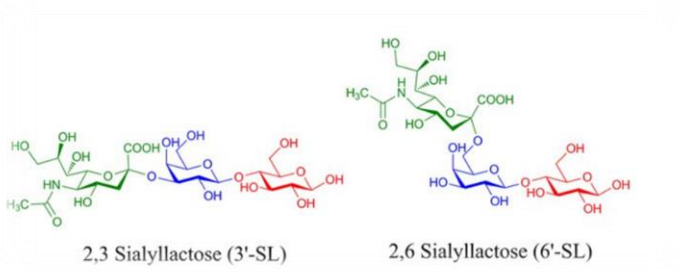
2020-2021

Are the immune-modulating effects of sialyllactose beneficial in osteoarthritis treatment in dogs?

Research Project Veterinary Medicine University Utrecht, Master thesis, the Netherlands



N.D. Pruijssers BSc, 6070280, 31-05-2021

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PREFACE

This study is part of the Master of Veterinary Science in the Netherlands. For my master thesis I really wanted to have a study that matched with my interest. My bachelor thesis was about hydrotherapy and osteoarthritis, this master thesis fits in nicely with that. Conducting a research is different than I first thought and surrounded by a good team I improved many of my skills. This research thought me to be more independent, but at the same time reminds me of the value of everyone who helped me. I have learned to be patient and to adjust myself in different situations. Although this study is conducted during the covid-19 outbreak, it has been a great learning experience and above all a memorable time.

As mentioned, I value everyone who helped me. I would like to thank my supervisor, Ronald Corbee, for the patience, time, effort, delicious apple pies, laughs, teaching and all the feedback during the study. As I am not great in statistics, I asked Hans Vernooij to support me, who helped me discovering that statistics can be fun and understandable, something I thought would be impossible. Furthermore, I would like to thank Doenja van Mourik and Ewout Hoogendoorn for supervising the conditions of the dogs, helping me with conducting the research and the good conversations. To limit the amount of stress to the dogs, the experienced Harry van Engelen collected the blood samples. Force plate analyses were explained, and graphs were made by Arie Doornenbal. I would like to thank you all for the help. Furthermore, I would like to thank Marianna Tryfonidou for being the second reviewer. In addition, I would like to mention the Utrecht University, Faculty of Veterinary medicine, Department of Clinical Sciences, and the Animal Welfare Office for giving permission for conducting this research.

Finally, I would like to thank my family, boyfriend and friends for re-reading my thesis to make sure it was good. Thankyou all for putting the time and effort in helping me with this study.

As my study is finished, I can only say, enjoy reading it!

31-5-2021 12:43 Utrecht, Netherlands Picture at the frontpage [1].

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ABSTRACT

Background: Secondary osteoarthritis is caused by abnormal stresses on the cartilage or as a consequence of other joint diseases. Osteoarthritis can cause lameness in dogs, furthermore it is one of the most common chronic musculoskeletal diseases in dogs. Sialyllactose is an acidic oligosaccharide, which can be found in human milk. It consists out of lactose and sialic acid. Sialyllactose has multiple effects on: intestines microbiota, glycosylation processes, resistance on respiratory and gut pathogens, brain and cognitive development and immune function and inflammation. These effects with emphasis on the immunologic effects could potentially have an effect in osteoarthritis in dogs.

Aim: To test sialyllactose in adult dogs with osteoarthritis and establish whether beneficial immunologic effects can be determined when this prebiotic is supplemented to a canine diet.

Study Design: Randomized, double-blinded, placebo-controlled proof of principle study.

Methods: Pilot study of 12 laboratory dogs whereof 6 with osteoarthritis, confirmed by Helsinki Chronic Pain Index, Canine Osteoarthritis Staging Tool and Force Plate. The dogs were divided into 4 groups; Healthy - Control, Healthy - Sialyllactose, Osteoarthritis - Control, Osteoarthritis - Sialyllactose. Adjusted diet started 5th November 2020, with or without sialyllactose. The supplemented sialyllactose existed out of 3' sialyllactose and 6' sialyllactose, ratio 9:1. At the start (time 1), after 2 weeks (time 2), 2 months (time 3) and after 4 months (time 4) all parameters were measured. These parameters are: Helsinki Chronic Pain Index, Canine Osteoarthritis Staging Tool, force plate analysis, body condition score and weight.

Results: The study was uneventful, thus the laboratory dogs tolerated the sialyllactose. The outcome of the minimum cranio-caudal force of the left- and right hindlimb after 2 months significantly changed. Furthermore, the maximum cranio-caudal force of the right hindlimb also changed significantly. After 4 months, the outcomes of the maximum vertical ground reaction force of the left hindlimb and the vertical impulse of the left-and right hindlimb significantly changed. These changes occurred in both the sialyllactose group and placebo group. However, significant changes between the diets were seen at the maximum cranio-caudal force of the left front limb, the maximum vertical ground reaction force of the left front limb and the symmetry between the vertical impulse of the hindlimbs. In addition, the outcomes of the minimum cranio-caudal force of the left hindlimb had multiple significant differences. At the start and after 2 months a significant difference was seen between the treatment and no treatment group. Furthermore, a significant difference was seen between the dogs with osteoarthritis and without. Moreover, the outcome of the minimum cranio-caudal force of the left hindlimb was significantly changed after 4 months. At last, the body condition score lowered and the dogs lost weight.

Conclusion: In this study, analysis was conducted about the centered outcome on pain and mobility of supplementing sialyllactose to adult dogs with OA. Analysis shows an effect of sialyllactose at the minimum cranio-caudal force of the left hindlimb, the maximum cranio-caudal force of the left front limb, the maximum vertical ground reaction force of the left front limb and the symmetry between the vertical impulse of the hindlimbs. Consequently, an effect of sialyllactose is determined. However, due too scarce significant results the exact effect of Sialyllactose is unknown, therefore a follow-up investigation is recommended. Hence, a longer, more diverse investigation including the separation of the 3'sialyllactose and 6'siallylactose is recommended.

ABBREVIATIONS

AD = Atopic Dermatitis

CILP = Cartilage Intermediate Layer Protein

Col2A1 = Type II alpha1 collagen

COMP = Cartilage Oligomeric Matrix Protein

HMO = Human milk Oligosaccharides

IGF-1 = Insulin-like Growth Factor -1

IL-1 = Interleukin-1

IL-6 = Interleukin-6

IL-1β = Interleukin – 1 beta

Kg = Kilogram

Ml = Milliliter

Mmp3 = Metalloproteinmatrix-3

Mmp13 =Metalloproteinmatrix-13

Neu5Ac = N-Acetylneuraminic acid

NF-kB = Nuclear Factor-kB

Neu5Gc = N-Glycolylneuraminic acid

NSAID = Non Steroid Anti Inflammatory Drug

OA = Osteoarthritis

PBMC = Peripheral Blood Mononuclear Cells

PGE2 = Prostaglandin E2

TNF = Tumor Necrosis Factor

INTRODUCTION

The main causes of osteoarthritis are thought to be found in excessive production of interleukin-1β (IL-1β). IL-1β causes up-regulation of metalloproteinases and activation of cyclooxygenase, furthermore, it causes down regulation of Col2a1 synthesis [2]–[4]. *Depletion of Col2a1 causes dedifferentiation of the chondrocytes and therefore results into osteoarthritis combined with possible lameness. The study of Jeon et al* [2], *used 3'-sialyllactose in cartilage cells damaged by IL-1β, from mice. After treatment with 3'-sialyllactose the synthesis of Col2a1 was restored* [2]. *The results of this study are promising, however the effects in dogs with osteoarthritis are still unknown. This research will attempt to investigate whether Sialyllactose has beneficial immunologic effects in adult dogs with osteoarthritis. The focus of this particular thesis was to assess the functional interference of pain in dogs with OA combined with the sialyllactose.*

OSTEOARTHRITIS

There are two main groups of osteoarthritis (OA), primary and secondary. Primary OA is caused by defective articular cartilage structure and biosynthesis [5]. Primary OA is considered uncommon in dogs. Therefore, this report focusses on the secondary OA [5]. Secondary OA is caused by abnormal stresses on the cartilage or as a consequence of other joint diseases [5]. OA can cause lameness in dogs, furthermore it is one of the most common chronic musculoskeletal diseases in dogs. Ninety percent of dogs older than five years are reported to get affected [5]–[7].

Johnston's [8] definition of OA is a typically slowly progressive, degenerative condition that most frequently involves the highly movable, or diarthrodial, joints [8]. It occurs when the normal cartilage structure and homeostasis are disrupted. These changes can result in pain and/or decreased ability to use the joint. The joint is normally covered by joint cartilages. This cartilage is produced by chondrocytes which are embedded together in a self-produced matrix [5]. This matrix consists mostly of collagen, water and proteoglycans. The proteoglycans act together with hyaluronic acid as a border to hold the water between the collagen strands [5]. Besides functioning as border, proteoglycans can absorb shocks with the presence of water [5]. Holding water is important, as articular cartilage is primarily composed out of it: 60 to 85 percent of articular cartilage consist out of water. Provided that, only 15 to 22 percent of the cartilage consists out of type II collagen (Col2A1) and four to seven percent of proteoglycans [9]. Proteoglycans are negatively charged which give the tissue an ion induced swelling and moreover proteoglycans can give stiffness. Collagen gives stiffness and strength to the cartilage [9]. Collagen exists in many forms, however they all contain the same characteristic triple helical structure [10]. The most common forms are type II, V, VI, XI and XVI. Additionally, the most present collagen type in articular cartilage is type II (Col2A1) [10]. Extra cellular matrix is not only made of collagen, there are many more components to it. For example, a cartilage intermediate layer protein (CILP) whereof the function is unknown. However, the synthesis of CILP increases in early OA [10]. A cartilage oligomeric matrix protein, also known as COMP, increases in mice which have experimental induced OA [10], [11]. Also, an increased amount of COMP is found in synovial fluid in dogs with OA [10], [12]. Moreover, COMP if mutated, is thought to be the cause of pseudo chondrodysplasia and multiple epiphyseal dysplasia in humans [10], [13].

An insult to the cartilage results in a cytokine release combined with a catabolic enzyme production. These enzymes damage the cartilage and influence the proteoglycan synthesis [5]. Enzymes involved in cartilage degradation are several proteinases, like: aspartic, serine, cysteine and metalloproteinases. Another important factor is Prostaglandin E2 [5]. Prostaglandin E2 (PGE2) is a lipid mediator that contributes to inflammation [14]. An inflammation consists of rubor (redness), calor (heat), tumor (swelling), dolor (pain) and function laesa (loss of function) [15].

The mentioned enzymes are being produced by synovial cells and chondrocytes under the influence of several cytokines. The most important cytokines are: tumor necrosis factor, interleukin-1 and interleukin-6 [5]. The tumor necrosis factor is thought to increase leukocyte adhesion on the vascular endothelium from synovial cavities [5]. Furthermore, the influx of leukocytes and thus release of inflammatory substances are thought to have a contributing role to the degeneration of cartilage found in OA [5]. The result of this insult weakness of the cartilage, which ultimately can cause flaking and fissuring [5].

These changes are partly caused by the phenotypic modifications of chondrocytes. A chondrocyte can transform to a hypertrophic phase, macrophage-like or apoptotic phase [6]. During a hypertrophic cartilage phase the chondrocytes produce multiple substances such as: aggrecan, tenascin and several types of collagen (type I, II, IIA, III, X) [6], [16]. This ultimately leads to cartilage hypertrophy. Consequently, the cartilage exists out of a formation of cell clusters, increased matrix hydration and an increased matrix turnover, showed in fig 1ab [6].

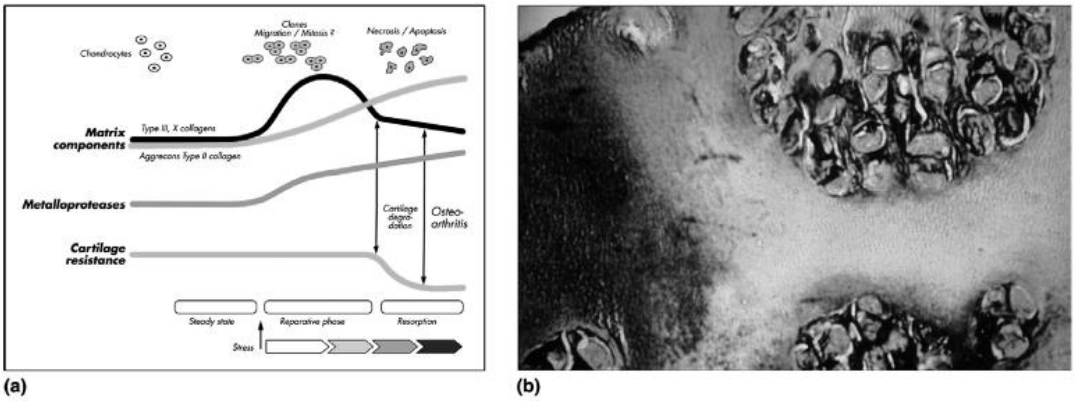


Figure 1ab : (a) Schematic representation of the repair process after mechanically induced cartilage lesion. (b) Micrography of chondrocyte clusters observed in human osteoarthritic cartilage [6].

This hypertrophic phase is an attempt to repair damaged cartilage, however the damages can only partly be repaired. As damage increases, the next phase follows. In this phase, also known as the overt stage of OA, the affected chondrocytes produce more metalloproteinases and less cartilage matrix molecules [6], [17], [18]. This ultimately leads to more cartilage degeneration, so much that OA is a progressive disease [19], [20].

OA is often described as a non-inflammatory disease, however recent research supports the presence of inflammation in OA [21]–[23]. For example, the most abundant inflammatory cell in chronic synovitis in OA are the macrophages and lymphocytic infiltrates [23]. Besides that, in most OA patients, increased mononuclear cells as macrophages and T-lymphocytes are found in the synovial fluid. Moreover, levels of cytokines, immunoglobulins and complement factors are increased [6], [24]. Given these points, inflammation is a part of the development of OA.

As discussed before, chondrocytes are essential to cartilage production. The loss of Col2a1 in chondrocytes induces OA by dedifferentiation of the chondrocytes [2], [25]. Col2a1 is regulated by Sox9 [25]. Metalloproteinmatrix-3 (Mmp3) and metalloproteinmatrix-13 (Mmp13) can cleave Col2a1 which causes degradation of Col2a1 and ultimately leads to OA development [2], [26], [27]. Moreover, nuclear factor-kB (NF-kB) is important, however it suppresses Sox9 expression [2], [25], [26], [28]. In conclusion, NF-kB signals the expression of Mmp3 and Mmp13, downregulated Sox9, which downregulated the expression of Col2a1, resulting into OA. This is important because the expression of NF-kB could lead to OA development. As previously stated, IL-β is thought to be one of the main causes of OA. Il-β has several functions, in particular the activation of NF-kB, which thus ultimately can cause OA [6], [25], [26], [28], [29]. These changes are illustrated in figure 2 [6].

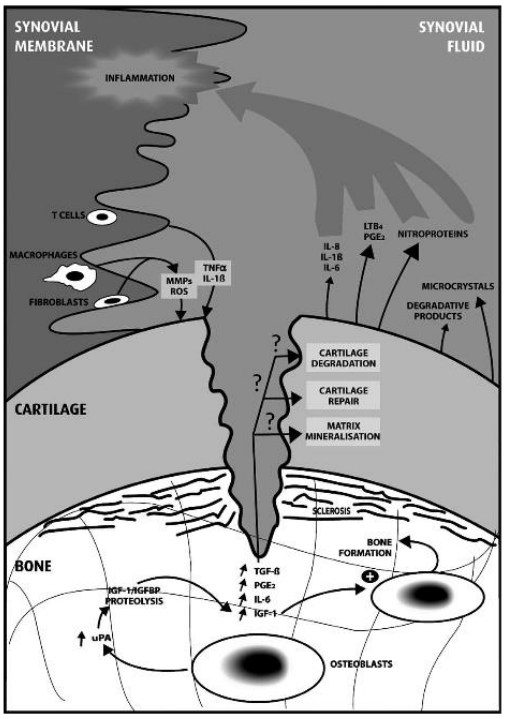


Figure 2 : Pathophysiological pathways involved in the OA disease process [6]. Abbreviations: MMP, metalloproteases; ROS, reactive oxygen species; TNFα, tumor necrosis factor alpha; IL-1β, interleukin-beta; IL-6, interleukin-6; Il-8, Interleukin-8; PGE2, prostaglandin E2; LTB4, leukotriene B4; uPA, urokinase; TGF, transforming growth factor [6].

The cause of OA is thought to be found in upregulation of Interleukin-1, tumor necrosis factor receptors and decreasing the expression of transforming growth factor (TGF) beta-II receptors [6]. However, Clements et al [30], showed that Interleukin-1 knock -out mice do get spontaneous osteolytic-lesions and increased cartilage degeneration in comparison to wild-type mice [6], [30]. Moreover, Massicotte et al [31], found similar levels of IL-β in normal osteoblasts and OA osteoblasts [31]. Therefore, the exact role of IL-β remains poorly understood.

Many treatments for OA exist, such as: physical rehabilitation, weight management, surgery, pharmacological treatment and supplementation with nutraceuticals [7]. Physical rehabilitation can result into a moderate level of activity, which is important for dogs with OA [7], [32]. Improving mobility can be achieved by different activities like: walking in water, swimming and leash walking [7], [32]. Dogs with a Body Condition Score (BCS) of 7 or more are obese. Obese dogs are more sensitive to developing OA than dogs without obesity [7], [33]–[37]. Subsequently, avoiding overweight dogs could prevent OA due to overweight. Another treatment is surgery. Surgical options are aimed at eliminating OA, in mature patients it can be considered a as salvage procedure. However, effectiveness is difficult to determine due to different used criteria postoperatively [32]. The most commonly used pharmacological agents are the NSAID's. It reduces pain caused by OA, however with long-term use it can contribute to cartilage degeneration [6], [7]. Moreover, drugs such as NSAID's are associated with side effects, such as negative gastrointestinal or cardiovascular effects [38]. By way of contrast, there are therapies with supplementation with nutraceuticals which may improve the clinical signs for dogs with OA, without enhancing the degeneration of cartilage, seen in table 1 [7]. However, current treatments are not yet able to reverse the cartilage changes [8]. A new treatment for OA in dogs is therefore required.

Table 1: Examples of nutritional and nutraceutical strategies with improvement in clinical signs for dogs with OA [7].

Study design (treatment duration)	Animals	Intervention	Control	Main outcomes	Reference
Prospective, randomized, blinded clinical trial (90 d)	Client-owned dogs with chronic osteoarthritis of hip or stifle joints (n = 177)	Diet A: 0.8% EPA-DHA (n = 55) Diet B: 2% EPA-DHA (n = 62) Diet C: 2.9% EPA-DHA (n = 60)	NA	Significant improvements in lameness, weight bearing, clinical signs of arthritis progression, and overall condition for dogs fed diet C, compared with dogs fed diet A	1
Randomized, double-blinded, controlled clinical trial (6 mo)	Client-owned dogs with osteoarthritis in ≥ 1 joint (n = 127)	Test diet: 3.47% omega-3 fatty acids (n = 71)	Control diet: commercial adult dog food containing 0.11% omega-3 fatty acids (n = 56)	Dogs fed the test diet had significantly increased ability to rise from rest and play at 6 wk and ability to walk at 12 and 24 wk, compared with dogs that received the control diet	3
Randomized, controlled clinical trial (12 wk)	Dogs with chronic osteoarthritis (n = 109) receiving a standardized carprofen dose (approx 4.4 mg/kg, PO, q 24 h) at the start of the study	Fish oil–enriched therapeutic diet: 3.5% omega-3 fatty acids (n = 52)	Control diet: commercial adult dog food containing 0.1% omega-3 fatty acids (n = 57)	Carprofen dose was decreased (on the basis of investigator and owner assessments) significantly earlier for dogs fed the fish oil–enriched diet than for dogs fed the control diet	18
Randomized, double-blinded, controlled clinical trial (90 d)	Client-owned dogs with osteoarthritis (n = 38)	Test diet: 3.5% omega-3 fatty acids (n = 22)	Control diet (commercial adult dog food): 0.11% omega-3 fatty acids (n = 16)	Dogs fed the test diet had a significantly greater increase in peak vertical force in affected limbs from baseline, compared with results for dogs fed the control diet	19
Randomized, controlled, double-blinded clinical trial (13 wk)	Privately owned dogs with hip or stifle joint osteoarthritis (n = 30)	Therapeutic diet: 1.08% omega-3 fatty acids (n = 15)	Control diet: 0.07% omega-3 fatty acids (n = 15)	Dogs fed the therapeutic diet had significantly greater peak vertical force in affected limbs during weeks 7 and 13 than dogs fed the control diet	20
Double-blinded, placebo-controlled clinical trial (28 d)*	Client-owned dogs with osteoarthritis (n = 59)	CFB low dose: 69 mg (n = 14) CFB high dose: 127 mg (n = 14) Combination treatment: 69 mg of CFB, 500 mg of glucosamine hydrochloride, and 200 mg of chondroitin sulfate (n = 16)	Placebo: 60 mg of fructose (n = 15)	Dogs in CFB low-dose and high-dose groups had significantly improved ability to rise from a lying position, compared with placebo group dogs	10
Randomized, double-blinded, positive-controlled clinical trial (70 d)*	Privately owned dogs with hip or elbow joint osteoarthritis (n = 35)	Glucosamine hydrochloride (475 mg/g), chondroitin sulfate (350 mg/g), N-acetyl-D-glucosamine (50 mg/g), ascorbic acid (50 mg/g), and Zn sulfate (30 mg/g) in a gelatin capsule: 1 g for dogs that weighed 5–19.9 kg, 1.5 g for dogs that weighed 20–39.9 kg, and 2 g for dogs that weighed > 40 kg; products given twice daily for 42 days, then dose was reduced by one-third for 28 d (n = 16)	Carprofen: 2 mg/kg, PO, twice daily for 7 days, followed by 2 mg/kg, PO, once daily (n = 19)	Dogs in the glucosamine-chondroitin group had significantly improved veterinarian-assessed scores for pain, weight bearing, and overall condition by day 70; dogs in the carprofen group had significantly improved veterinarian-assessed scores for lameness, joint mobility, pain, weight bearing, and overall condition by day 42	23
Field trial (50 d)†	Privately owned dogs with osteoarthritis (rated mild to medium; n = 85)	Nutritionally complete dog food supplemented with 0.3% GLM (n = 85)	NA; data were compared with pretreatment values when each dog received its routine diet	Significant reductions in visual (mobility impairment) scores, manipulation (clinical signs on manipulation of joints) scores, and total arthritic (visual plus manipulation) scores on day 50 of the dietary treatment, compared with pretreatment results	4
Double-blinded, longitudinal, controlled clinical trial (90 d)‡	Client-owned dogs with osteoarthritis (n = 23)	GLM-supplemented diet for 60 days (n = 23)	Control diet: commercial adult dog food for 30 days (n = 23)	Peak vertical force significantly improved during the GLM-supplemented diet phase, compared with the control diet phase and pretreatment value (day 0)	21

*Dogs were fed a commercial adult dog food during the study. †Dogs received the GLM-supplemented diet exclusively for 45 days after a 5-day transition period in which they received their normal diet mixed with increasing amounts of the study food. ‡All dogs received the control diet on days 1 to 30 and the GLM-supplemented diet on days 31 to 90 of the 90-day study.
CFB = Calcium fructoborate. NA = Not applicable.
To convert mg/kg to mg/lb, divide by 2.2.

SIALYLLACTOSE

Sialyllactose is an acidic oligosaccharide, which can be found in human milk [39], [40]. It consists out of lactose and sialic acid. Sialic acids are a family of monosaccharides with nine carbon atoms with a keto acid functional group. The milk composition varies for different mammals. This suggests that each mammal has its own milk composition according to the specific needs for their neonates [39]. In mammals the most common sialic acids are N-Acetylneuraminic acid (Neu5Ac) and N-Glycolylneuraminic acid (Neu5Gc). However, humans can only synthesize Neu5Ac because of a deletion mutation [39]. Currently, most research focuses on Neu5Ac only.

In neonates, sialyllactose is thought to have significant health benefits. It supports resistance to pathogens, maturation of the gut, immune function and the development of the cognitive system [40]. Sialyllactose has been identified in the milk of a beagle dog [41]. Furthermore, sialyllactose was seen in milk samples of the Alaskan husky, Alaskan husky German pointer, Alaskan husky English pointer, Labrador retriever and the Schnauzer, also seen in figure 3 AB [41].

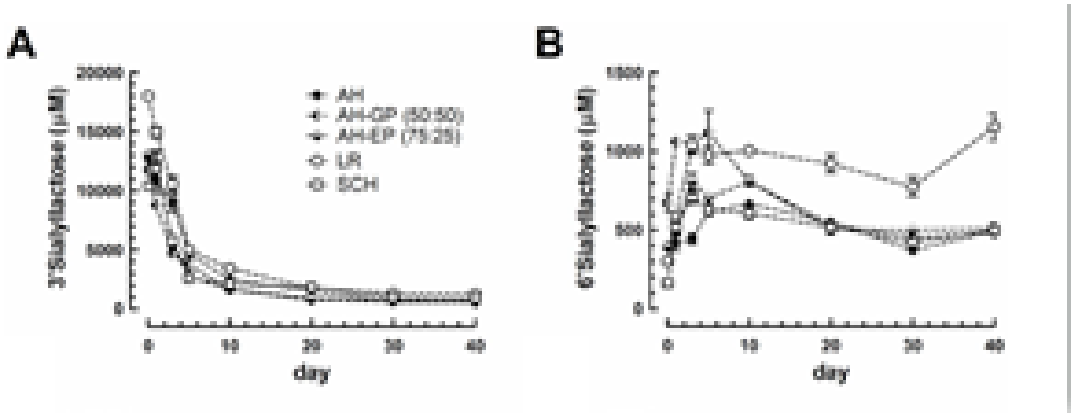


Figure 3AB: Levels of major oligosaccharides over time of lactation in milk samples from 5 different dog breeds (Alaskan husky (AH); Alaska husky German Pointer (AH-GP); Alaskan husky English pointer (AH-EP); Labrador retriever (LR); Schnauzer (SCH)). A. 3' sialyllactose; B. 6' sialyllactose [41].

By modulation of specific gut microbiota establishment and mucosal immunity, sialyllactose is thought to have a supporting function of the gut. Furthermore, it might serve as a sialic acid carrier providing fuel for glycosylation processes [39], [41]. Given these points sialyllactose is an important oligosaccharide for humans. However, the matter of importance of sialyllactose in dogs is still unknown.

The metabolism of sialyllactose is largely unknown. Most research focusses on elements instead of the bigger picture. All those different elements will be shown in the next paragraph attempting to display the bigger picture. Sialyllactose is an oligosaccharide which can be given orally. Normally it does not hydrolyze in the gastrointestinal tract, however in the intestinal mucosa of humans and rats it can be hydrolyzed by mucosal sialidases [40]. The sialidases are probably from lysosomal origin due to the lack of sialidases in the brush borders in infant rats [40]. Considering this, the sialyllactose can be absorbed by enterocytes and used as a nutrient for neonatal tissue and organ development [40]. Whereas in rats the absorption is investigated, the absorption of sialyllactose in dogs is still unknown.

Sialyllactose is partly absorbed by the paracellular route, transported by blood and excreted in the urine [40], [42]. Moreover, the research of Gnoth et al [42], show that acidic oligosaccharides such as 6'-sialyllactose only take the unspecific paracellular route, whereas the transcellular route can also be used by neutral oligosaccharides [40], [42]. In other words, oligosaccharides such as 6' sialyllactose get transported by passing through the intercellular space between cells. However, neutral oligosaccharides can be transported through the cell, by passing both the apical- and basolateral membrane.

Jantscher-Krenn et al [43], investigated the metabolism of several human milk oligosaccharides, including 3'siallylactose in rats. Surprisingly, from all the oligosaccharides in human milk, only 3'siallylactose was found in the bloodserum and urine, to conclude the existence of selective absorption pathways of rat milk-specific oligosaccharides [40], [43]. When in fact, in humans the founded oligosaccharides in serum and urine were less the same [40], [44].

EXISTING RESEARCH

The research of Kang et al [45], concluded that 3'-sialyllactose inhibited Il-1B, IL-6, IgE, and TNA-α secretion. Furthermore, it downregulated several atopic dermatitis (AD)-related cytokines. These cytokines include IL-4, Il-5, Il-6, IL-13, IL-17, TNF-α, IFN-Y, and Tslp by regulation of NK-kB in ear tissue. The research also included that 3'sialylactose induced TGF-β mediated Treg differentiation directly [45]. In conclusion, 3'sialyllactose induces Treg differentiation and down-regulated several AD-related cytokines, and thus inhibits the rise of an inflammatory reaction. The effects of sialyllactose were investigated in rabbits by Idota et al [46], they concluded that sialyllactose inhibited the cholera toxin inducing fluid accumulation [46]. However, if they split the sialyllactose in sialic acid and lactose, none of them influenced the cholera toxin [46]. The experiment from Tarr et al [47], included three feeding groups with mice, with laboratory diet or laboratory diet with 3'sialyllactose or 6'sialyllactose for two weeks. Tarr et al [47], showed that the Shannon Diversity Index was significantly higher by the control group compared with sialyllactose groups. For example, the Firmicutes and Cyanobacteria were significantly decreased. However, the Bacteroidetes increased in the 3'sialyllactose mice [47]. Thus, dietary sialyllactose significantly effects the colonic microbiota. Moreover, the mice in the experiment showed an increased spleen, serum levels of IL-6 and serum cortisone levels after stressor exposure. However, 3'sialyllactose and 6'sialyllactose did not significantly affected the mentioned levels [47].

Fuhrer et al [48], used normal, alpha2,3- and alpha2,6-sialyltransferase-deficient mother mice, each mother mice got newborn mice to foster. Thus, every group newborn mice got different milk content. They concluded that exposure of newborn mice to milk with sialyllactose or without gave no difference in development of mucosal leukocyte population [48]. Given these points, it seems sialyllactose does not enhance the mucosal leukocyte population. However, when Further et al [48], applied dextran sulfate sodium to the mice drinking water there was a difference. The newborn mice, at 7 weeks of age, were exposed to dextran sulfate sodium in the drinking water. The mice which got the sialyl(alpha2,3) lactose-deficient milk were more resistant to colitis as compared to the mice fed with normal milk or sialyl(alpha2,6) lactose-deficient milk [48]. In conclusion, exposure to sialyl(alpha2,3) lactose during infancy can affect the bacterial population of the intestine, which can change the susceptibility of the mice against dextran sulfate sodium induced colitis in adult mice [48].

As mentioned before, sialyllactose has multiple effects. Bruggencate et al [40], investigated these multiple potential effects of sialyllactose and sialylated oligosaccharides, displayed in figure 4 [40].

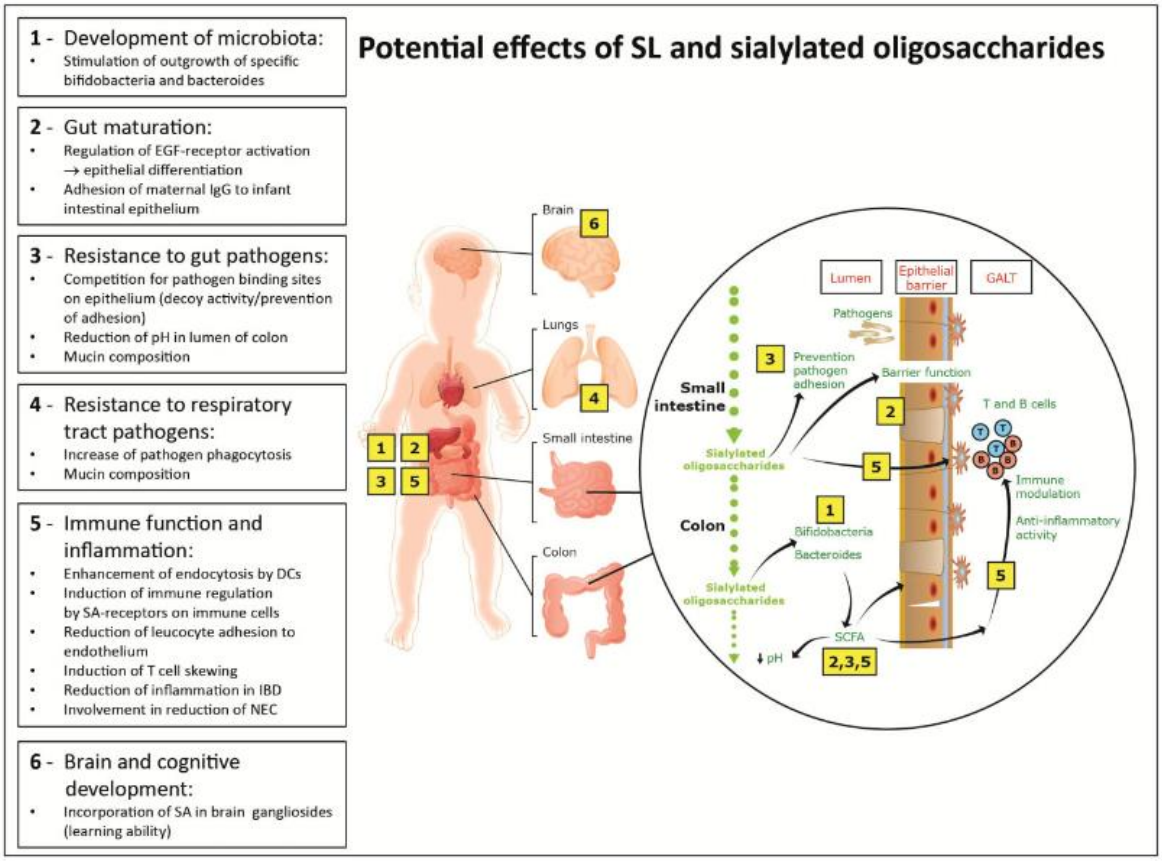


Figure 4: Potential effects and mechanism of SL and sialylated oligosaccharides [37]. ' Abbreviations: DCs, dendritic cells; EGF, epidermal growth factor; GALT, gut-associated lymphoid tissue; IBD, inflammatory bowel disease, IgG, Immunoglobulin G; NEC, necrotizing enterocolitis; SA, sialic acid; SCFA, short-chain fatty acids; SL, sialyllactose.' [40].

As seen in figure 4, sialyllactose influences microbiota, gut maturation and gut pathogens. In addition to this, sialyllactose has an effect to the respiratory tract and the brain [40]. In this study the effect of sialyllactose on the immune system is more important. As stated in the study of Bruggencate [40], 3' sialyllactose had anti-inflammatory properties due to reducing mRNA levels of proinflammatory cytokines [40], [49]. The research of Zenhom et al [49], investigated the mRNA levels of several pro-inflammatory cytokines. After supplementing alpha-3-Sialyllactose IL-12 levels decreased and NF-kB significantly decreased [49]. The alpha-3-Sialyllactose enhanced the expression of peptidoglycan recognition protein 3 (PGlyRP3), which is a pathogen recognition receptor regulating inflammatory responses [40], [49], [50]. Furthermore, in the study of Comstock et al [51], peripheral blood mononuclear cells (PBMC) were collected from 10-day old pigs. A group piglets were sow-reared (SR), the other group were fed with formula deprived colostrum (FF). Both groups got 72 hours long human milk oligosaccharides (HMO) administered. The HMOs increased IL-10 production and altered proliferation [40], [50], [51].

Another anti-inflammation effect was investigated by Eiwegger et al [52]. After exposing human naïve cord blood mononuclear cells to acidic oligosaccharides IL-10 and IFN-y production increases, IL-13 did not increase. Moreover, when peripheral blood mononuclear cells from human patients with peanut-allergic got stimulated with peanut allergen in the presence of acidic oligosaccharides a same effect was measured. The IL-4 was decreased and the IFN-y increased. Altogether, acidic oligosaccharides can result into a Th1-polarized regulatory immune response [40], [52].

Secondary OA is due to abnormal stresses on the cartilage or as a consequence of other joint diseases. Ninety percent of dogs older than five years are reported to get affected. Several treatments for OA exist, however the most used has side effects. Moreover, current treatments are not yet able to reverse cartilage changes. Therefore, a new treatment for dogs with OA is required. A possible treatment could involve sialyllactose, an acidic oligosaccharide. Sialyllactose has multiple effects on: intestines microbiota, glycosylation processes, resistance on respiratory and gut pathogens, brain and cognitive development and immune function and inflammation. These effects with emphasis on the immunologic effects could potentially have an effect in OA in dogs. The objective in this research is to test sialyllactose in adult dogs with OA and establish whether beneficial immunologic effects can be determined when this prebiotic is supplemented to a canine diet.

HYPOTHESIS

H0= There is no significant effect on the centered outcome of pain and mobility established by supplementing Sialyllactose to adult dogs with osteoarthritis.

H1= There is a significant effect on the centered outcome of pain and mobility established by supplementing Sialyllactose to adult dogs with osteoarthritis.

RESEARCH GOAL

Can the immune-modulating effects of Sialyllactose be beneficial for centered outcome on pain and mobility in adult dogs with osteoarthritis?

MATERIAL AND METHODS

The objective is to test sialyllactose in adult laboratory dogs with naturally OA and establish whether beneficial immunologic effects can be determined when this prebiotic is supplemented to a canine diet. This study is seen as a Proof of Principle study where the effect of a preselected dosage will be determined. At first there is a pilot study including a 4-month feeding trial with the product and placebo with 3 dogs per group. This pilot study will include 12 dogs in total. At the start, after 2 weeks, 2 months and after 4 months all parameters are being checked, except for the blood for the heparin tubes. These parameters are: force plate analysis, Helsinki Chronic Pain index score (HCPI), Canine Osteoarthritis Staging Tool score (COAST), and BCS.

To test sialyllactose in dogs with OA, force plate analysis, a questionnaire (HCPI), and a validated clinical examination score (COAST) will be used in dogs that are fed a diet enriched with either sialyllactose or placebo for 4 months.

ETHICAL STATEMENT

Project number (CCD) : AVD1080020184847

Work protocol number: 4847-1-13

Level of discomfort: Mild

Guideline work protocol: www.ivd-utrecht.nl/en

Animal facility: VET- Companion Animals

LABORATORY ANIMALS

At first, suited laboratory animals had to be selected. The selection process consisted out of HCPI, COAST, force plate analyses and body condition score. Fifteen dogs were examined, to determine whether they had an abnormality in the walk or not. The dogs with abnormalities were placed in de OA group and the group without abnormalities were placed in the healthy group. Furthermore, dogs which were difficult to handle are considered not suitable for this study.

6 dogs with OA older than 4 years old, not under treatment, multiple joints affected, no spondylosis.

These dogs are divided into two groups:

- OA dogs under treatment with sialyllactose (OA-SL): 3 dogs
- OA dogs without treatment with sialyllactose (OA-placebo): 3 dogs

Furthermore, there are 6 healthy dogs of same breed, similar age as the OA dogs, also divided into two groups:

- Healthy dogs fed with sialyllactose (Control-SL): 3 dogs
- Healthy dogs fed without sialyllactose (Control-placebo): 3 dogs

6 healthy dogs of same breed, similar age as the OA dogs: healthy-placebo

The included dogs were divides into the different groups by an animal caretaker. So, this research is double-blinded.

In conclusion, the number of laboratory animals used in this pilot study is 12, seen in table 2.

Table 2: Information Laboratory dogs.

Name	Ras	Gender	Birthdate	Food (1 cup= 200 gram)	Weight (at start)
093	Beagleton	Bitch	29-11-2010	¾ from 3/12 1 cup	10.65
777	Beagleton	Bitch	29-11-2010	¾	12.50
085	Beagleton	Bitch	29-11-2010	¾	11.75
Antonio	Beagleton	Male	17-10-2014	1	15.80
Rex	Beagle	Male castrated	24-04-2013	1	17.70
09.02	Beagle	Bitch	27-07-2009	1 ¼	16.35
09.04	Beagle	Bitch	20-11-2009	1	12.75
09.06	Beagle	Bitch	20-11-2009	1 ¼	15.65
Houston	Beagle	Male	20-05-2015	1	11.85
Austin	Beagle	Male	20-05-2015	1	10.90
Texas	Beagle	Male	20-05-2015	1	13.05
Denver	Beagle	Male	20-05-2015	1	10.50
13.01	Beagle	Bitch	05-11-2013	1	12.20

Table 3: Distribution OA-SL, OA-placebo, Control-SL and Control- placebo group.

OA-SL	OA-placebo	Control-SL	Control-placebo
093	Houston	085	1301
777	Texas	0902	Antonio
Rex	0904	Denver	0906

Table 4: Distribution group OA and group Control.

Group OA	Group Control (healthy)
Texas	085
Houston	1301
093	Antonio
777	0902
Rex	Denver
0904	0906

GENETICS

This research used laboratory dogs, therefore some dogs are related. Texas, Denver and Houston are brothers. De dogs 0904 and 0906 are sisters, furthermore the dogs 093, 777 and 085 are sisters. Moreover, 085 is the mother of Antonio and 0904 is the mother of 1301.

HOUSING AND HUSBANDRY

The dogs were placed in group housing. The housing existed out of a kennel, inside and outside. Grass fields were present outside, were de dogs were placed everyday for some hours, dependent on the weather. Food was given once a day between 7 and 8 pm. Water was given without restrictions. The dogs were individual or with two placed in a kennel, dependent on the behavior to each other. Enrichment was present like toys, balls and blankets. Each animal was checked every day by for abnormalities. Each week the dogs were checked on behavior, posture, gate/mobility, food intake/weight loss, grooming/auto mutilation and other striking clinical symptoms. Findings of these welfare checks were noted in the Welfare Logbook.

WELFARE-ASSESMENTS DURING EXPERIMENT

Welfare assessments were made during the experiment to ensure the welfare of the laboratory dogs. After a week into the experiment a mass was found in dog 093 (presumably a tumor, however further diagnosis was not been made). Extra welfare checks were scheduled to supervise the mass. Furthermore, dog 093 lost weight, therefore the food was altered. From December 2nd 2020, 093 got 1 whole mug instead of ¾ cup food. Dog 0906 had an ingrown toenail and got Carporal 30mg twice a day.

Some dogs already got medication before the experiment started, these medications were also given during the experiment, to ensure their welfare. Antonio got metalcapase 150mg twice a day. All dogs got deworming once a month, Drontal Large Dog Tasty from Bayer B.V.

DIETS

There are two different types of diets: a diet with Sialyllactose and a placebo diet. The diet with sialyllactose consist out of 3'Siallyllactose and 6'Siallylactose, in ratio 1:9. The concentration of the sialyllactose is 100 mg/kg.

The dogs' weight varied from 10.5 to 17.7 kg. This gives 1.05-1.77 gram. The mean of the dogs' weights was 14.1 kg which roughly resulted in a dosage of 1.5-gram sialyllactose per day. Not every dog had the same weight, which resulted in a different amount of food. For this reason, the smallest amount of food was considered average for the calculation. The smallest amount was ¾ cup thus 150 grams. In conclusion for 150-gram, 1.5-gram sialyllactose was needed, for 27 kg thus 270 grams. The ratio between the different sialyllactose is 9:1. In conclusion for 27 kg food, 27 grams of 6' sialyllactose and 243 grams of 3' sialyllactose. The powder was measured with a Sartorius GMBH Gottingen scale, type 1006 MP9.

The sialyllactose was used as powder. This powder is mixed with the normal food, for even distribution.

The diet without sialyllactose consisted out of:

Hill's Science plan Medium Adult [53]

- Composition: corn, wheat, lamb meal, soy flour, animal fat, corn gluten meal, brewer's rice, protein hydrolysate, vegetable oil, linseed, minerals [53].

Altogether, two boxes were made, one with normal food and the other with Sialyllactose added. If necessary, extra boxes were made and old ones refilled. The grouping is seen in the table below, table 5.

Table 5: Distribution of the food boxes.

Box 1	Box 2
Texas	093
Houston	777
0904	Rex
1301	085
Antonio	0902
0906	Denver

HELSINKI CHRONIC PAIN INDEX (HCPI)

The HCPI is a questionnaire to evaluate chronic pain in dogs. This research investigates the immune-modulating effect of sialyllactose, this effect could reduce pain in dogs with OA. Therefore, the HCPI questionnaire is an appropriate measurement tool for evaluating the pain of the dogs during the research.

The Helsinki chronic pain index was measured every measurement moment. The dogs are laboratory animals which do not have the opportunity to jump onto a couch in their kennels. Therefore, the 'jump' is removed afterwards and crossed out.

Name owner:
Name dog:
Date:
Control moment: 1 / 2 / 3

Question	**10**		**Gradation**		**1**	**Points**
Rate your dog's mood.	Very alert	Alert	Neutral	Indifferent	Very indifferent	
Rate your dog's willingness to participate in play.	Very willing	Willing	Reluctantly	Very reluctantly	Does not play at all	
How often does your dog groan, squeal or whine?	Never	Hardly ever	Sometimes	Often	Very often	
Rate your dog's willingness to walk.	Very willing	Willingly	Reluctantly	Very reluctantly	Does not walk at all	
Rate your dog's willingness/ability to walk up and down stairs.	Very willing	Willingly	Reluctantly	Very reluctantly	Does not want to at all	
Rate your dog's willingness to run.	Very willing	Willingly	Reluctantly	Very reluctantly	Does not run at all	

~~**Rate your dog's willingness to jump (e.g. into the car or onto the sofa).**~~	~~Very willing~~	~~Willingly~~	~~Reluctantly~~	~~Very reluctantly~~	~~Does not jump at all~~	
Rate your dog's ease in lying down.	With great ease	Easily	Neutral	With difficulty	With great difficulty	
Rate your dog's ease in rising from a lying position.	With great ease	Easily	Neutral	With difficulty	With great difficulty	
How difficult is it for your dog to move after a long period of rest?	Never difficult	Hardly ever difficult	Sometimes difficult	Often difficult	Very often or always difficult	
How difficult is it for your dog to move after major activity or heavy exercise?	Never difficult	Hardly ever difficult	Sometimes difficult	Often difficult	Very often or always difficult	
					TOTAL	

CANINE OSTEOARTHRITIS STAGING TOOL (COAST)

The COAST score was measured each measuring moment. The radiography measurement was not possible, due to extra costs and increase in dog use. Moreover, the radiography is not necessary to determine OA in dogs. Therefore, the decision was made to cross out the radiography. The COAST is a standardized method for diagnosing OA or joint diseases in dogs. This research involves dogs with OA and uses the COAST for determination of OA and evaluation of the joints during the research. The immune-modulating effects of Sialyllactose could reduce the stage of OA, therefore, the COAST is an appropriate measurement tool for this research.

'Grade the dog'						**GRADE**
I	**Effect on posture (static)**	**Normal;** Static posture appropriate for breed Appropriate limb loading Appropriate body weight distribution between forelimbs and hindlimbs	**Mildly abnormal;** Subtle abnormality of limb loading Subtle shift in static body weight distribution	**Moderately abnormal;** Obvious abnormality in limb loading Obvious shift in static body weight distribution	**Severely abnormal;** Restless when standing Reluctance (difficulty) to stay standing Severe shift in static body weight distribution Severely abnormal limb loading	(1-4)
II	**Effect on motion**	**Normal;** Symmetry Appropriate weight bearing Appropriate body weight distribution Fluent gait	**Mildly abnormal;** Motion possibly affected at some gaits or with some activities Subtle stiffness in gait Subtle changes in body weight distribution Subtle asymmetry Subtle lameness	**Moderately abnormal;** Consistent abnormalities in motion at all gaits and activities Obvious stiffness in gait Obvious changes in body weight distribution Obvious reduction in use of affected limb Obvious decrease in stance phase	**Severely abnormal;** Struggles to move/reluctant to move Severe lameness usually present Severe weight shift Marked difficulty rising (getting up)	(1-4)

			No difficulty rising (getting up)	Some difficulty rising (getting up)		
'Grade the joint'						**GRADE**
III	**Pain upon manipulation**	**None**	**Mild**	**Moderate**	**Severe**	(1-4)
IV	**Passive range of motion**	**Normal**	**Mildly abnormal;** Minimally reduced ROM No crepitus Slight joint thickening	**Moderately abnormal;** Obvious decrease in ROM Muscle atrophy Obvious joint thickening	**Severely abnormal;** Extremely limited ROM Crepitus Extreme muscle atrophy Severe joint thickening Loss of anatomical normality upon palpation Anatomical misalignment	(1-4)
~~**V**~~	~~**Radiography**~~	~~**No radiographic signs of OA;** If preclinical 'at risk', the dog may have radiographic evidence of risk factors such as dysplasia and/or trauma~~	~~**Mildly abnormal;** Early signs of OA Minimal osteophytes~~	~~**Moderately abnormal;** Obvious osteophytes~~	~~**Severely abnormal;** Advanced osteophytes Remodeling~~	~~(1-4)~~

Stage of OA	**Description**	
0	**Preclinical**	No risk factors apparent
1		'At risk': At least one predisposing factor for OA apparent e.g. breed predisposition, joint injury, obesity, intense activity and/or radiographic signs of dysplasia or joint trauma
2	**Mild**	
3	**Moderate**	
4	**Severe**	

FORCEPLATE ANALYSIS

A valid and reliable method to test differences in chronic pain in dogs with OA is force plate analysis [54]–[56]. Hence, with force plate analysis the effect of sialyllactose in dogs with OA can be determined.

Force plate analysis will be conducted at start, after two weeks, 2 months and 4 months. Each analysis will exist out of at least ten useful measurements at each side. In conclusion, each dog will have at least twenty measurements each force plate session.

The Force plate measurements went as followed. Each dog was taken separately from the kennel to the force plate. At first the bodyweight of the dog was determined by the DIWAC VS150 electronic scale, the weight was filled into the computer system. The type of force plate being used was a Kistler type 9261, a quartz piezoelectric force plate. Furthermore, mounted flush Kistler 9865B amplifiers were used. These amplifiers were connected to the nearby computer; thus signals were directly displayed on the screen. These signals consist out of a vertical (FZ), cranio-caudal (FY) and medio-lateral (FX) direction. The signals were transferred into graphs and numbers after each measurement moment. The graphs and numbers of the cranio-caudal, vertical and vertical impulse will be used for analysis. The walkway of the dog was surrounded by fence to guide the dog in the right direction. The handler walked or run beside the animal, outside of the surrounded fence. If necessary, the handler vocally encouraged the dog. The walkway was 11 meters long and the force plate itself 40 cm long and 60 cm wide. The force plate can be adapted in size if needed, for most of the dogs a smaller plate was more suitable. These size-adaptations can be seen in figure 1 of Corbee et al [57].

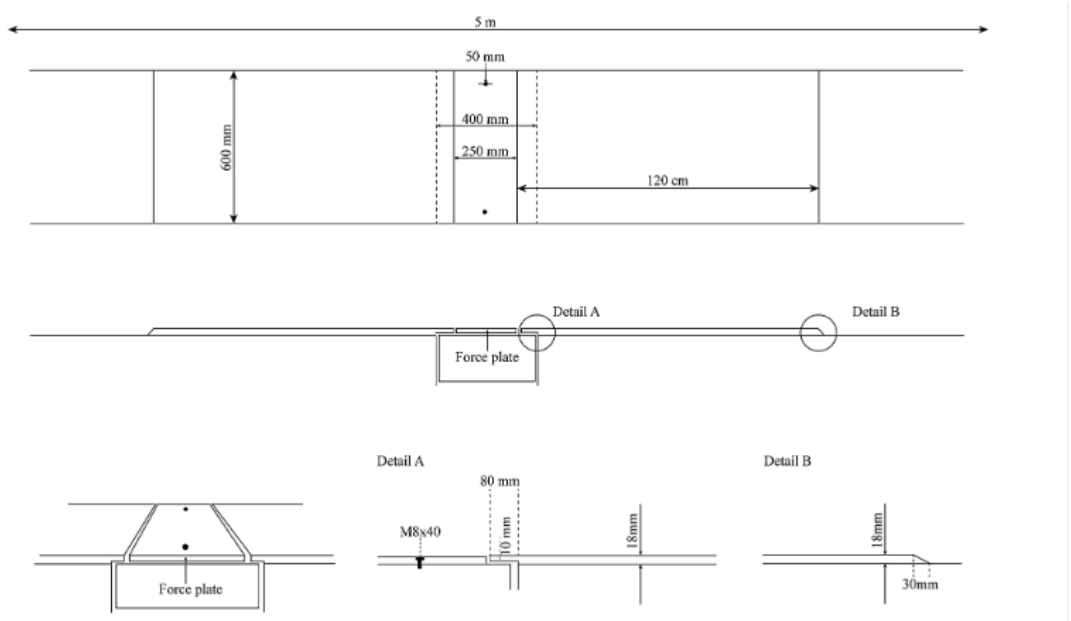
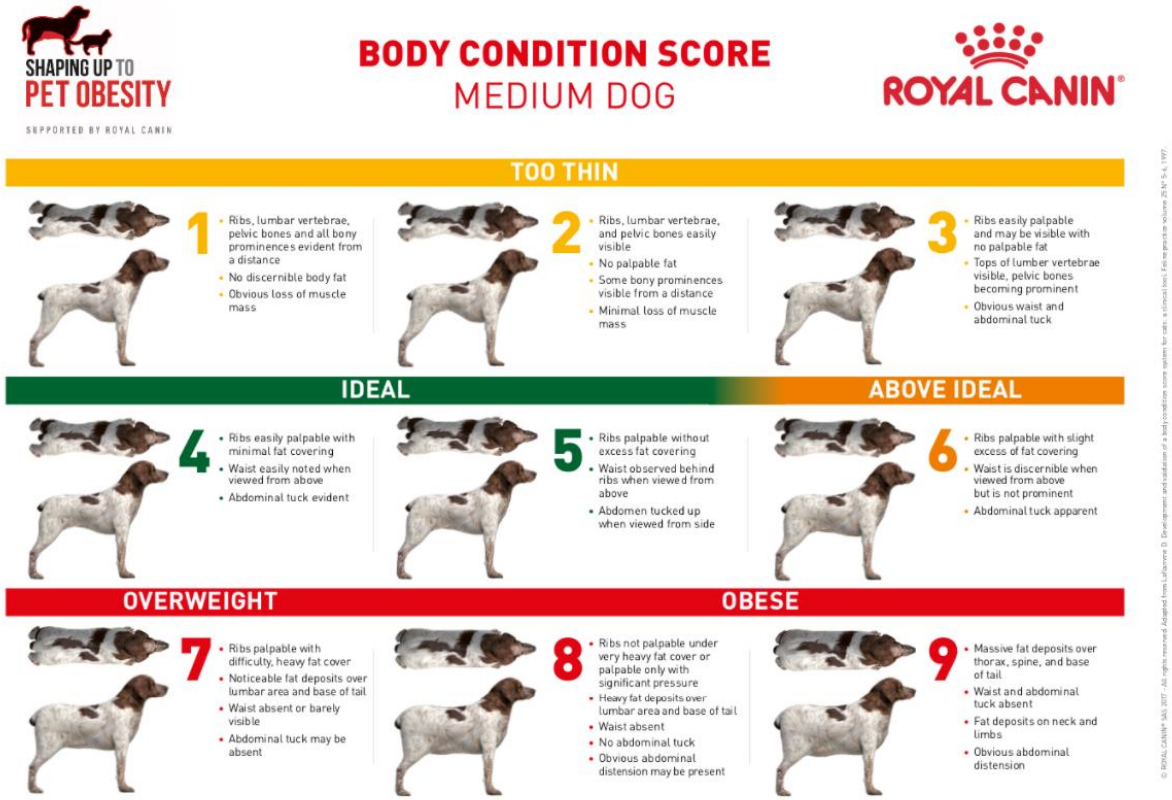


Figure 5 : Adaption of the force plate for force plate analysis in dogs. The force plate can be adapted by using a smaller plate on top of the force plate. To ensure exact measurements two plates were places next to the smaller plate, without interfering with the force plate, seen in detail A and B. Furthermore, M8 x 40 bolts were used to attach the small plate [57].

BODY CONDITION SCORE

The BCS was measured to determine and supervise the condition of the dog, during the research. The BCS of each dog was determined by a table as seen below [58]. The BCS was determined by the same veterinary student each measurement.

Table 6: Body Condition Score from Royal Canin [58].



STATISTICS

Statistical analysis was performed using R studio version 1.4.1103. R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/. A P-value < 0.05 was considered significant. The analyst determents some results, however the analyst was blinded to the treatments for the dogs. Parameters being analyzed were the HCPI, COAST, Forceplate, BCS and weight. Normal distribution was determined by using normal probability plots; all data were normally distributed and were checked for homoscedasticity. The final model used for analysis was a multiple regression model, all assumptions checked. Furthermore, to determine the best fitted model a drop1-Pearson chi-square model was used. Moreover, to determine the significance a 95% confidence interval was used. Due to the binary outcome of the COAST, a Fisher's Exact Test for Count Data was also used.

RESULTS

HELSINKI CHRONIC PAIN INDEX (HCPI)

No significant results were found in the analyses of the HCPI, however looking at figure 6, there is a difference between the placebo group (treatment 0 in figure 6) and the sialyllactose group (treatment 1 in figure 6). Whereas the line of placebo group is almost straight, the line of sialyllactose group is slanted.

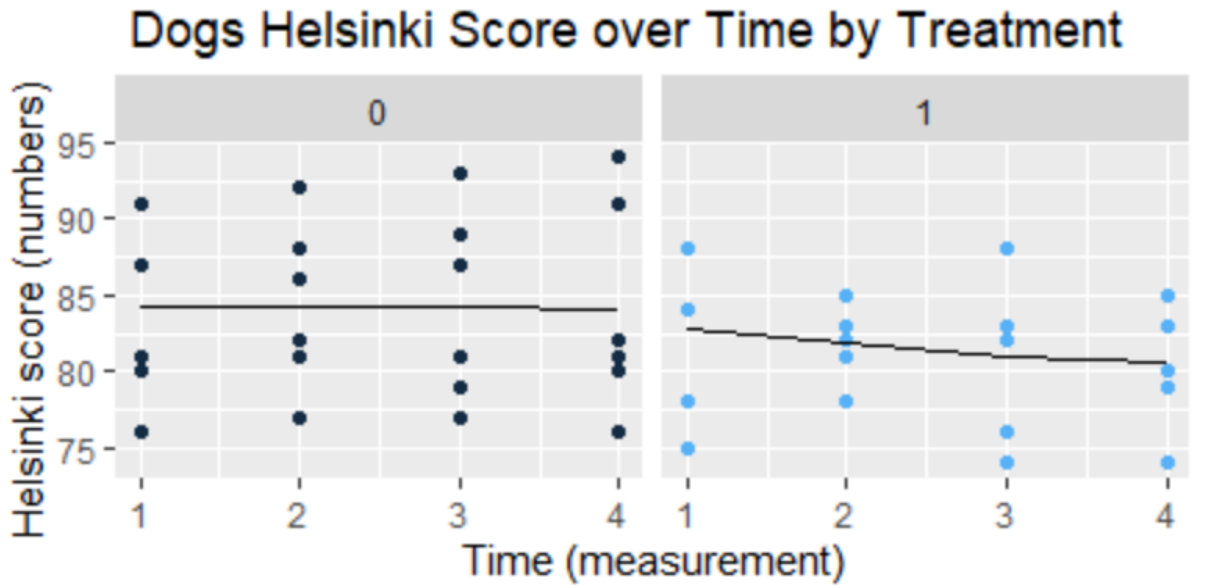
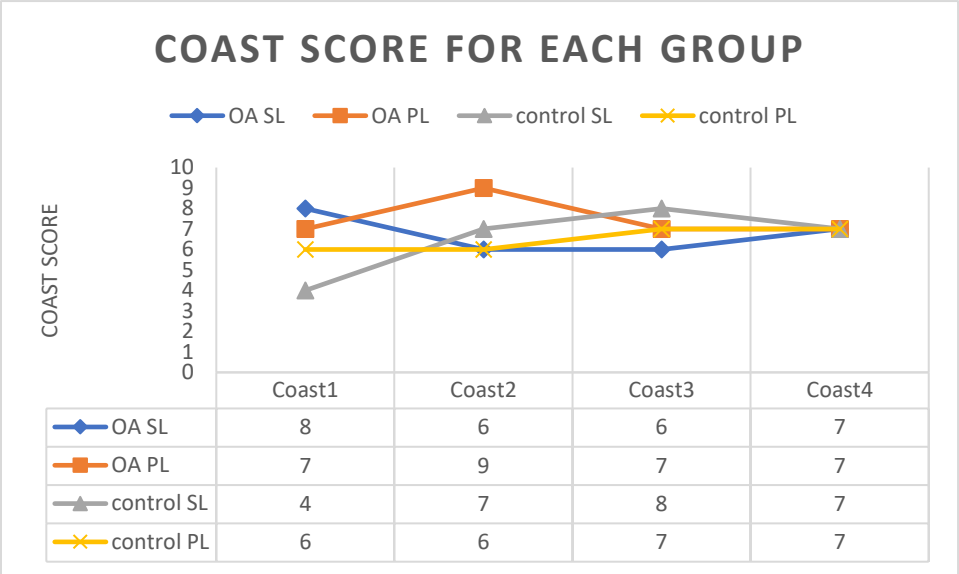


Figure 6: Ggplot HCPI.

CANINE OSTEOARTHRITIS STAGING TOOL (COAST)

The results of the Coast are determined by a Fisher test, shown in table 15 in the attachments. No significant results were found when comparing test day 1 and 4. The total Coast score for all the 4 groups are displayed in figure 6, as seen below. The start Coast score is different for each group, however the end score is the same.



	Coast1	Coast2	Coast3	Coast4
OA SL	8	6	6	7
OA PL	7	9	7	7
control SL	4	7	8	7
control PL	6	6	7	7

Figure 7: Total Coast Score for each group. Abbreviations: 'OA SL', Osteoarthritis Sialyllactose; 'OA PL', Osteoarthritis Placebo; 'Control SL', Control Sialyllactose; 'Control PL', Control Placebo.

FORCE PLATE

Forceplate analysis was conducted to determine the possible effect of sialyllactose. The maximum cranio-caudal force, minimum cranio-caudal force and maximum vertical force were measured of each dogs' feet. Furthermore, to compare the left an right side, the symmetry of each force was measured. The closer the symmetry approached the number 1, the more symmetry is present. To compare all feet, measurements of all were needed. Furthermore, the difference between the front and back of the dog can be analyzed. The time is added to determine the speed of the dog and the ground reaction time. Due to lameness, a dog will have a shorter ground reaction time on the lame foot, compared to a non-lame foot. Moreover, comparison between the sick/healthy and the treatment/no treatment groups can be made. In conclusion, the force plate analysis was done to determine if dogs with OA could have a better overall symmetry in the walk, moreover to conclude the walk would not get less symmetric in case of the healthy dogs.

Abbreviations list Force plate:

F= front

FYmax= maximum cranio-caudal force

FYmin = minimum cranio-caudal force

FZmax = maximum vertical force

H= hind

IZ= vertical impulze

LF= left front limb

LH= left hind limb

RF= right front limb

RH= right hind limb

S= symmetry

Significant effects were found by: FYminLH, FYminRH, FYmaxLF, FYmaxLH, FYmaxRH, FZmaxLF, FZmaxLH, IZLH, IZRH and SIZH. No significant effect was determined by: FYminLF, FZmaxRF, FZmaxRH IZLF, IZRF, SFYminF, SFYminH, SFYmaxF, SFYmaxH, SFZmaxF, SFZmaxH, SIZF. Furthermore, except for SIZH all the symmetry measurements are not significant.

FYMINLH

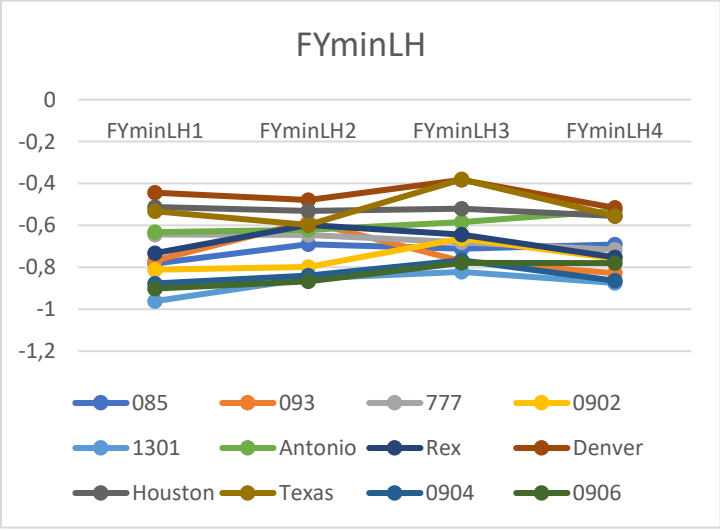
FYminLH, the minimum cranio-caudal force (FYmin) of the left hind limb (LH).

Table 7:FYminLH results.

FYminLH results	estimate	2.5 %	97.5 %
(Intercept)	-0.7406	-0.8925	-0.5887
factor(time)2	0.0441	-0.0306	0.1188
factor(time)3	**0.1139**	**0.0392***	**0.1886***
factor(time)4	0.0595	-0.0151	0.1342
factor(treatment)1	0.0199	-0.1488	0.1886
factor(time)1:factor(sick)1	0.0676	-0.1111	0.2462
factor(time)2:factor(sick)1	0.0603	-0.1183	0.2390
factor(time)3:factor(sick)1	0.0399	-0.1388	0.2185
factor(time)4:factor(sick)1	0.0213	-0.1574	0.1999

**Significant, p<0.05*

A significant effect was found at time 3, FYminLH was significant higher at time 3 in comparison to time 1, seen in table 7. Furthermore, some interaction was found between time and sick.



The FYminLH score each test day is displayed in figure 7. As seen in table 7, time 3 is significant, the significance in the figure can be seen at FYminLH3.

Figure 8: FYminLH.

Significance for time 3 is also found for FYminRH and FYmaxRH, these results can be found in the attachments.

FYMAXLF

FYmaxLF, the maximum cranio-caudal force (FYmax) of the left front limb (LF).

A significant effect was found in factor treatment. As seen in the table 8 and figure 9, The group with sialyllactose (treatment 1) is significantly lower than the placebo group (treatment 0).

Table 8: Results FYmaxLF.

FYmaxLF	estimate	2.5 %	97.5 %
(Intercept)	1.2596	1.1366	1.3825
factor(time)2	0.0425	-0.0276	0.1126
factor(time)3	0.0573	-0.0128	0.1274
factor(time)4	0.0028	-0.0673	0.0729
factor(treatment)1	**-0.1681**	**-0.3028 ***	**-0.0335 ***
factor(sick)1	0.0583	-0.0763	0.1929

**Significant, p<0.05*

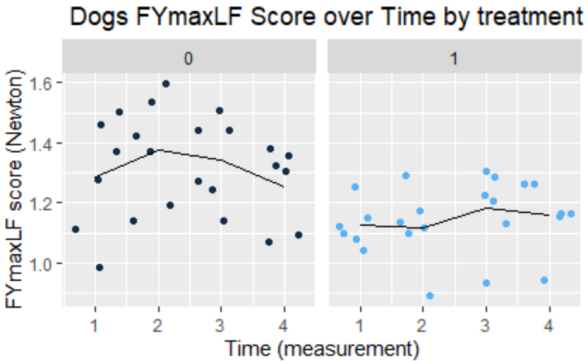


Figure 9: Ggplot FYmaxLF.

FYMAXLH

FYmaxLH, the maximum cranio-caudal force (FYmax) of the left hind limb (LH).

Multiple significant effects were found. As seen in table 9, at time 4 FYmaxLH was significantly lower than time 1. Furthermore, the FYmaxLH from the OA groups (sick 1) was significantly lower than the healthy groups (sick 0). At time 1 the difference between the placebo group (treatment 0) and the sialyllactose group (treatment 1) was significant, the same applies to time 3.

Table 9: Results FYmaxLH.

FYmaxLH results treatment	estimate	2.5 %	97.5 %
(Intercept)	0.8452	0.7071	0.9834
factor(time)2	-0.0121	-0.0755	0.0513
factor(time)3	-0.0041	-0.0675	0.0594
factor(time)4	**-0.0792**	**-0.1426***	**-0.0157***
factor(sick)1	**-0.1554**	**-0.3097***	**-0.0011***
factor(time)1:factor(treatment)1	**-0.1907**	**-0.3529***	**-0.0286***
factor(time)2:factor(treatment)1	-0.0689	-0.2311	0.0933
factor(time)3:factor(treatment)1	**-0.1744**	**-0.3365***	**-0.0122***
factor(time)4:factor(treatment)1	-0.0935	-0.2557	0.0687

**Significant, p<0.05*

The difference between the sialyllactose group (treatment 1) and the placebo group (treatment 0) at time 1 and 3 is displayed in figure 11. In Figure 10 the difference between healthy group/OA group (sick 0/1) can be seen, the line of healthy dogs (sick 0) is higher than that from the OA dogs (sick1). Therefore, the outcome of FYmaxLH is higher of healthy dogs (sick 0) in comparison to OA dogs (sick 1), there is a difference between the healthy and OA-dogs. As seen in table 9, time 4 was significant. This is also displayed in figure 10, both groups have a downward trend from time 2. Remarkable is the rise between time 1 and 2.

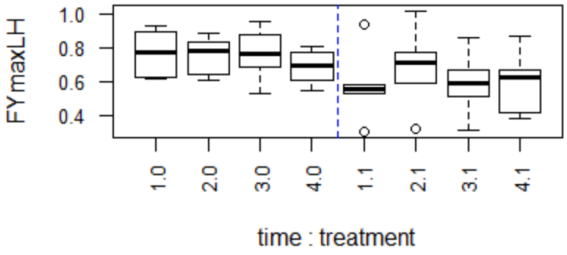


Figure 11: Boxplot FYmaxLH with time and treatment.

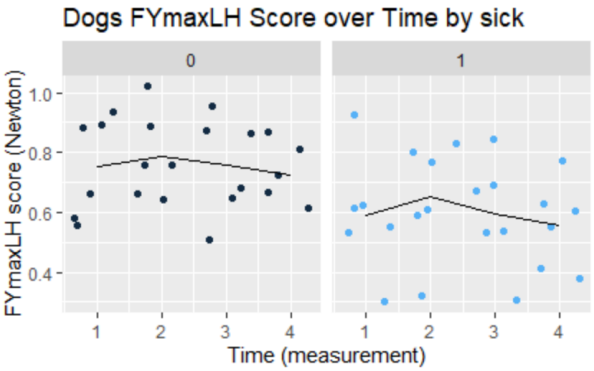


Figure 10: Ggplot FYmaxLH.

FZMAXLF

FZmaxLF, the vertical ground reaction force (FZmax) of the left front limb (LF).

The table of FZmaxLF, table 10, shows a significant effect of the sialyllactose group (treatment 1) in comparison to placebo group (treatment 0). This effect is showed in figure 12, the line of the sialyllactose group (treatment 1) is higher than that from the placebo group (treatment 0).

Table 10: Results FZmaxLF.

FZmaxLF	estimate	2.5 %	97.5 %
(Intercept)	6.6743	6.4282	6.9204
factor(time)2	0.1558	-0.0184	0.3300
factor(time)3	0.1363	-0.0380	0.3105
factor(time)4	-0.0931	-0.2673	0.0811
factor(treatment)1	**0.2616**	**0.0007***	**0.5224***
factor(sick)1	-0.1169	-0.3778	0.1440

**Significant, p<0.05*

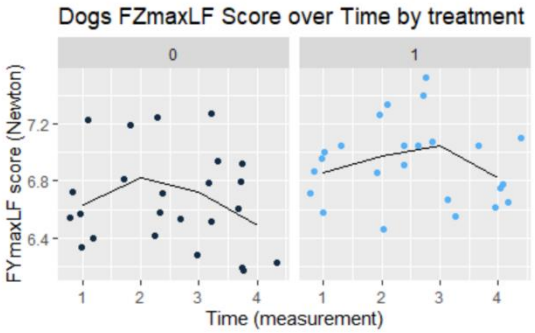


Figure 12: Ggplot FZmaxLF.

FZMAXLH

FZmaxLH, the maximum vertical ground reaction force (FZmax) of the left hind limb (LH).

At time 4, FZmaxLH is significantly changed, it is 0.3623 lower than at time 1, seen in table 11. Remarkable is the difference between FZmaxLH in the placebo group (treatment 0) and sialyllactose group (treatment 1) as displayed in figure 13, however these differences are not significant.

Table 11: Results FZmaxLH.

FZmaxLH	estimate	2.5 %	97.5 %
(Intercept)	4.9023	4.3506	5.4539
factor(time)2	0.0980	-0.1285	0.3245
factor(time)3	-0.1685	-0.3950	0.0581
factor(time)4	**-0.3623**	**-0.5888***	**-0.1358***
factor(treatment)1	0.0379	-0.5824	0.6582
factor(sick)1	-0.0566	-0.6769	0.5637

**Significant, p<0.05*

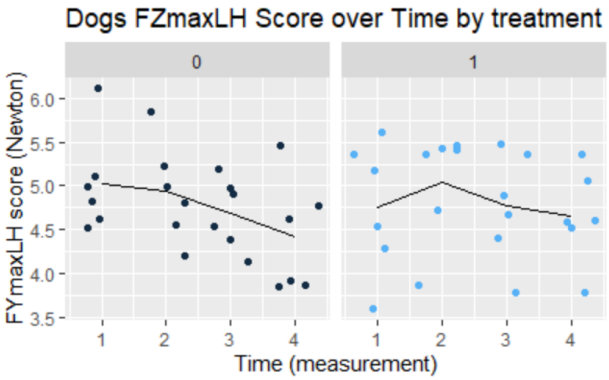


Figure 13: Ggplot FZmaxLH.

Time 4 is also significant by IZLH and IZRH.

SIZH

SIZH, the symmetry (S) between the vertical impulse (IZ) of the hindlimbs (H).

A significant effect found in the difference between the sialyllactose group (treatment 1) and placebo group (treatment 0), displayed in table 12.

Table 12: Results SIZH.

SIZH	estimate	2.5 %	97.5 %
(Intercept)	0.9781	0.9548	1.0014
factor(time)2	-0.0111	-0.0332	0.0109
factor(time)3	-0.0210	-0.0431	0.0010
factor(time)4	-0.0021	-0.0241	0.0199
factor(treatment)1	**-0.0292**	**-0.0570***	**-0.0014***
factor(sick)1	-0.0096	-0.0374	0.0182
factor(treatment)1:factor(sick)1	0.0328	-0.0065	0.0722

**Significant, p<0.05*

The difference of the symmetry in the IZ from the hindlimbs between the sialyllactose and placebo group can be seen in figure 14. As seen in the figure the sialyllactose group is closer to the 1.00 than the treatment group. Remarkable is the rise of both lines between time 3 (SIZH3) and time 4 (SIZH4).

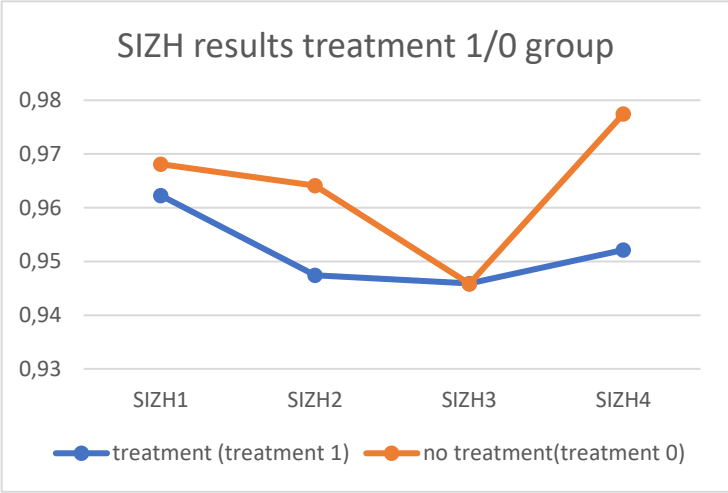


Figure 14: SIZH results treatment 1/0 group.

WEIGHT

The results of weight are displayed in table 16 in the detachments. Visible in this table is the significance of the weight in time. The estimate lowers each measurement day. Therefore, the dogs' weight is significantly lowered during the experiment. Moreover, the decrease of weight is also visible in the boxplot and Ggplot, figure 15 and 16. The boxplot shows a trend of decrease, remarkable is the small scatter in the treatment 0, sick 1 group. Furthermore, the ggplot shows a downward trend.

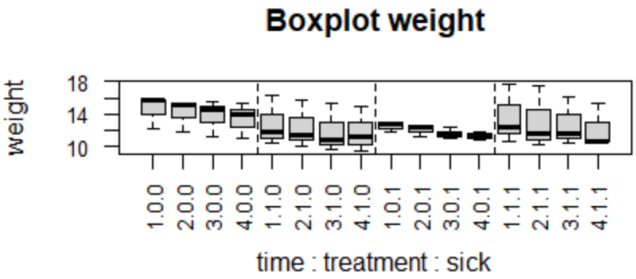


Figure 15: Boxplot weight.

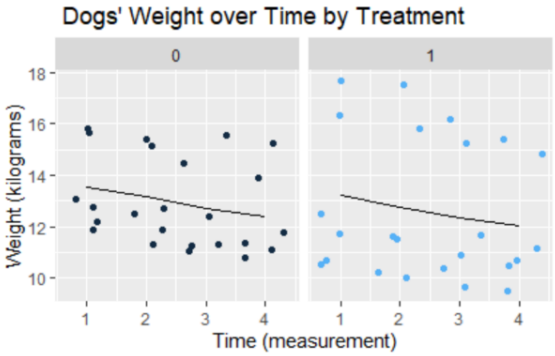


Figure 16: Ggplot 'Dogs' Weight over Time by Treatment'.

BODY CONDITION SCORE

The BCS was only for supervising, however due to significant result seen in table 13, the BCS is discussed. As seen in figure 17, both lines are decreased at time 4 in comparison to time 1. The significance is most visible at time 3, the line is decreased in the placebo group as well as in the OA group. Besides this significance, a remarkable rise between time 3 and 4 is displayed.

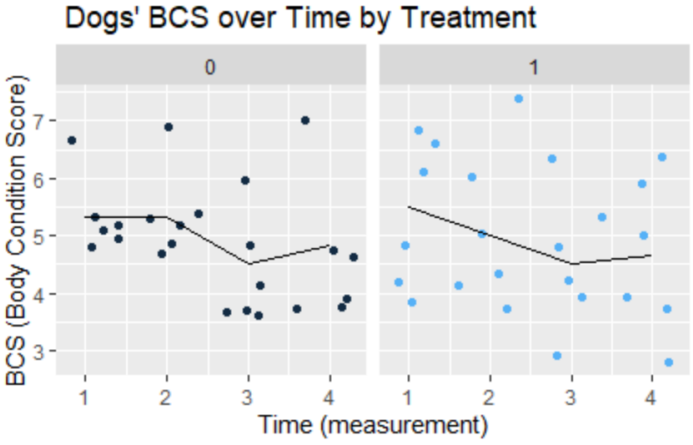


Figure 17: Ggplot ' Dogs' BCS over Time by Treatment'.

Table 13: Results BCS.

BCS	estimate	2.5 %	97.5 %
(Intercept)	5.5417	4.5310	6.5523
factor(time)2	-0.2500	-0.5688	0.0688
factor(time)3	**-0.9167**	**-1.2355***	**-0.5978***
factor(time)4	**-0.6667**	**-0.9855***	**-0.3478***
factor(treatment)1	-0.0833	-1.2325	1.0658
factor(sick)1	-0.1667	-1.3158	0.9825

**Significant, p<0.05*

DISCUSSION

LIMITATIONS

For this research 12 dogs were used, which is a small group. A larger number of dogs may have resulted in different outcomes. Not only the group of dogs is small but also the diversity. In the experiment Beagles and Beagleton were used, however other breeds are not investigated. Consequently, no conclusion can be made about using sialyllactose in other breeds. Besides these limitations, the used dogs are laboratory dogs. Laboratory animals live in different circumstances as pets. Moreover, these laboratory dogs were also largely genetically linked. Whether the outcome of this research stays the same in different circumstances is unknown.

The results were found with feeding schedule of 150 mg/kg sialyllactose per dog, for 4 months. The limitations in this part contain the concentration and the time schedule. For instance, it is possible that the concentration of sialyllactose was too low for a significant effect. Moreover, the research is conducted in only 4 months.

Moreover, two kinds of sialyllactose were used, 3'Siallylactose and 6'Sialyllactose, in a 9:1 ratio. Therefore, results cannot be attributed to either 3' sialyllactose or 6'siallaylactose. Indeed, using both Sialyllactose eliminates the opportunity to test effects of the two kinds Sialyllactose separately.

The measurements were conducted at start, two weeks, 2 months and 4 months. However, these laboratory dogs are also used for educational purposes. This resulted into spreading the measurements over some days. Some accuracy can be lost if the measurement time is spread. As seen in the results BCS and weight are significantly lower than in the beginning. The research is conducted in the winter, which can influence the BCS. However, it is unsure if the Sialyllactose also influenced the BCS.

DISCUSSION RESULTS

In this study, analysis was conducted about beneficial immune-modulating effects of sialyllactose in OA treatment in dogs. This analysis existed out of: HCPI, Coast, Force plate, and BCS. These parameters are patient centered and evaluate the effect of oral supplementation of sialyllactose at a dose of 100mg/kg for 4 months on pain perception and pain interference in daily life activities.

The HCPI questionnaire is used to evaluate chronic pain in dogs. A significant change in HCPI score can indicate an increase or decrease in chronic pain; the higher the HCPI score is, the lesser the pain. As previously stated, HCPI analyses gave no significant results, however a difference between treatment groups was visible, the dogs with the sialyllactose diet had a lower HCPI score than the placebo group, figure 6. The figure shows a slanted line in the treatment group, the placebo group stayed about the same. The cause of these differences cannot be determined in this study, therefore more study is needed.

Another questionnaire used is the COAST. The COAST is a standardized method for diagnosing OA or joint diseases in dogs, effects of Sialyllactose could reduce the stage of OA, giving a lower COAST score. The COAST results can be seen in figure 7, no significant effect was determined. Due to the fact that the COAST only has 5 stages, zero to 4, each stage has a high threshold, resulting into less differences. Consequently, only huge differences could give a significant result, which are less common in short studies. In other words, a longer study has to be conducted to find out if significant results can be found.

The most extensive part of the study consists out of the force plate analysis. In this study, multiple forces were analyzed as: FYmin, FYmax, FZmax and IZ. The analysis gave multiple significant outcomes, however these outcomes are widespread. Firstly, multiple forces were significant at time 3 or 4. At time 3: FYminLH, FYminRH and FYmaxRH were significant higher in comparison to time 1. Briefly, the cranio-caudal force has significant increased at time 3. However, due to lack of significance in the symmetry of the FY forces no statement can be made about improvement or deterioration. Furthermore, the mentioned forces did not have significant changes between the treatment/placebo group. Whether the significant changes can be attributed to the treatment are therefore unlikely.

Not only the results at time 3, but also some results at time 4 were significant in several forces: FYmaxLH lowered, FZmaxLH lowered, IZLH increased and IZRH increased. Thus, the cranio-caudal force, vertical force and vertical impulse were significantly changed. Though a significance is found, due to the lack of symmetry, except for IZ hind, no statement can be made about improvement or deterioration. By way of contrast, a significant symmetry change is found for IZ hind. However, this significance is found for the treatment groups, not at time 4. That being the case, a difference between the treatment and placebo group is found, which could indicate an effect of sialyllactose. As displayed in figure 14, the placebo group has more symmetry than the treatment group. This result could indicate a negative effect of Sialyllactose instead of the positive effect expected. Either way, as seen in figure 14, a slanted line is seen for the placebo group till time 3. Only between time 3 and 4 a remarkable rise is seen, due to the constant feeding schedule there is no validation for a negative Sialyllactose effect. To conclude, more research has to been done to validate or deny the negative effect of Sialyllactose.

As the study exists out of a feeding trial, the most interest goes to the treatment. . Not only was SIZH significant for treatment, the FYmaxLF and FZmaxLF were also significant. So, the cranio-caudal force significantly changed in the treatment group in comparison to the placebo group. The FYmaxLF is lower in the treatment group, while on the other hand the treatment group became higher by FZmaxLF. Despite this significance, no validation can be made about improvement or deterioration, due to the lack of significance in symmetry. However, sialyllactose having an effect, becomes more plausible.

The significant results are mostly limited to one factor. However, FYmaxLH has 4 significant outcomes; Time 4, sick1, time 1:treatment1 and time 3:treatment1, displayed in table 9, figure 10,11. As mentioned before FYmaxLH is significant for time 4 as are more forces and yet the only one with significance for sick1, time 1:treatment1 and time 3:treatment1. The maximum cranio-caudal force is significantly different between the groups healthy and OA. Furthermore, at time 1 and 3 the treatment group has on average a lower maximum cranio-caudal force in the left hindlimb than the placebo group. Notwithstanding, these significance results, a validation about improvement or deterioration cannot be made, due to the lack of significance in symmetry. Moreover, more research is needed to provide enough evidence and results to make statements about the effects of sialyllactose.

Although weight measurements were only measured to make more accurate force plate measurements, some noteworthy results were found. As mentioned in the results, the weight significantly decreased during the study. Each measurement day the weight dropped significantly. In this study, the cause can be found in one or both of the following: Sialyllactose and Weather. The only difference with normal circumstances is the addition of Sialyllactose in the diet. Admittedly, sialyllactose could be the cause of the weight loss, however it is not the best fitting cause. As displayed in figure 16, both groups, treatment and placebo, had weight loss. Furthermore, no significance is seen in relation to the treatment. More important, the lines are fairly parallel, implying an about even weight loss. With this is mind and the lack of sialyllactose in the placebo group, the cause is presumably found in the weather. The study is conducted from November to March in the Netherlands. On the subject of weather, the KNMI gives mean temperatures [59], [60]. These temperature decreases from October 2020 to January 2021, a slight rise is seen form January to March, seen in table 14 [59], [60]. However, the last measurements were done the first week of March.

Table 14: Mean temperatures in the Netherlands [59], [60].

Date	Mean temperature (degrees Celsius)
October 2020	11.3
November 2020	8.9
December 2020	5.5
January 2021	3.4
February 2021	4.3
March 2021	6.4

The BCS was measured to determine and supervise the condition of the dog, during the research. However, due to noteworthy results the BCS will be discussed. The results of the BCS are seen in figure 17 and table 13. The BCS is significant at time 3 and time 4, both times the BCS decreased in comparison to time 1. Besides this, an increase is also visible in figure 17 between time 3 and 4. Although this study is a feeding trial, the Sialyllactose is presumably not the cause of the significant difference. For the reason that in figure 17 and table 13, no significant difference is seen between treatment and placebo group. As previously stated, could the cause of weight loss be found in the weather, due to the fact that the BCS is partly based on fat storage [58]–[60]. However, due to the lack of similar studies and limitations of study design, only further studies can deny or agree to this statement.

As mentioned in the introduction, the effects of sialyllactose seemed promising in the research of Jeon J [2]. However, such promising results are not found in this study. Differences in the studies can be found in the animal, furthermore the cause of OA can be found elsewhere. In the research of Jeon et al [2], mouses were just with induced OA, while this study just dogs with naturally occurring OA. Furthermore, the study of Jeon et al [2], only used 3 sialyllactose instead of the 3’ Sialyllactose and 6’Siallylactose in this study. The differences in the study could be the cause of scarce significant results, however only more research could give clear answers on this matter.

This study is one of a kind, therefore no good comparisons can be made with other studies. Therefore, more research is needed about this subject, particularly in dogs.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSION

In this study, analysis was conducted about the centered outcome on pain and mobility of supplementing sialyllactose to adult dogs with OA. Analysis shows an effect of sialyllactose on the FYmaxLF, FYmaxLH, FZmaxLF and SIZH. Consequently, an effect of sialyllactose is determined. However, due too scarce significant results the exact effect of sialyllactose is unknown, therefore a follow-up investigation is recommended.

RECOMMENDATIONS

A more diverse research should use more dogs, breeds and different kinds of housing facilities. A higher concentration of both sialyllactose and a longer feeding time is recommended to determine if the found outcomes remain the same. In particular, it is recommended to separate the two kinds of sialyllactose to determine them independently. Furthermore, to eliminate potential cofounders the research should be conducted in the summer. Moreover, to increase the accuracy of the measurements, the used dogs should be available on every measurement day. Since this research is a proof of principle study a follow-up study should be conducted, the recommendation should be taking into account. Regarding the group size, no number can be given, since this number should be investigated in the follow-up research.

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ATTACHMENTS

COAST OSTEOARTHRITIS STAGING TOOL (COAST)

Results of the COAST are shown in table 15, no significant effect was found.

Table 15: Results COAST.

COAST, Fisher's Exact Test for Count Data	
data:	m = matrix(c(0,3, 0,3, 3,0, 1,2), byrow=T, nrow=4)
p-value =	0.5455
alternative hypothesis:	true odds ratio is not equal to 1
95 percent confidence interval:	0.2222013 313.0419931
sample estimates:	odds ratio 4.341094

WEIGHT

The results of weight are seen in table 16, a significant effect was seen at time 2,3 and 4.

Table 16: Results weight.

Weight	estimate	2.5 %	97.5 %
(Intercept)	13.9250	11.6598	16.1903
factor(time)2	**-0.4333**	**-0.7046***	**-0.1621***
factor(time)3	**-0.8792**	**-1.1504***	**-0.6079***
factor(time)4	**-1.2000**	**-1.4712***	**-0.9288***
factor(treatment)1	-0.3521	-2.9621	2.2579
factor(sick)1	-0.7062	-3.3162	1.9037

**Significant, p<0.05*

FORCE PLATE ATTACHMENTS

FYMINLF

FYminLF, the minimum cranio-caudal force (FYmin) of the left front limb (LF). Results are shown in table 17. No significant effect was found, however some interaction is found between time and treatment.

Table 17: Results FYminLF.

FYminLF	estimate	2.5 %	97.5 %
(Intercept)	-0.9599	-1.1142	-0.8056
factor(time)2	-0.0357	-0.0962	0.0249
factor(time)3	0.0333	-0.0273	0.0938
factor(time)4	-0.0274	-0.0879	0.0331
factor(sick)1	0.0842	-0.0898	0.2581
factor(time)1:factor(treatment)1	0.0934	-0.0870	0.2737
factor(time)2:factor(treatment)1	0.1306	-0.0497	0.3109
factor(time)3:factor(treatment)1	0.0073	-0.1730	0.1877
factor(time)4:factor(treatment)1	0.0378	-0.1425	0.2181

FYMINRF

FYminRF, the minimum cranio-caudal force (FYmin) of the right front limb (RF). Results are shown in table 18. No significant effect was found.

Table 18: Results FYminRF.

FYminRF	estimate	2.5 %	97.5 %
(Intercept)	-0.9233	-1.0438	-0.8028
factor(time)2	-0.0106	-0.0690	0.0477
factor(time)3	0.0319	-0.0264	0.0903
factor(time)4	-0.0518	-0.1101	0.0065
factor(treatment)1	0.0778	-0.0561	0.2118
factor(sick)1	0.1262	-0.0078	0.2602

FYMINRH

FYminRH, the minimum cranio-caudal force (FYmin) of the right hind limb (RH). As shown in table 19, significance is seen at factor(time)3, at time 3 FYminRH significant changed in comparison to time 1. At time 3 FYminRH is 0.1001 higher than -0.7305 at time 1. However, correlation with the treatment is not seen.

Table 19: Results FYminRH.

FYminRH	estimate	2.5 %	97.5 %
(Intercept)	-0.7305	-0.8795	-0.5814
factor(time)2	0.0405	-0.0130	0.0940
factor(time)3	**0.1001**	**0.0466***	**0.1536***
factor(time)4	0.0364	-0.0171	0.0899
factor(treatment)1	0.0199	-0.1488	0.1886
factor(sick)1	0.0473	-0.1214	0.2160

**Significant, p<0.05*

FYMAXRF

FYminRF, the minimum cranio-caudal force (FYmin) of the right front limb (RF). Results are shown in table 20. No significant effect was found.

Table 20: Results FYminRF.

FYminRF	estimate	2.5 %	97.5 %
(Intercept)	1.2333	1.1140	1.3527
factor(time)2	-0.0116	-0.0895	0.0662
factor(time)3	0.0442	-0.0337	0.1220
factor(time)4	-0.0205	-0.0983	0.0574
factor(treatment)1	-0.0943	-0.2226	0.0341
factor(sick)1	0.0056	-0.1228	0.1339

FYMAXRH

FYmaxRH, the maximum cranio-caudal force (FYmax) of the right hind limb (RH). As shown in table 21, significance is seen at factor(time)3, at time 3 FYmaxRH significant changed in comparison to time 1. At time 3, FYmaxRH is 0.0854 higher than 0.7585 at time 1. However, correlation with the treatment is not seen.

Table 21 : Results FYmaxRH.

FYmaxRH	estimate	2.5 %	97.5 %
(Intercept)	0.7585	0.5157	1.0012
factor(time)2	0.0024	-0.0549	0.0597
factor(time)3	**0.0854**	**0.0282***	**0.1427***
factor(time)4	-0.0033	-0.0606	0.0540
factor(treatment)1	-0.0488	-0.3267	0.2292
factor(sick)1	-0.0559	-0.3338	0.2221

**Significant, p<0.05*

FZMAXRF

FZmaxRF, the maximum vertical ground reaction force (FZmax) of the right front limb (RF). Results are shown in table 22. No significant effect was found.

Table 22: Results FZmaxRF.

FZmaxRF	estimate	2.5 %	97.5 %
(Intercept)	6.6069	6.3012	6.9126
factor(time)2	0.1054	-0.0682	0.2790
factor(time)3	0.0924	-0.0812	0.2660
factor(time)4	-0.1101	-0.2837	0.0635
factor(treatment)1	0.2569	-0.0780	0.5919
factor(sick)1	-0.0375	-0.3725	0.2974

FZMAXRH

FZmaxRH, the maximum vertical ground reaction force of the right hind limb. Results are shown in table 23. No significant effect was found, however some interaction between treatment and time was found.

Table 23: Results FZmaxRH.

FZmaxRH results treatment	estimate	2.5 %	97.5 %
(Intercept)	0.7927	0.5483	1.0371
factor(time)2	-0.0401	-0.1165	0.0362
factor(time)3	0.0346	-0.0418	0.1110
factor(time)4	-0.0470	-0.1233	0.0294
factor(sick)1	-0.0559	-0.3338	0.2221
factor(time)1:factor(treatment)1	-0.1173	-0.4016	0.1670
factor(time)2:factor(treatment)1	-0.0322	-0.3165	0.2521
factor(time)3:factor(treatment)1	-0.0157	-0.3000	0.2686
factor(time)4:factor(treatment)1	-0.0300	-0.3143	0.2543

IZLF

IZLH, the vertical impulse (IZ) of the left front limb (LF). Results are shown in table 24. No significant effect was found.

Table 24: Results IZLF.

IZLF	estimate	2.5 %	97.5 %
(Intercept)	0.7141	0.6910	0.7371
factor(time)2	-0.0111	-0.0236	0.0013
factor(time)3	0.0018	-0.0107	0.0143
factor(time)4	0.0067	-0.0057	0.0192
factor(treatment)1	-0.0276	-0.0587	0.0035
factor(sick)1	-0.0132	-0.0443	0.0179
factor(treatment)1:factor(sick)1	0.0312	-0.0128	0.0751

IZRF

IZLH, the vertical impulse (IZ) of the right front limb (RF). Results are shown in table 25. No significant effect was found.

Table 25: Results IZRF.

IZRF	estimate	2.5 %	97.5 %
(Intercept)	0.7056	0.6798	0.7315
factor(time)2	-0.0085	-0.0223	0.0053
factor(time)3	0.0012	-0.0126	0.0150
factor(time)4	0.0007	-0.0131	0.0145
factor(treatment)1	-0.0084	-0.0369	0.0201
factor(sick)1	-0.0062	-0.0347	0.0223

IZLH

IZLH, the vertical impulse (IZ) of the left hind limb (LH). A significant effect was found at time 4, IZLH was significant higher at time 4 in comparison to time 1, shown in table 26.

Table 26: Results IZLH.

IZLH results treatment	estimate	2.5 %	97.5 %
(Intercept)	0.5803	0.5182	0.6424
factor(time)2	0.0039	-0.0266	0.0344
factor(time)3	0.0190	-0.0116	0.0495
factor(time)4	**0.0415**	**0.0109***	**0.0720***
factor(sick)1	0.0014	-0.0676	0.0704
factor(time)1:factor(treatment)1	0.0353	-0.0378	0.1084
factor(time)2:factor(treatment)1	-0.0193	-0.0923	0.0538
factor(time)3:factor(treatment)1	-0.0041	-0.0771	0.0690
factor(time)4:factor(treatment)1	-0.0184	-0.0915	0.0546

**Significant, p<0.05*

IZRH

IZRH, the vertical impulse (IZ) of the right hind limb (RH). A significant effect was found at time 4, shown in table 27. IZRH was significant higher at time 4 than in comparison with time 1.

Table 27: Results IZRH.

IZRH Results treatment	estimate	2.5 %	97.5 %
(Intercept)	0.5877	0.5191	0.6562
factor(time)2	0.0010	-0.0203	0.0224
factor(time)3	-0.0093	-0.0306	0.0121
factor(time)4	**0.0431**	**0.0218***	**0.0645***
factor(sick)1	0.0126	-0.0653	0.0905
factor(time)1:factor(treatment)1	0.0129	-0.0668	0.0927
factor(time)2:factor(treatment)1	-0.0215	-0.1012	0.0582
factor(time)3:factor(treatment)1	-0.0084	-0.0881	0.0713
factor(time)4:factor(treatment)1	-0.0518	-0.1315	0.0279

SFYMINF

SFYminF, the symmetry of the minimal cranio-caudal force (SFYmin) between the front limbs (F). Results are shown in table 28. No significant effect was found.

Table 28: Results SFYminF.

SFYminF	estimate	2.5 %	97.5 %
(Intercept)	0.8958	0.8475	0.9441
factor(time)2	0.0088	-0.0506	0.0681
factor(time)3	0.0486	-0.0108	0.1080
factor(time)4	0.0215	-0.0378	0.0809
factor(treatment)1	-0.0422	-0.1105	0.0260
factor(sick)1	-0.0406	-0.0883	0.0072
factor(time)2:factor(treatment)1	0.0687	-0.0153	0.1527
factor(time)3:factor(treatment)1	-0.0432	-0.1272	0.0407
factor(time)4:factor(treatment)1	0.0563	-0.0276	0.1403
factor(treatment)1:factor(sick)1	0.0471	-0.0204	0.1146

SFYMINH

SFYminH, the symmetry of the minimal cranio-caudal force (SFYmin) between the hind limbs (H). Results are shown in table 29. No significant effect was found.

Table 29: Results SFYminH.

SFYminH	estimate	2.5 %	97.5 %
(Intercept)	0.9601	0.8979	1.0223
factor(time)2	-0.0162	-0.0736	0.0413
factor(time)3	-0.0483	-0.1058	0.0092
factor(time)4	-0.0469	-0.1043	0.0106
factor(treatment)1	-0.0099	-0.0711	0.0513
factor(sick)1	-0.0332	-0.0944	0.0280

SFYMAXF

SFYmaxF, the symmetry of the maximum cranio-caudal force (SFYmax) between the front limbs (F). Results are shown in table 30. No significant effect was found.

Table 30: Results SFYmaxF.

SFYmaxF	estimate	2.5 %	97.5 %
(Intercept)	0.9408	0.8958	0.9858
factor(time)2	-0.0464	-0.0942	0.0014
factor(time)3	-0.0292	-0.0770	0.0186
factor(time)4	0.0012	-0.0466	0.0490
factor(treatment)1	0.0089	-0.0324	0.0502
factor(sick)1	-0.0258	-0.0671	0.0155

SFYMAXH

SFYmaxH, the symmetry of the maximum cranio-caudal force (SFYmax) between the hind limbs (H). Results are shown in table 31. No significant effect was found.

Table 31: Results SFYmaxH.

SFYmaxH	estimate	2.5 %	97.5 %
(Intercept)	0.8694	0.7713	0.9675
factor(time)2	-0.0653	-0.1506	0.0200
factor(time)3	-0.0535	-0.1388	0.0318
factor(time)4	0.0071	-0.0782	0.0924
factor(treatment)1	0.0345	-0.0641	0.1332
factor(sick)1	-0.1031	-0.2234	0.0171
factor(time)2:factor(sick)1	0.0871	-0.0335	0.2078
factor(time)3:factor(sick)1	0.0230	-0.0976	0.1437
factor(time)4:factor(sick)1	-0.0771	-0.1978	0.0436

SFZMAXF

SFZmaxF, the symmetry of the maximum vertical ground reaction force (SFZmax) between the front limbs (F). Results are shown in table 32. No significant effect was found.

Table 32: Results SFZmaxF.

SFZmaxF	estimate	2.5 %	97.5 %
(Intercept)	0.9814	0.9681	0.9947
factor(time)2	-0.0063	-0.0213	0.0088
factor(time)3	-0.0040	-0.0190	0.0111
factor(time)4	-0.0083	-0.0233	0.0068
factor(treatment)1	-0.0058	-0.0174	0.0058
factor(sick)1	0.0028	-0.0089	0.0144

SFZMAXH

SFZmaxH, the symmetry of the maximum vertical ground reaction force (SFZmax) between the hind limbs (H). Results are shown in table 33. No significant effect was found.

Table 33: Results SFZmaxH.

SFZmaxH	estimate	2.5 %	97.5 %
(Intercept)	0.9557	0.9205	0.9908
factor(time)2	-0.0218	-0.0516	0.0081
factor(time)3	-0.0234	-0.0533	0.0064
factor(time)4	-0.0044	-0.0343	0.0254
factor(treatment)1	-0.0062	-0.0418	0.0294
factor(sick)1	-0.0061	-0.0417	0.0295

SIZF

SIZF, the symmetry of the vertical impulse force (SIZ) between the front limbs (F). Results are shown in table 34. No significant effect was found.

Table 34: Results SIZF.

SIZF	estimate	2.5 %	97.5 %
(Intercept)	0.9856	0.9707	1.0004
factor(time)2	-0.0087	-0.0228	0.0054
factor(time)3	0.0008	-0.0133	0.0150
factor(time)4	-0.0107	-0.0248	0.0034
factor(treatment)1	-0.0053	-0.0198	0.0091
factor(sick)1	-0.0045	-0.0190	0.0099