile*, *E. coli* general, and *E. coli* K-12, were not significant ($P \geq 0.20$) across treatments. Numerically, 25 had the lowest concentration of *E. coli* general, and 0 had the lowest concentration of *C. difficile*. Although increasing the dose of LIPC decreased *C. perfringens* the observation that increasing the dose also increases SBEC would suggest that there may be no additional benefit of dosing LIPC

above the recommended rate. However, this would also suggest that the nutraceutical reaches the cecum without being compromised in the stomach during the digestion process.

Parameters of inflammation, including ESR ($P = 0.87$) and range of motion ($P \geq 0.24$) were not significant among treatments (Table 5.2). Additionally, all blood work was within normal ranges (data not shown), suggesting that increasing the dose of LIPC has no negative effect. Interestingly, a numerical basis suggested that, 35 had the least amount of inflammation detected on the ESR test on d 8. The acclimation period of eight days may not have been long enough to show a significant improvement in inflammation blood parameters and range of motion. Therefore, further research needs to be conducted using LIPC over an extended period of time, such as 30 days, when looking at potential for decreasing inflammation parameters. A longer acclimation period may give the body enough time to respond to the anti-inflammatory properties and may yield a noticeable change in lameness, stiffness/soreness, and inflammation (Farinacci et al., 2009).

There was no treatment x time interactions so only treatment means are reported. Cecal fluid pH was not significant among treatments ($P = 0.82$) (Table 5.3). All cecal pH measurements were within the normal range for the horse cecum, 6.5 - 7.1 (Anard et al., 2009). Cecal fluid ammonia concentrations were not significant among treatments ($P = 0.21$); however, concentrations tended ($P = 0.11$) to decrease linearly as LIPC dose increased. Valerate was significantly different among treatments ($P = 0.02$) with 0 having the greatest concentration compared to all other treatments. Moreover, valerate decreased linearly ($P = 0.005$) as LIPC dose increased. As LIPC dose increased, butyrate and iso-valerate decreased linearly ($P \leq 0.03$). However, acetate tended to increase linearly ($P = 0.10$), as the dose of LIPC increased. Treatment did not affect any of the other individual VFAs measured ($P \geq 0.19$), but increasing

doses of LIPC tended ($P = 0.10$) to increase total VFA concentrations. Additionally, LIPC tended ($P = 0.11$) to increase total VFA concentrations when compared to 0. This data not only suggests that administering LIPC at the recommended rate, 15 g, 25 g, or 35 g, for eight days, does not cause adverse side effects on digestion, inflammation, and blood parameters, but that when dosed for a longer period of time may increase digestibility (Bergman, 1990). Therefore it is being suggested that this nutraceutical is relatively safe, even at higher dosages (El-Bahy and Bazh, 2015).

CONCLUSION

In conclusion, based on the literature and previous work on turmeric and its active ingredient, curcumin, and their medicinal properties, this study supports the theory that encapsulating curcumin in liposomes can increase its bioavailability, potentially resulting in heightened medicinal benefits, specifically, antimicrobial properties, compared with non-encapsulated forms. In the current investigation, the lack of significant concentration differences across selected opportunistic bacteria may be due to the potential need for a longer duration of the acclimation period. Further research needs to be conducted looking at similar dosages over an extended period of time to evaluate the anti-microbial and anti-inflammatory properties of 500 mg/g of liposomal-curcumin. Overall, oral administration of curcumin at different dosages did not have any adverse side effects on digestion, blood parameters, or range of motion; therefore, it could potentially be used as an alternative treatment for GI conditions and inflammation

Table 5.1. Effects of 500 mg/g of 95% liposomal-curcumin at 0 g, 15 g, 25 g, and 35 g, on

	Treatment[1]						
						P-value	
Strains	0	15	25	35	SEM	TRT[2]	LIN[3]
SBEC[4]	13.00[ab]	12.73[a]	13.68[bc]	14.12[c]	0.25	0.02	0.008
E. coli K-12	20.46	19.16	19.73	20.50	1.49	0.20	0.96
E. coli general	32.64	32.77	32.21	33.27	0.37	0.94	0.79
C. difficile	29.61	29.68	30.63	31.15	1.01	0.62	0.25
C. perfringens	49.18	46.36	45.97	43.51	1.36	0.12	0.03

[a-c] Treatment means ± SEM within a row with different superscripts differ ($P < 0.05$).

[1]Treatments: 0 = control (no nutraceutical); 15 = 15 g of 500 mg/g 95% liposomal-curcumin; 25 = 25 g of 500 mg/g 95% liposomal-curcumin; 35 = 35 g of 500 mg/g 95% liposomal-curcumin.

[2]*P*-value for treatment means.

[3]*P*-value for linear contrast.

[4] *Streptococcus bovis/equinus* complex

Table 5.2. Effects of 500 mg/g of 95% liposomal-curcumin at 15 g, 25 g, and 35 g, on inflammation in the blood and degree of range of motion

	Treatment[1]						
						P-value	
Item	0	15	25	35	SEM	TRT[2]	LIN[3]
ESR[4]	86.36	89.88	87.85	80.89	7.22	0.87	0.67
Right Knee	100.00	97.89	93.75	98.75	3.44	0.44	0.56
Right Hock	86.25	90.00	87.50	81.25	3.46	0.51	0.36
Left Knee	95.35	92.96	92.77	92.66	3.12	0.88	0.54
Left Hock	87.51	91.12	84.90	84.90	3.95	0.36	0.24

[1]Treatments: 0 = control (no nutraceutical); 15 = 15 g of 500 mg/g 95% liposomal-curcumin; 25 = 25 g of 500 mg/g 95% liposomal-curcumin; 35 = 35 g of 500 mg/g 95% liposomal-curcumin.

[2]*P*-value for treatment means.

[3] *P*-value for linear contrast.

[4]Erythrocyte sedimentation rate test, mm/hr.

Table 5.3. Effects of 500 mg/g of 95% liposomal-curcumin at 0 g, 15 g, 25 g, and 35 g, on cecal fluid characteristics

	Treatment[1]					*P*-value	
Item	0	15	25	35	SEM	TRT[2]	LIN[3]
pH	6.71	6.68	6.68	6.67	0.03	0.82	0.38
Ammonia, mg/dL	15.89	15.6	9.94	12.25	2.12	0.21	0.11
Total VFA, mM	51.59	71.15	73.68	65.32	6.14	0.11	0.10
VFA, mol/100mol							
Acetate	35.53	36.64	36.64	40.91	1.98	0.28	0.10
Propionate	49.68	52.75	54.75	50.15	1.96	0.34	0.62
Isobutyrate	3.97	1.67	1.31	0.59	1.90	0.54	0.19
Butyrate	10.63	9.57	8.09	8.46	0.62	0.06	0.01
Isovalerate	0.40	0.13	0.14	0.08	0.09	0.10	0.03
Valerate	0.68[a]	0.32[b]	0.28[b]	0.24[b]	0.09	0.02	0.005

[a-c] Treatment means ± SEM within a row with different superscripts differ ($P < 0.05$).

[1]Treatments: 0 = control (no nutraceutical); 15 = 15 g of 500 mg/g 95% liposomal-curcumin; 25 = 25 g of 500 mg/g 95% liposomal-curcumin; 35 = 35 g of 500 mg/g 95% liposomal-curcumin.

[2]*P*-value for treatment means.

[3] *P*-value for linear contrast.

CHAPTER 6

CONCLUSION

Turmeric is a rhizomatous herbaceous perennial plant, *Curcuma longa* Linn, belonging to the ginger family, *Zingiberaceae* (Chan et al., 2009). Curcumin, the active ingredient in turmeric, is gaining popularity for its anti-oxidant, anti-microbial, and anti-inflammatory properties. Curcumin has been used as medicine in Eastern countries for over a thousand years. In the past 30 years, studies have shown that curcumin has properties that can treat and prevent a magnitude of inflammatory disease while being nontoxic to metabolic pathways (Prasad et al., 2014). Curcumin inhibits inflammation by down regulating pro-inflammatory adipokines, tumor necrosis factor (TNF), and interleukin (IL)-6 (Prasad et al., 2014). Curcumin also possesses antimicrobial properties, which can aid in decreasing harmful and opportunistic bacteria found in the equine hindgut, including *SBEC, E. coli* general and K-12, *C. perfringens* and *C. difficile*. However, while curcumin seems to be the ideal alternative treatment for a wide variety of diseases, it has poor bioavailability. Curcumin's poor bioavailability is related to its hydrophobic properties and quick elimination in the body. Therefore, to increase curcumin's bioavailability, studies have tried to encapsulate curcumin in liposomes to increase its hydrophilic properties (Li et al., 2007 and Li et al., 2011).

In a series of studies, investigating curcumin's antimicrobial and anti-inflammatory properties in both, canines and equines. In the first study, the investigators observed that 95% curcumin at 500 mg, SID, can significantly reduce overall pain on day 60 and pain after physical exertion on day 90 in moderately arthritic canines. 95% curcumin (500 mg, SID) and 95% liposomal-curcumin (250 mg, BID) can decrease pain during manipulation at day 150 in

moderately arthritic canines. In the second project, a follow-up study to project one, 95% curcumin at 500 mg, SID, significantly decreased overall pain in moderately arthritic canines by day 60. 95% curcumin at 100 mg, SID, significantly decreased pain during limb manipulation by day 60. Both 95% curcumin at 100 mg and 500 mg, SID significantly decreased pain after physical exertion by day 60. By studying the anti-inflammatory properties of curcumin in moderately arthritic dogs, we concluded that both could significantly reduce overall pain, pain upon limb manipulation, and pain after physical exertion by day 60 out of a 150-day study. During both studies all 20 dogs had blood and chemistry parameters within normal range, the dogs did not experience any adverse side effects, concluding that giving curcumin as a nutraceutical to alleviate arthritic symptoms is safe.

In a two-part study, investigating the antimicrobial and anti-inflammatory properties of turmeric and curcumin on the bacteria found in the equine hindgut. Two *in vitro*, closed system, 24 h batch culture studies were conducted, first to look at which form of turmeric, 95% turmeric, 95% curcumin, and 95% liposomal-curcumin had the greatest effect on decreasing the opportunistic bacteria found in the equine hindgut, including *SBEC, E. coli* general and K-12, *C. perfringens* and *C. difficile*. Based on the results found in the first *in vitro*, a follow-up study was conducted looking at different dosages, 15 g, 20 g, 25 g, and 30 g, of 500 mg/g of 95% liposomal-curcumin had the greatest effect on decreasing the opportunistic bacteria found in the equine hindgut, including *SBEC, E. coli* general and K-12, *C. perfringens* and *C. difficile.* From these *in vitro* studies, it was concluded that the flasks that had the highest concentrations, SBEC, *C. difficile*, and *C. perfringens*, were the flasks with higher dosages of 95% liposomal-curcumin. Based on the results of the batch cultures, the authors decided to investigate 15 g, 25 g, and 35 g of 500 mg/g of 95% liposomal-curcumin for the *in vivo* study. This data not only suggests that

administering liposomal-curcumin at the recommended rate, 15 g, 25 g, or 35 g, for eight days, does not cause adverse side effects on digestion, inflammation, and blood parameters, but that when dosed for a longer period of time may increase digestibility (Bergman, 1990). Therefore, it is being suggested that curcumin is relatively safe, even at higher dosages, and could potentially be used as an alternative treatment for GI conditions and inflammation. In addition, the lack of significant concentrations differences in all selected opportunistic bacteria may be due to the potential need for a longer duration of the acclimation period. Further research needs to be conducted looking at similar dosages over an extended period of time to evaluate the anti-microbial and anti-inflammatory properties of 500 mg/g of liposomal-curcumin, similar to the previous studies conducted on moderately arthritic canines.

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APPENDICES

Appendix A – Murray State University IACUC Approval Form

MURRAY STATE UNIVERSITY

Institutional Animal Care and Use Committee
328 Wells Hall
Murray, KY 42071
phone: 270.809.5336
fax: 270.809.3535
www.murraystate.edu

June 30, 2014

Dr. Ramesh Gupta
Ms. Stephanie Bland
Breathitt Veterinary Center
Murray State University
715 North Drive
Hopkinsville, KY 42240

Dear Dr. Gupta:

It is with pleasure I inform you that the Murray State University Institutional Animal Care and Use Committee (IACUC) has approved your protocol for the project titled, "Therapeutic and Safety Evaluation of Curcumin in Moderately Arthritic Dogs." The period of performance will be from July 1 ,2014 through January 31, 2015.
If you have any questions , please contact me at (270) 809-3534.

Sincerely,

Kristi Stockdale

Kristi Stockdale
Coordinator

cc:
IACUC File

Equal education and employment opportunities M/F/D, AA employer

yourworld to explore

Appendix B – Southern Illinois University IACUC Approval Form

SIU Southern Illinois University
CARBONDALE

IACUC
OFFICE OF SPONSORED PROJECTS ADMINISTRATION
WOODY HALL, C WING
MAIL CODE 4709
900 SOUTH NORMAL AVENUE
CARBONDALE, ILLINOIS 62901

iacuc@siu.edu
618/453-4533
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MEMORANDUM TO: Erin Venable

FROM: Institutional Animal Care and Use Committee (IACUC)

DATE: December 4, 2014

SUBJECT: *Safety and effect of turmeric on equine digestibility*

PROTOCOL NUMBER: 14-048

SIUC ANIMAL ASSURANCE NUMBER: A-3078-01

ACTION: The above referenced protocol was approved by the members of the IACUC on December 4, 2014.

Protocol approval will expire at the end of three (3) years. If you wish to continue your research beyond 12/3/2017 you must re-submit. Re-submissions should be submitted at least two (2) months prior to the expiration date to allow adequate time for IACUC review and approval.

To protect the health and safety of personnel, all individuals having contact with animals as part of this protocol are required to have a current medical history, physical examination and appropriate vaccinations/testing. Please be sure you have complied with these regulations and update records as needed. SIUC IACUC Medical History Forms are available on the IACUC web page (http://www.siu.edu/~iacuc/).

Please use your protocol number when ordering animals or services from the Laboratory Animal Program.

Thank you for your cooperation.

LA/kr

cc: Laboratory Animal Program office

SIU.EDU

VITA

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Southern Illinois University

Stephanie D. Bland

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Murray State University
Bachelor of Science, Animal Health Technology, May 2012

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Special Honors and Awards:
Outstanding Ph.D. Researcher, Southern Illinois University, April 2015
Outstanding Graduate Student, Murray State University, May 2012

Dissertation Title:
THERAPEUTIC AND SAFETY EVALUATION OF CURCUMIN'S ANTIMICROBIAL AND ANTI-INFLAMMATORY PROPERTIES IN CANINE AND EQUINE

Major Professor: Dr. Rebecca Atkinson

Publications:

Bland, S. D. 2015. Equine colic: A review of the equine hindgut and colic. Vet. Sci. Develop. Ahead of press, accepted.

Haplin, M., **S. Bland**, V. Braner, N. Gulson, and E. Venable. 2015. Effect of grazing muzzles on the rate of pelleted feed intake in horses. J. Vet. Behav.

Bland, S. D. 2015. Canine osteoarthritis and treatments: A review. Vet. Sci. Develop. 5(2).

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