ŽIGA GERBEC

MAGISTRSKA NALOGA

ENOVITI MAGISTRSKI ŠTUDIJ FARMACIJA

Ljubljana, 2016
ŽIGA GERBEC

PREUČEVANJE TERAPEVTSKEGA POTENCIALA VZPOSTAVITVE GASTROINTESTINALNE HOMEOSTAZE S TRANSPLANTACIJO FEKALNE MIKROBIOTE NA PSIH

EVALUATION OF THERAPEUTIC POTENTIAL OF RESTORING GASTROINTESTINAL HOMEOSTASIS BY A FECAL MICROBIOTA TRANSPLANT IN DOGS

ENOVITI MAGISTRSKI ŠTUDIJ FARMACIJA

Ljubljana, 2016
Research work was carried out at the University of Helsinki, Faculty of Veterinary Medicine, at the department of Equine and Small Animal Medicine under the supervision of Assist. Prof. Anna Hielm-Björkman DVM, PhD and home university mentorship of Assist. Prof. Žiga Jakopin, PhD. Fecal samples were analyzed at the Veterinary Medical Teaching Hospital, Texas A&M University, Texas, USA.

**Acknowledgements**

I would like to extend my gratitude to Assist. Prof. Anna Hielm-Björkman DVM, PhD, whose supervision made it possible for me to perform my master’s thesis at the University of Helsinki, Faculty of Veterinary Medicine. I would like to thank her for supporting and guiding me throughout the research, enabling me to experience how current research is carried out. I also thank Prof. Thomas Spillmann, DVM, Ingrid Hang, DVM, PhD and everyone else participating in the study for their help and guidance.

I honestly thank my mentor Assist. Prof. Žiga Jakopin, PhD, for his advice concerning the writing of the thesis and for being accessible.

**Statement**

I declare that I have made this thesis independently under the mentorship of Assist. Prof. Žiga Jakopin, PhD, and the co-mentorship of Assist. Prof. Anna Hielm-Björkman, DVM.

Žiga Gerbec

Chairman of committee: Prof. Dr. Borut Štrukelj, PhD

Member of committee: Assist. Dr. Martina Gobec, PhD
Table of contents

Abstract .................................................................................................................................. 7

Razširjeni povzetek ................................................................................................................ 8

List of abbreviations ............................................................................................................ 11

1 Introduction .................................................................................................................. 12

1.1 Microbiota ............................................................................................................. 12

1.1 Microbiota Diversity ............................................................................................. 13

1.2 Microbiota Functions ............................................................................................ 15

1.3 Factors influencing microbiota ............................................................................. 17

1.4 Microbiota in human disease .............................................................................. 19

1.5 Fecal microbiota transplant ................................................................................ 22

1.6 Microbiota research in dogs ................................................................................ 25

2 Research plan ............................................................................................................... 27

3 Materials and methods ................................................................................................. 27

3.1 Dogs used in study ................................................................................................. 27

3.2 FMT procedure ....................................................................................................... 29

3.3 Sampling and analysis .......................................................................................... 32

4 Results and Discussion ................................................................................................. 33

5 Conclusions .................................................................................................................. 46

6 Literature ...................................................................................................................... 47

7 Attachments .................................................................................................................. 51
List of figures

Figure 1. Phyla and metabolic pathways at seven different body sites. .............................. 14
Figure 2. Exemplary interactions of microbiota and host mechanisms.............................. 15
Figure 3. Microbiota-related diseases outside the GI-tract.................................................. 20
Figure 4. Selected pathways by which intestinal bacteria affect host health ..................... 21
Figure 5. Various disorders associated with the modulation of microbiota. ....................... 23
Figure 6. FMT material preparation A: Metal strainers. B: 50 mL syringes...................... 30
Figure 7. Insertion of endoscope into duodenum through mouth....................................... 31
Figure 8. Administration of FMT material through endoscopic tube. (Jassu) .................... 31
Figure 9. Dysbiosis indices of FMT recipients throughout the study. ............................ 36
Figure 10. Fecal sample sequencing results presented at bacterial phylum level. ......... 37
Figure 11. Observed species in sequenced fecal samples grouped by week.................... 41
Figure 12. EMPeror display of sequencing data PCoA..................................................... 43
List of tables

Table I: Dogs used in study ................................................................. 28
Table II: Dysbiosis index of donor samples .................................................. 35
Table III: Sequencing labels through observational period (Figures 10, 12) .............. 37
Table IV: Bacterial taxa associated with GI health ........................................ 38
Table V: Bacterial taxa associated with dysbiosis ........................................ 39
Abstract

Dogs and humans are hosts to with a wide array of microorganisms termed the microbiota of the host. Gastrointestinal microbiota is most numerous and contributes directly to gastrointestinal homeostasis, its key roles being played in the metabolism and in regulation of immune response. The normal functioning of microbiota is obstructed when its microbial constituents are in a state of imbalance (dysbiosis). A fecal microbiota transplant is the procedure of infusing donor fecal material into the patient's intestine and has shown potential to treat gastrointestinal dysbiosis in humans. The purpose of this research is to evaluate the therapeutic potential of fecal microbiota transplants in restoring gastrointestinal homeostasis in dogs. The screening of nine suitable donors for fecal pathogens yielded only one dog fitting the donor criteria. Donor feces were mixed with Ringer's solution using a commercial blender and filtered through a metal strainer to provide fecal transplant material. Three recipient dogs with a similar history of inflammatory bowel disease and tylosin treatment were chosen as recipients and received a fecal microbiota transplant via a nasoduodenal tube while sedated. The condition of two dogs improved considerably following the transplant, while the third dog did not exhibit any observable changes in health. Fecal samples, taken weekly for 2 months, were characterized by the sequencing of the 16S rRNA gene and quantitative polymerase chain reaction analysis. Compared to the donor dog the microbiome of all three pre-transplant fecal samples had substantially lower species richness and other indications of dysbiosis. The microbial composition and species richness of recipient dogs' fecal samples resembled the donor's immediately following transplantation, similar to what has been observed in human fecal microbiota transplant studies. The results of this trial study indicate that a fecal microbiota transplant has therapeutic potential to restore gastrointestinal homeostasis, although confounding factors such as dog age, environment, diet and a limited number of patients may have considerably influenced our findings. Further extensive studies are needed to properly evaluate fecal microbiota transplantation as a therapeutic procedure option in dogs.

Keywords: dog, dysbiosis, fecal microbiota transplant, inflammatory bowel disease, microbiota
Razširjeni povzetek

Viruse, glive, bakterije in ostale mikroorganizme, ki se nahajajo na telesnih površinah in znotraj gostitelja, imenujemo mikrobiota. Novejše metode sekvenciranja, ki omogočajo celovit vpogled v sestavo mikrobiote, so prinesle številna spoznanja glede njenega delovanja in pomena za zdravje gostitelja. Najbolj raziskana je človeška bakterijska mikrobiota, ki tvori različne lokalizirane ekosisteme v človeškem telesu. Število bakterijskih celic, ki sestavljajo človeško mikrobioto, je desetkrat večje od števila somatskih celic, ki tvorijo človeško telo.

Najbolj zaslužna za to številčno prevlado je gastrointestinalna mikrobiota, kjer je zgoščena večina mikrobiote. Znotraj prebavili bakterije izboljšujejo presnovo, omogočajo večji vnos hrani iz zaužitih jedi, preprečujejo razrast patogenih organizmov, uravnavajo imunski sistem ter tvorijo telesu potrebne vitamine. Za običajno delovanje in doprinos k zdravju gostitelja je potrebna raznolika in funkcionalno stabilna mikrobiota. Porušeno ravnovesje, zaradi katerega mikrobiota lahko prenese normalno delovati, imenujemo disbioza. Raziskave sestave mikrobiote so povezale številna bolezenska stanja, uporabo antibiotikov, infekcije patogenih organizmov, debelost in druge bolezni zunanj črevesja, s specifičnimi spremembami v gastrointestinalni mikrobioti. Ponavljajoča infekcija z bakterijo Clostridium difficile v ljudeh, povzroči hudo gastrointestinalno disbiozo, ki pa jo običajna zdravljenja z antibiotiki in imunosupresivi ne odpravijo. Najnovejše smernice za zdravljenje te infekcije predlagajo transplantacijo fekalne mikrobiote, kar označuje vnos fekalne mikrobiote zdravega darovalca v prebavni trakt pacienta. Transplantacija fekalne mikrobiote je tako najbolj obsežno raziskana pri ljudeh s ponavljajočo se infekcijo Clostridium difficile, kjer transplantacija privede do zvišanja vrste raznolikosti mikrobiote in občutnega olajšanja ali odprave disbioze.

Namen naloge je preučiti terapevtski potencial vzpostavitve gastrointestinalne homeostaze s postopkom transplantacije fekalne mikrobiote na psih. Zaradi velikega uspeha zdravljenja infekcije Clostridium difficile pri ljudeh smo se odločili za transplantacijo fekalne mikrobiote na psih s podobnimi stanji. Devet zdravih psov, primernih za donatorstvo fekalij, je bilo testiranih za prisotnost fekalnih patogenov in parazitov. Osem psov ni prestalo omenjenega testa, zato je bila za donatorstvo izbrana le šest let stara avstralska ovčarka, edina brez znanih dejavnikov tveganja za okrnjenost mikrobiote, za prejemnike
transplantacije pa trije psi različnih pasem, stari štiri, sedem in dvanajst let, s kronično vnetno črevesno boleznijo. Poleg bolezni so imeli podobno zgodovino kroničnih gastrointestinalnih znakov (bruhanje, diareja, zaprtost), ki so se pri dveh psih (Idefix in Sisu) blažili z uporabo antibiotika, pri tretjem (Jassu) pa z imunosupresivom. Teden dni pred transplantacijo so vsi psi prejemniki prenehali z uporabo antibiotikov ter imunosupresivov in bili preventivno zdravljeni z antihelmintikom (fenbendazol).

Fekalije donatorskega psa so bile priskrbljene do šest ur pred transplantacijo. Za pripravo fekalnega transplantanta so bile v mešalku razredčene z Ringerjevo raztopino, ter filtrirane skozi čajno sito. Dobljena raztopina je bila nato injicirana skozi kanal endoskopa, vstavljenega skozi usta anesteziranih psov, ki je segel do sredine dvanajstnika. Psi so tako v povprečju prejeli 1,5 g donatorjevih fekalij oziroma 10 mL raztopine za vsak kilogram telesne teže. Lastniki psov so pred transplantacijo in sledeča dva meseca zbirali telesne vzorke fekalij svojih psov ter ob začetku in koncu tega spremljevalnega obdobja izpolnili vprašalnik o splošnem zdravju, prehranjevanju in gastrointestinalnih znakh.

Po transplantaciji se je zdravstveno stanje dveh psov (Idefix in Sisu) občutno izboljšalo. Lastnika obeh psov sta poročala o povečani energiji in zmanjšanju gastrointestinalnih znakov (diareja, bruhanje, napihnjenost trebuha). Zdravstveno stanje Idefixa se je mesec po transplantaciji poslabšalo, vendar je bil skozi spremljevalno obdobje še vedno v boljšem stanju, kot pred transplantacijo. Lastnik tretjega psa (Jassu) je poročal, da se zdravstveno stanje psa ni izboljšalo.

Vzorci fekalij psov so bili preučeni s sekvenciranjem prisotnih 16S rRNA genov, ki so bili nato primerjani z gensko knjižnico 16S genov, kar nam je omogočalo vpogled v bakterijsko filogenetsko sestavo fekalne mikrobiote psov. Vsakemu vzorcu fekalij je bila dodeljena določena vrednost indeksa disbioze, ki je bil definiran glede na številčnost specifičnih bakterijskih skupin v vzorcu. Kot pričakovano so bile vrednosti indeksa disbioze najnižje v vzorcih donatorskega psa, kar namiguje na odsotnost disbioze v njegovi fekalni mikrobioti. Psi prejemniki so imeli pred transplantacijo najvišje vrednosti indeksa disbioze, kar se ujema z njihovimi gastrointestinalnimi znahi. Vrednosti indeksa disbioze pri vzorcih, vzeti takoj po transplantaciji, so se pričakovano znižale in približale vrednostim, opaženim pri donatorskem psu. Skozi opazovalno obdobje je nato vrednost indeksa disbioze vzorcev počasi naraščala, vendar se je samo pri enem psu (Jassu) vrnila v območje vrednosti, povezanih z disbiozo.
Filogenetska sestava fekalne mikrobiote psov glede na prevladujoče bakterijske skupine se ujema s podatki drugih študij, ki so preučevali fekalno mikrobioto psov. V primerjavi z donatorskim psm so imeli ostali psi pred transplantacijo zmanjšano prisotnost predstavnikov bakterijskega debla Bacteroidetes in Firmicutes, poleg tega sta imela dva psa (Idefix in Sisu) občutno večjo prisotnost bakterijskega debla Proteobacteria. Po transplantaciji so se omenjene razlike v sestavi mikrobiote prejemnikov zmanjšale in približale sestavi donatorskega psa, podobno kot poročajo raziskave transplantacije fekalne mikrobiote na ljudeh. Primerjava števila predstavnikov nižjih taksonomskih bakterijskih skupin, povezanih z disbiozo, je v splošnem pokazala ugodne spremembe v sestavi mikrobiote dveh psov (Idefix in Sisu) ter manj ugodne spremembe za enega psa (Jassu).

Na podlagi podatkov, pridobljenih iz sekvenciranja, je bila izračunana krivulja redkih vrst za vsak vzorec, kar je omogočalo primerjavo njihove predvidene vrstne raznolikosti v fekalni mikrobioti. Vzorci donatorskega psa so imeli dvakrat večjo vrstno raznolikost kot vzorci ostalih psov pred transplantacijo. Po prejemu donatorskih fekalij so se psi prejemniki takoj približali donatorskemu psu v vrstni raznolikosti, nato pa se je raznolikost postopno zmanjševala skozi spremljevalno obdobje. Meritev filogenetske daljave med vzorci je omogočala tudi primerjavo podobnosti sestave mikrobiote. Vzorci mikrobiote posameznega psa so bili tako bolj podobni preostalim vzorcem istega psa, razen takoj po transplantaciji, ko je bila mikrobiota psov prejemnikov bolj podobna donatorskemu psu.

Poseg transplantacije fekalne mikrobiote je izkazal potencial vzpostavitve gastrointestinalne homeostaze v psih, čeprav je statistična moč dobljenih rezultatov močno okrnjena zaradi nizkega števila psov, vključenih v študijo. Hkrati se psi zelo razlikujejo v starosti, pasmi, tezi, spolu, dieti in domačem okolju, kar dodatno omejuje vrednotenje rezultatov. Iz tega razloga so za podrobno preučitev transplantacije fekalne mikrobiote kot učinkovitega terapevtskega posega na psih potrebne nadaljnje, obsežnejše raziskave.

Ključne besede: disbioza, kronična vnetna črevesna bolezen, mikrobiota, pes, transplantacija fekalne mikrobiote
List of abbreviations

CDI – Clostridium Difficile Infection

CIBDAI - canine inflammatory bowel disease activity index

DC – dendritic cell

FMT - fecal microbiota transplant

GF - germ free

GI – gastrointestinal

IBD – inflammatory bowel disease

IEC – intestinal epithelial cell

OTU - operational taxonomic unit

PCoA - principal coordinates analysis

QUIIME - Quantitative Insights Into Microbial Ecology

RC - retroauricular crease

RCT - randomized controlled trial

SCFA - short chain fatty acid

SIBO - small intestinal bacterial overgrowth

TJ – tight junction

TRD - tylosin responsive diarrhoea
1 Introduction

1.1 Microbiota

Since the discovery of microorganisms in the 17th century much has been revealed about these first forms of life, which dominate our biosphere. Their adaptability to environmental change is unmatched and that is why they can be found nearly anywhere on Earth. Mammals, as well as other animals, share their body with microorganisms. These microorganisms, defined as “the microbiota of the host”, thrive on body surfaces and predominantly reside in the gastrointestinal (GI) tract.

The microbiota of the human GI tract is the most studied so far. From the upper GI towards the colon, microbiota increases in concentration and diversity and in rises up to $10^{12}$ bacteria per gram of colonic content in the colon (1). Anaerobic bacteria predominantly inhabit the GI tract, while the presence of aerobes and facultative aerobes drops towards the colon as well as from the GI endothelial surface towards the intestinal lumen. The human microbiota is made up of 100 trillion cells, which outnumber human host cells 10 to 1 (2). On the other hand, the combined genes of the microbiota, defined as the microbiome (3), outnumber the human genome 150 times over.

Humans are 99.9 % identical in their human genes, however it is our microbiome that shows greater inter-individual variation. This variation has been observed even between identical twins and holds enormous potential in areas such as personalized medicine or even forensic identification. In a study comprising the sampling of computer keyboard keys and fingertips of subjects, researchers were able to show the subjects’ skin bacterial communities transferred to specific keys as well as matching the palm of the hand to the computer mouse that person uses with up to 90 % certainty (4).

Gut bacteria have very demanding growth requirements and most cannot grow on culture media, mainly due to the inability of recreating the gut environment, interdependence on other microbiota components and stresses imposed by the culture process (5). Novel, culture independent, molecular techniques show a more complicated picture of the microbiota. Because of the varying conditions at different host sites, microorganisms establish ecosystems in their residing part, where they use local nutrients and in return provide metabolites for host intake (6).
Research investigating the microbiome has recently grown both in quantity and broadness, due to the development of new sequencing technologies and related analytical software for the vast amounts of acquired data. Using new sequencing techniques, such as pyrosequencing, enables researchers to gain thousands of sequences per sample as opposed to a mere dozen using Sanger-based sequencing technologies for the same price (2). The general approach in these studies is to define the bacterial phylogeny using 16S rRNA gene sequencing, a prokaryotic ribosome component found in bacteria and archaea. This particular gene is highly conserved between species and allows for the classification of microbes using 16S ribosomal databases.

1.1 Microbiota Diversity

Bacteria, archaea, viruses, fungi and other eukaryotes that inhabit the gut are an integral part of the microbiota, cross-influencing each other. A recent study using germ-free and antibiotic treated mice has shown that enteric viruses can bring about the same benefits to the host’s immune system as some bacterial constituents of the microbiota (7). Phage attacks are also one of the biggest influences on the composition of the bacterial community. The virome has not been as thoroughly researched as the bacterial microbiome, since it is harder to characterize with sequencing techniques, but its importance to host physiology may be just as great, because of its systemic presence within the host (8).

Microbiota significantly differs between different body sites, people and populations. According to research done by the human microbiome project (2), more than 90% of human GI bacteria belong to the phyla Firmicutes and Bacteroidetes, with the majority of the human population having similar proportions of the two phyla. On the species level, there are more than 10,000 different species of bacteria living in the human gut, with each human hosting approximately 1,000 species in their gut microbiota (9). Early studies of microbiome diversity were based on a presumption that a common "core" of microbial organisms would be found in every human. Later studies have pointed towards this being true on the level of function, rather than specific microorganisms.

Although the majority of the human GI bacteria fall into a very limited number of phyla, the content of their genomes, even within the same bacterial species, can be very different. Microbiota exhibit a great deal of functional redundancy, meaning different microbiota
members can perform same functions and are thus replaceable, contributing to the stability of the host environment (10). Many other cell-level selective pressures force towards the differentiation of microbial genes in order to gain specific advantages against other bacteria. This implies microbiota have a core group of host specific microbiome functions with various arrangements of species, covering those functions (11). This functional redundancy was nicely illustrated by data obtained from the human microbiome project shown below (12).

**Figure 1. Phyla and metabolic pathways at seven different body sites.**
Vertical bars depict relative proportions of a.) microbial phyla from grouped operational taxonomic units (OTU) and b.) metabolic pathways. Predominant phyla and metabolic pathways are listed in legend by colour. The majority of microbial communities include a dominant phylum (and also genus), although this does not hold true for all body sites or individuals. On the other hand, metabolic pathways are uniformly shared and common between individuals and body sites. RC: retroauricular crease (behind the ear); TM7: a major lineage of Bacteria, candidate phylum
1.2 Microbiota Functions

**Figure 2. Exemplary interactions of microbiota and host mechanisms.**
Selected microbiota constituents (green) impact the host through various mechanisms which affect host health (black). As microbiota commonly displays functional redundancy, these effects are not limited to particular microbiota constituents as well as other microbiota factors that influence these phenotypes. (figure 2 was modified from (9))

Due to its many roles in basic body functions, GI microbiota has been termed either an organ (1) or a tissue (13) by different researchers. It maintains health by regulating immunity, protecting against invading pathogens, improving metabolism as well as producing many substances essential to the host’s health (figure 2). It performs many functions that the host itself is unable to do and eases the host’s adaptation to environmental changes.

Although gut microbiota utilize host ingested food for their own growth and fitness, they reduce the caloric intake needed to maintain host body weight. This is achieved through producing nutrients from compounds, which cannot be digested without specific microbiota components (mainly oligosaccharides and polysaccharides) and increasing the overall nutrient absorption through the intestine. Products of bacterial metabolism, such as short-chain fatty acids (SCFAs) from dietary fibres, represent a major energy source for the host and directly influence intestinal health. Many bacterial metabolites directly affect host
metabolism, inflammation, adiposity, energy balance and satiety (14). The ratio of
different SCFAs (e.g. acetic/propionic/butyric acid) is determined by diet and metabolic
pathways set up by residing gut microbiota communities.

Xenobiotics, such as therapeutic drugs, are also metabolized by microbiota which may lead
to individual variations in their biological activity, while some drugs rely on microbial
metabolism to generate the desired effects. Certain toxic compounds (e.g. hydrazine) are
prevented from causing great damage to the host after the GI microbiota convert them into
less harmful metabolites (9). Differences in microbiota composition also lead to different
capacities of metabolic pathways. In a recent study in humans, the variation in metabolic
capacity for sulfation between individuals was shown using a metabolite profile obtained
by urine analysis (15). These microbiota differences were shown to affect the individual's
capacity for acetaminophen sulfation thus leading to differences in pharmacokinetics.

The gravity of microbiota importance to the host is well-illustrated by research conducted
with germ-free (GF) animals and those given different constituents of microbiota. GF
animals have decreased blood vessel formation (14), lower absorption of nutrients, altered
brain functions and behaviour (9), as well as many immune system irregularities (16), due
to the lack of microbiota throughout life. Various experiments where GF mice were given
bacteria, even just a single bacterial species or molecules like polysaccharide A (17),
resulted in extensive improvements of such irregularities.

Further, the impact of microbiota on fat storage was shown in a study using leptin-mutant
mice (18), which are fatter and also have a distinctly different microbiota than normal
mice. The microbiota of leptin-mutant mice was shown to cause weight gain when
transferred to GF mice, pointing to the fact that the microbiota is changing their energy
balance phenotype, in turn, leading to obesity. The same weight gain occurs when
transferring the microbiota of mice lacking toll-like receptor 5, a normal component of the
immune system, into GF mice (19). However, this gain in weight was due to an increased
intake of food by the mice, thus indicating that a behavioural change was caused by the
transfer of microbiota into GF mice. The development and normal function of the
hypothalamic-pituitary-adrenal axis was shown to be strongly linked to microbiota in
another similar study (20). The study showed that GF mice responded with more stress to
being restrained than specific pathogen free mice (mice with microbiota lacking specific
pathogens). GF mice, which were given feces from specific pathogen free mice, were able to exert a normal stress response only if they were given feces at an early age.

The results of a study comparing North American and Japanese human subjects have shown that the Japanese had the capacity to metabolise marine algae, while the North Americans could not. Microbiota of Japanese subjects was shown to contain genes coding enzymes which enabled them to metabolize the seaweed. These genes were most likely transferred from marine bacteria on ingested seaweeds, which are part of the daily diet in Japan (21). This study led to an investigation of similar transfers in the human microbiota, showing that horizontal gene transfer between bacteria occurs at an immense rate in the human body. The rate of horizontal gene transfer between bacteria was even larger in those having similar oxygen tolerance, pathogenicity and occupying the same body site (22). Antibiotic resistance, virulence factors and different enzymes are some of many bacterial characteristics that can be shared between microbiota through this mechanism.

In order to perform its numerous functions, GI microbiota must maintain a balance between the host forces which strive to homogenize microbiota composition and on the other hand the microbial competition which diversifies it (23). This state of gastrointestinal homeostasis establishes the ability to perform needed functions with a diverse microbiota, resistant to perturbations from various factors.

1.3 Factors influencing microbiota

The amount of factors influencing the microbiota is immense, yet only a few factors are considered as essential. Basic microbiota characteristics are generally maintained in spite of small, gradual, daily changes in microbiota composition. The most influential factors of GI microbiome composition are the initial colonizers at birth, host diet, host genetics, environment and exposure to antibiotic substances (9; 12). Since there is great variation in microbiota between individuals, there are also great inter-individual variations in response to these factors.

The gut is considered to be sterile before birth and is very dynamic in its microbial composition after environmental exposure of the host. Certain bacterial species have been shown to transfer from mother to infant while passing through the birth canal as opposed to caesarean delivery (24). These initial colonizers thrive with little competition, stimulate gut development and prime the developing immune system for defence against pathogens.
After the initial colonizers, species which thrive on milk take hold and later, as solid foods are introduced, it quickly changes to obligate anaerobes and more diversity in the microbiota (25). Throughout the adult life, microbiota remains relatively stable, with short perturbations followed by quick recovery resembling previous states. With old age, microbiota gradually becomes unstable and less diverse (26).

Antibiotic treatments although aimed to reduce growth or kill specific bacteria, often cause great damage to untargeted microbiota members. Antibiotics thus lead to a decline of microbiota diversity lasting longer than the treatment period, but this decline is often restored to the previous state in a matter of days or weeks. Full recovery is not always achieved, since certain microbiota members may never reach pre-treatment counts or even disappear completely.

The gut microbiota is profoundly influenced by the diet of its host. Long-term ratio and structure of fibre, polysaccharide, protein and fat, determine which metabolic pathways are dominant and lead to niche specialization across different body sites. Microbiota shifts imposed by diet composition are consistent across various mammalian species, implying that diet may be more important than host phylogeny (27). Many studies have examined the direct effect of a diet intervention on microbiota composition (26-28). Certain dietary components (e.g. inulin, fructo-oligosaccharides) have been shown to boost growth of specific bacterial groups and are termed prebiotics (26; 29).

Another microbiota factor is the community that we share our home with, since it represents other microbial hosts we interact with the most. A research involving observation of the oral, skin and fecal microbiota of 17 families revealed that families which co-habited had more related microbial communities, especially those of the skin. The inclusion of dogs in households elevated the common skin microbiota in adults living together, as well as the microbiota shared with the dog (30).
1.4 Microbiota in human disease

The microbiota has been implicated in the progression and etiology of many human diseases. Intestinal dysbiosis may disrupt any of the aforementioned benefits of the microbiota to health. Diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome, GI malignancies, obesity, type 2 diabetes and enteric infections are evidently related to gut microbiota, exhibiting disease-specific microbiota alterations and response to antibiotic treatments (9; 31; 32). Although many of these diseases have been associated with dysbiosis, it remains open whether this is the cause or consequence of the related disease.

IBD is a chronic inflammatory disease and its development has been strongly linked to gut microbiota. Human IBD has many subtypes (e.g. Crohn's disease, ulcerative colitis), which differ in the affected part of the GI and the degree of changes induced by inflammation. Malignancies of the GI tract can be set off by residing microbiota through direct or indirect inflammatory and cytotoxic effects on intestinal epithelial cells. *Helicobacter pylori-* associated gastric carcinoma is the most studied malignancy correlated with a specific microbiota member. Although *H. pylori* has been a member of the human GI microbiota for at least 60,000 years (likely throughout mammalian evolution) and is present in approximately half of the population, it is being eradicated worldwide (33). This is because the gastric inflammation and cancer can be easily prevented by eliminating *H. pylori* with antibiotics and suppressing gastric acid secretions. In colorectal cancer, both microbiota composition and diet have been shown to influence its development (9).

Besides diseases directly linked to the intestinal tract, a great number of non-GI diseases (e.g. atopy, mood disorders, activation of chronic immunodeficiency virus infection) have been linked to the microbiota in recent years. Loss of intestinal homeostasis can lead to reduced intestinal barrier function, which causes increased influx of microbiota components to the liver and into systemic circulation, if the liver fails to handle this influx. This may lead to various liver diseases, formation of gallstones, minimal hepatic encelopathy and endotoxemia. Through systemic circulation microbiota components can reach remote organs and cause dysfunction far from the intestine as seen in figure 3 below (9).
Antibiotics have shed light on many links between microbiota and disease, due to their direct influence on microbiota composition and function. Antibiotic-associated diarrhoea is a common condition following GI dysbiosis caused by antibiotic treatment. The rate of its occurrence increases with the duration of antibiotic treatment and is most often caused by the overgrowth of a specific pathogen, such as *Clostridium difficile*. Antibiotics greatly affect microbiota and its development when used to treat infants. They can disturb the

*Figure 3. Microbiota-related diseases outside the GI-tract.*
normal expansion of microbiota in the developing host and have been associated with an increased risk of asthma, autism, paediatric GI disturbances as well as other illnesses related to improper development of the immune system (34).

Another link between microbiota and disease is observed through the use of probiotics, which are defined as live microorganisms which confer health benefit to the host when administered in adequate amounts. These health benefits can be the result of various, separate microbiota functions (figure 4) and do not necessarily require that the probiotic alters microbiota composition (26).

Figure 4. Selected pathways by which intestinal bacteria affect host health
1) Resident bacteria combat pathogens with bacteriocins 2) Synthesis of SCFA leads to a lower pH value 3) Competitive inhibition of pathogen growth through nutrient utilization 4) Prevention of pathogen adhesion by occupying IEC surface 5) Inducing growth of IEC (e.g. SCFA, vitamins) 6) Increased mucus, IEC cytoskeleton and TJ alteration lead to intestinal barrier function improvement 7) Downregulation of aberrant immune response and enhanced innate immunity. IEC, intestinal epithelial cells; DC, dendritic cells; TJ, tight junction; B, B cells; T, T cells; Th, T helper cells; Tn, naive T cells; Treg, regulatory T cells.

Probiotics have recently gained popularity with the implementation of bacteria and fungi in commercial food products and probiotic supplements as promoters of health. In studies using mouse models, many of the links between disease and microbiota are being
confirmed as viable probiotic treatment options (35). Administration of various probiotic formulations in human patients has shown their efficacy in both treating and preventing several diarrhoeal diseases, obesity and increasing remission rates of gut related inflammatory conditions (31; 36). Although specific probiotics have shown therapeutic effectiveness in extensive studies, not a single probiotic health claim has been approved by the European Food Safety Authority yet.

1.5 Fecal microbiota transplant

A fecal microbiota transplant (FMT) is the procedure of infusing suspended fecal material from a healthy donor into the gastrointestinal tract of the patient, with the intention of curing a disease. The idea of using donor fecal material as a remedy for a certain disease, however, is nothing new. In fact, more than seventeen centuries ago, a traditional Chinese medical doctor by the name of Ge Hong wrote that a human fecal suspension or "yellow soup" should be ingested by patients ailing from food poisoning or severe diarrhoea (37). This reportedly yielded positive results and fecal transplantation was also used in traditional Chinese medicine for treatment of fever, vomiting, pain and constipation. Recently, this procedure has gained much attention due to its high effectiveness in curing recurrent Clostridium difficile infections (CDI) and holds promise as a treatment option for many other diseases (38).

CDI manifests as severe GI dysbiosis due to an overgrowth of a single bacterial species and has mainly been treated with antibiotics (e.g. vancomycin, metronidazole). Unfortunately, antibiotic therapy induces further dysbiosis, in turn leading to CDI recurrences in more than 20 % of cases (13). In the first randomized, controlled FMT trial, the infusion of donor feces cured 94 % of recurrent CDI patients, who discontinued their vancomycin treatment (38). The patients in the infusion group received a bowel lavage before FMT and were compared to patients in two other vancomycin-treated groups. The first group resulted in a 31 % cure rate, while the second group received a bowel lavage besides its vancomycin treatment resulting in a 23 % cure rate. The fecal microbiota diversity of patients was shown to be considerably lower than that of the healthy donors. After the infusion of donor feces the patients’ microbiota diversity resembled that of their donors. The use of FMT in recurrent CDI has been studied in more than 500 patients worldwide and has achieved similar disease resolution rates (>90 %). As a treatment
option, FMT procedure is most frequently performed for recurrent CDI and is supported in the latest guidelines by the American College of Gastroenterology (13).

FMT also shows a lot of promise in the treatment of ulcerative colitis, irritable bowel syndrome, obesity, metabolic syndrome, type 2 diabetes and many other non-GI diseases (13). These claims have been studied to different extents (figure 5).

**Figure 5. Various disorders associated with the modulation of microbiota.**
*RCT, randomized controlled trial (39)*

For a FMT procedure it is required to obtain feces from a healthy donor. Choosing a healthy donor is a process involving evaluation of medical history and testing for transmissible diseases. Donor medical history should be devoid of any factors which
profoundly alter microbiota composition, lower diversity or increase risk of disease transmission. There is lack of research data to establish optimal donor characteristics, but risk factors of possible transmissible diseases have to be excluded (e.g. test for pathogens, parasites).

For a long time, the preferred route for FMT administration was through a retention enema. Recently, there has been an increase in self-administered enemas, especially in patients who have unresolved GI disorders and cannot get a FMT from their doctor. In the last two decades, the nasogastric tube and colonoscopy have been included in use for FMT administration as well. According to a recent review of over 400 human FMTs by Aroniadis et al., 3 out of 4 are administered by colonoscopy or retention enema, while the rest are by nasogastric or duodenal tube (32). Enemas can only reach the splenic flexure, while colonoscopies go past into the entire colon and ileum while also revealing the condition of the intestine.

Stool preparation is described in many publications which reveal many variations in this method. Certain principles, particularly those dealing with quick use of fresh stool (<6 h preferably), dilution, filtration and homogenization, are similar though. A review of FMT procedures for recurrent CDI concluded that the dilution of donor feces in water compared to saline yields a higher disease resolution (98.5 % vs. 86 %), yet the relapse of CDI appears to be more than 2 times higher with water than saline (8 % vs. 3 % for saline) (40). Authors also noted that other diluents can be used, with similar resolution rates, like saline with psyllium, milk or yogurt. Another observation was the increase in disease resolutions with increased volume of transplant (97 % with volumes above 500 mL vs. 80 % with volumes under 200 mL). The relapse of patients was also higher when 50 g or less of stool was used (4 % vs. 1 %).

A recent case series used standardized frozen stool samples for treatment of recurrent CDI and provided similar results to those of the fresh stool samples (41). More than half of the dry weight of feces is ascribed to microbes, while fibres and solubles account for the rest. Only half of these microbes are viable, since they are dead or damaged to an extent which would probably not remain viable upon FMT procedure (42). The impact of non-viable microbes and non-living components found in fecal material have on FMT procedure outcome is not well researched and could very well be crucial.
1.6 Microbiota research in dogs

Fecal transplantation has also been used in veterinary medicine and is still used for cattle, horses and other animals. Dogs, however, are known to be coprophagic; they voluntarily eat feces either from other dogs or from other species such as wild rodent feces, horse manure or cat feces. Compared to humans, the canine microbiota has been less thoroughly researched, but shows many similarities (43).

Dogs are carnivorous, have shorter intestines and a slower intestinal motility than humans. The complexity of microbiome composition and influence by host or environmental factors is parallel to that of the human microbiota (44). Chronic diarrhoea, IBD and other canine GI diseases have been associated with intestinal microbiome dysbiosis, with acute diarrhoea showing the most severe changes in GI microbial composition (5; 45). Some studies have also noted a difference in small intestinal bacterial overgrowth (SIBO) classification compared to humans, since dogs appear to have higher normal bacterial counts in the small intestine (46). Due to these differences the terms antibiotic-associated diarrhoea or small intestinal dysbiosis are used in dogs (43).

A common antibiotic used for canine diarrhoeal disorders is tylosin, which can lead to tylosin responsive diarrhoea (TRD). TRD most commonly affects middle aged, large breed dogs and affects both the small and large intestine according to clinical signs. Dogs encounter diarrhoea within a few weeks after ending tylosin administration and must therefore stay on tylosin indefinitely. Dogs are diagnosed with TRD when they experience diarrhoea at least twice as a result of tylosin discontinuation.

Tylosin is a macrolide antibiotic with a broad spectrum of activity against gram-positive and a narrow spectrum against gram-negative organisms. It binds to the 50S subunit of the bacterial ribosome which causes the inhibition of protein synthesis and leads to a bacteriostatic effect. Tylosin is naturally produced by *Streptomyces fradiae* as a product of fermentation. The effect of tylosin was shown to be different from other antibiotics, since some diarrhoeal disorders respond very well to tylosin and the effect does not seem to diminish through time (47; 48). Studies on TRD dogs have shown that tylosin induces significant, but temporary shifts in the small intestinal microbiota (49). This effect is in accordance with the researchers hypothesis that tylosin increases the abundance of beneficial commensal bacterial, while reducing abundance of harmful bacteria. Besides the
antimicrobial effect of tylosin, evidence of a direct anti-inflammatory effect has been found as well. Decrease in synthesis of several inflammatory mediators and cytokines as the direct effect of increasing in vitro concentrations of tylosin and tilmicosin (a semi-synthetic macrolide, derived from tylosin) was observed in one study (50).

The effect of diet, prebiotics and probiotics on microbiome composition has mostly been studied in canine microbiome studies. Variation in dog foods such as ratio and source of nutrients influences canine microbiome composition and can be explained by the metabolic properties of affected microbiome constituents. This way dietary fibre and certain prebiotics are used to alleviate GI symptoms in dogs as a treatment option.

In a study using dogs diagnosed with IBD, the effect of a daily administration of the probiotic VSL#3 was compared to the commonly used prednisone and metronidazole combination therapy (29). VSL#3 is a probiotic mixture of 9 different bacterial strains, shown to be capable of inducing and sustaining remission in the treatment of human ulcerative colitis. Both treatments led to similar improvements in histology and CIBDAI (canine IBD activity index) scores. Although there were no significant differences on the scale of remission, the probiotic treatment did take longer to reduce the incidence of clinical signs (e.g. vomiting, diarrhoea) than the combination therapy. Additionally, the probiotic treatment provided a general protective effect, greatly increasing regulatory T-cell markers and expression of two proteins related to intestinal tight junction well-being (occludin and claudin-2). This protective effect, induced by the probiotic, was also correlated to the normalization of GI dysbiosis. Regarding FMT in dogs, there is a lack of studies evaluating its potential as a treatment option, with rare case reports and some conference abstracts presenting the initial findings (51). One such abstract reports of eight dogs with refractory Clostridium perfringens associated diarrhoea that were unsuccessfully treated with antibiotic therapy while all of the eight dogs ceased experiencing diarrhoea immediately following FMT procedure (52). To achieve this, feces from one healthy donor was blended with saline and given as an enema, anywhere up to three times for each dog.
2 Research plan
The implementation of FMT treatments in dogs seems almost intuitive, since dogs are coprophagic. Although there is much data implicating therapeutic modulation of microbiota in dogs as a valid treatment option, a FMT procedure has not yet been properly evaluated in dogs.

The purpose of this pilot study is to evaluate the therapeutic potential of FMT in restoring dog GI homeostasis. We hypothesize that a FMT procedure will have a great therapeutic effect in dogs with GI dysbiosis, where other treatment options have been exhausted.

3 Materials and methods
Ringer's solution: Ringer acetate, isotonic (Fresenius Kabi, Uppsala Sweden)

Given that FMT procedure has had its most promising results in treating recurrent CDI, we chose a similar disorder in dogs - antibiotic-responsive diarrhoea. After sifting through many possible patients, which could benefit from the FMT procedure, three dogs were selected for this pilot study at the Veterinary Teaching Hospital in Helsinki. The first task was to obtain a suitable, readily available donor which would regularly provide the healthy donor feces needed for the FMT procedures.

3.1 Dogs used in study
We adapted our protocol for donor selection from human FMT guidelines, excluding human-specific diseases and adding canine-specific risk factors. Our goal was to obtain at least two donor dogs in case one should become ill or otherwise unavailable. We searched for donor dogs with the best indicators of microbiota health and low risk of transmitting disease through FMT. This was established by: no history of GI disease, GI symptoms (such as vomiting, diarrhoea, loose stools, flatulence, abdominal pain) or GI-associated disorders, dogs that have never used systemic antibiotics, naturally born, fed by mother, no immune disorders, no immunosuppressive therapy, no malignancies, and no atopic diseases.

Nine potential donor dogs were recruited to readily provide donor feces for the upcoming FMTs. The Faculty of Veterinary Medicine's central clinical laboratory tested the potential donor dogs for parasites. Fecal samples were tested for Giardia duodenalis with FASTest GIARDIA® (Megacor diagnostik, 6912 Hörbranz, Austria) and for various parasites
(Cestoda, Trematoda, Acanthocephala and Nematoda) using a fecal floatation method. The clinical microbiology laboratory also cultivated the feces for the following specific pathogens: Salmonella, Yersinia enterocolitica, Campylobacter, Clostridium perfringens, Clostridium difficile. The fecal samples were positive for Clostridium perfringens in five dogs, while Campylobacter was found in two dogs and Yersinia enterocolitica in one dog. Only one dog (Donor) had no pathogens found in its feces. This donor dog was selected to provide FMT material for all 3 recipient dogs. All FMT material was provided on the same day as the corresponding procedure, while part of the fecal matter was stored in freezer for fecal microbiota analysis.

We presumed that dogs with GI-related illnesses in which conventional means of treatment have been unsuccessful would benefit most from a FMT procedure. Such conditions were found in several IBD dogs, a few of which were exhibiting signs of TRD. All three of the chosen FMT recipients had received or were receiving tylosin to alleviate their GI signs. FMT recipient dogs were screened with the fecal pathogen and parasite tests as described before for donor testing. Since antibiotic treatment may impede the success of FMT procedure, the recipients were to be taken off their antibiotic treatments a week before the procedure. The dogs were also treated with fenbendazole (3 days, 50 mg per kg of body mass each day) a week prior to the FMT in order to eliminate possibly present parasites. Basic information regarding the dogs used in this study can be seen below (table I).

**Table I: Dogs used in study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Age (years)</th>
<th>BW (kg)</th>
<th>Sex</th>
<th>Breed</th>
<th>Date of birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>6</td>
<td>15,4</td>
<td>F, spayed</td>
<td>Australian Shepherd</td>
<td>08.04.2008</td>
</tr>
<tr>
<td>Idefix</td>
<td>4</td>
<td>2,7</td>
<td>M</td>
<td>Yorkshire Terrier</td>
<td>14.04.2010</td>
</tr>
<tr>
<td>Sisu</td>
<td>7</td>
<td>43,4</td>
<td>M</td>
<td>Rhodesian Ridgeback</td>
<td>21.09.2007</td>
</tr>
<tr>
<td>Jassu</td>
<td>12</td>
<td>21,9</td>
<td>M</td>
<td>Schapendoes</td>
<td>14.01.2002</td>
</tr>
</tbody>
</table>

**Donor:** No history of chronic disease or antibiotic use. Dog also never exhibited any signs of GI disease or any other disease. Fed a commercial dry food diet (Royal Canine German Shepherd adult®) before and during to the study. The dog tested negative for fecal parasites and specific microbial pathogens 10 days prior to the first FMT.
Idefix: History of chronic GI signs; vomiting and diarrhoea, anorexia, nausea as well as abnormal sounds from the stomach and intestines. It scored 10 on CIBDAI and was put on a daily antibiotic regimen (tylosin tartrate 240 mg daily) in December 2013 to alleviate GI signs. It was also on an elimination diet (chicken, fish and rice only) to avoid GI signs. Tylosine treatment was discontinued a week before the FMT and resulted in clinical GI signs 4 days later; which emerged as constipation for 2 days, followed by diarrhoea and vomiting. For these GI signs the dog was given Imodium® (loperamide; for diarrhoea) and Cerenia® (maropitant citrate; against vomiting) tablets.

Sisu: This dog had a long history of vomiting and diarrhoea as well as chronic pancreatitis and was put on antibiotic treatment (tylosin tartrate 720 mg daily) to alleviate GI signs. In the two months prior to the FMT procedure, the dog was showing signs of GI health deterioration (looser stools, increased anal licking and visceral pain). Tylosine treatment was discontinued 7 days before FMT procedure, but did not lead to any diarrhoea or other GI signs before FMT procedure was carried out.

Jassu: History of chronic GI inflammation and diagnosed with gall-bladder stones. Because of the chronic GI inflammation it was treated with cortisone (prednisolone 5 mg, twice a day) and was put on an elimination diet. In May 2014 it was presented with another diagnosis - foreign body in stomach. Following this, the dog was kept on the elimination diet and its cortisone dose was doubled (10 mg, twice a day) as the GI-signs were persisting. The dog had been receiving tylosin treatment before, but ceased taking tylosin more than a month prior to the study. A week before FMT, the dog was taken off cortisone and developed diarrhea.

3.2 FMT procedure
Human FMT procedures vary quite considerably in protocol, therefore we adapted it for our procedure in dogs. The chosen route of administration was through an endoscope into the upper GI canal. Although colonic retention enemas would have been easier to administer, compliance to retain the transplant by the patient would be difficult to achieve in dogs. Donor feces were obtained and prepared for administration within 6 h of defecation. Approximately 7 mL of Ringer's solution per gram of fecal material was added and then mixed with a commercial blender to produce a liquid slurry. The slurry was then filtered through a metal tea strainer to exclude particles (mostly hair and other solid
particles) which may later clog the endoscope liquid canal (figure 6a). The filtered slurry was then drawn into several 50 mL syringes (figure 6b), ready for administration through the endoscope liquid canal. We decided to use a volume of 10 ml of suspended fecal material per kg of body weight, since similar ratios proved to be successful in human studies.

Figure 6. FMT material preparation A: Metal strainers. B: 50 mL syringes.

The recipient dogs were taken off antibiotic treatment a week before FMT procedure. Dogs were fasted for 12 h before their arrival to the veterinary hospital and the owners also brought in their dog's most recent fecal samples. Dogs were then sedated (inj. Dextranitor®; dexmedetomidine hydrochloride 0.5mg/ml at a dose of 0.5mg/kg + inj. Butorfanol®; butorfanol 10mg/ml at a dose of 0.1mg/kg) and placed on their left side with an elevated upper body. They were then intubated and attached to an inhalator inducing them into anaesthesia using an isoflurane inhalant (Inhalant Isoflo Vet.® 100%) at a rate of 1-2%. When the dog was stabilized and its breathing normalized, an endoscope was introduced through the mouth (figure 7) to reach the small intestine.
After placing the endoscope about half way through the duodenum, the FMT material from the syringes was fed through the endoscopic tube into the GI of the recipient dog at a steady pace in order to normally pass through the intestinal tract (figure 8). In the case of obstructed flow of the FMT liquid, the abdomen was slowly massaged from the outside. Dogs were left lying in this elevated position for another 30 minutes after all of the FMT material had been administered.
Two dogs, Idefix and Sisu, had their FMT procedure on the same day. The FMT material for both dogs was prepared with Ringer's solution and 75 g of donor feces (sample Donor.1) to provide 500 mL of suspended fecal material. According to their body weight, Idefix received 24 mL of the suspended fecal material, while Sisu received 450 mL. At a later date, Jassu received a total of 250 mL of suspended fecal material prepared from 60 g of donor feces (sample Donor.2) and 350 mL of Ringer's solution.

3.3 Sampling and analysis

The owners of FMT-recipient dogs were asked to fill out a questionnaire concerning the dog's GI health, diet and general wellbeing (attachment 1). The questionnaire was filled out right before and two months after the FMT procedure.

Owners were also instructed to keep daily score of their dogs feces based on the 9-point WALTHAM stool-scoring system throughout the observation period (attachment 2) (53). This provided us with information regarding the dogs' stool consistency scores and if diarrhoea had recurred. Since there are many factors influencing stool consistency (diet, physical activity, stress, water intake, inflammation), the stool consistency scores were not used to directly evaluate FMT outcome.

Fecal samples were collected by dog owners before FMT administration and during 1-2 consecutive days every following week for a duration of 8 weeks. Fecal samples were immediately stored by the dog owner at home in a commercial freezer at -20 °C and transferred to the Veterinary Teaching Hospital of the University of Helsinki for storage at -80 °C, when they visited the hospital for the FMT procedure or at the final evaluation, 8 weeks after.

Fecal samples collected from donor and FMT-recipient dogs were sent for analysis to the Veterinary Medical Teaching Hospital at Texas A&M University. Using a DNA isolation kit, 100 mg of DNA were extracted from each fecal sample. A region of the 16S rRNA gene was amplified using specific primers. The samples were then prepared using sequencing adapters and distinct sample-barcodes to provide a DNA library and were then sequenced using an Illumina MiSeq system. To analyse the raw sequences obtained, the Quantitative Insights Into Microbial Ecology (QIIME) pipeline was used and sequences were denoised, chimeras and low quality sequence reads removed, while quality sequences were grouped into OTUs and taxonomically assigned (54).
A dysbiosis index was calculated for each fecal sample using quantitative PCR analysis data. The index takes into account individual bacterial groups which are usually altered in dogs with chronic enteropathies and is expressed as a single number. The dysbiosis index used is based on the QIIME dysbiosis index and is currently being developed, with a paper on its validation being published within a few months.

For expanded interpretation and comparison of sequencing results between each fecal sample, 51,000 sequences were randomly selected for each sample, in order to avoid an unequal sequencing depth among samples. To compare the microbiota communities between each sample, UniFrac, a distance metric evaluating phylogenetic differences between microbial communities, was applied (55). Principal coordinates analysis (PCoA) plots and rarefaction curves produced by QIIME were used to compare diversity of fecal samples (54). EMPeror was used to visualize the PCoA plots in a 3-dimensional system (56).

4 Results and Discussion

Screening the feces of potential donors and patients for bacterial pathogens and parasites provided some unexpected results. All but one of the nine healthy dogs, which were screened for suitability as donors, were positive for bacterial pathogens or parasites. On the other hand, all three dogs with constant GI illnesses (FMT-recipient dogs) were negative using the same tests. The suitability of such fecal tests for assessing GI health has been put into question by other studies, since many of these bacterial pathogens and parasites are routinely found in healthy dogs. Such is the case with Clostridium perfringens, as it was found present in all dogs included in a recent study (95 healthy and 104 GI disease dogs) (58). The study, however, did find that the presence of both Clostridium perfringens enterotoxin and fecal dysbiosis correlated with GI disease (58). Bacterial pathogens present in the microbiota are often kept in check by other microbiota constituents, preventing the colonization of a pathogen and countering its pathogenicity (9). Yet, using these dogs would add unnecessary risks regarding the procedure, therefore only one donor dog was used. Some studies using multiple donors for FMT material have observed donor specific responses, as was the case in a recent human FMT trial in patients with active ulcerative colitis, where most of the successful treatments were attributed to 1 out of 6 donors used (59).
According to data from questionnaires given to the dog owners (attachment 1), the condition of Idefix greatly improved following the FMT for a duration of 1 month. During this time, the owner reported increased energy, general wellbeing and improvement of GI signs. The dog’s condition then deteriorated towards its previous state, however, it was still better than before receiving the donor feces. Based on daily data on the dog’s stool consistency scores, Idefix developed diarrhoea two days prior to FMT and maintained a normal stool consistency afterwards throughout the observational period.

Sisu's owner reported an improvement in the dog’s general wellbeing and energy following the FMT procedure. The dog exhibited less abdominal signs, vomiting, diarrhoea, skin problems and had a less bloated abdomen. The condition of the dog did not deteriorate during the two months following FMT procedure. Contrary to our instructions, Sisu gradually changed its diet from a dry food diet on the day of receiving FMT to a raw food diet two months later. Diet influences GI microbiota composition and is an important confounding factor in assessing the therapeutic potential of FMT in this dog (28). Yet, the scale of this influence is presumably rather small, since the available studies on dog fecal microbiome composition or diversity mostly report small or no significant changes due to diet (60). Another confounding factor is that Sisu did not exhibit any GI signs after it was taken of tylosin. In a recent study, dogs treated with tylosin developed diarrhoea at a median number of 8 days (range 1-60 days) after tylosin was discontinued (49). According to data from the same research group, approximately 15 % of TRD dogs do not exhibit diarrhoea after ending tylosin treatment. Since Sisu had TRD, it most likely would have developed diarrhoea in the following days, should the FMT procedure be delayed. Based on the dog's daily stool consistency scores, no diarrhoea was observed before FMT administration and the dog maintained a normal score throughout the observational period.

Following the FMT procedure of Jassu, its owner provided no answered questionnaires or information regarding fecal consistency scores. At the 2 month follow up, the owner reported that the dog's condition did not improve in any observable way. Recent human studies have shown that a FMT also restores the normal fecal bile acid composition by restoring microbiota bile acid metabolism (61). We had hoped that an improvement would be seen in Jassu due to its medical history of gallstones. However, bile in dogs is different from that in humans, as it normally has a lower cholesterol saturation.
Fecal samples analyzed at the Veterinary Medical Teaching Hospital at Texas A&M University each received a dysbiosis index value, based on their quantitative PCR analysis results of specific bacterial groups. Two fecal samples from Idefix (week 1 and week 5) were not provided by the dog's owner and therefore the related data could not be obtained for those two samples. The dysbiosis index of all four donor samples provided from the donor dog are shown in table II.

**Table II: Dysbiosis index of donor samples**

<table>
<thead>
<tr>
<th>Date</th>
<th>Dysbiosis index</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>24.10.2014</td>
<td>-5.07</td>
<td>Donor.1</td>
</tr>
<tr>
<td>18.12.2014</td>
<td>-5.71</td>
<td>Donor.2</td>
</tr>
<tr>
<td>28.5.2014</td>
<td>-4.46</td>
<td>Donor.3</td>
</tr>
<tr>
<td>2.6.2014</td>
<td>-6.30</td>
<td>Donor.4</td>
</tr>
</tbody>
</table>

A negative index value indicates normal microbiota and a positive index value indicates dysbiosis, while the values between 0 and 2 are inconclusive. All four samples taken from our used donor are negative, implying a normal microbiota without dysbiosis. The dysbiosis index of all three FMT recipient dogs can be seen in figure 9.
Dysbiosis index values of all three dogs quickly move towards more negative, donor-like, values, implying the lessening of dysbiosis, following FMT procedure. This quick decrease in value indicates a shift in specific microbial taxa abundances, used to calculate the index, towards those found in donor feces. Idefix and Sisu maintain a dysbiosis index mostly below zero, which is in agreement with their improvement in general wellbeing and energy as assessed by their owners. The dysbiosis index values of Jassu are almost entirely within the dysbiosis area and/or inconclusive values, which is in agreement with the lack of observable changes in health reported by its owner. In future studies, the dysbiosis index could be used as a quick screening tool for GI dysbiosis and GI disease in addition to fecal parasites and microbial pathogen tests.

Sequencing analysis of fecal samples analysed at the Veterinary Medical Teaching Hospital at Texas A&M University provided us with detailed phylogenetic data on all of the 29 fecal samples. Sequencing labels assigned to each fecal sample according to week can be seen below (table III).
Table III: Sequencing labels through observational period (Figures 10, 12)

<table>
<thead>
<tr>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
<th>Week 7</th>
<th>Week 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDEFIX.1</td>
<td>/</td>
<td>IDEFIX.2</td>
<td>IDEFIX.3</td>
<td>IDEFIX.4</td>
<td>/</td>
<td>IDEFIX.5</td>
<td>IDEFIX.6</td>
<td>IDEFIX.7</td>
</tr>
<tr>
<td>SISU.1</td>
<td>SISU.2</td>
<td>SISU.3</td>
<td>SISU.4</td>
<td>SISU.5</td>
<td>SISU.6</td>
<td>SISU.7</td>
<td>SISU.8</td>
<td>SISU.9</td>
</tr>
<tr>
<td>JASSU.0</td>
<td>JASSU.1</td>
<td>JASSU.2</td>
<td>JASSU.4</td>
<td>JASSU.5</td>
<td>JASSU.6</td>
<td>JASSU.7</td>
<td>JASSU.8</td>
<td>JASSU.9</td>
</tr>
</tbody>
</table>

Figure 10. Fecal sample sequencing results presented at bacterial phylum level. Dotted lines indicate time of FMT procedure for each dog according to samples.

The relative abundances of bacterial phyla observed across all 29 fecal samples indicate a prevalence of Firmicutes (45.2 %), Fusobacteria (22.1 %), Bacteroidetes (17.9 %), Proteobacteria (10.5 %) and Actinobacteria (4.0 %). This distribution of relative abundances between phyla is in accordance with data reported in other studies sequencing the canine fecal microbiota (51; 62; 63). All three fecal samples taken before FMT are less abundant in Bacteroidetes and Fusobacteria compared to donor samples. Fecal microbiota composition, following FMT procedure in humans (mostly after CDI infections), shifts towards higher diversity with a general increase in Bacteroidetes and Firmicutes, while Proteobacteria decreases (42). Such a shift in microbiota composition (but with an increase of Fusobacteria and not Firmicutes) can be seen in all three recipient dogs following FMT.
and is persistent throughout the observation period. This difference is not surprising, since Fusobacteria are one of the predominant bacterial phyla in dogs, but not in humans.

Pre-FMT fecal samples of two dogs (Idefix1 and Sisu1) are significantly more abundant in Proteobacteria (almost entirely due to its family Enterobacteriaceae) and less abundant in Actinobacteria compared to donor fecal samples. A higher abundance of Enterobacteriaceae relative to healthy dogs has also been reported in the duodenum of IBD dogs (43). Fecal samples of IBD dogs in another study also revealed higher Gammaproteobacteria and decreased Erysipelotrichia, Clostridia and Bacteroidia abundances compared to healthy dogs (64). During diarrhoea, higher Enterococcus, Sutterella, Clostridium and lower Ruminococcaceae (and its genus Faecalibacterium), Fusobacteria and Blautia have been reported in dogs. Before and after FMT sequencing abundances of several bacterial taxa are shown in tables IV and V. For better comparison, all shown sequencing abundances were divided by the abundances of the same bacterial taxa found in the Donor.1 fecal sample. This enabled us to directly compare bacterial abundances found in the FMT recipient dogs' fecal samples to those found in the Donor.1 sample. Since Jassu received FMT material prepared from Donor.2 fecal material, the abundances of bacterial taxa within Donor.2 sample are divided by the abundances in Donor.1 sample as well.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Donor.1</th>
<th>Idefix</th>
<th>FMT</th>
<th>Sisu</th>
<th>FMT</th>
<th>Donor.2</th>
<th>Jassu</th>
<th>FMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusobacteria</td>
<td>0.1992</td>
<td>3,446%</td>
<td>90,95%</td>
<td>3,101%</td>
<td>190,1%</td>
<td>128,2%</td>
<td>12,69%</td>
<td>85,10%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>0,008333</td>
<td>33,65%</td>
<td>856,0%</td>
<td>18,12%</td>
<td>300,6%</td>
<td>142,6%</td>
<td>1170%</td>
<td>577,1%</td>
</tr>
<tr>
<td>(A. Collinsella)</td>
<td>0,008020</td>
<td>16,63%</td>
<td>877,5%</td>
<td>17,11%</td>
<td>311,5%</td>
<td>143,0%</td>
<td>1015%</td>
<td>597,2%</td>
</tr>
<tr>
<td>Bacteroidia</td>
<td>0,4459</td>
<td>1,231%</td>
<td>34,09%</td>
<td>0,998%</td>
<td>43,89%</td>
<td>54,21%</td>
<td>2,867%</td>
<td>34,84%</td>
</tr>
<tr>
<td>Clostridia</td>
<td>0,2490</td>
<td>131,0%</td>
<td>205,3%</td>
<td>174,1%</td>
<td>117,9%</td>
<td>175,9%</td>
<td>314,9%</td>
<td>187,7%</td>
</tr>
<tr>
<td>(C. Ruminococcaceae)</td>
<td>0,05043</td>
<td>3,810%</td>
<td>9,979%</td>
<td>1,400%</td>
<td>28,86%</td>
<td>76,87%</td>
<td>1,711%</td>
<td>3,135%</td>
</tr>
<tr>
<td>(C. Faecalibacterium)</td>
<td>0,009412</td>
<td>0,625%</td>
<td>6,771%</td>
<td>0,625%</td>
<td>27,16%</td>
<td>56,25%</td>
<td>4,025%</td>
<td>1,901%</td>
</tr>
<tr>
<td>Blautia</td>
<td>0,06125</td>
<td>144,9%</td>
<td>72,24%</td>
<td>177,0%</td>
<td>95,82%</td>
<td>174,1%</td>
<td>205,1%</td>
<td>175,1%</td>
</tr>
<tr>
<td>Erysipelotrichia</td>
<td>0,005922</td>
<td>81,46%</td>
<td>233,6%</td>
<td>9,93%</td>
<td>45,24%</td>
<td>134,1%</td>
<td>476,8%</td>
<td>111,2%</td>
</tr>
</tbody>
</table>

Donor.1 = sequencing abundances found in Donor.1 fecal sample (all other rows were divided by these abundances); Donor.2 = relative abundances found in Donor.2 fecal sample; Idefix/Sisu/Jassu = relative abundances found in each dog's pre-FMT fecal sample (week 0); FMT = average of all post-FMT sample's relative abundances for each dog (weeks 1-8); Taxon in brackets indicates it is subordinate to the taxon above it.
Colours indicate the increase or decrease of abundance following FMT. For example: the change in relative abundances of Actinobacteria for Idefix is from 33.65% (pre-FMT) to 856.0% (average of post-FMT samples), which is more than a 10-fold increase - colouring both cells dark green. (NOTE: colours are opposite in table IV, since these taxa are oppositely associated with GI-health)

**Table V: Bacterial taxa associated with dysbiosis**

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Donor.1</th>
<th>Idefix</th>
<th>FMT</th>
<th>Sisu</th>
<th>FMT</th>
<th>Donor.2</th>
<th>Jassu</th>
<th>FMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sutterella</td>
<td>0.04247</td>
<td>4.755%</td>
<td>32.61%</td>
<td>5.217%</td>
<td>29.66%</td>
<td>25.16%</td>
<td>5.956%</td>
<td>74.67%</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>0.003549</td>
<td>17292%</td>
<td>988.7%</td>
<td>13912%</td>
<td>1961%</td>
<td>91.16%</td>
<td>353.0%</td>
<td>640.7%</td>
</tr>
<tr>
<td>(G. Enterobacteriaceae)</td>
<td>0.003333</td>
<td>18409%</td>
<td>1052%</td>
<td>14809%</td>
<td>2087%</td>
<td>89.41%</td>
<td>375.3%</td>
<td>673.4%</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>0.001176</td>
<td>24217%</td>
<td>86.11%</td>
<td>233.3%</td>
<td>420.8%</td>
<td>0</td>
<td>100.0%</td>
<td>239.6%</td>
</tr>
<tr>
<td>Clostridium</td>
<td>0.007431</td>
<td>270.2%</td>
<td>442.2%</td>
<td>955.15%</td>
<td>273.55%</td>
<td>271.2%</td>
<td>473.9%</td>
<td>194.3%</td>
</tr>
<tr>
<td>(C. perfringens)</td>
<td>0.0003529</td>
<td>233.3%</td>
<td>678.7%</td>
<td>18994%</td>
<td>1356.25%</td>
<td>50.00%</td>
<td>61.11%</td>
<td>436.1%</td>
</tr>
</tbody>
</table>

Based on data shown in tables IV and V, we have observed a general increase in abundance of taxa associated with GI health and comparatively less uniform shifts in taxa associated with dysbiosis following FMT procedure. Pre-FMT samples of recipient dogs were commonly less abundant (< 100%) in GI health-associated and more abundant (> 100%) in dysbiosis-associated taxa compared to Donor.1 fecal sample. These differences were generally greatly narrowed or even widened by a sweeping shift towards donor abundances (as with *Collinsella* in Idefix and Sisu) following FMT procedure. Jassu shows the least favourable changes following FMT, with mostly decreasing abundances of taxa associated with GI health and increasing in taxa associated with dysbiosis. Idefix and Sisu, on the other hand, experienced great increases in abundance of taxa associated with GI health (*Fusobacteria*, *Collinsella*, Bacteroidia, *Faecalibacterium*) and a large decrease in some of the taxa associated with dysbiosis (*Enterobacteriaceae*, *Enterococcus*).

However, these differences and shifts only describe the phylogenetic composition of fecal microbiota before and after FMT procedure, while the fecal microbiota itself is used to estimate the actual state of the microbiota within the intestine. Also, data on specific bacterial taxa associated with GI health or dysbiosis is not easily comparable due to differences in PCR primers, sequencing preparation and method, which may lead to an increase or decrease in specific bacterial taxa (43). Although the exact mechanisms of how many bacterial taxa promote dysbiosis or GI health, the link between phylogenetic shifts in microbiota composition and GI health is presumed to be mainly due to influencing metabolic functions of the microbiota. Changes in GI amino and bile acid metabolism,
SCFA levels and redox state are some of the ways microbiota may influence GI health (51). The general health improvement of dogs 1 and 2 following FMT could very well be due to the observed shifts in bacterial composition and the associated improvements in metabolic function, leading to a decrease in GI inflammation and, in turn, resulting in a move towards GI homeostasis.

Sequencing data from fecal samples was grouped based on sample collection time following FMT procedure (see table III for samples combined for each week). To evaluate the species richness of the dogs' feces, rarefaction curves were calculated for each sample. Rarefaction curves can be used to estimate the total number of different species found within a sample, taken from a pool of randomized samples. Observed species are a measure which add up every unique OTU (novel species) found within an individual sample. An increase in observed species signifies an increase in diversity for a bacterial community. A rarefaction curve for the observed species according to sample week following FMT procedure is shown (figure 11).
Figure 11. Observed species in sequenced fecal samples grouped by week. Lines show mean values, while error bars indicate the standard deviation for each line. D - all four donor samples combined; 0-8 – joined samples for each corresponding week following FMT.

The rarefaction curve for observed species illustrates the alpha diversity (number of different species within a sample) between fecal samples based on their sequencing results. Observed species drastically increase following the FMT procedure, with week 1 fecal samples approaching the bacterial community diversity found in donor samples. This diversity then slowly deteriorates towards pre-FMT levels (week 0). The progressive lowering of observed species with time implies that many of the unique OTUs gained with FMT are later excluded from the microbiota or drop to undetectable levels. Such a shift is in agreement with human FMT studies, where the FMT-recipient's fecal microbiota composition most resembles its donor's immediately following FMT procedure (65).

A PCoA plot founded on the unweighted UniFrac distances generated from sequencing data, enabled us to evaluate beta diversity (diversity within samples) of fecal samples. The
unweighted UniFrac PCoA plot provided 10 axes which together displayed 55 % of variance. The first three axes of the PCoA plot were used to generate a 3-dimensional configuration of each fecal sample's position with regards to other fecal samples. The PCoA plot was unweighted, meaning the relative abundance of bacterial groups did not affect the plot, but only if the bacterial group is present or not (it does not give more significance to highly abundant bacterial groups). An unweighted PCoA plot better displays the changes in microbiota community following FMT, since the bacterial groups most affected by FMT are the less abundant ones.

EMPeror was used to visualize the relative positions of fecal samples in a coordinate system defined by the first three axes of the PCoA plot. Since this is a measure of beta diversity, it positions samples in the plot based on their similarity of microbial composition (samples more similar in microbial community composition will be positioned closer to each other).
12.1. PC2 vs. PC1

First three axes of PCoA plot displaying beta diversity between fecal samples. Axis labels show the percentage of variance explained by each principal component.

In the PCoA plot (figure 12), big shifts in the dogs’ fecal microbiota composition following the FMT procedure can be observed. Pre-FMT samples of dogs 1 and 2 (IDEFIX.1 and SISU.1) are positioned far apart from all other samples and very close to each other, thus implying great similarity in fecal microbiota composition between the two dogs. This close
similarity could be due to both dogs receiving tylosin up to a week before FMT procedure as well as their similar medical history. Directly following FMT (week 1), samples SISU.2 and JASSU.1 came closest to the position of donor samples, compared to all other samples, indicating their fecal microbiota composition was most similar to that of the donor dog. All three dogs were shown to approach the donor dog’s microbiota composition following the FMT procedure and later gradually drifted away from donor samples, but not further than pre-FMT samples. We have also observed a clustering of individual dog samples, with all four dogs forming their own individual clusters. This means fecal samples of individual dogs were generally more similar to their own fecal samples than to those of other dogs.

Shifts observed in the PCoA plot are in accordance with previous studies following post-FMT and general microbiota community dynamics in humans (65). The large increase in similarity of microbiota composition in all three dogs with donor samples following FMT is presumably due to the successful transplantation of many microbiota constituents originating from donor fecal material. The following gradual decrease in similarity and the formation of individual dog-specific sample clusters can be attributed to the dynamic nature of the microbiota and the many factors which constantly influence its microbial community. The dogs' difference in genetic background, age, diet and environment are some of the factors responsible for the formation of these individual dog-specific clusters.

The limitations of the study were mostly due to its small scale and difficulties in obtaining appropriate donor and recipient dogs. The small number of FMT recipient dogs in this study strongly limits the statistical power of its results. The dogs varied greatly in breed, age, weight and sex while also inhabiting different home environments throughout the study. Regarding the influence of sex, a recent study including 26 dogs (13 healthy and 13 with diarrhoea), found no significant differences between the individual dogs' fecal microbial communities ascribed to sex or sex status (spayed/castrated or intact) (57). Obesity, on the other hand, has been shown to influence microbiota composition in humans, however, studies comparing fecal microbiota composition of lean and obese dogs showed no significant connection due to obesity (11; 66). The dogs also varied in diet, but the scale of this influence may be rather small compared to the shifts observed following FMT procedures. Another issue was the missing data regarding Jassu's condition, since its owner provided no response to our numerous inquiries. The dogs also received different amounts of donor fecal material according to their body weight, yet there is no available
data implying that more donor fecal material results in greater or more health outcomes. A repeated FMT procedure or using a different donor could yield positive outcomes in Jassu, as some FMT studies have shown donor specific responses and recoveries, which occurred only following repeated FMT procedures.

Studying the role of microbiota in health and researching treatments such as FMT, promise a wide array of possibilities in the near future. Microbiota sequencing could soon be used to diagnose and customize the treatment of conditions associated with alterations in microbiota structure, such as IBD. Tailored synthetic microbial communities could be used instead of donor feces for FMT, giving higher viability, reproducibility and more controlled formulations (42).

Using a higher patient number, multiple donors and additional methods the therapeutic potential of FMT can be properly evaluated to allow its use in various diseases and not just as a last resort, when other treatment options have been exhausted. This study was meant to be a pilot trial of FMT procedure as a potential treatment option in dogs and we hope studies on a larger scale will follow to shed more light on this issue.
5 Conclusions

- FMT procedure, through nasoduodenal tube into upper GI, shows potential to correct GI dysbiosis in dogs, shifting fecal microbiome composition towards donor's and improving microbial diversity in three FMT-recipient dogs, all suffering from IBD.

- A general improvement in condition, energy and less GI signs were reported following FMT for two dogs, while the third dog showed no observable changes.

- Confounding factors influencing microbiota composition, such as dog age, environment, breed and diet may have significantly altered the results in this limited number of patients.

- Studies with a higher number of patients, multiple donors and additional methods are needed to optimize and properly evaluate the therapeutic potential of FMT in dogs.
6 Literature

1. O'Hara AM, Shanahan F: The gut flora as a forgotten organ. EMBO Rep 2006;7:688-693
28. Beloshapka AN, Dowd SE, Suchodolski JS, Steiner JM, Duclos L, Swanson KS: Fecal microbial communities of healthy adult dogs fed raw meat-based diets with or without inulin or yeast cell wall extracts as assessed by 454 pyrosequencing. FEMS Microbiol Ecol 2013;84:532-541
42. de Vos WM: Fame and future of faecal transplantations--developing next-generation therapies with synthetic microbiomes. Microb Biotechnol 2013;6:316-325
51. Schmitz S, Suchodolski J: Understanding the canine intestinal microbiota and its modification by pro-, pre- and synbiotics – what is the evidence? Veterinary Medicine and Science 2016:n/a-n/a


63. Beloshapka AN, Dowd SE, Suchodolski JS, Steiner JM, Duclos L, Swanson KS: Fecal microbial communities of healthy adult dogs fed raw meat-based diets with or without inulin or yeast cell wall extracts as assessed by 454 pyrosequencing. Fems Microbiology Ecology 2013;84:532-541


7 Attachments

Attachment 1: Dog follow-up questionnaire (in Finnish and English)

Attachment 2: WALTHAM feces scoring chart