



Archivos de Medicina Veterinaria

ISSN: 0301-732X

archmv@uach.cl

Universidad Austral de Chile

Chile

Garcia-Mazcorro, JF; Minamoto, Y
Gastrointestinal microorganisms in cats and dogs: a brief review
Archivos de Medicina Veterinaria, vol. 45, núm. 2, 2013, pp. 111-124
Universidad Austral de Chile
Valdivia, Chile

Available in: <http://www.redalyc.org/articulo.oa?id=173028392002>

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Gastrointestinal microorganisms in cats and dogs: a brief review

Microorganismos gastrointestinales en gatos y perros: una revisión breve

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RESUMEN

El tracto gastrointestinal (GI) de animales contiene diferentes tipos de microorganismos conocido como la microbiota GI. Por mucho tiempo, la microbiota GI ha generado interés porque los microorganismos GI están involucrados en múltiples procesos fisiológicos en el hospedero, así perpetuando salud o enfermedad. Estudios recientes han demostrado que la microbiota GI de gatos y perros es tan compleja como en humanos y otros animales, revelado con el uso de tecnologías de secuencia modernas y otras técnicas moleculares. La microbiota GI incluye miembros de todos los tres dominios principales de vida (Archaea, Bacterias y Eucariotas), pero las bacterias son el grupo de microorganismos más abundante y metabólicamente activo. El estómago de gatos y perros esta principalmente poblado de *Helicobacter* spp., el cual en perros puede representar tanto como el 98% de toda la microbiota bacteriana en el estómago. El intestino delgado contiene una microbiota más diversa, conteniendo representantes de al menos cinco diferentes filos bacterianos (principalmente Firmicutes y Bacteroidetes). El intestino grueso contiene el grupo de bacterias más abundante ($\sim 10^{11}$ células bacterianas por gramo de contenido intestinal), diverso (al menos diez diferentes filos han sido detectados) y metabólicamente relevante en el tracto GI. La mayoría de las bacterias en el intestino grueso son anaerobios estrictos, los cuales dependen de la fermentación de sustancias no digeridas para subsistir. Aunque estudios recientes han dilucidado las complejidades de la microbiota GI en gatos y perros, más investigación todavía es necesaria para encontrar maneras de manipular exitosamente los microorganismos GI para prevenir y/o tratar enfermedades GI.

Palabras clave: gastrointestinal, microbiota, gatos, perros.

SUMMARY

The gastrointestinal (GI) tract of animals contains different types of microorganisms known as the GI microbiota. The GI microbiota has long been of interest because of its involvement in multiple physiological processes in the host, influencing health or disease. Recent studies have shown that the GI microbiota of cats and dogs is as complex as the one present in humans and other animals, according to state-of-the-art sequencing technologies and other molecular techniques. The GI microbiota includes members of all three main life domains (Archaea, Bacteria, and Eukaryotes), with bacteria being the most abundant and metabolically active group of microorganisms. The stomach of cats and dogs is mainly inhabited by *Helicobacter* spp., which in dogs may account for as much as 98% of all gastric bacterial microbiota. The small intestine harbors a more diverse microbiota as it contains representatives from at least five bacterial phyla (mainly Firmicutes and Bacteroidetes). The large intestine harbors the most abundant ($\sim 10^{11}$ bacterial cells per gram of intestinal content), diverse (at least 10 bacterial phyla have been identified) and physiologically relevant group of bacteria in the GI tract. Most bacteria in the large intestine are strict anaerobes that depend on fermentation of non-digested dietary substances to subsist. Although recent studies are shedding light into the complexity of the GI microbiota in cats and dogs, further research is needed to find ways to successfully manipulate GI microorganisms to prevent and/or treat GI diseases.

Key words: gastrointestinal, microbiota, cats, dogs.

INTRODUCTION

The gastrointestinal (GI) tract of animals is colonized by a dense and heterogeneous group of microorganisms known as the GI microbiota, which supply more than nine million unique genes to the gene repertoire in the eukaryotic host (Yang *et al* 2009). The GI microbiota has long been of interest because of its involvement in multiple physiological processes in the host, including

resistance against colonization by pathogens (Stecher and Hardt 2011), production of useful substances that act as energy source for intestinal epithelial cells (Louis and Flint 2009), modulation of the intestinal immune system (Hooper and Macpherson 2010), salvage of energy from undigested dietary components (Cummings and Macfarlane 1997), and stimulation of intestinal angiogenesis (Stappenbeck *et al* 2002).

Most of the current information about the composition and activity of the GI microbiota comes from studies in human populations. However, an increasing number of investigations have also studied intestinal microbes in other animals, especially cats and dogs (Suchodolski 2011). This review summarises current information

Accepted: 10.01.2013.

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about the GI microbiota with emphasis on the GI bacterial microbiota of cats and dogs.

THE GI MICROBIOTA

The GI tract of animals is one of the most complex microbial ecosystems on Earth, and it is continuously affected by factors associated with the host (Spor *et al* 2011, Van den Abbeele *et al* 2011) and the outside environment (Claesson *et al* 2012). This complexity has been an obstacle to study single independent factors associated with its changes over time and among different populations of animals (e.g., healthy and diseased). Also, it is often difficult to determine the nature of the interactions among the microorganisms during health or disease, although recent advances in mathematical modeling could help understand this phenomenon (Hellweger and Bucci 2009, Arciero *et al* 2010). Moreover, there are controversies with regards to the way we classify microbial species (Staley 2006, Schleifer 2009). Despite this complexity, there is a growing body of literature suggesting that the GI microbiota can be studied objectively, and that health could be enhanced in the host through manipulation of its constitutive intestinal microbial populations.

CHARACTERIZATION OF THE GI MICROBIOTA

CULTURE METHODS

The characterization of the GI microbiota is the first step in determining its role in health or disease. Classic culture methods have the advantages of being relatively inexpensive, widely available, and suitable for biochemical and physiological studies, and therefore have been extensively used to characterise the GI microbiota of cats and dogs (see below). However, the usefulness of culture techniques to characterise microorganisms in the gut and elsewhere has long been questioned because it is not representative enough regarding both enumeration and community structure (Ritz 2007). While experts generally agree that about 99% of all GI microorganisms have not been successfully cultured (Tap *et al* 2009), a recent article showed that about 70% of all fecal bacterial genera (as determined by pyrosequencing) could be successfully cultured using an in-house culture media containing a mixture of several commercially available ingredients (Goodman *et al* 2011). Modifications to this universal gut microbiota media will facilitate the culture of more intestinal microorganisms and make possible a correlation between microbial abundance and utilization of dietary substances.

MOLECULAR METHODS

In contrast to culture methods, which rely on the identification of GI microorganisms by means of a phenotypic

characterization, molecular methodologies aim to identify and categorise microorganisms by means of detecting specific molecules inside the cells (e.g., DNA or RNA) (Zuckerandl and Pauling 1965). The 16S rRNA gene has often been used to identify bacteria because it is universally distributed and appears to have undergone a relatively slow change in base pair composition throughout evolution (Fox *et al* 1980). In other words, the 16S rRNA gene contains conserved regions (same among all bacteria) as well as variable and highly variable regions that allow the distinction and classification of bacterial phylotypes, according to theories of molecular evolution (Lemey *et al* 2009). Some examples of methodological differences between culture-based and culture-independent approaches include survey depth (tens to hundreds of cultural isolates versus thousands to millions of 16S rRNA gene sequences), accuracy of bacterial 16S rRNA gene assignments, and documentation of the generated data (Goodman *et al* 2011). A summary of the most commonly used methods and techniques to study the gastrointestinal microbiota is presented in table 1.

Polymerase chain reaction (PCR). PCR is a common and often indispensable molecular technique to characterize the GI microbiota. Currently, PCR is performed using a heat-stable DNA polymerase which can generate millions of copies of a given target sequence (e.g., a 16S rRNA gene fragment) in one hour or less. Some sequencing techniques require the use of PCR for generating amplicons (i.e., DNA fragments amplified by PCR). It is important to note that all PCR-based techniques suffer from several biases, including the fact that the generated 16S rRNA gene copies cannot be accurately extrapolated to the number of the microorganisms themselves, in part because different bacteria have different number of copies of this gene even within the same species (Acinas *et al* 2004, Lee *et al* 2008). Interestingly, these differences in the number of copies of the 16S rRNA gene may reflect ecological strategies of bacteria in response to resource availability (Klappenbach *et al* 2000).

Fingerprinting methods. The obtained 16S rRNA gene amplicons (e.g., from intestinal contents) are often the same size in number of base pairs, and therefore would appear as a single band in an agarose or polyacrylamide gel. However, these amplicons are likely to differ from one another in their base pair composition. When exposed to a denaturing agent or to increasing temperatures, these differences in base pair composition make the amplicons migrate at a different speed throughout a gel matrix. Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE) are examples of molecular fingerprinting methods that separate amplicons based on this principle. In particular, DGGE has been shown to be useful to assess qualitative variations in the GI microbiota of dogs among different

Table 1. Summary of the most commonly used methods and techniques to study the gastrointestinal microbiota.
Resumen de los métodos y técnicas mas comúnmente usados para estudiar la microbiota gastrointestinal.

Method/Technique	Target	Main Advantage (s)	Main Disadvantage (s)	Useful References
Culture	Microorganisms	Low-cost and allows for phenotypic characterization	Time consuming and not representative	Ritz (2007) Goodman <i>et al</i> (2011)
PCR	Specific genes	Low-cost	Qualitative (presence or absence of the gene)	Rådström <i>et al</i> (2004)
qPCR	Specific genes	Allows quantification	It is inaccurate to extrapolate gene copies to bacterial cell numbers	Klein (2002) Mackay (2004)
DGGE/TGGE	Specific genes	Relatively fast and low-cost	PCR amplicons often do not separate well	Nikolausz <i>et al</i> (2005)
TRFLP	Specific genes	Relatively fast and low-cost	Secondary restriction fragments	Liu <i>et al</i> (1997) Egert and Friedrich (2003)
Sanger Sequencing	Specific genes	Widely available	Low throughput	Sanger <i>et al</i> (1977)
Pyrosequencing	Specific genes	High throughput with an accuracy of more than 99.9%	Similar to other high-throughput sequencing technologies, errors during the sequencing procedure could artificially inflate diversity estimates	Ronaghi <i>et al</i> (1996) Margulies <i>et al</i> (2005) Kunin <i>et al</i> (2010)
Illumina	Specific genes	Higher throughput than 454-pyrosequencing with a similar accuracy	Shorter read length (150 bases for the MiSeq) than 454-pyrosequencing	Pettersson <i>et al</i> (2009) Caporaso <i>et al</i> (2012) Luo <i>et al</i> (2012)
ION Torrent	Specific genes	High throughput; per-base accuracy of 99.6% within the first 50 bases and 98.9% within the first 100 bases	When the paper was published in 2011, only 20-40% of the sensors in a given run yielded mappable reads	Rothberg <i>et al</i> (2011)
Microarrays	Specific genes	High throughput; fast and reproducible	Fails to detect microbial sequences that were not represented in the reference sequences used for probe design	Rajilic-Stojanovic <i>et al</i> (2009) Van den Bogert <i>et al</i> (2011)
Whole genome sequencing	All DNA of a microorganism	Provides a view of metabolic potential and a better phylogenetic resolution	The total amount of sequencing required to sequence all microbial genomes in a sample is unfeasible	Bentley (2006)
Metagenomics	All genes in a sample	Provides a view of community structure and metabolic potential	The total amount of sequencing required to sequence all microbial genomes in a sample is unfeasible	Huson <i>et al</i> (2009) Preidis and Versalovic (2009) Hugenholtz and Tyson (2008)
Proteomics	All proteins in a sample	Provides a view of metabolic activity	Similar to high-throughput sequencing technologies, the depth of analysis affects the coverage of all coding capacity of the gut microbiota	Wilkins <i>et al</i> (1996) Kolmeder <i>et al</i> (2012)

compartments of the intestinal tract (Suchodolski *et al* 2005). The result of DGGE or TGGE analysis is a specific banding pattern for every sample analysed, therefore providing a qualitative view of the microbial composition of the sample. However, fingerprinting techniques are not very useful to characterise microbial ecosystems because they often fail to accurately separate 16S rRNA gene fragments (Jackson *et al* 2000, Nikolausz *et al* 2005), thus underestimating the true bacterial diversity and its changes against external perturbations.

Quantitative real-time PCR (qPCR). In conventional PCR, the amplicons are routinely detected using electrophoresis on agarose gels after the PCR has finished. Because of this, traditional PCR is not capable of quantifying the genomic targets; it only provides information about the presence (band in the gel) or absence (no band in the gel) of the target. In contrast, PCR has been adapted to also allow the quantification of the unknown genomic targets as the PCR progresses (in real-time). This is possible by including in the PCR reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently-labeled sequence-specific primers or probes. qPCR has been widely used to assess the effect of different treatments on the abundance of the GI microbiota in cats and dogs (Gronvold *et al* 2010, Garcia-Mazcorro *et al* 2011) as well as in humans (Malinen *et al* 2005, Larsen *et al* 2011). However, bacterial cell numbers cannot directly be estimated from qPCR data in part because the cellular genome content varies with the growth phase of the organisms and bacteria have different number of copies of the 16S rRNA gene (see PCR above).

Fluorescence in situ hybridization (FISH). The detection of bacterial genomic targets using qPCR is useful when evaluating changes in the quantitative abundance of the microbiota, for example during administration of probiotics or therapeutic agents. However, the accurate extrapolation from amplified genomic targets to the actual numbers of bacterial cells is often difficult, mainly because bacteria have different copy numbers of the 16S rRNA gene. Unlike qPCR, FISH is capable of quantifying the actual bacterial cells by direct labeling of the 16S rRNA using fluorescently-labeled oligonucleotides. The FISH technique takes advantage of the fact that each bacterium usually contains thousands of ribosomes spread throughout the cell. Theoretically, like in PCR, it is possible to develop oligonucleotides that are capable to detect microorganisms at all taxonomic levels (i.e., Phylum, Class, Order, Family, Genus). However, this is often challenging due to high similarities in the 16S rRNA gene composition among phylogenetically related microorganisms. FISH can also provide important information about the morphology and spatial distribution of microorganisms in the GI tract (Simpson *et al* 2006).

Sequencing technologies. The identity of each 16S rRNA gene amplicon can be determined by estimating the order of the base pairs (sequencing). This has been traditionally done using nucleotides base analogs (dideoxynucleotides) that lack the 3'-hydroxyl group essential in phosphodiester bond formation, which act as specific chain-terminating inhibitors of DNA polymerase (Sanger *et al* 1977). This Sanger method is still used routinely in many laboratories for sequencing low number of samples. However, complex microbial ecosystems such as the intestinal tract contain millions of microorganisms, which makes necessary to clone and sequence thousands of individual PCR amplicons in order to obtain a representative view of the microbial composition. Recently developed high-throughput techniques such as 454-pyrosequencing (Margulies *et al* 2005) are capable of sequencing millions of base pairs in one hour or less, and have shown to be useful to study the feline and canine GI microbiota (Suchodolski *et al* 2009, Middelbos *et al* 2010, Garcia-Mazcorro *et al* 2011, Handl *et al* 2011). Other high-throughput techniques are based on different principles (e.g., reverse termination) and are discussed elsewhere (Pettersson *et al* 2009). Interestingly, a non-optical genome sequencing has been developed (Rothberg *et al* 2011), which promises a better performance than traditional optical-based sequencing. Nonetheless, the cost and necessary expertise for both sequencing and after-sequencing analysis procedures make most of these techniques inaccessible for many scientists around the globe. Fortunately, a number of freely available software platforms have been developed such as QIIME (Quantitative Insights into Microbial Ecology¹), which is capable of analyzing thousands of sequences in short periods of time. QIIME also offers free comprehensive guides for beginners as well as expert advice for more advanced users.

THE COMPOSITION OF THE GI MICROBIOTA

The composition and metabolic activity of the GI microbiota varies along the GI tract, in part reflecting anatomical and physiological conditions inherent to each of the intestinal sections. In cats and dogs, as well as in other monogastric animals, both the bacterial diversity (an index that incorporates the number of species in an area and their relative abundance) and richness (number of species) are higher in the large intestine when compared to the stomach and all regions of the small intestine (Ritchie *et al* 2008, Suchodolski *et al* 2008). The GI microbiota includes all three major domains of life (Archaea, Bacteria, and Eukaryotes), but bacteria make up the most abundant and metabolically active group of microorganisms in the GI tract. For example, a recent metagenomic study showed that bacteria may represent as much as 98% of all fecal microbiota in dogs, with Archaea, Eukaryotes, and viruses representing only

¹ <http://www.qiime.org/>

about 2% (Swanson *et al* 2011). Similarly, a recent study also used a metagenomic approach and showed that Eukaryotes, Archaea, and viruses were minor constituents (< 3%) of the fecal microbiota in cats, while bacteria represented the great majority (97.8%) (Tun *et al* 2012).

In monogastric animals, the large intestine contains the most abundant, diverse and metabolically relevant group of bacteria in the GI tract. The large intestine contains bacterial groups mainly within the phyla Firmicutes and Bacteroidetes. Other phyla such as Actinobacteria, Proteobacteria, Fusobacteria, Spirochaetes, Verrucomicrobia, Cyanobacteria, and Tenericutes are also frequently identified but their proportions are usually low. However, the exact proportions of each bacterial group vary widely throughout the literature. For example, one study showed that healthy cats and dogs may harbor > 90% of Firmicutes in faeces (Handl *et al* 2011), while others have shown that these animal species may only harbor ~ 13% (cats) and ~ 35% (dogs) of this phylum also in faeces (Swanson *et al* 2011, Tun *et al* 2012). The reasons for these discrepancies (see below) are unknown but may include differences in DNA extraction protocols (Zoetendal *et al* 2001), intra-stool variability of intestinal microorganisms (Garcia-Mazcorro *et al* 2009), inter-individual differences (Handl *et al* 2011), the target region of the

16S rRNA gene (Baker *et al* 2003), as well as inherent differences among the techniques utilized to characterize the microbiota (Zoetendal *et al* 2004, Kunin *et al* 2010).

Early culture-based studies suggested that the distal part of the human intestinal tract may harbor about 300 different bacterial species (Moore and Holdeman 1975, Savage 1977). However, recent culture-independent studies suggest that on average humans have an estimated richness of 943 bacterial species (operational taxonomic units or OTUs at 98% similarity) in faeces per subject (Tap *et al* 2009). In contrast, one study suggested that cats and dogs may harbor only 60 (cats) and 39 (dogs) OTUs (97% similarity) in faeces per subject (Handl *et al* 2011). This agrees with other studies that showed the presence of only 84 and 52 OTUs in the colon of cats and dogs based on a 98% similarity criterion (Ritchie *et al* 2008, Suchodolski *et al* 2008).

THE GI MICROBIOTA OF CATS AND DOGS

An overview of some of the most relevant investigations of the GI microbiota in cats and dogs is presented in table 2. Among all regions of the GI tract of these and other animal species, the distal part of the intestinal tract (i.e. fecal microbiota) has been the most widely studied to date (figure 1), mainly because of the ease of sampling.

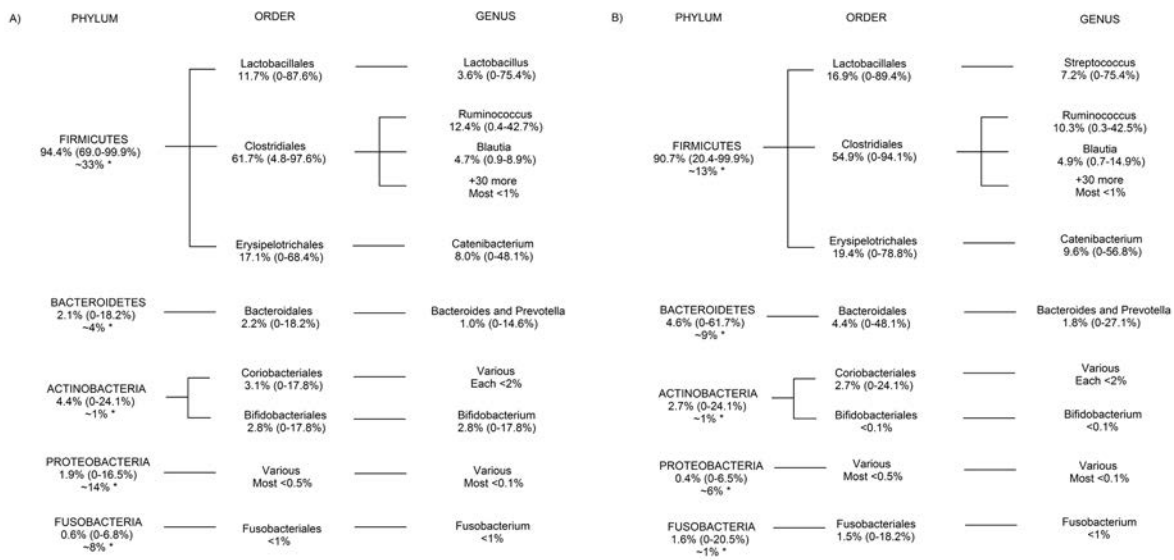


Figure 1. Simplified view of the faecal bacterial composition of dogs (A, left) and cats (B, right) at phylum, order and genus level. Numbers represent the average (minimum-maximum) of the relative proportions of sequences (number of sequences obtained from the bacterial group divided by the total number of sequences obtained), calculated according to both a published (Garcia-Mazcorro *et al* 2011, 12 dogs and 12 cats) and an unpublished (Weber *et al* 10 dogs and 10 cats) study using 454-pyrosequencing with the same primer set. *Clostridium* was the most abundant genus in both cats and dogs (> 20% on average in both studies) but it does not appear in this figure due to uncertain taxonomic classification. At the phylum level, we also included the approximate estimates (*) published by Swanson *et al* (2011) and Tun *et al* (2012) using a metagenomics approach (please see main text for more details).

Visión simplificada de la composición bacteriana fecal en perros (A, izquierda) y gatos (B, derecha) al nivel de filo, orden y género. Los números son promedios (mínimo-máximo) de proporciones relativas de secuencias (número de secuencias obtenidas del grupo bacteriano dividido entre el número total de secuencias obtenidas) calculado de un estudio publicado (García-Mazcorro *et al* 2011, 12 perros y 12 gatos) y un estudio no publicado (Weber *et al* 10 perros y 10 gatos) usando 454-pirosecuenciación con el mismo par de oligonucleótidos. *Clostridium* fue el género más abundante en gatos y perros (> 20% en promedio en ambos estudios) pero fue omitido en esta figura debido a clasificación taxonómica incierta. Al nivel de filo, también se incluyeron los estimados aproximados (*) publicados por Swanson *et al* (2011) y Tun *et al* (2012) usando un método metagenómico (ver texto para más detalles).

Table 2. Overview of some of the most relevant investigations about the gastrointestinal (GI) microbiota in cats and dogs. Visión general de algunas de las investigaciones más relevantes acerca de la microbiota gastrointestinal (GI) en gatos y perros.

Topic	Animal species	Number of subjects	Highlights	Study main conclusion (s)	Reference
Post-natal changes in the GI microbiota	Dog	16 (healthy)	First study evaluating age-related differences in the canine GI microbiota	Advances in age of beagle dogs yielded some changes in the microbiota of the large bowel	Benno <i>et al</i> (1992)
Enteric pathogens	Dog	58 (healthy), 32 (hospitalized with diarrhea), and 42 (hospitalized without diarrhea)	First study characterizing genotype and phenotype of two potentially pathogenic bacteria	A significant association was found for the presence of diarrhea and detection of CPE or toxin A via ELISA for <i>C. perfringens</i> and <i>C. difficile</i>	Marks <i>et al</i> (2002)
Effect of age, breed and fiber	Dog	18 (healthy)	First study using DGGE to evaluate canine fecal microbiota	Individual dogs have a unique and stable fecal microbiota	Simpson <i>et al</i> (2002)
Postnatal changes in the GI microbiota	Dog	110 (healthy)	Most comprehensive study describing postnatal changes in the canine GI microbiota	Age-related changes in the GI microbiota coincide with changes in diet and physiological processes	Buddington (2003)
Diagnostic yield of routine fecal panel	Dog	177 (healthy) and 260 (diarrhea)	First study evaluating the diagnostic value of presence or absence of microbes in feces of dogs	The diagnostic value of a fecal panel in dogs with diarrhea appears to be low	Cave <i>et al</i> (2006)
Characterization of the GI microbiota	Dog	6 (healthy)	First study to analyze different regions of the canine GI tract using molecular techniques	Dogs harbor a higher microbial diversity than previously thought	Suchodolski <i>et al</i> (2008)
Characterization of the GI microbiota	Cat	4 (healthy) and 1 (specific pathogen-free)	First study to analyze different regions of the feline GI tract using molecular techniques	Cats harbor a higher microbial diversity than previously thought	Ritchie <i>et al</i> (2008)
Inflammatory Bowel Disease (IBD)	Dog	9 (healthy) and 10 (diseased)	First study to evaluate microbiota of the small intestine in dogs with IBD	Canine IBD is associated with altered duodenal microbial communities	Xenoulis <i>et al</i> (2008)
Fungal DNA in the small intestine	Dog	64 (healthy) and 71 (diseased)	First study showing fungal diversity with molecular techniques in the canine small intestine	High prevalence and diversity of fungal DNA in the canine small intestine	Suchodolski <i>et al</i> (2008)
IBD	Cat	10 (healthy) and 17 (diseased)	First study to evaluate the relationship between mucosal bacteria and IBD in cats	IBD is associated with changes in the small intestine microbiota, abnormalities in mucosal architecture, immune upregulation and clinical signs	Janeczko <i>et al</i> (2008)

(continued)
(continuación)

Characterization of the GI microbiota	Cat	5 (indoor) and 4 (outdoor, predatory) all healthy	One of the first studies evaluating the feline fecal microbiota, first using <i>cpn60</i> gene (encoding the universally conserved 60 kDa chaperonin)	Substantial animal-animal variation, <i>Bifidobacterium</i> spp. seems to be highly abundant in feces of cats, based on the <i>cpn60</i> gene	Desai <i>et al</i> (2009)
Effect of synbiotic	Cat/dog	24 (healthy)	First study using pyrosequencing to evaluate the effect of a synbiotic on fecal microbiota of cats and dogs	The administered synbiotic led to increases in probiotic bacteria in feces without modifying the overall phylogenetic composition of the fecal microbiota	Garcia-Mazcorro <i>et al</i> (2011)
Metagenomics	Dog	6 (healthy)	First study using metagenomics to characterize the canine fecal microbiota	Mice, humans, and dogs share phylogenetic and metabolic similarities	Swanson <i>et al</i> (2011)
Effect of a proton-pump inhibitor	Dog	8 (healthy)	First study evaluating the effect of a PPI on the canine GI microbiota	Omeprazole leads to quantitative disturbances on specific groups of the GI microbiota in dogs	Garcia-Mazcorro <i>et al</i> (2012 ^a)
Short-term temporal variability	Dog	6 (healthy)	First study using different molecular techniques to investigate temporal variability in fecal microbiota of dogs	Different molecular techniques give different views of the fecal microbial composition in dogs	Garcia-Mazcorro <i>et al</i> (2012 ^b)
Metagenomics	Cat	5 (healthy)	First study using metagenomics to characterize the feline fecal microbiota	Cat metagenome clustered with the chicken metagenome both phylogenetically and metabolically	Tun <i>et al</i> (2012)
Fecal microbiome	Dog	32 (healthy), 12 (acute non-hemorrhagic diarrhea), 13 (acute hemorrhagic diarrhea), 9 and 10 (active and controlled IBD, respectively)	Most comprehensive study of the fecal microbiome in dogs with intestinal disease	Gastrointestinal disorders are associated with bacterial dysbiosis in feces of dogs. The observed changes differed between acute and chronic disease states.	Suchodolski <i>et al</i> (2012)

It is important to keep in mind that there are important differences in the reported abundances of GI microorganisms among different studies. This may be due to the DNA extraction method employed as well as the number of copies and the target region within the 16S rRNA gene (see above). A good example of these discrepancies is the recent summary of Armougom and Raoult (2008) about Firmicutes and Bacteroidetes in humans and mice.

THE GI MICROBIOTA OF CATS

Stomach. The stomach of animals was traditionally thought to lack a complex microbial ecosystem. This belief was sustained in part by the observation that gastric acid kills several microorganisms instantly (Giannella *et al* 1972). However, culture-independent molecular techniques are revealing a different story. An early study used several detection techniques and showed that the stomach of 91% of pet cats (n=58) were positive for the genus *Helicobacter* (Neiger *et al* 1998), suggesting a high occurrence of this bacterial group in the feline stomach. In another study, it was shown that cats and dogs are predominantly colonized by *H. heilmannii* (Priestnall *et al* 2004) but other species (e.g., *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. pametensis*) have also been identified in these animal species (Neiger and Simpson 2000).

Small intestine. Osbaldiston and Stowe (1971) were among the first to investigate the composition of the GI microbiota in cats (n = 12) using a wide variety of culture media. In this study, coliforms, *Streptococcus*, *Enterococcus*, and *Lactobacillus* were the predominant groups of bacteria along the feline GI tract. Other earlier studies suggested that *Bacteroides* and *Clostridium* spp. were the most common bacteria in the duodenum of cats (Papasoulotis *et al* 1998, Johnston *et al* 2001), also based on cultural isolates. Similarly, a recent study used molecular techniques and suggested that the small intestine (i.e., jejunum) of cats harbors mainly the orders Clostridiales and Lactobacillales (~ 90%) but also small proportions of at least five more orders, while the ileum and the colon both harbored a high proportion (> 50%) of Clostridiales with low proportions of Actinobacteria (~ 5%) (Ritchie *et al* 2008).

Large intestine. A recent study sequenced the gene encoding the universal 60 kDa chaperonin and showed that the faecal microbiota of cats was dominated by Actinobacteria (~ 53%) and Firmicutes (~ 40%) (Desai *et al* 2009). Recent studies using 454-pyrosequencing suggest that > 90% of all sequences obtained from feces of healthy cats belong to the Phylum Firmicutes (Garcia-Mazcorro *et al* 2011, Handl *et al* 2011), especially members of the family Clostridiaceae, while a metagenomic study suggests that Bacteroidetes/Chlorobi is the most abundant bacterial group (~ 68%), followed by Firmicutes (~ 13%) and

Proteobacteria (~ 6%) also in faeces of cats (Tun *et al* 2012). However, it is important to point out that the study by Tun *et al* also reports that Bacteroidetes only represented ~ 9% of the obtained sequence reads, while the phyla Chlorobi and Chloroflexi represented less than 1% of the reads. Thus, it is not clear which group represented the remaining ~ 59% of difference between the reported percentage of the Bacteroidetes/Chlorobi group (~ 68%) and the reported percentage of Bacteroidetes alone (~ 9%). We speculate that these discrepancies in the reported percentages may be due in part to the pipeline used to assign taxonomies.

In part because most GI microorganisms have not been successfully cultured, little is known about the phenotype of the GI microbiota in cats. The three major short-chain fatty acids (SCFA) found in cats are butyrate, acetate, and propionate (Brosey *et al* 2000). In particular, butyrate is considered to play a vital role in colonic human health (Hamer *et al* 2008, Louis and Flint, 2009) but little is known about its role in intestinal health of cats. Nonetheless, butyrate-producing bacteria are commonly found in feces of cats (Handl *et al* 2011).

THE GI MICROBIOTA OF DOGS

Stomach. A study published last year showed that the stomach of healthy dogs is home of a diverse microbiota (at least 4 phyla were identified), as evaluated by 454-pyrosequencing (Garcia-Mazcorro *et al* 2012^a). Despite this diversity, one single genus (i.e., *Helicobacter*) was by far the most predominant (~ 98% of all gastric microbiota). These results are in accordance with one study that showed that the human stomach is also home of a diverse microbiota, although the genus *Helicobacter* (*H. pylori* only) constituted only 42% of all sequences analyzed (Bik *et al* 2006).

Small intestine. Clapper and Meade (1963) attempted one of the first characterizations of bacteria and fungi in the lower intestinal tract of dogs using twelve different types of culture media. Using rectal swabs from 25 healthy Beagle dogs, the authors isolated 20 species of bacteria and 10 species of fungi (Clapper and Meade 1963). More recent studies using molecular techniques have shown the presence of at least four different bacterial phyla in the intestinal tract of dogs, namely Firmicutes (47.7%), Proteobacteria (23.3%), Fusobacteria (16.6%), and Bacteroidetes (16.6%) (Suchodolski *et al* 2008). Interestingly, these proportions differed depending on the intestinal compartment analyzed, with duodenum and jejunum containing more than 50% Firmicutes, while the ileum and colon only harbored ~ 30% of this phylum (Suchodolski *et al* 2008). Still, a more recent study used 454-pyrosequencing and identified ten bacterial phyla in the jejunum of dogs (Suchodolski *et al* 2009), although more than half of these groups were

only found in very low proportions (< 1% of all microbiota). A recent article used FISH to quantify bacteria in the duodenal biopsies of dogs and found a median of zero bacteria (range: 0-3) per microscopic field using almost 1000 microscopic fields (Garcia-Mazcorro *et al* 2012^a). In contrast, the same article found a high bacterial diversity (median: 173 OTUs) using 454-pyrosequencing also in duodenal biopsies from the same dogs. The reasons for this discrepancy are unknown but it may relate to the destruction of intestinal mucus during formalin fixation of the biopsies before paraffin embedding.

Large intestine. Some studies suggest that, in faeces, Firmicutes represent the great majority (> 90%) of the faecal microbiota in dogs (Garcia-Mazcorro *et al* 2011, Handl *et al* 2011). On the other hand, a recent metagenomic study suggested that the Bacteroidetes/Chlorobi group and Firmicutes were the dominant bacterial phyla (~ 35%), followed by Proteobacteria (~ 15%) and Fusobacteria (~ 8%) also in faeces of dogs (Swanson *et al* 2011). However, the results of this study show, just as in the reports of Tun *et al*, that Bacteroidetes only represented ~ 3% of all the obtained reads, while the Chloroflexi and the Chlorobi groups represented less than 1% of the reads. Therefore, it is not clear which group represented the difference between the reported percentage of the Bacteroidetes/Chlorobi group (~ 35%) and the percentage of Bacteroidetes alone (~ 3%). It is possible that the remaining percentage represents unclassified members of Bacteroidetes, but this has been scarcely discussed in the available literature.

As mentioned above, little is known about the phenotype of GI microorganisms in cats and dogs. As in cats, the major SCFA in dogs are butyrate, acetate, and propionate (Swanson *et al* 2002). A butyrate-producer bacterium that has attracted much attention for its role in intestinal health of humans is *Faecalibacterium prausnitzii* (Sokol *et al* 2009). A recent article suggests that *Faecalibacterium*-relatives are also abundant in faeces of dogs (Garcia-Mazcorro *et al* 2012^b), although it has been suggested that canine *Faecalibacterium spp.* may not be *F. prausnitzii*, based on phylogenetic analysis of near-full-length 16S rRNA gene sequences belonging to a canine clone and a human strain (Suchodolski *et al* 2008). Other butyrate-producers bacteria such as *Eubacterium* and *Roseburia* have been found in dogs and cats (Handl *et al* 2011).

MANIPULATION OF THE GI MICROBIOTA

Acknowledging that GI microbiota is closely involved in the wellbeing of the host led to the idea of manipulating intestinal microorganisms to enhance health. Several approaches have been used to accomplish this goal in cats and dogs (see below). In contrast, the consumption of therapeutic agents such as antibiotics can also lead to

unintended modifications of the GI microbiota, although less research on this topic is available in cats and dogs.

PROBIOTICS AND PREBIOTICS

Probiotics can be defined as live microorganisms that, if consumed in adequate amounts, would provide a health benefit to the host (FAO/WHO, 2002). On the other hand, prebiotics are selectively fermented ingredients that cause specific changes in the composition and/or activity of the gastrointestinal microbiota (Gibson *et al* 2010), thus also conferring health benefits on the host, while synbiotics are preparations containing both probiotics and prebiotics.

Sunvold *et al* (1995) were among the first to evaluate the *in vitro* effect of a prebiotic on faecal fermentation patterns of cats and dogs. In this study, the addition of fiber (citrus pulp) led to a higher organic matter disappearance and lower acetate to propionate ratio in both dogs and cats; however, these changes were not correlated with modification of the faecal microbiota. While other studies have also researched the properties and effects of probiotics and prebiotics on the composition and/or activity of the canine and feline intestinal microbiota *in vitro* (Strompfová *et al* 2004, Cutrignelli *et al* 2009) and *in vivo* (Vanhoutte *et al* 2005, Biagi *et al* 2007), most of these investigations have only studied selected intestinal bacterial groups, an approach that does not fully assess the effect of probiotics and prebiotics on the intestinal microbial ecosystem. A recent study investigated the effect of a commercial preparation of probiotics and prebiotics on the faecal microbial composition of healthy cats and dogs using several molecular techniques, including a high-throughput sequencing technique (Garcia-Mazcorro *et al* 2011). Similarly to other studies, the authors of this investigation showed that the consumption of the formulation leads to increases in faecal abundance of the ingested microorganisms, a change that rapidly disappears 2-3 days after consumption of the preparation. Interestingly, these quantitative changes in specific bacterial groups did not seem to lead to major modifications in the overall phylogenetic composition of the fecal microbiota, as evaluated by 454-pyrosequencing. This is an interesting observation because probiotics are thought to modulate the intestinal microbiota, including other, unrelated to the ingested microorganisms, bacteria. This modulation effect of probiotics on the intestinal microbiota has also been suggested in humans, as evaluated by culture (Venturi *et al* 1999) and molecular techniques (Larsen *et al* 2011), although the results are also controversial. For example, one study showed that the consumption of a synbiotic preparation leads to changes in bacterial populations but no significant differences in fecal chemistry (Worthley *et al* 2009), while others propose that the intake of a synbiotic food leads to modulation of the gut metabolic activities with a maintenance of gut “biostruc-

ture” (Vitali *et al* 2010). The discrepancy among different investigations may be due to the amount and types of probiotics administered (Pagnini *et al* 2010), as well as the combination and potential synergistic effect of different microorganisms. While some researchers encourage the design and use of several strains and/or species of microorganisms in probiotic formulations (Timmerman *et al* 2004), few data support a more beneficial effect of these multi-strains/species preparations compared to single strain preparations.

ANTIBIOTICS

Antibiotics are commonly used in Veterinary Medicine, but concerns have been raised about the potential reservoir of antibiotic resistance among the native intestinal microbiota of animals (Moyaert *et al* 2006). While in humans the effect of antibiotics on the intestinal microbiota has been characterised in depth (Dethlefsen and Relman 2011), little is known about the effect of antibiotics on the GI microbiota of cats and dogs. Johnston *et al* (1999) evaluated changes in duodenal bacteria of cats (n = 6) during metronidazole treatment, but this study only used culture techniques. Suchodolski *et al* (2009) analyzed changes in the small intestinal microbiota of dogs (n = 5) during administration of tylosin using 454-pyrosequencing. In this study, several changes in the abundance of different bacterial groups were observed, including an increase in the proportions of *Enterococcus* spp. which have been reported to be resistant to tylosin. However, these changes in bacterial amounts were not accompanied by any obvious clinical effect. Grønvold *et al* (2010) studied changes in faecal microbiota of healthy dogs (n = 7) during administration of amoxicillin using DGGE and qPCR. In this study, most of the variation in DGGE band profiles could be attributed to dog-specific factors, suggesting a minimal change in the composition of the fecal microbiota, as determined by the employed techniques.

INHIBITORS OF GASTRIC ACID SECRETION

Gastric acid is one of the first physiological barriers to impede the passage of potentially harmful agents into the intestinal tract. It is believed that inhibitors of gastric acid secretion can change the composition of the GI microbiota (Heidelbaugh *et al* 2009, Lombardo *et al* 2010), but only few studies support this statement and have mainly used culture techniques to study specific microorganisms (e.g. *Helicobacter pylori*). A recent study used a combination of several molecular techniques and concluded that the proton-pump inhibitor omeprazole can change the quantitative abundance of several gastric, duodenal and faecal microorganisms in healthy dogs, a change that did not seem to lead to major shifts in the overall phylogenetic composition of the gastric and small

intestinal microbiota (Garcia-Mazcorro *et al* 2012^a). Interestingly, the observed effect of omeprazole on the canine GI microbiota was dependent on the gender of the animals, perhaps suggesting a distinctive metabolism of the drug in male and female dogs.

CONCLUDING REMARKS

The GI tract of cats and dogs harbors a complex microbiota. The study of GI microorganisms is of interest because of its close relationship with the wellbeing of the host. Also, an increasing number of investigations suggest that GI microorganisms may play a role in the etiology of various GI disorders. However, little is known about what represents a healthy microbiota, its normal biological variations within and among individuals, and how to successfully manipulate it to prevent or treat GI disease. In order to achieve this goal, future collaborative studies should complement phylogenetic characterizations of the GI microbiota with functional (metabolic) analyses.

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