Immunity, Microbiota & Immune-related Disorders in German Shepherd dogs

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Abstract

In an epidemiological study based on insurance data we described a breed-specific pattern of diseases in German Shepherd dogs (GSD) and confirmed that this breed is predisposed to immune-related disorders. A prospective study was performed in order to further investigate immunological changes in the GSD using a large number of dogs, 30 bitches and their litters, from the same kennel under well controlled natural conditions. Changes in fecal and serum immunoglobulins were followed from birth to young adult age and possible relationships between these parameters in dams and their offspring were identified. We also described the composition of gut microbiome in dogs and how it changes in different life stages including pregnancy, lactation and growth. The levels of serum IgE, serum IgA and fecal IgA increased from seven weeks of age and were then stabilized at one year of age, there was no relationship in immunoglobulin concentrations between bitches and their 7 weeks old puppies. Dogs with high fecal IgA had a better vaccine response indicating a favorable systemic immune status. We found profound differences in the gut microbiome between mothers and young dogs. Litter mates had a more similar fecal microbiome compared to unrelated dogs. The 7 weeks old puppies were no more similar to the mothers than to unrelated bitches at partum. However, the fecal microbiome of the puppies were significantly more similar to their mothers than to unrelated bitches at 7 weeks postpartum. We observed a change in the relative abundance of different bacteria during lactation, and an increase of diversity from pregnancy to end of lactation. We also found that the diversity of fecal microbiome was affected by living environment but we were unable to demonstrate an effect of pre- and postnatal exposure to the chosen strain of probiotics. Our results provide information to an area within canine microbiology and immunology which is not studied before -this is the first study to describe the gut microbiome as well as immunoglobulins (and their relation to each other) in pregnant and lactating bitches and their offspring. This information is a needed foundation for further research on the relationship between the microbiome at an early age and immune function later in life and of value for the evaluation of interventions.

Keywords; immune-related disorders, German Shepherd dogs, immunoglobulins, fecal microbiome, probiotics

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To my family

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Vilson Å., Bonnett B., Hansson-Hamlin H. & Hedhammar Å. (2013) Disease patterns in 32,486 insured German shepherd dogs in Sweden: 1995-2006. *Vet Rec*. 177(3):74. doi: 10.1136/vr.102960.

II. Vilson Å., Hedhammar Å., Reynolds A., Spears J., Satyaraj E., Pelker R., Rottman., Björkstén B. & Hansson-Hamlin H. (2016) Immunoglobulins in dogs: correspondence and maturation in 15 litters of German shepherd dogs and their dams. *Vet Rec Open.* 3(1): e000173. doi: 10.1136/vetreco-2016-000173.

III. Vilson Å., Ramadan X., Li Q., Hedhammar Å., Reynolds A., Spears J., Labuda J.,Pelker R., Björkstén, Dicksved J & Hansson-Hamlin H. (2016) Disentangling factors that shape the gut microbiome in German Shepherd dogs (submitted manuscript).

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Abbreviations

APC	Antigen presenting cell
CAD	Canine atopic dermatitis
CSK	Chronic superficial keratitis
DC	Dendritic cell
EPA	Exocrine pancreatic acinar atrophy
EPI	Exocrine pancreatic insufficiency
GALT	Gut-associated lymphoid tissue
GSD	German Shepherd dog
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
PAMP	Pathogen-associated molecular pattern
SLE	Systemic lupus erythematosus
TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor alpha

1 Introduction

The immune system is a network of cells, tissues and organs that primarily work to defend the body against foreign invaders such as bacteria, viruses, parasites and fungi. The immune system works against invaders through layered defences of increasing specificity. The first line of defence are the mechanical or physical barriers (skin and mucosa). The next line of defence includes innate and acquired immunity. Innate immunity responds immediately, without needing to learn to recognize the invaders. In acquired immunity, lymphocytes meet an invader, learn how to attack it, and built up a memory of the specific invader so they can attack it more efficiently the next time they encounter it. Innate and acquired immunity interact with other molecules in the body fluids, including cytokines, antibodies and complement proteins (Janeway et al. 2005). The gut is the largest immune organ in the body (Chassaing et al. 2014). The gut microbes are essential for the immune system (Björkstén et al. 2001), for the general health, brain and even behaviour (Diaz Heijtz 2016).

In order to work appropriate, the immune system must have the ability to distinguish between what belongs in the body (self) or what does not (non-self). Any substances that are identified as non-self stimulate an immune response in the body. Immune-related disorders occur when the body generates an immune response against itself (autoimmune disorders), the body cannot generate sufficient immune response against invading organisms (immunodeficiency disorders), or the body react to harmless foreign antigens with an inappropriate immune regulation (allergic disorders).

For humans living in industrialized countries, the prevalence of immune-related disorders has increased rapidly the past decades (de Silva et al. 2008; Herbert et al. 2009) and there are indications that this is also the case in dogs. Since we share the same environment with the dogs, it is presumed that the influences of environment on the prevalence of immune-related disorders may be similar across these two species.

The relation between early microbial exposure and development of the canine immune system is not well described. In humans, this area has evoked great interest in the research of prevention and treatment of chronic immune-related disorders. The mammalian immune system is a unique example of symbiosis were microbes, as potential enemies, have been integrated into intestinal function, giving vital information to the host and protecting it from

hostile interventions. Microbes were there from the start of mammalian evolution (Ley et al. 2008). In a short time we have moved from a lifestyle with high load of microbial exposure to a comparable sterile environment which has resulted in a selection for a microbiota that do not always elicit balanced immune responses.

Despite their popularity, the overall disease pattern in the German Shepherd dog (GSD) is not well described. Breed-specific studies of disease patterns in companion animals are scarce since it is difficult to obtain accurate population-based estimates from records presented by veterinary clinics. However, there are indications that GSD are predisposed to a number of immune-related disorders compared to other breeds, (Wisselink et al. 1985; Day et al. 1986; Batt et al. 1991; Clark et al. 2005; Nødtvedt et al. 2006; Vilson et al. 2013). This predisposition is suggested to be a result of inherited defects in the immune system (Whitbread et al. 1984; Day et al. 1988; Batt et al. 1991; Griot-Wenk et al. 1999).

In this thesis, unique Swedish features have made it possible to supply breed and disease specific estimates on the extent of veterinary care and death risk through access to the database at Agria Insurance. Furthermore, we monitored immunological parameters in a large number of German Shepherd litters and their dams born and raised under well controlled conditions at the kennel of the Swedish Armed Forces. This breeding colony also gave us an opportunity to study the influences on the immune system and the gut microbiome from environmental factors, including probiotic supplementation, from an early age.

This large and well controlled study population has given us valuable information about the immune system as well as the gut microbiome during pregnancy, lactation and during the first stage of life. This information will be of great importance for further studies regarding immune-stimulation and disease-prevention.

2 Aims of the thesis

- Describe the disease patterns in GSDs in Sweden and to test the hypothesis that GSDs are predisposed to immune-related disorders.

- Monitor changes in serum immunoglobulins from birth to young adult age and evaluating the relationship between dams and their offspring reared under well controlled conditions.

- Explore the development of the gut microbiome and immunological parameters in German Shepherd dogs from puppy to adulthood and furthermore, to study the effect of relatedness, maternal microbiome composition and living environment in a large and well-defined population of dogs.

- Evaluate whether administration of probiotics (*Lactobacillus johnsonii NCC533 (*La1*)*) enhance IgG antibody responses against Canine Distemper Virus in serum, and assess the effects on the fecal microbiome of early age probiotic supplementation to bitches and puppies.

The results will increase our understanding of different factors involved in the development of immune related disorders and potentially provide a means to improve the overall health of our dog population. The results may also be useful in comparative studies by increasing our understanding of processes driving the development of immune regulation and potential to prevent allergies and other immune related disorders.

3 Background

3.1 Immune-related disorders in German Shepherd dogs

The German Shepherd dog (GSD) is one of the most popular working and companion dog breeds worldwide (*Figure 1)*. When compared to other breeds, they have higher morbidity due to immune-related disorders compared to other breeds, (Wisselink et al. 1985; Day et al. 1986; Batt et al; 1991; Clark et al. 2005; Nødtvedt et al. 2006; Vilson et al. 2013). This predisposition is suggested to be a result of inherited defects in the immune system (Whitbread et al. 1984; Day et al. 1988; Batt et al. 1991; Griot-Wenk et al. 1999).

Immune-related disorders can be divided into three major categories; allergy, autoimmunity and immune deficiency. Allergy and autoimmunity are characterized by a hyperactive immune system while immune deficiency occurs when the immune system is lacking parts necessary for normal function.

Figure 1. The German Shepherd dog is predisposed to immune-related disorders.

3.1.1 Allergic disease

3.1.1.1 Clinical features

The most common allergy in dogs is canine atopic dermatitis (CAD), defined as genetically-predisposed inflammatory and pruritic allergic skin disease commonly associated with IgE antibodies to environmental allergens (Olivry et al. 2001). The age of onset is typically between 6 months and 3 years. Initial clinical signs of CAD can be seasonal or nonseasonal, depending upon the allergens involved. Clinical signs include pruritis of the face, ears, paws, extremities, and/or ventrum. Some dogs have no visible primary lesions, but when they are present they will consist primarily of erythema. Secondary lesions are common as a result of chronic pruritis and trauma and may include chronic inflammation, and concurrent secondary infections or microbial overgrowth. Otitis externa and conjunctivitis is commonly seen in atopic dogs (Griffin et al. 2001).

3.1.1.2 Risk factors

Breed predilections are well known in CAD, with a marked variation depending on geographical location. The top-five breeds with highest incidence rates in Sweden are Bull terrier, Welsh terrier, Boxer, West Highland white terrier and Staffordshire bullterrier. The German Shepherd dog was number nine in that study (Nødtvedt et al. 2006). Genome-wide analysis in GSD reveals associations of a locus on CFA27 with atopic dermatitis and haplotype association analysis from the fine-mapping data indicated association to the gene, *plakophilin 2* (*PKP2*), known to be important for skin structure. Low(er) IgA levels in that study were correlated to increased risk for atopy (Tengvall et al. 2013).

Environmental factors such as living area and diet affect the development of CAD. The risk of CAD is higher in major cities in Sweden, however this may be biased by a higher number of dermatologists in these areas (Nødtvedt et al. 2006). Nødtvedt et al. (2007) showed a protective effect of feeding home-made diets to the lactating bitch on the subsequent development of CAD in her offspring. It is speculated that this is a result of increased microbial exposure early in life, which is shown to have importance for the development of the immune system of the neonate.

3.1.2 Autoimmune disease

In autoimmune disease is an immune response generated towards self-tissues. Depending on which of the self-antigens the immune response is directed toward, clinical signs of disease may occur and are related to the functions of those target tissues or organs. Autoimmune diseases are classified into organ-specific and systemic diseases depending on where the target antigen is expressed. Examples of autoimmune diseases overrepresented in GSD are; chronic superficial keratitis (CSK), exocrine pancreatic acinar atrophy (EPA) and systemic lupus erythematosus (SLE).

Chronic superficial keratitis or pannus is an autoimmune inflammation of the cornea that can lead to blindness. The disease can occur in any breed but most cases are reported in GSD (Slatter et al. 1977; Chavkin et al. 1994). The clinical signs are progressive, bilateral lesions in the anterior corneal stroma with vascularization, formation of fibrous tissue and often pigmentation at the chronic stage (Bedford and Longstaffe 1979). The inflammatory lesions involve stromal infiltration by CD4+ T lymphocytes. There is also an increased expression of major histocompatibility complex (MHC) class II in the cornea of affected dogs, which may play an important part in prolonging the inflammatory process in the cornea (Williams 2005).

Exocrine pancreatic acinar atrophy is characterized by the selective atrophy of pancreatic acinar cells as a result of lymphocytic pancreatitis. It is the most common cause of exocrine pancreatic insufficiency (EPI) in dogs (Westermarck et al. 1993) with high predisposition in GSD (German 2012). Clinical signs include an increased appetite, weight loss, and voluminous soft stools (Westermarck et al. 1989). Exocrine pancreatic acinar atrophy is diagnosed through measurement of serum trypsin-like immunoreactivity (TLI) which is decreased in diseased dogs (Williams et al. 1988). The destruction of acinar tissue is largely T-cell-mediated, although some humoral mechanisms are involved as well (Wiberg et al. 2000; Tsai et al. 2013).

Systemic lupus erythematosus (SLE) is an example of systemic autoimmune diseases in dogs. In humans, SLE is a heterogeneous disease with a classification based on well-defined criteria (Tan et al. 1982). In dogs, a consistent list of criteria does not exist –making it difficult to diagnose canine SLE. One hallmark of SLE is high titer of circulating antinuclear antibodies (ANA). In a study by Hansson-Hamlin et al. (2006), all ANA-positive dogs had symmetrical polyarthritis and/or myositis, lethargy, and fever. This is in contrast to human SLE where other organs often are involved. Thus, the canine disease is referred to as SLE-related disease. German Shepherd dogs were

clearly predominated among the ANA-positive dogs (32%) in this study. It has been shown that the majority of the German Shepherds with serum positive for ANA by indirect immunofluorescence also had an identical but unidentified immunodiffusion ANA-subgroup specificity which may indicate a breed-specific disorder, reflecting a hereditary susceptibility among German Shepherds to a certain subtype of SLE-related disease (Hansson & Karlsson-Parra et al. 1999).

3.1.3 Immunodeficiency

Immunodeficiency disorders are uncommonly reported in the dog. These are usually inherited and have been recognized in a number of breeds including the German Shepherd dog (Day et al. 1988; Batt et al. 1991; Day 1994; Chabanne et al. 1995).

Selective IgA deficiency is the most common primary immunodeficiency disease in humans (Cunningham-rundles 1999). In most cases the disease will not cause any illness, but some patients have a tendency to develop recurrent infections at mucosal sites, autoimmune disease, allergy and gastrointestinal infections (Yel 2010). Low concentrations of serum IgA have been reported in selected dog breeds, including the GSD (Whitbread et al. 1984), Beagle (Felsburg et al. 1985) and Shar-Pei (Moroff et al. 1986). Olsson et al. (2014) identified eight breeds that could be classified as IgA deficient according to the human cut off value for IgA deficiency. The clinical signs in dogs have been reported to resemble the human disease by recurrent infections and immune mediated disease (Felsburg et al. 1985; Moroff et al. 1986). Tengvall et al. (2013) studied a large cohort of GSD in Sweden and found significantly lower serum IgA levels in dogs with atopic dermatitis compared to the control dogs, suggesting a functional role of IgA in the aetiology of canine atopic dermatitis.

3.2 Immunoglobulins –basic concepts

Antibodies were the first product of the adaptive immune response to be discovered. They are found in the body fluids and secreted by activated B-lymphocytes. Antibodies are Y-shaped molecules where the stem of the Y defines the class, or isotype, of the antibody. There are five different isotypes found in different compartments of the body where each of them has distinct effector mechanisms for disposing the recognized antigen. Immunoglobulin A (IgA) dominates in secretions, while IgG is dominant in the blood and extracellular fluids. Although IgA is a relatively small component of the serum antibodies, the high abundance of IgA-secreting cells in mucosae makes IgA one of the most prevalent antibodies as they constitute 70% of all

immunoglobulins produced in mammals (Macpherson et al. 2008). Immunoglobulin G opsonizes pathogens for engulfment by phagocytes and activates the complement system, while IgA works mainly as a neutralizing antibody. While IgM is the first antibody to be produced in the humoral immune response process, these early antibodies have low affinity for their corresponding antigens. However, the IgM molecules can form pentamers that makes them able to bind simultaneously to multivalent antigens, compensating for the low affinity. This pentameric structure makes them especially effective in activating the complement system. Because of the large size of the pentamers, IgM is found mainly in the blood, while the other isomers are smaller and diffuse easily out of the blood into the tissues. Immunoglobulin E antibodies are present in very low concentrations in blood and extracellular fluid, they are bound to mast cells beneath the skin and mucosa and in the connective tissue along the blood vessels. Antigen-binding to IgE triggers mast cells to release chemical mediators that induce allergic reactions. Immunoglobulin D is the less abundant antibody, its function is unknown. (Janeway et al. 2005)

3.2.1 Immunoglobulins in growing dogs

Dogs have an endotheliochorial placenta, which constitutes a relatively impermeable barrier between the maternal and fetal circulation. Puppies are therefore born hypo-gammaglobulinemic and they receive passive immunity from the mother by antibodies transferred through colostrum. Only a small amount of IgG is transferred over the placenta. The variation in the efficiency of uptake of colostral immunoglobulin may be related to the size and strength of the newborn puppy and the maternal abilities of the bitch, as well as the concentration of specific antibodies in the colostrum of the individual bitch (Mila et al. 2015).

The serum levels of immunoglobulins increase during first year of life and reaches adult levels at one year of age (Schreiber et al. 1992). How the concentration of maternal immunoglobulins in serum and feces affects the immunoglobulin levels in their offspring is poorly studied in dogs.

3.3 The gastrointestinal immune system in dogs

3.3.1 Mucosa

The gastrointestinal tract is the largest immunologic organ in the body and the gut mucosa represents the primary point of contact between pathogens and the innate and adaptive immune response. In addition to acting as a physical and chemical barrier to mucosal pathogens, mucosal surfaces are highly active immunologically. Mucosal delivery of antigen has the potential to induce potent local and systemic immunity responses. The epithelial layer with intracellular tight junctions constitutes a physical barrier, preventing passage of pathogens. Other defence mechanisms in the gastrointestinal tract include low pH in the stomach, peristalsis, proteolytic enzymes and normal gut microbiota (Gelberg 2014).

The gut-associated lymphoid tissue (GALT) is comprised of Peyer´s patches and diffuse lymphoid tissue. Peyer´s patches are located throughout the small intestine and have an important role in the production of IgA by plasma cells in the lamina propria (Hogenesch et al. 1992). Immunoglobulin A is the most abundant immunoglobulin in the body and has an important role in mucosal immunity by preventing the transfer of specific pathogens and their toxins across the intestinal epithelium. Microfold (M) cells (overlying the GALT), dendritic cells (DC) and the epithelial cells themselves are examples of antigen presenting cells (APC) in the gut epithelium that are responsible for the uptake of antigens in the gut (Knoop et al. 2013).

3.3.2 Innate immune system

The barrier function of intestinal epithelial cells is considered to be their most important contribution in preventing activation of the mucosal immune system by commensal bacteria and protein antigens (Shen et al. 2011). Intestinal epithelial cells possess many properties of cells of the innate immune system, in particular the ability to recognize and respond to pathogens. Antigen presenting cells recognize molecules on the pathogens called pathogen-associated molecular patterns (PAMP), by pattern recognition receptors (PRR). Activation by microbes of a PRR called Toll-like receptor (TLR) in intestinal epithelium increases recruitment of B-cells to the lamina propria and results in increased secretion of IgA into the intestinal lumen (Shang et al. 2008).

3.4 The influence of environmental factors on the development of the immune system

3.4.1 Epigenetics

The term epigenetics was stated by Conrad Waddington (1957) to describe the process by which the inherited genotype could be influenced under development to produce a range of phenotypes. More recently the term has been defined as the molecular processes by which traits can persist across mitotic cell division without involving changes in the nucleotide sequence of the DNA (Bateson & Gluckman 2011).

Environment plays a critical role during early life development and has ability to influence long-term health. Epigenetic programming during development, resulting in functional changes in gene expression, is thought to form underlying mechanisms in early-life programming. These epigenetic changes are likely to have great importance in contributing to phenotypic expression of complex disease and can be triggered by both environmental and genetic factors. Events or exposure during pregnancy can modify gene expression through epigenetic mechanisms, determining the functionality of the immune system (Haathela et al. 2013; Nauta et al. 2013).

3.4.2 Microbial exposure

The relation between early microbial exposure and development of the canine immune system is not well described. In humans, this area has evoked great interest in the research of allergy prevention and this text is therefore primary based on human research.

The increase in allergy is paralleled with a reduced prevalence of infectious diseases (Aaby et al. 2009). The altered disease pattern is most likely associated with changes in environmental factors such as improvements in public health, disease treatment, vaccination programs and hygiene. Strachan proposed the so called hygiene hypothesis in 1989, explaining that the lack of microbial exposure as a result of hygienic conditions in early life may have an impact on the balance of the immune system, resulting in development of allergic diseases. In his study from 1989, Strachan suggests an association between the prevalence of hay fever/atopic dermatitis and the family size. He concluded that the higher infection rate of children with older siblings may have a protective effect upon allergic diseases. The World Allergy Organisation (WAO) Special Committee on Climate Change and Biodiversity has stated an extension of the hygiene hypothesis; the biodiversity hypothesis

where urbanization is proposed to lead to loss of biodiversity, dysbiosis, immune dysfunction and inflammatory disease (Haathela et al. 2013). One example of this is a study by Laatikanen et al. (2011) who showed that allergies were more common in Finnish children and adults than in their Russian counterparts during 1997-2007. Furthermore, immigrant studies have shown that when immigrants move from non-affluent areas of low prevalence of disease to affluent areas of high prevalence of disease, their good health status declines and converges to that of the natives, indicating that tolerance mechanisms can rapidly become impaired in microbe-poor environments (Haathela et al. 2013).

Several studies have shown association between microbial exposure and protection against allergy and asthma. Children growing up in farming environment had reduced risk of asthma and wheezing, prenatal exposure to such environments had the same effects (Braun-fahrländer et al. 1999; von Ehrenstein et al. 2000; Kilpeläinen et al. 2000; Portengen et al. 2002; Downs et al. 2001; Riedler et al. 2001; Douwes et al. 2007; Valkonen et al. 2015). Björkstén et al. (2001) found that infants who will develop allergy have a different composition of their gut flora compared to healthy infants, even before the development of any clinical manifestations of atopy. Furthermore, children treated with broad-spectrum antibiotics during early life have been shown to have an increased risk of allergic disease. Early exposure to antibiotics results in the decrease of microbial stimulation and subsequent reduction of the Th1-response. In addition, the disruption of intestinal microflora by antibiotics suppresses the Treg- response and thereby an upregulation the Th2-response (Kuo et al. 2013). Studies in germ-free mice have shown that exposure to commensal microbiota is critical for appropriate immune development (Sudo et al. 1997). The germ-free mice had significantly increased allergic airway inflammation and exhibited altered allergen-presenting cell populations (Herbst et al. 2011). Tulic et al. (2000) showed that exposure to LPS could modify the development of allergic inflammation in sensitized rats.

The importance of microbial exposure during the first moments of life is illustrated by findings that delivery mode may affect immunological function in infants. Under normal conditions of delivery there is transfer of fecal and vaginal bacteria from the mother to the baby. It is shown that children born through sterile caesarean section have a different gut microbiota compared to vaginally born children (Grönlund et al. 1999; Huure et al. 2008; Azad et al. 2013). These children may have increased risk of asthma and atopy –indicating

a disturbance in their immune function (Kero et al. 2002; van Nimwegen et al. 2011).

Microbial exposure activates pattern-recognition receptors, leading to downstream suppression of Th2-cell expansion and therefore Th2-mediated diseases such as allergies (Loss et al. 2012). Altered immunoregulatory mechanisms may also be important in this process; increased numbers of Treg cells have been found in newborn exposed to farming *in utero* (Schaub et al. 2009).

The hygiene hypothesis is challenged by increased prevalence of Th1-autoimmune diseases and that Th2-skewed parasitic worm infections are not associated with allergy. So allergy development is not only a balance of Th1/Th2 responses, and Treg may potentially play a substantial role in the regulation of this process (Bauer et al. 2007).

3.5 Gut microbiota in growing dogs

Microbes are present throughout the entire gastrointestinal tract but their concentration is highest in the colon. Despite the simple gut of dogs, the canine gut microbiome is highly diverse with several hundred phylotypes represented (Handl et al. 2011; Swanson et al. 2011). Every individual dog has a unique and stable microbial ecosystem. All dogs have similar bacterial groups on a higher phylogenetic level, but the microbiome differs substantially on a species/strain level between each individual dog (Simpson et al. 2002) and genetically related dogs have a more similar fecal microflora than unrelated dogs (Hand et al. 2013). Most of the information about the gut microbiota in dogs is from mature dogs, only a few studies have investigated age-related changes in the microbiota and they showed dramatic changes in quantitative and qualitative characteristics of the microflora during postnatal development (Matsumoto et al. 1976; Benno et al. 1992). The sterile gastrointestinal tract of neonates is rapidly colonized by bacteria in the birth canal and the surrounding environment. Buddington (2003) showed that already 24 hours after birth, the numbers of bacteria in the GI-tract were comparable to those in adult dogs. After the numbers of bacteria have stabilized, further age-related changes involve changed relative proportions of the various groups of bacteria in the gut microflora, with an increase of anaerobic bacteria.

In humans (Jost et al. 2014) and mice (Perez et al. 2007) it has been shown that during lactation, intestinally derived bacterial components were transported within cells of the intestinal lymphoid tissue to the mammary glands through the lymphatic system and peripheral blood. This transfer of

maternal microbiota to the newborn via milk was suggested to program the neonatal immune system to recognize specific bacterial molecular patterns and to respond appropriately to pathogens and commensal organisms.

3.6 Probiotics

3.6.1 Safety

Probiotics are defined as living microorganisms, which when administered in adequate amounts confer health benefits on the host (FAO/WHO 2002). Bacteria used as probiotics should have ability to express their activities in the host. Commercial probiotic preparations are mainly based on lactic acid bacteria (*Lactobacillus, Bifidobacterium* and *Enterococcus)* -important components of the gastrointestinal microflora and relatively harmless (Ishibashi et al. 2001). Since probiotics are considered to be food supplements, not drugs, they are not regulated with respect to efficacy and quality, and few studies have been conducted examining the safety of probiotic supplementation in dogs (Weese 2003). One of these studies investigated the safety and tolerance of dietary supplementation with the probiotic *Bifidobacterium animalis* AHC7 fed to growing dogs demonstrated that 5 $x10^{10}$ CFU once per day for at least 12 weeks was well tolerated by growing dogs with no safety concerns (Kelley et al. 2010).

The discovery of probiotic bacteria in human infection sites has evoked debate in recent years over the safety of probiotics (Adams et al. 1995). However, cases of infection due to the commonly used probiotics are extremely rare in humans and most of the cases occur in patients with severe underlying conditions (Cannon et al. 2005).

Another issue regarding probiotic safety is antibiotic resistance. This is not a hazard unless it makes the probiotic untreatable in cases where it has caused infection or unless the resistance can be transferred to potential pathogens. Resistance plasmids with antibiotic-resistance genes including genes encoding resistance to tetracycline, gentamicin, chloramphenicol, and macrolide-lincosamide-streptogamin have been found in *L. reuteri, L. fermentum, L. acidophilus* and *L. plantarum* (Gevers et al. 2003).

3.6.2 Proposed immunologic mechanisms of probiotics

It is well documented that probiotics have immunomodulatory effects upon its host, influencing both the innate and adaptive immune systems. The health benefits of probiotics are strain specific and there is no universal strain that provide all proposed benefits (Morita et al. 2002). Intestinal epithelial cells and

dendritic cells (DC) interact extensively with probiotics. These cells respond to the gut microbiota through their pathogen recognition receptors (PRRs). Toll-like receptor (TLR) signaling is essential in mediating the anti-inflammatory effects of probiotics (Rachmilewitz et al. 2004). Probiotic microorganisms activate the DCs via TLR, and DC initiates the appropriate response as differentiation of Th0 to Treg (Hart et al. 2004; Di Giacinto et al. 2005).

Probiotic organisms can modulate gastrointestinal permeability and enhance epithelial barrier function *in vitro* and in whole animal models. Intestinal barrier defence consists of the mucous layer, antimicrobial peptides, secretory IgA, and the epithelial junction adhesion complex. Mucins are constituents of epithelial mucus. Probiotics may promote mucous secretion and enhance mucin expression *in vivo* and *in vitro* (Caballero-Franco et al. 2007), improving the barrier function and the exclusion of pathogens.

Mazmanian et al. (2005) studied the effect of the symbiotic *Bacteroides fragilis* on the immune maturation in germfree mice and showed that maturation of the mammalian immune system requires direction of an immunomodulatory molecule provided by symbiotic bacteria. The bacteria contains an immunomodulatory molecule that directed the development of CD4+ T cells and elicited appropriate cytokine production which may correct the immunologic defects found in the absence of bacterial colonization. Administration o *Lactobacillus reuteri* CRL-1098 to vitamin B12 deficient mice during pregnancy and lactation, prevented serum vitamin B12 deficiency and increased IgA producing cells within the small intestine (Molina et al. 2009). In another mouse study (De Moreno de LeBlanc et al. 2008), administration of *Lactobacillus casei* DN-114001 during lactation resulted in increased secretory IgA in the intestinal fluid, and decreased macrophages, dendritic cells and IgA+ cells in the offspring. The mothers that received probiotics during lactation had higher levels of secretory IgA in their milk. The increase in secretory IgA in the offspring during the suckling period was therefore suggested to be transferred through lactation. Kaburagi et al. (2007) showed that administration of *Lactobacillus johnsonii* La1 enhanced intestinal IgA production and helped recovery from malnutrition-related immune problems in aged mice.

Increased levels of fecal IgA have also been shown in healthy adult humans after three weeks treatment with *Bifidobaterium lactis* (Kabeerdoss et al. 2011).

Defensins are antimicrobial peptides, active against bacteria, fungi and viruses. Specific probiotic strains including lactobacilli upregulate the

expression of defensins and thus impact on the antibacterial activity of the gut (Schlee et al. 2008).

The cytokine-secretion from intestinal epithelial cells is induced by probiotics in a strain-specific manner. Morita et al. (2002) studied eleven strains of lactobacilli for their ability to induce the murine macrophage-like cell line J774 to secrete cytokines. Some of the bacteria induced the production of IL-6, IL-12 and TNF-α, the majority of the strains also induced secretion of IL-10. Rangavajhyala et al. (1997) showed that *L.acidophilus* stimulated murine macrophages and induced the production of IL-1a and TNF-a, which co-stimulate the activation of T-helper cells. In an *in vitro* study by Kainulainen et al. (2015), canine *Lactobacillus acidophilus* LAB20 decreased LPS-stimulated IL-8 production and strengthened the epithelial barrier function by increasing transepithelial electrical resistance (TER).

3.6.3 Probiotics in clinical trials with dogs as study object

The effect of probiotic supplementation on canine microbiota, as well as its survival in the gastrointestinal tract, has been studied. It has been shown that different strains of probiotics survive transit through the canine gastrointestinal tract and temporary, but not permanent, influence the composition of the microflora in the adult healthy dog (Baillon et al. 2004; Manninen et al. 2006; Biagi et al. 2007; Garcia-Mazcorro et al. 2011; Strompfová et al. 2014).

The majority of the clinical trials where probiotics are tested in dogs, are focused on the treatment of diarrhea. Probiotic treatment has been shown to reduce the convalescence time in acute gastroenteritis (Kelley et al. 2009; Herstad et al. 2010). In dogs with non-specific dietary sensitivity, probiotic treatment improved fecal consistency, fecal dry matter and defecation frequency (Pascher et al. 2008). Sauter et al. (2006) studied the effects of probiotic supplementation in dogs with food responsive diarrhea, all dogs clinically improved after treatment. Treatment with symbiotic bacteria reduced the prevalence of diarrhea during periods of stress in healthy dogs (Gagné et al. 2013).

The immune stimulatory effect of probiotic supplementation is not well studied in dogs and a positive effect has mainly been in puppies. Benyacoub et al. (2003) studied the immune stimulatory effects of *Enterococcus faecium* (SF68) when supplemented to beagles from weaning to one year of age. The probiotic treatment increased fecal IgA and canine distemper virus (CDV) vaccine-specific circulating IgG and IgA. However, this probiotic strain (SF68) was also used in a study by Simpson et al. (2009) who supplemented it

to adult dogs with chronic Giardiosis for sex weeks. They could not find any effect on fecal IgA concentrations or circulating leukocyte phagocytic activity. Chung et al. (2009) showed that 7 weeks treatment with recombinant *Lactobacillus casei* to beagle puppies, starting at 7 weeks of age, increased monocytes in blood as well as serum IgA, and enhanced vaccine response. Baillon et al. (2004) treated 15 adult dogs with *Lactobacillus acidophilus* for 2 weeks; this treatment increased the concentration of neutrophils, monocytes, and serum IgG.

The immunological mechanisms of probiotics have made their use interesting in the prevention and treatment of immune-related diseases, such as allergies. There are few studies focusing on probiotic treatment in the prevention of canine atopic dermatitis (CAD) and these are based on laboratory dogs. In a study by Marsella (2009), 2 beagles with severe atopic dermatitis were bred twice. The puppies in the second litter received *Lactobacillus rhamnosus GG* between 3 weeks and 6 months and the mother was treated during pregnancy and lactation. Both litters were sensitized to *Dermatophagoides farinae.* The litter treated with probiotics had significantly lower serum titer of allergen-specific IgE and milder reaction to intradermal testing compared to the first litter, but the treatment had no effect on clinical signs. In a follow-up study (Marsella et al. 2012) 3 years after discontinuation of the probiotic treatment, the atopic dogs treated with probiotics early in life had decreased severity of clinical signs and lower production of IL-10 in PBMCs compared to the placebo-group. These results indicate a long-term effect of early probiotic treatment upon clinical signs of CAD. The effect of *Lactobacillus sakei* probio-65 upon clinical signs of CAD was studied in a placebo-controlled trial where 42 dogs with CAD were included, 32 of the dogs were treated with the probiotics for two months. The treatment resulted in a significant improvement of clinical signs (Kim et al. 2015).

4 Material and methods

This section outlines overall issues regarding the material and methods used in the separate papers in this thesis. For detailed descriptions, the reader is referred to each individual paper.

4.1 Study populations

Two populations were studied in this thesis; German Shepherd dogs insured in the Swedish insurance company Agria (paper I), and German Shepherd dogs born and raised at the Swedish Armed Forces kennel (paper II-III).

4.1.1 Insured population (I)

A large proportion of the Swedish dog population is covered by an insurance plan. About one-third of the Swedish purebred dog population is insured by the insurance company Agria. This company offers insurance that covers the costs for veterinary care as well as life insurance for pets (Egenvall et al. 1998). Most dogs insured for veterinary care also have a life insurance policy. The Agria database has been validated as a useful tool for epidemiological research of overall morbidity and mortality, as well as specific diseases in dogs (Bonnett et al. 1997).

The study population consisted of 445,336 dogs enrolled in both veterinary care and life insurance before 12 months of age between 1995 and 2006 in Agria, 32,486 (7.3 per cent) of these were GSDs.

The insurance database used for this study was developed to register claims, and it cannot be considered a perfect source of information on the disease prevalence. However, the number of dogs in the database is large, and high statistical power can be achieved, making it well suited to describe the occurrence of disease and death within a breed and differences across breeds. In paper I, only the first veterinary claim for each animal has been included, which is most appropriate for examining and comparing risk across breeds, but underestimates the entire burden of disease, in that, individual dogs with chronic conditions might have many veterinary visits over the course of their lives. So, given that these are data from one country, and there are limitations of the insurance database, exact rates and risks should be extrapolated cautiously.

4.1.2 Swedish Armed Forces kennel (II-III)

Paper II and III are based on a large clinical trial where the study population consisted of GSD litters from the Swedish Armed Forces (SAF) kennel in

Sollefteå, Sweden, which breeds GSD for service duties. This well controlled population of dogs which are bred in special facilities constitutes an ideal population for research aims. The majority of the bitches used in the breeding were imported or internally recruited from the kennel. The bitches lived with their families and arrived at the kennel at pregnancy day 37 or earlier. All bitches and their litters were housed and treated with the same routines at the kennel. When the puppies were 8 weeks old, they were moved from the kennel to live with families throughout Sweden. The families wrote a diary during the observation period where signs of disease, fecal score, body condition score, changes in environment etc. were noted. Mothers and puppies were restricted to the same diet during the entire study period. Mothers and their litters were separated from other dogs at the kennel and they were not exposed to other food.

Paper II included 15 litters (the placebo group in paper III) while paper III included 30 litters.

4.2 Study designs

In paper I we performed a retrospective longitudinal cohort study in order to describe the disease patterns for morbidity and mortality in GSDs in Sweden, based on insurance data from the years 1995–2006. This included determination of the most common disorders within the breed, as well as a comparison with other breeds in order to identify disorders where the GSD is relatively over-represented. We also tested the hypothesis that GSDs are predisposed to immune-related disorders.

To establish well defined data for comparison in further studies on the effect of relatedness as well as interventions in the environment, in paper II we performed a prospective study where we followed changes in serum and fecal IgA and serum IgE from birth to young adult age in entire litters and their dams. We also tested if dogs with lower serum IgA have low fecal IgA and/or serum immunoglobulin IgE. To reveal if any of the parameters could be proven to influence the immune response, we further measured serum IgG response to vaccination against canine distemper virus (CDV).

Paper III was based on a double-blinded placebo-controlled retrospective trial where the aim was to explore the development of the gut microbiome in GSD from puppy to adulthood and furthermore, to study the relation to maternal microbiome composition and living environment in a large and well-defined population of dogs. Additionally, we evaluated whether administration of probiotics (*Lactobacillus johnsonii NCC533,* La1) would enhance IgG

antibody responses against CDV in serum, and assessed the effects on the fecal microbiome of early age probiotic supplementation to bitches and puppies.

In this paper, the pregnant bitches were divided into two equally sized groups through block randomization (block size 6), where one group received probiotic supplementation (*Lactobacillus johnsonii NCC533*, La1) and the other group placebo (maltodextrin). The bitches started on treatment three weeks prior to estimated parturition (pregnancy day 42), and continued until the puppies where eight weeks old. Puppies received oral treatment (same as their mother) at the age of 3 weeks at the onset of exposure to solid food. The treatment continued until the puppies were 12 weeks old (*Figure 2)*.

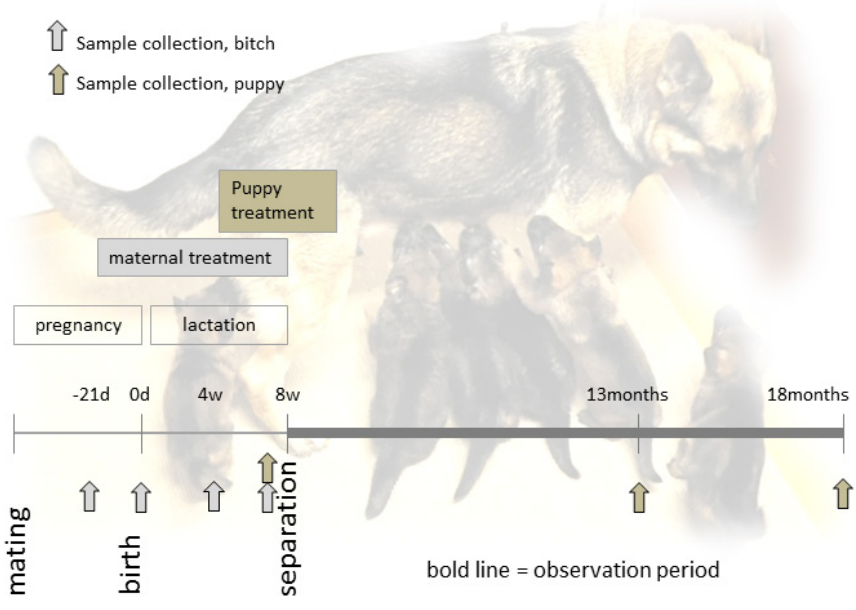

Figure 2. Flow diagram showing time for sampling, treatment and observation period.

4.3 Sampling (II-III)

Blood and feces were collected from bitches at pregnancy day 42, 12-24h after completed whelping, 4 and 7 weeks postpartum. Blood and feces were collected from puppies at 7 weeks, 12-13 months (median 12 months and 20 days) and 15-18 months (median 16 months and 16 days) of age (*Figure 2)*.

4.3.1 Blood samples

Blood samples were collected from the cephalic vein. Before centrifugation, samples were left to clot for at least 30 min in room temperature. After centrifugation (10 min, 7200 rpm), serum was collected and frozen. All samples were frozen at -80°C within 48 hours. Between collection and freezing at -80°C, the samples were stored in dry ice or in -25°C freezer.

4.3.2 Fecal samples

Fecal samples used for the 454 pyrosequencing (paper III) were collected by rectal swabs. Fecal IgA was extracted from 0.5g of fresh (mothers) or frozen (puppies) feces, collected in relation to defecation. The frozen fecal samples were frozen within two hours of collection and extracted in association with analysis, while the fresh fecal samples were extracted within 45 min and then frozen. All fecal samples were frozen at -80°C within 48 hours. Between collection and freezing at -80°C, the samples were stored in dry ice or in -25°C freezer.

4.4 Laboratory analyses (II-III)

All laboratory analyses were performed at Nestlé Purina Research Facilities in St. Louis, Missouri, USA.

4.4.1 Immunoglobulins in serum and feces

Serum and fecal extracts were assayed for fecal IgA, serum IgA, total serum IgE (Bethyl laboratories, Inc.), and IgG against CDV (VMRD) within 45 months by ELISA (Bethyl laboratories, Inc.). The concentration of fecal IgA was adjusted against total protein content in feces and expressed as µg IgA/µg total protein (TP). The concentration of serum immunoglobulins were expressed as g/L.

4.4.2 Assessment of fecal microbiome

The fecal microbiome was assessed by 454-pyrosequencing in all samples from puppies (three samples per puppy) and from bitches at pregnancy day 42, partum and 7 weeks postpartum.

Fecal genomic DNA was extracted using Qiagen genomic DNA extraction kit (Uppsala, Sweden) following manufacturer's protocol. 16S rRNA gene pyrosequencing was performed at the Core for Applied Genomics and Ecology, University of Nebraska, Lincoln. All samples were multiplexed with equal amount and sequenced using Roche GS FLX pyrosequencer. The raw

data from 454-pyrosequencing were processed using QIIME version 1.8.0 (Caporaso et al. 2010). Data were filtered to remove low-quality reads not meeting the quality criteria. Sequencing errors characteristic to pyrosequencing were removed by flowgram clustering (Reeder et al. 2010). Chimeric sequences generated due to PCR amplification of multiple sequences were removed using UCHIME (Edgar 2010). Processed reads were then demultiplexed into barcode-indexed samples. The barcode, forward primer, and reverse primer were subsequently trimmed from each read. This yielded a total of 1502990 reads from 507 samples from puppies and 246464 reads from 90 samples from bitches. The average length of the reads was 427 and 338 bps for bitches and puppies respectively.

Reads were clustered into operational taxonomic units (OTU) using a closed reference-based UCLUST algorithm at a 97% sequence similarity level implemented in QIIME (Edgar et al. 2010). The reference sequences and taxonomy assignment map were constructed from the greengenes database [www.greengenes.lbl.gov].

4.5 Statistical analyses

In paper I the morbidity in GSDs insured in Agria was calculated as true incidence rates (IR) where the denominator was the sum of each animal's total time in the register and number of disease events, where only the first claim for each dog within each general or specific category was included, was the numerator. The IR were multiplied by 10,000 and presented as number of events per 10,000 dog years at risk (DYAR). The mortality in GSDs insured in Agria was calculated as mortality rates (MR) where the number of deaths was divided by the sum of each animal's total time in the register. Mortality rates were multiplied by 10,000 to be interpreted as the number of deaths per 10,000 DYAR. Both MR and IR in GSDs were compared with those for all other breeds (combined) and presented as relative risk (RR). Standard error multiplied by 1.96 yielded 95% CI for IR, MR and mean ages and GSDs were considered to have a significant difference for the comparison for conditions where the CIs did not overlap those for all other breeds. Significant differences in the proportion of males and females within conditions (diagnoses) were detected using Proc Freq and the exact two-sided binomial test (SAS). The analysis was done using the software package SAS V.9.3 (SAS Institute, Cary, North Carolina, USA).

In paper II, R (R Core Team, 2012) and *lme4* (Bates et al. 2012) were used to perform linear mixed effect analyses of 1) the relationships between Ig-

concentrations in mother and offspring, 2) the relationships between the different immunoglobulins, 3) the change of immunoglobulins over time, 4) the impact of sex on immunoglobulins, and 5) the impact of litter size on immunoglobulins. The results were presented as the estimated population mean differences based on the model (β).

Intra Class Coefficients (ICC) was used to quantify the degree to which individuals with a fixed degree of relatedness resemble each other in terms of a quantitative trait (immunoglobulin concentrations).

In paper III, the relationships between the microbiome and metadata variables were explored using Orthogonal partial least square method with discriminant analysis (OPLS-DA) (Trygg et al. 2002; Ramdan et al. 2014) using SIMCA-P+ and MATLAB routines. In OPLS-DA models the cross-validation parameter, Q^2 (which can range from -1 to +1), is reported. Analysis of variance testing of cross-validated predictive residuals (CV-ANOVA) was also applied for each model. Principal components analysis (PCA) and pairwise OPLS-DA were applied to sample clusters (age, treatment, living area) with unit-variance scaling.

Shannon index was calculated for each rarefied OTU table. Mann Whitney U test was performed to compare the difference in diversity indexes between groups and the P values and mean +/- SD were reported. The OTU table was normalized by the total sum for each sample and was expressed as relative abundance. Linear discriminant analysis (LDA) effect size (LEfSe) was utilized to identify differentially abundant bacterial taxa.

In order to study if littermates had a more similar composition of the fecal microbiome than unrelated dogs of same age, an analysis of similarity (ANOSIM) was performed, where a p-value of <0.05 was regarded as a significant difference between litters (litter effect).

R (R Core Team, 2012) and *lme4* (Bates et al. 2012) were used to perform linear mixed effect analyses on the change of immunoglobulins over time and the effect of probiotic treatment upon immunoglobulins.

4.6 Ethical considerations

The study was approved by the Local Animal Ethical Committee in Uppsala, Sweden (C355/9).

5. Results and discussion

5.1 The German Shepherd dog is predisposed to immune-related disorders (I)

In paper I we showed that German Shepherd dogs were over-represented for immune-related disorders, it was 2.7 times more common that a GSD had immune-related disorders compared with other breeds (*Figure 3)*. Our result is in agreement with other epidemiological studies and case studies showing a predisposition for many of these disorders in GSDs (Wisselink et al. 1985; Day et al. 1986; Batt et al. 1991; Clark et al. 2005; Nødtvedt et al. 2006). This predisposition is suggested to be a result of inherited defects in the immune system (Whitbread et al. 1984; Day and Penhale 1988; Batt et al. 1991; Griot-Wenk et al. 1999). A predisposition for low serum IgA levels have earlier been reported in GSDs (Whitbread et al. 1984).

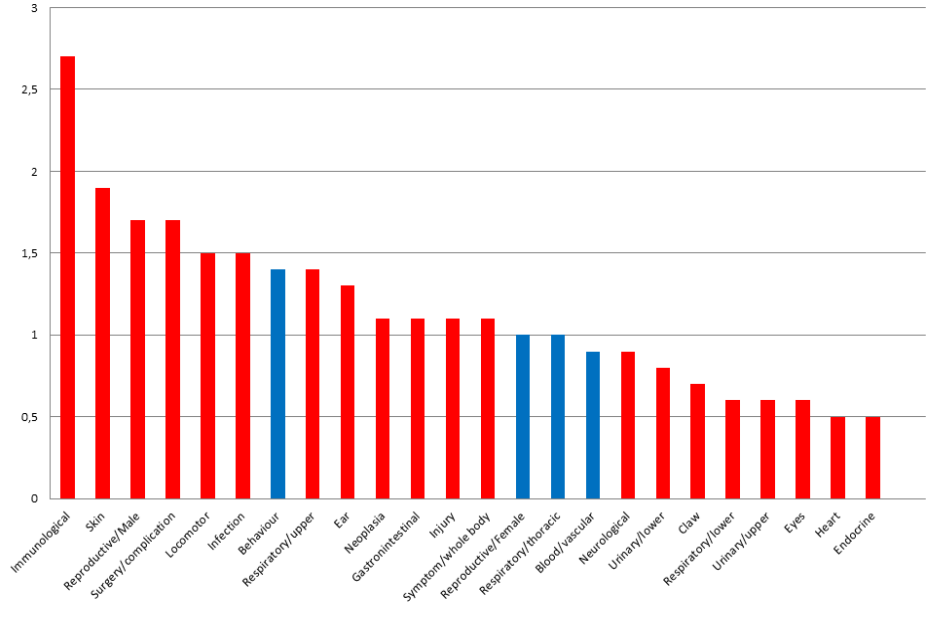

Figure 3. Relative Risk (RR) for general causes of veterinary care in the German Shepherd dog compared with allother breeds. Disease categories with a significant difference (non-overlapping CI intervals) between IRs in GSDs and all other breeds have red bars.

In paper I, we used a standardised diagnostic registry (Swedish Animal Hospital Organisation 1993), including both specific and general codes in hierarchical order. In general, only one diagnostic code was used per claim. The original diagnosis (provided by the veterinarian) was then collated into specific and then more general categories of diagnoses. The 24 general disease categories were based on body system or disease process. The general disease category 'immunological' was divided into three major groups; 'allergic', 'autoimmune' and 'various immune-related diseases' (*Table 1*). Of these three groups, allergic diseases were the most common among GSDs and this group also had the highest RR regarding morbidity, GSDs were 3.1 times more likely to have at least one veterinary care event for allergic disease compared with other breeds. Allergic disease was also the group of immune-related disorders where the mortality rate in GSDs was highest. It was 6.3 times more common that a GSD died/was euthanized due to allergic disease, when compared with all other breeds. 'Atopy' was the specific diagnosis with the highest RR, being 3.6 times more common than in other breeds, regarding mortality it was the seventh most common specific cause of death and the RR was 7.3 compared with all other breeds. The recorded diagnosis of atopy in the insurance database has been validated against practice records and the agreement was considered acceptable (Nødtvedt et al. 2006). Recently, genome wide analysis in GSDs reveals associations of a locus on CFA27 with atopic dermatitis and haplotype association analysis from the fine mapping data indicated association to the gene, *plakophilin 2* (*PKP2*), known to be important for skin structure. Low(er) IgA levels were, in that study, correlated to increased risk for atopy (Tengvall et al. 2013).

Including some diagnoses that are suspected to be immune-related, but not included in the category 'immunological', there are some breed-specific diseases, such as 'German shepherd dog pyoderma' 'exocrine pancreatic insufficiency' (EPI) and ´circumanal fistulae'. Not surprisingly, the GSD pyoderma was a diagnosis seen almost exclusively in GSDs. Exocrine pancreatic insufficiency, or the more specific diagnosis exocrine pancreas atrophy, is a disease mainly seen in a few breeds, including GSD which had a strong predisposition to this diagnosis in our study both regarding mortality and morbidity. 'Circumanal fistulae' was the specific skin diagnosis with the highest mortality rate, as well as the highest RR of mortality. It was 87.9 times more common that 'circumanal fistulae' was the cause of death in GSDs compared with all other breeds. . ´Circumanal fistulae' was also the specific skin diagnosis with highest RR regarding morbidity, this diagnosis had 46 times higher incidence rate in GSDs compared with all other breeds. This is a

disorder with a proposed immune-related aetiology (Day and Weaver 1992) with high breed predisposition in GSD (Massey et al. 2014).

The most common general disease category in GSDs was skin disorders, this category had almost twice the risk compared with all other breeds. 'Itching' was the most common specific diagnosis within the general category 'skin'. Many of the dogs in the 'itching' group were also represented in the group of allergic diagnoses indicating that the allergic dog was diagnosed with 'itching' before an investigation for allergic disease was completed. Although skin problems are common morbidities among dogs in general, the GSD stands out as a breed in which the problems are severe enough to result in euthanasia. 'Skin' is the general category where the GSD was most over-represented with a RR of mortality at 7.8 compared with other breeds.

Dogs with a condition that presents a challenging diagnostic problem are likely to be hidden within various diagnostic codes, this would result in an underestimation if only the rate based solely on the specific diagnosis was examined. Also, a dog may be given multiple different diagnoses over the course of a chronic problem, and show up in different categories. In order to handle these diagnostic considerations described, we used hierarchical classifications and presented both general and specific diagnostic categories (*Table 1)*.

Table 1. An example of the hierarchical classification of diagnoses used in study I.

Level 3	Level 2	Level 1	Specific diagnosis	Original diagnosis
Immunological	Allergic	Allergy/atopy	Allergy/atopy	Allergy with involvement of skin
				Urticaria/ angioderma
				Contact dermatitis/ allergy
				Food allergy
				Drug allergy
				Hormonal hypersensitivity
				Hypersensitivity to parasites, bacteria and insects
				Allergy to fleas
				Hypersensitivity to parasites
			Atopy	Atopy
			Hyposensitisation	Hyposensitisation
		Anaphylactic shock	Anaphylactic shock	Anaphylactic shock, whole animal
		Bronchitis/asthma	Bronchitis/asthma	Allergic bronchitis (asthma)
		Symptom of allergic disease	Symptom of allergic disease	Symptom of allergic disease

5.2 Immunoglobulins in the growing GSD (II)

In this extensive study of long-term changes of different immunoglobulins in a well-defined population of 74 growing GSD we identified a positive relationship between fecal IgA and serum IgG against CDV at all ages that might have clinical implications. We also found that the levels of serum IgE, serum IgA and fecal IgA are significantly lower in puppies and that levels are stabilized after one year of age. At 7 weeks, fecal IgA levels ranged between 0.010-0.175 µg IgA/µg TP, serum IgA ranged between 0.039-1.299 g/L and serum IgE between 0.0004-0.0242 g/L. At 12-13 months, fecal IgA levels ranged between 0.002-0.347 µg IgA/µg TP, to be compared with the maternal

levels (0.011-0.165 µg IgA/µg TP, 7w postpartum). Serum IgA levels at 12-13 months ranged between 0.165-2.921 g/L, to be compared with the maternal levels between 0.534-0.845 g/L. At 12-13 months, serum IgE ranged from 0.0005 to 0.0711 g/L, to be compared to the maternal levels (0.002-0.145 g/L).

The finding of lower levels of serum IgE, serum IgA and fecal IgA in puppies that stabilizes after one year of age is in accordance with previous studies (Glickman et al. 1988; Schreiber et al. 1992; Racine et al. 1999; Zaine et al. 2011; Olsson et al. 2014). The variation increased by age which could be a result of increased exposure to varied environmental factors.

Serum IgE and IgA were positively related to each other at 15-18 months. Fecal IgA and serum IgA was not related to each other.

There was no relationship between the mother and her puppies at seven weeks regarding serum IgE, serum IgA, or fecal IgA. The correlation within litters (intra class coefficients, ICC) for fecal IgA and serum IgG against CDV decreased with age, while serum IgA increased by age. In serum IgE, the ICC was close to 0 at all time-points.

We measured CDV vaccine response as a marker for the systemic immune status. Vaccine responses demonstrate clinically relevant alternations in an immune response to a challenge under well-controlled conditions and therefore are often used as a surrogate for responses to an infectious challenge. Fecal IgA was positively related to vaccine response at all ages, which means that dogs with high fecal IgA have a better vaccine response also indicating a favorable systemic immune status. Satyaraj et al. (2013) showed that colostrum supplementation increased fecal IgA as well as vaccine response, but it is unknown if the increased vaccine response resulted from the increase in fecal IgA.

Griot-Wenk et al. (1999) showed a negative correlation between serum IgE and serum IgA and the authors suggested that increased IgE levels might represent an important immune response to reduced IgA levels. We could only find a relationship between these immunoglobulins at 15-18 months (positive) and not in the other age groups which is in accordance with a study by Hill et al. (1995) where they did not find any correlations between serum IgA, IgE and IgG in a population of adult dogs.

How the concentration of maternal immunoglobulins in serum and feces affects the immunoglobulin levels in their offspring is not well studied in dogs. We could not find any relationship in immunoglobulin concentrations between bitches and their puppies when the puppies were seven weeks old. The puppies

however strongly resembled each other in fecal IgA, indicating that even if there is no relationship between mother and puppies, there is a strong relation between litter mates. This could be a result of an oral transfer through ingestion of each other´s feces within the litters. The ICC for IgG against CDV was high in the 7 weeks old puppies, but decreased when the dogs were older. Since the puppies were unvaccinated at sample collection at 7 weeks of age, IgG are supposed to have maternal origin and the transfer through colostrum should be equal within the litter. However, when the dogs are older, there might be environmental factors that affect the results and the increased variation might influence the ICC.

From 8 weeks of age, puppies lived with families which resulted in an increase in the variation arising from environmental factors. These results would be more applicable to pet dogs living in the society as compared to data from laboratory dogs and of great value when comparing immunoglobulins levels studies on immune stimulation in natural populations of growing dogs.

5.3 The fecal microbiome in the growing GSD (III)

The third paper included 30 bitches and their 168 offspring. The composition of the microbiota in puppies showed a clear age-related structure with a significant difference between 7 weeks old puppies and dogs at 15-18 months of age (OPLA-DA; Q^2=0.61) (*Figure 4)*. Firmicutes was the most dominant phylum at all ages with a relative abundance of 78–89%. Actinobacteria was the second most dominant phylum at all ages with a relative abundance of 4-9%. At 7 weeks of age, Bacteroidetes was the third most dominant phylum with a relative abundance of 7%, whereas at 12-13 months and 15-18 months of age, Fusobacteria were the third most dominant phyla (4%). Earlier studies have described a similar pattern in adult dogs using pyrosequencing of fecal samples (Middelbos et al. 2010; Handl et al. 2011; Garcia-Mazcorro et al. 2012). Although the predominant phyla are similar, proportions vary among these studies. Several sources can contribute to such variability, including breed, age, living conditions, diet and methodology. The maturation of microbiota by age was restricted to the composition of the fecal microbiome and was not reflected in the microbial alpha diversity.

There was a strong litter effect at 7 weeks of age when the puppies lived in the same environment (ANOSIM analysis on weighted UniFrac distances: R=0.49, p=0.001). This litter effect was less obvious, but still significant, at 18 months of age (weighted UniFrac R=0.17, p=0.001). This could be explained by a more heterogenic environment at 13 and 18 months of age, compared to 7

weeks where the puppies lived in the same place. Our results are in accordance with the results of Hand et al. (2013) who showed that 11 closely related miniature Schnauzer dogs had a more similar fecal microbiome profile compared to unrelated dogs within the same breed. By contrast, Middelbos et al. (2010) could not find any correlation in a study where they compared 3 pairs of littermates. However, our study compared a much larger number of litters (n=30) and this may account for the discrepancies between studies. There were more pronounced differences in the composition of the fecal microbiome among younger (7 weeks old) than older (>12 months) dogs. This could be explained by a greater variation in diet between litters at 7 weeks of age, were some of the litters were weaned and only had solid food, while others were still suckling. The composition of microbes and oligosaccharides in the milk may also have had an effect.

5.4 The fecal microbiome in pregnant and lactating bitches (III)

Firmicutes was the predominating phylum also in the bitches at all sampling points with relative abundances of 50-75%. The bacterial community structure in bitches was stable from pregnancy day 42 to partum, but was shifted after whelping (between partum and 7 weeks postpartum; OPLS-DA, Q^2=0.64 and pregnancy day 42 to 7 weeks postpartum; OPLS-DA, Q^2=0.57. *Lactobacillus* spp. was one of the species that increased during lactation, this species was also higher in relative abundance in 7 week old puppies compared to young adults (*Figure 4)*. Canine milk contains lactobacilli and may be a natural source of these potentially probiotic bacteria for the suckling puppy (Martin et al. 2010). In humans (Donnet-Hughes et al. 2010; Jost et al. 2014) and mice (Perez et al. 2007) it was shown that during lactation, cells of the intestinal lymphoid tissue travelled to the mammary glands through the lymphatic system and peripheral blood, transferring maternal microbiota to the new-born via milk. This entero-mammary pathway could be a possible route in which maternal probiotic treatment during lactation affected the microbiota in suckling puppies. However, we could not detect a difference in the amount of lactobacilli in the fecal microbiome of puppies between the La1-group and the placebo group.

The microbial alpha diversity increased from pregnancy day 42 to 7 weeks postpartum (Shannon´s diversity index: 3.76±0.41 to 4.03±0.37, $p<0.01$). The diversity was higher in the mothers than in the puppies at all ages. Interestingly, the composition of the microbiota also differed between the mothers 7 weeks postpartum and the 15-18 months old dogs (Q^2=0.84). The

composition of the fecal microbiota in bitches was more similar to the microbiota of puppies at 7 weeks postpartum than at partum. The 7 weeks old puppies were no more similar to the mothers than to unrelated bitches at partum. However, the puppies were significantly more similar to their mothers than to unrelated bitches at 7 weeks postpartum.

It was no difference in composition of the fecal microbiome between mothers and unrelated bitches at partum when comparing with 7 week old puppies. Our findings are similar with what Koren et al. (2012) found in humans where they showed that children´s microbiomes (at all ages) were no more similar to their own mothers´ than unrelated mothers´. However, the puppies were significantly more similar to their mothers than to unrelated bitches at 7weeks postpartum. This could be explained by the mothers´ behaviour of eating their puppies stool, making the maternal microbiome more similar to the microbiome of their puppies. That could also explain why the relative abundance of *Lactobacillus* spp. increased in the bitches during lactation.

As far as we know, this is the first study so far comparing the gut microbiome in pregnant and lactating bitches. Koren et al. (2012) showed that the composition of the gut microbiome in women changes dramatically during pregnancy but are then stabilized during first month postpartum. This finding was supported by Carrothers et al. (2015), Hesla et al. (2014) and Jost et al. (2014) who found that the microbiome in lactating women was relatively stable in the postpartum period. The results from the human studies are in contrast to our study where we found a significant change in the bitches fecal microbiome during lactation, which –once again –could be explained by the canine mothers´ behaviour of eating their puppies stool.

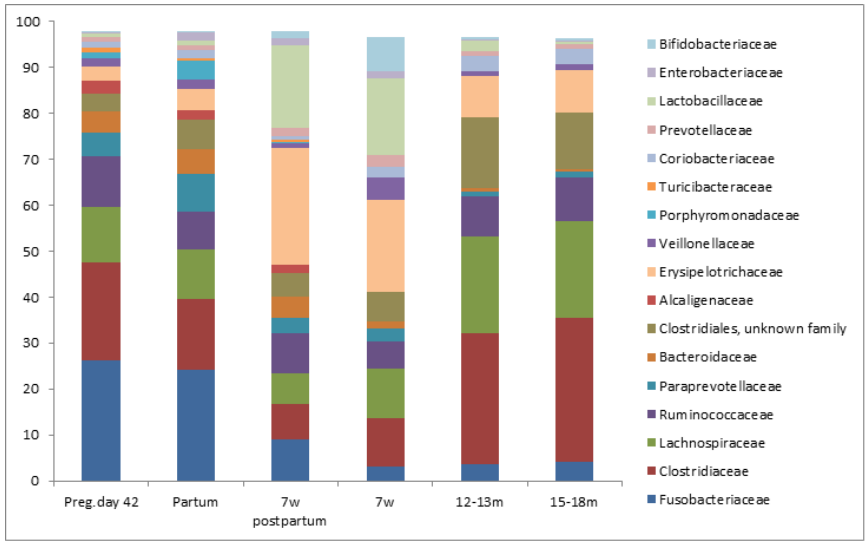

Figure 4. Relative abundance of bacteria family in feces from bitches at pregnancy day 42, partum and 7 weeks postpartum and from puppies at 7weeks, 12-13months and 15-18months of age. Families with relative abundance >1% are included.

5.5 The effect of environmental factors upon the fecal microbiome (III)

The bitches were treated daily with *Lactobacillus johnsonii* NCC533(La1) from pregnancy day 42 until the puppies where 8 weeks old. Puppies were treated between 3-12 weeks of age. Supplementation of the probiotic strain (La1) did not influence the diversity or composition of the microbiome in either the bitches or the puppies. The probiotic treatment did not affect the levels of serum IgA, total serum IgE, or the fecal IgA in bitches or puppies. There were no differences between the microbiome in dogs with low fecal IgA-levels and those with high fecal IgA-levels.

Alpha diversity was significantly affected by living environment (countryside, small cities or big cities). Dogs living in big cities had higher diversity compared to dogs living in small cities (3.36±0.63 vs 2.95±0.81, $p<0.01$) or at the countryside (3.36±0.63 vs 2.91±0.83, $p<0.01$). These differences were not observed at 7 weeks of age when all puppies shared the

same environment at the kennel, indicating that living area is the affecting factor in this aspect. Dicksved et al. (2007) showed that anthroposophically raised children had higher diversity of their fecal microbiome compared to farm children, indicating that living conditions affect the diversity of human fecal microbiome. It was, however, not possible to pinpoint the responsible factors in their lifestyle that contributed to this difference. One important factor could be the diet, since the different lifestyles are related to consumption of different food. In our study, food was standardized throughout the whole study period which minimized the effect of diet. However, dogs ingest more environmental microbes than humans because of their grooming habits, which might affect their fecal microbiome. Dogs living in big cities are often exposed to many different environments and a wide range of microbes, which might affect the microbial diversity.

Pre- and postnatal treatment with the probiotic La1 did not alter the composition of the fecal microbiome or diversity in either puppies or bitches. This is in accordance with results from the study of Garcia-Mazcorro et al. (2011), they could not observe any changes in the fecal microbiome of healthy adult dogs (n=12) after 3 weeks of treatment with a multi-species symbiotic (Proviable®-DC). Roos et al. (2013) treated infants with *Lactobacillus reuteri* for three weeks and could not detect any significant changes in the composition of their fecal microbiome. However, the fecal microbiome may not be a representative marker to display how the microbiome in different parts of the gut is affected by the treatment. Samples from different parts of the gut would be needed to answer that question. Beside the absence of impact on the intestinal microbiome, probiotic supplementation did not affect the levels of serum IgA and fecal IgA in our study, which is in accordance with the findings of Garcia-Mazcorro et al. (2011). Benyacoub et al. (2003) treated laboratory dogs with *Enterococcus faecium* (SF68) daily from weaning up to one year of age. They showed that the treatment increased vaccine (CDV) response and amount of circulating IgA and fecal IgA, indicating an immune-stimulatory effect. However, we used another probiotic strain and a shorter treatment period which might account for our absence of a treatment effect.

5.6 Strengths and limitations

The number of dogs in the insurance database was large, the high statistical power made it well suited to describe the occurrence of disease and death across breeds. The study population with dogs from the Swedish Armed Forces kennel included a large number of dogs born and raised under well-controlled conditions with standardized daily routines, diet, deworming and vaccination

schedules. Additionally, all samples were collected by the same person and analysed at the same laboratory, minimizing human induced variation. From 8 weeks of age, puppies lived with families which resulted in an increase in the variation arising from environmental factors. These results thereby would be more applicable to pet dogs living in the society as compared to data from laboratory dogs.

The insurance database used for our epidemiological study was developed to register claims, and it cannot be considered a perfect source of information on the disease prevalence. Veterinarians assign diagnoses using a standardized diagnostic registry, providing a level of consistency, but their underlying accuracy is unknown. In this study, only the first veterinary claim for each animal has been included, which is most appropriate for examining and comparing risk across breeds, but underestimates the entire burden of disease, in that, individual dogs with chronic conditions might have many veterinary visits over the course of their lives. So, given that these are data from one country, and there are limitations of the insurance database, exact rates and risks should be extrapolated cautiously.

Limitations of the study in paper II and III include the long time interval between first and second sample collection in puppies, the differences different length of the lactation period between litters, and too short treatment period in puppies. The treatment of puppies could have been more effective if started at birth, and in a larger study population with enough statistical power. That might have made it possible to detect the effect of probiotic treatment upon outcome of immune-related disorders. Biopsies from the small intestine would also give valuable information regarding the effects of probiotic treatment upon the gut microbiome.

6 Future perspectives

Prevalence of immune-related disorders, such as allergies, is an increasing problem in dogs as well as in man living in industrialized countries. The exposure of microorganisms early in life in order to stimulate the immune system, and thereby preventing immune-related disorders, is an interesting area of research that, besides improving the immune system in certain breeds (i.e. GSD) predisposed to immune-related disorders also might shed light on possibilities for interventions of comparative interest.

Further studies should be focused on investigating immune function in GSD and searching for genes involved in certain immune-related disorders within the breed. Molecular genetic studies would give more information regarding the genetics as a reason for variation in immunologic parameters as well as the gut microbiome.

The next generation sequencing has given us great possibilities to discover the gut microbiome in dogs. Little is known about the clinical importance of different bacteria and it´s relation to immune-related disorders. Further studies should focus on detecting differences in establishment of the gut microbiome between dogs developing immune-related disorders and those that remain healthy. With the knowledge about these differences in mind, a probiotic adapted for these conditions could then be chosen. Parallel to these studies, and irrespective of microbiome data, probiotic strains with immunologically important properties should be identified and tested in clinical trials. These trials could be tested during pregnancy and lactation as wells as treatment of the puppies directly from birth and where gut microbiome are investigated in different parts of the intestine through biopsies. Clinical trials with a larger study population than we studied here, or in a population with higher prevalence of immune-related disorders, would make it possible to reveal the effect on early probiotic treatment upon the prevalence of immune-related disorders.

7 Summary and concluding remarks

In an epidemiological study based on insurance data we described a breed-specific pattern of diseases in GSDs and confirmed that the GSD is predisposed to immune-related disorders such as allergies, circumanal fistulae and exocrine pancreas atrophy. These disorders are common causes of both morbidity and mortality in GSDs. With that in mind, we used this breed in a large prospective study including 30 GSD bitches and their entire litters, in order to follow changes in fecal and serum immunoglobulins from birth to young adult age and the relationship between dams and their offspring.

The levels of serum IgE, serum IgA and fecal IgA increased from seven weeks of age and were then stabilized at one year of age, there was no relationship in immunoglobulin concentrations between bitches and their puppies when the puppies were seven weeks old. We showed that dogs with high fecal IgA had a better vaccine response indicating a favorable systemic immune status.

In these 30 litters of GSD we also described the composition of gut microbiome in dogs and how it changes in different life stages including pregnancy, lactation and growth. We found profound differences between mothers and young dogs, with highest similarity 7 weeks postpartum. Litter mates had a more similar fecal microbiome compared to unrelated dogs. The 7 weeks old puppies were no more similar to the mothers than to unrelated bitches at partum. However, the puppies were significantly more similar to their mothers than to unrelated bitches at 7 weeks postpartum. We observed a change in the relative abundance of different bacteria during lactation, and an increase of diversity from pregnancy to end of lactation. We also found that the diversity of fecal microbiome was affected by living environment (if the dogs grew up on the countryside, in a small city or big city) but we were unable to demonstrate an effect of pre- and postnatal exposure to the chosen strain of probiotics.

Our findings provide a better understanding of the canine fecal microbiome and immunoglobulins in growing dogs as well as in pregnant and lactating bitches, but also the environmental influence upon these systems. Our results provide information to an area within canine microbiology and immunology which is not studied before -this is the first study to describe the gut microbiome as well as immunoglobulins (and their relation to each other) in pregnant and lactating bitches and their offspring in a large well-defined study population. This extensive trial, with a large study population, born and raised

under controlled conditions, provided us with a large amount of data, useful for further research on the relationship between the microbiome influences at an early age and immune function later in life.

8. Populärvetenskaplig sammanfattning

I en epidemiologisk studie baserad på försäkringsdata undersöktes sjukdomsmönstret hos schäferhundar, varvid en predisposition för olika immunrelaterade sjukdomar kunde identifieras. I en stor prospektiv studie med 30 schäfertikar och deras kullar, studerades immunförsvaret vidare i syfte att följa förändringar i immunoglobuliner från födsel upp till ung vuxen ålder samt förhållandet mellan dessa hos tik och avkomma.

Koncentrationen av IgA och IgE serum samt fecalt IgA ökade från sju veckors ålder och stabiliserades vid ett års ålder. Något samband avseende immunoglobulinkoncentrationer mellan tikarna och deras sju veckor gamla valpar kunde inte påvisas. Hundar med höga nivåer av fecal IgA visade sig ha bättre vaccinationsrespons, vilket kan indikera en bättre systemisk immunstatus hos dessa individer.

I de 30 schäferkullar som studerades analyserades även tarmflorans sammansättning och hur den förändras i olika livsstadier såsom dräktighet, digivning och tillväxt. Tydliga skillnader i tarmfloran mellan mödrar och unghundar kunde påvisas, medan den var mer lika mellan mödrarna och de sju veckor gamla valparna. Tarmfloran var också mer likartad bland kullsyskonen jämfört med obesläktade hundar. De sju veckor gamla valparnas tarmflora var inte mer lika de nyvalpade mödrarnas tarmflora än obesläktade tikar. Däremot var de sju veckor gamla valparnas tarmflora signifikant mer lika deras mödrar än de obesläktade tikarna sju veckor efter valpning. En skillnad i den relativa förekomsten av olika bakterier under digivning kunde identifieras, liksom en ökning av diversitet från dräktighet till slutet av digivningen. Vi såg även att tarmflorans diversitet påverkades av uppväxtmilön (om hundarna växte upp på landet, i en småstad eller i en storstad) men vi kunde inte visa någon effekt av probiotika-tillskott under dräktighet, digivning och första levnadstiden.

Våra fynd bidrar till en bättre förståelse för hundens tarmflora och immunoglobuliner hos såväl växande hundar som dräktiga och digivande tikar, men även hur dessa system påverkas av olika miljöfaktorer. Våra resultat tillför information till ett område inom hundars mikrobiologi och immunologi som inte studerats tidigare, det här är den första studien som beskriver tarmfloran samt immunoglobulinnivåer hos dräktiga och digivande tikar samt deras avkomma i en stor och välkontrollerad studiepopulation.

Denna kunskap är en viktig bas för fortsatta studier av sambandet mellan bakteriefloran tidigt och immunförsvaret senare i livet samt av betydelse för utvärdering av möjligheter till preventiva åtgärder

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