EFFECTS OF A *SACCHAROMYCES CEREVISIAE* FERMENTATION PRODUCT-SUPPLEMENTED DIET ON IMMUNE RESPONSE, OXIDATIVE STRESS MARKERS, AND SKIN AND COAT HEALTH OF DOGS

BY

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THESIS

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ABSTRACT

Feeding *Saccharomyces cerevisiae* fermentation product (SCFP) has previously altered fecal microbiota, fecal metabolites, and immune cell function of adult dogs. The objective of this study was to investigate measures of skin and coat health for the first time and further measure circulating immune cell numbers and activity, antioxidant status, and oxidative stress marker concentrations of adult dogs fed a SCFP-supplemented extruded diet. All procedures were approved by the facility's IACUC prior to experimentation. 16 adult pointer dogs (8 M, 8 F; mean age = 6.7 ± 2.1 y; mean BW = 25.9 ± 4.5 kg) were used in a randomized crossover design study. All dogs were fed a control diet for 4 wk, then randomly assigned to the control or SCFP-supplemented diet and fed to maintain BW for 10 wk. A 6-wk washout preceded the second 10-wk experimental period with dogs receiving opposite treatments. After baseline/washout and treatment phases, skin and coat were scored, and pre-and post-prandial blood samples were collected. Transepidermal water loss (TEWL), hydration status, and sebum concentrations were measured (back, inguinal, ear) using external probes. Oxidative stress and immune cell function were measured using commercial ELISA kits, circulating immune cell numbers were analyzed using flow cytometry, and mRNA expression of oxidative stress genes were analyzed using RT-PCR. Change from baseline data were analyzed using the Mixed Models procedure of SAS 9.4, with $P<0.05$ being significant and $P<0.10$ being trends. Sebum concentration changes tended to be higher ($P<0.10$; inguinal, ear) in dogs fed SCFP than control. TEWL change on the back was lower ($P<0.05$) in controls, but lower ($P=0.054$) on the ear in dogs fed SCFP. DTH response was not measured at baseline, but was affected by diet and time (i.e., post-inoculation) at week 10. As

expected, wheal diameter increased over time for all injections. For phytohaemagglutinin (PHA), diet and time interactions were observed in SCFP-fed dogs ($P=0.02$). Diet tended ($P<0.10$) to have an effect on DTH response in SCFP-fed dogs after concanavalin A (ConA) inoculation. Other skin and coat measures and scores were not affected by diet. Changes in unstimulated lymphocytes and stimulated IFN-γ secreting T cells were lower ($P<0.05$) in SCFP-fed dogs, while change in stimulated T cells were lower ($P<0.05$) in control-fed dogs. The stimulated cytotoxic T cells delta trended lower ($P<0.10$) in SCFP-fed dogs. Change in serum superoxide dismutase (SOD) concentrations were higher ($P<0.05$) and change in catalase mRNA expression was lower ($P<0.05$) in SCFP-fed dogs. All other immune cells, oxidative stress markers, and gene expression were unaffected by treatment. In conclusion, our data suggest that SCFP positively impacts indicators of skin and coat health of dogs, modulates immune responses, and enhances some key antioxidant defense mechanisms.

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CHAPTER 1: INTRODUCTION

The 2021-2022 National Pet Owners Survey conducted by the American Pet Products Association (APPA) reported that 70% of all households in the United States own a pet, and of those households, 69 million own a dog. Pet ownership has increased steadily since 1988 when this survey was first conducted, when only about 56% of households owned a pet (APPA, 2022). Along with the rise in pet ownership, a change in owner attitudes has occurred in recent decades, where dogs are now considered a part of the family. As members of the family, pet owners are taking more care to provide the best possible veterinary care, enrichment, and nutrition for their pets. Despite the COVID-19 pandemic that impacted many market sectors negatively, pet spending increased during and following the pandemic, with 35% of pet owners stating that they spent more on their pets or pet supplies (i.e., food, wellness-related products, and other pet care items) in the last twelve months than in the preceding year (APPA, 2022). Functional ingredients in pet foods and treats fall into the category of "wellness-related products," as they provide health benefits beyond the provision of essential nutrients (i.e., water, vitamins, minerals, proteins, and fats). Several studies have investigated the health benefits of functional ingredients in humans, which has led pet owners to believe functional ingredients will exert the same positive effects on their pets. However, the mechanisms of action, optimal inclusion levels, and possible harmful effects of most functional ingredients are not completely understood in dogs (Swanson et al., 2003).

Saccharomyces cerevisiae fermentation product (SCFP) is a dry product produced via *S. cerevisiae* fermentation and includes residual yeast cells, yeast cell wall fragments, fermentation metabolites and media used during fermentation. SCFP may aid as a functional ingredient in pet foods due to its positive impacts on performance, health, and immunity in humans and several

animal species, including swine, poultry, and cattle (Moyad et al., 2009; Shen et al., 2011; Kidd et al., 2013; Zaworski et al., 2014). Components of the yeast cell wall (i.e., mannanoligosaccharides and β-glucans) and metabolites from yeast fermentation have been implicated in yeast's gastrointestinal (GI)- and immune-modulatory properties. Moreover, the fermentation process is thought to promote health through modifying food constituents, synthesizing metabolites and other end-products, and providing living microorganisms to the GI tract. In adult dogs, supplementation of SCFP has been shown to positively impact GI health and immune function. Lin et al. (2019) demonstrated that SCFP (125, 250, and 500 mg/d) modulates fecal microbiota and fermentative end-products in adult beagles by increasing *Bifidobacterium*, decreasing *Fusobacterium*, and decreasing phenol and indole concentrations. SCFP was also shown to elevate immune capacity by enhancing T helper-1 cell responses and decreasing inflammation through inhibition of toll-like receptor responses (Lin et al., 2019). In another study, supplementing adult Labrador Retrievers with SCFP (500 mg/d) led to decreased thiobarbituric acid reactive substances and improved total antioxidant capacity in serum, suggesting a reduction in lipid peroxidation and increased antioxidant capacity when subjected to exercise and transport stress (Varney et al., 2021). SCFP-fed Labradors also exhibited increased serum concentrations of tumor necrosis factor-α and decreased serum concentrations of immunoglobulin E and immunoglobulin G, suggesting immunomodulatory effects (Varney et al., 2021). SCFP has been shown to alter fecal microbiota, fecal metabolites, antioxidant status, and immune cell function of adult dogs, however, SCFP supplementation has previously been administered via gelatin capsule or top dressing. Further investigation of oxidative stress, antioxidant status, and immune responses in dogs fed an SCFP-supplemented extruded kibble diet is warranted, and impacts on skin and coat health have not been determined.

Therefore, the objective of this study was to determine the effects of a SCFP-supplemented extruded diet on the circulating immune cell numbers and activity, antioxidant status and oxidative stress marker concentrations, and measures of skin and coat health of adult dogs. We hypothesized that dogs fed the SCFP-supplemented diet would have enhanced immune cell numbers and functionality, reduced concentrations of markers associated with oxidative stress and inflammation, and improved skin and coat health compared with dogs fed a control diet.

REFERENCES

American Pet Products Association (APPA). 2022. National pet owners survey. APPA, Greenwich, CT.

Kidd, M. T., L. Araujo, C. Araujo, C. D. McDaniel, and D. McIntyre. 2013. A study assessing hen and progeny performance through dam diet fortification with a *Saccharomyces cerevisiae* fermentation product. J. Appl. Poul. Res. 22:872-877. doi:10.3382/japr.2013-00774.

Lin, C. Y., C. Alexander, A. J. Steelman, and C. M. Warzecha, M. R. C. de Godoy, and K. S. Swanson. 2019. Effects of a *Saccharomyces cerevisiae* fermentation product on fecal characteristics, nutrient digestibility, fecal fermentative end-products, fecal microbial populations, immune function, and diet palatability in adult dogs. J. Anim. Sci. 97:1586–1599. doi:10.1093/jas/skz064.

Moyad, M. A., L. E. Robinson, J. M. Kittelsrud, S. G. Reeves, S. E. Weaver, A. I. Guzman, and M. E. Bubak. 2009. Immunogenic yeast-based fermentation product reduces allergic rhinitis-induced nasal congestion: a randomized, double-blind, placebo-controlled trial. Adv. Ther. 26:795–804. doi:10.1007/s12325-009-0057-y.

Shen, Y. B., J. A. Carroll, I. Yoon, R. D. Mateo, and S. W. Kim. 2011. Effects of supplementing *Saccharomyces cerevisiae* fermentation product in sow diets on performance of sows and nursing piglets. J. Anim. Sci. 89:2462–2471. doi:10.2527/jas.2010-3642.

Swanson, K. S., L. B. Schook, and G. C. Fahey. 2003. Nutritional genomics: implications for companion animals. J. Nutr. 10:3033-3040. doi:10.1093/jn/133.10.3033.

Varney, J. L., C. N. Coon, and S. A. Norton. 2021. PSV-B-20 Effects of *Saccharomyces cerevisiae* fermentation product (SCFP) postbiotic in Labrador retrievers during exercise and

transport stress. J. Anim. Sci. 99(Suppl. 3):332–333. (Abstr.) doi:10.1093/jas/skab235.611.

Zaworski, E. M., C. M. Shriver-Munsch, N. A. Fadden, W. K. Sanchez, I. Yoon, and G. Bobe. 2014. Effects of feeding various dosages of *Saccharomyces cerevisiae* fermentation product in transition dairy cows. J. Dairy Sci. 97:3081–3098. doi:10.3168/jds.2013-7692.

CHAPTER 2: LITERATURE REVIEW

OXIDATIVE STRESS

Oxidative stress refers to "an imbalance between the generation of oxidants and their elimination systems, i.e., antioxidants, in favor of oxidants, leading to a disruption of redox signaling and control and/or molecular damage" (Sies et al., 2017). Oxidation-reduction (redox) homeostasis is essential to all biological processes, including metabolism, and the regulation of redox reactions varies widely between different cellular systems. The major oxidant and antioxidant systems (i.e., the major sources and sinks of enzymatic hydrogen peroxide) are shown in **Table 2.1**.

Reactive oxygen species

Reactive oxygen species (ROS) are highly reactive molecules, with free radicals containing at least one unpaired electron (e.g., superoxide $O2^{\bullet-}$, hydroxyl radical •OH) and hydrogen peroxide (H_2O_2) considered to be the primary contributors of endogenous oxidative stress damage (Liochev, 2013). ROS are produced endogenously and exogenously, and aerobic organisms have developed complex antioxidant defense systems to limit oxidative damage caused by them. Intracellular ROS-generating systems are often directly adjacent to antioxidant defense mechanisms and are compartmentalized within cellular structures such as mitochondria, lysosomes, and peroxisomes. The major endogenous source of ROS generation is from electron leakage in the mitochondrial electron transport chain during normal oxidative respiration. Other endogenous sources of ROS include cytochrome P450 enzymes in the endoplasmic reticulum, peroxisomes, lipoxygenases, cyclooxygenases, xanthine oxidase, NADPH oxidase, and

inflammatory immune cells (i.e., neutrophils, macrophages, and eosinophils) (Curtin et al., 2002; McMichael, 2007). Major exogenous sources of ROS include radiation, environmental pollution, drugs, certain foods, and cigarette smoke. Both endogenous and exogenous ROS can lead to oxidative damage, which affects lipids, proteins, and nucleotides.

Oxidative damage

One of the consequences caused by oxidative stress is lipid peroxidation, which occurs in three steps: initiation, propagation, and termination. Lipid peroxidation is initiated by removing a proton from polyunsaturated fatty acids (PUFA) in the cell membrane. This causes a new radical to form, which can then attack more PUFA in the cell membrane and propagate a chain reaction of lipid peroxidation. This chain reaction is terminated when the lipids are eliminated, or when the radical encounters a chain-breaking antioxidant, such as vitamin E. PUFA with a high number of double bonds, such as arachidonic acid and linoleic acid, are highly susceptible to free radicals and ROS (Yin et al., 2011). Lipid peroxidation severely damages cell membranes, causing changes to enzyme systems, inactivating receptors, altering the function of ion channels, and increasing permeability to ions, ultimately leading to inflammation and apoptosis (Montuschi et al., 2007; Sousa et al., 2017). Moreover, lipid radicals generated in the oxidation process can form harmful end-products, including lipid hydroperoxides, lipid hydroxides and epoxides, F_2-isoprostanes, malondialdehyde (MDA) and other aldehydes, ketones, and alkanes (Niki, 2014; Spickett and Pitt, 2015).

Proteins are also important targets for ROS, which form oxidation products from amino acid side chains (Griffiths et al., 2014; Kim et al., 2015; Davies, 2016). Protein oxidation is not always deleterious. Normal redox signaling and control largely involves oxidative modification of

amino acid side chains in proteins by hydrogen peroxide, and activity of enzymes can be modulated by reversible redox changes of specific amino acids (Sies et al., 2017). However, uncontrolled protein oxidation can lead to changes to protein structure and folding, modification of physiological properties such as enzyme activities and signal transduction networks, and proteolytic degradation (Höhn et al., 2014; Davies, 2016). Carbonyl derivatives are a major source of end-products generated in protein oxidation and are considered the most widely used biomarkers of oxidative damage to protein (Luo et al., 2020).

Finally, ROS can cause oxidative damage to DNA, causing base mutations and strand breaking, DNA-protein cross-links, and formation of DNA-adducts (Luo et al., 2020). These changes can be particularly hazardous, as they can block gene transcription and DNA replication and lead to the development of cancerous cells. RNA is subject to oxidation as well. Damage from DNA and RNA oxidation is a major contributor to disease progression and instability of the genome (Cadet et al., 2012; Sies et al., 2017). Major DNA/RNA oxidation products include 8-oxoguanine, glutathione, protein thiols, and methionine sulfoxide. DNA damage from ROS can have far-reaching health implications, such as the initiation, promotion, and progression of cancer (Karihtala and Soini, 2007). For example, accumulation of 8-oxoguanine in neurons causes mitochondrial dysfunction and may contribute to tumor growth (Leon et al., 2016). Furthermore, ROS damage RNA through oxidative modification of microRNAs, which bind to target mRNA and either affect mRNA degradation or inhibit protein translation. A subset microRNA (i.e., redoximiRs) are responsible for regulating redox pathways, thus damage to redoximiRs can cause blocked translation, apoptosis, and production of ROS (Cheng et al., 2013; Wang et al., 2015; Lang et al., 2016).

Endogenous antioxidant defenses

Given the wide range of reactivity of oxidants and targets, living cells and organisms use multiple strategies to counteract oxidative damage, referred to as antioxidant defense (Sies et al., 2017). Antioxidants can delay or prevent oxidation of lipids, proteins, and nucleic acids through the scavenging process, producing a more stable compound after reacting with ROS. Antioxidant scavenging of ROS involves the antioxidant donating a single electron to a free-radical species (Hermans et al., 2007). During homeostatic redox conditions, ROS play an important role in regulating cellular function. The homeostatic balance of ROS is modulated by three main categories of endogenous antioxidant defense systems: enzymatic antioxidants, antioxidant proteins, and low-molecular-weight antioxidants (McMichael, 2007). To maintain a homeostatic level of ROS, powerful enzymatic systems, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, directly counteract ROS formation by chemical reduction of oxidants (Sies et al., 2017). Superoxide is the major oxidant produced during physiologic and pathophysiologic states and is scavenged by the enzyme SOD. The reaction creates hydrogen peroxide, which is subsequently detoxified by glutathione or catalase (Eaton, 2006). Antioxidant proteins, such as the aquaporin channel proteins or the glutathione disulfide (GSSG) transporters, facilitate transport of ROS and their products across biological membranes (Sies et al., 2017; Tamma et al., 2018). Low-molecular-weight antioxidants, such as α-tocopherol (vitamin E) and ascorbate (vitamin C), work non-enzymatically to maintain ROS balance. Vitamin E is a chain-breaking antioxidant during lipid peroxidation, and vitamin C is the most abundant water-soluble antioxidant that can directly scavenge ROS or regenerate vitamin E (McMichael et al., 2007).

Oxidative stress, aging, and disease in dogs

As mammals age, the accumulation of ROS causes oxidative damage to lipids, proteins, and nucleotides. Moreover, the robust antioxidant defense systems in place to maintain redox homeostasis may degenerate with age. Studies of dogs have investigated the role of aging on oxidative stress in the canine brain, showing oxidative damage to DNA and RNA increases in the aged dog brain (Rofina et al., 2006). Moreover, the activity of enzymatic antioxidants such as SOD appear to decline with age, while the output of lipid peroxidation end-products such as MDA are shown to increase with age (Kiatipattanasakul et al., 1997; Head et al., 2002). Increased oxidative stress is implicated in the pathophysiology and progression of disease in dogs, including chronic kidney disease, liver disease, anemia, congestive heart failure, and intervertebral disk disease (Center et al., 2002; Freeman et al., 2005; McMichael et al., 2006; Brown, 2008; Kendall et al., 2017).

IMMUNITY

The immune system is the body's defense against infectious agents, their toxins, and the damage they cause through interacting networks of effector cells and molecules (Murphy and Weaver, 2017; Tizard, 2018). The three major barriers used to protect the body against invasion from pathogens are physical barriers, innate immunity, and adaptive immunity. Physical barriers, such as intact skin, are the initial defense against infection and function to prevent exposure of internal tissues to microbes. When physical barriers are breached, the innate immune response is quickly activated to produce various chemical mediators that either directly kill invading pathogens or act on other cells to propagate the immune response (Tizard, 2018). Innate immune cells become activated by several pattern recognition receptors (PRR) that sense and detect pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns

(DAMP). These molecular patterns are associated with pathogens or cellular damage, but not the host's own cells. Examples of PRR include membrane bound toll-like receptors (TLR) and C-type lectin receptors, as well as cytoplasmic nucleotide-binding and oligomerization domain (NOD)-like receptors (NLR). Activation of PRR on sensor cells can induce phagocytosis or amplify the immune response by producing inflammatory mediators such as cytokines and chemokines to convey signals to other immune cells (Beutler, 2004; Murphy and Weaver, 2017; McComb et al., 2019). If the innate immune response fails, adaptive immunity is initiated when antigen-presenting cells (APC) phagocytize pathogens and present pathogen peptide fragments (i.e., antigens) via major histocompatibility complex (MHC) to T cells. APC form the bridge between innate and adaptive immunity, enabling the adaptive immune cells, called lymphocytes, to learn to recognize and destroy specific invaders (Murphy and Weaver, 2017; Tizard, 2018; McComb et al., 2019). The adaptive immune system mounts a slower, but more efficient response than the innate immune system due to the specificity of antigen recognition by lymphocytes.

Immune cell types

Innate immune cells include macrophages, dendritic cells, neutrophils, eosinophils, basophils, mast cells, and natural killer (NK) cells. PAMP and DAMP activate PRR on innate immune cells to induce effector functions, such as cytokine production or phagocytosis (McComb et al., 2019). Macrophages and neutrophils are the primary phagocytic cells that engulf pathogens and destroy them in intracellular vesicles. Dendritic cells mainly function as specialized APC that present antigens to lymphocytes, initiating adaptive immune responses. Macrophages can also act as APC. NK cells can recognize and kill some virus-infected cells and tumor cells via cell lysis (Tizard, 2018). Eosinophils, basophils, and mast cells are primarily secretory cells that release the

contents of their cytoplasmic granules upon antibody activation during an adaptive immune response (Beutler, 2004, Murphy and Weaver, 2017; McComb et al., 2019).

The two major components of adaptive immunity are the antigen-specific lymphocytes: B cells and T cells. When a T cell encounters an antigen that its receptor can bind, it proliferates and differentiates into one of several types of effector T cells with different functions. When an antigen is detected from an infected APC, effector T cells can develop into cytotoxic T cells (T_C cells) and helper T cells (T_H cells) (Tizard, 2018; McComb et al., 2019). T_C cells kill other cells that are infected with viruses or other intracellular pathogens bearing the same antigen. T_H cells provide signals (i.e., cytokines) to enhance the immune response of other cells by activating B cells to produce antibodies or activating phagocytes to engulf pathogens (Murphy and Weaver, 2017). After a B cell becomes activated in the presence of their target antigen, it will proliferate and differentiate into a plasma cell. Plasma cells produce large amounts of the specific antibody needed to bind to the target antigen and neutralize it (Murphy and Weaver, 2017; McComb et al., 2019). Some B cells and T cells activated by antigens will differentiate into memory cells, which provide long-lasting immunity from subsequent exposure to the specific antigens introduced by a previous infection or vaccination (Murphy and Weaver, 2017; Tizard, 2018).

Toll-like receptors

TLR are a family of PRR located on the surface or within cells that bind PAMP from extracellular and intracellular invaders. TLR are present on innate and adaptive immune cells such as macrophages, neutrophils, mast cells, dendritic cells, T cells, and B cells, as well as non-immune cells such as epithelial cells. The cell surface TLR (TLR 1, 2, 4, 5, 6, and 11) primarily bind bacterial and fungal proteins, lipoproteins, and lipopolysaccharides, whereas the intracellular TLR

(TLR 3, 7, 8, 9, and 10) bind viral and bacterial nucleic acids. Genome sequencing has confirmed that dogs express TLR 1-10 and possess pseudogenes for TLR 11 and 12 (Leulier and Lemaitre, 2008). Currently, complete canine cDNA sequences are available for TLR 2, 4, 7, and 9 (Asahina et al., 2003; Hashimoto et al., 2005; Ishii et al., 2006; Okui et al., 2008). TLR recognize and bind to specific ligands, which are summarized in **Table 2.2**. TLR ligands have been used in several studies for stimulating canine tissues and cells to determine their expression and function (Swerdlow et al., 2006; Kathrani et al., 2012; Schmitz et al., 2014; Yokoyama et al., 2017; Reineking et al., 2018). When activated by PAMP or tissue damage, TLR turn on genes involved in producing proinflammatory cytokines, chemotactic factors, antimicrobial peptides, and antiviral cytokines (i.e., interferon-α and interferon-β). TLR activate several different signaling pathways via cytoplasmic adaptor molecules that each activate different transcription factors (Murphy and Weaver, 2017). The four signaling adaptor molecules used by mammalian TLR are myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (MAL), TIR domain-containing adaptor-inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM). Most TLR use the adaptor MyD88 paired with MAL, or TRIF paired with TRAM. Signaling by most TLR activates the transcription factor nuclear factor κB (NFκB), as well as other members of the interferon regulatory factor (IRF) transcription factor family, which activates members of the activator protein 1 (AP-1) family, such as mitogen-activated protein kinases (MAPK). NFκB and AP-1 primarily function to induce the expression of proinflammatory cytokines and chemotactic factors.

Flow cytometry

Flow cytometry is a powerful tool for defining and counting immune cell populations. Flow cytometers detect and count individual cells passing through a laser beam, identifying properties of cell subsets by using monoclonal antibodies (MAb) against cell surface or intracellular proteins. Individual cells within a mixture of peripheral blood mononuclear cells (PBMC) are labeled with specific MAb attached to fluorescent dyes, or by specific antibodies coupled with fluorescent anti-immunoglobulin antibodies (Murphy and Weaver, 2017). As individual cells pass through the laser, they are exposed to two types of light scatter: forward-angle scatter (FSC) and side-angle scatter (SSC), which provides information about the size and granularity of the cell. Light scatter also excites the cells, resulting in fluorescence emissions that provide information about the binding of the labeled MAb. These fluorescent emissions signify the expression of cell-surface or intracellular proteins by each cell (Wilkerson et al., 2012; Murphy and Weaver, 2017). Cellular proteins recognized by a cluster of MAb have been given "cluster of differentiation" (CD) marker numbers, which are used as targets for immunophenotyping (Weis and Wardrop, 2010). By running large numbers of cells through a flow cytometer, quantitative data on the percentage of cells bearing different CD markers (i.e., phenotypes) can be generated.

Several studies have investigated the distribution of circulating lymphocytes in dogs using flow cytometry (Baumgarth et al., 2000; Byrne et al., 2000; Fujiwara et al., 2005; Platt et al., 2013; Rütgen et al., 2015). Cell distribution differed among studies, likely due to differences in the age, gender, and breed of dogs sampled, laboratory methods, and/or antibodies used (Byrne et al., 2000; Faldyna et al., 2005; Villaescusa et al., 2012). Byrne et al. (2000) reported the following cell population ranges for peripheral blood samples of normal adult dogs using flow cytometry: CD5 (%), 83.3 ± 3.5; CD4 (%), 45 ± 8.3; CD8 (%), 28.8 ± 5.6; CD21 (%), 12.9 ± 3.9. Faldyna et al.

(2005) reported slightly different ranges for the same cell types in normal adult dogs: CD3 (%), 83.9 ± 1.7; CD4 (%), 58.6 ± 3.7; CD8 (%), 15.1 ± 4.4; CD21 (%), 15.7 ± 1.8. In regard to the CD markers that facilitate immunophenotyping, canine T cells all express CD3, with some T cell subsets expressing CD4 or CD8. CD4 molecules are receptors for MHC class II molecules on APC and are present only on T_H cells. CD8 molecules are receptors for MHC class I molecules and are only expressed on T_C cells. The proportion of CD4 and CD8 cells differ between mammals, but the ratio of CD4+ to CD8+ T cells can be used to estimate lymphocyte function. An elevated CD4 count suggests increased lymphocyte reactivity, whereas a high CD8 count implies depressed lymphocyte reactivity (Tizard, 2018). CD21 and MHC II are common markers used for identifying B cell populations (Byrne et al., 2000). B cells express MHC II to present antigens to CD4+ T_H cells, and CD21 is closely associated with the B cell receptor, which regulates B cell responses to antigens (Murphy and Weaver, 2017; Tizard, 2018). It has been shown that canine monocytes can be subdivided into three populations based on the CD14 and MHC II surface markers: CD14+MHC II-, CD14+MHC II+, and CD14-MHC II+. These cell types are equivalent to human classical monocytes, intermediate monocytes, and non-classical monocytes, respectively (Gibbons et al., 2017). Classical monocytes circulate freely in the blood and enter infected tissues. Once in tissues, classical monocytes can differentiate into activated inflammatory monocytes or macrophages, whereas the intermediate and non-classical "patrolling monocytes" move along the endothelium to survey for injury rather than circulating in blood and do not differentiate (Murphy and Weaver, 2017). CD markers are not well defined for canine NK cells, but it has been demonstrated that CD5 is expressed at low density on canine lymphocytes that show NK cell characteristics (Huang et al., 2008).

SKIN AND COAT HEALTH

Canine skin and hair coat

The skin is a large, metabolically active organ that serves as an anatomical barrier to protect the body from injury and infection and communicates with the outside environment to aid in thermo- and immuno-regulation. The skin is made up of three main layers: the epidermis, dermis, and hypodermis. Keratinocytes are the most abundant cells in the epidermis and function to produce keratin, providing strength to the skin. As keratinocytes mature, keratin forms the protective outermost layer of the epidermis, the stratum corneum (SC). Melanocytes produce melanin, the major pigment of the epidermis, from the amino acid tyrosine. Hair follicles and sebaceous glands are also associated with the epidermis (Reinhart and Carey, 2000).

The barrier function of mammalian skin is maintained by lipids in the epidermis, especially within the layers of the SC. In mammalian SC, epidermal lipids consist of ceramides, cholesterol, cholesterol sulfate, fatty acid esters of cholesterol, free fatty acids, and sphingosine (Downing, 1992). Disruption of skin barrier function by application of detergents and/or solvents or disease states (e.g., atopic dermatitis) can alter the production of these lipids, resulting in increased transepidermal water loss (TEWL) (Inman et al., 2001). Sebaceous glands produce and secrete sebum, which is comprised of lipid compounds that cover and protect the hair coat and give it a glossy appearance. Sebum also functions to lubricate and protect the skin, and many of sebum's fatty acid constituents (linoleic, myristic, oleic, and palmitic acids) are known to have antimicrobial actions. During periods of illness or malnutrition, the hair coat may become dull and brittle as a result of inadequate sebaceous gland function (Scott et al., 2001). Furthermore, some dermatological disorders are characterized by abnormal sebum production and are often associated

with an increased predisposition to bacterial infections of the skin (Reinhart and Carey, 2000; Inman et al., 2001).

The hair coat of mammals insulates the body, aids in sensory perception, and acts as a barrier against chemical, physical, and microbial injury to the skin (Scott et al., 2001). Hair follicles are the basic unit of hair production in dogs, consisting of the follicular sheath and hair bulb. Dog hair follicles contain a single stiff primary or "guard" hair and a variable number of fine secondary hairs, comprising the undercoat. In all animals, hair is mainly comprised of keratin, containing a high proportion of sulfur-containing amino acids, methionine and cysteine. Hair growth is not continuous, but rather a growth cycle characterized by four distinct phases. The "anagen" (i.e., active) growth phase begins the cycle, followed by a "telogen" phase of no growth. There is a brief transitional "catagen" period in between the anagen and telogen stages in which hair follicles shrink and hair growth slows. During the final, or "exogen," phase, hair will shed from the hair follicle while new hairs are already starting to grow (Galbraith, 1998; Scott et al., 2001). Genetics, photoperiod, and environmental temperature, as well as an animal's age, health, and reproductive status can affect the length of these periods (Butler and Wright, 1981).

Impact of nutrition on skin and hair coat

As a metabolically active organ, the skin has high requirements for energy, protein, fatty acids, and other essential nutrients such as vitamin E. The health of a dog's skin and quality of the hair coat are affected by nutrient imbalances, although today most pet foods are formulated to meet the nutrient profiles set by the Association of American Feed Control Officials (AAFCO, 2022). However, nutrient imbalances may still occur due to improper feeding or storage of commercial

pet foods, or because of a disorder that affects the ability to digest, absorb, or utilize nutrients (Sousa et al., 1988; Reinhart and Carey, 2000).

Nutrition can also affect the health of the skin through the development of a food allergy or hypersensitivity. Although not completely understood, common dietary proteins can serve as potential allergens, eliciting type-I and/or type-III hypersensitivity responses when ingested (White, 1995). In dogs, hypersensitivity reactions around the feet, axillae, and inguinal region commonly manifest dermatological signs of pruritis, inflammation, and development of secondary infections. Atopic dermatitis is one of the most common chronic inflammatory skin diseases of dogs, with a prevalence of 3–15% in the general dog population and representing between 3% and 58% of dogs affected with skin disease presented to veterinarians (Saridomichelakis et al., 2016). There is increasing evidence that skin barrier dysfunction exists in dogs with AD, characterized by abnormal intercellular SC lipids, abnormal SC morphology, and in some dogs, abnormal SC protein expression (Inman et al., 2001; Shimada et al., 2009; Marsella et al., 2010; Popa et al., 2011). Allergen-induced atopic inflammation appears to further worsen these anomalies.

Evaluation of skin using biophysical parameters

Noninvasive skin biophysical methods have been used in clinical and experimental dermatology in humans and in companion animals (Dunstan et al., 2000; Groux and Bensignor, 2000; Marsella et al., 2012). Dog age, breed, sex, and photoperiod have been shown to influence various skin measurements, such as skin hydration, sebum concentrations, elasticity, TEWL, and pH (Young et al., 2002). Although their measurement varies among animals within and across species, as well as body sites, skin biophysical parameters can provide an objective evaluation of a dog's relative skin health. Changes in SC hydration, which provides an estimate of cutaneous

capacity to retain moisture, have been shown to affect the balance of epidermal cell proliferation and differentiation (Proksch et al., 1993). Higher skin hydration values indicate greater cutaneous water capacitance. TEWL measurements provide valuable information for assessing the integrity of the skin barrier and estimating the skin's moisture retention properties (Pinnagoda et al., 1990, Watson et al., 2002; Fluhr et al., 2006). Higher TEWL values indicate greater water loss and are consistent with increased damage of barrier function of the SC that can occur during irritant exposure, self-excoriation, or AD (Fluhr et al., 2006). Sebum is thought to protect the epidermis by lubricating the SC and hair follicle and through bacteriostatic activity (Dunstan et al., 2000). Studies on dogs have shown that sebum concentrations vary by breed (Sharaf et al., 1977; Young et al., 2002). For example, Young et al. (2002) reported the following sebum concentrations on the lumbar regions of different dog breeds: Beagle, 2.21 ± 0.78; Fox Terrier, 3.58 ± 1.10; Labrador Retriever, 6.97 ± 1.91; Manchester Terrier, 6.72 ± 1.69. Furthermore, investigations on the effects of dietary treatment on skin sebum concentrations in dogs have shown contradictory results (Campbell et al., 2000; Dunstan et al., 2000). Dustan et al. (2000) reported that a high-quality diet formulated to mimic a commercially available premium dog food was associated with increased sebum secretions (i.e., greater rate of increase for cholesterol; increased triglyceride secretions from baseline) when compared with a low-quality diet formulated to mimic a commercially available basic dog food. However, Campbell et al. (2000) reported that sebum concentrations were not associated with dietary treatment.

SACCHAROMYCES CEREVISIAE FERMENTATION PRODUCT

Yeast products are ingredients that contain yeast cells or yeast derivatives, commonly derived from baker's yeast, *Saccharomyces cerevisiae,* as well as other strains of yeast. Yeast

byproducts from the brewing industry, such as dried brewer's yeast, have a long history of use in the pet food industry to increase palatability (Swanson and Fahey, 2006). As demand for functional ingredients that go beyond basic nutrition (i.e., "superfoods") increases, yeast products containing live microorganisms and/or microbially-derived products are being investigated for use in commercial livestock and companion animal nutrition for their potential health-enhancing effects. The characteristics of yeast products dictate their physiological benefits, and the components of yeast ingredients can differ widely depending on the preparation.

Yeast products

There are numerous variations of yeast products used in companion animal nutrition. This section focuses on yeast products originating from *S. cerevisiae,* including direct-fed *S. cerevisiae* yeasts, *S. cerevisiae* fermentation products (SCFP), yeast culture, yeast cell walls, yeast-derived mannanoligosaccharides (MOS), and yeast-derived β-glucans. In addition to providing nutrient guidelines for dogs and cats, AAFCO (2022) defines ingredients that are currently allowed in pet foods. In the Official Names and Definitions of Feed Ingredients section of AAFCO, Section 36 defines "Fermentation Products," or animal feed ingredients derived from spent fermentation processes, while Section 96 on "Yeast" defines yeast products. Direct-fed microbial products (*S. cerevisiae* or other strains of yeast and bacteria) are viable microorganisms that are fed to animals directly. The term "probiotic" is typically used to when referring to direct-fed microbials in humans and companion animal nutrition, which is defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Direct-fed microbials in feed must only contain strains of yeast and/or bacteria that are approved for safety by the Food and Drug Administration (FDA) Center for Veterinary Medicine (AAFCO, 2022).

Yeast culture is defined as, "a dried product composed of yeast and the media on which it was grown, dried in such a manner as to preserve the fermenting activity of the yeast. The media must be stated on the label unless the yeast culture is a component of a proprietary mixed feed (AAFCO, 2022). Yeast fermentation products are derived from culturing yeast on appropriate media for the production of enzymes, fermentation substances, or microbial metabolites (AAFCO, 2022). One example of a yeast fermentation product currently used in commercial pet foods is SCFP. SCFP is a dry product produced via *S. cerevisiae* fermentation and includes residual yeast cells, yeast cell wall fragments, fermentation metabolites, and media used during fermentation. The *S. cerevisiae* cell wall is comprised of β-glucans (5-10% 1,6-β-glucans and 30-45% 1,3-β-glucans), mannoproteins (30-50%), and chitin (1.5-6%) (Kollár et al., 1997; Klis et al., 2006). Yeast-derived MOS from mannoproteins and β-glucans can be extracted and supplemented in pet foods as functional ingredients to support GI or immune health.

Potential benefits of consuming yeast products

Yeast products have been shown to possess functional properties that reach beyond basic nutrition in both humans and animals. Researchers have evaluated changes in intestinal health markers, immune markers, and fecal fermentative-end products in dogs after supplementation with various yeast products. The dietary treatments and main outcomes of these studies are summarized in **Table 2.3.**

Intestinal health

Yeast products have been suggested to improve intestinal health through modulation of GI microbiota, strengthening of intestinal barrier function, and/or altering the production of fecal

fermentative end-products (e.g., butyrate, ammonia, phenol, indole) by GI microbes. Components of the yeast cell wall, such as β-glucans, can improve intestinal barrier function by inhibiting the expression of inflammatory mediators and enhancing the expression of tight junction proteins associated with intestinal permeability (Han et al., 2017). Furthermore, yeast products have the potential to modulate the GI microbiota through elimination of pathogenic bacteria by inhibiting intestinal binding. Bacteria bind to the mucosal surface via bacterial fimbriae to colonize the host's intestine. Many *E. coli* and *Salmonella spp.* possess the mannose-sensitive type 1 fimbriae, allowing these pathogenic bacteria to bind to adhesion sites on MOS instead of the host's mucosa (Grieshop, 2002). In two dog studies, MOS supplementation tended to positively influence microbial populations by decreasing fecal total aerobe concentrations (Swanson et al., 2002a, $P = 0.05$; Swanson et al., 2002b; $P = 0.054$). Dogs supplemented with MOS also tended to have greater ($P = 0.13$) fecal *Lactobacillus* populations (Swanson et al., 2002a). Another study in dogs demonstrated that yeast cell wall supplementation with solubilized MOS increased ($P < 0.05$) fecal butyrate and putrescine concentrations, and reduced ($P < 0.05$) lactate, suggesting that yeast products may alter the metabolism of the GI microbiota and that increased solubility of MOS may enhance fermentation (Theodoro et al., 2019). In dogs supplemented with SCFP, fecal phenol and total phenol + indole concentrations decreased linearly with SCFP dosage ($P < 0.05$), and relative abundance of *Bifidobacterium* was greater ($P < 0.05$), while *Fusobacterium* was lower ($P < 0.05$) in SCFP-supplemented dogs (Lin et al., 2019).

Immunity

Components of the yeast cell wall (i.e., MOS and β-glucans) and metabolites from yeast fermentation have been implicated in yeast's immunomodulatory properties. Through digestion,

these components come in contact with the gut-associated lymphoid tissue, influencing its immune function and systemic immunity (Field et al., 1999). All characteristics, including solubility, molecular mass, degree of branching, and polymer charge can influence the immune-modulating effects of yeast products (Brown and Gordon, 2005; Che et al., 2012). In dogs supplemented with MOS, total lymphocytes as a percentage of white blood cells were greater ($P < 0.05$) and serum IgA concentrations tended to be greater ($P = 0.13$) compared with other treatments evaluated (Swanson et al., 2002a). Furthermore, supplementation of active fractions of mannoproteins (AFM; 400 mg/kg and 800 mg/kg) have been shown to stimulate the innate and acquired immune system of adult and elderly dogs. Interestingly, dogs fed AFM at 400 mg/kg tended to have higher neutrophilic phagocytic activity than dogs fed AFM at 800 mg/kg ($P = 0.073$) (Kroll et al., 2020). Lin et al. (2019) reported that supplementation with SCFP enhanced immunity and reduced inflammation in adult beagles. SCFP-supplemented dogs had greater ($P < 0.05$) MHC II-presenting B cell and monocyte populations than control dogs, and IFN-γ-secreting T_H and T_C cells increased linearly with SCFP consumption ($P < 0.05$). PBMC derived from SCFP-supplemented dogs produced less ($P < 0.05$) TNF-α than those from control dogs when cells were stimulated with TLR agonists 2, 3, 4, and 7/8 (Lin et al., 2019). However, yeast products have not consistently shown immunomodulatory effects in dogs. Supplementing dogs with a *S. cerevisiae* hydrolysate did not affect circulating lymphocyte populations and did not change the populations or phagocytic capacity of neutrophils and monocytes (Strompfová et al., 2021). Effects on immunological indices were limited in dogs supplemented with yeast cell wall preparations (Middelbos et al., 2007), and decreased serum lymphocyte numbers were observed in MOS-supplemented dogs (Grieshop et al., 2004). Therefore, yeast products may have variable efficacy on immunity and inflammation and may depend on yeast component, processing conditions, and dose.

Fermentation

Fermented foods and beverages date back to early human civilization, representing the first processed food products produced and consumed by humans. Fermented foods and beverages are made through controlled microbial growth and enzymatic conversions of major and minor food components (Marco et al., 2017). Food fermentation processes can be classified by the primary metabolites and microorganisms involved (e.g., alcohol or carbon dioxide and *Saccharomyces*) or described based on the food substrates (e.g., wine, beer, or bread). Raw materials high in monosaccharides, disaccharides, and sometimes starch are fermented by yeasts or lactic acid bacteria (Marco et al., 2017). Fermented foods were initially valued due to their role in food preservation, as there is a lower risk of microbial contamination in fermented foods due to the presence of anti-microbial end-products, such as ethanol. Fermented foods are also desired for their unique taste and texture when compared with their original characteristics. However, it is now understood that consumption of fermented foods and beverages may present additional nutritional and functional properties.

Fermented foods are thought to promote health through modifying food constituents, synthesizing metabolites and other end-products, and providing living microorganisms to the GI tract. For example, sourdough fermentation and extended fermentation times on wheat bread can reduce fermentable oligosaccharides, disaccharides, monosaccharides, and polyols, which have been previously shown to aggravate symptoms of irritable bowel syndrome (Ziegler et al., 2016). Fermentation results in the formation of new products with health-enhancing potential, some of which can be strain-dependent. Lactate, an end-product in lactic acid bacteria fermentations, has been shown to alter redox status by reducing ROS burden in enterocytes (Kahlert et al., 2016). The

way in which many fermented foods and beverages are processed kills most live microorganisms before consumption, but many well-known fermented foods (e.g., yogurt, miso, kombucha) typically contain viable cells in concentrations ranging between 10^6 and 10^9 cells/g or cells/ml (Marco et al., 2017). However, like the consumption of probiotics, consumption of microbes from fermentation culture are likely to be affected by host diet and are not likely to have a prolonged effects on the resident microbiota (Tachon et al., 2014; Zhang et al., 2016).

Postbiotics

Research on microbiota-modulating dietary interventions and their effects on host health has largely focused on supplementation of probiotics, prebiotics, dietary fibers, and fermented foods. Within this family of dietary interventions, the term “postbiotics” has been increasingly used in publications and commercial products. An International Scientific Association for Probiotics and Prebiotics expert consensus panel recently defined postbiotics as a “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host” (Salimen et al., 2021). In addition to modulating GI microbiota, the potential functions of postbiotics on host health include enhancing epithelial barrier function, modulation of local and systemic immune responses, and modulation of systemic metabolism. There is evidence that *Bifidobacterium* species may enhance barrier function through induction of signaling pathways, such as MAPK, that promote tight junction functioning via autophagy and calcium signaling pathways (Engevik et al., 2019), and cell surface exopolysaccharides produced by certain bifidobacterial strains can promote barrier function by reducing proinflammatory responses (Schiavi et al., 2016). PAMP that remain in postbiotic preparations interact with PRR on immune cells to activate expression of immune modulators, such as cytokines. Interactions between distinct

molecular patterns and specific PRR, such as TLR, have been established. For example, β-glucans derived from *S. cerevisiae* have been shown to interact with TLR2 and stimulate an immune response via the TLR-2-MyD88-NFκB/MAPK pathway (Jin et al., 2019). Modulators of systemic metabolism include microbial derived vitamins and short-chain fatty acids (SCFA). For example, the SCFA butyrate is able to upregulate the antioxidant glutathione and can reduce oxidative stress in the colon of healthy humans (Hamer et al., 2009). Sources of postbiotics include inactivated bacteria (lyophilized and/or heat inactivated), bacterial lysates, and fermentation products. Technological factors that may influence the characterization and bioactivity of postbiotics include the microorganisms used as the starting material for the postbiotic; description of the inactivation procedure or technique; and a description and quantification of the final postbiotic composition (Salimen et al., 2021). By definition, viable cells in postbiotics should be heat inactivated, although some cells may inevitably survive depending on the processing conditions. The concentration of live microorganisms was examined in two types of commercial products: a product containing live *S. cerevisiae* and a SCFP that is marketed as a postbiotic. The SCFP contained an average of 4.67×10^3 (min: 3×10^2, max: 1.9×10^4) viable cells, albeit 10^6 times less than the concentration of yeasts in the product with live *S. cerevisiae* (Garcia-Mazcorro et al., 2019). It is possible that viable cells may interact differently with the host's immune system, metabolism, and/or microbiota compared with inactivated cells, cell fragments, or metabolites. Therefore, it is important that postbiotics are produced using processing methods that are consistent with methods used in the study in which a health benefit was demonstrated (Salimen et al., 2021).

SUMMARY

In recent decades, pet owners have grown to consider their pets as members of the family. This has driven pet owners to provide the best care and nutrition for their pets. Functional ingredients, such as fermented foods, have grown in popularity among pet owners as a way to provide benefits beyond basic nutrition to their pets. SCFP may serve as a functional ingredient in pet foods due to its positive impacts on performance, health, and immunity in humans and several animal species, including dogs. SCFP has been shown to alter fecal microbiota, fecal metabolites, antioxidant status, and immune cell function of adult dogs; however, SCFP supplementation has previously been administered via gelatin capsule or top dressing in these studies. Further investigation of oxidative stress, antioxidant status, and immune responses in dogs fed an SCFP-supplemented extruded kibble diet is warranted, and impacts on skin and coat health are justified as they have not been previously investigated.

TABLES

Table 2.1 Major oxidant and antioxidant systems: enzymatic hydrogen peroxide (H_2O_2) sources and sinks[1]

Sources	**Sinks**
NADPH oxidases	Catalases (catalatic and peroxidatic)
NADH oxidases	Thioredoxin system
Mitochondrial complexes I, II, and III	Glutathione peroxidases (GPx 1–8)
2-Oxoacid dehydrogenases	Peroxiredoxins (Prx 1-6)
Superoxide dismutases (SOD1 and SOD2)	Eosinophil peroxidase
Extracellular SOD (SOD3)	Myeloperoxidase
	Lactoperoxidase
Cytochrome P450 enzymes	
Monoamine oxidases	
Xanthine oxidase	
Glycolate oxidase	Transport systems
L-α-hydroxyacid oxidase	Aquaporins, peroxiporins
Aldehyde oxidase	GSSG transport
D-amino acid oxidase	

[1]Based on Sies et al. (2017).

Table 2.2 Pathogen-associated molecular patterns and functions of mammalian toll-like receptors (TLR) and cellular TLR distribution in mammals[1]

TLR	Cell location	Ligand	Cellular distribution
TLR 1/2 **TLR 2/6**	Cell surface	Lipoproteins (bacteria), zymosan (fungi), cell-wall β-glucan (bacteria, fungi)	Monocytes, dendritic cells (DC), mast cells, eosinophils, basophils
TLR 3	Intracellular	Double-stranded RNA (viruses)	Macrophages, DC, intestinal epithelium
TLR 4	Cell surface	Lipopolysaccharide (bacteria)	Macrophages, DC, mast cells, eosinophils
TLR 5	Cell surface	Flagellin (bacteria)	Intestinal epithelium, macrophages, DC
TLR 7	Intracellular	Single-stranded RNA (viruses, bacteria)	Macrophages, eosinophils, B cells, plasmacytoid DC
TLR 8	Intracellular	Single-stranded RNA (viruses, bacteria)	Macrophages, neutrophils
TLR 9	Intracellular	Unmethylated DNA with CpG[2] (bacteria and viruses)	Plasmacytoid DC, macrophages, eosinophils, B cells, basophils
TLR 10[3]	Intracellular	Unknown	Plasmacytoid DC, macrophages, eosinophils, B cells, basophils
TLR 11[4]	Cell surface	Profilin and profilin-like proteins (bacteria)	Macrophages, DC
TLR 12[4]	Cell surface	Profilin (bacteria)	Macrophages, DC
TLR 13[4]	Intracellular	Single-stranded RNA (bacteria)	Macrophages, DC

[1]Based on Murphy and Weaver (2017) and Tizard (2018).
[2]CpG: cytosine-guanosine.
[3]Found in dogs and humans only.
[4]Found in mice only.

Table 2.3 Summary of publications testing yeast products in dogs

Reference	Intervention and Duration	Animals	Outcomes Measured	Relevant Findings
Swanson et al., 2002a	Duration: 10 d Control (no supplement) 1 g FOS[1] capsule 1 g MOS[1] capsule 1 g FOS + 1 g MOS capsule	Hounds n=4 BW: 22.5 kg Age: 3.3 y	Ileal digestibility and ATTD[1] Fecal fermentative end-products Fecal microbiota Fecal characteristics Fecal and ileal IgA Serum IgA, IgG and IgM Complete blood count	**MOS:** Decreased fecal aerobes (P=0.05) *Lactobacillus* populations tended to increase (P=0.13) Increased lymphocytes (% WBC[1]; (P<0.05) Serum IgA tended to increase (P=0.13) **FOS + MOS:** Increased ileal IgA (P=0.05) Decreased total fecal indoles and phenols (P<0.05)
Grieshop et al., 2004	Duration: 28 d Control (no supplement) Chicory (1% of diet) MOS (1% of diet) Chicory (1% of diet) + MOS (1% of diet) Supplemented in kibble diet	Pointers n = 18 BW: 23.5 kg Age: 9.5 Beagles n = 16 BW: 13.2 kg Age: 10 y	ATTD Fecal microbiota Fecal characteristics Serum immunoglobulins Complete blood count	**MOS:** Increased fecal bifidobacteria (P<0.05) Decreased fecal *E. coli* (P<0.05) Lymphocytes tended to decrease (P=0.06) **Chicory + MOS:** Neutrophil concentrations tended to increase (P= 0.10) Decreased lymphocytes (P<0.05)
Middelbos et al., 2007	Duration: 10 d Control (no supplement) Yeast cell wall capsules (0.05, 0.25, 0.45, and 0.65% of the diet)	Hounds n = 5 BW: 23 kg Age: 4 y	Ileal digestibility and ATTD[1] Fecal microbiota Fecal characteristics Ileal IgA Serum IgA, IgG and IgM Complete blood count	Fecal *C. perfringens* tended to respond cubically (P=0.09) Fecal *E. coli* decreased linearly (P=0.01) *E. coli* (P=0.10) and lactobacilli (P=0.08) tended to respond cubically Total WBC and eosinophils tended to respond quadratically (lowest at 0.25% YCW; P<0.09) Monocyte counts in the blood decreased linearly with YCW (P<0.05) Serum IgA tended to respond cubically (P=0.09) Ileal IgA tended to respond quadratically (greatest at 0.25% YCW; P<0.09)
Stercova et al., 2016	Duration: 42 d Control (no supplement) Live *S. cerevisiae* capsule (1 g/kg BW)	Beagles n = 24 BW: 7.2 – 12.3 kg Age: 5 –9 mo	ATTD Fecal fermentative end-products Fecal microbiota Fecal characteristics Serum chemistry Complete blood count	Increased weight gain (P<0.05) Decreased fecal *E. coli* and fecal *Enterococcus* (P<0.05) Higher ATTD of neutral detergent fiber (P<0.05) Increased MCH[1] and MCHC[1] (P<0.05) Fecal ammonia tended to increase (P<0.10)
Pawar et al., 2017	Duration: 60 d Control (no supplement) MOS (15 g/kg of the diet) top dressed	Spitz dogs n = 10 BW: 4.2kg Age: 4 mo	ATTD Fecal fermentative end-products Fecal characteristics Palatability Blood lymphocyte populations DTH[1] test Serum IgG Serum lipid profile Erythrocyte antioxidant indices	Increased DTH response (P < 0.05) Increased CD4+lymphocytes in the blood (P < 0.05) Increased CD4+:CD8+ lymphocyte ratios (P <0.05) Decreased serum total cholesterol concentration (P < 0.05) Decreased serum LDL[1]-cholesterol concentration (P < 0.05

Table 2.3 (cont.)

Reference	Intervention and Duration	Animals	Outcomes Measured	Relevant Findings
Theodoro et al., 2019	Duration: 32 d Control (no supplement) Yeast cell wall ([YCW]0.3% of the diet) High-MOS-soluble YCW (0.3% of the diet) (YCW supplementation added prior to extrusion)	Beagles n = 24 BW: 11.95 kg Age: 3.5 y	ATTD Fecal fermentative end-products Fecal characteristics Serum cytokine concentrations Immune cell function Fecal IgA	**Yeast cell wall:** Higher phagocytic index for peripheral monocytes (P=0.01) Serum TNF-α tended to be lower (P=0.08) **Yeast cell wall with high MOS solubility:** Decreased fat digestibility (P<0.05) Increased fecal butyrate and putrescine (P<0.05) Decreased fecal lactate (P<0.05) Lower serum IL-6 (P<0.05)
Lin et al., 2019	Duration: 28 d Control (sucrose) 125 mg/d SCFP 250 mg/d SCFP 500 mg/d SCFP via gelatin capsules	Beagles n = 12 BW: 10.3 kg Age 3.3 y	ATTD Diet palatability Fecal fermentative end-products Fecal characteristics Fecal microbiota Immune function Immune cell counts Complete blood count Serum chemistry Serum IgA, IgG, IgM, and IgE Serum SOD/MDA	Fecal phenol and total phenol + indole decreased linearly (P<0.05) Increased relative abundance of *Bifidobacterium* (P<0.05), while *Fusobacterium* decreased (P< 0.05) WBC counts decreased (P<0.05) Increased MHC II+ B cell and monocyte populations (P<0.05) IFN-γ secreting helper and cytotoxic T cells increased linearly (P<0.05) Immune cells produced less TNF-α when stimulated with TLR agonists (P<0.05) Linear increase in serum IgE (P<0.05) 1.9:1 consumption ratio for the SCFP-containing vs. control diet (P < 0.05)
Kroll et al., 2020	Duration: 28 d Control (0 mg/kg active fractions of mannoproteins [AFMs] in diet) (T0) 400mg/kg AFM in diet (T400) 800 mg/kg AFM in diet (T800) AFM supplementation added prior to extrusion	Beagles n = 36 BW: 11.95 kg Age: 4-11 y	Immune cell function Immune cell counts DTH test	Elderly dogs, when compared with adult dogs, had lower total T and B cell counts, lower auxiliary T cell counts, and higher cytotoxic T cell counts (P<0.05) DTH showed effect of diet, age, and time with saline inoculation T400 tended to have higher neutrophilic phagocytic activity than T800 (P = 0.073) **T400:** (LPS[1])-stimulated neutrophils tended to produce more H_2O_2 (P=0.09)
Strompfová et al., 2021	Duration: 14 d Control (no supplement) *S. cerevisiae* hydrolysate (0.3% of the diet)	German Shepherds n=20 BW: 28.5-34.7 kg Age: 1.5-8.7 y	Fecal characteristics Fecal microbiota Complete blood count Serum chemistry Immune cell counts Immune cell function	Increase relative abundance of *Bifidobacterium sp.* (d 14), lactic acid bacteria (d 42) and *Clostridium sp.* (d 42) (P<0.05) Increased fecal pH (d 28) (P<0.05) Decreased serum triglycerides and cholesterol (d 42) (P<0.05) Increased serum ALT[1] (d 14) and AST[1] (d 28 + d 42) (P<0.05) Onset of results mostly seen in the post-supplementation period

[1]ATTD, apparent total tract digestibility; FOS, fructooligosacchrides; MOS, mannanoligosaccharides; IgA, immunoglobulin A; WBC, white blood cell; MCH, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; DTH, delayed-type hypersensitivity; LDL, low-density lipoproteins; LPS, lipopolysaccharide; ALT, alanine aminotransferase; AST, aspartate aminotransferase;

REFERENCES

Asahina, Y., N. Yoshioka, R. Kano, T. Moritomo, and A. Hasegawa. 2003. Full-length cDNA cloning of toll-like receptor 4 in dogs and cats. Vet. Immunol. Immunopathol. 96:159–167. doi:10.1016/s0165-2427(03)00159-4.

Association of American Feed Control Officials (AAFCO). 2022. Official publication. AAFCO, Champaign, IL.

Baumgarth, N., and M. Roederer. 2000. A practical approach to multicolor flow cytometry for immunophenotyping. J. Immunol. Methods 243:77–97. doi:10.1016/s0022-1759(00)00229-5.

Brown, G. D., and S. Gordon. 2005. Immune recognition of fungal beta-glucans. Cell. Microbiol. 7:471–479. doi:10.1111/j.1462-5822.2005.00505.x.

Brown, S. A. 2008. Oxidative stress and chronic kidney disease. Vet. Clin. North. Am. Small. Anim. Pract. 38:157–166. doi:10.1016/j.cvsm.2007.11.001.

Bulteau, A. L., L. I. Szweda, and B. Friguet. 2006. Mitochondrial protein oxidation and degradation in response to oxidative stress and aging. Exp. Gerontol. 41:653–657. doi:10.1016/j.exger.2006.03.013.

Butler, W. F., and A. I. Wright. 1981. Hair growth in the greyhound. J. Small. Anim. Pract. 22:655–661. doi:10.1111/j.1748-5827.1981.tb00569.x.

Byrne, K. M., H. W. Kim, B. P. Chew, G. A. Reinhart, and M. G. Hayek. 2000. A standardized gating technique for the generation of flow cytometry data for normal canine and normal feline blood lymphocytes. Vet. Immunol. Immunopathol. 73:167–182. doi:10.1016/s0165-2427(99)00163-4.

Cadet, J., S. Loft, R. Olinski, M. D. Evans, and K. Bialkowski. 2012. Biologically relevant oxidants and terminology, classification and nomenclature of oxidatively generated damage to nucleobases and 2-deoxyribose in nucleic acids. Free Radic. Res. 46:367–381. doi:10.3109/10715762.2012.659248.

Campbell, K. L., D. P. Laflamme, and J. Harrison. 2000. Effects of four diets with varying omega 6: omega 3 ratios on the health and appearance of the skin and hair coat in normal dogs. Vet. Dermatol. 11(Suppl. 1):18 (Abstr.).

Center, S. A., K. L. Warner, and H. N. Erb. 2002. Liver glutathione concentrations in dogs and cats with naturally occurring liver disease. Am. J. Vet. Res. 63:1187–1197. doi:10.2460/ajvr.2002.63.1187.

Che, T. M., M. Song, Y. Liu, R. W. Johnson, K. W. Kelley, W. G. Van Alstine, K. A. Dawson, and J. E. Pettigrew. 2012. Mannan oligosaccharide increases serum concentrations of antibodies and inflammatory mediators in weanling pigs experimentally infected with porcine reproductive and respiratory syndrome virus. J. Anim. Sci. 90:2784–2793. doi:10.2527/jas.2011-4518.

Cheng, X., C. H. Ku, and R. Siow. C. 2013. Regulation of the Nrf2 antioxidant pathway by microRNAs: new players in micromanaging redox homeostasis. Free Radic. Biol. Med. 64:4–11. doi:10.1016/j.freeradbiomed.2013.07.025.

Curtin, J. F., M. Donovan, and T. G. Cotter. 2002. Regulation and measurement of oxidative stress in apoptosis. J. Immunol. Methods 265:49–72. doi:10.1016/s0022-1759(02)00070-4.

Davies, M. J. 2016. Protein oxidation and peroxidation. Biochem. J. 473:805–825. doi:10.1042/BJ20151227.

Downing, D. T. 1992. Lipid and protein structures in the permeability barrier of mammalian epidermis. J. Lipid Res. 33:301–313.

Dunstan, R. W., T. H. Herdt, L. Mei, K. M. Credille, R. A. Kennis, R. L. Maier, J. Dziezyc, S. Castle, G. A. Reinhart, and G. M. Davenport. 2000. The role of nutrition on canine sebum secretion: a preliminary report. Vet. Dermatol. 11(Suppl. 1): 2–3 (Abstr.).

Eaton, S. 2006. The biochemical basis of antioxidant therapy in critical illness. Proc. Nutr. Soc. 65:242–249. doi:10.1079/pns2006501.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), V. Bampidis, G. Azimonti, M. L. Bastos, H. Christensen, B. Dusemund, M. Fašmon Durjava, M. Kouba, M. López-Alonso, S. López Puente, F. Marcon, B. Mayo, A. Pechová, M. Petkova, F. Ramos, Y. Sanz, R. E. Villa, R. Woutersen, G. Martelli, M. Anguita, J. Galobart, P. Manini, J. Tarrés-Call, E. Pettenati, and F. Pizzo. 2021. Safety and efficacy of a feed additive consisting of *Saccharomyces cerevisiae* MUCL 39885 (Biosprint®) for cats and dogs (Prosol S.p.A.). EFSA J. 19:e06699. doi:10.2903/j.efsa.2021.6699.

Engevik, M. A., B. Luk, A. L. Chang-Graham, A. Hall, B. Herrmann, W. Ruan, B. T. Endres, Z. Shi, K. W. Garey, J. M. Hyser, and J. Versalovic. 2019. *Bifidobacterium dentium* fortifies the intestinal mucus layer via autophagy and calcium signaling pathways. mBio 10:e01087-19. doi:10.1128/mBio.01087-19.

Faldyna, M., J. Sinkora, P. Knotigova, L. Leva, and M. Toman. 2005. Lymphatic organ development in dogs: major lymphocyte subsets and activity. Vet. Immunol. Immunopathol. 104:239–247. doi:10.1016/j.vetimm.2004.12.002.

Field, C. J., M. I. McBurney, S. Massimino, M. G. Hayek, and G. D. Sunvold. 1999. The fermentable fiber content of the diet alters the function and composition of canine gut

associated lymphoid tissue. Vet. Immunol. Immunopathol. 72:325–341. doi:10.1016/s0165-2427(99)00148-8.

Fluhr, J. W., K. R. Feingold, and P. M. Elias. 2006. Transepidermal water loss reflects permeability barrier status: validation in human and rodent in vivo and ex vivo models. Exp. Derm. 15:483–492. doi:10.1111/j.1600-0625.2006.00437.x.

Freeman, L. M., J. E. Rush, P. E. Milbury, and J. B. Blumberg. 2005. Antioxidant status and biomarkers of oxidative stress in dogs with congestive heart failure. J. Vet. Intern. Med. 19:537–541. doi:10.1892/0891-6640(2005)19[537:asaboo]2.0.co;2.

Fujiwara, R. T., L. L. Bueno, A. M. Vale, J. C. Franca-Silva, R. T. da Costa, J. Quetz, G. L. Machado-Coelho, A. B. Reis, O. A. Martins Filho, O. Genaro, E. Nascimento, and W. Mayrink. 2005. Flow cytometric assay in peripheral blood of dogs--reference values for leukocytes from Brazilian beagles. Pol. J. Vet. Sci. 8:17–22.

Galbraith, H. 1998. Nutritional and hormonal regulation of hair follicle growth and development. Proc. Nutr. Soc. 57:195–205. doi:10.1079/pns19980032.

Garcia-Mazcorro, J. F., M. V. Rodriguez-Herrera, A. G. Marroquin-Cardona, and J. R. Kawas. 2019. The health enhancer yeast *Saccharomyces cerevisiae* in two types of commercial products for animal nutrition. Lett. Appl. Microbiol. 68:472–478. doi:10.1111/lam.13141.

Gibbons, N., M. R. Goulart, Y. M. Chang, K. Efstathiou, R. Purcell, Y. Wu, L. M. Peters, M. Turmaine, B. Szladovits, and O. A. Garden. 2017. Phenotypic heterogeneity of peripheral monocytes in healthy dogs. Vet. Immunol. Immunopathol. 190:26–30. doi:10.1016/j.vetimm.2017.06.007.

Grieshop, C. M. 2002. The interaction of nutrition and the immune system: the role of fatty acids, antioxidants and carbohydrates. Proc. Alltech 18th Ann. Symp. 481–487.

Grieshop, C. M., E. A. Flickinger, K. J. Bruce, A. R. Patil, G. L. Czarnecki-Maulden, and G. C. Fahey, Jr. 2004. Gastrointestinal and immunological responses of senior dogs to chicory and mannan-oligosaccharides. Arch. Anim. Nutr. 58:483–493. doi:10.1080/00039420400019977.

Griffiths, H. R., I. H. Dias, R. S. Willetts, and A. Devitt. 2014. Redox regulation of protein damage in plasma. Redox Biol. 2:430–435. doi:10.1016/j.redox.2014.01.010.

Groux, D., and E. Bensignor. 2000. Skin characteristics measured by trans-epidermal water loss, corneometry and sebometry are not valuable tools to evaluate the effects of topical treatments in dogs. Vet. Dermatol. 11(Suppl. 1):60. (Abstr.).

Hamer, H. M., D. M. Jonkers, A. Bast, S. A. Vanhoutvin, M. A. Fischer, A. Kodde, F. J. Troost, K. Venema, and R. J. Brummer. 2009. Butyrate modulates oxidative stress in the colonic mucosa of healthy humans. Clin. Nutr. 28:88–93. doi:10.1016/j.clnu.2008.11.002.

Harman, D. 1972. The biologic clock: the mitochondria? J. Am. Geriatr. Soc. 20:145–147. doi:10.1111/j.1532-5415.1972.tb00787.x.

Hashimoto, M., Y. Asahina, J. Sano, R. Kano, T. Moritomo, and A. Hasegawa. 2005. Cloning of canine toll-like receptor 9 and its expression in dog tissues. Vet. Immunol. Immunopathol. 106:159–163. doi:10.1016/J.VETIMM.2005.01.010.

Head, E., J. Liu, T. M. Hagen, B. A. Muggenburg, N. W. Milgram, B. N. Ames, and C. W. Cotman. 2002. Oxidative damage increases with age in a canine model of human brain aging. J. Neurochem. 82:375–381. doi:10.1046/j.1471-4159.2002.00969.x.

Hermans, N., P. Cos, L. Maes, T. De Bruyne, D. Vanden Berghe, A. J. Vlietinck, and L. Pieters. 2007. Challenges and pitfalls in antioxidant research. Curr. Med. Chem. 14:417–430. doi:10.2174/092986707779941005.

Hill, C., F. Guarner, G. Reid, G. R. Gibson, D. J. Merenstein, B. Pot, L. Morelli, R. B. Canani, H. J. Flint, S. Salminen, P. C. Calder, and M. E. Sanders. 2014. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. Rev. Gastroenterol. Hepatol. 11:506–514. doi:10.1038/nrgastro.2014.66.

Höhn, A., R. Jung, and T Grune. 2014. Pathophysiological importance of aggregated damaged proteins. Free. Radic. Biol. Med. 71:70–89. doi:10.1016/j.freeradbiomed.2014.02.028.

Huang, Y. C., S. W. Hung, T. R. Jan, K. W. Liao, C. H. Cheng, Y. S. Wang, and R. M. Chu. 2008. CD5-low expression lymphocytes in canine peripheral blood show characteristics of natural killer cells. J. Leukoc. Biol. 84:1501–1510. doi:10.1189/jlb.0408255.

Inman, A. O., T. Olivry, S. M. Dunston, N. A. Monteiro-Riviere, and H. Gatto. 2001. Electron microscopic observations of stratum corneum intercellular lipids in normal and atopic dogs. Vet. Pathol. 38:720–723. doi:10.1354/vp.38-6-720.

Ishii, M., M. Hashimoto, K. Oguma, R. Kano, T. Moritomo, and A. Hasegawa. 2006. Molecular cloning and tissue expression of canine toll-like receptor 2 (TLR2). Vet. Immunol. Immunopathol. 110:87–95. doi:10.1016/J.VETIMM.2005.09.007.

Jin, X., M. Zhang, and Y. F. Yang. 2019. *Saccharomyces cerevisiae* β-glucan-induced SBD-1 expression in ovine ruminal epithelial cells is mediated through the TLR-2-MyD88-NF-κB/MAPK pathway. Vet. Res. Commun. 43:77–89. doi:10.1007/s11259-019-09747-x.

Kahlert, S., S. Junnikkala, L. Renner, U. Hynönen, R. Hartig, C. Nossol, A. Barta-Böszörményi, S. Dänicke, W. B. Souffrant, A. Palva, H. J. Rothkötter, and J. Kluess. 2016. Physiological concentration of exogenous lactate reduces antimycin a triggered oxidative stress in

intestinal epithelial cell line IPEC-1 and IPEC-J2 in vitro. PLoS ONE 11:e0153135. doi:10.1371/journal.pone.0153135.

Karihtala, P., and Y. Soini. 2007. Reactive oxygen species and antioxidant mechanisms in human tissues and their relation to malignancies. APMIS. 115:81–103. doi:10.1111/j.1600-0463.2007.apm_514.x.

Kathrani, A., A. Holder, B. Catchpole, L. Alvarez, K. Simpson, D. Werling, and K. Allenspach. 2012. TLR5 risk-associated haplotype for canine inflammatory bowel disease confers hyper-responsiveness to flagellin. PLoS ONE 7:e30117. doi:10.1371/journal.pone.0030117.

Kendall, A., A. Woolcock, A. Brooks, and G. E. Moore. 2017. Glutathione peroxidase activity, plasma total antioxidant capacity, and urinary F2-isoprostanes as markers of oxidative stress in anemic dogs. J. Vet. Intern. Med. 31:1700–1707. doi:10.1111/jvim.14847.

Kiatipattanasakul, W., S. I. Nakamura, K. Kuroki, H. Nakayama, and K. Doi. 1997. Immunohistochemical detection of anti-oxidative stress enzymes in the dog brain. Neuropathol. 17:307-312.

Kim, H. J., S. Ha, H. Y. Lee, and K. J. Lee. 2015. ROSics: chemistry and proteomics of cysteine modifications in redox biology. Mass. Spectrom. Rev. 34:184–208. doi:10.1002/mas.21430.

Klis, F. M., A. Boorsma, and P. W. De Groot. 2006. Cell wall construction in *Saccharomyces cerevisiae*. Yeast 23:185–202. doi:10.1002/yea.1349.

Kroll, F., T. C. Putarov, L. Zaine, K. S. Venturini, C. G. Aoki, J. Santos, V. Pedrinelli, T. Vendramini, M. A. Brunetto, and A. C. Carciofi. 2020. Active fractions of mannoproteins

derived from yeast cell wall stimulate innate and acquired immunity of adult and elderly dogs. Anim. Feed. Sci. Technol. 261:114392. doi:10.1016/j.anifeedsci.2020.114392.

Lang, A., S. Grether-Beck, M. Singh, F. Kuck, S. Jakob, A. Kefalas, S. Altinoluk-Hambüchen, N. Graffmann, M. Schneider, A. Lindecke, H. Brenden, I. Felsner, H. Ezzahoini, A. Marini, S. Weinhold, A. Vierkötter, J. Tigges, S. Schmidt, K. Stühler, K. Köhrer, and R. P. Piekorz. 2016. MicroRNA-15b regulates mitochondrial ROS production and the senescence-associated secretory phenotype through sirtuin 4/SIRT4. Aging 8:484–505. doi:10.18632/aging.100905.

Leon, J., K. Sakumi, E. Castillo, Z. Sheng, S. Oka, and Y. Nakabeppu. 2016. 8-Oxoguanine accumulation in mitochondrial DNA causes mitochondrial dysfunction and impairs neuritogenesis in cultured adult mouse cortical neurons under oxidative conditions. Sci. Rep. 6:22086. doi:10.1038/srep22086.

Leulier, F., and B. Lemaitre. 2008. Toll-like receptors--taking an evolutionary approach. Nat. Rev. Genet. 9:165–178. doi:10.1038/nrg2303.

Lin, C. Y., C. Alexander, A. J. Steelman, and C. M. Warzecha, M. R. C. de Godoy, and K. S. Swanson. 2019. Effects of a *Saccharomyces cerevisiae* fermentation product on fecal characteristics, nutrient digestibility, fecal fermentative end-products, fecal microbial populations, immune function, and diet palatability in adult dogs J. Anim. Sci. 97:1586–1599. doi:10.1093/jas/skz064.

Liochev, S. I. 2013. Reactive oxygen species and the free radical theory of aging. Free Radic. Biol. Med. 60:1–4. doi:10.1016/j.freeradbiomed.2013.02.011.

Luo, J., K. Mills, S. le Cessie, R. Noordam, and D. van Heemst. 2020. Ageing, age-related diseases and oxidative stress: What to do next? Ageing Res. Rev. 57:100982. doi:10.1016/j.arr.2019.100982.

Marsella, R. 2012. Are transepidermal water loss and clinical signs correlated in canine atopic dermatitis? A compilation of studies. Vet. Dermatol. 23:238–249. doi:10.1111/j.1365-3164.2012.01055.x.

Marsella, R., D. Samuelson, and K. Doerr. 2010. Transmission electron microscopy studies in an experimental model of canine atopic dermatitis. Vet. Dermatol. 21:81–88. doi:10.1111/j.1365-3164.2009.00802.x.

McMichael, M. A. 2007. Oxidative stress, antioxidants, and assessment of oxidative stress in dogs and cats. J. Am. Vet. Med. Assoc. 231:714–720. doi:10.2460/javma.231.5.714.

McMichael, M. A., C. G. Ruaux, W. I. Baltzer, S. C. Kerwin, G. L. Hosgood, J. M. Steiner, and D. A. Williams. 2006. Concentrations of 15F2t isoprostane in urine of dogs with intervertebral disk disease. Am. J. Vet. Res. 67:1226–1231. doi:10.2460/ajvr.67.7.1226.

Middelbos, I. S., M. R. Godoy, N. D. Fastinger, and G. C. Fahey, Jr. 2007. A dose-response evaluation of spray-dried yeast cell wall supplementation of diets fed to adult dogs: effects on nutrient digestibility, immune indices, and fecal microbial populations. J. Anim. Sci. 85:3022–3032. doi:10.2527/jas.2007-0079.

Montuschi, P., P. J. Barnes, and L. J. Roberts 2nd. 2004. Isoprostanes: markers and mediators of oxidative stress. FASEB. J. 18:1791–1800. doi:10.1096/fj.04-2330rev.

Murphy, K., and C. Weaver. 2017. Janeway's immunobiology. 9th edition. Garland Science, New York, NY.

Niki, E. 2014. Biomarkers of lipid peroxidation in clinical material. Biochim. Biophys. Acta. 1840:809–817. doi:10.1016/j.bbagen.2013.03.020.

Okui, Y., R. Kano, H. Maruyama, and A. Hasegawa. 2008. Cloning of canine toll-like receptor 7 gene and its expression in dog tissues. Vet. Immunol. Immunopathol. 121:156–160. doi:10.1016/j.vetimm.2007.08.012.

Pawar, M. M., A. K. Pattanaik, D. K. Sinha, T. K. Goswami, and K. Sharma. 2017. Effect of dietary mannanoligosaccharide supplementation on nutrient digestibility, hindgut fermentation, immune response and antioxidant indices in dogs. J. Anim. Sci. Technol. 59:11. doi:10.1186/s40781-017-0136-6.

Pinnagoda, J., R. A. Tupker, T. Agner, and J. Serup. 1990. Guidelines for transepidermal water loss (TEWL) measurement. Contact Derm. 22:164–178. doi:10.1111/j.1600-0536.1990.tb01553.x.

Platt, R., T. Ng, S. Glover, M. Roof, K. Kimura, and J. A. Roth. 2013. Canine peripheral blood lymphocyte phenotyping by 7-color multiparameter flow cytometry. Anal. Quant. Cytol. Histol. 35:197–204.

Popa, I., N. Remoue, L. T. Hoang, D. Pin, H. Gatto, M. Haftek, and J. Portoukalian. 2011. Atopic dermatitis in dogs is associated with a high heterogeneity in the distribution of protein-bound lipids within the stratum corneum. Arch. Dermatol. Res. 303:433–440. doi:10.1007/s00403-011-1120-5.

Proksch, E., W. M. Holleran, G. K. Menon, P. M. Elias, and K. R. Feingold. 1993. Barrier function regulates epidermal lipid and DNA synthesis. Br. J. Dermatol. 128:473–482. doi:10.1111/j.1365-2133.1993.tb00222.x.

Reineking, W., J. Junginger, R. Mischke, and M. Hewicker-Trautwein. 2018. Canine primary jejunal and colonic epithelial cells predominantly express TLR5 and TLR9 but do not change TLR expression pattern after stimulation with certain toll-like receptor ligands. Vet. Immunol. Immunopathol. 206:16–24. doi:10.1016/j.vetimm.2018.11.003.

Reinhart, G. A., and D. P. Carey. 2000. Recent advances in canine and feline nutrition. Volume III: 2000 Iams Nutrition Symposium proceedings. Orange Frazer Press. Wilmington, OH.

Rofina, J. E., A. M. van Ederen, M. J. Toussaint, M. Secrève, A. van der Spek, I. van der Meer, F. J. Van Eerdenburg, and E. Gruys. 2006. Cognitive disturbances in old dogs suffering from the canine counterpart of Alzheimer's disease. Brain Res. 1069:216–226. doi:10.1016/j.brainres.2005.11.021.

Rütgen, B. C., R. König, S. E. Hammer, S. Groiss, A. Saalmüller, and I. Schwendenwein. 2015. Composition of lymphocyte subpopulations in normal canine lymph nodes. Vet. Clin. Pathol. 44:58–69. doi:10.1111/vcp.12221.

Saridomichelakis, M. N., and T. Olivry. 2016. An update on the treatment of canine atopic dermatitis. Vet. J. 207:29–37. doi:10.1016/j.tvjl.2015.09.016.

Schiavi, E., M. Gleinser, E. Molloy, D. Groeger, R. Frei, R. Ferstl, N. Rodriguez-Perez, M. Ziegler, R. Grant, T. F. Moriarty, S. Plattner, S. Healy, M. O'Connell Motherway, C. A. Akdis, J. Roper, F. Altmann, D. van Sinderen, and L. O'Mahony. 2016. The surface-associated exopolysaccharide of *Bifidobacterium longum* 35624 plays an essential role in dampening host proinflammatory responses and repressing local Th17 responses. Appl. Environ. Microbiol. 82:7185–7196. doi:10.1128/AEM.02238-16.

Schmitz, S., M. Henrich, R. Neiger, D. Werling, and K. Allenspach. 2014. Stimulation of duodenal biopsies and whole blood from dogs with food-responsive chronic enteropathy and healthy

dogs with Toll-like receptor ligands and probiotic *Enterococcus faecium*. Scand. J. Immunol. 80:85–94. doi:10.1111/sji.12186.

Scott, D. W., W. H. Miller, and C. E. Griffin. 2001. Muller and Kirk's Small Animal Dermatology. 6th ed. W.B. Saunders. doi:10.1016/B978-0-7216-7618-0.50005-5.

Sharaf, D. M., S. J. Clark, and D. T. Downing. 1977. Skin surface lipids of the dog. Lipids 12:786. doi:10.1007/BF02533265.

Shimada, K., J. S. Yoon, T. Yoshihara, T. Iwasaki, and K. Nishifuji. 2009. Increased transepidermal water loss and decreased ceramide content in lesional and non-lesional skin of dogs with atopic dermatitis. Vet. Dermatol. 20:541–546. doi:10.1111/j.1365-3164.2009.00847.x.

Sies, H., C. Berndt, and D. P. Jones. 2017. Oxidative stress. Annu. Rev. Biochem. 86:715–748. doi:10.1146/annurev-biochem-061516-045037.

Sousa, B. C., A. R. Pitt, and C. M. Spickett. 2017. Chemistry and analysis of HNE and other prominent carbonyl-containing lipid oxidation compounds. Free Radic. Biol. Med. 111:294–308. doi:10.1016/j.freeradbiomed.2017.02.003.

Sousa, C. A., A. A. Stannard, P. J. Ihrke, S. I. Reinke, and L. P. Schmeitzel. 1988. Dermatosis associated with feeding generic dog food: 13 cases (1981-1982). J. Am. Vet. Med. Assoc. 192:676–680.

Spickett, C. M., and A. R. Pitt. 2015. Oxidative lipidomics coming of age: advances in analysis of oxidized phospholipids in physiology and pathology. Antioxid. Redox. Signal. 22:1646–1666. doi:10.1089/ars.2014.6098.

Stercova, E., D. Kumprechtova, E. Auclair, and J. Novakova. 2016. Effects of live yeast dietary supplementation on nutrient digestibility and fecal microflora in beagle dogs. J. Anim. Sci. 94:2909–2918. doi:10.2527/jas.2016-0584.

Strompfová, V., I. Kubašová, D. Mudroňová, L. Štempelová, M. Takáčová, B. Gąsowski, K. Čobanová, and A. Maďari. 2021. Effect of hydrolyzed yeast administration on faecal microbiota, haematology, serum biochemistry and cellular immunity in healthy dogs. Probiotics Antimicrob. Proteins 13:1267–1276. doi:10.1007/s12602-021-09765-9.

Swanson, K. S., and G. C. Fahey. 2006. Potential role of yeast and yeast by-products in pet foods. In: D. K. Laue and L. A. Tucker, editors. Recent Advances in Pet Nutrition. Nottingham University Press, Thrumpton, UK. p. 19–35.

Swanson, K. S., C. M. Grieshop, E. A. Flickinger, L. L. Bauer, H.-P. Healy, K. A. Dawson, N. R. Merchen, and G. C. Fahey. 2002a. Supplemental fructooligosaccharides and mannanoligosaccharides influence immune function, ileal and total tract nutrient digestibilities, microbial populations and concentrations of protein catabolites in the large bowel of dogs. J. Nutr. 132:980–989. doi:10.1093/jn/132.5.980.

Swanson, K. S., C. M. Grieshop, E. A. Flickinger, N. R. Merchen, and G. C. Fahey. 2002b. Effects of supplemental fructooligosaccharides and mannanoligosaccharides on colonic microbial populations, immune function and fecal odor components in the canine. J. Nutr. 132:1717S-1719S. doi:10.1080/0003942021000019126.

Swerdlow, M. P., D. R. Kennedy, J. S. Kennedy, R. J. Washabau, P. S. Henthorn, P. F. Moore, S. R. Carding, and P. J. Felsburg. 2006. Expression and function of TLR2, TLR4, and Nod2 in primary canine colonic epithelial cells. Vet. Immunol. Immunopathol. 114:313–319. doi:10.1016/J.VETIMM.2006.09.003.

Tachon, S., B. Lee, and M. L. Marco. 2014. Diet alters probiotic *Lactobacillus* persistence and function in the intestine. Environ. Microbiol. 16:2915–2926. doi:10.1111/1462-2920.12297.

Tamma, G., G. Valenti, E. Grossini, S. Donnini, A. Marino, R. A. Marinelli, and G. Calamita. 2018. Aquaporin membrane channels in oxidative stress, cell signaling, and aging: recent advances and research trends. Oxid. Med. Cell Longev. 2018:1501847. doi:10.1155/2018/1501847.

Theodoro, S. S., T. C. Putarov, C. Tiemi, L. M. Volpe, C. de Oliveira, M. Glória, and A. C. Carciofi. 2019. Effects of the solubility of yeast cell wall preparations on their potential prebiotic properties in dogs. PLoS ONE 14:e0225659. doi:10.1371/journal.pone.0225659.

Tizard, I. 2018. Veterinary Immunology. 10th ed. Elsevier, St. Louis, MO.

Villaescusa, A., M. García-Sancho, A. M. Delgado, M. Á. Tesouro, F. Rodríguez-Franco, and A. Sainz. 2012. Immunophenotypic evaluation of working Labrador Retrievers and German Shepherd dogs living in the same environment. Vet. J. 193:602–605. doi:10.1016/j.tvjl.2011.12.020.

Wang, J. X., J. Gao, S. L. Ding, K. Wang, J. Q. Jiao, Y. Wang, T. Sun, L. Y. Zhou, B. Long, X. J. Zhang, Q. Li, J. P. Liu, C. Feng, J. Liu, Y. Gong, Z. Zhou, and P. F. Li. 2015. Oxidative modification of miR-184 enables it to target Bcl-xL and Bcl-w. Mol. Cell. 59:50–61. doi:10.1016/j.molcel.2015.05.003.

Watson, A., T. Fray, S. Clarke, D. Yates, and P. Markwell. 2002. Reliable use of the ServoMed Evaporimeter EP-2 to assess transepidermal water loss in the canine. J. Nutr. 132:1661S–1664S. doi:10.1093/jn/132.6.1661S.

Wei, Y. H. 1998. Oxidative stress and mitochondrial DNA mutations in human aging. Proc. Soc. Exp. Biol. Med. 217:53–63. doi:10.3181/00379727-217-44205.

Weiss, D. J., and K. J. Wardrop. 2010. Schalm's Veterinary Hematology. 6th edition. Wiley-Blackwell, Ames, IA.

White, S. D. 1995. Food hypersensitivity in the dog: 30 cases. J. Am. Vet. Med. Assoc. 188:695–698.

Wilkerson, M. J. 2012. Principles and applications of flow cytometry and cell sorting in companion animal medicine. Vet. Clin. North. Am. Small Anim. Pract. 42:53–71. doi:10.1016/j.cvsm.2011.09.012.

Yin, H., L. Xu, and N. A. Porter. 2011. Free radical lipid peroxidation: mechanisms and analysis. Chem. Rev. 111:5944–5972. doi:10.1021/cr200084z.

Yokoyama, N., H. Ohta, Y. Kagawa, N. Nagata, K. Nisa, T. Morita, T. Osuga, N. Sasaki, K. Morishita, K. Nakamura, and M. Takiguchi. 2017. Stimulation of colorectal biopsies from miniature dachshunds with inflammatory colorectal polyps with toll-like receptor ligands: a pilot study. Vet. Immunol. Immunopathol. 188:78–83. doi:10.1016/j.vetimm.2017.05.006.

Young, L. A., J. C. Dodge, K. J. Guest, J. L. Cline, and W. W. Kerr. 2002. Age, breed, sex and period effects on skin biophysical parameters for dogs fed canned dog food. J. Nutr. 132(Suppl. 2):1695S–1697S. doi:10.1093/jn/132.6.1695S.

Zhang, C., M. Derrien, F. Levenez, R. Brazeilles, S. A. Ballal, J. Kim, M. Degivry, G. Quéré, P. Garault, J. E. T. van Hylckama Vlieg, W. S. Garrett, J. Doré, and P. Veiga. 2016. Ecological robustness of the gut microbiota in response to ingestion of transient food-borne microbes. ISME J. 10:2235–2245. doi:10.1038/ismej.2016.13.

Ziegler, J. U., D. Steiner, C. F. H. Longin, T. Würschum, R. M. Schweiggert, and R. Carle. 2016. Wheat and the irritable bowel syndrome – FODMAP levels of modern and ancient species and their retention during bread making. J. Funct. Foods 25:257-266. doi:10.1016/j.jff.2016.05.019.

CHAPTER 3: EFFECTS OF A *SACCHAROMYCES CEREVISIAE* FERMENTATION PRODUCT-SUPPLEMENTED DIET ON IMMUNE RESPONSE, OXIDATIVE STRESS MARKERS, AND SKIN AND COAT HEALTH OF DOGS

ABSTRACT

Feeding *Saccharomyces cerevisiae* fermentation product (SCFP) has previously altered fecal microbiota, fecal metabolites, and immune cell function of adult dogs. The objective of this study was to investigate measures of skin and coat health for the first time and further measure circulating immune cell numbers and activity, antioxidant status, and oxidative stress marker concentrations of adult dogs fed a SCFP-supplemented extruded diet. All procedures were approved by the facility's IACUC prior to experimentation. 16 adult pointer dogs (8 M, 8 F; mean age = 6.7 ± 2.1 y; mean BW = 25.9 ± 4.5 kg) were used in a randomized crossover design study. All dogs were fed a control diet for 4 wk, then randomly assigned to the control or SCFP-supplemented diet and fed to maintain BW for 10 wk. A 6-wk washout preceded the second 10-wk experimental period with dogs receiving opposite treatments. After baseline/washout and treatment phases, skin and coat were scored, and pre-and post-prandial blood samples were collected. Transepidermal water loss (TEWL), hydration status, and sebum concentrations were measured (back, inguinal, ear) using external probes. Oxidative stress and immune cell function were measured using commercial ELISA kits, circulating immune cell numbers were analyzed using flow cytometry, and mRNA expression of oxidative stress genes were analyzed using RT-PCR. Change from baseline data were analyzed using the Mixed Models procedure of SAS 9.4, with $P<0.05$ being significant and $P<0.10$ being trends. Sebum concentration changes tended to be higher ($P<0.10$; inguinal, ear) in dogs fed SCFP than control. TEWL change on the back was lower

($P<0.05$) in controls, but lower ($P=0.054$) on the ear in dogs fed SCFP. DTH response was not measured at baseline, but was affected by diet and time (i.e., post-inoculation) at week 10. As expected, wheal diameter increased over time for all injections. For phytohaemagglutinin (PHA), diet and time interactions were observed in SCFP-fed dogs ($P=0.02$). Diet tended ($P<0.10$) to have an effect on DTH response in SCFP-fed dogs after concanavalin A (ConA) inoculation. Other skin and coat measures and scores were not affected by diet. Changes in unstimulated lymphocytes and stimulated IFN-γ secreting T cells were lower ($P<0.05$) in SCFP-fed dogs, while change in stimulated T cells were lower ($P<0.05$) in control-fed dogs. The stimulated cytotoxic T cells delta trended lower ($P<0.10$) in SCFP-fed dogs. Change in serum superoxide dismutase (SOD) concentrations were higher ($P<0.05$) and change in catalase mRNA expression was lower ($P<0.05$) in SCFP-fed dogs. All other immune cells, oxidative stress markers, and gene expression were unaffected by treatment. In conclusion, our data suggest that SCFP positively impacts indicators of skin and coat health of dogs, modulates immune responses, and enhances some key antioxidant defense mechanisms.

INTRODUCTION

Functional ingredients in pet foods and treats fall into the category of "wellness-related products," as they provide health benefits beyond the provision of essential nutrients (i.e., water, vitamins, minerals, proteins, and fats). Several studies have investigated the health benefits of functional ingredients in humans, which has led pet owners to believe functional ingredients will exert the same positive effects on their pets. However, the mechanisms of action, optimal inclusion levels, and possible harmful effects of most functional ingredients are not completely understood in dogs (Swanson et al., 2003).

Saccharomyces cerevisiae fermentation product (SCFP) is a dry product produced via *S. cerevisiae* fermentation and includes residual yeast cells, yeast cell wall fragments, fermentation metabolites and media used during fermentation. SCFP may aid as a functional ingredient in pet foods due to its positive impacts on performance, health, and immunity in humans and several animal species, including swine, poultry, and cattle (Moyad et al., 2009; Shen et al., 2011; Kidd et al., 2013; Zaworski et al., 2014). Components of the yeast cell wall [(i.e., mannanoligosaccharides (MOS) and β-glucans] and metabolites from yeast fermentation have been implicated in yeast's gastrointestinal (GI)- and immune-modulatory properties. Moreover, the fermentation process is thought to promote health through modifying food constituents, synthesizing metabolites and other end-products, and providing living microorganisms to the GI tract. In adult dogs, supplementation of SCFP has been shown to positively impact GI health and immune function. Lin et al. (2019) demonstrated that SCFP (125, 250, and 500 mg/d) modulates fecal microbiota and fermentative end-products in adult beagles by increasing *Bifidobacterium*, decreasing *Fusobacterium*, and decreasing phenol and indole concentrations. SCFP was also shown to elevate immune capacity by enhancing T helper-1 cell responses and decreasing inflammation through inhibition of toll-like receptor (TLR) responses (Lin et al., 2019). In another study, supplementing adult Labrador Retrievers with SCFP (500 mg/d) led to decreased thiobarbituric acid reactive substances (TBARS) and improved total antioxidant capacity (TAC) in serum, suggesting a reduction in lipid peroxidation and increased antioxidant capacity when subjected to exercise and transport stress (Varney et al., 2021). SCFP-fed Labradors also exhibited increased serum concentrations of TNF-α and decreased serum concentrations of immunoglobulin E and immunoglobulin G, suggesting immunomodulatory effects (Varney et al., 2021). SCFP has been shown to alter fecal microbiota, fecal metabolites, antioxidant status, and immune cell function of adult dogs; however, SCFP

supplementation has previously been administered via gelatin capsule or top dressing. Further investigation of oxidative stress, antioxidant status, and immune responses in dogs fed an SCFP-supplemented extruded kibble diet is warranted, and impacts on skin and coat health have not been determined.

The objective of this study was to determine the effects of a SCFP-supplemented extruded diet on the circulating immune cell numbers and activity, antioxidant status and oxidative stress marker concentrations, and measures of skin and coat health of adult dogs. We hypothesized that dogs fed the SCFP-supplemented diet would have enhanced immune cell numbers and functionality, reduced concentrations of markers associated with oxidative stress and inflammation, and improved skin and coat health compared with dogs fed a control diet.

MATERIALS AND METHODS

Animals and Housing: Sixteen adult pointer dogs [8 intact males, 8 intact females; mean age = 6.7 ± 2.1 y; mean body weight (BW) = 25.9 ± 4.5 kg] were used in a crossover design. All dogs were housed individually (inside run = 1.17 m x 1.42 m; outside run = 1.08 m x 3.05 m) at Kennelwood, Inc. (Champaign, IL). Dogs had free access to fresh water and were fed once daily to maintain BW throughout the study. The amount of food offered was based on previous feeding records and the estimated caloric content of the diets. All experimental procedures were approved by the Kennelwood Inc. IACUC prior to experimentation.

Experimental Timeline and Diets: Prior to the study, dogs were vaccinated (Duramune Max 5 Dog Vaccine; Elanco Animal Health, Greenfield, IN), and blood samples were collected for serum chemistry measures and complete blood count (CBC) to confirm health. Dogs were vaccinated with Duramune Max 5 Dog Vaccine prior to the start of the adaption phase to serve as

a specific antigen for the delayed-type hypersensitivity test (described later). A crossover study began with a 4-wk adaptation phase (wk -4 to -1) followed by two 10-wk experimental periods. There was a 6-wk washout phase (wk -6 to -1) between experimental periods. Dogs were fed the control diet during the adaptation and washout phases, then randomly allotted to a SCFP-supplemented or control diet after baseline measurements (wk 0) were taken. Both dietary treatments tested were extruded kibble diets formulated to meet all Association of American Feed Control Officials (AAFCO, 2022) nutrient recommendations for adult dogs at maintenance and were formulated with similar ingredients and nutrient targets (Blue Buffalo Co. Ltd., Wilton, CT; **Table 3.1**). The SCFP-supplemented diet was formulated to include approximately 0.13% of the active SCFP ingredient (0.4942% of TruMune; Diamond V Mills, Inc., Cedar Rapids, IA). Based on the food intake measured in the study, this inclusion level resulted in an average intake of 30.1 ± 3.6 mg SCFP/kg BW per day (ranged from 21.7 to 38.1 mg SCFP/kg BW). At baseline (wk 0), after wk 5, and after wk 10, blood samples were collected, and skin measurements were conducted. Dogs were weighed and body condition scores were assessed using a 9-point scale (Laflamme, 1997) each week prior to the morning feeding.

Blood Sample Collection: Fasted (12 h overnight) blood samples for serum chemistry and CBC were collected at baseline, wk 5, and wk 10 of each experimental period. Blood samples for immune cell numbers and functionality, antioxidant status, and oxidative stress measures were collected at baseline and wk 10 of each experimental period. Blood samples for the cell-based antioxidant protection in erythrocytes (CAP-e) assay were collected at baseline (before and 2 h after meal), and wk 10 (before and 2 h after meal) of each experimental period. Blood samples were collected via jugular or cephalic puncture using 19- to 22-gauge needles. Samples were immediately transferred to appropriate vacutainer tubes, with 0.5 mL going into BD Vacutainer

Plus plastic whole blood tubes (#363706; Lavender with K_2EDTA additive; Becton Dickinson, Franklin Lakes, NJ), 17.5 mL going into BD Vacutainer SST™ tubes (#367988 and #367983; Becton Dickinson) for serum separation, 20 mL going into BD Vacutainer Heparin tubes (#366480; Becton Dickinson), and 7.5 mL going into PAXgene Blood Tubes (#762165; Qiagen, Valencia, CA).

Serum Chemistry and CBC: Serum was isolated by centrifuging tubes at 2,000 × g at 4°C for 15 min (Beckman CS-6R centrifuge; Beckman Coulter Inc., Brea, CA). Once serum was harvested, it was transported to the University of Illinois Veterinary Medicine Diagnostics Laboratory for serum chemistry analysis. The K_2EDTA tubes were cooled (but not frozen) and transported to the University of Illinois Veterinary Medicine Diagnostics Laboratory for CBC analyses.

Immune Cell Populations: Ficoll (Sigma, St. Louis, MO) was added to blood in a 1:1 volume ratio and centrifuged at 300 × g at 4°C for 30 min to separate peripheral blood mononuclear cells (PBMC) from blood samples. The percentage of T cells, natural killer (NK) cells, and antigen-presenting (AP) cells was evaluated by flow cytometry. For T cell populations, PBMC were distributed into 2 tubes (1×10^6 cells/tube). One tube was incubated with cell stimulation cocktail [phorbol 12-myristate 13-acetate, ionomycin, brefeldin A and monensin (eBioscience, San Diego, CA)]. Both tubes of cells were incubated at 37°C in 5% CO_2 for 4 h followed by surface marker labeling on ice with fluorophore conjugated antibodies to CD3 (FITC; Clone CA17.2A12; BioRad, Cat. No. MCA1774F), CD4 (APC; Clone YKIX302.9; BioRad, Cat. No. MCA1038APC), and CD8 (Pacific Blue; Clone YCATE55.9; BioRad, Cat. No. MCA1039PB) to identify T cells. After staining, samples were fixed with fixation buffer (eBioscience, San Diego, CA) and then stained with IFN-γ (PE; Clone; BioRad, Cat. No. MCA1783PE). NK cells (1×10^6 cells/tube) were

identified using fluorophore conjugated antibodies to CD3 (FITC; Clone CA17.2A12; BioRad, Cat. No. MCA1774F), and CD5 (APC; Clone YKIX322.3; BioRad, Cat. No. MCA1037APC). For AP cells, the cells of interest included B cells and monocytes presenting major histocompatibility complex class II (MHC-II) on the cell surface. AP cells (1×10^6 cells/tube) were identified using fluorophore conjugated antibodies to CD14 (Pacific Blue; Clone TUK4; BioRad, Cat. No. MCA1568PB), CD21 (PE; Clone CA2.1D6; BioRad, Cat. No. MCA1781R), and MHC II (FITC; Clone YKIX334.2 BioRad, Cat. No. MCA1044F). Populations of T cells, NK cells, and APC were then acquired on a LSRII Flow Cytometer (Becton Dickinson). Gates were determined using unstained and single-stained samples obtained from the same PBMC of origin. Results were analyzed using FlowJo version 10.6.2 flow cytometry software (Becton Dickinson). The gating strategy used to determine immune cell populations is shown in **Supplementary Figures 3.1-3.3**. For NK cells, the population was determined according to Huang et al. (2008).

Responsiveness of Lymphocytes to Toll-Like Receptor (TLR) Agonists: PBMC (1×10^5 cells/well) were seeded into 96-well plates. Agonists of TLR2 (100 µg/mL zymosan; Invivogen, San Diego, CA), TLR3 [50 µg/mL polyinosinic–polycytidylic acid sodium salt, poly(I:C); Sigma], TLR4 (100 ng/mL LPS; Sigma), and TLR7/8 (5 µg/mL resiquimod, Invivogen) were added to assigned wells separately. After 24 h of incubation, supernatants were collected for measurement of TNF-α concentration using a commercial ELISA kit (#MBS761131; MyBioSource, San Diego, CA).

Serum Oxidative Stress and Immune Markers: Circulating oxidative stress and immune markers [8-isoprostane, Trolox equivalent antioxidant capacity (TEAC); malondialdehyde (MDA); superoxide dismutase (SOD)], LPS-binding protein (LBP), and lysozyme concentrations were measured using commercial ELISA kits (8-isoprostane: #MBS2611970, TEAC: #

MBS169313, MDA: #MBS2605193, SOD: #MBS2104718, LBP: #MBS093112, Lysozyme: #MBS2604408, MyBioSource, San Diego, CA) according to the manufacturer's instructions. Remaining serum samples were shipped overnight on dry ice to Michigan State University Veterinary Diagnostic Laboratory (Lansing, MI) for serum α-tocopherol analysis and to NIS labs (Klamath Falls, OR) for the CAP-e assay.

Cellular Antioxidant Protection and Bioavailability: To test for antioxidants in serum that were likely to be bioavailable at the cellular level in vivo, the canine serum samples were tested ex vivo using the CAP-e assay (Jensen et al., 2008; Phillips et al., 2019).Human erythrocytes were purified and washed 4 times in phosphate-buffered saline, and stored at 4°C until use, and used for testing within 4 d. For the CAP-e cellular antioxidant protection assay, each canine serum sample was tested in quadruplicate, using human erythrocytes to detect antioxidant compounds present in canine serum. The canine serum samples were kept at -80°C until testing. All long-term samples from each dog were tested in the same run, and all acute samples from each dog were tested in a parallel run using the same batch of human erythrocytes. Serum samples were thawed, briefly vortexed, and kept at 4°C until testing was initiated within the hour. To avoid antibody-mediated lysing of the human erythrocytes by Ig present in the canine serum samples, a 10x solution of EDTA buffer was added to each serum sample immediately prior to testing. The erythrocytes were treated with the canine serum samples in quadruplicate for 20 min. During this incubation time, antioxidant compounds able to cross the cell membrane enter the erythrocyte cell. Following the incubation of erythrocytes with serum, the erythrocytes were washed twice with PBS to remove any compounds from the test products that were not absorbed by the cells. Cell cultures were then treated with the indicator dye 2',7' –dichlorofluorescin diacetate (DCFDA), which becomes fluorescent when oxidized. The peroxyl-free radical generator 2,2′-azobis-2-methyl-

propanimidamide, dihydrochloride (AAPH) was added to trigger oxidation. Control cultures were performed in hexaplicate and included untreated erythrocytes as a negative control (not exposed to serum or AAPH) and erythrocytes treated with AAPH in the absence of serum (positive control). After exposure to AAPH for 1 h, the fluorescence intensity was measured at 488 nm using a Tecan Spectrafluor plate reader (Tecan, Männedorf, Switzerland). When a reduction of fluorescence intensity was observed in erythrocytes exposed to a serum sample prior to exposure to AAPH, this was indicative that the serum contained antioxidants that were able to penetrate the erythrocyte cells and protect them from AAPH-mediated oxidative damage.

Whole Blood Gene Expression: Total RNA from blood cells were isolated using a PAXgene Blood RNA Kit (#762331; Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentrations were determined using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). cDNA were synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Gene expression was measured by real-time two-step RT-PCR using an Applied Biosystems 7900HT real-time PCR system (Applied Biosystems, Waltham, MA) and was carried out with SYBR Green chemistry (Bio-Rad Laboratories, Hercules, CA) in a QuantStudio 7 instrument (Thermo Fisher Scientific, Waltham, MA) using validated forward and reverse primers (Bio-Rad Laboratories). Genes of interest included the following: glutathione peroxidase (UniqueAssayID: qCfaCED0030791), glutathione reductase (UniqueAssayID: qCfaCED0031064), catalase (UniqueAssayID: qCfaCED0028561), SOD (UniqueAssayID: qCfaCED0038911), myeloperoxidase (MPO, UniqueAssayID: qCfaCID0034597), and cyclooxygenase-2 (COX-2, UniqueAssayID: qCfaCED0024663) (PrimePCR SYBR Green Assay, Bio-Rad Laboratories). All gene expression data were analyzed

using the $2^{-\Delta\Delta Ct}$ method, represented as gene expression relative to the housekeeping gene (*RPS5*, UniqueAssayID: qCfaCED0028510).

Skin and Hair Coat Condition: At baseline and wk 10, skin and coat were scored by three blinded researchers according to Rees et al. (2001). Hair was scored using the following scale: 1: dull, coarse, dry; 2: poorly reflective, non-soft; 3: medium reflective, medium soft; 4: highly reflective, very soft; 5: greasy. Skin condition was scored using the following scale: 1: dry; 2: slightly dry; 3: normal; 4: slightly greasy; 5: greasy. After skin and coat scoring was done, dogs were sedated by an intramuscular injection of a combination of butorphanol (Torbugesic; 0.2 mg/kg BW) and dexmedetomidine (0.02 mg/kg BW) so that transepidermal water loss [TEWL; Tewameter TM 300 MDD (Courage + Khazaka Electronic GmbH, Cologne, Germany)], hydration status [Corneometer CM 825 (Courage + Khazaka Electronic GmbH, Cologne, Germany)], and sebum concentrations [external Sebumeter SM 815 (Courage + Khazaka Electronic GmbH, Cologne, Germany)], and delayed-type hypersensitivity (DTH) response could be measured.

Delayed-Type Hypersensitivity: DTH response was tested at wk 10 as described by Kim et al. (2000). Briefly, dogs were injected intradermally in the flank area with 100 µl of saline (8.5 mg/mL; functioned as control), an attenuated vaccine (Duramune Max 5 Dog Vaccine; Elanco Animal Health; functioned as specific antigen), and phytohaemagglutinin (PHA; 0.5 mg/mL) and concanavalin A (ConA; 0.5 mg/mL), which both functioned as non-specific antigens. Injections were performed in triplicate along the flank area after the injection site was clipped and wiped with 70% ethyl alcohol. After skin measurements and DTH injections were completed, an injection of the reversal agent for dexmedetomidine, atipamezole (0.2 mg/kg BW), was administered intramuscularly. Skin induration was measured at baseline, 15, 30, 45, and 60 min after injection,

and 24, 48, and 72 h after injection using a digital caliper. DTH responses were reported as average wheal diameter (mm) as described by van der Valk et al. (2016).

Diet Chemical Analyses: Both diets were ground in a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen and then analyzed for dry matter (DM) and ash according to AOAC (2006; methods 934.01 and 942.05), with organic matter (OM) being calculated. Crude protein was calculated from Leco (FP2000 and TruMac) total nitrogen values according to AOAC (2006; method 992.15). Total lipid content (acid-hydrolyzed fat) was determined according to the methods of the American Association of Cereal Chemists (1983) and Budde (1952). Total dietary fiber was determined according to Prosky et al. (1988). Gross energy was measured using an oxygen bomb calorimeter (model 6200, Parr Instruments, Moline, IL).

Statistical Analyses: Data were analyzed using the Mixed Models procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC). The fixed effect of treatment was tested, and dog was considered a random effect. Change from baseline differences between treatments were determined using a Fisher-protected least significant difference with a Tukey adjustment to control for experiment-wise error. A probability of $P < 0.05$ was accepted as statistically significant and $P < 0.10$ being trends when considering the effects of diet, time, and their interactions. Reported pooled standard errors of the mean was determined according to the Mixed Models procedure of SAS 9.4.

RESULTS

One dog was removed from the study immediately following the washout phase for medical reasons (pain and enlarged mammary tissue). Therefore, one less dog was allotted to the control group before the second treatment period began. The remaining 15 dogs completed the study. Most dogs maintained their BW, food intake, and health throughout the study. Two dogs

were administered oral antibiotics during the study period: cephalexin (525 mg, twice per day) for 10 d during the adaptation phase to treat an infected wound in one dog, and enrofloxacin (136 mg, twice per day) for 15 d during the first treatment period to treat a urinary tract infection in the other dog.

Most of the baseline measures are presented in **Table 3.2**. At baseline, T cells (% of lymphocytes) were higher ($P < 0.05$), and helper T cells (% of lymphocytes) tended to be lower ($P < 0.10$) in stimulated cells from dogs later allotted to the SCFP diet than those later allotted to the control diet. Baseline MHC+ B cells (% of B cells) and monocytes (% of white blood cells) were also higher ($P < 0.01$) in dogs later allotted to the SCFP diet than the control diet. Baseline hydration status and TEWL in the ear were higher ($P < 0.05$) in dogs later allotted to the SCFP diet than those later allotted to the control diet. Finally, baseline blood glutathione reductase mRNA expression tended to be lower ($P < 0.10$) in dogs later allotted to the SCFP diet than those later allotted to the control diet. Baseline CBC measures were not different (**Table 3.3**). Baseline serum creatine phosphokinase and triglycerides were higher ($P < 0.05$) in dogs later allotted to the SCFP diet than those later allotted to the control diet, but all other baseline serum chemistry measures were not different between groups (**Table 3.4**). Baseline serum corticosteroid-induced alkaline phosphatase and alanine transaminase were not different between groups but were slightly above the reference ranges. Due to differences observed at baseline, change from baseline values were used to evaluate outcomes in the present study.

Change from baseline CBC and serum chemistry profiles were slightly affected by dietary treatment and time (**Table 3.12 and 3.13**). Change from baseline platelet concentrations and basophil % were affected ($P < 0.05$) by a time*diet interaction (**Table 3.12**). Platelet concentrations were variable over time in dogs fed the control diet but decreased over time in dogs fed the SCFP

diet. Basophil % increased slightly over time in dogs fed the control diet but was more variable in dogs fed the SCFP diet. Red blood cell concentrations, hemoglobin concentrations, and hematocrit % changed ($P < 0.01$) over time but were not affected by diet. Change from baseline serum total protein, globulin, gamma glutamyltransferase, and triglyceride concentrations were lower ($P < 0.05$), while the albumin:globulin ratio tended to be higher ($P < 0.10$) in dogs fed the SCFP diet than those fed the control diet (**Table 3.13**). Change from baseline albumin:globulin ratio was higher ($P < 0.05$), while change from baseline triglyceride concentrations were lower ($P < 0.05$) over time in dogs fed the SCFP diet. Change from baseline globulin concentrations tended to be lower ($P < 0.10$), while change from baseline total bilirubin and creatine phosphokinase concentrations tended to be higher ($P < 0.10$) over time in dogs fed the SCFP diet. The other serum chemistry measures were not affected by diet or time.

Change from baseline of unstimulated lymphocytes (% of PBMC) and stimulated IFN-γ secreting T cells (% of lymphocytes) were lower ($P < 0.05$), while stimulated T cells (% of lymphocytes) were higher ($P < 0.05$) in dogs fed the SCFP diet than those fed the control diet (**Table 3.5**). Change from baseline stimulated cytotoxic T cells (% of lymphocytes) tended to be lower ($P < 0.10$) in dogs fed the SCFP diet than those fed the control diet. All other T cell populations were not different between groups. Change from baseline NK cell and antigen-presenting cell (B cells and monocytes) populations were not altered by treatment (**Table 3.6**). Change from baseline TNF-α concentrations of control cell culture supernatants tended to be lower ($P < 0.10$) in dogs fed the SCFP diet than those fed the control diet, but cells stimulated with TLR agonists were not affected by diet (**Table 3.7**).

Change from baseline serum SOD concentrations were higher ($P < 0.05$) in dogs fed the SCFP diet than those fed the control diet (**Table 3.8**). None of the other serum immune or oxidative

stress markers were affected by treatment. Change from baseline mRNA expression of catalase was lower ($P < 0.05$) in dogs fed the SCFP diet than those fed the control diet (**Table 3.9**), but the expression of other genes was not affected by diet.

Change from baseline measures of skin and coat health were variable and region-specific. Change from baseline TEWL in the back region was lower ($P < 0.05$) in dogs fed the control diet than those fed the SCFP diet (**Table 3.10**). In contrast, change from baseline TEWL in the ear region tended to be lower ($P < 0.10$) in dogs fed the SCFP diet than those fed the control diet. Change from baseline sebum concentrations tended to be higher ($P < 0.10$) in the inguinal and ear regions of dogs fed the SCFP diet than those fed the control diet. Other change from baseline skin and coat measures were not affected by diet.

DTH response was not measured at baseline but was affected by diet and time at the end of the treatment phases (**Table 3.11**). As expected, wheal diameter increased over time for all injections. The response to PHA was higher ($P < 0.05$) and the response to ConA tended to be higher ($P < 0.10$) in dogs fed the SCFP diet than those fed the control diet.

DISCUSSION

In recent decades, pet humanization has transformed the ways in which pet owners choose to feed their pets. Dogs are now considered a part of the family, driving owners to seek out premium, high-quality formulas often made with functional ingredients. Functional ingredients claim to provide health benefits beyond basic nutrition, such as immune support or anti-inflammatory properties. Yeast-based products are commonly added for their functional properties. Improvements to feed efficiency, growth performance, and quality of animal products have been observed in several livestock species due to SCFP supplementation (Hristov et al., 2010;

Kidd et al., 2013; Zaworski et al., 2014; Chen et al., 2020) and has stimulated interest in its use as a functional ingredient in companion animal diets. Previous research on yeast products in dogs has demonstrated that supplementation of yeast cell walls and their components (i.e., MOS and β-glucans) may modulate host microbiota (Swanson et al., 2002a; Grieshop et al., 2004) and alter fecal fermentative-end products (Swanson et al., 2002a; Theodoro et al., 2019), but the effects on immune function are not well defined. Early studies investigating the effects of yeast product supplementation on immune cell numbers and function were limited (Grieshop et al., 2004; Middelbos et al., 2007), possibly due to the lack of canine-specific research techniques at the time. However, supplementation of MOS in dogs has recently been shown to increase circulating CD4+ lymphocytes (43.7% vs. 45.6%) and the CD4+:CD8+ lymphocyte ratio (2.5 vs. 2.9) (Pawar et al., 2017) and yeast cell wall supplementation has resulted in a higher (37% increase) phagocytic index in peripheral monocytes (Theodoro et al., 2019).

There is currently limited information on the functional properties of SCFP in companion animal diets. Lin et al. (2019) supplemented SCFP to dogs via gelatin capsules (0, 125, 250, and 500 mg/d) and reported positive effects on GI health outcomes, immune function outcomes, and inflammatory markers. However, our study investigated a slightly different dosage and delivery method of SCFP than the dogs evaluated by Lin et al. (2019). When SCFP intake was calculated based on the average BW (10.3 kg) reported by Lin et al. (2019), supplementation was equal to approximately 12.1, 24.3, and 48.5 mg SCFP/kg BW in the 125, 250, and 500 mg/d treatment groups, respectively. In the present study, the active SCFP ingredient was included as 27% of the TruMune product (i.e., active ingredient + carrier). Because the SCFP product was formulated into an extruded kibble diet and dogs were fed to maintain BW, this resulted in dogs consuming slightly different doses of SCFP depending on their energy requirements. When calculating the dose of

SCFP based on daily food intake, dogs in the present study consumed an average of 30.1 ± 3.6 mg SCFP/kg BW per day throughout the treatment period. Furthermore, effects on oxidative stress and skin and coat health have not been thoroughly investigated, and to our knowledge, the functionality of SCFP has not been tested when included as an ingredient in an extruded kibble diet. In the present study, we investigated measures of skin and coat health and further measured circulating immune cell numbers and activity, antioxidant status, and oxidative stress marker concentrations of adult dogs fed a SCFP-supplemented extruded diet. All dogs remained healthy throughout the study. CBC values were within reference ranges for healthy dogs. A couple serum chemistry markers (corticosteroid-induced alkaline phosphatase; alanine transaminase) were slightly above the reference ranges, but no clinical signs were noted during the study.

To our knowledge, the effects of SCFP supplementation on skin and hair coat scores or skin biophysical parameters have not been previously investigated in dogs. Supplementation of SCFP did not affect subjective skin and coat scores or hydration status in the current study. The tendency for higher change from baseline in sebum concentrations in dogs fed SCFP, however, may provide benefits. Sebum is thought to protect the epidermis by lubricating the stratum corneum and hair follicle (Dunstan et al., 2000). It also gives the hair coat a glossy appearance, which is desired by pet owners. Although sebum concentrations increased in SCFP-fed dogs, the change did not result in an excessively greasy hair coat, as mean hair coat scores were "medium reflective/medium soft" for both groups.

TEWL measurements provide valuable information for assessing the integrity of the skin barrier and estimating the skin's moisture retention properties (Pinnagoda et al., 1990, Watson et al., 2002; Fluhr et al., 2006). TEWL outcomes varied by body site in our study, where TEWL values were higher on the back region but lower on the ears of SCFP-fed dogs. Higher TEWL

values indicate greater water loss and are consistent with increased damage of epidermal barrier function. TEWL measures must be interpreted carefully, however, as they can be altered by sampling methods (e.g., open chamber probe, closed chamber probe, hair clipping, and the use of anesthesia) (Watson et al., 2002; Oh and Oh, 2009). It has also been observed that TEWL measurements are subject to significant site-to-site, day-to-day, and dog-to-dog variation (Lau-Gillard et al., 2009), which may account for the discrepancy in TEWL data between body sites and treatment groups in the present study. Throughout our TEWL pilot studies, however, the ear region produced the most consistent values among dogs in our cohort, which we suspect was due to a lack of interference from the hair coat. Despite clipping the hair coat prior to measurement, it was difficult to ensure the TEWL probe maintained direct contact with skin on the back region. Thus, TEWL values from the ear region may be more reliable for interpretation, indicating that reduced TEWL on the ears of SCFP-fed dogs may be due to enhanced skin integrity.

The DTH is an intradermal test that indicates the *in vivo* cell-mediated immune response (Kim et al., 2000). This response is characterized by the activation of phagocytes and antigen-specific cytotoxic T cells and the production of proinflammatory cytokines. It has been shown that mannoproteins, a component of yeast cell walls, can induce hypersensitivity reactions and release of cytokines, such as TNF-α and IFN-γ (Chaka et al., 1997; Pietrella et al., 2001). In our study, DTH response was not measured at baseline, but was affected by diet and time at wk 10. As expected, wheal diameter increased over time for all injections. In response to PHA and ConA, a greater increase in wheal diameter was observed in dogs fed the SCFP-supplemented diet than those fed the control diet. Diet and time interactions were observed for DTH response to PHA (nonspecific immunity) in SCFP-fed dogs, whereas only diet tended to influence DTH response in SCFP-fed dogs after ConA (nonspecific immunity) inoculation. Similar DTH results were reported

in dogs supplemented with MOS (Pawar et al., 2017) and mannoproteins (Kroll et al., 2020) following intradermal inoculation with PHA. Pawar et al. (2017) reported that DTH response (i.e., skin thickness) to PHA inoculation was significantly higher in the MOS group when compared with controls, and Kroll et al. (2020) reported diet and time effects as well as time and age interactions after PHA injection. Increased DTH response to nonspecific antigens PHA and ConA may suggest an enhanced cell-mediated immune response due to SCFP supplementation.

Dietary supplementation with yeast products has been previously shown to modulate circulating immune cell populations and immune function in dogs (Swanson et al., 2002a; Grieshop et al., 2004; Middelbos et al., 2007; Pawar et al., 2017; Theodoro et al., 2019; Lin et al., 2019; Kroll et al., 2020). The lower change from baseline unstimulated lymphocytes observed in SCFP-supplemented dogs in the current study were similar to that reported in previous dog studies investigating yeast supplementation. Supplementation with MOS increased (15.55% vs. 20.40%; Swanson et al., 2002a) or tended to decrease (2.2 10^3/μL vs. 1.7 10^3/μL; Grieshop et al., 2004) lymphocyte populations. Lin et al. (2019) did not report changes to lymphocyte populations. However, Lin and colleagues reported dogs having lower total WBC counts, showing a linear effect with increasing SCFP dose (0 vs. 125, 250, and 500 mg/d), potentially due to an enhanced immune capacity requiring fewer immune cells. This was supported by immune cell population data demonstrating increases in MHC II presenting B cells, effector T cells (IFN-γ secreting helper and cytotoxic T cells) and monocytes (Lin et al., 2019) in SCFP-supplemented dogs, which is not consistent with our data. In the present study, change from baseline populations of stimulated IFN-γ secreting T cells and cytotoxic T cells decreased in SCFP-fed dogs. Change from baseline stimulated T cells decreased in both treatment groups, although populations were significantly lower in the control group. It is difficult to interpret the changes observed in immune cell

populations among SCFP-supplemented and control dogs in the present study. However, the decrease in circulating lymphocytes among SCFP-fed dogs may suggest a more balanced immune response. In addition to functional TLR stimulation, utilizing additional assays to investigate immune function (i.e., lymphocyte proliferation and/or phagocytic activity of leukocytes) may aid future studies in interpreting the changes observed in immune cell numbers.

An additional immune response observed in the study by Lin et al. (2019) was a significant reduction in TNF-α concentrations in the cell supernatant of SCFP-supplemented dogs. In cells stimulated with TLR2, TLR3, TLR4, and TLR7/8 agonists, an overall effect of controls (0 mg/d SCFP) vs. all SCFP treatments (125, 250, and 500 mg/d) was observed, with cells obtained from dogs supplemented with SCFP producing less TNF-α than cells collected from control dogs (Lin et al., 2019). These agonists represent yeast (zymosan, TLR2), bacterial (lipopolysaccharide, TLR4) and viral (polyinosinic:polycytidylic acid, TLR3; resiquimod, TLR7/8) challenges to the immune system. A reduction in the pro-inflammatory cytokine TNF-α coupled with reduced lymphocyte populations may have suggested a more moderated immune response due to SCFP supplementation. However, despite some numeric differences in TNF-α production in our study, this hypothesis cannot be confirmed due to the lack of significant differences in TNF-α concentrations between treatment groups and high biological variation in TNF-α concentrations between dogs.

Mannans isolated from *S. cerevisiae* have been shown to have antioxidant properties *in vitro* (Krizková et al., 2001), and zymosan derived from glucans in the yeast cell wall have been shown to increase antioxidant function in tumor-bearing mice (Liu et al., 2011). In the present study, SCFP supplementation increased serum SOD concentrations and decreased catalase mRNA expression, suggesting that antioxidant defenses may function more efficiently in SCFP-fed dogs.

To maintain a homeostatic level of oxidative stress, powerful enzymatic systems such as SOD and catalase directly counteract the formation of reactive oxygen species. Superoxide is a major oxidant produced during oxidative stress and is scavenged by the enzyme SOD. The reaction creates hydrogen peroxide, which is subsequently detoxified by glutathione or catalase (Eaton, 2006). Previous data on dogs supplemented with MOS (15 g/kg of the diet) indicated no influence of MOS on erythrocytic antioxidants (Pawar et al., 2017), and oxidative stress markers (i.e., SOD and MDA) were not different in dogs supplemented with SCFP (125, 250, and 500 mg/d) or the placebo (Lin et al., 2019). However, SCFP-supplemented dogs (500 mg/d) challenged with exercise and transport stress exhibited increased concentrations of serum TAC and reduced concentrations of serum TBARS, suggesting that SCFP may provide some protection from oxidative damage under conditions of stress (Varney et al., 2021). Using an induced environmental stress model, such as exercise or transport stress, may aid future studies in detecting changes to antioxidant status or oxidative stress in healthy dogs. Further research on the antioxidant status and oxidative stress markers of dogs fed SCFP is warranted, as our data suggests SCFP-supplementation increases the activity of antioxidant enzymes SOD and catalase and may reduce oxidative damage.

The treatment duration and experimental design of the present study allowed for a thorough, longitudinal assessment of the effects of SCFP supplementation in which each dog could serve as their own control. However, the experimental design can also be considered a limitation of the study, as it may have increased variability due to seasonal changes. Seasonal changes (e.g., relative humidity and room temperature) may have impacted skin and hair coat measures, as dogs in the present study had daily outdoor access across multiple seasons. However, giving dogs in the study outdoor access provided a more accurate comparison to pet dogs. Variability in skin and hair

coat outcomes may also be attributed to the use of anesthesia (Oh and Oh, 2019) or interference from the hair coat (Momota et al., 2013). Another potential limitation of the study was the measurement of 8-isoprostane in serum rather than urinary samples. Measurement of urinary rather than plasma or serum 8-isoprostane has been proposed as a better indicator of oxidative stress in humans because arachidonic acid, the metabolic precursor of isoprostanes, is widely distributed in cell membranes throughout tissues, providing a comprehensive reflection of oxidative stress activation in the entire body (Montero et al., 2000; Monnier et al., 2006). Moreover, 8-isoprostane concentrations fluctuate throughout the day, hence urinary determinations are likely to provide a more reliable estimation of the 24 h fluctuations in isoprostane production than blood sampling at fixed time points (Monnier et al., 2006; Ito et al., 2019). Logistically, metabolic cages would likely be required to carry out urine sampling over a 24-h period in dogs. Although such methodology is more difficult to use, it would allow for the accurate measurement of urinary 8-isoprostane and a more accurate measurement of oxidative stress.

CONCLUSIONS

In conclusion, our data suggest SCFP may act as a functional ingredient in dog foods to beneficially impact skin and coat health, immunity, and antioxidant status when included in an extruded diet. SCFP was shown to improve skin and coat health by improving skin barrier function and increasing sebum concentrations, which acts to lubricate and protect skin while giving the hair coat a glossy appearance. SCFP had effects on some parameters of cell-mediated and humoral immunity by enhancing the DTH response to nonspecific antigens (PHA and ConA) and modulating circulating lymphocyte populations. Finally, SCFP supplementation was shown to support key antioxidant defense systems through modulation of enzymes SOD and catalase. Our

data suggests that SCFP can be included in extruded dog foods as a functional ingredient to support antioxidant status and skin integrity. SCFP supplementation may be most beneficial to geriatric and/or working dogs and to dogs with skin sensitivities.

TABLES

Table 3.1 Ingredient and chemical composition of experimental diets tested

Ingredient	Control	SCFP
	--- %, as-basis ---	
Chicken, deboned	18.23	18.23
Chicken meal	18.05	18.05
Barley	18.05	18.00
Oats	18.05	18.00
Brown rice	16.58	16.54
Chicken fat	4.79	4.79
Liquid digest	1.71	1.71
Powdered cellulose	1.61	1.61
Powder digest	0.86	0.86
Potassium chloride	0.52	0.52
TruMune (SCFP)	-	0.49
Calcium carbonate	0.36	-
Salt	0.42	0.42
Trace mineral mix	0.23	0.23
Vitamin mix	0.23	0.23
Choline chloride	0.19	0.19
Mixed tocopherols	0.07	0.07
DL-methionine	0.07	0.07
Analyzed composition		
Dry matter (DM), %	90.97	90.59
	--- %, DM basis ---	
Acid-hydrolyzed fat	12.81	12.62
Crude protein	25.61	25.32
Total dietary fiber	14.03	12.94
Insoluble fiber	9.50	7.91
Soluble fiber	4.53	5.03
Ash	8.76	8.34
NFE[1]	38.79	40.61
Gross energy, kcal/kg	5,076	5,125
Calculated metabolizable energy[2], kcal/kg	3,342	3,386

[1]Nitrogen-Free Extract % = 100 % – (% Acid Hydrolyzed Fat + % Crude Protein + % Moisture + % Ash + % Total Dietary Fiber)

[2]Metabolizable energy estimated with modified Atwater factors: 10 × [(3.5 × % Crude Protein) + (8.5 × % Crude Fat) + (3.5 × % NFE)]

Table 3.2. Baseline measures of dogs consuming a SCFP-supplemented or control diet[1]

Item	Control	SCFP	SEM	p-value
Natural killer cell, % of lymphocyte	18.66	17.06	1.82	0.5288
Unstimulated (control)				
Lymphocyte, % of PBMC[2]	71.57	80.28	3.89	0.5141
T cell, % of lymphocyte	66.49	65.95	3.59	0.8742
Helper T cell, % of lymphocyte	39.83	41.81	2.17	0.4019
Cytotoxic T cell, % of lymphocyte	21.01	22.57	5.31	0.3835
Helper: cytotoxic T cell ratio	7.30	11.38	4.01	0.7164
IFN-γ secreting T cell, % of lymphocyte	4.46	6.75	1.90	0.6494
IFN-γ secreting helper T cell, % of lymphocyte	0.01	0.01	0.01	0.3547
IFN-γ secreting cytotoxic T cell, % of lymphocyte	22.32	21.49	4.35	0.8940
Stimulated[3]				
Lymphocyte, % of PBMC[2]	77.92	81.51	2.99	0.4033
T cell, % of lymphocyte	66.33[b]	71.66[a]	2.44	**0.0406**
Helper T cell, % of lymphocyte	45.34	42.58	2.05	*0.0763*
Cytotoxic T cell, % of lymphocyte	28.43	31.46	4.20	0.6142
Helper: cytotoxic T cell ratio	2.18	2.74	0.68	0.3954
IFN-γ secreting T cell, % of lymphocyte	19.27	17.86	3.39	0.7604
IFN-γ secreting helper T cell, % of lymphocyte	6.50	7.17	2.23	0.2770
IFN-γ secreting cytotoxic T cell, % of lymphocyte	23.46	25.05	3.18	0.7272
Antigen-Presenting Cells				
B cell, % of lymphocyte	8.37	8.18	0.82	0.8550
B cell, MHC II+, % of B cell[4]	95.97[b]	98.41[a]	0.63	**0.0032**
Monocyte, % of white blood cell	14.81[b]	22.20[a]	3.10	**0.0023**
Monocyte, MHC II+, % of monocyte[4]	48.36	56.96	4.60	0.1174
Immune Cell Markers				
Lipopolysaccharide-binding protein, μmol/L	112.86	117.20	8.37	0.7162
Lysozyme, ng/mL	2.83	3.00	0.13	0.2957

Table 3.2. (cont.)

Item	Control	SCFP	SEM	p-value
Oxidative Stress Markers				
Malondialdehyde, nmol/mL	37.92	39.50	1.31	0.2249
Superoxide dismutase, ng/ml	19.11	15.94	1.26	0.1726
8-isoprostane, pg/mL	291.03	301.17	9.11	0.2194
α-tocopherol, µg/mL	35.76	35.75	2.82	0.9207
Trolox equivalent antioxidant capacity (TEAC), µM Trolox	2685.27	2797.34	601.34	0.9842
CAP-e fasted[5]	28182.0	28670.0	872.3	0.3010
CAP-e fed[5]	28339.0	28895.0	862.5	0.2607
Tumor Necrosis Factor-Alpha (TNF-α) Concentrations (in cell culture supernatant)[6]				
Control, pg/mL	321.14	420.14	128.73	0.4780
Lipopolysaccharide (LPS; TLR4), pg/mL	1084.27	1087.31	155.52	0.9830
Zymosan (TLR2), pg/mL	2321.62	2028.85	438.87	0.4242
Poly I:C (TLR3), pg/mL	635.78	632.93	104.43	0.8779
R848 (TLR 7/8), pg/mL	4197.02	5361.31	800.86	0.5964
Hair and Skin Measurements				
Skin score[8]	2.86	2.93	0.10	0.4831
Hair score[9]	2.83	2.87	0.08	0.6118
Sebum[10] concentration (back)[11], arbitrary unit	1.45	1.02	0.51	0.4173
Sebum[10] concentration (inguinal)[12], arbitrary unit	0.81	0.96	0.32	0.4440
Sebum[10] concentration (ear)[13], arbitrary unit	43.36	41.25	5.20	0.6648
Hydration[14] (back)[11], arbitrary unit	0.21	0.95	0.42	0.2795
Hydration[14] (inguinal)[12], arbitrary unit	13.64	15.39	2.12	0.4372
Hydration[14] (ear)[13], arbitrary unit	27.14[b]	33.73[a]	2.50	**0.0325**
Transepidermal water loss (TEWL)[15] (back)[11], g/h/m^2	15.40	14.01	0.97	0.1484
TEWL[15] (inguinal)[12], g/h/m^2	11.67	10.68	0.77	0.2552
TEWL[15] (ear)[13], g/h/m^2	10.56[b]	11.88[a]	0.48	**0.0499**

Table 3.2. (cont.)

Item	Control	SCFP	SEM	p-value
Blood Gene Expression				
Cyclooxygenase-2	0.92	0.97	0.07	0.8588
Glutathione peroxidase	1.19	1.18	0.08	0.5723
Myeloperoxidase	1.20	1.00	0.11	0.9096
Superoxide dismutase	0.93	0.91	0.10	0.9709
Catalase	0.86	0.92	0.08	0.4630
Glutathione reductase	0.62	0.54	0.25	*0.0855*

[1]All dogs consumed control diet at baseline and then were allotted to control or SCFP-supplemented diet after baseline measurements.
[2]PBMC = peripheral blood mononuclear cells.
[3]Cells were stimulated with cell stimulation cocktail (phorbol 12-myristate 13-acetate, ionomycin, brefeldin A, and monensin) for 4 h.
[4]B cells or monocytes that present major histocompatibility complex class II (MHC II).
[5]Cell-based antioxidant protection in erythrocytes assay. Results provided as mean fluorescence intensity, where low fluorescence indicates less oxidative stress to RBC, and higher fluorescence indicates a higher level of stress to RBC.
[6]LPS mimics a bacterial challenge, zymosan mimics a yeast challenge, and poly I:C and resiquimod (R848) mimic a viral challenge.
[7]Fecal scores: 1 = hard, dry pellets; small hard mass; 2 = hard formed, remains firm and soft; 3 = soft, formed and moist stool, retains shape; 4 = soft, unformed stool; assumes shape of container; 5 = watery, liquid that can be poured.
[8] Skin scores: 1 = dry; 2 = slightly dry; 3 = normal; 4 = slightly greasy; 5 = greasy.
[9] Hair scores: 1 = dull, coarse, dry; 2 = poorly reflective, non-soft; 3 = medium reflective, medium soft; 4 = highly reflective, very soft; 5 = greasy.
[10]Measured using a sebumeter, μg/cm².
[11]Back measurements were taken on the right side along the spine between the rib and hip bone.
[12]Inguinal measurements were taken on the left side.
[13]Ear measurements were taken on the left side towards the inside of the ear.
[14]Measured using a corneometer, arbitrary units.
[15]Measured using a tewameter, g/h/m².
[ab]Mean values within a row with unlike superscript letters differ ($P < 0.05$). Bolded numbers are significant ($P < 0.05$) and italicized numbers tended to be significant ($P < 0.10$).

Table 3.3. Baseline complete blood count of dogs consuming a SCFP-supplemented or control diet[1]

Measure	Reference Range	Control	SCFP	SEM	p-value
Red blood cells, 10^6/uL	5.50-8.50	7.28	7.24	0.15	0.6714
Reticulocyte count, %	---	1.28	1.10	0.09	0.1075
A reticulocyte count, /uL	---	92478.00	79831.00	6974.12	0.1105
Hemoglobin, g/dL	12.0-18.0	16.66	16.53	0.35	0.6590
Hematocrit, %	35.0-52.0	48.52	48.19	0.89	0.6529
Mean cell volume, fl	58.0-76.0	66.82	66.64	0.54	0.4727
Mean corpuscular hemoglobin, pg	20.0-25.0	22.94	22.85	0.24	0.4791
Mean corpuscular hemoglobin, g/dL	33.0-38.6	34.34	34.27	0.15	0.5693
Platelets, 10^3/uL	200-700	374.87	366.67	16.58	0.5732
Mean platelet volume, fl	---	9.39	9.36	0.20	0.7231
White blood cell count, 10^3/uL	6.00-17.00	8.52	8.62	0.39	0.8330
Lymphocytes, %	---	16.53	18.19	1.49	0.4764
Monocytes, %	---	4.98	5.13	0.61	0.8401
Eosinophils, %	---	3.46	3.23	0.48	0.7020
Basophils, %	---	0.13	0.12	0.06	0.9472
A Lymphocytes, 10^3/uL	1.00-4.80	1.40	1.55	0.13	0.1743
A Monocytes, 10^3/uL	0.20-1.40	0.43	0.45	0.06	0.7551
A Eosinophils, 10^3/uL	0.10-1.00	0.30	0.28	0.05	0.7270
A Basophils, 10^3/uL	0.00-2.00	0.01	0.01	0.00	0.8944

[1]All dogs consumed control diet at baseline and then were allotted to control or SCFP-supplemented diet after baseline measurements.

Table 3.4. Baseline serum chemistry of dogs consuming a SCFP-supplemented or control diet[1]

Measure	Reference Range	Control	SCFP	SEM	p-value
Creatinine, mg/dL	0.5–1.5	0.79	0.80	0.03	0.7179
Blood urea nitrogen, mg/dL	6–30	14.46	14.81	0.57	0.4125
Total protein, g/dL	5.1–7.0	5.99	6.06	0.10	0.3297
Albumin, g/dL	2.5–3.8	3.03	3.01	0.06	0.8004
Globulin, g/dL	2.7–4.4	2.97	3.05	0.10	0.3006
Albumin:globulin ratio	0.6–1.1	1.05	1.00	0.04	0.1612
Ca, mg/dL	7.6–11.4	9.50	9.50	0.07	0.9415
P, mg/dL	2.7–5.2	3.69	3.73	0.14	0.7746
Na, mmol/L	141–152	144.59	144.94	0.30	0.3165
K, mmol/L	3.9–5.5	4.48	4.41	0.06	0.1582
Na:K ratio	28–36	32.32	32.94	0.47	0.1399
Cl, mmol/L	107–118	110.04	110.06	0.41	0.9509
Glucose, mg/dL	68–126	98.22	95.88	2.42	0.3438
Alkaline phosphatase (ALP), U/L	7–92	65.85	61.94	6.36	0.2341
Corticosteroid-induced ALP, U/L	0–40	43.07	40.81	6.20	0.4890
Alanine transaminase, U/L	8–65	87.99	65.75	20.27	0.7217
Gamma glutamyltransferase, U/L	0–7	4.68	4.50	0.44	0.6680
Total bilirubin, mg/dL	0.1–0.3	0.23	0.22	0.02	0.4685
Creatine phosphokinase, U/L	26–310	125.63[b]	159.44[a]	11.64	**0.0116**
Cholesterol, mg/dL	129–297	169.03	179.31	13.69	0.7072
Triglycerides, mg/dL	32–154	43.40[b]	50.44[a]	2.52	**0.0451**
Bicarbonate, mmol/L	16–24	21.61	22.25	0.41	0.2671
Anion gap	8–25	17.47	16.88	0.40	0.3030

[1]All dogs consumed control diet at baseline and then were allotted to control or SCFP-supplemented diet after baseline measurements.
[ab]Mean values within a row with unlike superscript letters differ ($P < 0.05$). Bolded numbers are significant ($P < 0.05$).

Table 3.5. Week 10 change from baseline T cell populations of dogs consuming a SCFP-supplemented or control diet

Item	Δ Control	Δ SCFP	SEM	p-value[3]
Unstimulated				
Lymphocyte, % of PBMC[1]	8.30[a]	-3.89[b]	4.19	**0.0440**
T cell, % of lymphocyte	-8.17	-3.20	3.12	0.2721
Helper T cell, % of lymphocyte	3.93	3.17	2.02	0.7911
Cytotoxic T cell, % of lymphocyte	-15.61	-20.40	5.42	0.2858
Helper: cytotoxic T cell ratio	10.48	13.96	9.54	0.7993
IFN-γ secreting T cell, % of lymphocyte	3.75	0.88	1.50	0.3568
IFN-γ secreting helper T cell, % of lymphocyte	0.00	0.01	0.01	0.2220
IFN-γ secreting cytotoxic T cell, % of lymphocyte	12.82	14.32	4.56	0.8174
Stimulated[2]				
Lymphocyte, % of PBMC[1]	-1.75	-6.28	2.05	0.1301
T cell, % of lymphocyte	-12.29[b]	-4.01[a]	3.23	**0.0350**
Helper T cell, % of lymphocyte	4.88	0.56	2.28	0.1388
Cytotoxic T cell, % of lymphocyte	-6.32	-21.13	5.68	*0.0759*
Helper: cytotoxic T cell ratio	2.15	13.32	4.30	0.1989
IFN-γ secreting T cell, % of lymphocyte	2.18[a]	-10.94[b]	4.09	**0.0309**
IFN-γ secreting helper T cell, % of lymphocyte	-1.53	-3.76	2.27	0.4916
IFN-γ secreting cytotoxic T cell, % of lymphocyte	8.31	10.87	4.46	0.6876

[1]PBMC = peripheral blood mononuclear cells.
[2]Cells were stimulated with cell stimulation cocktail (phorbol 12-myristate 13-acetate, ionomycin, brefeldin A, and monensin) for 4 h.
[3]Bolded numbers are significant ($P < 0.05$) and italicized numbers tended to be significant ($P < 0.10$).

Table 3.6. Week 10 change from baseline natural killer cell and antigen-presenting cell populations of dogs consuming a SCFP-supplemented or control diet

Item	Δ Control	Δ SCFP	SEM	p-value
Natural killer cell, % of lymphocyte	-0.53	3.74	1.78	0.1006
Antigen Presenting Cells				
B cell, % of lymphocyte	2.10	1.57	0.92	0.6526
B cell, MHC II+, % of B cell[1]	0.06	-0.41	0.62	0.5892
Monocyte, % of white blood cell	6.90	1.60	2.56	0.1541
Monocyte, MHC II+, % of monocyte[1]	2.11	-3.43	4.94	0.4339

[1]B cells or monocytes that present major histocompatibility complex class II (MHC II).

Table 3.7. Week 10 change from baseline TNF- α concentrations (pg/mL) in cell culture supernatant of dogs consuming a SCFP-supplemented or control diet

Agonist[1]	Δ Control	Δ SCFP	SEM	p-value[2]
Control, pg/mL	-116.72	-342.11	92.02	*0.0957*
LPS (TLR4), pg/mL	525.98	276.48	167.47	0.3011
Zymosan (TLR2), pg/mL	25.20	301.50	666.41	0.7716
Poly I:C (TLR3), pg/mL	545.55	132.56	172.15	0.1007
R848 (TLR 7/8), pg/mL	2515.40	1505.74	1078.27	0.5169

[1]LPS mimics a bacterial challenge, zymosan mimics a yeast challenge, and poly I:C and resiquimod (R848) mimics a viral challenge.
[2]Italicized numbers tended to be significant ($P < 0.10$).

Table 3.8. Week 10 change from baseline oxidative stress and immune function marker concentrations of dogs consuming a SCFP-supplemented or control diet

Measure	Δ Control	Δ SCFP	SEM	p-value
Lipopolysaccharide-binding protein, μmol/L	-4.38	2.37	4.72	0.3197
Lysozyme, ng/mL	-0.24	-0.21	0.08	0.7826
Malondialdehyde, nmol/mL	-1.32	-0.64	0.8	0.5521
Superoxide dismutase, ng/mL	-1.21[b]	2.84[a]	1.39	**0.0304**
8-isoprostane, pg/mL	20.18	20.85	8.57	0.9560
α-tocopherol, μg/mL	-0.76	-2.42	0.92	0.2160
Trolox equivalent antioxidant capacity (TEAC), μM Trolox	-67.42	26.16	40.37	0.1120
CAP-e fasted[1]	712.1	329.1	415.6	0.5204
CAP-e 2 hrs. postprandial[1]	507.1	223.3	450.1	0.6602

[1]Cell-based antioxidant protection in erythrocytes assay. Results provided as mean fluorescence intensity, where low fluorescence indicates less oxidative stress to RBC, and higher fluorescence indicates a higher level of stress to RBC.

[ab]Mean values within a row with unlike superscript letters differ ($P < 0.05$). Bolded numbers are significant ($P < 0.05$) and italicized numbers tended to be significant ($P < 0.10$).

Table 3.9. Week 10 change from baseline whole blood gene expression fold change of dogs consuming a SCFP-supplemented or control diet

Measure	Δ Control	Δ SCFP	SEM	p-value[1]
Cyclooxygenase-2	0.08	-0.06	0.09	0.2039
Glutathione peroxidase	0.20	0.22	0.08	0.1995
Myeloperoxidase	0.25	0.08	0.16	0.3532
Superoxide dismutase	0.27	0.50	0.11	0.5570
Catalase	0.20[a]	0.17[b]	0.10	**0.0156**
Glutathione reductase	-0.42	-0.27	0.22	0.5444

[1]Statistics were conducted using ΔΔCt values to generate p-values; data are reported as fold change (2^-ΔΔCt) in relation to a housekeeping gene (RPS5).
[ab]Mean values within a row with unlike superscript letters differ ($P < 0.05$). Bolded numbers are significant ($P < 0.05$).

Table 3.10. Week 10 change from baseline hair and skin scores, sebum concentrations[1], hydration status[2], and transepidermal water loss (TEWL)[3] of dogs consuming a SCFP-supplemented or control diet

Measure	Δ Control	Δ SCFP	SEM	p-value
Skin score[4]	-0.73	-0.71	0.23	0.8874
Hair score[5]	-0.73	-0.71	0.23	0.9762
Sebum concentration (back)[6], arbitrary unit	3.00	1.44	0.93	0.3276
Sebum concentration (inguinal)[7], arbitrary unit	-0.04	1.48	0.61	*0.0893*
Sebum concentration (ear)[8], arbitrary unit	23.40	44.38	8.77	*0.0572*
Hydration (back)[6], arbitrary unit	0.05	-0.88	0.43	0.1468
Hydration (inguinal)[7], arbitrary unit	0.44	2.30	2.65	0.4583
Hydration (ear)[8], arbitrary unit	-1.58	-4.49	3.29	0.5076
TEWL (back)[6], $g/h/m^2$	-6.47[b]	-3.82[a]	0.99	**0.0410**
TEWL (inguinal)[7], $g/h/m^2$	1.06	0.84	1.45	0.3465
TEWL (ear)[8], $g/h/m^2$	0.85	-1.18	0.77	*0.0545*

[1]Measured using a sebumeter, $\mu g/cm^2$.
[2]Measured using a corneometer, arbitrary units.
[3]Measured using a tewameter, $g/h/m^2$.
[4]Skin scores: 1 = dry; 2 = slightly dry; 3 = normal; 4 = slightly greasy; 5 = greasy.
[5] Hair scores: 1 = dull, coarse, dry; 2 = poorly reflective, non-soft; 3 = medium reflective, medium soft; 4 = highly reflective, very soft; 5 = greasy.
[6] Back measurements were taken on the right side along the spine between the rib and hip bone.
[7] Inguinal measurements were taken on the left side.
[8] Ear measurements were taken on the left side towards the inside of the ear.
[ab]Mean values within a row with unlike superscript letters differ ($P < 0.05$). Bolded numbers are significant ($P < 0.05$) and italicized numbers tended to be significant ($P < 0.10$).

Table 3.11. Week 10 delayed-type hypersensitivity measurements[1] of dogs consuming a SCFP-supplemented or control diet

				p-value[3]		
Measure	Control	SCFP	SEM	Diet	Time	Diet*Time
Saline			0.24	0.6751	**<0.0001**	0.9452
15 min	1.92	2.38				
30 min	1.38	1.74				
45 min	0.86	0.69				
60 min	0.09	0.18				
24 hr	0.01	0.00				
48 hr	0.01	0.00				
72 hr	0.01	0.00				
PHA[2]			0.65	**<0.0001**	**<0.0001**	**0.0200**
15 min	3.40	5.94				
30 min	2.60	5.59				
45 min	1.53	4.69				
60 min	0.65[b]	3.37[a]				
24 hr	0.90	1.69				
48 hr	0.07	0.00				
72 hr	0.07	0.00				
ConA[2]			1.27	*0.0653*	**<0.0001**	0.8623
15 min	4.74	5.99				
30 min	3.17	5.09				
45 min	1.83	3.60				
60 min	1.13	1.57				
24 hr	7.32	9.55				
48 hr	5.47	7.36				
72 hr	2.31	2.47				
Vaccine[2]			0.73	0.7794	**<0.0001**	0.9395
15 min	4.26	4.95				
30 min	2.96	4.30				
45 min	1.77	3.53				
60 min	1.10	2.15				
24 hr	0.42	0.20				
48 hr	1.03	1.08				
72 hr	0.68	0.48				

[1]Average wheal diameter, mm.
[2]PHA, phytohaemagglutinin; ConA, concanavalin A; Vaccine, Duramune Max5 Dog Vaccine (Elanco Animal Health, Greenfield, IN).
[3]Bolded numbers are significant (P < 0.05) and italicized numbers tended to be significant (P < 0.10).

Table 3.12. Week 5 and 10 change from baseline complete blood count of dogs consuming a SCFP-supplemented or control diet

Measure	Control Δ Wk 5-Wk 0	Control Δ Wk 10-Wk 0	SCFP Δ Wk 5-Wk 0	SCFP Δ Wk 10-Wk 0	SEM	p-value		
						Diet	Time	Diet*Time
Red blood cells, 10^6/uL	0.26	-0.11	0.11	-0.17	0.12	0.2426	**0.0008**	0.6388
Reticulocyte count, %	-0.09	-0.37	-0.07	-0.07	0.11	0.1282	0.1874	0.1815
A reticulocyte count, /uL	-8235.73	-28112.00	-4496.26	-6458.44	8518.39	*0.0970*	0.1473	0.2337
Hemoglobin, g/dL	0.51	-0.31	0.26	-0.39	0.26	0.4096	**0.0005**	0.6801
Hematocrit, %	1.49	-1.19	0.57	-1.24	0.67	0.3733	**0.0001**	0.4193
Mean cell volume, fl	-0.34	-0.74	-0.20	-0.08	0.31	0.1284	0.5886	0.3169
Mean corpuscular hemoglobin, pg	-0.08	-0.07	0.06	0.03	0.12	0.2867	0.8855	0.8365
Mean corpuscular hemoglobin, g/dL	0.02	0.22	0.19	0.09	0.12	0.8281	0.6696	0.1677
Platelets, 10^3/uL	19.00[a]	-60.93[b]	-3.33[a]	-25.60[ab]	18.63	0.6427	**0.0007**	**0.0444**
Mean platelet volume, fl	-0.09	-0.02	0.02	-0.03	0.10	0.6121	0.9238	0.5215
White blood cell count, 10^3/uL	-0.20	0.71	0.35	0.41	0.34	0.6969	0.1311	0.1877
Lymphocytes, %	0.58	-0.55	0.41	0.73	1.29	0.6420	0.7370	0.5409
Monocytes, %	0.69	0.57	-0.84	0.72	0.64	0.2611	0.2347	0.1671
Eosinophils, %	-0.68	-1.02	-0.73	-0.61	0.54	0.7054	0.8128	0.6290
Basophils, %	0.09[a]	0.04[ab]	-0.01[b]	0.09[a]	0.04	0.4995	0.4913	**0.0286**
A Lymphocytes, 10^3/uL	0.03	0.08	0.05	0.02	0.11	0.8108	0.9332	0.6864
A Monocytes, 10^3/uL	0.03	0.08	-0.04	0.08	0.06	0.5714	0.2147	0.5631
A Eosinophils, 10^3/uL	-0.06	-0.09	-0.05	-0.05	0.05	0.6263	0.8212	0.7678
A Basophils, 10^3/uL	0.01	0.00	0.00	0.01	3E-03	0.4108	0.8404	0.1556

[ab]Mean values within a row with unlike superscript letters differ (P < 0.05). Bolded numbers are significant (P < 0.05) and italicized numbers tended to be significant (P < 0.10).

Table 3.13. Week 5 and 10 change from baseline serum chemistry of dogs consuming a SCFP-supplemented or control diet

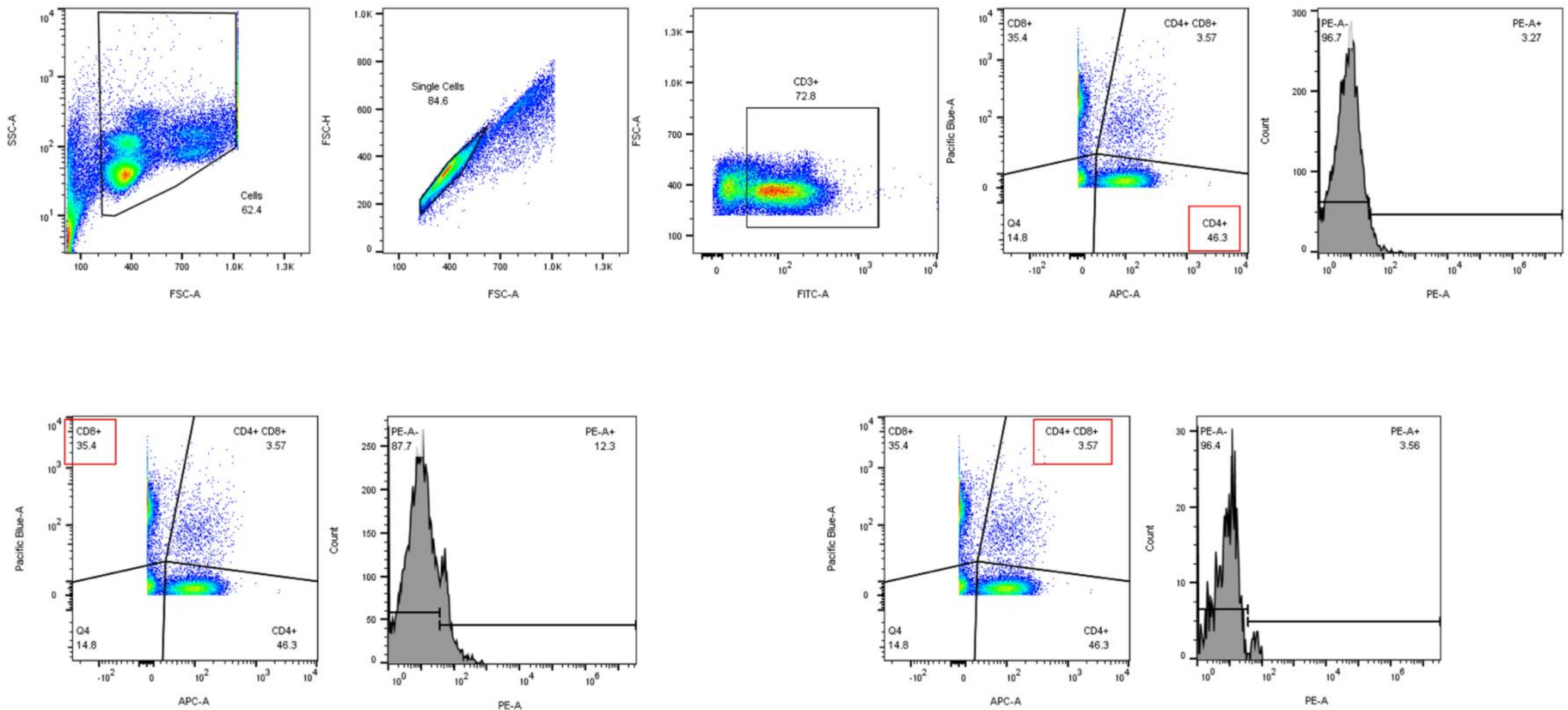
Measure	Control		SCFP		SEM	p-value[1]		
	Δ Wk 5-Wk 0	Δ Wk 10-Wk 0	Δ Wk 5-Wk 0	Δ Wk 10-Wk 0		Diet	Time	Diet*Time
Creatinine, mg/dL	0.00	0.01	-0.01	-0.03	0.02	0.4290	0.8005	0.5954
Blood urea nitrogen, mg/dL	0.86	0.99	1.19	0.31	0.53	0.7314	0.4660	0.3229
Total protein, g/dL	0.08	-0.04	-0.15	-0.09	0.06	**0.0252**	0.6660	0.1348
Albumin, g/dL	0.12	0.02	0.06	0.03	0.04	0.4812	0.1078	0.3945
Globulin, g/dL	-0.09	-0.06	-0.24	-0.11	0.05	**0.0371**	*0.0907*	0.3089
Albumin:globulin ratio	0.06	0.02	0.11	0.06	0.02	*0.0582*	**0.0293**	0.8033
Ca, mg/dL	0.04	0.09	0.02	0.07	0.07	0.7433	0.4777	0.9817
P, mg/dL	-0.01	0.11	-0.14	-0.16	0.15	0.1789	0.7191	0.6280
Na, mmol/L	-0.40	0.13	-0.81	-0.06	0.39	0.4421	0.1079	0.7838
K, mmol/L	-0.01	-0.12	-0.04	-0.05	0.06	0.6754	0.2597	0.3155
Na:K ratio	0.21	1.01	0.00	0.50	0.46	0.3734	0.1061	0.7052
Cl, mmol/L	-1.10	-0.30	-1.00	-0.81	0.37	0.5074	0.1079	0.3142
Glucose, mg/dL	-2.47	-3.13	-3.63	-1.81	2.28	0.9718	0.8030	0.5897
Alkaline phosphatase (ALP)2, U/L	-0.46	-0.13	0.63	-0.31	3.43	0.8879	0.9240	0.8410
Corticosteroid-induced ALP, U/L	0.58	0.87	-0.56	-1.69	2.06	0.3708	0.8392	0.7286
Alanine transaminase, U/L	-10.27	-11.70	2.47	1.31	10.35	0.4290	0.8001	0.5952
Gamma glutamyltransferase, U/L	0.86	1.02	0.25	0.31	0.32	**0.0385**	0.7248	0.8818
Total bilirubin, mg/dL	0.02	-0.01	0.01	0.00	0.02	0.7852	*0.0517*	0.3682
Creatine phosphokinase, U/L	6.53	21.39	-15.19	18.69	14.34	0.3706	*0.0765*	0.4828
Cholesterol, mg/dL	-3.20	4.18	2.25	0.51	5.76	0.8734	0.6116	0.4128
Triglycerides, mg/dL	0.36	-6.67	-5.20	-11.56	2.59	**0.0482**	**0.0123**	0.8988
Bicarbonate, mmol/L	1.27	1.53	0.69	0.94	0.61	0.3372	0.6721	0.9891
Anion gap	-0.73	-1.47	-0.44	-0.13	0.53	0.1264	0.6917	0.3261

[1]Bolded numbers are significant (P < 0.05) and italicized numbers tended to be significant (P < 0.10).

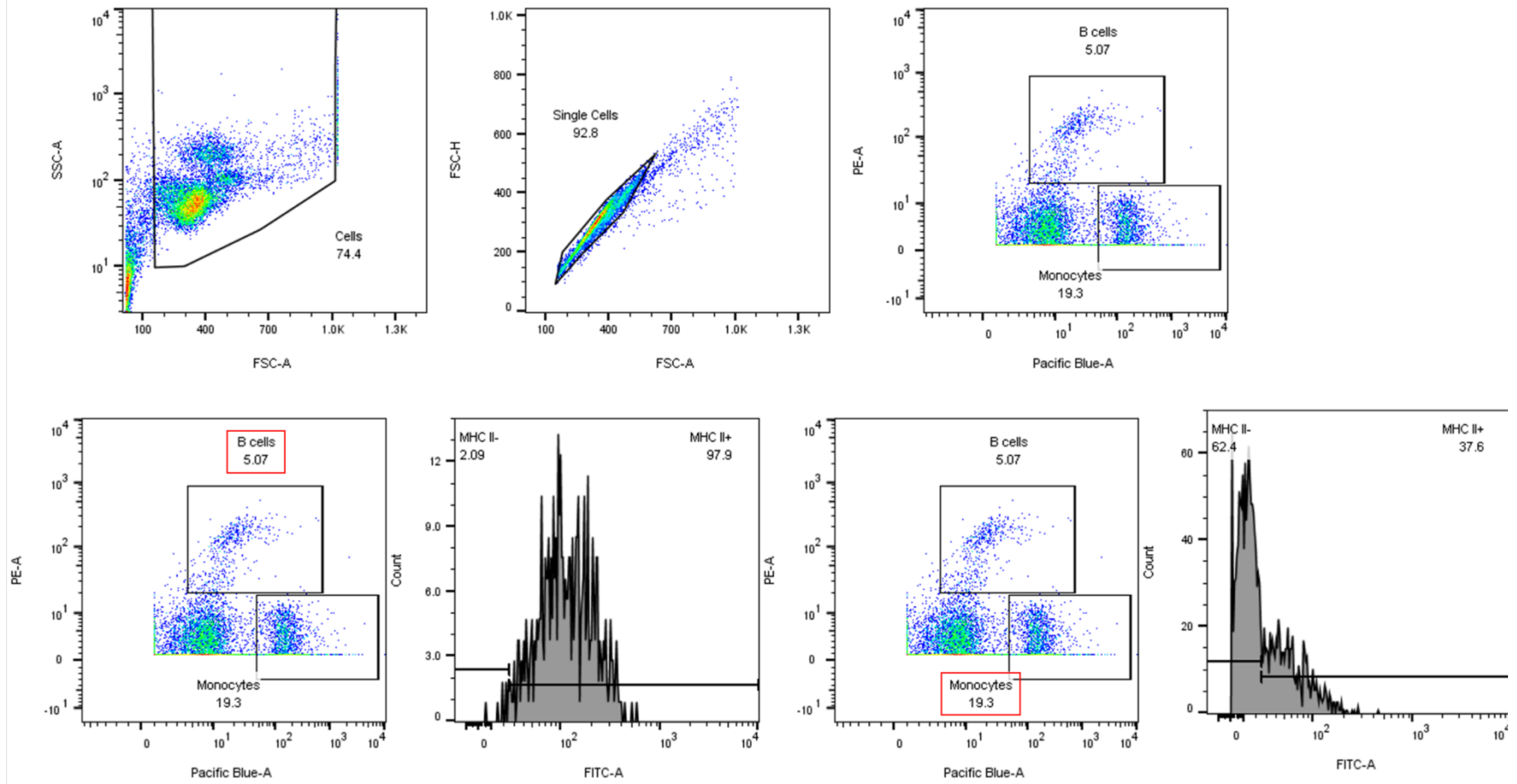
SUPPLEMENTARY FIGURES

Supplementary Figure 3.1-3.3. Gating strategy used to determine the cell populations of (1) T cells, (2) antigen-presenting cells (B cells and monocytes) and (3) natural killer cells. SSC: side scatter; FSC: forward scatter; -A: the pulse area; -H: the pulse height. Analyzed using FlowJo software (Ashland, OR).

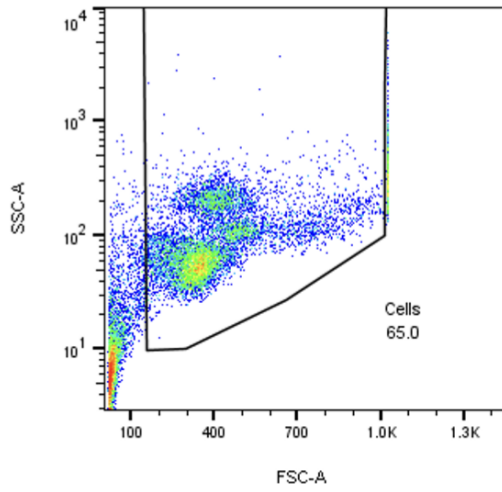
Supplementary Figure 3.1

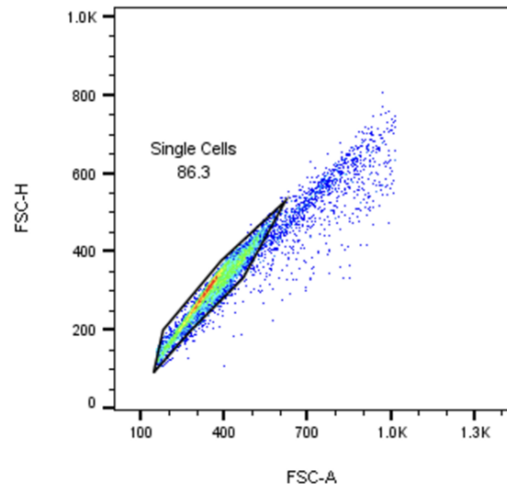


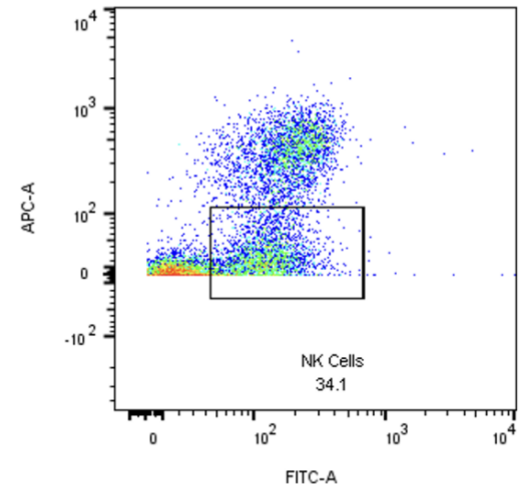
Supplementary Figure 3.2



Supplementary Figure 3.3







REFERENCES

American Association of Cereal Chemists (AACC). 1983. Approved methods. 8th ed. American Association of Cereal Chemists, St Paul, MN

American Pet Products Association (APPA). 2022. National pet owners survey. APPA, Greenwich, CT.

Association of American Feed Control Officials (AAFCO). 2022. Official publication. AAFCO, Champaign, IL.

Association of Official Analytical Chemists (AOAC). 2006. Official methods of analysis. 17th ed. Association of Official Analytical Chemists, Gaithersburg, MD.

Budde, E. F. 1952. The determination of fat in baked biscuit type dog foods. J. Assoc. Off. Agric. Chem. 35:799–805.

Chaka, W., A. F. Verheul, V. V. Vaishnav, R. Cherniak, J. Scharringa, J. Verhoef, H. Snippe, and A. I. Hoepelman. 1997. Induction of TNF-alpha in human peripheral blood mononuclear cells by the mannoprotein of *Cryptococcus neoformans* involves human mannose binding protein. J. Immunol. 159:2979–2985.

Chen, J., Y. Zhang, J. You, H. Song, Y. Zhang, Y. Lv, H. Qiao, M. Tian, F. Chen, S. Zhang, and W. Guan. 2020. The effects of dietary supplementation of *Saccharomyces cerevisiae* fermentation product during late pregnancy and lactation on sow productivity, colostrum and milk composition, and antioxidant status of sows in a subtropical climate. Front. Vet. Sci. 7:71. doi:10.3389/fvets.2020.00071.

Dunstan, R. W., T. H. Herdt, L. Mei, K. M. Credille, R. A. Kennis, R. L. Maier, J. Dziezyc, S. Castle, G. A. Reinhart, and G. M. Davenport. 2000. The role of nutrition on canine sebum secretion: a preliminary report. Vet. Dermatol. 11(Suppl. 1):2–3. (Abstr.).

Eaton, S. 2006. The biochemical basis of antioxidant therapy in critical illness. Proc. Nutr. Soc. 65:242–249. doi:10.1079/pns2006501.

Fluhr, J. W., K. R. Feingold, and P. M. Elias. 2006. Transepidermal water loss reflects permeability barrier status: validation in human and rodent *in vivo* and *ex vivo* models. Exp. Dermatol. 15:483–492. doi:10.1111/j.1600-0625.2006.00437.x.

Hristov, A. N., G. Varga, T. Cassidy, M. Long, K. Heyler, S. K. R. Karnati, B. Corl, C. J. Hovde, and I. Yoon. 2010. Effect of *Saccharomyces cerevisiae* fermentation product on ruminal fermentation and nutrient utilization in dairy cows. J. Dairy Sci. 93:682-692. doi:10.3168/jds.2009-2379.

Ito, F., Y. Sono, and T. Ito. 2019. Measurement and clinical significance of lipid peroxidation as a biomarker of oxidative stress: oxidative stress in diabetes, atherosclerosis, and chronic inflammation. Antioxidants 8:72. doi:10.3390/antiox8030072.

Jensen, G. S., X. Wu, K. M. Patterson, J. Barnes, S. G. Carter, L. Scherwitz, R. Beaman, J. R. Endres, and A. G. Schauss. 2008. In vitro and in vivo antioxidant and anti-inflammatory capacities of an antioxidant-rich fruit and berry juice blend. Results of a pilot and randomized, double-blinded, placebo-controlled, crossover study. J. Agric. Food Chem. 56:8326–8333. doi:10.1021/jf8016157.

Kidd, M. T., L. Araujo, C. Araujo, C. D. McDaniel, and D. McIntyre. 2013. A study assessing hen and progeny performance through dam diet fortification with a *Saccharomyces cerevisiae* fermentation product. J. Appl. Poul. Res. 22:872-877. doi:10.3382/japr.2013-00774.

Kim, H. W., B. P. Chew, T. S. Wong, J. S. Park, B. B. Weng, K. M. Byrne, M. G. Hayek, and G. A. Reinhart. 2000. Dietary lutein stimulates immune response in the canine. Vet. Immunol. Immunopathol. 74:315–327. doi:10.1016/s0165-2427(00)00180-x.

Krizková, L., Z. Duracková, J. Sandula, V. Sasinková, and J. Krajcovic. 2001. Antioxidative and antimutagenic activity of yeast cell wall mannans in vitro. Mutat. Res. 497:213–222. doi:10.1016/s1383-5718(01)00257-1.

Laflamme, D. P. 1997. Development and validation of a body condition score system for dogs: a clinical tool. Canine Pract. 25:10–15.

Lau-Gillard, P. J., P. B. Hill, C. J. Chesney, C. Budleigh, and A. Immonen. 2010. Evaluation of a hand-held evaporimeter (VapoMeter) for the measurement of transepidermal water loss in healthy dogs. Vet. Dermatol. 21:136–145. doi:10.1111/j.1365-3164.2009.00738.x.

Lin, C. Y., C. Alexander, A. J. Steelman, and C. M. Warzecha, M. R. C. de Godoy, and K. S. Swanson. 2019. Effects of a *Saccharomyces cerevisiae* fermentation product on fecal characteristics, nutrient digestibility, fecal fermentative end-products, fecal microbial populations, immune function, and diet palatability in adult dogs J. Anim. Sci. 97: 1586–1599. doi:10.1093/jas/skz064.

Liu, X. L., N. Lin, D. Zan, J. J. Yuan, and D. L. Cai. 2011. Effect of zymosan on antioxidant and immune function of S180 tumor-bearing mice. Cell. Biochem. Biophys. 60:225–229. doi:10.1007/s12013-010-9143-7.

Luo, J., K. Mills, S. le Cessie, R. Noordam, and D. van Heemst. 2020. Ageing, age-related diseases and oxidative stress: What to do next? Ageing Res. Rev. 57:100982. doi:10.1016/j.arr.2019.100982.

Momota, Y., K. Shimada, A. Takami, H. Akaogi, M. Takasaki, K. Mimura, D. Azakami, K. Ishioka, Y. Nakamura, and T. Sako. 2013. Transepidermal water loss in cats: comparison of three differently clipped sites to assess the influence of hair coat on transepidermal water loss values. Vet. Dermatol. 24:450–452. doi:10.1111/vde.12050.

Monnier, L., E. Mas, C. Ginet, F. Michel, L. Villon, J. P. Cristol, and C. Colett. 2006. Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes. JAMA. 295:1681–1687. doi:10.1001/jama.295.14.1681.

Montero, A., K. A. Munger, R. Z. Khan, J. M. Valdivielso, J. D. Morrow, A. Guasch, F. N. Ziyadeh, and K. F. Badr. 2000. F(2)-isoprostanes mediate high glucose-induced TGF-beta synthesis and glomerular proteinuria in experimental type I diabetes. Kidney Int. 58:1963–1972. doi:10.1111/j.1523-1755.2000.00368.x

Moyad, M. A., L. E. Robinson, J. M. Kittelsrud, S. G. Reeves, S. E. Weaver, A. I. Guzman, and M. E. Bubak. 2009. Immunogenic yeast-based fermentation product reduces allergic rhinitis-induced nasal congestion: a randomized, double-blind, placebo-controlled trial. Adv. Ther. 26:795–804. doi:10.1007/s12325-009-0057-y.

Pawar, M. M., A. K. Pattanaik, D. K. Sinha, T. K. Goswami, and K. Sharma. 2017. Effect of dietary mannanoligosaccharide supplementation on nutrient digestibility, hindgut fermentation, immune response and antioxidant indices in dogs. J. Anim. Sci. Technol. 59:11. doi:10.1186/s40781-017-0136-6.

Phillips, F. C., G. S. Jensen, L. Showman, R. Tonda, G. Horst, and R. Levine. 2019. Particulate and solubilized β-glucan and non-β-glucan fractions of *Euglena gracilis* induce pro-and anti-inflammatory innate immune cell responses and exhibit antioxidant properties. J. Inflamm. Res. 12:49–64. doi:10.2147/JIR.S191824.

Pietrella, D., R. Cherniak, C. Strappini, S. Perito, P. Mosci, F. Bistoni, and A. Vecchiarelli. 2001. Role of mannoprotein in induction and regulation of immunity to *Cryptococcus neoformans*. Infect. Immun. 69:2808–2814. doi:10.1128/IAI.69.5.2808-2814.2001.

Pinnagoda, J., R. A. Tupker, T. Agner, and J. Serup. 1990. Guidelines for transepidermal water loss (TEWL) measurement. Contact Derm. 22:164–178. doi:10.1111/j.1600-0536.1990.tb01553.x.

Prosky, L., N. G. Asp, T. F. Schweizer, J. W. DeVries, and I. Furda. 1988. Determination of insoluble, soluble, and total dietary fiber in foods and food products: interlaboratory study. J. Assoc. Off. Anal. Chem. 71:1017–1023.

Rees, C. A., J. E. Bauer, W. J. Burkholder, R. A. Kennis, B. L. Dunbar, and K. E. Bigley. 2001. Effects of dietary flax seed and sunflower seed supplementation on normal canine serum polyunsaturated fatty acids and skin and hair coat condition scores. Vet. Dermatol. 12:111–117. doi:10.1046/j.1365-3164.2001.00234.x.

Shen, Y. B., J. A. Carroll, I. Yoon, R. D. Mateo, and S. W. Kim. 2011. Effects of supplementing *Saccharomyces cerevisiae* fermentation product in sow diets on performance of sows and nursing piglets. J. Anim. Sci. 89:2462–2471. doi:10.2527/jas.2010-3642.

Swanson K. S., L. B. Schook, and G. C. Fahey. 2003. Nutritional genomics: implications for companion animals. J. Nutr. 133:3033-3040. doi:10.1093/jn/133.10.3033.

Varney, J. L., C. N. Coon, and S. A. Norton. 2021. PSV-B-20 Effects of *Saccharomyces cerevisiae* fermentation product (SCFP) postbiotic in Labrador retrievers during exercise and transport stress. J. Anim. Sci. 99(Suppl.3):332–333. (Abstr.) doi:10.1093/jas/skab235.611.

Watson, A., T. Fray, S. Clarke, D. Yates, and P. Markwell. 2002. Reliable use of the ServoMed Evaporimeter EP-2 to assess transepidermal water loss in the canine. J. Nutr. 132(6 Suppl. 2):1661S–1664S. doi:10.1093/jn/132.6.1661S.

Zaworski, E. M., C. M. Shriver-Munsch, N. A. Fadden, W. K. Sanchez, I. Yoon, and G. Bobe. 2014. Effects of feeding various dosages of *Saccharomyces cerevisiae* fermentation product in transition dairy cows. J. Dairy Sci. 97:3081–3098. doi:10.3168/jds.2013-7692.