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GUT-BRAIN AXIS AND THE IMMUNE MODULATION

Improving immune health through gut microbiota

PURINA SYMPOSIUM

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INTRODUCTION TO PURINA SYMPOSIUM 2019

The original observation of the potential health benefits of certain bacteria can be credited to the pioneering work of Metchnikoff in the early part of the 20th century. He claimed that the intake of yoghurt cointaining lactobacilli resulted in a reduction of toxin-producing bacteria in the gut.

Since then, Purina has undertaken extensive studies on how probiotics can improve healthy gut microbiota and support health, researching the various mechanism of action. For this research, leverage on Nestlé has been key in sharing expertise and exploring the nutritional connections between pet and human health. This close relationship , along with external collaborations and partnerships with local and international academic institutions, help to further strengthen Purina´s research capabilities and pave the way toward impactful scientific discoveries for the probiotic and microbiome research areas.

Purina has always been a strong supporter of veterinary education, and we are very proud to have organized the Purina Symposium "GUT-BRAIN AXIS AND IMMUNE MODULATION. Improving immune health through gut microbiota". We are sure this will be a good scenario to discuss the various mechanisms of action for probiotics, including the general mechanism of action and how probiotics can benefit gastrointestinal conditions such as diarrhea; as well as their proposed mechanism of action for the stimulation of immunity, and their role in the microbiota-gut-brain axis.

Rosa Carbonell
Head of the Veterinary Channel

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ROLE OF THE MICROBIOME FOR GENERAL HEALTH STATUS OF ANIMALS

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IMPORTANCE OF THE INTESTINAL MICROBIOTA

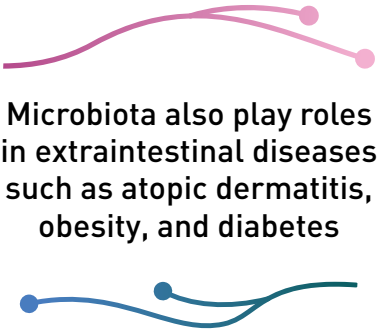
The intestinal microbiota consists of viruses, bacteria, fungi, and protozoa. Molecular methods are now the standard for the identification of intestinal bacteria. An estimated 100 trillion microbial cells are present in the gastrointestinal (GI) tract, which is approximately 10 times more than the number of host cells. This complex ecosystem of gut bacteria has a tremendous influence on host health. A balanced microbiota regulates the immune system, helps in the defense against enteropathogens, and provides nutritional benefits. Interactions between intestinal bacteria and the host immune system are mediated through direct contact between microbes and the immune system (e.g., dendritic cells, Toll-like receptors), and through microbiota derived metabolites. Anaerobic bacteria, such as *Ruminococcus, Faecalibacterium, Lachnospiraceae, Collinsella*, and *Clostridiales* produce metabolites that have direct beneficial effects on the host. For example, nutrient sources such as complex carbohydrates (e.g., starch, cellulose, pectin) are fermented by bacteria, resulting in the production of short chain fatty acids (SCFA). These act as energy sources for the host, regulate intestinal motility, and are important growth factors for epithelial cells. SCFA also have direct anti-inflammatory properties through expansion of immunoregulatory lymphocytes. Other bacterially derived metabolites such as indole, a byproduct of tryptophan degradation, or secondary bile acids, are also immunomodulatory, thereby maintaining immune homeostasis and strengthening intestinal barrier function. These beneficial effects of the gut microbiota reach beyond the GI tract. Recent research has shown that alterations in gut microbes play a role in the pathogenesis of diabetes mellitus and obesity.

Intestinal microbiota is considered a metabolic organ with important impact on host health

ALTERATIONS IN THE MICROBIOME AND EFFECTS ON THE HOST

Intestinal dysbiosis, defined as an alteration in the intestinal microbiota composition and/or richness, is associated with acute and chronic GI disorders.[1,2] A dysbiotic microbiome may be deleterious due to the production of bacterial toxins or due to reductions in anti-inflammatory metabolites derived from bacteria. Dysbiosis may also serve as a risk factor for the development of chronic GI disease in susceptible individuals. For example, antibiotic-induced dysbiosis in early childhood is one of the most important risk factors for the development of allergies, obesity and inflammatory bowel disease (IBD) in adult humans. These initial data in humans combined with better understanding of the immunomodulatory properties of the gut microbiota emphasizes that proper diagnosis and correction of dysbiosis are important. Changes in the microbiota result in functional and immunological consequences for the host. For example,

mucosa-adherent bacteria in the small intestine are an important stimulator of mucosal immunity, and changes in microbial composition may have significant effects on the host immune response. Small intestinal dysbiosis, often also termed antibiotic-responsive diarrhea, is suspected to be caused by an unspecific alteration in composition or an increase in numbers of small intestinal microbiota.[3] Dietary changes, or alterations in intestinal motility (i.e., architectural changes such as those occurring after surgical creation of intestinal loops, short bowel syndrome, and resection of the ileocolic valve) may alter small intestinal bacterial populations. Exocrine pancreatic insufficiency (EPI) leads to an increase in duodenal bacterial counts and also to dysbiosis observed in feces. These microbiota changes negatively impact the proper function of the GI tract through changes in various metabolic pathways (e.g., increase in lactate, increase in fecal bile acids). Examples are increased intestinal permeability, and damages to the intestinal brush border and enterocytes, subsequently causing malabsorption.



Microbiota also play roles in extraintestinal diseases such as atopic dermatitis, obesity, and diabetes

Large intestinal dysbiosis leads to decreases in the major abundant bacterial groups such as *Blautia, Faecalibacterium, Ruminococcaceae,* and *Turicibacter*. These bacterial groups are producers of many immunomodulatory metabolites such as SCFA, indoles, and secondary bile acids. Initial studies using wide screening for bacterial metabolites have associated the dysbiosis in dogs with acute and chronic diarrhea with a reduction of immuno-modulatory secondary bile acids and tryptophan-indole pathways.[4,5] Therefore, reduction of normal microbiota and their respective immunomodulatory metabolites does not allow the host to down-regulate the aberrant intestinal immune response in intestinal disease.

Dysbiosis can also be induced trough medications. For example, antibiotic administration such as metronidazole to healthy dogs induced major dysbiosis, with reductions in the normal commensal microbiota and substantial increases in *E. coli*. Furthermore, the microbiota dysbiosis goes hand in hand with extensive changes in bacterial metabolites (e.g., increase in oxidative stress, reductions in secondary bile acids).[6] Nine out of 16 healthy dogs receiving metronidazole developed loose stools while on antibiotics, but the remaining dogs did not exhibit any clinical signs, despite having similar microbial and biochemical changes. This suggests that an interplay of multiple microbial and host factors need to occur for clinical signs to develop (e.g., underlying genetic susceptibility of the host and dietary and environmental triggers). Nevertheless, antibiotic-induced dysbiosis is an example how changes in microbial composition and metabolism can affect host health in some patients. Recent epidemiological studies in humans have linked antibiotic-induced dysbiosis in early childhood or repeated pulse therapy to the development of allergies, obesity, and IBD.

These emerging epidemiological data in humans and our evolving understanding of the immunomodulatory and metabolic properties of the gut microbiota suggests that proper diagnosis and correction of dysbiosis will be an important therapeutic goal in various diseases.

Recent studies have described links between dysbiosis and changes in various biochemical pathways (e.g., dysmetabolism in bile acids, amino acids, and tryptophan pathways) that affect the host immune system and also metabolism. Therefore, many novel metabolic biomarkers, for example fecal concentrations of bile acids, are being investigated for better assessment of the etiology and treatment of GI diseases, and these may soon become useful for routine practice. Recent data suggest that a subset of dogs with chronic enteropathies have altered intestinal bile acid metabolism, with increases in fecal concentrations of primary and reductions in secondary bile acids. Based on human studies, it is speculated that these alterations in bile acids ratios are due to the dysbiosis, as the bacterial groups that convert primary to secondary bile acids are depleted in chronic GI disease, but also in antibiotic induced dysbiosis. Under physiological conditions, secondary bile acids bind to various receptors on epithelial cells but also macrophages and monocytes, and induce anti-inflammatory signals. Therefore, a lack of secondary bile acids due to dysbiosis may be an important pathological component of gut dysbiosis and inflammation. The measurement of fecal bile acids is available through specialized laboratories and may have diagnostic utility in some patients with chronic enteropathies. In humans, it is speculated that approx. 40% of patients with IBD would benefit from the use of bile acid sequestrants, which bind the increased primary bile acids in the gut lumen and improve bile acid-induced diarrhea. Anecdotal data suggest that also some dogs and cats with chronic diarrhea respond favorably to bile acids sequestrants, and clinical studies in dogs are currently ongoing evaluating their clinical utility.[8]

Novel therapeutics such as fecal microbiota transplant show promise

CONCLUSIONS AND HIGHLIGHTS

- Intestinal microbiota is considered a metabolic organ with important impact on host health.
- Microbiota dysbiosis is a component of intestinal disease.
- Microbiota dysbiosis is linked with metabolic changes in dogs and cats with GI disease.
- Microbiota also play roles in extraintestinal diseases such as atopic dermatitis, obesity, and diabetes.
- Antibiotic use has negative long-term consequences on intestinal microbiome
- Novel therapeutics such as fecal microbiota transplant show promise.

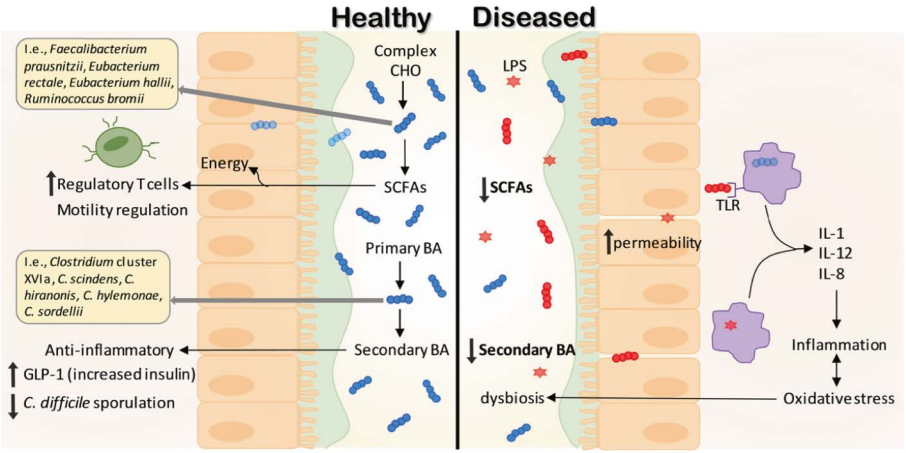


Figure 1. Schematic diagram representing some major microbiota associated pathways in health and disease.

Commensal bacteria in a healthy state convert complex carbohydrates (CHO) into beneficial short-chain fatty acids (SCFAs) that provide energy for endothelial cells, increase anti-inflammatory regulatory T-cells, and modulate intestinal motility. Commensal bacteria in the colon also drive the conversion from primary bile to secondary bile acids, and these have anti-inflammatory properties, induce GLP-1 (increases insulin), and decrease, for example, sporulation of *Clostridium difficile*. In a diseased state, the decreased production of antimicrobial peptides and mucus lead to an increase in the permeability of the endothelium and the translocation of bacteria. Toll-like receptors (TLR) on macrophages and other cells recognize specific pathogen-associated molecular patterns, such as lipopolysaccharides in bacterial cell walls (LPS), and trigger inflammatory reactions. Macrophages phagocytize pathogenic microbes, which also triggers an immune response in the host that can lead to oxidative stress. Oxidative stress in turn can cause intestinal dysbiosis. (Reprinted with permission, Blake AB and Suchodolski JS, Animal Frontiers, 6:37-42, 2016)

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MICROBIOME GUT-BRAIN AXIS

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NAAMA GEVA-ZATORSKY, is an Assistant Professor at the Technion, Faculty of Medicine. Geva-Zatorsky received her B.Sc. from Tel Aviv University, in Chemistry and Biology, summa cum laude, and her M.Sc. and Ph.D., from the Weizmann Institute, in systems-biology with Prof. Uri Alon, completed with honors. For her Ph.D studies she received the John F. Kennedy, Teva and the Barenholz prizes, for academic excellence. She pursued her postdoctoral studies, at Harvard Medical School in the lab of Prof. Dennis Kasper. During her postdoctoral studies, under the supervision of Profs. Kasper, Mathis and Benoist, and in a team they have characterized the host response to over 60 different gut bacteria during homeostasis, and upon disease. She also applied a metabolic labeling approach to enable, for the first time, visualization of anaerobic gut microbes, at real time, and in association with the host.

In her lab, at the Technion, she is applying Systems-Biology thinking strategies together with Microbiology, Immunology, metabolomics and bacteriophage biology to study the mechanistic interactions of the gut microbiota with mammalian host physiology at health and disease.

Geva-Zatorsky received the Alon and Horev fellowships, the UNESCO-L'Oreal award, Human Frontiers, EMBO and Fulbright fellowships, the CIFAR-Azrieli Global Scholar award of the Humans&Microbiome Program and the Jonshon&Johnson WiSTEM2D award.

SYNOPSIS

The gastrointestinal tract is colonized by trillions of microorganisms collectively called "the gut microbiota". The past decade has witnessed an immense amount of studies on their role in host physiology. Nevertheless, unraveling the complex interactions between the host and gut microbiota in the human body is still in its infancy. Studies have shown that our intestinal microbes support essential host functions. They play critical roles in metabolism, host behavior, immune system and more[1-5].

The gut is the most populated niche of commensal microbes

Over the past few years there is an increasing understanding of the important role that gut microbiota plays towards immune system maturation and function[6-8]. Much less is known about their effects on the human brain. On the immune side, the microbiota has been shown to be able to modulate host immune function along the gastrointestinal tract as well as peripheral lymphoid organs. There is an increasing number of studies that associate gut microbiota with immune-related diseases and therapies (e.g. autoimmune diseases, allergies, infectious diseases, efficacy of cancer immunotherapies etc...)[8-11]. Interestingly, **it is becoming increasingly evident that a bidirectional communication exists between the gut microbiota and the brain**. This relationship is commonly termed the **"microbiota-gut-brain axis"**. Disruption of gut microbiota composition (termed "dysbiosis") has been shown to affect behavior and to be involved in the pathogenesis of several neurodevelopmental, neurodegenerative and neuropsychiatric diseases such as: Parkinson disease, Autism spectrum disorder and schizophrenia. In addition, the ways in which the gut microbiota can affect brain development and physiology are in the process of being unraveled and include neural signaling, hormone signaling, microbial metabolites and the immune system.

One of the first evidences of the effects of gut microbiota on host physiology was the significant effect of transplanted microbiota from obese people to Germ-Free (GF) mice (i.e. mice without any microbes). Intriguingly, these mice became obese, but also had demonstrated behavioral disorders, such as anxiety. Moreover, in a direct manner, studies show that high-fat diet changed the microbiome composition and also increased anxiety in mice[12]. It has been shown that the prevalence of mental illness, particularly depression and dementia, is increased by obesity. This study[12], interestingly, demonstrated that obesity-associated changes in the composition of the gut microbes lead to impaired neurocognitive behavior in mice. The researchers have given "high-fat-diet" (HFD) to mice, analyzed the change in the microbiota as a consequence of the diet, and studied the effects on behavioral parameters. They found that the HFD mice had significant and selective disruptions in exploratory, cognitive, and stereotypical behavior compared with mice with control diet. They analyzed the microbiome composition and found differences in specific bacteria that are metabolically active. In addition, they found physiological

changes both in the gut (such as disrupted intestinal barrier), and also in the brain (e.g. neuroinflammation). Their data reinforces the link between gut microbial dysbiosis and neurologic dysfunction and suggests mechanistic explanations to this connection.

The gut-brain axis consists of bidirectional communication between the central and the enteric nervous system, linking emotional and cognitive centers of the brain with peripheral intestinal functions. Recent advances in research have described the importance of gut microbiota in influencing these interactions. This interaction between microbiota and the brain, or gut-brain axis, appear to be bidirectional, namely through signaling from gut-microbiota to brain and from brain to gut-microbiota by means of neural, endocrine, immune, and humoral links.

Recent studies demonstrate a correlation between the gut microbes and brain disorders, including behavioral and psychiatric states.

Both clinical and experimental evidence suggest that gut microbiota has an important impact on the gut-brain axis. They seem to be interacting not only locally with intestinal cells and the enteric nervous system, but also directly with the central nervous system through neuroendocrine and metabolic pathways. Both neural and hormonal lines of communication combine to allow the brain to influence the activities of intestinal cells, such as immune cells, epithelial cells, enteric neurons, smooth muscle cells, etc. These same cells, on the other hand, are under the influence of the residential gut microbiota whose contributing role in brain-gut reciprocal communications is recently been realized and assessed. The concept of a microbiome-gut-brain-axis is now emerging. These gut microbes modulate key neurobiological systems that are dysregulated in stress-related disorders. Preclinical studies show that the gut microbiota exerts an influence over neuroimmune and neuroendocrine signaling pathways, among others such as neurogenesis or neurotransmission. Preliminary evidence in humans suggests that the gut microbiota profile is altered in depression. Studies show that in dogs, the gut microbiome correlates with aggressive behaviors[13]. The full impact of microbiota-based treatments, at different neurodevelopmental time points, has yet to be fully explored. The integration of the gut microbiota, as a mediator, in the complex trajectory of depression and other neurological and neuro-behavioral disorders, may enhance the possibility of personalized precision medicine to affect the brain and related illnesses.

CONCLUSIONS AND HIGHLIGHTS

- The gut is the most populated niche of commensal microbes.
- The enteric microbiota is distributed in the mammalian gastrointestinal tract
- This microbial community has important metabolic and physiological functions for the host and contributes to its homeostasis during life.
- Changes in abundance and composition are related to disease states.
- Recent studies demonstrate a correlation between the gut microbes and brain disorders, including behavioral and psychiatric states.
- There is a strong potential for precision and personalized medicine based on the gut microbes and their derived molecules.

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Notes

OXIDATIVE STRESS AND THE MICROBIOTA-GUT-BRAIN AXIS

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She is an associate editor of the journal BMC Biochemistry (Protein and Enzyme Biochemistry).
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In 1994 she was a visiting scientist in the laboratory of prof. Marian Orlowski, dept. of Pharmacology, Mount Sinai School of Medicine, New York, USA.
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A total of 78 peer-reviewed articles on journals with a wide scientific impact in the category Biochemistry and Molecular Biology and two book chapters are the results of her scientific contribution, which resulted in a H-index 25 (Scopus) and around 1500 citations.

A bidirectional communication exists between the gastrointestinal tract and the brain, the so-called gut-brain axis, mediated by different pathways including hormonal, neural and immune stimuli[1]. The modulation of gut microbiota using the probiotic formulation SLAB51 (a mixture of lactic acid bacteria and bifidobacteria) affects numerous neuronal pathways, with a significant delay of Alzheimer's disease (AD) progression in a triple transgenic mouse model (3xTg-AD mice)[2]. In details, SLAB51 changes microbiota composition and metabolites, favouring the proliferation of anti-inflammatory species, and positively interferes with the concentration of gut hormones, able to regulate energy homeostasis, food intake and to affect the central nervous system modulating nervous function. The downstream effects of these changes include the influence on neuronal proteolysis, the reduction of Amyloid beta peptides (Aβ) load and the improvement of cognitive functions, suggesting a role for probiotics in the prevention of AD[1].

Gut microbiota modulation, through probiotics administration, increases antioxidant enzymes activities in AD mice brains.

The ability of SLAB51 to counteract oxidative stress, a condition that is exacerbated in 3xTg-AD mice brains, investigating the molecular mechanisms involved, is explored. The role of sirtuin 1 (SIRT1), a deacetylase with a strong neuroprotective and antioxidant potential[3] is elucidated. A great, age-dependent loss of SIRT1 functionality and expression levels that negatively mediates a series of processes related to cell survival and metabolism is observed in the brain of untreated AD mice. In fact, the deleterious effects of decreased SIRT1 expression are widely recognized, including the accumulation of Aβ and tau in the cerebral cortex of AD patients. Our data demonstrate that both SIRT1 activity and expression are significantly increased in the brain of AD mice administered with the probiotic formulation. The activation of SIRT1 is also confirmed by the acetylation levels of its substrate retinoic acid receptor beta (RARβ). In fact, if an age-dependent increase in the degree of acetylation can be detected in untreated AD mice, in line with SIRT1 diminished expression, SLAB51 strongly reduces the amount of RARβ acetylated lysines by restoring SIRT1 levels. Interestingly, the deacetylation and the consequent activation of RARβ stimulate a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) gene transcription stimulating the amyloid precursor protein (APP) non-amyloidogenic pathway, and preventing Aβ peptide generation and deposition[4], in accordance with our previous studies reporting diminished deposition of Aβ toxic fragments in the brain of AD mice treated with SLAB51[2]. SIRT1 activity also regulates p53 acetylation.

In treated AD mice increased levels of SIRT1 inversely correlates with acetylated p53, suggesting a protective action of SLAB51 against p53-mediated apoptosis. Moreover, p53 deacetylation by activated SIRT1 is also related

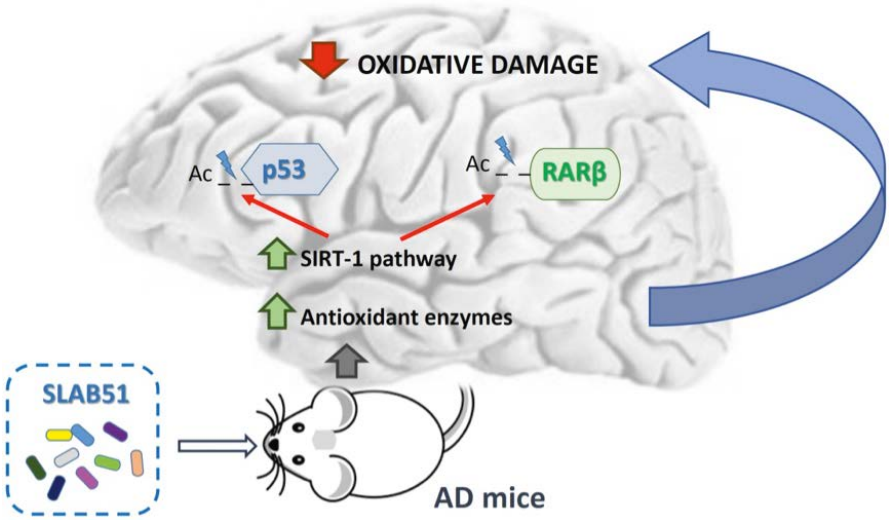
to ghrelin increased plasma levels in the same AD mouse model upon SLAB51 oral administration, as we have previously shown[2]. In fact, ghrelin has been demonstrated to promote the hypothalamic SIRT1-p53 pathway, causing changes in fatty acids metabolism and feeding behaviour[5].

Ghrelin promotes the hypothalamic SIRT1-p53 pathway, changing fatty acids metabolism and feeding behaviour.

These data are in agreement with our previous work reporting an enriched gut concentration of anti-inflammatory short chain fatty acids and a decrease of pro-inflammatory cytokines levels in SLAB51 treated AD mice[2]. Being SIRT1 directly involved in the regulation of oxidative stress, whose levels are extremely high in the brain of AD subjects, the functionality of redox enzymes and the amount of well-established markers of proteins, lipids and DNA oxidation are measured. We observe severe age-dependent alterations of the oxidative status in AD transgenic mice brains. Compared to 8-weeks old animals, elder AD individuals show decreased activities of antioxidant enzymes, mainly glutathione peroxidase and catalase, despite their increased expression levels and an enhancement of carbonyls, 3-nitrotyrosine, 4-hydroxy-2-nonenal, 8-oxo-2'-deoxyguanosine and advanced glycation end products. In AD mice SLAB51 markedly mitigates oxidative stress-related damages as indicated by the increase in antioxidant enzymes activity and insulin growth factor- 1β receptor, together with the diminished levels of oxidation markers. Finally, Poly(ADP-ribose) Polymerase (PARP) cleavage and 8-Oxoguanine-glycosylase levels increase in AD untreated mice in response to oxidized DNA[6], in agreement with the SIRT1 decreased expression. In fact, PARP and SIRT1 share NAD+ as cofactor, thus PARP activation can inhibit SIRT1 functionality. The great impact of SIRT1 pathway re-activation upon SLAB51 treatment in preserving brain redox homeostasis, with positive outcomes for AD, is demonstrated; SLAB51 acts at different levels in the cell, exerting beneficial effects that definitely ameliorate AD symptoms.

CONCLUSIONS AND HIGHLIGHTS

- Gut microbiota modulation, through probiotics administration, increases antioxidant enzymes activities in AD mice brains.
- Oxidized protein, lipid and DNA markers decrease in AD brain upon probiotics administration.
- Probiotics favour reestablishment of DNA repairing mechanisms as PARP1 and OGG1
- Probiotics activate SIRT1 pathway exerting antioxidant and neuroprotective effects in AD .
- SIRT1 activation is responsible for both p53 and RARβ deacetylation.
- Ghrelin promotes the hypothalamic SIRT1-p53 pathway, changing fatty acids metabolism and feeding behaviour.
- Probiotics counteract AD progression by influencing gut-brain axis.



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PROBIOTICS: GASTROINTESTINAL AND IMMUNE STIMULATING EFFECTS

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Probiotics are live microorganisms that when administered in adequate amounts confer a beneficial health effect on the host. There have been many studies and reviews of the effects of probiotics on the health of humans, but fewer in small animals.[1-4] Evidence supporting the use of probiotics is generally strongest for managing gastrointestinal syndromes such as acute or chronic diarrhea due to infectious diseases or inflammatory bowel disease. There is also some evidence that probiotics might be beneficial for mitigating antibiotic-associated vomiting or diarrhea.[5-7] It is known that some probiotics help balance the endogenous microbiota and that some can inhibit replication of pathogenic bacteria.[8-10] Some of the proposed mechanisms of action include competition for essential nutrients or receptor sites, binding of pathogenic bacteria, and production of inhibitory substances. There is also evidence that some probiotics induce immune modulating effects with potential benefits for the management of inflammatory bowel disease, atopy, or infectious diseases with systemic involvement (e.g., feline herpesvirus-1; FHV-1).[8,11-15]

Infectious diseases are common in small animals, so the potential beneficial effects of probiotics could impact veterinary practice significantly. It is also now known that some probiotics can beneficially influence innate and acquired immunity systemically.[11,12,16] Not all of the methods by which probiotics modulate the immune system have been characterized and it is likely that these effects vary by probiotic.[15,17] Thus, a clinical effect that is shown to be induced by one probiotic may not be induced by others, even in the same genus and species of bacteria. For example, there are many strains of *Enterococcus faecium*, but positive clinical effects in dogs or cats have only been shown with the *E. faecium* strain SF68.[5,18,19]

There is an evidence that some probiotics induce immune modulating effects with potential benefits for the management of inflammatory bowel disease, atopy, or infectious diseases with systemic involvement.

While probiotic use is generally considered safe, consumers should ask companies marketing probiotics for the product's safety information.[20] One of the other major issues concerning probiotic use in small animal practice is quality control. In recent veterinary studies, the majority of products claiming to contain probiotics generally did not meet the label claim when evaluated.[21,22] Other unresolved issues for probiotic use in small animals is the optimal number of bacteria to be used and whether there are clinical benefits to having multiple strains of bacteria in a product. Since each bacterium in a probiotic can have unique effects, products containing multiple bacteria actually may have competing mechanisms of action lessening potential beneficial effects.

There are several veterinary probiotic products marketed in the United States that have been shown to be

safe, meet label claims, and have clinical studies supporting potential beneficial effects, including *E. faecium* strain SF68 (FortiFlora®-Nestlé Purina PetCare). Recently, studies evaluating gastrointestinal effects of other commercially available probiotics or synbiotics have been published and can be used to help veterinarians choose which products to make available to their clients.[14,23-28]

Enterococcus faecium strain SF68 (NCIMB 10415) was originally isolated from the feces of a healthy baby and was initially shown to inhibit the growth of a number of enteropathogens.[29] The purpose of these proceedings is to summarize key studies regarding the potential effects of this probiotic in the management of different canine or feline clinical syndromes.

SF68 prevented the decline in antibody titers observed in the controls by maintaining high levels of antibodies in the supplemented puppies.

IMMUNE MODULATION STUDIES

In one of the first companion animal studies, *E. faecium* strain SF68 was fed to a group of puppies vaccinated for canine distemper virus (CDV) and compared over time with a control group that had the exact same vaccination protocol but was not fed the probiotic.[11] 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 an increased percentage of circulating B lymphocytes when compared with 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 the supplemented puppies.

A follow up study was performed to determine whether the immune modulating effects of *E. faecium* strain SF68 could be apparent as soon as 4 weeks after starting supplementation.[16] Age-matched, clinically healthy, laboratory-reared young adult beagles (n = 7) housed in a research facility were chosen for study. Blood and serum samples were collected from each dog prior to supplementation and then monthly for 12 weeks. Flow cytometry was used to measure percentage of B cells expressing surface-bound IgG or major histocompatibility complex class II (MHC II,) the percentage of CD4+ T cells expressing MHC II, and the percentage of CD8+ T cells expressing MHC II or CD11a. T lymphocyte proliferative responses of all dogs to a non-specific mitogen (Concanavalin A) were assessed by flow cytometry. For some B and T lymphocyte parameters, statistically significant differences compared with baseline were detected as early as 4 weeks after *E. faecium* strain SF68 supplementation was started.

A similar experiment to evaluate the safety and immunological effects of *E. faecium* strain SF68 when fed to healthy research kittens was performed.[12]

That study investigated whether feeding *E. faecium* strain SF68 to kittens would enhance their non-specific immune responses or specific immune responses to FHV-1, feline calicivirus, and feline panleukopenia virus vaccination.[12] Starting at 7 weeks of age, one group of 10 kittens was fed E. *faecium* strain SF68 daily and the other group was fed a placebo. All kittens were monitored to 27 weeks of age. A number of the measured humoral immune responses were numerically greater in the kittens that were fed *E. faecium* strain SF68 when compared to the placebo group but the differences did not reach statistical significance. For example, the mean FHV-1-specific serum IgG concentrations were greater in the treatment group when compared with the placebo group at 15, 21, and 27 weeks of age. 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 = 0.022). It was concluded that *E. faecium* strain SF68 was safe and evidence for immune modulation occurred.[12]

The cats fed *E. faecium* strain SF68 had significantly fewer episodes of conjunctivitis by FHV-1 than the placebo group during the supplementation period.

CHRONIC FHV-1 STUDY

To determine whether the immune modulating effects of *E.* faecium strain SF68 noted in the healthy kitten trial could be of clinical benefit, we chose to study FHV-1. This viral infection is extremely common in cats and is frequently associated with morbidity because of recurrent ocular and respiratory clinical signs of disease. In this pilot study, it was hypothesized that feeding *E.* faecium strain SF68 would decrease the incidence and severity of clinical disease, frequency of episodes of FHV-1 shedding, and the total number of FHV-1 DNA copies shed over time in cats with chronic FHV-1 infection.[8]

Twelve cats with chronic FHV-1 infection were fed either *E. faecium* strain SF68 or a palatability enhancer as a placebo. Clinical signs of disease, FHV-1 shedding, FHV-1-specific humoral and cell-mediated immune responses, and changes in the fecal microbiome were monitored and evaluated. After an equilibration period, mild stress was induced over time by changing the cats' housing from cages to group housing multiple times over a five-month period.

The *E. faecium* strain SF68 was well tolerated by all cats. Fecal microbial diversity was maintained throughout the study in cats supplemented with E. *faecium* strain SF68, but diversity was decreased in cats fed the placebo, indicating a more stable microbiome in cats fed E. *faecium* strain SF68. The cats fed *E. faecium* strain SF68 had significantly fewer episodes of conjunctivitis than the placebo group during the supplementation period, suggesting that probiotic administration lessened morbidity associated with chronic FHV-1 infection exacerbated by stress. Further work will be required to determine if the clinical effects induced by *E. faecium* strain SF68 in this study relates

to changes induced in the respiratory microbiota as noted in another study of healthy cats with a different probiotic.[30]

MURINE ACUTE GIARDIA STUDY

In previous work, mice fed *E. faecium* strain SF68 and then infected with *Giardia intestinalis* shed fewer trophozoites and less *Giardia* antigen than the placebo group.[31] In addition, supplemented mice had increased concentrations of CD4+ T cells in Peyer's patches and the spleen as well as increased anti-*Giardia* intestinal IgA and serum IgG concentrations when compared with untreated mice.

CHRONIC SUBCLINICAL *GIARDIA* STUDY IN DOGS

When *E. faecium* strain SF68 was fed to 10 adult dogs with chronic, subclinical *Giardia* infection, no differences in fecal antigen concentrations or levels of cyst shedding were found when compared with 10 placebo treated dogs.[32] In addition, there were no differences between groups in fecal IgA concentrations. In contrast to the mouse study, the dogs were clinically normal and had chronic, subclinical *Giardia* infection prior to administration of *E. faecium* strain SF68, which may explain the differences between studies.

The administration of *E. faecium* strain SF68 to cats housed in shelters may lessen the numbers of days with non-specific diarrhea.

SHELTER ANIMAL ACUTE NON-SPECIFIC DIARRHEA STUDY

In another study, it was hypothesized that cats and dogs housed in an animal shelter that were fed *E. faecium* strain SF68 would have decreased episodes of diarrhea and improved fecal scores compared with untreated cats and dogs in the same environment.[18] The cats were housed in two different rooms and the dogs were housed 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 *E. faecium* strain SF68 and animals in the alternate room were supplemented daily with a placebo. To reduce risk of a room influence on study results, the room in which cats or dogs were being supplemented with *E. faecium* strain SF68 was switched after one month, with a one-week washout period. Prior to cleaning the room each morning, feces in the cage of each animal were scored by a masked investigator using the Purina Fecal Scoring System for Dogs and Cats (Table 1). The percentages of dogs or 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. This result was significantly different ($p = 0.0297$) and

suggests that administration of *E. faecium* strain SF68 to cats housed in shelters may lessen the numbers of days with diarrhea. As this was a short-term study, this beneficial effect was likely from probiotic influences on intestinal flora rather than systemic immune enhancing effects.

METRONIDAZOLE AND *E. FAECIUM* STRAIN SF68 STUDY

In this study, it was hypothesized that dogs with non-specific diarrhea fed *E. faecium* strain SF68 with metronidazole would have better clinical outcomes than dogs administered metronidazole alone. *Enterococcus faecium* strain SF68 is resistant to metronidazole, so the two compounds were administered together.[19] Stray dogs housed in an open-admission shelter that had a normal appetite, no vomiting, and a fecal score of > 4 were studied. All dogs were fed a standardized diet and were administered metronidazole at 25 mg/kg orally twice daily for 7 days. The dogs were randomized be fed either *E. faecium* strain SF68 or a placebo mixed with their food daily for 7 days. The individuals doing the daily fecal scores (Table 1) were masked to the groups.

The percentages of days with normal stools were significantly higher for dogs administered dual therapy when compared with those administered metronidazole alone.

By day 7, a normal stool (fecal score < 5) was detected in 37.5% of the dogs administered metronidazole and 68.8% of the dogs administered dual therapy, but the result was not significant (P = .1556). The percentages of days with normal stools were significantly higher (P = .0496) for dogs administered dual therapy (65.6%) when compared with those administered metronidazole alone (46.9%). *Giardia* cysts were eliminated and diarrhea resolved in both dogs that were infected in the treatment group. In contrast, of the 7 *Giardia*-positive dogs in the placebo group, 6 (85.7%) were still positive for *Giardia* cysts on day 7, and 4 of those dogs still had diarrhea on day 7.

AMOXICILLIN-CLAVULANATE AND *E. FAECIUM* STRAIN SF68 STUDY

Amoxicillin-clavulanate is frequently prescribed by veterinarians for a wide variety of feline conditions, including bite wound abscesses and upper respiratory infections. As the drug has a broad spectrum against many bacteria, including anaerobes, diarrhea, vomiting, and loss of appetite can occur when administered to cats. One objective of the study was to quantify the frequency of gastrointestinal signs induced by amoxicillin-clavulanate. The second objective was to determine if feeding of *E. faecium* strain SF68 could ameliorate these side effects as this probiotic has been shown to be effective in the management of diarrhea in cats.[5]

In this double-blinded, placebo-controlled study, a total of 34 young adult, healthy, purpose-bred cats of both sexes were randomized into two rooms by their body condition score. The cats were caged so that individual clinical findings could be monitored daily. This included appetite, attitude, hydration,

vomiting, and consistency of feces score (FS) using the previously described scale (Table 1). After evaluation the fecal scores during a 10-day equilibration period, cats that registered a fecal score of > 4 more than twice were blocked from the study as those predisposed to stress diarrhea. All cats were administered amoxicillin-clavulanate (Clavamox®; Zoetis) at 62.5 mg/cat orally twice a day for seven days. Two hours prior to the antibiotic administration, 1/4 can of canned food mixed with the dose of *E. faecium* strain SF68 that was equivalent to a commercial probiotic product (13 cats) or only the palatability enhancer used in the same commercial product (placebo; 14 cats). Administration of the placebo or *E. faecium* strain SF68 and clinical fecal scoring were continued for an additional five days after stopping the antibiotic.

While vomiting was not noted during the equilibration period, during the antibiotic administration period, 7 cats in the treatment group and 6 cats in the placebo group vomited at least once. However, the total number of vomiting episodes during the antibiotic administration period were not significantly different between the groups, and vomiting resolved in all cats after stopping the antibiotic.

Administering amoxicillin-clavulanate orally to cats can induce vomiting or diarrhea and can alter the gastrointestinal microbiome. In addition, feeding the probiotic *E. faecium* strain SF68 can lessen some associated clinical abnormalities.

Fecal scores > 5 on a 7-point scale (Table 1) were detected in 9 of 13 cats (69.2%) in the treatment group compared with 12 of 14 cats in the placebo group (85.7%). Fecal scores of 7 were only detected in the placebo group. When compared, the cats in the treatment group had significantly lower total fecal scores than the cats in the placebo group for days 1 through 11 ($p = 0.0058$). Administration of amoxicillin-clavulanate led to decreased microbiome diversity in both groups, but differences between cats in the treatment group and the cats in the placebo group were not detected.

The results show that administering amoxicillin-clavulanate orally to cats can induce vomiting or diarrhea and can alter the gastrointestinal microbiome. In addition, feeding the probiotic *E. faecium* strain SF68 can lessen some associated clinical abnormalities.

In a separate study, feeding a synbiotic with clindamycin was associated with improvement in appetite and decreased incidences of vomiting, but not improvement in fecal scores when compared with the placebo group.[7] While the two studies cannot be directly compared, both show evidence for use of this probiotic or synbiotic when prescribing these two antibiotics, which are associated with a relatively high incidence of gastrointestinal signs.

CONCLUSIONS AND HIGHLIGHTS

Two other notable studies with probiotics or synbiotics marketed in the United States include an open trial showing potential clinical benefits including a synbiotic for management of chronic diarrhea in cats[25] and use of a probiotic in the management of inflammatory bowel disease in dogs.[14] Overall, the evidence gathered to date suggests that various probiotics or synbiotics may be of benefit in the management of certain clinical abnormalities in dogs or cats. Veterinarians should seek out products that have been proven to be safe, to meet the label claims, and to have a high level of evidence of clinical efficacy.

- There is an evidence that some probiotics induce immune modulating effects with potential benefits for the management of inflammatory bowel disease, atopy, or infectious diseases with systemic involvement
- SF68 prevented the decline in antibody titers observed in the controls by maintaining high levels of antibodies in the supplemented puppies.
- The cats fed E. faecium strain SF68 had significantly fewer episodes of conjunctivitis by FHV-1 than the placebo group during the supplementation period.
- The administration of E. faecium strain SF68 to cats housed in shelters may lessen the numbers of days with non-specific diarrhea.
- The percentages of days with normal stools were significantly higher for dogs administered dual therapy when compared with those administered metronidazole alone.
- Administering amoxicillin-clavulanate orally to cats can induce vomiting or diarrhea and can alter the gastrointestinal microbiome. In addition, feeding the probiotic E. faecium strain SF68 can lessen some associated clinical abnormalities.

Table 1. Purina Fecal Scoring System - with descriptions of stool characteristics

SCORE 1
- Stool very hard and dry
- No residue left when picked up

SCORE 2
- Stool firm but not hard
- Little or no residue left when picked up

SCORE 3
- Stool log shape
- No segmentation visible
- Moist surface
- Leaves residue but remains firm when picked up

SCORE 4
- Stool moist throughout
- Distinct log shape
- Leaves residue and loses form when picked up

SCORE 5
- Stool very moist
- Piles rather than log shape
- Leaves residue when picked up

SCORE 6
- Stool has texture but no defined shape
- Occurs in piles or looks like spots
- Leaves residue when picked up

SCORE 7
- Stool is watery, flat, with no texture
- Occurs in puddles
- Leaves residue when picked up

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Notes

IMMUNOMODULATORY EFFECT OF BOVINE COLOSTRUM

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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 co-evolved organ systems and signaling pathways during evolution. This close relationship can explain that chronic nutrient deficiency or excess can negatively impact immune health and consequently overall health. The good news is that this relationship can also be used to proactively enhance immune health.

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[1].

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 infections agent involving physiological changes including fever, inflammation, and cellular responses such as generation of T cells and antibodies that can specifically target the pathogen. This immune activation comes with a price, as it is a metabolically costly endeavor that uses precious resources. Repeated immune activation to combat infection can be a significant drain on metabolic resources and will unfavorably compete with energy-demanding processes, as well as increase oxidative stress. A healthy immune system capable of preventing infections, thus has profound positive metabolic implications[2].

HOW CAN DIET INFLUENCE THE IMMUNE SYSTEM? THE GUT IS THE LARGEST "IMMUNE ORGAN"

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.

The gut is the largest immune organ. Thus, a significant part of the immune system can interact with what we eat or feed our pets.

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 Ig-producing cells[3,4]. 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 the environmental antigens, especially microbiota in the gut, are essential for the development of a healthy immune system. Germ-free animals tend to have a very 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.

NUTRITION INTERACTS WITH THE IMMUNE SYSTEM AT MULTIPLE LEVELS

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.

Interaction between diet, environment, and genome ultimately defines health status and can be critical in influencing chronic disease2.

Nutrition and the immune system interact at multiple levels and, for simplicity, can be considered in a framework of 4 stages. Stages I and II are passive because they involve providing the immune system with essential nutrients. Stage I aims to provide all key nutrients needed to sustain immune cells and process. Stage II aims to modulate the immune system to appropriately respond to specific but broad areas of concern. Stages III and IV is more focused on predictive, preventive, and personalized nutrition. Interaction between diet, environment, and genome ultimately defines health status and can be critical in influencing chronic disease[2].

SUPPLEMENTATION OF DIETS WITH BOVINE COLOSTRUM INFLUENCES IMMUNE FUNCTION IN DOGS AND CATS

While the need for colostrum in neonates is well established, the systemic effect of feeding bovine colostrum (BC) to adult humans is gaining increasing attention[5].

Colostrum (early milk produced during the first few days after parturition) not only meets the unique nutritional needs of neonates, but also transfers passive immunity and promotes the growth and development of the gastrointestinal tract[6,7].

While the need for colostrum in neonates is well established, the systemic effect of feeding BC orally to adult humans is gaining increasing attention[8]. Bovine colostrum (BC) contains several bioactive components[9], including growth factors such as insulin-like growth factor-1, insulin-like growth factor-2, transforming growth factor-b and epidermal growth factor, antimicrobial compounds such as lactoferrin, and immunomodulatory compounds such as Ig, transferrin and cytokines. The presence of these closely homologous bioactive ingredients in BC has led to its use in the treatment and prevention of diseases in humans and animals[7,10]. In several studies, BC has been shown to be effective in treating gastrointestinal disorders (for a review, see Playford et al.[11]) as well as helping athletes in endurance and speed training[12,13].

In human trials, BC containing specific antibodies has also been shown to be effective against enteropathogenic and enterotoxigenic *Escherichia coli*[14,15], *Cryptosporidium*[16], *Helicobacter pylori*[17], *Rotavirus*[18,19] and *Shigella flexneri*[20].

There are two main studies evaluating the immunomodulatory effect of BC in dogs and cats.

The study in dogs[5], as well as the one in cats, was conducted in two phases: pre-test (8 weeks) and test (40 weeks). Twenty-four dogs (mean age 2·5 years) were randomly assigned into two groups. In the 'pretest' phase, both groups were fed a nutritionally complete diet. At the end of the 'pre-test' phase, all dogs received a canine distemper virus (CDV) vaccine, and dogs in the 'test group' were switched to a diet supplemented with 0·1% spray-dried BC. Response to the CDV vaccine was evaluated by measuring vaccine-specific plasma IgG levels. Gut-associated lymphoid tissue response was assessed by measuring faecal IgA levels. Gut microbiota was evaluated by the temporal temperature gel electrophoresis methodology. Dogs fed the BC-supplemented diet demonstrated a significantly higher vaccine response and higher levels of faecal IgA when compared with the control group. Supplementing diets with BC also resulted in significantly increased gut microbiota diversity and stability in the test group. In conclusion, diets supplemented with BC significantly influence immune response in dogs.

Supplementing diets with BC can result in significantly increased gut microbiota diversity and stability in the face of a mildly challenging life event.

In early life, a kitten faces many significant events including separation from its mother, re-homing and vaccination. The kitten is also slowly adapting to their post-weaning diet. In the study developed by Nestlé Purina[21] we report for the first time the effect of feeding a diet containing 0.1% spray dried bovine colostrum (BC) to growing kittens on gut-associated lymphoid (GALT) tissue responses, systemic immune responses, and on intestinal microbiota stability. BC supplementation induced increased faecal IgA expression ($p<0.05$), and induced a faster and stronger antibody response to a rabies vaccine booster, indicative of better localised and systemic immune function ($p<0.05$) (2), and helped to maintain kittens' intestinal microbiota stability in the face of a mildly challenging life event (3). These results show that BC supplementation can help strengthen the immune system and enhance the gut microbiota stability of growing kittens.

CONCLUSIONS AND HIGHLIGHTS

- Nutrient metabolism and immunity have co-evolved organ systems and signaling pathways during evolution. This relationship can also be used to proactively enhance immune health capable of preventing infections, thus has profound positive metabolic implications[2].
- The gut is the largest immune organ. Thus, a significant part of the immune system can interact with what we eat or feed our pets.
- Interaction between diet, environment, and genome ultimately defines health status and can be critical in influencing chronic disease[2].
- The systemic effect of feeding bovine colostrum (BC) to adult humans and animals is gaining increasing attention5. The presence of closely homologous bioactive ingredients in BC has led to its use in the treatment and prevention of diseases in humans and animals[7].
- Supplementing diets with BC can result in significantly increased gut microbiota diversity and stability in the face of a mildly challenging life event.

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