UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

CHANGES IN THE FELINE GUT MICROBIOTA ASSOCIATED TO *TOXOCARA CATI* INFECTIONS

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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I dedicate this dissertation to my mother, who I owe everything.

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Lucky that I had such amazing parents, specially my mother who fixed me ever since I was born and always believed in me. Lucky for my sister who was always there for me, and who spend hours with me studying. Lucky for my friends, especially Cristina Pereira who encouraged me and gave me the last push I needed to finish the degree.

Thank you all.

Abstract

Changes in the feline gut microbiota associated to *Toxocara cati* infections

Investigations of the relationships between the gut microbiota and gastrointestinal parasitic nematodes are attracting growing interest by the scientific community. These studies have however been carried out mainly in humans and experimental animals, while knowledge of the make-up of the gut commensal microbiota in presence or absence of infection by parasitic nematodes in domestic animals is limited. In this study, we investigate the qualitative and quantitative impact that infections by a widespread parasite of cats (i.e. *Toxocara cati*) exert on the gut microbiota of feline hosts.

The faecal microbiota of cats with patent infection by *T. cati* (= Tc+), as well as that of negative controls (= Tc-) was examined via high-throughput sequencing of the V3-V4 hypervariable region of the bacterial 16S rRNA gene, followed by bioinformatics and biostatistical analyses of sequence data.

A total of 2,325,366 useable high-quality sequences were generated from the faecal samples analysed in this study and subjected to further bioinformatics analyses, which led to the identification of 128 OTUs and nine bacterial phyla, respectively. The phylum Firmicutes was predominant in all samples analysed (mean of 53.0%), followed by the phyla Proteobacteria (13.8%), Actinobacteria (13.7%) and Bacteroidetes (10.1%). Among others, bacteria of the order Lactobacillales, the family Enterococcaceae and genera *Enterococcus* and *Dorea* showed a trend towards increased abundance in Tc+ compared with Tc- samples, while no significant differences in OTU richness and diversity were recorded between Tc+ and Tc- samples ($P = 0.485$ and $P = 0.581$, respectively). However, Canonical Correlation and Redundancy Analyses were able to separate samples by infection status ($P = 0.030$ and $P = 0.015$, respectively), which suggests a correlation between the latter and the composition of the feline faecal microbiota.

Keywords**:** Gut microbiota, Cat, *Toxocara cati*, Lactobacilli, Microbial richness and diversity, 16S rRNA

Resumo

Alterações na microbiota intestinal felina associadas a infecções por *Toxocara cati*

A investigação das interações entre a microbiota intestinal e os nematodes intestinais tem vindo a atrair o interesse da comunidade cientifica. No entanto, a maioria destes estudos tem sido desenvolvida em humanos e animais de laboratório, e deste modo o conhecimento da composição da microbiota comensal do intestino na presença de nematodes intestinais é reduzido. Neste estudo, foi investigado o impacto qualitativo e quantitativo da infeção pelo parasita comum dos gatos (i.e. *Toxocara cati*) na microbiota intestinal do hospedeiro felino.

A microbiota fecal de gatos com infeção patente por *T. cati* (Tc+), bem como controlos negativos (Tc-) foi avaliada através de sequenciação de alto débito da região hiper-variável V3-V4 do gene 16S, seguido de análise bioinformática e bioestatística.

Das amostras fecais incluídas no estudo foram obtidas um total de 2 325 366 sequências de alta qualidade e sujeitas a analise bioinformática, o que levou à identificação de 128 OTUs e nove filos bacterianos. O filo Firmicutes foi encontrado em predominância em todas as amostras (média de 53,0%), seguido do filo Proteobacteria (13,8%), Actinobacteria (13,7%) e por fim Bacteroidetes (10,1%). A abundância de determinados grupos de bactérias tendeu a aumentar nas amostras Tc+ quando comparadas com as amostras Tc-, tais como a ordem Lactobacillales, a família Enterococaceae e o género *Enterococcus* e *Dorea*. No entanto, a riqueza e diversidade das OTUs não apresentou diferenças significativas entre as amostras Tc+ e Tc- (P=0,485 e P=0,581, respetivamente). Todavia, a análise canónica de redundância demonstrou uma separação das amostras de acordo com o estado de infeção (P=0,030 e P=0,015, respetivamente), o que sugere uma correlação entre este e a composição da microbiota fecal felina.

Palavras chave: Microbiota intestinal, gato, *Toxocara cati*, Lactobacilli, Riqueza e diversidade microbiana, 16S rRNA

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List of Abbreviations and Symbols:

~ - Approximately
% - Percentage
= - Equal to
± – Plus or minus
ALT - Alanine Transaminase
AMPs - Antimicrobial Proteins
APRIL - proliferation-inducing ligand
Akt – protein kinase B
bp – base pairs
CCA – Canonical Correlation Analysis
CLRs – C-type lectin receptors
CTL – C-type Lectin
CP – Polysaccharides
DC – Dendritic Cell
DNA – Deoxyribonucleic Acid
dsDNA – double strain DNA
EPG – eggs per gram
ES – Excretory-secretory
FEC – faecal egg counts
g – grams
GALT – Gut Associated Lymphoid Tissues
GF - Germ Free
GI – Gastrointestinal
HMI – Helminth-microbiota interactions
ICD – Idiopathic Chronic Diarrhoea
IFN-g – Interferon-g
Ig - Immunoglobulins
IL – Interleukins
kg – Kilogram
L1 – First stage larva
L3 – Third stage larva
L4 – Fourth stage larva
L5 – Immature adult stage
LDH - glutamate dehydrogenase
LEfSe – Linear discriminant analysis effect size
LPS – Lipopolysaccharides
m – minutes
MAMPS – Microbe-Associated Molecular Pattern
ml – Millilitre
MLN – Mesenteric Lymph Nodes
MUC – Mucin
NCBI: National Centre for Biotechnology Information
ng/µl – nanograms per microlitre
NGS - next-generation sequencing
NLRs – NOD-like Receptors
NOD - Oligomerisation Domains
ºC – Centigrade degrees
OTU – Operational Taxonomic Units
P – p-value
PCR – Polymerase Chain Reaction
PGN – Peptidoglycan
PRR – Pattern Recognition Receptors
QIIME – Quantitative Insights Into Microbial Ecology

® – Registered trademark
RDA – Redundancy Analysis
RNA – Ribonucleic Acid
rRNA – recombinant RNA
s – seconds
sIgA - secretory IgA
SPF - specific pathogen free
sprr2A - small proline-rich protein 2A
Tc+ - *Toxocara cati*-positive
Tc- - *Toxocara cati*-negative
Tc-PEB-1 – *Toxocara* phosphatidylethanolamine binding protein 1
TES - *Toxocara* ES
Th2 - T-helper type 2 cells
TLR - toll-like receptor
™- Trade mark
Treg - T regulatory cells
µl – microlitre

Introductory Note

The present work was carried out in the Department of Veterinary Medicine of Cambridge University in England as part of the author's training period during the 6th year of the Integrated Master in Veterinary Medicine. The training period went from October 2015 to June 2016 and was guided by Dr. Cinzia Cantacessi. This research has resulted in a paper published on *Parasites and Vectors* on December 2016 (Annex A).

During this time the student had also the opportunity to participate in a basic epidemiological study to verify the occurrence of cat lungworm infections in the Cambridge area. This study was sponsored by Merial. Faecal samples from 54 cats were collected from November of 2015 to March of 2016 from a cat shelter in Cambridge. All samples were also tested for other intestinal parasites using the floatation method. To test for the presence of the lungworm the Baermann technique was used. All samples were found negative for *Aelurostrongylus abstrusus* but some were positive for other intestinal parasites (Annex B).

Chapter 1 – Literature Review

1. Introduction

The human gastrointestinal (GI) tract harbours ~100 trillion (10^{13}–10^{14}) bacterial cells belonging to over 500 species, which together are referred to as the 'gut microbiota' (Brown, Sadarangani & Finlay, 2013; Giacomin, Croese, Krause, Loukas & Cantacessi, 2015). Particularly in veterinary species, the gut microbiota often shares its environment with large multicellular organisms, i.e. parasitic helminths (Peachey, Jenkins & Cantacessi, 2017). Moreover, both helminths and commensal bacteria have developed a range of strategies to modulate host immunity in order to establish themselves in the host gut (Reynolds, Finlay & Maizels, 2015). It is therefore plausible that the successful establishment of parasitic nematodes in the vertebrate gut is achieved, at least in part and directly and/or indirectly, *via* physical, molecular, and/or immunological interactions with the resident commensal microbiota (Glendinning, Nausch, Free, Taylor & Mutapi, 2014; Reynolds, Finlay & Maizels, 2015) and recent research on relations between the microbiome and parasites has shown a variety of interactions with implications to the host (Reynolds et al., 2014; Kreisinger, Bastien, Hauffe, Marchesi & Perkins, 2015; Cattadori et al., 2016).

However, most studies studying these interactions have been conducted on humans and rodents (Walk, Blum, Ewing, Weinstock & Young, 2010; Cooper et al., 2013; Rausch et al., 2013; Cantacessi et al., 2014; Lee et al., 2014; Reynolds et al., 2014; Giacomin et al., 2015a; Holm et al., 2015; Houlden et al., 2015; McKenney et al., 2015; Cattadori et al., 2016; Giacomin et al., 2016) and only a handful of studies have explored the relationships between GI parasitic nematodes and the commensal gut microbiota in non-experimental animals (Li, Wu, Li, Huang & Gasbarre, 2011; Li et al., 2012; Wu et al., 2012; Slapeta, Dowd, Alanazi, Westman & Brown, 2015; Li et al., 2016). Furthermore, to the best of our knowledge, so far the helminths involved in the studies investigating this interaction do not include ascarids.

Ascarids are among the largest and commonest nematode parasites infecting the intestinal tract of domestic animals (Overgaauw, 1997). *Toxocara canis* and *T. cati* are large ascarids that infect dogs and cats, respectively (Overgaauw, 1997). Due to the zoonotic potential of *T. canis*, much is known of the pathogenicity and immunological features of this parasite, as well as of mechanisms of interaction with its hosts (Schneider, Laabs & Welz, 2011; Maizels, 2011; Resende et al., 2015). On the other hand, little is known of the complement of interactions occurring at the gut interface of cats infected by *T. cati*.

In this chapter in order to provide background information on the three way interaction between parasite, microbiota and host, a review of the relevant literature on the genus *Toxocara*, the microbiota and the helminth-microbiota interaction in veterinary species was undertaken. At the end of the chapter, conclusions drawn from the literature review to set the research aims of the present study will be presented.

2. Intestinal parasites of cats and dogs

Domestic cats and dogs can be infected with a wide range of intestinal parasites. Briefly, the most common parasites of cats are the hookworm *Ancylostoma tubaeforme*, the roundworms *Toxascaris leonina* and *Toxocara cati*, the tapeworms *Dipylidium caninum* and *Taenia taeniaformis* and protozoan parasites *Giardia* spp. and *Cytoisospora* spp. (Foreyt, 2001).
Conversely, in dogs, the commonest intestinal parasites are the hookworms *Ancylostoma caninum, A. braziliense* and *Uncinaria stenocephala*, the roundworms *Toxocara canis* and *Toxascaris leonina*, the tapeworm *Dipylidium caninum*, the whipworm *Trichuris vulpis* and protozoans *Cytoisospora* spp. and *Giardia* spp. (Foreyt, 2001).
Amongst these, ascarids are one of the most prevalent intestinal parasites in Europe (Näreaho et al., 2012; Beugnet et al., 2014; Zanzani, Gazzonis, Scarpa, Berrilli & Manfredi, 2014; Paoletti et al., 2015) and other parts of the world (Savilla, Joy, May & Somerville, 2011; Hoopes et al., 2015; Tun et al., 2015; Yang & Liang, 2015).
Both larval and adult stages of ascarids can potentially be harmful to the vertebrate hosts: adult worms often cause poor growth and diarrhoea and, occasionally, intestinal obstruction in young animals, while larvae damage host tissues during the somatic migration phase of their life cycle (Taylor, Coop & Wall, 2007; Elsheika and Khan, 2011; Bowman, 2014).

2.1. *Toxocara* spp.

2.1.1. Epidemiology

In Europe, a study of privately owned cats from Austria, Belgium, France, Hungary, Italy, Romania and Spain, reported a prevalence of *T. cati* infection of 19.7% (Beugnet et al., 2014), while other studies reported a prevalence ranging from 5.4% in Finland (Näreaho et al., 2012) to 15.7% in the UK (Gow et al., 2009).

In dogs, reported prevalence of infection by *T. canis* range from 1.3% in Portugal (Ferreira et al., 2011), to 4.48%-22.2% in Italy (Zanzani et al., 2014) to 8% in Albania (Shukullari et al., 2015).

In Canada, ascarid infections were present in 16.5% and 12.7% in cat and dog populations, respectively (Villeneuve et al., 2015), although the prevalence in Canadian free-roaming cats was reported to vary from 1% to 15% in the same year (Hoopes et al., 2015). Another study in North America, observed a prevalence of 6.9% in dogs in south central West Virginia (Savilla et al., 2010). In Mexico, the prevalence of ascarid infections in owned dogs was 2.3% (Torres-Chablé et al., 2015).

In Australia, a study involving cats and dogs, reported a prevalence of ascarid infections of 3.2% and 1.2% respectively (Palmer, Thompson, Traub, Rees & Robertson, 2008). In Malaysia, infection prevalence was 11.9% and 9.9% in stray dogs and cats, respectively (Tun et al., 2015). A higher prevalence of 17.78% was detected in stray and shelter cats from central China (Yang & Liang, 2015).

Several factors have been associated with higher rates of *Toxocara* infections. For instance, infection rates are higher for dogs and cats with outdoor access, due to the likelihood of being exposed to contaminated soil and infected paratenic hosts (Näreaho et al., 2012; Beugnet et al., 2014; Shukullari et al., 2015). Studies have also shown that younger cats and dogs have a higher risk of being infected (Palmer et al., 2008; Savilla et al., 2010; Näreaho et al., 2012; Zanzani et al., 2014; Villeneuve et al., 2015), due to activation of latent larvae in the tissues of the bitch and queen and migration across the placenta and to mammary gland and milk infecting the puppies and kittens (Overgaauw, 1997).

The extensive distribution and strong concentration of *Toxocara* infections are mainly associated to: (i) female fecundity (~700 eggs per gram of faeces per day per worm); (ii) egg resilience to environmental challenge and (iii) the life cycle of the parasite, that involves encystment of larvae in the somatic tissue of the female hosts that thus act as reservoirs of infection for the offspring (Taylor et al., 2007; Bowman, 2014).

2.1.2. Morphology

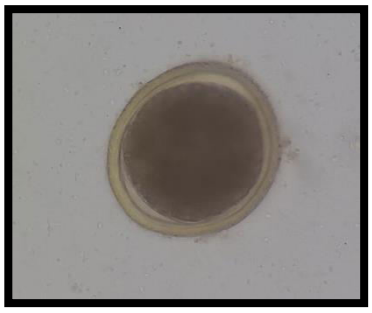
Adult ascarids are large white opaque worms with no buccal capsule, and a mouth surrounded by three lips, one dorsal and two subventral. Some genera, like *Toxocara*, are characterised by two lateral cervical alae that make the anterior end of the worm resemble an arrowhead (Figure 1) (Taylor et al., 2007).

Ascarids have a few characteristic features: a mouth surrounded by three lips, one dorsal and two subventral, a large cervical alae and a glandular oesophageal bulb and the *ventriculus*, located at the junction between oesophagus and the intestine (Bowman, 2014). Male *T. canis* is around 10 cm long, while the female can be up to 18 cm long (Taylor et al., 2007). Males also feature a terminal narrow appendage and caudal alae. The female genital organs prolong from the anterior end to the vulvar region (Taylor et al., 2007). Adult *Toxocara cati* are smaller than *T. canis*, i.e. males 3-6 cm and females 4-10 cm long (Taylor et al., 2007).

Figure 1. Anterior end of *Toxocara cati* adult worm (x200) (Alho, 2010, p.36)

Toxocara eggs are characterised by a thick external layer, an evolutionary adaptation that provides a protective feature against harsh environmental conditions (Bowman, 2014). Eggs of *T. canis* and *T. cati* are morphologically undistinguishable, in spite of slight size differences. Indeed, *T. canis* eggs are 90 x 75 µm in average, whilst *T. cati* eggs are ~65 x 75 µm (Fahrion, Schnyder, Wichert & Deplazes, 2011). Eggs in faeces appear dark brown and subglobular, with a central unicellular embryo (Figure 2) (Araújo, 1972).

Figure 2. *Toxocara cati* egg showing the thick external layer, internal wall and unicellular embryo (aprox. x230) (original)



2.1.3. Life Cycle

The adult worm resides in the intestine of the definitive host, that can shed up to 200 000 eggs per day (Bowman, 2014). When shed, the eggs containing the first stage larvae (L1), are not infective, and undergo development for 3 – 6 weeks before becoming infectious (Overgaauw, 1997). The time needed for the unembryonated egg to moult into the infective stage larvae varies depending on environmental conditions. Azam, Ukpai, Said, Abd-Allah and Morgan (2011) reported that *T. canis* larvae can resist at a wide range of temperatures, as low as -2ºC for 6 weeks. Relative humidity and oxygen tension also play a role in the maturation into the infective stage (Araujo, 1972; Zajac & Conboy, 2012). While, for decades, the second larval stage was traditionally considered the ascarid infective stage, recent studies have shown that the latter is indeed a third stage larva (L3) (Schnieder, Laabs & Welz, 2011). L3s encapsulated in the eggshell can remain viable in the environment for at least 1 year (Overgaauw, 1997).

Following the ingestion by the definitive host, the eggs reach the duodenum and hatch within 2 to 4h. The infective larvae are then released and infiltrate the mucosa of the intestine. How the penetration occurs is still unclear, but a parasite-secreted elastase-like protease was hypothesized to play a role in digestion of host tissues (Schnieder et al., 2011). Direct mechanical disruption has also been hypothesized (Schnieder et al., 2011). After penetrating the intestinal wall, the larvae enter the circulatory system and reach the liver, via portal circulation, 24h post-infection (Webster, 1958). Within the subsequent 12h, the larvae continue their migration and reach the heart through the *vena cava* and the lungs *via* the pulmonary artery (Webster, 1958). Here, according to the age and immune status of the host, as well as the infecting burden, the larvae may infiltrate the alveoli and continue their migration through the bronchioles and trachea to the pharynx, where they are swallowed, thus reaching the intestine and developing into adults in this site ("tracheal route") (Schnieder et al., 2011). Moult to a fourth stage larva (L4) is thought to occur in the bronchioles (Schnieder et al., 2011), while moult to the immature adult stage (L5) occurs after the larvae reach the small intestine (Schneider et al., 2011). The chance of the "tracheal route" happening was found to decrease after the puppy reaches 3 months old (Greve, 1971; Oshima, 1976) representing the phenomenon known as 'age resistance' (Schnieder et al., 2011), which is attributed to the development of immune competence and acquired immunity (Barriga, 1988).

In animals >6 months of age, larvae also infiltrate the alveoli and return to the circulatory system, to reach various somatic tissues ("somatic route") (Schnieder et al., 2011), including skeletal muscles, kidneys, but also liver and central nervous system. In these tissues, the L3s encyst, remaining infective (Schnieder et al., 2011).

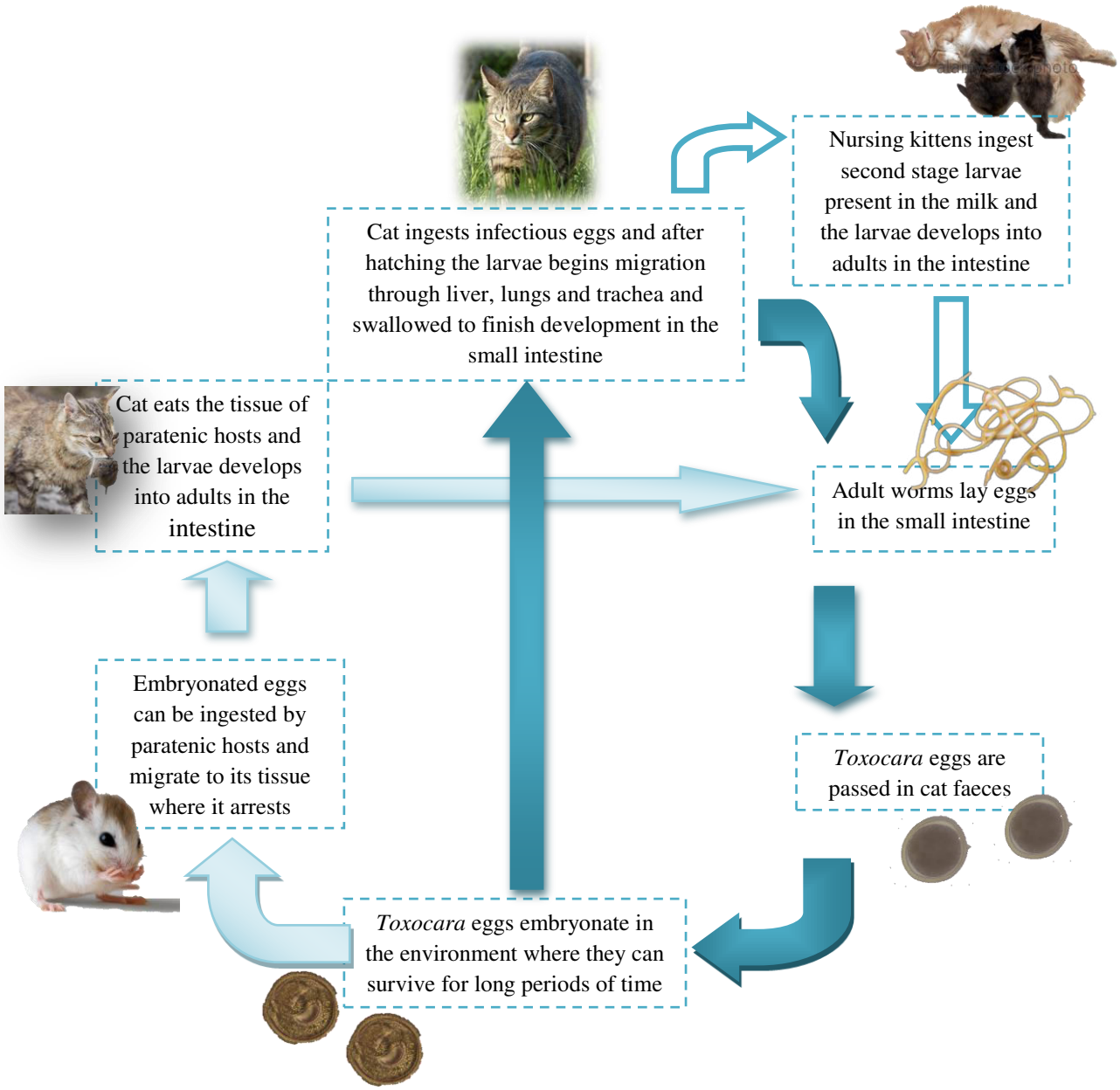
In female dogs, encysted larvae 'reactivate' during pregnancy and reach the foetus via the circulation and through the placenta (Schnieder et al., 2011). The larvae may also be transmitted via the lactogenic route, although it has been suggested that the latter way of transmission plays a minor role in the epidemiology of canine toxocarosis (Strube, Heuer & Janecek, 2013). The mechanism that determine reactivation of the larvae is yet unclear, but hormonal changes during pregnancy were hypothesized to play a role (Schnieder et al., 2011). After passing through the placenta, the larvae move to the liver of the foetus, where they remain until birth. At birth, larvae migrate to the lungs and follow the tracheal route (Schnieder et al., 2011).

In cats, reactivation of *T. cati* larvae encysted in somatic tissues of pregnant queen is unlikely and has only been shown to be lactogenically transmitted after acute infection during late pregnancy (Coati, Schnieder & Epe, 2004). Figure 3 illustrates the main stages of the *Toxocara cati* development in cats.

Besides ingestion of eggs, the definitive host may also become infected via ingestion of paratenic hosts that harbour *Toxocara* spp. larvae in their somatic tissues (Strube et al., 2013). Paratenic hosts include birds, mice, rabbits, foxes and humans (Schnieder et al., 2011). The migration pattern of *Toxocara* spp. larvae, especially *T. canis*, has been characterised in a variety of animal models (Strube et al., 2013), including rodents (mice, rats and gerbils), pigs, birds and primates (Strube et al., 2013). Tissues affected by encysted larvae, and distribution and survival of parasites are species dependent (Strube et al., 2013). However, for all of these species, somatic migration always affects the liver and the lungs (Strube et al., 2013). After ingestion of embryonated eggs, larvae penetrate the intestine wall and follow the same route as in the definitive host. After reaching the liver, some continue migration to the heart and lungs, whereas the others remain in the liver (Strube et al., 2013). After reaching the lungs, distribution to somatic tissues varies according to species of paratenic host (Strube et al., 2013). Larvae of *T. cati* migrate to the muscles of the latter, while *T. canis* larvae are more frequently detected in the central nervous system of these hosts (Overgaauw & van Knapen 2013).

No conclusive data is available regarding transmission of infective larvae to the offspring of paratenic hosts (Strube et al., 2013). However, lactogenic transmission is possible in mice infected by *T. canis* or *T. cati* (Strube et al., 2013). Interestingly, infection of mice with *Toxocara* resulted in behavioural changes, including a decreased activity and aversion to open areas, which makes them more likely to be caught by predators (Schneider et al., 2011).

Figure 3. *Toxocara cati* life cycle (original)



2.1.4. Pathogenesis and clinical signs

Clinical signs of toxocarosis vary with the age of the animal and the number, location and parasite developmental stage (Overgaauw, 1997). The infection with *Toxocara* spp. is highest in puppies and kittens until they are 6 months old (Overgaauw, 1997).

At birth, puppies can develop pneumonia and oedema as a result of larval migration during the pulmonary phase (Urquhart, 1996).

The migration of larvae through the lungs can be accompanied by coughing and dyspnoea and a bubbly nasal discharge (Urquhart, 1996; Overgaauw, 1997). Presence of adult worms in the intestine is associated to a limited to moderate mucoid enteritis that may cause diarrhoea and vomiting. Distension of the abdomen (pot-belly) may occur, likely as a result of gas formation caused by dysbacteriosis (Overgaauw, 1997). In cases of heavy burdens of infection, the adult worms may cause obstruction and rupture of the intestine, as well as invasion of the bile ducts, perforation of the liver parenchyma and invasions of the abdominal cavity causing peritonitis (Overgaauw, 1997; Schnieder et al., 2011). However, it is during the pulmonary phase and pups which are heavily infected that most deaths happen, sometimes just a few days from birth (Urquhart, 1996).

As a result of age resistance, adult dogs are rarely heavily infected, and therefore clinical signs are rare, except in the case of immunocompromised subjects (Overgaauw, 1997).

In puppies infected transplacentally, haematological findings include anaemia and eosinophilia (Schnieder et al., 2011). For both puppies and adult dogs, an increase of liver enzymes such as glutamate dehydrogenase (LDH) and alanine transaminase (ALT) can occur as a result of larval migration through the liver (Schnieder et al., 2011).

Histopathological changes observed in the tissues affected by the parasite include eosinophil infiltration and formation of granulomas, sometimes surrounding a larva (Schneider et al., 2011).

Kittens usually do not show clinical signs because infection is thought to be mainly acquired via the lactogenic route (Coati et al., 2004). Adult worms can only be found in the intestine ~28 days from birth, with clinical signs similar to those mentioned for dogs, albeit usually less severe (Overgaauw, 1997). If present, clinical signs in cats are usually limited to the intestine, and include pot-belly, diarrhoea, poor coat and failure to thrive (Urquhart, 1996). However, lung disease can still occur, even in the absence of symptoms (Dillon et al., 2013).

2.1.5. Immunological responses and evasion of the immune system

Data collected in paratenic hosts (i.e. murine models of infection) indicate that larval migration is rapidly followed by the onset of the host adaptive immune response. In particular, *Toxocara*-specific antibodies are produced, accompanied by an expansion of CD4+ T-helper type 2 cells (Th2) (Maizels, 2013). The Th2 response results in the production of the interleukins (IL)-4,-5, -10 and -13 (Maizels, 2013). IL-4 drives B cell differentiation and

switch from IgM into IgG, IgA and IgE (Maizels, 2013), while IL-5 drives the differentiation of eosinophils (Maizels, 2013).

Increased levels of IgE and eosinophils are an important feature of *Toxocara* infection (Schneider et al., 2011; Maizels, 2013). Host responses are partially stimulated by exposure to *Toxocara* excretory-secretory (TES) antigens (Maizels, Schabussova, Callister & Nicoll, 2006).

Prolonged larvae migration and long-term survival of arrested larvae in the host tissue occur as a result of an impressive ability of *Toxocara* to repeatedly slough off antibodies and cells from the outer cuticle (Maizels, 2013). This mechanism is preserved by constant expression and turnover of TES antigens, which results in the formation of an antigenic coat (Schnieder et al., 2011; Maizels, 2013). *T. canis* is also able to survive the activity of eosinophils (Maizels, 2013); while these rapidly adhere to the parasite surface, in the presence of specific antibodies, within 24 hours the larvae shed the outer cuticle to which immune cells are attached (Maizels, 2013). Indeed, electronic microscopy analysis of *Toxocara* larvae allowed to observe thickening and condensation of the surface coat upon incubation with immune serum for at least 30 minutes (Badley, Grieve, Rockey & Glickman, 1987).

Using different tools, such as peptide sequencing, monoclonal antibody binding and recombinant DNA techniques, three components of the TES-molecules were characterized, and two were completely identified (Maizels, 2013). TES-26 is a functional phosphatidyl-ethanolamine (PE)-binding protein with homology to a similar mammalian protein, and it was thus renamed as Tc-PEB-1 (Maizels et al., 2006; Maizels, 2013). Its function in the biology of the parasite remains thus far unclear (Maizels et al., 2006; Maizels, 2013). TES-32 and TES-70 are both members of the C-type lectin (CTL) family (Maizels et al., 2006; Maizels, 2013). Host lectins are known to play central roles in pathogen recognition and activation of the mammalian innate immune system and are therefore involved in host-host communications leading to the induction of inflammation (Maizels et al., 2006). Parasites may use soluble CTLs to compete for carbohydrates binding sites, thus blocking the inflammatory process (Maizels et al., 2006). TES-32 (CTL-1) is one of the major larval surface proteins; its 219-amino acid sequence is characterised by a carbohydrate-recognition domain with similarity to mammalian CTLs (Maizels et al., 2006). Recombinant TES-32 has been shown to bind monosaccharides in the presence of calcium, similarly to its mammalian homologue (Maizels et al., 2006). TES-70 (CTL-4), a larger protein than TES-32, is produced in smaller amounts; this protein binds to mammalian cells although its precise function is unclear (Maizels et al., 2006).

Several studies have shown that *T. canis* larvae express a range of mucin-like glycoproteins containing Serine-Threonine rich domains which act as a site for glycosylation, and that form the antigenic coat (Maizels, 2013).

Five different mucins (MUCs) have been identified using a combination of monoclonal antibody analysis and proteomics approaches (Maizels et al., 2006; Maizels, 2013); MUC-1 and MUC-3, together with MUC-2, form the TES-120 complex (Maizels et al., 2006).

In older definitive hosts, e.g. dogs >6 months of age, as previously mentioned, a phenomenon occurs known as 'age resistance' (Schnieder et al., 2011), which follows the development of immune competency and acquired immunity to the parasite. The onset of the latter occurs in the lung as a delayed hypersensitivity response (Schnieder et al., 2011). Also in immune dogs, L3s are, in some extent, prevented from penetrating the intestinal mucosa as a result of an inflammatory allergic reaction in which vasoactive amines are released by sensibilised mastocytes (Schnieder et al., 2011). Nevertheless, in immune dogs, somatic tissues still represent important reservoirs of hypobiotic larvae, which remain undetected by the host immune system (Schnieder et al., 2011).

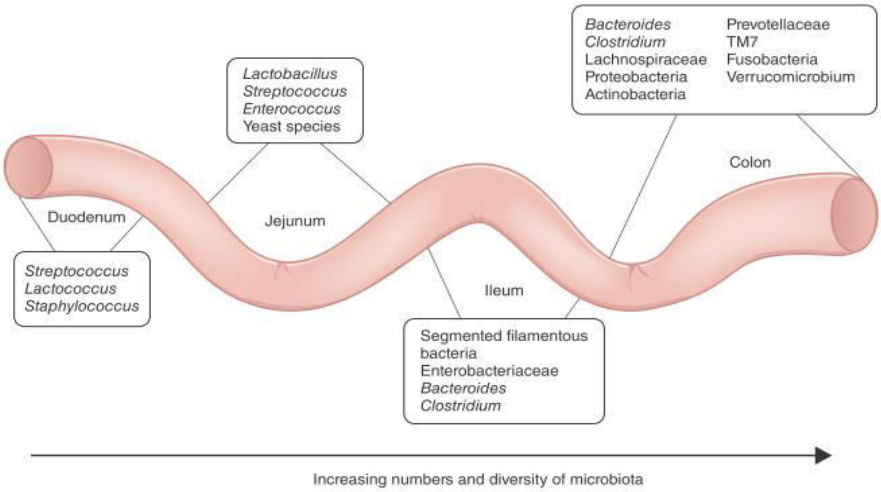
3. Gut microbiota in dogs and cats: composition and functions

The intestinal microbiota is a compound and shifting ecosystem, made of 10^{10} – 10^{14} microbes residing in the gastrointestinal tract (Handl, Dowd, Garcia-Mazcorro, Steiner & Suchodolski, 2011; Swanson et al., 2011). Mammals are colonized by different species of bacteria starting from birth (Round & Mazmanian, 2009). The feline gut microbiota undergoes significant changes occurring in parallel with growth and dietary modifications (Jia et al., 2011). While the intestinal microbiota of adult cats and dogs is believed to stay more or less constant over time, some studies have suggested that older animals harbour a slightly modified commensal microbiota (Benno, Nakao, Uchida & Mitsuoka, 1992; Jia et al., 2011).

In addition, that number of species (richness) and diversity of the gut microbiota increase substantially from duodenum to colon (Minamoto, Hooda, Swanson & Suchodolski, 2012). Species diversity can be defined as the number of different species that are found in a population (Tuomiso, 2010a); and it is dependent of the species richness and species enveness. Whereas the species richness is the sum of species present, the species evenness quantifies how similar the abundance of the species are (Tuomiso, 2010a; Tuomiso, 2010b).

Each bacterial species colonizes a specific niche; for example, species belonging to *Lactobacillus* and *Enterococcus* mainly occur in duodenum, while those belonging to *Bacteroides* and *Prevotellaceae* are predominantly found in the colon (Figure 4) (Brown et al., 2013).

Figure 4. Composition and spatial distribution of microbiota along the mammalian intestinal tract (Brown et al., 2013)



Traditionally, culture-based methods have been used to identify and describe the bacterial groups that comprehend the gut microbiota of mammals (Minamoto et al., 2012). However these methods are extremely time-consuming and do not permit the identification of most bacterial groups present in the GI tract, which are currently uncultivable (i.e. 99%) (Nocker, Burr & Camper, 2007). High-throughput sequencing technologies, combined with bioinformatics tools for analyses of large-scale sequence data developed in the past decades have allowed to characterise several hundred bacterial genera present in the vertebrate GI tract (Minamoto et al., 2012).

The most predominant phyla in the canine and feline gut are the Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria and Actinobacteria (Deng & Swanson, 2015). The relative proportions of each of these phyla vary between studies, likely as a result of methodological differences (Deng & Swanson, 2015) alongside differences in animal breed, diet, age and living conditions (Deng and Swanson, 2015).

Details concerning the study design, animal characteristics and method of analyses are described in Table 1 and 2.

Table 1 – GI microbiota of healthy dogs based on 16S rRNA gene sequencing methods

Sample type	Type of animal and breed	Predominant phyla (expressed as percentage of sequences)	Reference
Gastric biopsies	Research dogs/ Mix-breed	Proteobacteria (99.6%); Firmicutes (0.3%)	Garcia-Mazcorro et al., 2012
Duodenal biopsies	Client owned dogs/ Greyhounds and Beagles	Firmicutes (46.4%); Proteobacteria (26.6%); Bacteroidetes (11.2%); Spirochaetes (10.3%); Fusobacteria (3.6%); Actinobacteria (1%)	Xenoulis et al., 2008
Duodenal biopsies	Research dogs/ Mix-breed	Firmicutes (46.9%); Proteobacteria (21.2%); Bacteroidetes (6.9%)	Garcia-Mazcorro et al., 2012
Duodenal digesta	Research dogs/ Hound dogs	Firmicutes (~60%); Proteobacteria (32%); Fusobacteriales and Bacteroidales only present sporadically	Suchodolski, Camacho & Steiner, 2008
Jejunal digesta	Research dogs/ Hound dogs	Firmicutes (~80%); Fusobacteriales (~10%)	Suchodolski et al., 2008
Jejunal brush samples	Research dogs	Proteobacteria (46.7%); Firmicutes (15%); Actinobacteria (11.2%); Spirochaetes (14.2%); Bacteroidetes (6.2%); Fusobacteria (5.4%)	Suchodolski et al., 2009
Ileal digesta	Research dogs/ Hound dogs	Fusobacteria (32.6%); Firmicutes; Bacteroidetes	Suchodolski et al., 2008
Colon contents	Research dogs	Firmicutes (47.7%); Proteobacteria (23.3%); Fusobacteria (16.6%); Bacteroidetes (12.4%)	Suchodolski et al., 2008
Faecal	Research dogs/ mongrels and hound-crosses	Fusobacteria (23-40%); Bacteroidetes (31-34%); Firmicutes (14-28%); Proteobacteria (5-7%); Actinobacteria (0.8-1.4%)	Milddelbos et al., 2010
Faecal	Research dogs/ mongrels and hound-crosses	Bacteroidetes (37-38%); Firmicutes (31-35%); Proteobacteria (13-15%); Fusobacteria (7-9%); Actinobacteria (1%)	Swanson et al., 2011
Faecal	Client owned dogs/ ten different breeds	Firmicutes (95%); Bacteroidetes (2.2%); Actinobacteria (1.8%); Fusobacteria (0.3%)	Handl et al., 2011
Faecal	Client owned dogs/ ten different breeds	Firmicutes (97.5%); Actinobacteria (0.9-2.0%); Bacteroidetes (0.1-1.1%); Fusobacteria (0.1-0.8%); Proteobacteria (0.1%)	Garcia-Mazcorro et al., 2011
Faecal	Research dogs/ Hound bloodlines	Firmicutes (56.66%); Fusobacteria (27.44%); Tenericutes (12.74%); Proteobacteria (1.53%); Bacteroidetes (1.17%); Actinobacteria (0.23%)	Panasevich et al., 2014

Table 2 – GI microbiota of healthy cats based on sequencing methods

Sample type	Type of animal and breed	Method	Predominant phyla (expressed as percentage of sequences)	Reference
Intestinal content	Conventionally raised cats	Sanger sequencing on constructed 16S rRNA gene clone libraries	Firmicutes (68%); Proteobacteria (14%); Bacteroidetes (10%); Fusobacteria (5%); Actinobacteria (4%)	Ritchie, Steiner & Suchodolski (2008)
Faecal	Client owned indoor and outdoor cats	*cpn*60 gene clone libraries sequencing	Firmicutes (41 and 72% from indoors and outdoors cats respectively); Actinobacteria; Bacteroidetes and Proteobacteria	Desai, Musil, Carr & Hill (2008)
Faecal	Pet cats	High-throughput 454-pyrosequencing	Firmicutes (92%); Actinobacteria (7.3%); Bacteroidetes (0.45%); Fusobacteria (0.04%)	Handl *et al*. (2011)
Faecal	Privately owned cats	Next generation 16S rRNA gene 454-pyrosequencing	Firmicutes (92-95%); Actinobacteria (4-7%); Proteobacteria + Bacteroidetes + Fusobacteria (<1%)	Garcia-Mazcorro *et al*. (2011)
Faecal	Research cats	Shotgun 454-pyrosequencing	Firmicutes (36.3); Bacteroidetes (36.1%); Proteobacteria (12.4%); Actinobacteria (7.7%)	Barry *et al*. (2010)
Faecal	House-hold cats	GS Junior titanium shotgun pyrosequencing	Bacteoidetes (68%); Firmicutes (13%); Proteobacteria (6%); Actinobacteria (1.2%) and Fusobaceria (0.7%)	Tun *et al.* (2012)

Albeit variations according the method, type of animal/breed and sample type, in dogs, within the phylum Firmicutes, the most abundant orders are Bacillales, Lactobacillales and Clostridiales (Suchodolski et al., 2008; Xenoulis et al., 2008; Suchodolski et al., 2009; Handl et al., 2011; Swanson et al., 2011; Panasevich et al., 2014). Within the order Clostridiales, the family Clostridiaceae is always reported as one of the most abundant (Suchodolski et al., 2008; Xenoulis et al., 2008; Suchodolski et al., 2009; Handl et al., 2011; Swanson et al., 2011; Panasevich et al., 2014); and Handl et al. (2011) was able to demonstrate the genus *Clostridium* as the most abundant within the family, followed by the genus *Ruminococcus* from the Ruminococcaceae family (Handl et al., 2011; Panasevich et al., 2014). The family Carnobacteriaceae is reported to be one of the most abundant within the order Lactobacillales (Xenoulis et al., 2008). Still within the Firmicutes, one further family within the Clostridiales and one further order were reported by Handl et al. (2011): the family Lachnospiraceae, represented mainly by the genus *Dorea* and *Roseburia* and the order Erysipelotrichia, represented mainly by the family Erisipelotrichaceae, respectively (Handl et al., 2011). According to Suchodolski et al. (2009) the phylum Bacteroidetes is found to be mainly represented by the order Bacteroidales; the phylum Fusobacteria by the family Fusobacteriaceae and the phylum Actinobacteria by the family Corynebacteriaceae. At last, within the phylum Proteobacteria the most abundant class is Gamma-proteobacteria, which is mainly represented by the families Pasteurellaceae, Moraxellaceae and Enterobacteriaceae (Suchodolski et al., 2008; Suchodolski et al., 2009 and Xenoulis et al., 2012).

Again, despite variations according to the method, type of animal/breed and sample type, in cats, within the phylum Firmicutes, the most abundant reported orders are Clostridiales, Lactobacillales and Erysipelotrichales (Desai et al., 2008; Ritchie et al., 2008; Handl et al., 2011). In the study performed by Handl et al. (2011) the order Lactobacillales consisted mostly of the genera *Enterococcus* and *Lactobacillus*, the order Erysipelotrichales, included primarily the genera *Turicibacter*, *Catenibacterium*, and *Coprobacillus*. Still in this study the phylum Actinobacteria, the second most abundant phylum, was mainly represented by the order Coriobacteriales (Handl et al., 2011). Within this order, all sequences belonged to the family Coriobacteriaceae, which consisted mainly of the genera *Eggerthella* and *Olsenella* (Handl et al., 2011).

The development of next-generation sequencing (NGS) and hence high-throughput comparative metagenomics have been responsible for the fast increase of research in this area that have added valuable information of the composition and function of bacterial populations in various environments (Jovel et al., 2016).

Tun et al. (2012) analysed pooled faecal samples from five healthy privately owned cats; the most predominant phylum was Bacteoidetes (68%), followed by Firmicutes (13%); Proteobacteria (6%); Actinobacteria (1.2%) and Fusobaceria (0.7%) (Tun et al., 2012). Within Bacteroidetes, the order Bacteroidetes was predominant whereas, within Firmicutes, the most abundant class was Clostridia (65%), followed by Bacilli and Mollicutes (Tun et al., 2012). The metagenomic approach used by these authors also allowed the characterization of individual microbial genes; microbial carbohydrate and protein metabolism represented 13 and 9% of the feline metagenome, respectively, while other key functional metabolic groups included DNA metabolism (8%), virulence factors (7%), and amino acid metabolism (6%) (Tun et al., 2012).

Barry et al. (2010) undertook a metagenomcis analysis of 12 individual faecal samples from four healthy cats; the predominant phyla were Firmicutes (36.1%) and Bacteroidetes (24-36%), followed by Proteobacteria (12.4%) and Actinobacteria (7.7%). The major functional metabolic categories identified were carbohydrates (15%); clustering-based subsystems (14%); protein metabolism (8%); amino acids and derivatives (8%); cell wall and capsule (7%); DNA metabolism (7%); virulence (6%); and cofactors, vitamins, prosthetic groups and pigments (6%) (Barry et al., 2010).

The symbiotic relationship between the intestinal microbiota and host is pivotal for host metabolism, organ development, immune system maturation, and defence against pathogens (Sommer & Bäckhed, 2013). In return, the microbiota inhabits a safe and nutrient-rich environment (Lee & Mazmanian, 2010).

A number of studies have demonstrated that the development of the gastrointestinal tract is dependent upon the gut microbiota (Sommer & Bäckhed, 2013). Comparative studies between wild-type and germ free (GF) mice reported that the intestine of the latter is characterised by an underdeveloped brush border and thinner intestinal villi (Abrams, Bauer & Sprinz, 1963; Reinhardt et al., 2012).

Other studies performed in GF mice support the part played by gut microbiota in preserving the structure and function of the intestinal villi (Crawford & Gordon, 2005). Furthermore, microbial wall peptidoglycan stimulates toll-like receptor 2 (TLR2) signalling, which is pivotal for the maintenance of tight junctions (Cario, Gerken & Podolsky, 2007). Also involved in the maintenance of the tight junctions is *Bacteroides thetaiotaomicron*, reported to prompt the expression of the small proline-rich protein 2A (sprr2A), that is required for the maintenance of the epithelial villus desmosomes (Hooper et al., 2001).

Prevention of cytokine apoptosis of the intestinal epithelial cells is another mechanism involved in the maintenance of structure and function of the intestinal villi by the gut microbiota (Rackoff-Nahoum, Paglino, Eslami-Varzaneh, Edberg & Medzhitov, 2004). For instance, *Lactobacillus rhamnosus GG* strain produces p40 and p75, this two soluble proteins are able to activate the Akt pathway, inactivate proapoptotic pathways (Hanada, Feng & Hemmings, 2004), reduce the apoptosis of the epithelial cell induced by cytokines and stimulate cell growth of the epithelial cells, both in humans and mice (Yan et al., 2007).

The gut microbiota can also control gut barrier functions (Sommer & Bäckhed, 2013); for instance, *Akkermansia muciniphilia* leads to an increase in endocannabinoids levels which, in turn, results in a decrease of metabolic endotoxemia (Everard et al., 2013).

The gut microbiota is also important for the homeostasis of other tissues (Sommer & Bäckhed, 2013). Indeed, bone density is increased in GF mice when compared with wild-type mice, likely as a result of a decrease in number of serotonin receptors in the bone cells of the former (Sjögren et al., 2012). Bacterial colonization of GF mice resulted in the normalization of the bone density (Sjögren et al., 2012; Sommer & Bäckhed, 2013).

The presence of a large number of bacteria in the intestine of the animal, together with their proximity to epithelial tissues, represents a challenge to the mucosal immune system, which in one hand needs to tolerate the valuable commensals and on the other hand prevent the excessive expansion of the resident bacteria (Jandhyala et al., 2015). Despite this, the gut microbiota plays an important role in shaping both innate and adaptive immune systems and regulating intestinal immunity (Sommer & Bäckhed, 2013), via communications with the gut associated lymphoid tissues (GALT), effector and regulatory T cells, IgA producing B cells, and, resident macrophages and dentritic cells (DCs) in the lamina propria (Jandhyala et al., 2015). A role of the gut microbiota in promoting development of a 'normal' GALT has been demonstrated in studies conducted in GF mice (Round & Mazmanian, 2009). In these studies, the GALT of GF mice was characterised by reduced number of B, T and DCs and mesenteric lymph nodes (MLN), as well as by undeveloped Peyer´s patches (Round & Mazmanian, 2009). Colonization with commensal microorganisms resulted in the development of the GALT (Sommer e Bäckhed, 2013).

The surface of microorganisms presents molecules associated with different groups of microbes, such as peptidoglycans, lipopolysaccharides (LPS), lipid A, flagellin and bacterial RNA/DNA, fungal cell wall β-glucans, among others (Takeuchi & Akira, 2010). These components are part of microbe-associate molecular patterns (MAMPs), which activate immune responses (Takeuchi & Akira, 2010).

TLRs, C-type lectin receptors (CLRs) such as Dectin-1, and the cytosolic nucleotide-binding and oligomerisation domains (NOD) like receptors (NLRs) [all together, the pattern recognition receptors (PPR)] are present in the surface of macrophages, neutrophils and DCs (Takeuchi & Akira, 2010). The cross-talk between the PRRs and MAMPs leads to the activation of numerous signalling cascades, promoting the mucosal barrier functions and production of antimicrobial proteins (AMPs) such as cathelicidins, C-type lectins, and (pro)defensins by Paneth cells, and mucin glycoproteins and IgA (Takeuchi & Akira, 2010; Carvalho, Aitken, Vijay-Kumar & Gewirtz, 2012). *Bacteroides thetaiotaomicron* and *Lactobacillus innocua* are among the species responsible for increased production of AMPs, in the presence of a healthy microbiota (Hooper, Stappenbeck, Hong & Gordon, 2003; Cash, Whitman, Behrendt & Hooper, 2006).

By inducing production of IgA, the gut microbiota also prevents the overgrowth of pathogenic bacteria (Jandhyala et al., 2015). Gram negative bacteria like *Bacteroides* have been implicated in the activation of DCs leading, in turn, to the release of secretory IgA (sIgA) by the intestinal mucosa (He et al., 2007). sIgA binds to the gut microbiota, thus preventing the microbiota from entering the circulation (Jandhyala et al., 2015). Additionally, the cross-talk between the TLRs and MAMPs can induce the production of a proliferation-inducing ligand (APRIL) by the intestinal epithelial cells, which has the ability to switch a systemic sIgA1 phenotype to the intestinal mucosal sIgA2, more resistant to bacterial proteases (Macpherson & Uhr, 2004).

T cells differentiate into different subclasses implicated in different pro-inflammatory and anti-inflammatory pathways (Zhu, Yamane & Paul, 2010). Th1, Th2 and Th17, T helper cells, promote inflammation while $Foxp3^+$ T regulatory cells (Treg) are contrast inflammatory responses (Sommer and Bäckhed, 2013). The Gram negative bacteria *Bacteroides fragillis* induces the differentiation of T cells CD4+ into Tregs, which leads to the production of anti-inflammatory cytokines like interleukin-10 (IL10) contrasting the pro-inflammatory response mediated by Th17 cells (Maynard et al., 2007; Round et al., 2011). Induction of Tregs seems to be mediated by TLR2 signalling via the polysaccharide A on the surface of the bacterial capsule (Round et al., 2011).

4. Helminth-microbiota interactions (HMI)

The interactions between parasitic helminths and the gut microbiota have been investigated in a range of animal species, from mice (Walk et al., 2010; Reynolds et al., 2014; Holm et al., 2015; Houlden et al., 2015; Zaiss et al., 2015), pigs (Li et al., 2012), goats (Li et al., 2016) macaques (Broadhurst et al., 2012), to cats and dogs (Šlapeta et al., 2015).

Despite variations attributed to different range of animals and parasites, experimental designs and techniques, a few consistent alterations in the composition of the gut microbiota of the animals infected with helminths have been reported (Peachey et al., 2017).

For example, the abundance of Lactobacillaceae populations with an important role in carbohydrate metabolism (Felis & Pot, 2014) is frequently increased in the presence of helminths in the GI tract of animals.

In 2010, Walk et al. reported that infection of mice with the parasitic worm *Heligmosomoides polygyrus*, alters the relative abundance and distribution of gastrointestinal bacteria which, in turn, modulates host immunity. The authors also reported an increase in bacterial load/abundance in the ileum – but not in the cecum – of infected mice (Walk et al., 2010) and, in particular, an expansion of *Lactobacilliaceae* in the ileum (Walk et al., 2010).

Similarly, in 2015, Holm et al. also reported an increased abundance in Lactobacilli in response to *Trichuris muris* infection, also accompanied by a decrease in the diversity of intestinal microbiota (Holm et al., 2015). Other downstream effects of chronic infection included a skewed regulatory/inflammatory T cell balance in the intestine (Holm et al., 2015). Following on from this study, Houlden et al. (2015) demonstrated that parasite-associated changes in the metabolome and microbiota are reversed by anthelmintics.

In a study by Fricke et al. (2015) mice infected with *Nippostrongylus brasiliensis* presented a significantly increase of the members of the family Lactobacilliaceae in the ileum. These authors were also able to demonstrate the role of Th2 responses in parasite-associated modifications of the commensal microbiota (Fricke et al., 2015).

Lactobacilli by binding to PRRs expressed on immune cells prompt innate and adaptive immune responses in the host and several other tissues including the intestinal epithelium resulting in the expansion of Treg cells (Wells, 2011). Studies on mice indicate that specific strains of probiotics can be used to prevent or treat inflammatory diseases (Wells, 2011).

Interestingly, Reynolds et al. (2014) reported that administration of the bacteria, commensal of rodents, *Lactobacillus taiwanensis* to BALB/c mice was associated with increased establishment of the parasitic nematode, *H. polygyrus*.

In addition, infections by the latter were associated with increased abundance of *Lactobacillus* species in the duodenum of C57BL/6 mice whereas SPF (specific pathogen free) BALB/c mice showed high levels of worm expulsion after the same days post infection (Reynolds et al., 2014). These data led the authors to hypothesize that the microbiota composition in the duodenum increases the persistence of *H. polygyrus* within the host, and that the parasite may actively change the microbiota in order to guarantee its own survival (Reynolds et al., 2014). In this cooperation, both promote the activation of T regulatory mechanisms, decreasing the chances of the host immune acting on the counterpart (Reynolds et al., 2014).

Other findings not as consistent as those found for Lactobacillaceae have been described. Broadhurst et al. (2012) reported that clinical improvements of macaques affected by idiopathic chronic diarrhoea (ICD) and experimentally infected with the whipworm, *Trichuris trichiura*, were associated to significant alterations in the composition of the microbiota of the colon, i.e. a decrease in bacteria belonging to the genus *Streptophyta* of phylum Cyanobacteria – which were increased in ICD macaques before helminth infection – and an increase in members of phylum Tenericutes (Broadhurst et al., 2012). Change in inflammatory signatures were profiled by flow cytometry analysis of leukocytes from pinch biopsies, as well as global analyses of gene expression, that showed reduced immune activation and increased mucosal T_H2 responses (Broadhurst et al., 2012).

In small animals, a study by Šlapeta et al. (2015) showed infections of healthy cats and dogs by protozoa and helminths (*Cystoisospora* sp. in cats, and *Ancylostoma caninum* in dogs) and only protozoa (*Giardia* sp.) are associated with shifts in the composition of the host gut microbiota and its functions. In dogs, a significant difference between the *Giardia*-positive and -negative groups when assessing bacterial genera, was found. Unlike the faecal microbiomes of dogs infected with the canine hookworm (*A. caninum*) where no such difference was found. The exclusion of dogs infected by hookworms led to the separation of *Giardia*-positive and -negative groups. In *Giardia*-positive cats, no significant difference based on bacterial genera was found but the opposite was found in *Cystoisospora*-positive cats, which showed significantly different microbial structure when compared with *Cystoisospora*-negative cats. The genera *Catenibacterium, Pseudobutyrivibrio* and *Subdoligranulum*, significant producers of short-chain fatty acids, like butyric acid, which are associated with protection against pathogens and overall welfare of the gut (Kageyama & Benno, 2000; Wong, de Souza, Kendall, Emam & Jenkins, 2006; Fernandez-Rubio et al., 2009), presented significantly different abundances in parasitized animals.

Pseudobutyrivibrio and *Subdoligranulum* abundances were significantly lower in asymptomatic dogs and cats with *Giardia*, respectively, and *Catenibacterium* was significantly more abundant in dogs with *Giardia*. This led the authors to suggest an indirect effect of the *Giardia* infection in the upper gastrointestinal tract on the colon metabolism even in non-diarrhoeic animals and that *Giardia* may be stimulating growth of *Catenibacterium.*

In 2012, Li et al. reported that experimental infection of pigs with the intestinal parasitic nematode *Trichuris suis* was associated with changes in abundances of 15 phyla and 13% of the bacterial genera. Approximately 26% of all identified metabolic pathways identified from the luminal content from proximal colon revealed were affected, including carbohydrate metabolism, lysine biosynthesis, and fatty acid absorption (Li et al., 2012).

Other studies have also reported changes in specific groups of bacteria and metabolic indicators linked to the fibrolytic potential and carbohydrate and protein transport and metabolism, following parasite infections (Li et al., 2012; Wu et al., 2012; Houlden et al., 2015; Li et al., 2016). For instance, studies in pigs infected with *T. suis* and mice infected with *T. muris* have implied a depression of metabolic pathways aforementioned in the colon (Li et al., 2012; Wu et al., 2012; Houlden et al., 2015). However the opposite, i.e. an increase in carbohydrate, protein and lipid metabolism, has been suggested to happened as a result of *H. polygirus* and *Haemonchus contortus* infections in mice colons and caprine abomasa, respectively (Kreisinger et al., 2015; Li et al., 2016).

Alpha diversity usually associated with a healthy gut homeostasis has been found to be reduced in many inflammatory bowel and/or systemic diseases in humans (Ott et al., 2004; Manichanh et al., 2006; Sepehri, Kottowski, Bernstein & Krause, 2007; Giloteaux et al., 2016) and thus is subject to investigation in many researches. Studies in rabbits and mice reported a clear reduction of alpha diversity during the acute phase of infection with *Trichostrongylus retortaeformis* and *T. muris*, respectively (Holm et al., 2015; Houlden et al., 2015; Cattadori et al., 2016). On the other hand, the alpha diversity was found to increase in humans and primates naturally or experimentally infected with nematodes like *Trichuris trichiura* and *Necator americanus* (Broadhurst et al., 2012; Lee et al., 2014; Giacomin et al., 2015; Giacomin et al., 2016), and remained unaffected in most studies conducted in a variety of animals/parasites (Li et al., 2011; Fricke et al., 2015; Kreisinger et al., 2015; McKenney et al., 2015; Slapeta et al., 2015; Li et al., 2016). The different findings on these studies might not only be explained by variations in the implicated animal and parasite species, and experimental circumstances but also by the phase of parasite infection in which samples are collected (Peachey et al., 2017).

It is reasonable to expect the decrease of microbial alpha diversity in the acute inflammatory phase of the infection, which can be restored or augmented during the development of chronic infections (Peachey et al., 2017).

Explanations on the causality of these interactions have arisen. For instance, helminth-associated changes in gut microbiota could be explained as a host immune response to the infection (Reynolds et al., 2014; Fricke et al., 2015; Holm et al., 2015; Cattadori et al., 2016). Indeed several studies describe an association between upregulation of cytokines after parasite invasion with changes in microbial composition (Rausch et al., 2013; Reynolds et al., 2014; Holm et al., 2015; Cattadori et al., 2016). For example, in a study where rabbits were infected with *T. retortaeformis* the increase in the abundance of Pasteurellaceae, Clostridiaceae, Ruminococcaceae, Peptos-treptococcaceae, and Flammenovirgaceae was related with the gene expression of interferon-g (IFN-g), whereas the reduction of Enterobacteriaceae was in negatively related with the expression of IL13 and IL14 (Cattadori et al., 2016).

Another hypothesis, that the changes in the microbiota are induced directly by the host in an attempt to generate an unfriendly environment for the parasite, has been supported by the increased production of AMPs in response to helminth infection in cattle and mice (D'Elia et al., 2009; Li et al., 2015), although it has been suggested that these responses could be the result of the Th2-mediated immunity (Fricke et al., 2015).

Finally, although the direct interaction between the TES products and the microbiota has not been reported yet, it would not be surprising if this products, some of them known to contain lysozymes (Hewitson et al., 2011), had an effect on the gut microbiota (Peachey et al., 2017).

5. Summary and research aims

Despite these efforts, knowledge HMI remains fragmentary. Capitalising on the sampling opportunities provided by a large European multicentric clinical study, with the aim to assess the efficacy of a combination of fipronil, (S)-methoprene, eprinomectin and praziquantel (i.e. Broadline^{TM}) against feline lungworm infections (Giannelli et al., 2016) the goal of investigate the qualitative and quantitative impact that infections by GI nematodes (i.e. *Toxocara cati*) exerts on the gut microbiota of cats was set. More accurately, the ultimate aim was to collect information on the effects of parasite infections on the composition of the commensal microbiota of domestic animals.

Chapter 2 - Materials and methods

1. Sample collection and parasitological testing

Individual faecal samples from household cats with acess to outdoor areas and able to hunt, living in Tessaloniki, Greece, were collected as part of a broader study on feline lungworm infection (Giannelli et al., 2016). All the cats were fed with identical dry commercial cat food (i.e. Purina Friskies®) for at least 6 months prior to sampling, were clinically healthy (e.g. absence of signs of GI disease or any other concomitant disease) and received no antibiotic and/or antihelminths treatment in the 12 and 3 months preceding the sample collection., respectively. None of the female cats included in this study was pregnant or lactating at the time of sample collection.

After collection the samples were immediately transported to the Laboratory of Parasitology and Parasitic Diseases, School of Veterinary Medicine of the Aristotle University of Thessaloniki (Greece) and kept at 4°C.

Here, individual samples were aliquoted for use in standard parasitological procedures (i.e. faecal egg counts [FEC] using a standard McMaster technique), as well as DNA extraction and high-throughput sequencing of the bacterial 16S rRNA gene (see below). Briefly, aliquots of 2 g of faeces were suspended in 28 ml zinc sulphate solution ($ZnSo_4$, specific gravity = 1.180); the suspension was homogenised, filtered using a double-layer gauze, and pipetted into McMaster chambers for microscopical examination and let to stand, allowing the eggs to float to the surface and the residues to reach the bottom of the chamber (Euzeby, 1981).

Only cats with or without patent *T. cati* infection and negative for other helminths and protozoan parasites at the fecal examination were included. Thus of the examined samples, 24 were positive for *T. cati* only and 21 were negative for any intestinal helminth parasite. The negative samples were used from this point onward as a negative control. Of the 24 cats that tested positive for *T. cati*, seven were males and 17 were females. The egg count showed an arithmetic mean of 254.2 eggs/g of faeces (EPG). Five cats that tested negative for *T. cati* were males and 16 were females (Table 3). The age of the cats ranged from 5 months to 10 years, with an arithmetic mean and median of 2 years.

Table 3. Sex, age (months), and weight (kg) in evaluated cats. For those infected by *T. cati* (left column), the infection burden has been expressed in EPG.

Infected animals					**Uninfected animals**			
ID	Sex	Age	Weight	EPG	ID	Sex	Age	Weight
I001	F	24	>2,5	200	C002	F	12	<2,5
I005	M	5	<2,5	750	C003	F	36	>2,5
I007	F	24	>2,5	200	C004	F	120	>2,5
I008	M	30	>2,5	300	C006	M	12	>2,5
I009	M	18	-	150	C010	F	24	-
I012	F	36	>2,5	150	C011	F	30	-
I015	F	18	>2,5	200	C013	F	36	>2,5
I017	F	12	<2,5	300	C014	M	12	>2,5
I018	F	30	>2,5	200	C016	F	18	>2,5
I019	F	6	<2,5	400	C021	F	24	>2,5
I020	F	12	>2,5	300	C022	F	24	>2,5
I023	M	12	>2,5	300	C025	M	12	>2,5
I024	F	18	>2,5	450	C028	F	30	>2,5
I026	F	24	>2,5	300	C029	F	24	>2,5
I027	F	24	>2,5	100	C030	F	36	>2,5
I031	F	60	>2,5	100	C034	M	12	>2,5
I032	F	36	>2,5	200	C035	F	36	>2,5
I033	F	24	>2,5	150	C037	F	18	>2,5
I036	F	12	>2,5	100	C043	F	30	>2,5
I038	M	24	>2,5	300	C044	M	24	>2,5
I039	M	12	>2,5	350	C045	F	24	>2,5
I040	F	24	>2,5	100				
I041	F	18	>2,5	100				
I042	M	18	>2,5	400				

The remaining samples were again aliquoted (aprox. 4g) and preserved in ethanol, and shipped to the Parasitology Unit of the University of Bari (Italy), where they were kept at -80°C until DNA extraction.

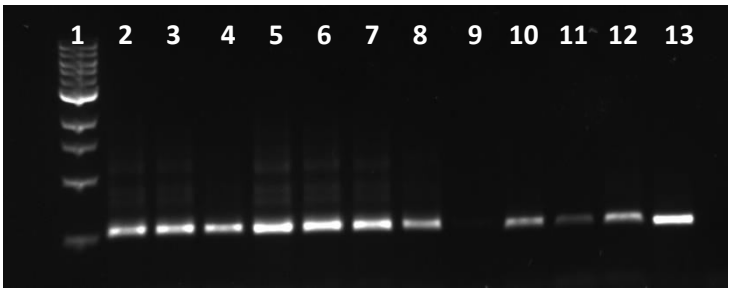
2. DNA extraction

Total genomic DNA was extracted directly from each *T. cati*-positive (*Tc*+) and –negative (*Tc*-) sample, as well as from two negative 'blank' (= no DNA) controls, using the PowerSoil® DNA Isolation Kit (MoBio, USA), according to manufacturers' instructions.
The extracted DNA concentration was assessed using Qubit Quant-iT™ dsDNA Broad-Range Assay Kit (Life Technologies, California, USA) and all samples were diluted to the final concentration of 0.2 ng/µl.

3. Library construction and 16S rRNA gene sequencing

High-throughput sequencing of the V3-V4 hypervariable region of the bacterial 16S rRNA gene was performed on an Illumina MiSeq platform according to the manufacturers' protocols with minor adjustments. Briefly, the V3-V4 region was PCR amplified using universal primers (Forward, 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGCAG-3; Reverse, 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′) (Klindworth et al., 2013), that contained the Illumina adapter overhang nucleotide sequences, using the NEBNext® Q5® Hot Start HiFi DNA polymerase (New England Biolabs® Inc, Massachusetts, USA) and the following thermocycling protocol: 98 °C/2 m, followed by 35 cycles of 98 °C/15 s, 63 °C/30 s, and 72 °C/30 s, and a final elongation step of 72°C/5 m. The quantity and quality of the amplicons were evaluated by agarose gel electrophoresis (Figure 5).

Figure 5. Agarose gel electrophoresis showing amplicons produced with universal primers for the V3-V4 region of the bacterial 16S rRNA gene (lane 1, marker; lane 9, negative control; lane 2 to 8 and 10 to 13 samples amplicons).



Amplicons were purified using AMPure XP PCR Purification beads (Beckman Coulter), and the NEBNext hot start high-fidelity DNA polymerase was used for the index PCR with Nextera XT index primers (Illumina) according to the following thermocycling protocol: 98 °C/30 s, 8 cycles of 98 °C/10 s, 65 °C/75 s, and at 65 °C/5 min. The indexing PCR reaction included 2.5 µl of each index primer, 2.5 µl of template DNA, 12.5 µl of NEBNext Hot Start HiFi DNA polymerase and 2.5 µl of molecular grade water.

The indexed samples were purified using AMPure XP beads, quantified using the Qubit Quant-iT™ dsDNA Broad-Range Assay Kit (Life Technologies, California, USA), and equal quantities from each sample pooled. The resulting pooled library was quantified using the NEBNext® Library Quant Kit for Illumina® (New England Biolabs) and sequenced on an Illumina MiSeq platform using the v3 chemistry (301 bp paired-end reads). Raw sequence data have been deposited in the NCBI Sequence Read Archive database under accession number RJNA349988.

4. Bioinformatics and data analysis

Raw paired-end Illumina reads were trimmed for 16S rRNA gene primer sequences using Cutadapt (https://cutadapt.readthedocs.org/en/stable/) and reads were joined using the Quantitative Insights Into Microbial Ecology (QIIME) software suite (Kuczynski et al., 2011). Successfully joined sequences were quality filtered in QIIME using default settings.

Then, sequences were clustered into Operational Taxonomic Units (OTUs) on the basis of similarity to known bacterial sequences available in the Greengenes database (v13.8; http://greengenes.secondgenome.com/; 97 % sequence similarity cut-off) using the UCLUST software; sequences that could not be matched to references in the Greengenes database were clustered de novo based on pair-wise sequence identity (97 % sequence similarity cut-off). The first selected cluster seed was considered as the representative sequence of each OTU.

Then, representative sequences were assigned to taxonomy using the UCLUST software. Singleton OTUs were removed prior to downstream analysis.

Normalisation was carried out by generating a subsampled OTU table by random sampling (without replacement) of the input OTU table using an implementation of the Mersenne twister algorithm (http://www.numpy.org/).

Subsequently, OTU tables were rarefied to accommodate for different sampling depths. Samples characterised by fewer than the requested rarefaction depth (i.e. 26036 sequences) were omitted from the output OTU table.

Statistical analyses were executed in R version 3.1.2 (http://www.r-project.org/); normality of variables was tested by Shapiro test and equality of variance by Levene test.

Differences in the composition of the feline gut microbiota between *Tc*+ and *Tc*- samples were assessed using the Linear discriminant *analysis* effect size (LEfSe) workflow (Segata et al., 2011), by assigning 'helminth infection status' as comparison class, and verified by paired t-test for taxa with normal distribution and equal variance.

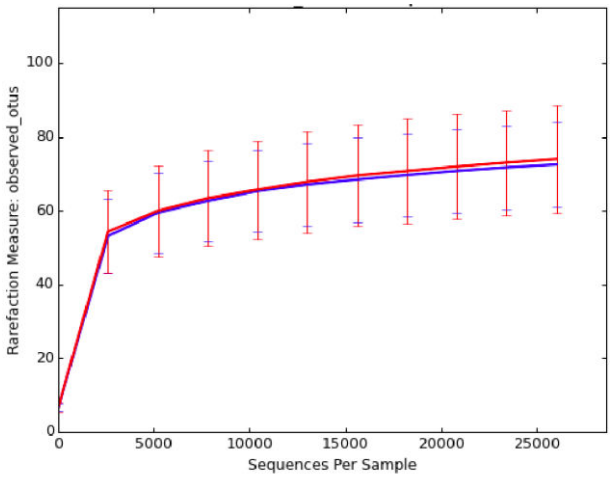
Differences in bacterial diversity and richness in control and infected samples were evaluated using paired t-test, with a significant level of $P<0.05$.

Chapter 3 - Results

A total of 7,063,321 paired-end reads were generated from the 45 samples analysed in the present study (per sample mean 160,530 ± 76,194) (not shown). After primer trimming, joining of paired-end reads and filtering of low-quality sequences, a total of 2,329,889 high-quality sequences were subjected to further bioinformatics analyses. Of these, 4523 reads (1597 reads in blank 1 and 2926 in blank 2) were assigned to OTUs also identified amongst the sequences obtained from two no-DNA control samples (not shown). OTUs assigned to these reads belonged mainly to the phyla Proteobacteria (52.4 % of reads), Firmicutes (26.0 % of reads), Bacteroidetes (8.5 % of reads) and Actinobacteria (7.5 % of reads); these reads were computationally subtracted from the dataset.

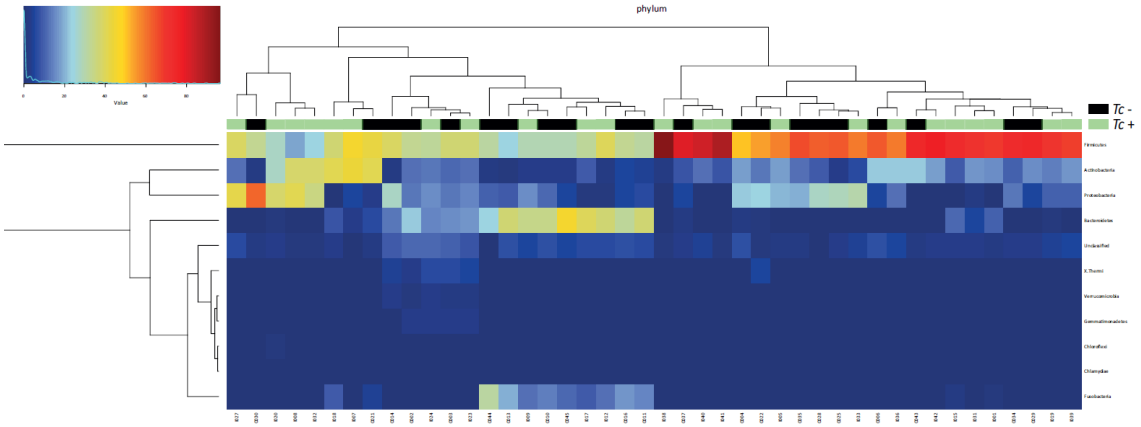
Nonetheless, the rarefaction curves (Figure 6) generated following *in silico* subtraction of these sequences indicated that the vast majority of feline faecal bacterial communities were represented in the remaining sequence data, thus allowing us to undertake further analyses.

Figure 6. Rarefaction curves (number of observed OTUs on Y axis) for *Tc*+ (blue curve) versus *Tc*- (red curve)



Due to low read counts sample I26 was excluded from further analyses. The remaining 2,325,366 sequences were assigned to 128 OTUs and 9 bacterial phyla, respectively. The composition of the faecal microbiota of *Tc*+ and *Tc*- cats is shown in Figure 7. The phylum Firmicutes was predominant in all samples analysed in the present study (mean of 53.0 %), followed by the phyla Protebacteria (13.8 %), Actinobacteria (13.7 %) and Bacteroidetes (10.1 %).

Figure 7. Hierarchical clustering heatmap of the composition of the faecal microbiota phyla of *Toxocara cati*-positive (*Tc*+) and *T. cati*-negative (*Tc*-) cats. Dendrograms at the top of the heatmap indicate relationships between samples. Colour intensity represents the relative abundance of sequences representing the corresponding bacterial family in each sample.



Analysis by LefSe, also supported by paired t-test, identified differences in abundance of individual taxa at the family, genus and species level between *Tc*+ and *Tc*- cats (Figure 8). In particular, Actinobacteria (phylum), Coriobacteriia (class), Coriobacteriales (order), Coriobacteriaceae, Enterococcaceae, Gammaproteobacteria and Lactobacillales (family), *Collinsella*, *Enterococcus*, *Dorea*, *Ruminococcus* and *Lactobacillus* (genus) showed a trend towards increased abundance in *Tc*+ subjects compared with *Tc*- samples (Figure 8). Conversely, sequences belonging to Gammaproteobacteria (order, family, genus and species) and the genera *Bulleidia* and *Jeotgalicoccus* were less abundant in *Tc*+ samples compared with *Tc*- (Figure 3). No significant differences in OTU diversity and richness were recorded between *Tc*+ and *Tc*- samples ($P = 0.581$; $P = 0.485$, t-test) (Figure 9).

Figure 8 - Rank plot of differentially abundant faecal bacteria (at the phylum - I, class - II, order - III, family - IV, genus - V and species - VI level) between *Toxocara cati*-positive (*Tc*+) and -negative (*Tc*-) cats, based on Linear Discriminant Analysis Effect Size (LEfSe) analysis. Taxa highlighted in green/black indicate overrepresentation in *Tc*+ and *Tc*- samples, respectively.

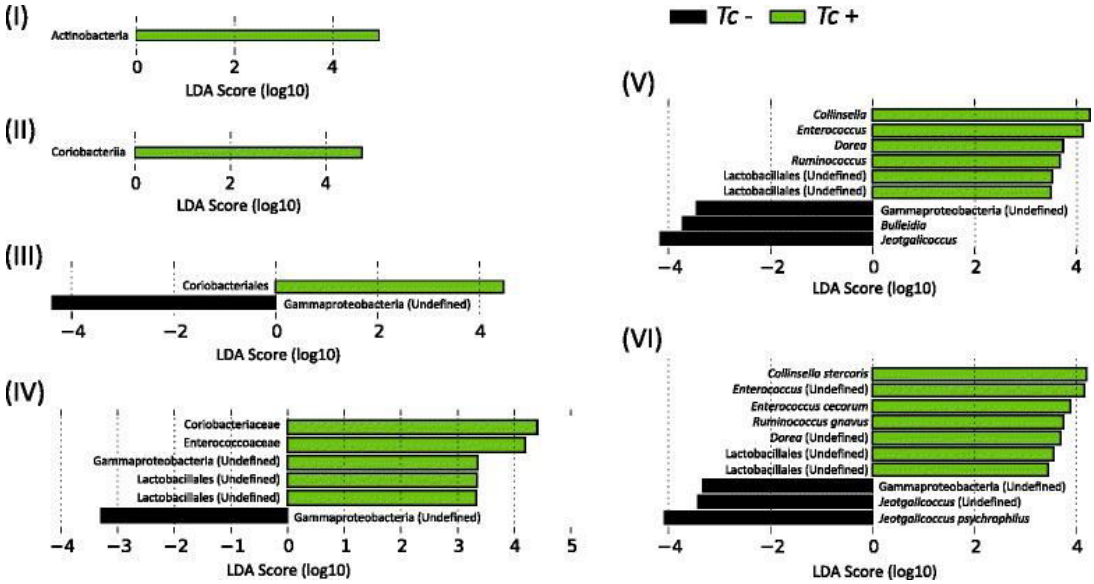
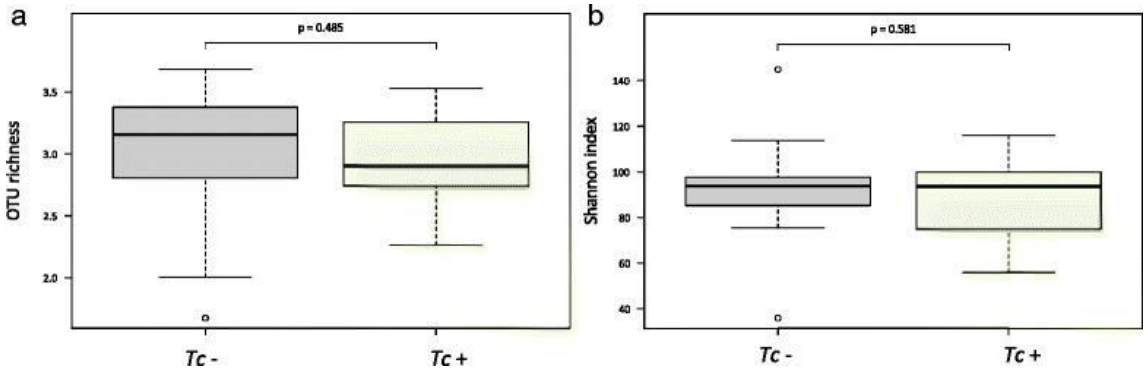
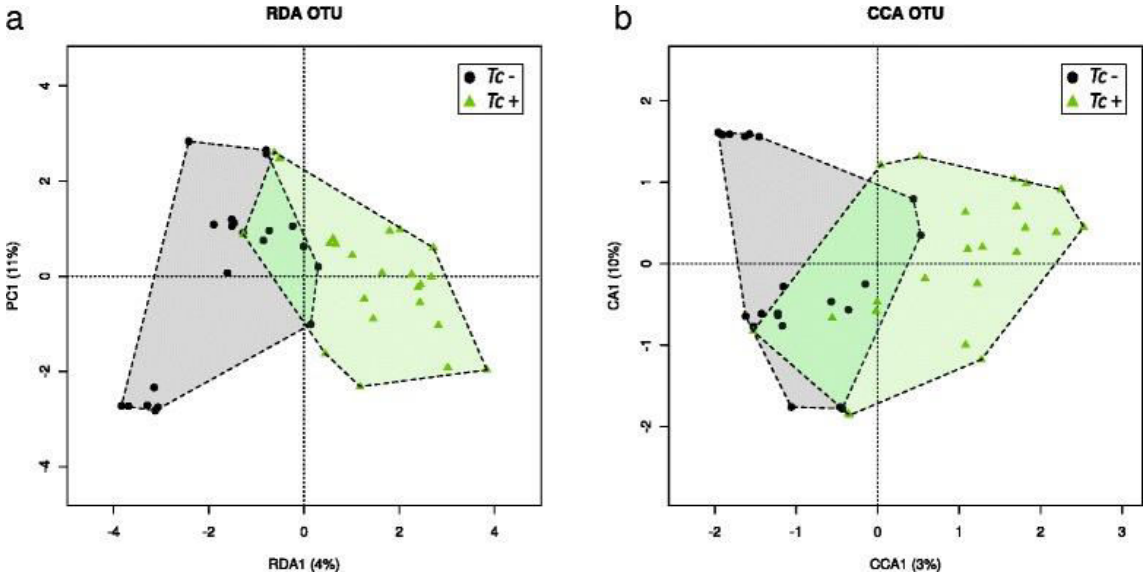


Figure 9 – Differences in overall taxonomic species richness (a) and diversity (b) between the faecal microbiota of *Toxocara cati*-positive (*Tc*+) and *T. cati*-negative (*Tc*-) cats.



Feline faecal microbial communities were grouped by hierarchical clustering and ordinated by supervised Redundancy analysis (RDA) and canonical correlation analysis (CCA). Microbial samples from *Tc*+ and *Tc*- cats formed separate clusters (Figure 10). A significant association between community composition and *T. cati* infection was detected by CCA and RDA analyses (P = 0.030, P = 0.015). They were both able to separate samples by infection status (cf. Figure 10), which suggests a correlation between the latter and the composition of the feline faecal microbiota.

Figure 10 - Redundancy analysis (RDA) and canonical correlation analysis (CCA) show that microbial samples from *Tc*+ and *Tc*- cats form separate clusters



Chapter 4 - Discussion/Conclusion

This study investigated, for the first time, the composition of the gut microbiota of feline hosts in presence or absence of *T. cati* infection. Our choice to specifically explore the relationships between the latter parasite and the feline commensal microbiota was motivated primarily by knowledge that *T. cati* is amongst the most prevalent gastrointestinal parasites of cats worldwide (Dillon et al., 2013), and unequivocal diagnosis of patent infection by *T. cati* can be achieved *via* observation of characteristic dark-brown coloured eggs with thick pitted shells in faecal samples (Overgaauw, 1997).

In addition, while limited information is available on the relationships between the composition of the gut microbiota and infections by selected species of strongylids (i.e. *A. caninum* and *H. contortus*) (Slapeta et al., 2015; Li et al., 2016) and enoplids (i.e. *T. suis*) (Li et al., 2012), no study, to the best of our knowledge, has analysed the effects of ascarid infections on the make-up of the gut commensal microbiota of domestic animals.

Besides providing the opportunity to characterise the cat gut microbiota associated with *T. cati* infection, this study allowed us to gather an overview of the faecal microbial populations of a relatively large cohort of cats. According to the observations from this investigation, the feline gut microbiota is characterised by a significant prevalence of bacteria of the phylum Firmicutes, followed by those of the phyla Proteobacteria, Actinobacteria and Bacteroidetes.

This data is in general agreement with the results of previous studies on the feline gut microbiota, albeit with some discrepancies linked to the relative contribution of individual phyla of bacteria to the overall composition of the feline commensal microbiota (Desai et al., 2008; Ritchie et al., 2008; Barry et al., 2010; Handl et al., 2011; Tun et al., 2012) . For instance, the phylum Firmicutes was mostly prevalent in the faecal microbiota of domestic cats fed either a chicken-based extruded diet or raw chicken (Kerr, Dowd & Swanson, 2014), while bacteria belonging to the Bacteroides group were the most prevalent in the intestinal microbiota of healthy felines fed a commercially available diet whose specific composition was not specified (Tun et al., 2012).

While these inconsistencies are likely to be linked to dietary differences between the cat cohorts enrolled in our and previous studies (Tun et al., 2012; Kerr et al., 2014), methodological differences may also have contributed to these discrepancies.

Indeed, while data from our study was generated using Illumina sequencing of the V3-V4 region of the bacterial 16S rRNA, data from the study by Tun et al. (2012) was generated using whole metagenome sequencing (i.e. shotgun pyrosequencing, 454) of bacterial DNA; finally, V3-V4 sequences characterised in the investigation by Kerr et al. (2014) were generated by pyrosequencing (454). Given the profound differences in detection of bacterial species by whole genome shotgun versus 16S rRNA amplicon sequencing (Ranjan, Rani, Metwally, McGee & Perkins, 2016), as well as the fact that the Illumina and 454 technologies are characterised by known differences in sequencing coverage (Cantacessi, Campbell & Gasser, 2012; Holroyd & Sanchez-Flores, 2012), which ultimately affects the overall number of times each OTU is sampled in a metagenomic survey (Myer, Kim, Freetly & Smith, 2016), direct comparisons between our results and those from previous studies are unwarranted.

Comparison of 16S rRNA sequence data between *Tc*+ and *Tc*- cats enrolled in this study by LefSe analysis (Segata et al., 2011), revealed that, albeit marginally, bacteria belonging to the families Lactobacillales and Enterococcaceae, both belonging to the phylum Firmicutes, were more abundant in helminth-infected felines.

Lactobacilli are Gram positive, members of the lactic acid bacteria, a large group of autochthonous microbes in the gut of humans and animals, that have been largely studied due to their potential properties as probiotics (Walter, 2008).

Research on the possible applications of probiotics as led to obtain more information about the ability of lactobacilli to regulate the immune system of an organism. These bacteria, like others, show in their cytoplasmatic membrane and cell wall conserved microbe-associated molecular patterns (MAMPs), such as specific cell wall polysaccharides (CPs), peptidoglycan (PGN), lipoprotein anchors and lipoteichoic acids (van Barleen, Wells & Kleerebezem, 2013). Different MAMPs have been found and studied in different species and strains of lactobacilli, suggesting considerably different immunomodulatory reactions between them (van Baarlen et al., 2013).

Interestingly, populations of lactobacilli are expanded in the duodenum of C57BL/6 mice following infection with *H. polygyrus*, while the opposite was observed in the microbiota of infected BALB/c mice, which are relatively resistant to the infection (Reynolds et al., 2014). In addition, a positive correlation was observed between the burden of *H. polygyrus* infection in BALB/c mice and the abundance of lactobacilli in the duodenum of these rodents, which was linked to expansion of Treg cells by the gut-associated lymphoid tissue and production of IL-17A by cells in the MLNs in response to helminth infection (Reynolds et al., 2014).

Since IL-17A was the only cytokine to show a positive correlation with the bacterial load, the authors have suggested that IL-17A responses might be induced by the microbiota and not the parasite. Furthermore, the experimental administration of a specific commensal species of *Lactobacillus* (*L. taiwanesis*) caused the rise of Tregs frequency in the GALT and prolonged the infection with *H. polygyrus* (Reynolds *et al*., 2014).

Furthermore, lactobacilli were markedly increased in the large intestine of C57BL/6 mice chronically infected with *T. muris*, albeit a reduction of frequency of Treg cells in MLN was also observed following infection, but prior to the expansion of this bacterial group, which led the authors to hypothesize the existence of different mechanisms of differentiation and development of adaptive immune responses against *T. muris*, compared with *H. polygyrus* (Holm et al., 2015).

Obvious ethical concerns prevented us from investigating the frequency of populations of inflammatory and regulatory cell populations of the gut and associated tissues of the *T. cati* infected cats sampled in our study. However, given the known immune-regulatory properties of a range of nematode parasites (Navarro, Ferreira & Loukas, 2013; Grencis, Humphreys & Bancroft, 2014), as well as those of certain groups of lactobacilli (van Barleen et al., 2013), the role of expanded populations of these microbes in helminth-associated modulation of the immune response of human and animal hosts deserves further consideration.

Another difference found between the two populations was regarding *Bulleidia* and *Jeotgalicoccus*, two genera found less abundant in cats infected with *T. cati*. Thus far, no information is available on possible associations between bacteria of the genera *Bulleidia* and *Jeotgalicoccus* and infection by GI nematodes. However, the genus *Bulleidia* is frequently associated with the microbiota of the oral cavity of vertebrate hosts (Downes et al., 2000) while *Jeotgalicoccus* is often isolated from environmental samples (Schwaiger, Holzel, Mayer & Bauer, 2010). Therefore, the possibility that these genera represent contaminants of the feline faecal samples examined in this investigation cannot be excluded.

In the present study, no significant differences in OTU richness and diversity were recorded between *Tc*+ and *Tc*- samples; this finding is in contrast with the results of previous studies of parasite-associated changes in gut microbiota (Broadhurst et al., 2012; Cantacessi et al., 2014; Lee et al., 2014; Giacomin et al., 2015; Kreisinger et al., 2015), albeit with some exceptions (Holm et al., 2015; Li et al., 2016).

For instance, helminth-associated increases in microbial diversities were recorded in studies aimed to elucidate the role of the gut microbiota in parasite-induced suppression if inflammation, both in humans with Coeliac Disease experimentally infected with *N. americanus* (Giacomin et al., 2015) and in macaques with idiopathic chronic diarrhoea experimentally infected with *T. suis* (Broadhurst et al., 2012). Furthermore, Lee et al. (2014) examining the composition of faecal samples from rural Malaysians, found that naturally helminth infected individuals, like in the present study, have greater species richness and diversity.

Given that a higher microbial diversity is generally associated with a 'healthier' homeostasis of the GI tract (Singh et al., 2015) and that the gut microbiota of individuals with chronic inflammatory disorders is characterised by a low diversity, when compared to healthy individuals (Nistal et al., 2012; Wills et al., 2014), these findings led to the hypothesis that the therapeutic properties of parasitic helminths may be due, at least in part, to their ability to promote the restoration of microbial diversity in the gut (Giacomin et al., 2015).

However, Holm et al. (2015) demonstrated that chronic *Trichuris muris* infection caused a general decrease in the microbial diversity of the murine large intestine. Likewise, the results of a study with the aim to characterize alterations in microbial composition of the caprine abomasal microbiome induced by *Haemonchus contortus* showed no alterations on the microbial diversity and richness between the uninfected control and infected goats (Li et al., 2016).

Nonetheless, the lack of observed differences in microbial richness and diversity between *Tc*+ and *Tc*- cats is likely associated to the relatively small number of animals enrolled in our trial, which may have affected the statistical power and thus the ability to detect qualitative and quantitative changes in their gut microbiota.

Indeed, whilst the samples analysed were included amongst a much larger number of specimens collected as part of a large multicentric clinical study to assess the efficacy of new association of parasiticides against feline lungworms (Giannelli et al., 2016), a number of exclusion criteria were applied that limited the selection of faecal samples to be subjected to analysis of the microbiota. For instance, cats enrolled in this study originate from a restricted geographical area (i.e. Thessaloniki, Greece) and were fed an identical diet of commercial dry food to minimise the effects of these variables on the composition of their faecal microbiota (Zoetendal & de Vos, 2014).

In addition, cats diagnosed as infected by gastrointestinal parasites (i.e. helminths and protozoans) other than *T. cati*, and/or with clinical signs associated with gastrointestinal disease (e.g. diarrhoea), and/or that had been subjected to antibiotic treatment over the last 12 months prior to sampling, were excluded, as these elements may have represented significant confounding factors in our analyses (Slapeta et al., 2015).

Thus, the limited number of samples analysed in our studies prevents us from drawing conclusions on the effects of *T. cati* infection on gut microbial richness and diversity; furthermore, the exact burden of *T. cati* infection in individual cats enrolled in our trial could not be established. The latter is likely to play a major role in investigations of quantitative and qualitative changes in gut microbiota associated to parasite infection, as heavy parasite burdens are likely to be linked to more pronounced shifts in the composition of the commensal microbiota.

Future studies conducted in larger cohorts of felines subjected to a homogeneous diet and for other ascarid nematodes of domestic animals (e.g. experimental infections with *Ascaris suum* in pigs, which would allow an estimate of the parasite burden in each animal host), as well as including samples pre- and post-anthelmintic treatment, are necessary to address these point.

Nonetheless, more knowledge emerging in experimental animal models has been added about the alterations in the microbiota induced by helminths. As mentioned before, a number of studies have reported the increase of the abundance of species belonging to the Lactobacillaceae family in the site where the helminth, in this case the murine helminth *H. polygyrus bakeri*, resides (small intestine) (Walk et al., 2010; Reynolds et al., 2014). In addition another study explored the impact of the same worm on the microbiome of the caecum, concluding the infection resulted in the increase of bacterial species belonging to γ-Proteobacteria/Enterobacteriaceae and Bacteroides families, although other resulted alterations could have been missed due to the PCR-based approach used in the study (Rausch et al., 2013). Comparable results were obtained with another helminth species, *Trichuris muris*, and in this case the relative abundance of *Lactobacilli* was found to increase (Holm et al., 2013).

In pigs the infection with *T. suis* resulted in the increase of bacterial species belonging to the phyla Proteobacteria and Deferribacteres, being that the first was mainly due to the outgrowth of the mucus dwelling bacteria *Mucispirillum* sp. (Li et al., 2012).

The same group continued the study and in addition reported a reduced abundance of the bacteria belonging to the phylum Ruminococcus in infected pigs with changes occurring unexpectedly independently of the burden of infection, leading the authors to speculate that the microbial changes may result from the initial infection and might persist after the host starts to expel the worm (Li et al., 2012).

Although variations in method make the comparison difficult, together this studies point towards a likely involvement of the helminths in modulating the gut microbiome and these changes in specific groups of bacteria can easily explain the results found with the RDA and CCA in our study. In fact, the separate clusters from infected and non-infected cats and the association between community composition and *T. cati* infection suggests a correlation between the infection and the composition of the feline microbiota as a result of the fore mentioned changes in specific groups of bacteria.

Future challenges will present on how the helminth infections affects the microbial communities and ultimately the host. Regarding the impact on the microbial communities it is important to remember the large collection of products that are likely to interfere with the growth or metabolism of microbial bacteria residents; instead or in addition direct competition for energy-rich nutrients or essential minerals may take place (Zaiss & Harris, 2016) and direct impact on the intestinal physiology may also contribute, such as increased fluid secretion, altered mucus production and infiltration of host immune cells (Finkelman et al., 2004).

For instance, the increased production of antimicrobial peptides in mice following helminth infection by intestinal cells may be called down by the ES products (D'Elia et al., 2009; Su et al., 2014). Helminth ES products have also been reported to alter TLR expression (Venugopal, Nutman & Semnani, 2009; Sun et al., 2011) and manipulate inflammatory responses of DCs and macrophages after TLR ligands stimulus (Ludwig-Portugall & Layland, 2012). An example of this is the ability of the whipworm *T. suis*, through its ES products, to downregulate DCs TLR responses (Klaver et al., 2013). This information has led to conjure about the importance of these modulatory responses in the promotion of host tolerance against certain groups within the gut microbiota during helminth infection (Reynolds et al. 2015).

Interestingly, changes in the gut microbiota may also occur as a result of modulation of the adaptive immune pathway. Examples of this are the production of microbiota-specific T cells during acute infection with the protozoan parasite *Toxoplasma gondii* (Hand et al., 2012), the suppression of cholera toxin IgA antibodies in patients also infected with helminths (Harris et al., 2009) and the increased percentage of IL-4 producing $CD4^+$ T cells in colonic biopsies from Rhesus macaques after oral administration of *T. trichuria* ova (Broadhurst et al., 2012).

The alteration of the physical microenvironment caused by the helminth may also result on the outgrowth of specific species, whether as a result of epithelial barrier disruption and/or stimulation of mucus production, which might alter the capacity of different bacterial species to persist in the intestinal tract (Hasnain *et al.*, 2011; Li et al., 2012; Turner, Stockinger & Helmby, 2013).

Such alterations, mainly credited to the ES products, may result in the improvement of disease clinical signs seen in mouse models of inflammatory diseases (Trujillo-Vargas et al., 2007; Ruyssers et al., 2009; Cançado et al., 2011; Ferreira et al., 2013), as well as the suppression of allergic airway diseases. Although these ES products are unlikely to intervene separately in modulating the gut microbiota, the extent of their contribution to the changes in the intestinal biology still needs more exploring, not forgetting that the microbiota itself is known to have immunomodulatory abilities (McSorley & Maizels, 2012; Belkaid & Hand, 2014).

Furthermore, such alterations may have implications on the host metabolism and in fact a few studies have found changes in the metabolic products not only in the faeces (Li et al., 2012; Wu et al., 2012; Houlden et al., 2015;), but also in the urinary metabolite levels (Li et al., 2008).

In conclusion, recent studies in both humans and animals point toward a helminth infection impact on the gut microbiota and some actually demonstrate the close relationship between helminths and bacteria with consequences for both. Therefore, the data gathered in this study adds valuable knowledge to the vast research field of parasite-microbiota interactions and can provide a basis for the elucidation of the role such interactions play in pathogenic as well as immune-modulatory properties of parasitic nematodes in both human and hosts.

Chapter 4 – Final Considerations and Future Directions

The information on the mechanisms behind the interactions between the gut microbiota and helminths in veterinary species is fragmentary and research findings variable. This is due part to the variations on the methodology used for each study, that range from different time a location of sampling to different sequencing techniques. Also, likely to result from distinctive changes in the gut microbiota associated with different host-helminth system. Finally, the differences in the composition of the gut microbiota at the starting point and may also influence the research results.

The problems in accounting for all these variables make it difficult to draw conclusions, and thus the studies with comparable conditions involving HMI will be necessary to allow conclusions that are more permanent.

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HELMINTH INFECTIONS AND GUT MICROBIOTA – A FELINE PERSPECTIVE

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Abstract

Background: Investigations of the relationships between the gut microbiota and gastrointestinal parasiticnematodes are attracting growing interest by the scientific community, driven by the need to better understand the contribution of parasite-associated changes in the composition of the gut flora to both host malnutrition and immune modulation. These studies have however been carried out mainly in humans and experimental animals, while knowledge of the make-up of the gut commensal flora in presence or absence of infection by parasitic nematodes in domestic animals is limited. In this study, we investigate the qualitative and quantitative impact that infections by a widespread parasite of cats (i.e. Toxocara cati) exert on the gut microbiota of feline hosts.

Methods: The faecal microbiota of cats with patent infection by T. cati (= Tc+), as well as that of negative controls (= Tc-) was examined via high-throughput sequencing of the V3-V4 hypervariable region of the bacterial 16S rRNA gene, followed by bioinformatics and biostatistical analyses of sequence data.

Results: A total of 2,325,366 useable high-quality sequences were generated from the faecal samples analysed in this study and subjected to further bioinformatics analyses, which led to the identification of 128 OTUs and nine bacterial phyla, respectively. The phylum Firmicutes was predominant in all samples analysed (mean of 53.0%), followed by the phyla Proteobacteria (13.8%), Actinobacteria (13.7%) and Bacteroidetes (10.1%). Among others, bacteria of the order Lactobacillales, the family Enterococcaceae and genera Enterococcus and Dorea showed a trend towards increased abundance in Tc+ compared with Tc- samples, while no significant differences in OTU richness and diversity were recorded between Tc+ and Tc- samples ($P = 0.485$ and $P = 0.581$, respectively). However, Canonical Correlation and Redundancy Analyses were able to separate samples by infection status ($P = 0.030$ and $P = 0.015$, respectively), which suggests a correlation between the latter and the composition of the feline faecal microbiota.

Conclusions: In spite of the relatively small number of samples analysed, subtle differences in the composition of the gut microbiota of Tc+ vs Tc- cats could be identified, some of which in accordance with current data from humans and laboratory animal hosts. Nevertheless, the findings from this study contribute valuable knowledge to the yet little explored area of parasite-microbiota interactions in domestic animals.

Keywords: Gut microbiota, Cat, Toxocara cati, Lactobacilli, Microbial richness and diversity, 16S rRNA

Background

The gastrointestinal (GI) tract of humans and animals is inhabited by a myriad of microorganisms, the gut microbiota, which are essential for the maintenance of the homeostasis of the digestive system [1, 2] and whose functions span nutrition and metabolism, protection againstpathogens and regulation of both innate and adaptive immune responses [1, 2]. In parallel, a number of nematode parasites of vertebrates have developed, over millions of years of evolution, a range of strategies to evade or dampen host immunity, thus providing them with the ability to chronically colonise the GI tract of humans and animals [3]. It is therefore plausible that the successful establishment of parasitic nematodes in the vertebrate gut is achieved, at least in part and directly and/or indirectly, via physical, molecular and/or immunological interactions with the resident commensal flora [3, 4]. Indeed, over the past few years, investigations of the relationships between the gut microbiota and parasitic nematodes have attracted growing interest by the scientific community, mainly driven by the need to better understand the contribution of parasite-associated changes in the composition of microbial populations to host malnutrition [4]. In addition, a number of studies have focused on the immunemodulatory properties shared by both commensal bacteria and GI parasitic nematodes, in a bid to address questionson the possible role/s of helminth-induced fluctuations in gut microbiota in parasite-driven suppression of inflammation [3, 5, 6]. However, these studies have been carried out in a limited range of vertebrate hosts and for a few speciesof GI nematodes; these include humans experimentally infected with hookworms (i.e. *Necator americanus*) [7, 8] or naturally infected with hookworms and/or whipworms (Trichuris spp.) and/or roundworms (Ascaris spp.) [9, 10], and laboratory animals infected with strains of *Heligmosomoides polygyrus bakeri, Trichuris muris* [11–15] or *T. suis* [16]. The specific findings from these studies differ considerably, with some pointing towards an overall increase in microbial species richness and diversity in response to nematode infection [7, 8, 10, 16] and others recording detectable shifts in the abundance of specific populations of bacteria following parasite establishment [3]. Given these inconsistencies, further studies in other host-parasite systems are required in order to determine whether changes in the composition of the commensal flora that occur in concomitance with colonisation by GI parasitic nematodes are dependent upon the animal host and/or the parasite involved and/or the burden of infection.

Domestic animals, for instance, provide useful systems for the collection of data on helminth-microbiota interactions under natural conditions, since they are often infected by a range of species of GI parasitic nematodes (i.e. enoplids, strongylids and ascarids) and by varying parasite loads [17–20]. However, thus far, only a handful of studies have explored the relationships between GI enoplids and strongylids and the commensal gut flora in nonexperimental animals. These studies include recent investigations of changes in the composition of the microbiota of the proximal colon of pigs infected with *T. suis* [21], of the abomasum of goats infected with *Haemonchus contortus* [22] and of dogs infected with *Ancylostoma caninum* [23]. However, despite these efforts, knowledge of this area remains fragmentary. In addition, to the best of our knowledge, no studies have thus far investigated the relationships between ascarid parasites and the gut commensal flora. This link is of particular interest, given the known immune-modulatory properties of these large GI nematodes [24] as well as their association with the onset of allergy in at-risk populations [25] Therefore, the elucidation of the relationships between ascarids of domestic animals and their gut microbiota may provide useful information towards elucidating the relative contribution of parasiteassociated changes in gut commensal microbes to host immune-modulation.

In this study, capitalising on the sampling opportunities provided by a recent clinical trial [26], we investigated the qualitative and quantitative impact that patent infections by Toxocara cati exert on the gut microbiota of the cat hosts.

Methods

Study cohorts

Cats enrolled in this study were initially selected based on the following criteria: (i) Owned and living in a relatively restricted area of Thessaloniki (Greece); (ii) Weaned; (iii) Fed an identical diet of commercial dry food (i.e. Purina Friskies®) for at least 6 months prior to sampling; (iv) Allowed to roam free in outdoor areas and hunt; (v) Clinically healthy (e.g. absence of signs of GI disease or any other concomitant disease); (vi) Not treated with antibiotics and/or anthelmintics over 12 and 3 months prior to sample collection, respectively. Only cats with or without patent T. cati infection (= Tc+ and Tc-, respectively) and negative for other helminths (i.e. hookworms and tapeworms) and protozoan parasites (i.e. *Isospora* spp., *Giardia* spp. and *Cryptosporidium* spp.) at the faecal examination (see below) were included. A total number of 45 cats (Tc+ = 24, 7 males and 17 females; and Tc- = 21, 5 males, 16 females) matched these criteria (Table 1). None of the female cats included in this study was pregnant or lactating at the time of sample collection.

Sample collection, DNA extraction and high-throughput sequencing

Once collected, fresh faecal samples were stored in sterile tubes at room temperature, and immediately transported to the Laboratory of Parasitology and Parasitic Diseases, School of Veterinary Medicine of the Aristotle University of Thessaloniki (Greece), where they were refrigerated (at 4 °C) prior to processing.

Table 1 Gender, age (months) and weight (kg) of Toxocara catipositive (Tc+) and T. cati-negative (Tc-) cats enrolled in this study. The infection burden of Tc+ cats is indicated in eggs per gram of faeces (EPG)

Tc+					*Tc-*			
ID	Gender	Age	Weight	EPG	ID	Gender	Age	Weight
I001	F	24	> 25	200	C002	F	12	< 25
I005	M	5	< 25	750	C003	F	36	> 25
I007	F	24	> 25	200	C004	F	120	> 25
I008	M	30	> 25	300	C006	M	12	> 25
I009	M	18	–	150	C010	F	24	–
I012	F	36	> 25	150	C011	F	30	–
I015	F	18	> 25	200	C013	F	36	> 25
I017	F	12	< 25	300	C014	M	12	> 25
I018	F	30	> 25	200	C016	F	18	> 25
I019	F	6	< 25	400	C021	F	24	> 25
I020	F	12	> 25	300	C022	F	24	> 25
I023	M	12	> 25	300	C025	M	12	> 25
I024	F	18	> 25	450	C028	F	30	> 25
I026	F	24	> 25	300	C029	F	24	> 25
I027	F	24	> 25	100	C030	F	36	> 25
I031	F	60	> 25	100	C034	M	12	> 25
I032	F	36	> 25	200	C035	F	36	> 25
I033	F	24	> 25	150	C037	F	18	> 25
I036	F	12	> 25	100	C043	F	30	> 25
I038	M	24	> 25	300	C044	M	24	> 25
I039	M	12	> 25	350	C045	F	24	> 25
I040	F	24	> 25	100				
I041	F	18	> 25	100				
I042	M	18	> 25	400				

Abbreviations: F female, *M* male

Briefly, individual samples were aliquoted for use in standard parasitological procedures, i.e. faecal egg counts (FEC) using a standard McMaster technique, as well as DNA extraction followed by highthroughput sequencing of the bacterial 16S rRNA gene (see below). For microscopical examination, aliquots of 2 g of faeces were suspended in 28 ml zinc sulphate solution (ZnSo4, specific gravity = 1.180); the suspension was homogenised, filtered using a double-layer gauze, and pipetted into McMaster chambers for microscopical examination.
The remaining aliquots from these faecal samples (approximately 4 g for each sample) were homogenized, preserved in 70% ethanol, and shipped to the Parasitology Unit of the University of Bari (Italy), where they were kept at -80 °C, until DNA extraction.
Total genomic DNA was extracted directly from two technical replicates of each Tc+ and Tc- sample, as well as from two negative 'blank' (= no DNA) controls, using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), according to the manufacturers' instructions. High-throughput sequencing of the V3-V4 hypervariable region of the bacterial 16S rRNA gene was performed on an Illumina MiSeq platform according to the manufacturers' protocols with minor adjustments. Briefly, the V3-V4 region was PCR amplified using universal primers (Forward, 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′; Reverse, 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′) [27], hat contained the Illumina adapter overhang nucleotide sequences, using the NEBNext® Q5® Hot Start HiFi DNA polymerase (New England Biolabs® Inc, Massachusetts, USA) and the following thermocycling protocol: 98 °C/2 min, followed by 35 cycles of 98 °C/15 s, 63 °C/30 s, and 72 °C/30 s, and a final elongation step of 72 °C/5 min. Amplicons were purified using AMPure XP PCR Purification beads (Beckman Coulter, Brea, California, USA), and the NEBNext hot start high-fidelity DNA polymerase was used for the index PCR with Nextera XT index primers (Illumina, San Diego, California, USA) according to the following thermocycling protocol: 98 °C/30 s, 8 cycles of 98 °C/10 s, 65 °C/75 s and at 65 °C/5 min. The indexed samples were purified using AMPure XP beads, quantified using the Qubit Quant-iT™ dsDNA Broad-Range Assay Kit (Life Technologies, Carlsbad, California, USA), and equal quantities from each sample pooled. The resulting pooled library was quantified using the NEBNext® Library Quant Kit for Illumina® (New England Biolabs® Inc) and sequenced on an Illumina MiSeq platform using the v3 chemistry (301 bp paired-end reads). Raw sequence data have been deposited in the NCBI Sequence Read Archive database under accession number PRJNA349988.

Bioinformatics analyses
Raw paired-end Illumina reads were trimmed for 16S rRNA gene primer sequences using Cutadapt (https://cutadapt.readthedocs.org/en/stable/) and reads were joined using the Quantitative Insights Into Microbial Ecology (QIIME) software suite (version 1.9.0) [28]. Successfully joined sequences were quality filtered in QIIME using the usearch_qf script with default settings. Then, sequences were clustered into Operational Taxonomic Units (OTUs) on the basis of similarity to known bacterial sequences available in the Greengenes database (v13.8; http://greengenes.secondgenome.com/; 97% sequence similarity cut-off) using the UCLUST software; sequences that could not be matched to references in the Greengenes database were clustered de novo based on pair-wise sequence identity (97% sequence similarity cut-off). The first selected cluster seed was considered as the representative sequence of each OTU. Then, representative sequences were assigned to taxonomy using the UCLUST software. OTUs assigned to sequences obtained from the no-DNA control samples, as well as singleton OTUs, were removed prior to downstream analysis. Normalisation was carried out by generating a subsampled OTU table by random sampling (without replacement) of the input OUT table using an implementation of the Mersenne twister algorithm (http://www.numpy.org/). Subsequently, OTU tables were rarefied to accommodate for different sampling depths. Samples characterised by fewer than the requested rarefaction

depth (i.e. 26,036 sequences) were omitted from the output OTU table. Statistical analyses were executed in R version 3.1.2 (http://www.r-project.org/); normality of variables was tested by Shapiro test and equality of variance by Levene test. Differences in the composition of the feline gut microbiota between Tc+ and Tc- samples were assessed using the LDA Effect Size (LEfSe) workflow [29], by assigning 'helminth infection status' as comparison class, and verified by paired t-test for taxa with normal distribution and equal variance. Differences in bacterial diversity and richness in control and infected samples were evaluated using paired t-test.

Results

A total of 7,063,321 paired-end reads were generated from the 45 samples analyzed in the present study (per sample mean 160,530 ± 76,194) (not shown). However, due to low read counts, sample I26 (i.e. 1,438 sequences) was excluded. The remaining 7,061,883 reads were subjected to further processing. Following primer trimming, joining of pairedend reads, filtering of low-quality sequences and removal of 'contaminant' and singleton OTUs, a total of 2,325,366 high-quality sequences were retained for further bioinformatics analyses. The rarefaction curves generated following *in silico* subtraction of low-quality and contaminant sequences indicated that the vast majority of feline faecal bacterial communities were represented in the remaining sequence data, thus allowing us to undertake further analyses.

These sequences were assigned to 128 OTUs and 9 bacterial phyla, respectively (Additional file 1: Dataset S1). The composition of the faecal microbiota of Tc+ and Tccats is shown in Additional file 2: Figure S1. The phylum Firmicutes was predominant in all samples analysed in the present study (mean of 53.0%), followed by the phyla Protebacteria (13.8%), Actinobacteria (13.7%) and Bacteroidetes (10.1%) (Additional file 2: Figure S1). Analysis by LefSe, also supported by paired t-test, identified differences in abundance of individual taxa at the family, genus and species level between Tc+ and Tc- cats (Fig. 1). In particular, Actinobacteria (phylum), Coriobacteriia (class), Coriobacteriales (order), Coriobacteriaceae, Enterococcaceae, Gammaproteobacteria and undefined Lactobacillales (family), Collinsella, Enterococcus, Dorea, Ruminococcus and undefined Lactobacillales (genus) showed a trend towards increased abundance in Tc+ subjects compared with Tc- samples (Fig. 1). Conversely, sequences belonging to Gammaproteobacteria (order, family, genus and species) and the genera Bulleidia and Jeotgalicoccus were less abundant in Tc+ samples compared with Tc- (Fig. 1). No significant differences in OTU diversity and richness were recorded between Tc+ and Tc- samples (Shannon index, P = 0.581 and richness, P = 0.485, respectively) (Fig. 2).

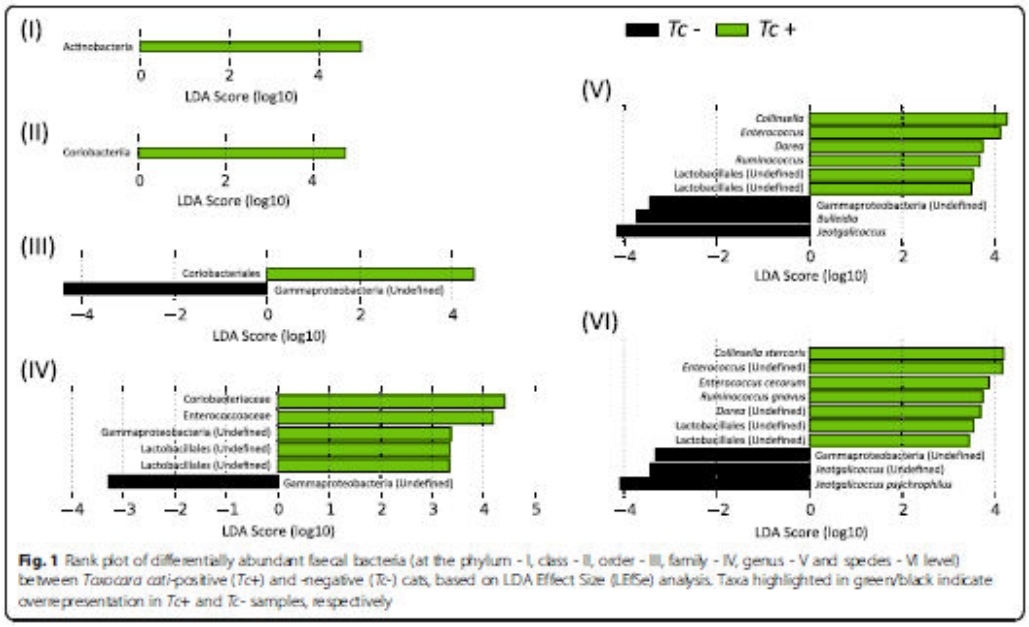

Fig. 1 Rank plot of differentially abundant faecal bacteria (at the phylum - I, class - II, order - III, family - IV, genus - V and species - VI level) between *Toxocara cati*-positive (*Tc*+) and -negative (*Tc*-) cats, based on LDA Effect Size (LEfSe) analysis. Taxa highlighted in green/black indicate overepresentation in *Tc*+ and *Tc*- samples, respectively

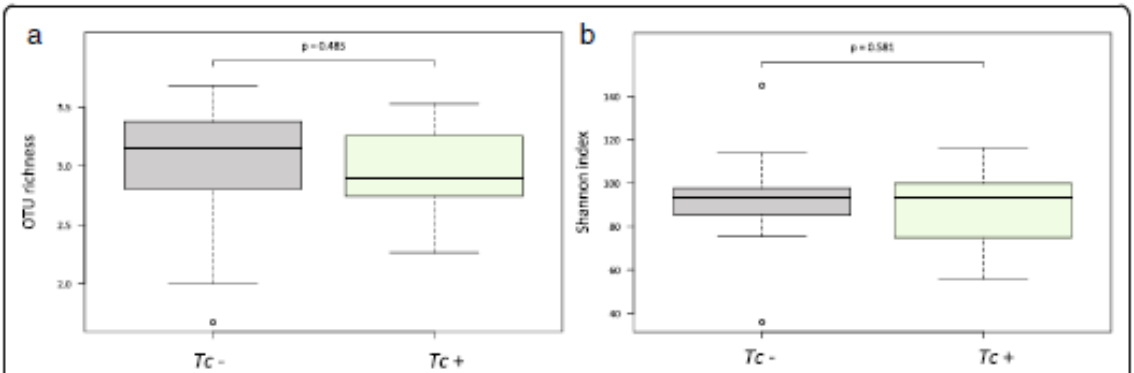

Fig. 2 Differences in overall taxonomic species richness (**a**) and diversity (**b**) between the faecal microbiota of *Toxocara cati*-positive (*Tc*+) and *T. cati*-negative (*Tc*-) cats

Feline faecal microbial communities were grouped by hierarchical clustering and ordinated by supervised Redundancy Analysis (RDA) and Canonical Correlation Analysis (CCA). Microbial samples from Tc+ and Tc- cats formed separate clusters (Fig. 3). A significant association between community composition and T. cati infection was detected by CCA and RDA analyses (CCA, P = 0.030 and RDA, P = 0.015, respectively). They were both able to separate samples by infection status (Fig. 3), which suggests a correlation between the latter and the composition of the feline faecal microbiota. The results obtained from the technical replicate of each sample were consistent with the data described above (data available from the corresponding author upon request).

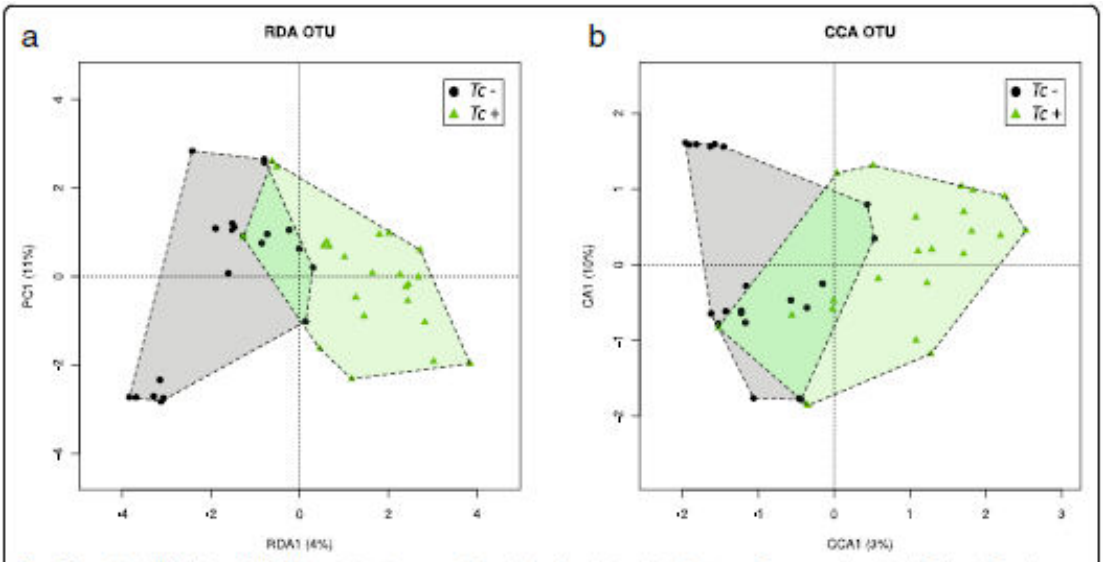

Fig. 3 Supervised RDA (**a**) and CCA (**b**) depicting the composition of the faecal microbiota between *Toxocara cati*-positive (*Tc*+) and *T. cati*-negative (*Tc*-) cats

Discussion

This study investigated, for the first time, the composition of the gut microbiota of feline hosts in presence or absence of patent *T. cati* infection. Our choice to specifically explore the relationships between the latter parasite and the feline commensal flora was motivated primarily by knowledge that T. cati is amongst the most prevalent GI parasites of cats worldwide [30], and unequivocal diagnosis of patent infection can be achieved via observation of characteristic dark-brown coloured eggs with thick pitted shells in faecal samples [31]. In addition, while limited information is available on the relationships between the composition of the gut microbiota and infections by selected species of strongylids (i.e. *A. caninum* and *H. contortus*) [22, 23] and enoplids (i.e. *T. suis*) [21], no study, to the best of our

knowledge, has analysed the effects of ascarid infections on the make-up of the gut commensal flora of domestic animals.

Besides providing the opportunity to characterise the gut microbiota of cats infected by *T. cati*, this study allowed us to gather an overview of the faecal microbial populations of a relatively homogeneous cohort of feline hosts. According to our observations, the feline gut microbiota is characterised by a significant prevalence of bacteria of the phylum Firmicutes, followed by those of the phyla Proteobacteria, Actinobacteria and Bacteroidetes.

These data are in general agreement with the results of previous studies, albeit with some discrepancies linked to the relative contribution of individual phyla of bacteria to the overall composition of the feline commensal flora [32–35]. For instance, the phylum Firmicutes was mostly prevalent in the faecal microbiota of domestic cats fed either a chicken-based extruded diet or raw chicken [36], while bacteria belonging to the Bacteroides group were the most prevalent in the intestinal microbiota of healthy felines fed a commercially available diet whose specific composition was not specified [32]. While these inconsistencies are likely to be linked to dietary differences between the cat cohorts enrolled in our study (fed an identical diet of commercial dry food but allowed to hunt small preys) and those from previous trials [32, 36], methodological differences may also have contributed to these discrepancies. Indeed, while data from our study was generated using Illumina sequencing of the V3-V4 region of the bacterial 16S rRNA, Tun et al. [32] utilised whole metagenome sequencing (i.e. shotgun pyrosequencing, 454) of bacterial DNA; finally, V3-V4 sequences characterised in the investigation by Kerr et al. [36] were generated by pyrosequencing (454). Given the profound differences in detection of bacterial species by whole metagenome shotgun vs 16S rRNA amplicon sequencing [37], as well as the fact that the Illumina and 454 technologies are characterised by known differences in sequencing coverage [38, 39], which ultimately affects the overall number of times each OTU is sampled in a metagenomic survey [40], direct comparisons between our results and those from previous studies [32, 36], are unwarranted.

In our study, the gut microbiota of cats harbouring patent infection by *T. cati* was compared with that of cats that were negative for this parasite at the time of sampling.

Indeed, it is highly likely that all cats enrolled in this trial had been repeatedly exposed to *T. cati* infections at various stages of their lives, i.e. via (i) infective larvae transmitted by the queens during lactation, (ii) eggs contaminating the environment, and (iii) larvae encysted in the tissues of paratenic hosts. Therefore, we speculate that most if not all Tc- cats had had a history of previous infection by this parasite. However, since in this study we aimed to assess the impact that the presence of live *T. cati* exerts on the composition of the feline gut microbiota, previous exposure to the parasite was not taken into account. Comparison of 16S rRNA sequence data between Tc+ and Tc- cats enrolled in this study by LefSe analysis [29], revealed that, albeit marginally, bacteria belonging to the families *Lactobacillales* and *Enterococcaceae*, both belonging to the phylum Firmicutes, were more abundant in helminth-infected felines. Lactobacilli are members of the lactic acid bacteria, a large group of autochthonous microbes in the gut of humans and animals and that are especially known for their probiotic properties [41]. Interestingly, populations of lactobacilli are expanded in the duodenum of C57BL/6 mice following infection with *H. polygyrus*, while the opposite was observed in the microbiota of infected BALB/c mice, which are relatively resistant to the infection [12]. In addition, a positive correlation was observed between the burden of H. polygyrus infection in BALB/c mice and the abundance of lactobacilli in the duodenum of these rodents, which was linked to expansion of Treg cells by the gut-associated lymphoid tissue and production of IL-17A by cells in the mesenteric lymph nodes (MLN) in response to helminth infection [12]. In addition, lactobacilli were markedly increased in the large intestine of C57BL/6 mice chronically infected with *T. muris*, albeit a reduction of frequency of Treg cells in MLN was also observed following infection but prior to the expansion of this bacterial group, which led the authors to hypothesize the existence of different mechanisms of differentiation and development of adaptive immune responses against *T. muris* compared

with *H. polygyrus* [15]. Obvious ethical concerns prevented us from investigating the frequency of populations of inflammatory and regulatory cell populations of the gut and associated tissues of the *T. cati* infected cats sampled in our study. However, given the known immune-regulatory properties of a range of nematode parasites [42, 43], as well as those of certain groups of lactobacilli [44], the role of expanded populations of these microbes in helminth-associated modulation of the immune response of human and animal hosts deserves further consideration. Thus far, no information is available on possible associations between bacteria of the genera *Bulleidia* and *Jeotgalicoccus* and infection by GI nematodes. However, the genus *Bulleidia* is frequently associated with the microbiota of the oral cavity of vertebrate hosts [45] while *Jeotgalicoccus* is often isolated from environmental samples [46]. Therefore, the possibility that these genera represent contaminants of the feline faecal samples examined in this investigation cannot be excluded.

In this study, no significant differences in OTU richness and diversity were recorded between Tc+ and Tc- samples; this finding is in contrast with the results of previous studies of parasite-associated changes in gut microbiota [7, 8, 16, 47], with some exceptions [15, 22].

For instance, helminth-associated increases in microbial diversity were recorded in studies aimed to elucidate the role of the gut microbiota in parasite-induced suppression of inflammation, both in humans with coeliac disease experimentally infected with *N. americanus* [8] and in macaques with idiopathic chronic diarrhoea experimentally infected with T. suis [16]. Given that a higher microbial diversity is generally associated with a 'healthier' homeostasis of the GI tract [48] and that the gut microbiota of individuals with chronic inflammatory disorders is characterised by a low diversity when compared to healthy individuals [49, 50], these findings led to the hypothesis that the therapeutic properties of parasitic helminths may be due, at least in part, to their ability to promote the restoration of microbial diversity in the gut [8]. The lack of observed differences in microbial richness and diversity between Tc+ and Tc- cats is likely associated to the relatively small number of animals enrolled in our study, which may have affected the statistical power and thus the ability to detect qualitative and quantitative changes in their gut microbiota. Indeed, whilst the samples analysed were included amongst a much larger number of specimens collected as part of a large multicentric clinical study to assess the efficacy of a new association of parasiticides against feline lungworms [26], a number of 'filters' were applied that limited the selection of faecal samples to be subjected to analysis of the microbiota. For instance, cats enrolled in this study originate from a restricted geographical area (i.e. Thessaloniki, Greece) and were fed an identical diet of commercial dry food to minimise the effects of these variables on the composition of their faecal microbiota [51]. In addition, cats diagnosed as infected by GI parasites (i.e. helminths and protozoans) other than *T. cati*, and/or with clinical signs associated with GI disease (e.g. diarrhoea) or any other disease and/or that had been subjected to antibiotic and/or anthelmintic treatment over the 12 months prior to sampling, were excluded, as these elements may have represented significant confounding factors in our analyses [23]. Thus, the limited number of samples analysed prevents us from drawing conclusions on the effects of *T. cati* infection on gut microbial richness and diversity; furthermore, the exact burden of T. cati infection in individual cats enrolled in our trial could not be established. The latter is likely to play a major role in investigations of quantitative and qualitative changes in gut microbiota associated to parasite infection, as heavy parasite burdens are likely to be linked to more pronounced shifts in the composition of the commensal flora. Future studies conducted in larger cohorts of felines subjected to a homogeneous diet and for other ascarid nematodes of domestic animals (e.g. experimental infections with *Ascaris suum* in pigs, which would allow an estimate of the parasite burden in each animal host), as well as including samples pre- and postanthelmintic treatment, are necessary to address these points.

Conclusions

Data from this study add valuable knowledge to the vast, and yet little explored, research field of parasite-microbiota interactions and will provide a basis for the elucidation of the role such interactions play in pathogenic as well as immune-modulatory properties of parasitic nematodes in both human and animal hosts.

Additional files

Additional file 1: Dataset S1. Sequence data representing the V3-V4 hypervariable region of the bacterial rRNA amplified from faecal samples from Toxocara cati-positive (Tc+) and T. cati-negative (Tc-) cats, and taxonomic assignment. (XLSX 3501 kb)

Additional file 2: Figure S1. Hierarchical clustering heatmap of the composition of the faecal microbiota phyla of Toxocara cati-positive (Tc+) and T. cati-negative (Tc-) cats. Dendrograms at the top of the heatmap indicate relationships between samples. Colour intensity represents the relative abundance of sequences representing the corresponding bacterial family in each sample. (PDF 63 kb)

Abbreviations

16S rRNA: 16S ribosomal RNA gene; BP: Base pairs; CCA: Canonical Correlation Analysis; DNA: Deoxyribonucleic acid; EPG: Eggs per gram of faeces; FEC: Faecal egg counts; GI: Gastrointestinal; LDA: Linear discriminative analysis; MLN: Mesenteric lymph nodes; NCBI: National Centre for Biotechnology Information; OTU: Operational Taxonomic Unit; PCR: Polymerase chain reaction; QIIME: Quantitative Insights Into Microbial Ecology; RDA: Redundancy Analysis; RNA: Ribonucleic acid; Tc-: Toxocara catinegative; Tc+: Toxocara cati-positive

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Availability of data and materials

Raw sequence data analysed in this article have been deposited in the NCBI Sequence Read Archive database under accession number PRJNA349988.

Annotated sequence data are available from Additional File 1: Dataset S1.

Authors' contributions

AMD and CC conceived the study; AMD, TPJ, MSL and AG collected the samples, performed the experiments and analysed the data; AMD, TPJ and CC interpreted the data and wrote the article, with corrections and suggestions from EP, LMdC, MJN and DO. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Enrolment in the study and collection of cat faecal samples was approved by the Ethics and Welfare Panel of the Department of Veterinary Medicine of the University of Cambridge (ref.: CR137).

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Annex B – Results from the epidemiological study to verify the occurrence of cat lungworm infections in the Cambridge area.

Patient ID	Microship	Breed	Age (months)	Sex	Weight	Life style	Predatory activity (Y/N)	A. abstrusus (LPG)	T. brevior (LPG)	Other parasites species
1	Absent	DSH tabby grey	12M	F	> 2,5	Stray	Not Known	Negative	Negative	NA
2	Absent	DSH red tabby	4Y	M	> 2,5	Stray	Not Known	Negative	Negative	Negative
3	960,011000104946	DSH tabby/black	3Y	M	> 2,5	Outdoor	Yes	Negative	Negative	Negative
4	Absent	DSH black	3Y	M	>2,5	Stray	Not Known	Negative	Negative	Negative
5	Absent	DSH tort	10M	F	>2,5	Outdoor acess	Yes	Negative	Negative	Negative
6	958,000010016407	DSH black and white	3Y	F	>2,5	Stray	Yes	Negative	Negative	Negative
7	978,101080245247	DSH grey	6Y	M	>2,5	Outdoor acess	Yes	Negative	Negative	Negative
8	900,008800104794	DSLH tabby/white	5Y	M	>2,5	Outdoor acess	Yes	Negative	Negative	Negative
9	960011000161759	DLH black/white	6y	M	5,4	Stray	N/A	Negative	Negative	*Toxocara cati* (eggs)
10	981000008297904	DSH tabby	3y	M	5,1	Stray	N/A	Negative	Negative	Negative
11	960011000463084	Bengal	4Y	M	4.8	Outdoor acess	Yes	Negative	Negative	Negative
12	985120031027320	DSH Grey tort	9y	F	4.3	Outdoor acess	Yes	Negative	Negative	Negative
13	958000002380002	DSH Black	4y	M	6.1	Outdoor acess	Yes	Negative	Negative	Negative
14	Absent	DSH Grey	5y	F	4	Outdoor acess	Yes	Negative	Negative	Negative
15	Absent	DSH black	6Y	M	4,6	Outdoor acess	Yes	Negative	Negative	Negative
16	Absent	DSH grey and tabby	6Y	M	5,1	Outdoor acess	Yes	Negative	Negative	Negative
17	958000001888888852	DSH black and white	5,6	M	5,6	Outdoor acess	No	Negative	Negative	Negative
18	Absent	DLH black/white	13Y	F	4,25	Indoor	No	Negative	Negative	Negative
19	Absent	DSH tabby	8Y	M	4,25	Outdoor acess	Yes	Negative	Negative	Negative

20	Absent	DSH black	3M	U	1,05	Indoor	No	Negative	Negative	Negative
21	Absent	DSH black and white	4/5M	M	1,7	Stray	N/A	Negative	Negative	Negative
22	Absent	DSH tart	4,5M	F	1,54	Stray	N/A	Negative	Negative	Negative
23	Absent	DSLH tabby	8M	F	2,7	Outdoor acess	Not Known	Negative	Negative	Negative
24	Absent	DSH grey and white	8Y	F	4,4	Outdoor acess	Yes	Negative	Negative	Negative
25	Absent	DSH tart and white	6M	F	2,9	Indoor	No	Negative	Negative	Negative
26	Absent	DSH tabby	10Y	F	4	Outdoor acess	No	Negative	Negative	Negative
27	958000010263754	DSH black	5Y	M	5	Indoor	No	Negative	Negative	Negative
28	Absent	DSH tabby	10M	F	2,6	Indoor	No	Negative	Negative	Negative
29	Absent	DSH black and white	18M	F	2,4	Outdoor	Yes	Negative	Negative	Negative
30	Absent	DSLH black and white	8W	U		N/A	N/A	Negative	Negative	Positive for *Isospora* spp. (12/01/2016)
31	Absent	DSH white and tabby						Negative	Negative	
32	Absent	DSH black and white						Negative	Negative	
33	Absent	DSH white and tabby	7Y	F	3,7	Stray	N/A	Negative	Negative	Negative
34	Absent	DSH black and white	8M	F	2,9	Outdoor acess	Not Known	Negative	Negative	Negative
35	Absent	DSLH grey	2Y	M	3,7	Outdoor acess	No	Negative	Negative	Negative
36	958000010126416	DSH Black	4Y	F	2,8	Outdoor acess	Yes	Negative	Negative	Negative
37	Absent	DSH Black	4Y	F	4,1	Stray	N/A	Negative	Negative	Negative
38	Absent	DSH black and white	2,5Y	F	3,5	Outdoor acess	Yes	Negative	Negative	Negative
39	981000006512115	DLH black/white	1,5Y	M	4,2	Outdoor acess	Yes	Negative	Negative	Negative
41	Absent	DSH corgy	8Y	F	3,25	Outdoor acess	No	Negative	Negative	Negative
42	Absent	DSH black	5Y	M	3,1	Stray	N/A	Negative	Negative	Negative

43	981000100015959	DSH tabby	12Y	F	4,2	Outdoor acess	In the past	Negative	Negative	Negative
44	985100009936424	DSH tabby	16Y	F	3,5	Stray	N/A	Negative	Negative	Positive for *Toxocara cati*
45	Absent	DSH black and white	11Y	M	4,5	Outdoor acess	Yes	Negative	Negative	Negative
46	Absent	DLH black/white	3Y	M	4,6	Outdoor acess	Not Known	Negative	Negative	Negative
47	Absent	DSH tort	13Y	F	2,6	Stray	N/A	Negative	Negative	Negative
48	Absent	DSH black and white	7Y	M	3,9	Stray	N/A	Negative	Negative	Negative
49	Absent	DSH ginger	2Y	M	4,3	Stray	N/A	Negative	Negative	Negative
50	Absent	DSH black and white	3Y	M	5,9	Outdoor acess	Yes	Negative	Negative	Positive for Toxocara cati
51	Absent	DSH Black	8Y	M	7,2	Outdoor acess	No	Negative	Negative	Negative
52	Absent	DSH Black	8Y	F	7,6	Outdoor acess	NO	Negative	Negative	Negative
53	985120028911376	DLH black	13Y	F	5,5	Indoor	No	Negative	Negative	Negative
54	Absent	DSH ginger and white	11Y	F	4,1	Outdoor acess	No	Negative	Negative	Negative