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## **Application of Molecular Tools for Gut Health of Pet Animals: A Review**

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### **Abstract**

Gut health is an important facet of well being of pet animals; it is in this context, various nutritional and biotechnological approaches have been proposed to manipulate the gut health by specifically targeting the colonic microbiota. Nutritional approaches include supplementation of antioxidants and phytochemicals like flavonoids, isoflavonoids and carotenoids. Biotechnological approaches include supplementation of probiotics, prebiotics, synbiotics in the diet and potential application of molecular tools like fluorescent in situ hybridization, denaturing gradient gel electrophoresis, quantitative dot blot hybridization, and restriction fragment length polymorphism etc. in studying the fecal microbiota composition. Post-genomic and related technologies, i.e. genomics, nutrigenomics, transcriptomics, proteomics, metabolomics and epigenomics in the study of gastrointestinal tract also put forward challenges for nutritionists and microbiologists to elucidate the complex interactions between gut microbiota and host.

*Keywords:* Molecular tools; Gut health; Pets; Prebiotics; Probiotics

### **Introduction**

Gut health depends on availability of a large number of diverse molecules. Many of these molecules are supplied through the food and made available in the large intestine (particularly colon) of pet animals by fermentative actions. The colon plays a major role in host nutrition and welfare through the activities of its resident microbiota (Manning and Gibson, 2004). It is the most heavily colonized region with a total population of  $10^{11}$ - $10^{12}$  cfu/ml of contents and therefore, the colonic microbiota is the predominant target for dietary intervention in the gut ecology, and consequently, the gut health. In this regard, dietary-management tools already exist in the shape of phytochemicals, antioxidants, probiotic microorganisms, prebiotic oligosaccharides, and synbiotic mixtures of the two. There is evidence that these tools do work in pet dogs. However, at present, our picture of the dog GI-tract ecology is far from complete. Rapid development of new DNA-based methods is under way for studying the composition of complex colonic mi-

crobial ecosystem and these have not yet been systematically applied to the study of pet animals. Such studies are required if we are to realize the potential of dietary strategies to alter the colonic microbiota. Various nutritional and biotechnological approaches have been proposed to resolve this issue. Some of them are discussed below.

### **Nutritional Approaches**

Dogs need a perfectly balanced diet for their optimum health; some fifty nutrients are now considered essential for dogs, and the quality of a particular dog food is based on how well it matches the requirements for each of those fifty nutrients. The amount of these nutrients must reflect the dog's size, physiological condition, and health status. However, the typical pet diet mainly contains high concentrations of protein, which can lead to increased concentrations of undigested amino acids and fecal putrefactive compounds in the colon. Many of these protein catabolites may have negative influences on gut health. So, the diet should be adequate and balanced in all respect. Active food compounds like phytochemicals and antioxidants can be used as one of the practical approaches to improve diet.

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Phytochemicals are present in plant based foods, e.g. fruits, vegetables and whole grain cereals. They constitute a large number of chemically diverse substances, which are grouped into various categories like flavonoids, isoflavonoids and carotenoids. The bioavailability of flavonoids in the small intestine is generally considered to be small. Thus a large portion of the ingested flavonoids may reach the large intestine. In the large intestine, considerable metabolism by the gut microbiota occurs. In the first step, the bound carbohydrates are cleaved and used as substrates by the microbiota. In further steps, the polyphenolic structures are degraded. Flavonoid - aglycones have been shown to potentially affect the functional properties of colonic epithelial cells thereby modulating cell proliferation, apoptosis and signal transduction in colonic carcinoma cell lines. Thus the metabolic activity of the microbiota may have a profound effect on the biological activity of these compounds in the large intestine. Carotenoids are lipid-soluble pigments with a high bioavailability in the small intestine depending on the presence of lipids in the diet. However, the release of carotenoids from the respective food matrices depends on the processing conditions. In raw fruits and vegetables, the carotenoid bioavailability is low. So, the processing of carotenoid-containing foods, e.g. heat treatment (cooking), for a large part determines its bioavailability in the small intestine. In the large intestine, the microbial degradation of dietary fiber contributes to the release of so far unavailable carotenoids.

Free radicals are a major cause of many degenerative diseases of GIT. Epidemiological data and randomized clinical trials provide ample indications that antioxidants play a fundamental role in the prevention of these diseases. They act as scavengers of reactive oxygen species and metal chelators that protect cells and reduce oxidative damages. Brussels sprouts, kale, cabbage, onions, cauliflower, red beets, cranberries, cocoa, blackberry, blueberry, red grapes, prunes, and citrus fruits are the richest sources of antioxidants.

### Biotechnological Approaches

The gastrointestinal tract is a multidimensional system ideal for an integrated, non-biased, system biology approach, which could be combined with clinical and health information to enable debilitat-

ing health issues to be addressed. A true system biology approach needs to integrate both the host and microbial components of the gastrointestinal tract, but as yet these techniques have seen a little application in pet animals.

### Conceptual Approaches

A dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health (Roberfroid *et al.*, 2010). It must be particularly available to some groups of bacteria (of which bifidobacteria and lactobacilli are considered indicator organisms) that are beneficial for the health of the intestine but less available to potentially pathogenic bacteria such as toxin producing clostridia, proteolytic bacteroides and toxogenic *Escherichia coli* (Manning and Gibson, 2004). In this manner, a 'healthier' microbiota composition can be obtained whereby the bifidobacteria and/or lactobacilli become predominant in the intestine and exert possible health-promoting effects. These prebiotics include resistant non-digestible oligosaccharides, resistant starch, and non-starch polysaccharides (dietary fiber).

Probiotics are the 'live microorganisms which when administered in adequate amounts confer a health benefit to the host' (FAO/WHO, 2002). Members of the genera *Lactobacillus* and *Bifidobacterium* have a long and safe history in the manufacture of dairy products and are also found as a part of gastrointestinal microbiota. Probiotics have been consumed in foods for perhaps thousands of years. However, because of the reported variability in viability of live bacteria (probiotics) in food products and during transit through the gastrointestinal (GI) tract, the microencapsulation technology has been developed.

This technology aims at encapsulating a selective probiotic within starch granules that is then coated with amylose thereby protecting the bacteria during processing, storage, and passage through the gastrointestinal tract (Myllä rinen *et al.*, 1999). Binding of adherent strains to the resistant starch core may facilitate encapsulation of the bacteria. Further investigations are necessary to determine the degrees of adhesion of bifidobacteria and other bacteria to different types of resistant starch in the gastrointestinal tract and the impact of adhesion on

substrate utilization, colonization, and competition in the oligotrophic colonic environment.

A synbiotic result from the combination of a probiotic and a prebiotic in a single product that is used as a healthy dietary supplement in the restoration and maintenance of colonic microbiota (Nova *et al.*, 2008). An exciting development in the field of pet animals is that of synbiotics targeted to particular species. This has been attempted for the first time with canine synbiotics. Five candidate lactobacilli, *L. acidophilus*, *L. murinus*, *L. reuteri*, *L. mucosae*, and *L. rhamnosus* were isolated from a Labrador dog (Tzortzis *et al.*, 2004). Three of these strains, *L. mucosae*, *L. acidophilus*, and *L. reuteri*, were then evaluated for their growth on various carbohydrates and antimicrobial activity against *Salmonella enterica* serotype typhimurium, enteropathogenic *E. coli*, and the toxin negative mutant of *E. coli* O157:H7. Based on of these data, a candidate synbiotic forms can be identified with activity against specific target pathogens. This canine synbiotic concept was taken further in an attempt to manufacture a prebiotic targeted to a particular probiotic organism.

Many different environmental factors may affect the gut microbial ecology; these include diet, medication, stress, age and general living conditions. Knowledge of the gut microbiota and its interactions has led to the development of dietary strategies that serve to sustain, or even improve normal gastrointestinal microbiology. However, it is clear that microbiota management through diet is achievable. Importantly, the scientific tools for determining probiotic and prebiotic effects now exist and should be exploited. This has led to a vibrant, global, functional food industry that is introducing new products for gut health into markets targeted to pet animals.

### Methodological Approaches

The gastrointestinal microbiota comprises of a very complex consortium of different types of microorganisms. A major drawback is the fact that scientific knowledge on gut bacteria is too scarce to determine what is a 'balanced' microbiota or even a 'normal' microbiota. The gut microbiota includes microbial communities assembled on the mucosal surfaces and in the lumen of the gut. These communities include both native species, that permanently colonize the tract-'persistent'/'resident'

strains, and a variable set of living microorganisms, that transit temporarily through the tract-'occasional'/'transient' strains (Guarner and Malagelada, 2003). However, most bacteria in the gut are not culturable and their phenotypic characteristics are unknown. They are acquired after birth and initial colonization largely depend on environmental factors. In addition, transient bacteria are continuously acquired from the environment. Recent evidence suggests that host genotype may influence the final composition, which is host-specific and relatively stable over time in a given individual (Zoetendal *et al.*, 2004).

Molecular tools based on 16S rDNA sequence similarities such as fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), quantitative dot blot hybridization, restriction fragment length polymorphism (RFLP) and large scale 16S rDNA sequencing have helped to overcome the limitations of conventional microbiological plating methods in studying the fecal microbiota composition. However, these tools are just now beginning to be applied to understand the dynamics of this complex community, and its relationship to diet and gut health. So, there is a need to understand both the limitations of the current data and the importance of moving forward with the best feasible molecular techniques.

The application of 'omics' technologies, i.e. genomics (study of genome), nutrigenomics (study of effects of diet on gene expression), transcriptomics (study of mRNA), proteomics (study of expressed proteins), metabolomics (study of metabolites), and epigenomics (study of genome modification) represents perhaps the greatest opportunity and challenge to date for nutritionists and microbiologists to elucidate the complex interactions between gut microbiota and host (German and Young, 2004). These biotechnological approaches are mainly targeted at 4 levels: adequate nutrient supply, healthy intestinal architecture, balanced microbiota composition and host-commensal interactions.

#### *Adequate nutrient supply*

Nutrigenomics puts forward a totally new approach to the monitoring of biological phenomena, associated with nutrition (Roberts *et al.*, 2001, Ommen van and Stierum, 2002). This new approach consists of analysis of many minute, even discrete changes associated with the genetic response to nu-

tritive stimuli. This kind of approach requires prior knowledge of unknown biochemical and physiological effects, which are difficult to identify with the help of the developed markers (a single gene, a single protein or a single metabolite).

### *Healthy intestinal architecture*

Mucosal surfaces represent the main sites in which environmental microorganisms and antigens interact with the host. In particular, the intestinal mucosal surfaces are in continuous contact with a heterogeneous population of microorganisms of the endogenous as well as exogenous origin. Moreover, the intestinal morphology changes with nutritional variations, stress, aging, and disease conditions. Because the absorptive functions of the intestine are related to its morphology, alteration in morphology may predispose the intestine to functional disorders. Villous height, crypt depth and epithelium thickness of intestinal segments are direct representations of the intestinal environment and may be used as indicators of gut health. These parameters can be measured by scanning electron microscopy (SEM) and by image analysis software, Microsoft Olympus.

For the critical events in gut mucosal health (e.g. cell proliferation or apoptosis), novel functional biomarkers are also developed based on transcriptomics.

### *Balanced microbiota composition*

Only limited literature exists on the normal gut microbiota of pets (Rastall, 2004). So, challenges with this approach include the difficulties of assessing and sampling many regions of the gastrointestinal tract without altering gene expression of microorganisms and issues of variability between individuals. Much of the information presently available regarding colonic microbiota comes from studies that employed conventional microbiological cultivation techniques based on agar plates. This poses a problem, however, as colonic microbiota are thought to contain a high level of biodiversity including many species that cannot be cultured using present techniques. Moreover, this approach is rather laborious, time consuming and often inaccurate. It is also limited in scope, as majorities of the bacterial species present in feces are not culturable using standard microbiological techniques. Conse-

quently, our picture of the intestinal microbiota has been biased in favor of the more easily cultured members of the community. Even among those that are culturable, species identification by traditional identification methods is often difficult, if not impossible; only a limited number of species has been fully characterized. In order to overcome the limitations associated with culturing techniques, molecular biological methods are increasingly being applied to study the GI tract ecology. This unculturable flora can only be characterized by using DNA-based microbiology methods (Langendijk *et al.*, 1995, McCartney *et al.*, 1996). Most of these methods rely on amplification, detection, and/or sequencing of diagnostic regions of 16S rRNA genes (Harmsen *et al.*, 2000). This culture problem is particularly acute in studies on canines. A study conducted by Greetham *et al.* (2002) illustrated the unreliability of apparently selective agar media for enumeration of canine fecal bacteria; many of the selective media used did not support the growth of the target population. The image of the canine gut flora, then, is based largely on traditional methods of investigation (Simpson *et al.*, 2002). In one study (Tzortzis *et al.*, 2004); fluorescent in situ hybridization (FISH) was used to describe the flora of Labrador's dog (Fig. 1). However, the most significant aspect of the canine microbiota is the much lower level of bifidobacteria found in canines than in other animals due to their dietary habit. According to their diet, herbivores contain a higher number of bacterial phyla, while carnivores the fewest number, and omnivores are at an intermediate level (Ley *et al.*, 2008).

### **Total genomic DNA hybridization method**

It utilizes the whole genomes rather than small genomic regions to determine the degree of similarity between two microbes. It formed the basis for molecular microbial phylogeny before the advent of the 16S rDNA revolution.

### **Genus- and species-specific PCR primers**

Use of 16S rDNA or rRNA and its encoding genes as target molecules are one of the most widely used approaches in ecological studies (Amann *et al.*, 1995). Specific PCR primers and probes can be designed based on the variable regions of this molecule to detect certain species or groups of bacteria.

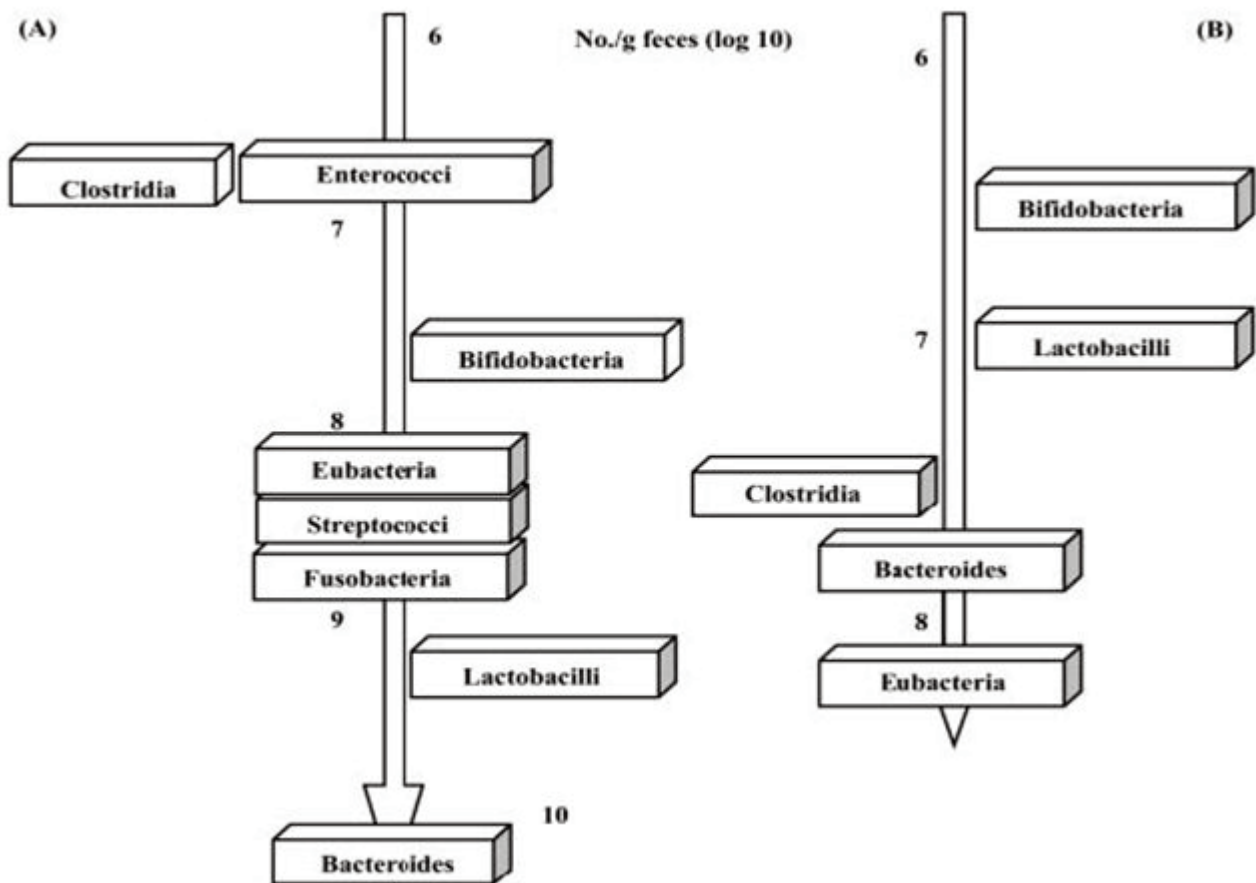


Fig. 1: Overview of the canine colonic microbiota.

(A): Bacterial groups were enumerated by selective media and confirmed by DGGE in adult Shepherd dogs (Simpson *et al.*, 2002).

(B): Bacterial groups were enumerated by FISH in adult Labrador dog (Tzortis *et al.*, 2004).

Numerous genus- and species-specific PCR primers and probes have been developed for bifidobacteria (Yamamoto *et al.*, 1992, Langendijk *et al.*, 1995, Kaufmann *et al.*, 1997, Matsuki *et al.*, 1999).

Species-specific primers and probes are excellent tools for targeting certain *Bifidobacterium* spp. in mixed populations, providing valuable help in identification, which is laborious and sometimes unreliable by phenotypic characterization. However, the use of specific primers and probes in ecological studies rules out the possibility of finding other than the target *Bifidobacterium* spp. possibly also present in the sample. On the other hand, genus-specific primers or probes can give a good overall picture of the bifidobacterial population, but no information is obtained about the species or strain composition. Another way of utilizing the rRNA sequence heterogeneity in microbial ecology is to use universal bacterial PCR primers to amplify a fragment of rRNA or rDNA and then separate the obtained PCR products in a sequence specific manner in temperature gradient gel electrophoresis

(TGGE) or denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993, Muyzer and Smalla, 1998). The gut bacterial profiling obtained by TGGE or DGGE represents the prominent bacteria in the community. This technique has already been successfully applied to monitor the bifidobacterial communities in human fecal samples (Zoetendal *et al.*, 1998). Recently, genus-specific primers were designed for *Lactobacillus* spp. and also used successfully in combination with DGGE to analyze communities of lactobacilli. This approach opens new possibilities to follow the qualitative changes in the bifidobacterial and lactobacilli populations in response to probiotic or prebiotic administration as well as to study the effect of age, genetic background and other factors on the composition and diversity of these bacterial groups.

#### Species specific quantitative real time PCR:

Currently, traditional plating methods, conventional PCR, and FISH are used for the enumeration of lactobacilli. Traditional plating methods have

some major disadvantages compared to modern molecular techniques, such as insufficient selectivity and the presence of 'non-culturable' bacteria in fecal samples (O'Sullivan, 2000). The FISH technique provides reliable quantitative data for physiologically related groups of bacteria and detects non-culturable organisms. It is currently used to quantify the genus *Lactobacillus* in feces. However, with the commonly used FISH probe (S-G-Lab-0158-a-A20) for quantification of the genus *Lactobacillus*, genera such as *Enterococcus*, *Pediococcus*, *Weissella*, *Vagococcus*, *Leuconostoc*, and *Oenococcus* are also detected (Harmsen *et al.*, 1999). In addition, the detection limit of FISH is rather high, which disables the quantification of very low bacterial numbers present in fecal samples, for example, different *Lactobacillus* spp. The conventional PCR is sufficiently sensitive for the detection of the different *Lactobacillus* spp. (Song *et al.*, 2000, Walter *et al.*, 2001). However, the conventional PCR can only be used for semi quantitative assessment, due to endpoint analyses limitations such as the plateau phase (Morrison and Gannon, 1994) and diminishing effects of differences in PCR product abundance (Mathieu-Daude *et al.*, 1996). Contemporary quantitative real-time PCR allows the monitoring of the complete amplification and as a consequence, overcomes the limitations correlated with endpoint analysis of the PCR process. To follow the PCR process, the use of specific fluorescently labeled probes or a minor groove binding dye, like SYBR Green, can be utilized (Bustin, 2000). A major disadvantage of the minor groove binding dye is that these bind non-specifically to all double-stranded DNA and may therefore, reduce the specificity of a PCR reaction. For enumeration of the relatively small amounts of the different *Lactobacillus* spp. in fecal samples duplex 5' nuclease assays were developed. These assays use a specific fluorescently labeled (TaqMan) probe during the amplification to ensure a high specificity as well as sensitivity.

#### **Microchip approach:**

While the feasibility of it has been established, currently available chips are still limited in scope for pets. These microchips are specifically designed to analyze the human fecal microbiota.

#### **Chromatography**

The beneficial bacteria exert their action by secreting enzymes. These enzymes of a particular bacterium can be purified from its cell extract by anion-exchange chromatography, adsorption chromatography, and size-exclusion chromatography. Apparent molecular masses can be judged by size-exclusion chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Moreover, the bacterial metabolites like the total short chain fatty acids (SCFA) and their fractions - acetate, propionate and butyrate concentrations can be determined by gas-liquid chromatography (GLC).

#### **Pulsed-field gel electrophoresis**

The health benefits imparted by probiotic bacteria are strain specific, and not species or genus specific. This usually requires the presence of receptors on the bacterial cell wall that permit attachment to the gastrointestinal epithelium. This strain or clonal relationship is determined by pulsed-field gel electrophoresis. The results suggest that a clone need not be present in a food in high numbers to establish itself in the intestine.

#### **Microarrays**

Short-chain fructo-oligosaccharides (scFOS) and other prebiotics are used to stimulate selectively the growth and activity of lactobacilli and bifidobacteria in the colon. However, there is little information on the mechanisms whereby prebiotics exert their specific effects upon such microorganisms. To study the genomic basis of scFOS metabolism in *Lactobacillus plantarum* WCFS1, two-color microarrays were used to screen for differentially expressed genes when grown on scFOS compared to glucose (control).

#### **Denaturing gradient gel electrophoresis (DGGE)**

Both prebiotics (fructooligosaccharides) and probiotics (*Bifidobacterium lactis* and *Streptococcus thermophilus*) supplemented diets induced a significant reduction of *Clostridium* and *Bacteroides* spp. compared to control diet, whereas prebiotics were also able to reduce the number of coliforms and to

increase the presence of Bifidobacterium spp. DGGE analysis showed a significant increase of 16S rRNA gene fragments in rats fed with either probiotics or prebiotics (Alejandra et al., 2005).

### **Fluorescent in situ hybridization (FISH) and terminal restriction fragment length polymorphism (t-RFLP)**

Dinoto *et al.* (2006) determined the bacterial populations in cecal samples of rats by FISH and terminal restriction fragment length polymorphism (t-RFLP).

#### *Host-commensal interactions*

The ability of bacteria to communicate with each other, and with the host, has been known for over a quarter of a century. However, genetic and molecular tools have made it possible to isolate the signals, study their mechanisms and potentially manipulate their effects for promoting gut health. The ability of probiotic organisms to affect host gene expression has been demonstrated in different systems. For example, the turning on of mucin expression by *Lactobacillus* spp. (Mack *et al.*, 1999) and by activating transcription factors involved in cytokine signaling directly, leading to NF  $\kappa$ B activation, and indirectly via cytokines, leading to signal transducer and activator of transcription (STAT) activation (Miettinen *et al.*, 2000). Human genome arrays now provide a means to study the effect of introduction of a probiotic organism on host gene expression. Such systems can document changes in differential gene expression (Cox *et al.*, 2001). In addition, quantitative morphometrix (numbers of T cells, B cells, macrophages, and peripheral lymphocyte sub populations like CD3, CD4, and CD8) can provide a means to study the key gene events changed with prebiotic use, thereby leading to identification of site of action and molecules involved.

Communication between bacteria has also been studied. One method involves molecules called autoinducers that are secreted by organisms to regulate gene expression and control behavior (Henke and Bassier, 2004). In many lactic acid bacterial strains, bacteriocins function as quorum sensing molecules, in that they are produced, and are controlled in a cell-density dependent manner, using a secreted peptide-pheromone that can enable the or-

ganism to switch on bacteriocin production at times when competition for nutrients is likely to become more severe (Eijsink *et al.*, 2002).

### **Conclusions**

These molecular tools need to be validated and standardized and should then be utilized to build a database of the pet intestinal microbiota, which will form the basis for determining the degree to which the microbiota can be influenced by dietary alterations. A concerted, multidisciplinary effort, incorporating molecular microbiology techniques in the setting of well-designed prospective studies, is needed to advance our knowledge of the complex interactions between host and microbiota to the point that we can design effective dietary interventions. Ultimately, this should lead to clinical intervention studies to determine if diet-induced microbiota changes can reduce the risk of major gastrointestinal disorders thereby maintaining gut health. Such intensive studies will exploit many new functional foods in the pet-care field.

### **Future Perspectives**

Outcomes tested with feeding studies in pet animals are frequently based on targets used in human research. The effectiveness of probiotics and prebiotics are likely to differ for different species, based in part on varying nutritional needs. Human probiotics have been used successfully in pet animals (Rastall, 2004), but a more effective approach would likely be use of strains found naturally in the target animal (Fuller, 1989). New bacterial genera may be discovered to be effective probiotics in pet animals. Understanding at the cellular level the interactions of specific probiotics with other intestinal bacteria and with different types of host cells is fundamental to understanding how the probiotics and indirectly prebiotics function, the basis for observed effects on canine physiology. Genetic technologies will be essential to providing necessary precision for revealing the secrets of probiotic and prebiotic actions as well as driving invention of strains with targeted functions.

Moreover, the focus of most experiments using dogs has been narrow, with most groups focusing on microbial populations rather than on indices of gut health. Future research should aim at the establishment of accurate relationships among the com-

position of the colonic microbiota, gut health and clinical outcomes observed in the animal. The various molecular tools will lead to a comprehensive, unbiased database documenting gastrointestinal commensal bacteria- the canine microbiome. The crucial component would be the development of a metagenomic database of canine microbes, preferably all regions of the gut and all stages of microbe development. In practice this can be achieved by using magic angle spinning (MAS), nuclear magnetic resonance (NMR), high pressure liquid chromatography (HPLC) and mass spectroscopy (MS). These techniques are well developed and are indeed being used to establish a metabolomics database of the rat intestinal tract. This approach will result in a tremendous increase of knowledge of the physiology and genetics of disease, and it will pave the way for defining the underlying molecular mechanisms in health and disease associated with GIT, and ultimately developing functional biomarkers for assessing disease risk and for use in dietary trials in pet animals. The current culture methods also need to be modified for pet animal applications. A polyphasic strategy based on selective plate culture for screening and colony isolation, with molecular techniques for speciation should be used.

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