The significance of fungal organisms in canine enteropathies

"Establishing a qPCR and determining the prevalence of fungal organisms in canine feces"

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November 2009 - February 2010
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Summary

Until now little research has been done evaluating the prevalence of fungal organisms in the gastrointestinal tract of dogs. Even less is known about the effects of fungal organisms on gastrointestinal health and disease. The aim of this study was to establish a quantitative real time PCR protocol (qPCR) to investigate the prevalence of fungal organisms in healthy dogs and dogs with signs of gastrointestinal disease, and to quantify fungal organisms in the canine feces.

Fecal samples from a total of 119 dogs (49 healthy dogs and 70 dogs with signs of gastrointestinal disease) of different age, breed, and sex were obtained. DNA was extracted from 100 mg of fecal sample by a modified bead beating method using phenol/chloroform. On all samples qPCR's were performed using universal fungal and universal bacterial primers to amplify the eukaryotic ITS2 region and parts of the bacterial 16S rRNA gene, respectively. Standard 1:10 serial dilutions containing 8,400 to 0.0084 pg of fungal DNA and 4,000 to 0.04 pg of bacterial DNA were used to calculate the starting DNA quantity for each specific sample. The obtained universal fungal data was normalized by the universal bacterial data to account for intersample variation during DNA extraction. All samples were run in duplicates on a commercially available real time PCR thermocycler. (iCylcer iQ, BioRad). The negative DNA extraction control was further investigated after PCR amplification by cloning and sequencing.

The negative DNA extraction control yielded a positive signal after 33 cycles during the universal fungal qPCR assay. A cut-off value of 27 cycle times (corresponding to approximately 100-fold more starting DNA quantity than present in the negative DNA extraction control) was used to determine which samples could truly be considered positive. From all analyzed samples, 26 of the healthy dogs (53%) and 23 of the dogs with signs of gastrointestinal disease (33%) were positive for fungal DNA ($p=0.0373$). For all positive samples the ratio of universal fungal to universal bacterial DNA was calculated: healthy dogs had a median ratio of 0.0002779 (range 1.701×10^{-5} – 0.02543, $p = 0.0013$), and the median for the diseased animals was 0.0001879 (range 1.116×10^{-5} – 0.1405, $p = 0.0009$).

This study suggests that healthy dogs have a significantly higher prevalence of fungal organisms compared to dogs with gastrointestinal signs. The relative quantities in fungal DNA between these two groups did not differ. Fungal organisms appear to be part of the normal intestinal microbiota, and the intestinal ecosystem may be disrupted in dogs with gastrointestinal disease. More research is warranted to obtain a more accurate prevalence and determine the role of fungal organisms in the gastrointestinal tract of dogs.

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Introduction

1.1 Gastrointestinal disease in dogs

Gastrointestinal disease in dogs can be classified in many different ways. Current means of classification include chronic versus acute, and small intestinal versus large intestinal disease. The causes of gastrointestinal disease in dogs can be as diverse as the classifications. One of the not yet fully understood gastrointestinal disease complexes is inflammatory bowel disease (IBD). IBD is a disease that in dogs and cats can affect any part of the gastrointestinal (GI) tract, and of which the exact pathogenesis is unknown to this day.[1] Over the past years, however, extensive research has been done to further elucidate this disease complex in companion animals.[1, 2]
Based on human and mouse models, several hypothesis about the possible cause have been formed (Box 1). Human IBD is a multi-factorial disease in which the innate and adaptive immune system along with the luminal microbiota and food antigens are being recognized as possible causes for the deviant immune response.[1-5]
Although there are obvious clinical and histopathological differences between human IBD and IBD in companion animals, there is evidence that the molecular pathogenesis of the two might be similar.[1, 2]

The pathogenesis of inflammatory bowel disease in humans is believed to encompass the following groups of causative factors:
1) The mucosal barrier
2) The host immune system (comprising the innate and adaptive immune system)
3) Microbes and food antigens in the intestinal lumen

The hypothesized pathogenesis of IBD starts with the innate immune system failing to distinguish commensals from pathogens. This leads to a large increase in IL-23 production, with differentiation of naïve T cells into Th17 cells. These cells then start production of large amounts of pro-inflammatory cytokines (e.g. IL-17, IL-22 and TNF), which leads to tissue destruction and epithelial injury, hereby letting more antigens pass into the lamina propria and restarting the entire procedure over again.

Box 1 Brief summary of the hypothesis of the cause(s) of inflammatory bowel disease

1.1.1 Role of fungal organisms in enteropathies in humans and dogs

Fungal organisms have been proposed as a secondary player in IBD.[6] Yeasts have been shown in the small intestine of both healthy humans and human IBD-patients[7, 8], and increased anti-*Saccharomyces* serum antibodies in Crohn's disease have been noted.[7, 8] Also an improvement of the disease after treatment with the oral anti-fungal nystatin has been reported.[1]
The presence and possible infection of fungal organisms of the intestines have been mentioned before.[1, 6, 9] Most fungal organisms present in the intestines are currently not considered pathogenic, although increased numbers have been reported in animal IBD.[1] In humans, however, a higher diversity of fungal organisms is found in active Crohn's disease.[6] Still, very little is known about the true prevalence and quantity of fungal organisms in the GI tract of dogs and their role within the intestinal ecosystem. Recent work by Suchodolski et al (2008) described fungal organisms in the GI tract of healthy dogs and dogs with chronic enteropathies by use of a nested-PCR method. No significant difference in the prevalence

between the two groups was found. Based on this study we expect a high prevalence and diversity of fungal organisms in dogs

1.2 Fungal organisms

The characterization of fungal organisms remains challenging. This is partly due to the morphology of fungal organisms, their growth requirements, and difficulty in species identification. Conventional methods of identification are usually based on culturing, morphology and biochemical tests.[10] All of these methods, however, can prove to be difficult in determining if and which fungal organism is present. Culture is time consuming, since morphological features and reproductive structures can take days or weeks to develop, and culture methods have low sensitivity.[12] Furthermore, identification requires skill and experience in the field of mycology.[11, 12]

Over the past decades, there has been an increasing move towards molecular (non-cultivation) methods for identifying fungal organisms.[10, 13] Some of these methods are conventional PCR[9, 11, 14, 15], DNA or RNA probe technology, real time PCR[13, 16-19] and Fluorescent In Situ Hybridization (FISH)[3, 20].

Currently the most used targets for molecular fungal diagnostics are the ribosomal genes, which are present in all fungal organisms at high copy numbers[21-24], thereby aiding in detection of these organisms.[17, 25] The ribosomal RNA genes of all eukaryotes are organized in groups of tandem repeats, each with their transcribed/coding and non-transcribed regions.[22] As seen in figure 1, the fungal nuclear ribosomal DNA (rDNA) consists of 3 genes: the large subunit gene (28S) and two small subunit genes (18S and 5.8S). Located between these coding regions are internal transcribed spacer (ITS) regions.[11, 21-24] These have been used extensively for characterization of fungal organisms over the past decades after first being proposed as targets for phylogenetic relationships between fungi by White et al 1990.[14-16, 22, 24, 26-32]

Figure 1 Graphic representation of the genes and spacer regions of the ribosomal DNA of eukaryotes. ITS=internal transcribed spacer, rDNA = ribosomal DNA

Other genes of interest for molecular diagnostics of fungal organisms have been the β-tubulin gene, mitochondrial genes, lanosterol demethylase gene, aspartic proteinase genes, mating genes, CaMP65 gene, RNase P RNA gene, 18S rDNA, and genes encoding the fungal heat shock proteins.[10, 12, 13]

However, most studies performed usually concern applications in humans and are typically targeting only one fungal genus or fungal species to maintain specificity.[15] As noted by Suchodolski et al (2008) and Scupham et al (2006), still little is known about the true prevalence and diversity of fungal species in the mammalian GI tract. Therefore, a panfungal assay would be a reasonable starting point of investigation with the ITS region as target.

1.2.1 ITS region

The particular interest for the ITS regions as targets for fungal molecular diagnostics originated because these regions are supposed to evolve more rapidly than the actual ribosomal coding regions. They therefore diverge more between different species of fungal organisms, making it easier to distinguish between species,[11, 14, 21, 23, 24, 26-29, 32, 33] although a conserved core within all fungal organisms is supposed to be present.[15, 23, 28, 33] The ITS1 region is known to have more sequence divergence than the ITS2 region.[14, 23, 28] When targeting highly conserved sequences within the 5.8S gene and the 28S gene flanking the spacer region a useful attachment site for universal primers is to be expected.[24]
The exact role of the ITS regions is until today not fully understood[24, 28], but is has been suggested by Van der Sande et al (1992) and Musters et al (1990), among others, that these regions play a role in the primary rRNA processing.[24] The ITS region is however not referred to as an intron but as an intervening non-coding or pseudo-intro.[24]
Several primers used to target the ITS region rDNA sequences in the fungal genome can be seen in figure 2.

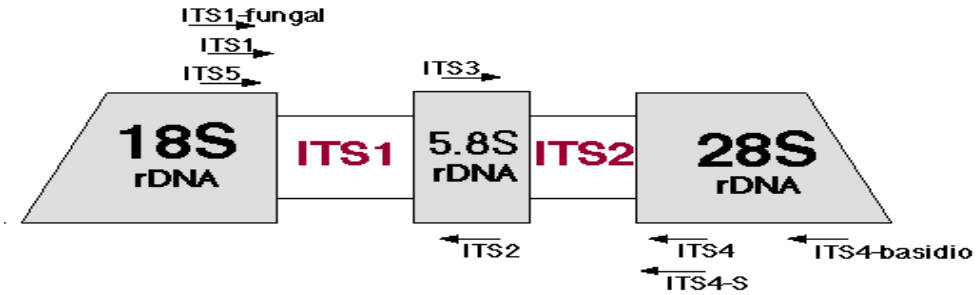

Figure 2 The various ribosomal genes, internal transcribed spacer regions and several primers known to be used for diagnostics.

1.3 Polymerase chain reactions[34]

Polymerase chain reaction (PCR) is a technique where by use of a thermostable DNA polymerase, a pair of DNA oligonucleotides (primers) and deoxyribonucleoside triphosphates, a nucleotide sequence on a desired gene is amplified several billion fold. This approach provides an easy way to detect and investigate the specific sequence. The strength of the PCR method is, however, not visible until after repeated rounds of DNA synthesis. With each round (cycle) the newly generated DNA fragments act as a template for the following cycle, thereby increasing the amount of target DNA sequence every cycle in a exponential manner, hence the term "chain reaction".
PCRs usually consist of three steps in each cycle. The first step (denaturation) employs a heat treatment to separate the two DNA strands, the second step (annealing) allows the oligonucleotide primers to hybridize to the complementary sequences on the two DNA strands by cooling of the DNA, followed by the third step (extension) where the DNA is synthesized starting from the two primers. The entire procedure is automated by use of thermal cyclers and widely used in genetic research.

1.3.1 Real-time PCR

The traditional PCR process does, however, have limitations. With traditional PCR, the end (or amplification) product, also known as amplicon, is usually analyzed for presence of the target sequence by gel electrophoresis, hereby making it a qualitative means of research. Quantification of the target gene sequence is only possible to a certain extent with traditional

PCR, since one original target copy can in theory yield a billion fold of copies by PCR. For some applications, these qualitative nucleic acid analyses are, however, sufficient.
Development of the PCR technology led by use of fluorophores to simultaneous amplification and quantification of the DNA target molecules in "real time", measuring the quantity of amplified product at each cycle of the PCR. This technique is known as real-time (quantitative) polymerase chain reaction, real-time qPCR, or qPCR. Real-time PCR can be either qualitative or quantitative and therefore used for many different studies: from relative gene expression, to calculation of copy numbers and detection and quantification of DNA in samples to determine the abundance of a particular organism or DNA sequence in a sample. An advantage of real-time PCR is evaluation of the end product without gel electrophoresis, resulting in a reduction of experiment time.
In real time PCR a fluorescent molecule is added to the reaction mixture. The fluorescent signal increases as the amount of generated DNA increases during the PCR. Fluorescent chemicals used in real time PCR are fluorescently labeled primers or probes, and DNA binding dyes (e.g. ethidium bromide, YO-PRO-1 and SYBR Green I)[35]. The latter dyes bind to all double-stranded DNA (dsDNA) including non-specific PCR products (like amplified non-target sequences and primer dimers).[35] This is therefore a limitation, since the properties of the fluorophore might interfere with the accurate quantification process when a non-optimized PCR is being employed.
The measured fluorescence in a real-time qPCR is expressed without an absolute unit. Standard dilutions with known quantities of DNA or target sequence are used in the same assay to provide a ratio of the sample relative to that standard, so the starting quantity (SQ) of the target DNA in the unknown sample can be calculated. During any PCR there is always an exponential phase and a non-exponential (plateau) phase of the reaction. By plotting the measured fluorescence from the exponential phase of the PCR to the cycle number (known as an amplification plot, figure 3), a point where the fluorescence exceeds the background signal (or set threshold) can be obtained, called the cycle threshold (C_t).

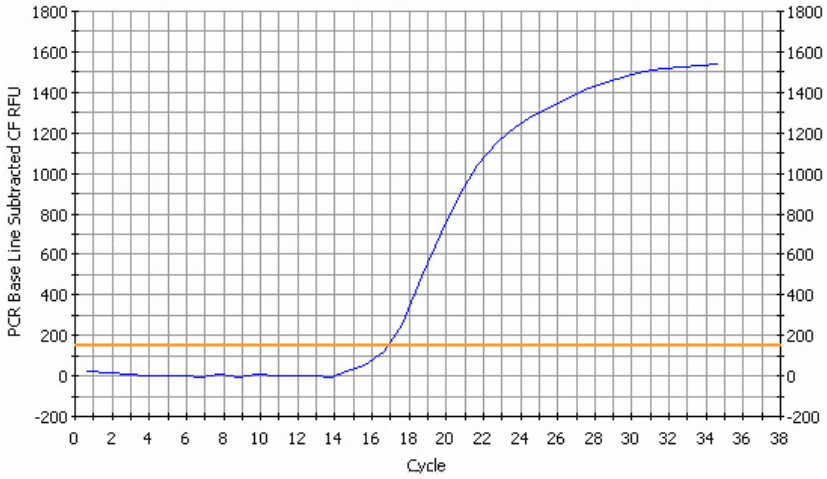

Figure 3 Example of an amplification plot with the threshold set at 150.0 RFU. The curve passes the threshold at Ct = 17. RFU = relative fluorescent units, Ct = cycle threshold

During this exponential phase of the reaction, the amount of PCR product approximately doubles in each cycle. The spacing of fluorescence curves in an amplification plot is therefore determined by the following equation: 2^n = dilution factor (where n is the number of cycles between the curves at the set fluorescent threshold). A ten fold serial dilution of DNA (2^n = 10) yields a 3.32 difference in C_t-values. Reactions with a large quantity of starting template

will require fewer cycles to exceed the threshold than reactions with a smaller starting (template) quantity.
When the log of a series of known starting quantities is plotted against the acquired C_t-value, a linear regression line (figure 4) is obtained that can be used to determine the starting quantity of an unknown sample.

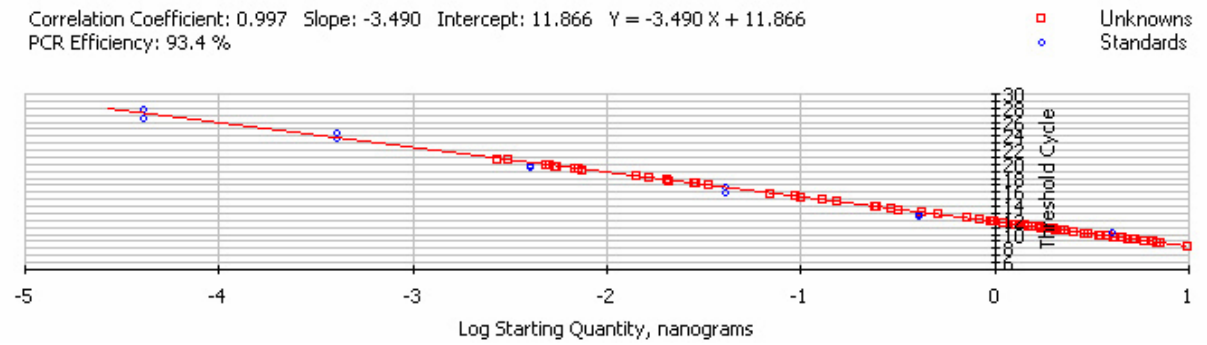

Figure 4 Example of a regression line where the log of the known starting quantities of the standards (here in nanograms) is plotted against the cycle threshold.

To accurately quantify starting material with a real-time PCR assay, a normalization is usually required. This normalization permits a more accurate means for inter-sample comparison, provided that the normalization factor is known to be relatively constant across the investigated samples. In microbiological real-time qPCR assays a known normalization factor is the total amount of bacterial DNA.[36, 37] Since the total amount of bacteria and therefore the amount of DNA is considered to be relatively constant in fecal samples, this normalization factor seems reasonable for fecal samples.
As mentioned before, the end product of the PCR can also be evaluated in the same assay without use of gel electrophoresis. This is done during the part of the assay called melt curve analysis. The concept of melt curve analysis is based on amplicon length, sequence, and difference in GC-content. With melt curve analysis, PCR end products can therefore be evaluated.[35] Different melt-curves will be obtained when the temperature is gradually raised through the melting temperature (T_m) of that specific product. Melt-curves can be plotted as either a function of the decline of fluorescence to the temperature (RFU vs. T), or as a plot of the negative derivative of fluorescence with respect to the temperature (-dF/dT vs. T). Melt curve analysis potentially allows differentiation between specific and non-specific PCR products. Since non-specific products can not be distinguished from the intended product by just monitoring the fluorescence during the PCR, a melt curve analysis is necessary.[35]

Aim of the study

The aim of this research was to establish a qPCR-protocol for fungal organisms that can be used on fecal DNA samples, and determine the prevalence and quantity of fungal organisms within the gastrointestinal tract of healthy dogs and dogs with signs of gastrointestinal disease by the established protocol.

Hypothesis

The hypothesis of this research is that dogs with signs of gastrointestinal disease will have a significantly higher prevalence of fungal organisms within their intestinal tract than healthy dogs. The quantity of DNA is also expected to be higher in dogs with signs of gastrointestinal disease.

Materials and methods

4.1 Animals & Samples

For this research DNA extracted from fecal samples from a total of 119 dogs was used. These dogs were divided into two groups: 49 healthy and 70 diseased dogs. Samples from all 70 diseased dogs were submitted to the laboratory by veterinarians for testing for specific pathogens because the animals showed signs of gastrointestinal disease (e.g. diarrhea). The healthy fecal samples were obtained from the US and the UK. The fecal samples from the diseased dogs came from several locations in the US. The diseased dog samples were submitted to the Gastrointestinal Laboratory at Texas A&M University over the period of November 2006 to March 2008. The healthy dog samples were all collected within the past six months. The animals used in this research were of different age, breed and sex. Information about weight, diet, living conditions or medical history of the animals was not always available, but at this stage of the project not found to be relevant.

4.2 DNA extractions

For DNA extraction from the 119 fecal samples a (modified) bead beating method with phenol chloroform was used. This modified bead beating method was also used in combination with a commercial DNA extraction kit (ZR Fecal DNA kit, Zymo, Orange, CA). A significant difference in the quality of the extraction methods was found. The negative extraction control from the Zymo kit yielded a significantly earlier cycle threshold than the negative extraction control from the phenol chloroform method. Fecal samples used for this study where therefore all extracted with the phenol chloroform method.

As a positive control for this research, DNA extracted from $2x10^9$ colony forming units of *Saccharomyces boulardii* (Florastor® Biocodex Inc., Oregon) was used. Standards for the real time qPCR assay were prepared from the extracted *Saccharomyces boulardii* DNA.

4.2.1 Phenol extraction

The phenol DNA extraction procedure is as follows: 100 mg from each sample was placed in a serum tube. To each of these tubes 300-400 µL of 0.1 mm diameter zirconia beads (BioSpec Products Inc, Bartlesville, Okla) and 700 µL of cell lysis solution (Qiagen, Valencia, CA) were added. The tubes were vortexed in a Fastprep-Homogenizer set at 4.0 m/s for 1 minute, and afterwards centrifuged at 20,817g for 7 minutes. The supernatant was transferred into a new tube to which 700 µL of phenol chloroform was added. Each sample was vortexed and then centrifuged at 20,817g for 5 minutes. The top layer of the supernatant was transferred to a new tube, which already contained 10 µL of RNase solution (Sigma Aldrich®, St. Luis, MO). These tubes were inverted manually to mix the solution and then placed in an incubator set at 37°C for 10 minutes. To each tube 500 µL of phenol chloroform was added, they were manually inverted to mix and then centrifuged at 20,817g for 5 minutes. The top layer of the supernatant was transferred into a new tube to which DNA binding buffer (Zymo, Orange, CA) in 2x volumes of the supernatant was added. The entire mixture was added to a Zymo-Spin III binding column and centrifuged at 6,462g for 1 minute. The columns were transferred to a new collection tube, and 500 µL of DNA wash buffer (Zymo, Orange, CA) was added to the column and the mixture was centrifuged at 6,462g for 1 minute. The columns were moved to a new collection tube and 500 µL of DNA wash buffer was added to the column and centrifuged at 11,934g for 3 minutes. The column was transferred to a new collection tube and centrifuged at 11,934g for 3 minutes to clear the column of any remaining ethanol. The

columns were transferred to clean micro-centrifuge tubes and 100 µL of elution solution (OmniPur, Gibbstown, NJ) was added directly to the membrane and incubated for 5 minutes at room temperature. After incubation, the columns with collection tubes were centrifuged at 6,462g for 2 minutes to elute the DNA from the column. This step of eluting DNA was performed twice. The flow-through now contains the DNA ready for use in the further steps of the study and the DNA was stored at -80°C. During the PCR experiments the samples were, however, stored at 4°C to prevent repeated freeze thaw cycles.

4.2.3 Extraction negative

During each DNA extraction procedure, one tube that contained only extraction reagents was added to the extraction process to serve as a control of the DNA extraction process. This DNA extraction negative control was used to determine a baseline signal and cut-off point for the real time qPCR assay.

4.3 PCR amplification

All real time PCR assays were run on a real time PCR thermocycler, iCycler iQ (Bio-Rad Laboratories Inc., California). The PCR master mix was prepared in a separate room with only low amounts of light to prevent any negative influence on the light sensitive fluorescent substances used for real time PCR. The DNA template was added to the reaction mixture in a specially designated biohazard hood. All necessary measures were taken to prevent any contamination while preparing or loading the reaction mixtures. For every real time PCR assay a PCR negative control was added by using a well, that contained the same amount of DNase/RNase free H_2O instead of the DNA template. This was done to evaluate the possibility of contamination during the preparation of the polymerase chain reaction mixture. All samples used in a real time PCR assay were run in duplicates within that same assay, including the reaction negatives.

For each fecal sample two assays were performed: one amplifying and measuring the total amount of fungal DNA, and a second one amplifying and measuring the total amount of bacterial DNA. The latter was used to normalize the amount of fungal DNA to the amount of bacterial DNA, which gives a more standardized view of quantification.[36, 37]

The primers used to amplify the fungal DNA were ITS3 forward (5'-GCA TCG ATG AAG AAC GCA GC-3') and ITS4 reverse (5'-TCC TCC GCT TAT TGA TAT GC-3'), which amplify the universal fungal ITS2 region. This region is conserved in all fungal organisms.[9, 21]

For the amplification of total bacterial DNA, universal bacterial primers F341 (5'-CCT ACG GGA GGC AGC AG-3') and R518 (5'-ATT ACC GCG GCT GCT GG-3') targeting parts of the 16S rRNA gene were used.

4.3.1 Real Time PCR protocol

Each PCR reaction mixture amplifying the ITS2 region consisted of a final volume of 17 µL, containing 2 µL of DNA template, 0.45 µL of each of the primers (each 0.1 mM), 8.5 µL of iQ™ SYBR® green Supermix (100 mM KCl, 40 mM Tris-HCl pH 8.4, 0.4 mM of each dNTP, iTaq DNA polymerase 50 units/mL, 6 mM $MgCl_2$, SYBR Green I (20 nM fluorescein); Bio-Rad Laboratories, Hercules, California)), with the remainder of the volume consisting of DNase/RNase free water (Teknova).

For fungal organisms the following real time PCR conditions were used: an initial 3 minute denaturation step at 95°C to activate the polymerase enzyme, followed by 40 cycles (denaturation 94°C for 30 seconds, annealing 57°C for 30 seconds, extension 72°C for 30 seconds during which the fluorescence was acquired), a final elongation step at 72°C for 10 minutes, a final 1 minute at 95°C and 1 minute at 55°C, followed by a melt curve

measurement where the temperature was raised from 55°C to 95°C in steps of 0.5°C measuring the decrease in fluorescence during each temperature change.

For each universal bacterial PCR reaction mixture the final volume was also 17 µL containing 2 µL of DNA template, 0.25 µL of each of the primers (each 0.1 mM in concentration), 8.5 µL of iQ™ SYBR® green Supermix (Bio-Rad Laboratories, Hercules, California), and again the remaining part of the volume containing DNase/RNase free water (Teknova).
The real time PCR protocol for universal bacterial DNA consisted of the same initial 3 minute denaturation step, followed by 35 cycles (denaturation 95°C for 10 seconds, annealing 57°C for 15 seconds, extension 72°C for 10 seconds during which the fluorescence was acquired), a final elongation step at 72°C for 10 minutes, 1 minute at 95°C and 1 minute at 50°C, followed by the same melt curve measurement.

4.3.2 Standards for the Real Time PCR
All fungal real time qPCR assays contained 7 standards (run in duplicates) consisting of 1:10 serial dilutions of DNA from *Saccharomyces boulardii*. These standards contained template DNA ranging from 8,400 pg to 0.0084 pg per reaction mixture. The standards were used during the qPCR assay to calculate the starting quantity of fungal DNA of the unknown samples. The threshold cycle values of the DNA standards were used to create a standard (regression) curve for that assay, which was used to evaluate the efficiency of the PCR reaction and to estimate the starting quantity of DNA based on the cycle time the unknown sample needed to exceed the set threshold of 150.0 Relative Fluorescent Units (RFU).

The same was done for all universal bacterial real time qPCR assays, which contained six 1:10 serial dilution standards These standard dilutions contained template DNA ranging from 4,000 pg to 0.04 pg per reaction mixture, which was used to calculate the starting quantity of bacterial DNA of the unknown sample.

4.3.3 Control of extraction negatives
As mentioned earlier, a negative control was extracted during each DNA extraction process. It was found that all of the extraction negatives yielded a positive signal at some stage during the fungal real time PCR assay by exceeding the set threshold of 150.0 RFU's. This has been well described for (universal) bacterial (quantitative) PCR-assays[17, 38], but less for fungal (q)PCR-assays. These extraction negatives were therefore further investigated to get an estimated baseline signal (i.a. during melt curve analysis) and to acquire a threshold cycle (C_t) value during the real time PCR-amplification, which was used later in the study to determine a cut-off C_t-value.
The real time PCR amplification product of these extraction negatives was assessed under UV-light on a 2% agarose gel, which was prestained with GelRed™ (Biotium, Hayward, California) and run for 60 minutes with 130V. That same amplification product was further identified by cloning and sequencing.

4.4 Cloning, Plasmid extraction and Sequencing
4.4.1 Cloning
The PCR amplification product of the extraction negative was ligated into a vector by adding 2 µL of the product to 1 µL of pCR® 4-TOPO-vector (Invitrogen, Carlsbad, California) and incubated for 25 minutes at room temperature. Afterwards, 2 µL of the ligation product were transformed into DH5-α *Escherichia coli* cells (Invitrogen) by heat shock in a water bath set at 42°C for 30 seconds. The transformed *E. coli* cells were cultured for approximately 24

hours at 37°C on a Luria Bertani medium plate with supplemented ampicillin (75 mg/mL) After 24 hours incubation, a total of 20 colonies were randomly selected and transferred to their own glass tube containing 1.4 mL of Luria-Bertani broth each supplemented with 1.4 µL of ampicillin (75 mg/mL), and left to grow at 37°C for 15 hours. From each tube the content was transferred to a clean micro-centrifuge tube and centrifuged at 1,900g for 5 minutes. The supernatant was decanted and the pellet was used for plasmid extraction.

4.4.2 Plasmid extraction & sequencing

Plasmid extraction was performed by use of PerfectPrep Spin Kit (Prime, Gaithersburg, MD) according to the manufacturer's instructions. The plasmid DNA was eluted in 75 µL of DNase/RNase free water.

The extraction negative products inserted into the plasmid were sequenced by an automated cycle sequencing method using ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, PerkinElmer Corporation, Foster City, California) according to the manufacturer's instructions. The sequencing products were analyzed by an automated sequence analyzer, ABI PRISM 377 DNA Sequencer (Applied Biosystems).

4.4.3 Sequence analysis

The sequences of the cloned PCR amplification products were compared with the NCBI Genbank database (BLASTn) to acquire information on sequence homology with known organisms to determine the possible cause of the positive signal in the DNA extraction negative.

4.5 Statistical analysis

For qualitative analysis of the samples a cut-off point of 27 cycle times (C_t) to exceed the set fluorescent threshold of 150.0 RFU was used to determine the proportion of positive samples in each group (i.e. healthy and diseased dogs). A Fisher's exact test was used to evaluate the proportions of positives in the healthy and diseased groups. Significance was set at a P-value of 0.05.

For each fecal sample with a positive signal before the set cut-off point of 27 C_t's the mean starting quantity of fungal DNA was determined. This quantity was normalized by the quantity of total bacterial DNA. Normalization was done to achieve an objective means of inter-sample comparison. The calculated ratios were used to determine if there was a statistically significant difference in fungal DNA between the healthy and diseased dogs. For comparative analysis of the fungal DNA between the groups, a non-parametric Mann-Whitney test was used with a P-value set at 0.05. All statistical analyses were performed with Prism5 (GraphPad Prism Software, San Diego, California)

Results

5.1 Evaluation of the DNA extraction procedure

Two methods of DNA extraction were initially evaluated for their usability in this study. As figure 5 a and b show, there was a difference in cycle times (C_t's) required for either of the two DNA extraction negatives to exceed the set threshold value of 150.0 RFU's. DNA extraction controls obtained with the commercial kit (Zymo) exceeded the set threshold at 27 C_t's (mean 26.95, SD 1.19) on average, while DNA extraction controls obtained with the phenol chloroform method did not exceed the threshold until 33 C_t's (mean 33.9 , SD 1.32). Because the commercial kit (Zymo) extraction negatives exceeded the threshold significantly earlier then the phenol chloroform negatives, all study samples were extracted by the phenol chloroform method.

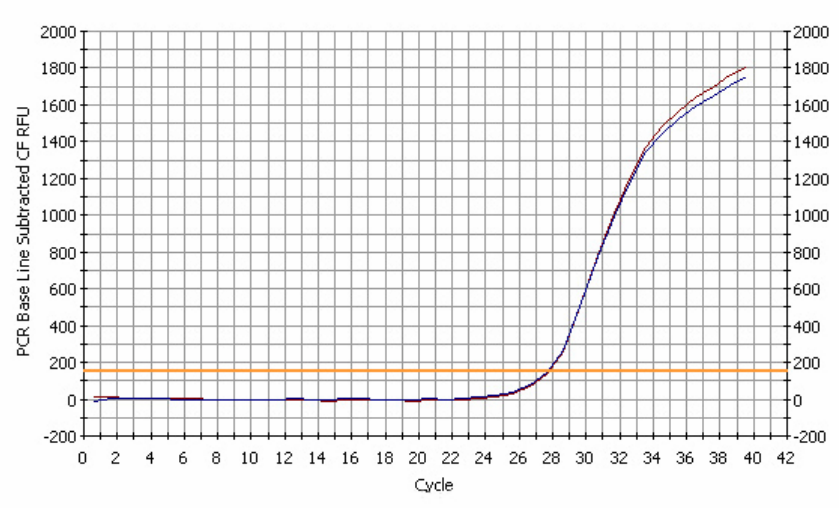

Figure 5 a
Amplification curves from the qPCR with extraction negatives obtained with the commercial kit (Zymo) method.
Mean Ct = 27

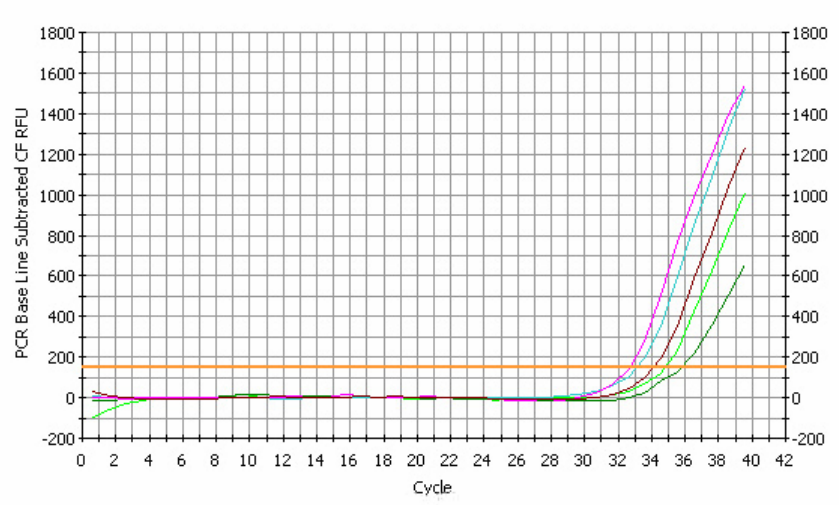

Figure 5 b
Amplification curves from the qPCR with extraction negatives obtained with the phenol chloroform method.
Mean Ct = 33

As seen in figure 6 there was a distinctive band at approximately 250 base pairs in the DNA extraction negative samples when run on a 2% agarose gel. The PCR amplification product of the extraction negatives was, therefore, further investigated by cloning and sequencing.

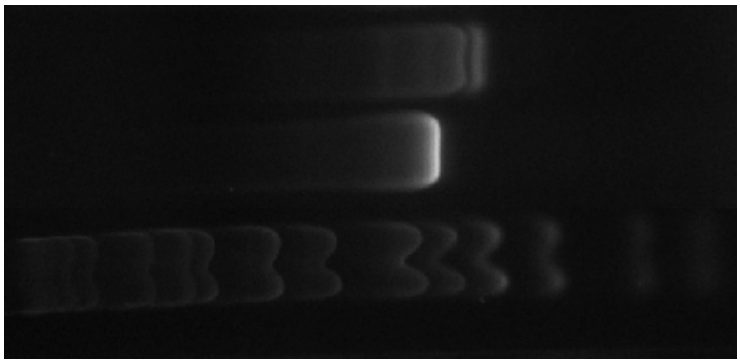

Figure 6 Results of the extraction negative and a positive control run on a 2% agarose gel for 60 min from left to right.

For the further proceedings in this study a cut-off value of 27 C_t's was chosen. Any sample exceeding the threshold of 150.0 RFU's before this cycle time value was considered to be truly positive. This cut-off of six cycle times earlier than when the extraction negative exceeds the threshold provides roughly a hundred-fold difference in starting quantity of DNA in the investigated sample if PCR-efficiencies in every assay would be 100%.

5.2 Qualitative analysis of the real time PCR

For this research DNA extracted from fecal samples from 119 dogs was investigated. Forty-nine of these animals were considered as healthy and seventy as diseased (defined as having signs of gastrointestinal disease such as diarrhea).
Forty-seven out of the forty-nine healthy dogs (96%) exceeded the set threshold value of 150.0 RFU's before the extraction negative exceeded the threshold at thirty-three cycle times. Only fifty-five out of seventy diseased animals (79%) showed a positive signal for fungal DNA before that same time. However, given the previously mentioned cut-off value of twenty seven cycle times, for 26 (53.1%) of the 49 healthy dog samples a positive signal was detected with this real time PCR assay. For the 70 diseased dog samples only 23 (32.9%) provided a positive signal before the cut-off value ($C_t < 27$). This data is summarized in table I. Table II shows the number of dogs found to be positive when a cut-off value of 30 cycle times would be used.

	Positive	Negative
Healthy	26	23
Diseased	23	47

Table I: In this contingency table the number of healthy and diseased dogs with positive and negative test results for a cut-off value of 27 Ct's can be seen ($p = 0.0373$).

	Positive	Negative
Healthy	42	7
Diseased	37	33

Table II: In this contingency table the number of healthy and diseased dogs with positive and negative test results for a cut-off value of 30 Ct's can be seen ($p = 0.0002$).

The difference in prevalence of fungal DNA between the two groups was found to be statistically significant with a Fisher's exact test, regardless if a cut-off value of 27 C_t's or of 30 C_t's was used ($p = 0.0373$, or $p = 0.0002$ respectively). The Odds ratio between the two groups was 2.31 (95% confidence interval 1.09 – 4.90) for the chosen cut-off value of 27 C_t's, which means that healthy dogs were 2.31 times more likely to yield a positive PCR result than diseased dogs.

5.3 Quantitative analysis of the real time PCR

For the quantitative analysis of the positive fecal DNA samples by real time polymerase chain reaction, 1:10 serial dilution standards were used. The seven standards for the fungal real time PCR assays contained a starting DNA template ranging from 8,400 to 0.0084 pg per reaction, and the six standards for the universal bacterial real time PCR assays contained 4,000 to 0.04 pg per reaction of starting template DNA. Figure 7 illustrates an example of the standard amplification curve for a fungal qPCR assay.

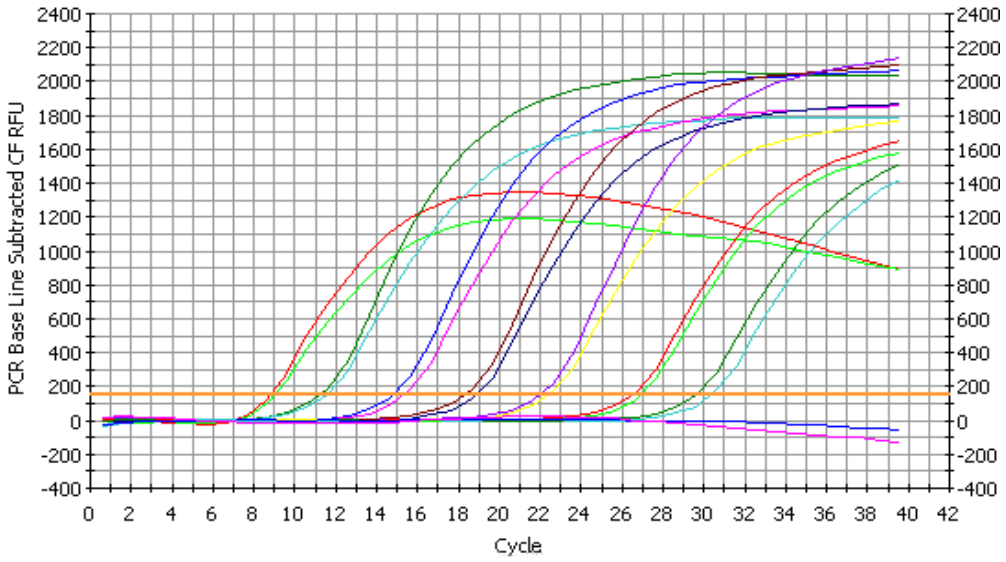

Figure 7 Amplification curves of the fungal serial standard dilutions 1 through 7, containing 8,400 pg to 0.0084 pg of template DNA per reaction.

The amplification curves for a bacterial qPCR assay in figure 8 look similar. Note however the following difference: the PCR negative (no-template) control for universal bacterial qPCR also exceeds the set threshold at approximately 32 cycles, whereas the same no-template control for the universal fungal qPCR does not. From these standard curves the C_t was plotted against the log of the starting quantity of standard templates and thereby used to create a qPCR regression line. From this regression line the starting quantity of the unknown samples could be calculated by the iCylcer (Bio-Rad Laboratories Inc., California) program. The starting quantity of fungal DNA for each sample was normalized by the quantity of total bacterial DNA in that sample, providing a ratio of fungal DNA to bacterial DNA for each sample.

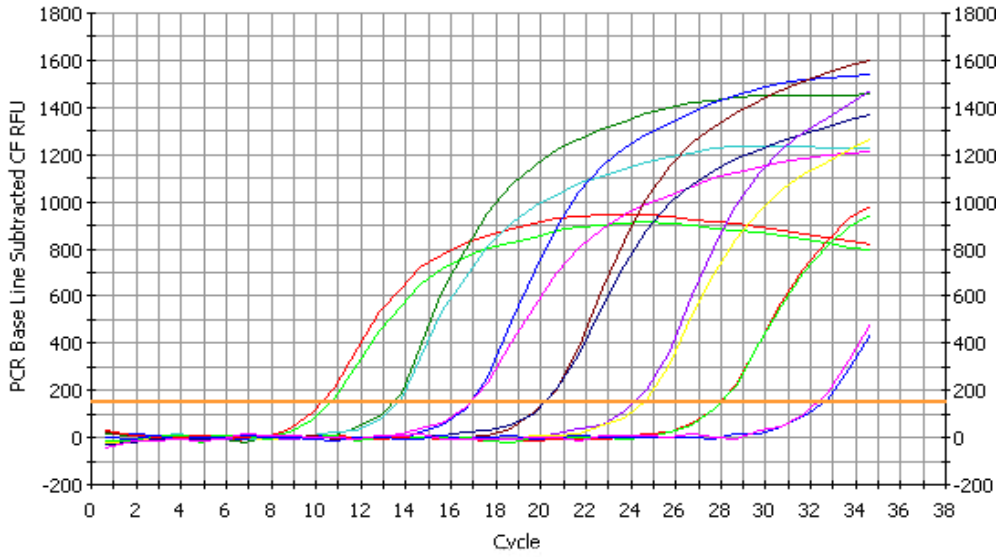

Figure 8 Amplification curves of the fungal serial standard dilutions 1 through 7, containing 8400pg to 0.0084pg of template DNA per reaction.

Data was only used for quantitative comparative analysis of the two groups from those samples that exceeded the set threshold before 27 C_t's. Table III shows the ratios of the starting quantity of fungal DNA divided by the starting quantity of bacterial DNA along with the cycle threshold time for all positive tested animals. The median of the healthy animals was 0.0002779 (range 1.701×10^{-5} – 0.02543, p = 0.0013), and the median for the diseased animals was 0.0001879 (range 1.116×10^{-5} – 0.1405, p = 0.0009). The medians were not significantly different (p = 0.4288) between the two groups using a two-sided non-parametric Mann Whitney test.

5.4 Sequence analysis of the extraction negatives

The result of cloning and sequence analysis of the DNA extraction negative amplification product was analyzed by BLASTn in the NCBI Database. This revealed that members of the genus *Saccharomyces* were present in the extraction negatives. The exact cause of the presence of these *Saccharomyces spp.* in the DNA extraction negative, however, remains obscure.

Animal	Univ. Fungal SQ	Univ. Bact SQ	Univ. Fung SQ / Univ. Bact SQ	Fungal Ct
D4	0.2035	68.85	0.002955701	24.73
D7	0.1595	4955	3.21897E-05	25.12
D11	0.8015	4265	0.000187925	22.56
D12	0.4235	1490	0.000284228	23.57
D15	0.07565	6780	1.11578E-05	26.29
D20	0.2045	9830	2.08037E-05	24.72
D21	0.492	3495	0.000140773	23.33
D22	0.0682	4825	1.41347E-05	26.46
D23	35.8	6800	0.005264706	16.54
D28	3.805	1875	0.002029333	20.11
D30	14.9	1695	0.00879056	17.93
D33	0.06695	774.5	8.64429E-05	26.5
D34	0.504	1375	0.000366545	23.29
D40	0.702	2935	0.000239182	22.76
D45	0.143	1230	0.00011626	25.74
D46	0.4295	3620	0.000118646	23.92
D48	0.0751	986.5	7.61277E-05	26.76
D52	0.07545	5470	1.37934E-05	26.74
D54	2.815	4005	0.000702871	20.88
D57	58.25	414.5	0.14053076	15.99
D62	0.407	16.25	0.025046154	24.03
D64	0.3415	1990	0.000171608	24.29
D68	3.795	7190	0.000527816	20.4
H81	0.0948	2040	4.64706E-05	26.05
H82	13.6	1555	0.008745981	18.26
H83	0.2305	1930	0.00011943	24.65
H84	0.07855	188.5	0.000416711	26.35
H88	0.1036	1080	9.59259E-05	25.91
H94	0.123	4100	0.00003	25.64
H95	0.5345	2320	0.000230388	23.32
H96	0.335	1290	0.00025969	24.07
H97	1.83	2490	0.00073494	21.38
H98	0.09245	5435	1.70101E-05	26.13
H99	0.0874	3980	2.19598E-05	26.18
H100	0.457	4700	9.7234E-05	23.57
H101	1.77	3800	0.000465789	21.44
H106	57.6	2975	0.019361345	15.95
H107	0.21625	1325	0.000163208	25.05
H109	0.09205	405	0.000227284	26.12
H110	2.155	424.5	0.005076561	21.19
H112	12.35	1440	0.008576389	18.37
H115	0.05945	1150	5.16957E-05	26.98
H121	0.9375	3165	0.000296209	22.78
H124	129.5	7510	0.017243675	14.7
H125	12.3	493	0.02494929	18.51
H126	0.1798	2520	7.13492E-05	25.56
H127	9.07	8825	0.001027762	19
H128	9.18	361	0.025429363	19.17
H129	6.07	386.5	0.015705045	19.73

Table III: The starting quantities (SQ) for the unknown samples calculated by universal fungal and universal bacterial qPCR, the ratio of the universal fungal to the universal bacterial and the cycle threshold (C_t). D = dogs with signs of gastrointestinal disease. H = healthy dogs. All starting quantities are in picogram's.

Discussion

Until now little research has been done on the prevalence of fungal organisms in the gastrointestinal tract of dogs.[9, 39-41] Even less is known about the effects of fungal organisms on the GI tract and their role in canine gastrointestinal diseases. Suchodolski et al (2008) used a nested-PCR assay and cloning and sequencing to describe fungal organisms in the GI tract of healthy dogs and dogs with chronic enteropathies. To our knowledge, no studies have been done to quantify fungal organisms in the canine GI tract by use of real time qPCR. Scupham et al (2006) showed and quantified fungal organisms in the intestinal tract of the mouse by use of PCR, oligonucleotide fingerprinting and FISH. Most studies for fungal organisms are until this day done on environmental samples, human blood or human bronchoalveolar lavage fluid. The genetic diversity in the latter two is of course much lower than in feces, where several orders of bacteria are present.[42]
In this study, a real time qPCR protocol was established to investigate the prevalence of fungal organisms in healthy dogs and dogs with signs of gastrointestinal disease, and an attempt to quantify fungal organisms in the GI tract in the previously mentioned groups was made. It was shown that the prevalence of fungal organisms in the canine GI tract is significantly higher for healthy animals than for animals with signs of GI disease. This would be in concordance with previously shown results for bacteria in the intestinal tract by Xenoulis et al (2008) and by Ott et al (2004), where reduced species richness in dogs with IBD and reduced diversity in human patients with IBD, respectively have been shown. Suchodolski et al (2008), however, showed by use of a nested-PCR that there was no statistical significant difference in the prevalence of fungal organisms in dogs with chronic enteropathies and healthy dogs. Ettinger et al (2010) and Ott et al (2008), on the other hand, support our hypothesis that an increased quantity of fungal organisms in the GI tract would be expected when signs of gastrointestinal disease are present. An increased richness and diversity are for instance also seen in other inflammatory barrier diseases (i.e. psoriasis and atopic dermatitis).[6]
Even though a statistically significant difference in prevalence between the two groups has been found by Fisher's exact test ($p<0.05$), the lower limit of the 95% confidence interval of the Odds Ratio approaches 1. Therefore, studies with higher sample sizes would be useful to confirm these results. In a personal communication with J.S. Suchodolski, a recent unpublished study has been mentioned that suggests a diverse fungal microbiota in the gastrointestinal tract of all dogs and that fungal organisms should be considered part of the normal intestinal ecosystem. This data would be in concordance with research done in humans, where fungi are being considered a rich and diverse microbial community of the human intestine.[6, 46, 47]
Quantitative analysis of the samples by the established real time PCR method showed no statistically significant difference in fungal DNA starting quantities between the two groups, even though the median of the diseased dogs was slightly lower than that of the healthy dogs. However, this study does show that fungal organisms only form a relatively small part of the intestinal microbiota in dogs. For mice and humans it has also been noted that fungal organisms only comprise 2% of the mucosal biofilm and 0.02% of the fecal microbiota, respectively.[3, 6] Scupham et al (2006) was unfortunately not able to determine the fungal abundance in murine feces due to a sampling problem, but the same study by Scupham et al (2006) showed a diverse array of fungi in the murine intestine. All major fungal taxa were present in both the large and small murine intestines, with the highest fungal abundance in the cecal mucosal mucus biofilm. Ott et al (2008) showed that the spectrum of fungal taxa differed between healthy controls and humans with Crohn's disease or Ulcerative Colitis and

reported a significant increase in diversity and composition of the fungal community with Crohn's disease.
The discovery of *Saccharomyces spp.* contaminating the DNA extraction negatives is also not surprising, since contamination by *Aspergillus fumigatus, Saccharomyces cerevisiae* and *Acremonium spp.* has been noted by Loeffler et al (1999) before.

The established qPCR protocol has, however, certain limitations for investigating fungal prevalence in the canine gastrointestinal tract. First, some of the healthy dog samples were obtained from dogs in the UK instead of the US. All the diseased samples were on the other hand obtained from various parts of the US. It is not known whether the geographical location of the obtained samples has any influence on the prevalence of fungal organisms in the canine GI tract, but it cannot be excluded. Secondly, DNA extraction negatives yielded a positive signal at some stage during the PCR, thereby requiring a cut-off point where the signal acquired from the investigated DNA fecal samples can truly be considered positive. This cut-off point was currently set at 27 C_t's, which would indicate a roughly hundred-fold higher amount of DNA starting quantity of a positive sample compared to that found in the DNA extraction negatives. To our knowledge no good references or guidelines are present for establishing certain cut-off points when investigating fungal organisms. This cut-off point might therefore be seen as stringent, but is to be considered necessary. The stringency of the cut-off point did, however, not influence the significant difference in prevalence between the two groups.
A possible explanation for the observed difference could also be due to the difference of how long the samples have been stored in the freezer (2-3 years versus 6 months for diseased and healthy animals, respectively). Samples stored for a longer period might have been exposed to more freeze thaw cycles than more recent samples. It is possible that repeated freeze thaw cycles may have had a negative impact on DNA quality.
It has been shown that for eubacterial primers a positive result will also be seen in no-template controls.[17, 38] This is mostly due to spurious contamination of the polymerase with bacterial DNA. We also observed a fungal contamination of our negative extraction controls. A possible cause for this could be external contamination with fungal organisms during the DNA extraction process or contamination of extraction reagents. Since fungal contamination might arise from many different sources, a panfungal PCR-based assay remains challenging.[43] It has been shown that several commercially available DNA extraction reagents are contaminated.[12, 15, 44] Most of the time the manufacturer is not aware that their products could potentially be used for fungal molecular diagnostics. Contamination could potentially occur by airborne fungal spores during the DNA extraction, carry over or by presence of fungal spores in equipment that is being reused.[12, 15] Loeffler et al (1999), however, showed that the risk of contamination can be kept to the same level for fungal PCR assay as for other diagnostic PCR-based assays. However, precautionary measures are then required, raising the question if these measures would be reasonable, attainable and effective when working with fecal samples. Because of the highly sensitive nature of real-time PCR technology contamination does need to be eliminated as much as possible for further fungal qPCR assays. It has been proposed that when working with universal bacterial primers contamination could interfere considerably with the detection of low copy numbers, thereby disproportionately over representing the contaminants.[38] Corless et al (2000) described several ways to eliminate contaminating agents like UV irradiation and restriction endonucleases. Both of these methods are not completely effective and also decrease analytical sensitivity. Further studies to determine the causative reagents and ways of eliminating contamination are required.
DNA extraction methods can also have great influence on the DNA yield.[13, 43, 45] Previous studies have shown that certain DNA extraction methods provide more total fungal DNA than

others.[43, 45] Fredricks et al (2005) even concluded that there are differences in yield between yeast-like organisms and filamentous fungi when the same extraction method is being used. He concluded that there is no optimal extraction method for all fungi. During this study it was not yet investigated which species are most commonly found in healthy and diseased canine intestines. A possible bias towards over- or underrepresentation of certain species can not be excluded. It must also be noted that the highest sensitivity for the detection of fungi by PCR-based assays can only be obtained when one also strives for optimization of the DNA extraction method by efficient lysis of fungal cells and elimination of PCR inhibitors.[13, 43, 45] Another limitation of the current method for accurately quantifying fungal organisms in DNA fecal samples is the use of only one species of fungal organisms, *S. boulardii*, for the standards in the assay. Fecal DNA samples from dogs probably contain several different species of fungal organisms.[9,48] All of these species have different lengths and sequences for the targeted ITS2 region[5, 11, 14-16, 22, 26-29], and different genome sizes and copy numbers of the rDNA genes for each species.[49] Amplicon length and GC-content are known factors to influence the SYBR green binding properties.[35, 50] For example, a two to four-fold difference in copy number is supposed to already give a single cycle time difference.[16] Therefore, all these factors should ideally be taken into account because they could cause a difference in the amplification efficiency.[16, 35, 50] The differences in length and sequence of the ITS regions are on the other hand also known to be used in molecular diagnostics as a tool for discriminating between different fungal species.[5, 11, 14-16, 22, 23, 27-31] Since a variety of different characteristics during the PCR amplification might be expected when dealing with mixed template samples, clear interpretation of the acquired data might be more challenging.
If certain species might be favored by the current protocol remains unknown, but Manter and Vivanco (2007) noted a preferential amplification of Ascomycota when a mixture of five different fungi (i.e. Fusarium equiseti, Alternaria solani, Verticillium sp., Rhizoctonia solani, Scletorina sclerotium) was used.
One of the strong points of this study was the normalization of the determined starting quantities of fungal DNA by universal bacterial DNA.[36, 37] This normalization provided a ratio of panfungal to eubacterial DNA starting quantity in stead of an actual DNA starting quantity, which gives a more accurate way of investigating intersample differences. Especially, since canine intrastool variability of three lactic acid bacterial genera has been shown by J. Garcia (2008).

For the future we would suggest further investigation and prevention of contamination of the DNA extraction as a main priority. Also, the effect of using standards which contain more than one species should be further investigated, so that different species could be determined in one sample by their melt curve profile. Other ideas to determine if specific species are present in a sample would be by use of species-specific probes or restriction length polymorphisms. The latter has already been successfully used for human samples by Chen et al (2000 and 2001). Furthermore, it would be interesting to investigate the fungal microbiota in different parts of the intestines and in biopsies. This could be done either by the already established real time PCR protocol or by means of FISH, which has shown that the mucosal mucus biofilm adjacent to the cecal epithelium harbours the highest abundance of fungal organisms.[3]

This study suggests that fungal organisms have a significantly higher prevalence in healthy dogs compared to dogs with signs of gastrointestinal disease. The relative quantities between these two groups, however, do not seem to differ. More research is warranted to obtain a more accurate prevalence and to determine the role of fungal organisms in the gastrointestinal tract of dogs. There is evidence that fungal organisms are part of the highly complex normal

intestinal ecosystem, but their exact role in gastrointestinal health and disease remains obscure.

Acknowledgements

Hereby I would like to express my gratitude to the entire Gastrointestinal Laboratory at Texas A&M University for all the help, good advice, and hospitality, and of course the wonderful time they gave me while doing my research. In particular I would like to thank Dr. Jan Suchodolski and Dr. Jörg Steiner for offering me the possibility to do this research at their lab and all their effort and good advice during my research period, and also Nicole Gossett for all the help and instructions she has given me in the past four months and Jose Garcia for his bright ideas, great enthusiasm and his assistance during my project.
Further more I would like to thank Dr. Gaby Hoffmann for here help and guidance with my research period and drs. Ronald van Noort and dr. Jan Mol for taking over the task of supervisor.

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