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The Juvenile Canine Intestinal Microbiota: Development from Birth to 12 Weeks of Age

Valpens tarmmikrobiota: Utvikling fra fødsel til 12 ukers alder

Anna Hilmersson, Glódís Sigmundsdóttir and Vilde Bjaaland Siljan
Class of 2015

Kristin Marie Valand Herstad and Ellen Skancke

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Summary

Title: The Juvenile Canine Intestinal Microbiota: Development from Birth to 12 Weeks of Age

Authors: Anna Hilmersson, Glódís Sigmundsdóttir and Vilde Bjaaland Siljan

Supervisors: Kristin Marie Valand Herstad and Ellen Skancke, Department of Companion Animal Clinical Science

A literature review was performed to characterize the development of the intestinal microbiota (IM) in puppies from birth to 12 weeks of age. Puppies born with meconium/placenta harbouring cultivable bacteria, and puppies born vaginally gain more relative weight compared to puppies born without cultivable bacteria in meconium/placenta or delivered by caesarean section. During the first 3 weeks, the puppy's IM is dominated by Firmicutes and the most common lactobacilli are *Lactobacillus johnsonii* and *L. animalis*. Prior to weaning the puppies IM is dissimilar to their mothers IM but at weaning (approximately age 6-8 weeks), it becomes more similar, likely due to the transition from milk to solid food together with behavioural factors. At this time the diversity has increased, the main phyla found are Fusobacteria, Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria. *L. johnsonii* is no longer dominant while *L. animalis* is still abundant. Small study populations and lack of information regarding sample handling can be improved in future studies of the IM in dogs. Whatever the role of the rearing environment and microbiota in milk has on the IM development in puppies needs to be studied further. Age related changes of lactobacilli strains should be considered when designing probiotics for dogs.

Definitions and abbreviations

Ad libitum	«As much or as often as desired»
Alpha diversity	Variation of microbes in a single sample. Includes measurement of species richness and species diversity.
CAD	Canine atopic dermatitis
CE	Chronic enteropathy
CPV	Canine parvovirus
CS	Caesarean section
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Enteritis	Inflammation in the small intestine
FMT	Faecal material transfer
IM	Intestinal microbiota
Dysbiosis	Microbial imbalance
NGS	Next-generation sequencing
OTU	Operational Taxonomic Units

Pathogen	Organism that can cause disease
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SCFA	Short-chain-fatty-acids
Unweighted UniFrac	Variation of microbial communities between samples based on sequence distances, does not include information about abundance

Introduction

Microorganisms and the intestinal microbiota in humans and dogs

Microorganisms, or microbes, are organisms such as Bacteria, Archaea and eukaryotic organisms that are invisible to our bare human eye, hence the prefix "micro- ". But even though we can't see them without magnifying equipment, the world is full of them (Deng & Swanson, 2015).

Cultivation techniques have for a long time been common methods for studying this myriad of microbes. With a particular interest in those microbes found residing in our bodies however, we are only able to culture around 1% (Deng & Swanson, 2015). This means that the vast majority of the microorganisms in the body would probably still be unknown to us if it weren't for the discovery and use of genomic analysis. A broad spectrum of unknown microorganisms has been identified by these methods (Martinez et al., 2017).

The mammalian body hosts a surprisingly large number of microbes and just in the digestive tract alone the total microbial load is estimated to be between 10^{12} to 10^{14}organisms (Suchodolski, 2011). Following the identification of so many unknown microbes, the focus over the last years has increasingly been on understanding these microbes, namely studying the microbiota. In general, all the microorganisms found in an ecosystem make up a microbiota (Berg et al., 2020). Different microbiotas are found in different regions of the mammalian body. Over the last two decades a particular interest has been on the intestinal region, home to the intestinal microbiota (IM).

An important first step has been to characterize the IM and learn about its behaviour in healthy individuals. This is a fundamental knowledge to establish, making it possible to further comment anything about its importance to the host.

In adult humans the IM composition has been studied using faecal samples and found to be composed differently in each individual (Human Microbiome Project, 2012). But although each person has its own unique composition of the IM, it is most often composed of the same bacterial phyla being Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria (Human Microbiome Project, 2012; Rajilić-Stojanović et al., 2013; Tap et al., 2009).

Like humans, dogs also exhibit individual composition of the IM (Handl et al., 2011; Suchodolski et al., 2005) Based on faecal samples, the most commonly accounted phyla are Firmicutes, Bacteroidetes and Actinobacteria, the same as in humans, along with the additional phylum of Fusobacteria that appears to be more abundant in dogs than in humans (Hand et al., 2013; Handl et al., 2011; Jha et al., 2020). The abundance of the different phyla in adult dogs varies greatly between studies though. Handl et al., (2011) found that Firmicutes dominated the IM and Bacteroidetes, Actinobacteria and Fusobacteria were only found in low abundance. This was not in a total agreement with another study finding Fusobacteria to be the most abundant phylum, followed by moderate abundance of Bacteroidetes, Firmicutes and Proteobacteria and low abundance of Actinobacteria (Hand et al., 2013). Yet another study found Firmicutes to be the most abundant phylum with a moderate abundance of Proteobacteria and Bacteroidetes (Jha et al., 2020).

The core-faecal microbiota is therefore difficult to describe but the phyla Firmicutes and Bacteroidetes appear to be found more often in high abundances, compared to Proteobacteria, Actinobacteria and Fusobacteria that show more variations in abundances.

Remembering the fact that the intestinal microbiota is composed of living microbes, its existence is both influenceable and influential, as other living things are. No two individuals are exactly the same and neither is their IM, as has been mentioned.

Age (Odamaki et al., 2016), genetics (Erwin G. Zoetendal, 2001) and nutrition (Koenig et al., 2011) are examples of factors that influence the IM in humans. Same factors have also been shown to be important influencers on the IM in dogs (as reviewed in Pilla & Suchodolski, 2020). The microbiota itself is involved in many processes, both in dogs and humans, with protecting the host against invading pathogens, aiding in digestion and training the immune system being particularly important roles (as reviewed in Pilla & Suchodolski, 2020; Selber-Hnatiw et al., 2017). This is accomplished through complicated pathways where the IM and the host try to live in a balance, since that benefits both parts. For example, short-chain-fatty-acids (SCFA) are a product of microbial breakdown of fibres ingested by the host that are important for nurturing the intestinal cells (Donohoe et al., 2011). The intestinal cells provide similar service to the microbiota by producing mucus which the microbes can utilize as a nutritional source for themselves (Schroeder, 2019).

Intestinal Dysbiosis

Intestinal dysbiosis is a term used to describe a negative disruption of the composition of the intestinal microbiota. These negative alterations of the microbiota can happen following exposure to factors like diet, toxins, drugs or pathogens. As a result of these changes, the immune system of the host starts to interact with stimuli and antigens from the intestinal lumen, in a disturbed manner. This can further trigger an uncontrolled inflammation in the intestinal mucosa which in turn can give rise to a number of different diseases in the host (Carding et al., 2015). In exactly which way this happens has not yet been determined. Altered gene expression, following dysbiosis, has been implicated as a possible cause. Genes

that encode special proteins can be switched on and off under the influence of the environment in the intestinal microbiota. Dysbiosis can therefore lead to activation of genes that code for diseases (Phillips, 2008).

It is believed that the development of diseases such as Canine Atopic dermatitis (CAD) and Canine Chronic inflammatory enteropathy (CE) in dogs, is strongly linked to a dysbiotic condition in the intestine (Craig, 2016; Jergens & Simpson, 2012). In humans, it has been described that a disorder of the intestinal mucosal barrier, which may be due to a dysbiotic condition in the intestine, appears to be involved in the pathogenesis of human AD (Majamaa & Isolauri, 1996).

Chronic enteropathy (CE) is a term used for gastrointestinal diseases that last for more than 3 weeks or longer, when other causes of intestinal disease are ruled out (Dandrieux & Mansfield, 2019). While CAD is a common skin disorder that is characterized by pruritus and secondary skin lesions typically affecting young dogs. It is associated with IgE antibodies, which are usually triggered by environmental allergens (Hensel et al., 2015).

The microbiota development in human infants

How the microbiome develops in infants is very important as it sets the ground for the composition of the microbiome in adulthood (as reviewed in Rodríguez et al., 2015). Development of disease later in life has been linked to abnormal gastrointestinal microbiota composition and colonization in early infancy (as reviewed in Collado et al., 2016). Several factors, such as mode of birth, gestational age, breastfeeding or formula feeding, administration of antibiotics and introduction to solid food, seem to influence the early establishment of the IM in infants (as reviewed in Francino, 2014).

The IM of neonates is particularly sensitive to changes during a "critical time window", due to high adaptability and plasticity of the microbiota (as reviewed in Sanidad & Zeng, 2020). A disruption and alteration of the IM during this "critical time window" can lead to diseases that appear later in life (as reviewed in Rodríguez et al., 2015; Sanidad & Zeng, 2020). Alteration and disruption of the IM early in life has been associated with functional alterations in various systems of the body resulting in diseases such as allergy, autoimmune disease and overweight etc. (as reviewed in Meropol & Edwards, 2015; Rodríguez et al., 2015).

It has been considered for more than a century that the intestinal microbiome in human neonates is acquired after birth, and that the fetal environment is sterile (as reviewed in Perez-Muñoz et al., 2017). This has been the traditional view of the uterine environment, and the expression "sterile womb paradigm" became a dogma. Novel research however has challenged this statement. Collado et al., (2016) supports this new theory "in utero colonization" by saying that the fetus is already in contact with microbes residing in the uterine environment during gestation, by findings of microbes from the placenta, amniotic fluid and meconium of 15 infants in their study. Perez-Muñoz et al., (2017) thinks otherwise, and argued in their review, that the milieu of the fetus is sterile and that an early colonization is not taking place, due to aspects of the placenta and the fetus physiology, anatomy and its immunological system that will act in protection from microbes.

The first microbes to colonize the intestine of newborns are facultative anaerobic bacteria which later on become strict anaerobic bacteria (as reviewed in Arboleya et al., 2016). Facultative anaerobic and aerobic bacteria are also found in the microbiota, but they are outnumbered by 100- to 1000-fold of anaerobic bacteria (as reviewed in Sommer & Bäckhed, 2013). The phylum Firmicutes seems to be the predominating phylum in the meconium of

human neonates (Moles et al., 2013), and as mentioned, birth method affects the intestinal microbiota. Babies born vaginally are exposed to the vaginal microbiota and a fecal-oral transmission is possible. Babies born by caesarean section (CS) are however more likely to become colonized with bacteria from their mother´s skin and oral cavity, and with bacteria found in the delivery room (as reviewed in Meropol & Edwards, 2015). Bacteria normally found in infants born vaginally are dominated by the genus *Lactobacillus, Prevotella* and *Sneathia*, while infants born by CS are dominated by the genus *Corynebacterium*, *Staphylococcus* and *Propionibacterium* spp. (Dominguez-Bello et al., 2010). A view of the most common genera, class and phyla from the study by Dominiguez-Bello et al., (2010), is listed in Table 1.

Table 1. Differences in faecal microbiota between vaginally born (VB) infants and infants born by caesarean section (CS) (Dominguez-Bello et al., 2010)

Delivery mode	**Phylum**	**Class**	**Genus**
CS	Firmicutes Actinobacteria	Bacilli Actinobacteria	*Staphylococcus* *Corynebacterium,* *Propionibacterium* spp.
VB	Firmicutes Bacteroidetes Fusobacteria	Bacilli Bacteroidia Fusobacteriia	*Lactobacillus* *Prevotella* *Sneathia*

Firmicutes along with the phylum Bacteroidetes, are by far the most common phyla in the IM seen in human adults (Eckburg, 2005; Fujio-Vejar et al., 2017). These are phyla found in the

IM of the infants as well, but in a lower abundance (Mariat et al., 2009). Other phyla that are also found in the IM are for example Proteobacteria, Actinobacteria and Fusobacteria (Eckburg, 2005; Fujio-Vejar et al., 2017).

Rautava et al., (2012) pointed out in their review that it is important to know how the microbiome develops in infants, and what factors that might influence it, since an abnormal IM colonization and composition can lead to diseases later in life. This is something that we believe is important in the canine species as well, but we are unfamiliar with the extent of information available, particularly regarding puppies.

Aim of study

The aim of this student thesis was to do a literature review on the development of the intestinal microbiota in dogs. Our focus will be on characterizing the development of the intestinal microbiota in puppies from birth to 12 weeks of age.

Material and methods

To perform our literature review we used two search-databases, Oria and PubMed. We established criteria and limitation-standards to be able to guide ourselves in the search, see Table 2. The literature search took place in September 2020.

Table 2. Standards for the literature search

Criteria and limitations	
Criteria for inclusion	Peer reviewed articles Language: English, Swedish, Norwegian Species: dog Age: puppies from birth to 12 weeks of age
Criteria for exclusion	Review articles Books
Database	Oria (www.oria.no) PubMed (www.pubmed.gov)
Keywords	See Table 3
Time period	Published in the period of 2010-2020

We started with some initial screening using different combinations of keywords. After that we ended up using two different combinations of keywords, shown in Table 3, and applied the search to both PubMed and Oria.

Table 3. Combinations of keywords

Keyword combination	**PubMed results (n)**	**Oria results (n)**
Canine OR dog AND pupp* AND development AND microbio* AND newborn OR juvenile OR young	n=20	n=1295
Puppies AND canine AND intestinal AND microbiota	n=5	n=416

The search in PubMed yielded very few papers compared to Oria. To reduce the number of results that came up in Oria, we followed our established search criteria and installed additional search limitations available in the database.

After identifying potential records in both databases, we systematically worked us through the pile as can be seen by the flowchart in Figure 1 below. We ended up with 6 papers that met our criteria for inclusion and exclusion.

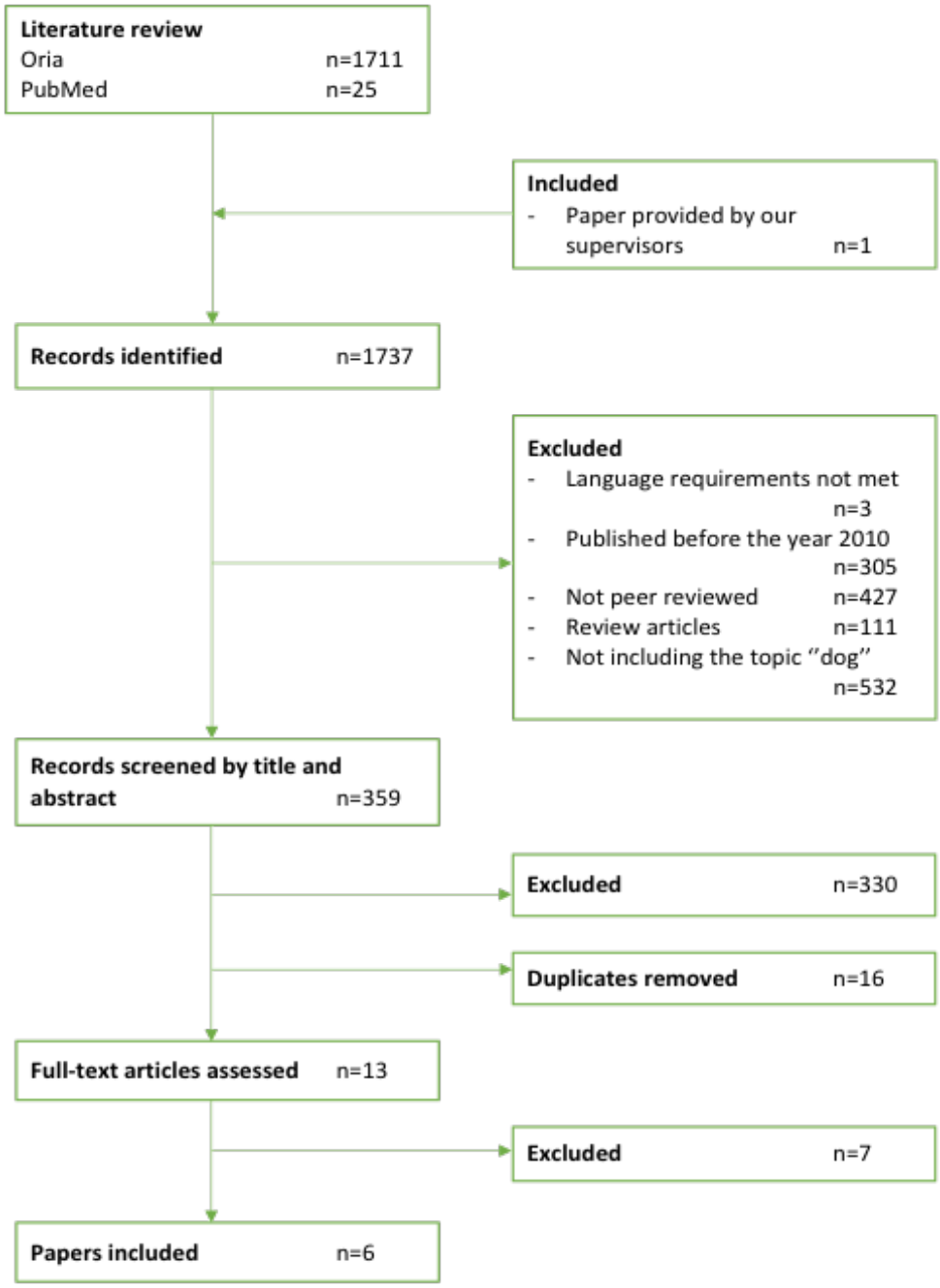


Figure 1. Literature review flowchart

Results

Paper 1. Transition of the intestinal microbiota of dogs with age (Masuoka et al., 2017)

Authors: Hiroaki Masuoka, Kouya Shimada, Tomoyo Kiyosue-Yasuda, Masaharu Kiyosue, Yukie Oishi, Seiji Kimura, Akio Yamad and Kazuhiro Hirayama

Background for the study

In humans, it is known that the intestinal microbiota changes with age. In animals, this is not as well studied. In this study, they tried to find out how the dog's intestinal microbiota changes with age.

Aim of study

The aim of the study was to analyze the composition of intestinal microbiota from different age groups of dogs.

Material and methods

The study population was composed of 50 dogs belonging to 5 different age groups (pre-weanling, weanling, young, aged, senile), see Table 4. In all age groups but one, the senile group, the dogs were of Beagle-breed, bred and maintained at Kitayama Labes Co., Ltd. (Nagano, Japan). The senile group was composed of mixed breeds kept in ordinary households. The pre-weanling group nursed from their mothers while weanling, young and aged dogs were reared individually and fed DS-E diet (Oriental Yeast Co., Ltd., Tokyo, Japan). Senile dogs were fed without any food restrictions.

Table 4. Remade from Table 1 in (Masuoka et al., 2017)

Group	Number of dogs in the group	Age
Pre-weanling	10	13,2 ±1,8 days
Weanling	10	6,8 ± 0,4 weeks
Young	10	2,0 ± 0,0 years
Aged	10	11,5 ± 0,9 years
Senile	10	16,7 ± 0,5 years

Fresh fecal samples were collected from all of the 50 dogs and culture-based methods were used to analyze the composition of the intestinal microbiota. The bacteria were identified at genus,- and family level based on colony form, gram staining, cell morphology and growth. Bacterial numbers were expressed as the $\log_{10}$ number of bacteria per gram wet weight of feces. Isolated colonies of Bifidobacterium and Lactobacilli were further identified by amplifying the 16S rRNA gene from the DNA extracted.

Results and conclusions/take home message

The number of *Enterobacteriaceae* was significantly lower in young and aged dogs compared with pre-weanlings, and significantly higher numbers of *Enterobacteriaceae* were detected in dogs of the senile group compared with those of the aged group. Compared with pre-weanling and weanling dogs, the number of *Bacteroidaceae* was significantly lower in young dogs. The highest number of eubacteria was found in feces of weanling dogs.

The family of bifidobacteria was detected in 50% of weanlings and 60% of pre-weanlings, it was however not detected in older dogs.

Staphylococci was not detected in any fecal samples from pre-weanlings and weanlings.

Isolated strains of lactobacilli were further identified by amplification of the 16S rRNA genes to delineate them at the species level. In the pre-weanling group, only the species of *Lactobacillus animalis* (detected in 70%) and *Lactobacillus johnsonii* (detected in 80%) were found. *L.johnsonii* was then only detected in 10% of the weaning and young group, but not in the senile group. In the weanlings *L.Reuteri* was found in 20% and *L.ruminis* was found in 50%. That means that the most widespread species of lactobacilli in the weanling were *L.ruminus.* In the young group *L.animalis* was found in 80%, *L.johnsonii and L.reuteri* in 10%.

In the aged group only *L.animalis* were detected. It was detected in 90% of the dogs. In the senil group bouth *L.gallinarum, L.paracasei subsp.paracasei* and *L.reuteri* were detected in 10 % of the dogs. The findings are listed in Table 5 below.

Table 5. Different strains of *Lactobacillus* spp. isolated between different age groups of dogs. The table was remade from Table 4 in (Masuoka et al., 2016)

Species	**Pre-weanling**	**Weanling**	**Young**	**Aged**	**Senile**
L. animalis	7	4	8	9	0
L. johnsonii	8	1	1	0	0
L. gallinarum	0	0	0	0	1
L. paracasei subsp. *paracasei*	0	0	0	0	1
L. reuteri	0	2	1	0	1
L. ruminis	0	5	0	0	0

The conclusion in this study is that composition of the canine intestinal microbiota changes over the dogs life span, from approximately 13 days of age to 17 years.

The study revealed that the bacterial families of *eubacteria, lactobacilli* and *enterococci,* together with the bacterial genera *Bacteroidaceae* and *Enterobacteriaceae* were found in most or all dogs belonging to the age groups pre-weanling and weanling.

The family of *clostridia* was found in all age groups apart from the group of pre-weanlings. Both the number and the prevalence of the family *lactobacilli* tended to decrease when dogs became older.

Paper 2. Disentangling factors that shape the gut microbiota in German Shepherd dogs (Vilson et al., 2018)

Authors: Åsa Vilson, Ziad Ramadan, Qinghong Li, Åke Hedhammar, Arleigh Reynolds, Julie Spears, Jeff Labuda, Robyn Pelker, Bengt Björkstén, Johan Dicksved and Helene Hansson-Hamlin

Background for the study

One sees an increase in allergy disorders in both humans and dogs. It is believed that one reason for this may be due to the fact that the immune system is not exposed to the same extent to bacteria as before. Exposure of different types of bacteria early in life is important for the maturation of the immune system and in humans. It is believed that exposure early in life to immunoregulatory bacteria that colonize the gastrointestinal tract is proposed to have lifelong consequences in humans.

This study wants to find out more about early gut colonization and immune function in dogs later in life, by giving a better understanding of the canine fecal microbiota in growing dogs

as well as in pregnant and lactating dams. They also want to try to reveal what impact different environmental factors have on the canine microbiota.

Aim of study

The aim of this study was to explore the development of the gut microbiota in German Shepherd dogs from 7 weeks to 18 months of age and furthermore, to study the effect of relatedness, maternal microbiota composition and living environment in these dogs. It was also assessed whether early probiotic supplementation to dams and puppies had an Immune stimulating effect against canine distemper virus (CAD).

Material and methods

The study included 30 dams and their litters, a total of 184 puppies from 7 weeks to 18 months of age. Fourteen dogs were excluded due to unrelated medical reasons and two dogs due to behaviour problems. Eleven of the 16 excluded puppies were excluded before 13 months of age. One litter (n = 2) in the La1-group and one litter (n = 2) in the placebo-group were delivered by caesarean section. There were too few dogs to do any separate analyses on this group. However, the four pups born by caesarean section were not outliers in the data.

The dams were treated with probiotics or placebo during the last trimester of pregnancy and until their puppies were 8 weeks old, the puppies received the same treatment as their mothers between 3–12 weeks of age. The dogs that were given probiotics were treated orally once daily with 0.55g (1010 CFU) powder (or poured on the food after 8 weeks of age). The number of active *L. johnsonii* was 1.9*1010CFU/g. The placebo group was given maltodextrin.

Samples from dams were collected at pregnancy day 42, at partum, and 4 & 7 weeks postpartum. The puppies samples were collected at the age of 4 & 7 weeks, 12–13 months and 15–18 months. Serum IgA, total serum IgE, fecal IgA and IgG antibody responses against canine distemper virus were analyzed by ELISA in order to detect any immune stimulating effects of the probiotic strain.

The dams lived with private families and arrived at the kennel at pregnancy day 37 or earlier. At the kennel, each dam and her litter had a separate room without any direct contact to other dogs. Upon arrival they were gradually introduced to their diet that was used throughout the study (Nestle´ Purina Pro Plan Puppy Sensitive Skin, Salmon & Rice Dry (32% protein, 20% fat, 1.2% omega 3). Twenty of the dams were imported, mainly from other European countries except one from the US, nine were born at the kennel and one at another Swedish kennel. Twenty-one sires were used. Four of the sires were imported, seven were from other Swedish kennels, and the rest were born at the SAF kennel. Mothers and puppies were restricted to the same diet during the entire study period. All dams and their litters were housed and reared with identical routines at the kennel. When the puppies were eight weeks old, they were moved from the kennel to live with private families.

Results and conclusions/take home message

Puppies during lactation

Lactobacillus was one of the genera that increased during lactation (in the probiotic as well as the placebo group). This genus was also higher in relative abundance in 7-week-old puppies (probiotic and placebo groups) compared to young adults (see Figure 2 below).

A difference was not detected in the number of lactobacilli in the fecal microbiota of puppies between the probiotic-treated group and the placebo group.

Compared to unrelated dogs the litters had a more similar fecal microbiota and 7 weeks old puppies were more similar to their mothers than to unrelated bitches at 7 weeks postpartum but not at partum.

Probiotic treatment did not increase *Lactobacillus* levels in the fecal microbiota in puppies. Lactobacilli increased during lactation. The puppies had more similar fecal microbiota to siblings and their mothers, than unrelated dogs.

Puppies from 7 weeks until 15-18 months of age.

The abundance of three families, *Clostridiaceae*, *Erysipelotrichaceae* (unidentified genus) and *Lachnospiraceae* increased from puppyhood to adulthood (15–18 months of age) whereas *Erysipelotrichaceae* (genus *Allobaculum*), *Lactobacillaceae* and *Bifidobacteriaceae* decreased from puppyhood to adulthood. *Erysipelotrichaceae* being the most abundant family at 7 weeks of age and *Clostridiaceae* at one year of age (see Figure 2 below).

The composition of the microbiota in puppies showed a clear age-related structure with a significant difference and diversity between 7 weeks old puppies and dogs at 15–18 months of age. The microbial diversity was affected by living areas where dogs living in big cities had higher diversity compared to dogs living in the countryside. This difference was not seen at 7 weeks when all the puppies lived at the kennel.

Dams

The bacterial community structure in the dams was stable from pregnancy day 42 to partum, but was shifted after whelping. During this period, *Erysipelotrichaceae* and *Lactobacillaceae* increased the most, while *Fusobacteriaceae* and *Clostridiaceae* decreased the most. The microbial diversity increased from pregnancy day 42 to 7 weeks postpartum in the dams. Firmicutes were in the bitches at all sampling points with relative abundances of 50–75%.

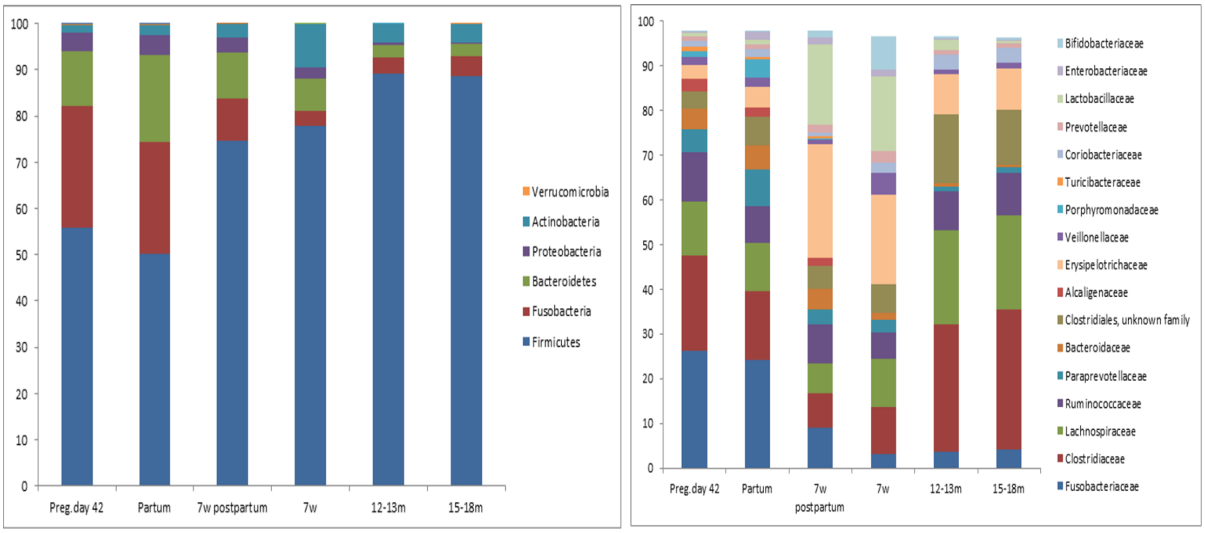


Figure 2. Relative abundance of bacteria phyla (figure a) and family (figure b) in feces from bitches at pregnancy day 42, partum and 7 weeks postpartum, and from puppies at 7 weeks, 12-13 months and 15-18 months of age. Families with relative abundance >1% are included.

The study showed that the bacterial community structure in the dams shifted after whelping and that the dams and the puppies at 7 weeks, showed the same predominant phyla (Firmicutes, Fusobacteria, Bacteroidetes). Probiotic treatment did not affect the levels of serum IgA, total serum IgE, fecal IgA in bitches. Firmicutes was the most dominant phylum at all ages, Actinobacteria was the second most dominant phylum at all ages.

Paper 3. Do newborn puppies have their own microbiota at birth? Influence of type of birth on newborn puppy microbiota (Zakošek Pipan et al., 2020)

Authors: Maja Zakošek Pipan, Leonida Kajdič, Anja Kalin, Tanja Plavec and Irena Zdovc

Background for the study

The hypothesis "sterile womb paradigm" is a dogma that now has been challenged by recent studies done on humans suggesting that an early colonization of intestinal microbiota in the fetus can be initiated already in the uterus. With modern sequencing technologies, human bacterial communities have been found in the placenta, amniotic fluid, meconium and umbilical blood in uncomplicated pregnancies. It has also been shown in humans, that mode of birth can influence the intestinal microbiota in newborn babies. Intestinal colonization of microbiota *in utero* and effect of mode of birth, has previously not been investigated in dogs.

Aim of study

To find out if presence of microbes could be found on the placenta or in the meconium of newborn puppies. To determine whether there was a difference in the microbiota between those born vaginally apart from those born by CS and see what effect the microbiota could have on the development of the puppy.

Material/methods

In this study a total of 96 puppies and 17 dams, who were all client owned, were sampled at different times during an 8-week period. Vaginal- and oral swabs were taken in half of the gestation time, and right before parturition. Swab samples from the placenta and meconium were taken directly after birth. All samples were taken with a sterile technique out in field

conditions. The puppies were also weighed every day in the first week and later on once a week for further 7 weeks. Samples were analysed with classical cultivation in anaerobe and aerobe conditions for 2-5 days in 37ºC.

Result and conclusions/take home message

The results in this study indicate that differences in birth method seems to influence the intestinal microbiota in the puppy, but also that bacteria were present on the placenta and in the meconium, suggesting presence of a microbiota early in life. It was observed that those born vaginally had an intestinal microbiota resembling the microbiota from the dam's vagina, and those born with CS had an intestinal microbiota that resembled the microbiota of the dam's vagina and oral microbiota.

Different bacterial genera were isolated from the meconium and the placenta sample in 86,5% and 57% respectively. The predominant bacteria found in the placenta, meconium, oral cavity and vagina were *Staphylococcus* spp. (phylum Firmicutes), *Streptococcus* spp. (phylum Firmicutes), *Actinomyces canis* (phylum Actinobacteria) and *Neisseria zoodegmatis* (phylum Proteobacteria). In Table 6, a view over the predominated bacteria isolated from placenta, meconium, oral cavity and vagina are shown. Regardless of birth method, the same predominant bacteria were isolated from the meconium, but a less diverse microbiota and more pathological bacteria could be found in those born by CS.

Table 6. Predominating bacteria isolated from placenta, meconium, oral cavity and vagina.

Bacteria	**Placenta**	**Meconium**	**Oral cavity**	**Vagina**
Staphylococcus spp.	x	x		x
Streptococcus spp.	x	x	x	x
Actinomyces canis			x	
Neisseria zoodegmatis	x	x	x	

Comparison between vaginally born puppies to those born by CS showed a statistically significant weight gain on day 2, 3 and 4. A statistically significant difference in weight gain was also noticed on day 2, 3 and 4 if a microbiome was found in the meconium or in the placenta.

The conclusion of this study was that the fetus is colonized with an intestinal microbiota during pregnancy, and that the placenta and meconium harbour different genera of bacteria. This challenges the dogma of the "sterile womb paradigm". Delivery mode and/or if a microbiome could be found in the placenta or meconium was also shown to be able to impact weight gain in puppies during the first days of life.

Paper 4. Characterization of **the fecal microbiome during neonatal and early pediatric development in puppies (Guard et al., 2017)**

Authors: Blake C. Guard, Hanna Mila, Jörg M. Steiner, Claire Mariani, Jan S. Suchodolski and Sylvie Chastant-Maillard.

Background for the study

Limited information about the development of intestinal microbiota in puppies is available, compared to studies done on human neonates, that are based on molecular techniques known as next-generation sequencing (NGS).

Aim of study

To characterize fecal microbiota in puppies when they were 2, 21, 42 and 56 days old with a technique known as 454-pyrosequencing where DNA is extracted from fecal samples to profile the 16S rRNA genes. The researchers also wanted to see if there was a difference in microbial communities between littermates, and if confounding factors like antibiotics or breed size could influence the intestinal microbiota.

Material/methods

A total of 30 puppies of different breed size participated in this study. They were all born vaginally and housed in a French breeding kennel. The study was a randomized two arm study, where the puppies acted as a control group. Collection of fecal samples were taken with rectal swabs when the puppies were 2, 21, 42 and 56 days of age, and one time in 24 hours after birth from 16 dams.

A food regime was set up for the dams, from one week before parturition until 2 months postpartum, where they were given a balanced dry food especially for growing dogs.

There was no food restriction for the dams, and no antibiotics were administered to them during the study. The puppies were given the opportunity to eat solid food when they were 3 weeks old but were still allowed to suckle freely during the 8-week test period.

Result and conclusions/take home message

During the test period, changes in both the composition and total amount of bacteria was observed in the puppies when they were 2 days of age compared to 56 days of age. The most distinctive changes were seen on phylum level where Firmicutes dominated at day 2 (64,3%), but shifted to a co-dominance between Bacteroidetes, Fusobacteria and Firmicutes at day 21 (37%, 16% and 26,11 respectively). These 3 phyla were also the most abundant phyla found in both the dams and the puppies by day 56. Differences in the fecal microbiota between the dams and the puppies 8-weeks after birth were still observed, but the differences were less obvious. No comparison of microbial communities between littermates could be done due to too few puppies in the same litter to get a satisfactory statistical power, and no changes in microbial communities could be seen between those puppies who had been administered antibiotics or not. Differences between small and large breeds were observed by day 42 when comparing microbial communities.

Observations in the study show that there is a clear shift in species richness and an increased microbial diversity between day 2 to 56 in fecal microbiota within the puppies. Also, that a stability in microbial communities was reached by day 42, and a major shift was more unlikely to be seen, but that a continuing diversification of the microbial communities could still be an ongoing process after day 56.

Paper 5. Evaluation of the fecal microbiota transfer as treatment for postweaning diarrhea in research-colony puppies (Burton et al., 2016)

Authors: Erin N. Burton, Erin O´Connor, Aaron C. Ericsson and Craig L. Franklin

Background for the study:

Temporary changes of the IM in puppies during the weaning period may cause the development of postweaning diarrhea. For puppies in research facilities, postweaning diarrhea can lead to reduced weight gain as well as increasing the risk of death.

Aim of study:

To investigate if the incidence of postweaning diarrhea in puppies could be decreased by performing oral faecal microbiota transfer (FMT) at the time of weaning.

Material/methods:

The study included 23 Dachshund-puppies from 7 litters, and their dams. The dogs lived under research settings and the experiment took time during the weaning period (at 6 to 8 weeks of age).

Puppies were randomly divided into two groups (FMT-treated or sham-treated). Eleven puppies were in the FMT-group and twelve in the sham-treated group. The puppies in the FMT-group were given faecal material prepared from their mothers feces. The treatment was provided daily for 5 days, at the time of weaning. During the same time, the puppies in the sham-group received a placebo treatment with bovine milk. The puppies had *ad libitum* access to dog food (ProPlan Puppy Dry and Wet Formulas, Purina, St Louis, MO) during the entire period of the study.

Fresh feces was collected at four time-points from each puppy and dam: 3 days before weaning and then 3, 10 and 24 days after weaning. The faecal samples were scored with Nestlé Purina Fecal Scoring System, to detect and monitor diarrhea, and analysed with 16S rRNA amplicon sequencing.

Results and conclusions/take home message:

The faecal microbiota in the dams varied throughout the study, but the 4 most commonly identified Operational Taxonomic Units (OTU) were *Fusobacterium* spp*., Bacteroides* spp*., Faecalibacterium prausnitzii* and *Prevotella copri.*

The faecal inoculum was analysed with 16S rRNA sequencing, only samples from days 1, 3 and 5 yielded sufficient amounts of data for interpretation. The 4 most common OTU in the faecal inoculum were *Fusobacterium, Prevotella copri, Bacteroides* spp. and *Prevotella* spp.

The most frequently identified OTU (mean relative abundance) in the sham-treated litters were *Fusobacterium* sp. (30.8%), *Bacteroides* sp. (12.5%), *Anaerobiospirillum* sp. (10.7%) and *Sutterella* sp. (7.6%). In the litters receiving FMT treatment, the most frequently identified OTU were *Fusobacterium* sp. (27.2%), *Bacteroides* sp. (12.8%), *Anaerobiospirillum* sp. (7.9%) and *Prevotella copri* (7.7%).

Neither group of puppies mirrored the dams at any time point. Both groups showed variability in microbial, composition between samples taken at different time points, and between individuals. Alpha diversity was however not detected, in spite of great variability, meaning that no difference in microbial diversity was between the two groups of puppies.

Although the puppy's faecal microbiota composition did not evolve into a composition more similar to the dams, *Prevotella copri* was among the 4 most commonly identified OTU in the FMT-treated puppies and in all of the dams. This indicates that the FMT was somewhat successful in the transfer of maternal faecal microbiota to puppies.

The study did not support the hypothesis that FMT treatment would speed up the transfer of a stable maternal microbiota to puppies and by that reduce the occurrence of postweaning diarrhea. The faecal inoculums used for the FMT treatments were analysed with 16S rRNA gene sequencing and their bacterial composition stayed consistent over the administration period. However, since genomic sequencing was used to assess the samples one could not say anything about the viability of the bacteria in the sample nor their capability to establish themselves in the intestine after oral administration.

The overall conclusion was that the faecal microbiota of puppies, at the time of weaning, had noticeable variations. Further studies are needed to better identify when the microbiota becomes stable in puppies after they have been weaned.

Paper 6. Intestinal microbial dysbiosis in beagles naturally infected with canine parvovirus (Park et al., 2019)

Authors: Jun Seok Park, Robin B. Guevarra, Bo-Ra Kim, Jun Hyung Lee, Sun Hee Lee, Jae Hyoung Cho, Hyeri Kim, Jin Ho Cho, Minho Song, Ju-Hoon Lee, Richard E. Isaacson, Kun Ho Song and Hyeun Bum Kim.

Background for the study:

Canine parvovirus (CPV) is a highly pathogenic virus in dogs. Infection with CPV can manifest itself as acute haemorrhagic enteritis with the following disruption of the intestinal barrier, or as myocarditis that can lead to abrupt death. The mortality rate is very high for young dogs infected with CPV (91%) while being much lower in adult dogs (10%). Dogs surviving CPV infection have been shown to have a greater risk of developing chronic gastrointestinal disease.

The effect that an infection with CPV has on the intestinal microbiota composition in dogs, has not been studied before with next-generation sequencing.

Aim of study:

To compare the composition of the intestinal microbiota in healthy puppies with the composition of the intestinal microbiota in CPV infected puppies.

Material/methods:

The study included two litters, composed of 4 Beagle-breed puppies each. One litter was naturally infected with CPV at the age of 6 weeks, while the other litter was healthy. Faecal samples were collected from the puppies using sterile faecal swabs at 4, 6, 8 and 12 weeks of

age. The faecal samples were analysed with 16S rRNA sequencing and then grouped into OTU with 97% similarity.

Results and conclusions/take home message:

At 4 weeks, all the puppies were healthy. The CPV-infected group started to show clinical signs in week 6 and the infection was then confirmed. Two CPV infected puppies died in week 8. The IM in all of the puppies, belonging to both groups, was composed of the following bacterial phyla: Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria. These five phyla accounted for approximately 99% of the total relative bacterial abundance. The microbiota however showed significantly different composition between the groups in week 6, based on unweighted UniFrac distance metrics.

At week 6, when clinical signs started, the CPV puppies displayed alpha diversity indices that were lower compared to the healthy puppies, and species richness was measured to be significantly higher in healthy puppies versus CPV puppies. There were also significant microbial differences between the groups. Puppies infected with CPV showed an increase in the phylum Proteobacteria and a decrease in the phyla Fusobacteria and Bacteroidetes.

Enterobacteriaceae, a bacterial family, was significantly more abundant in CPV infected puppies compared to healthy puppies. In CPV infected puppies the family accounted for 36,44% of the total bacterial population, while in healthy puppies it accounted for 0,21%. *Prevotellaceae* and *Lactobacillaceae* were more abundant in healthy puppies compared to CPV infected. The relative abundances of the genera *Prevotella* and *Lactobacillus* were significantly higher in healthy puppies compared to CPV infected puppies.

At 12 weeks of age, the proportion of Proteobacteria had decreased while Firmicutes and Actinobacteria increased in the CPV infected puppies. The microflora of the CPV infected puppies transformed back to a composition that was more similar to the microflora in the healthy puppies. In healthy puppies, Proteobacteria and Fusobacteria decreased with age while Firmicutes and Actinobacteria increased.

The results suggest that CPV infection can explain the variation in microbial composition between the two groups in week 6. Dysbiosis in the intestinal microbiota was linked to CPV infection. Statistical power of the study was limited by a small sample size.
Further studies are needed to evaluate the interaction of the microbiota with CPV.

Summarized results

The papers presented above are summarized in Tables 7 & 8, and Figure 3.
Table 7 provides a brief overview of the study populations, sample types, age for sampling, method for analysis and results. Table 8 provides information about handling and storing of faecal samples from four of the included papers that used genomic analysis as their main method of analysis. Figure 3 gives an overview of the origin of the dogs used in the studies showing that the dogs originated from America, Europe and Asia.

Table 7. Brief summary of the papers included

Paper 1. Transition of the intestinal microbiota of dogs with age (Masuoka et al., 2017)				
Study population	*Sample type*	*Age for sampling*	*Method for analysis*	*Results/Conclusions*
Total n=50 Pre-weanling n=10 Weanling n=10 Young n=10 Aged n=10 Senile n=10 Breed: Beagles (pre-weanling, weanling, young and aged) Mixed (senile group)	Faecal samples collected after defecation Kept under anaerobic conditions, refrigerated (temperature not mentioned)	Pre-weanling (13,2± 1,8) days Weanling (6,8±0,4) weeks Young (2,0±0,0) years Aged (11,5±0,9) years Senile (16,7±0,5) years	Cultivation under anaerobic and aerobic conditions Special species identification of bifidobacterial and lactobacilli with 16S rDNA amplicon sequencing	• The intestinal microbiota of dogs undergoes age-dependent changes at the levels of both bacterial groups and species. • Most of the classified bacteria belong to the phyla Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria, • Both the number and the prevalence of lactobacilli tended to decrease when dogs became older. • *Lactobacillus johnsonii* was mostly isolated from pre-weanling dogs, while *L. animalis* strains were isolated from dogs of almost all age groups except the senile group. • Bifidobacteria were only found in pre-weanling and weanlings, not in older age groups.
Paper 2. Disentangling factors that shape the gut microbiota in German Shepherd dogs (Vilson et al., 2018)				
Study population	*Sample type*	*Age for sampling*	*Method for analysis*	*Results/Conclusions*
Total n=214 Puppies n=184 Dams n=30 Breed: German Shepherd	Faecal samples taken with rectal swabs. Frozen at -80°C within 48 hours. Between collection and freezing at -80°C, the samples were stored on dry ice	Puppies: 7 weeks 12-13 months 15-18 months Dams: Gestation day 42 At partum 4- & 7-weeks post-partum	16S rRNA amplicon sequencing	• Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria were the predominant phyla in feces in puppies, as well as in pregnant and lactating bitches. • *Lactobacillus* increased during lactation (in the probiotic-group as well as in the placebo-group). • Significant changes were in the relative abundance of different fecal bacteria during lactation (from partum to 7 weeks postpartum), but not during the last trimester of pregnancy. • Litter mates had a more similar fecal microbiota

	or in -25˚C freezer			profile compared to unrelated dogs, especially at 7 weeks of age. • The composition of the fecal microbiota in bitches was more similar to the microbiota of puppies at 7 weeks postpartum than at partum. • The living environment affected the fecal microbiota. • Pre- and postnatal treatment with the probiotic *Lactobacillus johnsonii NCC533 (La1)* did not alter the composition of the fecal microbiota or diversity in either puppies or bitches.
Paper 3. Do newborn puppies have their own microbiota at birth? Influence of type of birth on newborn puppy microbiota (Zakošek Pipan et al., 2020)				
Study population	*Sample type*	*Age for sampling*	*Method for analysis*	*Results/Conclusions*
Total n=113 Puppies n=96 Dams n=17 Breed: Mixed	Meconium samples taken with sterile cotton swabs and immediately inoculated onto nutrient agar plates.	Collection immediately after birth	Cultivation under aerobe and anaerobe conditions	• Birth method seems to influence the GI microbiota in the puppies. • Early colonization of the GI tract is believed to take place. • GI microbiota in VB puppies resembles the dam's vaginal microbiota, and puppies born by CS have an GI microbiota resembling the dam's oral- and vaginal microbiota. • Predominant bacteria found from the placenta, meconium, oral cavity and vagina were *Staphylococcus spp., Streptococcus spp., Actinomyces canis* and *Neisseria zoodegmatis* • Weight gain was higher in puppies who were VB, and if a microbiome was to be found.
Paper 4. Characterization of the fecal microbiome during neonatal and early pediatric development in puppies (Guard et al., 2017)				
Study population	*Sample type*	*Age for sampling*	*Method for analysis*	*Results/Conclusions*

Total n=46 Puppies n=30 Dams n=16 Breed: Mixed	Faecal samples taken with rectal swabs. Stored immediately at -80°C until further processing	Puppies age: 2 days 21 days 42 days 56 days	16S rRNA amplicon sequencing	• Total amount of bacteria, and species richness, increased from day 2 to 56. • A shift at phylum level was noticed between day 2 and 56. • Puppies were getting more similar to the dam's IM by day 56. • Too few puppies to be able to compare microbial communities between littermates. • Differences in microbial communities between small and large breeds were noted. • Administration of antibiotics showed no difference in microbial communities compared to puppies who weren't administered antibiotics.
Paper 5. Evaluation of Fecal Microbiota Transfer as Treatment for Postweaning Diarrhea in Research-Colony Puppies (Burton et al., 2016)				
Study population	*Sample type*	*Age for sampling*	*Method for analysis*	*Results/Conclusions*
Total n=30 FMT treated puppies n=11 Sham-treated puppies n=12 Dams n=7 Breed: Dachshund	2g fresh faeces obtained with faecal loop from rectum. Stored at -80°C until further processing.	Puppies were weaned at the age of 6-8 weeks. Samples were taken: 3 days prior to weaning, and 3, 10 and 24 days postweaning. Puppies age: 39-80 days	16S rRNA amplicon sequencing	• Interindividual variability post-weaning • Both group of puppies dissimilar to their mothers • Most abundant phyla: Fusobacteria, Bacteroidetes and Proteobacteria

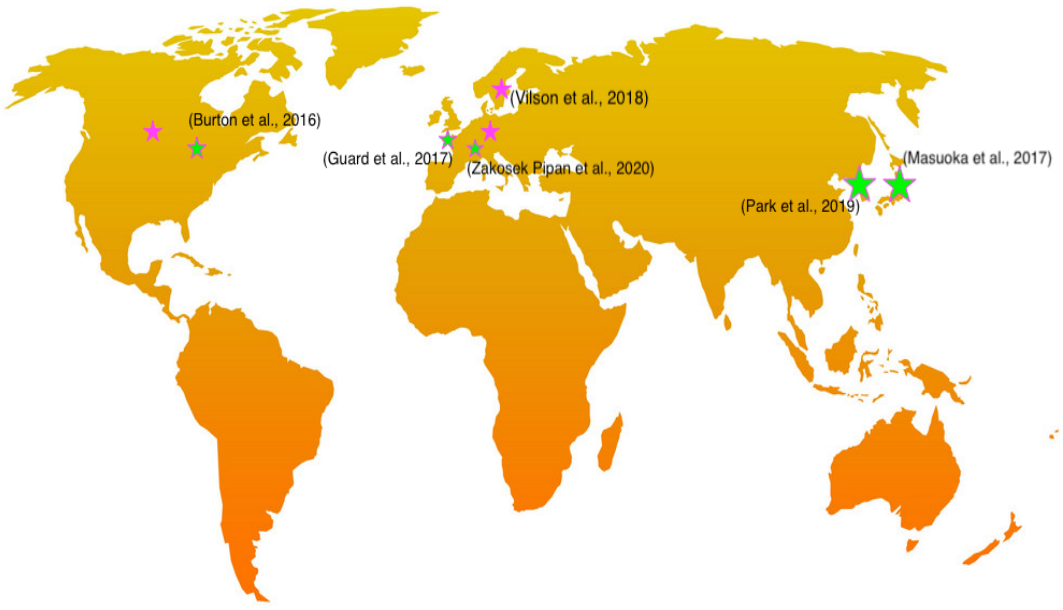
Paper 6. Intestinal Microbial Dysbiosis in Beagles Naturally Infected with Canine Parvovirus (Park et al., 2019)				
Study population	*Sample type*	*Age for sampling*	*Method for analysis*	*Results/Conclusions*

Total n=8
CPV infected puppies n=4
Healthy puppies n=4
 	
Breed: Beagle
	Sterile faecal swabs from rectum.
	
	Stored at -80°C until further processing
		4 weeks
		6 weeks
		8 weeks
		12 weeks
			16S rRNA amplicon sequencing	• ≈ 99% of bacteria belonged to phyla: Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria.
				• Shift in relative abundance of bacterial phyla in healthy puppies with increased age: Firmicutes, Actinobacteria ↑, Fusobacteria↓, Proteobacteria↓.
				• Shifts in relative abundance of bacterial phyla in CPV-infected puppies:
				  • Newly infected: Proteobacteria↑, Firmicutes↓, Actinobacteria↓, Bacteroidetes↓
				  • During recovery: Proteobacteria↓, Firmicutes↑, Actinobacteria↑
				• Significant difference between groups at 6 weeks of age: *Enterobacteriaceae*: 36,44% of the total bacterial in CPV-puppies and 0,21% in healthy puppies.
				• *Prevotella* and *Lactobacillus* more abundant in healthy puppies
				• CPV-survivors obtained similar microbiota to healthy puppies at 12 weeks of age

Table 8. Storage methods of faecal samples before analysis with 16S rRNA pyrosequencing. Four papers out of six are included in the table, see respective reference.

	(Guard et al., 2017)	**(Vilson et al., 2018)**	**(Park et al., 2019)**	**(Burton et al., 2016)**
Use of storing medium	No information	No information	No information	Yes. Type not mentioned.
Storing before deep-freezing (time and temperature)	Frozen immediately after collection	48 hours Stored either on dry ice or in a -25°C freezer	No information	Frozen immediately after processing
Storing temperature	-80 °C	-80°C	-80°C	-80°C
Time from freezing to analysis	No information	No information	No information	No information
Sample handling discussed as a potential bias by authors?	No	No	No	No

Figure 3. A world map showing where the dogs used in the different articles came from. Pink stars: dogs from the study made by Vilson et al., (2018) came from different places.



Discussion

Different aspects characterizing the development of the juvenile canine microbiota

Birth method

In the study done by Zakošek Pipan et al., (2020), differences in the intestinal microbiota were seen when comparing canine puppies born vaginally to those born by CS. In the same study, different bacteria genera were found in 57% of placental samples and in 86,5% of meconial samples. This suggests that the intestinal tract of the fetus is already colonized with microbes in the uterus.

In a review done by Perez-Muñoz et al., (2017) they pondered the two hypotheses “sterile womb paradigm” and “in utero colonization” against each other and concluded that the scientific evidence today is too scarce to support an early colonization.

Many human studies have shown that the mode of delivery has an impact on the bacterial microbiota, but how the transmission occurs is not fully understood (as reviewed in Meropol & Edwards, 2015). In a study done by Dominiguez-Bello et al., (2010), differences were noted when studying bacterial colonization profile in infants < 5 min of age, when comparing between babies born vaginally and babies born by CS. Babies born vaginally had an intestinal microbiota with bacterial species like *Lactobacillus, Prevotella* and *Sneathia,* which resembles the microbiota found in the mother's vagina, while those babies born by CS resembles the microbiota of the mothers’ skin with *Staphylococcus, Corynebacterium* and *Propionibacterium*.

Microbes have been detected in the meconium, which supports intestinal colonization in the uterus, but microbes in the meconium could also be a happening from a postnatal colonization (Hansen et al., 2015). Something that must be taken into consideration to the findings of microbes in the meconium, or from the placenta, is that modern DNA-based PCR and sequencing can detect microbes that don't have to be viable or cultivable. A weakness when using classical cultivation is that it fails to detect viable microbes that are not cultivable (as reviewed in Perez- Muñoz et al., 2017). Most of the studies that established the "sterile womb paradigm" are based on classical cultivation, but perhaps the best way is to combine both techniques to see if the "sterile womb paradigm" still stands strong, or if there really is a possibility that an early colonization can take place already in the uterus.

A wondering question to the study done by Zakošek Pipan et al., (2020) is why they could isolate bacteria from some of the placenta (57%) and meconium samples (86,5%), but not from all of the samples they took? Could it be due to that samples from the placenta were being taken from the wrong place or could it be because they used classical cultivation as an analytical technique? Human studies suggest that microbes are located at a specific area on the placenta called the "maternal basal plate" (Stout et al., 2013), and perhaps this is where samples should be taken. Zakošek Pipan et al., (2020) didn´t strive to take samples from this area and this could be a reason for why bacteria weren't isolated in some of the placental samples. Bacteria could be isolated from many samples of meconium and differences were noticed between puppies born vaginally compared to those born by CS in the study by Zakošek Pipan et al., (2020). But could microbes isolated from the meconium be from that the samples were taken after the puppies had been fed with colostrum, or were they there from the start, or is it because of contamination? A good start trying to answer this would be that

further studies used a form of standard procedure, when sampling meconium, or the placenta, to be able to compare results with each other.

The study done by Zakošek Pipan et al., (2020) is one of the first studies that have isolated bacteria from the meconium of puppies, which is something that we must build further on to get a better understanding on how the colonization of the microbiota begins, transfers and develops.

Could the rearing environment have an important role in the IM development?

Climatic conditions, geographic localisation, housing facilities and type of outdoor environment make up the rearing environment, the environment the puppies grow up in. None of our included studies were performed in the same country (see Figure 3) giving rise to both climatic and geographical variations between them. Considering housing facilities there were 3 studies performed in research settings, 2 in kennels and 1 used client-owned dogs living with their owners. This means that among the different studies the puppies were exposed to different rearing environments. Puppies being raised in a research setting are not exposed to the same environmental stimuli as puppies in a breeding kennel or typical privately-owned puppies. Puppies raised in Sweden are not exposed to the same climatic conditions as puppies raised in Japan. The studies therefore do not represent the typical family-owned puppy living in Norway. But could this have any special significance for the development of the puppies IM and overall health, being raised in different environments?

In people of the Western world, allergic diseases have become more common. The reasons for this have been puzzling for scientists but one explanatory hypothesis that has gotten much attention is the “hygiene-hypothesis”. According to that hypothesis early life exposure to microorganisms will minimize the risk of developing immune-mediated disease, such as atopy, later in life (Bloomfield et al., 2006). The hypothesis does match with the change in

our ways of living, a lot of people in the Western world nowadays spend the majority of their time inside and rarely go out and "get grubby". The IM diversity is decreasing in those people compared to other parts of the world (as reviewed by Tasnim et al., 2017). The diversity of the IM seems to play a role in the development of allergic disease, low diversity during the first year of life has for example been linked to the development of allergic rhinitis and allergic sensitization in children (Bisgaard et al., 2011).

Canine studies have not investigated the possible association between the rearing environment and the IM development in young puppies to our best knowledge, e.g., if being raised on a farm leads to higher IM diversity in puppies compared to those raised in the city. It may well be that the rearing environment is not so important for the IM development in puppies. One study found for example no association between the birth environment of puppies (breeders' home) and development of allergy in dogs. The same study found that if the current environment (owners' home) was in urban settings the dogs were more prone to developing allergy compared to dogs living in rural settings (Hakanen et al., 2018). In one of our included studies the diversity was significantly affected by the living environment (countryside, small cities, large cities) during early adulthood. Dogs that lived in large cities during the first 1,5 years of their lives had greater diversity compared to dogs living in small cities or in the countryside. This difference was however not observed in the same puppies while they were still with their mothers (Vilson et al., 2018).

Looking at the humane literature again, only one human study to our best knowledge has specifically addressed the impact of the environment on the IM development in infants. This study found that contact with nature and pets were factors that could alter the IM. Surprisingly maybe, the diversity of the IM in infants exposed to any natural environment, and particularly among infants that were formula-fed and were in contact with pets, was reduced. The authors

discussed that influence from the natural environment on infant IM could be attenuated by a stronger influence of breastfeeding, explaining this reduced diversity (Nielsen et al., 2020).

It is possible that the rearing environment is less important for the development of the IM in puppies and overall health later in life, compared to the environment at their permanent homes. Puppies are usually brought to new homes at the age of 8 weeks, before the IM has fully taken its adult form. Then again, these are only speculations. Further studies are needed that specifically assess the role of the rearing environment during the first weeks of the puppyhood and how it affects the IM composition. Additionally, further Scandinavian studies on the IM in puppies are needed as these would be better applicable for the dog population in this region of the world.

Similarities of the intestinal microbiota between puppies and their mothers

Vilson et al., (2018) highlight that whom the puppies interact with has an influence on the development of the IM. Their study revealed that puppies living in the same litter developed a more similar IM compared to puppies outside of the litter, showing a litter effect. The relationship of the mothers IM to its puppies IM was also explored. The IM of the dams in the study underwent changes during the time after whelping until 7 weeks postpartum. Puppies were first sampled at 7 weeks of age, so no comparison between younger puppies with their mothers was possible to make in the study. When comparing the IM of 7 weeks old puppies to the IM of the dams at partum the puppies IM was dissimilar to both the IM of their mothers and unrelated dams (i.e., not more similar to their mothers). However, when compared to the IM of the dams at 7 weeks postpartum, the puppies were significantly more similar to their mothers IM compared to unrelated dams (Vilson et al., 2018).

In human infants we don't find this similarity of intestinal microbial communities between mother and offspring as early or distinct as we do in dogs. Six-month-old infants appear to have their own uniquely composed IM that is dissimilar to the different microbiota of their mothers, including the intestinal, vaginal, oral, skin and milk microbiota (Drell et al., 2017). At the age of 4 years, the IM in children seems to obtain more similarities with their mothers IM. However, the children's IM at that age are not found to be more similar to their mothers IM compared to unrelated mothers IM (Koren et al., 2012).

Guard et al., (2017) also compared mothers IM to their puppies IM. The puppies IM was found dissimilar to their mother IM at every time point investigated (2, 21, 42 and 56 days). However, the puppies IM trended towards their mothers IM and the dissimilarities were the least at day 56, although still significantly different. This is in harmony with Vilson et al., (2018) that could not find similarities between the IM of puppies and the IM of their mother at partum. The mothers in Guard et al., (2017) were only sampled once, at partum. It would have been beneficial for the interpretation of the result if the mothers had been sampled again in the postpartum period. That would possibly have shown more similarities between the puppies IM and their mothers IM, like was shown in Vilson et al., (2018).

Why does the puppies IM not resemble the mothers IM until the puppies are around 7-8 weeks old and why is the IM in human infants not as distinctly similar to their mothers IM as is seen in canines? When puppies start eating solid food, the IM shifts into a more adult like IM. Increased diversity of phyla in the IM is seen as the puppies age (Guard et al., 2017) and is considered important for the more complex digestion of solid food compared to milk digestion. Human children start eating solid food later than puppies do, this may be one reason for why we do not see similarities between the mothers IM and the child's IM until the

child is 4 years old. The similarities are however not as pronounced as in dogs. We know that the way puppies and the dams live together, and the way the dams nurse their puppies, are different than in humans. The dams groom their puppies by licking them. In this way they ingest the puppy's feces, and puppies are more prone to ingest intestinal content from the mother and siblings, than human children are.

Burton et al., (2016) could not find similarities between the IM of puppies and their mothers at the time of weaning (6-8 weeks) or in the next 3 weeks following the weaning. In this study the puppies and their mother were placed on raised pens with a plastic slatted floor system, and feces were collected every morning. This clean environment in which the puppies and mothers were placed, may have meant that exchange between the puppies and the mother's faecal bacteria was not as great compared with puppies and mothers who live in a more normal environment. Perhaps this may indicate that the environment is of great importance for the intestinal microbiota to develop to become more similar between mother and offspring, something we see to a greater extent in dogs than in humans. However, this lastly mentioned idea is in a conflict with the result from the study itself. Half of the puppies in the study received faecal material transfer (FMT) prepared from their mothers feces and none of those puppies obtained a more similar microbiota to their mothers. Possibly the FMT was unsuccessful in altering the IM in the study due to practical reasons. Using the oral route to administer the faecal material can have altered its composition before it got to the intestine. It is also possible that the faecal inoculum had few viable bacteria, or that they were unable to establish themselves in the intestine. It is also possible that the IM already established in the intestine of the puppies outcompeted the flora found in the faecal material inoculum.

This might point out that the driving factor shifting the IM in puppies to a more adult like IM and more similar to their mothers IM, is the introduction of solid food. If the driving factor of the IM development is what the puppies ingest, it is reasonable to think that the dam’s milk

microbiota instead of its IM, steers the early development of the IM in puppies. Cultivation and following identification with 16s rRNA sequencing has shown that canine milk contains *Lactobacillus* spp. rebutting the idea that the milk is sterile (Martín et al., 2010). Sampling the dam's milk and comparing its microbiota to the puppies IM during the first weeks of life would be interesting to investigate and has not yet been done to our knowledge.

The role of *Lactobacillus* and *Bifidobacterium* in the intestinal microbiota of puppies

It is not exactly known what kind of bacteria that are needed for proper development of the microbiota in puppies. In humans, members of *Lactobacillus* and *Bifidobacterium* are considered important for the development of the microbiota in the gastrointestinal tract. These genera start to develop soon after birth, where they are believed to protect the host against development of various diseases (Bezirtzoglou & Stavropoulou, 2011).

In human infants it is believed that the colonization of *Bifidobacterium* has positive effects on the infant's vitality and development (Bezirtzoglou & Stavropoulou, 2011). The levels of the bacteria decrease with age, but remain relatively stable, and are considered an important part of the healthy microbiota also in adulthood (Arboleya et al., 2016). Masuoka et.al., (2016) found no evidence that *Bifidobacterium* were dominant in the dog’s intestine, especially not in older age groups. They question if this bacterium does play as big of a role in a dog's microbiota as it does in the microbiota of humans. But based on such a narrow research sample, it is hard to say whether this can apply to the remaining dog population.

Lactobacilli on the other hand seems to make up a larger part of the microbiota composition, especially in puppies. Vilson et al., (2018) detected that *Lactobacilli* increased during the lactation and that this genus was higher in 7 weeks old puppies compared to young dogs (2

years old). They also revealed that the bacterial community structure in the dams was stable from pregnancy day 42 to partum but shifted after whelping. During this period, *Erysipelotrichaceae* and *Lactobacillaceae* increased the most, while *Fusobacteriaceae* and *Clostridiaceae* decreased the most. The microbial diversity increased from pregnancy day 42 to 7 weeks postpartum in the dam.

It is known whether breast milk from dogs contains different strains of lactobacilli (Martín et al., 2010). In humans, the mother's microbiota has been shown to be transmitted to the newborn through the breast milk. Through the lymphatic system and peripheral blood, cells are transferred from intestinal lymphoid tissue to the mammary glands (Donnet-Hughes et al., 2010). This may indicate that breast milk leads to an increased establishment of lactobacilli in infants. In a review done by Zhuang et al., (2019) it was stated that infants that have been exclusively breastfed, are shown to have more *Lactobacillus* and *Bifidobacteria* in their stools compared to those infants who are given milk replacements. In human breast milk, “human milk oligosaccharides” (HMOs) are found and these are considered to be natural prebiotics by promoting growth of different microbes like *Bifidobacteria* in the intestinal microbiota of the infants (Zhuang et al., 2019).

Masuoka et.al., (2016) found that there was one specific strain of *Lactobacillus*, *L. johnsonii*, that seemed to be particularly prevalent in puppies until weaning. However, in the other age-groups of dogs, several different strains of lactobacilli, were detected. Also, in humans studies, intestine in infants is dominated by only one *Lactobacillus* strain, while in adults, the the intestine consists of diverse strains of *Lactobacillus*. The total change in the lactobacillus populations, both in diversity and complexity, changes from early life to adulthood (Wall et al., 2007). Several studies in humans also show that *Lactobacillus*

decrease with age. It is believed that this reduction, together with other changes in the composition of the bacterium in the gut, provides a greater opportunity for the development of diseases and infection in older people (Patel et al., 2014).

Establishment of *Lactobacillus* are in general recognized for engaging the intestinal barrier, and it is believed that this strain has an important role in development of a healthy immune system (Bezirtzoglou & Stavropoulou, 2011). In a study done by Inoue et al., (2007) it is shown that by giving oral probiotic with *Lactobacillus johnsonii* to weaning mice in a specific part of the weaning period, it was effective in preventing or inhibiting the development of atopic dermatitis. By giving *L. johnsonii* they stimulated and modulated the mice's immune system via the gut immune system.

As puppies until weaning appear to have an increased incidence of *L. johnsonii* in the microbiota, this may contribute to provide increased resistance to developing various diseases throughout life. This is due to the way *Lactobacillus* help to mature the puppies' immune system. It is possible that breast milk is an important contributor to the establishment of beneficial bacteria in the microbiota. Masuoka et.al., (2016) found that older dogs have particularly low levels of *Bifidobacterium* in their intestinal microbiota, and this may be cause the minimal levels they transfer to their litters during the puppy-period. The bitches will thus not be able to transmit as large amounts of these bacteria via milk as humans may be able to.

Limitations to the results

Study populations, limitations with small-group sizes in veterinary studies?

Our literature search made it apparent that small study populations can be a drawback in canine studies. Our articles have study populations ranging from a total of 8 to 214 dogs. Only

two articles have moderately large study populations (Vilson et al., 2018; Zakošek Pipan et al., 2020) while the rest have small study populations.

Many human studies aiming to characterize the microbiota in infants and adults are based on large study populations, often including hundreds of participants and thousands of samples. No two individuals have the exact same composition of microbiota, the natural variation is large. Since the natural variation is large, random errors will increase and the reliability for the results becomes low. To counteract low reliability, larger study populations are used to minimize these random errors and increase the reliability. When designing human metagenomic studies, it is recommended to collect pilot data and perform statistical calculations to get an idea about how many samples are required in the main study (Quince et al., 2017). When the aim is to investigate the potential significance, and the extent of it, one must look into the statistical power of the study. The definition of statistical power is the possibility that a study is going to show an expected result (Pettersson et al., 2013). If the power of a study is too low, we can miss finding an existing significant difference, so before performing a study it is important to find the necessary power (Donovan, 2016). In metagenomic studies the statistical power is affected by factors such as effect size and number of samples. Usually, acceptable power is 80% or higher (Odintsova et al., 2017). Effect size tells us about the size of a difference. If the effect size is large for a particular factor, a smaller number of samples are needed to get a significant results (Donovan, 2016).

Four out of six of the included studies discuss that their study populations were too small, and larger studies were called for (Burton et al., 2016; Guard et al., 2017; Park et al., 2019; Vilson et al., 2018). Finding the right size of study population and number of samples collected is challenging in metagenomic analysis. Since the study populations used in the different studies were found to be too small, it is possible to think of them as pilot studies. Pilot studies are

important, as has been stated. Exactly what the aim of the study is and definition of the target population, sets the cornerstone for estimating the size of the study population needed to get significant results.

After one has found the right size of the study population, other hindrances emerge. There are many reasons for why canine studies are mostly performed on small study populations, compared to human studies. If the idea is to research the microbiota development in puppies under controlled settings some important ethical problems arise. Where should those puppies come from, are they to be bred for the sole purpose of mapping their microbiota development and what happens to them afterwards? Would it be acceptable to house many puppies in refined settings, and how would the human-dog relationships be built if those dogs were to go to different families later on? Other factors to think of include the costliness of such studies along with the question of if the results would still be valid for the target populations: canine puppies.

Then there is the possibility to get breeders on board and collect samples from puppies living in many different places. The samples collected, if in large enough amounts, would possibly be better valid for the general population. The problems with this kind of study is the difficulty of finding breeders and the time-consuming job of collecting the samples and keeping the breeders on board during the whole sampling period. There is also the risk for systematic errors during sampling as it can be challenging to take samples from small puppies.

This dilemma is not so problematic in human studies. There is of course no discussion about keeping babies in research facilities, it is either health workers or the parents that collect the samples. Sampling infants is easier than sampling puppies as babies are much larger. There is also usually just one baby that is sampled each time, compared to maybe a litter of 6 puppies.

Sample materials

In the studies we included, faecal samples (meconium sample in one study) were the only sample type used. This is in harmony with other studies on the canine microbiota, most of them are based on faecal samples. Faecal samples are easy and rather inexpensive to collect and do not pose an ethical dilemma (Alessandri et al., 2020) as sampling of intestinal content can bring where subjects may have to be euthanized before sampling (Suchodolski et al., 2008).

But although faecal samples are easy to obtain and have the potential of giving representative results, they can also provide incorrect and misleading results. In a disfavour to IM studies in dogs, there is a lack of general instructions about the most appropriate collection and storing of faecal samples for the species. In order to gain sample integrity and yield reproducible data, appropriate sample handling and storing are undoubtedly important (Lin et al., 2020).

The storage methods of four of the included papers using 16S rRNA pyrosequencing, are summarized in Table 8. Only one of those studies mentions the use of lysis buffer for storing the faecal samples but fails to mention what type of buffer (e.g., ethanol or RNALater). The other 3 studies do not provide information about the use of storing medium, making it impossible for the reader to know if such medium was used or not. Information about handling before deep-freezing is provided in 3 papers, and all of the papers provide information about the storage temperature, being -80°C. No information about the time length from deep-freezing to analysis is provided. In the discussion section of each paper, the storage methods are not discussed as a potential source for bias in the results, that is unfortunate in our opinion.

Why this information is lacking in the different papers is unknown to us. It has been shown that methods for collection and storing of faecal samples can greatly affect the results of

genome sequencing analysis. The use of buffers (e.g. ethanol, RNALater or glycerol:PBS) or no buffer, temperature and storage time can alter the composition and diversity, affecting the results (Horng et al., 2018). We do therefore expect that the storage methods of the different studies have led to some bias in the results, but we cannot state the extent of this. Our conclusion is that sample handling is a possible source for bias in metagenomic studies and information about the handling and storing should always be included in the studies description.

Cultivation or genomic sequencing?

Zakošek Pipan et al., (2020) and Masuoka et al., (2017) used classical bacterial cultivation as an analytical technique. Zakošek Pipan et al., (2020) pointed out that this could be a weakness in their study due to the fact that important bacteria could have been missed.

Burton et al., (2016), Vilson et al., (2018), Guard et al., (2017) and Park et al., (2019) used the molecular technique 16S rRNA amplicon sequencing, Masuoka et al., (2017) used 16S rDNA amplicon sequencing for identification of *Bifidobacterium* and *Lactobacillus*. Vilson et al., (2018) reported that they used low sequencing depth and considered this a limitation to their study, the rest of the studies didn't point out other limitations in regard to the analytical method used.

Culturing bacteria from the intestinal tract for analytical purposes is not always the best available method. Some have even concluded that traditional cultivation of bacteria is not suitable to present the true bacterial diversity in faecal samples (Greetham et al., 2002). Preservation of the sample material is indeed especially challenging due to the fact that the vast majority of bacteria in the intestines are anaerobes.

Anaerobic bacteria are extremely sensitive to oxygen exposure which makes it difficult to keep them alive and in cultivable condition after sample collection. The enormous amount of

different bacteria species also offers challenges in finding the right growth medium and growth circumstances (Bellali et al., 2019).

On the other hand, molecular methods have provided new information about the diversity and composition of the intestinal microbiome. Such methods (e.g. metagenomics) have made it possible to characterize the microbiome at a much more detailed level compared to cultivation methods (Lagier et al., 2018). Different molecular diagnostic techniques are available today, such as PCR and Next-Generation Sequencing (NGS) and DNA fingerprinting, having the advantages of being a rapid, cost efficient and sensitive techniques to use. One of the major pros when 16S rRNA gene is used with NGS technique is that the bacteria doesn't have to be culturable and the abundance of bacteria within the sample can be determined. The possibility to obtain hundreds of parallel sequencing in one day is also a great advantage to this technique (Gupta et al., 2019).

Molecular methods do however have their limitations. An adverse effect when 16S rRNA gene amplicon sequencing is used is that a bias introduction can be established during amplification. This is due to the fact that some regions in the bacterial genome, that the primers bind to, are not 100% preserved across all bacteria. Due to high similarities of the 16S rRNA gene between bacteria, the genus level is the lowest taxonomic level that bacteria can be identified to (Gupta et al., 2019). Researchers nowadays have realized that it is the combination of cultivation and molecular methods that give the best and widest understanding of the intestinal microbiome (Lagier et al., 2018), since both classical cultivation and molecular diagnostic techniques have their own strengths and weaknesses, as mentioned above. The six included studies of this thesis used either classical cultivation or 16S rRNA amplicon sequencing, as has been mentioned, and this might not give us a totally fair picture of the IM in canine species. Future studies that will help to characterize the IM should perhaps try to combine both techniques to get the most "accurate" view of the IM.

Conclusion

Based on 6 published papers we have characterized the intestinal microbiota of puppies from birth to 12 weeks of age. There are indications that some puppies are colonized with intestinal microbiota *in utero,* based on samples taken from their placenta and meconium. Puppies having culturable microbiota in their placenta and/or meconium are more robust, expressed by increased relative weight gain compared to puppies having placenta and/or meconium without culturable microbiota.

As in humans, mode of birth is also important for the general health of puppies. Puppies born via the vaginal route have a more diverse meconium microbiota compared to puppies born with caesarean-section. Vaginally born puppies gain more relative weight during their first days compared to puppies delivered with caesarean-section. This increased relative weight gain is possibly linked to a more diverse meconial microbiota, although the explanation could also be less maturity of puppies delivered with caesarean-section compared to vaginal birth.

Over circa the 3 first weeks after birth, the puppies IM is dominated with the phylum Firmicutes, and *Lactobacillus johnsonii* and *L. animalis* appear to be the dominating lactobacilli during this time as well. This is the period when puppies are solely fed with milk and during this time the puppies IM composition is dissimilar to their mothers IM. At the time of weaning (around 6-8 weeks) the puppies however obtain an IM composition more similar to their mothers IM. Here, *Lactobacillus johnsonii* is no longer the dominant *Lactobacillus* spp. while *L. animalis* is still abundant. This can be linked to the transition from milk to solid food together with behavioural factors such as ingesting faecal material from family members. The IM at this time has gotten more diverse in the puppies with the main phyla found being Fusobacteria, Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria.

The role that the rearing environment has on the IM development remains undetermined but the living environment in the future homes of the puppies seems to influence the IM. Further

studies are needed to assess this in more depth though and pinpoint the meaning of this for general health in adulthood.

Limitations to the papers included are for example small study populations, making it difficult to obtain a sufficient statistical power for the diverse factors investigated. All of the papers can however be thought of as pilot studies and should be used as such when designing further studies on the IM of puppies and dogs. Sample handling was another factor that was poorly explained in most of the papers included, an obvious area for improvement in future studies since this is important to get the most reliable results.

The overall conclusion is that the development of the intestinal microbiota in puppies from birth to 12 weeks of age is dynamic and influenced by many factors. The area has not been researched extensively and many unknown aspects are yet to be investigated. Studying the possible role that the microbiota in milk has on the IM development in puppies, investigating the use of different strains of *Lactobacillus* spp. as probiotics and investigating the effect of the rearing environment are areas we have found to be open for further research.

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Sammendrag

Tittel: Valpens tarmmikrobiota: Utvikling fra fødsel til 12 ukers alder

Forfattere: Anna Hilmersson, Glódís Sigmundsdóttir and Vilde Bjaaland Siljan

Veiledere: Kristin Marie Valand Herstad and Ellen Skancke, Institutt for sports- og familiedyrmedisin

En litteraturgjennomgang ble foretatt for å karakterisere utviklingen av tarmmikrobiotaen hos valper fra fødsel til 12 ukers alder. Valper som fødes med mekonium/placenta som inneholder dyrkbare bakterier og valper som fødes vaginalt, oppnår økt tilvekst sammenlignet med valper som fødes med mekonium/placenta uten dyrkbare bakterier eller som tas med keisersnitt. I løpet av de 3 første ukene dominerer Firmicutes i valpens tarmmikrobiota og de vanligste melkesyrebakteriene er *Lactobacillus johnsonii* og *L. animalis*. Før avvenning er valpens tarmmikrobiota ulik morens tarmmikrobiota. Ved avvenning derimot (omtrent 6-8 ukers alder) vil valpens tarmmikrobiota bli likere moren tarmmikrobiota, dette er trolig på grunn av overgangen fra melk til fast føde sammen med atferdsmessige faktorer. På dette tidspunktet har diversiteten i tarmfloraen økt og de vanligste bakterielle rekkene er Fusobacteria, Bacteroidetes, Firmicutes, Proteobacteria og Actinobacteria. *L. johnsonii* er ikke lenger dominerende mens *L. animalis* har fortsatt hyppig forekomst.

Små studiepopulasjoner og mangelfull informasjon angående prøvehåndtering er områder som kan forbedres i fremtidige studier av hundens tarmmikrobiota. Hvilken rolle som oppvekstmiljøet og mikrobiotaen i melk har på utviklingen av tarmmikrobiotaen hos valper, krever videre studier. Aldersrelaterte endringer av de forskjellige artene av melkesyrebakterier burde tas hensyn til når probiotikum utformes til hund.

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