ASSESSMENT OF INTESTINAL MICROBIOTA IN DOGS WITH INFLAMMATORY BOWEL DISEASE IN CONTRAST TO HEALTHY DOGS

by

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Realising this masterproof was anything but easy. It turned out to be a period characterized by continuous frustration and doubts about my own abilities. Without the contribution and support of others, I think it would be nearly impossible to complete this work. I therefore would like to take the opportunity to thank some people

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ABSTRACT

Inflammatory bowel disease (IBD) is an inflammation of the gastro-intestinal tract with an unknown origin. The incidence of IBD is rising in both humans and animals and its aetiology and pathogenesis remains largely unknown, although it is demonstrated that microbiota play a key role. Previous publications have shown reduced species richness and a compositional change in the small and large intestine of dogs with IBD, especially an increase in the number of Proteobacteria and a decrease in the amount of Firmicutes. Since some members of the phyla Firmicutes produce butyrate and butyrate has a protective effect on the gastrointestinal tract, it is suggested that butyrate producing bacteria might play an important role in IBD. In this research, faecal samples of 11 healthy dogs and 15 dogs with IBD were collected. DGGE was used to compare microbial community; Firmicutes, Clostridium Cluster IV, Clostridium Cluster XIV and butyryl-CoA-acetate-CoA-transferase were quantified by qPCR. Out of our expectation, there were no significant differences in the faecal microbiota of IBD-dogs when compared with those of healthy dogs. However, interestingly, significant negative correlations of IBD-scores with Clostridium cluster IV (P=0.013), Clostridium cluster XIV (P=0.004) and butyryl-CoA-acetate-CoA-transferase (P=0.004) were found, which indicates that a lack of butyrate producing bacteria could be an indication of the severity of the disease. To gain more evidence supporting this theory, more research should however be conducted on biopsies before strong conclusions can be drawn.

KEYWORDS: Butyrate – Dog – Inflammatory bowel disease – Microbiota – Molecular technology
SAMENVATTING

IBD wordt gekenmerkt door ontstekingen in het gastro-intestinale stelsel zonder dat een exacte oorzaak kan worden geïdentificeerd. De incidentie van de ziekte in zowel mensen als dieren stijgt sterk, en de etiologie en pathogenese zijn grotendeels onbekend hoewel het wel is aangetoond dat microbiota een sleutelrol spelen. Eerdere publicaties over canine IBD hebben laten zien dat er een verminderde rijkdom aan soorten en een verandering van compositie van de microbiota in de dikke en dunne darm kan worden aangetoond bij IBD. Vooral de stijging van het aantal Proteobacteria en de daling in het aantal Firmicutes is opmerkelijk. Omdat de bacteriën van het phylum Firmicutes butyraat kunnen produceren en omdat butyraat een beschermende werking heeft in het gastro-intestinale stelsel kan gesuggereerd worden dat butyraat-producerende bacteriën een speciale rol spelen in IBD. Voor dit onderzoek zijn feces stalen van 11 gezonde en 15 honden met IBD gecollecteerd. DGGE is gebruikt voor het vergelijken van de microbiële gemeenschappen; Firmicutes, Clostridium Cluster IV and butyryl-CoA-acetate-CoA-transferase zijn gekwantificeerd door middel qPCR. De resultaten tonen geen significante verschillen tussen de microbiota van honden met IBD, vergeleken met die van gezonde honden wat tegen onze initiële verwachting indruist. Er is wel een significante correlatie waarneembaar tussen de IBD-scores van de honden en Clostridium Cluster IV (P=0,013), Clostridium cluster XIV (P=0.004) en butyryl-CoA-acetaat-CoA-transferase (P=0,004), wat een indicatie kan zijn dat een tekort aan butyraat-producerende bacteriën invloed heeft op de ernst van de symptomen. Voordat hier stevige uitspraken over worden gedaan moet dit echter nog verder onderzocht worden.

KEYWORDS: Butyrate – Dog – Inflammatory bowel disease – Microbiota – Moleculaire technieken
1. INTRODUCTION

Inflammatory bowel disease (IBD) is a disease which is located in the gastro-intestinal tract. IBD is described in several mammals, such as dogs, rats, cats, horses, ruminants and humans (Kirsner, 2001). Because of the resemblance of IBD between humans and animals, veterinarians often use the research done on humans with IBD. However, there is still variety of differences exists between IBD in human and in dogs (Cerquetella, 2010). More researches on canine IBD should be further conducted.

According to the severity of the disease a variety of clinical signs can be seen in dogs with IBD. The severity depends on location and extension of the affected region. Most common symptoms are vomiting and diarrhea, loss of appetite and weight (Sturgess, 2005). These symptoms have a negative effect on the quality of life of the patients and the disease can eventually lead to death. Unfortunately treatment is not yet available for curative purposes, but can be used to alleviate the symptoms of the disease.

Due to the severity and relapse of IBD, it is important to treat IBD, but the causes of IBD are still largely unknown which makes it difficult to be effectively treated. It is now known that the bacteria in the intestine play a role in causing IBD. There appears to be a difference in numbers and composition of the microbiota in the intestine between patients with IBD and a control group (Xenoulis et al., 2008; Suchodolski, 2010; Suchodolski et al., 2008). This difference, the so-called microbial lack of balance, has been found in both humans and dogs (Ott et al., 2004; Swidsinski et al., 2002; Takaishi et al., 2008; Marteau, 2010). The lack of balance mainly consists of an increase in the phylum Proteobacteria and a decrease in the phylum Firmicutes. Butyrate producing bacteria belongs to Firmicutes, which indicates that these strains of bacteria’s which are important in the metabolism of short chain fatty acids (SCFA) might play an important role in gastro-intestinal health.

In this research, we will try to expand our knowledge on the role of microbiota in the pathogenesis of IBD by performing a study in which compare the faecal microbiota of healthy dogs with those of dogs with IBD. Because butyrate plays an important role in the metabolism of the intestine and its protection against pathogens, our focus will be on the butyrate producing bacteria. This study is part of a larger project focused on identify dietary treatments to relieve the symptoms of IBD in dogs.

2. REVIEW

2.1 IBD CONCEPT

IBD is a collective name for idiopathic gastro-intestinal inflammatory disorders in a large variety of species. It has a chronic, relapsing nature and is immunologically mediated (Jergens, 2012).

The incidence in human IBD is still rising in the Western society, i.e. North-America and Europe (Hansen et al., 2010; Hou et al., 2011). An increase in incidence is recently also seen in other, less developed, parts of the world including China, South-Korea and Puerto Rico (Hou et al., 2011). In earlier years IBD was a rare condition in these countries and it is suggested that the change of diet into an aliment which consists of more protein and fat and less vegetables and fruits can be a possible
explanation for this increase in incidence (Hou et al., 2011). In humans IBD includes at least two forms of intestinal inflammation: ulcerative colitis (UC) and Crohn’s disease (CD). These two disorders can be divided into three subforms: regional enteritis, Crohn’s ileitis and granulomatous colitis (Podolsky, 1991). In UC, inflammation is limited to the colon and it can lead to mucosal ulceration. Histologically, this is shown by the infiltration of neutrophils in the lamina propria and infiltration of neutrophils in the crypts. The latter causes micro-abscesses which contributes to the severity of the disease (Xavier and Podolsky, 2007). CD on the other hand can be present in all parts of the gastro-intestinal tract but is most common in the terminal part of the ileum. The first mucosal lesions are often found over Peyer’s patches and these lesions consist of non-caseating granulomas caused by aggregations of macrophages (Xavier and Podolsky, 2007). People with CD can also develop strictures which can cause obstructions or fistulae which can be present between bowel segments, as well as between bowel and skin, or between other organs (Strober et al., 2007).

In contrast, canine IBD consists of different forms. The most common types that occur in the small intestines are lymphocytic-plasmacytic enteritis, lymphocytic-plasmacytic enterocolitis, lymphocytic plasmacytic colitis and eosinophilic gastroenterocolitis (Hall and Simpson, 2000). The type of IBD that affects the large intestine is histiocytic ulcerative colitis (HUC) (Cerquetella, 2010). Most cases of IBD are diagnosed in middle-aged dogs. There seems to be no difference in prevalence between male and female dogs (Cerquetella, 2010). In dogs other causative agents for IBD are proposed: meat proteins; food additives; artificial colouring; preservatives; milk proteins and gluten (Tilley and Smith, 2011). IBD more specifically in humans has already been described in humans for centuries. In 1612 a case report of a death was discussed of which the results of autopsy were very similar to those described in CD. In the latter part of the 18th century there are cases of people who died of ulcerative colitis (Kirsner, 2001). Kennedy Dalziel published in 1913 the classic paper which described CD in a way as we also know today (Kirsner, 2001; Tannock, 2005). He already ascribed CD to Johne’s disease, a chronic granulomatous ileitis in ruminants caused by Mycobacterium paratuberculosis, which is similar to CD (Hansen et al., 2010; Tannock, 2005). So Mycobacterium paratuberculosis became the first organism which was suggested as a specific infectious agent causing IBD (Hansen et al., 2010). In few CD tissues M. paratuberculosis was isolated, but further investigation eventually could not confirm the role of M. paratuberculosis (Danese et al., 2004).

Over the years, other organisms have been proposed as possible pathogens to cause IBD, such as Listeria monocytogenes, Chlamydia tracomatis, Escherichia coli, Cytomegalovirus, Saccharomyces cerevisiae (Danese and Fiocchi, 2006) various Helicobacter species (Hansen et al., 2010) as well as others. Another bacteria described in having a role in IBD is adherent-invasive E. Coli (AIEC) (Darfeuille-Michaud et al., 1998). AIEC is specifically observed in ileal CD (Darfeuille-Michaud et al., 2004; Danese and Fiocchi, 2006), but further research needs to be done to identify its potential etiological role in colonization of the ileum.

There is a possibility that classical infectious agents may cause IBD, but no strong evidence exists to support this theory. By know it is widely accepted that IBD originates from an inappropriate immune
response in genetically susceptible individuals as the result of a complex interaction between several components (Danese and Fiocchi, 2006).

2.2 AETIOLOGY AND PATHOGENESIS OF IBD

Although the aetiology still remains unknown (Jergens et al., 2009; Suchodolski et al., 2010), there are four components that are identified to play a role in the pathogenesis of IBD. These four components are dysbiosis of intestinal microbiota, environmental factors and genetic susceptibility (Xavier and Podolsky, 2007; Suchodolski, 2011) and an inappropriate immune response (Sartor, 2011) (see Figure 1.).

![Diagram showing the four components influencing the pathogenesis of IBD.](image)

Figure 1. Four components which influence the pathogenesis of IBD. One component alone is not sufficient enough to cause the disease. NSAID: nonsteroidal anti-inflammatory drug (Adapted from Sartor, 2011)

2.2.1 Immunology

The gastrointestinal tract is covered by surface epithelium, which forms an essential barrier to a broad range of potentially immunogenic and harmful factors within the lumen of the intestine (Dignass et al., 2004). The epithelium surface segregates the lumen from the specific mucosa-associated immune system. As a response to infectious factors or other harmful factors, intestinal inflammation may be induced, and the type of inflammatory response differs according to the specific type of antigen (Dignass et al., 2004). A big challenge for the intestinal immune system is to find an equilibrium in responding to antigens whilst not responding to harmless commensal bacteria and food antigens.
Various types of immune cells interact in complex ways to maintain inflammation (Dignass et al., 2004). Two forms of immunity exist: innate immunity (nonspecific) or adaptive immunity (highly specific) (Dignass et al., 2004). These forms of immunity interact, because the type of adaptive T cell response is, among other things, determined by components of the innate immune system (Cho, 2008). Although the malfunction in innate immunity is essential for the chronic inflammation which occurs in IBD, the malfunction of the adaptive immune system is also associated with IBD (Izcue et al., 2009).

The immunology of IBD can be seen as unbalanced populations of T cells. T cells can be subdivided into two populations: pro-inflammatory effector T cells (TEff) and regulatory T cells (Hansen et al., 2010). In IBD the TReg-cells response is inadequate, whilst the TEff-cell response is excessive abundant (Khor et al., 2011). In Crohn’s disease the most common described TEff-cells which are involved are T-helper 1 (TH1) and 17 (TH17), on the other hand in ulcerative colitis TH2 seems to be most regularly involved (Hansen et al., 2010; Khor et al., 2011). The most important influence for the differentiation of TH17 cells from CD4+ T cells is the presence of IL-23. TH17 is then largely responsible for the production of IL-17, which is an important pro-inflammatory cytokine and this cytokine is identified to be more present in IBD (Hansen et al., 2010). The development of TReg cells (which inadequately respond in IBD) are controlled by the transcription factor forkhead box P3 (FOXP3) and the TReg cells are subsequently responsible for the release of TGF-β, IL-10 and IL-35 (Hansen et al., 2010). TGF-β can either be pro-inflammatory or anti-inflammatory, which depends on the level of tissue inflammation existent. TGF-β has an anti-inflammatory role in a non-inflamed environment in which it promotes the FOXP3-mediated development of TReg cells (see Figure 2.). In an inflamed environment TGF-β turns out to be a wolf in sheep’s clothing because it promotes the differentiation of the TH17 cells which have an inflammatory role. (Hansen et al., 2010).

![Figure 2. This figure shows the balance between Treg and TEff cells in health and inflammation. IBD immunology is based on imbalance between anti-inflammatory cells Treg and inflammatory TH17 cells. (Adapted from Hansen et al., 2010)](image-url)
TGF-β and IL-10 counteract with the on-going TH1 and TH2 response and thereby inhibit the inflammatory process. Because of the eventual anti-inflammatory effect of this pathway TReg-cells have a protective role against detrimental reaction of microbial stimuli, which explains the abundant presence of these cells in the small intestine and colon. The abundant presence of TReg-cells also explain the immunologically suppressed milieu in the mucosa of the intestine (Izcue et al., 2009).

Another immunological finding, described in several studies, is that mucosal expression of different Toll-like receptors (TLR) is found in dogs with chronic enteropathies (Burgener et al., 2008). The TLR belong to the innate immune system and exist on the surface of cells, and because they are able to identify microbe-associated molecular patterns the TLR is able to activate the immune system (Suchodolski, 2010).

2.2.2 Genetic

It is widely acknowledged that genetics play a role in the development of human IBD, although the exact mechanisms have not yet been identified. Indications for this genetic susceptibility were first identified when it was described that first degree relatives of affected individuals have a relative risk of fivefold or greater to develop IBD (Xavier & Podolsky, 2007). Recently the genetic susceptibility to human IBD has been extensively reviewed (Khor et al., 2011).

Although the genetic inheritance in human IBD is not completely understood yet, it has been established that a complex polygenic model of inheritance underlie the development of IBD (Forabosco et al., 2000). Evidence of epistasis between genetic loci has been identified (Kathrani et al., 2011). Results from other studies also emphasize the importance of gene-environment interactions instead of the occurrence of a mendelian trait, because concordance rates in monozygotic twins are 10-15% for UC and 30-35% for CD (Spehlmann et al., 2008; Khor et al., 2011). Two meta-analyses of genome-wide association studies have been focused on these complex genetics. The first study revealed 71 positively associated loci for CD and 47 positively associated loci for UC (Kathrani et al., 2011) whilst the second study has identified 163 IBD susceptible loci (Khor et al., 2011). The majority of these loci can be assigned to both phenotypes which signifies that both diseases use common pathways (Jostins et al., 2012; Eeckhaut, 2013). These genes and loci play roles in the regulation of the epithelial barrier, innate immune response or the adaptive immune system which earlier in this paper have been described as key players for the protection of the host from bacterial invasion (Khor et al., 2011). The first gene which has been associated with human IBD is NOD2 (also known as CARD15 and IBD1), which due to the recognition of the muramyl dipeptide (MDP) modulates both innate and adaptive immune responses (Khor et al., 2011). Since the discovery of NOD2 other additional loci have been implicated in human IBD and confirmed by replication: IBD5, IL23R, ATG16L1 and IRGM (Xavier and Podolsky 2007; Khor et al., 2011). As mentioned earlier a complex polygenic model of inheritance underlies the development of human IBD which means that all the above genes are involved. It however goes beyond the scope of this study to fully elaborate on the exact pathways influenced by these genetic modifications.
In dogs, certain breeds are more susceptible to develop IBD although the exact mode of inheritance is incompletely understood (Simpson et al., 2006; Allenspach et al., 2010; Grützner et al., 2010). The breeds which have a significantly higher risk of developing IBD compared with mixed-breed dogs are Weimaraner, Rottweiler, German shepherd dog, Border Collie and Boxer (Allenspach et al., 2010; Kathrani et al., 2011). Specific forms of apparent genetic IBD have been described, such as immunoproliferative enteropathy in Basenji dogs (which represents analogies with human immunoproliferative enteritis) (Kathrani et al., 2011) and Lundehunds and Soft-Coated Weaten Terriers which suffer from IBD in which a hereditary aspect is also strongly suspected (Lecoindre, 2006). German Shepherd and Shar Pei dogs show a high incidence of blood and mucosal IgA immunodeficiency, which may explain their predisposition to the development of IBD (German et al., 2000). Another study shows that polymorphisms in Toll-like receptors (TLR) 4 and TLR5 are significantly associated with IBD in German Shepherd dogs (Kathrani et al., 2011). TLR-2, TLR-4 and TLR-9 were exaggerated in other different dog breeds with IBD. An increase in TLR-2 and a corresponding decrease in TLR-5 in German Shepherds with IBD when compared to healthy Greyhounds was also described (Allenspach et al., 2010).

Boxers are one of the most studied breeds when it comes to canine IBD and several interesting findings can be identified. It has been shown that the eradication of mucosally invasive E. Coli correlates with lasting remission of granulomatous colitis in boxers (Simpson et al., 2006). Genetic susceptibility has then been hypothesized, since this disease occurs almost solely in boxer dogs (Craven et al., 2010). A genome-wide analysis subsequently identified disease-associated single nucleotide polymorphisms (SNPs) in the gene NCF2, which is involved in killing intracellular bacteria (Kathrani et al., 2011). This genetic defect may result in difficulty to eliminate these bacteria and thereby making the host more prone to develop chronic infections (Suchodolski et al., 2010).

But although it is likely that human and canine IBD have similar etiological factors such as defects in TLR4 (Arbour et al., 2000; Kathrani et al., 2011) and that this importance of genetic factors in canine IBD has been emphasized in the above it remains a fact that detailed studies of genetic linkages or microsatellite markers are lacking in canine IBD (German et al., 2003). Therefore, few causal genetic defects have been identified to date (Simpson & Jergens, 2011) and the exact role of genetics in the pathogenesis of IBD remains ambiguous.

2.2.3 Intestinal microbiota
The intestinal microbiota, both in human beings and in animals, has various essential functions such as contributing to the metabolism and immune system. However, besides health-inducing properties, these bacteria can also play a role in the pathogenesis of various diseases. Various researchers suggested that these residential bacterial flora are also the most important factor for the development of IBD in genetically susceptible individuals (Rioux et al., 2005; Xenoulis et al., 2008). The precise interaction is however not yet discovered and two theories exist: either individual bacterial components of the intestine contribute to the pathogenesis of IBD or the composition and balance of the entire intestinal microbial communities are crucial for the pathogenesis of IBD. Several evidence is available
for both theories (Darfeuille-Michaud et al., 2004; Frank et al., 2007; Gophna et al., 2006; Mylonaki et al., 2005).

The various studies performed in this area led to multiple interesting conclusions on the role of microbiota in the pathogenesis of IBD. Most of the knowledge on gastrointestinal disease has arisen from the study of animal models (Blumberg et al., 1999; Nell et al., 2010; Rioux et al., 2005; Sellon et al., 1998). Animal experiments in rats showed that, when raised in a germ-free environment, inflammation only develops after colonisation with commensal bacteria (Taurog et al., 1994). In other studies several bacteria have been associated with the occurrence of inflammation: members of the normal flora such as Escherichia coli (Darfeuille-Michaud et al., 2004; Packey and Sartor, 2009; Rioux et al., 2005; Schuppler et al., 2004; Simpson et al., 2006a) and Enterococcus faecalis (Rioux et al., 2005; Schuppler et al., 2004). Other studies have indicated that a disturbance in the entire intestinal microbial communities play a crucial role in the pathogenesis of IBD (Darfeuille-Michaud et al., 2004; Hedin et al., 2007; Kotlowski et al., 2007; Swidsinski et al., 2002) which is endorsed by the higher concentrations of Bacteroides and Enterobacteria found in regions of inflammation in CD (Hedin et al., 2007; Swidsinski et al., 2002) and the enriched Enterobacteriaceae found in studies on canine en feline intestinal IBD (Janezcko et al., 2008). No evidence exists which indicates that a single pathogen, such as Mycobacterium avium paratuberculosis, causes either CD or UC (Packey and Sartor, 2009).

The last decade a shift from infectious to commensal agents in the aetiology of microbiota can be identified (Danese et al., 2004). There is mounting evidence that the commensal microbiota is the target of the immune response in IBD (Macdonald et al., 2005). In various animal models it was identified that normal microbiota must be present to develop IBD because IBD shall not arise in a germ-free environment. (Taurog et al., 1994). This is probably because an immune reaction against the commensal bacteria is essential for the pathogenesis of IBD (Strober et al., 2002). These discoveries emphasized the role of intestinal microbiota in the pathogenesis of IBD and the theory is supported by a variety of clinical observations in IBD patients (Danese and Fiocchi, 2006).

Both immune responses and intestinal microbiota seem to be a good target for treatment (Schirbel and Fiocchi, 2010), which caused many studies on microbiota and the use of pre- and probiotics for treatment of IBD to emerge and research in this field remains ongoing. The investigation of pre- and probiotics as a treatment for IBD has yielded contradictory results. This is caused by several limitations, amongst which the variety between the trials is the most striking. The variety is caused by different kinds of analysis used and different choices of pre- or probiotics used in the trials. Because of this variety it is difficult to compare the results obtained from the different studies. (Hedin et al., 2007). Some results from studies indicate that probiotics can alter intestinal microbiota and alleviate disease (Sartor, 2005) and that prebiotics improve luminal immune regulatory bacteria which can ultimately lead to a decrease of the inflammation (Hedin et al., 2007). Antibiotic treatment has shown efficacy, but cannot be used on a long-term because of the lack of long-term efficacy and the occurrence of side effects (Danese et al., 2004; Hedin et al., 2007; Sutherland et al., 1991). Since these pro- and antibiotics alter the intestinal microbial communities (Rioux et al., 2005; Xenoulis et al., 2008) these
not only offer indications of possible treatment but also stresses the importance of the microbiota in the pathogenesis of IBD.

In healthy dogs, more than 99% of the intestinal microbiota consists of the phyla **Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria** and **Actinobacteria**. The remaining <1% are the phyla **Spirochaetes, Tenericutes, Verrucomicrobia, Cyanobacteria, Chloroflexi** and some unclassified bacterial lineages (Suchodolski, 2011). These small intestinal bacteria are different from those found in dogs with IBD, which also indicates a correlation between these microbiota and IBD (Xenoulis et al., 2008). Studies on the microbiota showed reduced species richness and compositional changes in the small and large intestine of dogs with IBD (Xenoulis et al., 2008). Several studies showed very specific changes in the gut microbiota of dogs with IBD when compared to healthy dogs of which the most significant will be discussed: increased numbers of **Proteobacteria** were identified (Jergens et al., 2010; Suchodolski, 2011; Xenoulis et al., 2008) and a decrease in **Firmicutes**, especially Clostridiales (Jergens et al., 2010; Suchodolski, 2011). Another study however showed an increase in **Firmicutes** in dogs with IBD, but the authors also suggest that their result should be interpreted with caution because not all dogs showed these alterations and not all alterations were significant (Xenoulis et al., 2008). Studies have also showed a decrease in **Bacteroidetes**, more specifically its order **Bacteroidales** (Jergens et al. 2010; Xenoulis et al. 2008). It furthermore should be noted that dogs with IBD were significantly enriched in **Enterobacteriaceae** (Xenoulis et al., 2008) (see Table 1.).

Table 1. Microbial changes in dogs with IBD

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Dogs with IBD, compared to healthy dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteobacteria</td>
<td>Increase</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Decrease</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Decrease</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Strong increase</td>
</tr>
</tbody>
</table>

More details can be found in Appendix 1 which demonstrates microbial changes in dogs and cats.

2.2.3.4 Importance of butyrate

The before mentioned alterations in the composition of microbiota in dogs and humans with IBD has its consequences for the metabolic capabilities of the microbiota, because these intestinal bacteria metabolize dietary constituents to beneficial products (Thibault et al., 2010).

One of these products is butyrate or butyric acid, which is produced through bacterial fermentation of dietary carbohydrates (Breuer et al., 1997). Butyrate exerts a wide variety of effects on the function of the intestine: it firstly is the major source of energy for colonocytes, through which it affects cellular proliferation, differentiation and apoptosis (Hamer et al., 2008; Breuer et al., 1997). It is also shown that butyrate has anti-inflammatory potential (Hamer et al., 2008; Breuer et al., 1997; Hedin et al., 2007) and that it reinforces the mucosal barrier by both increasing the production of mucins and
antimicrobial peptides and decreasing intestinal epithelial permeability (Breuer et al., 1997; Peng et al., 2009).

These functions of butyrate are ideal properties for therapeutic purposes in IBD-like disease (Breuer et al., 1997). It seems that the changes which occur in the intestinal microbiota also impact the amount of butyrate and thereby its beneficial properties for the gastro-intestinal health. But despite the potential therapeutic value of butyrate, it still is problematic to actually deliver butyrate to the gut (Van Immerseel et al., 2010) although several options exist. Butyric acid-coated tablets is one of the possibilities, but the content may not always be released on the right location because of inter-individual differences in transit time and pH of the gut lumen. Alternatively butyrylated starch can be consumed and butyryl-L-carnitine as a prodrug can be used (Bajka et al., 2008; Srinivas et al., 2007). Another option is rectal butyric acid enemas, which have had positive results in treating UC, but the mucosa of the colon is only shortly and discontinuously exposed to the butyrate (Breuer et al., 1997). The third option is administering colonizing butyric acid-producing bacteria through which butyric acid will be in situ produced (Van Immerseel et al., 2010). The final option stems from the fact that butyrate is produced by the fermentation of dietary fiber. The direct stimulation of butyrate producers, such as the bacteria described below or metabolic cross-feeding of fermentation products from other bacteria could also lead to an increased butyrate production (Flint et al., 2007). Metabolic cross-feeding occurs when bacteria are able to produce butyrate by converting lactate or acetate. Lactate produced in healthy individuals can be used as a substrate for bacteria (e.g. Clostridial cluster IX or sulphate reducing bacteria). Several bacteria which are found in human faeces are able to produce butyrate by converting acetate and lactate into butyrate. Because of the presence of these bacteria high concentrations of lactate are not seen in healthy individuals, in contrast to ulcerative colitis in which an accumulation of lactate is described (Louis et al., 2007). Fructooligosaccharides (FOS) can be fermented into butyrate by e.g. Bifidobacteria. FOS can be administrated as a prebiotic and because of this fermentation an increase in butyrate is seen after the administration of prebiotics (Probert et al., 2004; Rossi et al., 2005).

Dietary changes, as well as supplements like prebiotics, can clearly affect the formation of butyrate (Pryde et al., 2002) through metabolic cross feeding in which the non-butyrate producing bacteria have an indirect effect on the butyrate concentration (Belenguer et al., 2006). Growth of butyrate producing bacteria can also occur more directly by dietary carbohydrates which have a positive impact on the formation of butyrate (Louis et al., 2007). Change of dietary pattern can therefore impact several metabolic mechanisms and thereby play an important role in the treatment of gastrointestinal diseases.

The most important butyrate producing bacterial phyla are the Firmicutes (Louis and Flint, 2009). This phylotype can be subdivided in other clusters, in which Clostridial cluster IV and Clostridial cluster XIV are the most important butyrate producers (Duncan et al., 2007). The importance of Firmicutes, more specifically its Clostridium clusters, can be derived from the fact that these are the second most
abundant group in the large and small intestine of both dogs and cats (Ritchie et al., 2008; Suchodolski et al., 2008) but also of humans (Louis and Flint, 2009).

As mentioned earlier, in humans with IBD (Sokol et al., 2008; Takaishi et al., 2008) and in dogs with IBD (Jergens, Nettleton, et al., 2010; Suchodolski, 2011b) a decrease in *Firmicutes* (including *Clostridiales*) is identified. The decrease of *Clostridiales* could also explain the decrease in butyrate, as observed in faecal extracts and/or colonic mucosa from patients with active IBD in comparison to those of healthy individuals (Eeckhaut, 2013). These findings indicate the important role of butyrate and butyrate producing bacteria in the pathogenesis of IBD.

### 2.2.4 Others

Although the importance of immunologic and genetic factors in the development of both humane and canine IBD was elaborated, several studies identified several environmental factors which might influence the development of IBD. The importance of these environmental factors is emphasized by the strong increase of the incidence of human IBD worldwide, because genetic variations are insignificant in such a short amount of time (Danese et al., 2004).

These potential environmental factors include: childhood infections (and early exposure to antibiotics), microbial agents, non-steroidal anti-inflammatory drugs (NSAIDs) smoking, diet, stress (Fiocchi, 1998; Danese et al., 2004). Amongst the most described environmental factors in humane IBD is smoking. Smoking both increases the risk of developing Crohn’s disease and aggravates its clinical course, but on the other hand seems to serve as protection in ulcerative colitis (Danese et al., 2004).

### 2.3 CLINICAL ASPECTS OF CANINE IBD

#### 2.3.1 Clinical signs

A variety of clinical signs can be present in dogs with inflammatory bowel disease (Sturgess, 2005). The affected area of the gastrointestinal tract and the magnitude of cellular infiltrate determine which clinical signs of IBD appear. The most frequent symptoms that arise in dogs are chronic diarrhea, vomiting, loss of weight and reduced appetite (Jergens et al., 1992). See Table 2. for other clinical signs which can be suggestive for gastrointestinal disease.
Table 2. Clinical signs which can suggest a gastrointestinal disease (Adapted from Sturgess, 2005)

<table>
<thead>
<tr>
<th><strong>Common signs</strong></th>
<th><strong>Less common signs</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight loss – present in nearly all cases of chronic small intestinal disease</td>
<td>Tenesmus, dyschezia and haematochezia – primarily features of large bowel disease, but can be associated with small intestinal bacterial overgrowth</td>
</tr>
<tr>
<td>Vomiting – very common in cats (more common than diarrhoea)</td>
<td>Weakness – tends to indicate severe disease but can be seen in hypoadrenocorticoid dogs with GI signs</td>
</tr>
<tr>
<td>Diarrhoea – very common in dogs (more common than vomiting)</td>
<td>Regurgitation – tends to suggest oesophageal disease but can occur secondarily to vomiting, causing oesophagitis</td>
</tr>
<tr>
<td>Variable appetite – many cases with chronic GI disease show a waxing and waning</td>
<td>Pallor – associated with GI tract haemorrhage</td>
</tr>
<tr>
<td>appetite related to the severity of clinical signs</td>
<td></td>
</tr>
<tr>
<td>Lethargy and anorexia – commonly seen when signs are severe</td>
<td>Jaundice – suggests involvement of the pancreas or bile duct as it enters the duodenum</td>
</tr>
<tr>
<td>Polyphagia – indicates malabsorption (primary or secondary to pancreatic</td>
<td>Abdominal enlargement – rare</td>
</tr>
<tr>
<td>insufficiency)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ptyalism – more commonly associated with swallowing disorders and hepatic disease</td>
</tr>
</tbody>
</table>

2.3.2 Diagnosis
Gastrointestinal (GI) diseases can have other causes rather than IBD. The differential diagnosis of IBD in dogs is: internal neoplasia, motility disorders, adverse food reaction, lymphangiectasia, exocrine pancreatic insufficiency, internal parasitism, antibiotic responsive diarrhea (Tilley and Smith, 2011).
Adequate diagnostic research (see Figure 3.) must be done first to exclude GI diseases with a known aetiology (Sturgess, 2005).

IBD should eventually be determined using clinical, pathogenic, imaging, pathophysiologic and genetic criteria together with histologic findings. Histologic findings only are not sufficient to determine IBD (Warshabau et al., 2010).

Five necessary requirements were demonstrated by Warshabau (2010) for a clinical diagnosis of IBD:

1. chronic gastrointestinal symptoms
2. histopathologic evidence of mucosal inflammation
3. inability to diagnose other causes
4. inadequate response to therapeutic trials
5. clinical response to anti-inflammatory or immunosuppressive agents

Because clinical signs of IBD in dogs often show many variations and the level and the location of manifestation of the disease can be quite different among dogs as well, a scoring index has been developed to assess the gravity of the disease and to be able to compare among patients (Jergens et al., 2003).
CIBDAI stands for Canine IBD Activity Index (Jergens et al., 2003). This is a scoring method in which 6 symptoms are registered with 4 levels, numbered from 0 to 3. Adding up the total score for the six symptoms is a measure of the severity of the disease. The symptoms used in CIBDAI are attitude/activity; appetite; vomiting; stool consistency; stool frequency and weight loss. The total score can range between 0 and 18 which can then be used to label the severity of the disease as either clinically insignificant (0-3 points); midi IBD (4-5 points); moderate IBD (6-8 points) or severe IBD (>9 points) (Jergens et al., 2003).

More recently the Canine Chronic Enteropathy Activity Index (CCECAI) was developed by Allenspach (see Figure 4). This index provides a better and more complete system for diagnosing and predicting IBD. The CCECAI contains three extra variables, i.e. albumin level; ascites/peripheral edema and pruritus which are ranked in the same way as CIBDAI (Allenspach et al., 2007).
Canine Chronic Enteropathy Activity Index (CCECAI) Scoring Sheet for Measuring Severity of Canine Inflammatory Bowel Disease

Canine IBD Scoring

Date:
History:

CCECAI Criteria:

<table>
<thead>
<tr>
<th>Criteria</th>
<th>0=normal</th>
<th>1=slightly decreased</th>
<th>2=moderately decreased</th>
<th>3=severely decreased</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Attitude/Activity:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Appetite:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Vomiting:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. Stool consistency:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. Stool frequency:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F. Weight Loss:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. Albumin levels</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. Ascites and peripheral edema</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Pruritus</td>
<td></td>
<td></td>
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</tbody>
</table>

CCECAI Score:

<table>
<thead>
<tr>
<th>Insignificant</th>
<th>Mild IBD</th>
<th>Moderate IBD</th>
<th>Severe IBD</th>
<th>Very Severe IBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total score of 0-3</td>
<td>Total score of 4-5</td>
<td>Total Score of 6-8</td>
<td>Total Score 9-11</td>
<td>Total Score &gt;12</td>
</tr>
</tbody>
</table>

Laboratory Tests:
CBC
Biochemistries
Urinalysis
Fecal

Comments:

Pathologic Markers:
CRP
Folate
TLi
Cobalamin

Endoscopy:
Upper GI
Lower GI
Both

Diet:

Drug:
CBC=complete blood count, CRP=c-reactive protein, TLi=trypsin-like immunoreactivity

Figure 4. Canine chronic enteropathy activity index (Adapted from Allenspach, 2007)
2.3.3 Treatment

Despite all effort it is not yet possible to treat, either human nor canine IBD, in a suitable way. The current therapeutic treatment of IBD is driven by a number of factors: the expectation that IBD is a race-related disease, the degree of the disease as measured by clinical indicators; the level of serum albumin and cobalamin; imaging appearance; the type of cellular infiltrate; the presence of bacteria or fungi; and changes in the architecture, such as atrophy, ulceration, lymphangiectasia and/or crypt cysts (Simpson and Jergens, 2011). Therapeutic intervention is aiming at correcting nutritional deficiencies like shortage of cobalamin, and alleviating inflammation and dysbiosis (Simpson and Jergens, 2011).

Different protocols are available for the handling of IBD. Most of the treatments consist of a combination of dietary approach and drug therapy, although it is possible that mild cases respond to dietary manipulation alone (Jergens, 1999). The specific treatment is selected based on the severity and chronicity of the patient's disease. The assessment of severity is based on both histological changes and clinical parameters, e.g. the frequency of vomiting/diarrhoea, the degree of weight loss, and the levels of serum protein (Sturgess, 2005).

It is possible to start therapy before a definitive diagnosis is established but it should be taken into consideration that the therapy should not influence the results of further tests (Sturgess, 2005). In canine IBD treatment usually starts with a change in diet. If this fails further steps in treatment which should be undertaken are the administration of prebiotics/probiotics, the administration of antimicrobials and eventually the administration of corticosteroids. The most common drugs administered in IBD are metronidazole (antibiotics); methylprednisolone (corticosteroids) (Sturgess, 2005); ciclosporin and azathioprine (immunosuppressant) (Allenspach et al., 2006).

2.4 TECHNOLOGY FOR MICROBIOTA ANALYSIS

Several different techniques to study the microbiota of the gut have been developed (see Figure 5.). These techniques and its advantages and disadvantages will be discussed in the following chapter.

2.4.1 Culture independent methods

Complex microbiota have classically been evaluated by using culture techniques, which is a duplicable and cost-effective technique. Although these are two major benefits, many drawbacks known for this technique are also described. With this technique it is challenging to distinguish between different bacterial phylogenetic groups, not to speak of recognizing different species or strains which is considered extremely difficult or maybe even unachievable (Sekirov et al., 2010). It is furthermore estimated that >80% of the gut microbiota cannot be cultured under standard laboratory conditions, because the majority of these bacteria are strict anaerobes conditions (Eckburg et al., 2005; Tannock, 2005). Another finding is that differences exist if bacteria in ecosystems are compared with those in
culture (Zoetendal et al., 2004). These disadvantages of culture techniques have led to more focus on the development of other techniques to study the complex microbiota.

Several techniques that have been developed uses the bacterial 16S ribosomal RNA (rRNA) gene as a marker of genetic diversity. This gene has been chosen because of two reasons: it has a relatively small size (1.5 kb) and it appears to have an appropriate equilibrium with enough similarity to identify members of the same larger phylogenetic group, but enough variation to identify different species and strains (Peterson et al., 2010). There are different methods that use this 16S rRNA gene which all have different advantages and disadvantages (Sekirov et al., 2010).

Regions of nucleotide base sequence are discovered in small ribosomal subunits of RNA (16S rRNA in bacteria) that were maintained across the bacterial world and that these sequences alternated between hypervariable regions (V regions) (Woese, 1987). Because these V regions signify phylogenetic groups and species, new methods for analyzing bacterial communities became available. After extraction of bacterial DNA or RNA it became possible to, partly or complete, perform polymerase chain reaction (PCR) amplification of the 16S rRNA gene (Tannock, 2005).

It now was possible to create clone libraries of the 16S rRNA genes and the sequenced clones, thereby producing a directory of the bacterial components of the ecosystem (Suau et al., 1999). It furthermore became possible to derive DNA probes from this sequence information, thereby identifying those probes that specifically focused on variable regions of the 16S RNA gene. This led to

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**Figure 5. Methods for the characterization of the intestinal microbiota (Adapted from Suchodolski, 2010)**

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the possibility to enumerate the phylogenetic groups of bacteria in the human gut, indifferent of whether they could be cultured (Sghir et al., 2000). Despite the possibility, it was logistically infeasible to make this library of hundreds of clones for every sample (Tannock, 2005).

2.4.1.1 Sequencing

Another possibility to analyze microbiota is Sanger sequencing, also known as full-length 16S rRNA sequencing (Sekirov et al., 2010), which was used by Eckburg et al. to characterize the diversity of gut microbial flora in three healthy humans (Eckburg et al., 2005). A major limitation of this method is the cost, since the budget required to perform the analysis increases, when the number of samples increases. Fortunately more cost-effective options for sequencing entire 16S rRNA genes have been developed.

Pyrosequencing has the possibility to produce large numbers of 16S rDNA sequence tags by expanding select variable regions within the 16S rRNA gene. With its capability to sequence 25 million bases at 99% or better accuracy in one 4-h run, it has a 100-fold higher throughput than Sanger sequencing (Margulies et al., 2006; Sekirov et al., 2010). Pyrosequencing requires only targeted amplification of the elect highly variable regions of the 16S rRNA gene (V2, V3 or V6 are the regions of interest). Because shorter sequence are being read taxonomic resolutions are not jeopardized (Hamady et al., 2012). Sequencing of multiple variable regions is very often performed so that what used to be a class-level taxonomic identification can be narrowed down to a certain genus or species (Sekirov et al., 2010; Sundquist et al., 2007).

Pyrosequencing has its advantages when compared to Sanger sequencing. It is not necessary to create clone libraries, which is a time-consuming step in Sanger sequencing, and bar-coded primers are used which makes it possible to mix multiple samples in a single batch sequencing run. At this point, new error-correcting bar codes are able to run hundreds of samples at the same time (Hamady et al., 2012; Sekirov et al., 2010). Although it seems that this method has important advantages compared to Sanger sequencing these two methods nevertheless share the major disadvantage of being massive endeavors that generate thousands of sequencing thereby requiring thorough data analysis (Sekirov et al., 2010).

2.4.1.2 Fingerprinting methods

DNA fingerprinting is a means by which can be used to generate a DNA profile of the microbial community in each sample, thereby allowing comparison between samples based on the differences between their genetic “fingerprints” (Sekirov et al., 2010). There are several different techniques available within the field of DNA fingerprinting, but they all share the advantages of reduces expense and effort when these techniques are employed (Sekirov et al., 2010).
2.4.1.2.1 Denaturing gradient gel electrophoresis (DGGE)

DGGE is amongst the best acknowledged molecular tools in microbial ecology (Marzorati et al., 2008; Muyzer and Smalla, 1998; Sekirov et al., 2010), gaining popularity in a relatively short period of time and now being used in most laboratories (Muyzer and Smalla, 1998).

The technique itself denatures the PCR-amplified gene of interest from the extracted community DNA. The gene products acquired from each community sample will subsequently migrate on an acrylamide gel correspondent to their G – C content. The most stable DNA will migrate further thereby creating a banding pattern that represents the amount of diversity in the sample. The PCR amplicons which are used are typically small in size (150 bp) and only areas with the greatest variability are accentuated. The results, the DGGE patterns, can be quantified according to the number and thickness of the bands on the gel (Sekirov et al., 2010).

The profile provided by DGGE represents the genetic structure and diversity of a microbial community from a specific environment (Muyzer and Smalla, 1998; Marzorati et al., 2008) and by coupling DGGE fingerprinting to statistical analysis and calculation of biodiversity indices (e.g. principal coordinate analysis (PCA) it can be used to measure differences between patterns and thereby comparing bacterial communities occurring in different environmental samples (Marzorati et al., 2008; Sekirov et al., 2010). This approach was first performed by Zoetendal et al. to compare the mucosa-associated bacterial communities in the colon and feces of patients with UC. These comparative purposes are still the major goal of DGGE, because the PCR amplicons are too small to obtain enough sequencing information to identify the bands of interest (Zoetendal et al., 2002).

Although DGGE is praised for its high versatility, reliability and reproducibility (Muyzer and Smalla, 1998; Marzorati et al., 2008) drawbacks can be identified as well. It is suggested that a single DGGE band can represent multiple bacterial strains from which a new bias for quantitative analysis based on the band intensities emerged. (Sekiguchi et al., 2001). Another limitation arises from the fact that DGGE is limited by the ability of the amplified fragment to be resolved on the gel, thereby heightening the detection limits compared to those obtained with Sanger or pyrosequencing methods. That means that a phylotype which is scare (<1% of the total membership) cannot be distinguished from background noise by the software used for fragment analysis (Sekirov et al., 2010). The third limitation lies in the fact that only relatively small fragments (≤ 500 base pairs) can be separated which restricts the quantity of sequence information for both phylogenetic inferences and probe design (Muyzer and Smalla, 1998).

2.4.1.2.2 Terminal restriction fragment length polymorphisms (TRLFP)

The difference between TRLFP and DGGE lies in the fact that the former uses full-length 16S rRNA PCR amplicons which have been obtained from the isolated community DNA. The terminal restriction fragment is created by adding restriction endonuclease to the PCR-amplified 16S rRNA gene products. This fragment is fluorescently tagged so it is detectable for a capillary sequencer and it
varies in length depending on the sequence of the 16S gene. The different fragment lengths subsequently migrate differently on the gel, through which a distinct pattern for each sample is created. The number and the size of the bands in the profile indicate variations between communities. An individual band represents an individual organism and by using a clone library this organism can be identified. TRFLP is considered to be rapid and reproducible and is used to measure variability within the human gut microbiota (Fei et al., 2007). A major drawback however is that co-migrating bands with PCR products with the same TRF will appear as a single phylotype, whilst this is not the case (Sekirov et al., 2010).

2.4.1.2.3 Ribosomal intergenic spacer analysis (RISA)

RISA is a technology commonly used by microbial ecologists to distinguish bacterial communities in marine and soil environment and is a relatively new technology when it comes to gut ecology (Sekirov et al., 2010). The novelty also causes one of the major drawbacks when it comes to the usage of RISA in gut ecology, because no extensive database on RISA taxonomy for gut micro-organisms yet exists. Before RISA can be used for a thorough analysis of gut microbiota this database should first be established. Another difficulty lies in the fact that bacteria have multiple RIS regions of variable length, thereby making it impossible to match one RIS fragment to a particular species which complicates bacterial identifications. However, despite these disadvantage, RISA also offers the major advantage of higher resolution when it comes to complex community analysis (Sekirov et al., 2010)

2.4.1.3 DNA microarrays

The first extensive DNA microarrays including probes designed for the detection of gastrointestinal microbiota were developed in the Brown laboratory. These were predicate upon an Agilent platform comprehending probes targeting up to 259 microbiota species and up to 316 noval operational taxonomic units (OTUs) (Palmer et al., 2006; Palmer et al., 2007) which is identified in studie of human colon microbial ecology (Eckburg et al., 2005). An even more sensitive microarray rendering 775 phylospecies was recently developed by Paliy et al., who used an Affymetrix Genechip platform and obtained their phylospecies clusters obtained from human feces and several sites in the colon. This platform was used because it has better sensitivity than other platforms. Comparative studies showed that this chip could detect and quantify differences in the intestinal microbiota of healthy adults and children and it also detected bacterial DNA present at 0.00025% of the total community DNA (Paliy et al., 2009). This phylogenetic microarray has several advantages: it is very powerful, because it was specifically designed for high through-put screening of the microbial communities in the human gut. Also it costs less time and is more profitable when compared to 16S sequencing methods, while maintaining similar levels of selectivity, sensitivity and quantification. Major disadvantages however stem from the fact that issues surrounding detection limits and hybridrization biases still exist (Sekirov et al., 2010).
2.4.1.4 Fluorence in situ hybridization (FISH) & quantitative real time polymerase chain reaction (qPCR)

Two other tools that can be used for screening of the microbiota of the gut are FISH and qPCR, which use fewer probes to focus on distinct groups of bacteria thereby being particularly beneficial when the focus lies on specific bacterial phylogenetic groups. FISH and qPCR are used together to compare and validate results obtained with other techniques (Sekirov et al., 2010; Kerckhoffs, 2009). Combining for instance PCR-DGGE could be used as a screening method to compare the bacterial composition of the samples. These and other nucleic acid-based methods are essential to the success of investigations of faecal microbiota, because they can detect every single bacterial species regardless of whether they have been cultivated (Tannock, 2005). Combining methods reduces potential biases and limitations of the individual techniques, thereby obtaining a more thorough understanding of the structure and function of the intestinal microbiota (Muyzer, 1999).

For the use of FISH, oligonucleotide probes are fluorescently labeled which hybridize to 16S rRNA sequences. This 16S rRNA sequence is unique to the targeted bacterial groups. It depends on the design of the probe whether FISH can be used more generally (to target large groups of bacteria) or more specific (genus or species-level classification) (Sekirov et al., 2010). FISH has been used in the case of enteric infection to characterize the associated phylum-level shifts which occur in the intestinal microbiota (Sekirov et al., 2008). It has also been used as a method for the comparison of intestinal microbiota between healthy and deceased individuals (Kerckhoffs, 2009). QPCR can also be used to target specific bacterial groups in complex mixtures and it also has the feature that it can be designed to be as general or specific as needed. The drawback with qPCR is that primers designed to magnify certain bacterial groups effects members of closely related groups as well (Sekirov et al., 2010).

The major disadvantage of these techniques and microarray lies in the fact that it is not feasible to identify new species or strains of bacteria because of the usage of chips, probes and primers which are designed for specific bacterial taxonomic groups. An additional drawback for qPCR it that a reference is strain is necessary for the interpretation of the results which can be problematic when no accurate culturable strain is available (Sekirov et al., 2010).

2.4.2 Function focused analysis

The techniques mentioned in the foregoing paragraphs study the composition and numbers of the bacterial communities in the gastrointestinal tract but it doesn’t study the function of these bacteria. To increase our knowledge on the ecology and function of the intestinal microbiota several additional tools are available which will be discussed in this paragraph (Sekirov et al., 2010).

2.4.2.1 Metagenomics

Metagenomics provides sequence information from the collective genomes of the intestinal microbial flora which can be used to identify the biological roles and function of this community in health and
disease. It does not depend on cloning and sequencing of individual genes, but rather provides a survey of all the genes existent within a community (Sekirov et al., 2010).

Metagenomics first uses shotgun sequencing in which cloned fragments isolated from community DNA are attached to each other into contigs which are subsequently assembled into scaffolds that are similar to whole genomes. In the case of intestinal microbiota, which is a complex bacterial community, whole genomes are rarely constructed because of the lack of sequence coverage obtained from a given sequencing run. There also is a lack of whole genomic sequence information in the public databases which also limits the possibility to identify gene function. The current, more feasible, way to use this technique is to enter metagenomic sequence reads directly into the database. The database subsequently gives information which helps determine the origins of particular genes (Tyson et al., 2004). Proteins are encoded by about 80% of the bacterial genome which makes it highly likely that a partial gene encoding a protein is included in each shotgun read (Noguchi et al., 2006). The protein-encoding genes are subsequently identified by BLASTp analysis and these identified genes are then given a COG (cluster of orthologous groups of proteins) based on the database (Moore et al., 1995). The COG database gives information about the function of the gene and other databases, such as KEGG, CAZymes and STRING can give additional functional information as well (Sekirov et al., 2010).

Although metagenomics can give various interesting insights into the intestinal microbiota, this technique also has its disadvantages. Plasmid preparation of the microbial DNA (required for shotgun sequencing) for instance can bias the number and type of genes sampled from the community. Furthermore, genes that are toxic to E.coli may not be included because they could not be reproduced in the host strain during cloning (Sorek et al., 2007; Sekirov et al., 2010).

2.4.2.2 Metaproteomics
Metaproteomics assesses diversity and abundance of proteins within the gut metaproteome by using non-targeted shotgun mass spectrometry. It is a relatively new technology and its results have been compared to the results of metagenomic analysis of two distinct sets of faecal human samples (Gill et al., 2011). Significant differences were identified which suggests that functional gene analysis does not necessarily correlate with gene expression levels (Verberkmoes et al., 2009). Metaproteomics has the advantage of the possibility to directly monitor the microbial protein expression levels. It furthermore can provide insight into the interactions which are required for the maintenance of host-microbiota symbiosis. Partly due to the novelty of technique several disadvantages however also exists and steps to optimize the technique are still pending (Sekirov et al., 2010).

2.4.2.3 Metabolomics
We can speak of metabolomics when we analyse simultaneously multiple small metabolites of a sample. This technique has been used to identify the profound and systematic impact of gut microbiota on the metabolism of the host. A major limitation lies in the fact that, due to the complexity of body fluids and tissues, it still is impossible to obtain a complete view of all the metabolites present in a sample (Sekirov et al., 2010).
2.4.2.4 Metatranscriptomics

This technique involves the characterization of the RNA content of samples which is also the major difference between metatranscriptomics and metagenomics which uses DNA content. The similarity between these techniques is the fact that both utilize high-throughput sequencing of nucleic acids derived from microbial populations and that they both can be used to generate structural information while obtaining functional insights. Due to the usage of RNA instead of DNA, metatranscriptomics has the advantage of providing knowledge on the dynamic nature of a community and the influences of changes of the environment on alterations in gene expressions. This technique has already been used to study microbiota in soil and aquatic environments but has not yet been applied to mammalian microbiota, so that the exact applicability of this technique remains to be seen (Sekirov et al., 2010).

A table is included in appendix 2 which contains all the mentioned techniques and its advantages and disadvantages.

3. MATERIAL AND METHODS

3.1 ANIMALS

The present study included two groups, the IBD group and the control group. The IBD group consisted of 15 dogs with IBD from the Faculty of Veterinary Medicine of Ghent University (Belgium) and from the private clinic Hond en Kat in Astene (Belgium). The control group consisted of 11 healthy dogs came from the Faculty of Veterinary Medicine of Utrecht University (The Netherlands) and from an animal shelter in Sint-Niklaas (Belgium).

The inclusion criteria of IBD in dogs consists of persistent (duration longer than 3 weeks) gastrointestinal signs, failed responses to dietary treatment (commercial intact protein elimination diet, hydrolysate elimination diet) and symptomatic therapies (paraciticides, antibiotics, anticholinergics, gastrointestinal protectants) alone, no systemic disease, exclusion of other possible causes of gastroenteritis, histopathological evidence of gastric, small intestinal or colonic inflammation on endoscopic biopsies (Jergens, 2004). Dogs with IBD were given a clinical score using the canine chronic enteropathy activity index (CCECAI) (Allenspach et al., 2007). The dogs were evaluated by nine variables: attitude and activity, appetite, vomiting, stool consistency, stool frequency, weight loss, ascites, pruritus and serum albumin concentration. The total score of the nine variables is determined to be clinically insignificant (score 0-3), mild (score 4-5), moderate (score 6-8), or severe (score 9 or greater)(Allenspach et al., 2010). None of the dogs were taken antibiotics for at least 3 weeks before sampling. Informed consent was obtained from the owners of all IBD dogs participating in the study.

The control dogs were clinical healthy or at least free of gastrointestinal signs. They were euthanized because of different reasons and purposes. No antibiotic was taken for at least 4 weeks before sampling.
3.2 SAMPLE COLLECTION
Samples were collected by the dog owners (IBD), during endoscopies (IBD) or after endoscopy (IBD), by the caretakers of the control dogs or obtained from the rectum after euthanasia was performed (control group). Although the methods of collection differ, samples are considered comparable because those collected after defecation were collected within 15 minutes thereby limiting the chance of contamination. All the faecal samples were immediately placed on dry ice and stored in a freezer at -80°C until assayed.

3.3 DNA EXTRACTION
DNA Extraction was achieved by use of the following protocol. Briefly, 0.5 g of RNase-free 0.1 mm-diameter zircon/silica beads (B.Braun Biotech International, Melsungen, Germany), 0.5 mL CTAB buffer (hexadecyltrimetlyammonium bromide 5% (w/v), 0.35 M NaCL, 120 mM K2HPO4 and 0.5 mL phenol-chloroform-isoamyl alcohol mixture (25:24:1) (BioUltra, Bornem, Belgium) were added to 100 mg faeces. The mixture was homogenized 3 times for 120 seconds using a Bead beater (B. Braun Biotech International) at 5,000 rpm. The supernatant was collected after centrifugation at 8000 rpm for 10 minutes. For a second time, 0.25 mL of CTAB buffer was added to original DNA sample, which was again homogenized in the Bead beater and supernatant was collected after centrifugation. Then, the phenol was removed by adding an equal volume of chloroform-isoamyl alcohol (24:1) followed by centrifugation at 16,000 g for 10 seconds. The aqueous phase was transferred to a new tube and 2 volumes of PEG-6000 solution (polyethyleenglycol 30% (w/v). 1.6 M NaCl) was added. The precipitation was performed for 2 h at room temperature. The pellet was obtained by centrifugation at 13,000 g for 20 minutes and washed with 1 mL of ice-cold 70% (v/v) ethanol. After centrifugation at 13,000 g for 20 minutes the pellet was dried and resuspend in 100 μL RNA free water. DNA concentration was measured with the Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

3.4 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)
DGGE based on the protocol of Muyzer et al. (1993) was performed using the Bio-Rad D gene system (Bio-Rad, Hercules, CA) (Muyzer et al.,1993). PCR fragments were loaded onto 8% (w/v) polyacrylamide gels in 1xTAE (20mM tris, 10 mM acetate, 0.5mM EDTA, pH 7.4). To separate the amplified DNA fragments, the polyacrylamide gels were made with denaturing gradients ranging from 45% to 60%. On each gel, a homemade marker of different PCR fragments was loaded, which was required for processing and comparing the different gels. The electrophoresis was run for 16h at 60 °C and 38 V. The obtained DGGE patterns were subsequently normalised and analysed with the BioNumerics software version 2.0 (Applied Maths, Kortrijk, Belgium). During this processing, the different lanes were defined, background was substracted, differences in the intensity of the lanes were compensated during normalisation, and the correlation matrix was calculated (Possemiers et al., 2004).

3.5 REAL-TIME QUANTITATIVE PCR (qPCR)
The quantification of DNA by real-time PCR was performed with the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The amplification and detection
were carried out in 96-well plates using SYBR-green low ROX 2x master mix (Applied Biosystems, Foster City, CA). Each reaction was done in triplicate in a 12 μl of appropriate dilutions of the DNA sample. The primer set used in this study are listed in Table 3. The final primer concentration used is different for the bacteria groups, the annealing temperature is also listed in Table 3. A melting curve analysis was done after amplification. Quantitation was done by using standard curves made from known concentrations of plasmid DNA containing the respective amplicon for each set of primers.

Table 3. PCR details

<table>
<thead>
<tr>
<th>Bacteria group (and reference)</th>
<th>Primers</th>
<th>Primer concentration</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Bacteria (Lee et al., 1996)</td>
<td>F: CCGYCCAGACTCCTACGGG R: TTACCGCGGCTGCTGGCA</td>
<td>0.5 μM</td>
<td>53°C, 1 min</td>
</tr>
<tr>
<td>Firmicutes (Guo et al., 2008)</td>
<td>F: GGAAYATGTGTATTTAATTCGAAGCA R: AGCTGACGACAACCATGCAC</td>
<td>0.5 μM</td>
<td>60°C, 1 min</td>
</tr>
<tr>
<td>Clostridium Cluster IV (Matsuki et al., 2004)</td>
<td>F: ATGCAAGTCGAGCGA(G/T)G R: TATGGGTATATATCT(C/T)CCTTT</td>
<td>0.5 μM</td>
<td>60°C, 1 min</td>
</tr>
<tr>
<td>Clostridium Cluster XIV (Song et al., 2004)</td>
<td>F: GAWGAAGTATYTCGTATGT R: CTACGCGWCCCTTTAC</td>
<td>0.5 μM</td>
<td>55°C, 1 min</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Primers</th>
<th>Primer concentration</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bytyryl-CoA acetaat-CoA transferase (BCoAT) (Charrier et al., 2006)</td>
<td>F: AAGGATCTCGGIRTICAYWSIGARATG R: GAGGTCGTCICKRAITYIGGRTGN</td>
<td>2.5 μM</td>
<td>53°C, 30 sec. (3 steps)</td>
</tr>
</tbody>
</table>

3.6 STATISTICAL ANALYSES
Bionumeric software version was used to obtain the results of the DGGE. Richness was analysed using the student T-test which is carried out using Microsoft Excel (Microsoft Corporation). All statistical analyses on PCR results were performed using SPSS software (SPSS Inc., Chicago, IL, USA). The data is expressed as a mean ± standard deviation. For the correlation between IBD score and bacterial species the Pearson correlation was applied using SPSS. A P-value <0.05 was considered significant.
4. RESULTS

4.1 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

In this study a DGGE, based on the protocol of Muyzer et al. (1993), was performed on the faeces of eleven healthy control dogs (C1 – C11) and fifteen dogs with IBD (D1 – D15). The banding patterns above visualize both the richness and abundance of the microbial communities in each individual sample (see Figure 6.). Each individual band in a lane represents a certain type of bacteria, and the intensity of the band represents the abundance of that certain type of bacteria. The quantity of bands in one lane then represents the richness of the microbiota (the number of species of bacteria) in the sample, whilst the intensity of the band represents the amount of that specific type of bacteria.

The cluster results from DGGE were analysed using Pearson correlation, which takes in account both richness and intensity of the bands. The higher the Pearson correlation between multiple bands, the more similar these samples are when it comes to richness and intensity of the microbial community. This correlation is visualized by the dendogram on the left side of the figure 6. The shorter the lines of the dendogram, the more similar the two samples are regarding the richness and the intensity. Similarity can range between 0% (no similarity) and 100% (identical).

The similarity between the samples in this research ranges from 42.5% (all samples) to 95% (cluster C2 – C3 and cluster D3 – D4). The clusters differ regarding to the total number of dogs, but also when it comes to whether or not control dogs are correlated to dogs with IBD. At first sight this means that control dogs have similarities with dogs with IBD regarding the intensity and richness of the faecal microbial community.
In order to have a more thorough look at the clusters, the Pearson correlation of each cluster and the members of each cluster were identified. The purpose was to have a closer look at the composition of the cluster, i.e. whether the cluster consisted of samples of healthy dogs, dogs with IBD or a combination of those. It is striking that clusters with the highest correlation rate consist of either dogs with IBD or healthy dogs. When clusters consist of four or more members they consist of a combination of both healthy and IBD dogs and the Pearson correlation drops significantly. This means that high similarities are only seen in cluster consisting of a few samples which indicates that there is high inter-individual variation when it comes to richness and abundance.

The samples can be compared on the richness of the microbiota as well, i.e. the number of bacterial species in the sample. The richness of the samples is visualized in the graph below (see Figure 7).

![Species richness of the control dogs (C1-C10) and IBD dogs (D1-D15)](image)

Figure 7 Species richness of the control dogs (C1-C10) and IBD dogs (D1-D15)

The student’s t-test indicated that there are no significant differences in the richness of the microbiota of IBD-dogs compared to healthy dogs ($P=0.476$). But although there are no major differences identifiable between the healthy and the IBD-dogs, interesting observations still can be made. Outliers defined as being most different from the average, can be seen in the sample D2 and D5, which indicates that richness of faecal microbiota in IBD-dogs can be quite varying amongst individuals. The control dogs also show inter-individual diversity with one (negative) outlier in the sample C8.

The foregoing description took into account the richness and intensity of the bacterial intestinal microbiota. The community evenness, i.e. a measurement of the stability and balance of the microbiota, can be visualized with a Lorenz Curve (Wittebolle et al., 2009). Perfect evenness, i.e. a stable and balanced microbiota, is visualized by a line which runs in a 45 degree angle, right through the middle. The horizontal axis represents the proportion of species (richness) in the sample and the vertical axis represents the abundance of the species (intensity) of the sample. Perfect evenness
exists when all existing species are equally abundant. The graph below (see Figure 8) shows that the C8-line deviates most of the perfect evenness-line, indicating that this sample has the most imbalanced and unstable microbiota (less richness and more intensity per species. It is furthermore striking that most of the C’s (samples from control dogs) deviate from the perfect evenness-line when compared with the D’s (samples from IBD-dogs). The order of the lines (from top to bottom) is approximately C8-C9-C10-D14-C7-D13-C3-C11-C5-C1-D12-C6-D3-C2-D7-D9-D5-C4-C7-D2-D4-D6-D10-D8-D1-D15-D11-D13-D9.

![Figure 8 Lorenz curve based on DGGE analysis derived from the samples C1-C10 and D1-D10](image-url)
The Lorenz curve furthermore can be used to calculate the Community Organisation (CO) (see Figure 9). CO is calculated by measuring GINI (the area between the Lorenz Curve and the perfect evenness-line) and multiplying GINI by a hundred. It thereby is another measurement of the equality, i.e. the stability and balance of the microbiota. The higher the GINI, and thereby the CO, the more the line deviates from the perfect evenness-line and the more unstable and imbalanced that specific microbial community is. As can be seen in the table below C8 shows the highest CO (and GINI) and this means that this specific microbial community is the most imbalanced and unstable. C6 on the other hand shows the lowest CO which represents the stability and balance of this specific microbial community. The student’s t-test furthermore shows that no significant differences ($P=0.234$) with respect to CO exist between the samples of the IBD-dogs and the samples of the healthy dogs.

![Figure 9 Community organisation of the control dogs (C1-C10) and IBD dogs (D1-D15)](image)

### 4.2 REAL-TIME QUANTITATIVE PCR (qPCR)

Detection of single bacterial species in the samples was obtained through qPCR and the results of the IBD-samples were compared with those of the control-samples. Significant differences between the control group and the IBD group did not exist in bacterial concentrations of Total Bacteria ($P=0.98$), *Firmicutes* ($P=0.87$), *Clostridium Cluster IV* ($P=0.82$), *Clostridium Cluster XIV* ($P=0.86$) and *Butyryl-CoA acetate-CoA transferase* ($P=0.86$) (see Table 4).
Table 4 qPCR results in faecal samples of dogs with IBD and healthy control dogs

<table>
<thead>
<tr>
<th></th>
<th>IBD</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacterial</td>
<td>10.48 ± 0.59</td>
<td>10.49 ± 0.74</td>
<td>0.98</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>10.57 ± 0.54</td>
<td>10.53 ± 0.70</td>
<td>0.87</td>
</tr>
<tr>
<td>Clostridium Cluster IV</td>
<td>8.87 ± 1.24</td>
<td>8.74 ± 1.66</td>
<td>0.82</td>
</tr>
<tr>
<td>Clostridium Cluster XIV</td>
<td>9.22 ± 0.43</td>
<td>8.87 ± 1.35</td>
<td>0.86</td>
</tr>
<tr>
<td>Butyryl-CoA acetate-CoA transferase</td>
<td>7.02 ± 0.81</td>
<td>6.94 ± 1.46</td>
<td>0.86</td>
</tr>
</tbody>
</table>

The amount of individual bacterial species were compared with the IBD-scores of the diseased dogs, which allowed a more thorough look at the correlation between the amount of bacteria in the faecal samples and the severity of the disease as expressed by the IBD-score (see Table 5.).

Table 5 Correlations between IBD score and

<table>
<thead>
<tr>
<th>IBD score</th>
<th>Total bacteria</th>
<th>Firmicutes</th>
<th>Cluster IV</th>
<th>Cluster XIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBD score</td>
<td>Pearson</td>
<td>Significance</td>
<td>Pearson</td>
<td>Significance</td>
</tr>
<tr>
<td></td>
<td>-.611*</td>
<td>.046</td>
<td>-.595</td>
<td>.053</td>
</tr>
<tr>
<td></td>
<td>-.718*</td>
<td>.013</td>
<td>-.783**</td>
<td>.004</td>
</tr>
<tr>
<td>Butyryl-CoA</td>
<td>-.787*</td>
<td>.004</td>
<td>.691*</td>
<td>.019</td>
</tr>
<tr>
<td></td>
<td>.684*</td>
<td>.020</td>
<td>.845**</td>
<td>.001</td>
</tr>
<tr>
<td></td>
<td>.757*</td>
<td>.007</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Significant negative correlations were found between the IBD-score and Total Bacteria (P=0.046) Clostridium Cluster IV (P=0.013), Clostridium Cluster XIV (P=0.004), and Butyryl-CoA acetate-CoA transferase (P=0.004). A positive correlation can be seen between Butyryl-CoA acetate-CoA transferase and Clostridium Cluster IV (P=0.001) and Clostridium Cluster XIV (P=0.007).

5. DISCUSSION

This study aimed at investigating the butyrate producing microbiota of IBD-dogs compared with that of healthy dogs by applying DGGE and qPCR on faecal samples. This thesis is part of a larger project studying the health benefits of dietary treatment of canine IBD. Identification of the intestinal microbiota in IBD-dogs therefore is important because changes caused by dietary treatment subsequently can be compared with the baseline measurements. The connection between a lack of butyrate producing bacteria and IBD, as described earlier in this thesis, is proven in human IBD but remains unstudied in canine IBD. The aim of this thesis is therefore primarily focused on the role of
butyrate producing bacteria in canine IBD. The phyla Firmicutes is the biggest producer of butyrate, and amongst others consists of Clostridium Cluster IV and Clostridium Cluster XIV which are important butyrate producing bacteria. Because of this high butyrate production we chose to highlight Firmicutes and Clostridium Cluster IV and XIV in this thesis. Butyryl CoA acetate-CoA transferase furthermore allows us to expose all different butyrate producing bacteria within the intestinal microbiota. For the purpose of determining the amount of the butyrate producing bacteria we therefore chose to include Butyryl CoA acetate-CoA transferase in our analysis. QPCR was used for the analysis of the aforementioned bacteria and function gene and because we used specific primers these could be easily determined. The combination of qPCR and DGGE was used to compare the bacterial composition of the samples.

Changes of the intestinal microbiota in both human and canine IBD when compared to healthy control groups are well-described in literature. In this study we therefore expected a decrease of Firmicutes (especially Clostridiales) in the faecal samples of IBD-dogs when compared to healthy dogs (Jergens et al., 2010; Suchodolski, 2011; Xenoulis et al, 2008). The Firmicutes, are especially interesting for this study because of their capacity to produce butyrate. In this study however the amount of these bacteria in the faeces of IBD-dogs were fairly similar to those of the healthy dogs. The DGGE showed several clusters which consisted of both healthy and IBD-samples, indicating that the samples in those distinct clusters were comparable in the richness and intensity of the microbiota. Research on microbiota has shown reduced species richness in the small and large intestine of dogs with IBD (Xenoulis et al., 2008), an observation which we would expect in the faecal samples we obtained and which would be represented by the clustering of the samples. We would therefore have expected a sharper distinction between clusters with healthy samples and clusters with IBD-samples, i.e. fewer clusters in which healthy and IBD-samples were combined, which indicated that the richness and intensity of the group of IBD-dogs substantially differ from the richness and intensity of the group of control dogs. Because of these unexpected results it was decided to have a more thorough look at the individual members of each cluster combined with the Pearson correlation of that cluster. This showed that clusters which contained solely IBD-samples or solely healthy samples had higher Pearson correlations, indicating that the members of those clusters were more comparable to each other when it comes to richness and intensity of the microbiota than the members of the clusters with both healthy and IBD-samples. We also looked at the richness of each individual sample. As mentioned earlier, richness is expected to be reduced in samples of the IBD-dogs. The results from our study did however not show significant differences in the richness of the microbiota in the samples of IBD-dogs when compared to those of healthy dogs. The Lorenz curve and CO also yielded results which we did not expect. The samples from healthy dogs deviated more from the perfect evenness-line than the samples of IBD-dogs, thereby implying that the dogs with IBD had a more stable and balanced microbiota when compared to healthy dogs. These differences however proved insignificant when a Student’s t-test was performed, thereby indicating that the stability and balance of the microbiota of IBD-dogs were comparable to those of healthy dogs. The Lorenz curve furthermore shows that a control sample (C8) has the least balanced and least stable microbiota of all samples, which we would have expected in an IBD-sample.
We can conclude that the obtained DGGE results did not meet our expectations and that the results do not yield reliable information. This is due to the several limitations of DGGE. The first limitation is that a single band can represent multiple bacterial strains. DGGE furthermore only has the ability to separate relatively small fragments which restricts the quantity of sequence information. The last disadvantage is that the detection limits for phylotypes present in a less large quantity are very low. For this study, DGGE was not able to produce desired results.

The fact that our results differ from the results obtained from other studies can be partly explained by the methods we used but also by the samples we chose. Faecal samples, as used in our study, are easily accessible but are not always considered as an adequate measure of the intestinal microbiota. It is indicated that faeces are unable to represent the microbiota of the upper gut and that it does not take into account the differences in the intestinal microbiota of the various compartments of the gastrointestinal tract (Mentula et al., 2005). Other studies mainly used biopsies of the gastrointestinal tract itself, thereby identifying mucosal microbiota instead of the luminal microbiota which is measured by the usage of faecal samples (see table 5 and appendix 1) (Allenspach et al., 2010; Jergens et al., 2010; Simpson et al., 2006; Suchodolski et al., 2010; Xenoulis et al., 2008). This difference could be fundamental for the unexpected results obtained in this study.

Table 5 Different studies on canine IBD and the samples and methods they use (Adapted from Suchodolski, 2011)

<table>
<thead>
<tr>
<th>Refs.</th>
<th>Sample material</th>
<th>Diagnosis</th>
<th>Method</th>
<th>Microbial alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suchodolski et al. 2010</td>
<td>Duodenal biopsies</td>
<td>IBD</td>
<td>Comparative 16s RNA gene analysis</td>
<td>↑ Proteobacteria</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ Clostridia (class)</td>
</tr>
<tr>
<td>Allenspach et al. 2010</td>
<td>Duodenal brush samples</td>
<td>German Shepard dogs with food-or antibiotic responsive diarrhea</td>
<td>Comparative 16s RNA gene analysis</td>
<td>↑ <em>Streptococcus</em> and <em>Abiotrophia</em> spp.</td>
</tr>
<tr>
<td>Jergens et al. 2010</td>
<td>Duodenal biopsies</td>
<td>IBD</td>
<td>16s RNA gene 454-pyrosequencing</td>
<td>↑ Proteobacteria</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ Clostridium cluster</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>XIV and IV</td>
</tr>
<tr>
<td>Xenoulis et al. 2008</td>
<td>Duodenal brush samples</td>
<td>IBD</td>
<td>Comparative 16s RNA gene analysis</td>
<td>↑ <em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ Microbial diversity</td>
</tr>
<tr>
<td>Craven et al. 2009</td>
<td>Duodenal biopsies</td>
<td>Chronic enteropathies (steroid-, food-, and antibiotic-responsive)</td>
<td>16s RNA gene 454-pyrosequencing</td>
<td>↓ Microbial diversity</td>
</tr>
<tr>
<td>Simpson et al. 2006</td>
<td>Colonic biopsies</td>
<td>Boxer dogs with granulomatous colitis</td>
<td>FISH</td>
<td>Intraepithelial invasion of adherend and invasive <em>E. coli</em></td>
</tr>
</tbody>
</table>
Corresponding protocols for both DGGE and qPCR were carefully followed, thereby reducing the risk of human error to a minimum and which allows the reliable comparison of the samples (Charrier et al., 2006; Guo et al., 2008; Lee et al., 1996; Matsuki et al., 2004; Muyzer et al., 1993).

By using qPCR we were able to detect single bacterial species and Butyryl CoA acetate-CoA transferase. Butyryl CoA acetate-CoA transferase plays an important role in butyrate fermentation and in protection against IBD (Pryde et al., 2002). The butyryl-CoA acetate-CoA transferase gene allows us to detect uncultured groups of butyrate-producing bacteria within the intestinal community (Louis et al., 2010). When we compare the IBD-samples with the control-samples no significant differences are found. This does not meet our expectations because we would expect a lower count of Total bacteria, Firmicutes, Clostridium Cluster IV, Clostridium Cluster XIV as described in earlier studies (Jergens et al., 2010; Suchodolski, 2010).

However when we correlate the IBD-scores from the IBD dogs with Total bacteria, Firmicutes, Clostridium Cluster IV, Clostridium Cluster XIV and Butyryl-CoA acetate-CoA transferase we can find some interesting results. Correlations between IBD-score and Total Bacteria \( (P=0.046) \) Clostridium Cluster IV \( (P=0.013) \), Clostridium Cluster XIV \( (P=0.004) \) are significantly negative which indicates that there is a negative link between the amount of these bacteria and IBD-score, i.e. the higher the IBD-score the lower the amount of these bacteria. When we correlate Butyryl-CoA acetate-CoA transferase with Clostridium Cluster IV \( (P=0.001) \) and Clostridium Cluster XIV \( (P=0.007) \) we find a positive correlation. This indicates a positive relation, i.e. the higher the existence of Butyryl-CoA acetate-CoA transferase the higher the amount of Clostridium Cluster IV and Clostridium Cluster XIV is. The last result is the negative correlation between the IBD-score and Butyryl-CoA acetate-CoA transferase \( (P=0.004) \). These results show us that if the IBD-score is high, the amount of investigated bacteria in general and Butyryl-CoA acetate-CoA transferase in particular is lower. These finding meet the results described in earlier studies on reduced richness and, since Butyryl-CoA acetate-CoA transferase is primarily responsible for its production, a lack of Butyrate in IBD.

6. CONCLUSION

In conclusion, no differences in total bacteria, Firmicutes, Clostridium cluster IV, Clostridium cluster XIV and Butyryl-CoA acetate-CoA transferase were found in the faecal microbiota of dogs with IBD compared to healthy dogs. Significant negative correlations were however found between the IBD-scores of the diseased dogs and the amount of Clostridium Cluster IV, Clostridium Cluster XIV and Butyryl-CoA acetate-CoA transferase. The first two are butyrate-producing bacteria and Butyryl-CoA acetate-CoA transferase is a function gene. This correlation could indicate that the concentration of butyrate-producing bacteria is linked to the severity of IBD, which suggests that administration of probiotics with butyrate producing bacteria in dogs with IBD might alleviate the severity of the disease.
7. REFERENCES


APPENDICES

Appendix 1. microbial changes in dogs and cats with gastrointestinal disease (adapted from Suchodolski, 2010)

Appendix 2. Analyses techniques for gut microbiota (adapted from Sekirov, 2010)
<table>
<thead>
<tr>
<th>Species</th>
<th>Sampling location</th>
<th>Tissue type</th>
<th>Disease</th>
<th>Method</th>
<th>Microbial changes in diseased animals</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cats</td>
<td>Small intestine</td>
<td>Biopsies</td>
<td>IBD</td>
<td>FISH</td>
<td>Increase in Enterobacteriaceae</td>
<td>Janeczko et al., 2008</td>
</tr>
<tr>
<td>Dogs</td>
<td>Duodenum</td>
<td>Biopsies</td>
<td>IBD</td>
<td>16S rRNA gene clone libraries</td>
<td>Increase in Proteobacteria; decrease in Clostridia</td>
<td>Suchodolski et al., 2010</td>
</tr>
<tr>
<td>Dogs</td>
<td>Duodenum</td>
<td>Mucosal/luminal brushings</td>
<td>IBD</td>
<td>16S rRNA gene clone libraries</td>
<td>Increase in Enterobacteriaceae (Escherichia coli); reduction in biodiversity</td>
<td>Xenaoulis et al., 2008</td>
</tr>
<tr>
<td>Dogs</td>
<td>Duodenum</td>
<td>Mucosal/luminal brushings</td>
<td>Chronic enteropathies (FRD, ARD)</td>
<td>16S rRNA gene clone libraries</td>
<td>Increase in Lactobacillales (Streptococcus and Abiotrophia)</td>
<td>Allenspach et al., 2010</td>
</tr>
<tr>
<td>Dogs</td>
<td>Duodenum</td>
<td>Biopsies</td>
<td>IBD</td>
<td>454-pyrosequencing of the 16S rRNA gene</td>
<td>Increase in Proteobacteria; decrease in Faecalibacterium, Ruminococcus, and Dorea spp. within the Clostridium clusters IV and XIVa</td>
<td>Jergens et al., 2010</td>
</tr>
<tr>
<td>Dogs</td>
<td>Duodenum</td>
<td>Biopsies</td>
<td>Chronic enteropathies (SRD, FRD, ARD)</td>
<td>454-pyrosequencing of the 16S rRNA gene</td>
<td>Reduced biodiversity</td>
<td>Craven et al., 2009</td>
</tr>
<tr>
<td>Cats</td>
<td>Feces</td>
<td>Fecal samples</td>
<td>Small and large bowel IRD</td>
<td>FISH</td>
<td>Decreased total bacteria, Bifidobacterium spp. and Bacteroides spp.; increase in Desulfovibrio spp.</td>
<td>Inness et al., 2007</td>
</tr>
<tr>
<td>Dogs</td>
<td>Feces</td>
<td>Fecal samples</td>
<td>Chronic diarrhea</td>
<td>FISH</td>
<td>Increase in Bacteroides</td>
<td>Jia et al., 2010</td>
</tr>
<tr>
<td>Dogs</td>
<td>Feces</td>
<td>Fecal samples</td>
<td>Diarrhea</td>
<td>T-RFLP</td>
<td>Increases in Clostridium perfringens, Enterococcus faecalis, and Enterococcus faecium</td>
<td>Bell et al., 2008</td>
</tr>
<tr>
<td>Dogs</td>
<td>Colon</td>
<td>Biopsies</td>
<td>Granulomatous colitis of Boxer dogs</td>
<td>FISH</td>
<td>Intracellular translocation of adherent and invasive E. coli</td>
<td>Simpson et al., 2006</td>
</tr>
</tbody>
</table>

1IBD = inflammatory bowel disease; FISH = fluorescence in situ hybridization; ARD = antibiotic responsive diarrhea; FRD = food-responsive diarrhea; SRD = steroid-responsive diarrhea; T-RFLP = terminal RFLP.

**Appendix 1.** Microbial changes in dogs and cats with gastrointestinal disease.
### Appendix 2. Analyses techniques for gut microbiota

<table>
<thead>
<tr>
<th>Technique</th>
<th>16s RNA based?</th>
<th>Cost (€)</th>
<th>Taxonomic Resolution/Sensitivity</th>
<th>Advantages</th>
<th>Disadvantages (limitations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture based</td>
<td>No</td>
<td>€</td>
<td>Moderate</td>
<td>- You have the organism “in hand”</td>
<td>- Most GI organism cannot be cultured in current defined media</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Functional information gained from what is known about the organism’s substrate utilization and other physiological parameters</td>
<td>- Labour intensive</td>
</tr>
<tr>
<td>Full-length (Sanger) sequencing</td>
<td>Yes</td>
<td>€€</td>
<td>Very good</td>
<td>Sequencing the entire 16S gene maximizes the taxonomic resolution offered by the gene</td>
<td>- Expensive (although mechanization is reducing the cost)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>€€</td>
<td></td>
<td></td>
<td>- Extensive bioinformatics analysis required</td>
</tr>
<tr>
<td>454 Pyrosequencing</td>
<td>Yes</td>
<td>€€</td>
<td>Good to very good</td>
<td>- Higher throughput than Sanger Sequencing (200,000 sequences versus 20,000)</td>
<td>- Can’t obtain the taxonomic resolution that can be achieved with full-length sequencing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>€</td>
<td></td>
<td>- More sensitive (can detect less abundant organisms due to the numbers of reads obtained)</td>
<td>- Shorter sequence reads (&lt;500 bp versus 1,5 kb)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Multiple samples can be analysed in a single sequencing run</td>
<td>- Extensive bioinformatics analysis required</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- No cloning bias introduced</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Less susceptible to PCR bias (shorter PCR amplicons, less influenced by G/C content)</td>
<td></td>
</tr>
<tr>
<td>DGGE</td>
<td>Yes</td>
<td>€</td>
<td>Poor</td>
<td>- Rapid</td>
<td>- Shorter PCR products mean less taxonomic information can be obtained</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Fingerprints provide a good basis to compare communities from different treatment groups</td>
<td>- Reproducibility between gels is difficult.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Bands of interest can be excised and sequenced</td>
<td></td>
</tr>
<tr>
<td>TRFLP</td>
<td>Yes</td>
<td>€€</td>
<td>Poor</td>
<td>- Fingerprints provide a good basis to compare communities</td>
<td>- Limited taxonomic resolution</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Multiple restriction enzymes can be used to provide greater resolution</td>
<td>- One ‘phylootype’ can represent more than one species</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Reproducible</td>
<td>- Capillary sequencer required</td>
</tr>
<tr>
<td>RISA</td>
<td>No</td>
<td>€€</td>
<td>Good</td>
<td>- Greater variability between species and strains than the 16S gene</td>
<td>- Limited phylogenetic data currently available; no extensive RIS database developed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- When a better database has been developed, taxonomic</td>
<td></td>
</tr>
<tr>
<td>Method</td>
<td>Validation</td>
<td>Cost</td>
<td>Quality</td>
<td>Advantages</td>
<td>Disadvantages</td>
</tr>
<tr>
<td>-----------------------</td>
<td>------------</td>
<td>------</td>
<td>---------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
</tbody>
</table>
| DNA microarrays       | Yes        | €€   | Very good | - Incredibly useful as a screening approach  
                      | - Fast, easy to use  
                      | - Clinical applications | - Detection limited by the sequences contained on the chip (no detection of uncharacterized phylotypes)  
                      | - Cross-hybridization issues |
| FISH                  | Yes        | €€   | Good     | - Can target specific bacterial groups/species of interest (they must be preselected)  
                      | - Flexible scope: probes can be designed to target groups of individual species  
                      | - Direct enumeration of bacteria-16S copy number is not an issue | - Can’t identify novel groups of bacteria  
                      | - It’s not a community-wide survey of “who’s there”  
                      | - Reference strains are required to validate the results  
                      | - Microscope work is time-consuming (however FACS options are becoming available) |
| qPCR                  | Yes        | €€   | Good     | - Can target specific bacterial groups/species of interest (they must be preselected)  
                      | - Flexible scope: primers can be designed to target groups or individual species | - Reference strains are required to validate the results  
                      | - Can’t identify novel groups of bacteria  
                      | - It’s not a community-wide survey of “who’s there”  
                      | - 16S copy number varies between 1 and 10 |
| Metagenomics          | Genome wide | €€   | Good     | - Provides a community-wide assessment of the functional genes present  
                      | - 16S gene sequences provide taxonomic identification of community members  
                      | - Sensitivity depends on the number of sequence reads obtained | - Shotgun reads are mapped to reference genomes; this is limited by the number of genomes available  
                      | - extensive bioinformatics analysis required  
<pre><code>                  | - Cloning biases could affect the |
</code></pre>
<table>
<thead>
<tr>
<th>Method</th>
<th>Level</th>
<th>Cost</th>
<th>Functionality</th>
<th>Advantages</th>
</tr>
</thead>
</table>
| Metabolomics        | No    | €€   | Poor          | - Metabolic profiles can be used to compare communities in a functional context  
- More direct functional information can be obtained  
- More rapid and less expensive than metagenomics  
- Nontargeted approach can also identify host metabolites associated with the gut microbiota                                                                   |
| Metaproteomics      | No    | €€   | Poor          | - Metaproteomes can be used to compare communities in a functional context  
- More direct functional information can be obtained  
- More rapid and less expensive than metagenomics  
- Nontargeted approach can also identify host proteins associated with the gut microbiota                                                                                           |
| Meta-transcriptomics| Genome wide | €€ | Good          | - Provides insights into community-wide structure and function  
- Can be used to detect changes in community-wide gene expression profiles in response to different environmental stimuli  
- No biases introduced by PCR or cloning steps (none required)  
- Transcripts can be measured quantitatively                                                                                       |

| Functional gene information obtained | - No direct information about which genes are expressed or functioning |
| No taxonomic information available | - The source of each metabolite is unknown; therefore, it is difficult to identify what organisms are producing what compound |
| Not all metabolites are detectable with current technology | - Protein abundance is difficult to estimate |
| Less abundant proteins (from populations making up <1% of the community) go undetected | - RNA is much more easily degraded than DNA; this could cause information loss |
| Sensitivity of community analysis depends on the number of sequence reads (via pyrosequencing) obtained | - Sensitivity of community analysis depends on the number of sequence reads (via pyrosequencing) obtained |