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The impact of probiotics on the canine gastrointestinal microbiota: A literature review

A probiotikumok hatása a kutya gasztrointesztinális mikrobiotáira: Irodalmi áttekintés

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Abstract

Gastrointestinal microbiota has been recently an appealing area of research due to its involvement in health. The microbial communities residing in the gut appear to regulate homeostasis and immune function, thus may preventing dysbiosis state. The development of advanced sequencing techniques allowed to reveal the complexity and diversity of the canine gut flora. Many factors modulate the composition of microbiota and among them, probiotics. These live microorganisms have been reported to confer benefits on the intestinal and immune system of the host. The aim of this literature review was to summarize and evaluate the effects of probiotics on the intestinal microbial communities in dogs. Studies investigating changes of microbiota composition following probiotics administration were collected. Despite an extreme heterogeneity of results, the administration of different strains led to some significant changes of bacterial communities from phylum to species level. This microbiota modulation appeared likely to be beneficial for the host. Further studies are warranted to confirm these observations.

Absztrakt

A gyomor-bélrendszerben élő mikrobióták kutatása egyre nagyobb hangsúlyt kap, ugyanis kiemelt hatásuk van a teljes szervezet egészségügyi állapotára vonatkozóan. Úgy tűnik, hogy a bélben élő mikrobiális közösségek szabályozzák a homeosztázist és az immunműködést, így megelőzhetik a dysbiosis kialakulását. A fejlett szekvenálási technikák lehetővé tették a kutya bélflóra összetettségének és sokféleségének feltárását. Számos tényező befolyásolja a mikrobiom összetételét, köztük a probiotikumok is. A kutatások szerint ezek az élő mikroorganizmusok jótékony hatással vannak a gazdaszervezet bélrendszerére és immunrendszerére. Ennek az irodalmi áttekintésnek a célja az volt, hogy összefoglalja és értékelje a probiotikumok hatását a kutyák bélrendszerében élő mikrobiotákra. Összegyűjtöttük azokat a tanulmányokat, amelyek a mikrobiom összetételének probiotikumok alkalmazását követő változásait vizsgálták. Az eredmények szélsőséges heterogenitása ellenére a különböző törzsek alkalmazása törzs és faj szinten is a baktériumközösségek jelentős változásához vezetett. Úgy tűnt, hogy ez a mikrobiota moduláció előnyös a gazdaszervezet számára, azonban további vizsgálatokra van szükség ezen megfigyelések megerősítésére.

Introduction

The gastrointestinal tract harbors a high diverse number of microorganisms. This complex ecosystem in constant interaction is termed as microbiota or microbiome. Microbiota refers to the taxonomical classification of these microorganisms whereas microbiome designates their function and gene content. The development of high-throughput sequencing techniques has allowed to assess the composition and diversity of microbiota in dogs. Alike a fingerprint, microbiota is unique to each individual. By establishing a symbiotic relationship, microbial communities contribute to the health of its host. The structure of the microbiota is dynamic, permanently modulated by intrinsic and extrinsic factors. Administration of live microorganisms also called probiotics has been associated with several health benefits, primarily on gastrointestinal disorders. Probiotics interact with the indigenous microbial communities, contributing to homeostasis of the gut. In case of disease, probiotics can then help restoring the balance of the gut microflora. Hence, over the last years, with the growing interest for natural therapies the use of probiotics became very appealing in human and veterinary medicine.

1. THE CANINE MICROBIOTA

1.1 Composition

Bacteria represent the largest component (98.08%) of the canine microbiota followed by archaea (1.09%), eukaryotes (0.37%, mostly fungi) and viruses (0.29%) according to the sequences analyzed by Swanson et al. [1].

In 1961, Smith and Crabb [2] were the first scientists attempting to characterize the microbiota of dogs. After culturing fecal samples, they could identify lactobacilli and *Escherichia coli* as predominant bacteria ($10^{4.6}$/g and in $10^{7.5}$/g of feces respectively). Later, Davis et al. [3] broadened the microbiota characterization to other parts of gastrointestinal tract (stomach, small and large intestine) and found that anaerobes bacteria dominated (10^{10}/g of samples). It is worth mentioning that most of the microbiota studies are based on the analysis of fecal microbiota. Divergences exist regarding this approach and will be developed later.

Among studies, differences are observed in the microbiota composition. Usually, bacterial groups are similar in a dog population when higher phylogenetic levels are observed (family or genus). In contrast, important divergences appear at species level between individuals. Handl et al. [4] reported that only 25% of dogs harbored the same *Bifidobacterium* species.

In order to clarify the following information, a classification of the main bacterial taxa of the dog microbiota is presented on Figure 1 (page 7) extracted from Barko et al. [5].

Canine microbiota is dominated by the phyla Fusobacteria, Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria. This finding is in accordance with the phyla commonly reported in the mammalian gut microbiota, for instance, in humans Firmicutes and Bacteroidetes predominate [6, 7]. The observed abundances of these phyla differ between studies. Firmicutes (14-44.8% of total average abundance), Bacteroidetes (27.7-34%), and Fusobacteria (14.26-40%) appear to be the predominant phyla [8–11]. These same studies reported lower abundances of phyla Proteobacteria (6.29 -11.31%) and Actinobacteria (0.33-3.4%).
Clostridia was reported to be the most prevalent class of dog microbiota (65% of all bacterial sequences) [4, 12]. Within the Clostridia class, the order Clostridiales was found to be the most

diverse order with a spatial geography predominating in the upper gut (duodenum (40% of sequences) and jejunum (39%)) [12]. This class was dominated by the genera *Clostridium* and *Ruminococcus*. The second most prevalent class was Bacilli which consisted almost exclusively of the order Lactobacillales (dominated by the genera *Streptococcus* and *Lactobacillus*). Lactobacillales were found in high abundance in duodenum, jejunum and colon [4, 12]. On the other hand, You and Kim [11] reported that *Fusobacterium* was the most abundant genera followed by *Bacteroides* and *Prevotella*. These observations could be due to a co-evolution between *Fusobacterium* and the canine gastrointestinal tract. As to *Bacteroides* and *Prevotella* genera, they have been associated with plant-based diet in humans. Their high abundances in dogs' microbiota could be the consequence of the shift from the meat-based diet of wolves to the omnivorous diet of domestic dogs [10].

The load of bacteria increases throughout the gastrointestinal tract. Ileum was reported to contain about 10^7 cfu (colony forming units) bacteria per gram or ml whereas in colon the range was 10^9 to 10^{11} cfu/g or ml of intestinal content [13]. Composition of microbiota appears to also greatly vary all along the gastrointestinal tract, suggesting that each segment of the gastrointestinal tract harbors a distinctive bacterial community. Stomach seems be dominated by phylum Proteobacteria with a high abundance of *Helicobacter* species whereas Firmicutes predominate in duodenum [14]. Colon exhibits the highest bacterial diversity [15] with dominance of Firmicutes and *Bifidobacterium* among others [16]. Overall, an increase of diversity and richness is observed from the stomach to the rectum.

Canine microbiota also contains in a smaller proportion other microorganisms than bacteria. By decreasing abundance, archaea represent about 1% of microbiota sequences. They mainly belong to Crenarchaeota and Euryarchaeota phyla [1]. Regarding fungi communities, phyla Ascomycota (the most abundant), Basidiomycota, Glomeromycota, and Zygomycota were recovered from canine faeces after pyrosequencing [4]. At class level, Saccharomycetes (recovered in 85.46% of dogs) was the most abundant represented mostly by Nakaseopmyces genus (in 76.7% of dogs) and Candida castelli species. Lastly, viruses identified in the dog microbiota belonged to Iridoviridae and Caudovirales orders. *Myoviridae*, *Podoviridae* and

Siphoviridae were the three observed families and the analyzed sequences were associated to bacteriophages [1].

Phylum	Class	Order	Family	Genus
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*
			Ruminococcaceae	*Ruminococcus*
				Faecalibacterium
			Eubacteraceae	*Eubacterium*
	Bacilli	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
			Streptococcaceae	*Streptococcus*
				Enterococcus
	Erysiphelotrichia	*Erisophelotrichales*	*Erysiphelotrichaceae*	*Turicibacter*
				Catenibacterium
				Coprobacillus
				Allobaculum
	Negativicutes	*Selenomonadales*	*Selenomonadaceae*	*Megamonas*
		Veillonellales	*Veillonellaceae*	*Dialister*
				Megasphaera
				Veillonella
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
			Bacteroidaceae	*Bacteroides*
Actinobacteria	*Coriobacteriia*	*Coriobacteriales*	*Coriobacteriaceae*	*Collinsella*
			Atopobiaceae	*Olsenella*
		Eggerthellales	*Eggerthellaceae*	*Slackia*
				Eggerthella
	Actinobacteria	*Bifidobacteriales*	*Bifidobacteriaceae*	*Bifidobacterium*
Fusobacteria	*Fusobacteriia*	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacterium*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteraceae*	*Escherichia*
				Shigella
		Aeromonadales	*Succinivibrionaceae*	*Succinivibrio*
				Anaerobiospirillum

Figure 1. Taxonomical and phylogenetical classification of the common constituents of the canine gastrointestinal microbiota. Extracted from Barko et al. [5]

1.2 Role

1.2.1 Digestion and metabolism

Dogs in contrast to humans do not rely on the microbiota for energy purposes and only up to 7% of their metabolic energy originates from microbial fermentation in colon [17] [18]. However, intestinal microbiota still plays a great role in their metabolism. Swanson et al. [1] observed that the metabolic profiles of canine gut microbes were carbohydrate metabolism (12–13% of all sequences), amino acids (6-7%), protein metabolism (8-9%), vitamins and cofactors synthesis (5-6%).

The microbial community has a key role in the digestive process. Undigested and/or unabsorbed nutrients by the host will be available for digestion by the gut microbes. Some of these end-products (amino acids, mono- and disaccharides and fatty acids) will be absorbed and used by the microbiota itself as source of energy. Nutrients exceeding the host and microbiome absorptive and digestive capacities will bypass to large intestine. As a result of bacterial fermentation, functional active compounds also called postbiotics will be produced [19, 20]. These postbiotics can impact locally or systemically the health of the host. Among them are short chain fatty acids, or SCFAs (mainly acetate, propionate and butyrate). SCFAs are beneficial as they provide energy for epithelial cells, support their growth and have a role in host defense [18, 21].

Commensal bacteria will also synthetize compounds such as bile acids and vitamins (vitamin K and B complex) [21].

1.2.2 Intestinal structure

Microbiota also plays a role in the development and maintenance of the intestinal anatomy. Al-Asmakh and Zadjali [22] showed that germ-free mice presented several anatomical differences with specific-pathogen-free and wild-type mice. Indeed, their small intestinal morphology had a decrease of surface area, shorter ileal villi and crypts and reduced epithelial cell turn-over time. Same observations were done earlier in germ free dogs [23].

Moreover, microbial communities reinforce the non-specific defenses of the host. On one hand, they strengthen the intestinal barrier. Resident bacterial flora competes for vital sources

(oxygen, nutrients and mucosal adhesion sites) with non-resident microbes, preventing the host from invasion by potential pathogens. It is the mechanism of colonization resistance [24]. On the other hand, commensal bacteria can produce anti-microbial compounds such as bacteriocins and colicins, creating a toxic environment to many pathogenic bacteria [21].

1.2.3 Immune system

Due to their location, the microbial communities are interacting with the gut-associated lymphoid tissue (GALT). Studies have reported that germ-free animals had a lower concentration of immunoglobulins (Ig) in their serum and a smaller abundance of lymphocytes in their intestinal mucosa. When these animals were exposed to commensal microbes, a rapid increase of Ig in their serum and lymphoid cells in their gut were observed [21]. Bacteria also play a role in development and regulation of immune cells (T-helper cells) and release of inflammatory mediators (cytokines) [21].

Microbiota may also contribute to other roles such as xenobiotics metabolism, maturation of gastrointestinal cells and behavior as it has been shown in rodents models [25]. Consequently, the microbiota by constantly interacting with the host, constitutes a metabolically active organ.

1.3 Influencing factors

1.3.1 Diet

Microbiota is resilient and can be influenced by nutrients. However, major shifts in dietary macronutrients are often necessary for inducing changes in bacterial taxa [26]. Moreover, durable changes of microbiota composition are possible only by maintaining a specific diet on a long term. Allaway et al. [27] fed Labrador retrievers with a highly purified diet for 36 weeks and the observed significant changes of microbiota returned to their original state as dogs went back to control diet. Absence of food induces also marked changes on canine microbiota. Kasiraj et al. [28] observed significant increase of Proteobacteria and *Fusobacterium* after food withdrawal in dogs.

The composition of diet has a great impact on the composition of microbiota in dogs. Coelho et al. [29] observed that a low-protein and high-carbohydrate diet, resulted in smaller shift in microbiota, and a higher *Prevotella* to *Bacteroides* ratio, than a high-protein and low-carbohydrate diet. *Prevotella* has been previously observed in high carbohydrates diet and *Bacteroides* in high protein diets [30]. Another example is the Bone and Raw Food (BARF) diet which contains a high protein and fat ratio compared to fibers and carbohydrates. Dogs fed with BARF foods had an increase of Lactobacillales and *Clostridium* compared to dogs fed with commercial diets [31].

As regards to fibers, most of them will act on microbiota by enriching Firmicutes, a fiber-fermenting phylum. A beet pulp-supplemented diet led to decrease of Fusobacteria and increase of Firmicutes and Clostridia in dogs [8]. A similar increase of Firmicutes in fecal microbiota was found by Alexander et al. [32] after supplementation with a commercial prebiotic (inulin-type fructans). Changes in bacterial abundances of some members of Proteobacteria were also noted. Prebiotics are non-digestible food which stimulate the growth of beneficial organisms within the intestines. By feeding the gut microbiota, prebiotics modulate its composition.

Synbiotics, mixture of probiotic and prebiotic, have shown to also influence microbiota. Gagné et al. [33] observed an increase in *Lactobacillaceae* and decrease of *Clostridiaceae* in sled dogs following a two-week treatment with a synbiotic. In another study, a significant shift in microbial diversity and decrease of *E.coli* were seen after synbiotics administration [34].

1.3.2 Diseases

Dysbiosis occurs when the homeostasis of the gut is disrupted. It is accompanied by changes in microbiota composition (decrease of bacterial diversity, loss of beneficial bacteria, and/or overgrowth of pathogens) and metabolic activities [21] [35]. Intestinal dysbiosis is often seen in dogs suffering from gastrointestinal diseases. It is still unclear if dysbiosis is the primary cause or a consequence of the disease process. A dysbiosis index has been created to track microbiota changes. It enumerates total bacteria and seven key bacterial taxa (*Faecalibacterium*, *Fusobacterium*, *Turicibacter*, *Streptococcus*, *Escherichia coli*, *Blautia* and *Clostridium hiranonis*). A higher dysbiosis index correlates with a lower microbial diversity [36].

In case of acute diarrhea, Suchodolski et al. [37] and Minamoto et al. [38] revealed that dogs exhibited a decrease of many members of the Firmicutes phylum (*Faecalibacterium*, *Blautia*, *Ruminococcaceae*). Dogs with acute hemorrhagic diarrhea (AHDS) had more profound changes and an increase abundance of *Clostridium perfringens*. It is worth mentioning that this latter species is not indicative of a disease as it is a commensal bacteria [38].

In dogs with inflammatory bowel diseases (IBD), hence suffering from chronic diarrhea, Suchodolski et al. [37] observed significant differences, in composition of duodenal microbiota, with a decrease of *Fusobacteria* and *Bacteroidaceae* and an increase of Proteobacteria, compared to healthy dogs. Xenoulis et al. [39] found a significant lower species richness in dogs suffering from IBD with increase of *Enterobacteriaceae* family (*Escherichia coli* like). A decrease of abundances of *Ruminococcaceae* and an increase of *Lactobacillus* and *E. coli* were reported in dogs suffering from chronic diarrhea [38]. Giaretta et al. [40] investigated the colonic microbiota of dogs with chronic inflammatory enteropathy (CIE) and showed a large depletion of the total number of bacteria within colonic crypts.

Several other extra-intestinal diseases such as exocrine pancreatic insufficiency (EPI), diabetes mellitus (DM) or cancer can also lead to severe dysbiosis.

For example, Isaiah et al. [41] showed that dogs with EPI exhibited a massive diminution of species richness as well as important shifts in their fecal microbiota. At level of families *Bifidobacteriaceae* and *Lactobacillaceae* were increased while *Lachnospiraceae* and *Ruminococcaceae* significantly decreased compared to healthy dogs. At species level, Blake et al. [42] reported an increased abundance of *Escherichia coli*, *Lactobacillus*, and *Bifidobacterium* in dogs with EPI.

In a similar way, canine diabetes mellitus has been shown to have the same pattern of dysbiosis as seen in humans with type 2 DM. Despite no significant changes in species richness, diabetic dogs had an increase of *Enterobacteriaceae* and a decrease of *Erysipelotrichia* [43].

Finally, other studies suggest the involvement of microbiota in cancer states. The fecal microbiota of dogs with multicentric lymphoma revealed a higher dysbiosis index and lower species richness compared to healthy control. It also had a lower abundance of *Faecalibacterium* and *Fusobacterium* spp. (“species plural”), and an increase of *Streptococcus* spp. [44].

Microbiota has appeared to be also altered in case of colorectal epithelial tumors [45] with marked abundances of *Enterobacteriaceae* and *Bacteroides*.

1.3.3 Drugs

Administration of certain drugs can also have a substantial impact on the gut microbiota. Prednisolone and metronidazole are commonly prescribed in the treatment of acute diarrhea and chronic enteropathy. Igarashi et al. [46] evaluated their effects on fecal microbiota of healthy dogs. In their study, prednisolone did not appear to have a significant impact on bacterial diversity or composition. On the contrary, metronidazole led to a significant decrease of bacterial diversity as well as marked changed in bacterial families. *Bacteroidaceae*, *Clostridiaceae* decreased whereas *Bifidobacteriaceae* and *Enterobacteriaceae* increased after a two-week administration. These changes reversed after the end of administration. Similar observations were done in another study by Pilla et al. [47].

Tylosin is another commonly used antibiotic in the treatment of canine chronic diarrhea. It can induce prolonged effects on the diversity and composition of canine microbiota. Suchodolski et al. [48] analyzed microbiota of healthy dogs and tylosin administration led to a progressive decrease in bacterial diversity and richness. Some bacterial taxa (*Spirochaetes*, *Streptomycetaceae*, and *Prevotellaceae*) did not return to baseline values 2 weeks after the end of treatment. Fecal microbiota of dogs receiving tylosin for one week was also significantly altered on the long term in another study of Manchester et al. [49].

Omeprazole could alter the abundance of bacterial groups in gut. Indeed, its administration was associated with a decrease of Helicobacter species and increase of Firmicutes and Fusobacteria phyla in gastric and duodenal biopsies. Abundance of *Lactobacillus* genus was also increased in fecal samples [14].

1.3.4 Other influencing factors

The impact of breed on the microbiota composition in dogs seems to diverge between studies.

A study comparing the fecal microbiota of German shepherds, Miniature schnauzers and English setters found significant differences in some bacterial taxa. German shepherds had a higher abundance of clostridia bacteria and in English setters Fusobacteria were enriched [50]. Reddy et al. [51] conducted a metagenomic analysis of fecal microbiota of three dog breeds

(Maltese, Miniature Schnauzer, Poodle dogs). Major differences at phylum level appeared. Indeed, Firmicutes abundance was significantly lower in the Maltese dogs compared to the other two breeds, whereas Fusobacteria was significantly greater in the Maltese than in the Miniature Schnauzer breed. On the contrary, other studies concluded that breed may only play a minor role in modulation of the microbiota [10, 52]. The study of Alessandri et al. [10] did not report any significant differences in microbiome after metagenomics analysis of the feces of about fifty canine breeds.

Besides, composition of microbiota appears to be significantly affected by age. Mizukami et al. [53] found that age was correlated with a decreasing tendency of microbial diversity in fecal microbiota. Moreover, Vilson et al. [54] also observed a great difference between 7-week-old puppies and adults (15-18 months). From puppyhood to adulthood there was an increase of *Clostridiaceae* and *Lachnospiraceae* families and a decrease *Lactobacillaceae* and *Bifidobacteriaceae*. Similar conclusions were done by Xu et al. [52] who found that the age of dogs, compared to the breed or gender, was the most significant factor affecting the microbiota. They revealed differences of structure of microbial communities at genus and species level. *Fusobacterium perfoetens* was enriched in the elderly dogs (5-13 years old) and young dogs (below 8 months of age) had a greater abundance of *Streptococcus* and *Lactobacillus acidophilus*.

Lastly, living environment appeared to significantly impact microbial diversity as reported by Vilson et al. [54]. Indeed, dogs living in large cities during their first 1.5 year of life showed higher diversity compared to dogs living in small cities or countryside. However, the structural composition of microbiome was not affected.

1.4 Analyzing techniques

1.4.1 Sampling choice

Microbiota screening is frequently realized from fecal samples, thus depicting the fecal microbiota. Fecal sampling has many advantages as it is a simple, non-invasive method, and raising no ethical concerns compared to intestinal biopsies or small intestine fistulations. Due

to its easiness, fecal sampling allows to investigate microbiota of a large population. In Jha et al. [55] the gut microbiota of nearly 200 dogs could be successfully screened using fecal sampling by owners. This direct-to-consumer approach has already demonstrated great results in humans. It could overcome the current limitations in canine microbiota research by permitting a larger collection of dog samples.

However fecal microbiota may be less precise and representative of the microbiota of upper parts of the intestinal tract as the small intestine. Mentula et al. [56] compared the findings of jejunal and fecal samples of dogs via culturing. After enumeration, they found that some bacteria taxa were more prevalent in the jejunum than feces (such as staphylococci) and that colon harbored a higher count of bacteria than jejunum (10^8 to 10^{11}cfu/g in feces vs 10^2 to 10^6cfu/g in jejunum). In addition, fecal sampling cannot give information on mucosa-adherent bacterial communities which differ from luminal communities [57]. Hence, the analysis of canine microbiota based only on fecal samples appears to give imprecise information of the bacterial composition in the different compartments of the gastrointestinal tract.

1.4.2 Culture approach

Methods for characterizing the gastrointestinal microbiota have greatly evolved over the years. Traditionally, bacterial cultures from mainly fecal samples have been used to investigate microbiota of dogs. Culturing media are selective and indicative and only the targeted and viable bacterial population will be counted. Microbial cultivation appears to be better adapted in clinical cases for targeting a specific pathogen (such as salmonella).

The cultivation approach has significant limits to assess the diversity of the microbiota as less than 5% of intestinal bacteria can be cultivated. This small percentage is explained, among others, by the lack of knowledge of optimal growth characteristics of many microorganisms, possible damage of bacteria during handling of sample (anaerobic bacteria are more sensitive), or lack of media specificity (developed primarily for humans [58]). All these factors let to only a limited number of microorganisms recovered from the growth media [13].

1.4.3 Molecular approach

Molecular tools have become the standard approach for screening the gastrointestinal microbiota.

High-throughput sequencing includes 16S rRNA genes sequencing (the most used approach in small animal studies), shotgun sequencing of DNA (metagenomics) and metatranscriptomics (more complex and rarely used, it focuses on the gene expression of microbiota).

First of all, 16S rRNA gene sequencing starts with the extraction of DNA or RNA from intestinal samples (feces, biopsies, luminal content). As the 16S ribosomal RNA (16S rRNA) gene is universally shared by bacteria, it is a common target for bacterial (and archaeal) identification. This gene consists of conserved and variable regions. Primers will target and amplify the conserved regions by PCR, resulting in 'amplicons'. Then, the flanked variable regions will be sequenced via 16S rRNA gene clones' libraries of different sequencing platforms (454-pyrosequencing, IlluminaMiSeq) [57]. After, the sequences are processed using a bioinformatics pipeline to eliminate low quality and chimeric sequences and to be compared to references databases [57]. Sequences can be grouped into Operational Taxonomic Units (OTUs) based on their similarity (typically above 97%). The total number of unique OTUs will give the richness of the sample. Basic statistical analyzes assesses alpha and beta diversity as well as individual bacterial groups of different phylogenetic levels (from phylum to species) between treatment groups. Alpha diversity ($\propto$-diversity) represents the "diversity within an individual sample " and Beta diversity (β-diversity) is "the diversity between different samples" [5].

In metagenomics, all DNA extracted from a sample will be sequenced without being first amplified by PCR. It allows identification of various genes at taxonomic and functional level (for example, carbohydrates or vitamin synthesis). Compared with 16S rRNA sequencing, the resolution is better at species and strain level. It can also identify fungi, DNA viruses (mainly bacteriophages) and archaea, the other components of the microbiota which are still poorly investigated in veterinary medicine. Metagenomics is considered as the best method for microbiota analysis but it's rarely used in small animal's research studies as it's expensive and requires a specific and high knowledge.

Quantification of bacteria can be done via quantitative PCR (qPCR) or Fluorescence In Situ Hybridization (FISH). Quantitative PCR is a rapid method for total bacteria or specific

individual taxa determination. Fluorescence in situ hybridization is mostly used and can indicate changes of targeted bacterial taxa or their localization [57]

2. PROBIOTICS IN VETERINARY MEDICINE

2.1 Definition

The concept of probiotics was first introduced in the early 20th century, as Professor Metchnikoff suggested that oral administration of fermented milk products could improve intestinal microbial balance as well as longevity. The idea was, as intestinal microbes interact with the food, that measures could be adopted "to modify the flora in our bodies and to replace the harmful microbes by useful microbes" [59]. The word probiotic appeared in 1965 to name substances synthetized by protozoa and capable of growth-promoting effects on other microorganisms [60].

The etymology "pros" and "bios", derived from Latin and Greek meaning literally "for life" summarizes well the beneficial effect of probiotics. The FAO/WHO definition states that probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host " [61] . More precisely and according to The International Scientific Association for Probiotics and Prebiotics (ISAPP), the term probiotic is appropriately used when a microorganism meets a few minimum criteria. Among these, a well-characterized identity (with designation at genus, species and strain level and name deposited in international culture collection), being safe for intended use and available studies demonstrating its health benefits in the target host [62].

The creation of a "probiotic framework" has aimed to further refine what could be qualified as probiotic. It excludes microbial preparations such as traditional fermented foods (which do not have a well-defined and stable microbial community) or fecal microbiota transplant. Frequently, misuse of the term probiotic is seen as claimed biological effects of probiotic are not always scientifically documented [63].

From the definition, the term probiotic only applies to products containing live microorganisms. Hence, dead microorganisms and other microbial products have the be excluded. A paradox exists as administration of dead probiotics was reported to produce beneficial responses in the host. Indeed, the administration of a heat-killed strain of *Enterococcus faecalis* stimulated immune responses via an increase of neutrophil phagocytic activity in dogs [64].

Probiotics can be bacteria, yeasts (*Saccharomyces cerevisiae*) or fungi (*Aspergillus oryzae*). The most commonly used probiotics in dogs are bacterial strains originated from healthy canine microbiota. They belong mainly to the group of lactic acid bacteria. Strains of *Enterococcus faecium, Lactobacillus acidophilus, Bifidobacterium lactis* to only cite a few are available as over-the-counter canine supplements [65].

2.2 Properties

In order to establish themselves within the resident gut microbiota, probiotics must possess several properties. First of all, they have to survive their passage through gastrointestinal tract. After ingestion, the first barriers to overcome are gastric juice, bile acids and pancreatic enzymes. The persistence to these harsh conditions will select the most resistant strains. Indeed, probiotics must still be alive, active and in sufficient numbers at their arrival to their target site, usually in the large intestine (colon).

To implant themselves in the gut they must first adhere to intestinal epithelial cells. Then, getting access to nutritive resources is essential to become metabolically active [66]. For doing so probiotics have to compete with indigenous microbiota and overcome what has been called as "colonization resistance" or “barrier effect”. These expressions refer to the mechanism by which resident bacteria maintain their presence in the gut and avoid external microorganisms (probiotics or pathogens) to conquer the same intestinal sites [61]. If the environment conditions meet their minimum growth requirements, probiotics will replicate within the gut. If this proliferation equalizes or exceeds the intestinal wash-out, the incoming probiotic has successfully established itself within the resident microbial community [66].

Other properties of probiotic include the ability to be easily cultivable for large scale commercial production and resist temperature fluctuations as well as low oxygen concentration in packaging and storage conditions [67]. Again, as stated by the FAO/WHO definition, probiotic strains have to be alive when consumed. Therefore, probiotics must remain viable during manufacturing and throughout their shelf life.

2.3 Mode of action

Mode of action of probiotics have been extensively studied in vitro in human and rodent models. Due to a lack of documentation, mode of action of probiotics in small animals remain theoretical and further in vivo investigations are warranted. Probiotics exert their beneficial effects through diverse complex pathways. Two probiotics can have a different mode of action and still confer the same health benefit. Probiotics can act directly on the microbial communities via production of given enzymes or metabolites, or can induce the host to produce a beneficial effect. Details of mechanisms at cellular and molecular levels can be found in reviews such as the one of Oelschlaeger [68]. Mechanisms of actions of probiotics at intestinal level are illustrated on Figure 2 (on page 20), extracted from Schmitz, [69].

Mode of action of probiotics tends to be strain specific and mainly include strengthening of the epithelial barrier, exclusion and inhibition of adhesion of pathogens, and modulation of the immune system of the host [69].

Probiotics are well-known to help enhancing the function of the gastrointestinal epithelial barrier as well as promoting a defense against pathogenic microorganisms. The integrity of the intestinal barrier is primordial as it is a first defense mechanism against environment pathogens ingested with food. Probiotics reinforce the function of the barrier, also called ‘colonization resistance’, by reducing its permeability and increasing lifespan of intestinal cells. Underlying mechanisms are still unclear but it could be via modulation of genes coding for the tight junction proteins between the epithelial cells. Lactobacilli have been shown to modulate genes encoding for E-cadherin and β-catenin proteins which are both involved in adhesive junctions between cells. Probiotics could also prevent damage and disruption of epithelial barrier caused by pro-

inflammatory cytokines [70]. Mucin production can be increased via stimulation of goblet cells and leads to the creation of a mucus barrier preventing pathogens passage [70, 71].

Probiotics decrease the pathogenic load in the intestines by competing for epithelial adhesion sites and nutrients. They also modify the environmental conditions making it hostile to others organisms via production of various antimicrobial substances like organic acids (acetic acid and lactic acid), fatty acids, bacteriocins or defensins [70].

Probiotics act locally and systemically to modulate the action of the immune system. They interact with the gut associated lymphoid system (GALT) via mainly intestinal epithelial cells and dendritic cells. Probiotics also lead to anti-inflammatory responses by binding to membrane-bound receptors as toll-like receptors and associated downstream signaling pathways [70]. Probiotics can stimulate immunoglobulin A (IgA) production and its secretion in the intestinal mucous layer or interact with regulatory T cells and other immune cells (lymphocytes and macrophages) [71].

Other frequent mechanisms of probiotics include vitamin synthesis, bile salt metabolism and enzymatic activity. Rarely but worth mentioning, probiotics can also act via endocrinology and neurologic mechanisms [63].

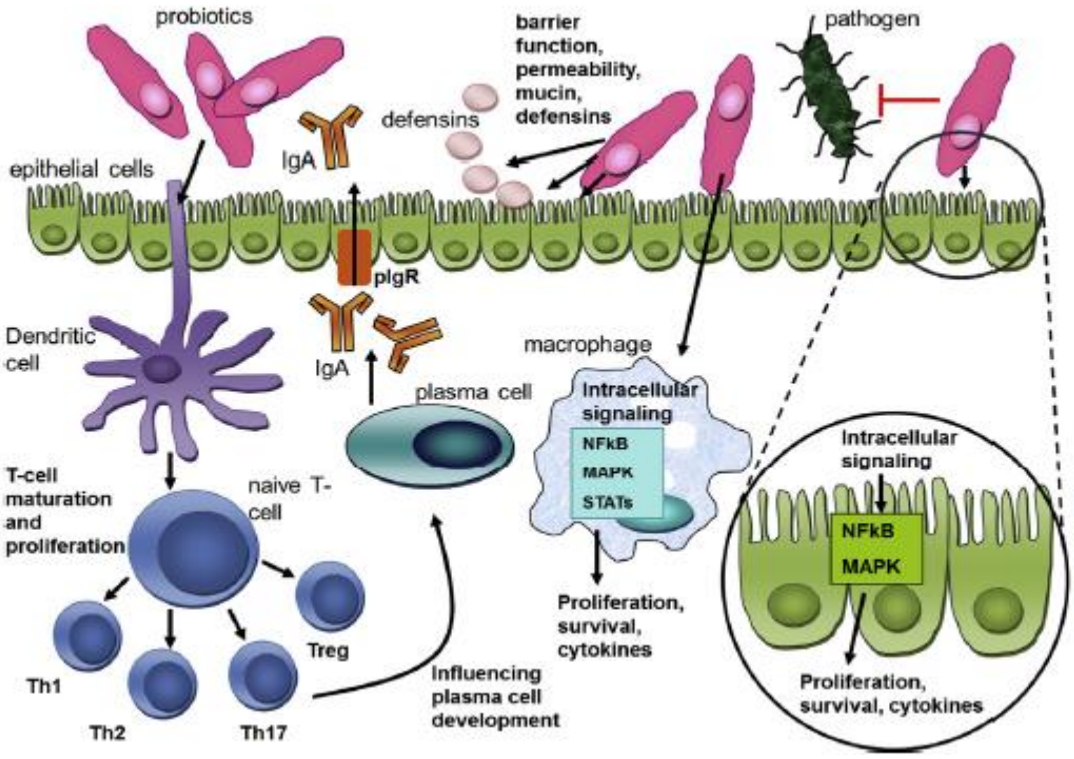

Figure 2: Drawing presenting the "mechanisms of action of probiotics on the intestinal mucosa (intestinal epithelial cells in green) and the associated local immune system. *Arrows* indicate direct promoting effects, red "T"-shaped bars indicate inhibitory effects. MAPK, mitogen-activated protein kinase; NFκB, nuclear factor kappa B; plgR, polymeric immune globulin receptor; STAT, signal transducer and activator of transcription; Th, T-helper lymphocyte cell." *Extracted from Schmitz* [69]

2.4 Clinical efficacy

Investigations of in vivo effects of probiotics remain limited in dogs. Indeed, a recent review gathered 17 studies with dogs with only 7 randomized controlled trials [72].

2.4.1 Gastrointestinal disorders

Inflammatory bowel disease (IBD) is a common cause of chronic gastrointestinal signs in dogs. Following administration of probiotics, clinical improvements were seen in dogs suffering from IBD. Rossi et al. [73] reported a decrease of the canine IBD activity index (CIBDAI), scoring system tracking the clinical disease activity. In this study, a cohort dogs received a multi-strain probiotic product (mixture of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* strains) for 3

months. In parallel, other diseased dogs had a combination therapy of prednisone and metronidazole. The results were a significant decrease in the clinical scores despite a slower recovery time (for diarrhea and vomiting) in dogs with probiotics compared to combination therapy dogs (median time of 10.6 days with probiotic treatment, compared to 4.8 days with drug treatment group). White et al. [74] reported a same reduced CIBDAI scores in IBD dog receiving a multi-strain probiotic, for two months, in addition to standard therapy with prednisone.

Acute gastroenteritis can be of infectious origin (viral, bacterial, parasitic), antibiotic induced or idiopathic. In case of acute hemorrhagic diarrhea syndrome (AHDS) probiotics could successfully supplement symptomatic treatment. Indeed, after a 3-week administration, a quicker clinical recovery and a significant reduced abundance of *Clostridium perfringens* shedding were observed in dogs supplemented with a lactic acid bacteria mixture [75].

Kelley et al. [76] reported that supplementation of *Bifidobacterium animalis* strain in dogs was associated with a better fecal score and shorter duration of diarrhea. This latter was significant with a mean number of days of diarrhea of 3.9 ± 2.3 versus 6.6 ±2.7 days in the control group. Improvement of stool consistency following probiotics administration are also reported by Gómez-Gallego et al. [77] and in the case of kenneling stress-related diarrhea [78].

Infectious origin diarrhea has also showed to be improved by the use of probiotics. Aksu [79] compared standard therapy with or without probiotics addition in dogs suffering from parvoviral enteritis. A higher survival rate and a faster improvement of clinical state (among which diarrhea and frequency of vomiting) were observed in the group of dogs supplemented with probiotics. It is worth mentioning that these observed positive effects only apply to the probiotic preparation used in this study (a commercial mixture) as the strain specificity is important.

Chronic gastrointestinal disorders can be also improved to some extents by the use of probiotics. D'Angelo et al. [80] studied the addition of *Saccharomyces boulardii* to standard treatment of dogs suffering from chronic enteropathy. They revealed a significant reduction of stool frequency, an improvement of stool consistency and body condition score compared to the

placebo group. In another study, CIBDAI scoring system was reported to decrease significantly in dogs suffering from food responsive diarrhea and receiving a lactobacilli mixture. Indeed, at the beginning of the trial the score was corresponding to a moderate degree of symptoms (5-8) and decrease to insignificant to mild degree range (0-5). But there was no between group effect, so, just based on these results it cannot be concluded that the probiotic supplementation impacted the course of the disease. Most likely the observed effects could be attributed to the elimination diet [81].

2.4.2 Immune system

Probiotics can also have a great efficacy in modulating the immune system of the host. Benyacoub et al. [82] administered the strain *Enterococcus faecium* SF68 to growing puppies (from weaning to one year of age). They demonstrated mucosal and systemic effects as circulating immunoglobulin G (IgG) and IgA, fecal IgA, and the number of mature B cells were higher in dogs with the probiotic. Rossi et al. [83] report similar significant increase of fecal IgA and circulating IgG following administration of a multi-strain probiotics. Baillon, Marshall-Jones and Butterwick [58] also observed improved immune functions with increased monocytes, neutrophils and IgG following *Lactobacillus acidophilus* DSM13241 strain in dogs. Addition of probiotics could be a good option for strengthening the immune system at critical time periods in the life of dogs as weaning, pregnancy or aging.

Clinical signs of atopic dermatitis could also be alleviated by probiotics. The supplementation of *Lactobacillus rhamnosus* strain GG was associated with a significant decrease of the immunologic indicator immunoglobulin E (IgE) in the first 6 months of life of dogs [84].

2.5 Safety

Compared to antibiotics, probiotics are generally perceived as safer substances. However, they are live microorganisms and hence, they could lead to harmful effects as infection or toxin production. Theoretically probiotics can have four types of side effects [85]:

- Systemic infections (septicemia, endocarditis)
- Harmful metabolic activities (leading to gastrointestinal side effects)

- Overstimulation of immune system in vulnerable animals (young, pregnant, immune depressed)
- Gene transfer (antibiotic resistance genes) with microbiota or other intestinal pathogens.

Concerning the last point, as observed with any bacteria, antibiotic resistance has been reported among some probiotics strains of lactic acid bacteria. This resistance could be encoded by chromosomal or plasmid genes. However, current information cannot establish if such resistance may lead to clinical problem [61]. Obviously, when selecting potential probiotic strains, bacteria containing specific drug resistance genes should be discarded. At this date there is a necessity to develop tests for determining drug insensitivity or resistance profiles of probiotics.

Enterocci (mainly *Enterococcus faecium* or *faecalis*) are found in several commercially available probiotics intended for small animals and might be harmful. Indeed, studies reported that these same enterococci could be pathogenic and diffuse antibiotic resistance as well as stimulating the development of other potential harmful microorganisms. [86].

To date, there are no specific contraindications for probiotics use in dogs. However, as they are live microorganisms, the zero risk does not exit. Further clinical studies are then required to ensure the safety of probiotic use in small animal patients. The use of nonviable form of microbial preparations could be a solution to prevent potential side effects of probiotics [86].

2.6 Regulation

As probiotics are considered dietary supplements their use as food additives are not regulated compared to other drugs. [87]

In the United States, the regulation of probiotics is under the scope of the Food and Drug Administration (FDA). Depending its intended use, a probiotic product is categorized as “food”, “medical food”, “dietary supplement” or “drug” and follows a specific set of regulations. Only products promoting health claims need a prior approval by the FDA. Hence, probiotics marketed as dietary supplements can be freely commercialized. Clinical proofs of safety and efficacy as well as consent of FDA are prerequisite for probiotics intended for use as drugs [88].

In the European legal framework, probiotics food is under the scope of the General Food law. Currently six probiotics strains have been authorized by the European Commission: *Lactobacillus fermentum* (NCIMB 41636), *Lactobacillus plantarum* (NCIMB 41638) and *Lactobacillus rhamnosus* (NCIMB 41640), *Lactobacillus acidophilus* CECT 4529, *Enterococcus faecium* DSM 10663/ NCIMB 10415 and *Bacillus subtilis* C-3102 (DSM 15544) [89]. However, many probiotics preparations are commercially available without a prior approval. These products often contain a mixture of probiotics strains such as lactobacilli (*L. acidophilus, L. lactis, L. rhamnosus*), bifidobacteria (*Bifidobacterium lactis* or *bifidum*), *Bacillus subtilis*, some yeasts (*Saccharomyces cerevisiae*) or fungi. Quite frequently not enough in vivo studies attest the efficacy and safety of these microorganisms [65].

Health claims of probiotics are authorized in the EU only after correct scientific assessment (panels adopted by the European Food Safety Authority). Nutrition and Health Claim Regulation 1924/2006 (NHCR) is followed by food business operators who want to emphasize a specific beneficial effect regarding health or nutrition of their products for purpose of labelling or advertising. The goal is to protect the consumers and ensuring that any claim is "clear, accurate and based on scientific evidence" [90].

2.7 Labelling and quality control

At an international level, practice guidelines have been developed by the Council for Responsible Nutrition and the International Probiotics Association [91]. These guidelines are scientifically based and support a 'responsible production and marketing'. Aiming to clarify these two processes, they address labelling, stability testing, and proper storage of dietary supplements or foods containing probiotics.

Labels of commercial probiotic preparations are frequently improper. Indeed, Weese and Arroyo [87] exposed many inaccuracies on the labels of dog and cat diets claiming to contain probiotics. Among the 19 diets tested, none of them contained all of the organisms labelled and bacterial names were misspelled on five labels. In addition, only a low number of viable organisms was recovered from the products (between 0 and 1.8×10^5 cfu/g). Same conclusions were drawn after a microbiologic study of commercial veterinary probiotics [92]. In only 3 over the 8 tested products the description of organisms present and their concentrations were

accurate. None of products contained the stated concentrations of organisms and less than 2% of the listed concentrations were actually found in the products. Inaccuracies in labelling and quality were also observed after evaluation of commercial probiotics marketed for use in animals [93]. They concluded that only two products had an adequate label and viable bacterial contents. These studies revealed that the quality control of commercial probiotics remains too poor. A continued pressure on the manufacturers as suggested by Weese and Martin [93] could be improved the situation. Indeed, some of these discrepancies could be due to damage of microorganisms during processing and storage.

3. STUDIES ON THE IMPACT OF PROBIOTICS ON MICROBIOTA

A total of 29 studies examining the impact of probiotics administration on the canine gut microbiota was collected. Two studies were exclusively focusing on the recovery of the probiotics in feces whereas the others also examined the changes at different phylogenetic levels. All trials, except the one of Manninen et al. [94] (analysis of jejunal chyme), were based on examination of fecal microbiota. In the following pages, an overview of the individual studies examining the impact of probiotics on the fecal microbiota is presented on table 1.

Here after is the corresponding caption:
cfu: colony forming units
D: number of dogs enrolled
S: number of samples
↑/↓: significant increase/decrease of tested microorganism
≈: no significant changes
T: trend to increase/decrease of tested microorganism.

Table 1: Summary of studies examining the effects of probiotic strains on canine microbiota composition

Probiotic strains	Dose (cfu/ day)	Form and Origin	Length of application	Sample size	Analysis technique	Tested microorganisms									Reference
						Lactobacillus	*Bifidobacterium*	*Enterococcus*	*Clostridium*	*Cl. difficile*	*Cl.perfringens*	Coliforms	*E.coli*	*Pseudomonas*	*Aeromonas*	
Lactobacillus animalis LA4	10^9 (0.5x 10^9)	Freeze dried, canine derived (faeces)	10d	D= 9 S= 27	Culturing	↑		↓ T			↓ T	≈				[95]
Lactobacillus acidophilus DSM 13241	10^9 (1.97-3.53 x10^9)	Freeze dried	28d	D= 15 S= 45	Culturing	↑		≈	≈		≈					[58]
					FISH	↑			↓							
						Total anaerobes: ≈										
Lactobacillus acidophilus NCFM	10^9 ($2x10^9$)	Freeze dried	28d	D= 30 S= 150	Culturing	≈	↑ T				≈		↓ T			[96]
Lactobacillus acidophilus D2/CSL (CECT/4529)	10^9 (1.75-1.85x 10^9)	Not mentioned	35d	D= 40 S= 80	Culturing	↑							↑			[97]
Lactobacillus fermentum CCM 7421	10^9 ($3x10^9$)	Fresh, canine derived (faeces)	7d	D= 15 S= 30	Culturing	↑		↑					≈			[98]
						Staphylococcus: ≈										
Lactobacillus fermentum CCM 7421	10^7	Freeze dried, lyophilized, canine derived (faeces)	7d	D= 11 S= 33	Culturing			≈					↓	↓	↓	[99]
						Lactic acid bacteria: ↑										
Lactobacillus fermentum CCM 7421	10^8 ($2x10^8$)	Freeze dried, lyophilized, canine derived (faeces)	14d	D= 12 S= 72	Culturing			≈	↓				↓ T	≈	↓ T	[99]
						Lactic acid bacteria: ↑										
Lactobacillus fermentum CCM 7421 +/- inulin	10^8 (2.37x 10^8) Inulin 1% of diet	Fresh, canine derived (faeces)	14d	D= 36 S= 252	Culturing Probiotic			≈	↓				≈	≈	≈	[100]
					Probiotic + inulin			≈	↓ T				≈	≈	≈	
						Lactic acid bacteria: ↑ in probiotic group										
Lactobacillus fermentum CCM 7421 +/- chlorophyllin	10^8 (1.3x 10^8)	Fresh, canine derived (faeces)	14d	D= 30 S= 120	Culturing Probiotic		≈	≈	≈			≈				[101]
					Probiotic + chlorophyllin		≈	≈	↓			↓ T				
						Lactic acid bacteria: $\uparrow_T$ in probiotic group ≈ in probiotic + chlorophyllin group										

(Continues)

Table 1: (Continued)

Probiotic strains	Dose (cfu/ day)	Form and Origin	Length of application	Sample size	Analysis technique	Tested microorganisms										Reference
						Lactobacillus	*Bifidobacterium*	*Enterococcus*	*Clostridium*	*Cl. difficile*	*Cl.perfringens*	Coliforms	*E.coli*	*Pseudomonas*	*Aeromonas*	
Lactobacillus fermentum CCM 7421 +/- Eleutherococcus senticosus	10^8 (1.92-4.4x 10^8)	Fresh, canine derived (faeces)	14d	D= 24 S= 144	Culturing			≈	≈				≈	≈	≈	[102]
					Probiotic			≈	≈				≈	↑	↑	
					Probiotic + E.senticosus	Lactic acid bacteria: ↑ in both groups										
Lactobacillus fermentum CCM 7421 +/- alginite	10^9 ($4x10^9$) alginite 1% of diet	Fresh, canine derived (faeces)	14d	D= 30 S= 120	Culturing		≈	≈	↓			↓ T				[103]
					Probiotic		≈	≈	↓			↓				
					Probiotic + alginite	Lactic acid bacteria: ↑ in both treatments										
Lactobacillus johnsonii NCC533	10^{10}	Powder, human origin	10w	D= 168 S= 504 puppies	16S rRNA gene sequencing	No effects on diversity or composition Impact of age										[54]
Lactobacillus kefiri LKF01 DSM32079	10^7 (3.2x 10^7)	Powder, human origin	30d	D= 9 S= 81	16S rRNA gene sequencing	No significant changes at phylum, family, genus level At T_{60}: $\downarrow_T$ of relative abundance of *Fusobacteriaceae* and *Ruminococcaceae* No significant difference in ∝-diversity										[104]
Lactic acid bacteria and yeast from KEFIR	10^{11} (2x 10^{11}) And 10^9 ($2x10^9$) for yeast	Fresh, from Kefir milk	14d	D= 6 S= 12	qPCR (quantitative Polymerase Chain Reaction)	qPCR: ↑ Lactic Acid bacteria ↑ LAB:*Enterobacteriaceae* ratio ↓ Firmicutes:Bacteroidetes ratio										[105]
					16S rRDNA gene sequencing	16S rRDNA: no significant change, same ∝-diversity Phylum: ↓ relative Fusobacteria Family: - ↑ *Prevotellaceae* - ↑ *Selenomonadaceae* - ↑ *Sutterellaceae* - ↓ *Clostridiaceae* - ↓ *Fusobacteriaceae* - ↓ *Ruminococcaceae* Species: ↓ *Bacteroides uniformis* ↓ *Fusobacterium perfoetens* and *mortiferum* ↓ *Faecalibacterium prausnitzii* ↓ *Pseudoflavonifractor capillosus* ↓ *Fusicatenibacter saccharivorans* ↓ *Anaerorhabdus furcosa* ↓ *Ruminococcus torques* ↓ *Intestinimonas butyriciproducens* ↓ *Herbinix hemicellulosilytica* ↑ *Catenibacterium mitsuokai*										

(Continues)

Table 1: (Continued)

Probiotic strains	Dose (cfu/ day)	Form and Origin	Length of application	Sample size	Analysis technique	Tested microorganisms									Reference
						Lactobacillus	*Bifidobacterium*	*Enterococcus*	*Clostridium*	*Cl. difficile*	*Cl.perfringens*	Coliforms	*E.coli*	*Pseudomonas*	*Aeromonas*	
Lactobacillus acidophilus + *Candida utilis*	$10^{9.6}$	Not mentioned	35d	D= 20 S= 60	Culturing	↑	↑	↓					↓			[106]
						Staphylococcus: ↓										
Streptococcus thermophilus DSM 32245 *Bifidobacterium lactis* DSM 32246 and 32247 *Lactobacillus acidophilus* DSM 32241 *L. helveticus* DSM 32242 *L. paracasei* DSM 32243 *L. plantarum* DSM 32244 *L. brevis* DSM 27961	10^{11} ($4x10^{11}$)	Lyophilized	60d	D= 20 S= 40	qPCR	↑	↑				↑		≈			[83]
						Streptococcus: ↑										
					16S rRNA gene sequencing	No significant ∝-diversity or β diversity difference										
Lactobacillus casei Zhang *L.plantarum* P8 *Bifidobacterium animalis subsp. lactis* V9	10^{9}-10^{10} (elderly: 2x 10^{10} young: $4x10^{9}$ training: $8x10^{9}$)	Lyophilized	60d	D= 90 S= 360	16S rRNA gene sequencing	Genus level: - Elderly: ↑*Bacteroides* and *Faecalibacterium* ↓ *Clostridium*, F*lavonifractor*, *Oscillibacter*, *Blautia* - Training: ↓ *Escherichia* Species level: - Elderly: ↑ *Faecalibacterium prausnitzii*, *Bacteroides clarus* ↑*Succinovibrio dextrinosol*vens ↑ T *Ruminococcus gravus*, lactaris ↓ *Blautia coccoides*, *hanseni* and product ↓ Clostridium species ↓ *Ruminococcus torques* - Training: ↑ *Lactobacillus animalis* and *acidophilus* ↓ *E. coli* ↓ *Collinsella stercoris* and *tanakaei* - Young: ↑ *Bacteroides coprophilus* ↑ *Lactobacillus animalis* and j*onhsonii* ↓ *Sutterrella stercoricanisin*										[52]

(Continues)

Table 1: (Continued)

Probiotic strains	Dose (cfu/ day)	Form and Origin	Length of application	Sample size	Analysis technique	Tested microorganisms									Reference
						Lactobacillus	*Bifidobacterium*	*Enterococcus*	*Clostridium*	*Cl. difficile*	*Cl.perfringens*	Coliforms	*E.coli*	*Pseudomonas*	*Aeromonas*	
Bifidobacterium animalis Subp. Lactis LKM512	10^{10}	Freeze dried, Powder	14d	D= 5 S= 20	16S rRNA gene sequencing	Genera: ↑ *Bifidobacterium* ↓ *Clostridium cluster XVIII* Family: ↑ *Bifidobacteriaceae* ↓ *Erysipelotrichaceae*										[107]
Bifidobacterium animalis AHC7	10^9 (1.5x 10^9)	Pill, canine derived (mucosa)	6w	D= 11 S= 55	Culturing	≈	≈		↓	↓	≈		≈			[108]
						Bacteroides: ≈ Total aerobes: ≈ Total anaerobes: ≈										
Bifidobacterium animalis AHC7	10^9 10^{10} (1.43x 10^9, 4.93x 10^{10})	In cocoa butter treats, canine derived (mucosa)	12w	D= 20 S= 80	Reverse transcription PCR	≈										[109]
						Total bacteria concentration: ≈										
Bifidobacterium animalis B/12	10^9 (1.04x 10^9)	In solution, canine derived (faeces)	14d	D= 20 S= 120	Culturing		≈	≈	≈			↓				[110]
						Lactic acid bacteria: ↑ at day 7										
Bifidobacterium longum KACC 91563	10^9 (4-6x10^9)	Added to cheese during making, human origin	8w	D= 12 S= 36	Culturing culturing 16S rRNA gene sequencing	≈	↑	≈			↓					[111]
						Culturing: ↓ *Enterobacteriaceae* *Eubacterium, Bacteroidaceae*: ≈ 16S rRNA: ↑ T *Bifidobacteriaceae* 8w: ↓ *Blautia, Collinsella, Fusobacterium*										
Bifidobacterium longum KACC 91563	10^9 10^{10} (1.25x 10^9, 1.75x 10^{10})	Added to cheese during making, human origin	8w	D= 15 S= 75	Culturing	≈	↑	≈	↓							[112]
						Enterobacteriaceae: ↓ *Eubacterium, Bacteroidaceae*: ≈										
Enterococcus faecium NCIB 10415	10^9 (9.2x 10^9)	Human origin	18d	D= 12 S= 24	Culturing				↓							[113]
						Salmonella, Campylobacter: ↑ T										
Enterococcus faecium EE3	10^9 (2-3x 10^9)	Isolated from canine feed	7d	D= 11 S= 66	Culturing	↑ T		↓ T					≈	↓		[114]
						Staphylococcus: ↓ T										

(Continues)

Table 1: (Continued)

Probiotic strains	Dose (cfu/ day)	Form and Origin	Length of application	Sample size	Analysis technique	Tested microorganisms										Reference
						Lactobacillus	*Bifidobacterium*	*Enterococcus*	*Clostridium*	*Cl. difficile*	*Cl.perfringens*	Coliforms	*E. coli*	*Pseudomonas*	*Aeromonas*	
Bacillus subtilis C-3102	10^6	Powder	33d	D= 15 S= 75	16S rRNA gene sequencing	Phylum level: in % - ↑ Firmicutes - ↑ Bacteroidetes - ↑ Fusobacteria - ↓ Actinobacteria Genus level: ↑ *Bacteroides* ↑ *Alloprevotella* ↑ *Faecalibacterium* ↑ *Allobaculum, Catenibacterium* ↓ *Lactobacillus* ↓ *Bifidobacterium* ↓ *Enterococcus* ↓ *Clostridium sensu stricto 1* ↓ *Eubacterium brachy, Anaerostipes* ↓ *Escherichia-Shigella, Enterohabdus*										[115]

3.1 Materials and methods of the studies

3.1.1 Experimental design of the studies

The studies were divided into 15 self-controlled studies and 14 randomized controlled studies (gold standard for evaluating treatment intervention in a group).

Overall, a low number of dogs was enrolled in clinical trials. It ranged between 5 and 168 dogs (most frequently from 9 to 30 dogs). It is evident that a higher sample size would have provided more detailed data regarding changes in microbiota. Indeed, a larger cohort of dogs could overcome the high variability of bacterial communities between individuals and allow to better detect significant changes in microbiome [109].

The co-morbidities of dogs enrolled in studies were very diverse. All the dogs recruited were healthy (no ongoing drug treatment), from different gender and breeds. The age and body weight of dogs greatly differed between and within the studies. Overall, the age gap between the trials was of 3 weeks to 13 years old. As it was developed in the first part, age can influence the composition of microbiota. The study of Xu et al. [52] showed that the elderly group (5-13 years

old) was more profoundly affected by the ingestion of the probiotic compared to the training (9-24 months old) or young (below 8 months old) group. Hence the age of dogs enrolled could have influence the response to the probiotics.

Some studies made the choice of a standardized diet (with or without a prior acclimation period of a variable duration) while others kept the previous one throughout the length of the experiment. Regarding housing, it greatly varied between studies, some dogs were housed in kennels (individually or in pairs) whereas others stayed at the owner house. They had outdoor and/or indoor access. Diet and housing do not appear to have a major impact on the response to probiotics. Indeed, even if animals are housed and fed identically their intestinal microbiota will still show major differences [15]. Moreover, it seems that a design with a non-standardized diet and housing allow obtained results to be more easily transposed into real life conditions.

3.1.2 Profile of probiotic used

A wide range of probiotic strains were used and most of the studies used strains derived from genera *Lactobacillus* and *Bifidobacterium*. Other strains derived from *Streptococcus*, *Enterococcus*, *Weissella* and *Bacillus* genera. One study used undefined strains as the ones contained in Kefir. Some strains were supplemented with food additives (inulin, chlorophyllin, E.senticosus, alginite) or enriched with trace elements such as Selenium and Zinc. The majority of the studies used single strain probiotics. The impact on microbiota with the use of a multi-strain probiotics could be attributed to the potential synergism between the strains.

It is to be pointed out that Ren et al. [106] was the only study which did not specify the bacterial and yeast strains administered. Nevertheless such precision is crucial as the effects of probiotics can be specific not only at the genus and species level but also at strain level [61, 69] . Strains derived from a same bacterial species do not always exhibit the same effects. For example, *Lactobacillus acidophilus* DSM 13241 used in Baillon, Marshall-Jones and Butterwick [58] led to a significant increase of fecal lactobacilli whereas *L. acidophilus* NCFM applied in Swanson et al. [96] did not have a significant impact on this lactic bacteria taxa even if both strains were administered for the same period of time (4 weeks). Hence, it could be thought that the effect on microbiota would be specific to a given strain.

The origin of probiotics used was very diverse and not always mentioned. Some were of human origin, canine origin (mostly from faeces, except O'Mahony et al. [108] and Kelley et al. [109] who isolated the probiotic strain from gastrointestinal tissue) or isolated from kefir milk [105]. The importance of the origin of a probiotic strain is unclear. Indeed, the two studies of Park et al. [111, 112] showed that *Bifidobacterium longum* KACC 91563 of human origin led to significant decrease of *Clostridium* genus and *Clostridium perfringens* respectively after administration. Concerning Kim et al. [105], strains isolated from kefir successfully improved canine microbiota (by an increase of lactic acid bacteria) and induced clear changes at various taxonomical levels. On the other hand, some studies recommend that probiotics should have the same origin than the target species as probiotics can have species-specific effects [116]. To reinforce this idea, Ley et al. [7] reports that the canine microbiota, despite being closely related, is distinct from the human microbiota. Hence, from these authors, bacterial strains of canine origin would be the best choice as use in probiotics in dogs. For O'Mahony et al. [108], isolation from gut mucosa is to be preferred as "certain commensal organisms may lose viability rapidly once shed in feces". The results from the collected studies are that non-canine and canine origin probiotics have both impact on canine microbiota. No difference related to the origin of probiotic is reflected in the present results.

Among studies, probiotic concentrations used ranged from 10^6 to 10^{11}cfu (colony forming units) with the amount of 10^9 was most often used. In humans, a daily dosage ranging from 10^7 to 10^9 cfu is commonly recommended and effect of probiotic appear to be dose-dependent [117]. To date, such dose ranges are not fully described in dogs (neither the frequency of administration) despite the fact that the FAO/WHO definition clearly highlights that probiotics must be provided "in adequate amounts". Consequently, in some of the collected studies, the absence of significant changes in bacterial taxa could be attributed to an insufficient dosage. Indeed, Gaspardo et al. [104] did not observe significant changes of fecal microbiota neither at family, neither or genus level. The daily dose of probiotic administered (10^7 live *Lactobacillus kefiri*) was extrapolated from recommendations of the manufacturer concerning an adult person. Hence, it can be hypothesized that a higher dosage would have led to more significant changes on microbial communities in dogs.

In addition, some errors of labelling about concentrations were reported by Gaspardo et al. [104] who used *Lactobacillus kefiri* originating from a commercial food supplement. Prior its administration, they proceeded to a quality control of the product which revealed that the number of viable *Lactobacilli kefiri* was 2-log fold lower than the claimed concentration of the product ($3.2\pm2.4\times10^7$cfu compared to the declared $\geq 10^9$cfu). As seen in the previous part, labelling errors are not rare events. A systemic quality control to assess exact concentrations of probiotics would be recommended for studies using commercial probiotics.

Length of probiotic administration widely ranged from days (7 days in Strompfová et al. [98]) to months (3 months in Kelley et al. [109]). This great variation could be explained by the fact that no information concerning the length of administration in dogs is clearly stated in the literature.

3.1.3 Analyzing methods

Samples were usually taken before (as a baseline) and after probiotics supplementation in order to compare the effects of probiotic strains. Some studies in addition also took samples during the supplementation phase. Repeated samplings after probiotic withdrawal allowed to follow the persistence of changes within the microbiota. Time of sampling largely differed between the studies. The frequency of sampling could impact the observed changes in microbiota, it is likely that a higher frequency would allow a more accurate insight of the microbiota variations. Except for Manninen et al. [94] who analyzed luminal microbiota via jejunal chyme sampling, fecal microbiota was assessed in the collected studies. Screening of adherent microbial populations from intestinal mucosa (via biopsies) are thought to give a better overview of the canine gut ecosystem [108].

Most of the studies analyzed the impact on microbiota via culture methods. However, as said previously, only limited results can be obtained with such methods. Only targeted bacterial taxa within the complex gastrointestinal microbiota could be recovered. The study of Baillon, Marshall-Jones and Butterwick [58] revealed that changes in clostridial population were only significantly detected by the use of FISH compared to the use of selective agars. Culture

approach does not bring a reliable identification of gut bacterial communities, specially it fails to reflect shifts at low phylogenetic levels.

The 16S rRNA sequencing was the method of choice for studies examining the microbiota with a molecular approach. Studies used different bacterial primers which consequently targeted different regions of the 16S rRNA gene. In addition, different sequencing platforms and bioinformatics processing were used. All these combined factors can have an impact on the bacterial groups detected and may have affected the studies' results. This further complicates comparison between the different studies [57].

To reduce the heterogeneity of the studies in terms of materials and methods a similar framework could be used. Ideally it would include a randomized controlled trial design, a large cohort of dogs, similar probiotic strains used for a same length of administration with similar analysis techniques (via sequencing).

Last but not least, it is important to emphasize that individual responses following probiotics supplementation cannot be predicted. This is could be attributed to intraindividual differences of microbiota composition prior experiment. As a matter of fact, Weese and Anderson [118] supposed that dogs having a preexisting high lactic acid bacteria population could show a lower enrichment with "foreign" lactobacilli. Underlying production of antibacterial products or competition for nutritional resources could explain this phenomenon. Hence, there is still a poor understanding of possible interactions between indigenous bacterial communities and administered probiotic strains.

All of the above points could explain why changes observed in microbiota were not always significant in the studies.

3.2 Summary of the findings

3.2.1 Recovery of the probiotic

Some studies successfully recovered the administered probiotic from feces and jejunal chyme [94] during the administration phase, proof that it could survive gastrointestinal passage. Interestingly, Nakamura et al. [107] reported that the amount of recovered probiotics ($10^{10.4}$ and $10^{10.8}$) was greater than the administration dose (10^{10}), indicating that this strain was able to proliferate in the canine intestine. After cessation of administration, the probiotic recovery in faeces was however variable. In some studies, the probiotic quickly disappeared after 3 days [119] or 1 week [107] suggesting that the strain was rapidly washed-out from the intestines. Other studies reported that the probiotic was recovered even several weeks or months after end of administration. *Lactobacillus fermentum* CCM 7421 was still detected three weeks and five weeks respectively after the end supplementation in [103] and [100]. Three and six months respectively after the end of administration, *Enterococcus faecium* strain EE3 in [120] and *Lactobacillus fermentum* CCM 7421 in [98] were still present in faeces. This shows that these strains successfully survived and colonized the canine gut, prerequisite for their efficacy [118].

3.2.2 Impact on microbiota composition

As the vast majority of studies used a culture approach to analyze the response of microbiota following probiotics administration, changes could only be seen for targeted genus or species. Interestingly, two studies could show significant changes at phylum level [105, 115]. Alpha-diversity (variation of microbes as species richness and evenness in a sample) and Beta-diversity (variation of microbial communities between samples) were not significantly changed following probiotics treatment [83, 104, 105].

Interestingly, Xu et al. [52] found that a two-month probiotic treatment could temporarily reduce the age index of the microbiota of elderly dogs, shifting towards that of the training dogs.

Strains derived from *Lactobacillus* genus have showed to have various impacts on microbiota. After only one week of administration of *Lactobacillus fermentum* CCM 7421, significant higher counts of fecal lactobacilli were observed by selective media [98]. *Lactobacillus animalis* LA4 administered for 10 days led to similar results (lactobacilli counts rose by about 4 log units). But this later change was temporary as 5 days after cessation of probiotic, counts

dropped close to initial values [121]. The same effect was observed after administration for 4 weeks of two strains of *Lactobacillus acidophilus* (*DSM 13241* and D2/CSL) by Baillon, Marshall-Jones and Butterwick [58] and Marelli et al. [97]. Despite a longer administration period this effect was also lost after cessation of probiotics administration [58]. It is very likely that this increase of fecal lactobacilli is due to enrichment by the probiotics. Probiotics seem to temporary colonize the gut as when the probiotic is washed-out from the intestines (time which seems to depend on the bacterial strain), its action on microbiota is lost. Hence these studies suggest that probiotics should be fed continuously to exert their effect on microbiota.
In a broader scope, lactic acid bacteria group was showed to be positively impacted by probiotics in all studies using the probiotic strain *Lactobacillus fermentum* CCM7421. For example, the counts significantly rose to 6.73±0.25 log at day 7 compared to day 0 5.15±0.33 mean $\log_{10}$ cfu/g±SEM in [99] . In another study, after one week of treatment, lactic acid bacteria were 7.86 ±0.42 mean $\log_{10}$ cfu/g±SE compared to control group (6.57±0.13) [100] .

The effect of the strain *Lactobacillus fermentum* CCM7421 seems to be prolonged as five weeks after cessation of administration, significant high counts of lactic acid bacteria were still observed by Strompfová et al. [99] and Strompfová et al. [100].
Lactobacillus was not impacted by the administration of *Bifidobacterium* derived strain expect in Strompfová et al. [110] where administration of *Bifidobacterium animalis* B/12 was associated with a significantly higher population of lactic acid bacteria (LAB).

The other genus considered as part of beneficial microbiota is *Bifidobacterium*. Significant increase was observed after administration of a multi-strain probiotics for one [106] or for two months [83]. Significant increase of bifidobacteria was also seen after inclusion of *Bifidobacterium longum* (KACC 91563) in queso blanco cheese at 4 weeks [111] or 8 weeks [112] of supplementation. Surprisingly, the ingestion of a commensal probiotic for a period of 6 weeks did not affect the level of bifidobacteria [108]. However, the strain *Bifidobacterium animalis* AHC7 was prepared from canine mucosa samples. The authors suggest that it could be due to the lack of selectivity of the media used for enumeration of bifidobacteria. Hence, this apparent lack of effect on bifidobacteria is to be taken with caution. *Lactobacillus fermentum*

CCM 7421 failed to significantly increase *Bifidobacterium* genus even with feed additives (chlorophyllin or alginite) [101, 103].

Probiotics showed to also decrease potential pathogenic bacterial taxa. With the use of strains of probiotic derived from *Lactobacillus* genus, significant lower counts of clostridia were observed. Indeed, a decrease to nearly half the original value was observed by Baillon, Marshall-Jones and Butterwick [58] after 4 weeks of *Lactobacillus acidophilus DSM 13241* administration. Similar results were obtained with a two-week supplementation of *Lactobacillus fermentum* CCM7421 [99] (which reported a decrease of clostridia counts up to 2 log cycle), [100, 103]. Probiotics derived from *Bifidobacterium* genus also showed to impact clostridia populations. O'Mahony et al. [108] reported a reduction of total fecal clostridia counts with a significant decrease of *Clostridium difficile* numbers after *Bifidobacterium animalis* AHC7 treatment. On the other hand, *Clostridium perfringens* was significantly decreased after administration of *Bifidobacterium longum* KACC 91563 added to cheese [111] or of a multi-strain probiotic [83]. Finally, the use of a *Enterococcus faecium* strain was associated with significant lower counts of *Clostridium* spp. in the majority of dogs [113].

Other potential pathogens as *Aeromonas* and *Pseudomonas* were successfully reduced by probiotics. Indeed, Strompfová et al. [99] reported that administration of *L. fermentum* CCM7421 for 7 days led to significant decrease of these two genera populations in feces. *Pseudomonas*-like bacteria were also significantly decreased by *Enterococcus faecium* EE3 administration [120].

Some trends of reduction of staphyloccoci were observed by Strompfová [101] and Marciňáková et al. [120]. The only significant decrease of this genus was with the administration of a probiotic mixture of *Lactobacillus acidophilus* and *Candida utilis* [106].

Summary

Current collected studies bring a first set of knowledge on the modulation of canine microbiota by probiotics. Many factors differ among the studies in terms of material and methods used to analyze the microbiota. This certainly explains the vast heterogeneity and sometimes conflicting nature of results obtained. However, some isolated but significant results were reported after supplementation with some probiotics strains. Beneficial bacterial flora (lactobacilli and bifidobacteria) was seen to be enriched as opposed to potential harmful bacteria (*Clostridium perfringens*, *Aeromonas*, *Pseudomonas*) which were decreased. Sometimes these changes persisted after the end of supplementation suggesting that probiotics can have a profound and durable effect on microbiota.

Nevertheless, there is a lack of information if changes observed in other bacterial taxa are positive or negative for the host. For example, Kim et al. [105] could not determine if the remarkable decrease of the abundance of Fusobacteria following kefir supplementation was beneficial. Further studies are required to better understand the clinical and physiological consequences of changes in bacterial taxa. Metabolomics, study of metabolites, could help deciphering the function of each microbial community within the microbiota.

Limitations of knowledge in the use of probiotics in dogs were emphasized by these studies. Choice of the bacterial strain, effective dose, or length of administration are key elements which are still unknown when probiotics are administered to dogs. The impact on microbiota could depend on these factors. In addition, probiotics are live microorganisms and rigorous assessment of their viability is crucial prior supplementation. Hence, quality control of commercial available probiotics need to be reinforced. Overall, a better knowledge on how and under which conditions probiotics can impact canine microbiota would provide an additional therapeutic choice to veterinarians in gastrointestinal disorders.

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