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Innovations in Canine and Feline Nutrition: Technologies for Food and Nutrition Assessment

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Abstract

Pet owners have increasing concerns about the nutrition of their pets, and they desire foods and treats that are safe, traceable, and of high nutritive value. To meet these high expectations, detailed chemical composition characterization of ingredients well beyond that provided by proximate analysis will be required, as will information about host physiology and metabolism. Use of faster and more precise analytical methodology and novel technologies that have the potential to improve pet food safety and quality will be implemented. In vitro and in vivo assays will continue to be used as screening tools to evaluate nutrient quality and adequacy in novel ingredients prior to their use in animal diets. The use of molecular and high-throughput technologies allows implementation of noninvasive studies in dogs and cats to investigate the impact of dietary interventions by using systems biology approaches. These approaches may further improve the health and longevity of pets.

INTRODUCTION

The companion animal population is increasing worldwide. Recent surveys show that more than 60% of all American households have at least one cat or dog, and there are approximately 83 million dogs and 96 million cats in the United States (1). Besides their popularity, the importance of companion animals has changed considerably in the past several decades. Previously, dogs and cats were kept mostly outdoors to work as guardians, livestock herders, hunters, and pest controllers. Although a few pet dogs and cats may still be used for these purposes, the importance of companion animals in today's society goes well beyond those roles. Pet owners today have a strong emotional bond with their animal companions. Many consider their pets to be members of the family and attribute human characteristics to them, a phenomenon known as anthropomorphism. There is also increasing evidence of the important role that companion animals play in the psychological, social, and physiological health of humans.

Contemporary pet owners, self-identified "pet parents," are focused on enhancing the health status, well-being, quality of life, and longevity of their pets. Concerns pertaining to the nutrition of companion animals far surpass concerns about the nutrient adequacy of commercial pet foods, but rather emphasize the safety, quality, and traceability of ingredients' processing transparency and use of functional ingredients to improve pet health. Among the different sectors of the pet food industry, organic, natural, grain-free, GMO-free, human-grade, holistic, wholesome ingredients, and raw and dehydrated products are in the spotlight. Despite consumers' (pet owners') interest in these products, very little scientific information is available about the health benefits of these ingredients and products for pets. Furthermore, many of these terms are poorly defined and have little, if any, regulatory supervision or meaning.

To continue to meet the high expectations of pet owners, and to further develop our knowledge in companion animal nutrition and pet food technology and safety, the generation of scientific evidence will be imperative. Research should be done to expand the portfolio of novel and alternative ingredients and to determine their chemical composition, gastrointestinal tolerance, and nutrient digestibility, as well as their potential health benefits and safety. As scientific efforts continue to be made in this field, researchers must consider the availability and acceptance of scientific methods and technology used in companion animal nutrition research. The close companion animal–human bond and the humanization of dogs and cats result in the lack of acceptance of invasive techniques and terminal studies, despite their scientific merit. Thus, in companion animal nutrition, technological innovation is not always related to development of new technologies or to use of the best available research methods, but rather to the ability to adjust to a new paradigm and apply acceptable scientific methods that meet the softer requirements and demands of the pet food industry and societal values.

In vitro systems have been used extensively to assess hydrolytic and (or) fermentative digestion capacity and kinetics of single ingredients or diet matrices (2). In addition, alternative animal models, such as the cecectomized rooster and young chick and rodent [used in protein efficiency ratio (PER) assays], also have been widely used in companion animal nutrition to estimate small intestinal and standardized amino acid (AA) digestibility and protein quality, respectively. In addition, the rapid development of analytical and research tools, such as near-infrared reflectance spectroscopy (NIRS) and nano- and -omics (e.g., nutrigenome, microbiome, and metabolome) technologies, along with advances in bioinformatics and data analysis, have offered new analytical platforms in companion animal nutrition. These technologies and research methods are the focus of this review.

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USE OF SELECT TECHNOLOGIES FOR INGREDIENT AND PET FOOD EVALUATIONS

Pet owners demand innovative, high-quality, and safe food products for their pets. The pet food industry has responded to this demand by embracing technological advances in the agro-food sector. Emerging technologies have presented new approaches to study pet foods and will allow the pet food sector to offer higher quality, safer products in the future.

Proximate Analysis, Near-Infrared Reflectance Spectroscopy, Nanotechnology

Proximate analysis. Originally, the most extensive information about the composition of foods was based on a system of analysis described as the Weende method of proximate analysis of foods, devised over 150 years ago (3). Recently, new analytical techniques have been introduced, and the information about food composition is expanding rapidly. However, proximate analysis still forms the basis for the mandatory declaration of nutrient composition on food labels in Europe and the United States.

The proximate, or Weende, system of feed analysis is a quantitative method to determine select macronutrients in feed. Basically, it is the partitioning of feed components into six categories according to their chemical properties: moisture (crude water), crude ash (CA), crude protein (CP), ether extract (EE; fats or lipids), crude fiber (CF), and nitrogen-free extract (NFE). This system is viewed by some as being archaic and imprecise, and in the majority of laboratories, it has been coupled with more sophisticated analytical procedures. Major concerns relate to the imprecision of CA, CF, CP, and NFE measurements. For example, the ash procedure provides no information about the mineral composition of the food, and when required, analytical techniques involving atomic absorption spectroscopy are generally used (4), along with ICP-MS (inductively coupled plasma mass spectrometry).

Knowledge of the CP content of a food likewise is an insufficient measure for use in pet nutrition. CP is a calculated value obtained from measuring total nitrogen (N) content of a sample that will include both protein and nonprotein N, leading to an overestimation of actual food protein content (5). The CP value should be reported along with the AA composition of the food or ingredient. This information is indicative of how a food or ingredient might meet the essential AA requirements of the animal.

Similarly, the total EE content of food or ingredients does not provide sufficient information about food lipids because it is important to know their fatty acid composition, mainly their essential fatty acid content. When detailed information about AA composition of protein or fatty acid composition of fat is required, then techniques involving chromatographic separation must be used. High-performance liquid chromatography (HPLC) and gas-liquid chromatography are used to analyze AA and fatty acid profiles of foods, including ion-exchange chromatography and reverse-phase liquid chromatography (6, 7).

Carbohydrate measurements have been the most difficult of all the proximate constituents. Digestible carbohydrates, mainly starch and sugars, are included as NFE; however, because the NFE fraction is estimated by difference, it includes those components of the cell wall that are incompletely recovered in the CF residue but are resistant to pancreatic enzymes, such as a portion of the hemicelluloses and lignin as well as a variable portion of nitrogen fractions that escape Kjeldahl analysis, resulting in a poor representation of the actual digestible carbohydrate fraction of the food. This agrees with studies showing that the apparent digestibility of NFE is usually somewhat lower than starch digestibility results using direct starch analysis methodology (8). Sugars can be determined colorimetrically and by HPLC. Starch usually is determined by dilute acid hydrolysis of

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the sample followed by spectrophotometric determination of the released sugars; other enzymatic and chromatographic techniques also have been described (9). CF includes most of the cellulose and a variable portion of the hemicelluloses and lignin, plant cell wall constituents not available to intestinal enzymes and with low to moderate fermentation in the hindgut of dogs and cats. It is well known that CF seriously underestimates dietary fiber content of food resistant to digestive enzymes (10–12) but includes certain N compounds from animal connective tissue (13). The CF value is mandatory information on the pet food label despite its lack of accuracy and usefulness.

The fiber content of animal feeds and pet foods may be determined by other fiber analysis methods, such as the total fiber method (14), the neutral detergent fiber method (15), the total dietary fiber (TDF) method (16), the nonstructural polysaccharide method (17), and several other methods (16, 18–20). These methods can be categorized as either gravimetric or chemical analyses (21). Gravimetric methods do not provide information about the monomeric sugar composition of the fiber. However, TDF analysis is capable of separating and quantifying soluble and insoluble dietary fiber fractions. The TDF procedure (16) is more difficult to set up, more labor intensive, and more expensive than CF, but it provides an accurate estimate of the fiber content of the ingredient or diet (22). Even if some soluble fibrous fractions, such as low-molecular weight nondigestible oligosaccharides or resistant starch, are not quantified in the analysis, most fermentable and nonfermentable fibers will be included in the TDF fraction. Today, methods exist to capture all fiber fractions that reach the large bowel of the dog and cat (e.g., AOAC methods 2009.01 and 2011.25) (23).

Approaches to estimate the energy value of dog and cat diets without the need for in vivo balance trials are based mainly on factorial models using proximate analysis of foods and empirical models with determined or estimated energy values of foods and estimation of energy digestibility from their fiber content. Measurement of energy content of pet foods is not currently mandatory for pet food manufacturers; thus, prediction methods based on proximate analysis [modified Atwater factors; National Research Council (24)] are currently accepted by the Association of American Feed Control Officials (AAFCO) (25) and by European law (EU Comm. Dir. 95/10/EC) as acceptable for determining the metabolizable energy (ME) content of dog and cat foods. As inaccuracy of modified Atwater factors has been demonstrated in dogs (12, 26, 27), improvements in accuracy have been achieved when food fiber content is included in the equations (28). Moreover, related studies (10) also demonstrated more accurate results using TDF rather than CF for digestible energy (DE) prediction of dry dog foods. In June 2015, AAFCO (25) recommended enforcement of caloric content for all complete and balanced foods, snacks, treats, and nonexempt chews for dogs and cats. Existing commercial products will have a grace period of until January 2017 to comply, whereas new products will have to comply promptly. The caloric content must be displayed as kcal/kg of product and kcal/unit of product (e.g., cup, treat). A statement about the method used to determine the caloric content also will be required to ensure compliance.

Near-infrared reflectance spectroscopy. Currently, NIRS is used routinely for evaluating the nutritional quality of a wide range of foods and food ingredients. The principles of NIRS were developed 40 years ago and were eventually established as a new branch of agricultural chemistry. Norris et al. (29) applied the technique to the evaluation of forage quality for the first time in 1976. NIRS quickly became a popular laboratory technique owing to its advantages (e.g., speed) compared with wet chemistry methods. NIRS is used in the food and feed industries to estimate total and available nutrient and energy content in both food ingredients and compound foods and is used in feed formulation and quality-control programs in the feed industry. It may be used as an alternative to in vitro or proximate analysis–based systems (30).

NIRS has been used to quantify a wide range of nutrients. Examples include estimation of moisture, protein, fat, starch, and fiber concentrations in grains (31–35); AA composition in wheat

(36–39); and other compounds in select ingredients. The use of NIRS to estimate available nutrient concentrations has been hampered by the lack of adequate databases of in vivo nutrient availabilities in feed ingredients. However, over the past few decades, NIRS has been used to estimate the ME content of feed ingredients and diets for poultry (40, 41), the DE content of feeds and feed ingredients for pigs (42, 43), the ileal digestible protein and AA concentrations in feed ingredients for pigs and poultry (44, 45), and the gross energy (GE) and ME concentrations of cereal food products (33).

NIRS technology belongs to the discipline of chemometrics that generates correlations between experimental data and chemical composition or physical properties of the tested samples by applying mathematical and statistical procedures. The absorption intensities that correlate with concentrations are determined, and standardization curves for the individual constituents are subsequently constructed by means of linear regression by using suitable software. By using the NIRS spectrum and the standard curves relating the spectrum with the sample concentration, unknown samples may be assessed for nutrient concentration.

Givens et al. (46) summarized the advantages and limitations of NIRS compared with traditional techniques. The advantages include the speed of analysis, which requires minimal sample preparation. In addition, NIRS analysis is a nondestructive method that allows simultaneous measurement of several components with high precision, and no reagent or chemical waste is generated. Moreover, in spite of the high instrument cost, high throughput makes NIRS a relatively inexpensive technique when expressed on a per-sample basis. However, its main limitation is the great care and effort needed in developing calibrations. Calibration procedures require a great number of reference method analyses, which are expensive and time consuming and worthwhile only if large sample numbers are available. Moreover, the complexity in the choice of data treatment demands highly trained personnel.

Although NIRS is used primarily in quality-assurance programs in the pet food industry for ingredients and final products, very few studies have been published on using NIRS in pet food assessment. Castrillo et al. (47) analyzed 56 extruded dog foods and generated calibration equations for nutrients and energy, as well as for apparent nutrient digestibilities and DE content, with good results. The coefficients for determining cross-validation were above 0.9 except for DE (0.87), and the standard errors also were relatively low. Alomar et al. (48) analyzed 59 extruded dog foods, obtaining calibration equations for GE of nutrients and National Research Council (NRC)-estimated (24) ME content of foods in addition to some AAs and trace elements. The results obtained for GE and for estimated ME were accurate. In those studies, a less precise estimation of available energy was expected compared with GE, and consequently DE and ME, because they depend on food characteristics as well as animal response to feeding. Indeed, Hervera et al. (49), using 71 commercial extruded dog food samples, found NIRS estimations accurate as estimated through application of the recommended equations from NRC (50) for prediction of GE and DE in pet food.

Nanotechnology. Nanotechnology is the study of the manipulation of matter on atomic and molecular scales. Nanotechnologies promise new benefits in many areas of science and technology. Their potential uses range from water purification to energy storage to multiple medical applications.

In food and agricultural systems, nanotechnologies cover many aspects, such as food safety, packaging materials, disease treatment, delivery systems, bioavailability, new tools for molecular and cellular biology, and new strategies for pathogen detection (51, 52). Actually, food nanotechnology started with the pasteurization process introduced by Pasteur to kill spoilage bacteria (nanoparticles of approximately 1,000 nm), which led to key advances in food processing and improvement in food quality (53).

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Nanotechnology applications for the food sector have been developing rapidly in recent years, offering several opportunities to provide better and safer consumer products that have been represented in pet food production technologies. The areas of key focus for the development of food industry applications of nanoparticles are (*a*) to size micronutrients as nanoparticles incorporating novel functional properties in products, (*b*) to encapsulate and deliver bioactive food components by using nanomaterials to enhance their bioavailability, (*c*) to functionalize nanoparticles for detection of food-related microbials, and (*d*) to develop innovative packaging to enhance food safety against pathogens by increasing the shelf life of foods (53).

In the pet food industry, nanotechnologies have been applied to improve textures and stability of ingredients (as nanoemulsions to improve thermodynamic stability and avoid addition of emulsifiers). Moreover, as nanoparticles are more efficiently absorbed and distributed throughout the body, the association of poorly bioavailable substances with nanoparticles results in enhanced digestibility and absorption rates, along with reduced spoilage and wastage and increased safety and quality of foods (54). Similarly, nanotechnology is being used to maintain the nutritional value and stability of food materials during processing, storage, and delivery by microencapsulation (e.g., probiotics passing intact through gastric acid).

Another important application of nanotechnology in the pet food industry is food preservation. Nanotechnology-derived food-packaging materials are the largest category of current applications for the food sector. A relatively low nanoparticle level is sufficient to change the properties of packaging materials. Applications for food packaging include (*a*) improving packaging properties (e.g., flexibility, gas barrier properties, temperature/moisture stability), (*b*) incorporating nanoparticles with antimicrobial or oxygen-scavenging properties, (*c*) incorporating nanosensors to monitor and report the condition of the food, and (*d*) producing biodegradable composites (54).

The same characteristics that may allow nanoparticles to provide healthier, safer, and higher-quality characteristics to foods may result in toxicological outcomes during their interaction with biological cells, tissues, and organs. Health implications for consumers may result owing to the greater absorption of certain nanoingredients or nanoadditives that may lead to better health outcomes. It is of concern that the introduction of nanoparticles into foods designed to carry dietary supplements could lead to introduction of foreign substances into the blood or to interactions with the normal microflora of the consumer in an unexpected way (52, 55). Such potential risks and knowledge gaps make it difficult to assess the overall risk of nanoparticle consumption and use in the food industry. The European Food Safety Authority and the US Food and Drug Administration analyzed and provided safety assessment guidance for nanotechnology applications to food and feed and stressed the need for suitable characterization and safety test approaches for such a fast-developing new field (56, 57).

In Vitro Nutrient Disappearance

In vitro enzyme digestibility methods have been used for years in an attempt to predict in vivo digestibility by both nonruminant and ruminant animals. Indeed, perhaps the most widely used in vitro method is that of Tilley & Terry (58), which simulates the degradation of dietary organic matter in the rumen by incubating test substrates in a vessel with rumen fluid. The method has proven to be reliable and remains widely used in the evaluation of ruminant feeds, mainly forages and high-fiber ingredients.

Boisen (59, p. 156) concluded that "in vitro digestibility methods have considerable potential for improving and optimizing the formulation of diets for farm animals," and the same may be said for diets for companion animals. In vitro models simulating gastric and small intestinal digestion and (or) fermentative digestion in the large bowel are a relatively inexpensive and rapid means of

simulating events occurring throughout the gastrointestinal tract of the dog and cat. Several types of in vitro models exist, including batch cultures, chemostat simulators, and computer-controlled systems. They range widely in complexity and capability, some systems are capable of simulating either hydrolytic or fermentative digestion, whereas others can simulate both. By monitoring digestive events at select time points, kinetic outcomes, such as rate of nutrient digestion, maximal rate of short-chain fatty acid production, and time to achieve maximal rate of short-chain fatty acid production, can be determined along with extent of dry matter or nutrient digestibility.

Longland (60) identified the major characteristics of an ideal gastrointestinal in vitro model: (*a*) Physiological quantities of enzymes should be used in sequence to digest the test substrate; (*b*) the pH of the compartment should allow the activation of enzymes and other cofactors; (*c*) digestive end-products should be removed from the system in a timely fashion; (*d*) at each stage, digesta should be adequately mixed; (*e*) the time that the digesta resides in any one compartment should simulate the residence time in the representative host animal species; and (*f*) a strict anaerobic environment is required for survival, growth, and activity of the gut microbiota used in the fermentation phase of the in vitro experiment. Most in vitro systems used today will not reach this high standard, with the major problem being inability to remove end-products after digestion has had a chance to proceed. All other criteria described above can be achieved, some more easily than others.

The simplest in vitro system to evaluate hydrolytically and fermentatively digestible substrates is a batch system that consists of a single, self-contained vessel in which samples are first hydrolytically digested for 6 h using simulated gastric juice (HCl-pepsin) and for 18 h using intestinal enzymes (pancreatin) at 39°C. Following hydrolytic digestion, fermentative digestion may be measured by filtering the sample, then adding a fecal suspension to the residue to act as a microbial inoculum. When simulating fermentative events in the dog or cat, time of fermentation usually is approximately 12 h to allow the microbiota some lag time at the beginning. This method does not allow addition or removal of test substrate from the vessel, and over time, this causes the microbes to enter the death phase, where cessation of fermentation occurs (2). This generally is not a problem provided shorter incubation times are studied. The three-stage method has been validated with in vivo data (61–63). Several other methods have been devised with a particular focus on protein digestibility. These are reviewed by Boisen (59).

An example of a complex digestion model is the TNO Intestinal Model (TIM-1) developed at the TNO Nutrition and Food Research Institute in Zeist, the Netherlands (64). This computer-controlled system is capable of simulating characteristics such as meal size, meal duration, peristalsis, pH, gastric and intestinal secretions, gastrointestinal transit time, and absorption of water and nutrients. It consists of four successive compartments simulating the stomach, duodenum, jejunum, and ileum. TIM-1 is designed to mimic physiological characteristics (i.e., gastrointestinal transit rate and gastric and intestinal secretions) based on in vivo data from the species of interest, in this case, the human.

The functional gastrointestinal dog model (FIDO) is similar to TIM-1 but has been modified to mimic the gastrointestinal tract of the dog. Smeets-Peeters (65) validated FIDO using data obtained from ileal cannulated dogs at the University of Illinois. Substrates evaluated were select carbohydrates, poultry by-product meal, and fresh poultry. FIDO proved to be an excellent model to rank substrates in terms of digestibility, but not to predict actual in vivo digestibility coefficients.

Some substrates need not be tested by using a three-stage in vitro method, as their hydrolytic digestibility is low or negligible. Dietary fibers would be prime examples in this category. Therefore, for fibrous substrates, only a fermentation phase is required. A microbial inoculum from the species of interest is necessary, and this is usually obtained from fresh feces. Again, simple batch systems can be used involving a single anaerobic vessel containing nutrient media mixed with the test substrate. The vessel then is inoculated with a fecal suspension containing microbiota and

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incubated at 37–39°C for various periods of time. Fermentability of the substrate is quantified by measurement of substrate dry matter disappearance, change in pH of the medium, production of short- and branched-chain fatty acids and gases, and changes in concentrations of microbiota present at the beginning and end of the fermentative phase.

A more complex in vitro model to simulate in vivo fermentative events is TIM-2, which simulates the proximal colon of the human (66). The system is inoculated with fresh fecal inoculum, and substrate is allowed to incubate for 16 h. The pH of the system is kept constant at 5.8, the pH of the proximal colon. Hollow fiber membranes connected to a dialysis machine remove fermentative end-products, such as short-chain fatty acids, and water to prevent build-up of metabolites that hinder bacterial growth and survival. Physiological characteristics of this system were established from data collected on victims of sudden death. Agreement has been noted with in vivo data where novel carbohydrates and prebiotics have been tested, but not in all cases. Unfortunately, to our knowledge, this system has not been tested with the canine or feline.

Other complex in vitro simulation models exist. These include SHIME (simulated human intestinal microbial ecosystem), a chemostatic system that evaluates the complete digestive process from stomach to colon in one closed system (67). Use of different vessels allows predictions of the hydrolytic and fermentative processes occurring in each region of the intestinal tract. However, despite the consistent movement of media through the vessels, this system lacks the ability to remove digested nutrients, all of which enter the simulated colon vessels, causing problems with the interpretation of these results as additional hydrolytic end-products are fermented at this site in vitro that would not be present in vivo.

Other fermentation-only models include the Reading Simulator, a three-stage continuous culture device consisting of three vessels mimicking the proximal, transverse, and distal colon of the human (68), and the EnteroMix Colon Simulator, a semicontinuous device that consists of four vessels representing the cecum and ascending, transverse, descending, and distal colon, respectively (69). Data obtained from both systems compared favorably with in vivo data, but these, too, have not been tested using experimental conditions and inoculum relevant to the pet animal.

In comparing these methods, batch culture is capable of simulating both hydrolytic and fermentative digestion events but does not allow steady-state conditions to be achieved in any gut compartment and, on occasion, will produce results far removed from what occurs in vivo. Chemostats mimic only the fermentative phase of digestion, so their use is limited. TIM-1 and -2 mimic both hydrolytic and fermentative phases of digestion but are labor intensive and expensive to maintain. Also, results obtained using these systems sometimes do not correlate well with in vivo results. Few validation studies have been conducted testing in vivo and in vitro responses in the same experiment, and so for now, it is recommended that in vitro systems be used as screening tools to reduce the number of substrates that must be evaluated in vivo (2) and to prevent unnecessary use of animals for experimentation.

In Vivo Nutrient Digestibility

As noted in the NRC (50, p. 12) publication *Nutrient Requirements of Dogs and Cats*, "nutrient digestibility values provide information on the relative amounts of nutrients in the diet that can be used for productive purposes and, additionally, serve as an index of overall quality of the ingredients of dog and cat diets." Nutrient digestibility is the difference between the amount of nutrient consumed by the animal and the amount of nutrient excreted in the stool divided by the amount of nutrient consumed and multiplied by 100% in order that the value may be expressed on a percentage basis. In conventional total tract digestibility studies, a distinction is not made between

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undigested dietary nutrients and endogenous nutrients secreted into the digestive tract that are not reabsorbed. Calculated digestibility values, then, should be referred to as "apparent." For AAs and fat, endogenous losses are quite substantial and are influenced by the presence of other nutrients as well as nonnutrients in the diet (70). Nyachoti et al. (71) concluded that endogenous nutrient losses interfere with the additivity of nutrient digestibilities in mixtures of ingredients present in complete foods, and with metabolic losses associated with the use of absorbed nutrients for production. To account for endogenous losses, ileal digestibility must be determined. This allows nutrients to be quantified before they reach the large bowel and are modified by the substantial microbiota population present at this site. In this procedure, animals are cannulated at the terminal ileum, and digesta are collected from this site at specific times throughout an experimental period. Inert digestion markers are used to track the passage of nutrients to this site. Ileal digestibility coefficients still should be considered "apparent" rather than "true," as endogenous secretions make up a portion of the ileal chyme. Nevertheless, these values are much more accurate and reflective of nutrient digestion events than are total tract digestibility measurements. Considerable research has been conducted at the University of Illinois on numerous pet food ingredients fed to ileal cannulated dogs (72–78).

Sauer & Ozimek (79) and Scott et al. (80) identified inherent inaccuracies in determining nutrient digestibilities and availabilities. Problems can occur with sampling of the food, estimation of nutrient intake, conduct of the chemical analyses, and improper collection and sampling of ileal digesta and (or) feces. Use of indigestible markers in the diet can help to overcome some of these problems, but they, too, have their shortcomings, as several assumptions must be made about marker use (i.e., homogeneous distribution of the marker in the gastrointestinal tract, marker recovery, marker analysis, proper sampling procedures). AAFCO (25), the organization that publishes protocols used to measure the nutritive value of dog and cat foods, recommends a five-day dietary adaptation period followed by a five-day fecal collection period to ensure accurate digestibility measurements. A shorter period (three and four days, respectively) may be used for dogs but not for cats because their consumption and excretion patterns often are more variable than that of dogs.

Shields (81) identified four major factors that can affect digestibility values. These include (*a*) food processing effects (ingredient particle size and modifications to the preconditioner, pellet mill, extruder, retort apparatus, or drying oven), (*b*) feeding management practices (previous diet fed, amount of food offered), (*c*) animal factors (breed, age, gender, activity level, physiological state), and (*d*) housing and environmental factors (metabolism cages versus covered kennels, effective environmental temperature, caretaker-animal relationship, photoperiod). More data exist for some of these factors than others, but all could have an effect on the extent of nutrient digestibility by the dog and cat.

Alternative Animal Models

The domestic dog and cat are the ideal animal models for evaluation of ingredients and foods intended for canine and feline pet nutrition. However, new societal values, increasing concerns about animal welfare, the high cost of animal care and specialized facilities, and ethical considerations have hindered their use in this field, increasing the need to seek alternative animal models. The PER assay, the cecectomized rooster, and the ileal cannulated pig are potential alternative animal models to screen ingredients that might be suited for canine and feline nutrition, especially those pertaining to protein quality and AA digestibility of select ingredients used in pet foods, as well as energy status of animals fed the select ingredients. These alternative animal models should complement in vivo apparent total tract nutrient digestibility and pet food palatability studies

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in the domestic dog or cat, as well as studies searching for beneficial effects of nutrition (e.g., nutraceutical ingredients) on canine and feline health.

The PER assay is a method used to evaluate the quality of protein in foodstuffs. This assay requires the use of a growing animal model (e.g., chicks or rat pups), as protein deficiency and lack of an adequate dietary supply of essential AAs can be more easily and rapidly detected during growth. The PER assay was used first to predict protein quality of human foods (82) and later to evaluate the protein quality of various proteinaceous ingredients used in animal feeding (75, 83, 84). The simplicity, low cost, and sensitivity of the PER assay make it a good screening tool for protein-quality evaluation and determination of biological value of novel ingredients with potential use in pet foods, prior to making large investments in diet manufacturing and in vivo testing using dogs and cats. The PER assay consists of feeding a test ingredient as the sole source of dietary protein in a diet containing 9–10% crude protein for a period of time ranging from 28 days in rats to 6–14 days in poultry (82, 85). A control diet often is used. This diet can be either N-free, which allows correction for maintenance weight loss (86), or casein-containing and can be used as a reference to report the relative PER value of the test ingredient (87). The PER value is calculated as the grams of body weight accreted divided by the grams of protein intake. A PER value equal to or greater than 2 is considered indicative of a good-quality protein. Whey, casein, and egg are considered three of the highest-quality proteins, with PER values above 3. Because animal- and plant-protein meals and other protein coproducts are commonly used in pet foods, a good understanding of variability and bioavailability of the AAs in these ingredients is important for formulation of complete and balanced diets for dogs and cats. Substantial research has been conducted at the University of Illinois on numerous protein sources as potential ingredients for pet foods using the PER assay (88–90).

In addition to the PER assay, the cecectomized rooster assay also can be used to estimate the AA digestibility of protein ingredients used in animal nutrition. In contrast to digestibility studies in dogs and cats, this assay is faster and less expensive and minimizes the confounding factor of protein degradation by bacterial populations in the hindgut (in this case, the ceca), providing a more accurate evaluation of different protein sources by enzymatic and hydrolytic digestion. This methodology involves the cecectomy, removal of the paired ceca of the bird, the major fermentative organ housing the gut microbiota of an adult rooster, followed by an 8-week recovery period (91). In this assay, the roosters are fasted for 24 h prior to crop intubation of approximately 30 g of the test ingredient (92). Excreta (urine and feces) are collected for a period of 48 h. Standardized AA digestibility also can be determined by measuring endogenous secretions of AA by roosters that are maintained in a fasted state throughout the experimental period. Excreta samples of crop-intubated fed and fasted birds then are lyophilized and ground prior to determination of gross energy, nitrogen, and AA concentrations. By applying these techniques, apparent and standardized AA digestibility, as well as true metabolizable energy corrected for nitrogen (TMEn), can be calculated, as described by Sibbald (92) and Parsons et al. (93), respectively.

Johnson and coworkers (75) used the cecectomized rooster and the ileal cannulated dog to compare the AA digestibility of dog foods made with different protein sources (meat and bone meal, poultry by-product meal, lamb meal). These authors found that AA digestibility values obtained by employing these two animal models were highly correlated, especially for lysine ($r = 0.89$), cystine ($r = 0.94$), threonine ($r = 0.87$), methionine ($r = 0.90$), total essential AA ($r = 0.90$), and total AA ($r = 0.92$). The protein quality of select protein ingredients suitable for pet food manufacturing, such as corn protein concentrates, fish and meat cuts, meals, and hydrolysates, has been determined with this model (78, 89, 90). Similar to the cecectomized rooster assay, the conventional (noncecectomized) rooster also has been used to determine the TMEn for various novel carbohydrates and fiber sources with potential use in pet and human foods (94–96). The

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conventional rooster assay has been a valuable animal model to evaluate the caloric content of foodstuffs produced for their low caloric content and low-density properties. In recent years, this area of research has gained increased attention owing to the prevalence of health complications associated with overweight or obesity. The use of the rooster model allows a better representation of the digestive process in contrast with in vitro assays for determining ME content of foodstuffs but also allows for a shorter, easier, and more accurate collection of data than occurs when human subjects and the canine models are employed (94).

The ileal cannulated pig is another animal model for studying hydrolytic digestion of novel ingredients that may have relevance to the companion animal. The pig shares more gastrointestinal tract similarities with the dog than does the cecectomized rooster. Similar to the ileal cannulated dog, the ileal cannulated pig allows sample collection before digesta passes through the large intestine (cecum and colon), where most fermentative activity takes place. Like the dog, the pig is an omnivore with similarities in feeding behavior and flexibility in types of foods and ingredients accepted (e.g., raw ingredients, meal diets, dry extruded pet foods, canned pet foods, pet treats). Also, the quantity of sample that can be collected from the ileum of the pig is sufficient to conduct numerous assays. Standardized AA digestibility measurements may be made, as well as DE content and metabolizable energy content if urine is collected.

IMPACT OF NUTRITION ON PET HEALTH

Advances in companion animal nutrition and veterinary care of pet dogs and cats have resulted in longer life expectancy than ever before (97). Recently, it was reported that over 90% of the canine and feline population of developed countries such as the United States and Australia consume at least half of their food intake from commercial products. Thus, the popularity of nutritionally complete and balanced pet foods seems to be a contributing factor to the improvement in pet health (98). Furthermore, companion animal nutrition practices go well beyond providing nutritionally adequate foods for dogs and cats. They focus on improving the health, longevity, and quality of life of pets by investigating the role of novel ingredients and understanding the relationships between the animal, its nutritional physiology, and the metagenome on the prevention and treatment of diseases.

Novel molecular and high-throughput methodologies allow for a faster, more informative, and holistic view of organisms and systems of interest (99). Some of these methodologies are currently being used in the field of companion animal nutrition, for example, nutrigenomic strategies that study nutrient-gene interactions and how diet may turn on or turn off genes related to nutrient metabolism or genes related to certain diseases (e.g., obesity); microbiome studies that focus on characterizing the population of microorganisms present in various body sites and understanding their role as it pertains to host health and (or) disease status; and metabolomics approaches that provide new platforms for the identification and qualitative or quantitative analysis of hundreds (potentially thousands) of metabolites that may be altered owing to nutritional interventions and (or) disease status. These methodologies are further explored in the following sections.

Nutrigenome

The genomics field includes the various high-throughput technologies used to generate, process, and apply molecular biology data to the composition and biological purposes of genomes (100). Broadly defined, genomics not only pertains to the study of genomes in terms of DNA sequence but also includes DNA/chromatin structure (epigenomics), transcription of DNA to messenger RNA (mRNA; transcriptomics), translation of mRNA to proteins (proteomics), and measures of metabolism (metabolite profiles; metabolomics). The use of genomic biology to study

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nutrient-gene interactions is referred to as nutrigenomics. Nutrigenomics is the study of how nutrients or bioactive dietary compounds affect host gene expression (mRNA). Nutrigenetics, a similar term that is defined as the study of how the genetic background of an animal affects nutrient absorption, metabolism, and transport, often is included in the nutrigenomics discussion.

Although traditional assays still may be used to measure and study DNA (e.g., Southern blotting), RNA (e.g., Northern blotting), and proteins (e.g., Western blotting), the availability of high-throughput molecular assays and the computer software programs and bioinformatics strategies required to interpret the large data sets they generate have dramatically altered the research landscape over the past decade and are most commonly used in nutrigenomics research projects today. For the measurement of mRNA, real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), microarrays, and RNA sequencing (RNA-Seq) are most common. RT-PCR is not high-throughput but is used to amplify and quantify a specific mRNA sequence that is known to be unique for the gene of interest. Because it is highly sensitive and has a rather low cost, it is most commonly used to test specific mechanisms of action where much data/knowledge already exists.

For biological processes or diseases where little is known, high-throughput assays that enable the measurement of thousands of genes simultaneously may be used not only to test hypotheses but also to generate them. Targeted assays such as RT-PCR then may be used to study specific mechanisms of action in more detail in subsequent experiments. Microarrays were developed in the mid-1990s (101, 102) and were used heavily for such purposes for 10–15 years. Several projects have used a commercial canine microarray to study aging and nutrition (103–107) and obesity (108–110) in dogs. A microarray is now available for cats, but few have used it for the study of nutrition (111). Microarrays had a dramatic impact on the field when they were first developed, but they are only semiquantitative in nature and are limited to measuring the mRNA sequences that have had fluorescent probes designed and included on the chip. RNA-Seq, however, enables the measurement of all mRNA sequences present in a sample. Because sequencing assays, especially those of the Illumina platform, also have improved greatly in terms of cost, sequence length and quality, and turnaround time over the past five years, RNA-Seq often is used instead of microarrays. RNA-Seq has been used sparingly to study canine and feline metabolism to date (112).

As regards nutrigenetics, in which DNA is the primary target, SNP chips have been developed and used for a variety of species, including the dog (113). Single-nucleotide polymorphisms (SNP) are single-nucleotide differences in the DNA sequence of a gene. Depending on the location and type of SNP, the effect on the animal may be undetectable, mild, or severe. The effects of SNP also may be additive, so even if they are not detected on an individual basis, SNP combinations may alter protein functionality significantly. Although many have successfully used SNP chips to identify a genetic basis for disease susceptibility or response to diet, their complexity and costs associated with the analysis of the millions of SNP that exist in mammalian genomes have limited their use. Similar to what has occurred with microarrays used for gene expression analysis, whole-genome sequencing strategies are now often used instead of SNP chips (114).

Gastrointestinal Microbiome

Many of the strategies and tools used to advance the field of nutrigenomics have been used to characterize and study the gastrointestinal microbiome of mammalian host species, including dogs and cats. Previously, our knowledge and understanding of microbial communities were based primarily on research using culture-based techniques. Because a small fraction of the microorganisms present in the gastrointestinal tract can be cultured and studied, progress in the field was greatly hindered. The recent availability of molecular assays has dramatically

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advanced this field and has enhanced greatly our understanding of the composition, dynamics, and functionality of the host-microbiota ecosystem in dogs and cats. Multiple DNA-based, culture-independent methods for microbiome analysis have emerged recently and may be useful tools to effectively identify and quantify microbial populations. These methods are summarized briefly in **Table 1**, with more in-depth reviews available in the literature for quantitative PCR (qPCR) (115, 116), fluorescent in situ hybridization (FISH) (117), restriction fragment length polymorphism (RFLP) (118, 119), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) (120, 121), and sequencing assays (122, 123).

Many of the molecular methods for determining the identity of bacteria are based on the 16S rRNA gene, including qPCR; FISH; and gel-based techniques, such as RFLP analysis, DGGE, and TGGE. Although they are not high throughput in nature, these procedures were an effective means by which to advance the field a decade ago; many research groups used these strategies to study the effects of gastrointestinal disease and diet on the microbiota of dogs (124–126) and cats (127–130), and vice versa. Sequencing assays were the next logical step, with clone libraries and Sanger sequencing being used initially (131, 132). Because sequencing assays are not dependent upon known primer sets or probes, these assays greatly increased the research scope and improved our vision of the microbiota communities that exist in the canine and feline gastrointestinal tracts. Although Sanger sequencing was used early on, the high-throughput sequencing techniques (i.e., 454 pyrosequencing, Illumina) were quickly adopted once available (133, 134).

Although 16S-based assays, especially those that include sequencing, have greatly expanded our vision of the gut microbiota, the rRNA gene provides information only on the identity of bacteria. It also does not allow for the study of archaea, fungi, and eukaryotes present in the gastrointestinal tract. Shotgun sequencing, which includes all DNA content in a sample, provides not only taxonomic information but also information as to the functional capacity of the microbiota community. A few recent shotgun sequencing studies focused on dietary intervention have been performed in dogs and cats and published (135–138). Although these experiments have provided much information, much more remains to be understood. Given the great functional redundancy that exists within the microbiome, a single taxonomic snapshot does not identify the mechanisms by which microbiota impact the health and nutritional status of the host and (or) how the host-microbe relationship may be altered by environmental factors, including diet. Hundreds of metabolites are synthesized by gut microbiota, including short-chain fatty acids, bile acids, indolic and phenolic compounds, vitamins, polyamines, lipids, and many others, that play a role in a wide variety of biological functions in the host (139). In the future, measurement of microbial shifts and metabolic by-products over time that will provide important data pertaining to microbial adaptability and efficiency is needed to increase our understanding of microbe-host relationships and how dietary intervention impacts health and disease.

Metabolome

Metabolomics has been defined as the qualitative and quantitative characterization of global metabolic profiles, and the study of the composition and interactions of the metabolites in response to interventions (e.g., diet) and environmental, cellular, tissue, and biofluid modifications (140, 141). In turn, metabolites can be defined as small molecules of intermediate or final metabolism (140) that provide a dynamic readout of biological processes and a potential link with transcriptomics and proteomics data (142). Metabolomics is a complex discipline, as mammalian and plant cells may contain 3,000 to 8,000 metabolites (143). Owing to its complexity, more specific disciplines have emerged to study smaller groups of metabolites of interest in biological systems. For example, lipidomics is a research field dedicated to studying the metabolites

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Table 1 Common molecular techniques used for microbiome analysis (summarized from References 115–123)

Method	Description	Characteristics	Primary advantages	Primary disadvantages
Quantitative polymerase chain reaction (qPCR)	Amplifies and quantifies a targeted DNA molecule	Dye or probe used to bind double-stranded DNA that causes intensity of fluorescent emissions to increase	Low cost; high sensitivity; allows for detection of sequences at low concentrations	Limited in scope
Fluorescent in situ hybridization	Sensitive detection of specific nucleic acid sequences in metaphase or interphase cells	Manual procedure of biological samples; fluorescence intensities measured using FLEX (a quantitative fluorescence microscope system)	Allows for localization and study of spatial organization of cells as they occur in their natural habitat	Costly; not easily scalable for disease screenings
Restriction fragment length polymorphism	High-throughput fingerprinting technique used to explore changes in structure and composition of microbial communities	DNA sample digested by restriction enzymes to characterize microbiota of specific regions; fragments then separated according to length by gel electrophoresis	Provides a broad view of microbial systems	Primers not specific
Denaturing gradient gel electrophoresis	PCR-amplified 16S rRNA fragments separated on polyacrylamide gel containing gradient of denaturant (e.g., urea, formamide)	Gel-based method of fingerprinting	Provides a broad view of microbial systems	Only semiquantitative and insensitive
Temperature gradient gel electrophoresis	PCR-amplified 16S rRNA fragments separated on polyacrylamide gel containing gradient of temperatures	Gel-based method of fingerprinting	Generates qualitative differences in microbial ecology	Only semiquantitative and insensitive
454 pyro-sequencing	Pyrosequencing light emission	500 base reads or higher	Decent 16S coverage	Costs much higher than Illumina, limiting coverage, especially with shotgun sequencing
Illumina sequencing	Fluorescent, stepwise sequencing	2 × 250–300 paired-end base reads	Very high coverage owing to high instrument output and very low cost	Shorter reads than other sequencing formats; increased bioinformatics costs and time owing to large volume of data
Sanger sequencing	Fluorescent, dideoxy terminator	750 base reads or higher	High read length and accuracy	Compared to next-generation sequencing, costly and has low throughput

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involved in pathways and networks related to the metabolism of lipids (144). The discipline of metabolomics can be applied in a myriad of scientific fields, such as crop sciences, toxicology, pharmacology, analytical chemistry, and nutritional and biomedical sciences (145, 146).

Despite its wide application, the field of metabolomics is relatively new; it was first applied in the 1970s by Pauling et al. (147), who investigated metabolites in urine samples to monitor health status in humans. In 2003, the National Institutes of Health's (148) Metabolomic Roadmap Initiative stimulated further metabolomics research. Because of the complexity of metabolite identification, and their qualitative and quantitative measurements, multiple analytical platforms are required. The most common analytical technologies employed to study metabolomics have been based on liquid chromatography–mass spectrometry (LC-MS), gas chromatography coupled with mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR). In addition, capillary electrophoresis–mass spectrometry, ultraperformance liquid chromatography (UPLC), and photodiode array also have been employed (146). Among the instruments used in metabolomics, GC-MS and LC-MS are able to detect a wide range of metabolites in complex mixtures, especially the LC technology that easily separates compounds of varying polarity. In contrast, the UPLC offers faster speed and better chromatographic resolution compared with HPLC. NMR is a highly selective and nondestructive analytical technique, provides reproducible measurements, and requires minimal sample preparation, but offers lower sensitivity. It can be used to identify a molecule and characterize its chemical structure (141, 146, 149). The ^{1}H NMR technology often is used in metabolomics studies, as the ^{1}H nucleus has a high natural abundance (>99.98%) (150). This technology also is used to identify metabolites of the same mass and configuration that cannot be differentiated by MS (151). Some advantages of the MS technologies are high sensitivity and selectivity; some are even able to provide information about the chemical structure of metabolites (141). The combination of LC-MS-NMR is perhaps the most efficient method to identify metabolites; however, it is not widely used owing to its complexity (152). Ultimately, the selection of a particular technology will depend on the scope of the study and compromises among instrumentation sensitivity, selectivity, and speed (141). Detailed descriptions of the technologies used in metabolomics are beyond the scope of this review, but these technologies have been described in great detail (141, 146, 149).

The hundreds or thousands of metabolites identified by applying variations in the technology described above can be quantified as a relative change or an absolute concentration, methods known as untargeted and targeted analyses, respectively. The untargeted analysis consists of the normalization of metabolite signal intensities in relation to internal standards or relative metabolites, whereas targeted analysis is performed by means of external or isotopically labeled standards (141). The high cost of targeted analysis has limited its use in exploratory studies evaluating large numbers of metabolites.

Metabolomics is a new discipline in companion animal nutrition, and only a few studies have been published based on this technology (153–155). Using GC-MS and LC-MS technology, Allaway et al. (153) investigated fasted plasma metabolic profile differences between adult dogs and cats fed a complete and balanced diet with or without glucose supplementation. Despite the idiosyncrasies in their metabolic adaptations, dogs and cats responded similarly when supplemented with dietary glucose. Using similar technology, Deng et al. (154) characterized the plasma metabolite profile of cats fed diets varying in their macronutrient composition. In that study, a distinct metabolite profile for cats fed a high-protein versus high-fat or high-carbohydrate diet was observed. Cats fed the high-protein diet had decreased nucleotide catabolism, whereas cats fed the high-fat diet demonstrated increased lipid metabolism and displayed markers of oxidative stress. More recently, de Godoy et al. (155) used untargeted plasma metabolite profiling to identify metabolic changes related to rapid body weight gain in adult dogs fed ad libitum.

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Distinct metabolite patterns were observed during early and late phases of body weight gain, with metabolites related to AA and lipid metabolism being most affected.

Additional studies that employed the metabolomics approach in companion animals have evaluated metabolite profile variations in dogs and cats (156), breed-specific metabolic fingerprints of dogs (157), the metabolite profile of toxicological markers in dogs (158), and the metabolite profile of cerebrospinal fluid in healthy and epileptic dogs (159, 160). Additionally, the Companion Animal Nutrition Program at the University of Illinois is currently applying metabolomics technology to investigate effects of exercise and diet, aging, gastrointestinal microbiota, and weight loss in dogs and cats.

FUTURE OF COMPANION ANIMAL NUTRITION AND TECHNOLOGICAL APPLICATIONS

Future advances in the field of companion animal nutrition will focus on understanding the impact of dietary interventions by using a systems biology approach, in an attempt to integrate large data sets from biological processes with nutrients and nonnutrient compounds present in a variety of ingredients and pet food matrices. Although this is an exciting area of research and seems to be a promising tool to further improve the health and quality of life of companion animals, the integration and application of these technologies will not be without challenges. First, the expansion of knowledge about the chemical composition of nutrient and nonnutrient compounds in novel ingredient sources used in pet foods will allow the creation of a database of compounds that may affect endogenous and xenobiotic metabolism of dogs and cats during and (or) after dietary interventions. Second, the effect of food processing (e.g., extrusion and retorting) is an area of research that must be explored. Similar to the other technologies covered in this review, food processing technologies are advancing. The vast majority of the commercially available pet foods are either extruded or retorted, and very little information is available on chemical transformations owing to processing conditions and their implications in animal metabolism. Third, the characterization and elucidation of the functional capacity of microbial communities inhabiting the gastrointestinal tract of dogs and cats will provide further insights into their role in and interaction with host metabolism. Fourth, the development of standardized protocols for nutritional studies pertaining to type of sample (e.g., food, blood, urine, stool, tissue), sample collection, handling, processing, methodology applied, and data curation and analyses will be crucial to develop an accurate public database linking transcriptomic, proteomic, metagenomic, and metabolomic data sets with specific nutritional interventions, host physiological status, and host disease status that can be used in future studies. Such endeavors are complex and will require scholarly collaborative efforts; however, they will be a major step toward identifying consistent biological signatures of relevance in the detection of small groups of metabolites that can be used as biomarkers of specific diseases, physiological status, and (or) characterization of nutritional interventions, as well as in the development of diagnostic tools for early disease detection or management.

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