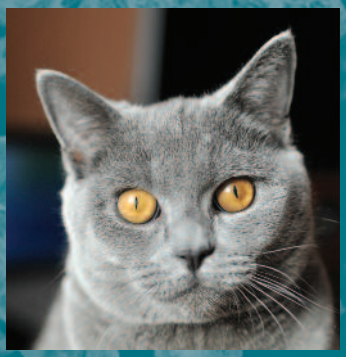
Nestlé PURINA

Companion Animal Nutrition Summit

The Gastrointestinal Tract in Health and Disease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We would like to thank Dr. Kenny Simpson of Cornell University
for providing the image of gastrointestinal tissue
that appears in the background of the cover.

Preface

The Nestlé Purina Companion Animal Nutrition (CAN) Summit is a scientific meeting where experts gather from around the world to explore an important topic in veterinary medicine. This year, the focus of the CAN Summit is the gastrointestinal (GI) tract in health and disease. The GI tract serves a critical role in the health of the body. It provides a physical barrier against the outside world on the inside of the body. Compromises in this barrier can be caused by, and can cause, disease.

Disturbances in the intestinal barrier function ("leaky gut") can lead to increased uptake of foreign proteins, contributing to immune and autoimmune diseases and alterations in body function. For example, in genetically predisposed people and rats, a leaking gut predisposes individuals to Type 1 diabetes mellitus. This is an autoimmune disorder common in humans and dogs, and dogs may share some common risk factors.

The GI tract is the largest immune organ in the body. Immune cells in the gut actively protect the body against invading organisms, such as bacteria and viruses, while also tolerating normal proteins, such as dietary proteins, and beneficial bacteria. The GI tract is home to millions of microorganisms, collectively called the microbiome. It has long been recognized that these organisms perform a number of functions that are beneficial to the host animal. For example, the microbiome is critical for normal development of a healthy immune system. However, in recent years, knowledge regarding the extent of the effects of the microbiome has been expanding. Recent findings have identified a link between microfloral patterns and psychological disorders, such as anxiety and depression, via a gut-brain axis. Studies in animals have confirmed that changes in the intestinal microflora, especially increases in certain *Lactobacillus* spp, result in behavioral changes associated with reduced anxiety and greater activity.

New research is exploring the fascinating extent of the effects the microbiome can have on its host. The microbiome tends to be somewhat unique for each individual, but there also are patterns influenced by the typical diet consumed. For example, a diet high in animal proteins will result in a different profile compared to a diet high in simple carbohydrates. Changes in the diet can result in changes to these patterns, but the individual differences in resident microflora help to explain why different patients respond differently to an antibiotic treatment or dietary change.

Gut inflammation, especially in inflammatory bowel disease, is associated with disturbances in the gut microbiome. Dietary changes may induce positive changes in the microflora and/or otherwise help reduce the clinical signs, such as diarrhea and weight loss.

We hope you enjoy this collection of papers from experts from around the world, providing current, practical information as well as emerging research findings.

D.P. Laflamme, DVM, PhD, DACVIM
Chair, Nestlé Purina Companion Animal Nutrition Summit

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"All Disease Begins in the Gut": Elucidating Disease Mechanism Related to Intestinal Barrier Dysfunction

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Abstract

Tight junctions between intestinal epithelial cells form a selective barrier, which regulate paracellular traffic of luminal substances into the lamina propria. As the gut is the primary site of exposure to antigens, this barrier function plays an important role in systemic immune function. Accumulating evidence suggests that the disturbance in intestinal barrier function has a causative role in the pathogenesis of several systemic diseases, including diabetes mellitus.

Glossary of Abbreviations
DH: Dermatitis Herpetiformis
GI: Gastrointestinal
IBD: Inflammatory Bowel Disease
IL-4: Interleukin-4
IFN-γ: Interferon-γ
IP: Intestinal Permeability
NF-κB: Nuclear Factor-κB
NSAIDs: Nonsteroidal Anti-Inflammatory Drugs
TNF-α: Tumor Necrosis Factor-α

Intestinal Barrier Function and the Role of Tight Junctions

Along the gastrointestinal (GI) tract, an adjacent layer of cells separates the internal body systems from the external environment. This separation ensures protection from a wide range of environmental pathogens entering the lumen, thereby preventing infection, inflammation and alteration of normal body functions. Besides the tight lining of epithelial cells, other products, such as mucus, immunoglobulins and other antimicrobial agents, are important in maintaining a proper barrier function. The absorptive functions of the small intestine are regulated through two mechanisms. The first is transcellular transportation across the enterocyte brush border, usually facilitated by transport carriers or by means of passive diffusion. The second path is movement through paracellular spaces, not mediated by carriers and thus based solely on passive diffusion of molecules.

Several recent reports have reviewed the structure and function of tight junctions, which appear to have a principal role in regulating paracellular transport across the intestinal epithelium.[1,2] In brief, the junctions between adjacent epithelial cells consist of the more luminally situated tight junctions. Tight junctions are composed of transmembrane proteins (occludins, claudins) and plaque proteins (ZO protein family, among others) and are associated with the intracellular actin-myosin cytoskeleton. Components of the diet, such as glucose and amino acids, are able to induce openings of the tight junctions and increase paracellular permeability. These openings are regulated through a series of signal transductory pathways, all resulting in the increased activity of myosin light chain kinase, which phosphorylates myosin and causes a contraction of cytoskeletal components and conformational changes in structures associated with it, such as the tight junctions. Hence, this dynamic process of the opening and closing of the tight junction complex regulates the paracellular transport of luminal substances into the lamina propria.

Measuring Intestinal Permeability

When evaluating intestinal permeability (IP), researchers are particularly interested in the regulatory mechanisms and properties concerning the intrinsic permeability of the gut barrier. To measure the barrier function, different sets of probes have been used, such as monosaccharides (mannitol, L-rhamnose), disaccharides (lactulose, sucralose), polyethylene glycol, and nondegraded radiolabeled chelates (^{51}Cr-EDTA). The probes share specific characteristics: They are small-sized, water-soluble, not degraded or metabolized in the gut lumen, nontoxic, totally excreted by the kidney, and therefore can easily be detected in urine samples. Measurements using a single molecule (such as ^{51}Cr-EDTA) may be influenced by inter-individual differences not related to permeability, such as intestinal transit or urinary excretion. Thus far, human intestinal permeability has been measured by urinary excretion of two probes of different sizes but similar transit and uptake processes, calculating the excretion ratio of a monosaccharide and a disaccharide, such as mannitol and lactulose, respectively.[3] These probes differ in manner of transport, i.e., paracellular or transcellular. In this way two routes of uptake are compared. The most widely accepted method of measuring IP in the small intestine in humans is the lactulose/mannitol or lactulose/rhamnose urine excretion test. In the healthy small bowel, the permeability for larger sugars, such as lactulose, is much lower than for smaller sugars, such as mannitol or rhamnose. Lactulose and other larger

molecules pass through the intercellular spaces, which are regulated by intercellular tight junctions. Under pathological conditions, such as mucosal inflammation, the permeability of the larger sugars increases, whereas the permeability of the smaller sugars remains stable or decreases. This results in an increased urinary excretion ratio of large to small sugars.[4]

The Role of Intestinal Barrier Function in Systemic Disease

An increased intestinal permeability, often referred to as a "leaky gut," has been proposed to be associated with several gastrointestinal disorders, including intestinal and liver diseases, such as inflammatory bowel disease (IBD)[5] and nonalcoholic steatohepatitis,[6] but also diseases that are not primarily related to GI malfunction, such as type 1 and type 2 diabetes.[7]

Although an altered intestinal barrier function can be a consequence of disease exacerbation, clinical evidence suggests that it may be a primary causative factor predisposing to disease development.[1] For example, healthy, first-degree relatives of patients with IBD and celiac disease have increased intestinal permeability.[8-10] Although the diseases associated with increased permeability differ in terms of pathogenesis and clinical presentation, there seems to be a common denominator: An altered barrier function is believed to facilitate increased exposure to antigens that can trigger immune reaction and autoimmune destruction and alter normal body function. Within this model, the specificity for disease location (target tissue) is provided by both the antigen and the genetic abnormality of the immune system. For instance, the target may be the beta cells of the pancreatic islets (diabetes), the epithelial cells of the gut (celiac disease), or the myelin sheaths surrounding nerves (multiple sclerosis).[11]

This model also does not place any requirements on how the increase in permeability arises. This increase can occur during an infectious process by activation of endogenous humoral pathways or by microbial manipulation of the host's epithelial cell pathways. It may also be a transient event, which may explain the lack of detectable permeability abnormalities in some patients.

Perhaps the most convincing evidence for such a disease model exists for type 1 diabetes mellitus. Moordian et al. were the first to demonstrate increased permeability in diabetic patients by measuring urinary secretion of lactulose and rhamnose.[12] Later, a significantly increased lactulose/mannitol ratio was observed in diabetic patients in comparison to controls, but no significant correlation was found with duration of disease or mean HbA1c values. These findings have been confirmed in other studies.[13,14] Prediabetic subjects had the greatest increase, suggesting that increased IP precedes the onset of clinical diabetes. Accordingly, Bosi et al.[15] observed no differences in enteropathy, measured by the lactulose/mannitol test, between preclinical and long-standing diabetes, suggesting that the duration of diabetes does not further influence IP and that an increased IP precedes, rather than is caused by, type 1 diabetes mellitus. Furthermore, studies in biobreed rats have indicated that the increased permeability detected in prediabetic rats is related to decreased expression of claudin-1 and occludin,[16,17] suggesting a role for tight junctions in altered barrier function in diabetes.

These findings demonstrated that increased IP is observed not only in patients who have developed type 1 diabetes but also in those who are already in preclinical condition. Subclinical inflammation, found in young diabetic patients and characterized by increased interleukin-4 (IL-4), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), is possibly involved in compromising the integrity of epithelial barrier leading to increased IP of the gut.[18-20] Whether subclinical inflammation precedes or is caused by increased IP requires further investigation. Nevertheless, increased IP makes the host more susceptible and prone to immune reactions against antigens from dietary (cow milk substances like bovine insulin[21] or wheat gliadins), viral or bacterial origin. These agents can activate humoral responses and provided there is genetic susceptibility may trigger autoimmune reactions against insulin-producing beta cells. According to this proposed disease model, expression of diabetes requires genetic predisposition, a dietary provocative agent and abnormal permeability. Removal of either the luminal antigen or the permeability defect prevents disease despite retaining the genetic predisposition. This offers an unprecedented opportunity to prevent disease by counteracting dysbalances in intestinal barrier function.

In case of celiac disease patients, for instance, removal of the antigen (gluten) prompts complete remission of all attributes of the disease, including a return of abnormal intestinal permeability to almost the normal range in the majority of subjects.[22] Furthermore, an inbred Irish Setter line was shown to develop a gluten-sensitive enteropathy that mimics human celiac disease. In these animals, the disease can be completely prevented by weaning the animal onto a gluten-free diet. However, subsequent exposure to the antigen immediately prompts development of the disease. Importantly, animals that have never been exposed to dietary gluten have increased small intestinal permeability.[23] This strongly suggests that in this animal model, abnormal permeability precedes disease. Patients with dermatitis herpetiformis (DH) provide an interesting perspective in this regard. Subjects with this condition exhibit an enormous range of associated bowel pathology from frank celiac disease to a completely normal intestinal biopsy and no evidence of bowel disease. DH patients exhibit increased intestinal permeability, including those patients without evidence of intestinal disease.[24] As some patients may go on to develop celiac disease, it would appear that, in these cases, increased permeability precedes development of disease.

Rheumatological conditions have long been associated with abnormalities of intestinal function, and the concept of abnormal reactivity to a luminal antigen in these conditions is prevalent. Perhaps the best evidence for this comes from the literature on ankylosing spondylitis. Increased gastrointestinal permeability had

been recognized in these patients for decades, but it was unclear whether this was due to the disease or treatment with nonsteroidal anti-inflammatory drugs (NSAIDs),[25] a drug group known to influence intestinal permeability. With more recent work, the effect of NSAIDs has been isolated, and it is apparent that these patients appear to have a primary defect in intestinal permeability that is shared by a subgroup of relatives.[26] Also, increased gut permeability was observed in patients with juvenile chronic arthritides[27] irrespective whether they were taking NSAIDs, indicating that the disrupted permeability is disease-related.

Accumulating evidence therefore suggests the involvement of barrier function in the pathogenesis of a wide variety of diseases. Another mechanism related to intestinal barrier dysfunction is bacterial translocation. An increase in intestinal barrier permeability can facilitate translocation of luminal bacteria. This can lead to macrophage activation and an increased systemic production of pro-inflammatory cytokines (interleukins, TNF-α) and C-reactive protein, resulting in a systemic inflammatory reaction. These cytokines can thereafter induce systemic changes, such as induction of peripheral insulin resistance by activating nuclear factor-κB (NF-κB), which results in serine phosphorylation of insulin receptor substrate-1 and insulin resistance.[28] Similarly, bacterial translocation has been implicated to play a role in other systemic diseases, as higher levels of antibodies to *Klebsiella pneumoniae* have been found in the serum of patients with ankylosing spondylitis, rheumatoid arthritis and IBD.[29] More recently, it has been proposed that translocation of endotoxin, a constituent of the wall of gram negative bacteria, through a "leaky gut" can exert cardiotoxic effects and contribute to the development of chronic heart failure.[30]

Novel Therapeutic Target: Reinforcement of the Intestinal Barrier Function

Although the diseases listed above clearly differ with respect to pathophysiological mechanisms and clinical presentation, they possibly share an important initiating organ in common: the gut. Reinforcement of the intestinal barrier may therefore become a major goal. There are several routes through which intervention on gut barrier can be established: (1) by altering exposure to nutrients (antigens, especially at young age); (2) by alterations in microbiota composition (pre-, pro- and antibiotics); (3) by modification of gut-barrier proteins and other regulatory proteins; and (4) by restraining the inflammation responsible for the autoimmune reaction. It has become apparent that when the finely tuned trafficking of macromolecules through the intestinal barrier is dysregulated, both intestinal and extraintestinal disorders can occur, particularly in genetically susceptible individuals. This new paradigm subverts traditional theories underlying the development of certain diseases, suggesting that the unfavorable immune activation can be counteracted if the interplay between genes and environmental triggers is prevented by re-establishing intestinal barrier function. Acknowledging the role of the intestinal barrier in the pathophysiology of systemic diseases, a limited number of studies, albeit with varying success, have attempted to reinforce the barrier function using nutritional interventions.[7] Further studies will be needed to verify the true therapeutic potential of enhancing intestinal barrier function.

Conclusion

The intestinal epithelial cells form a selective barrier and ensure the regulation of the trafficking of macromolecules between the environment and the host. Alteration in this barrier function can have profound effects on the interactions between the mucosal immune system and luminal contents, including dietary antigens and microbial products. Increased permeability can therefore contribute to systemic malfunctioning and disease development. Clinical and experimental evidence supports that diseases such as diabetes, celiac disease, IBD and rheumatoid disorders, among others, are associated with an increased intestinal permeability. Whether intestinal epithelial barrier function is a primary causative factor in the predisposition to disease development needs further elucidation. However, recent studies have identified a number of plausible mechanisms that could account for an increased exposure of luminal contents to immunoreactive host cells contributing to altered immune reactions. This increased exposure to luminal antigens can result in an autoimmune destruction of certain target cells leading to disease manifestation or can contribute to augmentation of a systemic immune reaction. Therefore, reinforcing intestinal barrier function may become an important objective to help prevent or counteract pathophysiological mechanisms. A more complete understanding of the molecular pathways involved in the regulation of intestinal barrier function will have important clinical implications by opening new horizons in the treatment and prevention of several systemic diseases, including diabetes mellitus.

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Amino Acids for Optimal Intestinal Mucin Synthesis and Gut Protection in Health and Disease

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Abstract

Amino acid requirements are defined in healthy conditions. In pathological situations, including intestinal inflammation, the body defense is associated with anabolic reactions involving the splanchnic area and especially the gut. Intestinal defense and repair processes dramatically increase the synthesis rate of proteins implicated in the gut barrier function, such as mucins. It augments the host's need of specific amino acids, particularly those enriched in mucins. A "healthy" diet is therefore not adapted. Increasing the dietary supply of threonine, serine, proline and cysteine is required to promote mucin synthesis and strengthen the non-immune intestinal barrier function.

Glossary of Abbreviations
ASR: Absolute Synthesis Rate
IBD: Inflammatory Bowel Disease
FSR: Fractional Synthesis Rate
MUC2: Mucin 2 Gene
Muc2: Mucin 2 Protein

Introduction

The gastrointestinal tract is one of the most metabolically active organs of the body, which reflects its important and numerous biological functions. Whereas the gastrointestinal tract contributes 3% to 6% of the mammalian body weight, it accounts for more than 20% of the whole-body protein turnover.[1] This is mainly due to a high protein synthesis rate and to a continuous and significant secretory activity. This translates into a high demand in certain amino acids required for the protein synthesis process. Such a high requirement has been ascribed to support the non-immune gut barrier, in particular the synthesis of intestinal mucins. Inflammatory situations further increase the intestinal protein synthesis and consequently the utilization of certain amino acids by the intestine. In this context, adequate nutritional management is required to maintain or repair the intestinal barrier integrity and function.

The Non-Immune Intestinal Barrier

The intestinal protection of the host is ensured by both the intestinal immune system and a physical, non-immune intestinal barrier. The intestinal barrier ensures protection of the host from the external environment (luminal pathogens, noxious agents, etc.) while allowing absorption of nutrients for adequate supply of the whole body. Its optimal function relies on the close interplay of several intestinal compartments. The major key players are: the commensal intestinal microbiota presence and equilibrium, which antagonizes the adhesion of potentially pathogenic bacteria[2]; the intestinal mucus layer, which covers and protects the delicate epithelial cells[3]; the intestinal epithelium itself, ensuring the separation between the luminal contents and the underlying tissue compartments;[4] the Paneth cells, producing antimicrobial peptides;[5] the tight junctions between epithelial cells, contributing to the modulation of paracellular pathways[6]; and the enteric nervous system, recently recognized as a key regulator of the epithelial barrier integrity.[7]

Complex regulatory mechanisms are taking place to ensure the subtle equilibrium among these different components of the non-immune intestinal barrier. Optimal nutritional support is crucial to maintain this intestinal homeostasis, favoring a global healthy status of the body and preventing gut-related diseases.

Composition and Role of the Intestinal Mucus Layer

The gastrointestinal epithelium is covered by a viscoelastic mucus gel layer composed of: a complex mixture of glycoproteins named mucins; peptides, including trefoil peptides and antimicrobial peptides; water; macromolecules, such as secretory immunoglobulin A; electrolytes; microorganisms; and sloughed cells.[3,8] The mucus gel constitutes the front line of innate host defense; one of its main documented functions is to protect delicate epithelial surfaces against mechanical stresses and constant attacks from digestive fluids, microorganisms and toxins.[3,9] Its protective effect is directly related to its thickness and composition. The unique protection capacity of the mucus gel is conferred, in part, by its high content in mucin glycoproteins, which are continuously synthesized and secreted by intestinal goblet cells and mucosal epithelial cells throughout the entire gastrointestinal tract.[3]

The mucus thickness, composition and protective effect vary along the gastrointestinal tract[10] as a result of the differential expression of various distinct mucins and the dynamic balance between opposing anabolic (expression, synthesis and secretion from goblet cells) and catabolic (physical and proteolytic degradation) processes. The mucus layer is thickest in the stomach and

large intestine in order to provide strong protection from acidic conditions (stomach) and microbiota (colon). It is thinnest in the small intestine likely to avoid interference with the absorption of nutrients.[10] An inner, firmly adherent mucus layer consisting of membrane-bound mucins adheres to the apical side of epithelial cells and contributes to the formation of glycocalyx, a polysaccharide matrix coating the surface of intestinal epithelial cells. A soluble, loosely adherent mucus outer layer, consisting of secreted gel-forming mucins, covers the inner mucus layer. This soluble layer favors the establishment and maintenance of a balanced commensal microbiota that antagonizes potentially pathogenic bacteria.[11,12]

Characteristics of Intestinal Mucins

To date, 21 mucin genes have been identified, of which 15 have been shown to be expressed in the human gastrointestinal tract.[13] Intestinal mucins share particular compositional features. They are usually large polypeptides (10%-20% of the mucin mass) that are heavily glycosylated (up to 80%-90% of the mucin mass). The oligosaccharide side chains are mainly composed of N-acetylgalactosamine, N-acetylglucosamine, galactose and fucose primarily linked to serine and threonine residues of the mucin polypeptide core via O-glycosidic bonds. Post-translational modifications, including sialylations and sulfations, complete the macromolecule.[3]

The mucin polypeptide size usually ranges from 200 kDa up to 900 kDa, with the exception of the salivary form MUC7 (39 kDa).[14] As compared to other mammalian proteins, mucins are particularly enriched in the amino acids threonine, serine and proline, which account for up to 28%, 14% and 13%, respectively, of the total amino acid composition of mucins.[3] For comparison, the average threonine content of body proteins ranges from 3% to 7% of total amino acids. The threonine, serine and proline residues are concentrated into central tandem repeat PTS (proline, threonine, serine) regions made of conserved sequences repeated about 100-fold. Cysteine-rich domains also are present on the mucin polypeptides.[14] They allow mucins to assemble into homo-oligomers via intermolecular disulphide bonds formed between the cysteine-rich domains, which confer the viscoelastic and protective property of the mucus gel.[13]

Among the 15 mucins expressed in the human gastrointestinal tract, MUC2, MUC5AC, MUC5B, MUC6, MUC7, and MUC19 are secreted mainly by specialized goblet cells.[14] In the small and large intestines, MUC2 is the predominant gel-forming mucin. Its critical role to protect the colonic epithelium from colitis has been clearly demonstrated in a Muc2-deficient mice model.[15] MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, and MUC17 are membrane-associated mucins expressed by mucosal epithelial cells of the human gastrointestinal tract.[13] In the small and large intestines, MUC3, MUC4, MUC13, and MUC17 are the predominant membrane-associated forms that have been identified.[13] They extend above the cell surface and form the glycocalyx. Specific roles in anti-adhesive and signaling mechanisms,[16] intestinal cell restitution[17] and protection of intestinal epithelial cells from infection[18] have been proposed for membrane-associated mucins.

Complex regulatory mechanisms are taking place to ensure adequate mucin expression and secretion for optimal intestinal protection. These mechanisms have been shown to involve neuronal, hormonal and paracrine pathways.[19-21] The nutritional status that allows the supply of adequate amounts of amino acids required for mucin synthesis[22-26] and the microbiota[11,27] also are key regulators of intestinal protection.

Metabolic Disorders in Intestinal Diseases Impair Mucin Production and Gut Protection

Many intestinal diseases involving chronic inflammation, such as inflammatory bowel disease (IBD), are associated with intestinal barrier dysfunctions. The two major types of IBD, ulcerative colitis and Crohn's disease, are accompanied by an increase in small and large intestinal permeability.[28,29] Among modifications observed at the gut barrier level, an altered gut microbiota composition[30,31] and qualitative and quantitative impairment of the mucus layer and mucin production have been reported.[13,32] In particular, the synthesis of a mature, glycosylated form of Muc2, the primary mucin secreted in the colon, is decreased in ulcerative colitis patients, which reduces the mucus barrier.

Abnormal expression of gastric-secreted mucins in ileum and colon also has been reported, which may reflect an adaptive response to strengthen the defense reaction.[13] The expression of membrane-bound mucins MUC3, MUC4 and MUC17 has been observed to be decreased, further corroborating the reduction of epithelial protection. However, and interestingly, the expression of MUC13, recently documented to inhibit toxin-induced apoptosis of the colonic epithelium,[33] has been shown to be increased in inflamed colonic mucosa biopsies, reflecting a defensive mechanism that remains nevertheless insufficient to maintain or restore the intestinal barrier function.

Metabolic disorders associated with acute systemic inflammatory reactions, as observed in sepsis, for instance, also impact the intestinal barrier function. Acute inflammation stimulates the synthesis of acute-phase proteins in the liver[34] and mucosal proteins and mucins in the intestines.[33] These anabolic reactions are important adaptations aiming at ensuring the body's defense against primary and secondary aggressions. A key factor in the initiation and maintenance of such body defenses is therefore the ability of the host to sustain such stimulation of protein synthesis. In this context, there is a strong increase in amino acid requirements,[36] especially in those present at high levels in mucins. In a disease state, food intake is often decreased, and the dietary amino acid supply is too low to meet the metabolic demand. Amino acids are thus obtained through increased muscle catabolism.[37]

Amino Acid Requirements for Optimal Mucin Synthesis and Gut Protection

The gastrointestinal tract contributes only 3% to 6% of the mammalian body weight, whereas it accounts for more than 20% of the whole-body protein turnover.[1] This is, in part, due to its high proliferative and secretory activities that support the non-immune gut barrier function, particularly the rapid renewal of intestinal epithelial cells and the continuous synthesis of intestinal mucins. The amino acid composition of synthesized and secreted proteins largely affects the amino acid requirements of the gut, which has to be met by dietary nutrition and endogenous synthesis (for nonessential amino acids).

Under Healthy Conditions

Threonine is an essential amino acid, which means it cannot be synthesized by the organism and must therefore be supplied in the diet. Under healthy conditions, threonine is key for the maintenance of the gut. Indeed, compared with other essential amino acids, a large proportion of dietary threonine (up to 60%) is retained by the healthy pig[38] or human[39] intestine. Since the core protein of intestinal mucins contains high amounts of threonine (up to 30% of their amino acid composition[3]), their continuous synthesis explains the high rate of threonine utilization by the gastrointestinal tract. Along this line, a lack of Muc2 in knock-out mice indeed inducies the metabolic oxidation of unused threonine,[40] which reflects an excessive supply of threonine occurring in the absence of Muc2 synthesis.

In contrast, when dietary threonine supply is below the requirements, threonine can become a limiting amino acid for the synthesis of intestinal mucins, as shown in rats[23] and in pigs and piglets.[24-26] Indeed, the mucin fractional synthesis rate, defined as the percentage of mucins synthesized per day, has been shown to decrease by half in the upper small intestine of rats fed a diet covering 30% of their threonine requirements for growth (Figure 1). Nevertheless, it has no major limiting effect on total mucosal protein synthesis[23] (Figure 1), with these proteins containing about seven times less threonine than Muc2.

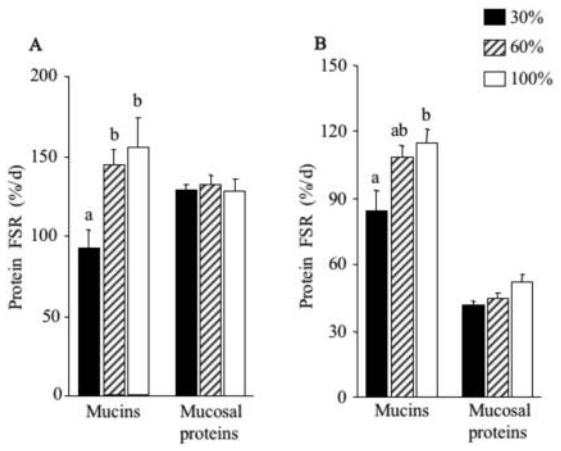

Figure 1. Fractional synthesis rate (FSR), expressed in %/day, of mucins and total mucosal proteins in the upper small intestine (A) and colon (B) of rats fed semisynthetic diets meeting 30%, 60% or 100% of their threonine requirements for growth. Diets were isonitrogenous (adjusted with alanine) and administered to the rats for 14 days. All groups of rats were pair-fed to the mean intake of rats from the group 30%. The *in vivo* protein synthesis was measured using the flooding dose method following injection of L-(1-13C)-valine. Values are means ± SEM, n=8. For each intestinal compartment (mucins or mucosal proteins), means without a common letter differ, $p<0.05$.

Because mucins are particularly resistant to digestive enzyme activities, the threonine recycling from mucins secreted in the upper gastrointestinal tract is very low[41] and the threonine loss is very high in respect to the whole body threonine requirement.[42] In summary, under healthy conditions, it is crucial that the dietary threonine supply accurately meets the body's threonine requirement in order to maintain optimal mucin synthesis and intestinal protection, to favor a global healthy status of the body, and to prevent gut-related diseases.

In Inflammatory Diseases

As shown in animal models and humans, inflammatory situations, such as those observed in IBD (chronic inflammation) and sepsis (acute inflammation), are associated with an overall increased anabolic reaction occurring mainly in the intestines and the liver, respectively.[43-46] This anabolic response increases the utilization of amino acids and, in particular, those present at high levels in intestinal and hepatic proteins. Therefore, the requirements for threonine and for other amino acids, such as serine and cysteine, is strongly increased.[47]

The availability of those amino acids for the synthesis of intestinal mucins for which they are primary (threonine) or likely secondarily (serine, cysteine) limiting[23-26,47] is probably too low because of a limited nutritional quality (insufficient levels of these amino acids) and quantity (poor appetite) of the dietary intake. As an example, two days after infection, the utilization of threonine for the synthesis of rat intestinal mucins has been shown to be 70% greater than in pair-fed rats.[35] Overall, the daily absolute threonine utilization for the synthesis of intestinal proteins (gut wall) plus the plasma proteins (minus albumin) increased by 23%, which represented 2.6 times the dietary intake of rats.[35] Similarly, proline, which is highly represented in the composition of intestinal mucins (13%[3,48] as compared to 4%–7% in body proteins, except collagen), also may be a secondary limiting amino acid for mucin synthesis.

In inflammatory situations, adequate and well-balanced nutritional support is therefore required to promote the defensive response, the repairing mechanisms and consequently the maintenance or restoration of an effective intestinal barrier function. The definition of "adequate and well-balanced nutritional support" will depend on the metabolic condition associated with the disease and therefore can't refer to that defined for the healthy condition.

As previously observed in IBD animal models, the intestinal

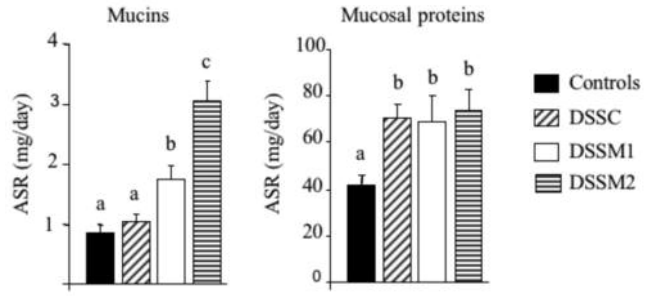

Figure 2. Absolute synthesis rates (ASR), expressed in mg/day, of mucins and mucosal proteins in the colons of dextran sodium sulfate (DSS) treated rats. The rats were fed for 28 days with isonitrogenous (adjusted with alanine) semisynthetic powder diets providing the following supplementation levels as compared to rat's requirements: DSSM1; twofold increases in threonine, proline, serine and cysteine; DSSM2; fourfold increases in threonine and proline; and threefold increases in serine and cysteine. Values are means ± SEM (n=8). For each intestinal compartment (mucins or mucosal proteins), means without a common letter differ, $p<0.05$.

mucin production is not stimulated with a healthy, balanced diet.[45,46,49-52] However, increasing the threonine, serine, proline and cysteine dietary supply in a rat model for colitis has been shown effective in promoting the colonic mucin synthesis in a dose-dependent manner, while having no effect on total mucosal proteins[52] (Figure 2). The higher dose of amino acids increased the presence of Muc2-containing goblet cells in the surface epithelium of the ulcerated area.[52] It also promoted the growth of all commensal bacterial populations tested, including *Lactobacillus*.[52]

Conclusion

The amino acids threonine, serine, proline and cysteine are relatively high in the composition of intestinal mucins, which explains, in part, their high utilization by the gut. Adapted nutritional support, in particular with accurate levels of these four amino acids, is therefore crucial to maintain an effective intestinal barrier function. Pathological situations, including intestinal inflammation, intestinal defense and tissue repair processes, further increase the host's need of such amino acids. In such situations, an increased dietary supply of threonine, serine, proline and cysteine is advised to promote the mucin synthesis and the growth and equilibrium of the commensal microbiota and consequently to strengthen the non-immune intestinal barrier function.

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What Is the Role of Diet in Canine Inflammatory Bowel Disease?

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Abstract

Inflammatory bowel disease (IBD) is the collective term applied to a group of chronic enteropathies characterized by persistent or recurrent gastrointestinal (GI) signs and inflammation of the GI tract. It is widely accepted that IBD involves a complex interplay among host genetics, the intestinal microenvironment (principally bacteria and dietary constituents), the immune system, and environmental "triggers" of intestinal inflammation.[1] However, the specific steps that lead to IBD and the basis for phenotypic variation and unpredictable responses to treatment are not known. This article will examine the role of diet in the etiopathogenesis and treatment of IBD in dogs.

Glossary of Abbreviations
GI: Gastrointestinal
IBD: Inflammatory Bowel Disease
pANCA: Perinuclear Antineutrophil Cytoplasmic Antibodies
PLE: Protein-Losing Enteropathy
PLN: Protein-Losing Nephropathy
SCWT: Soft Coated Wheaten Terrier

Evidence to Support the Role of Diet in the Etiopathogenesis of IBD

I. Clinical Responses in Breed-Specific Enteropathies

Irish Setters, as a breed, are predisposed to developing an enteropathy related to ingestion of gluten.[2] An interaction of genetics and diet in dogs is supported by the finding that gluten-sensitive enteropathy in Irish Setters is an autosomal recessive trait, but the casual mutation has not been identifed.[2]

Adverse reactions to corn, tofu, cottage cheese, milk, farina cream of wheat, and lamb have been described in Soft Coated Wheaton Terriers (SCWT) affected with protein-losing enteropathy (PLE) and protein-losing nephropathy (PLN).[3] In these dogs, serum albumin concentrations decreased and fecal alpha1-protease inhibitor concentration increased four days after the provocative trial when compared with baseline values. Antigen-specific fecal IgE varied throughout the provocative trial, with peak levels following ingestion of test meals. Pedigree analysis of 188 SCWT demonstrated a common male ancestor, although the mode of inheritance is unknown.[4]

Polymorphisms in nephrin and filtrin have recently been asociated with PLN in SCWT but do not segregate with PLE (Paula Henthorn, University of Pennsylvania, personal communication). Autoantibodies to perinuclear antineutrophil cytoplasmic antibodies (pANCA), associated with ulcerative colitis in people,[5] have been demonstrated in 20/21 SCWT and preceeded hypoalbuminemia by an average of 2.4 years.[6] Elevated pANCA was also described in 61% of 90 dogs of various breeds with food-responsive enteropathy versus 31% to 34% dogs with non-food responsive IBD.[7,8] These findings suggest that immune dysregulation as evidenced by autoantibody formation is a relatively common and early feature of food-responsive enteropathies in dogs.

II. Clinical Responses to Commercial Antigen-Restricted Diets

In controlled studies of 65 dogs with IBD and diarrhea of at least six weeks' duration, 39 dogs responded to an antigen-restricted diet of salmon and rice (10 days fed Purina Veterinary Diets® LA Limited Antigen® Canine Formula, now called Purina Veterinary Diets® DRM Dermatological Management® Canine Formula).[7] Only eight dogs relapsed when challenged with their original food, and none was sensitive to testing with beef, lamb, chicken or milk. The CIBDAI and histopathologic scores were similar (>70% moderate to severe in each group) in dogs that did and did not respond to diet. Dogs that responded to diet tended to be younger and have higher serum albumin than dogs that did not respond to diet. Dogs that did not respond to diet were treated with steroids. Interestingly, intestinal histopathology did not differ in either diet-responsive or steroid-responsive dogs before and after treatment. Ten of the 21 diet-unresponsive dogs responded to prednisolone with no relapse after taper for up to three years. Of the 11 diet and steroid unresponsive dogs, nine were euthanized after steroids, with only two of eight steroid refractory dogs responding to cyclosporine (5mg/kg PO q 24 hrs 10 wks).

In a study of 13 dogs with lymphocytic plasmacytic colitis, clinical signs resolved in all 13 dogs (2-28 month follow-up) after they were fed a low-residue, easily assimilated, relatively hypoallergenic diet.[9] In 11 dogs, two commercial diets not previously fed to these dogs were successfully substituted for the initial test diet, without causing recurrence of signs. Only two of these 11 dogs subsequently tolerated a switch to diets that had been fed at the onset of signs of colitis.

From a comparative standppoint, it is intersting to note that of 55 cats with chronic GI disease, 49% responded to dietary modification with limited antigen diets: Signs recurred in 16 of

26 cats challenged with the original food. The dominant groups of antigens eliciting a response in these cats were: cereals (wheat=corn gluten>barley) and meat proteins (beef>chicken=lamb), and 50% of cats were multiply allergic.[10]

III. Clinical Responses to Commercial Hydrolyzed Protein Diets

Six dogs with IBD received a commercially available hypoallergenic diet containing an enzymatically hydrolyzed defatted soy globulin as the only protein source. (Purina Veterinary Diets® HA Hypoallergic® Canine Formula)[12] Five of the six dogs had been refractory to a variety of control diets, and four dogs had failed to respond to previous medical therapy. Dietary therapy alone provided adequate clinical improvement in four dogs, and concurrent medical therapy was required in two dogs, one of which had exocrine pancreatic insufficiency. Mean fecal scores improved after therapy. Five dogs showed mild to moderate histologic improvement in duodenal biopsies after therapy.

In a recent study, 26 dogs with signs of chronic gastrointestinal disease (six had normal GI pathology) were fed either a soy and chicken hydrolysate (n=18, Royal Canin Hypoallergenic diet) or an intestinal diet (n=8, Royal Canin Intestinal diet).[13] The initial response to diet was 88% in both groups, and approximately 66% of the dogs in either group relapsed in response to the original diet. However, over a three-year period, only one of six dogs on the intestinal diet was maintained in remission versus 13 of 14 dogs on the hydrolysate diet.

In a prospective trial, we have observed positive responses to a hydrolyzed soy diet (Purina Veterinary Diets® HA Hypoallergenic® Canine Formula) in 18 of 25 dogs with IBD and normal serum albumin. All dogs responded within two weeks, with mean follow-up of 20 months. Those dogs not responding to food alone responded to food+antibiotics (n=2) or immunosuppression (n=5). It is noteworthy that marked perturbation of the duodenal microbiome "dysbiosis" were detected in a majority of dogs with IBD, including those with a response to diet.[14]

Taken as a whole, these studies reveal responses to antigen-restricted or hydrolyzed diets in 60% to 88% of dogs with lymphocytic plasmacytic IBD.

What Is the Basis of Clinical Responses to Dietary Intervention in IBD?

It has been promulgated for many years that dietary intervention for canine IBD is based on a careful dietary history, with an emphasis on determining exposure to proteins, particularly those of animal origin, e.g., beef, chicken, etc. Dietary intervention was then directed at feeding a diet containing proteins that had not been fed previously, i.e., an antigen-restricted diet. The more recent approach has been to hydrolyze proteins to a molecular weight that does not cross-link IgE on mast cells, which is reported to range from approximately 4.5-10 kDa,[11] i.e., a hypoallergenic diet. For soy, the smallest known allergens are 20 kDa and greater, so anything less is hypoallergenic.[15,16] Both of these approaches are based on the hypothesis that intestinal inflammation is driven by hypersensitivity or allergy to a dietary protein, frequently assumed to be animal in origin.[17]

However, the observation that many dogs do not relapse when rechallenged with their original diet or when fed proteins that are assumed from their diet history are likely to be allergens, e.g., "only 8/39 diet responsive dogs relapsed when challenged with their original food and none was sensitive to beef, lamb, chicken or milk,"[7] questions the role of "allergy" or "hypersensitivity" in canine IBD.

Until the relevant pathomechanisms have been elucidated, the diagnostic terms "food responsive" or "dietary intolerant" seem more appropriate than "food allergy," where an immunological basis for disease has not been identified.

Studies in Irish Setters suggest that cereal-based proteins, such as gluten, and toxic and nonhypersensitivity-based immunological mechanisms should be considered in the genesis of intestinal inflammation in dogs and cats with IBD. It is notable that cereal-based ingredients were just as likely as animal proteins to be responsible for food sensitivity in cats with gastrointestinal problems.[10]

The high response rates to diets that differ markedly in their composition (e.g., hydrolyzed soy versus salmon) but are formulated from relatively few ingredients raise the possibility that it is perhaps the absence of certain ingredients, rather than the modification

Table 1. Complete and balanced hydrolyzed protein diets available for dogs[11]

Diet[a]	Protein Source	Carbohydrate Source	Lipid Source
Hill's z/d Ultra Allergen Free	Chicken	Corn Starch, Cellulose	Soybean Oil
Hill's z/d Low Allergen	Chicken	Potato, Potato Starch, Cellulose	Soybean Oil
Nestlé Purina HA	Soy	Corn Starch, Cellulose, Vegetable Gums (Gum Arabic and Guar Gum)	Coconut Oil, Canola Oil, Corn Oil
Royal Canin Hypoallergenic	Soy, Poultry Liver	Rice, Beet Pulp, Fructo-Oligosaccharides	Poultry Fat, Soybean Oil, Borage Oil, Fish Oil

Ingredients listed from manufacturers' product guides (January 2006).

[a] Hill's Pet Nutrition Inc. Topeka, KS, USA; Nestlé Purina PetCare Co., St. Louis, MO, USA; Royal Canin, Aimargues, France.

or substitution of dietary protein, that has a beneficial effect. For instance, undegraded carrageena, a jelling agent used in the food industry, including pet foods, has been shown to induce GI inflammation and promote oncogenesis in animal models.[17-19] However it remains to be determined whether the carrageena is able to induce intestinal inflammation in dogs or cats.

Conclusion

Clinical response rates of 60% to 88% in dogs with lymphocytic plasmacytic IBD fed a restricted-antigen or hydrolyzed diet indicate that dietary modifcation is an important therapeutic tool in the management of canine IBD. An unexpected positive finding of recent studies is that few dogs require continuous treatment with corticosteroids or other imunosuppressive agents. The pathomechanisms underlying the positive responses to dietary manipulation in canine IBD remain to be elucidated, and it is important to consider possibilities other than IgE-mediated hypersensitivity to animal proteins.

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Assessment of Intestinal Permeability in Dogs

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Abstract

Intestinal permeability (IP) is part of the mucosal barrier function allowing small molecules to pass through the tight junctions between epithelial cells. In a healthy state, low IP contributes to a homeostatic immune response. In a diseased state, increased IP can lead to the permeation of luminal antigens that exacerbate intestinal immune responses. IP is tested by administrating inert IP markers orally and quantifying their percentage recoveries in urine or blood. Despite proving abnormal IP in a variety of canine intestinal disorders, IP tests have not found widespread clinical use. Currently, iohexol is regarded as a highly promising marker, as it avoids problems associated with radioactive or sugar IP markers.

Glossary of Abbreviations
^{51}Cr-EDTA: ^{51}Chromium-Labeled Ethylenediamine Tetra-Acetic Acid
IBD: Inflammatory Bowel Disease
IP: Intestinal Permeability
M: Microfold
TJs: Tight Junctions

Intestinal Permeability in Health

The gastrointestinal tract is the largest mucosal surface of the body. The epithelial monolayer covering the intestinal mucosa is the central mediator of the interaction between luminal environment and mucosa associated lymphoid tissue. It forms a leaky barrier allowing the flux of essential nutrients, ions and water but limiting the host's contact with potentially harmful intestinal contents, such as dietary allergens or microbes.[1,2] Intestinal permeability is part of this mucosal barrier function and refers to the passage of solutes mainly by paracellular diffusion through the tight junctions (TJs), adherens junction and desmosomes between adjacent epithelial cells.

TJs consist of structural and regulatory molecules, such as occludins, claudins and junctional adhesion molecule A connecting to the actomyosin ring through zonula occludens proteins. TJs form pores, which, in humans, have a size of 50 to 60 Å (5-6 nm) in the intestinal crypts and 4 to 9 Å (0.4-0.9 nm) in the villi.[1,2] Results of paracellular pathway studies suggest that dogs possess larger TJ pores than rats or humans.[3] This mucosal permeability is molecular-size selective with decreasing passage of larger solutes from the crypts to the villi. Due to the dimensions of the paracellular space, it has been suggested that under physiological circumstances solutes with a molecular radius exceeding 15 Å (~3.5 kDa) will be excluded from this uptake route.[4] However, paracellular IP also can adapt in response to extracellular stimuli, such as nutrients, cytokines and bacteria, leading to changes in the structure of the TJs.[1,2]

Permeability of macromolecules, such as food antigens and microbes, occurs to a limited extent transcellularly by endo- and exocytosis, mediated or not by membrane receptors. Cells capable of transcellular IP are microfold (M) cells, dentritic cells and columnar enterocytes. This physiologic process is important for the induction of a homeostatic immune response by the host, which includes induction of immune tolerance to dietary antigens and local production of secretory immunoglobulin A that prevents pathogenic and commensal microbiota from entering the host's body.[2]

Intestinal Permeability in Chronic Intestinal Disorders

Intestinal disorders can be a consequence or cause of abnormal IP. Chronic intestinal inflammation results in IP changes that are induced by the release of pro-inflammatory cytokines, such as interferon γ, tumor necrosis factor and interleukin 13, as shown in human inflammatory bowel disease (IBD). Induced structural and functional changes in the TJs increase paracellular IP, which often is associated with an increased transcellular permeation of macromolecules. In the final stage of inflammation, apoptosis and ulceration lead to nonspecific leakage. In digestive tract diseases, luminal antigens or microbes, therefore, can more easily access the subepithelial immune system, initiating pathological processes. Dietary antigens are associated with food allergy and celiac disease, while bacterial antigens are linked to IBDs.[2] Abnormal IP is considered a cause of disease in humans with Crohn's disease since it occurs in healthy relatives of affected people and before the onset of clinical signs.[5]

Also, in Irish Setters with gluten-sensitive enteropathy, abnormal IP preceded the development of clinical signs.[6] In dogs, abnormal IP was shown not only to be associated with gluten-sensitive enteropathy but also with a variety of disorders, including diet-responsive intestinal disease, proximal small intestinal bacterial overgrowth, IBD, sustained strenuous exercise, meloxicam treatment, and severe parvovirus infection.[7-15]

Assessment of Intestinal Permeability in Veterinary Clinical Research

Severity assessment of intestinal mucosal damage has proved valuable in dogs for clinical and research purposes. Clinical scoring

systems and laboratory test results (feces, blood, urine, absorption and permeability tests) have been used to investigate chronic enteropathies and the influence of extraintestinal disorders on the gut. Direct evaluation of intestinal damage is typically performed by collection and histologic interpretation of intestinal tissue biopsies. Histology of the intestine is of major importance for a qualitative and semiquantitative assessment of morphologic changes. However, its use for follow-up examinations is limited due to the need for invasive sampling methods requiring anesthesia (endoscopy, laparotomy).

In addition, clinical studies have repeatedly revealed a lack of improvement in histologic severity grades despite improvement of clinical and endoscopic scores.[16,17] Testing IP provides a noninvasive method to assess repeatedly the severity of intestinal mucosal barrier dysfunction associated with intestinal or extraintestinal diseases. It offers the advantages of being objective (provides numerical data) and of having no major welfare concerns due to its minimal invasiveness.

In dogs, intestinal permeability tests have been performed by administering one or two specific markers orally and quantifying their subsequent concentrations or percentage recoveries in urine or blood. When the intestinal mucosa is damaged, there is a greater translocation of orally administered probes from the intestinal lumen into the bloodstream and urine. This results in an increased recovery of IP markers.[6-17] Extraintestinal influences, such as renal function disorders, need to be considered when assessing the test results, as these can influence the rates of recovery of the markers. Prior IP tests applied in dogs have used small, inert radioactive or nonradioactive molecules as summarized in Table 1. They, therefore, reflect paracellular, rather than transcellular, transport.

^{51}Chromium-labeled ethylenediamine tetra-acetic acid (^{51}Cr-EDTA) was the first molecule used to assess paracellular IP in dogs.[6,7,20] It was initially introduced as a urinary excretion text. Recent studies also validated the ^{51}Cr-EDTA-IP test for canine serum or plasma testing.[21-23] The test is considered the gold standard method, but the use of radioactivity has severely restricted its widespread use.

Sugar probes for IP assessment by urinary excretion after gavage have used a combination of various saccharides.[24-28] The lactulose/rhamnose test has become the most commonly used sugar assay for assessing IP in dogs, but its use has been associated with inconsistent test results.[16] The method currently is considered insensitive and nonspecific, probably due to intestinal and bacterial degradation of the so-called "inert" sugars.[29] Sugar IP tests have not found broad use in veterinary clinical research and practice due to difficulties in performing the test, limited access to sample analysis and conflicting study results.

Iohexol is an iodine-contrast medium shown to have promise as an IP marker for humans, laboratory rats and dogs.[30-34] For dogs, it was shown that ^{51}Cr-EDTA and iohexol, despite having different molecular sizes and weights, share the same paracellular pathway.[34] Several studies have proved reliable determination of iohexol in canine serum by high-performance liquid chromatography.[36, 37] Experimental studies in healthy dogs suggest an optimal dose of iohexol (omnipaque 350®) at 2 ml/kg body weight.[35] Results of experimental studies in rats with induced colitis or small intestinal bacterial overgrowth, as well as clinical studies in humans with IBD, support the hypothesis that iohexol can become a valuable IP marker.[30-33] Clinical studies in canine patients with chronic enteropathies still are needed to show whether iohexol also will prove a simple and reliable alternative to radioactive or sugar-based IP tests in this species.

Table 1. Markers of intestinal permeability used in dogs, including molecular size and mass[2,18,19]

Marker	Molecular Size Å	Molecular Mass Da (g/mol)
Iohexol	12	821
^{51}Cr-EDTA	10.5	358
Cellobiose	10.5	342
Lactulose	9.5	342
Rhamnose	8.3	164
Mannitol	6.7	182

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The Gastrointestinal Tract: A Complex Immunological Organ?

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Abstract

Associated with the intestine is a well-developed local immune system that is dependent upon the establishment of a gut microbial flora for its development. The gut mucosal immune system is required to recognize and respond "appropriately" to different groups of antigens via a vigorous response to potential pathogens and by not overreacting to otherwise harmless dietary antigens. The high incidence of food allergic reactions and paucity of mucosal vaccines highlight the difficulties associated with controlling and targeting these responses.

Glossary of Abbreviations
GALT: Gut-Associated Lymphoid Tissue
IELs: Intraepithelial Lymphocytes
pIgR: Polymeric Immunogloblin Receptor
TLRs: Toll-Like Receptors

Introduction

The gastrointestinal tract is a major interface between a host and its environment. While the epithelial layers of other interfaces, such as the skin, are well-suited to preventing the absorption of harmful antigens, the gut is highly specialized for digestion and the absorption of nutrients. It has been calculated that in man the villi and micro-villi of the intestine provide a combined surface area greater than 400 m^2, ideal for a nutritional role but not for preventing the entry of potential pathogens or their products.[1] Associated with the gastrointestinal tract is a well-developed local immune system. The gut mucosal environment is complicated by the magnitude of challenge and the complex array of antigens that are presented, and the immune system that is associated with the gastrointestinal tract is required to recognize these different groups of antigens and respond "appropriately." It must, thus, be able to respond actively to potential pathogens while simultaneously not "overreacting" to harmless components of the diet.

In order to control such an extensive and diverse challenge, a complex battery of responses can be invoked. These include innate and acquired mechanisms, but it can be reasonably argued that the principal strategy adopted by both is a response directed toward preventing pathogens from interacting with epithelial cells and thereby closing a "potential gateway" into the body. The gut epithelial cells and their associated mucus layer, along with peristalsis and the low stomach pH, all contribute toward the barrier against the entry of harmful antigens.

Gut Immunological Architecture

The gastrointestinal tract is an extremely complex organ having multiple functions directed toward the digestion and absorption of nutrients and the control of potentially harmful pathogens and commensal microflora. It is not surprising, therefore, that a well-developed mucosal immune system has evolved to protect it. The mucosal immune system can be divided into two major compartments: the organized lymphoid structures (Peyer's patches, mesenteric lymph nodes, etc.) and tissues specialized for other functions (the intestinal lamina propria). In the conventional model, the organized tissues are "inductive" sites, populated by naive cells: Following priming, the cells migrate via the mesenteric lymph node before homing to the diffuse "effector" sites, such as the intestinal lamina propria. Lymphoid aggregates are found throughout the intestine, and it has been suggested that the numbers may reflect the bacterial load encountered in different areas of the large intestine.[2]

The Epithelial Barrier in Mucosal Immunity

The innate immune defense system is particularly important at host barriers, such as the gut mucosal surface, and gut epithelial cells play a major role. Mucosal "barrier function" is central to mucosal defense and is made up of a number of elements. Small intestinal epithelial cells arise from progenitor stem cells located in the crypts. As they migrate up the crypt and then the villus, these cells mature and differentiate, changing from immature secretory cells to mature absorptive cells. Cells reaching the tips of the villus are then shed into the gut lumen. Importantly, this occurs before the epithelial cells become effete and thus avoid any compromise to barrier function. Continuity of the barrier between adjacent epithelial cells is maintained through a series of specialized interactions made up of "tight junctions," adherens junctions and desmasomes (Figure 1).

Interposed between the epithelial cells are mucus-secreting cells that provide a "mucus protective blanket" over the epithelial surface (Figure 2). Besides forming a highly specialized physical and functional barrier to dietary and microbial antigens, epithelial cells recognize colonizing microorganisms through expression of diverse receptor systems. These include glycan receptors that recognize fimbrial lectins found on many pathogenic and commensal strains of bacteria and viruses and Toll-like receptors that recognize microbial molecular patterns and MHC class II molecules. There are significant differences between species in the expression of MHC class II molecules on gut epithelial cells. In the cat, there is no

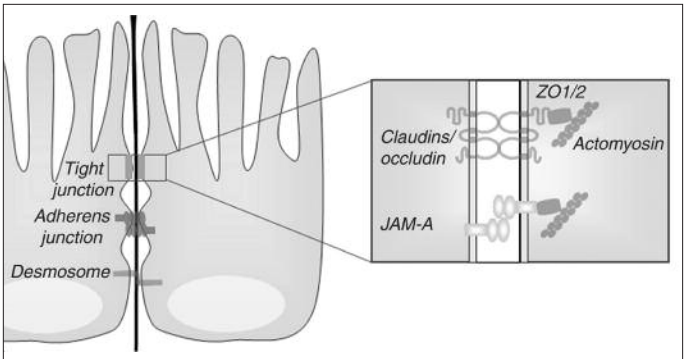

Figure 1: Tight junction proteins on epithelial cells play a pivotal role in the maintenance of "barrier function" in the intestinal wall.

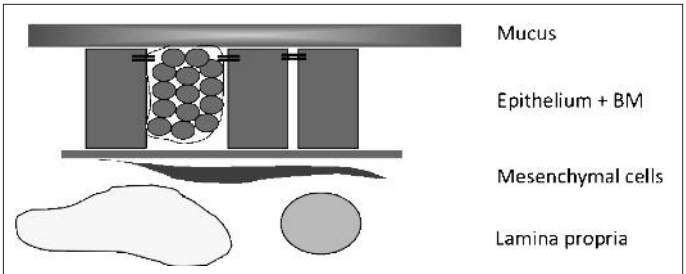

Figure 2: Gut-barrier function is an essential requirement for gut health. Many processes contribute to "the barrier," including tight junctions between epithelial cells and a layer of mucous secreted by goblet cells that are located between epithelial cells. Locally synthesized and secreted IgA may also become closely associated with the mucous layer.

expression by villous or crypt enterocytes, but granular cytoplasmic staining of epithelial cells adjacent to Peyer's patches has occasionally been observed. In contrast, in the dog, epithelial cell expression of MHC class II molecules has been shown taking the form of granular and epithelial staining.

The unique location of gut enterocytes at the interface between host and gut environment highlights their pivotal role in gut defense. It is then not surprising there is a growing body of literature on their expression of various "accessory molecules" that may help facilitate this role. Chemokine receptor mRNAs have been detected in the feline large intestine, expressed by epithelial cells (and some lamina propria cells) of the colon and rectum. Toll-like receptors (TLRs) are an evolutionary-conserved family of cell surface and cytosolic receptors that have an important role in microbial recognition. Recent studies have highlighted their importance in innate immunity against pathogens and in immune homeostasis.[3,4] TLR4 is a receptor for bacterial endotoxin (LPS), and TLR4 mRNA has been shown to be expressed in the canine stomach and small intestine and in the feline lung and small and large intestines. Immunohistochemical studies have also shown TLR4 in the canine lung and small intestinal macrophages.

Inductive & Effector Sites

A large body of evidence shows that while Peyer's patches (Figure 3) are the major site of induction of mucosal responses, the lamina propria and epithelial compartments are essentially involved in surveillance and assisting during the rapid responses to antigens that had been previously met (recall responses).

These effector responses include active protective responses against potential pathogens and the prevention of damaging allergic responses to dietary and environmental antigens. Studies on the distribution of immune-cell populations of cats and dogs primarily have focused on the lamina propria and epithelial compartments with relatively few studies of Peyer's patches. The distribution of cells of cat Peyer's patches are reported to be similar to other species, with the greatest number of B cells, then T cells, present.

Intraepithelial lymphocytes (IELs) are located in the epithelial compartment and are generally observed in close proximity to the basement membrane. They are an extremely complex population of cells that are capable of killing virus-infected epithelial cells. There are considerable differences between species in the numbers of small intestinal IELs that have been reported, ranging from 12 to 20 per 100 epithelial cells in dogs to 51 per 100 epithelial cells in pigs.[5] Cat IELs are more frequent in villus than crypt epithelium (<5 per 100 epithelial cells), and within the villus the number of IELs increases from duodenum (~50 per 100 epithelial cells) to ileum (~80 per 100 epithelial cells).[6] Studies in the dog also have shown a greater number of IELs in villus than crypt epithelium, but the numbers were similar in the duodenum and ileum. The phenotype of IELs has been investigated in cats and dogs. In both species, $CD8^+$ IELs greatly outnumbered $CD4^+$

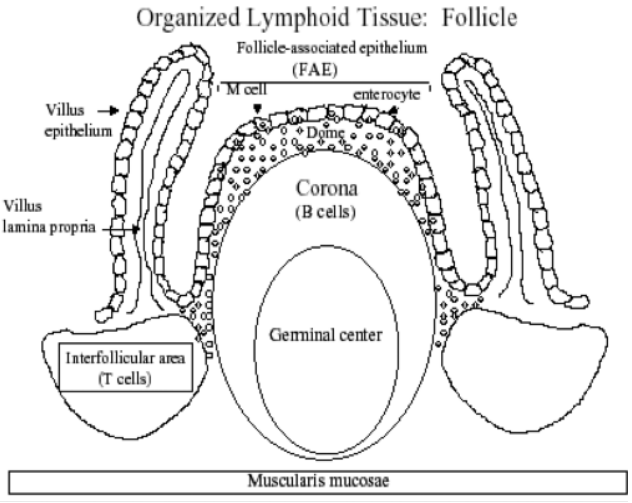

Figure 3: The Peyer's patches are the major site of induction for mucosal responses. The follicle-associated epithelium includes M-cells (microfold cells), which sample antigens in the intestinal lumen and transport them to dendritic cells in the dome region.

cells, supporting further that their primary role is likely to involve killing cells infected with virus.

The intestinal lamina propria is populated by a large number of different immunologically significant cell types. Mucosal macrophages are present in healthy and diseased guts, whereas neutrophils appear following some form of infectious or noninfectious challenge. Similarly, mucosal mast cells are not detected in the lamina propria of healthy individuals but are rapidly recruited during parasite infection. Recruitment is dependent upon the rapid induction of Th2 cytokines produced by T cells residing in the lamina propria. Naïve T cells are primed in the Peyer's patches and migrate from the gut via the mesenteric lymph node and thoracic duct before homing back to the intestinal lamina propria. Lymphoid effector cells re-enter the circulation and return to the lamina propria through altered integrin and chemokine receptor expression. In the small intestine, lamina propria T cells are distributed primarily in the upper villus, with gradually decreasing numbers to the crypts.

In contrast, the majority of B cells and plasma cells are present within the crypts with significantly fewer cells within the villus. The reasons underlying the different distributions of B and T cells are unclear, but it has been suggested that $CD4^+$ cells adjacent to the crypts are predominantly Th2, while a greater proportion of the $CD4^+$ cells present in the upper villus may be of the Th1 phenotype. The number of IgA-producing plasma cells greatly exceeds those expressing IgG and IgM in the intestinal lamina propria, which are preferentially located in the crypts. The polymeric immunogloblin receptor (pIgR), which is required for the selective transport of locally synthesized IgA across epithelial cells to the gut lumen, is also largely restricted to the crypt region. The major roles of IgA in mucosal secretions are listed in Table 1.

Reflecting their pivotal role as an inductive site for mucosal immune responses, Peyer's patches display the greatest expression of MHC class II antigens, with lower levels of expression in the epithelial and lamina propria compartments. Within the feline lamina propria, MHC class II molecules are expressed predominantly by cells with macrophage or dendritic cell morphology. The number of positive cells was greater in the villus than crypt areas. A similar pattern of staining has been described for the dog with no differences between anatomical regions of the small intestine.[7-9]

Table 1.

Functions of IgA
Inhibition of Adherence: SIgA antibodies to microbes shown to prevent adherence to pharyngeal, intestinal and genitourinary tract epithelia.
Mucus Trapping: SIgA antibodies may associate with mucins, thereby trapping SIgA-bound microbes in the mucin layer, preventing adherence.
Virus Neutralization: Binding to attachment receptors, preventing internalization inhibition of viral replication within infected cells.
Neutralization of Enzymes and Toxins: Intestinal SIgA to cholera toxin. Salivary antibodies to neuraminidases, IgA proteases. Serum IgA to Clostridial enterotoxin A.

Cell Trafficking & Homing

A large body of evidence supports the observation that mucosal immune cells are distinct from those found at nonmucosal sites. Such evidence includes, based on phenotypic analysis, migration and trafficking studies as well as functional properties. In order to mount an effective mucosal immune response, cells are required to traffic between inductive (Peyer's patch) and effector sites (lamina propria and epithelium). This migratory pathway requires the interaction between the ligand $\alpha4\beta7$ (expressed by "mucosal lymphocytes") and the mucosal cell addressin molecule, MAdCAM-1, (expressed on vascular endothelium in mucosal tissues).

Studies of the distribution of MAdCAM-1 in canine tissue have confirmed that its expression is restricted to endothelial cells in gut-associated lymphoid tissue (GALT), including Peyer's patches, mesenteric lymph node, intestinal mucosa, submucosa and muscularis, a pattern of expression similar to that reported for other species. While the expression $\alpha4\beta7$ has been associated with the homing of cells to the lamina propria, another member of the $\beta7$ subfamily of integrins has been implicated in the localization of IELs. Studies in other species have found $\alpha E\beta7$ expressed on the overwhelming majority of IELs but on a smaller number of lamina propria lymphocytes (LPL's ~50%), and very few peripheral blood cells are positive for this marker.

Induction of Mucosal Immune Responses

Two of the key reasons that underlie the need for better understanding of the mechanisms that operate at mucosal surfaces are an ability to control infections through the development of mucosal vaccines and the protection from allergic reactions through the development of oral tolerance. A large body of data shows that immune responses that are protective at mucosal surfaces are most effectively stimulated by local application of antigen. The expression of active immune responses against antigens presented to the mucosa is frequently disadvantageous for an individual organism. Induction of responses, proliferation of appropriate cell types, and synthesis and secretion of appropriate effector molecules require diversion of energy and resources from other systems.

The effector mechanisms of immune responses frequently result in tissue inflammation and damage independent of that generated by the pathogen. Presumably, the temporary disadvantage of expression of immune responses outweighs the long-term disadvantage of having to live or die with the pathogen. Since the pathogenicity of microorganisms varies from severe (e.g., *Vibrio cholerae*) to low or absent (true commensal flora, food), this also requires an ability to modulate immune responses dependent on the perceived threat and independent of the antigenic load. In other words, the magnitude and type of response should be dependent on the "quality" of the antigen, not solely on the quantity.

In most food antigens in normal individuals, this ideally would involve complete absence of immune responses or "immunological tolerance." Studies in cats and dogs would suggest that such complications as those described above are equally applicable to cats and dogs. Oral tolerance is a specific acquired mechanism whereby prior feeding reduces an individual's ability to respond to subsequent presentation of that antigen. The induction of oral tolerance has been extensively studied in rodents, and a number of regulatory processes have been characterized. Following feeding, small quantities of fed protein (<0.02%) are absorbed intact across the intestinal mucosa. While such levels may not be nutritionally significant, immunologically they are highly important and capable of eliciting both humoral and cellular immune responses that are comparable to that induced by injection,[10] as illustrated in Figure 4.

The absorption of intact proteins from the diet raises the potential of eliciting damaging allergic reactions and food allergy. In order to prevent tissue-damaging allergic responses to harmless dietary components, these responses must be controlled, and two regulatory mechanisms have been identified. The first involves the local production and secretion of IgA antibody into the intestinal mucus layer, where it may reduce the subsequent absorption of that dietary protein. This process, termed "immune exclusion,"[11] is rarely absolute,[12] and systemic tolerance to fed proteins ("oral tolerance") may develop. In contrast to the response to injected antigens, which prime for a secondary response of greater magnitude than the primary response, feeding after a transient primary response normally leads to the development of oral tolerance.

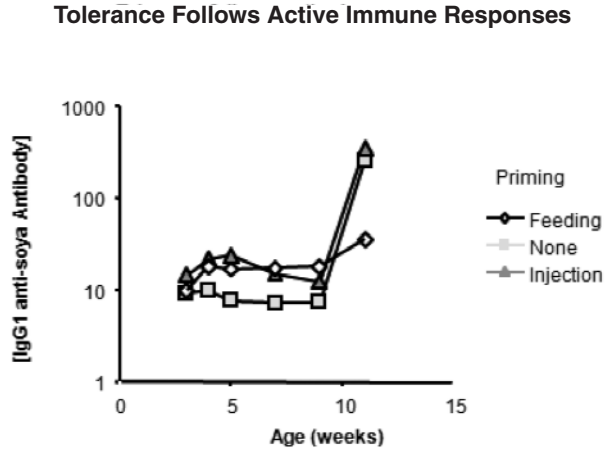

Figure 4: Serum IgG1 antibody to soya in piglets weaned at 3 weeks of age to diets containing either ovalbumin (triangles, squares) or soya (diamonds). At 7 weeks, all piglets were transferred on to a fish-based diet, and at 9 weeks, they were injected with soya in quil A adjuvant. The triangle group was also injected with soya in quil A at 3 weeks. The results show the means for groups of six piglets.

The latter is defined as a specific-acquired mechanism, whereby prior exposure reduces an individual's ability to respond to subsequent presentation of that antigen. Mucosally induced tolerance provides protection from the damaging allergic responses responsible for eczema, asthma, hay fever, and food allergy. Although fewer studies have been performed, it is clear that tolerance can be induced in cats[13] and dogs.[14]

The studies in rodents have identified a number of factors (e.g., age, genetics, dietary change, microbial flora, weaning) that can abrogate or delay the induction of mucosal tolerance. It is expected that a similar range of factors also may play a role in determining the outcome of feeding novel dietary proteins in cats and dogs. If so, then differences in the induction of tolerance in these species are likely to underlie a number of gut pathologies, including inflammatory bowel disease.

Further Reading

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Emerging Paradigms in Immunonutrition

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Abstract

Nutritional immunology is the study of the relationship between food and the immune system. It evolved with the study of immune deficiencies caused by malnutrition. However, due to technological advances over the past few decades, malnutrition is no longer the main cause of lowered immune status in otherwise healthy people/animals. Rather, life stage (neonate or old age) and natural stressors have taken over as the primary reasons for immune deficiency. Unlike malnutrition, immune deficiency due to life stage or natural stress cannot be addressed by correcting underlying nutritional problems. Lowered immune status because of life stage or naturally occurring stress is characterized by reduced capacity to process and present foreign antigens to immune cells, resulting in a less efficient or altered immune response that leads to increased susceptibility to infections and an increase in autoimmunity and cancers. Beyond providing essential nutrients, diet can actively influence the immune system. Over 65% of the immune cells in the body are present in the gut, making the gut the "largest immune organ." Receptors present on the immune cells in the gut are the primary targets for immunomodulation via diet. Diet interacts with the immune system at multiple levels, starting with providing basic nutrients, then moving on to providing higher levels of key nutrients such as protein, vitamins and minerals, and leading to a more focused modulation of the immune system. A framework outlining this interaction, along with relevant examples, will be presented in this paper.

Key Words
Nutritional Immunology
Natural Stress
Aging
Immunosenescence
Cytokines
Prebiotics
Vaccine Response
Caloric Restriction
Probiotics
Colostrums

Glossary of Abbreviations
AD: Alzheimer's Disease
APC: Antigen-Presenting Cells
CR: Caloric Restriction
DC: Dendritic Cells
GALT: Gut-Associated Lymphoid Tissue
GC: Glucocorticoid
GI: Gastrointestinal
IFN-γ: Interferon-γ
IG: Immunoglobulin
IL-6: Interkeukin-6
IRMs: Immune Response Modifiers
LPS: Lipopolysaccharide
MHC: Major Histocompatibility Complex
NAIDS: Nutritionally Acquired Immune Deficiency Syndrome
NF-kβ: Nuclear Factor-kβ
PAMPS: Pathogen-Associated Molecular Patterns
TGF-β: Tumor Growth Factor-β
Th1: T-Helper Type 1
Th2: T-Helper Type 2
TNF-α: Tumor Necrosis Factor-α

Nutrition & Immunity Are Evolutionarily Linked

Both nutrient metabolism and immunity (nutrient sensing and pathogen-sensing pathways) are essential for survival — the former to sustain and the latter to preserve life. Consequently, nutrient metabolism and immunity have codeveloped organ systems and signaling pathways during evolution.[1] We see many examples of this in nature. In the common fruit fly, *Drosophila melanogaster*, both immune and metabolic responses are controlled by the same organ, the "fat body."[2] Although higher animals have evolved different organ systems for immune and metabolic responses, the evolutionary relationship is apparent by: 1) the close proximity of immune cells, such as macrophages and Kupferr cells, in tissues actively involved in nutrient metabolism like adipose and liver tissue,[3] and 2) the observation that remodeling of adipose tissue often accompanies certain inflammatory diseases, such as development of panniculities during inflammatory bowel disease[4] and the inflammatory stress brought on by obesity.[5] Furthermore, this evolutionary relationship is hardwired at the molecular level in cells involved in both processes. Both adipocytes and macrophages secrete cytokines in response to bacterial products such as lipopolysaccharide (LPS).[6] Preadipocytes can differentiate into macrophages, and transcriptional profiling reveals that they are genetically related.[7,8] Given this close relationship, it is no surprise that chronic nutrient deficiency or excess can negatively impact immune health and consequently overall health. For example, adipose tissue in obesity has been shown to produce higher levels of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α),[9] resulting in low-grade inflammation that leads to metabolic syndrome and

associated diseases, such as insulin resistance, type 2 diabetes and arthrosclerosis. The good news is that this relationship can also be used to proactively enhance immune health.

Immunonutrition: History and Renewed Focus

The understanding that food impacts health goes back to antiquity with references in the writings of ancient Egyptians and Indians. Hippocrates, the father of Western medicine, is believed to have recommended that his students evaluate diet to understand disease. However, the earliest scientific evidence implicating the role of nutrition in immune function came from J. F. Menkel in 1810, describing thymic atrophy in malnourished people in England. These observations, among others, gave birth to nutritional immunology, which continued to evolve as a scientific discipline with the study of nutritional deficiencies caused by malnutrition, sometimes referred to as nutritionally acquired immune deficiency syndrome (NAIDS).[10] Since its beginning in the 1800s and with new information from the vitamin era of the early 1900s, the emphasis in nutritional immunology was on how nutrient deficiencies impact the immune system. Although malnutrition still remains a global problem, many of the detrimental effects of malnutrition can be addressed by correcting the specific underlying nutritional problem. The current challenge, however, is related to an aging population, increased natural stress and dietary overindulgence. Unlike immune deficiency caused by malnutrition, age-related immune deficiencies (life stage) and immune deficiency due to natural stress or dietary overindulgence need a more comprehensive strategy and cannot be simply addressed by correcting nutritional problems. Therefore, these problems are difficult to evaluate, understand and manage. More importantly, as a practicing clinician, one is more likely to see immune deficiencies of the latter kind (immune deficiency not related to malnutrition), hence the paradigm shift in today's research emphasis in nutritional immunology from malnutrition to addressing impaired immune status because of age, natural stress and diet.

Why Is It Important to Ensure Immune Health?

The benefits of good immune health go beyond protection from infections. Immune health or a lack thereof has profound metabolic consequences, and new research indicates that it can affect several body systems including brain aging and cognition.[11] At a fundamental level, a healthy immune system affords protection by preventing infectious agent(s) from entering the host and establishing an active infection. This is the critical "barrier" function, otherwise known as the "first line of defense" role of the immune system. When the immune system is compromised, this barrier weakens and pathogens invade, causing disease. This triggers an active immune response to neutralize and eliminate the infectious agent involving physiological changes, including fever, inflammation and cellular responses such as a generation of T cells and antibodies that can specifically target the pathogen. Although such a full-blown immune response is critical for survival, it nevertheless comes with a price; it's a metabolically costly endeavor that uses precious resources. To put this in perspective, a 1°C increase in body temperature (fever associated with active infection) involves energy expenditure equal to a 70-kg person walking 45 kilometers (9.4×10^6 J).[12] Clearly, repeated immune activation to combat infection can significantly drain metabolic resources and will unfavorably compete with energy-demanding processes like reproduction, lactation and growth, because evolutionarily "protection" is assigned a higher priority than these other processes. Repeated immune activation has other secondary consequences, such as increased oxidative stress, which is especially harmful in older animals. A healthy immune system capable of preventing infections thus has profound positive metabolic implications. Recent research done in rodents and people with age-related dementia suggests that poor immune health can negatively impact cognition and brain aging.[11] Clearly, a healthy immune system has implications that go beyond disease prevention.

In this review, I would like to: 1) discuss causes of immune deficiency in an otherwise healthy animal, 2) explore how food influences the immune system, and 3) propose a framework to understand how nutrition interacts with the immune system.

What Impacts Immune Health?

In the absence of disease, age and natural stress are two important factors influencing immune status. The immune response of a neonate or an older animal tends to be less vigorous than that of an adult, making them more susceptible to infection.[13] Aging is also characterized by low-level chronic inflammation that contributes to the declining ability of the immune system to respond and regulate immune response.[14] Stress and, in particular, chronic stress have been shown to have a significant negative impact on the immune system irrespective of the age of the subject.[15]

The Effect of Age on the Immune System

Immune Response in Neonates

Neonatal immune responses tend not to be as strong as those in an adult animal.[16] In Beagle pups between the ages of 0 to 4 weeks, mitogenic responses (a measure of how immune cells would respond during an immune challenge) was shown to be significantly lower than those in an adult animal.[17] Somberg et al. found that the *in vitro* lymphocyte proliferation activity (also a measure of immune response like the one above) of newborn pups was 50% lower than that of adults.[18]

Although neonates are capable of responding to an immune challenge, their immune responses tend to exhibit a T-helper type 2 (Th2) bias.[13] A T-helper type 1 (Th1) immune response is characterized by proinflammatory cytokines, such as interferon-γ (IFN-γ), interleukin-6 (IL-6), and TNF-α, and hence is more effective in preventing infectious diseases. In contrast, a Th2-biased immune response is predominated by anti-inflammatory cytokines

such as IL-10, IL-4 and tumor growth factor-β (TGF-β) and is not as effective in dealing with microbial infections, making neonates more susceptible to infections.

There are several cellular and molecular reasons for this Th2 bias. They include:

1 As compared to adult cells, neonatal antigen-presenting cells (APC) are less efficient in antigen presentation because of their reduced capacity to express crucial costimulatory molecules CD86 and CD40 and upregulate MHC Class II molecules.
2 The feto-placental environment tends to be immunosuppressive and Th2 biased because of locally acting cytokines and hormones, and these influence neonatal immune responses.[13]
3 Neonatal B cells, which also function as APC, have altered signaling due to lowered MHC Class II molecules as well as lowered accessory signaling molecules. Lack of upregulation of CD40 (accessory signaling molecules) and CD40L (receptor for CD40) tends to dampen B-cell response as well as its ability to class switch immunoglobulin (Ig) production, contributing to the Th2 bias.
4 Neonatal Th1 cells undergo apoptosis because of the unique receptors they express. In a recent study, Lee et al. have shown that although a primary immune response from neonatal T cell includes a significant Th1 component, the Th1 cells generated have unique characteristics. They tend to have high levels of IL-13Rα1, which heterodimerizes with IL-4Rα. As the immune response progresses, because of the lack of appropriate dendritic cells (DC), the immune response is dominated by lL-4, which binds the IL-13Rα(1)/IL-4Rα complex expressed on the Th1 cells and induces apoptosis, eliminating the Th1 cells, which results in a Th2 bias. As the neonate ages, a significant number of appropriate DCs start accumulating, especially in the spleen. These DCs produce IL-12, and this IL-12 triggers the downregulation IL-13Rα 1 on the Th1 cells, rescuing them from IL-4 induced apoptosis.[19]

Immune Response Changes with Aging

Aging brings changes to both the humoral and cellular immune responses. These include defects in the hematopoietic bone marrow and in lymphocyte migration, maturation and function. Aging also involves involution of the thymus, which contributes to loss of immune function with increasing age.[20]

With age, the immune system loses plasticity, resulting in lowered response. Immune plasticity is the ability of the immune system to remodel itself to respond appropriately to danger signals, which include pathogens, tissue damage and oxidative stress, and return to a quiescent state once the danger has passed. One of the reasons for this declining immune plasticity is chronic metabolic stress associated with aging.[21] This results in reduced immune response and a lower cellular capacity in DNA repair, leading to a condition described as immunosenescence, which increases the risk of age-related diseases, i.e., cancer and infection.[22,23] Declining immune plasticity leads the cells of the immune system to undergo cell death or necrosis triggered by oxidative stress.[24]

Age (life stage) of the animal has a significant impact on immune status and is one of the important reasons to consider nutritional strategies to address immune system effectiveness.

Naturally Occurring Stress

Naturally occurring stress, both physical and mental, has a significant negative impact on the immune system, irrespective of age. Both major and minor stressful events have been shown to have a profound influence on immune responses in both animal and human studies. One of the hallmarks of chronic stress is the general increase in levels of oxidative stress, and oxidative stress gradually erodes immune plasticity. Research in this area has spawned a new discipline called psychoneuroimmunology, the study of the interaction between the psychological process and the nervous and the immune systems.[25] Using vaccine responses as a indicator of immune status,[26-31] researchers have demonstrated that among medical students taking exams, stress levels lowered response to vaccine (virus-specific antibody and T-cell responses to hepatitis B vaccine), whereas the degree of social support increased vaccine response.[32]

Another good example of chronic stress is the stress associated with caregiving provided for a spouse with Alzheimer's disease (AD), which was associated with a poorer response to an influenza virus vaccine when compared to well-matched control subjects.[28] Vaccine responses demonstrate clinically relevant alterations in an immunological response to challenge under well-controlled conditions and therefore can be used as a surrogate for responses to an infectious challenge. Individuals who respond poorly to vaccines tend to have greater susceptibility to the pathogens compared to those with better vaccine responses. Burns et al., among others, have shown that adults who show poorer responses to vaccines also experience higher rates of clinical illness as well as longer-lasting infectious episodes.[33,34] Cohen and co-workers showed that human volunteers who were inoculated with five different strains of respiratory viruses showed a dose-dependent relationship between stress and clinical symptoms after infection.[35] Therefore, from these vaccine studies, it is clear that stress puts individuals at greater risk for more severe illnesses.

At the molecular level, stress delays inflammation by reducing efficiency of CD62L-mediated immune surveillance by phagocytes.[36] Stress decreases IFN-γ secretion by lymphocytes and may decrease antigen presentation efficiency by downregulating MHC Class II molecule expression on APC and may delay or impair immune responses to vaccination.

Hormones play an important role in the effect of stress on the immune system. Stress sets into motion physiological changes that help the organism cope with the stressor — the fight or flight response. However, chronic stress results in sustained activation of stress responses, which include activation of the hypothalamic-pituitary-adrenal axis and the sympathetic–adrenal-medullary axis, resulting in the production of glucocorticoid (GC) hormones

and catecholamine. GC receptors are expressed by a variety of immune cells that bind cortisol, interfering with nuclear factor-kβ (NF-κβ) function, which, in turn, regulates the activity of cytokine-producing immune cells. Sustained release of stress hormones negatively impacts the immune system. Several models have been proposed to explain the action mechanism of stress hormones on the immune cells.[37] GC impacts expression of cytokines, co-stimulatory molecules and adhesion molecules, which influences immune cell migration, differentiation, proliferation and effector function.[38-40] Adrenergic receptors bind epinephrine and norepinephrine and activate the cAMP response element-binding protein, inducing the transcription of genes that encode a variety of immune-response genes including genes for cytokines. Elevated levels of catecholamines produced during stress can modify immune-response genes.[41] Natural stress is another key factor that can negatively impact the immune status of an animal irrespective of its age.

Age and natural stress clearly can undermine the immune status in an otherwise healthy animal. Immunodeficiency, irrespective of its etiology, can severely undermine the health of the animal, triggering debilitating diseases, such as infections, malignancies and autoimmune diseases. Hence, there is a critical need to evaluate immune status and address deviations, which, if managed effectively, can significantly enhance the quality of life.

How Can Diet Influence the Immune System?

The Gut Is the Largest 'Immune Organ'

Besides being the gateway for nutrient intake, the gut is the largest immune organ, containing over 65% of all the immune cells in the body and over 90% of all immunoglobulin-producing cells.[42,43] In an adult human, the intestine contains threefold greater Ig-producing cells (about $7x10^{10}$) compared to the bone marrow ($2.5x10^{10}$).[44] It is estimated that a total of ~3 g of secretory IgA is secreted daily into the lumen of an adult human.[45] Thus, a significant part of the immune system can interact with what we eat or feed our pets.

Gut-Associated Immune Tissue Plays an Important Role in Development of the Immune System

Research conducted with germ-free animals has documented that stimuli from environmental antigens, especially microbiota in the gut, are essential for the development of a healthy immune system (J.J. Cebra, 1999). Germ-free animals tend to have an underdeveloped immune system, clearly underscoring the role played by symbiotic microflora and associated environmental antigens. The gut-associated lymphoid tissue (GALT), therefore, offers unique opportunity for immunomodulation via diets. The GALT is unique in its ability to be exposed to a diverse array of antigens from foods (roughly 10-15 kg/year/human) and from over 1,000 species of commensal microorganisms (10^{12} mL/mL of colon content, making them the most numerous cells in the body), yet remain quiescent until it encounters a threat, such as a pathogen. This is initiated by molecules called pathogen-associated molecular patterns (PAMPs) expressed by microbial pathogens. PAMPs are highly conserved motifs present in these microorganisms and include LPS from the gram-negative cell wall; peptidoglycan; lipotechoic acids from the gram-positive cell wall; the sugar mannose (common in microbial glycolipids and glycoproteins but rare in mammals); bacterial DNA; N-formylmethionine found in bacterial proteins; double-stranded RNA from viruses; and glucans from fungal cell walls. Most dietary immune-modulating strategies involve targeting PAMPs receptors of the GALT using appropriate ingredients.

Efficient Antigen Presentation Is Fundamental for Efficient Immune Response

Efficient antigen presentation to T lymphocytes by the APC-like macrophages is a prerequisite for an effective immune response. APCs set the tone of the immune response by the costimulatory molecules they express and the cytokines they secrete. APC function is central to the altered immune response that is characteristic of the neonatal immune system, the immune response of an aging immune system and the immune response during stress. In all three cases, because of the lack of immune-potentiating cytokines, such as IL-1 and IL-12, APCs responding to an immune challenge are not able to upregulate MHC Class II molecules as well as costimulatory molecules, such as CD86. Lack of these cytokine signals also modifies the immune response, reducing its efficiency and giving it a Th2 bias. The resulting immune response, therefore, tends to be not as efficient.

The approach to address this deficiency hinges on providing the required signaling to the APCs.[46] Receptors on immune cells present in the gut serve this function and are the primary targets of strategies for immunomodulation via diet. These receptors have evolved to respond to molecules in microbial pathogens collectively called PAMPs (described in paragraph above). Examples include yeast β-glucans,[47] yeast mannans[48] and nucleic acids.[49] Probiotics interact with the immune system by virtue of their PAMPs molecules, such as LPS.[50] These molecules, also referred to as immune response modifiers (IRMs), primarily initiate a local proinflammatory cytokine secretion that activates local APCs to upregulate MHC Class II and costimulatory molecules, enabling them to present antigens efficiently to T lymphocytes. IRMs provided via diet enhance APC efficiency, and APCs in the gut continually process and present antigens to T lymphocytes in the GALT. Although the GALT is quiescent to the myriad antigenic stimuli it receives via diet, when it encounters a pathogen it is able to initiate a more efficient immune response.

The enhanced immune activity induced by dietary IRMs in the GALT (mucosal immune system) spread to the entire immune system by the trafficking of activated lymphocytes and cytokines and the significant overlap with the nonmucosal immune system.[51]

Nutrition Interacts with the Immune System at Multiple Levels

Nutrition and the immune system interact at multiple levels

and, for simplicity, can be considered in a framework of four stages. Stages I and II are passive because they involve providing the immune system with essential nutrients. Stages III and IV focus on modifying the immune response using agents such as IRMs that primarily target the PAMPS receptors in the gut and involve more active approaches in enhancing immune status.

Stage I: Complete Nutrition

At the first stage, the focus revolves around dietary energy, protein, vitamins (vitamins A, C and E) and minerals (zinc, magnesium, iron, etc.).[52] Minerals such as Ca+ and Mg+ drive signaling mechanisms in the immune system and are therefore also important for enhanced immune response. Providing basic nutrition is the very least we can do for the immune system.

Stage II: Optimizing Macro and Micro Nutrients

The second stage involves optimized key nutrients that are critical for the immune cells. The immune system has a need for certain nutrients, and providing greater amounts of these key nutrients will optimize immune function. A temporary deficiency of a key nutrient can negatively impact the immune system. For example, during strenuous exercise, muscle cells preferentially use glutamine as their energy source, and, as a result, there is a reduction of glutamine levels in circulation. Glutamine also is the preferred energy source for immune cells, and because of low levels of glutamine in circulation following strenuous exercise, immune cells cannot function efficiently if challenged, making these athletes vulnerable to infections immediately after vigorous bouts of exercise.[53]

Key ingredients needed for a healthy immune system would include higher levels and higher quality proteins in diet. At a molecular level, proteins make up the structural components and mediate key processes of the immune system. Receptors, cytokines, Ig, complement components, and bactericidal proteins are all proteins. A source of high-quality protein in diets is therefore important for a healthy immune system. Vitamins and minerals are critical for the immune system. For this reason, dietary products for companion animals often exceed the required minimum for dietary energy, proteins, vitamins and minerals.

Addressing oxidative stress and subsequent damage to cellular DNA is another example of this strategy. Aging, along with other environmental stressors, tends to increase the levels of oxidative damage to cellular DNA, including immune cells. Cells have the ability to repair damage in response to injury or stress. However, beyond a point, the damage can be irreparable and results in cell death by apoptosis. Oxidative DNA damage due to free radicals produced during cellular metabolism is one of the primary causes of cell death.[54] Increased apoptosis can break immune tolerance to self-antigens resulting in autoimmunity.[55] Immunosenesence is characterized by a decreased response to mitogens and decreased cytokine production, and changes in signal transduction have been associated with aging (reviewed in[52]). Various strategies can help address senescence, tissue damage and apoptosis associated with aging, including the following:

1. Caloric Restriction (CR): Apart from increasing the life span,[56] data from laboratory animals have demonstrated that CR reduces immunosenescence.[57] Recent data from a CR study conducted in Labrador Retrievers clearly shows that CR can help retard immunosenescence.[58] A CR diet will help aging animals maintain a healthier immune system.

2. Antioxidants: Increased levels of antioxidants such as vitamin C (R. Anderson and co-workers, 1990), vitamin E[52] and carotenoids (β-carotene, α-carotene, lycopene, astaxanthin, etc.) can help prevent damage mediated by free radicals. A number of reports document the benefits of carotenoids in dogs, particularly in older animals.[59-61]

3. Prebiotics: Prebiotics that help maintain normal gut flora also fall into this category. Intestinal microflora play an important role in keeping the immune system primed to prevent colonization by pathogenic microbes. However, under certain conditions, such as after antibiotic therapy, gastrointestinal (GI) infections, stress or old age, the normal flora in the GI is perturbed, leading to a change in the bacterial flora due to overgrowth of harmful bacteria (e.g., *Clostrodium difficile*). Prebiotics such as inulin help maintain a healthy commensal population in the gut under stress.[62]

The first two stages are passive approaches in "immunonutrition." They are passive because they focus on providing dietary energy, protein, vitamins, minerals and antioxidants and managing caloric intake to help the immune system function optimally. Stages III and IV are considerably different and involve a more proactive approach at managing the immune system to obtain the desired outcome.

Stage III: Active Modulation of the Immune System

In Stage III, the emphasis is on active interaction with the immune system to modulate its function toward a desired goal. Examples include:

1. Reversing the Th2 Bias & Restoring Th1 Response by Enabling Efficient Antigen Presentation: A Th1 (pro-inflammatory) response is important for protection against microbial infections. The Th1 component of the immune system is boosted by stimulating the immune system with probiotic bacteria or PAMPS-expressing moieties (e. g., yeast β-glucans). Probiotics (*Enterococcus faceum, Lactobacilli* sp., *Bifidobacteria* sp., etc.) in diet have been shown to enhance immune status in dogs.[63] Milk bioactives from bovine colostrum have been show to have immune-enhancing effects in both human and murine studies, making bovine colostrums an interesting immunomodulating ingredient. Colostrum (and whey protein, which has a similar composition) contains Igs, cytokines, lactoferrin, and lactoperoxidase, each of which can influence the immune system.[64] Mice that were fed milk bioactives produced significantly higher serum and intestinal antibodies to several antigens (influenza virus, diphtheria and tetanus toxin,

poliomyelitis vaccine, ovalbumin, and cholera toxin subunit).[65] In another study, mice fed milk bioactives had enhanced resistance to pneumococcal infection.[66,67] In *in vitro* studies conducted with human monocytes, Biswas and co-workers[68] reported that coculture with bovine colostrum without antigenic stimulus induced a dose-dependent production of IL-12 by CD 14+ monocytes but did not induce IFNγ production. Interestingly, in the same study, bovine colostrum differentially affected stimuli-induced IFN-γ production; it enhanced IFN-γ in response to weak antigenic stimulation and inhibited IFN-γ in response to strong antigenic stimulation. As discussed earlier, IL-12 and IFN-γ are cytokines involved in the Th1 polarization required for a successful immune response toward intracellular pathogens, such as bacteria and viruses.

In a clinical study conducted in a highly trained cyclist, low-dose bovine colostral protein concentrate supplementation favorably modulated immune parameters during normal training and after an acute period of intense exercise, which contributed to lowering the incidence of upper respiratory illness.[69] In a research study conducted with adult dogs,[70] we evaluated the immune-enhancing effect of bovine colostrum. Our results demonstrated that adding bovine colostrum significantly enhanced their immune status as measured by their response to canine distemper vaccine as well as increased level of GALT activity measured by IgA production. Colostrum supplemented diets also enhanced immune status in cats as evidenced by increased rabies vaccine response and increased GALT activity measured by IgA production.[71] Stimulating the immune cells in the gut likely leads to a cascade of immune cell activation, which results in the secretion of cytokines that reach the rest of the immune cells via circulation and results in overall activation of the immune system and an increase in the production of IgA in the gut.

2. Better Management of Inflammation Will Prevent Further Damage: Chronic inflammation is central to the pathophysiology of a number of diseases, including cardiovascular and neurological diseases (Alzheimer's, impaired cognition).[72] Physiologically, the effects of inflammation are mediated by prostaglandins and leukotries, all end products of arachadonic acid metabolism. A diet rich in docosahexaenoic and omega-3 fatty acids can control the damaging effects of inflammation because of the reduced levels of active prostaglandins and leukotries and can be an effective strategy in addressing chronic inflammation. Reduced inflammation not only improves quality of life by preventing a number of cardiovascular and neurological diseases but also helps prevent autoimmunity by reducing exposure of the immune system to self-antigens.

Stage IV: Personalized Nutrition: Predictive, Preventive and Personalized Nutrition

Interaction among diet, environment and genome ultimately defines health status and can be critical in influencing chronic disease.[73-76] Over the last few decades, the science of pharmacogenomics, which deals with the genetic basis underlying disease susceptibility and variable drug response in individuals, has brought about a paradigm shift in the pharmaceutical industry by moving from a "one drug fits all" approach toward personalized therapy. This process has been greatly accelerated by advances in the -omics fields: single nucleotide polymorphisms analysis, transcriptomics (complementary DNA analysis), proteomics, and metabolomics. A good example of genetic variability affecting disease is breast cancer therapy using the drug transtuzumab (Herceptin, a humanized monoclonal antibody against the HER2 receptor developed by Genentech Inc.) linked to HER2 overexpression. Individuals expressing low levels of HER2 receptor respond poorly to Herceptin.[77,78] Another example is the influence of genetic variability on cytochorome P450 monooxygenase system enzymes (P450 family of enzymes is important for the metabolism of most drugs) and drug toxicity in individual patients.[79]

The concept of "personalized medicine" is now being explored in nutrition. Although personalized nutrition is still in its infancy, it is practiced in principle in dietary management of diabetes or in maintaining a healthy lipid profile to manage risk of cardiovascular disease. For a practical personalized diet strategy, there are two basic requirements: a clear understanding of the disease pathogenesis and the availability of cheap and reliable disease biomarkers to identify either susceptibility or diagnose disease. Biomarkers are an objectively measured characteristic that indicate normal biological processes, pathological processes or pharmacological responses to a therapeutic intervention. The ultimate goal is to modify physiology through personalized dietary regimen before the animal enters into the disease continuum, preventing disease or at least significantly delaying the onset of disease and thereby enhancing quality of life.

Induction of a local Th2 bias in animals with inflammatory bowel disease using dietary means is an example of a targeted approach to immunomodulation. Probiotic microbes have been characterized based on the cytokines responses they induce. Certain bacteria induce secretion of anti-inflammatory cytokines, such as IL-10, TGF-β and IL-13 (D. Ma and co-workers, 2004). These probiotic agents give us the opportunity to explore probiotic-fortified diets that will help animals suffering from inflammatory bowel diseases. Similarly, TGF-β-rich ingredients such as colostrum and whey proteins are being increasingly used to effectively address localized inflammatory conditions in the gut, especially with diets for inflammatory bowel diseases.

Conclusion

In summary, as research advances in understanding complex physiological networks in health and disease, the role played by the immune system and its interaction with diet takes a whole new meaning. As our understanding of the relationship between nutrition and the immune system matures, a variety of diet-based approaches to address immune needs will become available both

for us and our pets. The food we eat and feed our pets can clearly deliver several other benefits beyond basic nutrition, and therein lies the promise of immunonutrition.

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Microbiota in Health and Disease

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Abstract

Recent advances in molecular methods have revealed that the canine and feline gastrointestinal (GI) tracts harbor a highly complex microbial ecosystem, comprising several hundred different bacterial genera. We are just beginning to understand how these microbes interact with the host and thereby exhibit an influence that reaches beyond the GI tract. This paper reviews recent studies cataloging the microbial phylotypes identified in the GI tracts of healthy dogs and cats and how this intestinal ecosystem is altered in gastrointestinal diseases.

Glossary of Abbreviations
AIEC: Adherent and Invasive *Escherichia Coli*
EPI: Exocrine Pancreatic Insufficiency
GI: Gastrointestinal
IBD: Inflammatory Bowel Disease

Introduction

The intestinal microbiota is defined as the collection of all living microorganisms (i.e., bacteria, fungi, protozoa and viruses) that inhabit the gastrointestinal tract. With the development of novel molecular analysis tools (based most commonly on sequencing of the bacterial 16S rRNA gene), it is now appreciated that the gastrointestinal microbiota of mammals is highly diverse, comprising several hundred to over a thousand bacterial phylotypes.[1,2] It is estimated that the mammalian intestine harbors a total of 10^{10} to 10^{14} microbial cells, which is approximately 10 times more than the number of cells in the host body.

It is, therefore, obvious that this highly complex microbial ecosystem will play a crucial role in host health and disease. Gut microbes are useful to the host by acting as a defending barrier against transient pathogens, aiding in digestion and helping to harvest energy from the diet, providing nutrition for enterocytes, and playing an important role in the development and regulation of the host immune system. However, the intestinal microbiota also can have a detrimental influence on gastrointestinal health; in the last few years, convincing evidence has been gathered associating alterations in the composition of the intestinal microbiota with chronic enteropathies of humans, dogs and cats.[1,3]

We are at the beginning in being able to describe the microbial populations in the gastrointestinal tract and how they are influenced by environmental factors. In addition to recognizing which bacterial groups are present in the gastrointestinal tract, in the future, we will need to study the functional properties of the resident microbiota and their impact on the host. Newly developed high-throughput tools allow shot-gun sequencing of microbial DNA and provide a more in-depth picture of the functionality of the intestinal ecosystem in dogs and cats.[4]

Characterization of Gastrointestinal Microbiota

Until recently, traditional bacterial culture was the most commonly used method for describing the bacterial groups present in the GI tracts of dogs and cats.[5] Bacterial culture can be a useful technique for detecting specific intestinal pathogens (e.g., *Salmonella, Campylobacter jejuni*). However, it is now well-recognized that bacterial culture is not well-suited for in-depth characterization of complex environments, such as the mammalian gastrointestinal tract.[6]

Because the majority of intestinal bacteria cannot be cultured, a culture-based method underestimates total bacterial numbers and does not allow for identifying the majority of bacterial groups present in the GI tract. Some reasons for our inability to culture most intestinal bacteria include a lack of knowledge regarding their optimal growth requirements and the fact that the canine and feline gastrointestinal tracts harbor predominantly anaerobic bacteria, which are prone to sampling and handling damage. Furthermore, many selective culture media lack sufficient specificity, and other organisms than the targets often are enumerated.

Molecular Characterization of the Intestinal Microbiota

Molecular tools allow the identification of previously uncharacterized intestinal microbes, and these techniques also are able to provide information about the functionality of the microbiome by means of metagenomics and transcriptomics.[7]

Several methods are available, and all these approaches are ideally used in a complimentary fashion. A brief overview about these methods is provided in Table 1, and more detailed information is provided elsewhere.[8]

Table 1. Commonly Used Methods for Characterization of the Intestinal Microbiota

Method	Purpose	Description	Advantages/Disadvantages
Fluorescence in situ hybridization (FISH)	Identification, quantification, visualization of bacterial cells	Fluorescent dye-labeled oligonucleotide probes are hybridized to ribosomal RNA sequence in bacterial cells	Useful method for quantifying bacteria, allows visualization of bacteria in tissue. Labor intense, FISH probes need to be developed for groups of interest
Denaturing gradient gel electrophoresis (DGGE)	Profile of PCR amplicons represents the bacterial diversity (fingerprint) of the sample	A region of the 16S rRNA gene is amplified by PCR. Separation of the PCR amplicons (representing different bacteria) is achieved by migration through an increasing gradient of chemical denaturants	Inexpensive, requires sequencing of bands for identification of bacterial groups, limited phylogenetic resolution
Terminal restriction fragment length polymorphism (T-RFLP)	Profiling and quantifying the composition of the bacterial community in a Sample	PCR amplicons are generated using a fluorescent dye-labeled primer. The amplicons are then fragmented by digestion with restriction enzymes. These fragments are run over gels and the fluorescence can be detected and measured, providing a fingerprint that represents the composition of the community	Inexpensive, allows semiquantification and identification of bacterial groups, limited phylogenetic resolution
Quantitative real-time PCR (qPCR)	Quantification of bacterial groups	Target organisms are detected in real-time using fluorescent dye-labeled primers and/or probes	Rapid, inexpensive, quantitative. Primers and probes need to be designed for groups of interest
Next-generation sequencing	Identification of bacteria in a sample	Bacteria in a sample are amplified using universal primers, and PCR amplicons are separated and sequenced using a high-throughput sequencer	Rapid, relatively inexpensive, allows identification of bacteria. Semiquantitative, allows to describe changes within a community, requires advanced bioinformatics
Metagenomics (shotgun sequencing of genomic DNA)	Identification of microbial genes present in sample	Genomic DNA is fragmented and then randomly sequenced (without PCR amplification) on a high-throughput sequencer	Provides not only phylogenetic information but also what functional genes are present in sample, expensive. Requires advanced bioinformatics

Gastrointestinal Microbiota of Healthy Dogs and Cats

Due to differences in anatomical and physiological properties along the gastrointestinal tract (i.e., differences in pH, bile concentrations, intestinal motility), the microbial composition differs among the segments of the GI tract (Table 2). Furthermore, differences are observed between luminal and mucosa-adherent microbial populations. Of special note is that each dog and cat harbors a unique, individual microbial profile (Figure 1). These differences among individual animals are mostly notable on a

Table 2. Major phylogenetic lineages in different compartments of the canine intestinal tract. The proximal GI tract harbors a higher percentage of aerobic bacteria, whereas the colon harbors predominantly anaerobic bacterial groups.

	Duodenum	Jejunum	Ileum	Colon
Clostridiales	40.0%	38.8%	24.8%	26.1%
Lactobacillales	24.7%	12.6%	1.4%	13.4%
Fusobacteriales	3.3%	14.2%	32.6%	28.9%
Enterobacteriales	32.0%	27.3%	18.4%	1.4%
Bacteroidales	0.0%	7.1%	22.7%	30.0%

species and strain level, with typically only a minor overlap of bacterial species between individual animals. For example, in a recent study, it was shown that almost all evaluated cats harbored *Bifidobacterium* spp. in their GI tract, but only a small percentage of cats harbored the same species of *Bifidobacteria*.[9] These differences in bacterial composition among individual animals may explain, in part, why there is a highly individualized response to therapeutic approaches that are designed to modulate intestinal microbiota and why not every animal will respond similarly to dietary changes or administration of antibiotics or nutraceuticals (i.e., probiotics).

Differences also exist in the number of total bacteria in the different compartments of the GI tract. The stomach harbors between 10^1 and 10^6 cfu/g of contents. Bacterial counts in the duodenum and jejunum of dogs and cats can range from 10^2 to 10^9 cfu/mL of contents.[10] The distal small intestine (i.e., ileum) contains a more diverse microbiota and higher bacterial numbers (10^7 cfu/mL of contents) than the proximal small intestine. Bacterial counts in the colon range from 10^9 and 10^{11} cfu/ml of intestinal content.

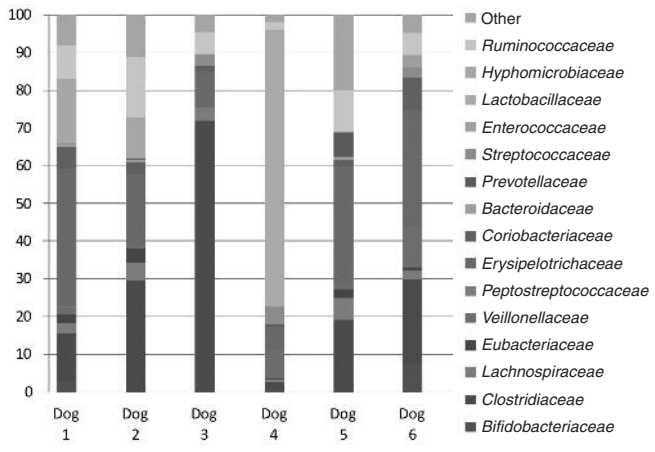

Figure 1: Predominant bacterial families observed in fecal samples of dogs. Note the differences in the abundance of bacterial groups among individual dogs. Similar observations in the composition of the intestinal microbiota are observed in cats.

Cultivation studies have reported *Bacteroides, Clostridium* spp., *Lactobacillus* spp., *Bifidobacterium* spp. and *Enterobacteriaceae* as the predominant bacterial groups in the canine and feline intestines. However, molecular tools have now greatly expanded our knowledge about the phylogenetic diversity within the canine and feline gut, and more recent results suggest that some of the bacterial groups (for example, *Bifidobacterium* spp.) believed to predominate the GI tract based on cultivation studies actually are less abundant.

The exact number of species or strains present in the GI tract is unknown, partly due to difficulties to comprehensively capture this diverse ecosystem. Recent studies have revealed approximately 200 bacterial phylotypes in the canine jejunum, and it is estimated that the canine and feline large intestines harbor a few hundred to over a thousand bacterial species.[2] Despite this vast bacterial diversity, only a few of the 55 known major phylogenetic lineages have been observed in the mammalian GI tract. The phyla *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes*, and *Fusobacteria* constitute almost 99% of all gut microbiota in dogs and cats.[4,11,12] A few less abundant phyla are *Tenericutes, Verrucomicrobia, Cyanobacteria*, and *Chloroflexi*. However, it is believed that other low abundant bacterial lineages have not yet been identified.

Aerobic bacteria occur in higher abundance in the small intestine, whereas anaerobic bacteria predominate in the large intestine. In the stomach, mucosa-adherent *Helicobacter* spp. is the major group, followed by various lactic acid bacteria (i.e., *Lactobacillus* and *Streptococcus* spp.) and *Clostridia* spp. The most abundant groups in the small intestine are *Clostridia, Lactobacillales* and *Proteobacteria*, whereas *Firmicutes, Bacteroidetes* and *Fusobacteria* are the predominant bacterial phyla in the large intestine.[12,13] Of interest is that the phylum *Firmicutes* comprises many phylogenetically distinct bacterial groups, the so-called *Clostridium* clusters. Clusters XIVa and IV encompass many important short-chain fatty acid producing bacteria (i.e., *Ruminococcus* spp., *Faecalibacterium* spp. and *Dorea* spp.) and are the major groups in the ileum and colon of cats and dogs.[9,13]

More recent studies are attempting to analyze the functional properties of the intestinal microbiota. This is important because it remains challenging to correlate the presence of specific bacterial groups with gastrointestinal health and disease. For example, administration of antibiotics to healthy dogs leads to decreases in some of the beneficial bacterial groups, but this change does not lead to obvious gastrointestinal problems. It is believed that a functional redundancy exists in the GI tract, with several members of the bacterial community performing similar functions, and if one group is displaced because of perturbations (e.g., antibiotic therapy), other members of the community appear to maintain a stable ecosystem function. Therefore, it is crucial to evaluate the intestinal microbiome as an entity, including phylogenetic relationships and metabolic functions (i.e., metagenome, transcriptome and metabolome).

Fungi, Archaea and Viruses

In addition to bacteria, the mammalian GI tract harbors various fungi, archaea, protozoa and viruses. Recent molecular studies have provided more in-depth analysis about the diversity of these microorganisms in healthy animals, but their interactions, their influence on the host and their role in disease remain unclear.[4,8,11,14]

Microbiota in Dogs and Cats with Gastrointestinal Disease

Microbial causes of gastrointestinal disease include colonization with invading pathogens, an imbalance (dysbiosis) caused by opportunistic bacterial residents, or an altered cross talk between the intestinal innate immune system and the commensal microbiota. Opportunistic pathogens can either directly invade the intestinal epithelium or cause gastrointestinal disease due to production of enterotoxins (i.e., enterotoxigenic *Clostridium perfringens*). A dysbiosis caused by opportunistic bacterial residents can affect the mucosal barrier in the GI tract with an increase in intestinal permeability and clinically significant bacterial translocation.

Unspecific alterations in the intestinal microbiota have been associated with several gastrointestinal diseases of dogs and cats. Small intestinal dysbiosis (often termed as small intestinal bacterial overgrowth or antibiotic-responsive diarrhea) is a disorder that is suspected to be caused by changes in the composition or numbers of bacteria present in the small intestine, but the exact pathogenesis remains unknown. It is believed that changes in intestinal motility or in the architecture of the intestine (i.e., surgical creation of intestinal loops, short bowel syndrome and resection of the ileocolic valve) will predispose to dysbiosis. Based on bacterial culture studies, increases in total bacterial counts in the small intestine have been observed in exocrine pancreatic insufficiency (EPI) due to the lack of antibacterial peptides that are secreted by the normal pancreas.[15] These increases are associated with altered intestinal barrier function, damage to the intestinal brush border and enterocytes, increased competition for nutrients and vitamins, and increased deconjugation of bile acids. This may result in nutrient and vitamin malabsorption and an increased concentration of potentially deleterious metabolites.

Microbiota in Chronic Enteropathies

In humans with inflammatory bowel disease (IBD), the intestinal microbiota has been implicated in the disease pathogenesis, often in combination with a genetic susceptibility of the host. These are often combined with underlying defects in the innate immune system that may result in impaired bacterial killing or removal of bacterial antigen (i.e., NOD2/CARD15 gene defects).

The cause-effect relationship between microbial alterations and inflammation is not well determined. It is suspected that intestinal inflammation causes a shift toward gram-negative bacteria (i.e., proteobacteria) that may perpetuate the disease in genetically susceptible individuals. A common finding in humans with Crohn's disease is a decrease in the bacterial phyla *Firmicutes* and *Bacteroidetes* and an increase in *Proteobacteria*. Within the *Firmicutes*, a reduction in the diversity of *Clostridium* clusters XIVa and IV (i.e., *Lachnospiraceae, Ruminococcaceae, Faecalibacterium prausnitzii* and *C. coccoides* subgroups) often is observed.

These findings suggest that these bacterial groups, important producers of short-chain fatty acids, may play an important role in maintenance of gastrointestinal health. It is also speculated that depletion of these commensal bacterial groups impairs the capability of the host to downregulate the aberrant intestinal immune response. The importance of some of these bacterial groups that are depleted in IBD have recently been demonstrated. For example, *Faecalibacterium prausnitzii* is consistently reduced in human IBD, and this bacterium has been shown to secrete metabolites with anti-inflammatory properties, thereby downregulating IL-12 and IFNγ and increasing IL-10 secretions.[16]

Few data are available characterizing the intestinal microbiota in acute and chronic gastrointestinal diseases of cats and dogs. However, recent molecular approaches performed in dogs and cats have also revealed differences in microbial composition between healthy animals and IBD patients, with some of these changes similar to those observed in humans. Dogs and cats with idiopathic IBD of the small intestine showed significant increases in *Enterobacteriaceae* and decreases of *Faecalibacterium* spp. when compared to control dogs.[17,18]

Changes in bacterial groups have also been reported in the large intestines of animals with chronic enteropathies. Cats with IBD had higher microscopic counts of *Desulfovibrio* spp., potential producers of toxic sulphides, and decreases in *Bifidobacterium* spp.[19] In Boxers with granulomatous colitis, the presence of adherent and invasive *Escherichia coli* (AIEC) and inflammation was observed.[20] Preliminary studies in the author's laboratory suggest that dogs with IBD also have decreases in *Faecalibacterium* spp. in their large intestines when compared to control dogs.

Recent studies suggest that dogs with chronic enteropathies have not only a dysbiosis as described above but also defects in their immune systems (i.e., differential expression of Toll-like receptors).[21,22]

Conclusion

Recent advances in molecular diagnostics have provided a better overview about the microbes present in the GI tract. However, our understanding of the complex interactions between microorganism and the host still are rudimentary. Future studies will need to encompass metagenomics, transcriptomics and metabolomics to understand the cross talk between microbes and the host. These may allow us to develop better treatment modalities targeted at modulating the intestinal microbiota.

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Prebiotics

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The Microbiota-Gut Brain Axis in Health and Disease

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Abstract

The "gut-brain axis" is a bidirectional communication system between the CNS and the gastrointestinal system that is comprised of neural and humoral pathways. There is accumulating evidence, mainly from animal studies using perturbation of the microbiota by antimicrobials and gnotobiotic models, that intestinal bacteria play an important role as modulators and signalling components of the gut-brain axis.

Glossary of Abbreviations
BDNF: Brain-Derived Neurotrophic Factor
CNS: Central Nervous System
ENS: Enteric Nervous System
IBS: Inflammatory Bowel Syndrome
ME: Median Eminence
NGF: Nerve Growth Factor
POMC: Pro-Opio-Melanocortin
SPF: Specific Pathogen-Free Flora

Introduction

Clinicians and researchers have long recognized the link between gastrointestinal function and the central nervous system (CNS). The "gut-brain axis" is a bidirectional communication system comprised of neural pathways, such as the enteric nervous system (ENS), vagus, sympathetic and spinal nerves, and humoral pathways, which include cytokines, hormones and neuropeptides as signalling molecules. Recently, results in animal models have generated great interest into the role of intestinal microbes as key players in gut-brain communication (Figure 1).

The intestinal microbiota involves a wide diversity of microbial species[1] and can be considered a postnatal acquired organ that performs different functions for the host. Intestinal microbes have developed a mutual relationship with their host, and they play a crucial role in the development of innate and adaptive immune responses[2,3] and influence physiological systems throughout life by modulating gut motility, intestinal barrier homeostasis,[4,5] absorption of nutrients, and the distribution of somatic and visceral fat.[6,7]

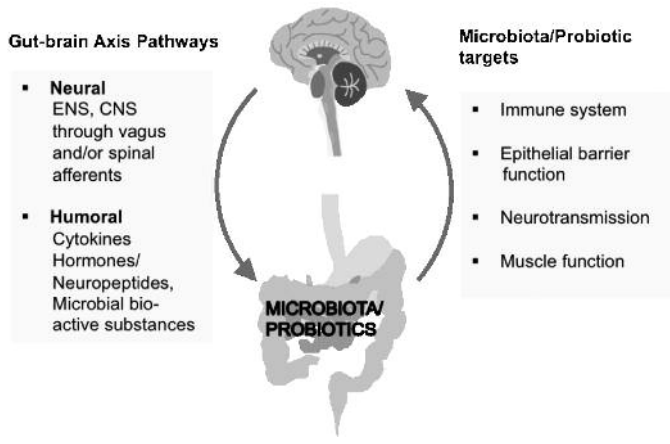

Figure 1: The gut-brain axis. Pathways of communication and probiotic targets.

Until recently, composition of this microbial community was considered unique for each individual and relatively stable over time.[8,9] However, using deep sequencing of stool samples from several hundred individuals, the European MetaHit consortium study has shown that human microbiota profiles can be grouped in three major bacterial enterotypes dominated by *Bacteroides, Prevotella* and *Ruminococcus*, respectively.[10] Distinct enterotypes strongly associated with long-term diets has been confirmed by Wu et al., linking protein and animal fat with *Bacteroides* and consumption of carbohydrates with *Prevotella*.[11] This indicates that despite large numbers of bacterial strains in the human intestine, there is a limited number of well-balanced host-microbial symbiotic states that might respond differently to diet and drug intake.

Microbiota-Gut-Brain Axis

The concept that gut bacteria are a driving force for immune maturation and gut function in the host is well accepted. The notion that bacteria could also influence brain function and behavior is seemingly implausible, but clinicians routinely use laxatives and oral antibiotics to treat patients with altered mental status due to hepatic encephalopathy.[12] Several clinical studies have also described altered composition of gut microbiota in patients with autism[13] and suggested at least a short-term beneficial effect of antibiotic treatment,[14,15] though no randomized clinical trial currently is available. There also are multiple reports of patients developing psychoses after administration of different antibiotics.[16] No current studies have characterized the gut microbiota associated with depression or anxiety, but earlier studies demonstrated that depression in females is associated with increased fermentation of carbohydrates, indirectly implicating changes in the composition or metabolic activity of the gut microbiota.[17]

Lessons from Animal Models: Effects of Bacteria on the CNS

At this point, the brunt of evidence linking microbes with behavior and brain biochemistry comes from animal studies. Pivotal experiments performed by Lyte et al. have shown that mice display altered, anxiety-like behavior during the early phase of acute infection with *Campylobacter jejuni*.[18] This abnormal behavior occurred within several hours after introduction of the intestinal pathogen into the GI tract, before any significant immune response was mounted, suggesting that this was not a consequence of cytokine-induced sickness behavior. Subsequent studies showed that presence of *C. jejuni* triggers activity of vagal ascending pathways and a specific activation pattern in multiple brain regions previously implicated in anxiety-like behavior[19,20] This clearly illustrates that the neural system can detect an acute change in the gut and can selectively identify a pathogen in the gut lumen.

Studies using chronic *H. pylori* infection in mice have shown that this pathogen alters gastric physiology, namely delayed gastric emptying and visceral sensitivity, with upregulation of SP and CGRP-containing nerves in the stomach and the spinal cord.[21,22] Furthermore, chronic *H. pylori* infection leads to abnormal feeding behavior characterized by frequent feeding bouts with less food consumed per feeding than controls, which is reminiscent of early satiety observed in patients with functional dyspepsia.[22] The abnormal feeding pattern was accompanied by downregulation of regulatory peptide pro-opio-melanocortin (POMC) in the arcuate nucleus and upregulation of the proinflammatory cytokine TNF-α in the median eminence (ME) of the hypothalamus. The ME is part of the circumventricular organ, an area of the brain where the blood-brain barrier is relatively leaky, enabling metabolites/molecules from the systemic circulation to enter the CNS. Interestingly, altered behavior and biochemical abnormalities persisted for at least two months post-bacterial eradication, suggesting that changes induced by chronic infection in the CNS may be long-lasting or permanent.

To establish a link between commensal bacteria and the CNS, several experimental approaches can be undertaken. One is to compare germ-free animals with animals colonized with specific pathogen-free flora (SPF). Sudo et al. demonstrated an abnormal HPA axis with elevated ACTH and corticosterone levels in response to restraint stress in germ-free mice, which normalized after colonization with commensal bacteria.[23] Furthermore, germ-free mice had lower brain-derived neurotrophic factor (BDNF) levels in the cortex and hippocampus. Several recent studies have compared behavior and brain biochemistry in germ-free and SPF mice.

Overall, using standard behavioral tests, such as elevated plus maze, open field and light/dark preference tests, germ-free mice displayed higher exploratory and lower anxiety-like behavior than SPF mice.[24,25] Heijtz et al. showed that compared to germ-free mice, SPF mice had higher central expression of neurotrophins, such as nerve growth factor (NGF) and BDNF.[24] Furthermore, there was differential expression of multiple genes involved in the secondary messenger pathways and synaptic long-term potentiation in the hippocampus, frontal cortex and striatum. Similarly, Neufeld et al. demonstrated increased expression of NMDA receptor subunit NR2B in the central amygdala and serotonin receptor 1A (5-HT 1A) expression in the hippocampus in SPF mice compared to germ-free mice.[25] The pronounced differences between germ-free mice and mice colonized with complex microbiota may relate to the ability of gut bacteria to affect multiple aspects of host metabolism, immunity and physiology. Colonization with a single commensal bacterium, *B. thetaiotomicron*, was shown to change expression of a vast array of genes in the intestine encoding for metabolism, intestinal permeability and angiogenesis as well as for glutamate uptake, GABA production and neurotransmitter release.[26]

A different approach to investigate the role of microbiota in gut-brain axis is to perturb a previously "stable" microbiota in healthy adult mice by oral administration of nonabsorbable antimicrobials. Combination of neomycin, bacitracin and pimaricin induced changes in colonic microbiota composition (gut dysbiosis) in SPF mice, with a marked increase in *Firmicutes*, mainly *Lactobacilli* spp, and decrease in γ-proteobacteria. This was accompanied by an increase in mouse exploratory behavior and altered BDNF levels in hippocampus and amygdala[27] (Figure 2A).

The same antimicrobial treatment failed to induce behavior abnormalities in germ-free mice or in mice treated intraperitoneally with antimicrobials. The antimicrobial regime used in this study did not induce measurable changes in gut inflammation or change levels of intestinal serotonin (5-HT), noradrenalin (NA) or dopamine. Interestingly, studies using subdiaphragmatic vagotomy or chemical sympathectomy before antimicrobials suggest that vagal and sympathetic pathways are not involved in gut-brain communication in this experimentally induced dysbiosis model of altered behavior.

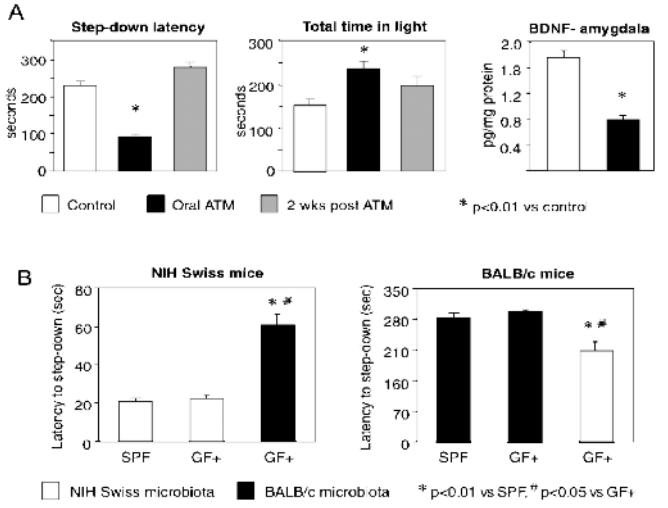

Figure 2: Effects of commensal bacteria on behavior.

Behavior has a genetic component, and it is known that mouse strains differ in their behavioral phenotype. There also is a difference in microbiota composition among mouse strains, and the "SPF" status does not indicate uniformity of the microbiota, only that mice have been screened for the most common murine pathogens. BALB/c and NIH Swiss mice are on opposite ends of the behavior phenotype: BALB/c mice are timid and less exploratory, while NIH Swiss mice display a high exploratory drive. BALB/c and NIH Swiss mice were reared under germ-free conditions and then colonized with SPF microbiota from either NIH Swiss or BALB/c mice. Germ-free mice colonized with microbiota from the same strain exhibited similar behavior as the SPF mice. However, mice colonized with microbiota from the other strain exhibited a behavior profile similar to the donor[27] (Figure 2B). This was not accompanied by measurable changes in systemic or gut immune activation or levels of intestinal 5-HT, NA or dopamine. A change in central neurotrophins was observed one week post-colonization. We can therefore speculate that host behavioral phenotype is also influenced by microbial factors.

Probiotics and the CNS Function

Psychiatric comorbidities, such as anxiety and depression, are common in patients with chronic bowel disorders, including inflammatory bowel syndrome (IBS) and inflammatory bowel disease.[28,29] Both disorders also are associated with abnormal intestinal microbiota profiles. In this respect, chronic infection with a noninvasive parasite or mild chemically induced colitis was shown to be associated with anxiety/depression-like behavior and decreased levels of hippocampal BDNF expression.[30,31] Interestingly, both abnormalities were normalized with treatment of the probiotic *B. longum* NC3001 but not with *L. rhamnosus* NCC4007. *B. longum* did not improve gut inflammation or circulating cytokines, however, its anxiolytic effect was absent in mice with previous vagotomy, suggesting that its action was neurally mediated. This was further confirmed by *ex vivo* studies, in which electroresponsiveness of enteric neurons was assessed after perfusion with *B. longum* supernatant. Compared to controls, *B. Longum*-treated neurons fired less action potentials in response to suprathreshold depolarizing current.[31]

The beneficial effect of probiotic bacteria may extend to healthy individuals. A study by Desbonnet et al. showed that administration of *Bifidobacterium infantis* to healthy Sprague-Dawley rats reduced concentrations of serotonin and dopamine metabolites in the frontal and the amygdaloid cortex, respectively.[32] The authors suggested that this bacterium may have an anxiolytic potential, although no difference in behavior was found in that study. Subsequent experiments with the same bacterium using maternal separation models demonstrated beneficial effects on altered behavior together with normalization of noradrenaline concentrations in the brainstem.[33]

Bravo et al. have recently demonstrated that administration of the probiotic *L. rhamnosus* JB1 promoted exploratory behavior and attenuated despair-like behavior, as assessed by an elevated plus maze and forced swim test, respectively, in healthy BALB/c mice. This was accompanied by region-dependent alterations in GABA(B1b) and GABA(Aα2) mRNA in the brain,[34] which was vagally dependent, as subdiaphragmatic vagotomy abolished both changes in brain biochemistry and behavior. Thus, animal studies support the notion that commensal bacteria and specific probiotics can influence brain chemistry and the function of the central nervous system.

Conclusion

While clinical observation and psychiatric comorbidity in various chronic intestinal disorders support a role of the intestinal microbiota in gut-brain axis communication, the strongest evidence for a role of microbes as signalling components in the gut-brain axis comes from animal studies using perturbation of the microbiota by antimicrobials and gnotobiotic models. Mechanisms of communication are likely to be multiple and involve neural, humoral and inflammatory pathways, depending on the host and environmental factors.

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Fecal Microbiota of Cats with Naturally Occurring Chronic Diarrhea

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Expanded Abstract

Metagenomics is the analysis of entire communities of microbes. The ability to perform this analysis, such as via 454-pyrosequencing, could lead to a more comprehensive understanding of the gut microbiome and could be used to aid in the development of effective dietary therapies and products to help maintain health and wellness for both humans[1-4] and companion animals.[5-7] It is increasingly recognized that gastrointestinal (GI) microflora has a strong impact on the health of cats and dogs[8] and that gut microbiome populations can be altered in GI disease[4-10] Prior research has confirmed alterations in intestinal microflora in cats with GI disease.[11,12] Microbial communities based on 16S rDNA sequence data can be analyzed on multiple fecal samples simultaneously utilizing 454-pyrosequencing with bar-coded primers to amplify particular 16S sequences.

Glossary of Abbreviations
FS: Fecal Scores
GI: Gastrointestinal
OPLS-DA: Orthogonal-Partial Least Squares

In this study, the 16S rDNA tag pyrosequencing was used to phylogenetically characterize the hindgut microbiome in cats with naturally occurring chronic diarrhea before and after response to dietary therapy. This controlled, crossover clinical study of cats with naturally occurring chronic diarrhea is the first to compare the metagenomic pyrosequencing profiles of cats before and after receiving dietary therapy.

Sixteen adult cats with chronic diarrhea were grouped and assigned to Diet X (Hill's® Prescription Diet® i/d® Feline, Hill's Pet Nutrition Inc., Topeka, KS, USA) or Diet Y (Purina Veterinary Diets® EN Gastroenteric® brand Feline Formula, Nestlé Purina PetCare Co., St. Louis, MO, USA). Diet X is a highly digestible diet marketed for cats with GI disease. Likewise, Diet Y is highly digestible and is formulated as a high-protein, low-carbohydrate diet with a blend of soluble and insoluble fibers and a source of omega-3 fatty acids (Table 1).

Table 1: Nutritional analysis of diets used in study

Nutrient	Baseline Diet		Diet X		Diet Y	
	% dm	g/100K	% dm	g/100K	% dm	g/100K
Protein	58.90	12.79	38.72	8.46	49.15	10.79
Fat	29.91	6.50	26.16	5.71	28.14	6.18
Total n3	3.44	0.75	0.37	0.08	1.01	0.22
Total n6	4.28	0.93	6.75	1.47	4.87	1.07
n6/n3	1.24		18.24		4.82	
Carbohydrate (NFE)	0.00	0.00	28.59	6.24	12.65	2.78
Crude Fiber	1.31	0.28	1.25	0.27	2.22	0.49
TDF	4.47	0.97	12.67	2.77	6.95	1.53
Sol	1.28	0.28	2.34	0.51	1.27	0.28
Insol	3.20	0.70	10.50	2.29	5.68	1.25

Baseline = Fancy Feast® Savory Salmon Feast cat food, Nestlé Purina PetCare Co.
Diet X = Hill's® Prescription Diet® Feline i/d,® Hill's Pet Nutrition Inc.
Diet Y = Purina Veterinary Diets® EN Enteral Management® brand Feline Formula, Nestlé Purina PetCare Co.
NFE = Nitrogen-free extract, determined as 100% (% water + % protein + % fat + % ash + % crude fiber)
TDF = Total dietary fiber

All cats were fed the same canned diet during baseline evaluations and then fed their assigned test diet for four weeks. Fecal scores (FS), ranging from 7=very watery to 1=extremely dry and firm, were recorded daily during the last week on each diet. Each cat was then switched to the alternate test diet, and the procedure was repeated. For each period, a three-week adaption to the diet was allowed before data collections began in the fourth week.

Both therapeutic diets resulted in a significant improvement in average FS over baseline, and Diet Y resulted in significantly better results compared to Diet X. FS improved at least one unit in 40% of the cats while fed Diet X and in 67% of the cats while fed Diet Y, resulting in normal stools (FS 2 to 3) in 13.3% of cats fed Diet X and 46.7% of cats fed Diet Y.

Table 2: O-PLS-DA model summary for discriminating metagenomics data at family, genus and species levels for Baseline, X and Y Diets

454 Level	Baseline Diet vs Diet Y	Baseline Diet vs Diet X	Diet X vs Diet Y
Family	0.126	-0.402	0.0588
Genus	0.399	-0.213	0.127
Species	0.61	-0.306	0.197

O-PLS-DA models were generated with 1 predictive and 2 orthogonal component to optimize microbial differences due to diet effects. The Q^2 value (sevenfold cross-validation) represents the predictability of the model and relates to its statistical validity. Statistical models where Q^2 value is negative were considered nonsignificant.

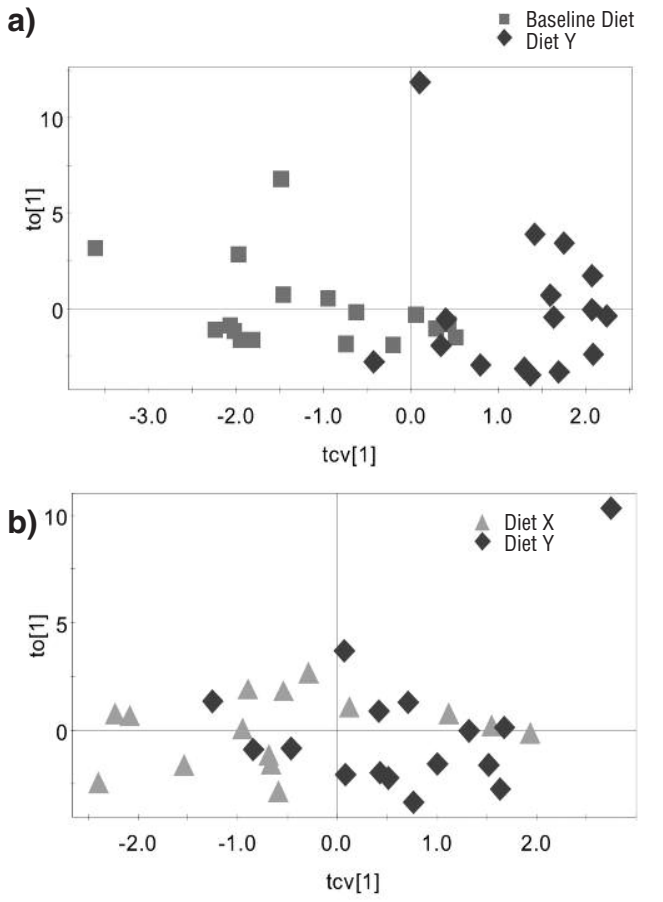

Figure 1. Multivariate analysis of the effects of dietary changes on fecal microbiota at the species level OPLS-DA scores' plot. Data were visualized by means of component scores plots, where each point represents an individual metagenomics profile of a sample. The score matrix (tcv and to) contains its projections onto the latent variables of the O-PLS-DA model instead of the original variables (16S rRNA sequence data. a) Scores plot of O-PLS model showed significant differences (P<0.01) between Diet Y vs. Baseline, and b) Diet Y vs. Diet X.

Fecal DNA samples from each cat were extracted, and the V1-V2 hypervariable regions of the microbial 16S rRNA gene amplified using primers suitable for 454-pyrosequencing generating 384255 sequences. DNA from the cats was sent to the Core for Applied Genomics and Ecology (University of Nebraska, Lincoln, USA), where equal amounts of each were sampled and sequenced by the Roche-454 GS-FLX Pyrosequencer. Data were analyzed to assess the phylogenetic changes induced by the therapeutic diets and to find which microbial communities showed clinical improvement in diarrhea. Dominant bacterial phyla included *Bacteroidetes* and *Firmicutes*, both comprising 30% to 34% of all sequences, followed by *Fusobacteria* (19%), *Proteobacteria/Tenericutes* (7% to 8%) and *Actinobacteria* (2%). Orthogonal-partial

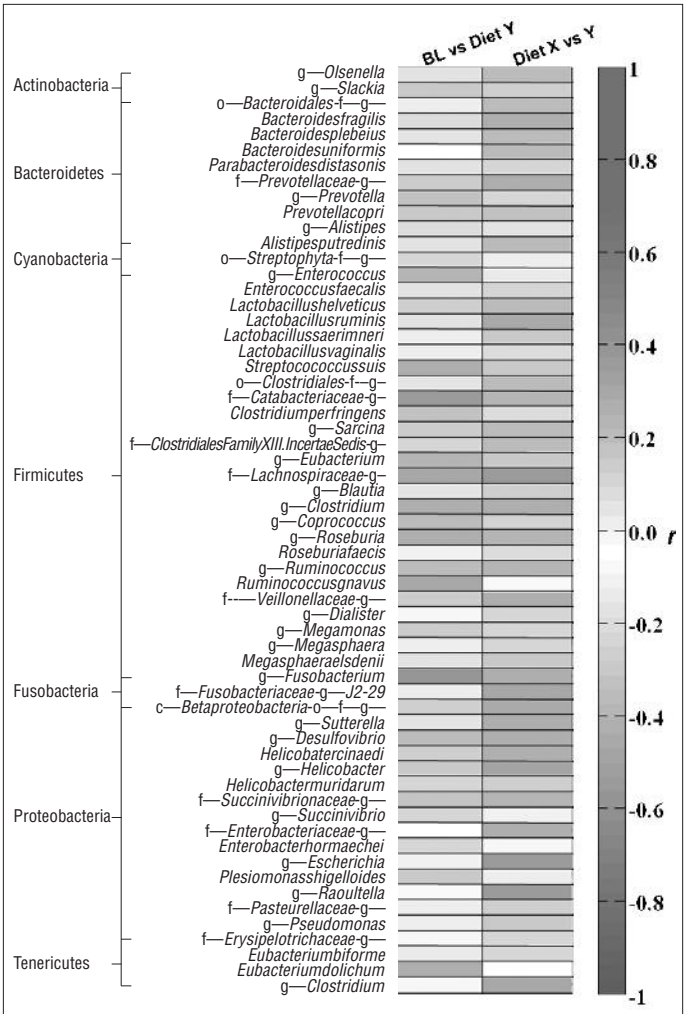

Figure 2. Heat map plot of significant bacterial microbiota. The heat plot (us r range -1 to +1) indicates the abundance of the bacteria that were up- or downregulated in cats after eating the two diets. Red corresponds to bacteria that are upregulated in Diet Y (high positive r correlation value), and green corresponds to bacteria that are downregulated in Diet Y (high negative r correlation value), which resulted from the O-PLS-DA model. The bacteria species were colored according to their phylum level.

least squares (OPLS-DA) clustering of metagenomics sequencing showed the greatest microbial differences between cats when fed Diet Y versus Baseline (Figure 1) and less significant differences (Q2 is positive but a lesser value) in cats fed Diet X versus Diet Y (Table 2). There were no differences between Baseline and Diet X, as shown by negative Q2 in Table 2. The data suggest that alterations in intestinal microflora are associated with improvement in diarrhea.

Figure 2 lists the bacterial populations that increased or decreased in cats fed Diet Y with comparison to the Baseline diet or Diet X, which was associated with improvement in diarrhea. For example, the species *Clostridium perfringens, Prevotella copri, Plesiomonas shigelloides, Enterobacter hormaechei, Helicobacter cinaedi, Helicobacter muridarum, Lactobacillus helveticus,* and *Bacteroides fragilis* were decreased in cats fed Diet Y compared to the Baseline diet. Some unidentified species in the genera *Enterobacter, Succinivibrio, Slackia, Helicobacter*, and *Prevotella* also were increased in Diet Y. On the other hand, the species *Ruminococcus gnavus*, *Streptococcus suis* and *Eubacterium dolichum* were increased in cats fed Diet Y compared to the Baseline diet. Some unidentified species in the genera *Desulfovibrio, Ruminococcus, Coprococcus, Eubacterium, Enterococcus, Roseburia, Clostridium*, and *Fusobacterium* also increased in cats fed Diet Y compared to the Baseline diet. Some of these alterations were also found with Diet X but not to the same extent, and this appears because Diet Y resulted in significantly better FS compared to Diet X.

In conclusion, the variations in taxonomic composition of cat-gut microbiota and the effects of therapeutic canned diets were quantified. Identifying these metagenomic changes will provide new insights into how therapeutic diets alter the feline-gut microbiota to manage diarrhea. This will provide a basis to further develop novel, more effective dietary interventions for the management of cats with chronic diarrhea.

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Research and Cinical Experience with Probiotics

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Abstract

There are many products in the veterinary market purported to contain probiotics that exert a beneficial effect on dogs and cats. *Enterococcus faecium* SF68 (FortiFlora,® Nestlé Purina PetCare Co., St. Louis, MO) is one of the most widely studied products. Administration of this product has been shown to have immunomodulating effects in dogs and cats. In addition, use of SF68 has been shown to aid in the management of dogs and cats with diarrhea in animal shelters. This paper will detail several studies describing the use of SF68 in dogs and cats with an emphasis on gastrointestinal diseases.

Glossary of Abbreviations
FHV-1: Feline Herpesvirus 1
IFA: Immunofluorescent Antibody Testing

Introduction

Probiotics are live microorganisms that when administered in adequate amounts confer a health effect on the host.[1] There have been many studies of the effects of probiotics on the health of humans but few in small animals. In a recent review of human studies involving probiotics,[2] it was stated that well-established probiotic effects include:

1. Prevention and/or reduction of duration and complaints of rotavirus-induced or antibiotic-associated diarrhea as well as alleviation of complaints due to lactose intolerance.
2. Reduction of the concentration of cancer-promoting enzymes and/or putrefactive (bacterial) metabolites in the gut.
3. Prevention and alleviation of unspecific and irregular complaints of the gastrointestinal tract in healthy people.
4. Beneficial effects on microbial aberrancies, inflammation and other complaints in connection with inflammatory diseases of the gastrointestinal tract, *Helicobacter pylori* infection or bacterial overgrowth.
5. Normalization of passing stool and stool consistency in subjects suffering from obstipation or an irritable colon.
6. Prevention or alleviation of allergies and atopic diseases in infants.
7. Prevention of respiratory tract infections (common cold, influenza) and other infectious diseases as well as treatment of urogenital infections.

Infectious diseases are common in small animals, so the potential beneficial effects of probiotics could significantly impact veterinary practice. All mechanisms of immune modulation have not been characterized, and it is likely these effects vary by probiotic. It is known that many probiotics in the lactic acid bacteria group help balance the endogenous microbiota, and some can inhibit replication of pathogenic bacteria. The proposed mechanisms of action include competition for essential nutrients or receptor sites, binding with pathogenic bacteria, and production of inhibitory substances. It also is known that some probiotics can beneficially influence innate and acquired immunity systemically by a variety of proposed mechanisms, including inducing cytokine production, natural killer cell activity, and specific and nonspecific immunoglobulin production.[2]

Several review articles in human medicine recently have suggested evidence that probiotics have provided a beneficial effect for a variety of conditions, such as *Clostridium difficile* diarrhea and hospital-acquired pneumonia, suggesting that larger, more rigorously controlled multicenter studies should be performed. These findings emphasize that the biological effects of individual probiotics vary and that each probiotic introduced should be rigorously evaluated in a controlled fashion to define the potential for clinical utility.[3-5] In addition, the source of the probiotic should be considered. For example, in recent veterinary studies, the majority of products claiming to contain probiotics generally did not meet the label claim when evaluated.[6,7] One exception is the Nestlé Purina PetCare probiotic, *Enterococcus faecium* SF68 (FortiFlora®).

The potential benefit of probiotics to animal health could be considerable.[8] There are several commercially available probiotics marked for use in dogs or cats in the United States. Several veterinary probiotic manufacturers have funded and continue to fund research studies evaluating the clinical effect of their products.[9-16]

Enterococcus faecium strain SF68 (NCIMB10415) was originally isolated from the feces of a healthy baby and was initially shown to inhibit the growth of a number of enteropathogens.[17] The purpose of this paper is to summarize key studies regarding the potential effects of this probiotic in the management of different canine or feline clinical syndromes.

Immune Modulation Studies

In one study, *Enterococcus faecium* strain SF68 was fed to a group of puppies vaccinated for canine distemper virus and compared over time to a control group that was similarly vaccinated but not fed the probiotic.[12] A number of findings suggested an immune-modulating effect of the probiotic. The puppies supplemented with SF68 had increased serum and fecal total IgA concentrations, increased CDV-specific IgG and IgA serum concentrations, and increased percentage of circulating B lymphocytes when compared to control puppies. The effect on canine distemper virus-specific IgG and IgA antibodies in serum was seen only after the puppies had been supplemented for 31 and 44 weeks, and it was believed that SF68 prevented the decline in antibody titers observed in the controls by maintaining high levels of antibodies.

In a follow-up study, a similar experimental design was applied to kittens. In that study, it was hypothesized that feeding *E. faecium* SF68 to kittens would enhance nonspecific immune responses; FHV-1-, FCV- and FPV-specific humoral immune responses; and FHV-1-specific cell-mediated immune responses.[10] Twenty 6-week-old SPF kittens were purchased from a commercial vendor and divided into two groups. One group was fed SF-68 daily, and the other group was fed the placebo starting at 7 weeks of age.

Table 1. Microbiota stability before and during supplementation with SF68 or a placebo[10]

Group	Equilibration	Supplementation	*P* value (vs equilibration)
Number of bands			
SF68 supplemented	22.40	22.09	0.880
Placebo supplemented	24.40	20.53	0.092
P value	0.449	0.593	
Simpson's index of microbiota diversity			
SF68 supplemented	0.863	0.869	0.851
Placebo supplemented	0.899	0.839	0.050
P value	0.114	0.513	
Shannon–Wiener index of microbiota diversity			
SF68 supplemented	2.457	2.538	0.624
Placebo supplemented	2.689	2.385	0.046
P value	0.079	0.492	

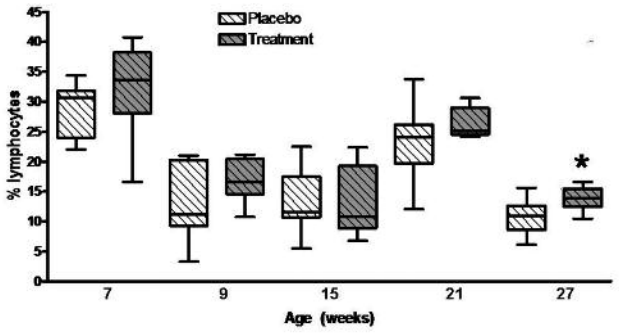
The equilibration period was 14 days and the supplementation period was 140 days. Results of supplementation samples were pooled within cat.

At 9 and 12 weeks of age, a commercially available FVRCP modified live vaccine was administered SQ, and the kittens were followed until 27 weeks of age. The attitudes and behaviors of the kittens were monitored daily throughout the study. Body weight was measured weekly. Blood, saliva, and feces were collected from all cats prior to starting the probiotic or placebo supplementation, at 7 weeks of age, and at 9, 15, 21 and 27 weeks of age. In addition, feces were collected from kittens in the treatment group after the study was completed at 28 weeks of age. For each group of kittens, five fecal samples per day were randomly selected from the shared litter box and scored using a standardized graphic scoring card.

Fecal extracts from samples taken at 9 and 27 weeks of age were analyzed for total IgA and total IgG. Other parameters monitored include randomly amplified polymorphic DNA RAPD-PCR on feces to determine if viable *E. faecium* SF68 was in the stools of treated cats and to assess whether the probiotic was transmitted from the treated kittens to the control kittens. Commercially available ELISAs were used to determine whether *Clostridium perfringens* enterotoxins or *C. difficile* toxins A/B were present in the feces of the kittens. Routine aerobic fecal cultures for *Salmonella* spp. and *Campylobacter* spp. were performed. Complete blood counts, serum biochemical panels and urinalyses were performed to assess adverse events induced by the probiotic. Antigen-specific humoral immune responses were estimated by measuring serum FHV-1-specific IgG, FHV-1-specific IgA, FCV-specific IgG and feline panleukopenia-specific IgG in sera as well as FHV-1-specific IgG and IgA levels in saliva using adaptations of previously published ELISA assays. Total IgG and IgA concentrations in sera, fecal extracts and saliva were estimated using commercially available ELISA assays or radial immunodiffusion assay. Cellular immune responses were assessed via flow cytometry and whole blood proliferation assays. Lymphocytes were stained for expression of CD4, CD8, CD44, MHC Class II, and B cells. In addition, lymphocyte proliferation in response to concanavalin A and FHV-1 antigens was assessed.

Body weight and fecal scores were not statistically different between the two groups over time or at individual time points. Feces from seven of nine treatment cats were positive for SF68 at least at one time point during the study, whereas feces from all control cats were negative for SF68 at all time points. SF68 DNA was not detectible from the feces of any treated cat one week after stopping supplementation (week 28). All samples from placebo cats were negative for SF68 by RAPD-PCR. Neither *Salmonella* spp. nor *Campylobacter* spp. was grown from feces. Numbers of positive samples for *C. difficile* toxins A/B or *C. perfringens* enterotoxin were not significantly different between the groups over the course of the study.

Complete blood counts and biochemical profiles were within normal limits for the age groups of all cats at all time points.



Percent of gated lymphocytes positive for CD4 in peripheral blood by flow cytometry

***Denotes time points at which treatment group was significantly higher than placebo group (p=0.022)**

Figure 1. CD4+/CD8+ cell counts over time in kittens fed either FortiFlora® or a placebo.[10]

A number of immune markers were numerically greater in the SF68 kittens versus the placebo group but did not reach statistical significance. For example, at 21 and 27 weeks of age, the mean levels of FHV-1-specific IgA in serum and saliva were greater in the treatment group when compared to the placebo group. Moreover, the mean FHV-1-specific serum IgG levels were greater in the treatment group when compared to the placebo group at 15, 21 and 27 weeks of age. At 15 weeks of age, the treatment group serum mean FPV-specific IgG levels were greater than those of the placebo group. There were no statistical differences between the groups for any cell surface markers at the first four time points. However, at 27 weeks of age, the treatment group had a significantly higher percentage of gated lymphocytes positive for CD4 (mean 13.87%) than the placebo group (mean 10.61%, p=0.0220, Figure 1).

In this study, we concluded that SF68 was safe to administer to cats and that the increase in CD4+ cell counts in the treatment group compared to the placebo group without a concurrent increase in CD8+ counts at 27 weeks of age demonstrated a systemic immune-modulating effect by the probiotic. Because we did not show a significant increase in lymphocyte stimulation by FHV-1 or an increase in the expression of the memory cell marker CD44 on the CD4+ lymphocytes in the treatment group, the increase in CD4+ T lymphocytes may have been nonspecific as the cells appeared to be unprimed. As the CD4+ T lymphocytes of kittens in this study were not additionally characterized via cytokine production profiles or additional cell surface marker characterization, it could not be determined whether a Th1 or Th2 response predominated. We believed that sample size and/or the duration of the study may have precluded detection of statistical differences between the groups in regard to FPV, FCV and FHV-1 antibody titers.

Chronic Feline Herpesvirus 1 Study

Feline herpesvirus 1 (FHV-1) is extremely common in cats and is frequently associated with morbidity because of recurrent ocular and respiratory disease. In addition, there is no known drug therapy that consistently eliminates the carrier state and vaccination does not provide sterilizing immunity. In this study, it was hypothesized that feeding SF68 would decrease clinical disease, episodes of FHV-1 shedding and numbers of FHV-1 DNA copies shed over time in cats with chronic FHV-1 infection.[11]

Overall, 12 cats with chronic FHV-1 infection were administered either SF68 or a palatability enhancer as a placebo. The cats were monitored for clinical signs of disease and FHV-1 shedding, and evaluated for FHV-1-specific humoral and cell-mediated immune responses as well as for fecal microbiome stability. After an equilibration period, mild stress was induced by changing the housing of the cats from cages to group housing multiple times over a five-month period.

The SF68 was well-tolerated by all cats. Fecal microbial diversity was maintained throughout the study in cats supplemented with SF68 but decreased in cats fed the placebo, indicating a more stable microbiome in cats fed SF68. Upper respiratory signs of disease were not exacerbated in this model of stress. While results varied among cats, those administered SF68 had fewer episodes of conjunctivitis than the placebo group during the supplementation period, suggesting that administration of the probiotic lessened morbidity associated with chronic FHV-1 infection exacerbated by stress (Figure 2).

Murine Acute *Giardia* Study

In previous work, mice administered SF68 and then infected with *Giardia intestinalis* shed fewer trophozoites and less *Giardia* antigen than the placebo group.[14] In addition, supplemented mice had increased CD4+ cells in Peyer's patches and the spleen as well as increased anti-*Giardia* intestinal IgA and serum IgG when compared to untreated mice.

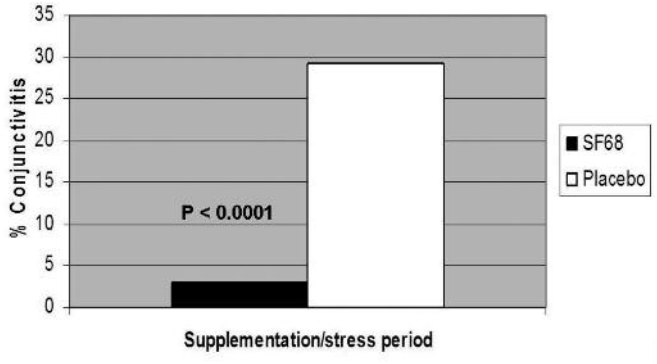
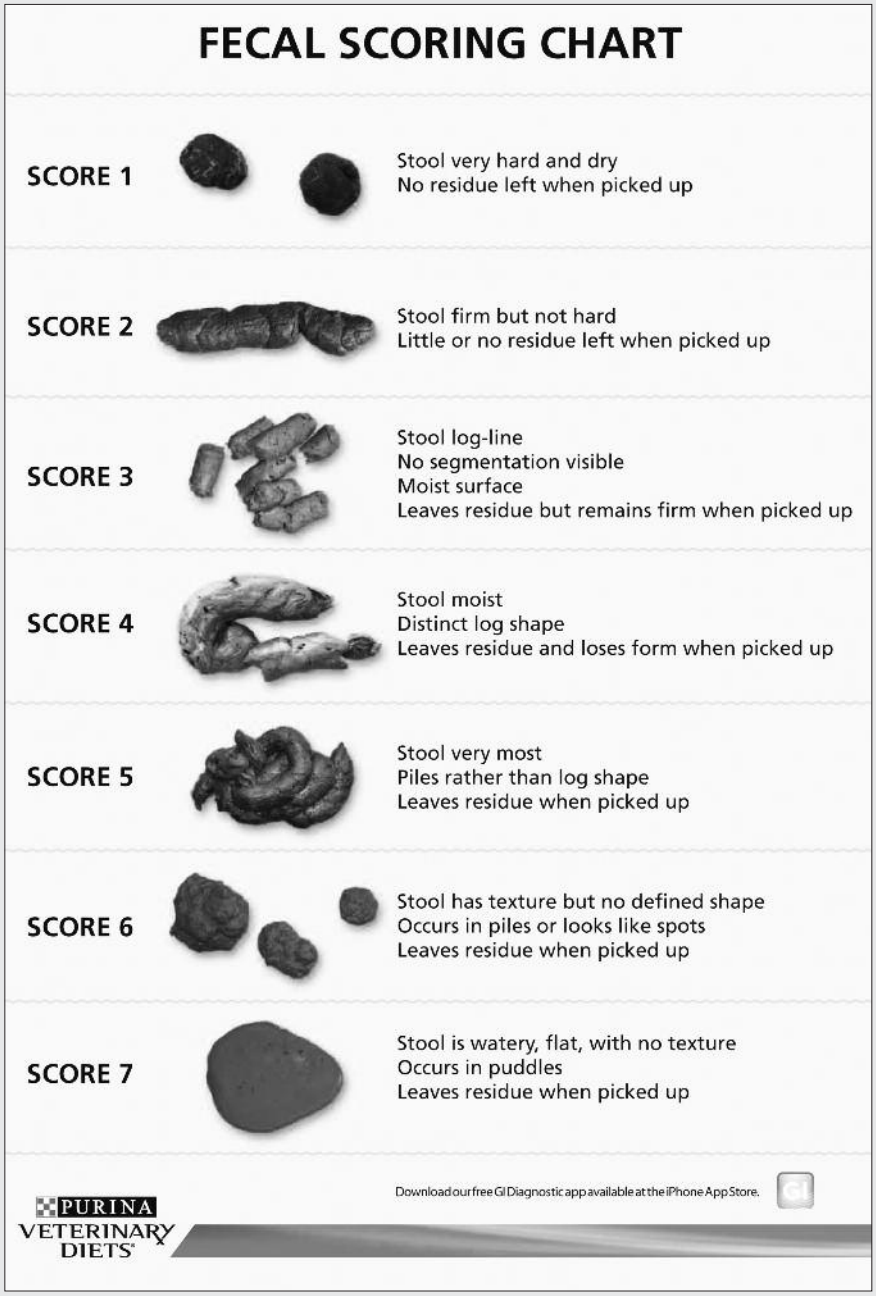


Figure 2. Differences in cumulative conjunctivitis scores from chronic feline herpesvirus 1 infection in cats administered either SF68 (FortiFlora®) or a placebo during the time periods where housing stress was induced as described.[11]

Chronic Subclinical *Giardia* Study in Dogs

When SF68 was administered to 10 adult dogs with chronic subclinical *Giardia* infection, no differences in cyst shedding or fecal antigen testing were found when compared to 10 placebo-treated dogs.[9] There also were no differences between groups in fecal IgA concentrations. In contrast to the mouse study, the dogs were previously infected by *Giardia*, which may have affected the results.[14] In addition, the study was only for six weeks; in the previously discussed puppy study, some of the significant immune-modulating effects were not seen until later in the supplementation period.[12]

Table 2. The Purina Fecal Scoring System for Dogs and Cats[13]



Shelter Animal Acute Nonspecific Diarrhea Study

In a recent study, we hypothesized that cats and dogs housed in an animal shelter that were fed SF68 would have decreased episodes of diarrhea and improved fecal scores compared to untreated cats and dogs in the same environment.[13] The cats and dogs were housed by species in two different rooms in a northern Colorado animal shelter. The cats and dogs were all fed a standardized diet by species. Animals in one room were supplemented daily with FortiFlora,® and animals in the alternate room were supplemented daily with a placebo. Otherwise, management of the rooms was identical for the duration of the study. To reduce risk of a room influence on the results of the study, the room in which cats or dogs were being supplemented with FortiFlora® was switched after one month, with a one-week washout period to lessen the possibility that SF68 surviving in the environment could influence the results of the study.

During the study, routine shelter cleaning and disinfection protocols were being followed. Prior to cleaning the room each morning, feces in each animal's cage were scored by an investigator using the Purina Fecal Scoring System for Dogs and Cats. This person was blinded to the treatment groups. After scoring, feces from dogs with scores from 4 to 7 (indicating mild to severe diarrhea) were collected and transported to Colorado State University for infectious disease testing, which included microscopic examination for parasite eggs, cysts and oocysts after zinc sulfate centrifugation flotation and immunofluorescent antibody testing (IFA) for *Cryptosporidium* oocysts and *Giardia* cysts (Merifluor® *Cryptosporidium/Giardia*, Meridian Bioscience Inc., Cincinnati, OH). The percentages of dogs and cats with diarrhea of >2 days duration were calculated over the course of the study. A generalized linear mixed model using a bionomial distribution with treatment being a fixed effect and the room being a random effect was used to assess for statistical differences between treatment groups. Presence of parasites was included as a covariate. Significance was defined as $p<0.05$.

Diarrhea prevalence rates were low for all dogs in the study, so statistical differences were not detected. However, the percentage of cats with diarrhea >2 days was 7.7% for the probiotic group and 20.7% for the

placebo group (Figure 3). This result was significantly different (p=0.0297). These results suggest that administration of SF68 to cats housed in shelters may lessen the number of days with diarrhea. As this was a short-term study, this effect was likely from probiotic influences on intestinal flora rather than systemic immune-enhancing effects.

Metronidazole and SF68 Study

In one study, dogs with *Giardia* were administered metronidazole alone or with silymarin.[18] While all dogs ceased shedding *Giardia* cysts, the dogs treated with metronidazole and silymarin had several positive clinical findings compared to dogs treated with metronidazole alone, suggesting a beneficial effect for dual therapy.

Based on that study, our research group hypothesized that dogs with nonspecific diarrhea administered SF68 with metronidazole would have better clinical outcomes than dogs administered metronidazole alone.

In the first experiment, we showed that SF68 is resistant to metronidazole, so the two compounds were administered together in the subsequent experiment. In the second experiment, a physical examination was performed on all dogs reported to have a fecal score >4 (Table 2) in an open admission shelter. Stray dogs with diarrhea without vomiting that had a fecal score of >4, interest in food and no clinical findings suggesting a foreign body were included. The fecal score was determined daily by a person masked to the treatment groups. All dogs were fed a standardized diet and were administered metronidazole USP at 25 mg/kg, PO twice daily for seven days. The dogs were randomized to be administered SF68 (treatment) or a placebo mixed with their food daily for seven days. SF68 and the placebo were provided in separate coded and marked capsules, and none of the investigators at the research facility knew which capsule contained which product.

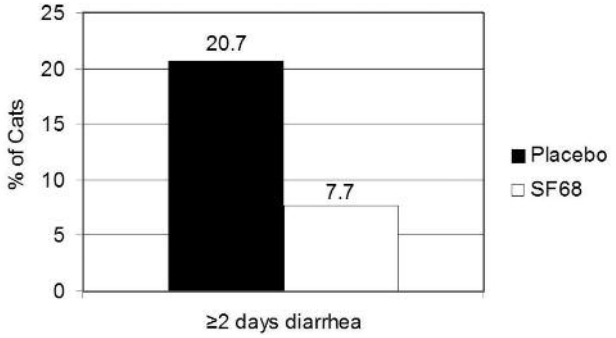


Figure 3. Differences in the prevalence rates of diarrhea for two days or longer in cats fed either FortiFlora® or placebo in an animal shelter in north central Colorado. There was a lower prevalence of diarrhea of two days or longer in the cats fed FortiFlora® in the stray room and when the results from the two rooms were combined. The results between groups are significantly different.[13]

Feces collected prior to treatment were analyzed by fecal flotation, fluorescent antibody assay for *Giardia* cysts and *Cryptosporidium* spp. oocysts, and *Clostridium perfringens* enterotoxin assay. Proportions of dogs in each group to have a fecal score of <4 by day seven were compared by Fisher's Exact Test. Speed to improvement was defined as the first day the score dropped two points from day 0 or a fecal score of 4 was reached and sustained for two consecutive days. Mean values were compared by two-tailed T test. Significance was defined as P<0.05 in both analyses.

A total of 48 dogs were entered into the study at the time this paper was submitted. Thirty-three dogs (16 treatment, 17 placebo) completed the study. Overall, 50% of the treatment group and 29.4% of the placebo group had fecal scores <3 by day seven (p=0.3). However, speed to improvement was faster (p=0.036) for the treatment group (mean=2.8 days) compared to the placebo group (mean=4.4 days). In these dogs, administration of SF68 resulted in a faster speed to improvement than administration of metronidazole alone, suggesting a positive effect induced by the probiotic.

Conclusion

The evidence gathered to date suggests that FortiFlora® has immune-modulating effects in dogs and cats and can be used to aid in the management of select gastrointestinal disorders.

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Useful GI Function Tests and Molecular Tools for Veterinary Clinicians

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Abstract

The mucosal immune system is at the forefront of defense against invading pathogens but, at the same time, must maintain tolerance toward commensals and food antigens in the intestinal lumen. The interplay between the innate immune response and commensal microorganisms is essential in this process. Great progress has been made to identify some of the genetic predispositions underlying inflammatory bowel disease (IBD) in certain breeds, such as the German Shepherd Dog. Some immunological markers, such as cytokine measurement, immunohistochemistry for p-glycoprotein expression, perinuclear anti-neutrophil cytoplasmic antibodies and PCR for antigen receptor rearrangement, are discussed for their clinical usefulness in the diagnosis and management of IBD.

Glossary of Abbreviations
ARD: Antibiotic Responsive Diarrhea
CCECAI: Canine Chronic Enteropathy Clinical Activity Index
DCs: Dendritic Cells
IBD: Inflammatory Bowel Disease
NOD2: Nucleotide Oligomerization Domain 2
PARR: Polymerase Chain Reaction for Antigen Receptor Rearrangement
PRR: Pathogen-Recognition Receptors
TLRs: Toll-Like Receptors

Introduction

Among the causes of chronic enteropathies in dogs, adverse reactions to food, idiopathic inflammatory bowel diseases and antibiotic responsive diarrhea (ARD) are common. These disorders are retrospectively diagnosed by their response to treatment.[1] The clinician faced with a case usually performs an extensive workup to exclude extragastrointestinal causes as well as treatable disorders, such as pancreatic diseases, chronic parasitic or bacterial infections, and tumors. After taking intestinal biopsies and reaching a tentative diagnosis, the gold standard approach to treatment is a trial therapy with elimination diet, antibiotic treatment for several weeks and finally immunosuppressive treatment with corticosteroids.

The last decade brought advances in knowledge about the pathogenesis of IBD in people; specifically, the interplay of innate immunity receptors with commensals of the intestinal microbiome is now implicated in the disease. Molecular studies have identified specific imbalances in the microbiome of people with IBD. In addition, genetic polymorphisms associated with an increased risk of development of IBD have been identified. These data promise to help in the development of new treatment options for IBD, including probiotics and targeted molecular treatment strategies. This article reviews the newest findings in canine IBD and discusses how they could lead to the development of new therapeutic targets.

Histology and Assessment of T-Cell Infiltration

Sampling of intestinal biopsies is considered an essential step to exclude neoplastic causes and confirm the presence of intestinal inflammation. However, the interpretation of intestinal biopsies is difficult and subject to controversy. In several recent studies looking at conventional histological interpretation of intestinal biopsy samples, no correlation of clinical activity with histological grading either before or after therapy was found.[1,2] In addition, total lymphocyte counts as well as the number of infiltrating CD_3 cells in the lamina propria were not good markers for clinical activity of disease, as there was no difference in cell counts before and after treatment.[2]

These findings suggest that the type and degree of histological infiltrates in canine IBD may not be as helpful as in human medicine, where the clinical scores correlate well with the histological grading. Therefore, a new grading scheme for the histological interpretation of endoscopically obtained biopsies from dogs and cats with IBD has recently been published by the WSAVA working group. The findings from this group suggest that microarchitectural changes seem to be significantly more important than cellular infiltrates when assessing histological severity of disease. However, so far, there is limited information on how well this new grading system correlates with clinical disease. Further prospective studies will assess this grading system in conjunction with clinical findings and outcomes in dogs and cats with IBD.

Evidence of Innate Immunity Hyper-Responsiveness in Canine IBD

Toll-like receptors (TLRs) are upregulated in the intestine of humans with Crohn's disease and ulcerative colitis. These receptors are responsible for recognizng specific microbe-assisted patterns of bacteria, viruses and fungi and are expressed on immune cells as well as intestinal epithelial cells. They form an important part of the barrier of the intestine toward the antigens in the intestinal lumen as they are intricately involved in the decision-making process of the gut immune system as to whether an antigen is self or non-self. The change in TLR expression may be either a consequence of the ongoing stimulation of TLRs by an altered

microbiota or may be a causal factor contributing to the pathogenesis of disease. Most human studies show that the mRNA and protein expression of TLR2 as well as TLR4 are increased in the intestines of people with active IBD.

In a recent clinical study at the Royal Veterinary College (RVC), London, we were able to show that dogs of any breed with clinically severe, active IBD express higher levels of TLR2 receptors in the duodenum compared to healthy dogs when measured by real-time PCR (polymerase chain reaction) in endoscopic biopsies. In addition, TLR2 expression was correlated with the clinical severity of IBD using the Canine Chronic Enteropathy Clinical Activity Index (CCECAI).[6,7] However, TLR4 expression levels were similar to those in healthy canine intestines.

Other studies have found that only a subgroup of dogs with IBD (the ones responding only to steroid administration) showed an increased expression of TLR2, TLR4 and TLR9, compared to healthy intestines when expression was measured by real-time PCR. In further studies looking at German Shepherd Dogs, we found that TLR4 expression was 60-fold higher in the duodenum, ileum and colon of dogs with IBD compared to samples from healthy dogs; however, TLR2 and TLR9 were similarly expressed.[8] These data show that it is important to look at similar phenotypes of dogs when choosing cases for such studies, as the results vary depending on the severity of disease, the treatment response and the specific breed of dog.

In addition, care must be taken to compare studies using real-time PCR as the standardization method depending on the reference genes, which need to be carefully chosen for each study. TLR2 has recently been shown to be overexpressed in the diseased intestine in mouse models of IBD.[9] TLR2 in this context is implicated in the homeostasis and repair of intestinal tissue after injury. It is therefore possible that the high expression of TLR2 found in dogs with IBD in the studies mentioned above could be a marker of intestinal inflammation, and its physiological action is to downregulate ongoing inflammation. TLR5 expression was consistently downregulated in the intestines of German Shepherd Dogs with IBD as compared to healthy dogs.[8]

In mice and humans, TLR5 is highly expressed in the healthy small intestine, with CD11c+ dendritic cells (DCs) in the lamina propria mucosa expressing most TLR5. It is believed that this tolerogenic phenotype of DCs induces T regulatory cells and stimulates the production of anti-inflammatory cytokines, such as IL-10 in response to flagellin. In contrast, in intestinal inflammation characterized by the upregulation of Th1– and Th17 cytokines, CD11c- DCs express low levels of TLR5 but high levels of TLR4. In this context, TLR4 is thought to be upregulated to compensate for the low TLR5 expression.

It could be speculated that the differentially low expression of TLR5 and high expression of TLR4 seen in the intestines of the German Shepherd Dogs in our study indicate a similar compensatory role of TLR4, as gram-negative flagellated bacteria can also be recognized through binding of LPS by TLR4. Figure 1 shows the current concept of how IBD develops in people and in dogs.[10]

PCR for Antigen Receptor Rearrangement to Diagnose Intestinal Lymphoma

Polymerase chain reaction for antigen receptor rearrangement (PARR) amplifies the highly variable T or B cell antigen receptor genes and is used to detect a clonally expanded population of lymphocytes. In a recent study at the RVC, we prospectively evaluated the accuracy of PARR in diagnosing lymphoma from biopsies obtained endoscopically compared to the gold standard of histopathology and clinical outcome (determined by follow-up information of at least two years) (Gajanajake, et. al. Abstract. ECVIM.2008).

Samples from 39 dogs were included in the study. Five dogs had a diagnosis of lymphoma, of which four were positive on PARR. One dog was diagnosed with an intestinal carcinoma, three with a gastric carcinoma (with concurrent inflammation in the intestine), and 30 with inflammatory bowel disease. Five dogs with IBD and two dogs with carcinoma were positive on PARR. Of the five dogs with IBD that were positive on PARR, four were clinically well on follow-up but one had been euthanized due to the development of jaundice. This indicated a sensitivity and specificity of 80% and 79%, respectively, for PARR to correctly identify cases of canine gastrointestinal lymphoma when

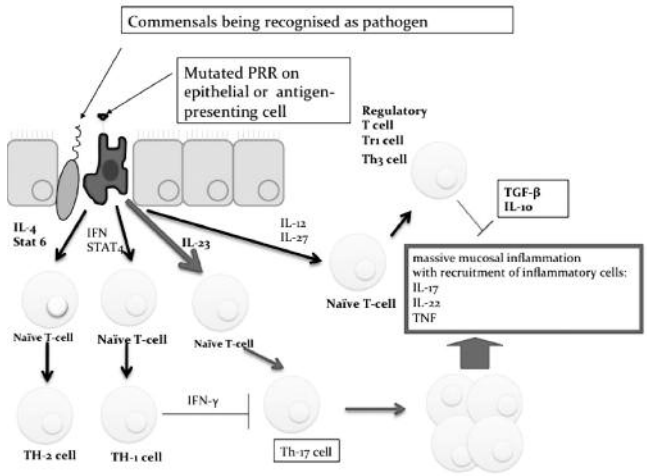

Figure 1. Proposed pathogenesis of inflammation in canine and feline IBD.[13] In the case of IBD, a primary defect in the recognition of commensals or pathogens by innate immunity receptors may play a role. Mutations in pathogen-recognition receptors (PRR) lead to misrepresentation of commensals as pathogens, which results in production of IL-23, driving naïve T cells to differentiate into Th17 cells. These Th17 cells now produce large amounts of proinflammatory cytokines, such as IL-17, and TNF. This leads to tissue destruction and epithelial cell injury, which lets even more antigens pass through to the lamina propria. This inflammatory response cannot be counter-regulated anymore by regulatory T cells, which leads to the characteristic inflammatory pattern seen in IBD.

compared to histopathology and clinical outcome as a gold standard. The data derived from this pilot study indicate a noteworthy false positive rate (7/36 cases) for PARR when used on endoscopic biopsies to diagnose cases of canine intestinal lymphoma. The conclusion that a positive PARR test on an endoscopic biopsy means a diagnosis of lymphoma must therefore be made cautiously in a clinical situation.

Imbalance of the Intestinal Microbiota in Canine IBD

Molecular studies on the intestinal microbiome in dogs of different breeds with IBD have found that members of the families *Enterobacteriaceae* and *Clostridiaceae* were enriched in the diseased intestine. These bacteria are thought to contribute to the pathogenesis of disease in dogs as well as humans with IBD. There seems to be differences in the microbiome of different dog breeds that are predisposed to the development of IBD. It appears that German Shepherd Dogs with CE have a distinctly different microbiome from healthy dogs, as well as from other breeds of dogs presenting with IBD, with overrepresentation of certain traditionally labelled "beneficial" bacteria in the duodenum, specifically sequences of the order of *Lactobacilalles*. This may indicate why many German Shepherd Dogs respond to dietary and/or antibiotic treatment alone, whereas in other breeds with CE, immunosuppressive treatment is often necessary to control clinical signs.

Genetic Predisposition in German Shepherd Dogs with IBD

Over the last decade, numerous genes have been found to be associated with an increased risk of development of IBD in humans, many of them implicated in the innate immune response in the intestine.[11] Mutations in pathogen recognition receptors, such as nucleotide oligomerization domain 2 (NOD2), toll-like receptor 4 (TLR4), IL-23 receptor and others, have all been associated with IBD.[11] In dogs, it has long been obvious to clinicians that IBD seems to have a genetic component. This is particularly evident in breeds like the Boxer, which is predisposed to histiocytic ulcerative colitis. Another breed with a predisposition to the development of IBD is the German Shepherd Dog, which seems to be predisposed to antibiotic-responsive diarrhea. We recently found that several polymorphisms in TLR4 and TLR5 are significantly associated with IBD in German Shepherd Dogs.[12] One polymorphism in TLR5 also seems to be more widely implicated in the pathogenesis of IBD in dogs in general. The next step will be to evaluate correlations between such polymorphisms and the particular phenotype expressed in different breeds in order to make the genetic assays useful to the practitioner.

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Protein-Losing Enteropathy: The Beginning of the End?

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Abstract

Intestinal protein loss is a sign of failure of digestive function that often results from severe acute or chronic inflammatory lesions or from a disruption of chyle absorption and intestinal lymph flow. Early recognition of the syndrome and identification of the cause are important. A systematic diagnostic approach is required to rule out other causes of hypoproteinemia and to identify the underlying intestinal disease. Significant hypoalbuminemia (serum albumin <20 g/l) has been identified as a negative prognostic factor for chronic idiopathic enteropathies in the dog. Treatment of protein-losing enteropathy focuses on optimizing the diet and addressing the underlying intestinal disease. Acute oncotic support may be required in animals with severe panhypoproteinemia.

Glossary of Abbreviations
GI: Gastrointestinal
IBD: Inflammatory Bowel Disease
IL: Intestinal Lymphangiectasia
PLE: Protein-Losing Enteropathy
PLN: Protein-Losing Nephropathy

Objectives

- To describe a systematic diagnostic approach to confirm the intestinal protein loss and identify the cause of the problem
- To review the main causes of protein-losing enteropathy (PLE) in dogs
- To provide updated therapeutic options and discuss the prognosis of various forms of PLE

Introduction

Intestinal protein loss is a sign of failure of digestive function that may result from severe acute or chronic inflammatory lesions or from a disruption of chyle absorption and intestinal lymph flow. While the exact mechanisms leading to intestinal protein loss have not been elucidated in the dog, the three basic mechanisms defined for humans with PLE likely also apply to canine PLE. Protein loss may result from: 1) erosive or ulcerative mucosal lesions causing secondary exudation of proteins; 2) lymphatic dysfunction causing leakage of protein rich lymph into the intestinal lumen; and/or 3) mucosal changes disturbing the "mucosal barrier," causing abnormal permeability and protein leakage into the lumen.[1]

This presentation will focus on chronic intestinal disorders associated with intestinal protein loss in dogs. PLE is much less prevalent in cats. In dogs, it is frequently associated with severe chronic idiopathic inflammatory enteropathies, such as inflammatory bowel disease (IBD), or with idiopathic intestinal lymphangiectasia in specific breeds.

Diagnostic Approach

Dogs with PLE often present with clinical signs typical of chronic intermittent small intestinal diarrhea with possible vomiting. In severe cases, dysorexia/anorexia and malnutrition with evidence of malabsorption and weight loss may be observed. However, significant intestinal protein loss and hypoalbuminemia may also occur without obvious diarrheic episodes. In some dogs, hypoalbuminemia may even be detected incidentally during regular health screens. In the presence of severe hypoalbuminemia (serum albumin <20 g/l, often ≤ 15 g/l), the main complaint may relate to signs suggestive of significantly decreased oncotic pressure (cavitary effusion, subcutaneous edema).

The first diagnostic challenge consists in establishing the origin of the protein loss. To this effect, a minimal diagnostic database should be collected (CBC, chemistry panel, urinalysis). Renal protein loss must be ruled out (urinalysis, urine protein-creatinine ratio), as well as liver dysfunction (postprandial serum bile acids). Additionally, third spacing of serum proteins should be considered (e.g., vasculitis). Generally, PLE is associated with panhypoproteinemia due to nonselective protein loss. Hypoalbuminemia with normal or increased globulin concentration is suggestive of protein-losing nephropathy or possibly liver dysfunction. While these rules of thumb are useful in practice, they should not be blindly relied upon since many exceptions occur. For instance, a dog with significant systemic inflammation (e.g., histoplasmosis) may present with hypoalbuminemia and hyperglobulinemia. Other common abnormalities of dogs with PLE include hypocholesterolemia, hypocalcemia (total and ionized), hypomagnesemia, and lymphopenia.

Once the GI tract has been confirmed as the site of protein loss, further workup should include abdominal ultrasound with a focus on the intestinal wall, in particular wall thickness and wall layering. The ultrasonographic appearance of the intestinal wall consists of five distinct layers. Hyperechogenic mucosal striations are frequently observed in dogs with PLE (Figure 1) and appear to be quite specific. It has been postulated that they may represent dilated lacteals, although they may also be due to dilated crypts often seen in PLE or to other mechanisms. Striations should not

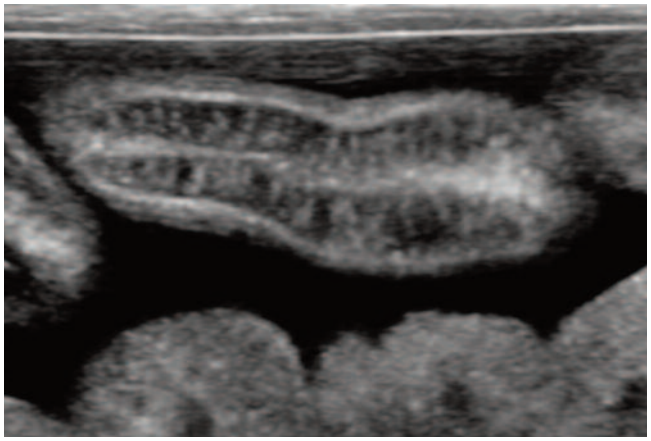

Figure 1: Abdominal ultrasound from a 5-year-old neutered male Yorkshire Terrier with lymphangiectasia and crypt disease. Note the presence of free abdominal fluid (ascites) between the organs. An oblique cut through a small intestinal loop is visible. The layering of the intestinal wall is abnormal, and vertical hyperechogenic striations can be seen in the otherwise hypoechogenic mucosal layer. Intestinal mucosal striations have been reliably associated with PLE in dogs.

be confused with hyperechogenic mucosal speckles that are only a nonspecific indicator of inflammation.[2]

However, the final diagnosis relies solely on histopathologic analysis of intestinal biopsies collected during endoscopy or exploratory laparotomy. Dogs with severe hypoalbuminemia are poor anesthetic candidates, and it is sometimes preferable to avoid taking excessive risks and postpone endoscopy or surgery. Additionally, many dogs with PLE have bicavitary effusion, and thoracic radiographs are recommended as a screening tool for the presence of thoracic effusion, which may represent an additional anesthetic risk. Synthetic (hydroxyethylated starches) and natural colloids (plasma, human or canine albumin concentrates) are very useful in order to acutely increase oncotic pressure in critical cases. Despite the risk of anaphylactic reaction or other complications, slow transfusion of 5% human albumin at 2 ml/kg/h during 10 h/day (total daily volume of 20 ml/kg/day) has been successful for partial restoration of serum albumin concentration in order to minimize the risks of general anesthesia.[3]

The decision regarding the preferred biopsy collection technique depends on a variety of factors, such as availability of the equipment and surgical or endoscopic skills of the veterinarian. Advantages of a surgical exploration include the possibility of sampling several sites along the small intestine and obtaining full thickness specimen. Surgical collection of intestinal biopsies was not shown to be more risky in hypoalbuminemic patients,[4] although a cautious approach is recommended (consider serosal patching). Endoscopy allows minimally invasive collection of biopsies limited to the mucosa, and good endoscopic skills are required to obtain quality specimen. However, visualization of the mucosa is an advantage, and it allows targeted sampling of mucosal lesions (Figure 2). Traditionally, only the duodenum was examined. Recent studies convincingly demonstrated that collecting both duodenal and ileal biopsies is essential, as lesion distribution may be irregular and severe ileal lesions may occur in a dog with only mild (or absent) duodenal lesions.[5] This added procedure may prolong anesthesia time since a colonoscopy is required to intubate the ileum or at least collect ileal mucosal biopsies by blindly passing a forceps through the ileo-colic junction. However, the improved diagnostic yield often outweighs the inconvenience of a prolonged procedure.

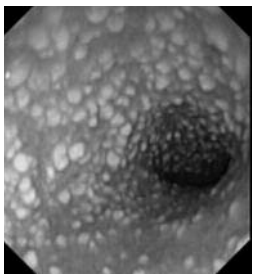

Figure 2: Appearance of the duodenal mucosa at the time of endoscopy in a 4 year-old spayed female Yorkshire Terrier with PLE due to primary IL. The numerous "with spots" are thought to represent enlarged villi secondary to lacteal dilation.

Differential Diagnosis

Diseases frequently associated with PLE include intestinal lymphangiectasia,[1,6] IBD[1,7] and chronic enteropathies characterized by significant mucosal architectural changes, such as dilation of small intestinal crypts.[1,8] Moreover, alimentary lymphoma[9] and intestinal histoplasmosis[10] may also cause PLE.

Intestinal Lymphangiectasia (IL): The following breeds have been shown to be prone to primary IL: Yorkshire Terriers, Chinese Shar-Pei, Maltese, Norwegian Lundehunds, and Rottweilers (in Europe).[1,6,11,12] The pathogenesis of primary IL is still poorly understood. It results from obstruction to the flow of lymph in the intestinal wall, which could conceivably be due to abnormal intestinal lymphangiogenesis. However, acquired obstruction to normal lymph flow appears to be a more common occurrence in granulomas associated with lymph leakage impinging on intestinal lymphatics and/or intestinal lymphangitis. Secondary IL is commonly associated with significant intestinal mucosal inflammation (e.g., IBD, fungal diseases) and neoplasia (alimentary lymphoma). Histopathologic mucosal changes include dilated lacteals in the mucosa (Figure 3) and deep-seated perilymphatic granulomas that can be seen in full thickness biopsies. Lacteals are essential for fat absorption, and their obstruction leads to severe dilation and tear. Damaged lacteals empty their lipid- and protein-rich content into the intestinal lumen.

Inflammatory Bowel Disease (IBD): A detailed review of IBD is beyond the scope of this paper. The term IBD describes "a group of chronic enteropathies characterized by persistent or recurrent gastrointestinal (GI) signs and inflammation of the GI tract."[7] The inflammatory process located in the GI mucosa may lead to protein loss both by preventing the absorption of nutrients and by compromising the integrity of the intestinal mucosal barrier

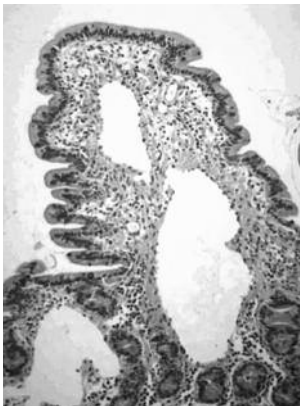

Figure 3: Same dog as in Figure 2. Histology of a duodenal mucosal biopsy specimen. Dilated lacteals are visible and cause dilation of the villus (H&E stain).

leading to exudation of proteins into the intestinal lumen.

PLE of Soft Coated Wheaten Terriers is a specific form of IBD affecting this breed worldwide. In approximately 50% of these dogs, PLE and protein-losing nephropathy (PLN) occur concurrently. Mucosal lesions can be severe and include inflammatory infiltration, dilated lacteals and deep-seated intestinal lymphangitis. While the pathogenesis is still poorly understood, a hypersensitivity component has been documented, as clinical episodes can be triggered by specific proteins.[13,14]

Crypt Disease: Since Willard first reported six dogs with crypt lesions 12 years ago,[8] crypt dilation and necrosis have been frequently associated with PLE.[12,15] Crypt dilation is a mucosal architectural change that is relatively frequently observed in dogs with IBD and IL (Figure 4). However, in some cases, crypt dilation and abscesses may be the only detectable mucosal lesions in dogs with PLE. In a recent, yet-unpublished study of 58 dogs with chronic enteropathies, the author's group showed that dogs with histologically documented small intestinal crypt abscesses are more likely than dogs with no such lesions to experience significant hypoalbuminemia due to PLE, to show ultrasound changes of their intestinal mucosa, and to experience more severe clinical signs.[a]

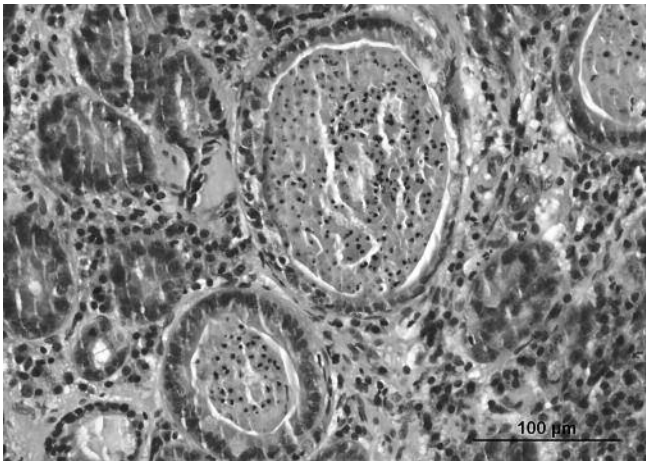

Figure 4: Histopathological appearance of the duodenal mucosa in a dog with PLE. Note the dilated crypts that are filled with mucus, cellular debris and neutrophils (crypt abscesses). Courtesy of Dr. N. Wakamatsu, LSU (H&E stain, 20x objective).

Therapy

The two main components of treatment in dogs with PLE are dietary modification and management of the inflammatory process.

Diet: Dogs with PLE are in a catabolic state, and adequate nutrition is essential. There currently are no published studies critically evaluating nutritional aspects of canine PLE; however, a large body of clinical experience is available. In dogs with *primary idiopathic IL*, dietary modification centers on feeding a highly digestible diet with low to very low fat content (10-15% on a dry matter basis) to prevent further dilation and rupture of lacteals. Additionally, the diet should contain highly bioavailable dietary proteins and be low in crude fiber. While drug therapy may be administered for a few months (see below) and then discontinued in some cases, dietary therapy should probably be maintained throughout the dog's life. In dogs with *PLE associated to underlying IBD*, many veterinary gastroenterologists report success with exclusive feeding of a diet consisting of hydrolyzed proteins. Novel protein diets are an alternative approach.

Acceptance of the diet is a critical issue in PLE dogs, particularly in the most severely affected animals, which may be anorexic. For each patient, the veterinary care team needs to identify the most palatable diet. Initially, it may be more important to feed a less optimal diet that the dog will be interested in eating and progressively transition to a more desirable diet. Elemental diets only contain free amino acids including tglutamine, carbohydrates and reduced fat (e.g., Vivonex TEN,® Peptamen HN®). In dogs with severe IBD and PLE, they may be administered via feeding tubes to provide the necessary nutrients with minimal risk of disease flare. Attention should be paid to their osmolality. Elemental diets are expensive, and there are no published studies documenting their benefits in dogs with IBD.

Management of Inflammation: In dogs with primary IL, anti-inflammatory glucocorticoid therapy (e.g., prednisone at 1 mg/kg/day) is useful and often required for proper management of the disease. Its main desired effect is to decrease inflammation associated with lipogranulomas secondary to chyle leakage and therefore help restore an adequate flow in intestinal lymphatics. In some dogs, anti-inflammatory treatment can be slowly weaned over two or three months or longer.

Immunosuppressive Therapy: Immunosuppression is the basis for treatment of severe IBD with PLE. As a side note, it is important to remember that chronic immunosuppression may make animals more susceptible to developing severe infections after contact with pathogens or opportunistic microorganisms.

The first approach consists of administering *prednisone* or *prednisolone* using the following protocol: Start with 2 mg/kg q12 h for three to five days, then switch to 2 mg/kg once daily until the dog's condition has significantly improved and appears stable. Subsequently, the dose can be decreased in two-week steps with 1 mg/kg/day, then 1 mg/kg every other day, and so on. However, side effects of steroid therapy may compromise owner's compliance.

Other corticosteroids: *Budesonide* has gained in popularity in the treatment of canine IBD. In humans, the drug is known to be locally efficient and undergo high first-pass hepatic metabolism. Therefore, systemic complications of steroid treatment are less likely. In dogs, the drug significantly influences the pituitary-adrenal axis.[16] To date, budesonide use in dogs or cats with IBD has not been evaluated critically and only anecdotal reports are available. Furthermore, there is no data on the pharmacokinetics of the orally administered drug in pets. The recommended doses are 0.5-3 mg/dog daily (depending on the dog's size). The drug needs to be reformulated by a compounding pharmacist for use in small dogs. Concurrent use with other glucocorticoids is not recommended.

Azathioprine is a thiopurine drug that may be used in dogs with steroid-refractory IBD and in those that relapse when weaned off prednisone treatment. It also may be combined with prednisone in the initial treatment of severe cases of IBD. Azathioprine is generally well-tolerated, but side effects include bone marrow suppression, hepatotoxicity and pancreatitis. Regular monitoring of CBC and biochemistry profile is advisable during the first weeks to months of treatment. The initial dose is 2 mg/kg daily for three weeks, then 1-2 mg/kg every 48 h. Up to three weeks of treatment may be necessary for the drug to reach maximal effect.

Chlorambucil is an alkylating agent. It is mostly used in conjunction with prednisolone in cats with low-grade alimentary lymphoma or refractory IBD. A recent study from the UK compared the survival of 27 dogs with chronic enteropathies and PLE with serum albumin concentration <18 g/l receiving a prednisolone and chlorambucil combination (n=14) versus dogs treated with prednisolone and azathioprine (n=13). At recheck, dogs receiving chlorambucil and prednisolone had gained more weight and their serum albumin concentration was significantly higher than in the other group. Also, the survival was greatly improved using the chlorambucil combination.[17] The recommended initial canine dose of chlorambucil is approximately 4 mg/m,^{2} q24-48h, and it comes in 2 mg tablets (the drug will need to be appropriately reformulated or compounded for small dogs). Side effects of chlorambucil are rare but include bone marrow suppression. A CBC should be performed after one and three weeks of treatment and repeated every two to three months or if the dog's condition deteriorates (look for signs of neutropenia).

Cyclosporine is an inhibitor of T-cell function. In a 2006 study, pharmacokinetics of cyclosporine in dogs with IBD were not significantly different from those of normal dogs. Fourteen dogs with steroid-refractory IBD were enrolled, and eight dogs (57%) went into complete remission within four weeks of cyclosporine treatment (5 mg/kg PO once daily). Additionally, three dogs experienced partial remission, while two dogs did not respond and were euthanized. Furthermore, one dog relapsed after 14 weeks despite initial successful treatment. Transient adverse effects were seen during the first two weeks of treatment in five dogs and included vomiting and loss of appetite in four dogs and hair coat changes and gingival hyperplasia in one dog. Most side effects responded to temporary discontinuation followed by dose reduction. Cyclosporine treatment was discontinued in eight of the 11 responders, which subsequently remained free of clinical signs. The owners of the remaining three dogs elected to continue treatment for several additional months, and the dogs remained apparently healthy.[18] Monitoring whole blood or plasma concentration of cyclosporine is controversial. In dogs that regularly vomit one to two hours after oral administration, it is possible that serum cyclosporine concentration peak reaches toxic levels, and splitting the daily dose may be beneficial.

Other immunosuppressive drugs, such as *mycophenylate mofetil, methotrexate* and *leflunomide*, have been used to treat immune-mediated or autoimmune diseases in dogs. Due to lack of data and possible side effects on the intestinal mucosa, their use for treatment of IBD in dogs cannot be recommended at this time.

Complications

Hypocobalaminemia: Low serum cobalamin (vitamin B12) concentrations are commonly found in dogs with PLE, especially in the presence of underlying IBD. Deficiency in vitamin B12 has negative effects on the intermediary metabolism and may delay proper healing of intestinal inflammation. Hypocobalaminemic dogs are initially treated with weekly SC injections of vitamin B12 (from 250 to 1500 μg/dog) for six weeks. If the treatment is successful, the interval between injections may be increased to two weeks for another six weeks.

Hypercoagulability: Recent studies using thromboelastography have revealed the high prevalence of hypercoagulability in dogs with PLE,[19] which significantly increases the risk of potentially fatal thromboembolic events. The problem may be compounded by the pro-thrombotic effects of glucocorticoids that are often used for treatment. Interestingly, hypercoagulability does not appear to resolve after successful treatment of PLE,[19] and this raises questions as to the pathogenesis of this complication. In dogs with documented hypercoagulability, administration of low doses of aspirin (0.5-1 mg/kg/day) and/or clopidogrel (1-5 mg/kg/day) should be considered in order to prevent thrombosis. However, there currently is no study confirming the beneficial effect of such a therapeutic regimen.

Hypocalcemia: A significant decrease of total calcium is expected in dogs with moderate to severe hypoalbuminemia since 50% of total calcium is bound to albumin. However, ionized calcium may also be abnormally low in dogs with PLE.[20, 21] Low serum ionized calcium concentration occurred in association with low 25-hydroxyvitamin D and increased levels of parathyroid hormone in a recent series of dogs with PLE.[21] The authors of the study postulated that hypovitaminosis D was due to intestinal loss rather than to malabsorption since a control group of dogs with IBD without PLE had normal 25-hydroxyvitamin D levels, and serum 25-hydroxyvitamin D concentration correlated with

serum albumin concentrations.[21] Correction of moderate to severe hypocalcemia with parenteral administration of 10% calcium gluconate (e.g., 1 ml/kg slowly IV over at least 15 to 30 minutes may also be administered SC after 1:1 dilution with saline to a maximum daily amount of 9 ml/kg given in three to four doses), and vitamin D is advisable to prevent the onset of clinical signs. Concurrent hypomagnesemia may compromise the success of treatment and should be corrected.[20]

Prognosis

In two European studies of a total of 150 dogs with chronic enteropathies, hypoalbuminemia (serum albumin <20 g/l) was associated with a less favorable outcome.[22, 23] This was confirmed in a preliminary report from a recent North American study, although outcome did not appear to be correlated to severity of hypoalbuminemia.[24]

Idiopathic Intestinal Lymphangiectasia: Preliminary reports from a few studies show a high mortality among Yorkshire Terriers with IL (50-60%).[25, 26] However, results from the UK revealed that the presence of dilated lacteals was associated with a better outcome in a group of 27 dogs with PLE.[17] In the author's practice, a significant proportion of Yorkshire Terriers with IL responds well to a strict diet alone or with anti-inflammatory doses of glucocorticoids. The proportion of refractory cases seems to vary according to geographical location. Unfortunately, there are no known parameters that allow early segregation of dogs likely to be refractory to dietary and steroid treatment. It would be useful to initiate early aggressive treatment in difficult cases.

Crypt Disease: In a series of 58 dogs with chronic enteropathies, the author's group found that the presence of crypt abscesses in the small intestine was associated with significantly shorter survival.[a]

Footnotes

[a] Stroda K, Wakamatsu N, Kearney M. and Gaschen F. Unpublished results (2012).

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Clinical Diagnosis and Management of Canine Acute Pancreatitis

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Abstract

Acute pancreatitis in dogs remains a challenging disease, with many unanswered questions regarding optimal diagnostic criteria and treatment options. Diagnostic assays with high sensitivity provide a good ability to detect disease. High sensitivity is associated with a low rate of false-negative diagnoses. The specificity of an assay refers to its ability to differentiate the disease from other influences. A high specificity is associated with a low frequency of false-positive diagnoses. This paper discusses recent research surrounding diagnosis and some potential areas of treatment where further investigation may be beneficial.

Glossary of Abbreviations

APACHE: Acute Physiology and Chronic Health Evaluation
CART: Classification and Regression Tree
CIRCI: Critical Illness-Related Corticosteroid Insufficiency
LRS: Lactated Ringer's Solution
NG: Nasogastric Feeding
NJ: Nasojejunal Feeding
NK1: Neurokinin 1
PAP: Pancreatitis-Associated Protein
PE-1: Pancreatic Elastase-1
PPIs: Proton Pump Inhibitors
SAA: Serum Amyloid A
SIRS: Systemic Inflammatory Response Syndrome
TPN: Total Parenteral Nutrition
XO: Xanthine Oxidase

Diagnostic Advances

Canine Pancreatic Lipase

Canine pancreatic lipase is a recently established laboratory test (first as a radioimmunoassay and then as an enzyme immunoassay) that has been well-validated and is now widely used.[1,2] The premise of this test is that it measures lipase originating in the pancreas, and therefore lipase values only should be increased in pancreatic inflammation.[3] Immunolocalization studies have detected pancreatic lipase only within pancreatic tissue of dogs, and serum concentrations in dogs with absent exocrine pancreatic function were decreased.[4,5]

The current commercially available test for specific canine pancreatic lipase (spec-CPL) is a sandwich ELISA, using a recombinant peptide as the antigen and monoclonal antibody. This new commercially available assay shows a good correlation to the original assay, as well as high reproducibility,[6] although there has been a shift in the reference intervals for the diagnosis of pancreatitis, with results ≤200 μg/L expected in healthy dogs and results >400 μg/L considered consistent with a diagnosis of pancreatitis.[7,8] A clinical rapid semiquantitative assay (SNAP-cP™) also has been developed and shows good alignment and reproducibility with spec-CPL.[9]

An early study of pancreatic lipase reported a sensitivity of 88%, much higher than total lipase in the same 11 dogs.[10] In a later study, 22 dogs with gross evidence of pancreatic disease were assessed post-mortem.[11] Both cPLI and spec-CPL had an overall sensitivity of 63.6%, compared to 40.9% and 31.8% for amylase and total lipase, respectively. The sensitivity for cPLI and spec-CPL increased with increasing severity of pancreatic inflammation.

Another study recently assessed 70 dogs presented consecutively for post-mortem at a tertiary referral center.[12] Sixty-three dogs were found to have pancreatic inflammation on histology (56 mild, seven moderate), while seven had no histological evidence of pancreatic inflammation. The estimated sensitivity of canine pancreatic lipase was 21% for mild disease and 71% for moderate disease. This was a lower sensitivity than for total lipase (54% and 71%, respectively) in the same cohort of dogs. Although only seven dogs were classified as having normal pancreatic histology, there was a specificity of 86% for spec-CPL as compared to 43% for total lipase.

The specificity of spec-CPL in dogs also has been recently assessed. In one study, 64 dogs (20 dogs with gross evidence of pancreatitis post-mortem and 44 dogs euthanized and submitted for post-mortem analysis) were assessed.[13] The pancreas from each dog was sectioned, and inflammation scored as previously described.[14] Forty dogs were classified as having no pancreatic disease due to an absence of clinical signs and no inflammation on histology. Thirty-eight of those 40 dogs had a spec-CPL value <200 μg/L, and 39 had values <400 μg/L. This resulted in a specificity using the lower cut-off value of 95% (95% CI 83.1-99.4) and using the higher cut-off value of 97.5% (95% CI 86.8-99.9). This study assessed a number of healthy dogs but no dogs with acute renal failure.

Another recently published paper assessed the specificity of spec-CPL in dogs that died or were euthanized for a variety of reasons in a tertiary referral center.[15] In this study, the investigators attempted to stratify the dogs with suspected pancreatitis based on the severity of disease in order to assign a diagnosis of positive pancreatitis to dogs that had a minimum amount of histological

pancreatitis present. They reported a specificity of 80% for spec-CPL (<200 μg/L).

These four studies that assessed sensitivity of spec-CPL (and cPLI), with pancreatic histology as the gold standard, were combined for analysis.[10-12,15] Using the diagnostic cutoff of >400 μg/L, a sensitivity of 43.8% (43/98) overall was determined, although it must be emphasized that many of these dogs had minimal inflammation. Conversely, spec-CPL appears to be highly specific for pancreatic inflammation, although dogs with acute renal failure require further investigation.

These studies are useful guides, but the clinical relevance of the test is not fully evaluated, as dogs that have histologic pancreatitis may be presented for another problem such as septic peritonitis. This is demonstrated in an unpublished study, assessing SNAP-cP™ in dogs with acute abdominal disease (abdominal pain, vomiting, diarrhea, abdominal distension) presenting to a veterinary emergency center.[16] This study showed a poor correlation between a positive test and primary presentation of acute pancreatitis in dogs presenting with acute abdomen (κ=0.33). A negative or low test had a good correlation in dogs with disease other than acute pancreatitis.

This is similar to a study using Bayesian statistics rather than pancreatic histology as a gold standard. In that study, it was determined that dogs with spec-CPL ≤200 μg/L and/or a negative SNAP-cP™ were unlikely to have clinical acute pancreatitis.[17] Therefore, a positive spec-CPL or SNAP-cPL™ should be considered in conjunction with other clinical signs and diagnostic imaging to ensure acute pancreatitis is the main cause of the clinical presentation. However, a negative result means acute pancreatitis is unlikely to be the cause of the dogs' presenting signs.

Serum Pancreatic Elastase

Pancreatic elastase-1 (PE-1) came to the attention of researchers in the late 1960s, when elastase was shown to be involved in the pathogenesis of hemorrhagic pancreatitis in experimental models. This later was confirmed to occur simultaneously or immediately after trypsin activation.[18] Studies have shown that when macrophages are exposed to pancreatic elastase, they upregulate the expression of TNF-α, and this supports the role of elastase in the systemic response to pancreatic inflammation.[19] Additionally, elastase has proteolytic effects, hydrolyzes scleroprotein elastin, is fibrinolytic, and increases the oxidative activity of neutrophils. It has been closely linked to the development of adult respiratory distress syndrome in severe pancreatitis.[20]

The most widely used application of pancreatic elastase in human medicine is measurement of the enzyme in feces as a determinant of exocrine pancreatic function.[21] Fecal cPE-1 appears to have limited use in dogs for the diagnosis of exocrine pancreatic insufficiency.[22]

There is some support for serum PE-1 as a diagnostic marker for pancreatitis in dogs.[23,24] A recent study determined that pancreatic elastase-1 had an overall sensitivity of 61%, comparable to published sensitivities for other pancreatic markers, such as lipase and pancreatic lipase.[25] If only dogs with severe acute pancreatitis were evaluated, this sensitivity increased to 92%. There is a strong suggestion that serum elastase is not affected by renal clearance[26] because elastase circulates in the serum bound to inhibitor proteins, such as α-macroglobulin, and is too large to pass through the glomeruli, relying on extra renal metabolic pathways for clearance. It also is suggested that there could be an age-related decline in non-renal clearance.[26]

Histopathology

Histological grading schemes have been developed for diagnosing pancreatitis in dogs and for assisting in assessing the sensitivity and specificity of diagnostic tests.[14,27] These have not been correlated to clinical severity, and therefore the clinical significance of those grading systems is not fully understood. It has been established that pancreatic histological changes can be unevenly distributed throughout the pancreas, necessitating frequent sectioning along the organ in order to rule out pancreatic inflammation.[28] Pancreatic biopsies are seldom obtained in dogs with acute disease and are probably most suitable for evaluation of chronic disease.

Assessing Severity

In people, early detection of severe pancreatitis, as compared to mild pancreatitis, is considered particularly important as this enables rapid transfer to intensive-care units and improves the outcome.[29] The most common system referenced in the medical literature is the Atlanta criteria produced in 1992, where severe pancreatitis is defined as the presence of local complications, organ failure or death.[30]

There also are multifactorial scoring systems, such as Ranson, Glasgow and APACHE II (Acute Physiology and Chronic Health Evaluation),[31] that take a large number of clinicopathologic variables into account;[32,33] that assess obesity, age and etiology;[34] and that use other methods assessing blood markers combined with the presence of pancreatic necrosis, age or respiratory status.[35,36] These multifactorial systems appear effective for predicting severity and the presence of necrosis but require assessment of dynamic changes and highly specialised assessments. This makes them most useful in tertiary intensive-care units.

There also has been a significant increase in measuring various blood markers, such as IL-6, -8, -18, PLA2, CRP, PMN-elastase, MMP-9, serum amyloid A (SAA), trypsinogen-2, TAP, and procalcitonin. Of these, IL-6 seems to have the most clinical relevance.[37,38] TNF-α has been investigated in dogs with presumed pancreatitis,[39] with no difference in dogs classified with severe pancreatitis compared to those with mild disease.

CRP is an acute phase protein that changes rapidly in the circulation when there is inflammation or tissue damage, and is the blood marker most commonly used in human medicine. CRP has been measured in dogs and shown to be increased in a number of inflammatory conditions, including pancreatitis.[40,41] Although CRP is increased in dogs with acute pancreatitis, a large variation results; thus, it may be more beneficial in dogs to assess a daily change in CRP to predict outcome.[41,42]

A large-scale recent study in people assessed over 18,000

patients with acute pancreatitis at over 200 centers.[43] This study used classification and regression tree (CART) analysis to predict hospital mortality. Five factors were determined to contribute to prognosis: azotemia, impaired mental status, the presence of systemic inflammatory response syndrome (SIRS), age >60 years, and pleural effusion. The mortality rate was significantly greater with a higher number of abnormalities present. This concept is similar to a severity score developed in canines using clinical and laboratory data obtained in general practice within 24 hours of admission.[42] The biggest contributor to this severity was fasting three or more days.

Treatment

There are a number of areas surrounding treatment of acute pancreatitis that have not been fully evaluated and have the potential to improve outcome in affected dogs.

Intravenous (IV) Fluid Therapy

One of the major factors that progresses mild pancreatitis to severe pancreatitis is disturbed pancreatic microcirculation.[44,45] This disturbance is usually multifactorial in origin and can occur as a result of increased vascular permeability due to inflammatory cytokines and microthrombi formation from hypercoagulability.[46] The increased capillary permeability leads to edematous changes in the acinar cells and further migration of inflammatory cells. In necrotizing pancreatitis, there is a progressive reduction in capillaries after acinar cell injury, which cannot be reversed by fluid resuscitation.[45]

In animal models, vasoconstriction within the pancreas appears to be an early event in severe cases.[47] In people, early-onset spasms of large pancreatic vessels have been shown to correlate with poorly perfused areas of the pancreas and subsequently high mortality rates.[48] Reperfusion injury is hypothesized to occur when the splanchnic flow is restored. Xanthine oxidase (XO) is produced upon reperfusion, which converts hypoxanthine to xanthine and subsequently leads to production of free radicals (O2-), amplifying the inflammatory response.

One study in people has shown that early fluid resuscitation (compared to fluid resuscitation 24 and 72 hours after the onset of pain) leads to a better clinical outcome.[49] It also has been shown that using Lactated Ringer's solution (LRS) produces better outcomes than using normal saline.[50] In the veterinary literature, there is no current recommended preference for using LRS or saline solution as the initial crystalloid of choice. Saline is considered a safe, first-line fluid choice for resuscitation, but studies in people have shown that hyperchloremic metabolic acidosis develops when it is used.[51] It is possible that acidosis directly contributes to the systemic inflammatory state by stimulation of cytokine production, especially NF-κB.

Experiments using rodent models also have identified that high acinar pH protects against secretagogue induction of pancreatitis.[52] However, crystalloid therapy may not be adequate or well-tolerated in severely affected dogs. An experimental study that induced acute pancreatitis in dogs showed that the dogs resuscitated with LRS alone required approximately 5L more fluid during resuscitation to maintain systemic pressures, and this resulted in pulmonary hypertension and pulmonary edema.[53] There are multiple experimental rodent studies that show dextrans exert a beneficial effect in acute pancreatitis.[54,55] The benefit appears to be independent of the molecular weight, concentration (6% or 10%), or in combination with hypertonic saline but has not been evaluated in dogs with pancreatitis.

Plasma

The use of plasma in dogs with acute pancreatitis is widely reported in review papers and textbooks, although the use in this condition has been declining over the past five years.[8,56,57] Administration of plasma was shown to be superior to both crystalloid and colloid administration in a rat experimental model of pancreatitis.[58] Purported benefits include replacement of circulating α-macroglobulins and coagulation factors and treatment of SIRS with anti-inflammatory factors.

There are no prospective controlled studies that prove the benefit (or lack thereof) of plasma transfusion in dogs with naturally occurring pancreatitis. One retrospective veterinary study analyzed data from a 10-year period and identified 77 dogs with pancreatitis that were admitted for treatment during that time.[59] There was a significant (P=0.008) difference in mortality between the dogs that received plasma (7/20) compared to those that did not (6/57). However, due to its retrospective nature and the lack of stratification of disease severity or standardization of other treatments, there was significant bias. The dogs that received plasma by inference were more severely affected and thus were inherently more likely to die as a result of their disease. However, the lack of benefit seen in this study does reflect much of the human literature on this same subject, and plasma is not currently recommended as a treatment in people with acute pancreatitis.[60-62]

Anti-Emetics

Anti-emetics are a commonly used group of drugs in the management of acute pancreatitis in dogs. Vomiting in dogs with pancreatitis is likely to be centrally mediated due to the presence of circulating emetic agents and peripherally mediated due to ileus, peritonitis and pancreatic distension.[63]

There are no studies published on the efficacy of individual anti-emetic drugs in canine pancreatitis. Experimental rodent models have shown that dopamine infusion improves the outcome in acute pancreatitis and ameliorates the inflammatory severity of the disease.[64] This does not appear to be related to blood flow to the pancreas; instead, it is postulated to be due to reduction of pancreatic microvascular permeability.[64] There is therefore a theoretical disadvantage in giving metoclopramide (a dopaminergic antagonist) to dogs with acute pancreatitis, although this has not been proved.

Maropitant, which blocks the Neurokinin 1(NK1)-receptor is

an effective anti-emetic agent that inhibits centrally and peripherally mediated emesis.[65-67] As well as being effective in controlling emesis, there is another theoretical benefit to NK1-receptor antagonism via reduced production of Substance P. Substance P is a mediator produced by nerve endings throughout the body that mediates capillary permeability and is involved in the pathogenesis of pain.[68] Substance P is intimately related to the NK1-receptor, which also has been shown to be upregulated in acinar cells during murine experimental pancreatitis. When the NK1-receptor was blocked in a genetic mouse model, there was no difference in the amount of pancreatic inflammation produced, but distantly mediated lung injury was reduced.[69]

Gastric Acid Suppression

The rationale for gastric acid suppression in management of acute pancreatitis is that a higher gastric pH will lead to decreased pancreatic exocrine stimulation and that acute pancreatitis predisposes to the development of gastric mucosal ulceration due to hypovolemia and local peritonitis. There have been no studies that report on the efficacy of gastric acid suppression in dogs with acute pancreatitis.

In people with mild to moderate disease, there have been randomized clinical trials assessing nasogastric suctioning. None of these has shown any benefit of this treatment in reducing pain or hospitalisation duration.[70-73] In fact, some of these have shown prolongation of pain and nausea.[70-72]

Should gastric acid suppression be required, it is theoretically preferable to administer proton pump inhibitors (PPIs). PPIs may have direct beneficial effects by blocking the vacuolar ATPase pump on pancreatic acinar cells. Lower pH within the acinar cell accelerates zymogen activation and amplifies the subsequent inflammatory damage.[52,74] One experimental study in rats showed that pantoprazole reduced inflammatory changes and leakage of acinar cells.[75] Additionally, recent work suggests that PPIs are the most effective at reducing gastric acidity in dogs.[76]

Corticosteroids

Historical reluctance to use corticosteroids in dogs, and to a lesser extent in people, resulted from the presumption that corticosteroids could lead to pancreatitis. The putative link between pancreatitis and corticosteroids in dogs may be attributed to early studies showing dexamethasone increased pancreatic enzyme concentrations, but it has been shown there was no effect on pancreatic tissue.[77,78] While theoretically any drug can cause pancreatitis in an individual dog, corticosteroids are no longer considered to be a high risk in people.

Corticosteroids are the one group of drugs that are known to counteract virtually all pathways of inflammation. Corticosteroids inhibit release of proinflammatory mediators; decrease sequestration of neutrophils in the pulmonary vasculature; reduce adhesion of primed neutrophils to the endothelial surface of pulmonary vasculature; reduce release of elastase and free radicals from adherent neutrophils; and reduce pulmonary vascular permeability.[79] A specific role of corticosteroids to enhance apoptosis and increase production of pancreatitis-associated protein (PAP), which confers a protective effect against inflammation, also has been proposed.[80]

In addition, dogs with acute pancreatitis may have relative adrenal insufficiency, which is now termed critical illness-related corticosteroid insufficiency (CIRCI).[81] CIRCI occurs when there is adrenal insufficiency along with tissue resistance to the effects of corticosteroids due to a prolonged and severe proinflammatory state. In particular, it causes hypotension and a poor response to fluid or vasopressor therapy in a subgroup of people. Low-dose hydrocortisone is the current recommended treatment for people with septic shock and CIRCI, while methylprednisolone is recommended for those with acute lung injury.[81] These recommendations have not been extended to people with acute pancreatitis but are being evaluated. This is an area that warrants evaluation in dogs.

Nutritional Management

Acute pancreatitis is a catabolic disease with significant nitrogen losses strongly associated with mortality.[82] Ileus often complicates feeding, the presence of necrosis conveys greater nutritional requirements, and acute pancreatitis is diabetogenic. In human and animal experimental models, it has been shown that fasting leads to intestinal mucosal atrophy,[83,84] an increased rate of enterocyte apoptosis in the intestine,[85] changes in mucin composition of goblet and deep crypt cells,[86] and decreased glutamine and arginine transport.[87] Overall, these changes result in a breakdown of the intestinal barrier and increased intestinal permeability, potentially leading to bacterial translocation. The gastrointestinal tract itself is now thought to be a major contributor to the systemic inflammatory state during acute pancreatitis, particularly if it is not supplied with luminal nutrients.[88]

Historically, in human and veterinary gastroenterology, the idea was to "rest" the pancreas and provide no exocrine stimulation during bouts of acute pancreatitis. Early studies in people and dogs showed that despite pancreatic secretion being less when nutrients were delivered to the jejunum rather than to the duodenum, it still occurred to some extent.[89,90]

Interest in enteral feeding for acute pancreatitis began to increase in the medical field over the past 15 years, due to the expense and complications associated with total parenteral nutrition (TPN).[83,91] A number of studies to assess enteral nutrition in experimental models have been performed. These studies, including some in dogs, show an array of benefits in enteral feeding compared to TPN.[92-95] There also are a number of clinical papers comparing enteral feeding to TPN in people. The results show that enteral feeding is well-tolerated, less expensive and provides some clinical benefits, although it is not conclusively associated with better survival.[96-98]

Perhaps the biggest failing of the majority of these clinical or experimental studies is the inability to compare enteral nutrition to fasting (or full pancreatic rest), instead of comparing enteral

nutrition to TPN. Meta-analysis that supports the use of early enteral nutrition in severe pancreatitis is based on the knowledge that TPN is probably harmful. In this way, the studies only compare a treatment of unknown efficacy to one with harmful side effects.

Due to technical difficulties associated with nasojejunal (NJ) feeding, human studies have assessed delivery into the stomach via nasogastric (NG) feeding. NG feeding was shown to be as well-tolerated as NJ feeding, and there was no increase in pain.[99-101] This has been investigated in a recent prospective pilot study that demonstrated esophageal tube feeding was well-tolerated in dogs with acute pancreatitis.[102] This study compared enteral feeding to TPN and was unable to show a statistically significant difference in the outcome or other parameters.

Even if the notion that enteral nutrition is well-tolerated and perhaps beneficial, it is still unclear as to what diet to feed. Intuitively, dogs are generally fed a low-fat diet. In one study of healthy dogs fed variable fat content,[103] there was no significant difference in measurable pancreatic adaptation. This also brings into question whether feeding a low-fat diet is essential in the management of acute pancreatitis in the dog. The addition of probiotics (not supported in medical literature) or omega-3 fatty acids also has not been fully investigated in dogs with acute pancreatitis. However, as there is no proven benefit in human medicine, this is an area that is not necessarily a high priority.

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