The role of the intestinal microbiota in the pathogenesis of chronic enteropathies and their interplay with the immune system

Lina María Martínez-López

ORCID ID: 0000-0002-2292-4613

Submitted in total fulfilment of the requirements

of the degree of Doctorate of Philosophy

Faculty of Veterinary and Agricultural Sciences

The University of Melbourne

2018

32   Abstract
33
34   The intestinal microbiota and its associated genome is collectively called the gastrointestinal (GI)

35   microbiome; and is composed of crucial components that help not only to determine host biology but also to

36   maintain host physiology. Dysregulation of the gastrointestinal microbiome has been associated with a

37   range of diseases in people such as inflammatory bowel disease (IBD), diabetes and obesity. Previous

38   studies have found dysbiosis and a reduced bacterial diversity in dogs with chronic enteropathies (CE).

39   However, the precise nature of the intestinal microbiota dysfunction and whether the microbiota has a

40   causative role or is secondarily affected remain to be elucidated.

41

42   The first step in understanding the relationship between the gut microbiota and disease is the

43   characterisation of the normal gut microbiota, how it is established and how stable it is during different

44   periods of life. In this work, we assessed the dynamics and stability of faecal microbiota over time in healthy

45   dogs of different age groups, and the development of the microbiota from birth in puppies, and the

46   association with the maternal microbiome.

47

48   In puppies the main findings were that (1) at birth dogs exhibit a low diversity and stability in their gut

49   microbiota that increases as the animal grows; (2) gut microbiota is closely associated with the maternal

50   faecal microbiota but not oral microbiota; (3) Influence is bidirectional and maternal gut microbiota is

51   affected by puppy microbiota; (4) delivery route has low influence on gut microbiota and (5) weaning and

52   introduction of solid food are the key determinants that drive maturation of the gut microbiota into an adult–

53   like phenotype.

54

55   In juvenile and adult dogs, we could determine that: (1) the maximum complexity and stability is reached in

56   adulthood, (2) there are permanent fluctuations in microbiota composition over time and (3) gut microbiota

57   is more stable within the subject than between subjects.

58   Next, we characterised highly immunoglobulin A and G coated bacteria in faecal samples from dogs with

59   chronic enteropathies using flow cytometry and 16S rRNA sequencing and assessed their correlation with

60   disease stage and resolution of the clinical signs. We found that (1) there were lower proportions of

61   immunoglobulin coated bacteria during remission compared to active disease; (2) Amount of

62   immunoglobulin coating in active disease are similar to those found in healthy dogs; (3) immunoglobulin

63   coating is highly individualised despite similar clinical presentations between dogs, but differed between

64   active and remission period in most of the individual dogs.

65

66   Finally, we characterised the expression of thymic stromal lymphopoetin (TSLP), a cytokine that is

67   produced in response to bacterial contact, in the intestine of healthy dogs and its correlation with disease

68   activity in dogs with chronic enteropathies. We found that (1) TSLP is constitutively expressed in the

69   intestine and (2) its expression is not correlated with disease or disease stage.

70

71   In summary, the results reported here, help to understand the assembly of the gut microbiota, its interaction

72   with the immune system and emphasises on the importance of longitudinal studies and personalised

73   approach in order to understand the pathogenesis and the role of the microbiota in intestinal diseases in

74   dogs.

75

Declaration

This is to certify that:

I. The thesis comprises only my original work towards the degree of doctorate of philosophy except where indicated in the preface.

II. Due acknowledgement has been made in the text to all other material used.

III. The thesis is fewer than 100,000 words in length, exclusive of tables, bibliographies and appendices.

Lina María Martínez-López

Preface

All experimental work, data analysis and writing carried out for this PhD was undertaken by Lina María Martínez-López aside from the following cases. Sample collection and clinical management of the cases was carried out by Dr. Caroline Mansfield, Dr. Nathalee Prakash and Dr. Julien Dandrieaux. Histopathology was performed by the Pathology Department from The Faculty of Veterinary and Agricultural Sciences of The University of Melbourne.

Flow cytometry analysis and cell sorting of bacteria was carried out in collaboration with Dr. Elizabeth Ann Washington and Dr. Alexis Gonzalez. Bioinformatics analysis was done in collaboration with Dr. Alexandra Schulze from the Walter & Eliza Hall Institute, Melbourne, Australia. Sequencing of samples was done in collaboration with Stephen Wilcox from the Walter & Eliza Hall Institute, Melbourne, Australia.

113   **Funding**

114

115   This work was supported by:

116   I.   The International and Postgraduate Research Scholarship (IPRS and The Australian Posgraduate

117   Award (APA) from The Australian Government.

118   II.   Comparative and Gastroenterology Society/Waltham Grant 2016

119

120

121

Abbreviations

Acetyl-CoA: Acetyl-coenzyme A

ACTH: Adrenocorticotropic hormone

ADH: Vasopresin

AhR: Aryl hydrocarbon receptor

AID: Activation-induction cytidine deaminase

AIEC: Adherent and invasive *E coli*

ALT: Alanine Aminotransferase

AMP: Antimicrobial peptide

AP-1: Activating protein 1

APCs: Antigen-presenting cells

APRIL: Apoptosis-inducing ligand

ARE: Antibiotic- responsive enteropathy

ATG16L1: Autophagy-related protein 16-1

BAFF-B: B cell-activating factor of the TNF family (BAFF)

BCMA: B cell maturation antigen

BSA: Bovine Serum Albumin

BSG: British Society of Gastroenterology

C-ALP: corticosteroid- alkaline phosphatase.

CCECAI: Canine chronic enteropathy clinical activity index

CCL: Chemokines chemokine (C-C motif) ligands

CCL16: Chemokine ligand 16

CCL2: Chemokine ligand 2

CCL20: Chemokine ligand 20

CCL24: Chemokine ligand 24

CCR1: Chemokine receptors type 1

CCR3: Chemokine receptors type 3

CCR4: C-C chemokine receptor type 4

CCR5: Chemokine receptor type 5

CCR6: C-C Motif Chemokine Receptor 6

CCR9: Chemokine receptor type-9

CD: Chron's disease

CD40L: CD40 ligand

cDNA: Complementary DNA

CE: Chronic enteropathy

CINC-1: Cytokine-induced neutrophil chemoattractant-1

CL17: Chemokine 17

CMTs: Chemically modified tetracyclines

cpn60: Chaperonin 60

CRS: IgA class-switch recombination

CSF1: Colony stimulating factor 1

CXCL: Chemokine (C-X-C motif) ligand

CXCL10: C-X-C motif chemokine 10

CXCL12: CXC-chemokine ligand 12

DCs: Dendritic cells

DRE: Diet – responsive enteropathy (also termed food-responsive enteropathy FRE)

dsRNA: Double-stranded RNA

DSS: Dextran sodium sulfate

ECCO: European Crohn's and colitis organisation

EoE: Eosinophilic esophagitis

ER: Endoplasmic reticulum

ES: excretory-secretory products

ESPGHAN: ECCO-European Society for Paediatric Gastroenterology Hepatology and Nutrition

FACS: Fluorescence-activated cell sorting

FFAR2: Free fatty acid receptor 2 (also called GPR43)

FFAR3: Free fatty acid receptor 3 (also called GPR41)

FISH: Fluorescence *in situ* hybridization

FSC: Forward scatter

FSH: Follicle-stimulating hormone

GALT: Gut associated lymphoid tissue

GATA-3: Transcription factor GATA-3

GC: Germinal centres

GCs: Glucocorticoids

GIT: Gastrointestinal tract

GRs: Glucocorticoid receptor

H_2S: Hydrogen sulphide

HATs: Histone acetyltransferases

HDAC: Histone deacetylase

HFGP: Human Functional Genomics Project

HMP: Human Microbiome Project

IBD: Inflammatory bowel disease

ICAM-1: Intercellular adhesion molecule-1

IEC: Intestinal epithelial cells (also termed enterocytes)

IFN-γ: Interferon-gamma

IgA: Immunoglobulin A

IgA-SEQ: IgA 16S rRNA sequencing

IHC: Immunohistochemistry

IKK-β: Kinase subunit beta

IL-10: Interleukin-10

IL-13: Interleukin-13

IL-17: Interleukin-17

IL-17A: Interleukin-17A

IL-1b: Interleukin-1b

IL-1β: Interleukin-1 beta

IL-22: Interleukin-22

IL-23: Interleukin-23

IL-25: Interleukin-25

IL-33: Interleukin-33

IL-4: Interleukin-4

IL-5: Interleukin-5

IL-6: Interleukin-6

ILC3: Type 3 innate lymphoid cells

ILFs: isolated lymphoid follicles

iNOS: Inducible nitric oxide synthase

IRE1α: Inositol-requiring enzyme 1α

IκBα: Inhibitor kappa B alpha

JAK: Janus kinase (1 and 2)

lfTSLP: Long isoform or variant 1 TSLP

LH: Luteinizing hormone

LL-TSLP: *Lactococcus lactis* strain producing TSLP

LL-WT: *Lactococcus lactis* wild type

LPS: Lipopolysaccharide

MACS: Magnetic-activated cell sorting

MAMPS: Microbe-associated molecular patterns

MCP-1: Monocyte chemotactic protein-1

MHC I: Major histocompatibility complex type I

MHC II: Major histocompatibility complex type II

MLCK: Myosin light chain kinase

MMPs: Matrix metallo proteinases

MNLs: Mesenteric lymph nodes

moDCs: Monocyte derived dentritic cells

MPO: Myeloperoxidase

Muc2: Mucin 2 gene

MUC-2: Mucin-2

MAFF: Mucus Associated Functional Factor

nFcR: MHC-class-I-like Fcgamma receptor

NF-κB: Nuclear factor kappa B

NO: Nitric oxide

NOD: Nuclear organization domain

OTU: operational taxonomic unit

OX40L: OX40 ligand

PAMPs: Pathogen-associated molecular patterns

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate buffered saline

PCoA: Principal coordinate analysis

PD: Phylogenetic diversity

PERK: Protein kinase R (PKR)-like endoplasmic reticulum kinase

PGE_2: Prostaglandin E_2

PI3K: Phosphoinositide 3-kinase

PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

PIgR: Polymeric immunoglobulin receptors

PPs: Peyer Patches

PRRs: Pattern recognition receptors

PSA: Polyssacharide A

PUL: polysaccharide utilization loci

qPCR-MSP: Quantitative real-time methylation-specific PCR

RPMI: Roswell Park Memorial Institute medium

rRNA: Ribosomal ribonucleic acid

RXR-α: Retinoid X receptors alpha

RXR-β: Retinoid X receptors beta

RXR-γ: Retinoid X receptors gamma

SAA: Serum amyloid A

SCFAs: Short-chain fatty acids

sfTSLP: Short isoform or variant 2

SHM: Somatic hypermutation

sIgA: Secretory IgA

siRNA: Small interfering RNA

SLPI: Secretory leukocyte peptidase inhibitor

SLPR: TSLP receptor chain

SRE: Steroid-responsive enteropathy

SSC: Forward scatter

SSU: Small subunit

STAT: Signal transducer and activator of transcription (1, 3, 4 and 5)

T3: Triiodothyronine

T4: Thyroxine

T6SSs: Type VI secretion systems

TACI: Transmembrane activator and calcium-modulating cyclophilin-ligand interactor

TCR: T-cell receptor

TD: T-cell dependent response

TFF-3: Trefoil factor 3

$T_{fh:}$ T follicular helper cells

TGF β: Transforming growth factor beta

TGF-β: Transforming growth factor beta

TGF-β: Transforming growth factor beta

$T_{h:}$ T helper cells

$T_{H1:}$ lymphocyte T helper 1

$T_{H17:}$ T helper 17 cells

$T_{H2:}$ lymphocyte T helper 2

$T_{H2:}$ T helper 2 cells

TI: T-cell independent response

TIMP-1: Tissue inhibitor of metallo proteinase-1

TLR-2: Toll-like receptor 2

TLR-3: Toll-like receptor 3

TLR-8: Toll-like receptor 8

TLR-9: Toll-like receptor 9

TLRs: Toll-like receptors

TNBS: Trinitrobenzene sulfonic acid

TNF: Tumour necrosis factor

TNF-α: Tumour necrosis factor alpha

T_{reg}: T lymphocyte regulatory cells

TSH: Thyroid-stimulating hormone

TSLP: Thymic stromal lymphopoietin

TSLPR KO: $TSLPR^{-/-}$ knockout mice

TSLPR: Thymic stromal lymphopoietin receptor

UC: Ulcerative colitis

VDR: Vitamin D receptor

WGS: Whole-genome shotgun

318   Chapter 1: Literature Review
319
320   1.1. Intestinal microbiota
321
322   The collection of bacteria, archaea and eukaryote present in the gastrointestinal tract is termed the 'gut
323   microbiota'. The gut microbiota profoundly influences mammalian biology and can elicit beneficial and
324   harmful effects on the host (Hooper & Macpherson, 2010). The gastrointestinal microbes perform multiple
325   functions that are vital for the host through different host-microbiota interaction pathways and production of
326   myriad of metabolites that help the host to maintain a homeostatic state. Thus, the gut microbiota must be
327   tightly regulated, as its alteration can drive or exacerbate disease not only in the gastrointestinal tract but
328   also systemically.

329
330   1.1.1 Function
331
332   The gut microbiota is composed of several organisms that form a complex network to exert a wide variety
333   of functions; they provide instructive signals for several aspects of intestinal development including
334   epithelial cell maturation, angiogenesis, lymphocyte development and play a crucial part in the digestion of
335   food (Backhed, Ley, Sonnenburg, Peterson, & Gordon, 2005) (Nicholson *et al.*, 2012).

336

337   The microbiota is also crucial for shaping the gastrointestinal immune system, and intimately interacts with
338   all the arms of the system. Either directly or through the production of a myriad of metabolites; microbes
339   can upregulate or downregulate the immune response, and thus; influence intestinal inflammation. In this
340   review, I will emphasize in the immune functions of the gut microbiota and how it can prevent or induce
341   inflammation and intestinal tissue damage.

342

343   1.1.1.1 Gut microbiota and its interaction with the immune system
344
345   The gut microbiota co-develops with the host immune system from birth and a complete interaction is
346   required to maintain homeostasis, mucosal barrier function and functional maturation of the immune system
347   (Kau et al., 2015). Intestinal homeostasis is achieved by the gut immune system exhibiting a state of hypo-
348   responsiveness against commensals, and active readiness against pathogens (Gutzeit, Magri, & Cerutti,
349   2014). The intestinal microbiota and its associated genome influence the metabolic environment of the
350   intestine, and the activity of both the innate and adaptive immune system (Round & Mazmanian, 2009).

351 Thus, depending on the genera, microbiota can decrease, promote or perpetuate the inflammatory
352 response in the intestine.

353

354 **1.1.1.1.1 Development of the immune system**
355
356 The first months and years of life constitute crucial moments that can influence health later in life (Cox et
357 al., 2014), particularly because microbial colonisation is responsible for educating the immune system and
358 induce tolerance; factors that are disrupted in several diseases (Picchianti-Diamanti, Rosado, & D'Amelio,
359 2017). Studies in mice have demonstrated that events in early microbiome development may have a role in
360 promoting susceptibility to or protection from disease later in life. For example, epidemiological and
361 experimental studies suggest that foetal microbial contact may be casually related to increased risk of
362 disease. The maternal microbial environment and antibiotic exposure during pregnancy are both reportedly
363 associated with a risk of developing asthma later in childhood (Loewen, Monchka, Mahmud, t Jong, &
364 Azad, 2018). Also, it has been reported that children born by caesarean-section have a higher risk for
365 atopic diseases such as allergic rhinitis, asthma and coeliac disease (Bager, Wohlfahrt, & Westergaard,
366 2008).

367

368 After birth, microbiota drives the development of the immune system, including the induction of oral
369 tolerance, maturation of secondary lymphoid organs, and strengthening of the barrier function through the
370 enhancement of the expression of antimicrobial factors by intestinal epithelial cells (IECs). Both
371 gastrointestinal and systemic immune responses are absent or markedly reduced in the absence of
372 commensal microorganisms. Previous studies have shown that dogs raised in germ-free conditions have
373 underdeveloped lymphoid systems and decreased immunoglobulin concentrations. Histologically, the
374 intestine of germ-free dogs is characterized by thinner villi and a reduction in both the lamina propria and
375 mucosal surface area (Cohn & Heneghan, 1991).

376

377 Early responses to microbial ligands such as lipopolysaccharide, the endotoxin found in the outer
378 membrane of gram-negative bacterial walls, condition gut epithelial cells to become hypo-responsive to
379 subsequent Toll-like receptor (TLR) stimulation, helping to promote oral tolerance; an effect that has long-
380 term consequences for host capacity to develop inflammatory diseases such as inflammatory bowel
381 disease (IBD) (Lotz *et al.*, 2006).

382

383   Locally the microbiome exerts different functions based on their location. For example, in the small intestine

384   the gut microbiome regulates enterocyte proliferation in the crypts whereas at the tips of the villi the

385   microbiota regulates the expression of genes involved in metabolic and immune functions. Throughout the

386   whole intestine, goblet cell differentiation and production of the protective mucosal mucus layer are

387   stimulated by commensal organisms (Jandhyala *et al.*, 2015), (Cohn & Heneghan, 1991).

388

389   Recently, Gomez de Aguero *et al* (2016) using a mouse model demonstrated that the gut microbiota not

390   only influences intestinal immune system development directly but also indirectly, through transfer of

391   bacterial metabolites via mother's milk. In their experiment, they colonised germ-free pregnant mothers

392   (from days 4 to 15 days of gestation) with an auxotrophic mutant of *Escherichia coli* that has a deletion of

393   three genes required for synthesis of peptidoglycan, a component of the cell wall. Colonisation with these

394   bacteria generally results in transient and reversible colonisation whereby no bacteria are detectable in the

395   gut 72 hours after administration. Compared to mice born from germ-free mothers, the litters were

396   characterised by a 10-fold more increase of type 3 innate lymphoid cells (ILC3), higher intestinal monocytes

397   belonging to the CD11c$^+$, F4/80$^+$ subset and higher expression of antimicrobial factors such as the Reg3

398   family of C-type lectins and defensin-related proteins. This is despite the mice being born germ-free

399   themselves. They found that bacterial metabolites such as kynurenine (a tryptophan metabolite), act as

400   ligands for the aryl hydrocarbon receptor (AhR), which is an important developmental regulator of the

401   immune system. Thus, bacteria present in the mother's intestine contribute to innate maturation of the

402   neonatal gut even in the absence of intestinal colonisation (Gomez de Aguero *et al.*, 2016) (Rakoff-

403   Nahoum, 2016).

404

405   **1.1.1.2 Colonisation resistance**

406

407   Another very important role of the gut microbiota for intestinal health is known as colonisation resistance.

408   This protective mechanism represses the growth of harmful microorganisms by commensal bacteria.

409   Direct inhibition occur via several pathways including competition for oxygen, nutrients and mucosal

410   adhesion sites, dampening of virulence-related gene expression and production of metabolites

411   (peroxidases, proteases and bacteriocins) that create a physiologically restrictive environment for non-

412   resident bacterial species (Buffie & Pamer, 2013). For example, the bacterium *Bacillus thuringiensis*

413   isolated from the human faeces produces the bacteriocin called thuricin CD, which has potent antimicrobial

414   activity against *Clostridium difficile* as well as *Listeria monocytogenes* but not on other components of the

415   intestinal microbiota (Rea *et al.*, 2010).

416

417   Various Gram-negative bacteria commonly express type VI secretion systems (T6SSs). This system

418   mediates contact-dependent killing by translocating toxic effector proteins into their targets; for example,

419   *Vibrio cholerae* targets *E. coli* for T6SS-dependent killing (Basler, Pilhofer, Henderson, Jensen, &

420   Mekalanos, 2012).

421

422   Indirect inhibition occurs by enhancing host immunity in the intestine. As described above and in

423   subsequent chapters, the gut microbiota stimulate the development and function of the innate and adaptive

424   immune system that help fight pathogenic microorganisms (Buffie & Pamer, 2013).

425

426   ### 1.1.1.3 Pro versus anti-inflammatory signalling

427

428   The competition between induction of productive systemic immunity, with the potential for inflammation and

429   damage to host tissue, or a tolerogenic response seems to be largely determined by the microbial effect on

430   antigen presenting cells and naïve T cells of gut-associated lymphoid tissue. In the absence of signals

431   triggered by microbe-associated molecular patterns, conditioned antigen-presenting cells might induce

432   various subsets of T_{reg} cells, which produce IL-10 and transforming growth factor beta (TGF β) and thus,

433   suppress effector lymphocytes T-helper T_{H1}, T_{H2} and T_{H17} responses, as well as innate

434   immunoinflammatory responses. This phenomenon is frequently called "bystander immune repression"

435   (Manichanh, Borruel, Casellas, & Guarner, 2012).

436

437   Several studies have elucidated the effect of specific bacteria on the regulation of the inflammatory

438   response. For example, *Bacteroides* and *Clostridium* induce the expansion of lymphocyte regulatory T cells

439   (T_{reg}) cells; *Bifidobacterium, Lactobacillus* and *Faecalobacterium* genera induce the down-regulation of

440   inflammatory cytokines and stimulate the production of interleukin-10 (IL-10) (Kostic, Xavier, & Gevers,

441   2014) and the commensal bacterial *Bacteroides fragilis* protects animals from experimental colitis induced

442   by *Helicobacter hepaticus*, through the expression of the microbial molecule polysaccharide A (PSA) which

443   induces the production of IL-10 (Mazmanian, Round, & Kasper, 2008).

444

445   On the other hand, pathogenic microorganisms can cause direct damage to the intestinal mucosa and

446   indirectly through the production of certain metabolites, thereby inducing an inflammatory response. For

447   example, anaerobic bacteria produce nitrate and nitrite that can repress the growth of beneficial bacteria

448   (Spiro, 2007). Intestinal bacteria also produce large amounts of hydrogen sulphide (H_2S), which is capable

449   of supressing mitochondrial metabolism in epithelial cells (Szabo *et al.,* 2014). Commensal bacteria can

450   also act as opportunistic pathogens under certain conditions. For example, *Bacteroides fragilis*, a prominent

451   gram-negative member of the microbiota that closely associates with mucosal surfaces and

452   opportunistically invades intestinal tissues (Belkaid & Hand, 2014)

453

454   Schirmer *et al* (2016), investigated the relationship between inter-individual variation in gut microbial

455   community composition and the inflammatory cytokine response to microbial stimulation in the Human

456   Functional Genomics Project (HFGP). Stool samples were collected from 500 healthy individuals of

457   Western-European genetic background, and metagenomics performed to assess microbial taxonomic and

458   functional profiles. Concurrently, blood samples were taken and peripheral blood mononuclear cells

459   (PBMCs) and whole blood were stimulated under five different microbial stimulations to assess cytokine

460   response. Three stimulations were bacteria-derived (purified *E. coli*-derived lipopolysaccharide [LPS]

461   and *B.fragilis* representing Gram-negative bacteria, and *Staphylococcus aureus* representing Gram-positive

462   bacteria) and two were fungal-derived (*Candida albicans* hyphae and conidia yeast). The cytokine

463   response to the stimulations was compared to the unstimulated state (Roswell Park Memorial

464   Institute medium (RPMI)) and three monocyte-derived cytokines (IL-1β, TNFα, IL-6) and three lymphocyte-

465   derived cytokines (IFNγ, IL-17, IL-22) were measured at different times to capture peak stimulation

466   (Schirmer *et al.,* 2016).The first cytokine response was stimulus-specific, the second one stimulus-

467   independent and microbial faecal composition-specific and the third response was stimulus and gut

468   microbial-dependent.

469

470   In contrast to the unstimulated cells that only exhibited a small degree of variation in cytokine levels, a

471   significant inter-individual variation in cytokine responses and gut microbial profiles under stimulation

472   conditions was observed, with the strongest changes induced by *C. albicans.* This response was

473   compatible with the third pattern of interaction, suggesting that inter-individual variation in cytokine

474   response is linked to specific microbial organisms present in the intestine. A large proportion of the

475   metabolites in the blood originate from the gut and they modulate inflammatory cytokine capacity by the

476   host immune cells that could potentially impact disease susceptibility (Schirmer *et al.,* 2016). Thus, gut
477   microbiota not only affect the immune response locally but systemically.

478

479   **1.1.1.4 Production of metabolites**
480
481   Gut microbiota exert direct effects on the immune system or indirectly through the production of
482   metabolites. Short-chain fatty acids (SCFAs) and products of the metabolism of bile acids are among the
483   most studied metabolites.

484

485   **1.1.1.4.1 Short-chain fatty acids: synthesis and function**
486
487   Microbes in the intestine produce enzymes that help the host to digest complex carbohydrates from the
488   diet, which otherwise would be not absorbed; and ferment endogenous products such as sloughed
489   epithelial cells and mucus (Flint, Scott, Duncan, Louis, & Forano, 2012). The primary end-products of this
490   digestion process are short-chain fatty acids (SCFAs), namely acetate, propionate and butyrate that
491   constitute 60, 25 and 10% of the total volatile fatty acids respectively in canine faecal samples (Sunvold,
492   Hussein, Fahey, Merchen, & Reinhart, 1995). They exert local and systemic effects.

493

494   Locally, in the intestine, SFCAs serve as energy sources for gut epithelial cells, strengthen epithelial cell
495   barriers, modulate intraluminal pH, regulate intestinal motility and have immunomodulatory properties
496   through expansion of T regulatory (T$_{reg}$) cells, modulation of cytokine production and inhibition of neutrophil
497   migration (Wang, Wang, Wang, Wan, & Liu, 2012) (Mathewson *et al.,* 2016). Butyrate has also been
498   proposed to enhance the intestinal barrier function and for exerting anti-cancer properties (Donohoe et al.,
499   2014).

500

501   Systemically, SCFAs play a dual role both as substrates for metabolism and as signalling molecules that
502   regulate the immune system and influence gene expression (den Besten *et al.,* 2013).

503

504   Once produced, SCFAs are metabolised by the liver (approximately 70% of acetate and 30% of
505   propionate), and then serve the host as a source of energy. In adult dogs, SCFAs provide an estimated 5 to
506   7% of metabolic energy.

507

508 In addition, SCFAs act as ligands of the G protein-coupled receptors free fatty acid receptor 2 (FFAR2 also

509 called GPR43) and free fatty acid receptor 3 (FFAR3 also called GPR41), and influence lipid and glucose

510 metabolism (Samuel *et al.,* 2008) (Y. Xiong *et al.,* 2004). Acetate can also serve as a substrate for

511 cholesterol, long-chain fatty acid, and glutamine and glutamate synthesis in the liver. The remainder of

512 acetate is metabolised by other tissues, including adipose tissue (den Besten *et al.,* 2013).

513

514 A recent study showed that SCFAs can alter host histone acetylation and methylation in multiple tissues,

515 and mediate global epigenetic programming. Histone-modifying enzymes control chromatin configuration,

516 making it accessible or non-accessible to factors necessary for gene transcription, replication,

517 recombination and DNA repair. In general, acetylation of histones is associated with active chromatin,

518 whereas methylation is associated with repression. On one hand, SCFAs can be either directly converted

519 (acetate) or oxidized (propionate and butyrate) to acetyl-coenzyme A (acetyl-CoA) (Duncan, Barcenilla,

520 Stewart, Pryde, & Flint, 2002), which is a necessary substrate for histone acetyltransferases (HATs), that

521 are in charge of increasing the acetylation of histones. But, on the other hand, butyrate can also act as a

522 histone deacetylase (HDAC) inhibitor.

523

524 **1.1.1.4.2 Bile Acid metabolism**
525
526 Gut bacteria are also important contributors to bile acid metabolism. Normally, over 95% of the conjugated

527 bile acids (cholic and chenodeoxycolic acid) are retained in the enterohepatic circulation, and those that

528 reach the colon are deconjugated by intestinal bacteria to produce secondary bile acids (lithocolic,

529 deoxycholic and ursodeoxycholic acid). It has been shown that lithocholic and deoxycholic acids can elicit

530 proinflammatory responses through the production of reactive oxygen and nitrogen species, and through

531 nuclear factor kappa B (NF-κB) activation in IECs. As ursodeoxycholic acid has immunomodulatory

532 properties, the ratio of secondary bile acids produced could have an impact in the development and

533 progression of intestinal disease (Sears & Garrett, 2014).

534

535 **1.1.2 Development of the gut microbiota**
536
537 The first step in understanding the symbiotic relationship between gut microbes and their host consist in the

538 characterisation of the baseline healthy microbiota and how the microbiota evolves and is established.

539   Defining what is consider a healthy gut microbiota is crucial to understand what would be the biological

540   significance of the different patterns of microbial colonisation associated with disease.

541

542   Marked age-associated changes in the gut microbiota occur throughout the life of an individual and are

543   driven by several ecological factors including selective pressure, rates of colonisation and rates of

544   extinction (Griffin et al., 2017). During the first years of life, studies in people have demonstrated that gut

545   microbiota possesses low diversity and stability but as the host grows, phylogenetic diversity increases and

546   reaches its maximum complexity and stability in adulthood (Vaishampayan *et al.*, 2010) (Palmer, Bik,

547   DiGiulio, Relman, & Brown, 2007).

548

549   Both internal and external factors contribute to the development and maintenance of the core intestinal

550   microbiota and influence inter-individual microbial variability (Marques *et al.,* 2010). On one hand, the

551   gastrointestinal tract possesses unique physiological and anatomical characteristics (pH, motility, mucus

552   layer, etc.) that make the core microbiota specific for each intestinal region (McConnell, Basit, & Murdan,

553   2008). The small intestine only possesses a thin mucus layer, however as it has high levels of acids,

554   oxygen and antimicrobial substances together with a short transit time, the growth of bacteria is limited and

555   only the rapid growing, facultative anaerobes with the ability to adhere to intestinal layer are capable to

556   survive. In contrast, the colon possesses a double mucus layer and environmental conditions that support a

557   dense and diverse community of bacteria. In general, total bacterial counts, species richness and diversity

558   increase along the gastrointestinal tract (Donaldson, Lee, & Mazmanian, 2016) (Mentula *et al.*, 2005).

559

560   On the other hand, host factors such as genetic background, age, sex, environmental exposure and diet

561   confer unique characteristics in the gut microbiota of every individual, despite the uniform properties at

562   anatomical level (Fragiadakis et al., 2018).

563

564   **1.1.2.1 Development of the gut microbiota in infants**

565

566

567   It is well known that proper development and structure of the immune system is crucial not only during the

568   first stages of life but also in adulthood. Thus, a complete understanding of microbial assembly is the first

569   step not only to determine what is considered normal and healthy for a species, but for also determining

570 strategies for manipulation of the gut microbiota and to guide the formation of health-promoting microbiota

571 (Koenig *et al.,* 2011).

572

573 **1.1.2.1.1 Pre-natal stage**

574

575 Previously, microbial gut colonisation was thought to start immediately after birth as the uterine

576 microenvironment was considered sterile. However recent studies have detected the presence of bacterial

577 DNA in the placenta and the amniotic fluid, suggesting that assembly of the gut microbiota could start

578 prenatally (Aagaard *et al.,* 2014).

579

580 Agaard *et al* (2014) collected placental specimens under sterile conditions from 320 mothers for

581 comparative 16*S* ribosomal DNA–based and whole-genome shotgun (WGS) metagenomic studies. The

582 group consisted of full-term births as well as pre-term births (< 35 weeks gestation). The study found that

583 the placenta harbors a low-abundance but metabolically rich microbiome. The placental microbiome is

584 largely composed of non-pathogenic commensal microbiota from the Firmicutes, Tenericutes,

585 Proteobacteria, Bacteroidetes, and Fusobacteria phyla. Pre-term birth placenta specimens were enriched

586 with taxa such as *Burkholderia;* while, term placental specimens were enriched with taxa of genera

587 *Paenibacillus.* In general, placental microbiome was significantly associated with the interval week of

588 delivery, whereas the week of gestation was closely associated with a specific pattern of microorganisms

589 (Aagaard *et al.,* 2014).

590

591 Furthering this concept, Stout *et al.* (2013) performed histological analysis of 195 placenta specimens and

592 demonstrated gross morphologic evidence of Gram-positive and Gram-negative intracellular bacteria at the

593 level of the basal plate (which comprises the tissue layer directly at and below the maternal-foetal

594 interface). Intracellular bacteria were found in the placental basal plates of 54%

595 spontaneous preterm deliveries <28 weeks, and in 26% of term spontaneous deliveries, with and without

596 clinical or histologic evidence of chorioamnionitis (Stout *et al.,* 2013).

597

598 Collado *et al* (2016) collected maternal faeces, placenta, amniotic fluid, colostrum, meconium and infant

599 faeces from 13 mothers and their full-term babies delivered by elective C-section. Through different

600 methods such as conventional bacterial culture, 16S rRNA gene pyrosequencing, quantitative PCR, and

601 denaturing gradient gel electrophoresis, microbial composition was assessed. Interestingly, bacterial DNA

602   was detected in the placenta and amniotic fluid; both sites were characterised by low richness, low diversity

603   and the predominance of Proteobacteria, particularly species belonging to Enterobacteriaceae family such

604   as *Enterobacter* and *Escherichia/Shigella.* These genera were also present in colostrum, meconium and

605   infant faeces but in lower abundance. *Propionibacterium* was the second most predominant genus present

606   in the amniotic fluid and placenta, and was also detectable in meconium. Other genera present in placenta

607   and amniotic fluid, although in lower abundance compared to other sites, were *Streptococcus,*

608   *Staphylococcus* and *Lactobacillus* (Collado, Rautava, Aakko, Isolauri, & Salminen, 2016).

609

610   Although the presence of bacterial DNA doesn't mean that they are viable and/or exerting an influence in

611   that environment, Onderdonk *et al* (2008) reported viable microbes of the genera *Staphylococci* and

612   *Propionobacteria* in the placenta parenchyma and amniotic fluid during the second trimester of pregnancy

613   (Onderdonk *et al.,* 2008). The origin of this prenatal microbiota is not known. In their study of placental

614   microbiome, Agaard *et al* (2014) integrated the placental microbiome taxonomy profile with those present in

615   other body site niches as reported by the HMP (Human Microbiome Project) such as oral, stool, skin,

616   airway (nasal), and vaginal sites, then calculated the Bray-Curtis distance at phylum-level abundance to

617   reflect the similarity among body sites. The placental microbiota show similarity to the oral phyla but not

618   from potential anatomic contaminants during delivery of the placenta (stool and vagina), derived from both

619   gravid and no gravid subjects (Bray-Curtis index <0.3 oral and placental microbiota), suggesting an oral

620   origin *(Aagaard et al.,* 2014).

621

622   However, studies in pregnant mice using labelled bacteria introduced in the intestine have shown that these

623   bacteria can be detected in the placenta (Jimenez *et al.,* 2005). Moreover, another study also found

624   increased intestinal bacterial translocation during pregnancy and lactation, and that intestinal microbes can

625   be transported to breast and milk through circulating immune cells; suggesting that the origin of intrauterine

626   and milk bacteria may come from the maternal gut (Perez *et al.,* 2007).

627

628   Regarding the origin of the meconium microbiota, animal models have recovered specific bacteria

629   introduced to the gut of pregnant animals in the meconium after sterile caesarean-section and it has been

630   suggested that it comes from the amniotic fluid, which is constantly being swallowed by the foetus

631   (Ardissone *et al.,* 2014).

632

633   Controversy about the presence of microorganisms in the placenta remains. A study by Lauder *et al.* (2016)

634   quantified total 16S rRNA gene copies using quantitative PCR and found that placental samples and

635   negative controls contained low and indistinguishable bacterial copy numbers. Oral and vaginal swab

636   samples, in contrast showed high copy numbers; 16S rRNA gene sequencing found no separation between

637   communities from placental samples and contamination controls (Lauder *et al.,* 2016).

638

639   Despite this latter study, overall it appears as if intestinal colonisation begins *in utero*, and that a transfer of

640   microbes from mother to baby could serve as the initial inoculum of bacteria in the neonatal intestine.

641   However, the significance of the presence of microbes during foetal life in microbial colonisation and how

642   they could impact further development of the intestinal community is still unclear. Some studies have

643   suggested that exposure to microbes during pregnancy may influence the metabolic and immunologic

644   profiles of the pregnant uterus and, hence, the risk of disease developing in the offspring later in life, a

645   theory termed foetal programming hypothesis (Collado et al., 2016) (Abrahamsson, Wu, & Jenmalm, 2015).

646

647   1.1.2.1.2 Post-natal stage

648

649

650   In the first few hours of life, vaginal and faecal microbes present in the birth canal are the most important

651   sources of inoculum in vaginally-delivered babies. In babies delivered by C-section, the skin and the

652   environment constitute the main source of bacteria (Dominguez-Bello *et al.,* 2010). Afterwards, diet plays a

653   predominant role in shaping the microbiome, and cessation of breast-feeding and introduction of solid food

654   are the key determinants that drive the maturation of the gut microbiota into an adult-like phenotype

655   (Backhed *et al.,* 2015).

656

657   1.1.2.1.2.1 Initial colonisation of the gut microbiota

658

659

660   Early studies in infants found that facultative anaerobic bacteria including *Staphylococcus s*pp,

661   *Streptococcus* spp, *E coli* and Enterobacteria are the first colonisers of the gut (Palmer *et al.,* 2007). It is

662   thought that the main purpose of the first wave of bacterial colonisation is to consume oxygen and create a

663   more suitable environment for obligate anaerobes. By one-two weeks of age, faeces are mainly dominated

664   by anaerobes of the genera *Bifidobacteria, Bacteroides, Clostridia* and *Eubacteria* (Mshvildadze & Neu,

665   2010) (Collado, Cernada, Bauerl, Vento, & Perez-Martinez, 2012).

666

667 However, another study found that almost from the beginning, the initial gut microbiota of newborns is

668 dominated by strict anaerobes and not facultative, suggesting that the anaerobic environment is quickly

669 established and gut bacterial colonisation is dictated by highly competitive and specialised gut anaerobic

670 bacteria (Koren *et al.*, 2012). Thus, although the predominance of anaerobic bacteria is rapidly established,

671 the speed could be influenced by other factors during the prenatal (maternal microbiota) and postnatal

672 period (environment).

673

674 **1.1.2.1.2.2 Diversity and functional dynamics of the infant gut microbiota**

675

676 The earlier stages of infant gut microbiota development are characterised by high levels of inter-individual

677 variability and a very uneven distribution of taxa. As infant development progresses, microbial assemblages

678 converge towards an adult-like composition with an increased α-diversity but reduced β-diversity; indicating

679 a more complex and less heterogeneous community (Backhed *et al.*, 2015).

680

681 Palmer *et al.* (2007), performed a time-series analysis using small subunit (SSU) rDNA microarray and

682 SSU rDNA clone library sequencing in 14 healthy, full-term human infants; and collected stool samples,

683 immediately after birth and during defined intervals throughout the first year of life. They also collected

684 vaginal (n=11) and milk samples (n=16), and stool samples from all the mothers (n=13), most of the fathers

685 (n=9), and two siblings (n=2). This study found high interpersonal variation in microbial diversity and in

686 functional gene content, large fluctuations in the abundances of the major bacterial taxonomic groups

687 (except for fraternal twins) and in the pattern of evolution (acquisition, maintenance and disappearance of

688 species) with abrupt shifts intercalated with intervals of relative stability, with no cause established in most

689 of the cases.

690

691 Organisms of the genera *Bacteroides* varied greatly from baby to baby in the timing of their first

692 appearance, but were consistently present by one year of age. Prior to 6 months of age, stool samples

693 tended to cluster by baby, however by one year of age they were more like that of adults but not

694 significantly more like that of their parents than to that of other adults. It was also found that some early

695 samples showed high similarity in bacterial composition to breast milk or vaginal swabs, and that twins

696 were like each other but not to their parents, emphasizing the importance of the environment (Palmer *et al.,*
697 2007).

698

699 Along those lines, a recent study performed in Spain collected faecal samples from 13 infants throughout
700 the first year of life and from the mothers before and one-year after childbirth to characterise phylogenetic
701 composition and gene repertoire of gut microbiota via metagenomics. The researchers evaluated the
702 correlation of the taxonomic composition with the functional development, and how similar the trends were
703 across individuals. The study determined that divided the microbial development is divided into two distinct
704 phases, firstly a decrease in taxonomic richness due to the loss of rare taxa, and then an increase in the
705 number of core genera (Valles *et al.,* 2014).

706

707 Another interesting finding of this study was that although one stage of microbial assemblage did not
708 determine the assemblage of the next one, microbial development followed a similar trend over time toward
709 a more adult-like microbiota, particularly toward mother's microbiota that was not yet completed by one
710 year-age (opposite results compared to the previous study). Results also indicated that the taxonomic
711 composition of the microbiota shapes its functional capacities, and therefore the observed inter-individual
712 variability in taxonomic composition during succession is not fully compensated by functional equivalence
713 amongst bacterial genera. Network analyses suggested that positive interactions among core genera
714 during community assembly could contribute to ensure their permanence within the gut (Valles *et al.,*
715 2014).

716

717 Furthermore, metagenomics analysis of 12 faecal samples identified that the earliest microbiome was
718 enriched in genes facilitating lactate utilisation, and that functional genes involved in plant polysaccharide
719 metabolism were present before the introduction of solid food, suggesting that gut microbiota is ready in
720 advance for processing simple derived plant carbohydrates. In accordance with this, introduction of rice
721 cereal did not cause a change in the microbial profile. However, the introduction of more complex plant
722 derived foods drove the expansion of the genera *Bacteroidetes*, an increase in the production of SCFAs
723 and an enrichment of genes associated with carbohydrate utilisation, vitamin biosynthesis, and xenobiotic
724 degradation (Koenig *et al.,* 2011). Collado *et al,* 2016 also demonstrated that at a functional level, genes in
725 charge of membrane transport, carbohydrate metabolism, amino acid metabolism, replication and repair,
726 energy metabolism were the most predominant pathways in the newborn (Collado *et al.,* 2016).

727

728 Kostic *et al* (2015) examined the gut microbiome of a cohort of 33 infants genetically predisposed to Type 1
729 diabetes at both functional and taxonomic level. This study corroborated the findings that age is the main
730 determinant of taxonomic diversity, whereas metabolic diversity does not exhibit an age trend, suggesting
731 that all metabolic pathways are present from birth. They also observed shared taxonomic trajectories that
732 were consistent across individuals that indicate a general change in abundance and occur at similar time
733 frames. For example, the abundance of Lachnospiraceae and Ruminococcaceae were positively correlated
734 but were inversely correlated with the Enterobacteriaceae and Bifidobacteriaceae; Thus, whereas the latter
735 ones decrease after breastfeeding cessation, the former ones increase. It was also determined that the
736 strain composition within an individual remains essentially constant throughout infancy for almost all
737 individuals (Kostic *et al.,* 2015).

738

739 **1.1.2.1.2.3 Factors that influence gut colonisation**
740
741
742 Several factors influence initial colonisation and early establishment of the infant gut such as maternal
743 microbiota, gestational age, delivery mode, feeding patterns, sanitary conditions and antibiotic
744 administration.

745

746 • Maternal microbiota during pregnancy

747

748 Significant differences in maternal microbiome between the perinatal period and one year after childbirth
749 have been found (Mor & Cardenas, 2010) (Newbern & Freemark, 2011). Maternal faecal microbiota during
750 pregnancy exhibits higher taxonomic richness but lower functional diversity, indicating redundancy; and
751 exhibits substantial inter-individual variation compared to postnatal samples (Koren et al., 2012). The
752 intestinal microbiota shifts between the first and third trimester of pregnancy and follows a unidirectional
753 shift characterised by increase of species such *Faecalibacterium prausnitzii* that facilitates the production of
754 T_{reg} cells. Then, in the third trimester, there is another shift in the gut community structure and every
755 mother acquires her own individual microbiota. However, this period is characterised by an increase in
756 certain taxa such as Enterobacteriaceae, Enterococci, and Streptococci; organisms that dominate in the
757 early days of the infant's life (Koren *et al.,* 2012).

758

759   During late pregnancy, immune, hormonal, physiologic and metabolic changes not only influence maternal
760   gut microbiota but also induce a low grade inflammatory response in the gastrointestinal tract, which could
761   facilitate the transport of gut bacteria to the uterus and foetal gastrointestinal system via systemic
762   circulation (Mor & Cardenas, 2010). In humanised germ-fee mice, the microbiota in the third semester
763   induces more intestinal inflammation and increases energy storage and promotes hyperglycaemia, which
764   could be useful for energy transfer. After delivery, the maternal gut microbiota diversity increases again,
765   especially with organisms of the *taxa Clostridia, Bifidobacteria* and *Bacteroides*, that will be present in the
766   child as well. Interestingly, the gut microbiota of the baby resembles the gut microbiota present in the first
767   trimester of pregnancy (Koren et al., 2012).
768
769   • Gestational Age
770
771   Gestational age has a profound role in determining the gut microbiota in newborn infants, especially those
772   prematurely born, who are hospitalised and therefore separated from their mothers for long periods
773   immediately after birth (La Rosa *et al.*, 2014). They are at higher risk of developing disease (especially
774   necrotizing enterocolitis), possess increased occurrence of potential pathogens and have higher rates of
775   drug treatment. The intestinal microbiota of pre-term infants is characterised by high inter-individual
776   variability and reduced microbial diversity, and the environment becomes the main source of colonising
777   bacteria. One study has found that *Leptotrichia* spp and other related bacterial species are detected in the
778   amniotic fluid of women in pre-term labor with a strong dose-dependent relationship between bacterial
779   abundance in the amniotic fluid and gestational age at delivery (DiGiulio, 2012).
780
781   • Delivery mode
782
783   Dominguez-Bello *et al* (2010) characterised the microbiota from nine mothers and their newborn babies,
784   four born vaginally and six born via C-section (male dizygotic twins). The mothers' skin (ventral forearms),
785   oral mucosa, and vagina were sampled 1 hour before delivery, and their babies' skin (before removing the
786   vernix caseosa), oral mucosa, and nasopharyngeal aspirate were sampled <5 min, and meconium <24 h,
787   after delivery. The variable region 2 (V2) of the bacterial 16S rRNA gene was PCR-amplified and
788   multiplexed 16S rRNA gene pyrosequencing was performed (Dominguez-Bello *et al.*, 2010).
789

790   In contrast to their mothers, who harboured bacterial communities distinctive to body location, the
791   newborns had bacterial communities that were undifferentiated across multiple body sites, regardless of
792   delivery mode. This suggests that in its earliest stage of community development, the human microbiota is
793   homogeneously distributed across the body. Moreover, vaginally delivered infants acquired bacterial
794   communities resembling their own mother's vaginal microbiota, dominated *by Lactobacillus, Prevotella,* or
795   *Sneathia* spp., and C-section infants harboured bacterial communities like those found on the skin surface,
796   dominated by *Staphylococcus, Corynebacterium,* and *Propionibacterium* spp. (72% of vaginally-delivered
797   infants' gut microbiota resembles that of their mothers' faecal microbiota, compared to only 41% of C-
798   section babies). This study showed that the primary determinant of a newborn's bacterial community
799   composition was his or her mode of delivery (Dominguez-Bello *et al.,* 2010).

800

801   Recently, the same group of researchers could partially replenish the microbiota of babies born by C-
802   section and make it more like the microbiota of those born vaginally through the exposure to maternal
803   vaginal fluids at birth (vaginal seed). Similarly, in vaginally delivered babies the gut, oral and skin bacterial
804   communities during the first 30 d of life was enriched in vaginal bacteria, which were underrepresented in
805   unexposed C-section-delivered infants (Dominguez-Bello *et al.,* 2016).

806

807   C-section influences the development of the immune system and sucession of the gut microbiota in several
808   ways: on one hand, if labor is lacking, the hormonal and immune responses activated at this period, will not
809   occur, affecting the immune environment of the neonate. Intra-uterine concentrations of inflammatory
810   cytokines are elevated during labor, and are tought to be responsible for the activation of the fetal immune
811   system during delivery. On the other hand, the lack of exposure to the vaginal and gut microbiotas from the
812   mother, will influence the type and diversity of bacteria that in turn, differentially will affect the colonization
813   of other bacteria (Francino, 2018).

814

815   Bäckhed *et al.* (2015) studied the functional characteristics of the gut microbiota in 98 full-term Swedish
816   infants and their mothers through metagenomics shotgun sequencing on faecal samples during the first
817   year of life. They found that the mode of delivery strongly affected microbiome species in neonates.
818   Compared with vaginally born infants, the faecal microbiome of C-section infants was enriched in
819   MetaOTUs such as *Enterobacter hormaechei/E. cancerogenus, Haemophilus*
820   *parainfluenzae/H.aegyptius/H. influenzae/H. haemolyticus, Staphylococcus saprophyticus/S.*

821   *lugdunensis/S. aureus, Streptococcus australis* and *Veillonella dispar/V. parvula*, indicating that skin and

822   oral microbes, bacteria from the surrounding environment during delivery, were the first colonisers in these

823   infants. In contrast, the gut microbiota of vaginally delivered newborns were enriched in microbes from the

824   genera *Bacteroides, Bifidobacterium, Parabacteroides, Escherichia/Shigella* (p <0.05).

825

826   However, differences in time of sampling could be a factor that contributes to these differences. Newborns

827   with *Escherichia/Shigella* as the most abundant genus were sampled earlier than those dominated

828   by *Bacteroides* or *Bifidobacterium. Escherichia* DNA is highly abundant in the meconium and the placenta,

829   but its amount decreases rapidly over time. Thus, by the time the babies dominated with

830   *Bacteroides/Bifidobacterium* were sampled, the abundance of *Escherichia/Shigella* could have decreased.

831

832   Although the differences in microbiome between C-section and vaginally delivered babies lessen in the first

833   year, the gut microbiota of C-section born infants remained more heterogeneous compared to vaginally

834   delivered infants (Backhed *et al.,* 2015).

835

836   •   Antibiotic treatment

837

838   Studies have shown that antibiotic treatment to the mother at birth influenced the pace of microbial

839   colonisation, but not the pattern of maturation (Cox *et al.,* 2014).

840

841   Koenig *et al (*2010) followed an infant for 2.5 years, to investigate how life events impact the assembly of

842   the gut microbiota. The infant was a full-term, vaginally delivered healthy male. A total of 60 faecal samples

843   were collected along with a detailed record regarding diet, health status and general activities. The infant

844   suffered from several ear infections for which he was treated with antibiotics, but was otherwise healthy.

845   The child received three antibiotic treatments during the study period. Two out of three treatments caused

846   major shifts in the gut microbiota composition. However, the second treatment with amoxicillin did not affect

847   the microbial pattern, which could indicate adaption of the gut microbiota to repetitive exposure (Koenig *et*

848   *al.,* 2011).

849

850   Bäckhed *et al.* (2015), assessed the pool of antibiotic resistance genes, known as the resistome; and found

851   that newborns already have a big pool of genes involved in resistance against bacitracin, tetracycline and

852   macrolides, possibly a consequence of the relative high abundance of Proteobacteria (Backhed *et al.,*
853   2015)

854

855   •   Diet

856

857   Overall, dietary and environmental changes constitute major drivers of gut microbiota maturation *(Koenig et*
858   *al.,* 2011).

859

860   Mammary glands and milk contain live bacteria, mainly *Bifidobacterium* spp. and *Lactobacillus* spp. that
861   could be a source of intestinal colonisation after birth. It is believed that milk is the main source of
862   *Bifidobacteria*. The most common *Bifidobacteria* present in infant faeces are *B. longum*, *B. infantis* and *B.*
863   *breve* (Roger, Costabile, Holland, Hoyles, & McCartney, 2010).

864

865   *Bifidobacteria* are important anaerobes for general health. It has been shown that reduced numbers of
866   *Bifidobacteria* or aberrant species of *Bifidobacteria* have been associated with later development of
867   immunological and inflammatory disorders and even obesity (Kalliomaki, Collado, Salminen, & Isolauri,
868   2008). Also, many strains of Bifidobacterium group are considered probiotics and they have been
869   associated with reduced incidence of serious postnatal complications in pre-term infants such as
870   necrotising enterocolitis (Aceti *et al.,* 2015; Grzeskowiak *et al.,* 2015).

871

872   Breast feeding also provides several bioactive substances such as oligosaccharides, lactoferrin and
873   lysozymes that inhibit the growth of many bacteria, and block the attachment of these microorganisms to
874   the intestinal mucosa. Lactoferrin also stimulates cytokine production, natural killer cell and macrophage
875   activity (Frese & Mills, 2015). Milk is also rich in glycans and drives the enrichment of specific functional
876   pathways needed to consume these glycans in breast-fed infants (Pacheco, Barile, Underwood, & Mills,
877   2015). Thus, milk acts not only as a prebiotic (providing nutrients and growth factors), but also as a
878   probiotic (providing microbes and modulate their population by regulating the immune system and host-
879   microbe interactions) (Pacheco *et al.,* 2015).

880

881   During the breastfeeding period, starts the colonization with Actinobacteria and Firmicutes [32];
882   actinobacteria are represented mainly by *Bifidobacterium (B.), B. breve, B. longum, B. dentium, B. infantis,*

883 *B. Pseudocatenulatum and Firmicutes by Lactobacillus, Enterococcus and Clostridia* (Harmsen et al.,
884 2000).

885

886 In line with the concept of milk as a synbiotic, studies have shown that exclusively breastfed infants exhibit
887 significant differences in gut microbiota compared with formula-fed infants. Breast-fed babies have a higher
888 amount of *Bifidobacterium* and other microbes such as *Staphylococcus, Streptococcus, Corynebacterium,*
889 *Lactobacillus, Micrococcus* and *Propionibacterium* that originates from the nipple, milk ducts and the
890 surrounding skin (Martin *et al.,* 2007). In contrast, the microbiome of formula-fed infants is characterised by
891 the predominance of facultative anaerobes such as *Bacteroides* and *Clostridium* followed by
892 *Staphylococcus, Streptococcus* and *Enterobacteriaceae* (Roger *et al.,* 2010). Although *Bifidobacterium* is
893 also present, its colonisation is delayed. In general, the gut microbiota of formula-fed infants is more
894 complex and like that of adults. Therefore, the changes in microbiota composition during weaning are more
895 drastic in breast-fed than in formula-fed infants.

896

897 Likewise, Backed *et al* found that formula-fed infants had higher populations
898 of *Clostridium, Franulicatella, Citrobacter, Enterobacter,* and *Bilophila* relative to breast-fed infants and,
899 functionally, these infants hosted higher proportions of antibiotic resistance genes, especially from *γ-*
900 *Proteobacteria* (Backhed *et al.,* 2015). The source of these bacteria is also unknown but some studies have
901 suggested gut, skin and oral origin. The transfer of gut bacteria could be through a route called entero-
902 mammary pathway via the mucosa-associated lymphoid system, via endocytosis due to increase of gut
903 permeability and the influence of the enteric nerve system that affects the sampling of bacteria in the gut
904 and their transfer to Peyer's patches (Rodriguez, 2014).

905

906 After weaning and introduction of solid foods, there is a significant diet-related shift in the gut microbiome
907 profiles. Weaning promotes higher levels of Bacteroidetes and Firmicutes as well as shifting to functional
908 genes characteristic of an adult microbiome. From 18 to 36 months, the infant gut microbiome undergoes
909 its final significant shift, attributed to the continued influence of a varied solid food diet and greater
910 environmental exposure. By the age of 2-3 years, the intestinal microbiota reaches a stable population like
911 that of an adult. The earlier that solid food is introduced into the diet, the more quickly the gut microbiome
912 begins to resemble to an adult microbiome. However maternal illness, milk and diet quality can cause

913   malnutrition that is associated with severe dysbiosis and persistent gut microbial immaturity which remains

914   refractory to treatment (Palmer e*t al.,* 2007).

915

916   • Other environmental factors

917

918   Other environmental factors such as geographic location, number of family members (Strachan, 1989) and

919   the presence and contact with animals (Penders *et al.,* 2006) (Dicksved *et al.,* 2007)   influence the

920   assembly and composition of gut microbiota. For example, children living in developing countries exhibit a

921   different profile compared to children that are born in developed-countries (Grzeskowiak *et al.,* 2012).

922   Sanitary conditions as well as higher exposure to infectious agents, parasites, different dietary diets and in

923   some instances, inadequate nutrition may contribute to this (Kau *et al.,* 2015).

924

925   In Europe, northern infants show higher levels of *Bifidobacteria*, *Atopobium, C. perfringens, C. difficile* while

926   Southern infants presented higher proportion of *Bacteroides*, *Eubacteria* and *Lactobacillus* (Fallani *et al.,*

927   2010). Higher counts of lactobacilli, eubacteria and enterococci have been described in Estonian infants

928   while Swedish infants showed high numbers of clostridia and bacteroides (Sepp *et al.,* 1997).It has also

929   been reported that *Bifidobacteria* genus, *Bacteroides-Prevotella* and *Cl. histolyticum* levels were higher in

930   Malawi infants than Finnish infants at age of 6 months (Grzeskowiak *et al.,* 2012).

931

932   Although one study found that infants living on a farm had a low diversity and a pronounced dominance of

933   some *Clostridium* and *Eubacterium* species (Dicksved et al., 2007) , another study found more diversity in

934   children living with pets (Song *et al.,* 2013) (Gupta, 2017). Further complicating the potential factors, one

935   study found that single children tend to have lower counts *of* bifidobacteria in the gut at one month of age,

936   non-*E. coli,* enterobacteria and clostridia, and a lower ratio of anaerobic to facultative bacteria by one year

937   of age compared to children living with siblings (Penders *et al.,* 2006).

938

939   It is worth to highlighting the fact that studies have been done using different methodologies, with different

940   environmental conditions and levels of exposure that could cause discordance in the results among

941   studies. Also, there are many confounding factors that can have an influence in the results. For example,

942   mothers consuming a high-fat diet during pregnancy are more likely to deliver via c-section but the diet

943   itself can also affect the gut microbiota; and babies that are born via c-section, spend more time at hospital

944   which can predispose them to higher exposure to hospital microbiota (Francavilla, Cristofori, Tripaldi, &

945   Indrio, 2018).

946

947   **1.1.2.2 Microbiota development in puppies**

948

949

950   In dogs, few studies have characterised the development of the gut microbiome. In general, it has been

951   found that every segment of the gastrointestinal tract harbours its unique type of bacteria, that diversity

952   increases over time and that diet constitute one of the key determinants in the assembly of the intestinal

953   microbiota.

954

955   However, it is well known that the developing gut microbiota changes continuously as the individual is

956   exposed to more environmental challenges and thus; key aspects of the microbial development could have

957   been missed by long intervals of sampling.

958

959   Buddington *et al.* (2003) described postnatal changes in ninety-five puppies representing 15 litters and the

960   15 dams of those litters. Populations of bacteria in the stomach, small intestine, and colon were evaluated

961   via cell culture of mucosal and luminal samples obtained from 6 groups of dogs determined by big changes

962   in diet parameters (unsuckled neonates within 1 hour after birth [n =14], 1-day-old neonates after initial

963   suckling [n=20], puppies at 21 days of age during suckling [n =19], puppies at 42 days of age during

964   feeding of a weaning diet in combination with suckling [n =21], puppies at 63 days of age that were 1 week

965   after weaning [n =21], and adult females (Buddington, 2003). Four specific groups of bacteria were studied:

966   *Clostridium* spp., *Lactobacillus* spp., *Bacteroides* spp. and enteric bacteria.

967

968   This study found that the intestine of neonatal dogs is rapidly colonised, apparently by bacteria in the birth

969   canal and the surrounding environment. By 24 hours after birth, numbers of bacteria in the various regions

970   of the GIT were comparable to those of the adult female Beagles. Moreover, weaning was the most

971   important factor associated with large-scale changes in bacteria of the GIT of dogs. In the stomach, the

972   numbers of anaerobic bacteria were significantly higher at day 1, compared with values at day 21. In the

973   small intestine, the highest numbers of aerobic and anaerobic bacteria in luminal contents of the small

974   intestine were detected at day 1. Thereafter, values were significantly lower and did not vary among older

975   puppies and adult dogs. The number of clostridia in the lumen of the small intestine was highest at days 1

976 and 21, with the lowest values at day 42. A significant effect of age was not detected for the number of

977 *lactobacilli*. The number of *bacteroides* increased significantly between days 1 and 63, but values for adult

978 dogs were lower and not different from those of puppies at day 1. The most noticeable changes in the

979 bacterial populations in the stomach and small intestine were detected between days 1 and 21, a period

980 when there are increases in secretion of pepsin from the stomach and digestive enzymes from the

981 pancreas of dogs (Buddington, 2003).

982

983 In the proximal colon, the numbers of luminal anaerobic and aerobic bacteria did not change between day 1

984 and 21. Although numbers of anaerobic and aerobic bacteria decreased after day 21, the rate and

985 magnitude of the decrease were greater for the aerobic bacteria. In the distal colon, no significant effects

986 were observed in the total number of bacteria, but there was a decrease over time of aerobic bacteria. In

987 general, significant effects of age were detected for each of the 4 specific groups of bacteria studied. In the

988 colon, numbers of enteric bacteria and clostridia decreased but not until after day 21, whereas the numbers

989 *of Lactobacilli* increased > 100- fold between days 1 and 21, with the highest numbers at day 63. Numbers

990 of *Bacteroides* increased approximately 1,000-fold between days 1 and 21 with no additional changes

991 thereafter (Buddington, 2003). This study relied on culture methodology, and therefore may have missed

992 many bacterial families. In addition, the sample intervals may have missed shifts or changes in the bacterial

993 composition.

994

995 Suchodolski *et al*, (2013), using 16S rRNA gene 454-pyrosequencing and quantitative PCR assays,

996 followed and characterised the gut microbiota of twelve colony-housed research puppies from three

997 different litters. Faecal samples were collected from each puppy at five different time points during periods

998 of nursing (N; < 2 days, 5–7 days, and 19–21 days after birth), weaning (W; 30–35 days after birth), and

999 post-weaning (PW; 9 to 13 weeks after birth). Within 2 days of birth, all puppies had established a complex

1000 microbiome, which was not significantly different in the abundance of total bacteria (p = 0.196), but differed

1001 in composition compared to later time points. In general, aerobic and facultative anaerobic bacteria (e.g.,

1002 Proteobacteria) were most abundant during the N period, while obligate anaerobic bacteria were more

1003 abundant in the PW period. *Escherichia* spp. were significantly increased in the N period compared to PW

1004 (median % of sequences; N = 20.0%; PW = 0.4%; p < 0.001). Bacteroidia (N = 0.5%; PW = 12.8%; p =

1005 0.024) and Firmicutes (N = 44.7%; PW = 70.8%; p = 0.002) were significantly more abundant in the PW

period. Important genera such as *Faecalibacterium*, *Lactobacillus*, and *Bifidobacterium*, were also significantly increased during PW compared to N (p < 0.001 for all) (Suchodolski JS, 2013).

Finally, Guard *et al.* (2017) collected faeces from 30 puppies of ten different breeds at 2, 21, 42, and 56 days after birth and from mothers (n=16) at a single time point within 24 hours after parturition. DNA was extracted and 454-pyrosequencing was performed. The study found that 5 out of 8 phyla, 12 out of 15 classes, 18 out of 23 orders, 27 out of 52 families, 48 out of 109 genera, and 56 out of 137 species were significantly different between time points. At the phylum level, the percent of sequences belonging to Firmicutes dominated the faecal microbiota at 2 days of age, and Bacteroidetes increased over time, reaching approximately 37% of all sequences. Fusobacteria and Proteobacteria were other prominent members of the faecal microbiota comprising approximately 16% and 11%, respectively, of sequences in all time points (Guard *et al.*, 2017).

Clostridium (belonging to the phylum Firmicutes) accounted for almost 10% of sequences identified in puppies at day 2 compared to approximately 1% of sequences identified at each time point thereafter. Other members of the family Clostridiaceae were also found to be prominent at day 2 and then decreased over time. Species richness increased significantly over time, and by 56 days of age, the faecal microbiota was dominated by Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria.

The dams harboured Bacteroidetes, Firmicutes, Fusobacteria, Actinobacteria, and Proteobacteria. The microbial communities belonging to dams clustered separately from that of puppies at any given time point, with a significant difference in microbial communities detected between large and small breed puppies at day 42; this is likely due to diet, as small breeds were still consuming milk at that time. Significant shifts were observed in microbial communities belonging to puppies during pre-weaning development (Guard *et al.*, 2017).

Inferred metagenomic analysis performed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) identified an increased presence of genes belonging to cellular motility, cellular processes, and ABC transporters at 2 days of age compared to day 56 in puppies. These genes are related to cellular components that stimulate the development of the innate immune system and development of the gut epithelium integrity. In puppies of 56 day-old, there is an increase in the

1037   genes related to metabolism that have been shown to be beneficial for the generation and maintenance of
1038   the immune system (Guard *et al.*, 2017).

1039

1040   **1.1.3 Gut microbiota in adulthood**
1041
1042

1043   The gut microbiota reaches its maximum complexity and phylogenetic diversity during adulthood, and it
1044   also reaches maximal stability, a phenomenon termed resilience.

1045

1046   **1.1.3.1 Gut microbiota in adult people**
1047
1048

1049   The same ecological factors that govern the gut microbiota in infant, govern the microbial patterns in adults.
1050   Selective pressures such as disease, geographical location, drugs and diet composition can alter the
1051   microbiome structure. For example, subjects sampled in the USA display reduced alpha-diversity and
1052   greater beta-diversity compared to subjects from Papua New Guinea (Martinez *et al.*, 2015). Highly variable
1053   selection pressures, particularly diet, may play major roles in geographical diversity. Western diets are
1054   associated with lower taxonomic and functional diversity, which may be partially restored by dietary
1055   interventions involving calorie restriction and addition of more vegetables into diet (Albenberg & Wu, 2014).

1056

1057   Combined data from the MetaHit and the Human Microbiome Project have provided the most
1058   comprehensive view of the human-associated microbial repertoire to date. Initially, the analysis of 33
1059   samples from different nationalities suggested the presence of three enterotypes according to the most
1060   prevalent genera: enterotype 1 dominated by Bacteroides, enterotype 2 dominated by Prevotella and
1061   enterotype 3 dominated by Ruminococcus (Arumugam *et al.*, 2011). However, several studies have shown
1062   that rather than discrete enterotypes, there are continuous gradients of dominant taxa, whereby an
1063   individual's enterotype can be highly variable and continuously change over time (Koren *et al.*, 2013).

1064

1065   An extensive catalogue of the functional capacity of the human gut microbiome was recently obtained,
1066   where 9.879,896 genes were identified through a combination of 249 newly sequenced and 1018 published
1067   samples (J. Li *et al.*, 2014). The study identified the presence of country-specific microbial signatures,
1068   suggesting that gut microbiota composition is shaped by environmental factors, such as diet, and possibly
1069   also by host genetics (J. Li *et al.*, 2014) (Thursby & Juge, 2017). Additionally, studies in people have shown

1070 that gut microbiota varies from individual to individual, over time and even between mucosa, luminal

1071 contents and faeces (Parthasarathy *et al.*, 2016).

1072

1073 Recent experiments in mice colonised with a diverse specific pathogen-free microbiota showed that

1074 bacterial species present in the lumen of the large intestine differed from the ones found in the mucus layer.

1075 The colon is composed of a double-layer of mucus that mainly contains a glycoprotein termed mucin

1076 secreted by the goblet cells. The inner layer is considered nearly sterile, whereas the outer mucus layer

1077 forms a unique microbial niche of different communities including bacteria without specialised mucolytic

1078 capability *(H. Li et al.*, 2015). In contrast, the species present in the intestinal lumen, have highly available

1079 resources of iron and epithelial-derived carbon that facilitate the growth of certain bacteria. The abundance

1080 of Bacteroidetes appears to be higher in faecal/luminal samples than in the mucosa, whilst Firmicutes,

1081 specifically *Clostridium* cluster XIVa, are enriched in the mucus layer (Van den Abbeele et al., 2013).

1082 Importantly, however, the microbiota of different colorectal mucosal regions within the same individual is

1083 spatially conserved in terms of both composition and diversity, even during periods of localised

1084 inflammation (Eckburg et al., 2005) (Lavelle et al., 2015).

1085

1086 **1.1.3.1.1 Stability of the human adult gut microbiome**
1087
1088

1089 Costello *et al* (2009) sampled different body sites in healthy adults on four occasions, two months apart and

1090 two days in a row each month. The V2 region of the 16S rRNA gene was amplified and pyrosequencing

1091 was performed. They observed that gut community structure was highly variable among people, but

1092 exhibited minimal variability within an individual over time (Costello *et al.*, 2009).

1093

1094 Furthermore, the human microbiome project consortium (2012) collected samples of 131 individuals twice

1095 (mean 219 sd. 69 days after first sampling, range 35–404 days) to evaluate within-subject stability of the

1096 microbiome. 16S profiling revealed that within-subject variation over time was consistently lower than

1097 between-subject variation (2012).

1098

1099 A study by Faith *et al.* (2013) followed changes in the microbiome of 37 adults over 5 years; 33 of these

1100 subjects were sampled 2–13 times up to 296 weeks apart. The remaining 4 individuals were sampled on

1101 average every 16 days for up to 32 weeks while consuming a liquid diet as part of a controlled in-patient

1102   weight-loss study. Amplicons of the V1V2 region of the 16S rRNA gene were amplified using an Illumina

1103   HiSeq2000 instrument. Using the Jaccard index, each individual's microbiota at a given time point was

1104   most like their own at other time points (Jaccard Index 0.82±0.022), followed by their family members

1105   (Jaccard Index 0.38±0.020), and then unrelated individuals (Jaccard Index 0.30±0.005). Moreover, more

1106   strains were shared between closer time intervals than with long intervals. On average, any two unrelated

1107   individuals share ~30% of strains in their microbiota. However, it is possible that unrelated individuals on

1108   average share no strains in their microbiota and this 30% represents the lower resolving limit of 16S rRNA

1109   amplicon sequencing. At an individual level, ˜60-70 % of the present bacterial strains remained unchanged

1110   over the course of the study and the most stable members of the microbiome tended to be the most

1111   abundant. At a phyla level, Bacteroidetes and Actinobacteria populations were less susceptible to

1112   perturbations whereas Firmicutes and Proteobacteria were significantly less stable (Faith *et al.,* 2013).

1113

1114   Another study repeatedly analysed the faecal microbial composition of five unrelated and healthy subjects

1115   over a period of 8 to 12 years using the human intestinal tract chip (HITChip), a comprehensive

1116   phylogenetic microarray that facilitates profiling and semi-quantitative analysis of over 1000 representative

1117   intestinal phylotypes. The results revealed that although the microbiota preserved subject-specific patterns

1118   over time; the similarity of the microbiota profile declined over time. Samples were hierarchically clustered

1119   and resulted in subject-specific grouping of samples and it seems that each person harbours a subject-

1120   specific core. This core consists of genera that include: *Allistipes, Bifidobacterium, Bacteroides,*

1121   *Faecalibacterium, Blautia, Dorea* and *Ruminoccoccus.* Interestingly, the use of antibiotics, change of diet

1122   and travelling had a limited impact on bacterial composition. There was more fluctuation in the number of

1123   bacteria at the phylum-level than at the genus-level (Rajilic-Stojanovic, Heilig, Tims, Zoetendal, & de Vos,

1124   2012).

1125

1126   Data from Rajilic-Stojanovic *et al.* suggests that larger fluctuations occur between samples taken at longer

1127   intervals while Faith *et al.* report the opposite trend, with larger fluctuations occurring in samples taken over

1128   shorter periods of time compared to those that are temporally farther apart. In fact, they found that weight

1129   stability of an individual was a better predictor of the faecal microbiome stability than time between sample

1130   collections (Faith *et al.,* 2013) (Rajilic-Stojanovic *et al.,* 2012). Further complicating interpretation of

1131   microbiome shifts, a study performed in two healthy people, sampled daily for 15 months and 6 months

1132 respectively, showed that there are permanent fluctuations in the composition of the faecal microbiota over
1133 time (Caporaso *et al.*, 2011).

1134

1135 In the study made by Caporaso *et al.*, the variable region 4 (V4) of 16S rRNA gene was amplified by PCR
1136 and subjected to multiplex sequencing on an Illumina Genome Analyzer IIx. They found a pronounced
1137 variability in an individual's microbiota across months, weeks and even days. Additionally, although many
1138 OTUs remained for an extended period, only a small fraction of the total taxa were present across all time
1139 points. This suggests that no core temporal microbiome exists at high abundance, and that bacteria are
1140 persistent but not permanent community members (Caporaso *et al.*, 2011).

1141

1142 In general, it seems that the proportion of incidental colonisers seems larger if only two samples per subject
1143 are compared, than if all analysed samples are considered. Therefore, the assessment of similarity
1144 between only two samples from a subject underestimates the component of subject-specific microbiota,
1145 since some of the core microbes may be below the detection limit of an applied technology *(Rajilic-*
1146 *Stojanovic et al.,* 2012). Fortunately, even though there are great fluctuations in the proportional
1147 representation of microbial lineages, the gene content tends to remain stable, and can give a better
1148 estimation of the metabolic functions of the microbiota at each time point. Bacterial functional redundancy
1149 provides an evolutionary advantage, as the loss of one species doesn't impact the function of the gut
1150 microbiota. Thus, community structure is better described in terms of functional diversity rather than
1151 taxonomic diversity alone (Weinstock, 2011).

1152

1153 1.**1.3.1.2 Gut microbiota in the elderly**
1154
1155
1156 As people age, the stability and diversity of their gut microbiota declines with the state of their health. Aging
1157 is associated with physiological changes in the gastrointestinal tract, as well as changes in dietary pattern
1158 and immune function. The imbalance between pro-inflammatory and anti-inflammatory molecules results in
1159 a low-grade chronic systemic inflammation known as "inflammaging". The main changes in the immune
1160 system during aging are reduced humoral responses, decreases in dendritic cell (DC) efficiency to activate
1161 T and B cells populations, decline in the generation of naïve T and B cells and natural killer activity.
1162 Additionally, aging is characterised by a persistent activation of innate immune mediate by NF- κB
1163 (Claesson *et al.*, 2011).

1164

1165 The main age-related intestinal microbiome changes are a reduction in species diversity and less

1166 resistance to environmental challenges. However, if elderly adults maintain their health status, the microbial

1167 composition often retains the stability and compositional diversity as a healthy younger adult, although with

1168 some unusual phylum proportions (Biagi, Candela, Fairweather-Tait, Franceschi, & Brigidi, 2012).

1169

1170 The ELDERMET consortium was established in 2007 to investigate the role of the intestinal microbiota in

1171 elderly Irish subjects as an agent and indicator of health. They reported the data for the microbiota

1172 composition of 161 elderly subjects, 26 of which were also sampled 3 months later. Amplification of the V4

1173 region of the 16S rRNA gene and analysis of β-Diversity using Unifrac analysis could clearly separate all

1174 samples by subject; and fine-detail analysis of genus confirmed individual-specific microbiota

1175 configurations. Temporally paired samples from elderly subjects were also more similar than randomly

1176 compared samples from different subjects. The core microbiota of elderly subjects exhibited a greater

1177 proportion of *Bacteroides* spp. compared to younger adults. In 68% of the individuals, the microbiota was

1178 dominated by phylum Bacteroides, whilst the phylum Firmicutes had an average proportion of 40%.

1179 Additionally, the *Clostridium* cluster IV was prevalent in elderly people, in contrast with younger subjects

1180 where cluster XIVa is more prevalent (Claesson *et al.*, 2011). This has been suggested to be implicated in

1181 nutrition and increased susceptibility to inflammatory diseases or it could reflect the body changes

1182 associated with aging.

1183

1184 Biagi *et al.* (2010) investigated the age-related differences in the gut microbiota composition among young

1185 adults (20–40 years old), elderly (60–80 years old) and centenarians using the Human Intestinal Tract Chip

1186 (HITChip) and quantitative PCR of 16S rRNA gene. Overall structure of the gut microbiota was very similar

1187 between young and elderly adults, with Bacteroidetes and Firmicutes being the most dominant phyla,

1188 contributing for approximately 95% of the total gut microbiota. In centenarians, although these two phyla

1189 are still dominant; there is enrichment with Proteobacteria. The centenarian microbiota also exhibited

1190 group-specific differences such as an increase in the abundance of facultative anaerobes (e.g. *Escherichia*

1191 *coli*) and rearrangement of the profile of butyrate producers. These included *Ruminococcus obeum et rel.,*

1192 *Roseburia intestinalis et rel., E. ventriosum et rel., E. rectale et rel., E. hallii et rel.* (all

1193 belonging *Clostridium* cluster XIVa), and *Papillibacter cinnamovorans et rel.,* and *F. prausnitzii et*

1194 *rel.* (*Clostridium* cluster IV). Conversely, the butyrate producers *Anaerotruncus colihominis et*

1195 *rel.* (*Clostridium* cluster IV), and *Eubacterium limosum et rel. (Clostridium* cluster XV) increased in
1196 centenarians (Biagi *et al.,* 2010). These changes have been implicated in an increase in the inflammatory
1197 status as the proportion of pathobionts increase and the butyrate production decreases (Biagi *et al.,* 2010).

1198

1199 Aging is associated with changes in physiology and lifestyle. Teeth loss and altered taste and smell can
1200 affect the dietary habits, with a decrease in the consumption of fibre-rich foods; also the intestinal motility is
1201 diminished and the intestinal permeability is increased. All these factors, can influence gut microbiota
1202 composition (Biagi *et al.,* 2017).

1203

1204 To summarise, the intestinal microbiota of individuals is composed of subject-specific species, and the
1205 relative proportions between the microbiota constituents change because of the ecosystem adaptation to
1206 different environmental factors. As much as a dysbiosis can cause disease, a healthy microbial community
1207 is also vital to maintain optimal wellness. Thus, it is essential to understand the factors that shape and alter
1208 the microbiome throughout the lifespan of an individual.

1209

1210 **1.1.4 Gut Microbiota in adult dogs**
1211
1212
1213 In dogs, studies have been made mostly at a single time point. In these studies, the major individual
1214 differences have been found on a bacterial species and strain level between individual animals (Ley *et al.,*
1215 2008). These studies also have shown that each dog harbors a very unique and individual microbial profile
1216 (J. S. Suchodolski, Ruaux, Steiner, Fetz, & Williams, 2004), that different intestinal compartments of
1217 individual dogs appear to host different bacterial populations and vary among dogs (J. S. Suchodolski,
1218 Ruaux, Steiner, Fetz, & Williams, 2005); and that there is also variation between the intestinal lumen and
1219 the mucosa (Mentula *et al.,* 2005).

1220

1221 Initial studies were performed using cultivation techniques. However, recent advances in DNA technology
1222 have allowed a better characterisation of the predominant bacterial flora in dogs. These molecular
1223 technologies such as 16S high-throughput sequencing studies have shown that the phyla Firmicutes,
1224 Bacteroidetes, Proteobacteria, Fusobacteria and Actinobacteria constitute more than 99% of all gut
1225 microbiota in dogs and cats. The remaining bacterial groups are represented by the phyla Spirochaetes,
1226 Tenericutes, Verrucomicrobia, Cyanobacteria, Chloroflexi and a few unclassified bacterial lineages. In the

1227   stomach, mucosa-adherent *Helicobacter* spp. predominates, followed by *Lactobacillus* spp., *Streptococcus*

1228   spp. and *Clostridium* spp. The proximal small intestine contains approximately 10 different phyla, with

1229   *Clostridium* spp*., Proteobacteria* spp*.* and *Lactobacillae* spp*.* being the most abundant. The phyla that

1230   predominate in the large intestine are Firmicutes, Bacteroides and Fusobacteria. However, the reported

1231   abundance of these bacterial groups differs between studies likely due to differences in sample collection

1232   methodology, diet, breed, age, DNA extraction techniques and analytical technology (J. S. Suchodolski,

1233   2011) (J. S. Suchodolski, Camacho, & Steiner, 2008) (Swanson *et al.*, 2011) (Handl, Dowd, Garcia-

1234   Mazcorro, Steiner, & Suchodolski, 2011).

1235

1236   Analysis of the metagenome of the canine intestinal microbiome has identified that the predominant

1237   bacterial gene categories in the canine gut are related to carbohydrate metabolism (12–13% of all

1238   sequences), protein and amino acid metabolism (8–9 and 7%, respectively), cell wall synthesis (7–8%),

1239   vitamin and cofactor synthesis (6%), and nucleic acid synthesis (7%); like that found in human beings.

1240   Genes for virulence and antibiotic resistance are also common features of human, dog, and cat microbial

1241   gene pools (Swanson *et al.*, 2011).

1242

1243   One study evaluated the short-term variability of gut microbiota in healthy dogs. Two faecal samples (15

1244   days apart) from six privately owned healthy dogs were collected. The microbiota was evaluated using

1245   fluorescence *in situ* hybridization (FISH) and 454-pyrosequencing. Aliquots of 100mg from each faecal

1246   sample were obtained and paraffin-embedded faecal blocks were prepared. Two serial sections of 5 μm

1247   were coated and placed on coated glass slides and FISH was performed. Pyrosequencing identified that

1248   15 families comprised > 80% of all microbiota, and over time intra-individual coefficients of variation (CV)

1249   ranged from 2 to 141% (median 55%). Ruminococcaceae had the lowest variability among individuals. In

1250   contrast, the inter-individual CV ranged from 62 to 230% (median 145%). The reason for these results is

1251   likely because individual dogs had differing genetics, environment and diet, but within dogs these did not

1252   alter during the 15 day period of faecal sample collection (Garcia-Mazcorro, Dowd, Poulsen, Steiner, &

1253   Suchodolski, 2012).

1254

1255   The dynamics, richness and evenness of the gut microbiota has not been explored at different age stages

1256   in dogs, and this would be useful to better understand the significance of the changes that can occur during

1257   disease.

1258

1259   **1.1.5 Gut virome and bacteriophages**

1260

1261

1262   Another aspect that it is being investigated and is increasing in importance is the population of viruses

1263   (bacteriophages) that influence the bacterial population structure. The phageome (total bacteriophage

1264   community) constitute the largest and less characterised part of the virome (total virus population); and they

1265   colonise the intestine from the beginning of life and influence gut ecology throughout all stages of life.

1266   Although the phageome exhibits a high degree of interpersonal variation, in people, it has been classified in

1267   three classes: a set of core bacteriophages shared among more than half of the human population, a

1268   common set of bacteriophages found in 20%–50% of individuals, and a set of bacteriophages that are

1269   unique to an individual (Manrique *et al.*, 2016).

1270

1271   In the healthy gut, most of the phages have a lysogenic lifestyle. In the lysogenic cycle, the viral DNA or

1272   RNA enters the cell and integrates into the host DNA as a new set of genes called prophage. These

1273   prophages serve as a major repository of mobile genetic elements in the gut and play key roles in the

1274   exchange of genetic material between bacterial species via horizontal gene transfer (HGT) (Manrique *et al.*,

1275   2016).

1276

1277   In   infants,   one   study   studied   the   development   of   the   gut   virome   from

1278   1 week to 3 months of age, and showed that the viral diversity was   extremely low and dynamic.   Direct

1279   epifluorescence microscopy in meconium samples did not detect any phage particle. However, by the end

1280   of the first week, there were ~10^8 virus particles per gram wet weight of faeces; the majority of which were

1281   phages (Breitbart *et al.*, 2008). Analysis of these sequences revealed that most of the sequences were not

1282   similar to previously reported sequences. During the second week, there was significant turnover of the

1283   sequences present and most communities were dominated by siphophages and prophages (which are the

1284   main particles also present in adults).

1285

1286   Although the initial source of viruses in the infant human gut is unknown, Breitbart *et al.* found that twenty-

1287   five percent of phage-like sequences were more like phages that infect lactic acid bacteria,

1288   including *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Enterococcus*, which are known to be abundant

1289   in breast milk (although they could not be identified in milk).

1290

1291 Recently, another study followed eight healthy infants (four twin pairs) for two years. Metagenomic

1292 sequencing found that during the first days of life, the diversity of the phage community increases rapidly

1293 (1-4 days post-birth) but at the end of the first week, the initial high diversity of bacteriophages disappears.

1294 The microbial abundance at the beginning is low and it is believed that it cannot support the high virome

1295 diversity, leading to a collapse of the phage diversity (Lotka-Volterra dynamics). The researchers also

1296 found that most of the bacteriophages belonged to the Caudovirales order (Siphoviridae, Inoviridae,

1297 Myoviridae and Podoviridae families) and Microviridae family, consistent with the previous study. However,

1298 there was a marked shift in the community composition toward an increased relative abundance of

1299 Microviridae bacteriophages by 24 months of age (Lim *et al.*, 2015)

1300

1301 In dogs, one study performed by Moreno *et al*, described the faecal virome of healthy dogs and dogs with

1302 acute diarrhoea in Australia, using shotgun metagenomics. Twelve viral families were identified, of which

1303 four were bacteriophages. Eight eukaryotic viral families were detected: Astroviridae, Coronaviridae,

1304 Reoviridae, Picornaviridae, Caliciviridae, Parvoviridae, Adenoviridae and Papillomaviridae. Families

1305 Astroviridae, Picornaviridae and Caliciviridae were found only in dogs with acute diarrhoea, with

1306 Astroviridae being the most common family identified in this group (Moreno *et al.*, 2017).

1307

1308 **1.2 Canine Chronic enteropathies**
1309
1310
1311 Canine chronic enteropathies (CE) constitute a group of disorders that cause gastrointestinal tract

1312 inflammation and persistent or recurrent gastrointestinal signs in dogs, and are also termed inflammatory

1313 bowel disease (IBD) (K. W. Simpson & Jergens, 2011). The pathogenesis of IBD is thought to involve a

1314 dysregulation of the mucosal immune response to enteric microorganisms in genetically predisposed

1315 individuals , which induces the activation of the inflammatory cascade causing direct and indirect intestinal

1316 mucosal damage (Elinav *et al.*, 2011) (Hooper & Macpherson, 2010).

1317

1318 **1.2.1 Aetiology**
1319
1320
1321 The triad of host genetics-immune system-environment (dietary antigens and gastrointestinal flora) are

1322 intimately related in the development of gastrointestinal disease. Alteration in any of these components can

1323 potentially deregulate the function of the other ones, predisposing the host to the development of disease

1324 (Manichanh *et al.*, 2012).

1325

1326 1.2.1.1 Genetics
1327
1328
1329 Individual genetic makeup dictates how the immune system reacts to antigen exposure. Genetic alterations

1330 can lead to a dysfunctional immune reactivity against commensal luminal antigens leading to aberrant cell

1331 signalling, activation and cytokine production by lymphoid and myeloid cells and intestinal inflammation of

1332 the enteric mucosa.

1333

1334 In the case of canine chronic enteropathies, it has been found that are breed differences in susceptibility to

1335 the disease and some forms are exclusive to certain breeds. For example, immunoproliferative enteropathy

1336 in Basenjis and gluten-sensitivity enteropathy in Irish Setters.(Fogle & Bissett, 2007).

1337

1338 Additionally, other studies have been performed in components of the immune system. Polymorphisms in

1339 TLR-4 have been associated with IBD in German shepherd dogs and polymorphisms in TLR-5 have been

1340 associated with IBD in several breeds (Kathrani *et al.*, 2010; Kathrani *et al.*, 2011). In Boxers, it has been

1341 discovered that a gene implicated in cellular autophagy is mutated in dogs with granulomatous colitis. This

1342 could imply that adherent and invasive *E.coli* may not be efficiently destroyed intracellularly if the enzyme

1343 for fusion of the autophagosome with the lysosome is functionally defective (Craven M, 2010).

1344

1345 A recent study also reported an association between SNPs in Major histocompatibility

1346 class (MHC) II haplotypes and a potentially increased resistance to IBD in GSD (Peiravan et al., 2016).

1347 Whereas another study have identified 16 candidate genes potentially associated with IBD in GSD, using a

1348 genome-wide association study (GWAS) (Peiravan et al., 2018).

1349

1350 1.2.1.2 Immune system
1351
1352
1353 In the intestine, the immune system is continuously challenged by a wide range of stimuli such as

1354 microbes, a high degree of microbial diversity, as well as an extensive variety of food and environmental

1355 antigens. The intestinal immune system therefore must be tolerant to food antigens and commensal

1356   bacteria, but at the same time able to resist tissue invasion by resident microbiota. Persistent inflammation

1357   also needs to be avoided as it can lead to damage of intestinal tissues or alter the symbiotic relationship of

1358   the intestine with the commensal microbiota. To achieve this purpose, the intestinal immune system is

1359   tightly regulated (Manichanh *et al.*, 2012).

1360

1361   The immune system is divided into two main components: the innate immune system and the adaptive

1362   immune system. The innate immune system consists of a set of cells and proteins that are readily available

1363   to fight against microbes and are not dependent on prior exposure to the antigen. On the other hand,

1364   adaptive immunity comprises a set of cells, mainly lymphocytes that depend on previous antigen

1365   expression to be activated, proliferate and create potent mechanisms for neutralising or eliminating

1366   pathogenic agents (Day, 2012).

1367

1368   The innate immune system is particularly active at those anatomical sites that are likely the first point of

1369   contact with pathogens such as the GI tract. It is mainly composed of the epithelial cell barriers with mucus-

1370   producing goblet cells and several secreted antimicrobial substances; and different type of leukocytes that

1371   become rapidly activated and are non-specific in targeting pathogens. These cells include phagocytic cells,

1372   DCs, the mast cells, the natural killer cells and intraepithelial lymphocytes. On the other hand, the adaptive

1373   immune system is considered more specific and potent than the innate system. It is mainly composed of T

1374   lymphocytes and B-lymphocytes that are capable of secreting immunoglobulins (Day, 2012).

1375

1376   Several experiments have been conducted to unravel the role of each component of the immune system in

1377   gastrointestinal inflammation. In this review, we will focus on two components: Immunoglobulins, that make

1378   part of the adaptive immune system and thymic stromal lymphopoetin that makes part of the innate immune

1379   system.

1380

1381   1.2.1.2.1 Immunoglobulin A and G

1382

1383   1.2.1.2.1.1 Immunoglobulin synthesis

1384

1385   Immunoglobulins are γ type of globulins produced as part of the immune response that can bind antigens

1386   and prevent their actions or promote their destruction, and are produced by plasma cells that come from B

1387   lymphocytes. Precursors of B lymphocytes are produced in the bone marrow and when they are released

1388 they express on their surface immunoglobulin M (IgM) and immunoglobulin D (IgD) chains; and are called

1389 naïve B cells. When they mature and differentiate into specific types of immunoglobulins, they get active

1390 and release plasma cells, containing the immunoglobulins (Day, 2012).

1391

1392 In the body, there are five types of immunoglobulins (Ig): Immunoglobulin G (IgG), immunoglobulin A (IgA),

1393 immunoglobulin M (IgM), immunoglobulin E (IgE) and immunoglobulin D (IgD); each of one with specific

1394 actions. However, all of them share the same basic structure. Immunoglobulins consist of a y-shaped unit

1395 of two polypeptide light chains (L) and two polypeptide heavy chains (H) bound by disulphide bonds. There

1396 are five forms of the heavy chain (γ,α,μ,δ and ξ) that give rise to each type of Ig, and they may associate

1397 with either of the two forms of the light chain (κ and λ). In the dog, the λ form is more commonly than the κ

1398 one (Day, 2012).

1399

1400 On each of the heavy and light chains, there are variable and constant regions. The variable regions are

1401 denoted by the letter V and the constant regions are denoted by the letter C. The light chain consists of

1402 two parts: the distal part (NH3 terminus) is the variable region (V_L) and the proximal part is the constant

1403 region (C_L) (COO terminus). Similarly, the heavy chain is divided into two parts. The distal quarter is the

1404 variable region (V_H), and the proximal three quarters are the constant region, numbered as 1, 2 and 3 (C_H1,

1405 C_H2, C_H3). The hinge region is a segment of the heavy chain between C_H1 and C_H2, where the interchain

1406 disulphide bonds occur (Day, 2012).

1407

1408 The constant regions confer specific properties to the immunoglobulin that determine which kind of cell they

1409 will bind, whereas the variable regions determine which type of antigens the antibody react to, and is also

1410 known as the antigen-binding site. Thus, the V region recognizes the pathogen and the C region attaches

1411 to different type of immune cells or activates the complement cascade against that pathogen (Day, 2012).

1412

1413 1.2.1.2.1.1.1 Immunoglobulin A

1414

1415

1416 Immunoglobulin A is the main Ig type present in body secretions and its main function is preventing the

1417 antigen from entering the cells. Mucosal IgA+ plasma cells can be generated by two mechanisms: one

1418 dependent of T-cells (TD) and the other independent of T-cells (TI) (Pabst, 2012). Responses dependent

1419 on T-cells occur in organised lymphoid structures associated with the gut associated lymphoid tissue

1420   (GALT), whereas TI responses occur in organised lymphoid tissue as well as in non-organised lymphoid

1421   tissues (Pabst, 2012).

1422

1423   Gut associated lymphoid tissue (GALT) is found within the lamina propria along the entire length of the

1424   gastrointestinal tract (GIT) (Pabst, 2012) (Fogle & Bissett, 2007). Anatomically, GALT consists of

1425   secondary lymphoid organs, which act as inductive sites of the immune response, termed Peyer Patches

1426   (PPs) in the small intestine, isolated lymphoid follicles (ILFs) throughout the whole intestine and the

1427   mesenteric lymph nodes; and the effector sites are comprised of the lamina propria (Fogle & Bissett, 2007).

1428   Components of an individual unit of GALT include the dome, follicle and parafollicular region. The dome is

1429   composed of M cells, which are enterocytes specialized in antigen capture, and transport antigens to all

1430   antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs). The follicles contain B

1431   lymphocytes, while the parafollicular region contains T lymphocytes, most of which are naïve (Pabst, 2012).

1432

1433   Lymphocytes can be divided in different types: T lymphocytes α-β, and T lymphocytes γ-σ. T cells α-β

1434   contain CD8+ymphocytes (cytotoxic T cells) and CD4+lymphocytes (T helper cells (T_h)). CD8+ lymphocytes

1435   recognise antigen presented by major histocompatibility complex type I (MHC I) on the target cells and can

1436   destroy that cell; CD4+ lymphocytes recognize antigen presented in conjunction with MHC II by APCs and

1437   help to promote memory and effector responses, including immunoglobulins (Fogle & Bissett, 2007).

1438

1439   In turn, CD4+ T cells contain another type of cell termed T regulatory (T_{reg}) cells that promote anti-

1440   inflammatory responses, and are vital for the maintenance of immunotolerance to gastrointestinal antigens

1441   (Allenspach, 2011).

1442

1443   Conversely, the γ-σ lymphocytes are CD4 and CD8 negative and are abundant on external surfaces (Day,

1444   2012). Although the role of γ-σ lymphocytes is unclear, they are particularly responsive to bacterial

1445   antigens and it is believed that they are in charge of eliminating stressed or infected gastrointestinal

1446   epithelial cells (Day, 2012; Fogle & Bissett, 2007).

1447

1448   Peyer patches (PPs) develop in the small intestine *in utero*, independently of bacterial gut colonisation and

1449   constitute the main site of IgA production (Pabst, 2012). However, after birth the full development of the

1450   intestinal IgA repertoire; depends on bacterial stimulation. Bacterial stimulation introduces additional

1451   mutations in highly expanded B-cell clones, promotes the generation of new mutated B-cell clones and the

1452   formation germinal centres (GCs) that foster the interaction of B cells with CD4+ T cells (Gutzeit *et al.,*

1453   2014).

1454

1455   Germinal centres are comprised of plasmablasts that eventually relocate to the lamina propria and displace

1456   resident old plasma cells (Pabst, 2012). By constantly receiving antigen stimulation and exposure, PPs

1457   generate a massive and diverse repertoire of high affinity IgA. Studies in mice have established that the

1458   majority (approx. >90%) of intestinal IgA production is microbiota-driven, since germ-free animals contain

1459   around 10-fold reduced numbers of mucosal IgA-producing cells. However, this can be easily reversed by

1460   the introduction of a normal microbiota (Crabbe, Nash, Bazin, Eyssen, & Heremans, 1970).

1461

1462   In contrast, ILFs develop after birth in response to bacterial colonisation and are scattered along the

1463   intestinal tract (Gutzeit *et al.,* 2014). They consist of solitary B-cell clusters built on a scaffold of stromal

1464   cells with a few interspersed CD4+ T cells and more abundant perifollicular DCs (Gutzeit *et al.,* 2014).

1465   Patches and ILFs lack afferent lymphatics, so antigens are delivered from the sub-epithelial dome to the

1466   follicles by DCs (Gutzeit *et al.,* 2014). Conversely, mesenteric lymph nodes (MLNs) receive lymph from the

1467   intestinal lamina propria, PPs and ILFs (Gutzeit *et al.,* 2014). Thus, DCs from the lamina propria can carry

1468   antigens into the MLNs directly and induce IgA responses; or activated B cells from PPs and ILFs can

1469   reach MLN and undergo further differentiation before travelling back to the lamina propria (Gutzeit *et al.,*

1470   2014).

1471

1472   Peyer patches are the main site for induction of antigen-specific responses, and are composed of B-cells

1473   and T-cells (ratio 6:1) that are covered by M cells (Cerutti & Rescigno, 2008). M cells sample IgA-free

1474   bacteria through an antigen recognition system involving the glycoprotein 2 receptor and IgA-coated

1475   bacteria by using a poorly characterised IgA receptor and dectin-1, a C-type lectin receptor that interacts

1476   with glycans associated with IgA (Gutzeit *et al.,* 2014). The antigen is then delivered from the gut lumen to

1477   DCs through a vesicular transport system (Cerutti & Rescigno, 2008).

1478

1479   Immunoglobulin-A induction in ILFs and the lamina propria occurs in the absence of segregated T-cell

1480   zones, suggesting that T-independent mechanisms of IgA induction may predominate in these sites. By

1481   contrast, PPs and MLNs can support both TI and TD modes of IgA induction (Pabst, 2012).

1482

1483 **1.2.1.2.1.1.1.1 T-cell dependent pathway**

1484

1485 T cell-dependent responses are termed classical and are essential for providing protection against invasive

1486 commensal microbes (pathobionts that possess strong immunostimulatory properties) such as filamentous

1487 bacteria or pathogens, and therefore are of high-affinity (Slack, Balmer, Fritz, & Hapfelmeier, 2012). The

1488 TD-responses require signals from CD4+T follicular helper (T$_{fh}$) cells to drive the selection and

1489 differentiation of high affinity B cells into long-lived plasma cells (N. Xiong & Hu, 2015).

1490

1491 In the TD-pathway, antigens presenting cells (APCs) located in the sub-epithelial dome, present and

1492 process the luminal antigens to the T- follicular helper lymphocytes (T$_{fh}$). **The naïve lymphocyte then**

1493 recognises the epitope and becomes activated. The T$_{fh}$ expresses CD40 ligand (CD40L, also known as

1494 CD154), a tumour necrosis factor (TNF) family member, on their surface that interacts with CD40

1495 expressed on the surface of B cells and induces them to undergo class switching to IgA (Allenspach et al.,

1496 2010). This switching process is enhanced by several cytokines. For example, Peyer Patch cells secrete

1497 transforming growth factor beta (TGF-β) **that cooperates with CD40 ligand to trigger IgA class**-switch

1498 recombination (CSR) and generates antigen-specific IgA+ B cells (N. Xiong & Hu, 2015). They also produce

1499 interleukin-4 (IL-4), IL-6, and IL-10, which facilitate the expansion of IgA-expressing B cells and their

1500 differentiation to IgA-secreting plasma cells. Meanwhile, dendritic cells secrete interleukin (IL)-6, which in

1501 turn induces B cells to preferentially undergo class switching to IgA (Gutzeit *et al.*, 2014).

1502

1503 **1.2.1.2.1.1.1.2 T-cell independent pathway**

1504

1505 T cell-independent responses are termed innate or primitive. Although the innate response is of low-affinity

1506 in regards to antigen specificity, it is sufficient to protect the host from mucosal invasion of harmless

1507 microbes, and therefore to avoid the activation of an inflammatory response (Pabst, 2012). The term

1508 'natural response' is applied to IgA that is produced in the absence of microbial and antigenic stimulation,

1509 therefore only refers to germ-free animals and newborns (Slack *et al.*, 2012) (although new studies have

1510 found the presence of bacterial DNA in meconium and placenta) (Aagaard *et al.*, 2014; Wassenaar &

1511 Panigrahi, 2014).

1512

1513   In the TI- pathway, class switching of B cells to IgA does not require the help of T cells, but instead is

1514   dependent on more direct interaction with the microbiome (Gutzeit *et al.*, 2014). Dendritic cells (DCs) in the

1515   lamina propria continuously sample antigens from the lumen, and present them directly to B cells, which in

1516   turn become activated. Toll-like receptors (TLRs) at the epithelial barrier and in DCs recognise bacterial

1517   components termed pathogen-associated molecular patterns (PAMPs), and stimulate the production of B-

1518   cell activating factor (BAFF), apoptosis-inducing ligand (APRIL), inducible nitric oxide synthase (iNOS) and

1519   retinoic acid (Pabst, 2012). In turn, BAFF and APRIL activate B cells, induce IgA class switching and

1520   promote plasma cell survival and differentiation through B cell maturation antigen (BCMA) and

1521   transmembrane activator and CAML interactor (TACI) (Cerutti, Chen, & Chorny, 2011).

1522

1523   Inducible nitric oxide synthase promotes the synthesis of BAFF and APRIL through the generation of nitric

1524   oxide and upregulates TGF-ß receptors in B cells, further enhancing the IgA production. Moreover,

1525   intestinal epithelial cells also produce thymic stromal lymphopoetin (TSLP), which can in turn upregulate

1526   the expression of BAFF and APRIL by DCs, TGF-ß and IL-6 (Cerutti *et al.*, 2011). Retinoic acid promotes

1527   the differentiation of IgA class-switched B cells into IgA-secreting plasma cells and confers gut-homing

1528   properties to IgA class-switched B cells through its ability to upregulate chemokine receptor type-9 (CCR9)

1529   and $\alpha 4\beta 7$ expression on these cells (N. Xiong & Hu, 2015).

1530

1531   Another factor that promotes IgA production is IgA-inducing protein (IGIP) that is expressed by DCs and is

1532   upregulated by the vasoactive intestinal peptide (VIP) (Pabst, 2012). This molecule, is a neuropeptide

1533   synthesized and released by immune cells, as well as by nerve endings that synapse on central and

1534   peripheral lymphoid organs that not only promotes IgA production indirectly but it also can trigger the

1535   expression of activation-induced cytidine deaminase (AID), a B cell-specific enzyme required for the

1536   diversification of Ig genes through class-switch DNA recombination (CSR) and somatic hypermutation

1537   (Abeles, Pillinger, & Abramson, 2015; Pabst, 2012).

1538

1539   Mucosal macrophages, stromal cells and epithelial cells secrete cytokines such as IL-6 and IL-10 and CXC-

1540   chemokine ligand 12 (CXCL12) that further promote the differentiation of B cells into IgA-secreting plasma

1541   cells (Pabst, 2012). The development of an antigen-specific SIgA response is a long process; 3 to 4 weeks

1542   are needed to detect an appreciable amount of SIgA antibodies in the faeces (reported in humans and

1543   mice) (Cerutti & Rescigno, 2008).

1544

1545 **1.2.1.2.1.1.1.3 The common pathway**
1546

1547 T-cell dependent and T-cell independent pathways share some steps in the production of immunoglobulins.
1548 After B cells are activated, they need to replace the immunoglobulin heavy chain composed of μ and ξ
1549 exons encoding IgM and IgD by Cα exons encoding IgA in a process termed class switch recombination
1550 (CSR). CRS alters the effector function of immunoglobulins without changing their antigen specificity and
1551 requires the enzyme AID (Gutzeit *et al.*, 2014). In both pathways, factors of the intestinal microenvironment
1552 such as TGF-β, IL-10 and retinoic acid direct the process (Bunker *et al.*, 2015).

1553

1554 In dogs, there is a single gene that encodes the α-heavy chain. However, it has been recently shown that
1555 there are four allelic variants within the part encoding the hinge region, suggesting that functional
1556 subclasses may exist (Day, 2012).

1557

1558 Another crucial process involved in IgA generation is termed somatic hypermutation (SHM). In this step,
1559 point mutations are introduced in the variable-region segments that encode part of the heavy and light
1560 chains of B cell immunoglobulins. This process generates structural changes that enhance antigen-affinity
1561 (Gutzeit *et al.*, 2014).

1562

1563 During differentiation from naïve B cells, the resultant IgA+ plasmablasts travel to the MLNs, where they are
1564 imprinted with gut homing integrins and chemokines that guide them to return to the lamina propria, a
1565 process known as lymphocyte homing. CCR9 and CCR10 are two major mucosa-specific chemokine
1566 receptors upregulated on IgA+ plasmablasts during their generation. CCL28, the mucosal ligand of CCR10,
1567 is expressed in both small and large intestines while CCL25, the ligand for CCR9, is predominantly
1568 expressed in the small intestines. Additionally the expression of α4β7 integrin is induced and interacts with
1569 its ligand mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed on postcapillary venules of
1570 the small and large intestines, helping the homing of IgA+ plasmablasts to the intestines (N. Xiong & Hu,
1571 2015).

1572

1573 In the lamina propria, IgA+ plasmablasts terminally differentiate into IgA-secreting plasma cells. These cells
1574 synthesise the polypeptide termed joining (J) chain, which allows the formation of stable IgA oligomers with

1575   increased avidity for antigen (Cerutti & Rescigno, 2008; Gutzeit *et al.*, 2014). Dimeric IgA interacts with

1576   polymeric immunoglobulin receptors (PIgR) located on the basolateral membrane of intestinal epithelial

1577   cells (IECs)(Pabst, 2012). Through a process called transcytosis, dimeric IgA is shuttled onto the luminal

1578   side and onto the surface of the gut in a modified form termed secretory IgA (sIgA) (Gutzeit *et al.*, 2014).

1579   SIgA includes a PIgR-derived polypeptide termed secretory component that increases the stability of SIgA

1580   in the intestinal lumen and anchors it to the mucus (Gutzeit *et al.*, 2014; Pabst, 2012). In the lamina propria,

1581   mature IgA$^+$ plasmablasts can survive for a long period of time, providing a local source for IgA antibodies

1582   (Allenspach, 2011; N. Xiong & Hu, 2015).

1583

1584   Although in people, IgA can exist as a monomer (in serum) or as a dimer (mucosal sites); in dogs, dimeric

1585   forms predominate in serum as well as in mucosa; this likely reflects the fact that serum IgA comes from

1586   plasma cells present in the respiratory, conjunctival, reproductive, and intestinal mucosa (Day,

1587   2012). Although, most serum IgA likely comes from intestine because it is the largest mucosal surface;

1588   serum level and intestinal levels are not always correlated (DeBey, 2010)

1589

1590   The development of an antigen-specific SIgA response is a long process; 3 to 4 weeks are needed to

1591   detect an appreciable amount of SIgA antibodies in the faeces of people (Cerutti & Rescigno, 2008). IgA-

1592   secreting plasma cells can survive, even in the absence of antigen stimulation, for long periods of time in

1593   the intestine. The maintenance of the IgA repertoire is modulated by the current status of the commensal

1594   microbiota, more microbiota stimulation will trigger the formation of new IgA-secreting plasma cells that will

1595   compete with the old ones (N. Xiong & Hu, 2015).

1596

1597   The repertoire of intestinal IgA comprises high-frequency clones that recognise highly prevalent and stable

1598   components of the microbiota that are cross-reactive, and low-frequency clones that recognize changes in

1599   the microbiota and exposure to pathogens (Gutzeit *et al.,* 2014). Thus, IgA responses to simple and

1600   frequent protein antigens involve the reutilization of pre-existing GCs in multiple PPs and the acquisition of

1601   new somatic mutations whereas new antigenically distinct proteins induce the generation of a new plasma

1602   cell pool (Gutzeit *et al.,* 2014). Generally, antibodies produced in a TI manner produce low-affinity

1603   oligoclonal antibodies, whereas antibodies produced in a TD manner are of greater affinity and greater

1604   specificity for a given antigen (Stephens & Round, 2014).

1605

1606   Recently, a study has given some new insights into the dominant site of microbial IgA induction. Using

1607   murine genetic models of immunodeficiency and IgA-sequencing, they found that IgA targets mainly

1608   bacteria residing in the small instestine (40-80% IgA+ in small intestine versus 10-30% IgA+ in the colon).

1609   Whereas in the colon, many taxa were entirely IgA negative; and those that were positive were present at

1610   >1% relative abundance in the duodenum. This correlated with significant higher titres of luminal free IgA in

1611   the small intestine and 10-15 times more IgA+ plasma cells in the small intestinal lamina propria relative to

1612   colonic lamina propria. A similar trend was found in intestinal aspirates from human patients during

1613   colonoscopy (Bunker *et al.*, 2015).

1614

1615   That same study also found that colonic bacteria markedly segregated into IgA+ and IgA- taxa. As opposed

1616   to the colon, duodenal bacteria were equally represented in both fractions. This highlights the importance of

1617   IgA induction in the small intestine, where the lack of a double mucus layer could predispose it to bacterial

1618   invasion. However, another reason could be that PPs and iLFs are primarily associated with the small

1619   intestine and thus, it possesses a higher IgA production capacity (Bunker *et al.,* 2015) (Macpherson &

1620   McCoy, 2015).

1621

1622   In addition, most small intestinal bacteria elicit IgA responses whereas the same bacteria in the colon are

1623   not major targets of IgA, at least under normal conditions; and the response most of the times is driven by

1624   TI pathways, except in atypical commensals that can penetrate the mucus layer and induce a TD-response

1625   (Bunker *et al.*, 2015).

1626

1627   **1.2.1.2.1.1.2 Immunoglobulin G**
1628
1629

1630   Immunoglobulin G is the most abundant type of antibody present in the serum and due to its small

1631   molecular weight (150000 Da) it can cross easily the blood vessels and exert its functions at the

1632   extravascular space. In dogs, IgG is present as a monomer and potentially binds two antigen epitopes, and

1633   there are four subclasses (IgG1-IgG4) with IgG1 the most abundant form (Day, 2012).

1634

1635   Immunoglobulin G is synthesized and secreted by B-cells present in the spleen and lymph nodes. These

1636   antibody secretory cells are also distributed throughout the gastrointestinal tract. To synthesise IgG, B cells

1637   **replace the immunoglobulin heavy chain composed of μ and ξ exons encoding IgM and IgD by Cγ exons**

1638   encoding IgG. Then, these molecules are actively transported to the intestinal mucosa where they undergo

1639   bidirectional transcytosis across epithelial cells by binding to the neonatal Fc receptor (nFcR) or passively,

1640   perhaps secondary to epithelial barrier disruption during the inflammatory response (Eckmann &

1641   Stappenbeck, 2015; Yoshida *et al.*, 2006).   The bidirectional transport of IgG retrieves intestinal luminal

1642   antigens as a complex with IgG and deposits them into the intestinal mucosa, where the antigen/IgG

1643   complexes can be captured by DCs for subsequent presentation to CD4^{+} T cells (Yoshida *et al.*, 2004).

1644

1645   **1.2.1.2.1.2 Function of immunoglobulins**
1646
1647
1648   Immunoglobulin A is secreted into the gut and has a variety of functions that influence microbial community

1649   composition and location; effectively regulating host epithelial physiology and inflammatory response

1650   (Cerutti *et al.*, 2011).   Through multiple mechanisms, IgA favours the maintenance of non-invasive

1651   commensal bacteria and neutralises pathogens (Cerutti & Rescigno, 2008).

1652

1653   One of the main functions of IgA is termed immune exclusion, where the antigen-binding variable (V) region

1654   of SIgA specifically blocks certain bacterial epitopes (Gutzeit *et al.*, 2014). This in turn modifies gene

1655   expression and motility, to prevent the adhesion of bacteria to the apical surface of IECs and entraps

1656   antigens in the mucus. Also, glycans associated with the secretory component and constant region α (Cα)

1657   of IgA can be responsible for this effect (Gutzeit *et al.*, 2014). Immune exclusion not only applies to

1658   bacterial antigens but also self-antigens, food components, toxins and viruses (Pabst, 2012).

1659

1660   As well as neutralising pathogens in the intestinal lumen, SIgA can intercept microbes and toxins inside

1661   IECs (N. Xiong & Hu, 2015). SIgA can neutralise antigens within epithelial cell endosomes and excrete

1662   antigens from the lamina propria into the intestinal lumen. This can be done through the pIgR receptor or by

1663   promoting their destruction via Fc fragment of IgA receptor (FcαRI) (also known as CD89). This receptor is

1664   expressed by dendritic cells (DCs), neutrophils, and other phagocytes. They also facilitate antigen sampling

1665   by binding to microfold (M) cells (Cerutti & Rescigno, 2008).

1666

1667   One of the key characteristics of IgA action is that it delivers its protective functions without activating the

1668   complement cascade, preventing inflammation and damage to the intestinal epithelial barrier (Slack *et al.*,

1669   2012).

1670

1671   Limitation and prevention of the inflammatory response not only impedes damage to the epithelial barrier,

1672   but also can limit the pathogenic potential of pathogens (Pabst, 2012). One example of this is *Salmonella*

1673   *typhimurium*, which takes advantage of host inflammatory response to successfully compete against

1674   commensal microbes. Endt *et al.* (2010) recently elucidated that high-affinity surface (predominantly

1675   lipopolysaccharide (LPS) O-antigen)-binding IgA protects against colitis upon re-infection with *S.*

1676   *typhimurium* in the non-typhoidal salmonellosis model; it does this by inducing bacterial coagulation and by

1677   hindering the proinflammatory response (Endt *et al.*, 2010).

1678

1679   Pathogens have developed mechanisms of resistance to host inflammatory response that are not present

1680   in commensals. This gives them an advantage to survive over commensals, and decrease the number of

1681   bacteria that could compete against them. Thus, IgA not only create a containing wall but also prevents the

1682   induction of inflammation and promotes the survival of commensals (Endt *et al.*, 2010).

1683

1684   Many different proteins have been suggested to bind IgA, in addition to the PolyIg receptor, which mediates

1685   its transport across epithelia. These include the Fcα/μR, CD89, asialoglycoprotein receptors, transferrin

1686   receptors and secretory component receptors (Slack *et al.*, 2012). The association of IgA- bacteria induces

1687   alteration in bacterial signalling when compared to recognition of bacteria alone and thus, modify the

1688   pathogenic properties of bacterial survival and growth rate (Slack *et al.*, 2012).

1689

1690   For example, studies using a monoclonal dimeric IgA specific for the outer membrane of *Helicobacter felis*

1691   could protect germ-free mice from Helicobacter infection (Czinn, Cai, & Nedrud, 1993). Forbes *et al,* 2011

1692   found that IgA directed against the LPS-O antigen of *Shigella flexneri* inhibited the type secretion III system,

1693   required for bacterial motility and invasion (Forbes, Bumpus, McCarthy, Corthesy, & Mantis, 2011). Another

1694   study performed by Cullender *et al,* using a toll like receptor 5 deficient (TLR5$^{-/-}$) mouse model discovered

1695   that flagellin- SIgA responses, downregulate flagellin transcription to decrease bacterial motility . TLR5

1696   deficient mice lack the receptor that recognizes flagellin and therefore the production of anti-flagellin

1697   antibodies is almost absent. Under these circumstances, bacteria can breach the mucosal barrier and

1698   cause intestinal damage and inflammation (Cullender *et al.*, 2013).

1699

1700   Recently, a study found that IgA alters the expression of the polysaccharide utilization loci (PUL), including

1701   a functionally uncharacterised molecular family provisionally named Mucus Associated Functional Factor

1702   (MAFF). In mice and people, MAFF is predominantly detected in mucus-resident bacteria and its

1703   expression facilitates symbiosis with other members of the phylum Fimicutes and promotes colon

1704   homeostasis. Nakajima et al., assessed whether "bystander" IgA (IgA elicited by antigens other than

1705   bacterial antigens) could modulate gut microbiota via glycan–glycan interactions among IgA, bacteria, and

1706   mucus. They generated a monoclonal IgA (7-6IgA) recognizing OVA, which is heavily glycosylated. IgA

1707   binded to *Bacteroides thetaiotaomicron* via glycan–glycan interactions (IgA-glycan and LPS) and enhanced

1708   the association of the bacteria with host mucus and/or diet-derived polysaccharide. The mucus-associated

1709   *B. theta* induced MAFF system expression dependent on interaction with Firmicutes members such as

1710   Clostridiales and enhanced the metabolic activity of *B. theta*. Metabolically active *B. theta* up-regulated the

1711   polysaccharide utilization activity of *B. theta* and stimulated the fatty acid utilization and expansion of

1712   butyrate-producing Clostridiales, altering the composition of the gut microbial community (Nakajima et al.,

1713   2018).

1714

1715   These metabolic changes may directly stimulate the proliferation and regeneration of colonic epithelial cells

1716   in response to epithelial damage and they could test these properties in a model of DSS induced colitis.

1717   Thus, IgA is not only altering the gene expression of mucus-associated, IgA-coated bacteria, but is also an

1718   essential component of the regulatory network modulating interphylum bacteria interaction.Mucus-specific

1719   MAFF expression was also observed in human colon biopsies (Nakajima et al., 2018).

1720

1721   All of these actions of IgA influence the function and composition of the microbiota, reinforce the integrity of

1722   the intestinal barrier and attenuate pro-inflammatory immune responses that help to maintain intestinal

1723   health and homeostasis.

1724

1725   Although IgA is the most important immunoglobulin in the mucosa, when invasive bacteria tresspass the

1726   epithelial border, IgG help IgA to repel invaders; and thus, constitute a second line of defense by eliciting a

1727   robust inflammatory reaction (Cerutti *et al.*, 2011).

1728

1729   In people, it has been shown IgG can be detected within the intestinal lumen and even, may reach levels

1730   approximating that observed for sIgA in certain locations such as rectum (Yoshida *et al.*, 2004).

1731

1732   The main mechanism of action of IgG is by complement mediated destruction of foreign cells. The

1733   complement system is composed of about 30 proteins, that when activated, interact sequentially to form an

1734   enzymatic cascade that causes the destruction of the pathogen. Although, there are four complement

1735   pathways: classical, lectin,alternative and termimal pathways; the classsical pathway is the one that is

1736   activated by the aggregation of immunoglobulins on the pathogen surface. After C1 binds to complexed

1737   antibody and antigen; it becomes activated and cleaves the second factors of the pathway: C4 and C2.

1738   They then, sequentially activate C3 and the terminal pathway initiates. The C3 activation finally leads to the

1739   formation of the membrane attack complex that causes lysis of the cell by osmotic imbalance. The

1740   complement also exerts other functions such as opsonisation and inflammation. IgG and C3b are

1741   recognized by receptors on the surface of phagocytic cells and signal phagocytosis. C3a and C5a can

1742   amplify inflammation by activating white cells and releasing inflammatory mediators (Day, 2012).

1743

1744   **1.2.1.2.1.3 Significance of immunoglobulin A and G in intestinal disease**
1745
1746
1747   • Studies performed in people and mice
1748
1749
1750   Mice and humans with defective IgA secretion have increased susceptibility to intestinal disease,

1751   autoimmune diseases and allergy (Cunningham-Rundles, 2001) (Brandtzaeg, 2010).However, many of

1752   these affected individuals have a normal life-span, probably due to compensatory mechanisms of other

1753   components of the immune system, such as polymeric IgM secretion that can compensate for the lack of

1754   SIgA (Cunningham-Rundles, 2001; Eckmann & Stappenbeck, 2015). However, this assumption has been

1755   refuted. Longitudinal studies have revealed that 80% of patients are symptomatic, when assessing

1756   complications more broadly or when follow-up is extended (Jorgensen et al., 2013) (Koskinen, 1996). In

1757   animals devoid of all Ig isotypes, serious intestinal disease occurs and cannot be compensated by the other

1758   components of the immune system (Cunningham-Rundles, 2001).

1759

1760   In Activation-induced cytidine deaminase (AID)-deficient mice, the main feature is dysbiosis characterised

1761   by the overgrowth of segmented filamentous bacteria and ileal inflammation (Suzuki *et al.*, 2004). Mice

1762   lacking the pIgR have no faecal IgA or IgM and although they have a normal microbiota, commensal

1763   bacteria can penetrate more easily the mucosa and induce systemic antibody responses. Also, knockout

1764   mice had increased serum IgG levels, including antibodies to *E. coli,* suggesting undue triggering of

1765   systemic immunity (Johansen *et al.*, 1999). In people, malfunction of cells in the PPs in production of SIgA

1766   has been considered a risk factor for coeliac disease, as gluten of wheat is neutralised in the intestine by

1767   SIgA (Mulder & Mulder-Bos, 2006).

1768

1769   In regards to IBD in people, studies have shown contradictory results. Some studies have shown that the

1770   frequency of IgA deficiency among IBD patients is significantly higher than that in the healthy population

1771   and that affected individuals exhibited decreased mucosal IgA concentrations (Brandtzaeg, 2010).

1772   However, another study have reported that IBD lesions exhibit excessive numbers of IgA$^+$ and IgG$^+$ plasma

1773   cells with a remarkable skewing toward IgG production, depending on the severity of inflammation

1774   (Brandtzaeg, Carlsen, & Halstensen, 2006).

1775

1776   The relative average increase is more prominent for IgG (×30) and IgM (×2.5) than for IgA (×1.7–2.0). In

1777   fact, adjacent to Crohn's disease ulcers, the number of plasma cells is increased 100–200-fold for the IgG

1778   class and 8–12-fold for the IgM class compared with 1.2–6.7-fold for the IgA class (Brandtzaeg *et al.*,

1779   2006).

1780

1781   Locally produced IgG in IBD lesions has been reported to react against cytoplasmic antigens from a range

1782   of Gram-positive and Gram-negative faecal bacteria, with higher activity in Crohn's disease than in

1783   ulcerative colitis, and higher in ulcerative colitis than in other types of intestinal inflammation (Brandtzaeg *et*

1784   *al.,* 2006). However, serum antibodies levels exhibit a considerable heterogeneity in microbial specificities

1785   among IBD patients; suggesting that rather a global loss of tolerance against intestinal bacteria, the

1786   response is individual and pathogen- specific (Landers *et al.*, 2002).

1787

1788   Another interesting aspect reported in people with IBD is that although the number of IgA producing cells is

1789   increased, the J-chain expression is decreased or deficient in inflammatory lesions (producing monomers

1790   that cannot be exported by the pIgR); and a shift from the IgA2 to the less-stable IgA1 subclass (more

1791   easily degraded by enzymes present in the intestinal lumen) occurs (Kett & Brandtzaeg, 1987).

1792

1793   In people, 90% of the plasma cells present in the colon secrete IgA, 4% secrete IgG and 6% secrete IgM

1794   (De Palma et al., 2010). In a previous study by Van der Waaij *et al.,* only a fraction of the faecal bacteria

1795   were coated with IgA, IgG or IgM in healthy people (L. P. Van der Waaij LA, Mesander G, van der Waaij D,

1796   1996), whereas IBD patients had higher concentrations of Ig-coated bacteria (IgA, IgG and IgM

1797   populations) in active disease and also shortly after remission compared to healthy patients. In healthy

1798   controls, approximately 40% of faecal anaerobic bacteria are coated with IgA, 12% with IgG and 12% with

1799   IgM. In IBD, the percentages are raised to 65, 45 and 50% respectively, with no difference between

1800   ulcerative colitis and Crohn's disease (K. F. van der Waaij LA, Jansen PLM, *et al*, 1997). In patients with

1801   long-term remission, the percentages of Ig-coated bacteria return to control values. Thus, clinical remission

1802   of IBD patients occurs before coating of bacteria returns to normal, and this could be an indication of a

1803   stage of sub clinically active IBD (van der Waaij *et al.*, 2004).

1804

1805   To assess whether an immune response against a person's own gut bacteria is present in CD, Harmsen *et*

1806   *al,* assessed IgG-coated bacteria by flow cytometry in faeces from CD patients (n=23) and healthy

1807   volunteers (n=11), with or without incubation with autologous serum. Their relationship with disease activity

1808   through the measurement of faecal calprotectin (a marker of intestinal inflammation) and the bacterial

1809   composition in faeces using fluorescence *in situ* hybridization was also assessed (Harmsen, Pouwels,

1810   Funke, Bos, & Dijkstra, 2012). They found that the *in vivo* IgG-coated fraction of faecal bacteria was higher

1811   in patients, particularly after incubation with either autologous or heterologous serum. This was dependent

1812   on the bacteria type (stronger against *E. coli* strains isolated from patients) and independent of disease

1813   activity or host serum (Harmsen *et al.*, 2012). The amount of IgG coating, however, did not correlate with

1814   calprotectin concentration, which could indicate that patients in remission still have an impairment in the

1815   intestinal barrier. These results suggest that patients with IBD harbour more immunogenic bacteria and/or

1816   loss of tolerance against normal intestinal microbiota (the exact difference between the *E. coli* strains found

1817   in the CD patients and those from healthy volunteers was not determined). The induction of inflammation

1818   may lead to ulceration, and subsequent leakage of IgG into the intestinal lumen, and entry of bacterial

1819   antigens to the bloodstream can induce and increase the levels of serum IgG (Harmsen *et al.*, 2012).

1820

1821   Conversely, in coeliac disease proportions of IgA, IgM and IgG-coated bacteria are significantly lower

1822   compared to healthy controls in both untreated and treated patients (De Palma *et al.*, 2010). At the

1823   mucosal level, IgA+ plasma cells remain numerous in the lamina propria in both treated and untreated adult

1824   coeliac disease, although the numbers of IgA+, IgM+ and IgG+ plasma cells per tissue unit increase only

1825 slightly (only 2.4, 4.6 and 6.5 times, respectively (Brandtzaeg, 2006)). This suggests that either low or high

1826 levels of Ig-coated bacteria can be indicative or predispose to disease.

1827

1828 Recently, Palm *et al.*, using flow cytometry and 16 ribosomal ribonucleic acid (rRNA) sequencing, showed

1829 that high IgA coating selectively marks specific members of the mouse and human intestinal microbiota that

1830 can drive or exacerbate intestinal inflammation in a mouse model. Traditional approaches to assay

1831 antibody production, such as enzyme-linked immunosorbent assay (ELISA) require a lower affinity for

1832 binding when compared to flow cytometry which requires 10^9i/mol. Thus, fluorescence-activated cell sorting

1833 (FACS)-based IgA approach enriches for the high-affinity antibody against microbiota that has been

1834 generated through TD interactions, and can potentially provide more information about the disease-causing

1835 microbes (Palm *et al.*, 2014) .

1836

1837 The study by Palm performed IgA 16S rRNA sequencing (IgA-SEQ) on faecal samples from 27 people with

1838 Crohn's disease (CD), 8 with ulcerative colitis (UC), and 20 healthy controls. As previously noted, the

1839 percentage of bacteria coated with IgA was significantly increased in people with CD or UC. Although

1840 several bacterial species were shared in both healthy people and patients, many of the bacteria were only

1841 highly coated in sick people. They also found specific taxa that were more highy coated specifically in UC

1842 or CD patients. As expected, there was a high amount of bacterial diversity between individuals (Palm *et*

1843 *al.*, 2014).

1844

1845 Next, the researchers selected and isolated representative members of the microbiota that were coated

1846 and uncoated with IgA from 11 patients wth IBD and grew them under laboratory conditions to create

1847 personalized culture collections. These faecal microbes were then transferred to germ-free mice. Although

1848 the transplants did not cause disease themselves, when colitis was induced using dextran sulfate sodium

1849 (DSS), mice colonised with the IgA+ consortia exhibited more severe intestinal disease and bacteria were

1850 more invasive compared to animals colonized with the IgA- consortia, indicating that the species present

1851 within the IgA+ group exacerbated disease (Palm *et al.*, 2014).

1852

1853 Another study was performed in germ-free mice transplanted with IgA-coated bacteria from faecal samples

1854 of undernourished children from Malawi. The intestinal microbiota of these children is characterised by

1855 disrupted normal postnatal assembly, dysbiosis and increased enteropathogen burden. When fed with a

1856   nutrient deficient diet similar to the Malawi children, mice exhibited disruption of the intestinal epithelial

1857   barrier, weight loss and sepsis. In contrast, when these mice were fed with a nutrient-sufficient diet, the

1858   pathological changes were not seen, suggesting that these bacteria can be a contributing or confounding

1859   factor for disease when other conditions are involved into the equation, such as diet and environmental

1860   conditions also occur.(Kau *et al.*, 2015)

1861

1862   In 2015, Kamada *et al.* showed that humoral immunity, in particular IgG, selectively targets virulent bacteria

1863   in the intestine, particularly those associated to the intestinal mucosa. Using mice models of *Citrobacter*

1864   *rodentium* infection, Kamada *et al.*, could show that IgG recognises virulent factors from bacteria attached

1865   to the mucosa and targets them for elimination; whereas avirulent bacteria that remain in the lumen are

1866   outcompeted by the resident microbiota. Thus, although IgG is not actively transported into mucosal

1867   secretions, unlike IgA, it could be particularly important in periods of intestinal disease and/or patients with

1868   IgA deficiency (Kamada *et al.*, 2015).

1869

1870   Recently, Vilamidiu *et al.* (2017), using IgA-SEQ in faecal samples from patients with CD and from patients

1871   with CD and spondyloarthritis (CD-SpA), discovered that CD-SpA patients had a a selective enrichment in

1872   IgA-coated *Escherichia coli* compared to CD alone patients. The *E. coli* isolates from CD-SpA–derived IgA-

1873   coated bacteria were similar in genotype and phenotype to an adherent-invasive *E. coli* (AIEC) pathotype.

1874   When germ-free C57BL/6 mice were colonized with AIEC isolated from patients and with non-AIEC *E. coli*;

1875   mice with AIEC induced T helper 17 cell (T_{H17}) mucosal immunity with no histopathological changes.

1876   However, when mice were challenged with DSS, clinical signs were more severe in AIEC mice; suggesting

1877   a pro-inflammatory effect of this strain (Viladomiu *et al.*, 2017).

1878

1879   The group also found that the presort population had significantly increased relative abundance of

1880   Proteobacteria in CD-SpA compared to CD, but no significant differences at the genus level. Moreover, the

1881   proportion of intestinal bacteria coated with IgA was increased in CD compared to healthy donors; however,

1882   no significant difference was seen in IgA coating between CD-SpA and CD (Viladomiu *et al.*, 2017).

1883

1884   •   Studies performed in dogs

1885

1886   In dogs, few studies have assessed the role of Igs in healthy conditions and during intestinal disease,
1887   although immunodeficiency due to low levels of IgA is considered common in dogs. Similar to people, many
1888   cases of IgA deficiency are not associated with clinical disease. Diseases such as pyoderma (Miller,
1889   Wellington, & Scott, 1992), atopy, otitis, demodicosis, chronic bronchitis (Campbell, 1991), recurrent
1890   pneumonia (Moroff, Hurvitz, Peterson, Saunders, & Noone, 1986), food allergy (Campbell, 1991), or
1891   enteritis (Batt, Barnes, Rutgers, & Carter, 1991)have been reported in dogs with IgA deficiency (DeBey,
1892   2010).

1893

1894   Peters *et al.* (2004), measured the faecal IgA, IgM, and IgG concentration in healthy colony German
1895   Shepherd dogs (GSD) (n = 209), Labrador Retrievers (n = 96), Beagles (n = 19), and Miniature Schnauzers
1896   (n = 32), via ELISA. Faecal IgG concentrations between the four breed groups were not significantly
1897   different. Conversely, IgA concentrations were significantly greater in Miniature Schnauzers than in GSD (P
1898   = 0.0003) and Labradors (P = 0.0004), but not significantly different from those in Beagles. As well,
1899   IgM concentrations were significantly greater in Miniature Schnauzers than in GSD (P < 0.0001), Labradors
1900   (P < 0.0001), and Beagles (P = 0.0098) (Peters, Calvert, Hall, & Day, 2004)

1901

1902   Peters *et al* also assessed the variation in the amount of faecal IgA, IgM and IgG during a 7-day collection
1903   period, and the effect of delayed freezing of the samples in two of the dogs. During the study period, there
1904   was no significant difference in the amount of faecal IgA, IgM or IgG and the average coefficients of
1905   variation for sampling of a single defecation were 25, 31, and 50% for IgA, IgM, and IgG, respectively.
1906   Regarding the freezing effect, multiple samples were taken from a single defecation and were frozen at 0,
1907   12, 24, 48, and 72 hours after collection. There was no significant difference in the amount of IgA, IgM, or
1908   IgG following delayed freezing of the faecal samples. They also found that the faecal concentration of IgA,
1909   IgM and IgG were significantly correlated with each other. The correlation of IgG with both IgA and IgM was
1910   unexpected, since IgG is not actively transported into mucosal secretions like IgA and IgM (Peters et al.,
1911   2004). This correlation may have reflected the contribution of bile to the faecal immunoglobulins measured,
1912   as the concentrations of IgA and IgG have previously been found to be similar in canine bile (German, Hall,
1913   & Day, 1998).

1914

1915   Litter *et al* (2006) assessed the concentration of immunoglobulins in faecal and duodenal organ cultures in
1916   76 GSD dogs and 63 healthy dogs of different breeds. They found a significant correlation between

1917   the concentrations in faecal extracts and the concentrations produced in duodenal organ cultures extracts.

1918   However, GSDs had significantly lower median IgA concentrations in their faecal extracts compared to the

1919   rest of the breeds. Sixteen of the GSD had IgA concentrations below the 95 per cent confidence limit of the

1920   control population, and six had no demonstrable faecal IgA. The faecal concentrations of IgG and albumin

1921   were significantly higher in the GSDs than in the controls, but their IgM concentrations were similar (Littler,

1922   Batt, & Lloyd, 2006). German shepherd dogs are one of the breeds that have been reported to be IgA-

1923   deficient and are highly susceptible to developing IBD (Allenspach *et al.*, 2010; German, Helps, Hall, &

1924   Day, 2000). It is possible that the deficiency of IgA in this breed predisposes to IBD, and the increased

1925   concentrations of IgG could be a compensatory mechanism for this deficiency.

1926

1927   Low concentrations of serum IgA have previously been reported in several dog breeds, but no generally

1928   accepted cut-off value has been established (Campbell, 1991). A large screening study of 1267 dogs

1929   representing 22 breeds was performed with both healthy and sick dogs (inflammatory and endocrine

1930   conditions) (Olsson *et al.*, 2014). Serum IgA concentrations were quantified using capture ELISA and

1931   varied widely between and within breeds (0.01 to 3.0g/L), and was positively correlated with age

1932   (p<0.0001). Apart from the two breeds previously reported as predisposed to low IgA (Sharp Pei and GSD),

1933   the study identified six additional breeds in which ≥ 10% of all tested dogs had very low (<0.07

1934   g/L) IgA concentrations (Hovawart (n=19), Norwegian Elkhound (n=14), Nova Scotia Duck Tolling Retriever

1935   (n=11), Bullterrier (n=14), Golden Retriever (n=168) and Labrador Retriever (n= 141)). In addition,

1936   low IgA concentrations were significantly associated with canine atopic dermatitis (CAD, p<0.0001) and

1937   pancreatic acinar atrophy (PAA, p=0.04) in GSDs (Olsson *et al.*, 2014).

1938

1939   It is important to point out that other studies have reported mutational changes in genes related to immunity

1940   in GSD, such as polymorphisms in toll-like receptor 5 and nucleotide-binding oligomerization domain-

1941   containing protein 2 (NOD2), that could predispose them to develop immune-mediated conditions (Aarti

1942   Kathrani, 210).

1943

1944   Batt *et al.* (1991) assessed serum immunoglobulin concentrations and number of IgA-producing cells

1945   in intestinal mucosa (using immunohistochemistry (IHC)) in a group of clinically healthy dogs of various

1946   breeds, a group of clinically healthy GSDs, and a group of GSDs diagnosed with bacterial overgrowth in the

1947   proximal small intestine . Serum concentrations of IgA, but not IgM or IgG, were significantly lower in GSD

1948   (healthy and sick) than in other purebred and mix breed dogs. However, densities of intestinal IgA-

1949   producing cells were not significantly different in any group (Batt *et al.*, 1991). Nowadays, the term small

1950   intestinal bacterial overgrowth (SIBO) is not used anymore in veterinary practice. Studies have shown that

1951   the normal bacterial counts are much higher in healthy dogs compared to humans, and dogs treated

1952   successfully with antibacterial agents do not necessarily show a decrease in duodenal bacterial numbers

1953   and so SIBO is now termed antibiotic-responsive diarrhoea/enteropathy (E. J. Hall, 2011).

1954

1955   Jergens *et al.* (1996) quantitated immunoglobulin-containing cells (IgA and IgG) and CD3+ T cells in the villi

1956   of duodenal mucosal biopsy specimens obtained from healthy dogs (n= 9), dogs with IBD (n=11) and dogs

1957   with non-specific gastroenteritis (n=8), using immunoperoxidase techniques and morphometric analyses,

1958   respectively. Healthy dogs had significantly higher T-cell counts, dogs with nonspecific gastroenteritis had a

1959   significantly higher concentration of IgA-containing cells; whereas, dogs with IBD had significantly

1960   lower cell counts for IgG-containing cells (Jergens, Moore, Kaiser, Haynes, & Kinyon, 1996). This could be

1961   an indication of an inappropriate immune response in dogs with IBD.

1962

1963   Using the same techniques, Jergens *et al* (1999) assessed the number of immunoglobulin-

1964   containing cells (IgA, IgG, and IgM) and CD3+ T cells in colonic biopsy specimens obtained

1965   from dogs with lymphocytic-plasmacytic colitis (LPC), and in healthy dogs. They found increased numbers

1966   of plasma cells in the intestinal mucosa (particularly IgA+ and IgG+ cells) and CD3+ T cells in dogs with LPC

1967   compared to healthy dogs, although the number of IgG+ cells was less numerous compared to IgA+ cells

1968   (Jergens *et al.*, 1999). This increase suggests that humoral immunity is stimulated in IBD. This could be

1969   secondary to a disruption of the intestinal barrier (extravasation of serum immunoglobulins or exposure of

1970   mucosal immune system to intestinal bacteria), presence of more immunogenic bacteria; or could be

1971   indicative of a breakdown of mucosal immune tolerance to intestinal flora (van der Waaij *et al.*, 2004).

1972   Previous studies have reported that mucosal T-cells from IBD patients with active disease proliferate

1973   intensely when incubated with commensal bacteria derived from the same patient, whereas mucosal T-

1974   cells from healthy controls do not respond (only in the presence of bacteria derived from another individual)

1975   (Duchmann *et al.*, 1995).

1976

1977   German *et al.* 2000, measured IgG, IgM, IgA and albumin concentrations in matched samples of serum,

1978   saliva and tears collected from four groups of dogs: GSD dogs with small intestinal disease (n=25), other

1979   breeds with intestinal disease (n=21), other breeds with disorders not related to TGI (n=36) and GSD with

1980   disorders not related to TGI (n=30). There were no significant differences in IgG, IgM or IgA concentrations

1981   in serum, saliva or tears between the different groups of dogs (German, Hall, & Day, 2000).

1982

1983   Additionally, the authors assessed the IgA production by 24-hour explant duodenal organ cultures in the

1984   dogs with small intestinal disease, and found that IgA production was significantly lower in GSDs compared

1985   with non-GSDs with small intestinal disease (German, Hall, *et al.*, 2000). . These findings suggest that

1986   there may be a relative deficiency of intestinal IgA in GSD that is not reflected in either serum or on

1987   secretion at unaffected mucosal sites; and that IgA deficiency could be breed-related or secondary to

1988   disease within the intestinal mucosa.

1989

1990   Maeda *et al* (2013), using ELISA measured the concentration of IgA and IgG in the serum, faeces and

1991   duodenal samples of 37 dogs with IBD (defined based on clinical signs and need of glucocorticoid therapy),

1992   10 dogs with alimentary lymphoma and 20 healthy dogs (colony of beagles). Additionally, IgA+ cells in

1993   duodenal lamina propria and IgA+ CD21+ peripheral blood mononuclear cells (PBMCs) were examined by

1994   immunohistochemistry and flow cytometry, respectively. Following that, duodenal expression of the IgA-

1995   inducing cytokine transforming growth factor β (TGF-β), B cell activating factor (BAFF), and a proliferation-

1996   inducing ligand (APRIL) was quantified by real-time RT-PCR (Maeda *et al.*, 2013).

1997

1998   Dogs with IBD had significantly decreased concentrations of IgA in faeces compared to healthy dogs

1999   (healthy median 116.2 µg/mg total protein, range 55.3–353.4; IBD, median 77.6 µg/mg total protein, range

2000   24–251.8. P = .0068) and duodenal samples compared to healthy and dogs with lymphoma (healthy,

2001   median 322.3 µg/mg total protein, range 24.7–759.1; IBD, median 152 µg/mg total protein, range 27.9–

2002   531.4. P = .0037, lymphoma median 260.5 µg/mg total protein, range 99.1–637.5, P = .0181). The number

2003   of IgA+ CD21+ PBMCs and IgA+ cells in duodenal lamina propria was significantly lower in dogs with IBD

2004   than in healthy dogs or dogs with intestinal lymphoma, suggesting a defect in IgA switching class. Overall,

2005   when assessing faecal and duodenal IgG concentrations and serum IgA or IgG concentrations there were

2006   no significant differences among the 3 clinical groups. There was also no significant correlation between

2007   IgA or IgG concentrations and the canine chronic enteropathy clinical activity index (CCECAI) in dogs with

2008   IBD or intestinal lymphoma.

2009

2010 Conversely, duodenal BAFF and APRIL mRNA expression was significantly higher in IBD dogs than in the

2011 healthy controls; whereas duodenal TGF-β mRNA expression was significantly lower in dogs with IBD than

2012 in healthy dogs and dogs with intestinal lymphoma. TGF-β is related to T cell-dependent IgA class

2013 switching whereas BAFF and APRIL are to T cell-independent IgA class switching. Decreased expression

2014 of TGF-β can reflect impaired IgA expression, as seen in mice and humans with IgA deficiency. While, the

2015 increased expression of BAFF and APRIL; could represent a physiologic compensatory mechanism.

2016

2017 In a subsequent study they delved into the pathophysiology of decreased levels of IgA and evaluated the

2018 mRNA expression of TACI and BAFF-receptor (BAFF-R) and the methylation levels of their corresponding

2019 genes TNFRSF13B and TNFRSF13C, in the duodenal mucosa of dogs with

2020 IBD. Duodenal mRNA expression levels of TACI and BAFF-R were significantly lower in dogs with IBD

2021 compared to healthy controls.

2022

2023 Quantitative real-time methylation-specific PCR (qPCR-MSP) showed significant hypermethylation of the

2024 CpG islands of TNFRSF13B and TNFRSF13C in the duodenal mucosa of dogs with IBD.

2025 mRNA expression levels of TACI were positively correlated with intestinal IgA expression, whereas

2026 the methylation level of its gene (TNFRSF13B) was negatively correlated with IgA expression, suggesting

2027 that epigenetic modifications could contribute to the concentrations of IgA (Maeda *et al.*, 2014).

2028

2029 In summary, results of studies of the role of IgA in dogs with intestinal disease have been discordant, and

2030 there is no definitive conclusion whether IgA plays a role in disease pathogenesis or is a simple

2031 consequence of inflammation. The discordance in results could be given by several factors such as

2032 sample site (duodenum vs, colon), methodology (ELISA and IHC), environmental conditions (privately-

2033 owned dogs versus colony dogs), different criteria of classification of disease status (small intestinal

2034 bacterial overgrowth [SIBO] versus antibiotic/responsive diarrhoea [ARD], or IBD versus CE) and breed of

2035 dogs (as some breeds have been reported to be IgA-deficient, particularly German-Shepherd dogs).

2036

2037 ### 1.2.1.2.2 Thymic Stromal Lymphopoetin

2038

2039 Thymic stromal lymphopoetin (TSLP) is a cytokine that is constitutively expressed in IECs in response to

2040 commensal bacterial colonisation, with highest expression in colonic epithelial cells (Rimoldi *et al.*, 2005).

2041

2042 **1.2.1.2.2.1 Definition and structure**

2043

2044 The mucosal epithelium is the main constituent of the intestinal physical barrier, and is composed of a

2045 diverse population of cells such as intestinal epithelial cells (IEC), goblet cells, endocrine cells and M cells.

2046 Enterocytes (IEC) comprise approximately 80% of mucosal epithelial cells and constantly regenerate

2047 (Fogle & Bissett, 2007). Immature enterocytes are located within the intestinal crypts and migrate from the

2048 crypt to the villus tip over a period of 3 to 5 days until they mature and are eventually sloughed off (Fogle &

2049 Bissett, 2007).

2050

2051 During physiologically normal conditions, IEC constitutively produce factors, such as interleukin-10 (IL-10),

2052 transforming growth factor beta (TGF-β), prostaglandin E$_2$ (PGE$_2$), which can inhibit and/or promote an anti-

2053 inflammatory response (Wells, Rossi, Meijerink, & van Baarlen, 2011). Another epithelial-derived factor is

2054 termed thymic stromal lymphopoietin (TSLP); TSLP is an IL-7 like cytokine that was initially identified and

2055 characterised as a B cell growth-promoting factor in conditioned supernatants of a mouse thymic stromal

2056 cell line (Friend *et al.*, 1994). Later, it was discovered that TSLP was not only expressed in the thymus but

2057 is also widely expressed in other epithelial cells lines of the lung, skin, intestine, mucosa-associated

2058 lymphoid tissue and tonsils (Ziegler *et al.*, 2013).

2059

2060 Two transcript variants of TSLP have been identified in people: the long isoform or variant 1 (lfTSLP,

2061 inducible isoform), and the short isoform or variant 2 (sfTSLP, constitutive isoform) *(*Bjerkan *et al., 2015;*

2062 Fornasa *et al.*, 2015). Under physiological conditions, sfTSLP is released by IEC to create a tolerant

2063 microenvironment in response to commensal bacterial colonisation (Fornasa *et al.*, 2015). Conversely,

2064 under pathological conditions the expression of the long isoform is up-regulated to promote an

2065 inflammatory response to clear the pathogen and avoid further damage to the intestinal mucosa (Fornasa

2066 *et al.*, 2015). Thus, TSLP is critical to maintain the balance between inflammatory responses and immune

2067 clearance.

2068

2069 Recently, a partial segment of the canine TSLP cDNA has been cloned and characterised (Klukowska-

2070 Rotzler *et al.*, 2013). The canine TSLP gene is located on chromosome 3 (1.503–1.507 Mbp) (Klukowska-

2071 Rotzler et al., 2013). The complementary DNA (cDNA) sequence contains 465 nucleotides and is

2072 organised in four exons (Klukowska-Rotzler *et al.,* 2013). The alignment of the canine TSLP cDNA with the

2073 orthologous human sequence (GenBank accession number NM_033035.4)01164063.1), gave a nucleotide

2074 identity of 70%, and covered the sequence encoding the signal peptide and full-length mature protein of

2075 human TSLP. The predicted amino acid sequence deduced from the canine TSLP cDNA shares 60.8%

2076 identity with human (NP_149024.1) TSLP protein (Klukowska-Rotzler *et al.,* 2013).

2077

2078 TSLP (lfTSLP) exerts its biologic activities by binding to a heterodimeric receptor consisting of the IL-7

2079 receptor α chain and the TSLP receptor chain (TSLPR); TSLPR is a member of the haematopoietic

2080 receptor family and binds TSLP at low affinity (Ziegler *et al.,* 2013). As a result, TSLPR is expressed on

2081 DCs, T cells, B cells, natural killer cells, monocytes, basophils and mast cells. When TSLPR and the IL-7α

2082 chain interact together, there is a high affinity binding to TSLP that leads to the activation of many pathways

2083 (Zhong *et al.,* 2014). The TSLP-TSLPR/IL-7Rα complex results in the activation of multiple signal

2084 transducer and activator of transcription STAT-1,3,4,5 and 6 and Janus kinase (JAK)1 and 2 (Arima *et al.,*

2085 2010). In fact, mapping of the TSLP signalling pathway has shown that TSLP can regulate the

2086 phosphorylation of approximately 226 proteins.(Zhong *et al.,* 2014).

2087

2088 The activation of the STAT-JAK pathway exerts its function in the nucleus, where they activate the

2089 promoter region of NF-κB that promote a pro-inflammatory environment and in turn, stimulate more TSLP

2090 production (Saenz, Taylor, & Artis, 2008)

2091

2092 In contrast to the lfTSLP, sfTSLP in people does not bind with TSLPR as it is not able to block the binding

2093 of lfTSLP to this receptor. Currently, it is unknown the sfTSLP-receptor although, it has been seen to

2094 induce the phosphorylation of p38α extracellular signal-regulated kinase 1 and 2 and lyn (Fornasa *et al.,*

2095 2015). Studies perfomed in LPS-stimulated monocyte derived dentritic cells (moDCs) previously

2096 conditioned or not with sfTSLP showed that the level of p38α and extracellular signal-regulated kinase 1/2

2097 phosphorylation was decreased when moDCs were preconditioned with sfTSLP. This suggests that sfTSLP

2098 might either desensitize the cells against further activation of the pathway or raise the threshold of moDC

2099 activation, regulating Toll-like receptor (TLR)–mediated signalling and inflammation (Fornasa *et al.,* 2015).

2100

2101 **1.2.1.2.2.2 Regulation of TSLP**
2102

2103   Multiple factors can regulate TSLP transcription. Besides TLRs, nuclear factor-kappa B (NF-κB), IL-1b, -4, -

2104   13, activating protein 1 (AP-1) and TNF-α positively regulate TSLP (Ziegler & Artis, 2010). Conversely, a

2105   heterodimer formed between retinoid X receptors (RXR) α and/or RXRβ and free vitamin D receptor (VDR)

2106   or the retinoic acid receptor-γ and glucocorticoids act as co-repressors; via blocking AP-1 or NF-κB activity.

2107   Vitamin D3 activates both regions and induces sfTSLP transcription but is not sufficient for lfTSLP

2108   transcription (Tsilingiri, Fornasa, & Rescigno, 2017).

2109

2110   Exposure to pro-inflammatory cytokines such as IL-1β and TNF-α, IL-4 and IL-13 alone or in combination

2111   has also been shown to induce the expression of TSLP (Saenz *et al.*, 2008).Also, parasites and common

2112   allergens induce the production of IL-25, that in turn, promote TSLP production by IECs.

2113

2114   New studies have discovered that endogenous proteases regulate the activity of TSLP. Protease

2115   upregulation is common in active coeliac disease. The effect of biopsy supernatants from refractory,

2116   untreated, treated patients with coeliac disease and controls on TSLP integrity was assessed. The protease

2117   furine can degrade the long isoform producing fragments of 10 and 4 kDa that show different activity on

2118   mononuclear cells compared to the intact isoform (long isoforms stimulate INF-γ, whereas fragments

2119   downregulate its expression) (Biancheri *et al.*, 2015). Another study found that these fragments enhance

2120   the pro T_{H2} activity of mast cells compared to the full-length protein (Tsilingiri *et al.*, 2017).

2121

2122   **1.2.1.2.2.3 Function**

2123

2124

2125   In the intestine, TSLP expression is mediated by bacteria membrane cell contact and in health, promotes

2126   the generation and maintenance of non-inflammatory resident dendritic cells (DCs), particularly to

2127   preferentially promote T lymphocyte helper 2 (T_{H2}) differentiation (Rimoldi *et al.*, 2005)

2128 T_{H2} cells are classified into two subtypes per their cytokine profile: non-inflammatory T_{H2} cells that release

2129   IL-4, -5, -13 and -10 and pro-inflammatory T_{H2} cells that release IL-13, -5 and tumour necrosis factor alpha

2130   (TNF-α) (Liu, 2009).

2131

2132   During physiologically normal conditions, sfTSLP promotes a T_{H2}-DC non-inflammatory phenotype,

2133   particularly the development of natural regulatory T cells; whereas under pathologic conditions, lfTSLP

2134   activates a pro-inflammatory phenotype (Fornasa *et al.*, 2015).

2135

2136   When IECs are exposed to viral, bacterial or parasites, toll-like receptors -2, -3, -8 and -9 can be activated

2137   and trigger the production of TSLP by epithelial cells. TLR-2 is involved in the recognition of cell-wall

2138   components, lipoteichoic acid and lipoprotein, from gram-positive bacteria; lipoarabinomannan, from

2139   mycobacteria; and zymosan, from yeast. TLR-3 recognizes double-stranded RNA (dsRNA), whereas TLR-8

2140   recognizes single stranded RNAs, both associated with viral infection. Finally, TLR-9 recognizes specific

2141   unmethylated CpG motifs prevalent in microbial genomic DNA . When commensal bacteria trigger TLRs,

2142   the sfTSLP is induced, that promote a T_{H2}-DC non-inflammatory phenotype. If, instead, pathogens are the

2143   ones that induce TLRs, lfTSLP is produced and inflammation is generated.

2144

2145   In contrast to constitutive production of TSLP by IECs, TSLP expression in innate cells requires stimulation,

2146   either by cytokines or directly by pathogens (Saenz *et al.*, 2008). The same scenario applies for TSLPR.

2147   DCs and mast cells appear to express TSLPR constitutively, however CD4$^+$ T cells require T-cell receptor

2148   (TCR) stimulation to induce receptor expression and become sensitive to TSLP treatment (Saenz *et al.*,

2149   2008).

2150

2151   Intestinal DCs are located within the lamina propria, and their main function is presenting antigens to T

2152   lymphocytes, thereby bringing the innate and adaptive immunity together (Fogle & Bissett, 2007). Intestinal

2153   DCs can open tight junctions between adjacent IECs and send dendrites to sample bacteria directly from

2154   the intestinal lumen. Encounter with bacteria or bacterial products triggers the functional maturation of DCs

2155   that leads to the generation of powerful antigen-presenting cells (Rimoldi *et al.*, 2005).

2156

2157   DCs themselves make significant amounts of TSLP in response to pattern-recognition receptor (PRR)

2158   engagement, but little is known about the key downstream signals that induce and modulate this TSLP

2159   secretion from human DCs. Studies in vitro have shown that in human monocytes and monocyte derived

2160   DC (moDCs); stimulation with TSLP increases the production of chemokine 17 (CL17), a known ligand for

2161   C-C chemokine receptor type 4 (CCR4), which is expressed on polarized T_{H2} cells. Further, TSLP

2162   enhances the spontaneous maturation moDC cells, increasing their expression of co-stimulatory molecules

2163   and their ability to promote naive CD4$^+$ T cell proliferation (Saenz *et al.*, 2008).

2164

2165   When DCs are stimulated with TSLP, they express the surface OX40 ligand (OX40L) that interacts with the

2166   OX40 receptor present in CD4$^+$T cells. The OX40 ligand and its cognate receptor belong to the TNF and

2167   TNF-receptor superfamily; OX40 is preferentially expressed on activated CD4$^+$T cells, whereas OX40L is

2168   mainly expressed by antigen-presenting cells. The interaction between OX40L and OX40 promotes the

2169   differentiation of naïve CD4$^+$T cells into T$_{H2}$ cells, particularly T$_{H2}$-DC non-inflammatory cells (Ziegler *et al.*,

2170   2013).

2171

2172   Simultaneously, TSLP inhibits IL-12/23p40 production by DCs, which not only allows T$_{H2}$ differentiation but

2173   also blocks a T$_{H1}$ response, allowing a full expression of T$_{H2}$ phenotype (Rimoldi *et al.*, 2005). Consistent

2174   with this, DCs isolated from the gut-associated lymphoid tissue (GALT) of TSLPR$^{-/-}$ mice exhibited

2175   elevated expression of IL-12/23p40 under steady state conditions and T$_{H1}$ responses (Zaph *et al.*, 2007).

2176

2177   In addition, TSLP gives DCs the ability to produce interleukin-6 (IL-6) and a proliferation-inducing ligand

2178   (APRIL), which drives the development of IgA-producing plasma cells. Thus, the homeostasis of the

2179   intestine is preserved through the continuous generation of non-inflammatory helper T cells and antibody

2180   responses that limit bacterial entrance and promote an environment tolerant to commensal bacteria.

2181

2182   Studies in humans have shown that during allergic inflammation, human epithelial cells release large

2183   amounts of TSLP and activate DCs to induce the differentiation of allogeneic pro-allergic T$_{H2}$ cells and

2184   cytotoxic T cells, and induce homeostatic proliferation of autologous CD4$^+$T cells (Ziegler *et al.*, 2013). So,

2185   it seems that the expression of TSLP must be tightly regulated to promote a non-inflammatory response

2186   and a tolerogenic environment following bacterial colonisation.

2187

2188   Recent *in vitro* studies in purified CD4$^+$T cells have demonstrated a direct effect of TSLP on T cells

2189   whereby TSLP induced T cell IL-4 production (Saenz *et al.*, 2008). In turn, IL-4 acts on the T-cells to

2190   activate STAT-6 and up-regulate transcription factor (GATA-3) expression, causing T$_{H2}$ cell differentiation

2191   (Omori & Ziegler, 2007).

2192

2193   Most of the studies in people to date have focused on the long form of TSLP, while translation of a short
2194   form has been reported only recently. The expression pattern of both isoforms is dependent on tissue
2195   localisation and disease state (Fornasa *et al.*, 2015). So far, the short isoform has only been identified in
2196   the gut, skin, oral epithelium and salivary glands (Tsilingiri *et al.*, 2017).

2197

2198   New studies are beginning to explore novel functions of TSLP. *In vitro* experiments have shown that the C-
2199   terminal region of the TSLP protein possesses potent antimicrobial activity against bacteria and fungi.

2200

2201   Sonesson *et al.* investigated the TSLP antibacterial activity against *E. coli* ATCC 25922. Purified
2202   recombinant human TSLP was added at different doses and viable count assay, as well as radial diffusion
2203   assay (RDA) were performed. The results demonstrated a dose-dependent killing of E. coli. They also
2204   evaluated the effect of TSLP on other bacterial and fungal specimens (*Staphylococcus aureus ATCC*
2205   *29213, Staphylococcus epidermidis ATCC 14990, Escherichia coli ATCC 25922, Pseudomonas aeruginosa*
2206   *ATCC 27853, Candida albicans ATCC 90028 and Candida parapsilosis ATCC, Pseudomonas aeruginosa*
2207   *27.1, S. aureus BD 14312 and S. epidermidis BD 1723*). Significant antimicrobial activity was observed
2208   against *E. coli* and *P. aeruginosa*, whereas TSLP exerted only a moderate antimicrobial effect on *S.*
2209   *epidermidis* and *Candida*, and little or no antibacterial activity against *S. aureus*).

2210

2211   They also found that TSLP was degraded by bacterial proteases and by human neutrophil elastase into
2212   several low–molecular weight peptides. Interestingly, *S. aureus* V8 proteinase fragmented TSLP, releasing
2213   C-terminal TSLP-derived fragments that retained antimicrobial activities. That could suggest that although
2214   bacteria can degrade TSLP and compromise its cytokine response; the microbial activity could be still
2215   retained (Sonesson *et al.*, 2011).

2216

2217   Bjerkan *et al*, added sfTSLP or lfTSLP peptide at a concentration of 1.35 mM to microbial suspensions
2218   containing a diverse panel of bacterial and fungal species (*Streptococcus mitis, Escherichia coli,*
2219   *Enterococcus faecalis, Bacillus cereus, Staphylococcus epidermidis and Candida albicans)* for 2 hours and
2220   then plated them on agar. Colony-forming units per mL were determined after incubation overnight. Results
2221   showed that sfTSLP exerted potent antimicrobial activity against all the tested species. Although, lfTSLP
2222   exerst antimicrobial effects, this form was not able to inhibit the growth of *Enterococcus faecalis* and
2223   *Staphylococcus epidermidis.*

2224

2225 The C-terminal region has penetrating effect on bacterial membranes and although the C-terminal region is

2226 common for both isoforms sfTSLP exhibits the strongest antimicrobial peptide (AMP) activity (Bjerkan *et al.*,

2227 2015).

2228

2229 It also has been shown that physical damage can induce TSLP production and could be involved in tissue

2230 healing, although the precise mechanism has not been explored (Allakhverdi *et al.*, 2007) Evaluation for

2231 genes related to resolution of inflammation, found that mRNA levels of secretory leukocyte peptidase

2232 inhibitor (SLPI) were significantly up-regulated following DSS colitis in *TSLP*$^{+/+}$, but not *TSLP*$^{-/-}$ mice. The

2233 inability to increase SLPI leads to unrestrained neutrophil elastase activity that oversees degradation of

2234 progranulin, a protein that induces IEC proliferation. Additionally, SLPI functions as a serine protease

2235 inhibitor, anti-microbial peptide, and inhibitor of NF-κB (Reardon *et al.*, 2011).

2236

2237 **1.2.1.2.2.4 Significance of TSLP during disease**
2238

2239

2240 In vitro and in vivo studies have confirmed that TSLP has a marked impact on regulating immune

2241 responses in health, but it also has a role in protection against enteric pathogens.

2242

2243 Mucosal DCs possess unique attributes that allow them to induce a non-inflammatory environment. They

2244 can preferentially promote T helper type 2 (T_{H2}) (in contrast to monocyte–derived DCs [MoDCs], that

2245 express T_{H1}) differentiation and to induce B cells to secrete immunoglobulin A (IgA). These properties in the

2246 intestine are given by the surrounding environment and by factors secreted by other cells such as IECs

2247 (Rimoldi *et al.*, 2005).

2248

2249 To assess the role of TSLP in T_{H2} polarization, Rimoldi *et al*, isolated DCs from healthy human colon tissue

2250 and incubated them first for 24 h with the T_{H1}-inducing pathogen *Salmonella enterica serovar typhimurium*

2251 *(SL-WT)* and then for 5 d with allogeneic naive CD4$^+$CD45RA$^+$ T cells.

2252

2253 During *Salmonella* infection, only unconditioned, newly recruited DCs can mount protective T_{H1} responses.

2254 Exposure to *Salmonella* induces the IECs to release chemokine ligand 20 (CCL20), which attracts C-C

2255   Motif Chemokine Receptor 6 (CCR6)-expressing immature DCs at various epithelial sites that have not

2256   been subjected to IEC conditioning (Sierro *et al.,* 2001).

2257

2258   As expected, mucosal DCs that are conditioned with TSLP, were unable to induce T_{H1} responses but

2259   instead induced T cell responses that were strongly polarized toward T_{H2}; releasing IL-10 but not IL-12

2260   *(Rimoldi et al.,* 2005). In contrast, when TSLP expression on mucosal DCs was suppressed by small

2261   interfering RNA (siRNA); mucosal DCs lost their ability to drive T_{H2} cell polarization and instead induced T_{H1}

2262   cells (Rimoldi *et al.,* 2005).

2263

2264   They also showed that bacteria can induce TSLP up-regulation only when attached to the basolateral

2265   membrane of epithelial cells, suggesting that invasion of the epithelial cell barrier is necessary to produce

2266   large quantities of TSLP (Rimoldi *et al.,* 2005).

2267

2268   The researchers also suggested that the effect of TSLP on DCs was dose-dependent. Small doses of

2269   *Salmonella* inhibited the expression of IL-12, a T_{H1} cytokine, thus promoting an environment permissive for

2270 T_{H2} differentiation. Whereas large doses of *Salmonella* promoted the production of IL-12 by DCs and shifted

2271   to a protective T_{H1} response (Rimoldi *et al.,* 2005). However, now it is known that the effect is not dose

2272   dependent but depends on the isoform expressed (Tsilingiri *et al.,* 2017).

2273

2274   *In vitro* studies in Caco-2 cells (human epithelial colorectal adenocarcinoma cells) showed that basal levels

2275   of expression of the 2 isoforms were identical to the one observed in primary intestinal epithelial cells,

2276   where sfTSLP is mainly expressed. When caco-2 cells were stimulated with *Salmonella thypimurium,* levels

2277   of sfTSLP mRNA and protein expression were downregulated with a concurrent increase of lfTSLP. A

2278   similar pattern was found when cells were challenged with the adherent-invasive *E coli* strain LF82,

2279   although this strain only increased lfTSLP but it did not have any effect on sfTSLP. Notably, the *E coli*

2280   nonpathogenic strain MG1655 had no effect on both isoforms.

2281

2282   *In vitro* studies also showed that lfTSLP-conditioned moDCs were inflammatory and significantly

2283   upregulated secretion of TNF-α from naive T cells, that resulted in the generation of the T_{H2}-inflammatory

2284   phenotype both in the presence and absence of sfTSLP. Whereas sfTSLP-conditioned moDCs, resulted in

2285   impaired induction of interferon gamma (IFN-γ) secretion by T cells (Fornasa *et al.,* 2015)

2286

2287   Further *in vivo* studies in human and mice; also have begun to elucidate the role of TSLP in inflammatory

2288   responses. Although most of the studies have assessed the skin and lung; studies in the gastrointestinal

2289   tract also indicate an immunoregulatory function for TSLP (Saenz *et al.*, 2008).

2290

2291   During parasitic infections, T_{H2} responses are vital to achieve parasite clearance and resolution of the

2292   clinical signs. IEC-intrinsic IkappaB kinase (IKK)-beta-dependent gene expression is a critical regulator

2293   of responses of dendritic cells and CD4$^+$ T cells in the GI tract. Mice with an IEC-specific deletion of IKK-

2294   beta failed to produce TSLP during *Trichuris* infection, leading to impaired protective T_{H2} responses.

2295   Instead, they expressed increased levels of dendritic-cell-derived interleukin-12/23p40 and tumour necrosis

2296   factor-alpha, increased levels of CD4$^+$ T-cell-derived interferon-gamma and interleukin-17 (IL-17), and

2297   developed severe intestinal inflammation due to uncontrolled T_{H1} and T_{H17} inflammatory responses (Zaph *et*

2298   *al.*, 2007).

2299

2300   These findings were corroborated in another study, where monoclonal antibody-mediated neutralization of

2301   TSLP or deletion of the TSLPR in normally resistant mice to *Trichuris* infection, resulted in defective

2302   expression of T_{H2} cytokines, elevated expression of interleukin (IL) 12/23p40, interferon (IFN) gamma, and

2303   IL-17A, development of severe intestinal inflammation and persistent infection.

2304

2305   Blockade of proinflammatory cytokines (p40 or INF-γ) during Trichuris infection mice restored expression of

2306 T_{H2} cytokines, host protective immunity and worm expulsion; suggesting that protective T_{H2} response can

2307   be compensated by TSLP- independent mechanisms (helminth-derived factors or production of IL-25 and

2308   IL-33). The TSLP promoter in humans and in mice contains a NF-κB site, and it is known that IKK-β

2309   activates NF-κB that in turn, enhances TSLP production. (Zaph *et al.*, 2007)

2310

2311   However, TSLP is not considered essential for the initiation of protective T_{H2} cytokine responses in the

2312   intestine in other parasitic infections. TSLPR-knockout mice exhibited normal protective T_{H2} responses after

2313   infection with *Heligmosomoides polygyrus* or *Nippostrongylus brasiliensis*. These parasites produce

2314   excretory-secretory (ES) products that are capable of directly suppressing dendritic cell (DC) production of

2315   IL-12p40, thus bypassing the need for TSLP (Massacand *et al.*, 2009).

2316

2317   Thus, it seems that although TSLP can condition DCs toward T_{H2} responses; in parasitic infestations the

2318   main role is to block the production of IL-12p40, limiting T_{H1} responses and allowing the expression of

2319   other T_{H2}-independent TSLP responses.

2320

2321   Recent clinical studies have also indicated that T_{H2} and T_{reg} responses induced by helminth infections in a

2322   TSLP partially dependent manner are beneficial in reducing the symptoms of IBD through the decrease of

2323   the inflammatory response. Helminths have evolved a wide variety of strategies to counteract inflammatory

2324   responses, promote immune suppression and prolong their survival. Parasites promote the generation

2325   of regulatory T cells and anti-inflammatory cytokines interleukin-10 and transforming growth factor-β. This is

2326   a very effective strategy for subverting protective immune responses to prolong their survival in the host but

2327   has the bystander effect of modulating immune responses to unrelated antigens (Finlay, Walsh, & Mills,

2328   2014).

2329

2330   **1.2.1.2.2.4.1 Role of TSLP in inflammatory bowel disease**
2331

2332   Further studies have also evaluated the role of TSLP in the pathogenesis of inflammatory bowel disease

2333   (IBD) in mice and people.

2334

2335   A dextran sodium sulfate (DSS) mouse model of colitis, using TSLP receptor knockout (TSLPR KO) mice,

2336   showed a rapid onset and more severe clinical signs (pronounced weight loss and increased disease

2337   activity index) compared to wild type mice, because of unrestrained T_{H1} responses. TSLPR KO mice

2338   exhibited severe thickened of the muscularis and inflammatory cell infiltration with loss of crypt architecture

2339   and epithelial cell sloughing and displayed elevated production of IL-12/23p40 and IFN-γ (Spadoni, Iliev,

2340   Rossi, & Rescigno, 2012).

2341

2342   However, when Reardon *et al.* compared the severity of clinical signs between *Tslp*$^{-/-}$ and *Tslp*$^{+/+}$ mice in a

2343   model of DSS-colitis; macroscopic score, colon length, concentration of the acute phase protein serum

2344   amyloid A (SAA), and histological damage scores, were not different among groups (Reardon *et al.,* 2011).

2345

2346   In this model, the difference was found during the recovery period. *Tslp*^{+/+} mice began to recover 9-10 days

2347   post-DSS, whereas TSLP deficiency prevented recovery from inflammation resulting in death (Reardon *et*

2348   *al.,* 2011).

2349

2350   Another study using recombinant lactic acid bacteria (that deliver TSLP), showed that TSLP exerted a

2351   protective effect in the mouse model of DSS-induced colitis (Aubry *et al.,* 2015; Aubry *et al.,* 2016).

2352

2353   Aubrey *et al.,* constructed *Lactococcus lactis* MG1363 strain producing TSLP (LL-TSLP) and investigated

2354   the effect of its administration on DSS-induced colitis model in mice. In acute DSS-induced colitis, LL-TSLP

2355   or *Lactococcus lactis* wild type (LL-WT) was administered orally 7 days before and 4 days after the

2356   induction of colitis followed by only LL-WT until the end of the experiment. LL-TSLP delayed the disease

2357   Activity Index and lowered histological score and colonic INF-γ production (Aubry *et al.,* 2015; Aubry *et al.,*

2358   2016).

2359

2360   To test the involvement of TSLP in the healing process, two groups of mice were treated 7 days before

2361   colitis or during the inflammation as well as the recovery period with LL-WT or LL-TSLP. In the DSS-

2362   recovery model, LL-TSLP induced a better protective effect if the strain was administered at the beginning

2363   of the colitis due to its anti-inflammatory effect than later on, where no significant effects were seen.

2364   Treatment with LL-TSLP, increases the amount of TGF-β secreted by T cells in mesenteric lymph nodes in

2365   healthy mice and exhibited a higher recruitment of T_{reg} in the intestinal mucosa (Aubry *et al.,* 2015; Aubry *et*

2366   *al.,* 2016).

2367

2368   Additionally, *Winnie* mice carrying a missense mutation in the mucin 2 gene (Muc2) that results in a

2369   epithelial barrier defect (endoplasmic reticulum (ER) stress, reduced goblet cell numbers, a depleted mucus

2370   layer, increased intestinal permeability) and spontaneous colitis, exhibited lower mRNA TSLP levels

2371   compared to wild-type mice, suggesting that deregulation of TSLP expression could be related to

2372   exaggerated inflammatory responses due to the lack of regulatory T cells production. However, these mice

2373   exhibit strong T_{H1}-, T_{H2}-, and T_{H17}-cell response and there is a possibility that the low TSLP levels are a

2374   consequence of the disease and not part of the pathogenesis (Eri *et al.,* 2011).

2375

2376   In mice, the short TSLP isoform has not been described or annotated in RefSeq. However, the C-terminal

2377   region of mouse TSLP shares 40% identity with human sfTSLP so its effect during experimental colitis in

2378   mice was assessed. C57/BL6 mice were injected intraperitoneally with 200 μg of sfTSLP every other day.

2379   Treated mice with sfTSLP displayed significantly reduced weight loss and faster recovery compared to

2380   untreated mice. Other group of mice were injected intraperitoneally with sfTSLP 18 and 2 hours before

2381   injection with lipopolysaccharide (LPS). Prophylactic injection with sfTSLP led to a significant decrease of

2382   IL-6, IL-12/23p40, and IFN-γ levels in a dose-dependent manner, suggesting a protective role of sf TSLP

2383   during gut-induced inflammation.(Fornasa *et al.,* 2015)

2384

2385   Consistent with findings in mice, colonic epithelial cells from patients with Chron's disease (CD) who exhibit

2386   a strong T_{H1} response, have a lower expression of TSLP gene; whereas patients with ulcerative colitis

2387   (UC), where a T_{H2} response predominates, have a higher expression of the TSLP (Fornasa *et al., 2015;*

2388   Rimoldi *et al.,* 2005).

2389

2390   Expression of TSLP mRNA in intestinal mucosa was undetectable in nearly 70% of the patients with Crohn

2391   disease (six of nine patients). IECs from patients with Crohn disease are not able to control IL-12 release

2392   by bacteria-activated DCs and have a propensity to drive T_{H1} responses without stimulation (Rimoldi *et al.,*

2393   2005), Another *in vitro* study, compared supernatants from primary healthy intestinal epithelial cells to

2394   supernatants from intestinal epithelial cells from Crohn's disease patients in their capacity to stimulate DCs

2395   to induce Foxp3 expression in naive T cells. Only cells from healthy patients boost the capacity of

2396   CD103+ DCs to induce Foxp3 expression in naive T cells and this was attributed to the presence of TSLP

2397   (Spadoni *et al.,* 2012) In other study, expression of both TSLP isoforms were assessed in patients with

2398   ulcerative colitis and celiac disease (Fornasa *et al.,* 2015).

2399

2400   Intestinal mucosa from patients with ulcerative colitis (UC) (n = 13) was compared to healthy tissue (at least

2401   7 cm away from neoplastic tissue) of patients undergoing surgery for colon cancer (n = 26). Also, intestinal

2402   mucosa from patients with celiac disease (patients under treatment (gluten-free diet) n = 15; patients with

2403   active disease n = 13) was compared to intestinal mucosa from healthy subjects (n = 13) (Fornasa *et al.,*

2404   2015).

2405

It was found that ITSLP and TSLPR were significantly upregulated in tissues from patients with UC compared with levels seen in healthy colonic mucosa, whereas sfTSLP expression was unchanged (Fornasa *et al.*, 2015).

In coeliac disease, both isoforms were significantly downregulated in patients with untreated disease compared with those with treatment and biopsy specimen from healthy subject (Fornasa *et al.*, 2015)

These results suggest that TSLP is constitutively expressed by gut IECs and that pathological conditions may result in the deregulated expression of TSLP.

Genes such as chemokine receptor type 5 (CCR5), C-X-C motif chemokine 10 (CXCL10), IL-4), colony stimulating factor 1 (CSF1), chemokine ligand 16, 24, 2 (CCL16, CCL24, CCL2) and IL-6 are influenced by TSLP, and have been implicated in the pathogenesis of IBD, particularly in relation to the inflammatory response and epithelial damage (Ziegler *et al.*, 2013).

TSLP has been also studied in other types of intestinal inflammation. Mice models of food allergy and oral tolerance showed that TSLP enhances pathogenic allergic T_{H2} responses by acting directly on CD4$^+$T lymphocytes but it was not required for primary sensitisation or tolerance to food antigens (Noti *et al.*, 2013). Likewise, there is a strong association between a gain-of-function polymorphism in the gene that encodes TSLP and the development of eosinophilic esophagitis (EoE) in children (Sherrill *et al.*, 2010).

Collectively, all these studies demonstrate that during pathologic states up-regulation of TSLP, or induction of the long isoform in people, promotes a T_{H2}-inflammatory phenotype; whereas down-regulation of TSLP results in exuberant T_{H1} responses. However, there are not many studies that relate specifically to the TSLP isoforms. To date, it has shown that lfTSLP is upregulated in conditions such as atopic dermatitis, ulcerative colitis, EoE and smokeless tobacco- exposed oral mucosa (Fornasa *et al.*, 2015). Furthermore, it is now believed that conditions leading to a reduction in shTSLP expression could lead to an uncontrolled T_{H1} type of responses, such as in patients with CD. By contrast, when lfTSLP is upregulated, such as in patients with ulcerative colitis, a T_{H2} component is induced, presumably through NF-κB activation. Thus, the ratio sfTSLP:lfTSLP is important to the balance of the intestinal immune system (Fornasa *et al.*, 2015).

2437   The role of TSLP in dogs has been only investigated in atopic dermatitis, where TSLP has been shown to

2438   be upregulated in the skin of dogs with the disease compared to healthy dogs, but its expression and its

2439   role in the intestine has not been investigated yet (Klukowska-Rotzler *et al.*, 2013).

2440

2441   **1.2.1.3 The role of bacteria in Inflammatory Bowel disease**

2442

2443   Chronic enteropathies in dogs share several characteristics with inflammatory bowel disease (IBD) in

2444   people; IBD is a complex and common disease and several studies have been performed in regards to the

2445   role of gut microbiota and host-microbe pathways during and after disease (Cerquetella *et al.*, 2010).

2446

2447   In both species, dysbiosis is commonly found. However, it has been shown that the organisms that are

2448   implicated in disease in people do not necessarily appear to play a big a role in dogs. For example,

2449   *Fusobacterium* is considered harmful in people whereas in dogs is considered to have a protective role

2450   (Vazquez-Baeza, Hyde, Suchodolski, & Knight, 2016). *Fusobacterium* genus exhibits a higher abundance

2451   in carnivores, so differences in diet, physiology and genetics could play a role in this inverse relationship.

2452

2453   **1.2.1.3.1 The role of bacteria in inflammatory bowel disease in people**

2454

2455

2456   In people, there are two forms of IBD, Crohn's disease (CD) and ulcerative colitis (UC); CD is a more

2457   severe form of the disease and affects the small as well as the large intestine whereas UC only affects the

2458   large intestine.

2459

2460   *In vitro* studies of inflamed mucosal samples from patients with CD and UC have shown that co-culture with

2461   non-pathogenic *E coli* strains strongly stimulates the release of pro-inflammatory cytokines (TNF, INF-γ, IL-

2462   6, IL-23p35 and IL-17) and chemokines (IL-8, chemokine (C-X-C motif) ligand (CXCL)1 and CXCL2), and

2463   the activation of the inflammatory cascade. The inflammatory process also activates matrix

2464   metalloproteinases that in turn provoke matrix degradation, epithelial cell detachment and ulceration

2465   (Manichanh *et al.*, 2012).

2466

2467 Additionally, diversion of the faecal stream induces inflammatory remission and mucosal healing in the

2468 excluded intestinal segment, whereas infusion of the intestinal contents reactivates the disease in patients

2469 with CD (Manichanh *et al.,* 2012) (Wright *et al.,* 2015).

2470

2471 Initially, microbiome studies focused on patients with well-established IBD using only faecal samples.

2472 Although dysbiosis was identified, with decreased overall diversity and increased number of bacteria with

2473 pathogenic and pro-inflammatory potency (e.g. *E. coli*, *Salmonella*, *Proteus*, *Klebsiella*, and *Shigella*), no

2474 associations between disease severity/treatment and a single bacterial phyla/family were found (Manichanh

2475 *et al.,* 2012).

2476

2477 Later studies focused on longitudinal and new-onset patient populations, which have provided a lot of

2478 information. Gevers *et al.,* studied paediatric patients in the early stage of the disease, particularly Chron's

2479 disease, and focused on mucosal samples. Significant dysbiosis existed in these children even at early

2480 stages, and species diversity was inversely correlated with disease severity. Additionally, disease status

2481 correlated strongly with an increased abundance of Enterobacteriaceae, Pasteurellaceae, Veillonellaceae,

2482 and Fusobacteriaceae and decreased abundance of Erysipelotrichales, Bacteroidales, and Clostridiales.

2483 From this study, a formula called the microbial dysbiosis index (MD-index) which is calculated as the log of

2484 [total abundance in organisms increased in CD] over [total abundance of organisms decreased in CD] was

2485 developed. The MD-index strongly correlated with clinical disease severity, and could distinguish healthy

2486 subjects from patients with CD, but could not predict the most appropriate therapeutic approach or

2487 response to treatment (Gevers *et al.,* 2017).

2488

2489 **1.2.1.3.2 The role of the microbiota in chronic enteropathies in dogs**

2490

2491 Molecular studies have been conducted in different gastrointestinal diseases in dogs in order to unravel the

2492 organisms responsible for disease; however, identification of specific pathogens or dysbiosis has remained

2493 challenging. Findings of studies are variable depending on the method used, sample collected and

2494 disease.

2495

2496   - Studies performed using faecal samples

2497

2498   One study, using fluorescence *in situ* hybridization (FISH) probes, found Bacteroides counts to be

2499   significantly increased in Beagle dogs with chronic diarrhoea (Jia *et al.*, 2010). In contrast, using 454-

2500   pyrosequencing of the chaperonin 60 (cpn60) gene, a significant decrease of the proportion of

2501   Bacteroidetes was observed in dogs with diarrhoea (aetiology was varied and included bacterial, parasitic

2502   infections and undetermined origin) (Chaban, Links, & Hill, 2012). Other study, using terminal restriction

2503   fragment length polymorphism (T-RFLP) analysis and quantitative PCR, found an increased abundance of

2504   *Clostridium perfringes*, *Enterococcus faecalis* and *E. faecium* in dogs during diarrheic episodes (Bell *et al.*,

2505   2008).

2506

2507   Regarding IBD, results have been discordant as well. In one study, evaluation of faeces from dogs

2508   demonstrated that there were no significant differences between dogs with idiopathic IBD and control dogs

2509   (J. S. Suchodolski *et al.*, 2012).

2510

2511   Another study compared the faecal microbiota in dogs with IBD, dogs with intestinal lymphoma, and

2512   healthy dogs. Eight dogs with IBD (active disease), eight dogs with intestinal lymphoma, and fifteen healthy

2513   dogs were included in the study and samples were analysed by 16S rRNA gene next-generation

2514   sequencing. Dogs were from different breeds, privately owned and came from diverse environments. Dogs

2515   that had been treated with corticosteroids, antibiotics, or prebiotics/probiotics within 2 weeks prior to the

2516   study were excluded. When compared with healthy dogs, dogs with intestinal lymphoma had significant

2517   increases in organisms belonging to the Eubacteriaceae family. The proportion of the family

2518   Paraprevotellaceae and the genus *Porphyromonas* was significantly higher in dogs with IBD compared to

2519   healthy dogs, although their clinical significance and functions are not known yet. Within the phylum

2520   Actinobacteria, an increased proportion of *Actinomyces* was observed in IBD compared to intestinal

2521   lymphoma (Omori *et al.*, 2017). They suggest that the increase in Eubacteriaceae might be involved in the

2522   increased number of T_{regs} in canine intestinal lymphoma through overproduction of butyrate but further

2523   studies are needed.

2524

2525   A study by Minamoto *et al,* 2015 assessed the serum metabolite profiles and faecal microbiota in dogs with

2526   IBD before and after treatment (n=12) and compared these to healthy dogs (n=10). Microbial diversity was

2527   decreased in dogs with IBD (steroid-responsive dogs) compared to the healthy control dogs. No major

2528   differences in microbial communities were observed between the IBD-PRE and IBD-POST groups,

2529   although diversity decreased with treatment. Serum metabolites 3-hydroxybutyrate, hexuronic acid, ribose

2530   and gluconic acid lactone were significantly more abundant in dogs with IBD, suggesting oxidative stress in

2531   dogs with the disease compared to healthy controls. Changes in microbial diversity and serum metabolites

2532   persisted even in the face of clinical resolution of the clinical signs (Minamoto *et al.,* 2015).

2533

2534   In a recent study, faecal samples from 85 healthy dogs and 65 dogs with chronic signs of gastrointestinal

2535   (GI) disease, and inflammatory changes confirmed by histopathology were analysed and an increase of

2536   Gammaproteobacteria (specifically Enterobacteriaceae) was significantly associated with IBD; whereas

2537   Firmicutes such as *Clostridium* and *Ruminococcus* were associated with healthy samples. The authors

2538   created a faecal dysbiosis index (DI), similar to the paediatric MD that was negatively correlated with alpha

2539   diversity. This index was later developed as a qPCR panel that consisted of total bacteria (16S) and

2540   *Faecalibacterium, Turicibacter, Escherichia coli, Streptococcus, Blautia, Fusobacterium and Clostridium*

2541   *hiranis.* A negative DI indicates normobiosis, whereas a positive DI indicates dysbiosis. For a threshold of

2542   0, the DI based on the combined dataset achieved 74% sensitivity and 95% specificity to differentiate

2543   healthy from CE dogs (Vazquez-Baeza *et al.,* 2016).

2544

2545   -   Studies performed using intestinal mucosa samples

2546

2547   Again, results were discordant among studies. In one study, there was a difference in members of

2548   Proteobacteria between healthy dogs and dogs with IBD in duodenal biopsies, but not in faeces (J. S.

2549   Suchodolski *et al.,* 2012).

2550

2551   In German shepherd dogs with chronic intestinal inflammation, mucosal-adherent microbiota were analysed

2552   in small intestinal brush samples and showed significant over-representation of *Bacilli* and *Erysipelotrichi*

2553   when compared to healthy Greyhounds (Allenspach *et al.,* 2010). However, the results differed from other

2554   studies where a more diverse population of dogs with chronic intestinal inflammation was evaluated. In

2555   these studies, the most frequently observed changes in the mucosa-adherent microbiota in the small

2556   intestine were increases in members of the Proteobacteria, especially *Escherichia coli*-like organisms or

2557   Pseudomonas with concurrent decreases of members of Firmicutes and Bacteroidetes (J. S. Suchodolski,

2558   Xenoulis, Paddock, Steiner, & Jergens, 2010) (Xenoulis *et al.,* 2008).

2559

2560   Cassmann *et al.,* 2016 performed *in situ* hybridization analysis (FISH) in ileal and colon mucosal biopsies
2561   from dogs with CE and healthy dogs. They found that dogs with CE harboured more (P < 0.05) mucosal
2562   bacteria belonging to the *Clostridium-coccoides/Eubacterium rectale* group, Bacteroides,
2563   Enterobacteriaceae, and *Escherichia coli* compared to healthy controls; and the numbers of total bacteria
2564   adherent to the colonic mucosa were associated with clinical disease severity in CE dogs (P < 0.05)
2565   (Cassmann *et al.,* 2016).

2566

2567   **1.2.3 Treatment of Chronic enteropathies in dogs**
2568
2569
2570   Currently, treatments of chronic enteropathies in dogs are directed to either counteract the inflammation or
2571   the dysbiosis associated with the disease. The main therapeutic modalities consist of dietary, antibiotic and
2572   glucocorticoid treatments. The chosen therapeutic modality depends on the severity of the clinical signs
2573   and the lack of response to prior treatments. Usually, the first line of choice consists in the modification of
2574   the diet to a hypoallergenic or hydrolysed antigenic load. This is then followed by antibiotic treatment and
2575   finally followed by immunosuppressive treatment. According to the clinical response the affected animals
2576   can be classified into diet – responsive (DRE), antibiotic- responsive (ARE) and steroid-responsive (SRE)
2577   dogs.

2578

2579   **1.2.3.1 Diet-responsive enteropathy**
2580
2581
2582   Diet constitutes the first choice of treatment and many dogs appear to respond to this treatment, even if
2583   previous dietary trials have been unsuccessful. Diets can be homemade, hypoallergenic or hydrolysed
2584   (Mandigers, Biourge, van den Ingh, Ankringa, & German, 2010) (Marks, Laflamme, & McAloose, 2002).
2585   Many of the commercial diets also modify fibre, digestibility and other macronutrients in their intestinal
2586   diets. Dietary fibre supplementation is a well-known strategy to influence the concentration of SCFAs and
2587   gut microbiota. The type of the fibre ingested as well as the composition of the intestinal microbiota,
2588   determine which type of SCFA is produced. While resistant starch promotes the production of relatively
2589   more butyrate, pectin leads to more acetate and propionate production. Regarding the gut microbiota,
2590   bacteria of the Bacteroidetes phylum produce more acetate and propionate, whereas bacteria of the
2591   Firmicutes phylum predominantly produce butyrate (Macfarlane & Macfarlane, 2003).

2592

2593 Additionally, omega-6 fatty acids, especially arachidonic acid, are potentially pro-inflammatory, whereas

2594 omega-3 fatty acids, such as α-linolenic acid from plants and eicosapentaenoic acid and docosahexaenoic

2595 acid from fish, are anti-inflammatory (Calder, 2005).

2596

2597 In experimental IBD, using a model of chemically induced colitis, selenium supplementation prevented

2598 tissue damage through the upregulation of the expression of mitochondrial transcription factors: nuclear

2599 respiratory factor-1 and mitochondrial transcription factor-A; and by interfering in the expression of key

2600 genes responsible for inflammation (Tirosh, Levy, & Reifen, 2007).

2601

2602 Recent research has emphasised the profound effect that diet have on the gut microbiome. Long-term as

2603 well as short-term dietary changes impact the intestinal bacterial flora by influencing the substrata available

2604 for them (Albenberg & Wu, 2014). In recent years, although studies have evaluated the effect of diet on the

2605 microbiota of healthy dogs (Hang *et al.*, 2013) (Kerr, Forster, Dowd, Ryan, & Swanson, 2013) (Gonzalez-

2606 Ortiz, Castillejos, Mallo, Angels Calvo-Torras, & Dolores Baucells, 2013), the nature of these changes is

2607 not known in dogs with chronic enteropathies. In IgA-deficient German Shepherd Dogs with small

2608 intestinal bacterial overgrowth, dogs fed with diets supplemented with 1% fructo-oligosaccharides exhibited

2609 fewer aerobic/facultative anaerobic bacterial colony-forming units in fluid from the duodenum/proximal part

2610 of the jejunum, as well as in the duodenal mucosa (Willard et al., 1994).

2611

2612 Recently, Kalenyak *et al.* compared the intestinal mucosal microbiota in dogs diagnosed with idiopathic

2613 inflammatory bowel disease and dogs with food-responsive diarrhea before and after treatment. All dogs

2614 received a standardized elimination diet for 14 days. The elimination diet was a selected protein diet based

2615 on codfish and rice only, with codfish being a novel source of protein for all dogs enrolled in the study

2616 (specially produced by (Biomill SA, Granges-Marnand, Switzerland). If clinical signs improved significantly

2617 or resolved within the first 14 days of feeding the diet, dogs were assigned to the FRD group. If not, dogs

2618 received additional steroid treatment and were assigned to the IBD group (Kalenyak, Isaiah, Heilmann,

2619 Suchodolski, & Burgener, 2018).

2620

2621 Twenty-four dogs were included in the study: fifteen of these dogs responded to the dietary modification

2622 only (FRD group) and nine dogs needed additional immunosuppressant treatment (IBD group). The FRD

2623   group of dogs was reassessed 4 weeks after starting the elimination diet, whereas the IBD group of dogs

2624   was re-evaluated at 10 weeks after starting treatment with steroids (Kalenyak *et al.*, 2018).

2625

2626   Alpha diversity analysis did not show any significant difference between dogs with FRD and dogs with IBD

2627   neither in the duodenum nor colon, or within each disease group or before and after treatment Beta

2628   diversity analysis also did not revealed any significant difference between groups. Analysis of the specific

2629   bacterial taxa in dogs with FRD and dogs with idiopathic IBD showed a differential abundance of mainly

2630   bacteria belonging to the phylum of Proteobacteria (e.g. *Bilophila* in the duodenum, *Burkholderia* and

2631   Unclassified_Helicobacteraceae in the colon of FRD dogs; Unclassified_Neisseriaceae and

2632   Unclassified_Rhizobiales in the duodenum of IBD dogs) (Kalenyak *et al.*, 2018).

2633

2634   Regarding treatment status, in dogs with FRD, only *Bacteroides* showed a significant abundance after

2635   treatment. In dogs with IBD, there was an increase in the abundance of mainly members of the phylum

2636   Proteobacteria (unclassified genus of the family Neisseriaceae in the duodenum; unclassified genus of the

2637   family Oxalobacteraceae, and the genera *Citrobacter* and *Burkholderia* in the colon) and one member of

2638   the phylum Firmicutes (family Planococcaceae in the colon) before treatment. Only an unclassified genus

2639   of the family Bradyrhizobiaceae was found to be enriched in the duodenum of dogs with IBD post-treatment

2640   (Kalenyak *et al.*, 2018).

2641

2642   Although some bacteria such as Proteobacteria have shown inflammatory potential, other members with no

2643   pathogenic role have been reported; so the relevance of the findings remains unclear. Also, the washout

2644   period of two weeks could have an effect as some studies in humans have suggested a 4-week wash-out

2645   period (Langdon, Crook, & Dantas, 2016).

2646

2647   Thus, diet modification not only helps to alleviate the clinical signs associated with IBD, through

2648   modification of the gut microbiota, but also by modifying the inflammatory response itself and even the

2649   digestion process.

2650

2651   **1.2.3.2 Antibiotic- responsive enteropathy**

2652

2653   Antibiotics are commonly used in humans as well as in animals due to their effects on the intestinal

2654   microbiota. Antibiotic activity has a profound effect on the intestinal microbiota and it is important to

2655 characterise the changes in bacterial diversity and species richness, their function and the resilience of the

2656 intestinal microbiota against antibiotic related modifications (Thompson-Chagoyan, Maldonado, & Gil,

2657 2005).

2658

2659 Antibiotic therapy causes changes in the gut microbiota that are asymmetric, as some bacteria are more

2660 susceptible than others and fitness varies among microorganisms. Some individuals return to pre-treatment

2661 states, whereas other individuals have an alteration in the composition of the microbiota to alternative

2662 stable states. The magnitude of the disturbance, speed and extent of recovery depend on drug related

2663 factors such as class, pharmacokinetics, pharmacodynamics and range of action, as well as their dosage,

2664 duration and administration route; and on host-related factors such as the initial composition of the

2665 microbiota, age and lifestyle (Dethlefsen, Huse, Sogin, & Relman, 2008). Studies in people have shown,

2666 that most of the taxa returned to baseline within 30 days of cessation of antibiotic treatment. However,

2667 some individuals failed to recover for up to 6 months (De La Cochetiere *et al.,* 2005).

2668

2669 In dogs, there is limited information about the effect of antibiotics on the intestinal microbiota. Suchodolski

2670 *et al* (2009), studied the effect of the macrolide antibiotic tylosin on the microbial diversity in the canine

2671 small intestine of healthy dogs (n=5, Beagle dogs). The response to tylosin administration varied among

2672 each dog, however there was a persistent reduction in diversity and bacterial richness in 40% of dogs and

2673 changes in GI microbiota were not reversed at 4 weeks (J. S. Suchodolski *et al.,* 2009).

2674

2675 Another study assessed the effect of metronidazole on the microbiota of healthy dogs using next-

2676 generation sequencing (Illumina MiSeq). Metronidazole is also often used as part of treatment of intestinal

2677 diseases. The drug was administered twice daily at 12.5 mg/kg to a group of five dogs for 14 days. Faecal

2678 samples were collected before and after administration (day 0 and 14), and 14 and 28 days after cessation

2679 (day 28 and 42). Metronidazole induced an alteration of the intestinal microbiota, noticeable at day 14. The

2680 proportions of Bacteroidaceae, Clostridiaceae, Fusobacteriaceae, Lachnospiraceae, Ruminococcaceae,

2681 Turicibacteraceae, and Veillonellaceae decreased, while Bifidobacteriaceae, Enterobacteriaceae,

2682 Enterococcaceae, and Streptococcaceae increased and turned to their initial proportions by day 42, after a

2683 2-weeks of cessation period (Igarashi *et al.,* 2014).

2684

2685   These studies have focused on healthy dogs, but currently it is not known what effects antibiotics have

2686   during periods of intestinal disease, where the bacterial composition and the microenvironment may be

2687   profoundly altered. Because of the nature of an ecosystem, the changes that are induced by an antibiotic

2688   on a set of organisms will affect directly or indirectly others. So, the initial microbial structure will shape the

2689   changes in microbiota during the antibiotic course.

2690

2691   Along those lines, a recent study in dogs with CE showed that a combination therapy of metronidazole at

2692   20 mg/kg q12 h and prednisone at 1 mg/kg/day administered for 60 days followed by a 30-day washout

2693   interval did not alter the proportions of several bacterial groups (as detected with quantitative PCR),

2694   including   Bacteroidetes,   Firmicutes,   Fusobacteria, *Bifidobacterium*,

2695   *Lactobacillus*, *Faecalibacterium*, *Escherichia coli*, and *C. perfringens*. However, the effect was assessed 30

2696   days following discontinuation of treatment, so short-period effects were not excluded (Rossi *et al.*, 2014).

2697

2698   Antibiotics have been proven to be efficacious in inducing and maintaining IBD remission in people so it

2699   can be suggested that modifying the pattern of intestinal bacteria may change intestinal disease status.

2700   Some antibiotics provide a so-called 'eubiotic' effect, by increasing abundance of beneficial bacteria

2701   (Gevers *et al.*, 2017). However, the long-term exposure of antibiotics can lead to side effects as severe

2702   diarrhoea, abdominal pain or drug-related allergies and the potential risk of developing microbial resistance

2703   or nosocomial infections (Dethlefsen *et al.*, 2008).

2704

2705   Additionally, the use of antibiotics is highly controversial due to the emergence of widespread antimicrobial

2706   resistance. Currently, the European Crohn's and colitis organisation (ECCO) guidelines for UC advise the

2707   use of antibiotics only if infectious complications are suspected or ongoing, and before surgical

2708   interventions. The ECCO-European Society for Paediatric Gastroenterology Hepatology and Nutrition

2709   (ESPGHAN) guidelines for the management of paediatric IBD has similar recommendations. Similarly,

2710   current ECCO guidelines do not recommend the use of antibiotics for uncomplicated CD, but only in the

2711   case of sepsis, abdominal or perianal abscess, or bacterial overgrowth. The British Society of

2712   Gastroenterology (BSG) guidelines suggest the use of antibiotics mainly for secondary complications in

2713   IBD; nevertheless, the use of antibiotics in short-term treatment of colonic CD may be used for patients with

2714   refractory disease or contraindications to other therapeutic options supported by stronger evidence.

2715

2716 ## 1.2.3.2.1 Use of Oxytetracycline during intestinal inflammation
2717

2718 Oxytetracycline is a drug that is used in dogs with chronic enteropathies. Oxytetracycline belongs to the
2719 tetracycline family, which is divided into three main groups consisting of the natural product, semi-synthetic
2720 compounds and chemically modified tetracyclines. Oxytetracycline belongs to the first group and it is a
2721 natural product of the metabolism of *Streptomyces* spp; it is a broad-spectrum antibiotic used in infections
2722 caused by both gram-negative and gram-positive bacteria. However, the widespread use of oxytetracycline
2723 in the past has resulted in an increase in the acquired resistance by many bacterial groups and nowadays
2724 its use is limited to few clinical circumstances such as rickettsia infections and chronic enteropathies
2725 (Plumb, 2011).

2726

2727 Oxytetracycline is a bacteriostatic agent that inhibits cell growth by blocking transduction; it binds to
2728 receptors of the 30S ribosomal bacterial subunit and prevents animo-acyl tRNA from binding to the A site of
2729 the ribosome. This binding prevents the addition of amino acids to the elongating peptide chain, blocking
2730 protein synthesis. Tetracyclines are also believed to reversibly bind to the 50S ribosomal subunit and alter
2731 cytoplasmic membrane permeability in susceptible organisms. In high concentrations, tetracyclines can
2732 also inhibit protein synthesis in mammalian cells (Giguere, 2006) (Plumb, 2011).

2733

2734 ## 1.2.3.2.1.1 Pharmacokinetic properties
2735
2736
2737 In dogs, most of the tetracyclines are absorbed from the gastrointestinal tract but systemic availability can
2738 vary widely among oral preparations. Most of the absorption takes place in the stomach and upper segment
2739 of the small intestine. The absorption of oxytetracycline can be decreased significantly by the presence of
2740 food, particularly milk and its products, with reductions of 50% of more. The presence of bivalent and
2741 trivalent cations (calcium, magnesium, iron, aluminium) in the stomach, decrease the absorption by
2742 chelating tetracyclines. Oxytetracycline is approximately 20-25% bound to plasma proteins. The volume of
2743 distribution in approximately 2.1 L/kg in small animals (Giguere, 2006) (Plumb, 2011).

2744

2745 Because of chelation with calcium, tetracyclines become bound at active sites of ossification and in
2746 developing teeth. Tetracyclines can cross the placenta, enter the foetal circulation and amniotic fluid, and
2747 can be secreted in milk, where they reach concentrations approximately those of serum (Plumb, 2011).

2748

2749   Oxytetracycline is excreted unchanged primarily via urine; as glomerular filtration is the mechanism of

2750   excretion, impaired renal function can increase its elimination half-life. Additionally, oxytetracyclines can be

2751   excreted via biliary and nonbiliary routes into the gastrointestinal tract where they undergo enterohepatic

2752   circulation, with most of the compound excreted in bile being reabsorbed from the intestine. This process

2753   contributes to the half-life of 6 -10 hours. The portion of the drug that remains in the gastrointestinal tract

2754   may become inactive after chelation with faecal materials (Plumb, 2011).

2755

2756   The exact role of the antimicrobial properties of oxytetracycline in CE is not known. There may be a direct

2757   effect on intestinal pathogens, or a more general modulation of the intestinal microbiota, with subsequent

2758   improvement of digestion and absorption by altering the production of bacterial metabolites. Likewise,

2759   oxytetracycline may decrease the competition for nutrients and vitamins, alter the cross talk with the

2760   intestinal immune system and modify cellular metabolism that can lead to an improvement of the clinical

2761   signs. It has been shown that the proposed mode of action of an antibiotic on different bacterial genera *in*

2762   *vitro* does not necessarily match the *in vivo* effects (Walsh, Guinane, O'Toole, & Cotter, 2014).

2763

2764   In addition to its antimicrobial properties, studies in vivo and in vitro have shown that tetracyclines can

2765   affect many cellular functions and depending on the member, they have shown to have antioxidant, anti-

2766   inflammatory, angiogenic and anti-apoptotic properties. All these effects together with their antimicrobial

2767   properties mean oxytetracycline may be beneficial in diseases such as IBD.

2768

2769   Most of the studies conducted so far, have used tetracyclines belonging to the second and third group, so

2770   little information is available regarding the non-antimicrobial effects of oxytetracycline. The transcription

2771   factor NF-κB is considered a master regulator of inflammation and immune responses; NF-κB activity is

2772   controlled by chemical modifications such as phosphorylation and by interactions with other proteins,

2773   notably members of the IκB kinase family (Ci *et al.*, 2011). In a model of allergic airway inflammation in

2774   mice, oxytetracycline inhibited phosphorylation and degradation of IκBα, which then depressed NF-κB p65

2775   translocation from the cytoplasm to the nucleus. Additionally, in this model oxytetracycline treatment

2776   significantly decreased the concentration of interleukin IL-4, IL-5 and IL-13; reduced the expression of the

2777   chemokines chemokine (C-C motif) ligands (CCL) such as CCL11, of the chemokine receptors type 1

2778   (CCR1) and type 3 (CCR3); decreased the level of phospho-Akt which is a direct downstream effector of

2779 phosphoinositide 3-kinase (PI3K) and decreased the inflammatory cell infiltration in the airways. It is

2780 important to highlight that the dose used in those experiments was much higher than the one used in

2781 clinical practice (Ci *et al.,* 2011).

2782

2783 Studies conducted in a rat model of Trinitrobenzene sulfonic acid (TNBS) colitis showed divergent results in

2784 the preventive and curative effect of the different members of the tetracycline family. In the preventive

2785 protocol, antibiotic administration (minocycline or tetracycline) was started 1 week before TNBS instillation

2786 and continued up to the day before the sacrifice, which took place 2 days after the induction of the colitis. In

2787 the curative protocol, the antibiotic was administered from the day of the colitis induction to 7 days after the

2788 induction of the colonic damage. Macroscopic and microscopic analysis of the colonic samples showed

2789 that only minocycline had an evident anti-inflammatory effect. However, colonic myeloperoxidase (MPO)

2790 activity, a marker of neutrophil infiltration, was reduced by both antibiotics and the colonic depletion of the

2791 antioxidant peptide glutathione was counteracted by both antibiotic treatments. The assessment of

2792 inflammatory cytokines revealed that tetracycline reduced TNF-α, IL-6 and IL-1B, although to a lesser

2793 extent than minocycline. Both antibiotics down regulated the expression of the chemokines: monocyte

2794 chemotactic protein-1 (MCP-1), cytokine-induced neutrophil chemoattractant-1 (CINC-1) and intercellular

2795 adhesion molecule-1 (ICAM-1) in the same manner. Although tetracycline and minocycline have a similar

2796 antimicrobial spectrum, tetracycline didn't show the same efficacy in ameliorating the intestinal

2797 inflammation at a macro or microscopic level, even though there were changes to some inflammatory

2798 cytokines and chemokines. The effects were only evident in the curative protocol not in the preventive one

2799 (N. Garrido-Mesa et al., 2011).

2800

2801 Studies *in vitro* of Caco- 2 cells (human epithelial colorectal adenocarcinoma) and in RAW 264.7 cells

2802 (mouse macrophages) showed that minocycline reduced the levels of IL-8, IL-17 and nitric oxide synthase

2803 (iNOS) expression, whereas tetracycline did not significantly affect this cytokine production. The T_{H17}

2804 pathway and its related cytokines IL-23 and IL-17 have been described to play a key role in the

2805 development of chronic intestinal inflammation; IL-17 contributes to neutrophil migration, expansion and

2806 function, and enhances dendritic cell maturation, T cell priming and the production of inflammatory

2807 mediators from different cell types. Furthermore, IL-17 can synergise with other cytokines to stimulate the

2808 release of additional pro-inflammatory cytokines. When the barrier function was studied, only minocycline

2809   reversed the decline of colonic mucus thickness during colitis. It increased the expression of both mucin 2

2810   (MUC-2) and trefoil factor 3 (TFF-3) (N. Garrido-Mesa et al., 2011).

2811

2812   Tetracyclines can combat oxidative stress, mop up free radicals and inhibit an excessive inflammatory

2813   response secondary to antigenic stimulus. Bacterial LPS causes marked upregulation and release of IL-β1,

2814   tumour necrosis factor alpha (TNF-α) and nitric oxide (NO), and this can be inhibited by minocycline.

2815   Investigation conducted in human aortic smooth muscle cell showed that doxycycline was more effective in

2816   inhibiting matrix metallo-proteinases (MMPs) than minocycline, by upregulating the MMP inhibitor tissue

2817   inhibitor of metallo-proteinase-1 (TIMP-1) (Garcia-Alvarez & Oteo, 2010) (Soory, 2008).

2818

2819   Minocycline and doxycyclines have been shown to inhibit angiogenesis by preventing endothelial growth

2820   and activity of collagenase. Inhibition of syntheses of MMP-8 and of MMP-9 by endothelial cells in response

2821   to doxycycline and to a lesser extent by the chemically modified tetracyclines (CMTs) has been

2822   demonstrated at the mRNA level. Elastin degradation and MMP activity are reduced by doxycycline in a

2823   model representing aneurismal disease. In a cell culture model of corneal epithelial cells treated with LPS,

2824   doxycycline inhibited the degree of formation of IL-1β to an extent that was like that of corticosteroids; it

2825   also prevents endotoxemia *in vivo.* Doxycycline can cause dose dependent reduction in the production of

2826   cytokines IL-1β, IL-6, TNF-α and interferon gamma (IFN-γ) (Soory, 2008).

2827

2828   Therefore, these studies lead to questions regarding whether the actual therapeutic effect of oxytetracycline

2829   in dogs with chronic enteropathies is due to its antimicrobial properties or its anti-inflammatory properties.

2830

2831   **1.2.3.3 Steroid-responsive enteropathy**

2832

2833

2834   Glucocorticoids (GCs) constitute potent anti-inflammatory agents and are first-line therapeutics for the

2835   induction of remission in moderate to severe IBD. In dogs, the most common corticosteroids used in IBD

2836   are dexamethasone, budesonide, prednisone and prednisolone, with the latter the most commonly used (K.

2837   W. Simpson & Jergens, 2011).

2838

2839   The same study that evaluated the effect of metronidazole in healthy dogs also assessed the effect of

2840   prednisolone in the faecal microbiota of five healthy dogs. Prednisolone was administered at a dose of 1.0

2841   mg/kg for 14 days. No effect of prednisolone was observed in either bacterial diversity or phylogeny

2842   (Igarashi *et al.*, 2014).

2843

2844   The effect of prednisolone on gut microbiota during disease was also evaluated but in conjunction with

2845   metronidazole in dogs with IBD. After 30 days following cessation of treatment, no changes were observed

2846   in major bacterial groups. However, higher dosages are occasionally used in animals and that could have

2847   an effect in the gut microbiome or reduction of the inflammatory response could impact on bacterial

2848   composition (Igarashi *et al.*, 2014).

2849

2850   ### 1.2.3.3.1 Mechanism of action
2851
2852
2853   Glucocorticoids mediate their actions by binding the intracellular glucocorticoid receptor GRα present in the

2854   cytoplasm of cells. This allows the formation of a homodimer of two activated GRs which is transported into

2855   the nucleus and mediates the transcriptional regulation of specific target genes by binding to specific DNA

2856   sequences that enhance or inhibit the promoter region of certain genes (Farrell & Kelleher, 2003).

2857

2858   The cellular effects of GCs are dose dependent. A low doses GCs exert an anti-inflammatory function,

2859   inhibiting phospholipase A2, the release of pro-inflammatory cytokines and stabilize the granulocyte cell

2860   membranes. In patients with IBD, it has been shown that glucocorticoids induce the expression of potent

2861   anti-inflammatory molecules such as IL-10, annexin 1 and the inhibitor kappa B alpha (IκBα) and repress

2862   the expression of several genes such as nuclear factor κB (NF-κB) and activator protein 1 (AP-1), that

2863   constitute potent pro-inflammatory molecules (Feldman E, 2004) (Viviano, 2013).

2864

2865   At high doses, GCs exert an immunosuppressive effect and impair macrophage function by down

2866   regulating Fc receptor expression, decreasing responsiveness to antibody-sensitized cells and decreasing

2867   antigen production. GCs suppress T-cell function and induce apoptosis of T cells and with chorine use B-

2868   cell antibody production may be inhibited in some dogs (Viviano, 2013).

2869

2870   In people, it has been shown that besides its anti-inflammatory properties, glucocorticoid therapy restores

2871   increased intestinal permeability in collagenous colitis. Previous assumptions considered that it was a

2872   secondary effect to the overall attenuation of the inflammatory response. However, studies in various

2873 endothelial and non-intestinal epithelial cell lines provided evidence for the ability of glucocorticoids to
2874 stimulate tight junction sealing in the absence of inflammatory stimuli. At the intestinal level, a recent report
2875 described upregulation of multiple tight junction proteins by corticosteroids in immature enterocytes as part
2876 of their well-known ability to promote intestinal maturation (Fischer *et al.*, 2014).   Another *in vitro* study
2877 conducted in Caco -2 cells showed that under normal conditions, corticosteroids did not have a significant
2878 effect on barrier function but that under the effect of TNF-α, corticosteroid helped to restore the permeability
2879 and epithelial barrier. Glucocorticoids also inhibit the transcription of the myosin light chain kinase (MLCK);
2880 MLCK catalyses the phosphorylation of myosin light chains that stimulates the energy-driven contraction of
2881 the prejunctional actinomyosin ring thereby opening tight junctions and increasing permeability.
2882 Additionally, it has been shown that glucocorticoids promote the production of mucus that can help to
2883 restore the epithelial intestinal barrier (Boivin *et al.*, 2007).

2884

2885 A defective intestinal epithelial tight junction barrier has been proposed as a crucial factor that contributes
2886 to or triggers intestinal inflammation. In people, clinical studies have shown that defects in the epithelial
2887 barrier in patients with CD precede the onset of active disease and are a positive predictive factor for early
2888 recurrence after treatment. Additionally, a direct correlation exists between normalisation of intestinal
2889 permeability and clinical improvement (Boivin *et al.*, 2007).

2890

2891 **1.2.3.3.2 Pharmacokinetic properties**
2892

2893 Glucocorticoids vary in their potency, route of administration and duration of action. Prednisolone is an
2894 intermediate acting corticosteroid with a biologic half-life of 12-36 hours and is administered orally.
2895 Prednisone is a prodrug that is metabolised to its active form (prednisolone). Glucocorticoids are unbound
2896 to plasma proteins. Prednisolone has four times the anti-inflammatory activity of endogenous cortisol but
2897 only 0.3 the mineralocorticoid activity (Feldman E, 2004) (Plumb, 2011).

2898

2899 **1.2.3.3.3 Side-effects**
2900
2901
2902 As every cell in the body possesses glucocorticoid receptors, the use of these drugs can induce multiple
2903 side effects that depend on the dosage, type of glucocorticoid administered and duration of the treatment.
2904 This detrimental side effects prevent their long-term use in many animals, and many become resistant to
2905 glucocorticoid therapy (Feldman E, 2004) (Plumb, 2011) (Black, 1988). (Table1.1)

2906   Table 1.1: Side-effects of corticosteroids. ACTH: Adrenocorticotropic hormone, TSH: Thyroid-stimulating

2907   hormone, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, T4: Thyroxine, T3: Triiodothyronine,

2908   ADH: Vasopressin, ALT: Alanine aminotransferase C-ALP: corticosteroid- alkaline phosphatase.

2909

Body System	Side-effect
Cardiovascular	Reduction capillary permeability
	Vasoconstriction
	Increase blood pressure
	Increase fragility of blood vessels
Nervous system	Lower seizure threshold
	Decrease response to pyrogens
	Stimulation of appetite
Endocrine	Suppression ACTH, TSH, FSH, LH and prolactin
	■ Males: testicular atrophy, decreased libido
	■ Females: Anestrus, clitoral hypertrophy
	Reduction in conversion of T4 to T3
	■ Sublinical hypothyroidism
	Inhibition of insulin binding to insulin-receptors
	■ Diabetes Mellitus (long-term)
Renal	Interference with the activity of ADH
	Inhibition COX-1
	■ Polyuria- Azotemia
Haematology	Neutrophilia
	Monocytosis
	Lymphopenia
Metabolic electrolytic	- Influence water and electrolyte balance (mineralocorticoid activity)
	Liver
	■ Increase gluconeogenesis
	■ Hepatomegaly
	■ Increase ALT, C-ALP
	Increase lipolysis

	▪ Hypercholesterolemia
Musculoskeletal	Protein catabolism
	Bone and cartilage growth inhibition
Ophthalmic	Increase intraocular pressure
	Cataracts
	exophthalmos
Gastrointestinal	Ulceration
	Inhibition ulcer healing
Dermatologic	Skin atrophy
	Alopecia
	Abdominal enlargement
	Inhibition healing process
	Calcinosis cutis

2910

2911

2912

2913   Despite the long list of clinically significant side effects associated with the use of glucocorticoids, the
2914   effective modulation of both the innate and acquired immune systems and their relative rapid onset of
2915   action make them the first choice in dogs with severe clinical manifestations of CE. Glucocorticoids given
2916   for days or weeks rarely have prolonged significant clinical effect if administered correctly. However, the
2917   degree of individual variation is response to GC administration among dogs and cats is significant so each
2918   animal must be evaluated independently.

2919

2920   Conversely, glucocorticoids inhibit cyclooxygenases, which in turn suppress the production of
2921   prostaglandins and can lead to damage to the intestinal mucosa (Black, 1988). It is also unknown how the
2922   glucocorticoids affect the microbiota. Maybe, the decrease of inflammation could favour a friendly
2923   environment for commensal bacteria or the restoration of the mucosal barrier impedes pathogenic bacteria
2924   penetrating into the mucosa. *Escheria coli*, particularly adherent-invasive *E. coli* strains are enriched in
2925   patients with ulcerative colitis. The increase in Enterobacteriaceae may indicate the preference of this clade
2926   for an inflammatory environment. In boxers with granulomatous colitis, mucosa adherent-invasive *E. coli*,
2927   play a key role in the pathogenesis of the disease. However, in boxers, the persistence of E.coli is
2928   associated with a neutrophil killing defect, and so immune suppression is not effective (Craven, Mansfield,
2929   & Simpson, 2011).

2930

2931   **1.3 Specific objectives and Hypothesis of the project**
2932
2933   Most of the studies done to date, have characterized the microbiota only at a single time-point. Due to the
2934   large amount of factors that can influence the bacterial diversity in a given individual, it is difficult to
2935   correlate specific microbial signatures with disease. In these cases, longitudinal studies that relate changes
2936   of the microbial community structure with the individual biology, have the potential to offer a better
2937   overview of the changes in the microbiota associated with health, disease, treatment and remission
2938   periods.

2939   The project has the potential to help determine how the gut microbiota is established, how it evolves over
2940   time and how stable it is during health. Additionally, has the potential to determine whether the microbial
2941   composition (especially immunoglobulin-coated bacteria) predicts subsequent risk of activity flares, whether
2942   the luminal flora predicts response to therapy and whether the treatments help to restore the core

2943   microbiome in the host. Clinical improvement is not always followed by significant improvement of the
2944   histopathologic lesions, so maybe changes in the microbiota could be related to improvement.

2945

2946   The aims of the projects are:

2947

2948   • To assess the development of the microbiota from birth in puppies, and its association with the
2949   maternal microbiome. We hypothesize that (1) at birth puppies exhibit a low diversity and stability
2950   in their gut microbiota that increases as the puppies grow. (2) The gut microbiota of puppies is
2951   closely associated with the maternal faecal and oral microbiota. (3) Weaning and introduction of
2952   solid food are key determinants that drive the maturation of the gut microbiota into an adult–like
2953   phenotype.

2954

2955   • To assess the dynamics and stability of the microbiota over time and age in healthy dogs. We
2956   hypothesize that (1) the diversity and stability of the microbiota vary according to age, reaching its
2957   maximum complexity and stability in adulthood. (2) The maximum complexity and stability is
2958   reached in adulthood. (3) There are permanent fluctuations in microbiota composition over time. (4)
2959   The stability and diversity of the microbiota declines in senior dogs.

2960

2961   • To characterise highly immunoglobulin A and G-coated bacteria in dogs with chronic enteropathies.
2962   We hypothesize that (1) dogs with CE possess a higher proportion of highly coated IgA-bacteria
2963   compared to healthy dogs and they can distinguish between members of the microbiota that impact
2964   disease susceptibility or severity in dogs. (2) Resolution of the clinical signs is associated with the
2965   eradication of these highly coated IgA-bacteria. (3) Ig+ bacteria in faeces are similar to bacteria
2966   found in small intestinal mucosa and cytology brush.

2967

2968   • To characterise the disease-associated changes in the microbiome and the relationship between
2969   the treatment, the microbiome and the resolution of the clinical signs. We hypothesize that (1) the
2970   intestinal microbiota of dogs with CE during periods of remission is the reflection of the core
2971   microbiota in pre-clinical states and differs from healthy individuals. (2) The intestinal microbiota of
2972   dogs with chronic enteropathy during periods of remission is highly unstable and could predispose
2973   to relapse of the clinical signs.

2974

2975   • To characterise the expression of TSLP in the intestine and its correlation with disease activity. We

2976   hypothesize that (1) TSLP is constitutively expressed in the intestine of dogs in response to

2977   commensal bacterial colonization. (2) TSLP expression is deregulated in dogs with CE. (3) TSLP

2978   activity is correlated with disease severity and remission of clinical signs.

2979

2980

2981

2982   **Chapter 2: Characterisation of gut microbiota at different age stages and its stability over time**

2983

2984   **2.1 Introduction**

2985

2986   The gut microbiota is essential for maintaining health as exerts several beneficial effects on the host and

2987   interacts with various organs and systems in the body, including the brain, liver, bone and cardiovascular

2988   system. However, it can cause harmful effects and it has been implicated to multiple conditions such as

2989   diabetes, IBD and even behavioural disorders (Hooda, Minamoto, Suchodolski, & Swanson, 2012; Hooper

2990   & Macpherson, 2010). Currently, it is not known whether the changes in the microbiota are primary and

2991   could be considered as causative factors of the condition (Manichanh *et al.,* 2012) or whether they are a

2992   consequence of the disease itself.

2993

2994   The first step in understanding the symbiotic relationship between gut microbes and their host consists in

2995   the characterization of the baseline healthy microbiota.

2996

2997   Various host factors such as genetic background, age, sex, environmental exposures and diet contribute to

2998   the development and maintenance of the core intestinal microbiota (Forbes *et al.,* 2011). Marked age-

2999   associated changes in the gut microbiota occur throughout the life of an individual human. In infants, the

3000   gut microbiota is quite volatile (exhibit fluctuation in the number and genera) and low in richness, with an

3001   increase in diversity and stability over the first three years. In adults the gut microbiota is highly diverse and

3002   stable whereas in the elderly, stability and diversity decrease again; (Lozupone, Stombaugh, Gordon,

3003   Jansson, & Knight, 2012).

3004

3005   Although there have been some studies in dogs (Handl *et al.,* 2011) (Buddington, 2003; Guard et al., 2017),

3006   the effects of age on the gut microbiota and how stable it is at different age-stages is not known. It is crucial

3007   to define the healthy gut microbiota, not only to understand the biological significance of the different

3008   patterns of microbial colonisation associated with disease, but for the establishment of new diagnostic,

3009   therapeutic and preventive strategies based on the gut microbiota. In people, the so-called "First thousand

3010   days", represent a true window of opportunity for microbiota reprogramming towards a healthy direction

3011   (Arboleya et al., 2018).

3012

3013　The aims of this study are to (1) Characterise the dynamics, stability, richness and evenness of the gut

3014　microbiota at different age-stages and to (2) characterise the succession of the microbiota in puppies.

3015

3016　　**2.2 Methodology**

3017

3018　**2.2.1 Animals**

3019

3020　Faecal samples from a total of twenty-nine healthy dogs were analysed. Adult dogs were privately owned

3021　and lived in diverse home environments and were on a variety of commercial diets. Most of the dogs

3022　belonged to staff of the University of Melbourne with the exception of dog1 (group 3_12 month). Dog 2 and

3023　3 from the group of young adults (1_7 year) and dog 1 and 2 from the group of senior dogs (7_10 year)

3024　were related (brother and sister). Criteria of inclusion included up to date vaccination and deworming

3025　status, and no signs of gastrointestinal disease or medication within the previous three months. Owners

3026　were requested to fill in a questionnaire at the beginning of the study, were asked to maintain the diet and

3027　daily routine as constant as possible and keep a record of any change during the duration of the study. We

3028　collected detailed information regarding diet (type, treats, changes within the previous three months,

3029　coprophagia, rubbish) health status, previous diseases, travel history, level and type of exercise, body

3030　condition score and increase or decrease in body weight in the previous three months. The level of

3031　exercise was considerate mild (walkings less than 30 minutes per day),moderate (walkings more than 30

3032　minutes per day or running twice per week) and severe (running every day or more than three times per

3033　week) Animals were divided in 5 groups according to age: from 1 day to 10 weeks old (Puppies), from 3

3034　months old to 1 year (Growth), from 1 to 7 year old (Young adult); from 8 to 10 years old (Mature) and older

3035　than 10 years old (Senior). Two litters were followed from birth until 10 weeks of age. Detailed information

3036　of the mothers was obtained as mentioned above, including previous pregnancies and size of the litters.

3037　Mothers were antibiotic-free in the previous three months. For detailed information, see tables 2.1, 2.2 and

3038　2.3. The diet transition scheme in puppies consisted in exclusive milk during the first three weeks, then a

3039　combination of milk ad libitum and Advance puppy growth® during 2 weeks, then milk once to twice per day

3040　and Advance puppy large growth® during 1 week, after which they are completely weaned. Owners gave

3041　written consent and were able to withdraw their animals from the trial at any point.

3042　All experimental procedures were approved from the Animal Ethic Committee of University of Melbourne.

3043　(Animal Ethics Committee approval AEC # 1413272.1).

3044

3045

3046

3047   Table 2.1: Metadata information of mothers
3048

Animal	Breed	Age	Diet	Worming	Previous litters
	Litter 1				
Mother	Labrador Retriever	5 years	Advance adult active/ transition to advance puppy growth®	Popental®	2 litter (8-6 puppies)
	Litter2				
Mother	Labrador Retriever	2 years	Advance adult active/ transition to advance puppy growth®	Popental®	1 litter (6 puppies)

3049

3050

3051

3052

3053

3054

3055   Table 2.2: Metadata information of puppies

Animal	Breed	Delivery method	Sex/neutering status	Diet
Litter1				
Puppy 1a	Labrador Retriever	Vaginal	Male/Entire	Breast-feeding
Puppy 1b	Labrador Retriever	Vaginal	Female/Entire	Breast-feeding
Puppy 1c	Labrador Retriever	c-section	Male/Entire	Breast-feeding
Puppy 1d	Labrador Retriever	c-section	Male/Entire	Breast-feeding
Puppy 1e	Labrador Retriever	c-section	Female/Entire	Breast-feeding
Litter2				
Puppy 2a	Labrador Retriever	Vaginal	Female/Entire	Breast-feeding
Puppy 2b	Labrador Retriever	Vaginal	Female/Entire	Breast-feeding
Puppy 2c	Labrador Retriever	Vaginal	Female/Entire	Breast-feeding
Puppy 2d	Labrador Retriever	Vaginal	Female/Entire	Breast-feeding
Puppy 2e	Labrador Retriever	Vaginal	Female/Entire	Breast-feeding

3056

3057

3058

3059

3060   Table 2.3: Metadata Information of growth, adult, senior and mature dogs
3061

Animal	Breed	Age	Sex/neutering status	BCS	Diet	Treats	Coprophagia/ rubbish	Worming	Level of exercise	Travel history	Other pets at home
	Dog 10w -1y										
Dog1	Havanese	6m	Female/entire	4/9	Artemis small breed puppy®	Chicken wings/necks (once per week) Liver treats (three small pieces per day)	NO	Drontal®	Moderate	None	1 ferret
Dog2	Australian kelpie cross	10m	Female/spayed	5/9	Hills puppy®	Dental chews 1/ once per month	Dog and rabbit faeces	Dontral®	Moderate	None	None
Dog3	Pomeranian x terrier cross	11m	Female/spayed	4/9	Advance dry food®	meat	NO	Drontal®	Moderate	None	None
Dog5	Griffon Bruxellois	12w	Male/entire	4/9	Advance puppy®	Chicken, liver treats Small piece/three times per week	Other dog's food	Drontal®	Moderate	None	1 dog,2cats,2 guinea pigs
	Dogs 1-7 y										
Dog1	Labrador Retriever	2	Male/neutered	4/9	J/d hills®	Pig ears, biscuits, cooked meat. 1 per day	NO	Drontal®	High	National	1dog, 2 cats,1 guinea pig
Dog2	German wirehaired pointer	6	Male/entire	4/9	Advance dry food®	Biscuits, raw meat, chicken necks, sardines Amount varies according to activity	Cow manure, kangaroo	Interceptor®	Moderate	National	2 dogs
Dog3	German wirehaired pointer	1	Female/entire	4/9	Advance dry food®	Biscuits, raw meat, chicken necks, sardines Amount varies according to activity	NO	Interceptor®	Moderate	National	2 dogs

Dog4	Terrier cross	5	Female/spayed	3/9	Hills adult light®	Pig ears, biscuit 1/twice per week	NO	Drontal®	Intense	Europe	2 cats
Dog5	Nova Scotia Duck Tolling Retriever	3	Female/spayed	5/9	T/d Hills®	Pig ears 1/once per week	NO	Sentinel®	Intense	None	None
Dog6	Cairn Terrier	5	Female/spayed	7/9	T/d Hills®	Carrot, brocoli ocassionally	NO	Sentinel®	Moderate	None	None
Dog7	Australian kelpie	3	Female/spayed	8/9	Hills large breed® + tinned food	Homemade food, cat food Amount varies per day	Eats everything	Sentinel®	Moderate	Unknown	1 dog, 2 cats
Dog8	Australian kelpie	4	Female/spayed	5/9	J/d Hills®	Meatballs ,liver treats 1/three times per week	NO	Canex®	Intense	None	2 cats
Dogs 7-10										
Dog1	Leonberger	8	Female/spayed	4/9	Black hawk chicken rice®	Pig ears, raw chicken frames, 1 once per weer	NO	Drontal®	Moderate	New Zealand	1 dog
Dog2	Leonberger	7	Female/spayed	4/9	Black hawk chicken rice®	Pig ears, raw chicken frames, 1 once per week	NO	Drontal®	Moderate	New Zealand	1 dog
Dog3	Australian kelpie	9	Female/spayed	4/9	Royal canin®	Dental chews, cow/sheep bones 1/ three times per week	NO	Comfortis®	Moderate	National	1 cat, reptiles
Dog4	Cocker spaniel	9	Female/spayed	4/9	Z/d ultra Hills®	Dental chews 1/twice per week	Dog and cat faeces. Facial tissues and toilet paper	Drontal®	Moderate	National	1 dog, 1 cat
Dog5	Australian cattle dog cross	8	Male/neutered	4/9	J/d Hills®	Pig ears, biscuits, cooked meat 1 per day	NO	Drontal®	Moderate	National	1 dog, 2 cats, 1 guinea pig
Dog >10y										
Dog1	Siberian husky cross	11	Male/neutered	5/9	J/d Hills®	Homemade food, biscuits 1/ once per day	Dog, cat and possum faeces	Drontal®	Mild	Canada	None
Dog2	French poodle	15	Female/spayed	3/9	K/d Hills®	None	None	Drontal®	Mild	National	None

3062

3063 .2.2 Samples
3064
3065
3066 Faecal samples from the two litters were collected at day 1 post birth, then every week until adoption
3067 (between 6 – 8 weeks of age) and then at 10 weeks of age. From one puppy, it was possible to collect an
3068 extra sample at week 14. Faecal samples from mothers were collected during the last week of pregnancy,
3069 first week post-partum and after weaning. Buccal swabs were also collected from mothers, one sample
3070 during the last week of pregnancy and another during the first post-partum week. From the remaining dogs,
3071 one faecal sample was collected once per month during 6 months. Some dogs were withdrawn early from
3072 the study.
3073
3074 One faecal sample was collected per dog during daily walk, upon voiding without contacting the
3075 environment (to avoid transfer genetic material) or via rectal examination. Samples were kept in ice
3076 immediately (most of samples were collected by the researcher and owners were provided with a faecal kit,
3077 containing gloves, sterile container and ice), aliquoted and stored at -80ºC before DNA extraction.
3078
3079 For the collection of the buccal swabs, mothers were not allowed to eat or drink for at least one hour prior
3080 to having the sample collected and were isolated from other dogs, toys, rawhides and other possible
3081 sources of oral contamination for at least 1 hour prior sampling (they were taken for a walk). Just prior to
3082 sampling, the dog's mouth was checked to see that no food or other material were present. The tip of a
3083 sterile cotton applicator was rotated along the inside cheek for 20 seconds and another sample was taken
3084 from the hard palate and tongue (rotated during 5-10s). The swab was hold for ten seconds to let it dry (do
3085 not blow) and then was put on ice for transportation and stored at -80°C within 2 hours.
3086
3087 2.2.3 Faecal DNA extraction
3088
3089 Faecal DNA was extracted using the Power soil DNA isolation kit (MoBio® laboratories). 250 mg of faeces
3090 (except in neonates where the first samples weighted between 80 mg – 220 mg) were processed using the
3091 protocol for DNA isolation, detailed in the manufacturer's instructions, with some modifications. Briefly, the
3092 faecal pellet was added to a glass bead tube (0.1mm) and 750 μL of bead solution and 60 μL of C1 solution
3093 were added. Then, samples were incubated at 94ºC during 10 minutes. Afterwards, tubes were placed in
3094 the PowerLyzer® 24 and were run at 3000 RPM for 45 seconds. Subsequent steps were done as indicated

3095 by the manufacturer. Extracted DNA was eluted from the spin column in 100μl of C6 solution from Mobio®

3096 (10 mM tris-Cl pH 8.0- 8.5). Extracted DNA was quantified and checked for purity, based on UV absorption

3097 ratios 260:280 nm and 260:230 nm, on a ND1000 spectrometer. Samples with highly aberrant absorption

3098 ratios were re-extracted.

3099

3100 Samples from puppies and mothers were extracted using the alternative protocol for DNA for low biomass

3101 soil that incorporates the phenol:chloroform method as indicated by the manufacturer. Briefly, the faecal

3102 pellet was placed into a glass bead tube (0.1mm) and 550 μL of bead solution followed by 200 μL of

3103 phenol:chloroform:isoamyl alcohol pH 7-8 (Amresco® cat. no. 0883) and 60 μL of C1 solution were added.

3104 Samples were mixed using a vortex for 10 minutes and then centrifuged at 10,000 x g for 30 sec. Next,

3105 supernatant or upper aqueous layer was removed and placed in a new tube. 100 μL of C2 solution and 100

3106 μL of C3 solution were added; samples were mixed and incubated for 5 minutes on ice. After this step,

3107 protocol was done as recommended in the classic protocol.

3108

3109 **2.2.4 Buccal swabs DNA extraction**

3110

3111 Two sites were sampled: the oral mucosa and tongue. DNA was extracted using the QIAamp® DNA

3112 microkit and the protocol for isolation of total DNA from surface and buccal swabs of QIAamp® DNA

3113 investigator. Carrier RNA was added to buffer AE to a final concentration of 1 ug/ul. The cotton swab was

3114 separated from its shaft using a sterile blade and placed in a 2ml centrifuge tube and DNA was isolated in

3115 accordance with manufacturer's instructions. DNA was finally eluted in 30 μl of AB buffer (provided in the

3116 kit). DNA purity was measured by spectrophotometry at 260 nm. Purity of DNA was assessed using the

3117 ratio of $OD_{260/280}$ with a ratio of 1.8–2.0 being of good purity. As sample contained RNA carrier, DNA

3118 concentration was measured using Qubit® 3.0 fluorometer.

3119

3120 All samples including the faecal and swabs were quantified using the Qubit® 3.0 Fluorometer

3121 (Thermofisher®) and checked for DNA integrity in agarose gel (loading ~100-150ng of genomic DNA per

3122 sample).

3123

3124 **2.2.5 16S DNA sequencing**

3125

3126   The V4 hypervariable region of the bacterial 16S rRNA gene (16Sv4) was PCR-amplified with primers

3127   515F-OH1   (GTGACCTATGAACTCAGGAGTCGGACTACNVGGGTWTCTAAT)   and   806R-OH2

3128   (CTGAGACTTGCACATCGCAGCGTGYCAGCMGCCGCGGTAA); ~100ng of DNA were loaded directly to

3129   a PCR master mix (20 µL reaction/sample). This primer pair amplifies the region 533–786 in the

3130   Escherichia coli strain 83972 sequence (greengenes accession no. prokMSA_id:470367). Cycling

3131   conditions consisted of 95°C for 3 minutes followed by 35 cycles of 95°C for 15 seconds, 60°C for 30

3132   seconds, 72°C for 30 seconds and 72°C for 7 minutes. Individual "barcode" sequences of 8 base pairs

3133   were added to each sample so they could be distinguished and sorted during data analysis. Specificity and

3134   amplicon size were verified by gel electrophoresis and the amplicons were checked and measured using

3135   the Agilent High Sensitivity DNA assay in Agilent 2100 Expert (samples for checking were chosen

3136   randomly). The 600 cycle kit was used for paired end sequencing (2x 311 cycles) using Illumina MiSeq.

3137   Raw data was demultiplexed and quality filtering using default parameters of the open source software

3138   package Quantitative Insights into Microbial Ecology (QIIME).

3139

3140   Taxonomic assignment was done using Operational taxonomic units (OTUs) using a threshold of 97%

3141   similarity and the open-reference OTU approach. Measurements of richness (Alpha (α) – diversity) and

3142   similarity (beta (ß)-diversity) were done using Microbiome and Phyloseq package from R. The correlation

3143   coefficient, using the Pearson's correlation method, was calculated per each pair of samples. Coefficients

3144   of ≥ 0.98 were considered as appropriate. For posterior analysis, samples were merged.

3145

3146   Gut microbiota composition can be characterized based on the microbiota abundance and diversity.

3147   Abundance of certain bacteria defines the amount of these bacteria in the community; whereas diversity is

3148   a measure of variety present. Diversity consists of species richness and evenness. Richness is a measure

3149   of a number of different species present and evenness is the relative abundance of the species. Alpha-

3150   diversity is a measure of diversity in individual level and beta diversity between subjects. β diversity also

3151   refers to the turnover of the community composition from place to place or from time to time.

3152

3153   Alpha-diversity was calculated using the Observed index (richness), the Inverse Simpson, Shannon and

3154   Fisher index (richness and evenness). ß-diversity was assessed qualitatively using unweighted UniFrac;

3155   and quantitatively using Bray-Curtis and Weighted UniFrac.

3156

3157   Raw data was loaded into QIIME 1.9.0 pipeline, and barcodes were extracted (Caporaso *et al.*, 2010).

3158   Next, paired-end reads were merged using the paired-end read merger program (PEAR) (Zhang, Kobert,

3159   Flouri, & Stamatakis, 2014). Subsequently, data was loaded into QIIME 1.8.0 to demultiplex and to filter out

3160   low quality sequencing reads by applying default settings, a minimum number of consecutive high quality

3161   base calls to include a read of 0.90 (default: 0.75) and a minimum acceptable Phred score of 33. Samples

3162   were run in duplicate.

3163

3164   After filtering, from a total of 486 samples, we obtained a total of 7.567.890 millions of high-quality

3165   sequences with a number of reads ranging from 48 to 40382 per sample.

3166

3167   In order to keep only sequences from the same region of the 16S rRNA gene, samples were aligned using

3168   the Silva database from MOTHUR (Schloss *et al.*, 2009).Next, we used USEARCH algorithm to cluster

3169   similar filtered sequences into Operational Taxonomic Units (OTUs) based on a 97% similarity threshold

3170   (Edgar, 2010). Taxonomy assignment to the uniqued sequences was done by aligning the sequences

3171   against Greengenes template alignment (DeSantis *et al.*, 2006). The script make_phylogeny_py was used

3172   to create phylogenetic tress using the FastTree program in Qiime (Price, Dehal, & Arkin, 2009).

3173

3174   To calculate the relative abundance of bacteria at different phylogenetic levels, the OTU table was rarified

3175   at 5000 sequences per sample. Five samples were excluded from the analysis: Mother Group: Two

3176   samples prepartum (Tongue: Mother1, 48 sequences; Oral mucosa: Mother1, 585 sequences); Puppies

3177   group, one sample (Puppy1b, litter1, Week_5, 1666 sequences), Young Adults group: one sample (Dog2,

3178   Month_2, 716 sequences) and Mature group: one sample (Dog5, Month_2, 4741 sequences).

3179

3180   To calculate richness and alpha diversity indexes, faecal (included meconium) and oral samples were

3181   analysed separately. Samples with less than 500 reads (counts) were removed. All Faecal samples

3182   contained more than 500 counts so none of them was removed (Total number of samples: 226). For the

3183   oral microbiota, one sample was removed (prepartum tongue sample, mother1, 48 reads). Additionally,

3184   taxa that were not present in any of the samples were removed. Richness was calculated based on the

3185   number of observed OTUs and diversity was calculated using Shannon, inverse_simpson and Fisher

3186   indexes. All diversity indexes take into account the abundance and evenness of the species present.

3187   However, each index gives different information and has advantages and disadvantages.

3188

3189 • Shannon Index: it measures the entropy and uncertainty of the sampling outcome (it assumes all
3190 species are represented in a sample and that they are randomly sampled). The Shannon diversity
3191 index is nonparametric, which allows for the simultaneous measurement of a richness estimation
3192 from heterogeneous samples and takes into account both the relative abundance and total number
3193 of species in a microbiome community. The Shannon index increases as both the richness and the
3194 evenness of the community increase (Zhu, Wang, Reyes-Gibby, & Shete, 2017).

3195

3196 • Inverse Simpson's Index: It describes the probability that that two randomly chosen reads from a
3197 sample of the given community come from different taxa. A higher value for the inverse Simpson's
3198 diversity index represents greater diversity. It is is considered a dominance index as it gives more
3199 weight to dominant or common species. Thus, the inverse Simpson's diversity index is biased
3200 when estimating numerous species that have low abundance within a community (Zhu et al.,
3201 2017).

3202

3203 The Shannon index emphasizes the richness component, while Simpson's index stresses the
3204 evenness component (Zhu et al., 2017). A Shannon index implies higher uncertainty in correctly
3205 predicting the identity of the next species chosen at random for the given sample

3206

3207 • Fisher Index: it describes the relationship between the number of species and the number of
3208 individuals of the corresponding species by logarithmic distribution. Compared with the Shannon
3209 index and Simpson's index; this index is not influenced by the sample size and is less affected by
3210 the abundance of the most common species. Fisher's α index depends more on the number of
3211 species of intermediate abundance. A higher value of Fisher's α index represents more diversity
3212 within the sample. The estimation of Fisher's α index gives an unbiased estimation of diversity (Zhu
3213 et al., 2017).

3214

3215 To calculate between-sample diversity, the OTU matrix was normalized to account for uneven column
3216 (sample) sums, through Cumulative sum scaling (CSS) using the command normalize table.py available in
3217 Qiime Weighted and unweighted Unifrac metrics were applied to build phylogenetic distance matrices
3218 (Lozupone *et al.,* 2012). We also assessed the Bray_curtis dissimilarity index.

3219

3220   The Bray–Curtis dissimilarity index measures the distance between two microbiome samples A and B; by

3221   accounting for the abundance information (i.e. diversity). The Bray–Curtis index varies between 0 and 1. If

3222   the two microbiome samples A and B are identical in composition, then the index is 0 (i.e., coincidence). If

3223   there are no species in common between the two samples, then the index is 1 (i.e., complementarity).

3224

3225   UniFrac distance: The unique fraction (UniFrac) measures the difference between microbial samples by

3226   incorporating the phylogenetic information. The phylogenetic distance is measured between sets of taxa as

3227   the fraction of branch length in the phylogenetic tree that leads to descendants from either sample A or B,

3228   but not both. The unweighted UniFrac UniFrac distance considers whether an OTU is present or absent in

3229   a sample but not the abundance information, so it does not account for the evenness component, and

3230   cannot address how much of the observed community is attributable to the phylogenetic tree. Weighted

3231   UniFrac on the other hand, account for the abundance information of taxa sets, by weighting the branch

3232   lengths with differences in abundance.

3233

3234   To perform microbial differential abundance testing between the different groups, we used the extension

3235   DESeq2 from the Phyloseq package (version1.18.1) (McMurdie & Holmes, 2013) (Love, Huber, & Anders,

3236   2014). DESeq2 estimates variance-mean dependence in count data from high-throughput sequencing

3237   assays and test for differential expression based on a model using the negative binomial distribution. Due

3238   to the small number of dogs in some of the groups, we focused the analysis on comparing puppies with

3239   young adults; puppies with their mothers, mothers with young adults and young adults with mature dogs.

3240   Samples with less than 500 reads (counts) were removed. The DESeq function performs multiple-inference

3241   correction with the Benjamini-Hochberg method.

3242

3243   **2.2.6 Statistical Analyses**

3244

3245   A Shapiro-Wilk test of normality was performed on alpha diversity and Richness to check whether the data

3246   was normally distributed or not. As the data was not normally distributed, differences in alpha diversity were

3247   calculated using the non-parametric Kruskal-Wallis test (more than two levels) and pairwise comparisons

3248   were calculated using the Wilcoxon rank sum test. Tests were carried out using the microbiome R package

3249   (version 1.0.2). Graphics were created using the package ggpubr in R. We use the following convention for

3250   symbols indicating statistical significance: ns: $p > 0.05$, *: $p <= 0.05$, **: $p <= 0.01$, ***: $p <= 0.001$ and ****:

3251 $p <= 0.0001$.

3252

3253   Differences in beta diversity were calculated in normalized data (CSS OTU table) based on Permutational

3254   Multivariate Analysis Of Variance using Distance Matrices and the function Adonis from the program

3255   Vegan 2.4.6. in R (Anderson, 2001). PERMANOVA is a distribution-free test that measures the overall

3256   difference for multiple responses based on permutation tests and partitions a symmetric distance matrix

3257   based on linear models. A P value < 0.05 was considered significant. For microbial differential abundance

3258   testing, DESeq2 from the Phyloseq package was used.

3259

3260   **2.3 Results**

3261

3262   **2.3.1 Sequencing summary**

3263

3264   The total number of sequences per group range from 48 to 40382. A summary per group can be seen in

3265   Figure 2.1.

3266

3267   Analysis of the negative samples revealed that some of the samples, had the presence of bacterial DNA,

3268   most of which corresponded to the phylum Proteobacteria (97%). At class level, 99% of the bacteria were

3269   Alphaproteobacteria. At order level Sphingomonadales (53%), Caulobacterales (46%), Burkholderiales

3270   (4%), Rhizobiales (3%), Lactobacillales (1%) and Clostridiales (1%) were the most abundant. At family

3271   level, Sphingomonadaceae (46%) and Caulobacteraceae (46%) predominated. Other groups presented

3272   belonged to the Bradyrhizobiaceae (3%), Oxalobacteraceae (1%) and Streptococcaceae (1%) family. The

3273   Enterobacteriaceae family was present in a very low percentage (0.6%).

3274

3275   Sphingomonadaceae and Caulobacteraceae bacteria are commonly isolated from soil, activated sludge, or

3276   marine   environments   what   reinforce   the   idea   of   contamination   of   these   samples

3277   (www.ncbi.nlm.nih.gov/mesh/?term=Sphingomonadaceae).

3278

3279

3280

3281

3282

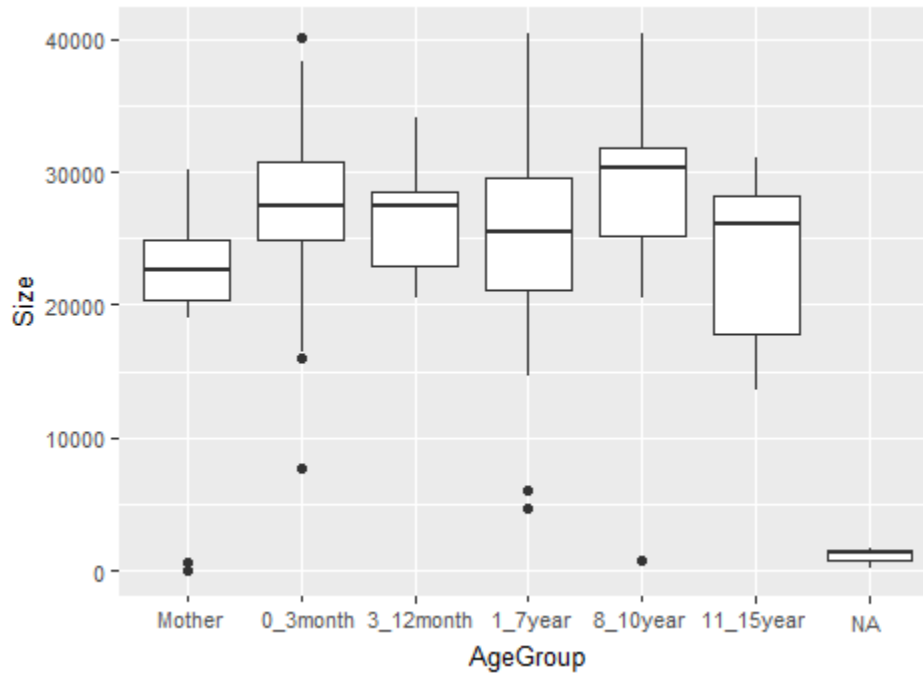Figure 2.1: Total number of sequences per group. NA corresponds to water samples. Mother N=2; n=19. Puppies (0_3 month) N=10; n=111. Growth (3_12 month) N=3; n=15. Young Adult (1_7 year) N=10. Mature (8_10 year) N=3; n=17. Senior (11_15 year) N=2; n=10. NA (Water) n=3. N: Number of Animals, n: number of samples.

3286

3287

3288

3289

3290 **2.3.2 Diversity analysis and Relative Abundance at different phylogenetic levels according to Age**
3291
3292
3293 The most abundant phyla, classes and orders of bacteria were compared among groups of different ages.
3294 Mothers and puppies (1 day old to 10 weeks old) showed a different profile compared to the other age
3295 groups. At phylum level, bacteria belonged mainly to Bacteroidetes, Firmicutes, Fusobacteria,
3296 Proteobacteria and Actinobacteria groups, comprised more than 99% of the phyla present; as it has been
3297 previously reported in dogs (Jan S. Suchodolski, 2013). However, the percentage of Proteobacteria was
3298 much higher in the group of mothers and puppies compared to the other groups (~ 10-34% versus ~2-5%);
3299 whereas the percentage of Fusobacteria was much lower (~2% versus 13-27%). Also, the percentage of
3300 Firmicutes comprised 52% of the sequences in mothers compared to 24-35% in the other groups. At Class
3301 level, the most abundant groups comprised bacteria belonging to the class Bacteroidia, followed by
3302 Clostridia, Fusobacteria and Gammaproteobacteria. However, the percentage of Gammaproteobacteria
3303 was significantly higher in mothers and puppies (Figure 2.2). At order level, bacteria belonging to
3304 Bacteroidales, Clotridiales and Fusobacteriales group, represented the majority. However, Mothers and
3305 puppies had a higher proportion of Lactobacillales (~7-33% versus 2% in the other groups). Additionally, in
3306 puppies, the percentage of Enterobacteriales (~30% versus ~5-10%) and in mothers, the percentage of
3307 Clostridiales (~41%versus 23-30%) were much higher compared to other groups (data not shown).
3308
3309 When alpha and beta diversity indexes were analysed, there was a significant difference between groups
3310 (P < 0,001). However, the significant difference in alpha diversity was driven by puppies. When the other
3311 groups were compared, there were not significant differences. Adults exhibited the highest diversity index
3312 whereas puppies exhibited the lowest one, as it has been published in people (Figure 2.3). Also, when the
3313 different type of samples were plotted in a principal coordinate analysis (PCoA) graph using UNIFRAC
3314 diversity analysis, faecal samples clustered together but separately from the samples coming from the oral
3315 cavity. A more detailed analysis of the faecal samples revealed that samples from puppies exhibited a
3316 wider range of distribution and only some of the samples clustered with samples coming from other groups
3317 (Figure 2.4).
3318
3319
3320

3321

3322
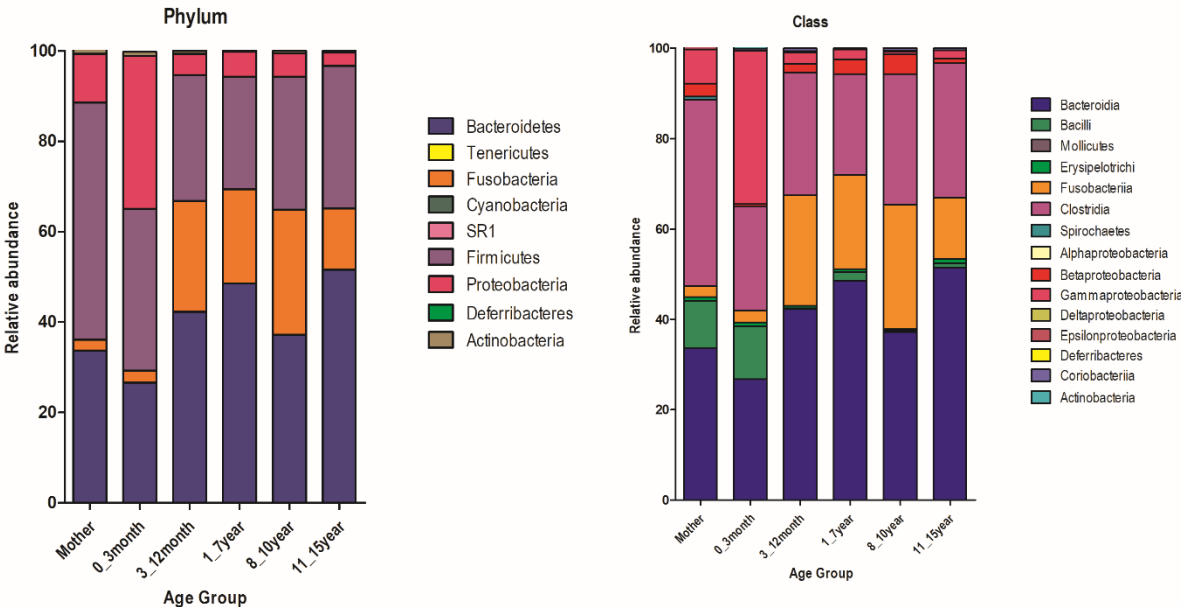
3323 Figure 2.2: Top 20 of the relative abundance of the major phylogenetic levels in the different Age Groups. Mother

3324 N=2, n = 11. Puppies (0_3month) N=10, n = 111; Growth (3_12month) N=3, n = 15 ; Young Adults (1_7year) N=10, n

3325 = 62 ; Mature (8_10y) N=3, n = 17 and Senior (11_15year) N=2,n = 10. Samples were rarified at 5000 sequences.

3326 N: Number of Animals, n: number of samples.

3327

3328

3329

3330
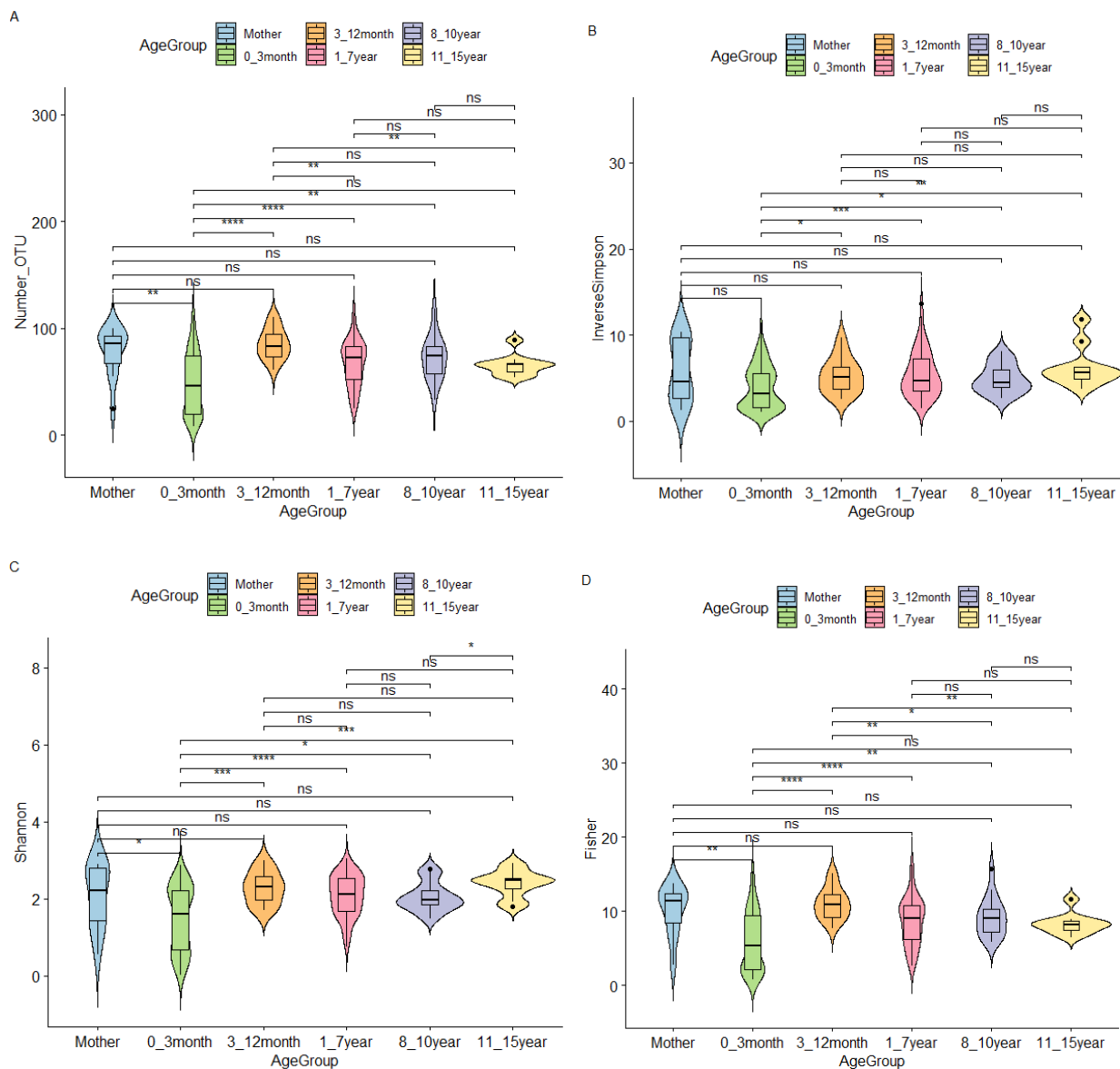
3331 Figure 2.3: Alpha diversity analysis of different Age Groups. A:Number of OTUs (Observed), B: Inversed Simpson's

3332 Index, C: Shannon's Index and D: Fisher's Index.. Mother N=2, n = 11 Puppies (0_3month) N=10, n = 111; Growth

3333 (3_12month) N=3, n = 15 ; Young Adults (1_7year) N=10, n = 62 ; Mature (8_10y) N=3 n = 17 and Senior

3334 (11_15year) N=2, n = 10. P value: ns: p > 0.05, *: p <= 0.05, **: p <= 0.01, ***: p <= 0.001 and ****: p <=

3335 0.0001. N: Number of Animals, n: number of samples.

3336

3337

3338
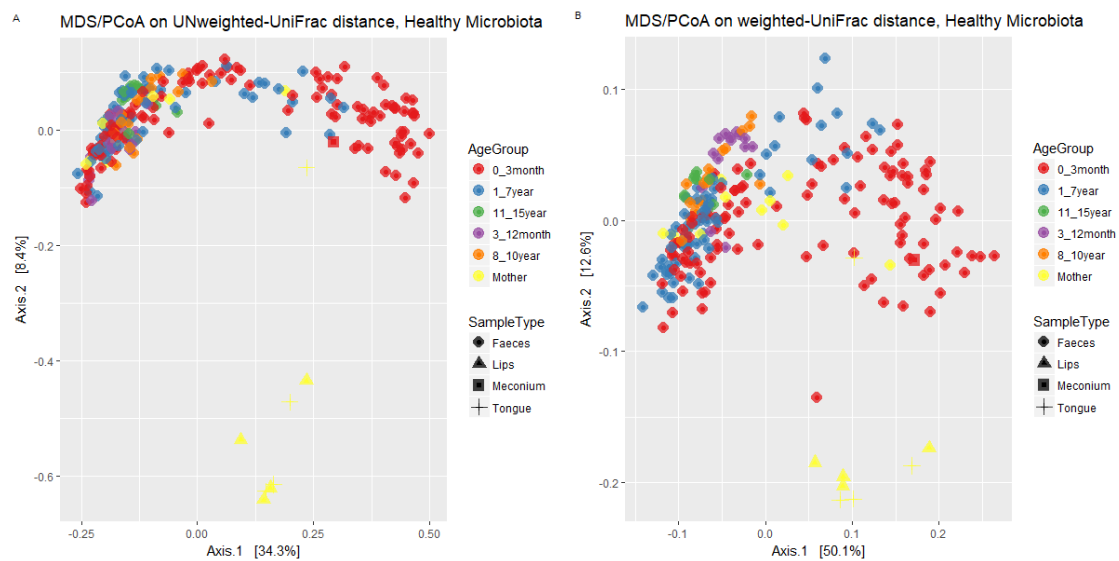
Figure 2.4: Beta diversity analysis. A: Unweighted and B: Weighted UNIFRAC analysis based on age group and sample type. Mother N=2, n = 18 Puppies (0_3month) N=10, n = 111; Growth (3_12month) N=3, n = 15 ; Young Adults (1_7year) N=10, n = 62 ; Mature (8_10y) N=3, n = 17 and Senior (11_15year) N=2, n = 10. N: Number of Animals, n: number of samples.

3349   Next, a more detailed analysis was performed in every age group based on the different time points

3350   assessed.

3351

3352   **2.3.2.1 Development of gut microbiota in puppies**

3353

3354   **2.3.2.1.1 Relative abundance of the major phylogenetic groups**

3355
3356

3357   The first weeks of life in a puppy are characterised by low richness and low diversity and the predominance

3358   of Proteobacteria (94%), particularly species belonging to the Enterobacteriaceae (69-92%) family.

3359

3360   Analysis of the relative abundance at the highest phylogenetic levels showed that the microbial

3361   development was divided in two distinct phases: 1 day-old – 4 weeks-old and 6-14 weeks-old. The first

3362   three weeks were characterised mainly by bacteria belonging to the phylum Proteobacteria (69-94%), and

3363   a much lower percentage of Firmicutes (5-20%), followed by Fusobacteria (0-20%, increasing over time). In

3364   the next two weeks, there was a transition period, where the proportion of Proteobacteria decreased

3365   substantially (8-10%), the levels of Firmicutes increased (66-73%) and Bacteroidetes started to become

3366   evident (8-23%). From week 6 onwards, the microbial population was dominated by Bacteroidetes (50-

3367   76%) and Firmicutes (18-45%), and only a small proportion belonged to the phyla Proteobacteria (1-3%)

3368   and Fusobacteria (1-4%) (Figure 2.5). All these changes were correlated with modifications in dietary

3369   patterns. The diet transition scheme in puppies consisted in exclusive milk during the first three weeks,

3370   followed by introduction to dry food and milk ad libitum for the next two weeks, then milk only once to twice

3371   per day and dry food ad libitum for one week, after which they were completed weaned. Thus, introduction

3372   of solid food and weaning promoted the predominance of Bacteroidetes and Firmicutes, as it has been

3373   seen in people (Palmer *et al.*, 2007).

3374

3375   At class and order level, the same distinct phases were evident. The first phase was dominated by bacteria

3376   belonging to the class Gammaproteobacteria (69-87%), the transition phase showed a predominance of

3377   Clostridia (55-28%) , Bacilli (17-36%), Bacteroidia (8-22%), and Gammaproteobacteria (6-10%) and after

3378   weaning, Bacteroidia (50-76%) and Clostridia (16-33%) overruled (Figure 2.5).

3379

3380   At order level, the main exponents were Enterobacteriales (70-92%) in the first phase, Lactobacillales (16-

3381   33%), Clotridiales (28-55%), Bacteroidales (0-20%) and Fusobacteriales (0-10%) in the transition phase

3382   and in the second phase; preponderance of Bacteroidales (50-76%) and Clostridiales (14-33%).

3383

3384   At family level, first phase was dominated by members of the Enterobacteriaceae (69-92%) family, the

3385   transition phase by Streptococcaceae (1-24%), Lachnospiraceae (23-39%), Bacteriodaceae (4-21%),

3386   Veillonellaceae (4-13%) and Lactobacillae (2-11%) and the second phase by Prevotellaceae (36-51%),

3387   Paraprevotellaceae (9-31%), Veillonellaceae (5-21%), Lachnospiraceae (9-11%). (Data not shown).

3388

3389   We followed two litters weekly from birth until 10 weeks old. From one of the dogs, it was possible to obtain

3390   a sample of meconium. Analysis of the sample revealed the presence of bacterial DNA, mainly from the

3391   Proteobacteria phylum (94%). At class level 93% of the bacteria corresponded to Gammaproteobacteria,

3392   followed by Clostridia (4%) and Bacilli (1%). At order level, the main groups were Enterobacteriales (92%),

3393   Clostridiales (4.5%) and Pasteurallales (1%) and at family level; Enterobacteriaceae predominated (92%),

3394   followed by Clostridiaceae (3.9%) and Pasteurellaceae (1%).

3395

3396

3397   **2.3.2.1.2 Microbial differential abundance testing**

3398

3399   Puppies were compared to Mothers and young adults to assess which taxonomic groups were significantly

3400   different between the groups. When puppies were compared to mothers, seven family groups were

3401   significantly enriched in puppies: Staphylococcaceae, Enterobacteriaceae, Clostridiaceae,

3402   Enterococcaceae, Streptococcaceae and Lachnospiraceae (Table 2.4) (Figure 2.5).

3403

3404   Compared to young adults, fourteen families were enriched in puppies: Enterobacteriaceae,

3405   Enterococcaceae, Streptococcaceae, Bifidobacteriaceae, Staphylococcaceae, Bacteroidaceae,

3406   Coriobacteriaceae, Clostridiaceae, Peptostreptococcaceae, Ruminococcaceae, Lactobacillaceae,

3407   Erysipelotrichaceae, Porphyromonadaceae and Lachnospiraceae (Table 2.5) (Figure 2.5).
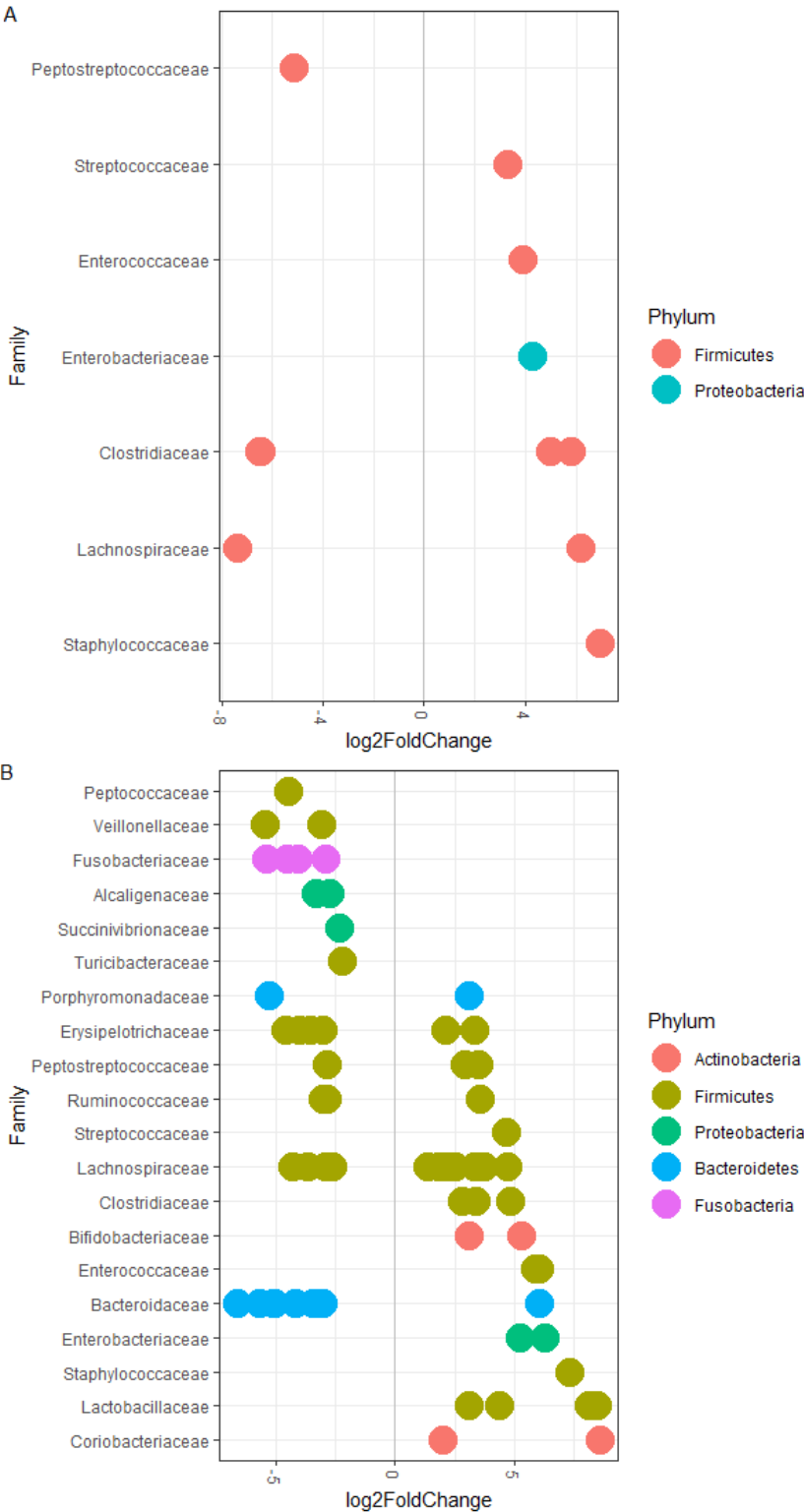
3408

3409
3410 Figure 2.5: Microbial differential abundance family groups. A: Puppies versus Mothers. Puppies versus Young Adults
3411 N=22 (N: 2 Mothers, 10 puppies and 10 young adults); n=173 (Mother n=11 Adults n= 73, Puppies n=111) N:
3412 Number of Animals, n: number of samples.

3413   Table 2.4 Microbial differential abundance in puppies compared to mothers

3414

OTU	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Phylum	Class	Order	Family	Genus	Species
Otu34	353.1693691938	-5.1454355071	0.9208813455	-5.5875119	2.30e-08	2.90e-06	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	NA	NA
Otu17	384.8406600801	-6.4240526413	1.3191656908	-4.8697845	1.11e-06	7.03e-05	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
Otu78	38.4047479959	-7.3668856417	1.5660030567	-4.7042600	2.54e-06	0.0001070111	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium	NA
Otu91	17.785052334	6.9470560278	1.6099000927	4.3152094	1.59e-05	0.0004133384	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	NA
Otu1	20360.4518891	4.2893078303	0.9954389699	4.3089611	1.64e-05	0.0004133384	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	NA
Otu15	335.7581286428	5.8014579181	1.379421097	4.2057192	2.60e-05	0.0005465315	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
Otu36	145.9881468735	3.9014205056	1.0252664399	3.8052747	0.0001416	0.0022309381	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	NA
Otu329	3.472488444	-6.4851117839	1.6784889918	-3.8636605	0.0001117	0.0020106071	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
Otu5	1636.3100092548	3.284600146	0.9014261773	3.6437816	0.0002686	0.0037612593	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA
Otu29	89.6409667926	4.9987615141	1.3939074802	3.5861501	0.0003355	0.0042285068	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
Otu52	15.5403568414	6.1743384627	1.7423598314	3.5436643	0.0003946	0.0045200465	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Dorea	NA

3415
3416

3417

3418   Table 2.5 Microbial differential abundance in puppies compared to young adults

3419

OTU	baseMean	log2FoldChange	lfcSE	stat	Pvalue	padj	Phylum	Class	Order	Family	Genus	Species
Otu1	21938.66	6.30180	0.5204226	12.1090062061	9.46334e-34	1.779114e-31	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	NA
Otu16	39.35623	8.47643	0.7153693	11.8490339167	2.17685e-32	2.046247e-30	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
Otu36	77.95887	6.04757	0.52880	11.4363834211	2.75116e-30	1.724062e-28	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	NA
Otu185	16.96936	-5.4075	0.477274	-11.3300108341	9.31931e-30	4.380088e-28	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	NA
Otu22	263.5918	8.15034	0.8372029	9.7352133326	2.13367e-22	8.022693e-21	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	ruminis
Otu5	1081.011	4.6771	0.4868035	9.6079465458	7.40119e-22	2.318986e-20	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA
Otu40	31.19537	5.31713	0.5814029	9.1453574427	5.94328e-20	1.596200e-18	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	NA
Otu91	18.08810	7.29700	0.8099563	9.0091359258	2.07683e-19	4.751001e-18	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	NA
Otu4	1925.482	-4.55048	0.5056566	-8.9991646627	2.27445e-19	4.751001e-18	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	NA
Otu9	230.9208	-5.1128	0.5754843	-8.8844114801	6.42603e-19	1.208088e-17	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
Otu54	25.483031	8.58920	0.9934426	8.6459011556	5.33821e-18	9.123522e-17	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Collinsella	aerofaciens
Otu105	56.370305	-3.63301	0.4251828	-8.5445865798	1.28992e-17	2.020956e-16	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
Otu15	222.32382	4.83809	0.6110818	7.91725993	2.42818e-15	3.511287e-14	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
Otu314	3.9266813	-2.881925	0.3826305	-7.5318750306	5.00181e-14	6.716544e-13	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
Otu98	2.621834	-4.6009103	0.6126997	-7.5092410077	5.94715e-14	7.453717e-13	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Allobaculum	NA
Otu55	12.2536898	-4.7933493	0.6497585	-7.377123577	1.61758e-13	1.900513e-12	Firmicutes	Clostridia	Clostridiales	NA	NA	NA
Otu34	58.7643853	2.9255744	0.4075526	7.1783956871	7.03417e-13	7.800249e-12	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	NA	NA
Otu365	7.6048836	5.8585414	0.8206969	7.1384957173	9.43570e-13	9.855149e-12	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	NA
Otu42	7.8992113	-6.591976	0.9326018	-7.0683708982	1.56630e-12	1.502070e-11	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
Otu79	4.3101269	-4.1813756	0.5917840	-7.0657118191	1.59747e-12	1.502070e-11	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
Otu86	13.3223261	-5.672336	0.8727277	-6.4995491855	8.05105e-11	7.212132e-10	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
Otu143	7.0842340	3.5277295	0.5746205	6.1392327263	8.29211e-10	7.085977e-09	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	NA	NA
Otu107	1.46066350	-4.3146561	0.7198442	-5.9938741928	2.04899e-09	1.674834e-08	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
Otu60	8.63422882	3.5653560	0.6032716	5.9100344564	3.42031e-09	2.572112e-08	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	NA
Otu389	2.87945883	-4.0626553	0.687375	-5.9103897638	3.41291e-09	2.572112e-08	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	NA
Otu85	3.30143764	-3.0496544	0.5247294	-5.8118611149	6.17828e-09	4.467320e-08	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	NA
Otu31	7.03179950	5.2619866	0.9289690	5.6643292927	1.46007e-08	1.027738e-07	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	NA
Otu10	298.3814884	-2.7422794	0.4894612	-5.6026488545	2.11006e-08	1.417389e-07	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella	NA

Otu21	220.7266911	-3.0456626	0.5562113	-5.4757291266	4.35737e-08	2.824627e-07	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
Otu24	71.6436380	-3.1445900	0.5821661	-5.4015338636	6.60734e-08	4.140603e-07	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
Otu41	57.6094349	-3.0950020	0.5740161	-5.3918378417	6.97406e-08	4.229435e-07	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	NA
Otu110	0.9068748	-3.0074199	0.5902095	-5.095512082	3.47798e-07	2.043324e-06	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	NA	NA
Otu29	58.943227	3.397330	0.6732555	5.0461243182	4.50621e-07	2.568547e-06	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
Otu8	79.6047214	6.0562790	1.2037037	5.0313700901	4.86987e-07	2.692751e-06	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	fragilis
Otu73	14.5919710	-2.8771196	0.6026957	-4.7737515882	1.80854e-06	9.712907e-06	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	NA	NA
Otu90	2.7898926	4.6882532	0.9879575	4.7453996346	2.08050e-06	1.086718e-05	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	NA
Otu70	8.3218808	-2.8481754	0.6061272	-4.698973126	2.61428e-06	1.328564e-05	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	NA
Otu17	28.4167543	2.8181063	0.6076862	4.6374364161	3.52750e-06	1.745219e-05	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
Otu342	1.7141939	-2.6127557	0.5681342	-4.5988352471	4.24855e-06	2.048041e-05	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Dorea	NA
Otu18	202.416367	-2.9109631	0.6569562	-4.4309850225	9.38030e-06	4.408769e-05	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	NA
Otu360	0.6519828	-2.716082	0.6327396	-4.2925747708	1.766130e-05	8.098354e-05	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
Otu116	41.9484060	2.5434362	0.5972339	4.25869344	2.05652e-05	9.204177e-05	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	producta
Otu339	1.6277657	3.1280652	0.7366927	4.2460922272	2.17531e-05	9.510662e-05	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	NA
Otu12	23.3571267	-3.408838	0.8068022	-4.2251229994	2.38813e-05	0.0001020371	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
Otu19	233.076764	1.8123810	0.4303133	4.2117704567	2.53369e-05	0.0001058553	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
Otu274	2.2077563	4.3481771	1.0470405	4.1528260823	3.28343e-05	0.0001342133	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
Otu111	2.40610622	-5.0228809	1.2470693	-4.0277477779	5.63170e-05	0.0002252548	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	uniformis
Otu27	97.7555422	2.1978258	0.5485716	4.0064517493	6.16370e-05	0.0002414144	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	NA
Otu431	0.919152	3.1142663	0.7822902	3.9809599122	6.86372e-05	0.000263344	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
Otu101	1.4274654	3.308168	0.8582400	3.8545960398	0.00059209	0.0004358625	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
Otu97	5.0809336	-4.463573	1.1879525	-3.7573666225	0.00017108	0.0006329733	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Peptococcus	NA
Otu23	120.99131	3.7333026	1.0077632	3.7045431694	0.00021772	0.0007656374	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	NA
Otu89	7.5726502	-3.3076609	0.9102970	-3.6336063238	0.00024872	0.0009913886	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella	NA
Otu66	11.3882896	1.9570565	0.5426218	3.6066677804	0.00001543	0.0010797966	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
Otu32	3.2264385	-5.44557393	1.56711604	-3.4749015128	0.00051409	0.0017468308	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Megasphaera	NA
Otu238	1.1869930	-4.00532928	1.15620955	-3.4641897325	0.00018313	0.0017854335	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	[Eubacterium]	dolichum
Otu168	0.819648	3.10430806	0.9083730	3.4174373131	0.00021365	0.0020849414	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	NA
Otu62	14.0793637	-2.3439292	0.72027719	-3.2542044876	0.0011104	0.0036857853	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	NA	NA
Otu114	1.5857154	2.00662644	0.61861367	3.2437473112	0.00117965	0.0037589915	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Collinsella	NA
Otu113	2.8889837	-5.2546686	1.63312038	-3.2175635886	0.00129437	0.0040509101	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	NA

Otu415 3.4678123	-2.2314185	0.6988966	-3.1927731291	0.04091361	0.0043127508	Firmicutes	Bacilli	Turicibacterales	Turicibacteraceae	Turicibacter	NA
Otu43 34.1275528	2.1047581	0.65978038	3.1900889369	0.00142229	0.0043127508	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	[Eubacterium]	biforme
Otu122 1.2821202	3.3175420	1.04812653	3.165211366	0.00154970	0.0046245115	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	[Eubacterium]	dolichum
Otu108 0.8762674	-3.5224505	1.14805618	-3.0681865772	0.00215362	0.006326262	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	NA	NA
Otu6 1230.185370	1.3907784	0.46539971	2.9883525243	0.00280485	0.0081125132	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	[Ruminococcus]	gnavus

3420

3421   **2.3.2.1.3 Diversity Analysis**

3422

3423   Analysis of alpha diversity showed a gradual increase in diversity over time. Thus, samples collected at

3424   **closer time points showed a more similar α diversity than samples collected at farther time points** (Table

3425   2.6). We observed a high species diversity in meconium compared with samples taken at day 1 post-birth

3426   (week_0). Although it is not possible to draw conclusions from one single sample; in people it has been

3427   observed that diversity tends to decrease during the first week postpartum. Probably, due to the presence

3428   of microbes poorly adapted to colonize the gastrointestinal tract that are easily lost or replaced (Ferretti et

3429   al., 2018). Interestingly, when Inverse simpson index was analysed, two distinct patterns were observed

3430   and coincided with the weaning period, where diversity and abundance increased remarkably (P value <

3431   0,001).In general, initiation of solid feeding increases species diversity and consequently with solid food

3432   introduction, gut microbiota composition starts to resemble that of an adult microbiota (Figure 2.7).

3433

3434   Beta diversity analysis showed that microbial communities belonging to puppies clustered separately from

3435   that of the mother in the first weeks, only resembling that of the mother in the last weeks after weaning (P <

3436   0,001) (Figure 2.8).

3437

3438

3439

3440
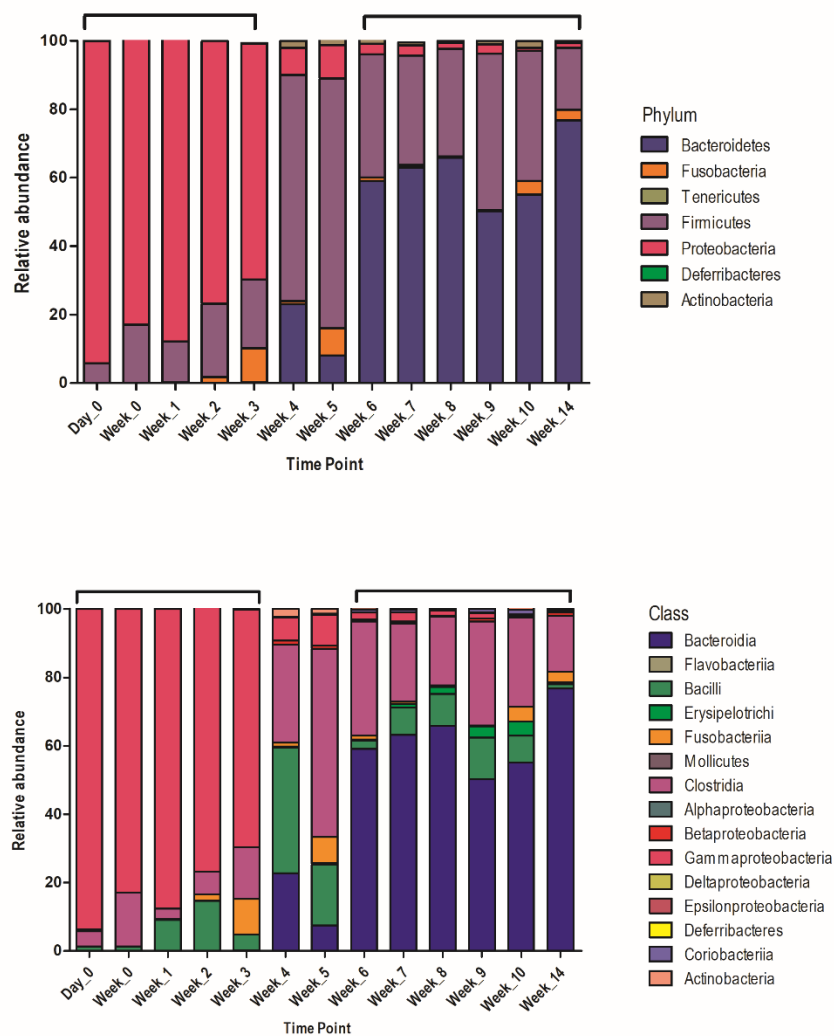
3441

3442

3443

3444

3445

Figure 2.6: Top 20 of the relative abundance of the major phylogenetic levels in puppies over time. Two distinct phases are highligthed: Nursing and weaning. Puppies N=10. Day_0, n=1; Week_0, n= 11; Week_1, n=11; Week_2, n=10; Week_3, , n=11; Week_4, , n=13; Week_5, , n=11; Week_6, , n=12; Week_7, , n=12; Week_8, , n=5; Week_9, , n=4; Week_10, , n=9; and Week_14, n=1. Samples were rarified at 5000 sequences. N: Number of Animals, n: number of samples.
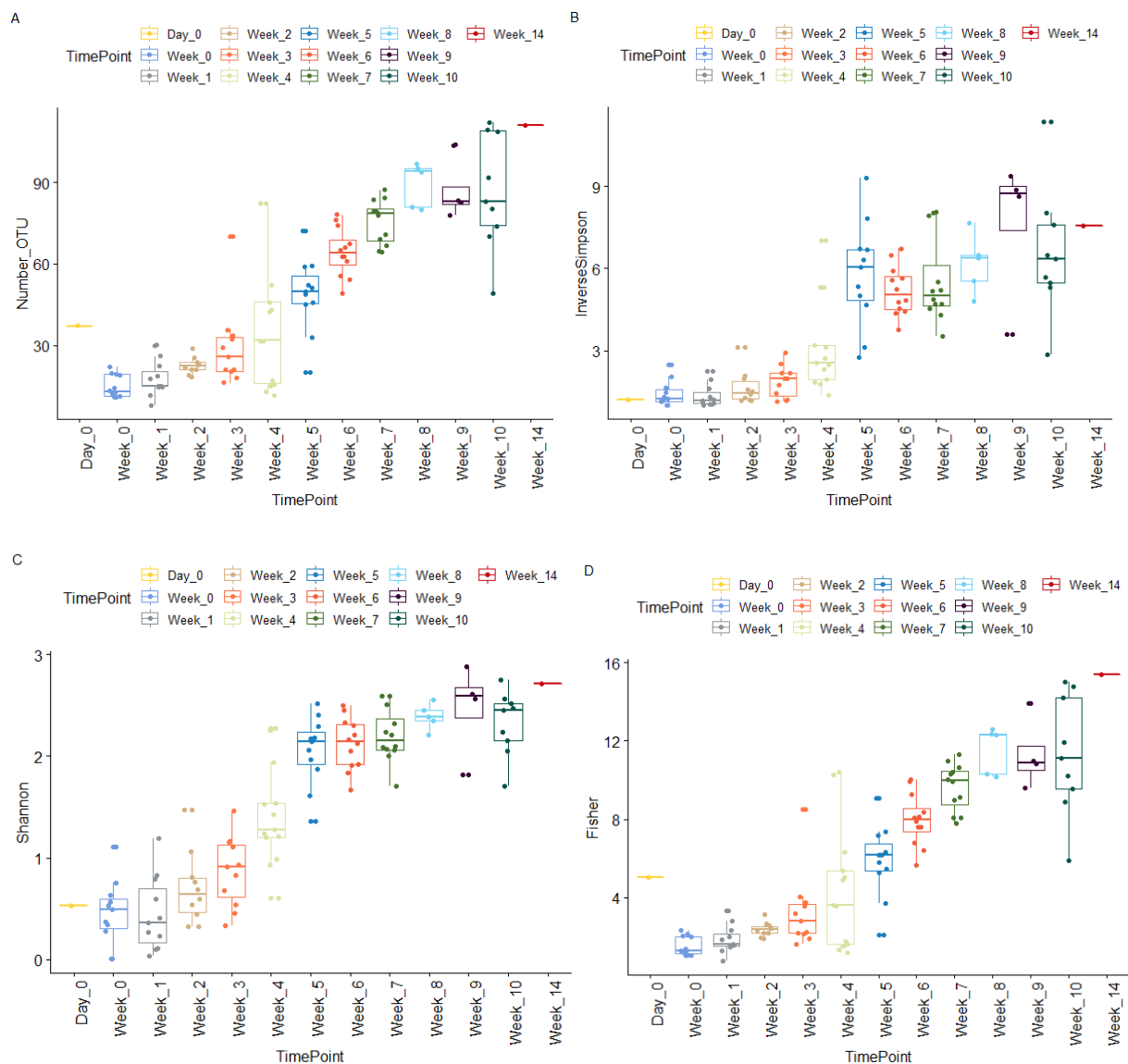
3451

3452

3453

Figure 2.7: Alpha diversity analysis of puppies (0_3month) at different time points. A:Number of OTUs (Observed), B:

Inversed Simpson's Index, C: Shannon's Index and D: Fisher's Index.. Day_0, n=1; Week_0, n= 11; Week_1, n=11;

Week_2, n=10; Week_3, , n=11; Week_4, , n=13; Week_5, , n=11; Week_6, , n=12; Week_7, , n=12; Week_8, ,

n=5; Week_9, , n=4; Week_10, , n=9; and Week_14, , n=1. N: 10 puppies. At some time-points, samples from the

same patient were collected twice per week during consecutive days. N: Number of Animals, n: number of samples.

3459

3460

3461

3462 Table 2.6: Alpha diversity analysis of puppies over time.
3463

Observed	Day_0	Week_0	Week_1	Week_2	Week_3	Week_4	Week_5	Week_6	Week_7	Week_8	Week_9	Week_10	Week_14
Day_0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Week_0		0	0,25	0,0017	0,0016	0,0082	0,00013	5,40E-05	5,30E-05	0,0021	0,0047	0,00019	NA
Week_1			0	0,04	0,01	0,039	0,00017	5,30E-05	5,20E-05	0,0021	0,0046	0,00019	NA
Week_2				0	0,42	0,37	0,001	8,60E-05	8,50E-05	0,0026	0,0057	0,00027	NA
Week_3					0	0,71	0,0078	5,00E-04	1,50E-04	0,0022	0,0049	3,00E-04	NA
Week_4						0	0,063	4,30E-04	1,20E-03	0,0057	0,0077	0,0011	NA
Week_5							0	0,0038	0,00019	0,0022	0,0049	0,0016	NA
Week_6								0	0,0038	0,0019	0,0052	0,0094	NA
Week_7									0	0,013	0,18	0,14	NA
Week_8										0	0,9	0,74	NA
Week_9											0	1	NA
Week_10												0	NA
Week_14													0

InvSimpson	Day_0	Week_0	Week_1	Week_2	Week_3	Week_4	Week_5	Week_6	Week_7	Week_8	Week_9	Week_10	Week_14
Day_0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Week_0		0	0,69	0,19	0,066	0,0005	8,20E-05	5,50E-05	5,50E-05	0,0022	0,005	2,00E-04	NA
Week_1			0	0,073	0,0018	0,00033	8,20E-05	5,50E-05	5,50E-05	0,0022	0,005	2,00E-04	NA
Week_2				0	0,5	0,0048	0,00016	8,70E-05	8,70E-05	0,0027	0,0058	0,00038	NA
Week_3					0	0,024	0,00011	5,50E-05	5,50E-05	0,0022	0,005	0,00027	NA
Week_4						0	0,0038	0,0051	0,0043	0,01	0,011	0,0013	NA
Week_5							0	0,31	0,64	0,73	0,17	0,49	NA
Week_6								0	0,98	0,15	0,16	0,082	NA
Week_7									0	0,27	0,13	0,15	NA
Week_8										0	0,27	0,89	NA
Week_9											0	0,32	NA
Week_10												0	NA
Week_14													0

Shannon	Day_0	Week_0	Week_1	Week_2	Week_3	Week_4	Week_5	Week_6	Week_7	Week_8	Week_9	Week_10	Week_14
Day_0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Week_0		0	0,9	0,15	0,015	0,00013	8,20E-05	5,50E-05	5,50E-05	0,0022	0,005	2,0E-04	NA
Week_1			0	0,11	0,018	0,00013	8,20E+05	5,50E-05	5,50E-05	0,0022	0,005	2,0E-04	NA
Week_2				0	0,19	0,0017	0,00016	8,70E-05	8,70E-05	0,0027	0,0058	0,00028	NA
Week_3					0	0,0045	0,00011	5,50E-05	5,50E-05	0,0022	0,005	2,0E-04	NA
Week_4						0	0,0045	1,80E-03	1,00E-03	0,0031	0,011	0,0011	NA
Week_5							0	0,78	0,34	0,031	0,1	0,095	NA
Week_6								0	0,47	0,051	0,13	0,095	NA
Week_7									0	0,19	0,2	0,41	NA
Week_8										0	0,27	1	NA
Week_9											0	0,25	NA
Week_10												0	NA
Week_14													0

Fisher	Day_0	Week_0	Week_1	Week_2	Week_3	Week_4	Week_5	Week_6	Week_7	Week_8	Week_9	Week_10	Week_14
Day_0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Week_0		0	0,21	0,0017	0,0016	0,0065	0,00014	5,50E-05	5,50E-05	0,0022	0,005	2,00E-04	NA
Week_1			0	0,038	0,013	0,049	0,00018	5,50E-05	5,50E-05	0,0022	0,005	2,00E-04	NA
Week_2				0	0,31	0,37	0,0011	8,70E-05	8,70E-05	0,0027	0,0058	0,00028	NA
Week_3					0	0,69	0,0086	5,10E-04	1,20E-04	0,0022	0,005	0,00027	NA

Week_4						0	0,064	4,30E-03	8,20E-04	0,0043	0,0078	0,0011	NA
Week_5							0	4,20E-03	1,50E-04	0,0022	0,005	0,0014	NA
Week_6								0	7,30E-03	0,0019	0,0091	0,0077	NA
Week_7									0	0,031	0,1	0,17	NA
Week_8										0	0,9	0,79	NA
Week_9											0	0,94	NA
Week_10												0	NA
Week_14													0
	n=1	n=11	n=11	n=10	n=11	n=13	n=11	n=12	n=12	n=5	n=4	n=9	n=1

3464

3465

3466

3467

3468
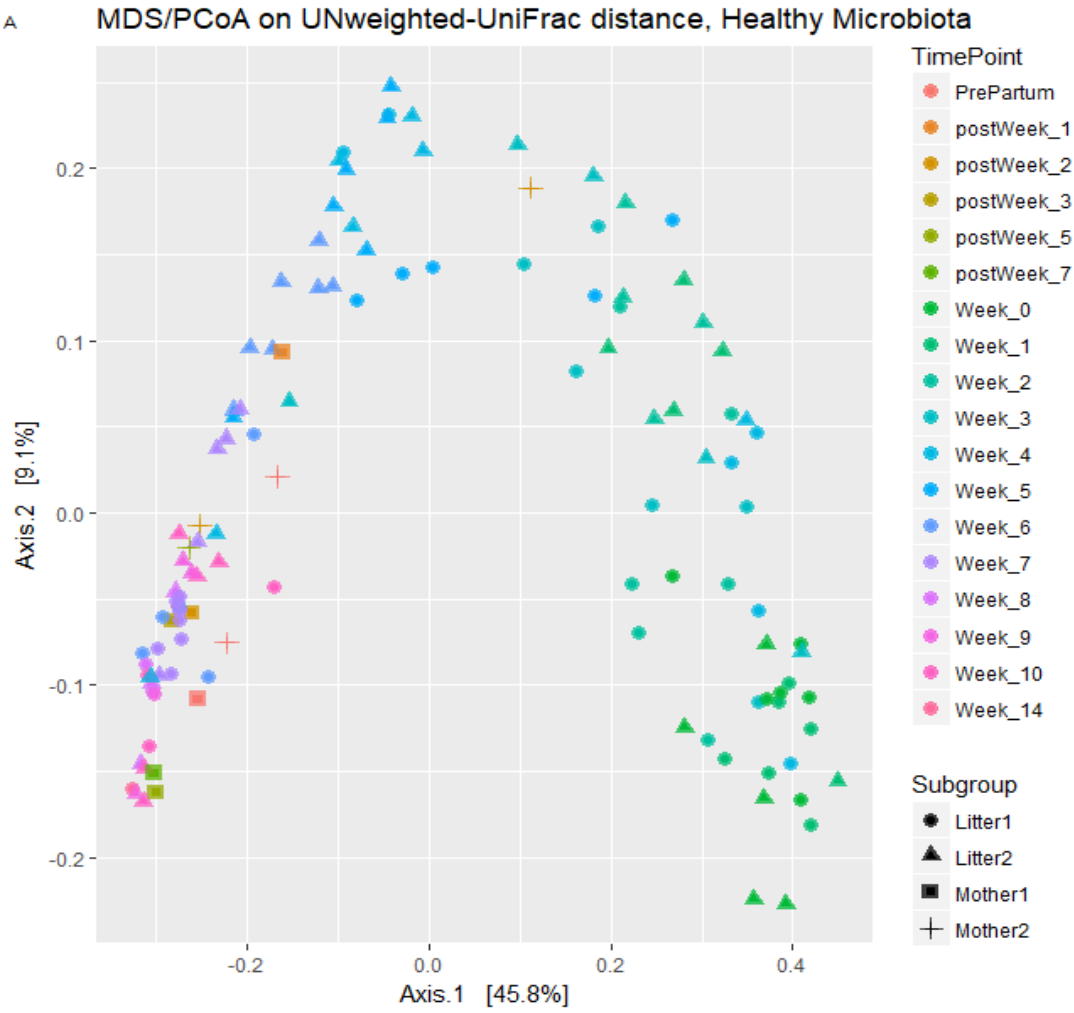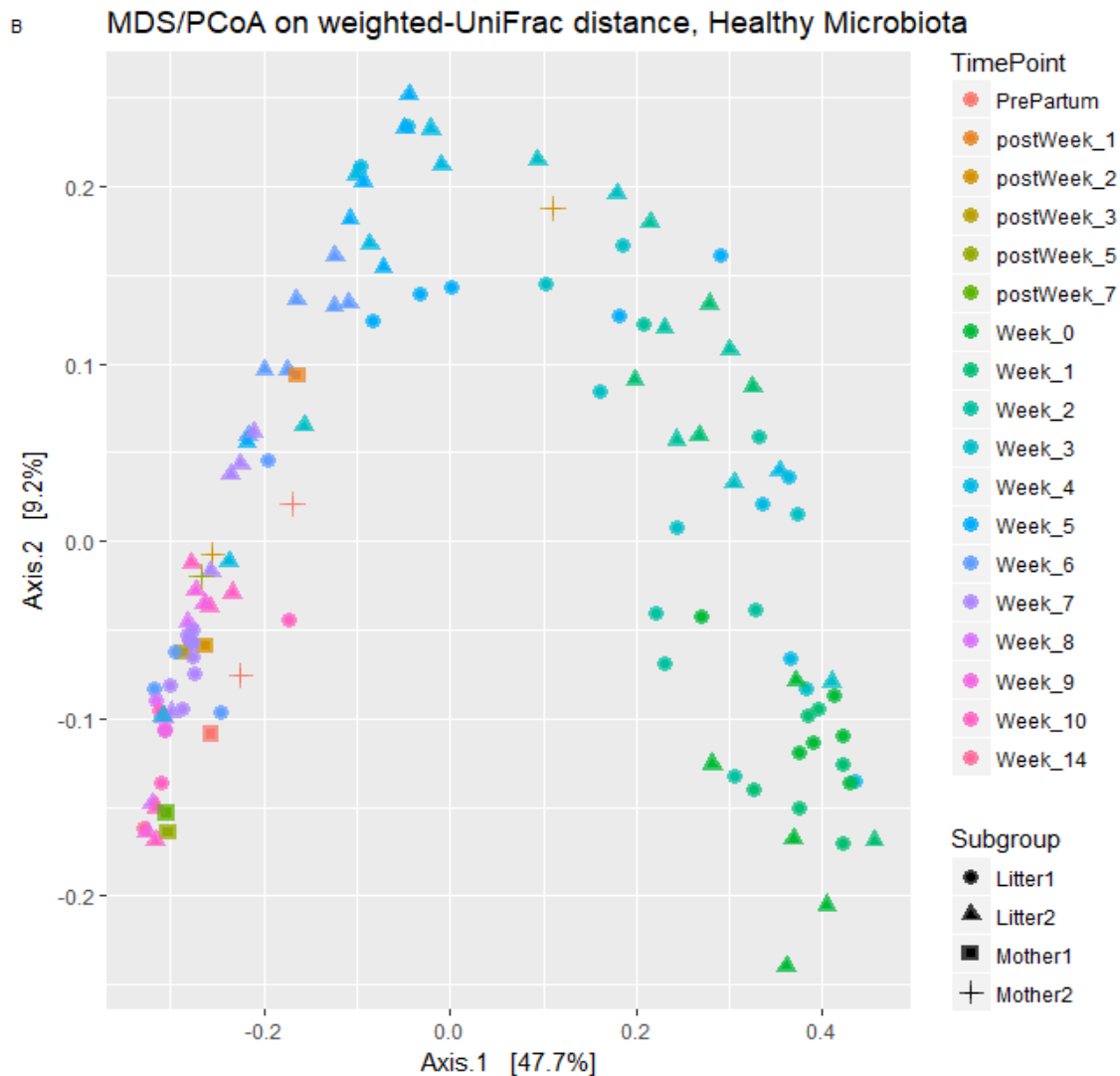
Figure 2.8: Beta diversity analysis in puppies. A: Unweighted and B: Weighted UNIFRAC analysis comparing mother and puppies and different time points. Puppies N=10; Day_0, n=1; Week_0, n= 11; Week_1, n=11; Week_2, n=10; Week_3, , n=11; Week_4, , n=13; Week_5, , n=11; Week_6, , n=12; Week_7, , n=12; Week_8, , n=5; Week_9, , n=4; Week_10, , n=9; and Week_14, , n=1. Mother N=2; n=11. N: Number of Animals, n: number of samples.

3474 **2.3.2.2 Characterisation of the maternal microbiota**
3475
3476 **2.3.2.2.1 Relative abundance of the major phylogenetic groups**
3477
3478 **2.3.2.2.1.2 Gut microbiota**
3479

3480 At prepartum, 94% of the mother's gut microbiota belonged to the Firmicutes phylum. Other phyla
3481 presented at that time were Proteobacteria (3,5%), Fusobacteria (2%) and Bacteroidetes (0,5%). During
3482 the first week after the partum, Firmicutes (57%) and Bacteroidetes (34%) predominated. Interestingly,
3483 Proteobacteria, the dominant phylum in puppies during the first weeks; was not present in high quantities
3484 during prepartum or immediately postpartum (first week post partum 8%). The phylum proteobacteria in
3485 mothers increased rapidly during the two weeks after partum (second week post partum proteobacteria
3486 accounted for 31% of all bacteria), suggesting that the influence is bidirectional, and puppies also affect
3487 their mothers' gut microbiota. This phylum decreased substantially at week three postpartum following the
3488 same pattern seen in puppies (third week postpartum 3%) (Figure 2.9).
3489

3490 At class level, prepartum period was characterised by the preponderance of Clostridia (72%) and Bacilli
3491 (21%) and at the order level by Clostridiales (72%) and Lactobacillales (21%). Postpartum was dominated
3492 by Bacteroidia (ranging from 31-34% in the first weeks up to 60-70% in the last weeks), Clostridia (21-28%)
3493 at class level and by Bacteroidales (ranging from 31-34% in the first weeks up to 60-70% in the last weeks),
3494 Clostridiales (28-40%) at order level. Noteworthy, week 1 postpartum was also characterised by a
3495 preponderance of Bacilli (28%) at class level, especifically of the order Lactobacilli; and week 2
3496 postpartum by a preponderance of Gammaproteobacteria (23%) at class level, especifically of the order
3497 Enterobacteriales (Figure 2.8). At week 7 postpartum, Fusobacteria accounted for ~10% of all bacteria at
3498 class level (Fusobacteriales at order level).
3499

3500 **2.3.2.3.1.2 Oral microbiota**
3501

3502 We investigated the contribution of the oral microbiome in the puppy gut microbiota. Although, in general
3503 there is a minimal overlap between the oral cavity and gut microbiota in adults (consortium, 2012); a study
3504 have shown that in infants; oral microbiome represented about a quarter of the total abundance of the stool
3505 microbiome (at least in the first three days) (Ferretti et al., 2018).

3506 When the relative abundance of the main phylogenetics groups were analysed in samples from the oral
3507 microbiota, it could be seen that the majority of the groups that were present in the oral mucosa; were
3508 present in the tongue with some differences in abundance of each group. Also, the same trend was
3509 observed when samples were compared at different time points (prepartum versus postpartum), with only
3510 differences in the abundance of each group (Figure 2.10).
3511
3512 At prepartum, between 60-75% of the bacteria belonged to the phylum Bacteroidetes, with other groups
3513 present at much lower percentages, such as Proteobacteria (18-33%), Firmicutes (4-6%), At class level,
3514 Bacteroidia (53-68%) predominated, followed by Gammaproteobacteria (13-26%).
3515
3516 Two weeks after labour, the phyla Proteobacteria (49%) and Bacteroidetes (38%) were the most abundant
3517 groups. At class level, the same groups were the most abundant, but the relative abundance of each group
3518 changed: Bacteroidia (36%) and Gammaproteobacteria (23%). In the tongue, Betaproteobacteria (23%)
3519 and Flavobacteria (20%) were present in high quantities (Figure 2.10).
3520
3521
3522
3523
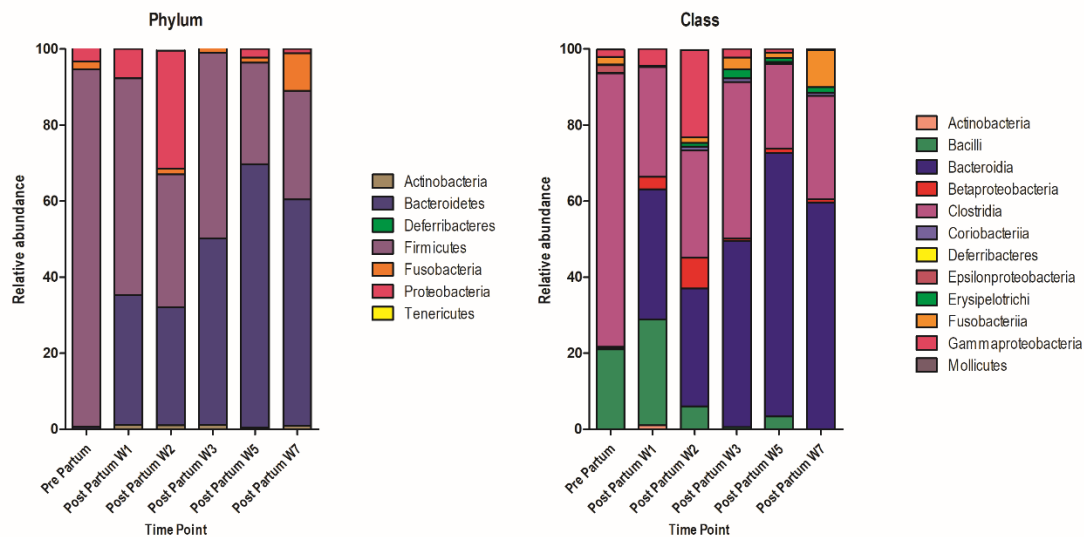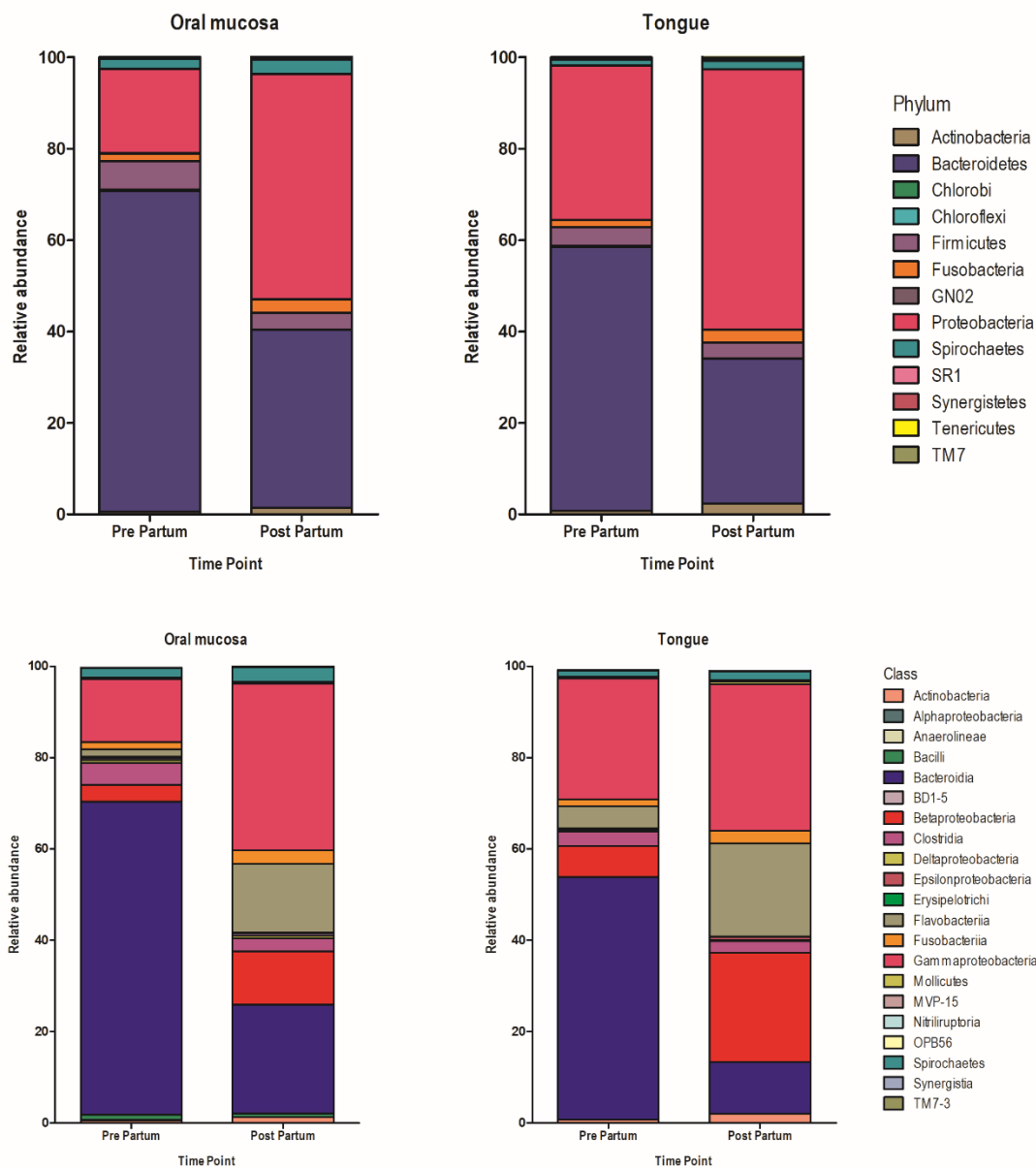3524

3525

3526

3527

3528 Figure 2.8: Relative abundance of the major phylogenetic levels in the gut microbiota in mothers before and after

3529 partum. Mother N=2; n=11. Samples were rarified at 5000 sequences. N: Number of Animals, n: number of samples.

3530

3531 Figure 2.9: Relative abundance of main phylogenetics groups in oral microbiota: Oral mucosa versus Tongue. Mother

3532 N=2; n=7 (oral mucosa n=4; tongue n=3). Samples were rarified at 5000 sequences.

3533 N: Number of Animals, n: number of samples.

3534

2.3.2.2.2 Microbial differential abundance testing

3535
3536
3537   Mothers were compared to young adults to assess which taxonomic groups in the intestine were

3538   significantly different between the groups. When mothers were compared to young adults, six family groups

3539   were significantly enriched in mothers: Clostridiaceae, Peptostreptococcaceae, Lachnospiraceae,

3540   Lactobacillaceae and Succinivibrionaceae (Table 2.7).

3541

3542
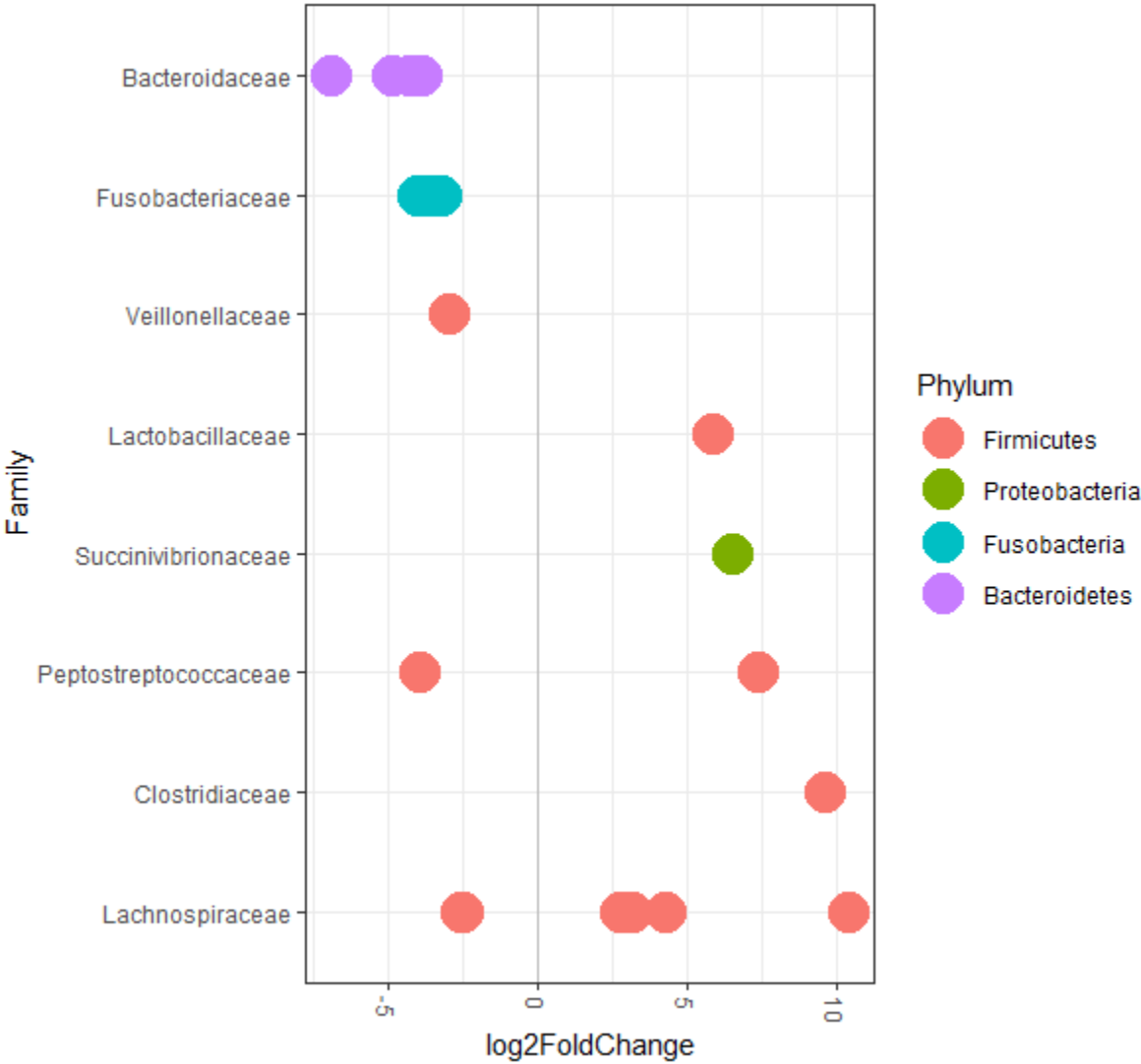
3543
3544   Figure 2.10: Microbial differential abundance family groups. Mother versus Young Adults. Mother N=2; n=11. Adults
3545   N=10, n=73.

3546 Table 2.7 Microbial differential abundance family groups. Mother versus Young Adults

3547

OTUs	base Mean	log2Fold Change	lfcSE	stat	P value	P adj	Phylum	Class	Order	Family	Genus	Species
Otu 17	712.3	9.609.704	11.854	8.106.	5,22E-10	6,84E-08	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<NA>	<NA>
Otu 34	586.6	7.369.011	0.9824	7.500.	6,37E-08	4,17E-06	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	<NA>	<NA>
Otu 78	83.8	10.422.383	17.476	5.963.	2,47E-03	1,08E-01	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium	<NA>
Otu 21	479.6	-3.914.828	0.725	-5.396.	6,80E-02	2,23E+00	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	<NA>
Otu 185	41.37	-3.984.128	0.761	-5.231.	1,68E-01	4,41E+00	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	<NA>
Otu 325	3.14	4.305.537	0.879	4.893.	9,91E-01	2,16E+01	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	<NA>
Otu 105	108.45	-2.479.658	0.540	-4.591.	4,40E+00	8,23E+01	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<NA>	<NA>
Otu 4	3.874.74	-3.200.193	0.701	-4.561	5,08E+00	8,32E+01	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	<NA>
Otu 24	163.7	-4.024.523	0.888	-4.531	5,86E+00	8,53E+01	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	<NA>
Otu 9	546.1	-4.220.209	10.045	-4.201	2,66E+01	3,48E+02	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	<NA>
Otu 27	77.9	3.178.349	0.765	4.151	3,30E+01	3,94E+02	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blauti	<NA>
Otu 42	19.9	-6.868.535	17.629	-3.895	9,78E+01	1,07E+03	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	<NA>
Otu 79	11.4	-3.878.843	10.218	-3.795.	1,47E+02	1,48E+03	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	<NA>
Otu 314	9.6	-2.549.188	0.675	-3.776	1,59E+02	1,49E+03	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<NA>	<NA>
Otu 73	33.6	-3.915.888	10.417	-3.759.	1,71E+02	1,49E+03	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	<NA>	<NA>
Otu 22	14.	5.892.301	16.024	3.676.	2,36E+02	1,93E+03	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	ruminis
Otu 12	46.0	-4.872.392	13.450	-3.622.	2,92E+02	2,12E+03	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	<NA>
Otu 3	2.667	-2.951.090	0.811	-3.635.	2,77E+02	2,12E+03	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Megamonas	<NA>
Otu 189	15.94	2.770.786	0.772	3.586.	3,35E+02	2,31E+03	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<NA>	<NA>
Otu 389	4.60	-3.544.481	10.581	-3.349	8,09E+02	5,30E+03	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	<NA>
Otu 402	1.70	2.740.856	0.8461	3.239	1,20E+03	7,48E+03	Firmicutes	Clostridia	Clostridiales	<NA>	NA>	<NA>
Otu 68	65.38	6.548.851	20.448	3.202	1,36E+03	8,11E+03	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	Anaerobiospirillum	<NA>

3548

3549 **2.3.2.2.3 Diversity Analysis**
3550

3551 Analysis of alpha diversity showed that the lowest diversity was present during the first week postpartum.

3552 Beta diversity analysis showed that microbial communities in mothers clustered together according to the

3553 type of sample, being faecal samples separated from oral microbiota as expected (P value: <0,001).

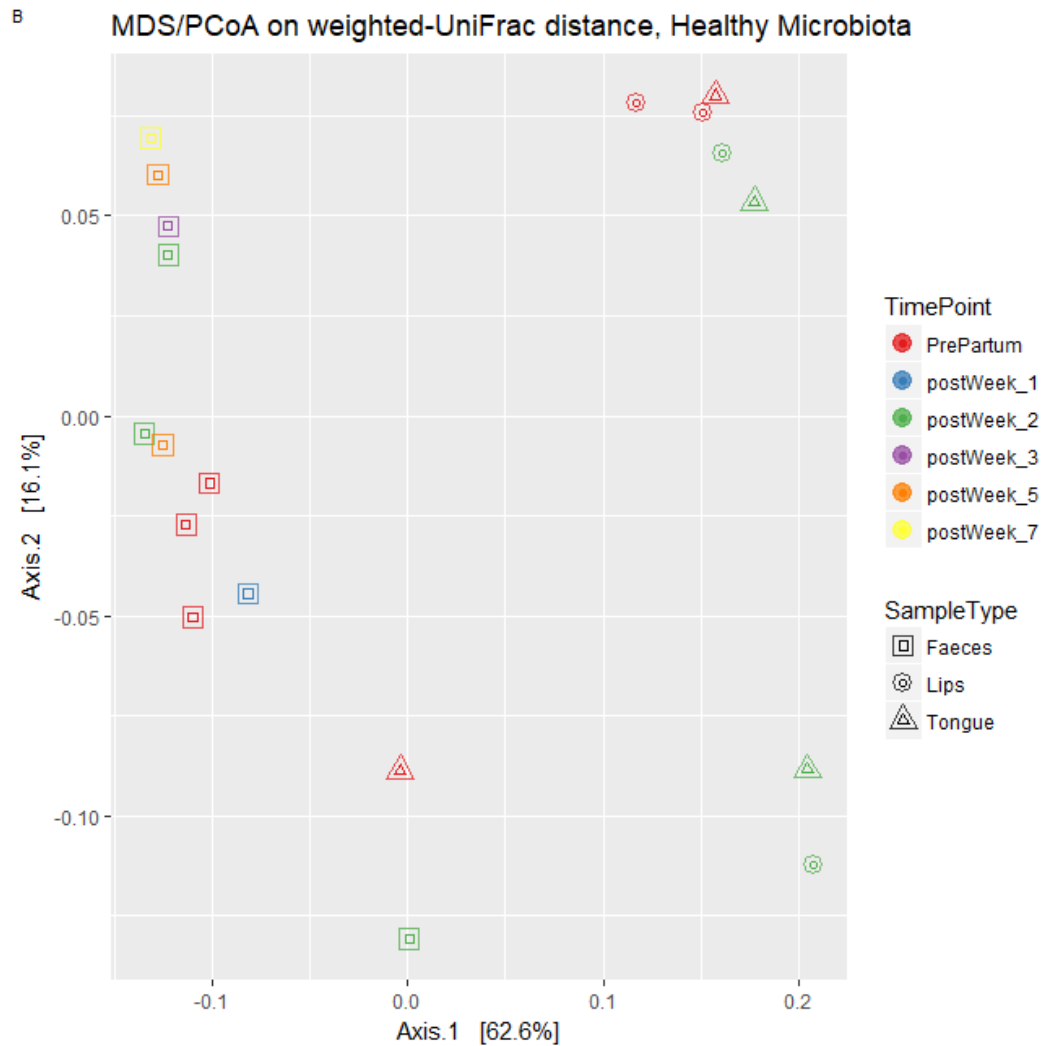3554 However, time point didn't account much in the observed differences (P value: 0.437) (Figure 2.11).

3555

3556
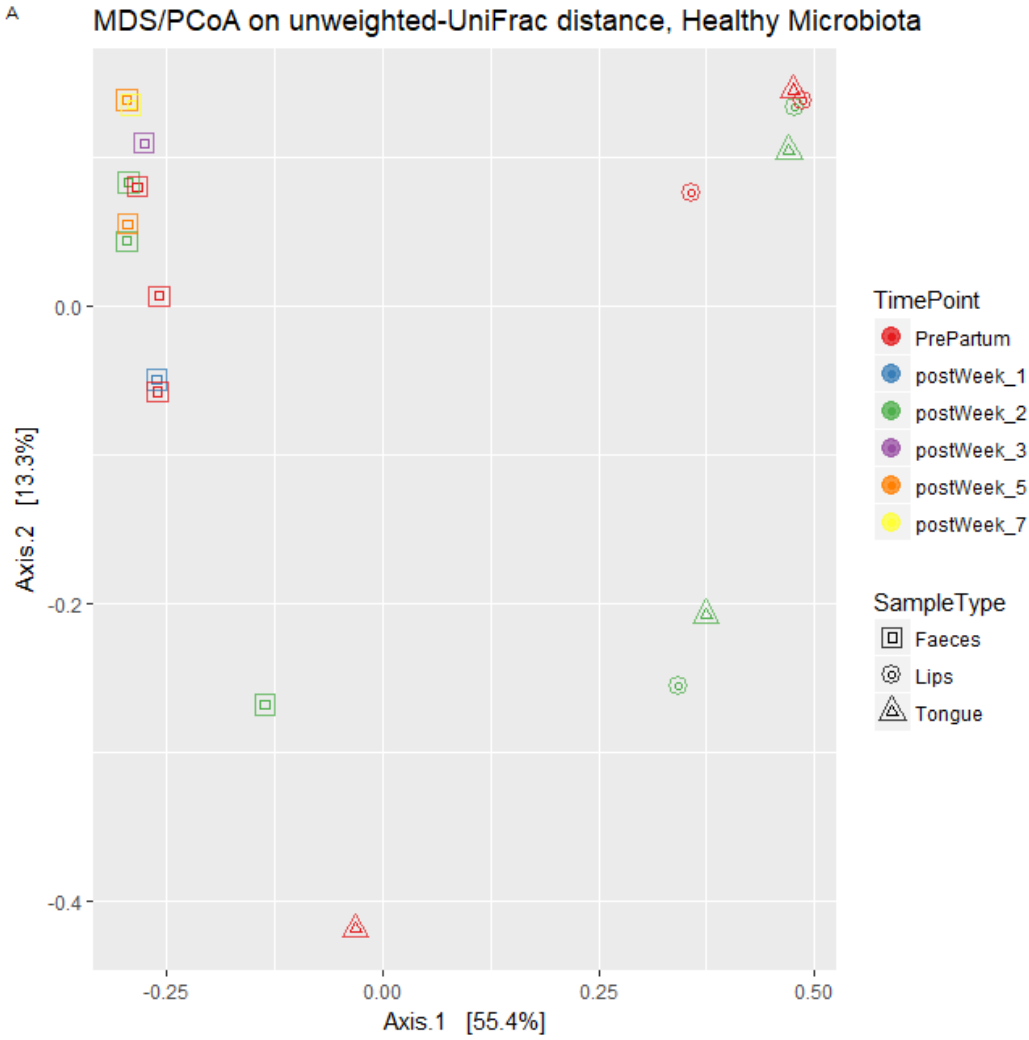
3557

3558

3559

3560

Figure 2.11: Beta diversity analysis in mothers. A: Unweighted and B: Weighted UNIFRAC analysis comparing different time points and type of samples. Mother N=2; n=19 (Faeces=11, oral mucosa n=4; tongue n=3).

3566 ## 2.3.2.3 Gut microbiota in growing dogs
3567
3568 ### 2.3.2.3.1 Relative abundance of the major phylogenetic groups
3569
3570
3571 During this period, the gut microbiota undergoes its final significant shift attributed to the continued
3572 influence of a varied solid food diet and especially to a greater environmental exposure. Although gut
3573 microbiota development followed a similar trend over time toward a more like adult-like microbiota; this was
3574 not yet completed by one year of age. Three dogs were assessed in this group. For one dog, it was
3575 possible to collect only three samples as the owner had to move out and it was difficult for her to bring her
3576 puppy. For dog2, five-time points were collected. The last sample could not be collected as the dog
3577 suffered from an urinary tract infection and received antibiotics. For dog1, seven samples were collected in
3578 total. Samples six corresponded to the transition from puppy to an adult diet (mix in a proportion 50:50
3579 same brand) and sample seven was adult diet exclusively. At phylum level, the first five time points were
3580 characterised by Bacteroidetes (38-53%), followed by Firmicutes (23-40%), Fusobacteria (16-26%) and
3581 Proteobacteria (2-10%). Time-point six and seven corresponded with the diet transition and it was
3582 characterised by the predominance of Fusobacteria (46-52%) followed by Firmicutes (29-15%),
3583 Bacteroidetes (14-19%) and Proteobacteria (4-18%) (Figure 2.12).
3584
3585 At class level, Bacteroidia (38-53%), followed by Clostridia (10-39%), Fusobacteria (16-26%),
3586 Gammaproteobacteria (1-7%) and Betaproteobacteria (1-2%) preponderated in the first five time-points.
3587 The last two-time points were ruled in decreasing order by Fusobacteria (46-52%), Clostridia (15-28%),
3588 Bacteroidia (19-14%), Gammaproteobacteria (2-15%) and Betaproteobacteria (1-3%) (Figure 2.12).
3589
3590 At order level, Bacteroidales (53-38%), Clostridiales (39-10%), Fusobacteriales (26-16%),
3591 Enterobacteriales (0-7%) and Burkholderiales (1-2%), The last two-time points had the same groups but
3592 the abundance changed. Fusobacteriales (46-52%), followed by Clostridiales (28-15%) and Bacteroidales
3593 (19-14%) predominated.
3594
3595 ### 2.3.2.3.1 Diversity Analysis
3596
3597 Alpha diversity analysis showed that diversity is still incresing over time and a lot of variation exists, (Figure
3598 2.13). When Beta diversity was studied, samples were dispersed over time; and the differences among

3599   subjects was significant (P value: < 0,001). However, samples coming from the same individual clustered
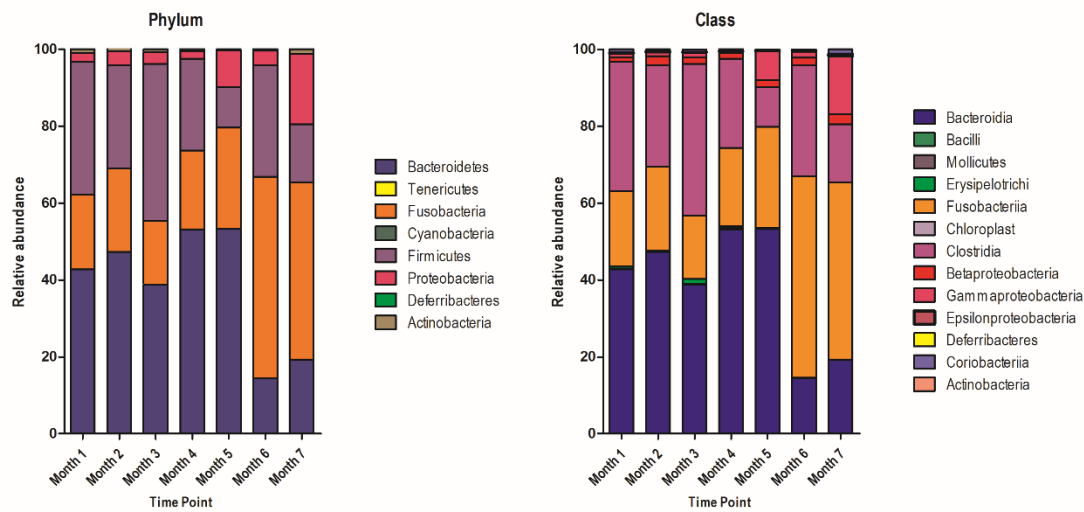
3600   together (Figure 2.14).

3601

3602

3603

3604

3605

3606   Figure 2.12: Top 20 of the relative abundance of main phylogenetics groups in the group 3 -12 months.

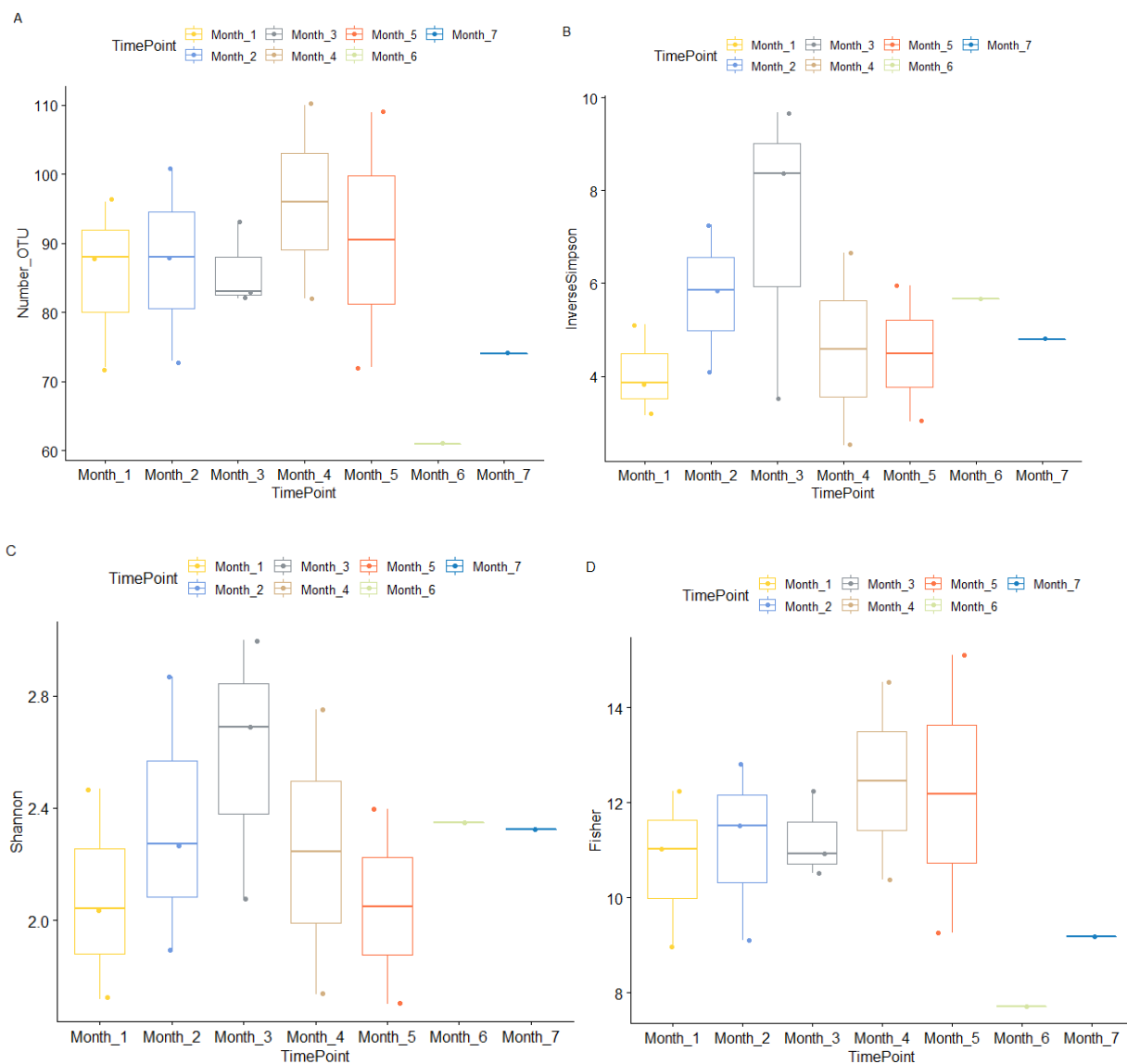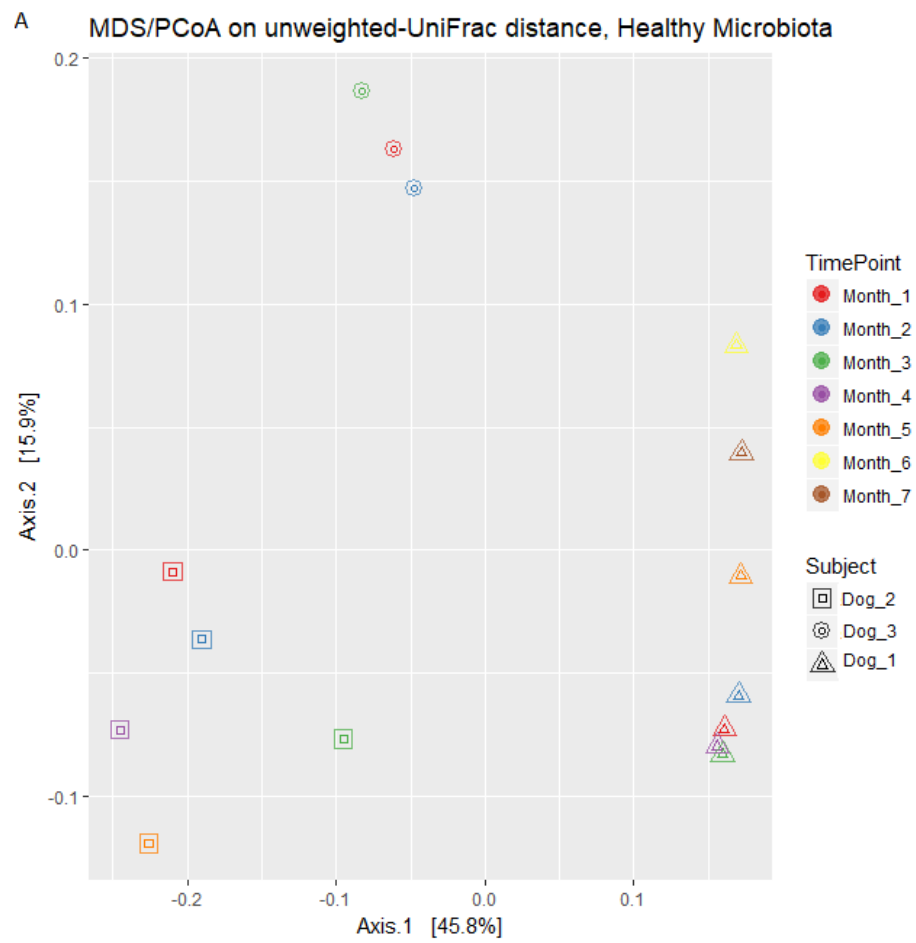3607   N=3, n=15. Samples were rarified at 5000 sequences.

3608

3609

3610

3611

3612

3613

Figure 2.13: Alpha diversity analysis of puppies (growth group) at different time points. A:Number of OTUs (Observed), B: Inversed Simpson's Index, C: Shannon's Index and D: Fisher's Index.. Month_1, n=3 Month_2, n= 3; Month_3, n=3; Month_4, n=2 Month_5, , n=2; Month_6, , n=1; Month_7, , n=1 N: 3 puppies.
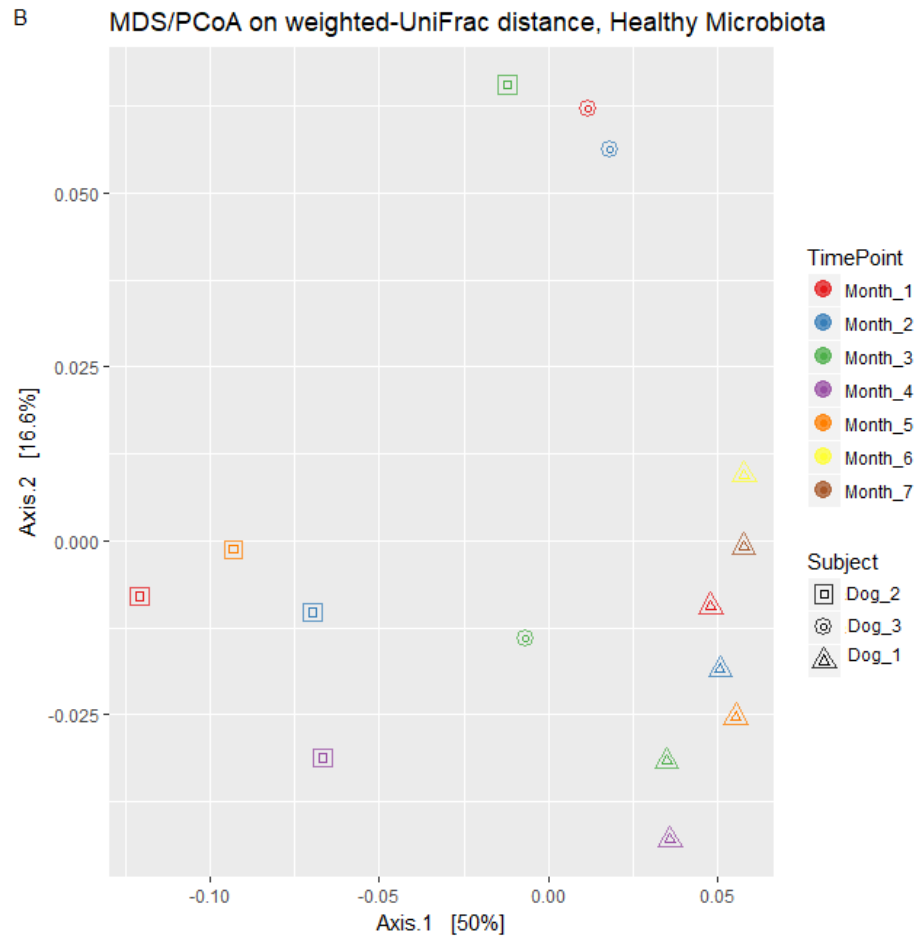
3617

3618

A

MDS/PCoA on unweighted-UniFrac distance, Healthy Microbiota

3619

3620

3621

3622

3623

B

MDS/PCoA on weighted-UniFrac distance, Healthy Microbiota

3624

3625

3626   Figure 2.14: Beta diversity analysis in Growth puppies. A: Unweighted and B: Weighted UNIFRAC analysis

3627   comparing different time points and subjects. N=3, n=15.

3628

3629

3630 **2.3.2.4 Gut microbiota in adulthood**
3631
3632 **2.3.2.4.1 Relative abundance of the major phylogenetic groups**
3633
3634 **2.3.2.4.1.1 Young adults**
3635

3636 At this age stage, higesth phylogenetic levels were constant during the sampling period and their relative
3637 abundance and classification were in accordance with previous reports in healthy dogs (Jan S.
3638 Suchodolski, 2013). At phylum level, the most abundant group in decreasing order were Bacteroidetes (41-
3639 56%), Firmicutes (18-32%), Fusobacteria (18-27%), Proteobacteria (3-9%) and Actinobacteria and
3640 Tenericutes (less < 1%) (Figure 2.15).
3641

3642 At Class level, Bacteroidia (41-56%) predominated, followed by Fusobacteria (18-27%), Clostridia (17-
3643 26%), Gammaproteobacteria (0,3-7%) and Betaproteobacteria (2-4%) (Figure 2.14). The order level,
3644 followed the same trend with Bacteroidales being the most abundant (41-56%). Other groups that were
3645 present were Fusobacteriales (18-27%), Clostridiales (17-26%), Lactobacillales (1-6%), Enterobacteriales
3646 (0,3-6%) and Burkholderiales (2-4%).
3647

3648 **2.3.2.4.1.2 Mature dogs**
3649

3650 Although the number of dogs was small in this group, the same characteristics that were observed in
3651 young adults were observed in mature dogs. At phylum level, the most abundant group in decreasing order
3652 were Bacteroidetes (20-50%), Firmicutes (20-43%), Fusobacteria (23-32%), Proteobacteria (2-10%) and
3653 Actinobacteria (less < 1%) (Figure 2.15).
3654

3655 At Class level, Bacteroidia 30-50%) predominated, folloved by Fusobacteriia (23-32%), Clostridia (19-32%),
3656 Betaproteobacteria (1-10%), Gammaproteobacteria (0-4%) and Bacilli (0-1%) (Figure 2.14). The order
3657 level, followed the same trend with Bacteroidales being the most abundant (20-50%). Other groups that
3658 were present were Fusobacteriales (23-32%), Clostridiales (19-44%), Lactobacillales (0-1%),
3659 Enterobacteriales (0-4%) and Burkholderiales (2-10%).
3660

3661 **2.3.2.4.1.3 Senior dogs**
3662

3663   Only two dogs in this group were followed over time. One of the dogs had a history of chronic kidney

3664   disease that has been under control over the last year with diet only. However, the dog was withdrawn from

3665   the study at month-3 due to a flare-up of clinical signs (dog2). In people, it has been reported that kidney

3666   disease is associated with alterations in the gut microbiota and thus could have an effect in the microbiota

3667   profile of this dog.

3668

3669   In general, the same groups that were observed in adult and mature dogs; were present in this group.

3670   (Figure 2.15) At phylum level, the most abundant group varied at different sample-points. The groups

3671   presented were Bacteroidetes (34-72%), Firmicutes (16-42%), Fusobacteria (3-39%), Proteobacteria (1-

3672   9%) and Actinobacteria and Tenericutes (less < 1%) (Figure 2.15).

3673

3674   At Class level, Bacteroidia 34-72%) predominated, followed by Clostridia (15-40%), Fusobacteriia (3-39%),

3675   Gammaproteobacteria (0-9%), Betaproteobacteria (0,5-2%), Erysipelotrichi (0,5-2%) (Figure 2.15). The

3676   order level, followed the same trend with variations at different time-points. The main group presented were

3677   Bacteroidales (34-72%), Fusobacteriales (3-39%), Clostridiales (15-40%), Lactobacillales (0-2%),

3678   Enterobacteriales (0-8%), Burkholderiales (0,5-2%) and Erysipelotrichales (0,5-2%).

3679

3680

3681
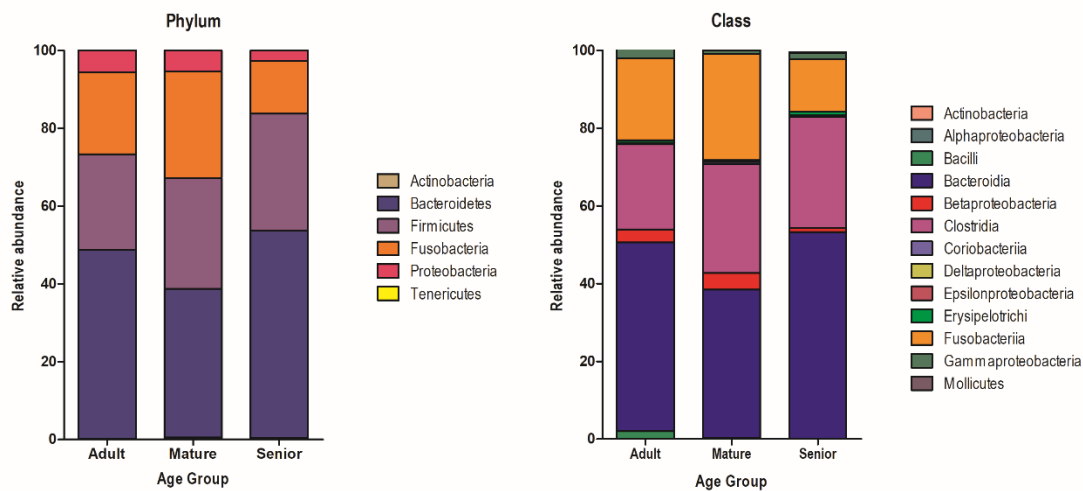
3682

Figure 2.15: Top 20 of the relative abundance of main phylogenetics groups during adulthood. Young Adult (1_7 year) N=10. Mature (8_10 year) N=3; n=17. Senior (11_15 year) N=2; n=10. N: Number of Animals, n: number of samples. Samples were rarified at 5000 sequences. N: Number of Animals, n: number of samples.

3686 **2.3.2.4.1 Microbial differential abundance testing**

3687

3688 Young adults were compared to mature dogs (senior were not compared as only two dogs were part of the

3689 group) to assess which taxonomic groups were significantly different between the groups. When young

3690 adults were compared to mature dogs, three family groups were significantly enriched in mature dogs:

3691 Lachnospiraceae, Bacteroidaceae and Erysipelotrichaceae (Table 2.8) (Figure2.16).
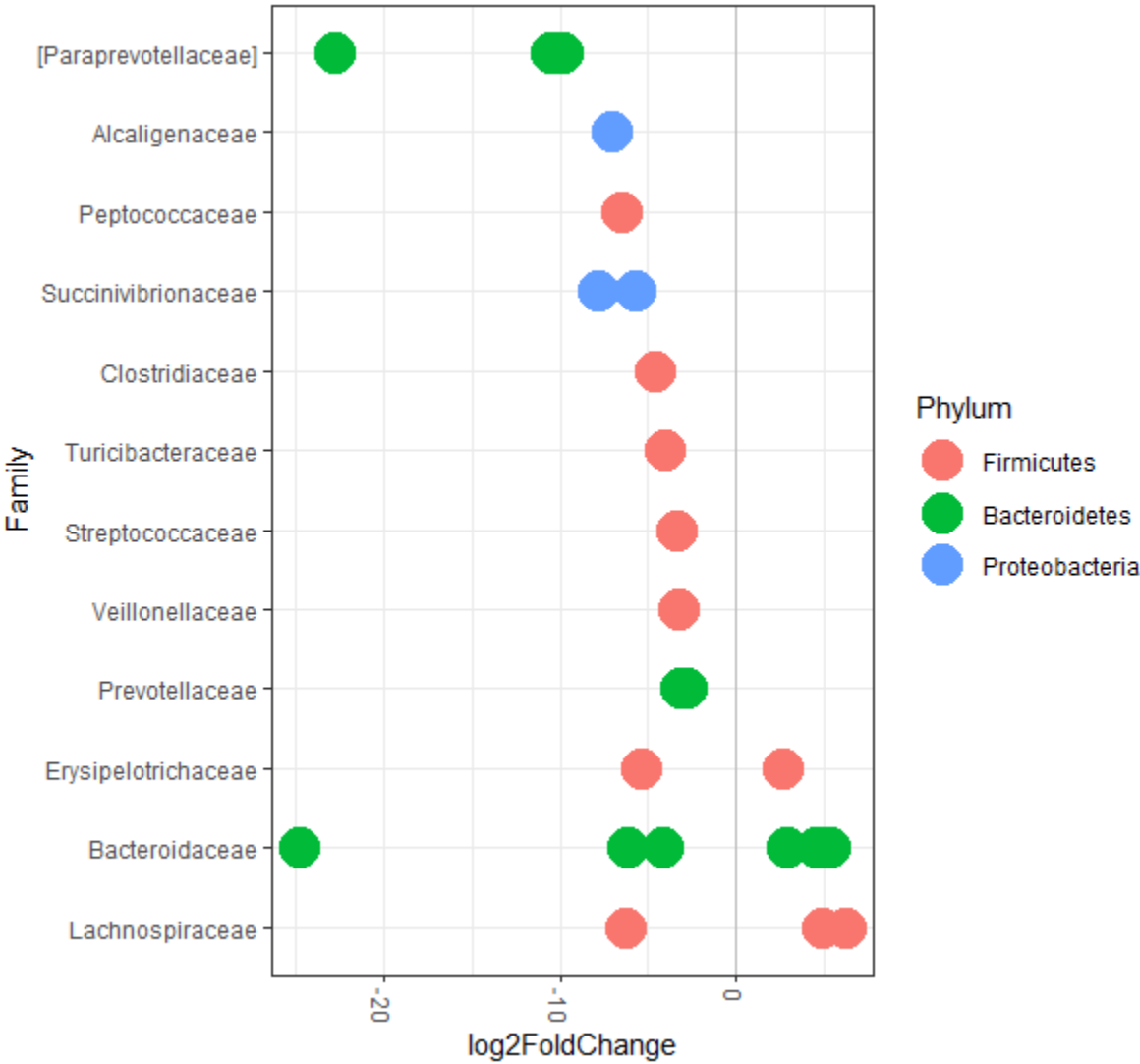
3692

3693

3694

3695

3696 Figure 2.16: Microbial differential abundance family groups. Adults versus Mature dogs. Young Adult (1_7 year)

3697 N=10. Mature (8_10 year) N=3; n=17.

3698
3699

3700

3701 Table 2.8 Microbial differential abundance. Adults versus Mature dogs.

3702

OTUs	baseMean	log2FoldChange	lfcSE	stat	pvalue	Padj	Phylum	Class	Order	Family	Genus	Species
Otu86	37.422.286	-24.821.940	16.706.000	-14.858.099	6,16E-44	9,06E-42	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	<NA>
Otu7	1.741.884.008	-10.486.024	0.9072448	-11.558.098	6,72E-25	4,94E-23	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	[Prevotella]	<NA>
Otu51	38.825.444	-22.810.414	20.222.312	-11.279.825	1,65E-23	8,09E-22	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	[Prevotella]	<NA>
Otu129	2.566.284	4.915.869	0.7411646	6.632.628	3,30E-05	1,21E-03	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<NA>	<NA>
Otu62	30.746.709	-7.792.746	12.808.824	-6.083.889	1,17E-03	3,45E-02	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	<NA>	<NA>
Otu101	4.554.658	6.286.515	11.990.539	5.242.896	1,58E-01	3,49E+00	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<NA>	<NA>
Otu262	5.719.285	5.339.105	10.201.168	5.233.818	1,66E-01	3,49E+00	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	<NA>
Otu46	35.485.640	-4.044.139	0.7876929	-5.134.157	2,83E-01	5,21E+00	Firmicutes	Bacilli	Turicibacterales	Turicibacteraceae	Turicibacter	<NA>
Otu25	86.700.152	-5.378.564	10.760.861	-4.998.266	5,78E-01	9,45E+00	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Catenibacterium	<NA>
Otu89	17.786.500	-7.003.729	14.415.957	-4.858.317	1,18E+00	1,74E+01	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella	<NA>
Otu26	14.921.234	-6.194.880	13.485.530	-4.593.723	4,35E+00	5,82E+01	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	coprophilus
Otu79	11.299.099	-4.171.525	0.9121217	-4.573.430	4,80E+00	5,88E+01	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	<NA>
Otu12	709.325.663	4.570.068	10.530.451	4.339.859	1,43E+01	1,61E+02	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	<NA>
Otu97	12.740.339	-6.524.413	15.376.574	-4.243.086	2,20E+01	2,31E+02	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Peptococcus	<NA>
Otu23	12.190.828	-6.307.198	15.072.258	-4.184.640	2,86E+01	2,80E+02	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	<NA>
Otu41	132.989.408	-3.283.415	0.7894174	-4.159.289	3,19E+01	2,93E+02	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	<NA>
Otu58	22.911.766	-9.789.246	24.305.426	-4.027.597	5,63E+01	4,87E+02	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	<NA>	<NA>
Otu110	5.674.794	2.655.857	0.6633804	4.003.521	6,24E+01	5,10E+02	Firmicute	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	<NA>	<NA>
Otu24	479.217.696	2.920.607	0.7505152	3.891.470	9,96E+01	7,71E+02	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	<NA>
Otu2	5.019.573.603	-3.160.872	0.8238806	-3.836.566	1,25E+02	9,17E+02	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	copri

Otu 61	5.966.725	-4.602.013	12.798.228	-3.595.820	3,23E+02	2,26E+03	Firmicute	Clostridia	Clostridiales	Clostridiaceae	Clostridium	<NA>
Otu 391	3.070.538.468	-2.797.306	0.8323433	-3.360.760	7,77E+02	5,19E+03	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	copri
Otu 35	7.885.452	-5.649.372	17.178.831	-3.288.566	1,01E+03	6,44E+03	Proteobacteri	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	Anaerobiospirillum	<NA>
Otu 5	51.363.321	-3.333.347	10.374.981	-3.212.870	1,31E+03	8,05E+03	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	<NA>

3703

3704   **2.3.2.4.2 Diversity Analysis**

3705

3706   When Alpha diversity was analysed, community structure was highly variable but exhibited less variability

3707   within the individual over time and it was not significantly different. Representative data from the young

3708   adult group is shown in figure 2.17.

3709

3710   Analysis of Beta diversity showed subject-specfic grouping of samples and its seems that each dog

3711   harbours a subject- specific core (Young adults versus mature dogs: P value: <0,001). (Figure 2.18).

3712

3713

3714

3715

3716
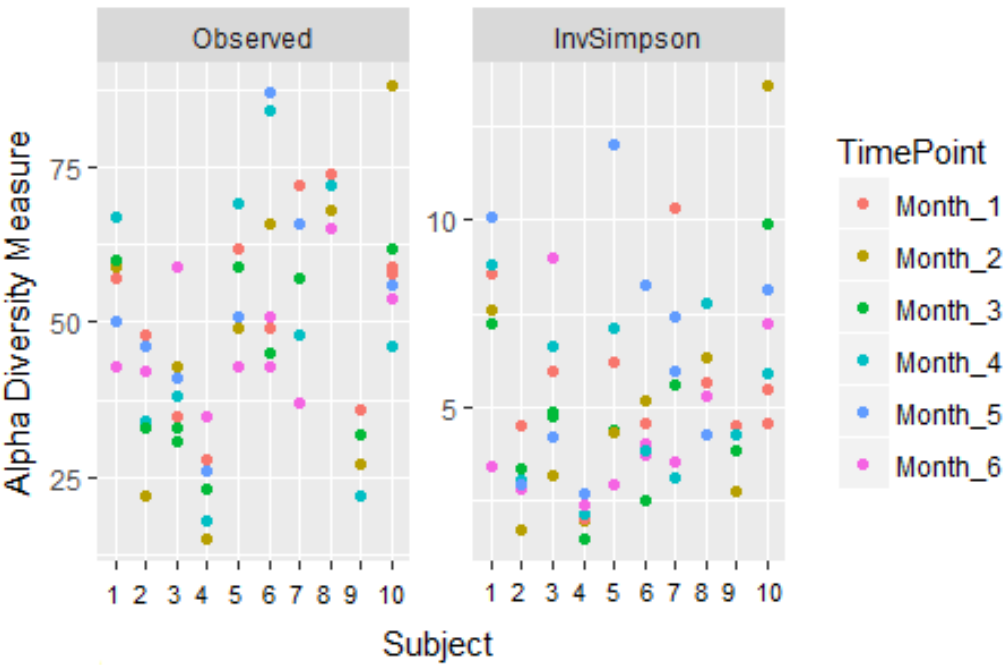
3717 Figure 2.17: Alpha diversity analysis of adult dogs at different time points and per subject. Observed

3718 (Number of OTUS) and Inversed Simpson index analysis.The same level of dispersion was seen in senior

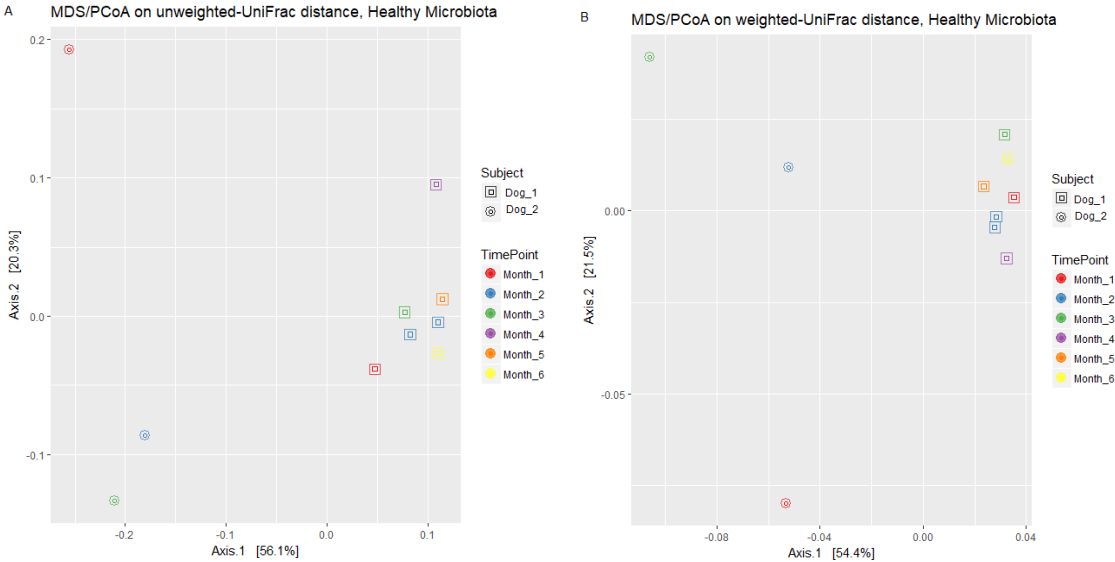3719 and mature dogs.

3720

3721

3722

3723

3724

3725
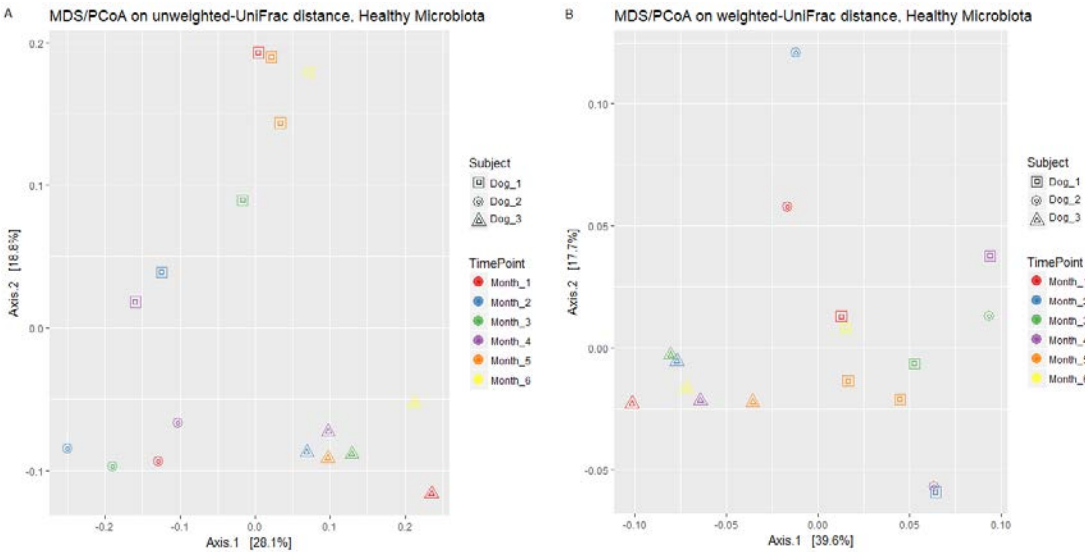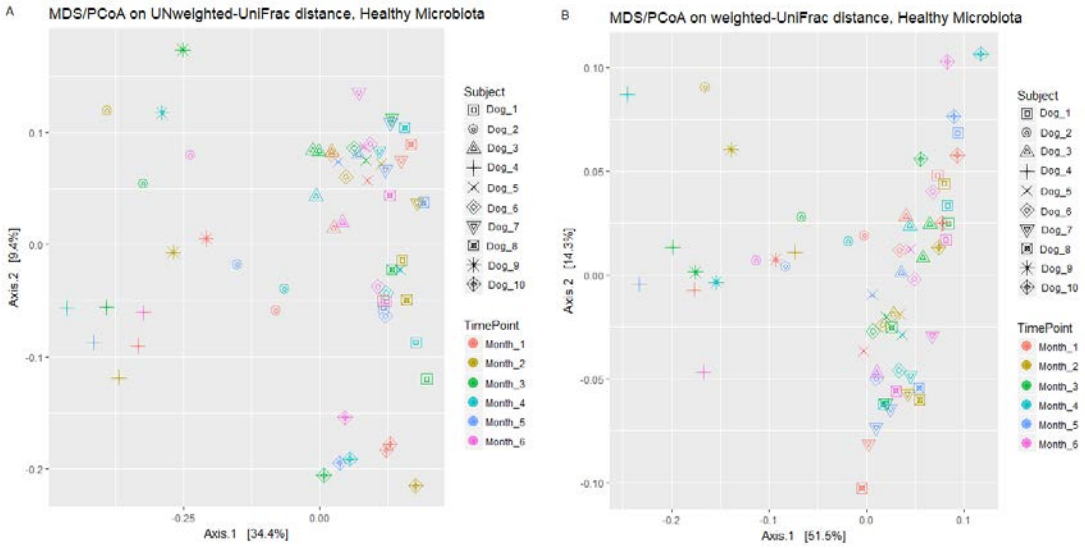
Figure 2.18 : Beta diversity analysis in adult (upper panel), mature (middle panel) and senior dogs (lower

panel). A: Unweighted and B: Weighted UNIFRAC analysis comparing different time points and subjects.

3726

3727

3728

3729

3730

3731

2.4 Discussion

Gut microbiota plays a crucial role in physiologic, metabolic and immunologic processes and thus; it helps the body to keep a homeostatic state. The first step in understanding the symbiotic relationship between gut microbes and their host consists in the characterisation of the baseline healthy microbiota and how the microbiota evolves and is established. Differences in microbial colonization patterns are not only important for microbial composition, but also because they influence the concomitant development of the immune system. The microbiome is dynamic and responds to a variety of factors including diet, environment, medical interventions, and disease states. Also, developmental milestones (such as infancy and ageing) mark important changes in the gut microbiota. These stages are accompanied by numerous physiological changes, including changes in hormonal levels, inflammation, and metabolic states.

Previously, it was believed that the first microbial contact took place immediately after birth. However, recent studies in humans, have found bacterial DNA in placenta, amniotic fluid and meconium (Aagaard *et al.*, 2014). It is suggested that in people, the foetal microbiome is acquired in the last trimester from the microbiome in the mother's amniotic fluid (Laing, Barnett, Marlow, Nasef, & Ferguson, 2018). Meconium are the first faeces produced after birth and are mainly composed of the amniotic fluid ingested during the foetal period. Bacterial composition of meconium is about 61% shared with the bacterial composition of amniotic fluid, further suggesting bacterial colonization *in utero* (Ardissone *et al.*, 2014).

We followed two litters weekly from birth until 10 weeks old. From one of the dogs, it was possible to obtain a sample of meconium. Analysis of the sample revealed the presence of bacterial DNA, mainly from the Proteobacteria phylum (94%). Although, it is impossible to draw conclusions from a single sample, findings are similar to those reported in babies. In babies, it has been found that approximately 67% of the meconium samples contained bacterial DNA (Ardissone *et al.*, 2014). In babies, meconium shows low species diversity, high between-subject variation, and enrichment of Proteobacteria at the expense of reduction of Bacteroidetes (Hu *et al.*, 2013).

Controversy about the presence of bacteria prenatally remains in people. First, the presence of bacterial DNA doesn't necessarily mean viable bacteria or that they or their products are really exerting a physiological role in the foetus and later on during the baby's life or in the assembly and succession of a

3764 microbial community. Currently, this is unknown. Also, Lauder *et al.* (2016) using quantitative PCR of 16S

3765 rRNA gene, found that placental samples and negative controls contained low and indistinguishable

3766 bacterial copy numbers, suggesting that findings could be more related to contamination introduced during

3767 the DNA purification or sequencing process (Lauder *et al.*, 2016). Additionally, it has been found that

3768 reagent and laboratory contamination can critically impact the results (Salter *et al.*, 2014). We sequenced

3769 some negative samples, including the water used for resuspension of the DNA. Although some of the

3770 samples, showed bacterial DNA, most of which corresponded to the phylum Proteobacteria (97%); at lower

3771 phylogenetic levels, bacteria were clustered in groups not found (or found less than 1% abundance) in

3772 meconium.

3773

3774 Numerous factors in early life contribute to the establishment and maintenance of the microbiome. This

3775 stage is characterised by enormous dietary changes, new environmental exposures, and maturation of the

3776 immune system; all of which shape the gut microbiota (Nuriel-Ohayon, Neuman, & Koren, 2016).

3777

3778 In people and mice, several studies have shown that bacterial gut colonization is influenced by delivery

3779 mode, gestational age at birth, feeding patterns, sanitary conditions, antibiotic administration and maternal

3780 diet. After birth, the intestine of puppies becomes rapidly colonized by bacteria present in the birth canal,

3781 skin, milk, and surrounding environment. Samples collected one day after birth (week 0) were characterised

3782 by the predominance of Proteobacteria (83%) and Firmicutes (17%). At class level, Gammaproteobacteria

3783 (82%), Clostridia (16%) and Bacilli (1%) were the most abundant groups and at family level,

3784 Enterobacteriaceae (83%), Clostridiaceae (15%) and Enterococcaceae (1%) predominated.

3785

3786 This was in accordance with previous studies performed in people and dogs. The first colonizers in the

3787 intestine are mainly facultative anaerobic bacteria (Enterobacteriaceae, Enterococcaceae and

3788 Pasteurellaceae). It is thought that their main purpose is to consume oxygen and create a more suitable

3789 environment for obligate anaerobes. However, they rapidly change to strict anaerobes (Palmer et al.,

3790 2007). In puppies, although Enterobacteriaceae was still abundant during the first weeks (69-93%),

3791 proportions of obligated anaerobes increased over time and predominated after three weeks postpartum. At

3792 this time-point, the main families comprised the groups Streptococcaceae (24%) (Facultative anaerobe),

3793 Lachnospiraceae (23%), Bacteroidaceae (21%) and Lactobacillaceae (11%) (anaerobes). The percentage

3794 of Enterobacteriaceae decreased to 7%.

3795

3796 When we compared puppies with young adults, fourteen bacterial groups were enriched in puppies. The

3797 Proteobacteria are thought to play a key role in preparing the gut for colonization by the strict anaerobes

3798 required for healthy gut function by consuming oxygen, and lowering redox potential in the gut environment

3799 (Moon, Young, Maclean, Cookson, & Bermingham, 2018) (Shin, Whon, & Bae, 2015).

3800

3801 On the other hand, when we analysed the relative abundance of the different bacterial groups at the

3802 highest phylogenetic levels, we found that the microbial development was divided into two distinct phases

3803 mainly correlated with diet. One phase characterised by mainly breast feeding (nursing) and another phase

3804 characterised by solid food as the main dietary source (weaning).

3805

3806 In babies, delivery method greatly influences the composition of their gut microbiota. In babies delivered by

3807 C-section, the skin and the environment constitute the main source of bacteria; whereas, vaginally-

3808 delivered infants' gut microbiota resembles that of their mothers' faecal microbiota (Dominguez-Bello *et al.*,

3809 2010). The infants born by C-section also exhibit delayed colonization of the phylum Bacteroidetes, and

3810 lower alpha diversity during the first 2 years of life (Jakobsson *et al.*, 2014). However, the differences in

3811 species diversity between delivery modes are decreased after 4 months, and almost disappear by the age

3812 of 12 months (Backhed *et al.*, 2015).

3813

3814 In one of the litters, the first two puppies were born vaginally and the other three via C-section. Contrary to

3815 what it has been observed in people, β-diversity analysis did not show any difference regarding delivery

3816 method at any time point (P value: 0.864). In dogs, mothers frequently lick their puppies during the first

3817 weeks. This constitute a critical step in the health of growing puppies. Immediately after birth, mothers do

3818 this to clean their puppies and encourage them to breathe; stimulate the reflex to urinate and defecate as

3819 well as for establishing a bond. We believe that this action could help to transfer gut microbiota among

3820 puppies and among mother and puppies, distributing the microbiota evenly. Additionally, puppies and their

3821 mothers are in close relation with other dogs living in the facility, which could help to increase the exposure

3822 to every type of bacteria. The same scenario may not apply to dogs that are born in private houses.

3823 Another factor that we have to consider is that the impact of a C-section on the gut microbiota and

3824 especially on the immune system development; is strongly dependent on whether the procedure is elective

3825 or an emergency. Now that, the onset of labor has been shown to have effects in immune response and

3826 microbial exposure (Arboleya et al., 2018).

3827

3828 Due to the aforementioned action, we hypothesised that the mother's oral microbiota could also influence

3829 the gut microbiota in puppies. However, β-diversity analysis showed that at any time point, oral microbiota

3830 clustered separately from those of the puppies and those from mother's gut microbiota. One limitation of

3831 this finding, is the use of different DNA extraction methods for faecal and oral samples. This could

3832 potentially have an effect on the bacterial population present. A recent study in babies showed that all

3833 maternal body sites contribute to the common mother-infant species, being the stool microbiome the major

3834 contributor. Stool microbiome accounts for 22.1% of the overall microbial abundance in the infant gut,

3835 followed by the vagina (16.3%), the oral cavity (7.2%) and the skin (5%). However, those bacteria coming

3836 from the vagina, the oral cavity and the skin are only transient colonizers as it seems that they are not

3837 adapted to the intestinal environment and also because of the selection forces that are happening within

3838 the intestinal environment (Ferretti et al., 2018).

3839

3840 Studies in humans, have reported spatial differences in the oral microbiota, so we collected two samples

3841 from the oral cavity, one coming from the tongue and another one coming from the cheek mucosa (M. W.

3842 Hall *et al.*, 2017). We did not find differences in the type of groups presented, although the relative

3843 abundance was slightly but not significantly different. Prepartum oral samples were mostly composed of

3844 Bacteroidetes (58-70%) followed by Proteobacteria (18-33%). Other phyla present comprised Firmicutes,

3845 Spirochaetes, Fusobacteria and Actinobacteria. This was in accordance with previous studies. (Elliott,

3846 Wilson, Buckley, & Spratt, 2005). Porphyromonas (64-44%) was the predominant genus in oral samples in

3847 our study, which is in agreement with previous studies (Oh *et al.*, 2015). Other genera present comprised

3848 Moraxella (4-16%) Treponema (1-2%), Pausterella (1-2%), [Prevotella] (2-6%), Fusobacterium (1.5%) and

3849 Capnocytophaga (0.5-1%).

3850

3851 Interestingly, samples taken from the oral microbiota two weeks after partum, showed a different profile. At

3852 phylum level, Proteobacteria (49%) followed by Bacteroidetes (38%) predominated. At genus level,

3853 Porphyromonas (10-22%), Moraxella (11-21%), Pausterella (3-12%), Treponema (2-3%), Capnocytophaga

3854 (3-8%), Fusobacterium (2%), Lampropedia (2-5%), Lautropia (2-3%), Conchiformibius (1,5-3%),

3855 Bacteroides (0,51%) and Actinomyces (0,5-1%).

3856

3857 Proteobacteria was the predominant phylum in puppies during the first few weeks, so there is a possibility

3858 that the constant action of cleaning the puppies could also influence oral microbiota. However, some

3859 studies have shown that Proteobacteria predominates in the oral microbiota. Thus, there is a possibility that

3860 the increase in Proteobacteria could be an indication of normalization of the oral bacterial population (Other

3861 studies have shown the predominance of Bacteroidetes) (Flancman, Singh, & Weese, 2018) (Isaiah et al.,

3862 2017). As our main purpose was the analysis of the gut microbiota in puppies and not the oral microbiota,

3863 we did not collect more samples. However, it would have been interesting to assess the oral microbiota

3864 after weaning, to check whether the oral microbiota returned to a normal profile or not. Also, it would be

3865 interesting to assess the oral microbiota during pregnancy and characterise it. In people, changes in the

3866 oral microbiota has been reported during pregnancy (Fujiwara *et al.*, 2017) (Borgo, Rodrigues, Feitosa,

3867 Xavier, & Avila-Campos, 2014).

3868

3869 Studies in humans have shown that the early stages of colonization are characterized by profound

3870 interindividual variation of the gut microbiota. However, we did not find significant differences in beta

3871 diversity between subjects at any time-point during the first 10 weeks of life (P value: 0.478). These findings

3872 don't necessarily mean that the gut microbiota is similar among all puppies at this age-stage. Our study has

3873 several limitations. We only analysed samples from one breed (Labrador Retriever), coming from the same

3874 genetic background that were born and grew up in the same facility under the same diet and environmental

3875 conditions. This could have contributed to the results obtained.

3876

3877 Also, development and establishment of the gut microbiota is characterised by temporal variation and

3878 increasing compositional diversity over time. We could evidence the same phenomenon in our study, where

3879 the richness increased gradually and the proportions and the type of bacteria kept changing over time;

3880 indicating that the gut microbiota is highly unstable during the first period of life.

3881

3882 On the other hand, when we analysed the relative abundance of the different bacterial groups at the

3883 highest phylogenetic levels, we found that the microbial development was divided into two distinct phases

3884 mainly correlated with diet. One phase, where the main diet consisted in milk and and another phase

3885 characterised by solid food as the main dietary source and where composition converges toward a mature

3886 configuration. Thus, diet plays a predominant role in shaping the microbiome, and weaning is one of the

3887 key determinants that drive the maturation of the gut microbiota into an adult-like phenotype, similar to what

3888 it is seen in babies and in other studies in dogs (Backhed *et al.*, 2015).

3889

3890 Microbial colonisation of the neonatal gut is greatly influenced by the maternal gut microbiota. We collected

3891 faecal samples from the mother, just few days before the partum and at different time-points after, until

3892 weaning. Pregnancy is characterised by extensive hormonal, metabolic and immunological changes aimed

3893 to support foetal growth and development (Nuriel-Ohayon *et al.*, 2016).

3894

3895 Early pregnancy is characterized by a maternal anabolic state; where maternal metabolic adaptations,

3896 mediated by pregnancy hormones, facilitate lipogenesis, glycogenesis, and adipocyte hypertrophy. Late

3897 pregnancy on the other hand, is characterised by a catabolic state where increasing production of

3898 progesterone, estradiol and placental lactogen participate in mediating insulin and leptin resistance that

3899 support growth of the foetus and prepare the body for the energetic demands of lactation *(Nuriel-Ohayon et*

3900 *al.,* 2016).

3901

3902 In people, the maternal gut microbiota is known to increase from early to late pregnancy and is

3903 accompanied by an increase in diversity between mothers (β-diversity) and reduction in richness (α-

3904 diversity). Some studies have reported increases of Proteobacteria and Actinobacteria in the last trimester

3905 (Koren et al., 2012), others increases of Firmicutes and decreases in Bacteroidetes (Xia *et al.*); and others

3906 no dramatic changes in gut microbiota composition during pregnancy (DiGiulio *et al.*, 2015) .

3907

3908 We observed that the majority of the bacterial population belonged to the Firmicutes phylum (94%) in the

3909 samples taken antepartum. Some of the proposed mechanisms by which gut microbiota play a role during

3910 pregnancy include enhanced absorption of glucose and fatty acids, increased fasting-induced adipocyte

3911 factor secretion, induction of catabolic pathways, and stimulation of the immune system (Collado, Isolauri,

3912 Laitinen, & Salminen, 2008; Koren *et al.*, 2012).

3913

3914 It has been shown that bacteria from the phylum Firmicutes increase the efficiency of energy harvest from

3915 host diet. Several mechanisms are implicated in this process. They have the ability to digest a larger

3916 diversity of dietary polysaccharides and modulate host genes that affect energy deposition in adipocytes

3917 and hepatic lipogenesis (Backhed *et al.*, 2004). In fact, it has been reported that an increase in the

3918   *Firmicutes* to *Bacteroidetes* ratio is correlated with obesity (Khan, Gerasimidis, Edwards, & Shaikh, 2016)

3919   (Million, Lagier, Yahav, & Paul, 2013).

3920

3921   In people, it has been observed that there is a depletion in the genus levels of *Faecalibacterium*, a butyrate-

3922   producing bacterium with anti-inflammatory activities, during the third trimester of pregnancy (Haro et al.,

3923   2016). We also found that the prepartum samples contained a significant lower proportion of this genus

3924   compared to the postpartum samples (0,02 versus 3-4%).

3925

3926   Prepartum and first week postpartum were also characterised by a higher proportion of Bacilli (28%) at

3927   class level, especifically of the order Lactobacilli compared to the other time-points. Lactobacilli are known

3928   due to their probiotic properties and it has been reported that during this period they are useful to prevent

3929   the growth of pathogenic bacteria, help digestion and for shaping host innate and adaptive immune system

3930   responses (Konstantinov, van der Woude, & Peppelenbosch, 2013).

3931

3932   The postpartum period is also characterised by significant changes not only at hormonal levels but in the

3933   gut microbiota. It has been reported that at least 1 month after birth, the mothers' microbiotas do not yet

3934   return to their baseline (Koren *et al.*, 2012). We collected some samples at different time points after the

3935   partum. In one of the mothers, we collected the last sample one week after weaning. Although the gut

3936   microbiota was resembling that of an adult; by that time-point, it had not returned to their baseline yet.

3937

3938   It would therefore be of interest to investigate what changes occur in the maternal gut microbiota during

3939   pregancy, partum and postpartum period; how they correlate with the microbiota of puppies, how long the

3940   postpartum transition period lasts, and whether a return to baseline microbial populations ever occurs.

3941   Since the postpartum period is also associated with dramatic hormonal changes including a significant

3942   decrease in progesterone and estrogen levels, it would be interesting to test the direct effects of the

3943   hormonal changes on the microbiome.

3944

3945   The microbial communities belonging to puppies clustered separated from that of the mothers during the

3946   first weeks, being more closely related after weaning. This is in contrast to a previous study made in

3947   puppies, where dams clustered separately from the puppies at any time point (Guard *et al.*, 2017).

3948

3949   Mother from litter 1 received two doses of antibiotic (Clavulox ® (amoxycillin and clavulanic acid), one

3950   during the c-section and another one next day. Studies in people and mice have shown that antibiotics in

3951   general reduce bacterial diversity. During pregnancy, the use of category B antibiotics (azithromycin,

3952   amoxicillin, and cefaclor) increased the fecal relative abundance of Proteobacteria and *Enterobacter*, while

3953   reducing the relative abundance of Firmicutes and *Lactobacillus*. Another study found that maternal

3954   antibiotic treatment during pregnancy and lactation in mice, reduced adaptive antiviral immune responses

3955   in the infant mice, suggesting a broad immune effect on the offspring (Gonzalez-Perez *et al.*,

3956   2016). However, we did not evidence significant changes in the gut microbiota in the mother or puppies,

3957   when we compared them with the other mother and litter. Future studies with more subjects and longer

3958   follow- up periods are needed; to unravel the effect of antibiotics on early stages of microbial development

3959   and later in life.

3960

3961   Next, we followed some puppies after weaning during six months and found that further changes in the

3962   developing microbiota still existed during this period. Although the configuration of the gut microbiota was

3963   resembling that of the adult, diversity was still increasing over time and it was converging towards a mature

3964   configuration.

3965

3966   Dogs at this stage exhibited a significant variation between individuals, however samples from the same

3967   individual clustered together. After weaning, animals are exposed to a variety of diets and environmental

3968   conditions, different lifestyles and hygienic practices; that add to the complexity in the microbiota and

3969   influence the individual assembly of the gut microbiota.

3970

3971   Adulthood microbiota is characterised by high stability and resilience over time. However, the gut

3972   microbiota differs among individuals and within individuals. When we analysed the samples from adult dogs

3973   (1 year – 7 years) we found that at the highest phylogenetic levels, the gut microbiota was highly stable.

3974   However, there was high variability within and between individuals at the lower phylogenetic levels.

3975   Noteworthy, samples from the same dog clustered together during β- diversity analysis. It has been

3976   reported that at the level of species and strain only 5% to 20% of bacterial species overlap between

3977   individual animals (J. S. Suchodolski *et al.*, 2005). Variabilities within the same subject could be due to the

3978   presence of incidental colonizers at the time of the sampling and between subjects could be a reflection of

3979   ecosystem adaptation to different environmental factors. In people, evidence suggests that there is not a

3980   core microbiome at species level but there may be one at phylum level. Each adults has its own profile,

3981   where bacterial species may vary between 12 and 2.187-fold (Laing et al., 2018) (Qin et al., 2010).

3982

3983   It has been reported that the Firmicutes is the major group represented in faeces (30% -95%), followed by

3984   Bacteroides, Actinobacteria, and Fusobacteria (Jan S. Suchodolski, 2013). Although we found the same

3985   groups in our study, Bacteroidetes (41-56%), was the predominant group, followed by Firmicutes (18-32%),

3986   This difference could be due to different methologies employed or it could be a characteristic of the

3987   Australian population. In people,variations have been reported at different geographic locations (De Filippo

3988   *et al.*, 2017). This could be in part, due to the continous horizontal gene transfer between gut microbes and

3989   environmental microbes (Laing et al., 2018).

3990

3991   At order level, we found bigger differences compared to previous reports. It has been reported that at class

3992   level, faeces samples are dominated by the groups Clostridiales (60-78%), Fusobacteriales (0,3-10%),

3993   Erysipelotrichales (0-8%), Lactobacillales (1-5%), Coriobacteriales (1-2,5%) and Enterobacteriales (0,1-2%)

3994   (Jan S. Suchodolski, 2013). In our study, we found that Fusobacteriales (18-27%), Clostridiales (17-26%),

3995   Lactobacillales (1-6%), Enterobacteriales (0,3-6%) and Burkholderiales (2-4%) predominated.

3996   Erysipelotrichales were present at very low levels (<1%). Within these orders, the genera Prevotella (22-

3997   44%), Fusobacterium (18-28%), [Prevotella] (4-13%), Megamonas (6-11%) and Bacteroides (4-11%).

3998

3999   The gut microbiota homeostasis is crucial for healthy aging and like infancy, application of intervention

4000   measures at this period could help to maintain longevity. As age advances, several physiological changes

4001   occur in the body. Ageing influences gut microbiota and vice versa. Ageing is linked to physiological

4002   changes that impact food digestion and absorption and to decline in the normal function of the immune

4003   system. In older people, the gut microbiota is extremely variable between individuals, and differs from the

4004   core microbiota and diversity levels of younger adults. When we analysed the dissimilarities in beta

4005   diversity between young adults and mature (P value: 0.001), young adults and senior (P value: 0.005), and

4006   mature versus senior (P value: 0,001), we found that the difference was statistical significant (despite the

4007   low number of dogs in the mature and senior group); suggesting that the gut microbiota is changing during

4008   ageing. However, we need to consider that biological age is not always related with chronological age (Kim

4009   & Jazwinski, 2018). Gut microbial diversity inversely correlates with biological age but not with

4010   chronological age. Studies in people have shown that with advancing chronological age, the diversity of the

4011   gut microbiota increases, whereas as biological age increases; overall richness decreases, and the

4012   abundance of some microbial genera such as Ruminococcus, Coprobacillus and Eggerthella increases

4013   (Jackson et al., 2016). Thus, it would be advisable to consider the use of biological or functional measures

4014   in future aging studies (Kim & Jazwinski, 2015).

4015

4016   In older people it has been reported that there are an elevated proportion of facultative anaerobes

4017   (Proliferation of Enterobacteriaceae) (Biagi *et al.*, 2010) and an alteration of the relative proportions of the

4018   Firmicutes and the Bacteroides. The elderly have a higher number of Bacteroides while younger individuals

4019   have a higher proportion of Firmicutes. In general, infants and elderly have a Firmicutes/Bacteroidetes ratio

4020   < 1 and adults a ratio > 1 (Mariat *et al.*, 2009). However, we did not see any of these changes in mature or

4021   senior dogs, when they were compared to adult dogs (Firmicutes/Bacteroidetes ratio: 0,5 – 0,7).

4022

4023   Studies in older dogs, have reported more *Clostridium perfringens* and *Streptococcus spp.*, and fewer

4024   *Bacteroides, Bifidobacterium, and Lactobacillus spp.* (Benno, Nakao, Uchida, & Mitsuoka, 1992). DGGE

4025   fingerprinting profiles also cluster according to age, and levels of *Bacteroides* are significantly lower in older

4026   dogs (J. M. Simpson, Martineau, Jones, Ballam, & Mackie, 2002). We also found lower proportions of

4027   *Lactobacillus*, however; levels of *Bacteroides* were higher in older dogs compared to adults, especially in

4028   mature dogs (8% versus 24%) (P value: <0,001).

4029

4030   In general, we found that the most intense development occurs during the first year of life. In adults,

4031   although the intestinal microbiota is stable, it presents variations over time and differs among individuals

4032   and within individuals, but it has the tendency to cluster by subject (Subejct-specific core). Thus, it would be

4033   advisable to collect various samples in order to establish the normal pattern in each subject and to have a

4034   better understanding of the dynamics of the gut microbiota during disease.

4035

4036   Fortunately, it is known that even though there are great fluctuations in the proportional representation of

4037   microbial lineages, the gene content tends to remain stable, and can give a better estimation of the

4038   metabolic functions of the microbiota at any given time point (Qin et al., 2010). Thus, metagenomis studies

4039   could be very useful to decribe community structure in terms of functional diversity rather than taxonomic

4040   diversity alone (Weinstock, 2011).

4041

4042   We had several limitations in our study. The number of subjects per group was small, especially for mature

4043   and senior dogs, which could have hindered some of the differences reported in previous studies.

4044   Although, differences in sample processing techniques, sequencing technologies, and statistical methods;

4045   complicate any direct comparison between different sequencing studies. Also, changes in gut microbiota

4046   have been reported between breeds. In puppies, we only assessed the gut microbiota in one particular

4047   breed. It would be interesting to assess the gut microbiota development in other breeds and/or other

4048   environmental conditions (privately owned). Also, it would be interesting to include the assessment of the

4049   microbiota in placenta, meconium, milk and maternal skin and vaginal microbiota in future studies and to

4050   determine their influence in the development and establishment of the gut microbiota in puppies. Another

4051   limitation of our study is that our taxonomic profiling was limited to species-level resolution. Strain- level

4052   metagenomic profiling is essential for identifying the instances of transmission from external sources to the

4053   puppies (Ferretti et al., 2018). Also, we need to take into account that the classification of dogs based on

4054   age, can be affected accoding to the kind of breed, as small and large breed mature and age at different

4055   rates.

4056

4057   Finally, it has been shown that timing and selection of the first solid foods are factors that have an

4058   important effect in the correct development of the gut microbiota (Arboleya et al., 2018). Thus,this aspect

4059   could be another interesting factor to investigate.

4060

4061

4062

4063 **Chapter 3: Characterisation of immunoglobulin-coated (Ig-coated) bacteria in faeces from dogs with**
4064 **chronic enteropathies**

4065
4066 **3.1 Introduction**
4067
4068
4069 Canine chronic enteropathies (CE) constitute a group of disorders that cause chronic gastrointestinal tract
4070 inflammation, persistent or recurrent gastrointestinal signs in dogs and are also termed inflammatory bowel
4071 disease (IBD) (Walker *et al.*, 2013). Although they occur frequently in dogs, many aspects of their
4072 pathogenesis remain to be unravelled and the therapeutic approach in some cases remains challenging
4073 (Craven *et al.*, 2011). In general, it is considered that the triad host genetics-immune system-
4074 microenvironment; particularly dietary antigens and the gastrointestinal flora; are closely related to the
4075 development of gastrointestinal disease (Koboziev, Reinoso Webb, Furr, & Grisham, 2014).

4076
4077 In CE, it is proposed that either an inappropriate response to a normal gut microbiota leads to excessive
4078 immunological responses; or alternatively changes in the composition of the gut microbiota elicit
4079 pathological responses from a normal mucosal immune system. It has been found that the intestinal
4080 microbiota and associate genome influence the metabolic environment of the intestine and the activity of
4081 both the innate and adaptive immune system. Thus, depending on the genera, they can decrease, promote
4082 or perpetuate the inflammatory response in the intestine (Pabst, 2012). Immunoglobulins (Igs) are part of
4083 the adaptive immune system and constitute crucial arms that directly influence the function and structure of
4084 the microbiota (Olsson *et al.*, 2014). Although, intestinal IgA is the predominant isotype produced in the
4085 intestine, other immunoglobulins such as IgM and IgG can be produced as well. This is particular important
4086 in dogs, where several breeds have been reported to be IgA deficient (Olsson *et al.*, 2014). In general, it is
4087 considered that pathogens and commensals with strong immune stimulatory activities trigger affinity
4088 maturation more effectively than other commensals that are less frequently sample and are under-
4089 represented in germinal centres. Thus, relative levels of bacterial coating with Igs might be predicted to
4090 correlate with the magnitude of the inflammatory response triggered by specific intestinal bacterial species
4091 (Palm *et al.*, 2014).

4092
4093 Few studies related to immunoglobulins have been done in dogs with CE and the results are mixed. One
4094 showed increased numbers of plasma cells in the intestinal mucosa (particularly IgA+ and IgG+ cells)

4095   (Jergens *et al.*, 1999) , another study found higher concentrations of IgA and lower concentration of IgG

4096   compared to healthy patients (Jergens *et al.*, 1996); whereas another study found no difference in the

4097   plasma cell populations in the mucosa compared to healthy patients (although they found lower levels of

4098   IgA in blood) (Batt *et al.*, 1991). More recently; Maeda et al have reported decreased IgA concentrations in

4099   faeces, duodenum and peripheral mononuclear cells of dogs with IBD (Maeda *et al.*, 2013). The

4100   discordance in results could be given by several factors such as methodology (duodenum vs, colon),

4101   different criteria of classification of the entities (Small intestinal bacterial overgrowth (SIBO),

4102   antibiotic/responsive diarrhoea (ARD), or IBD) and breed of dogs (as some breeds have been reported to

4103   be IgA-deficient, particularly German-Shepherd dogs).

4104

4105   Using 16S high-throughput sequencing methodologies, previous studies have found dysbiosis and reduced

4106   bacterial diversity in canines with chronic enteropathies (CE) (Garcia-Mazcorro *et al.*, 2012; J. S.

4107   Suchodolski *et al.*, 2008; Walker *et al.*, 2013). However, the identification of bacteria responsible of causing

4108   or exacerbating inflammation has remained challenging. Given the complexity, the large amount of factors

4109   that shape the intestinal microbiota, the interpersonal variation and the amount of specific susceptibility loci

4110   that can influence microbial composition; it is likely that some bacteria may cause problems only in few

4111   individuals while being harmless in others (Manichanh *et al.*, 2012). In these cases, longitudinal studies that

4112   relate changes in an individual biology to microbial community structure have the potential to offer a better

4113   overview wherein the subject itself represents the best control (internal control).

4114

4115   Due to the importance of IgA and IgG in the intestinal immune system and the ability of these

4116   immunoglobulins to detect microorganisms responsible for exacerbating or causing clinical inflammation;

4117   we hypothesize that (1) dogs with CE possess a higher proportion of highly coated IgA-bacteria compared

4118   to healthy dogs in the faeces; (2) we can use flow cytometry to distinguish between members of the

4119   microbiota that impact disease susceptibility or severity in dogs and (3) resolution of the clinical signs in

4120   dogs with CE is associated with the eradication of these highly coated IgA-bacteria.

4121

4122   The aim of the study is to characterise immunoglobulin A and G-coated bacteria in dogs with chronic

4123   enteropathies over time using flow cytometry and 16S RNA sequencing.

4124

4125   **3.2 Methodology**

4126

4127   **3.2.1 Study dogs**

4128

4129   Dogs with signs of chronic gastrointestinal disease (> 3 weeks), including persistent and/or recurrent

4130   vomiting and/or diarrhoea and/or weight loss; presented at the veterinary hospital of the University of

4131   Melbourne were enrolled into the prospective study. A total of 9 dogs were enrolled (New study). Dogs

4132   underwent a complete clinical evaluation by an internal medicine specialist. Dogs were evaluated for co-

4133   morbidities and extra-intestinal disease prior to inclusion by a combination of faecal analysis (faecal

4134   flotation and faecal cytology), blood testing (including canine pancreatic lipase immunoreactivity, cobalamin

4135   and canine trypsin-like immunoreactivity) and abdominal ultrasound. Dogs were not included in the trial if

4136   there was a history of dietary or medical therapy 3 weeks prior to analysis, or if hypoalbuminemia (albumin

4137   < 20 g/L) was present.

4138

4139   The disease activity was scored using the canine chronic enteropathy activity index (CCECAI). For this

4140   score, nine signs (attitude/activity, appetite, vomiting, stool consistency, stool frequency, weight loss,

4141   albumin levels, ascites/peripheral oedema and pruritus) are scored from 0 to 3 based on the magnitude of

4142   their alterations. The scores are added, yielding a total cumulative score. Five categories are defined:

4143   insignificant disease, 0–3; mild disease, 4– 5; moderate disease, 6–8; severe disease, 9–11; very severe

4144   disease >11 (Allenspach, Wieland, Grone, & Gaschen, 2007).

4145

4146   One animal withdrew from the study and the second endoscopy could not be performed (CE Dog 3). Three

4147   dogs did not fully participate in the study but owners agreed to donate some samples for the study (CE

4148   Dog22 [colon and ileum biopsy; cytology brush], CE dog 21 [cytology brush and faeces] and CE dog 12

4149   [faeces]). According to the classification based on response to treatment, five dogs had diet-responsive

4150   enteropathy (DRE); five dogs had antibiotic-responsive enteropathy (ARE) and two dogs had steroid-

4151   responsive enteropathy (SRE) (Dandrieux, 2016) Detailed information about the patients can be found in

4152   table 3.1.

4153

4154   Samples from dogs of a previous study performed between 2012 and 2014 were included (Old study).

4155   Selection criteria were the same as specified above. A total of 12 dogs were enrolled in this study.

4156   According to the classification based on response to treatment, five dogs had diet-responsive enteropathy

4157   (DRE); six dogs had antibiotic-responsive enteropathy (ARE) and one dog had steroid-responsive

4158   enteropathy (SRE) (Dandrieux, 2016). Detailed information about the dogs can be found in Table 3.1

4159

4160   Controls: Healthy pet-owned dogs with no signs of gastrointestinal disease, no antibiotic or other drug

4161   treatment or change of diet in the previous 6 weeks were also recruited into the study. Detailed information

4162   regarding diet (type, treats, and changes within the previous three months; coprophagia, and rubbish),

4163   health status, previous diseases, travel history, level and type of exercise, body condition score and

4164   increase or decrease in body weight in the previous three months was collected. Detailed information about

4165   the control dogs can be found in Table 3.2.

4166

4167   All experimental procedures were approved from the Animal Ethic committee of University of Melbourne.

4168   (Animal Ethics Committee approval AEC # 1112072.2). (Healthy dogs AEC #1413272.1)

4169

4170   Owners gave informed, written consent in which they agreed to participate in initial and follow-up diagnostic

4171   evaluation. They could withdraw their animals from the trial at any point.

4172

4173

4174   Table 3.1 Metadata information of dogs with Chronic Enteropathies

Patient	Breed	Age (y)	Neutering status	Type CE	Localisation	CCECAI-1	CCECAI-2	Duration Signs (month)	Concurrent diseases	Treatment	Study period
CE Dog 1	Spoodle	5	Male castrated	SRE	Mixed	5	0	24	Polyarthritis Bronchopathy	Z/D Hills® Sensitivity Royal canine® Hypoallergenic Royal canine® Prednisolone Clorambucil (Gabapentin)	New
CE Dog 2	Japanese Spitz	1.5	Female spayed	ARE	Mixed	8	2	4	None	Sensitivity Royal canine® Hypoallergenic Royal canine® Oxytetracycline 200mg	Old
CE Dog 3	Whippet	4	Female spayed	ARE	SI	6	NA	15	Separation anxiety	Sensitivity Royal canine® Hypoallergenic Royal canine® Oxytetracycline 200mg	New
CE Dog 4	Border Collie	5	Male castrated	ARE	SI	Unknown	Unknown	14	EPI	Hypoallergenic Royal canine® Oxytetracycline 200mg (Creon®)	New
CE Dog 5	Maltese Cross	6.5	Male castrated	ARE	SI	11	2	31	None	Sensitivity Royal canine® Oxytetracycline 200mg	Old
CE Dog 6	Labrador Retriever	2	Female spayed	ARE	SI	7	1	5	Incontinence (USMI)	Sensitivity Royal canine® Oxytetracycline 200mg (stilboestrol)	Old

CE Dog 7	Golder Retriever	5	Male castrated	FRE	SI	1	1	18	None	Hypoallergenic Royal canine®	New
CE Dog 8	Staffordhire Bull Terrier	2	Male castrated	FRE	Mixed	11	2	24	None	Hypoallergenic Royal canine®	Old
CE Dog 9	Labrador Retriever	10	Female spayed	SRE	Mixed	7	0	14	PLE EPI	Hypoallergenic Royal canine® Oxytetracycline Prednisolone Clorambucil (Creon®)	New
CE Dog 10	Labrador Retriever		Female spayed	FRE	Mixed	7	4	12	Otitis	Z/D Hills®	New
CE Dog 11	Great Dane	2,5	Male castrated	FRE	LI	4	0	28		Sensitivity Royal canine®	Old
CE Dog 12	Maltese Terrier	3,5	Female entire	FRE	Unknown	Unknown	Unknown	4	None	Z/D Hills® Hypoallergenic Royal canine® Omeprazole	New
CE Dog 13	GSD	14	Female spayed	FRE	Mixed	6	0	7	None	Z/D Hills® Sensitivity Royal canine® Hypoallergenic Royal canine®	New
CE Dog 14	GSD	1	Male entire	FRE	Mixed	3	0	2	None	Sensitivity Royal canine®	Old
CE Dog 15	Chihuahua	9	Male castrated	FRE	Mixed	Unknown	Unknown	24	None	Z/D Hills® Sensitivity Royal canine®	New

										Hypoallergenic Royal canine®	
CE Dog 16	GSD	1.5	Female spayed	ARE	Mixed	12	1	3	Anxiety	Sensitivity Royal canine® Hypoallergenic Royal canine® Oxytetracycline (Fluoxitine, diazepam)	Old
CE Dog 17	Maltese Cross	5	Female Spayed	FRE	SI	6	0	12	None	Sensitivity Royal canine® Hypoallergenic Royal canine®	Old
CE Dog 18	GSD	3.5	Male castrated	ARE	SI	9	0	3	None	Sensitivity Royal canine® Hypoallergenic Royal canine® Oxytetracycline	Old
CE Dog 19	Greyhound	2	Female spayed	ARE	Mixed	5	0	Unknown	Greyhound alopecia	Hypoallergenic Royal canine® Oxytetracycline	New
CE Dog 20	Basset Hound	3	Male entire	ARE	Mixed	5	0	24	None	Sensitivity Royal canine® Hypoallergenic Royal canine® Oxytetracycline	Old
CE Dog 22	Toy Poodle	2.5	Male castrated	ARE	LI	9	0	18	None	Sensitivity Royal canine® Oxytetracycline	Old
CE Dog 23	Flat Coated Retriever	9	Male castrated	SRE	SI	Unknown	Unknown	8	Trigeminal neuropathy	Z/D Hills® Hypoallergenic Royal canine® Oxytetracycline	New

										Prednisolone	
CE Dog 24	Labrador Retriever	3.6	Female spayed	ARE	Mixed	Unknown	Unknown	32	None	Hypoallergenic Royal canine® Oxytetracycline	New
CE Dog 25	Weimaraner	1.7	Male castraded	FRE	Mixed	4	0	3	None	Hypoallergenic Royal canine®	Old

4175

4176

4177

4178

4179

4180

4181

4182

4183   Table 3.2 Metadata information of dogs with Chronic Enteropathies

Dog	Breed	Age (years)	Diet	Neutering Status
H dog 1	Labrador	2	J/D Hills®	Male castrated
H dog 2	Leonberger	7	Black Hawk chicken rice®	Female spayed
H dog 3	German Wirehaired Pointer	6	Advance Dry Food® Homemade	Male entire
H dog 4	Nova Scotia Duck Tolling Retriever	3	T/D Hills®	Female spayed
H dog 5	Cairn Terrier	5	T/D Hills®	Female spayed
H dog 6	Siberian Husky Cross	11	J/D Hills®	Male castrated
H dog 7	Catledog Cross	8	J/D Hills®	Male castrated
H dog 8	Kelpie	4	T/D Hills®	Female Spayed
H dog 9	Kelpie	9	Adult Royal canin®	Female Spayed
H dog 10	German_Wirehaired_pointer	1	Advance Dry Food® Homemade	Female entire
H dog 11	Terrier Cross	5	Adult Light Hills®	Female Spayed

4184

4185

4186

3.2.2 Treatment

The treatment for the study consisted first of a diet trial (hydrolysed or hypoallergenic prescription diet determined on previous dietary history) (DRE), followed by antibiotic treatment in non-responders (oxytetracycline) (ARE), and then a prednisolone trial if needed until resolution of the clinical signs ensues (SRE). Dietary trial consisted in the administration of an elimination diet exclusively for two weeks. If the dog showed improvement of the clinical signs after this period (>75% reduction in CCEAI), treatment continued. If no response to treatment was seen, antibiotic treatment was added to the therapy. Oxytetracycline 10mg/kg twice a day for two weeks was prescribed. After this period or if clinical signs were worsening, dogs were prescribed prednisolone. Initially, prednisolone at a dose of 2mg/kg once per day for 10 days was instituted, followed by a tapering protocol.

3.2.3 Samples

3.2.3.1 Tissue samples

Two endoscopic examinations (upper and lower endoscopy) prior to treatment trial and after treatment success (defined as a decrease in the clinical IBD activity index of at least 75% for at least six weeks) were performed by an experienced endoscopist (CM – JD). Food was withheld from dogs 12 hours prior to the collection of tissue specimens. Biopsies from the stomach, duodenum, ileum and colon were collected for histology analysis. Two samples of each section and a cytology brush from the duodenum were stored at -80°C for further analysis. The cytology of the duodenum was performed using a Gastroscope cytology brush – Sheath diameter (1.8mm), length 160cm (Device technologies®). Cytology brush sample was collected before the biopsies were taken.

3.2.3.2 Faeces

Serial stool samples (at the beginning, during treatment and remission periods at different time points) were collected upon voiding without contacting the environment (to avoid transfer of genetic material) or via rectal examination and placed in a container at 4°C immediately. Aliquots of 250 mg were done and stored

4217   within four hours after collection at -80°C, until further analysis. In healthy dogs, two samples were

4218   collected, one month apart and aliquoted in the same way as described above.

4219

4220   **3.2.4 Histology**

4221

4222   Endoscopic biopsies of the stomach, duodenum and colon before and after treatment were placed in 10%

4223   neutral-buffered formalin, routinely processed and stained with haematoxylin and eosin (H&E). Specimens

4224   were evaluated by a board-certified veterinary pathologist with experience in gastrointestinal pathology and

4225   scored using published international guidelines. (World Small Animal Veterinary Association WSAVA).

4226

4227   **3.2.5 DNA isolation intestinal biopsies and cytology brush**

4228

4229   DNA from intestinal biopsies was done using the QIAamp DNA minikit (Qiagen®) using the protocol for

4230   DNA isolation from tissues of the handbook. For isolation of DNA from cytology brushes, the kit QIAamp

4231   DNA microkit (Qiagen®) was used using the protocol for Isolation of Total DNA from Surface and Buccal

4232   Swabs of QIAamp DNA Investigator Handbook. Carrier RNA was added to buffer AE to a final

4233   concentration of 1 ug/uL. The brush was separated from its shaft using a sterile blade and placed in a 2 mL

4234   centrifuge tube, and DNA was isolated in accordance with manufacturer's instructions. DNA was finally

4235   eluted in 30 µl of AB buffer (provided in the kit). DNA purity was measured by spectrophotometry at 260

4236   nm. Purity of DNA was assessed using the ratio of $OD_{260/280}$ with a ratio of 1.8–2.0 being of good purity. As

4237   sample contained RNA carrier, DNA quantitation was measured using Qubit® 3.0 fluorometer

4238   (Thermofisher scientific®). Additionally, 3ul of sample were run in agarose gell. DNA was stored at -80°C.

4239

4240   **3.2.6 Flow cytometry and Sorting of IgA$^+$ and IgA$^-$ bacteria**

4241

4242   One aliquot of faeces was placed in a sterile conical tube and 2.5 mL of cold (4°C), sterile, filtered (0.22

4243   µm, Millipore®) phosphate buffered saline (PBS) 1X was added (Phosphate buffered saline 10X

4244   concentrate, P5493 Sigma®). Samples were incubated on ice for 1 hour. Samples were homogenised by

4245   vortexing every 15 minutes during 1 minute until the faecal material was completely dissolved. Next,

4246   samples were centrifuged at 40 x *g* during twenty minutes at 4°C to separate larger faecal particles from

4247   bacteria. Supernatants were passed through a 70 µm sterile filter (Cell strainer, Z742103 Sigma®) into a

4248　new, sterile tube. Aliquots of 500 µL were stored at -80°C. 10 uL of the faecal bacterial supernatant was

4249　diluted in 500 uL of 5 µM Tris buffer containing 5 µM SYTO 17 and bacteria were counted using 50 µL of

4250　the CountBright™ Absolute Counting Beads, for flow cytometry (Catalog number: C36950) in FACSAria III

4251　(BD Biosciences) from the Faculty of Veterinary and Agricultural Sciences of University of Melbourne. From

4252　the aliquots stored at -80°C, 10^7 bacteria were washed with 1ml sterile and filtered PBS 1X containing

4253　1% (w/v) Bovine Serum Albumin (BSA) (Bovine Serum Albumin heat shock fraction, protease free, fatty

4254　acid free, essentially globulin free, pH 7, ≥98%, A7030 Sigma®) (staining buffer) and centrifuged for 5

4255　minutes (8000 x g, 4°C). A sample of this bacterial suspension was saved (100 µL) as the pre-sort sample

4256　for 16S rRNA sequencing analysis (before centrifugation). Next, Supernatant was removed and the pellet

4257　was resuspended in 1 mL of staining buffer and centrifuged again for 5 minutes (8000 x g, 4°C). Next,

4258　supernatant was removed and the bacterial pellet was resuspended in 100 µL of blocking buffer (Staining

4259　buffer containing 20% normal goat serum for IgA (G9023 Sigma®) or staining buffer containing 20% normal

4260　sheep serum for IgG (S3772 Sigma®), incubated for 20 minutes on ice and then stained with 100 µl of

4261　staining buffer containing Goat anti-dog IgA:FITC 1:200 (Serotec SEAA131F, Abacus ALS) or Sheep anti-

4262　dog IgG:FITC (Serotec SEAA132F, Abacus ALS) 1:200. Samples were incubated during 30 minutes on ice

4263　away from light. Samples were then washed with 1 mL of staining buffer and centrifuged for 10 minutes

4264　(8000 x g, 4°C). The procedure was repeated. Finally, the bacterial pellet was resuspended in 500 µL of 5

4265　µM sterile tris buffer containing 5 µM SYTO 17 (SYTO™ 17 Red Fluorescent Nucleic Acid Stain - 5 mM

4266　Solution in DMSO, catalog number: S7579, Invitrogen™), kept on ice and in the dark before cell sorting

4267　analysis. All buffers were prepared freshly under sterile conditions.

4268

4269　Threshold settings were set to the minimal allowable voltage for SSC. Gating was based on forward and

4270　side scattering properties; followed by their ability to bind the DNA dye Syto17. Ig+ and Ig− populations

4271　were then identified by their ability to bind fluorescently labelled antibodies; 100,000 events from each

4272　population were sorted (Figure 3.1: healthy dog; figure 3.2 dog CE) In addition, samples of sheath fluid

4273　were collected immediately before and after sorting to assess any potential contaminants in fluid lines. Cell

4274　sorting was carried out using MoFlo™ Astrios (Beckman Coulter, Inc) from the Faculty of Medicine,

4275　Dentistry and Health Sciences of University of Melbourne. Each fraction was stored at -80°C prior to PCR

4276　and sequencing of bacterial 16S rRNA genes.

4277

4278 Multiple precautions were taken to minimise potential contamination of sorted samples. Sterile filtered PBS
4279 1X was used for sheath fluid, the flow cytometer was sterilised according to the manufacture's
4280 recommended protocol, the sheath fluid filter was replaced routinely. Before commencing the cell sorting
4281 and between samples; sheath fluid system was washed with sodium hypochlorite 2.5% for 2 minutes,
4282 followed by sterile filtered PBS 1X during 4 minutes.

4283

4284 Every run was done using samples from healthy dogs, and dogs with CE belonging to the new study and
4285 old study. All visits from each dog were sorted the same day.

4286

4287   -   Initial testing:

4288

4289 The flow cytometer (FACSAria III (BD Biosciences)) was calibrated with
4290 BD FACSuite CS&T Research Beads (BD Biosciences).   Measurements were performed with 10,000
4291 events, at a flow rate of 1,000 events per second. The fluorescence was recorded bi-exponentially and
4292 forward scatter (FSC) recorded logarithmically. To determine the specificity of the antibodies fluorescein
4293 (FITC), ChromPure goat IgG, whole molecule (Jackson Laboratories) and Fluorescein (FITC)-conjugated
4294 AffiniPure F (ab') 2 fragment donkey anti-sheep (Jackson laboratories) was used for isotype control for IgA
4295 and IgG respectively. The following antibody concentrations were tested: 1:50, 1:100, 1:200 and 1:400.
4296 Syto 17 concentration were tested at the following concentrations: 1 μM and 5μM and 20 μM. The highest
4297 concentration resulted in bacterial death. Threshold was set up in FSC 200. Antibody concentrations were
4298 selected at 1:200 and syto17 at 5 μM.

4299

4300 MoFlo™ Astrios (Beckman Coulter, Inc) was calibrated with Astrios QC beads (Beckman Coulter, Inc) for
4301 the optical alignment and Flow-Check™ Pro Fluorospheres (Beckman Coulter, Inc) to confirm drop charge
4302 delay values.

4303

4304 **3.2.7 Bacterial 16S rRNA gene analysis**

4305

4306 The V4 hypervariable region of the bacterial 16S rRNA gene (16Sv4) was PCR-amplified with primers
4307 515F-OH1   (GTGACCTATGAACTCAGGAGTCGGACTACNVGGGTWTCTAAT)   and   806R-OH2
4308 (CTGAGACTTGCACATCGCAGCGTGYCAGCMGCCGCGGTAA); 2,5 μL were added directly to a PCR

4309   master mix (20 µL reaction/sample). This primer pair amplifies the region 533–786 in the *Escherichia coli*

4310   strain 83972 sequence (greengenes accession no. prokMSA_id:470367). Cycling conditions consisted of

4311   95°C for 3 minutes followed by 35 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30

4312   seconds and 72°C for 7 minutes. A 10 min 95°C step at the beginning of the PCR was added to heat lyse

4313   the bacteria. Individual "barcode" sequences of 8 base pairs were added to each sample so they could be

4314   distinguished and sorted during data analysis. Specificity and amplicon size were verified by gel

4315   electrophoresis and the amplicons were checked and measured using the Agilent High Sensitivity DNA

4316   assay in Agilent 2100 Expert (samples for checking were chosen randomly). The 600 cycle kit was used

4317   for paired end sequencing (2x 311 cycles) using Illumina MiSeq. Raw data was demultiplexed and quality

4318   filtering using default parameters of the open source software package Quantitative Insights into Microbial

4319   Ecology (QIIME).

4320

4321   Taxonomic assignment was done using Operational taxonomic units (OTUs) using a threshold of 97%

4322   similarity and the open-reference OTU approach. Measurements of richness (Alpha (α) – diversity) and

4323   similarity (beta (ß)-diversity) were done using Phyloseq from R.

4324

4325   Raw data was loaded into QIIME 1.9.0 pipeline, and barcodes were extracted (Caporaso *et al.*, 2010).

4326   Next, paired-end reads were merged using the paired-end read merger program (PEAR) (Zhang *et al.*,

4327   2014). Subsequently, data was loaded into QIIME 1.8.0 to demultiplex and to filter out low quality

4328   sequencing reads by applying default settings, a minimum number of consecutive high-quality base calls to

4329   include a read of 0.90 (default: 0.75) and a minimum acceptable Phred score of 33.

4330

4331   16S rRNA analysis was performed on samples collected from the flow cytometer droplet stream before and

4332   after every sort, thus permitting any identification of any sequences that did not originate from the sorted

4333   sample. Additionally, buffers and PCR water were sequenced to check for potential contamination.

4334   Samples were run in duplicate.

4335

4336   After filtering, from a total of 1400 samples, we obtained a total of 13,334,258 of high-quality sequences

4337   with reads ranging from 1 to 256,276 per sample.

4338

4339   In order to keep only sequences from the same region of the 16S rRNA gene, samples were aligned using
4340   the Silva database from MOTHUR (Schloss *et al.*, 2009). Next, we used USEARCH algorithm to cluster
4341   similar filtered sequences into Operational Taxonomic Units (OTUs) based on a 97% similarity threshold
4342   (Edgar, 2010). Taxonomy assignment to the unique sequences was done by aligning the sequences
4343   against Greengenes template alignment (DeSantis *et al.*, 2006). The script make_phylogeny_py was used
4344   to create phylogenetic tress using the FastTree program in Qiime *(Price et al.,* 2009).
4345
4346   Taxonomic assignment was done using Operational taxonomic units (OTUs) using a threshold of 97%
4347   similarity and the open-reference OTU approach. Measurements of richness (Alpha (α) – diversity) and
4348   similarity (beta (ß)-diversity) were done using Microbiome and Phyloseq package from R. The correlation
4349   coefficient, using the Pearson's correlation method, was calculated per each pair of samples. Coefficients
4350   of ≥ 0.98 were considered as appropriate. For posterior analysis, samples were merged.
4351
4352   To calculate the relative abundance of bacteria at different phylogenetic levels, the OTU table was rarified
4353   at 1000 sequences per sample. Five samples were excluded from the analysis: One faecal sample IgG-
4354   active, three small intestinal biopsies (2 active and 1 remission) and one colon biopsy (Remission).
4355
4356   To calculate richness and alpha diversity indexes, faecal and intestinal samples were analysed separately.
4357   Samples with less than 1000 reads (counts) were removed. Only one faecal sample was removed (CE dog
4358   11: IgG- visit 1) Thirty pre sorting water samples and two negative water samples were excluded as well.
4359   For the intestinal microbiota, four samples were removed (three small intestine biopsies: CE dog 7 active,
4360   CE dog 13 active, CE dog 14 remission; one Colon biopsy: CE dog 2 remission). Additionally, taxa that
4361   were not present in any of the samples were removed. Richness was calculated based on the number of
4362   observed OTUs and diversity was calculated using Shannon, inverse_simpson and Fisher indexes. (Total
4363   number of samples: 622).
4364
4365   To calculate between-sample diversity, the OTU matrix was normalized to account for uneven column
4366   (sample) sums, through Cumulative sum scaling (CSS) using the command normalize table.py available in
4367   Qiime Weighted and unweighted Unifrac metrics were applied to build phylogenetic distance matrices
4368   (Lozupone *et al.,* 2012). We also assessed the Bray_curtis dissimilarity index.
4369

4370 To perform microbial differential abundance testing between the different groups, we used the extension

4371 DESeq2 from the Phyloseq package (McMurdie & Holmes, 2013) (Love et al., 2014).

4372

4373 Hierarchical clustering in the IgA positive population was performed using the weighted UniFrac distance

4374 and the UPGMA method (hclust parameter method="average"). In the 'average' method the the distance

4375 between the two groups is the distance between the centers of gravity of the two groups. Results were

4376 plotted as an annotated dendogram.

4377

4378

4379 **3.2.8 Relative enrichment of IgA and IgG taxa**

4380

4381 Samples from individual animals subjected to FACS followed by bacterial 16S rRNA gene sequencing of

4382 the "input" (pre-sort), the IgA$^+$, and IgA$^-$ fractions. The differential representation of each taxon between the

4383 IgA$^+$ and IgA$^-$ fractions was expressed in the form of an Ig index enrichment.

4384

4385 First, phylum, class, order, family, genus and species taxonomy were summarised. The threshold used for

4386 **designating that a taxon was called as present was ≥ 0.1% relative abundance in either** the IgA$^+$, IgA$^+$ or

4387 input fractions. Enrichment at the family-level taxon in the IgA$^+$/IgG$^+$ or IgA$^-$/IgG$^-$ fraction was chosen; now

4388 that at species and genus level, many of the bacteria were not assigned to any group. A pseudo count

4389 (equal to 0.001, which was the lower limit of detection of fractional representation in the community) was

4390 then added to every taxon detected in both the IgA$^+$ and IgA$^-$ fractions generated from every faecal sample.

4391 The fractional abundance of a given taxon in the positive population was divided by the fractional

4392 abundance of that given taxon in the negative population and the result was log transformed.

4393

4394 A paired Wilcoxon test was done for comparing the significance of differences in abundances of that taxon

4395 in the IgA$^+$ to IgA$^-$ fractions prepared from a group of samples. If a taxon was not detected in a sample, that

4396 sample was excluded from analysis of that taxon. IgA index values of zero represent samples in which a

4397 taxon was detected in equal proportions in both the IgA$^+$ and IgA$^-$ fractions.

4398

4399   This value represents a relative measure of targeting; but does not provide an absolute quantitation of the

4400 IgA response since the total amount of IgA bound to each taxon is not determined.

4401

4402   -   Contamination samples

4403

4404 A sample of water was collected before the sorting of each sample. Group of families that were present in

4405 this sample, were removed from the analysis of the sorted sample. Samples with fewer than 1000 reads

4406 after removing contaminating sequences were not included in the analysis.

4407

4408 **3.2.9 Statistical Analyses**

4409

4410 Differences in the percentage of bacterial immunoglobulin coating were calculated with the unpaired t-test.

4411 Differences between samples of the same patient at different time points were evaluated with the paired t-

4412 test using the program GraphPad Prism 7.04. All data were distributed normally. A P value < 0.05 were

4413 considered significant.

4414

4415 A Shapiro-Wilk test of normality was performed on alpha diversity and Richess to check whether the data

4416 was normally distributed or not. As the data was not normally distributed, differences in alpha diversity were

4417 calculated using the non-parametric Kruskal-Wallis test (more than two levels) and pairwise comparisons

4418 were calculated using the Wilcoxon rank sum test. Tests were carried out using the microbiome R package.

4419 Graphics were created using the package ggpubr in R. We use the following convention for symbols

4420 indicating statistical significance: ns: $p > 0.05$, *: $p <= 0.05$, **: $p <= 0.01$, ***: $p <= 0.001$ and ****: $p <=$

4421 0.0001.

4422

4423 Differences in beta diversity were calculated in normalized data (CSS OTU table) based on Permutational

4424 Multivariate Analysis Of Variance using Distance Matrices and the function Adonis from the program

4425 Vegan 2.4.6. in R (Anderson, 2001). A P value < 0.05 was considered significant. For microbial differential

4426 abundance testing, DESeq2 from the Phyloseq package was used.

4427

4428 **3.3 Results**

4429

4430 **3.3.1 Immunoglobulin coating of faecal bacteria in healthy dogs**

4431

4432   The percentage of bacteria coated with immunoglobulin A and G was determined during cell sorting

4433   analysis. Healthy dogs contained on average 21 ± 9% of IgA+ bacteria versus 78 ± 10% IgA- bacteria and

4434   on average 17 ± 8% of IgG+ bacteria versus 82 ± 9% IgG- bacteria.

4435

4436   To assess the stability of immunoglobulin concentrations in healthy dogs, two faecal samples were

4437   collected one month apart. In the first visit, the average of bacteria coated with immunoglobulin A was 20 ±

4438   10% of IgA+ bacteria versus 78 ± 10% IgA- bacteria, whereas in the second visit the percentage was 21 ±

4439   9% of IgA+ bacteria versus 82 ± 10% IgA- bacteria. Coefficients of variation among visits per patient

4440   ranged between 5-63% for IgA+ and between 2-17% for IgA- and there were no significant differences

4441   among the visits in any of the parameters assessed. In the first visit, the average of bacteria coated with

4442   immunoglobulin G was 17 ±6% of IgG+ bacteria versus 81 ± 7% IgG- bacteria, whereas in the second visit

4443   the percentage was 17 ±10% of IgG+ bacteria versus 82 ± 10% IgG- bacteria. Coefficients of variation

4444   among visits per patient ranged between 2-17% for IgG+ and between 4-16% for IgG (Figure 3.3).

4445

4446   **3.3.2 Immunoglobulin coating of faecal bacteria in dogs with chronic enteropathies**

4447

4448   The percentage of bacteria coated with immunoglobulin A and G during active disease and shortly after

4449   remission of clinical signs was determined during cell sorting analysis and flow cytometry.

4450

4451   Clinical remission was associated with a decrease in immunoglobulin coating: IgA+ from 22 ± 12% to 12 ±

4452   10% (P <0.01) and IgG+ from 16 ± 10% to 8 ± 4% of IgG+ (P <0.01). In contrast, the percentage of

4453   uncoated population was inversely proportional to the positive population, increasing over time: IgA- from

4454   76 ± 13% to 87 ± 10% IgA (P <0.01) and IgG- from 82 ± 10% to 91 ± 4% (P <0.01) (Figure 3.4 and 3.5).

4455

4456   Contrary to what was expected, the active disease profile was like the profile of healthy dogs, whereas the

4457   remission period showed significant lower percentages of immunoglobulin coated bacteria compared to

4458   healthy population (P <0.01). Samples paired by dog showed the same trend and it was statistically

4459   significant in all cases (Figure 3.8 and 3.9).

4460

4461   Some dogs were followed during the treatment period to assess the dynamic of the coating over time. In

4462   general, the trend was dominated by decreases in the percentage of coated bacteria and increases in the

4463   percentage of non-coated bacteria, suggesting that treatment and response are associated with attenuation

4464   of the immune response. (Figure 3.6).

4465

4466   In one of the dogs, an extra sample was collected six months later after the resolution of the clinical signs.

4467   The percentage of the positive population increased in relation to the previous sample, without the

4468   presence of clinical signs. This could suggest that the dog was at a subclinical stage or the percentage of

4469   the populations were normalizing with regards to the healthy one. More studies will need to be analysed in

4470   order to unravel this question (Figure 3.7).

4471

4472

4473

4474

4475

4476

4477
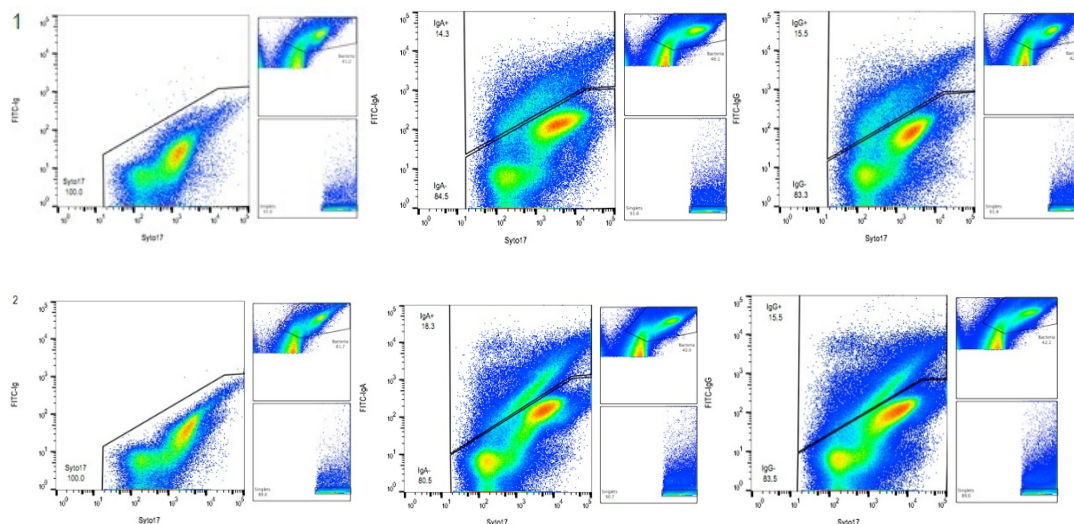
4478 Figure 3.1: Flow cytometry healthy dog. Representative samples. Left panel: samples stained only with syto

4479 17. Middle panel: Cells stained with syto17 and FITC-IgA. Right panel: Cells stained with syto17 and FITC-

4480 IgG. Upper line: visit 1. Lower line: Visit 2. For microbial fraction identification the trigger was set up based

4481 on side scatter properties (SSC) (small upper panel). Then, singlets were selected based on SSC-width vs

4482 SCC-height (small lower panel). FITC-negative window population was set up in samples containing only

4483 Syto 17. These windows were applied to samples stained with FITC-IgA or FITC-IgG to distinguish

4484 negative population from the positive one. Each sample was analyzed individually.

4485

4486

4487

4488

4489

4490

4491

4492

4493

Figure 3.2: Flow cytometry dog with CE. Representative samples. Left panel: samples stained only with syto 17. Middle panel: Cells stained with syto17 and FITC-IgA. Right panel: Cells stained with syto17 and FITC-IgG. Lines correspond to visit 1 - visit 4 (CE remission). For microbial fraction identification the trigger was set up based on side scatter properties (SSC) (small upper panel). Then, singlets were selected based on SSC-width vs SCC-height (small lower panel). FITC-negative window population was set up in samples containing only Syto 17. These windows were applied to samples stained with FITC-IgA or FITC-IgG to distinguish negative population from the positive one. Each sample was analyzed individually.

4501

4502

4503
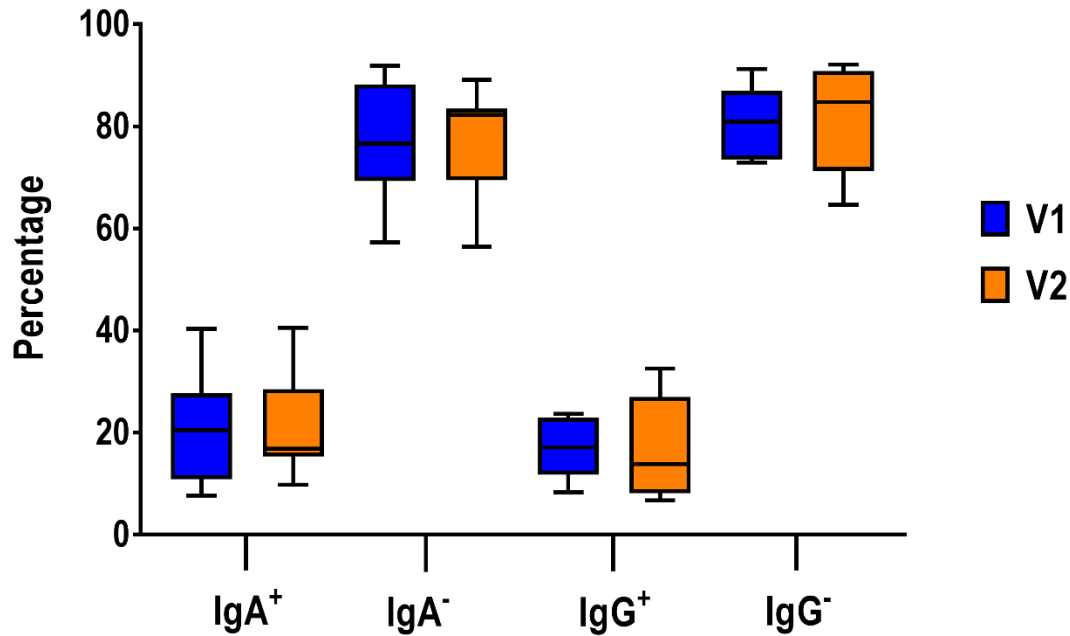
4504   Figure 3.3: Percentages of faecal bacteria coated with immunoglobulin A (IgA) and immunoglobulin (IgG)

4505   from healthy dogs (n=11). V1: Visit 1 V2: Visit 2.
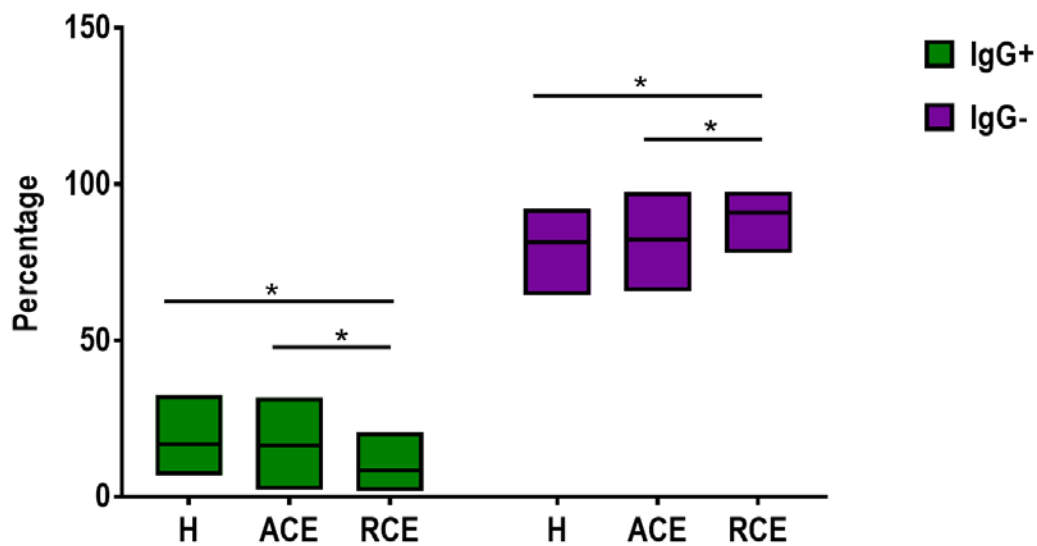
4506

4507

4508

4509

4510
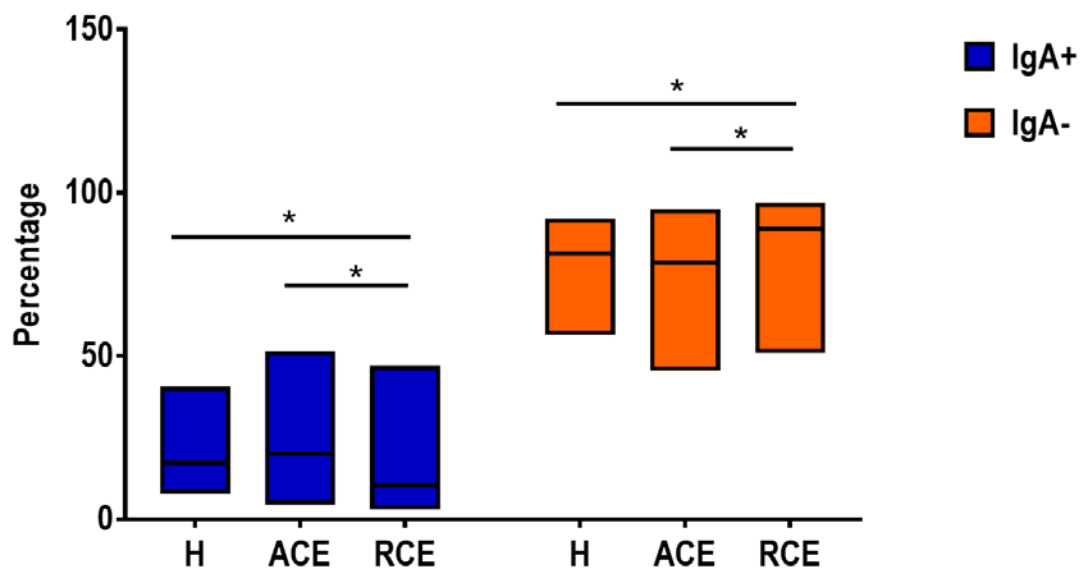
4511   Figure 3.4: A: Percentages of faecal bacteria coated with immunoglobulin A (IgA) from healthy dogs (H)

4512   with chronic enteropathies during active disease (pre-treatment (ACE)) and during remission (Post-

4513   treatment (RCE).* P value :< 0,01 student t-test. Horizontal bars shown with individual data points

4514   represent sample median.

4515

4516

4517

4518

4519

4520 Figure 3.5: Percentages of faecal bacteria coated with immunoglobulin G (IgG) from healthy dogs (H) with

4521 chronic enteropathies during active disease (pre-treatment (ACE)) and during remission (Post-treatment

4522 (RCE). * P value :< 0.01 student t-test. Horizontal bars shown with individual data points represent sample

4523 median.

4524

4525

4526

4527

4528

4529

4530 Figure 3.6: Time series analysis of the percentages of faecal bacteria coated with immunoglobulin A (IgA)

4531 and immunoglobulin (IgG). Representative patient (CE dog 7). Visit 1 corresponds to sample before

4532 treatment (first endoscopy) and visit 4 corresponds to sample after resolution of clinical signs (6 weeks

4533 later) (Second endoscopy).

4534

4535

4536

4537

4538

4539
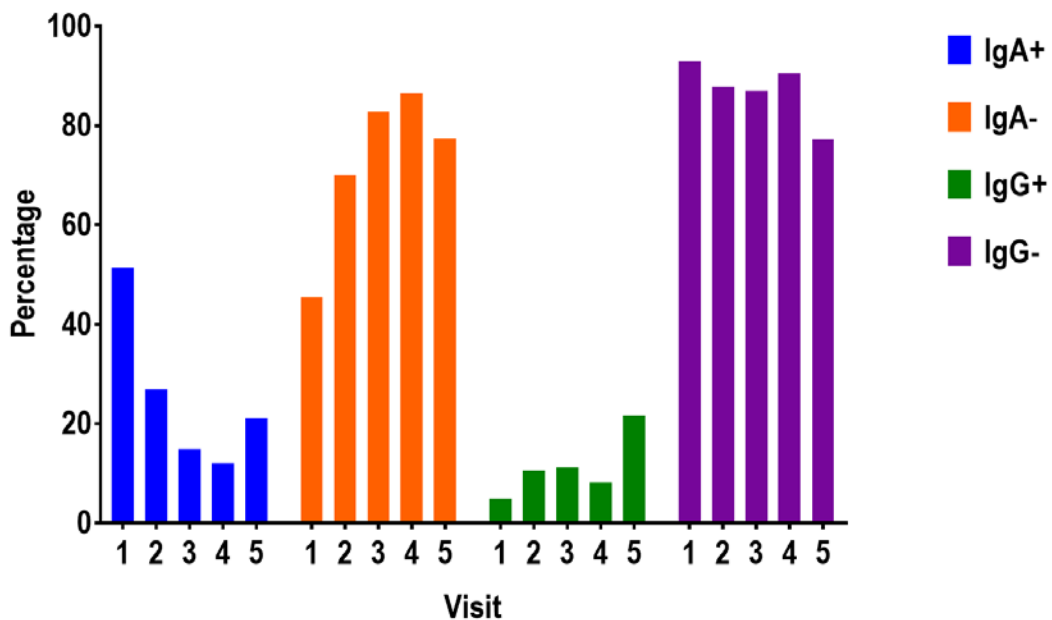
4540   Figure 3.7: Time series analysis of the percentages of faecal bacteria coated with immunoglobulin A (IgA)

4541   and immunoglobulin (IgG).   Representative patient (CE dog 1). Visit 1 corresponds to sample before

4542   treatment (first endoscopy) and visit 4 corresponds to sample after resolution of clinical signs (6 weeks

4543   later) (Second endoscopy) and visit 5 corresponds to sample taken after 6 months of resolution of clinical

4544   signs.

4545

4546

4547

4548

4549

4550
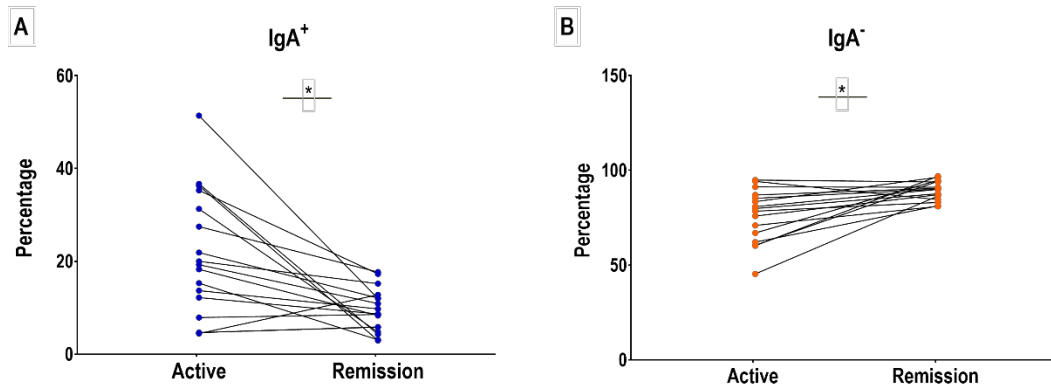
4551   Figure 3.8: A: Percentages of faecal bacteria coated with immunoglobulin A (IgA) and B uncoated with IgA.

4552   Paired samples IgA+: 17 samples. IgA-: 16 samples.). * P value :< 0.01 student t-test.

4553

4554

4555

4556

4557

4558
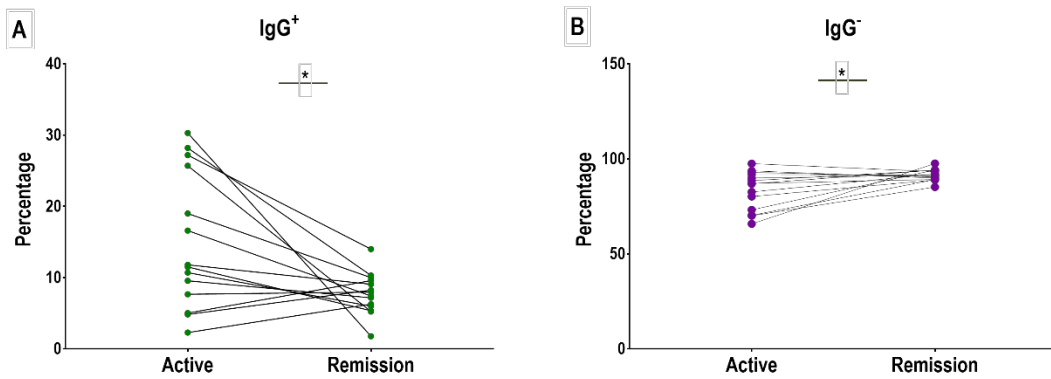
4559   Figure 3.9: A Percentages of faecal bacteria coated with immunoglobulin G (IgG) and B uncoated with IgG.

4560   Paired samples IgG$^+$: 14 samples. IgG$^-$: 14 samples.). * P value :< 0.01 student t-test.

4561

4562

4563

4564

4565   3.3.3 16S rRNA sequencing immunoglobulin coated population

4566

4567   3.3.3.1 Pre-sorting water

4568

4569   Although numerous precautions were taken to avoid or minimise sample contamination, samples of water

4570   collected before the pre-sorting (pre-sorting water) were contaminated with bacterial DNA. All water profiles

4571   were similar, suggesting that the bacteria were present in the system or sheath fluid. At phylum level, the

4572   groups present comprised Proteobacteria (59-70%), Firmicutes (12-26%), Actinobacteria (5-9%),

4573   Fusobacteria (0,02-4%), Cyanobacteria (0,17-2%); Gemmatimonadetes, Acidobacteria, [Thermi] and

4574   Chloroflexi (<1%). Other phyla that were present in some samples included FBP, Tenericutes, SR1,

4575   Spirochaetes and Planctomycetes (<0,1%).

4576

4577   When we analysed the samples at lower phylogenetic levels, we found that at genus level, only 37% of the

4578   samples in the water could be assigned to a genus group. Some of the genera that could be identified

4579   comprised *Sphingomonas* (0-12%), *Kaisobacter* (0,17-9%), *Methylobacterium* (1-7%),*Staphylococcus* (0,5-

4580   5%), *Enterococcus* (0-4%) and *Fusobacterium* (0-4%) *Blautia* (0,5-4%), *Corynebacterium* (1-3%), and

4581   *Deinococcus* (0,2-4%),*Clostridium* (0-3%).

4582

4583   3.3.3.2 Healthy group

4584

4585   3.3.3.2.1 Sequencing summary
4586

4587   The total number of sequences per group range from 620 to 75198. A summary per group can be seen in

4588   Figure 3.10. Although the number of sequences in the sheath fluid before sorting was low; this indicate a

4589   potential contamination of the samples.

4590
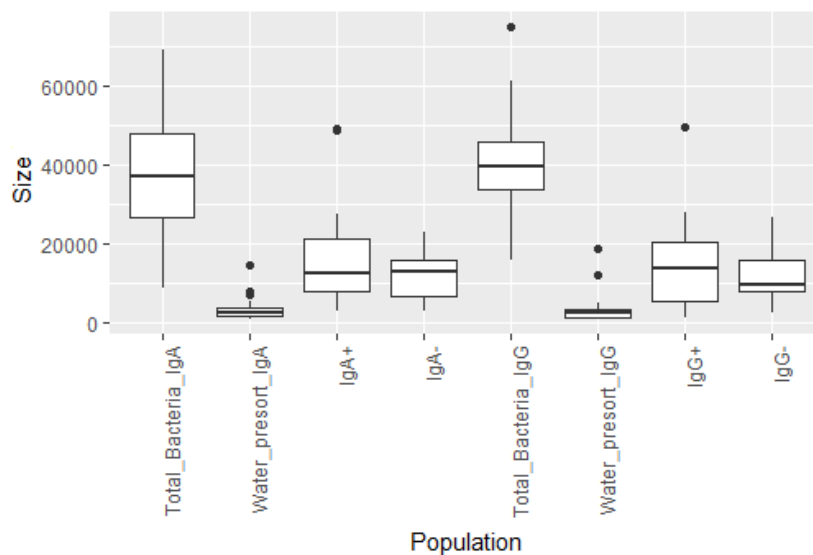
4591

4592

4593

4594

4595 Figure 3.10: Total number of sequences in healthy dogs per type of immunoglobulin. Total bacteria

4596 correspond to the pre-sorting sample; water pre-sort corresponds to the sheath fluid collected before

4597 sorting of bacterial coated (+) and uncoated (-) with Immunoglobulin A (IgA) and Immunoglobulin G (IgG).

4598 N: 11 dogs (visit 1 and 2). IgA: Total n =22, Pre-sorting water n =22, IgA+ = 22, IgA- =22. IgG: Total n =22, Pre-

4599 sorting water n =21, IgG+ = 21, IgG- =21.

4600

4601

4602

4603

4604 **3.3.3.2.2 Analysis of the relative abundance at different phylogenetic levels**
4605

4606 An aliquot of bacterial suspension was collected during the preparation of the samples (before the staining)
4607 and was classified as the total bacteria population for each immunoglobulin.
4608

4609 **3.3.3.2.2.1 Immunoglobulin A**
4610

4611 For the total population, Firmicutes predominated at phylum level, with 62% of the population belonging to
4612 this group. This was followed by Bacteroidetes (20%), Fusobacteria (11%), Proteobacteria (4%) and
4613 Actinobacteria (2%). This is in accordance to previous studies made in dogs (Figure 3.11).
4614

4615 Other groups were found, although at a very low percentage: Gemmatimonadetes (0,009%) and Chloroflexi
4616 (0,004%). Gemmatimonadetes and Chloroflexi have been reported in soil and marine environments,
4617 suggesting incidental colonisers or sample contamination. These groups were present in the water pre-
4618 sorting.
4619

4620 - Positive population
4621

4622 IgA-positive population was dominated by the phyla: Firmicutes (72%), Bacteroidetes (12%),
4623 Proteobacteria (10%), Fusobacteria (3%) and Actinobacteria (1,5%). Other phyla present were [Thermi],
4624 Chloroflexi, Tenericutes, Acidobacteria and FBP (less than <0.01%) (Figure 3.11).
4625

4626 At the genus level, IgA$^+$ population was dominated by [*Ruminococcus*] (29%), *Blautia* (13%), *[Prevotella]*
4627 (5%), *Dorea* (5%), *Prevotella* (3,6%), *Fusobacterium* (3%), *Bacteroides* (2%) and *[Eubacterium]* (2%)
4628 genera. Other groups presented were *Catenibacterium, Turicibacter, Sutterella, Clostridium* and *Collisenlla*
4629 (Figure 3.11).
4630

4631 - Negative Population
4632

4633 IgA-negative population was dominated by the phyla Firmicutes (56%), Fusobacteria (17%), Proteobacteria
4634 (14%), Bacteroidetes (11%) and Actinobacteria (2%). Other phyla present were [Thermi], Chloroflexi,
4635 Tenericutes, Cyanobacteria and Planctomycetes (less than <0.01%) (Figure 3.11).

4636

4637 IgA- population was dominated *by Fusobacterium* (17%), *Blautia* (16%), *Prevotella* (7%), *Clostridium* (6%),

4638 *Megamonas* (5%) and *Catenibacterium* (5%), *[Ruminococcus]* (2,5%), *Enterococcus* (2%) and *Bacteroides*

4639 (2%). Other groups present were *Phascolarctobacterium, Veillonella, Peptococcus, Sphingomonas* and

4640 *Streptococcus* (Figure 3.11).

4641

4642 Although the phyla groups that were present in the samples were also present in the pre-sorting water

4643 (making difficult to distinguish between the real population of bacteria present and contamination), analysis

4644 of the samples at lower phylogenetic levels found that different groups of bacteria were present between

4645 the pre-sorting water and samples or at much lower proportion in the water samples. Noteworthy, most of

4646 the sequences in the water could not be assigned to a specific group (Figure 3.11).

4647

4648 **3.3.3.2.2.2 Immunoglobulin G**
4649

4650 For IgG, the total population of bacteria was dominated by the same groups and in similar proportion as the

4651 ones present in the IgA total population. This is what it was expected as both populations come from the

4652 same samples. The pre-sorting water sample was again similar as the one collected for IgA, although it

4653 also contained the following phyla: FBP (<0,01%).

4654

4655 - Positive population

4656

4657 The IgG- positive population was dominated by the phyla: Firmicutes (74%), Bacteroidetes (9%),

4658 Proteobacteria (9%), Fusobacteria (5%) and Actinobacteria (2%). Other phyla present were [Thermi],

4659 Cyanobacteria, Tenericutes, Acidobacteria and Armatimonadetes (less than <0.01%) (Figure 3.11).

4660

4661 At lower phylogenetic levels, IgG+ population was dominated by *[Ruminococcus]* (30%), *Blautia* (8%),

4662 *Fusobacterium* (5%), *[Prevotella]* (5%), *[Eubacterium]* (3%), *Dorea* (3%), *Clostridium* (2,5%), *Prevotella*

4663 (2%), *Bacteroides* (1,8%), *Catenibacterium* (1,6%) and *Lactobacillus* (1%) (Figure 3.11).

4664

4665 - Negative population

4666

4667   The IgG-negative population was dominated by the phyla Firmicutes (60%), Fusobacteria (17%),

4668   Proteobacteria (10%), Bacteroidetes (11%) and Actinobacteria (2%). Other phyla present were [Thermi],

4669   Chloroflexi, Tenericutes, Cyanobacteria, Crenarchaeota, Verrucomicrobia, Nitrospirae, WPS-2 and

4670   Planctomycetes (less than <0.01%) (Figure 3.11).

4671

4672   At lower phylogenetic levels, IgG⁻ population was dominated *Blautia* (18%), *Fusobacterium* (17%),

4673   *Prevotella* (5%), *Clostridium* (5%), *Megamonas* (4,5%), *Catenibacterium* (3%), *Bacteroides* (3%),

4674   *[Ruminococcus]* (3%), *[Eubacterium]* (2%), *[Prevotella]* (2%), *Streptococcus* (1,5%) (Figure 3.11).

4675

4676

4677

4678

4679
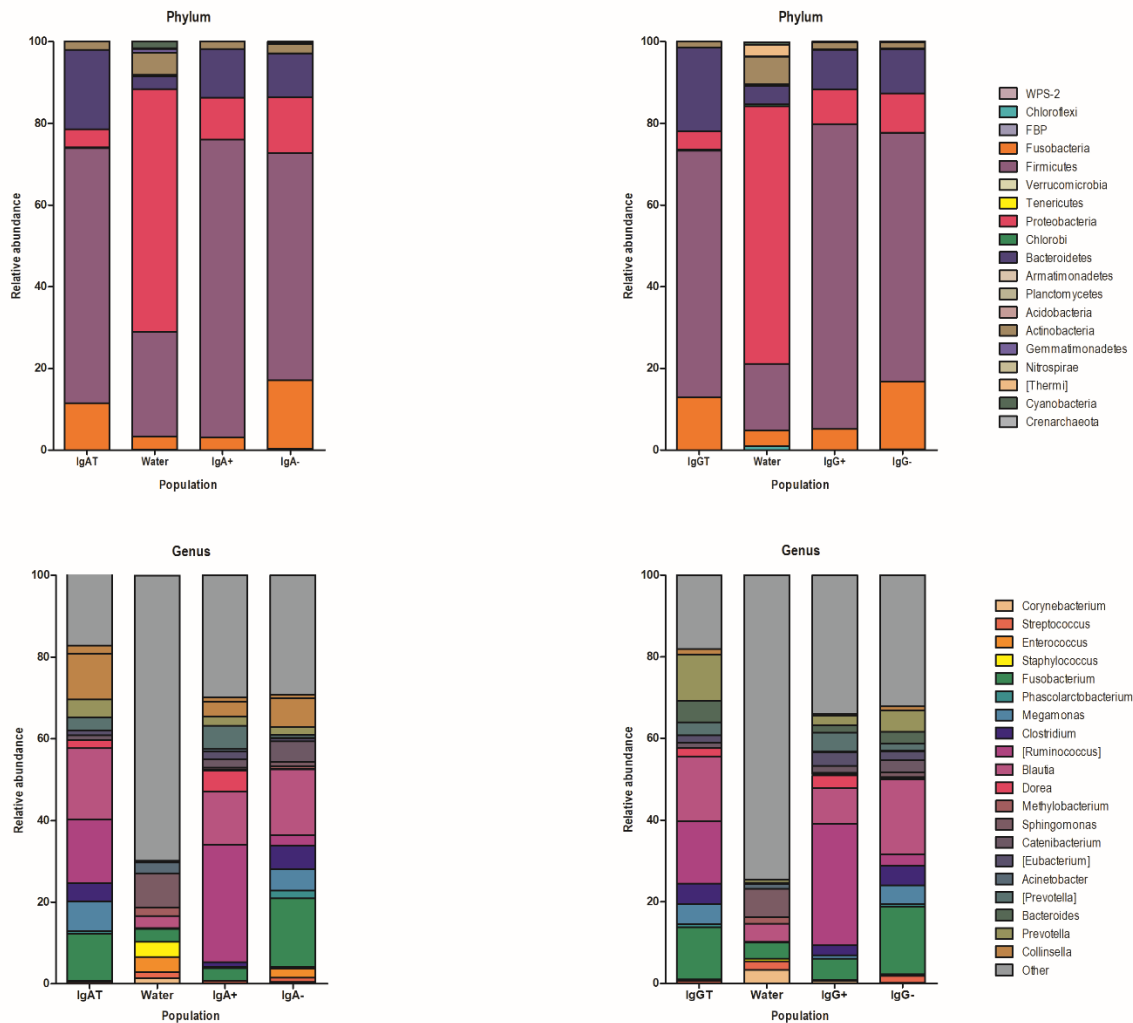
4680   Figure 3.11: Top 20 of the relative abundance of the major phylogenetic levels in healthy dogs. IgA:

4681   immunoglobulin A, IgG: immunoglobulin G. Phylum (Upper panel). Genus (Lower Panel) N: 11 dogs (visit 1

4682   and 2). IgA: Total n =22, Pre-sorting water n =20, IgA+ = 22, IgA- =22. IgG: Total n =22, Pre-sorting water n =19,

4683   IgG+ = 21, IgG- =21.

4684

4685 3.3.3.2.3 Diversity analysis

4686

4687 When alpha diversity analysis was performed, the highest diversity was found in the total population of

4688 bacteria and the lowest diversity in the pre-sorting water as it was expected. Interestingly, the differences

4689 were given by the richness more than the evenness component (Figure 3.12). However, when positive and

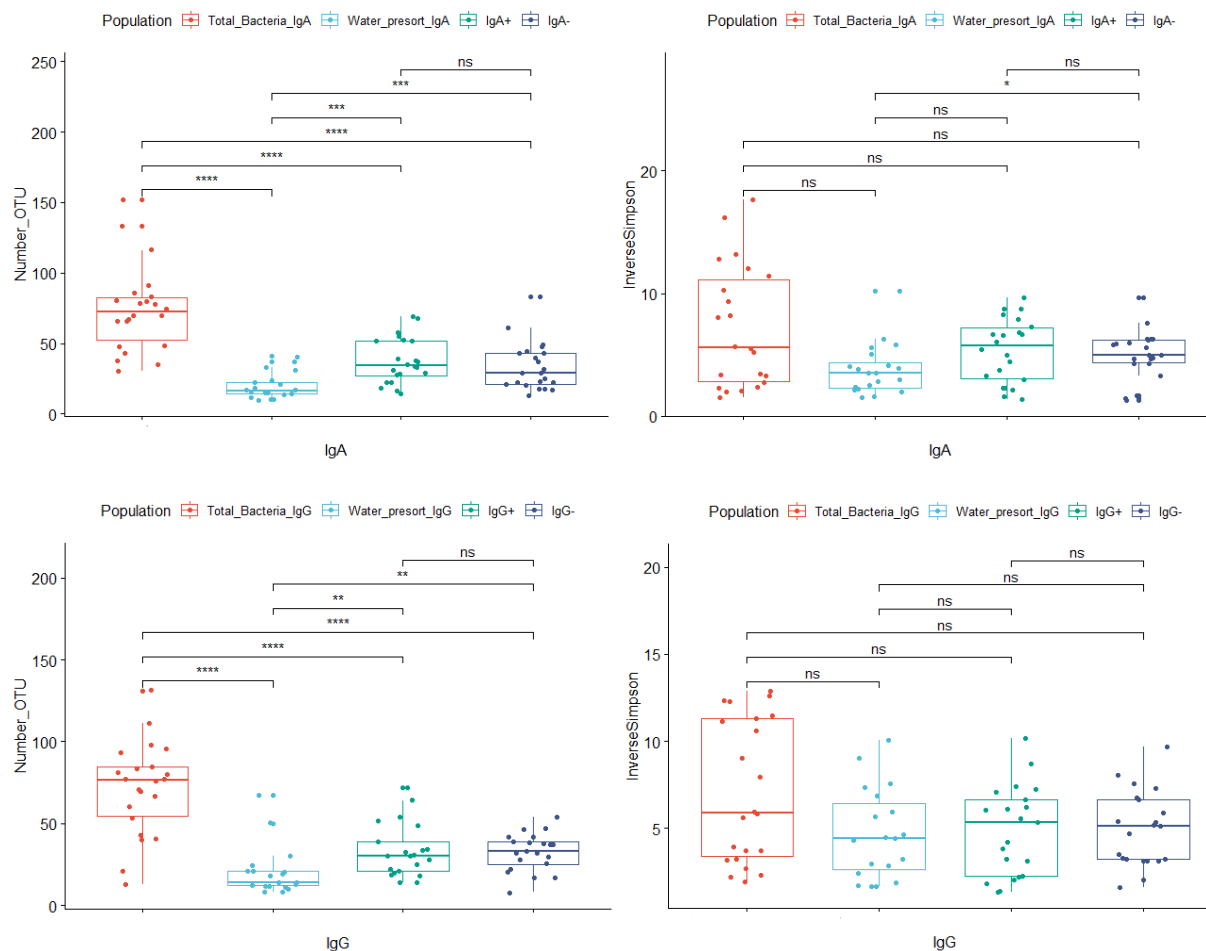4690 negative populations were compared; there was no significant difference in alpha diversity.

4691

4692

4693

4694

Figure 3.12: Alpha diversity analysis of Immunoglobulins in healthy dogs. Upper panel: Immunoglobulin A (IgA);

Lower panel (IgG). N: 11 dogs (visit 1 and 2). IgA: Total n =22, Pre-sorting water n =20, IgA+ = 22, IgA- =22. IgG:

Total n =22, Pre-sorting water n =19, IgG+ = 21, IgG- =21. Right panel: Number of OTUs (Observed), Left panel:

Inversed Simpson's Index. . P value: ns: p > 0.05, *: p <= 0.05, **: p <= 0.01, ***: p <= 0.001 and ****: p <=

0.0001.

4700

4701

4702   When samples were plotted using Unifrac analysis, pre-sorting samples clustered together, total population

4703   of bacteria also clustered together but separately from the pre-sorting samples and immunoglobulin

4704   samples were mixed in the plot (Figure 3.13). When microbial communities were compared there was a

4705   significant difference between the positive and negative population but not among positive IgA and IgG and

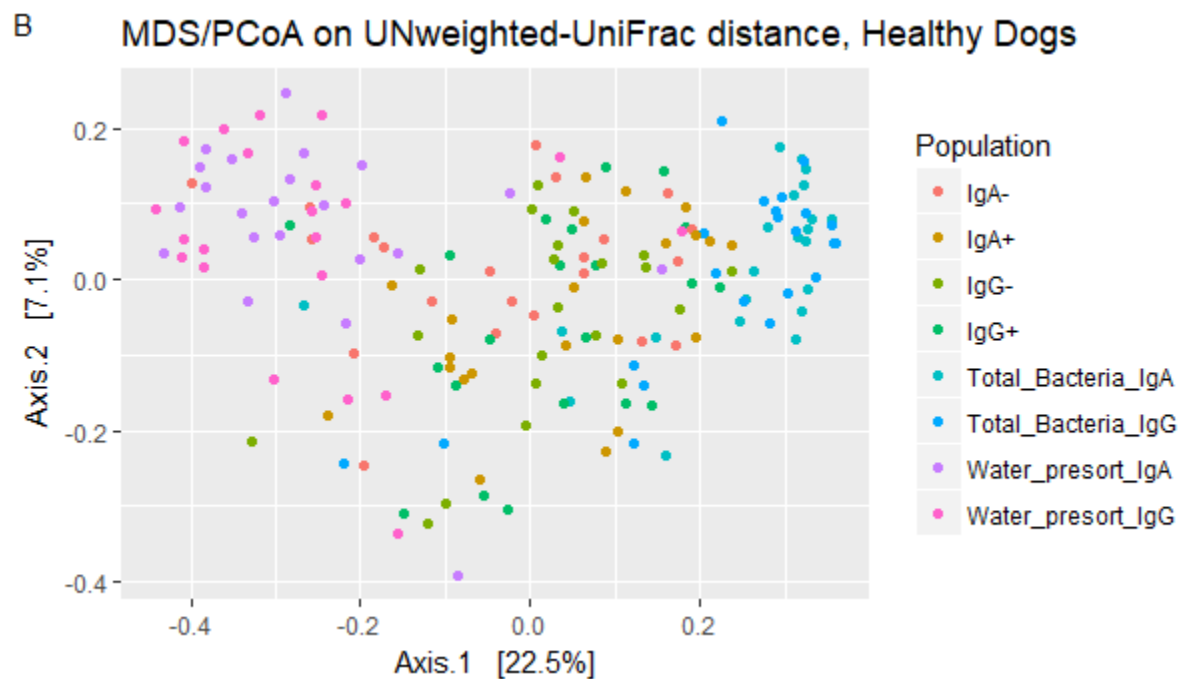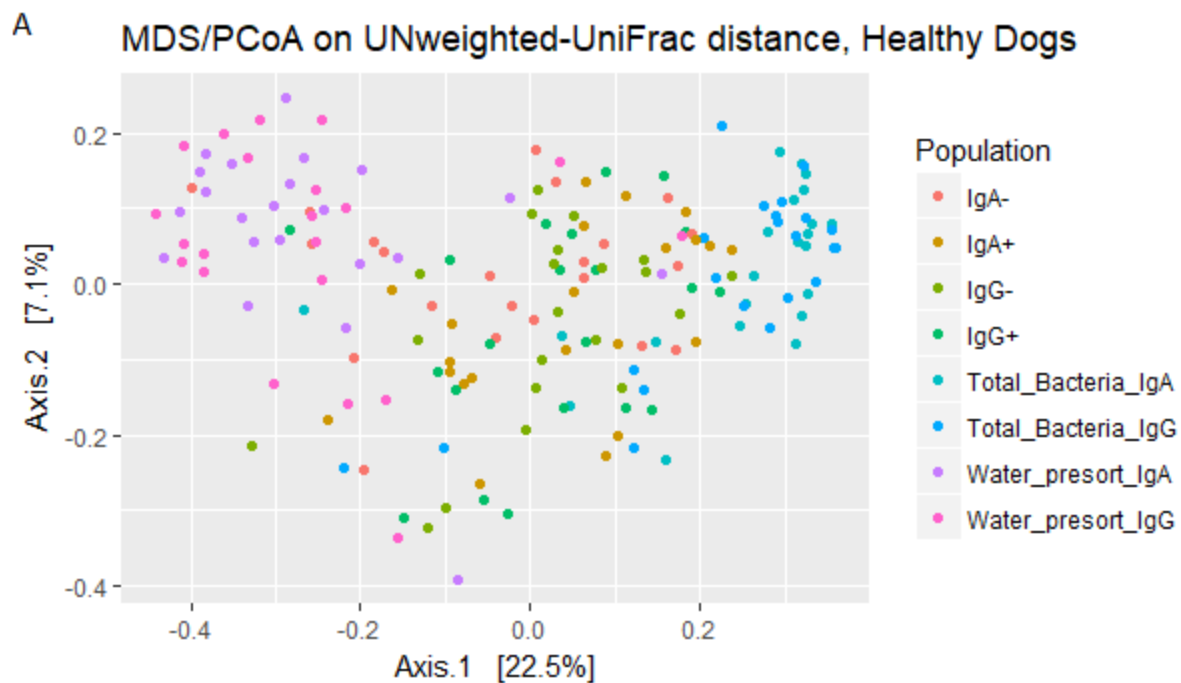4706   negative IgA and IgG population (Table 3.3).

4707

4708

4709

4710

4711

4712 Figure 3.13: Beta diversity analysis in healthy dogs. A: Unweighted and B: Weighted UNIFRAC analysis

4713 comparing different type of immunoglobulin populations. N: 11 dogs (visit 1 and 2). IgA: Total n =22, Pre-sorting

4714 water n =22, IgA+ = 22, IgA- =22. IgG: Total n =22, Pre-sorting water n =21, IgG+ = 21, IgG- =21.

4715

4716   Table 3.3: Comparison of microbial communities (Beta- diversity) according to population of

4717   immunoglobulins in healthy dogs.

Group comparison		R^2	p-value
Total bacteria IgA	Pre-sorting water	0.29691	0.001
vs.	IgA+	0.11102	0.001
	IgA-	0.15135	0.001
Total bacteria IgG	Pre-sorting water	0.27097	0.001
vs.	IgG+	0.09769	0.001
	IgG-	0.11885	0.001
IgA+ vs.	IgA-	0.03803	0.025
IgG+ vs.	IgG-	0.04937	0.002
IgA+ vs.	IgG+	0.01206	0.996
IgA- vs.	IgG-	0.01483	0.953

4718

4719

4720

4721

4722 **3.3.3.3 Chronic Enteropathy group**

4723

4724 **3.3.3.3.1 Faecal Samples**

4725

4726 **3.3.3.3.1.1 Sequence summary**

4727

4728 The total number of sequences per group in active disease range from 63 to 66143, whereas in the

4729 remission period, the number of sequences per group ranged from 179 to 66143. A summary per group

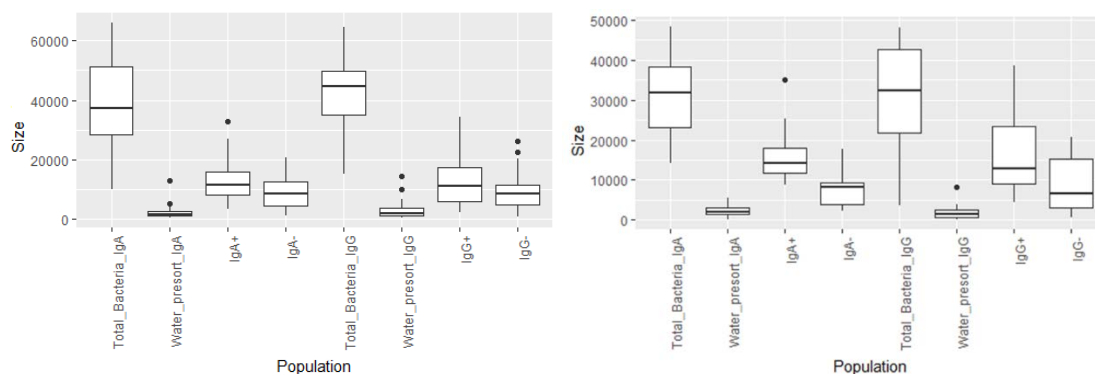4730 can be seen in Figure 3.14.

4731

4732

4733

4734

4735 Figure 3.14: Total number of sequences in dogs with Chronic Enteropathy, Active disease and Remission. Total
4736 bacteria corresponds to the pre-sorting sample; water pre-sort corresponds to the sheath fluid collected before
4737 sorting of bacterial coated (+) and uncoated (-) with Immunoglobulin A (IgA) and Immunoglobulin G (IgG). Active
4738 disease N: 18 dogs. IgA: Total n =28, Pre-sorting water n =28, IgA+ = 28, IgA- =28. IgG: Total n =28, Pre-sorting
4739 water n =28, IgG+ = 28, IgG- =28. Remission 17 dogs. IgA: Total n =18, Pre-sorting water n =17, IgA+ = 18, IgA-
4740 =18. IgG: Total n =18, Pre-sorting water n =18, IgG+ = 18, IgG- =18.

4741

4742

4743 **3.3.3.3.1.2 Analysis of the relative abundance at different phylogenetic levels**
4744

4745 **3.3.3.3.1.2.1 Immunoglobulin A**
4746
4747

4748 An aliquot of bacterial suspension was collected during the preparation of the samples (before the staining)
4749 and was classified as the total bacteria population for each immunoglobulin.

4750

4751 During active disease, Firmicutes predominated at phylum level, with 57% of the population belonging to
4752 this group. This was followed by Bacteroidetes (21%), Proteobacteria (11%), Fusobacteria (6%), and
4753 Actinobacteria (3%) (Figure 3.15).

4754

4755 Additionally, other groups were found, although at a very low percentage: Tenericutes (0,5%),
4756 Verrumicrobia (0,03%) Fibrobacteres (0,017%), [Thermi] (0,007) and Chloroflexi (0,004%). Fribrobacteres
4757 has been reported in the gastrointestinal tract of animals, but not in dogs before. [Thermi] has been
4758 reported in soil and marine environments, suggesting incidental colonizers or sample contamination. This
4759 group was present in the pre-sorting water.

4760

4761 During remission, the same groups at phylum level, were present and in similar proportions.

4762

4763   -   Positive population

4764

4765 During active disease, the IgA- positive population was dominated by the phyla: Firmicutes (72%),
4766 Proteobacteria (12%), Bacteroidetes (8%), Fusobacteria (4%) and Actinobacteria (3%). Other phyla present
4767 were [Thermi], Chloroflexi, Tenericutes, Acidobacteria and Cyanobacteria (less than <0.1%) (Figure 3.15).

4768

4769 At the genus level, the IgA+ population was dominated by *[Ruminococcus]* (20%), *Blautia* (17%),
4770 *Catenibacterium* (5%), *Dorea* (5%), *Fusobacterium* (4%), *[Prevotella]* (4%), *Collisenlla* (3%), *Prevotella*
4771 (2%), Faecalibacterium *(2%), Bacteroides* (2%) *Fusobacterium* (3%), *Bacteroides* (2%) and *[Eubacterium]*
4772 (2%) genera. Other groups presented were *Megamonas* and *Clostridium.* 26% of the bacteria could not be
4773 classified at genus level (Figure 3.15).

4774

4775   During remission, the phylum and genus profile conserved the same characteristics, with minor differences
4776   in proportion.

4777

4778   -   Negative population

4779

4780   During active disease, the main groups that were present in the positive population, were present in the
4781   negative population and in similar proportions, except for Actinobacteria (6%). Other phyla present were
4782   [Thermi], Chloroflexi, Cyanobacteria and Verrucomicrobia (less than <0.1%) (Figure 3.15).

4783

4784   At genus level, the IgA- population was dominated *by Blautia* (12%), *Fusobacterium* (6%), %), *Megamonas*
4785   (5%), *Prevotella* (7%), *Clotridium* (6%), *Megamonas* (6%), *Streptococcus (6%), Clostridium* (5%),
4786   *Faecalibacterium* (4%), *Catenibacterium* (4%), *[Ruminococcus]* (3%), *Enterococcus* (2%) and *Prevotella*
4787   (2%). Other groups present were *Lactobacillus, Collinsella, Enterococcus, Bacteroides* and *[Prevotella]*
4788   (Figure 3.15).

4789

4790   IgA- population was dominated by *Blautia* (13%), *[Ruminococcus]* (7%), *Bacteroides* (5%), *Fusobacterium*
4791   (5%), %), *Streptococcus* (4%), *Megasphaera* (4%), *Sphingomonas* (4%), *Staphylococcus* (2%),
4792   *Streptococcus* (6%), *Clostridium* (5%), *Faecalibacterium* (4%), *Catenibacterium* (4%), *Prevotella* (2%) and
4793   *Megamonas* (2%). Other groups present were *Corynebacterium, Collinsella, Micrococcus, [Prevotella],*
4794   *Allobaculum, Deinococcus.* 39% of the bacteria could not be classified at genus level (Figure 3.15).

4795

4796   During remission, the phylum and genus profile conserved the same characteristics, with minor differences
4797   in proportion.

4798

4799   **3.3.3.1.2.2 Immunoglobulin G**
4800

4801

4802
4803   For IgG, the total population of bacteria was dominated by the same groups and in similar proportion as the
4804   ones present in the IgA total population, during active disease and remission (Figure 3.15).

4805

4806   -   Positive population

4807

4808   In both stages, the IgG- positive population was dominated by the phyla: Firmicutes (74-76%),

4809   Proteobacteria (8-11%), Bacteroidetes (11%), Fusobacteria (1,5-3%) and Actinobacteria (2%). Other phyla

4810   present were [Thermi], Cyanobacteria, Chloroflexi, Acidobacteria, Verrucomicrobia and WPS-2 (less than

4811   <0.1%) (Figure 3.15).

4812

4813   At the genus level, the IgG+ population was dominated by *[Ruminococcus]* (20%), *Blautia* (20%), *Dorea*

4814   (4%), Megamonas (4%), *[Prevotella]* (4%), *Fusobacterium* (3%) and *Prevotella* (3%). Other groups present

4815   were C*lostridium*, *Bacteroides*, Faecalibacterium, *Dialister, Collinsella and, Catenibacterium* (Figure 3.15).

4816

4817   -   Negative Population

4818

4819   In both stages, the IgG-negative population was dominated by the phyla Firmicutes (63-74%),

4820   Proteobacteria (9-17%), Fusobacteria (7%), Bacteroidetes (7%) and Actinobacteria (2%). Other phyla

4821   present were [Thermi], Chloroflexi, Tenericutes, Cyanobacteria and Verrucomicrobia, (less than <0.1%)

4822   (Figure 3.15).

4823

4824   The IgG- population was dominated *Blautia* (24%), *Fusobacterium* (6%), Megamonas (5%), *Clostridium*

4825   (5%), *[Ruminococcus]* (4%), *Catenibacterium* (4%), Faecalibacterium (4%), *Prevotella* (3%), and

4826   Bacteroides (3%). Other present groups included *Streptococcus, Enterococcus, Roseburia* and

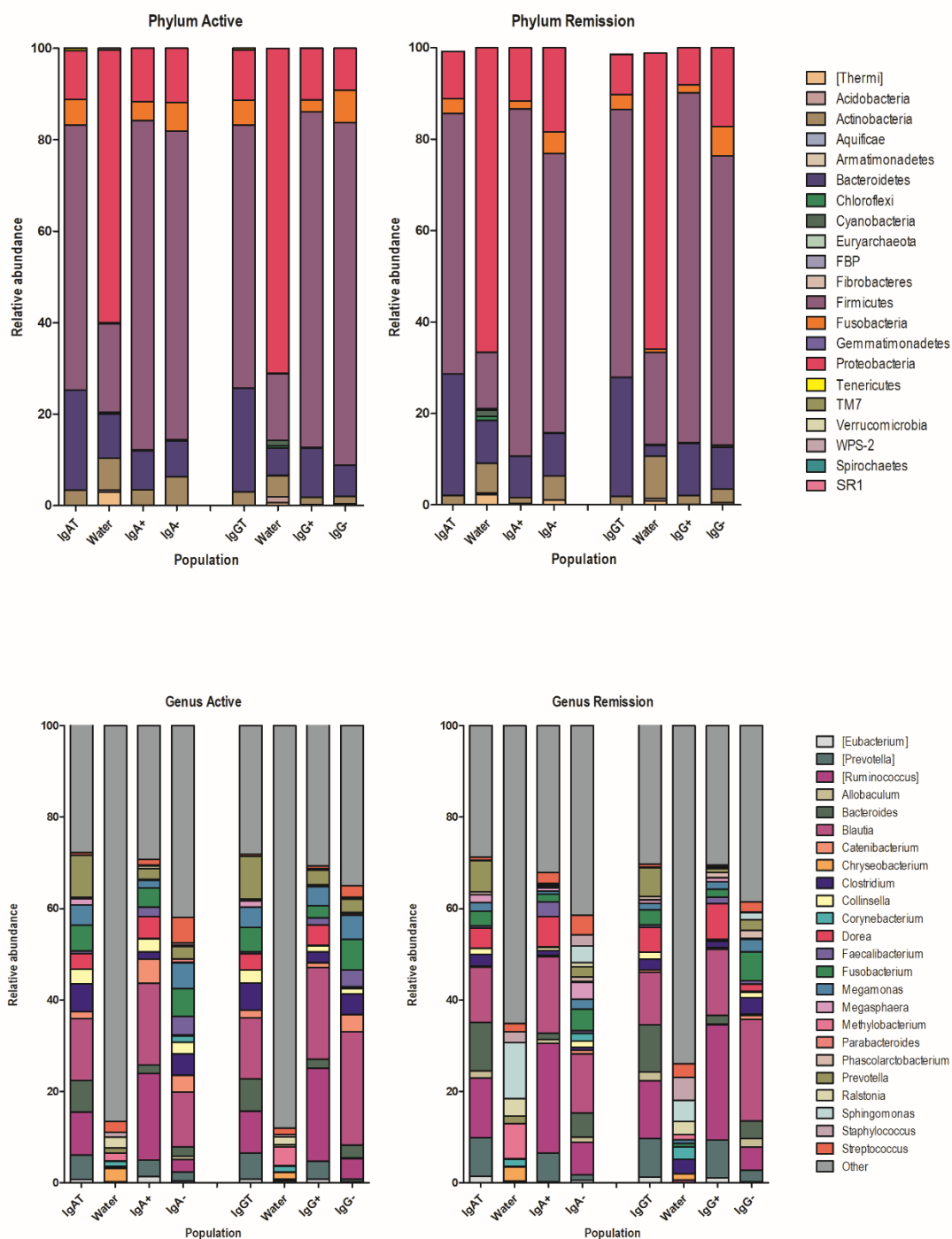4827   *Kaistobacter* (Figure 3.15).

4828

4829

4830

4831

4832

4833   Figure 3.15: Relative abundance of the major phylogenetic levels in dogs with chronic enteropathy, active disease

4834   versus remission. Phylum (Upper panel), Genus (Lower panel) IgAT: immunoglobulin A total bacteria (presort), IgA+:

4835   immunoglobulin A-positive population and IgA-: immunoglobulin A-negative population. IgGT: immunoglobulin A total

4836   bacteria (presort), IgG+: immunoglobulin G-positive population and IgG-: immunoglobulin G-negative population.

4837 **3.3.3.3.1.2 Diversity Analysis**
4838
4839

4840   -   Alpha diversity

4841

4842 When alpha diversity analysis was performed, both stages shared the same characteristics. The highest

4843 diversity was found in the total population of bacteria and the lowest diversity in the pre-sorting water.

4844 When negative and positive populations were compared; there was not a significant difference in alpha

4845 diversity (Supplementary figure 5.1 and 5.2); as well as, when populations were compared at different

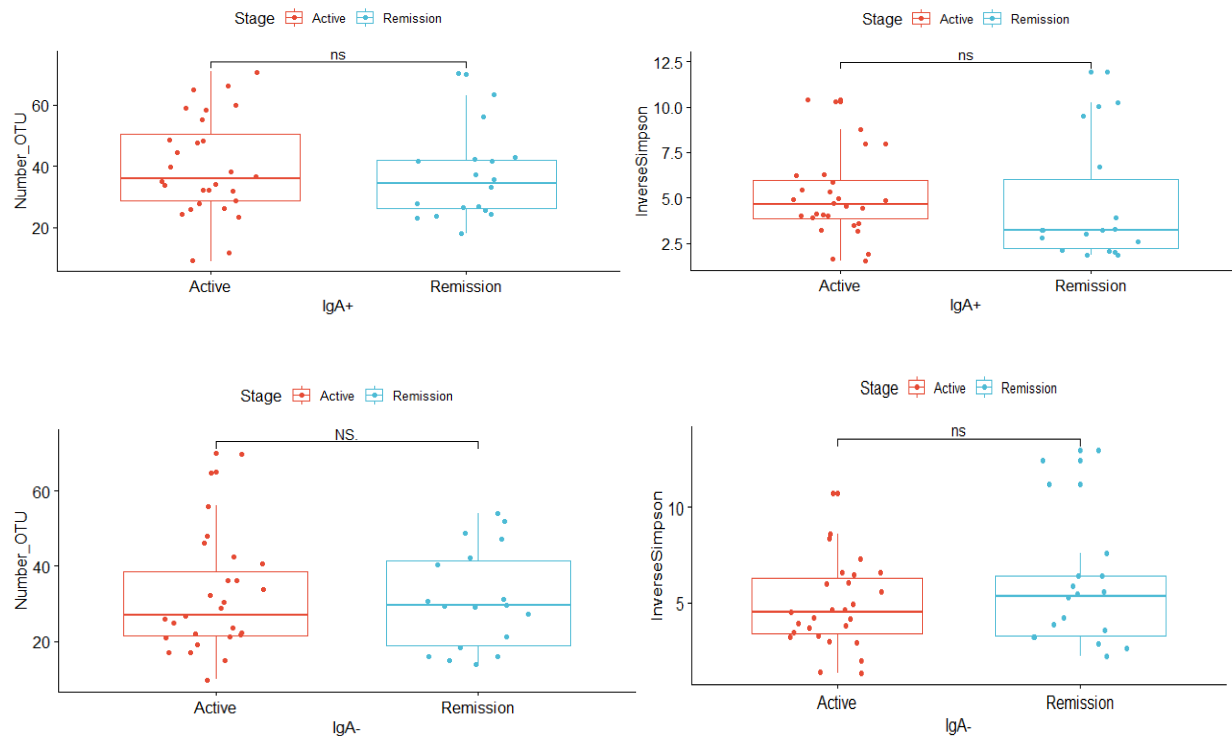4846 stages (Figure 3.16 and 3.17).

4847

Figure 3.16: Alpha diversity analysis of Immunoglobulin A (IgA) in dogs with Chronic Enteropathy at different stages. Active disease versus Remission. Upper panel: Immunoglobulin A positive population (IgA+); Lower panel Immunoglobulin A negative population (IgA-); N Active: 18 dogs; N Remission: 17 dogs. IgA+ Active n =27 IgA+ Remission n=18. IgA- Active n= 27; IgA- Remission =18. Right panel: Number of OTUs (Observed), Left panel: Inversed Simpson's Index.
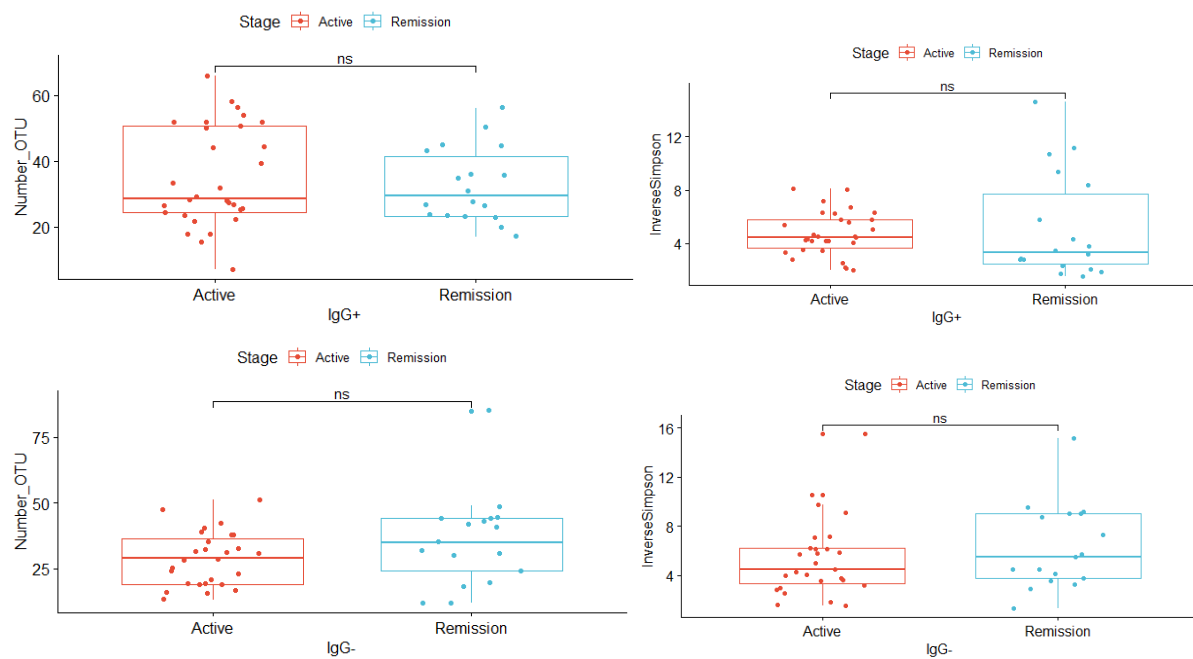
4857
4858

4859

4860
4861 Figure 3.17: Alpha diversity analysis of Immunoglobulin G in dogs with Chronic Enteropathy at different stages.

4862 Active disease versus Remission. Upper panel: Immunoglobulin G positive population (IgG+); Lower panel

4863 Immunoglobulin A negative population (IgG-); N Active: 18 dogs; N Remission: 17 dogs. IgG+ Active n = 30 IgG+

4864 Remission n=18. IgG- Active n= 27; IgG- Remission n=18. Right panel: Number of OTUs (Observed), Left panel:

4865 Inversed Simpson's Index.

4866

4867   -   Beta diversity

4868

4869 When samples were plotted using Unifrac analysis, the following pattern was observed in both stages of

4870 the disease: Clustering was evident for the water pre-sorting samples and total bacterial population. The

4871 positive and negative population were dispersed. When microbial communities were compared there was a

4872 significant difference between the positive and negative population but not among positive IgA and IgG and

4873 negative IgA and IgG population (Table 3.4, 3.5 and 3.6) (Figure 3.18) at any stage and compared to

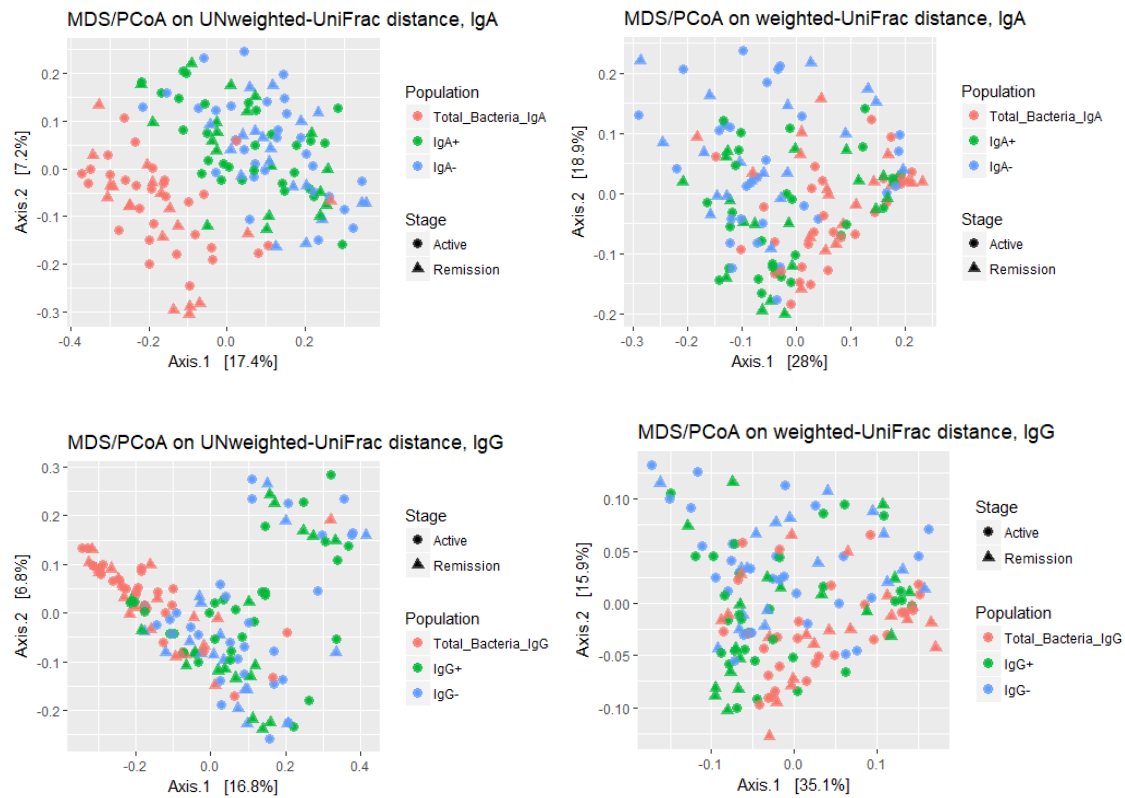4874 healthy dogs (Table 3.7).

4875

4876

4877

4878

Figure 3.18: Beta diversity analysis in dogs with chronic enteropathy, active versus remission. Unweighted and Weighted UNIFRAC analysis comparing different type of immunoglobulin A (upper panel) and G (lower panel).

4879

4880

4881

4882   Also, when samples were compared according to the type of disease, there was no a clear separation

4883   between different types or stages of the disease in the immunoglobulin populations. (Figure 3.19)
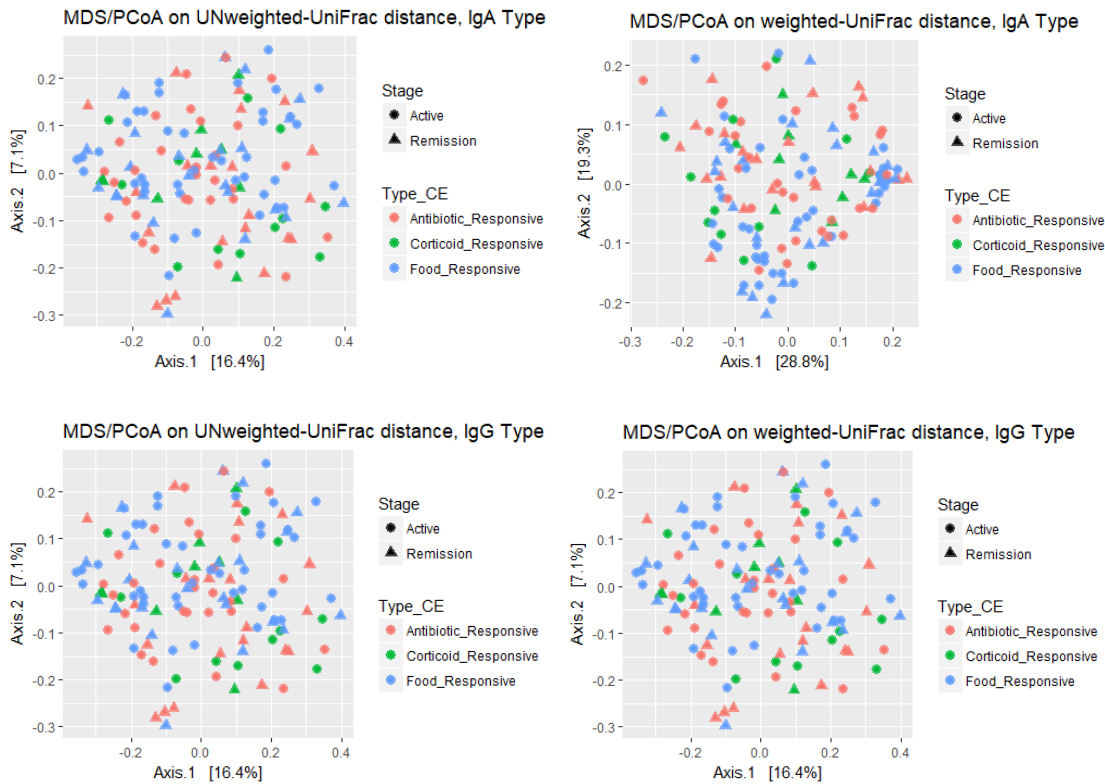
4884

4885

4886

4887

4888

4889

4890 Figure 3.19: Beta diversity analysis in dogs with chronic enteropathy, active versus remission and

4891 according to the type of disease. Unweighted and Weighted UNIFRAC analysis comparing different type of

4892 immunoglobulin A populations (upper panel) and immunoglobulin G populations (Lower panel).

4893

4894

4895

4896

4897   Table 3.4: Comparison of microbial communities (Beta- diversity) according to population of immunoglobulins in dogs

4898   with chronic enteropathy, Active disease.

4899

Group comparison		R2	p-value
Total bacteria IgA vs.	Pre-sorting water	0.26854	0.001
	IgA+	0.08094	0.001
	IgA-	0.12503	0.001
Total bacteria IgG vs.	Pre-sorting water	0.24715	0.001
	IgG+	0.09246	0.001
	IgG-	0.12747	0.001
IgA+ vs.	IgA-	0.05281	0.001
IgG+ vs.	IgG-	0.04964	0.001
IgA+ vs.	IgG+	0.00625	0.999
IgA- vs.	IgG-	0.01567	0.693

4900

4901

4902

4903

4904   Table 3.5: Comparison of microbial communities (Beta- diversity) according to population of immunoglobulins in dogs

4905   with chronic enteropathy, Remission.

4906

Group comparison		R2	p-value
Total bacteria IgA vs.	Pre-sorting water	0.26041	0.001
	IgA+	0.10075	0.001
	IgA-	0.12632	0.001
Total bacteria IgG vs.	Pre-sorting water	0.23886	0.001
	IgG+	0.0932	0.001
	IgG-	0.04702	0.024
IgA+ vs.	IgA-	0.06549	0.001
IgG+ vs.	IgG-	0.04964	0.001
IgA+ vs.	IgG+	0.01327	0.996
IgA- vs.	IgG-	0.02053	0.912

4907

4908

4909

4910

4911

4912 Table 3.6: Comparison of microbial communities (Beta- diversity) according to population of immunoglobulin A in
4913 dogs with chronic enteropathy, according to stage CEA: Active disease versus CER: remission; and versus healthy
4914 dogs.

4915

Group comparison			R2	p-value
IgA+ Healthy	vs.	IgA+ CEA	0.03482	0.492
		IgA+ CER	0.05132	0.566
IgA- Healthy	vs.	IgA- CEA	0.04507	0.154
		IgA- CER	0.02231	0.369
IgA+ CEA	vs.	IgA+ CER	0.01522	0.881
IgA- CEA	vs.	IgA- CER	0.06534	0.107

4916

4917

4918

4919

4920

4921 Table 3.7: Comparison of microbial communities (Beta- diversity) according to population of
4922 immunoglobulin G in dogs with chronic enteropathy, according to stage CEA: Active disease versus CER:
4923 remission; and versus healthy dogs.

4924

Group comparison			R^2	p-value
IgG+ Healthy	vs.	IgG+ CEA	0.0306	0.526
		IgG+ CER		0.001
IgG- Healthy	vs.	IgG- CEA	0.05434	0.072
		IgG- CER	0.06534	0.105
IgG+ CEA	vs.	IgG+ CER	0.01294	0.959
IgG- CEA	vs.	IgG- CER	0.0189	0.71

4925

4926

4927 ### 3.3.3.3.1.3 Microbial differential abundance testing

4928

4929 Positive populations of immunoglobulins were compared between different stages of the disease and between healthy and sick dogs. When IgA+

4930 population was compared between dogs with active disease and dogs in remission, only one family group was significantly enriched during active

4931 disease: Alcaligenaceae. None of the bacterial groups were enriched in the IgG+ population (Figure 3.20A).

4932

OTUs	base Mean	log2Fold Change	lfcSE	stat	P value	P adj	Phylum	Class	Order	Family	Genus	Species
Otu 20	23.512	10.00	2.143	4.666	3.06e-06	0.0008	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella	NA

4933
4934

4935 Analysis of the different immunoglobulin populations between dogs with the disease and healthy dogs, showed that two families were enriched in

4936 sick dogs in the IgA+ population: Clostridiaceae and Lachnospiraceae, whereas the IgG+ population did not show any enrichment (Figure 3.20B).

4937

4938

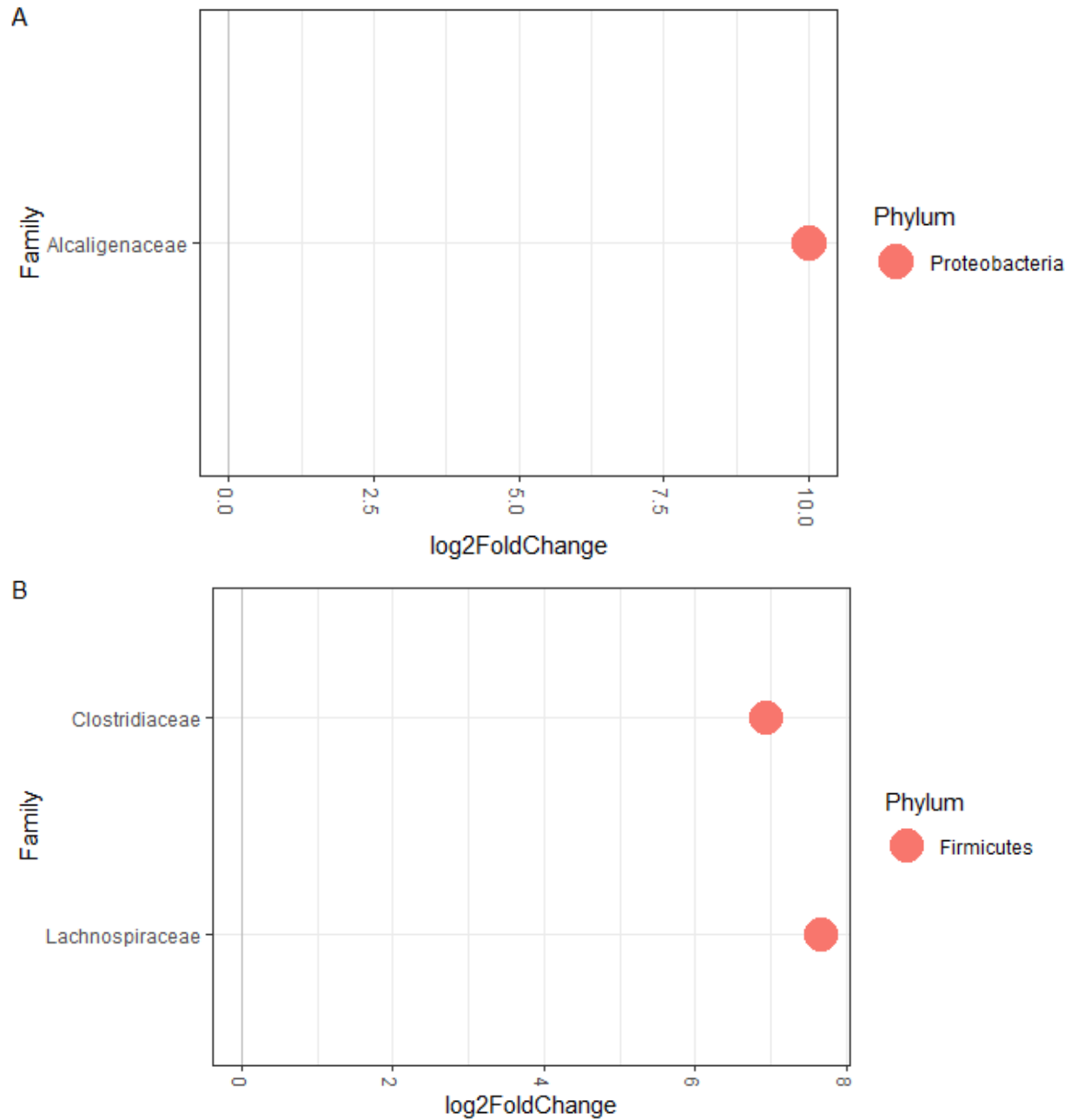| OTUs	base Mean	log2Fold Change	lfcSE	stat	P value	P adj	Phylum	Class	Order	Family	Genus	Species
Otu 11	69.822	6.940	1.501	4.622	3.79e-06	0.001	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
Otu 128	14.659	7.668	1.841	4.163	3.128e-05	0.005	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	producta

4939

4940
4941
4942 Figure 3.20 Microbial differential Abundance Testing in the Immunoglobulin A positive population. A: Active disease

4943 versus Remission. B Dogs with Chronic Enteropathy versus healthy dogs. DEseq2 method was used.

4944
4945
4946 **3.3.3.3.1.4 Hierarchical Cluster Analysis**
4947
4948
4949 Hierarchical cluster analysis was performed in order to compare IgA positive population in healthy and sick

4950 dogs, using UPGMA (unweighted pair group method with arithmetic mean) clustering and the weighted

4951 UniFrac distance. In healthy dogs, UniFrac distances between samples outlines strong structural

4952 intraindividual sample proximity, suggesting overall temporal stability of individual gut IgA microbiota

4953 profiles (Figure 3.21); wheras in sick dogs intraindividual samples were disperse, and in some dogs, only

4954 some samples were at close proximity (Figure 3.22).

4955
4956
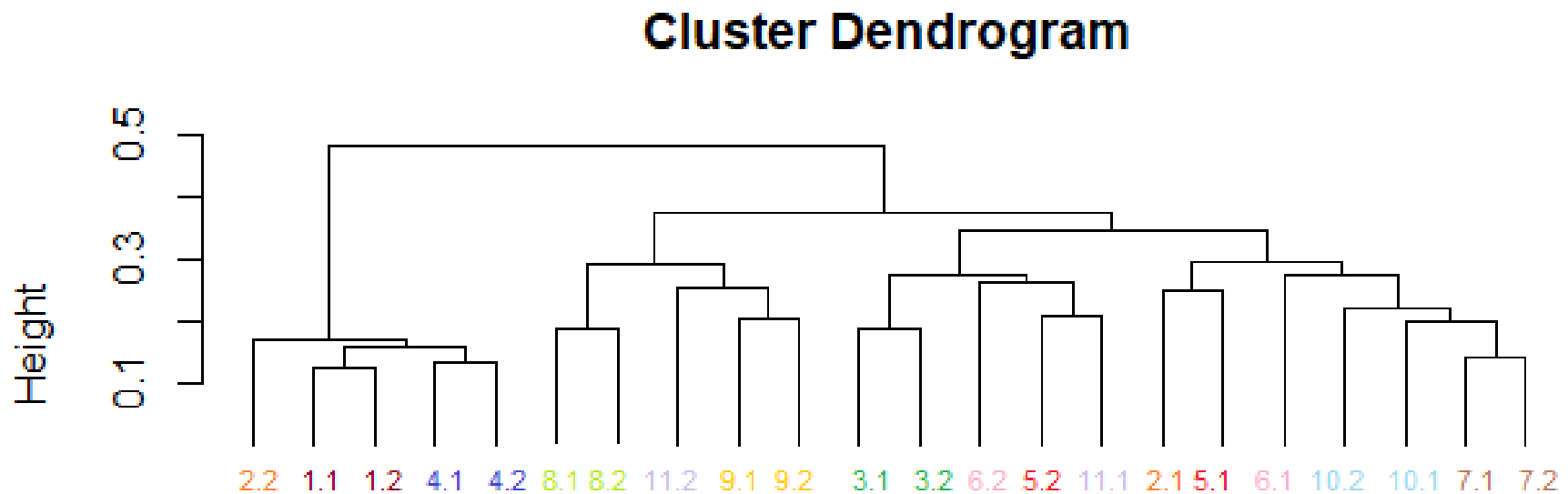
4957
4958
4959

Cluster Dendrogram

4960
4961
4962   Figure 3.21 Hierachical clustering of the microbiota profiles of Immunoglobulin A populations in healthy dogs on weighted Unifrac distances. Colors represent
4963   different dogs. The number corresponds to the number of the dog and visit (1.1 corresponds to dog1, visit1). Height reflects the distance between the samples
4964   (height of the common node). The closer the distance between the two samples, the more similar is their bacterial community The plot highlights the fact that
4965   samples tended to cluster by dog.

4966

Cluster Dendrogram
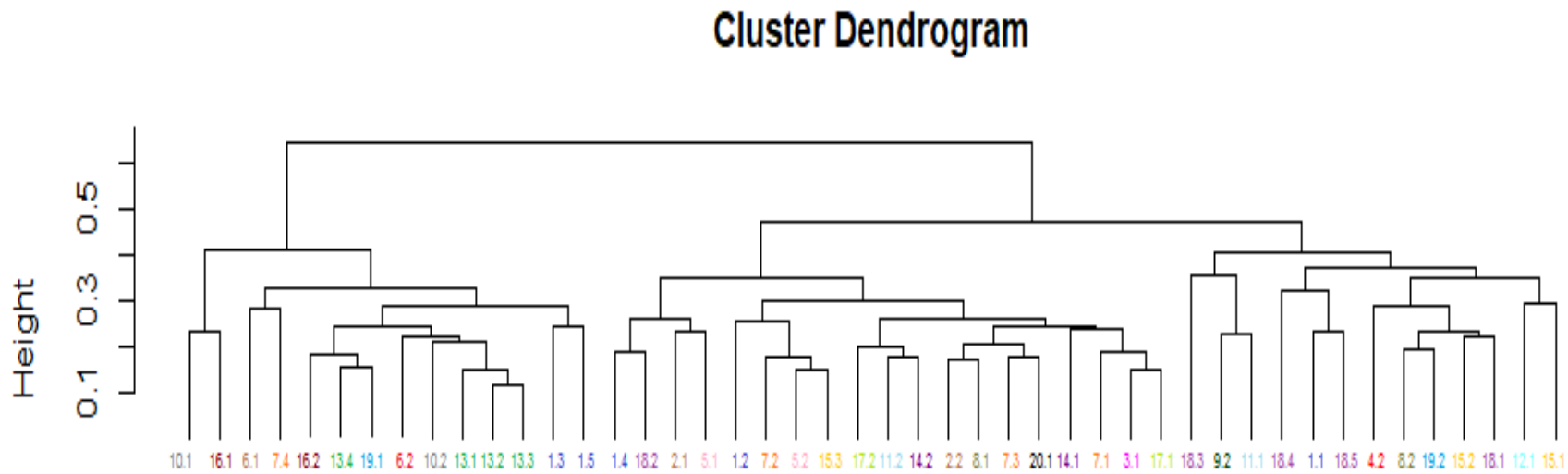
4967
4968
4969 Figure 3.22 Hierachical clustering of the microbiota profiles of Immunoglobulin A populations in dogs with Chronic Enteropathy. Colors represent different dogs.

4970 The number corresponds to the number of the dog and visit (1.1 corresponds to dog1, visit1). Last visit corresponds to the remission period, except for dog 1,

4971 where the last two visits corresponds to this period (The remaining samples correspond to active disease). Height reflects the distance between the samples

4972 (height of the common node.

4973 **3.3.3.3.2 Intestinal Samples**
4974

4975 **3.3.3.3.2.1 Sequencing summary**
4976
4977
4978 The total number of sequences during active disease per type of sample ranged from 11 to 75123 and from

4979 301 to 73199 during the remission period. A summary per group can be seen in Figure 3.23.
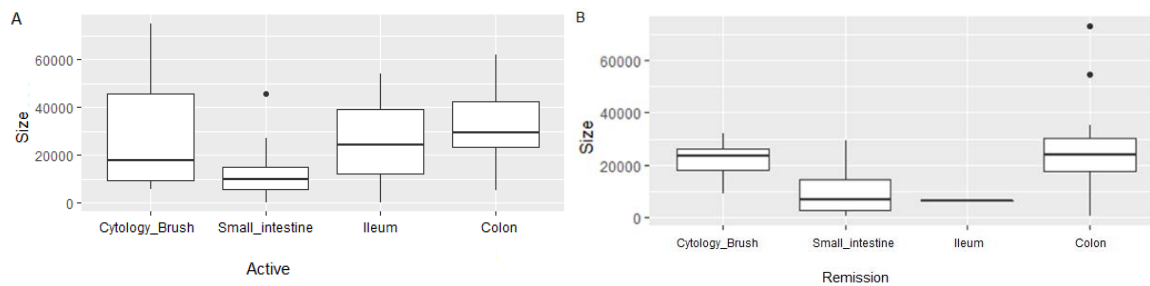
4980

4981

4982

4983

4984

4985

4986   Figure 3.23: Total number of sequences in dogs with chronic enteropathy. A: Active disease and B: Remission
4987   period. Different segments of the intestine were sampled. Cytology brush was collected from the duodenum. Small
4988   intestine (duodenum). Active N= 23 dogs. Cytology_Brush n=7; Small_intestine n=19; Ileum n=3 and Colon n=14.
4989   Remission N= 20 dogs. Cytology_Brush n=7; Small_intestine n=16; Ileum n=1 and Colon n=19.

4990

4991

4992

4993

4994

4995

4996

4997 **3.3.3.3.2.2 Analysis of the relative abundance at different phylogenetic levels**

4998

4999

5000   -   Small Intestine

5001

5002

5003 Samples of small intestine were characterised by a predominance of Proteobacteria at phylum level, with

5004 55% of the population belonging to this group. This was followed by Firmicutes (19%), Bacteroidetes

5005 (16%), Fusobacteria (4%) and Actinobacteria (3%). Other groups that were present comprised GN02,

5006 Tenericutes, SR1, Spirochaetes, TM7, Chloroflexi, Acidobacteria and Cyanobacteria (Figure 3.24).

5007

5008 At genus level, only 46-48% of the bacteria could be assigned to a specific group. The most common

5009 genera during active disease included *Porphyromonas* (4%), *Fusobacterium* (4%), *[Ruminococcus]* (4%),

5010 *Blautia (3%), Bacteroides* (3%), *Moraxella* (3%), *[Prevotella]* (3%), *Pasteurella* (2%), *Streptococcus* (2%),

5011 *Prevotella* (2%), *Ochrobactrum* (2%) and *Helicobacter* (2%).

5012

5013 During the remission period, the most common genera included *Helicobacter* (8%), *Clostridium* (4%),

5014 *Bacteroides* (4%), *Proteous (4%), Fusobacterium* (3%), *Porphyromonas* (3%), *Moraxella* (2,5%),

5015 *Pseudomonas* (2%), *Streptococcus* (2%), *Pausterella* (2%) and *Prevotella* (2%) (Figure 3.24).

5016

5017   -   Cytology Brush

5018

5019 As was expected, samples of cytology brush showed a similar profile to that found in the small intestine.

5020 Proteobacteria predominated at phylum level, with 62% of the population belonging to this group. This was

5021 followed by Firmicutes (18%), Bacteroidetes (10%), Actinobacteria (3%) and Fusobacteria (2%) During

5022 remission, Proteobacteria also predominated, followed by Bacteroidetes (18%), Firmicutes (9%),

5023 Fusobacteria (0,8%) and Tenericutes (0,8%). Other groups that were present comprised GN02,

5024 Tenericutes, SR1, Spirochaetes, TM7, Synergistetes, Chloroflexi, Chlorobi, [Thermi] and Cyanobacteria

5025 (Figure 3.24).

5026

5027 At genus level, 60% of the bacteria could be assigned to a group during active disease. The most common

5028 genera included *Helicobacter* (18%), *Moraxella* (11%), *Clotridium* (6%), *Porphyromonas* (5%), *Actinomyces*

5029   (4%), *Streptococcus* (2%), *Gemella* (2%), *Enterococcus* (2%), *Trichococcus* (2%) and *Fusobacterium*

5030   (1,2%) (Figure 3.18).

5031

5032   At genus level, 48% of the bacteria could be assigned to a group during the remission period. The most

5033   common genera included *Helicobacter* (20%), *Porphyromonas* (9%), *Moraxella* (5%), *Clostridium* (3%),

5034   *[Prevotella]* (2%), *Pasteurella* (1%), *Sphingomonas* (1%), *Prevotella* (0,8%), *Mycoplasma* (0,5%),

5035   *Bacteroides* (0,7%) and *Fusobacterium* (0,6%) (Figure 3.24).

5036

5037   -   Ileum

5038

5039   Samples of ileum were also characterised by a predominance of Proteobacteria at phylum level, with 57%

5040   of the population belonging to this group. This was followed by Fusobacteria (26%), Bacteroidetes (22%),

5041   Firmicutes (5,6%) and Actinobacteria (0,05%) (Figure 3.25).

5042

5043   Interestingly, although only one sample was available for analysis during the remission period, this sample

5044   was characterised by a predominance of Firmicutes at phylum level, with 44% of the population belonging

5045   to this group. This was followed by Fusobacteria (30%), Bacteroidetes (7%), Proteobacteria (5,6%) and

5046   Tenericutes (0,1%) (Figure 3.24).

5047

5048   At genus level, 56% of the bacteria could be assigned to a group during active disease. The most common

5049   genera included *Fusobacterium* (26%), *[Prevotella]* (14%), *Bacteroides* (7%), *Proteus* (3%), *Sutterella* (2%),

5050   *Clostridium* (1%), *Blautia* (1%), *Dorea* (0,7%), *[Ruminococcus]* (0,55%), *Phascolarctobacterium* (0,3%),

5051   *Pseudomonas* (0,25%) and *Prevotella* (0,2%) (Figure 3.24)

5052

5053   The remission period was characterised by the predominance of *Fusobacterium* (30%) followed by *Dorea*

5054   (11%), *Blautia* (9%), *Streptococcus* (4%), *Clotridium* (3%), *[Ruminococcus]* (3%), *Sutterella* (3%),

5055   *Actinomyces* (2%) and *Gemella* (1,2%) (Figure3.24). 70% of the bacteria could be assigned to a group.

5056

5057   -   Colon

5058

5059   Samples of colon were characterised by a predominance of Bacteroidetes at phylum level, with 41-43% of

5060   the population belonging to this group. This was followed by Firmicutes (20-25%), Proteobacteria (20%),

5061   Fusobacteria (12-14%) and Actinobacteria (0,005-1,5%) (Figure 3.24).

5062

5063   At genus level, both stages shared similar profiles, 73% of the bacteria could be assigned to a group. The

5064   most common genera included *Bacteroides* (16-19%), *Fusobacterium* (13-14%), *[Prevotella]* (13-18%),

5065   *Sutterella* (4-6%), *Megamonas* (3-4%), *[Ruminococcus]* (3-5%), *Prevotella* (3%), *Dorea* (2-3%), *Clostridium*

5066   (2%), *P Parabacteroides* (2%), *Proteus* (2%) *Blautia* (1%) and *Corynebacterium* (1%) (Figure 3.24).
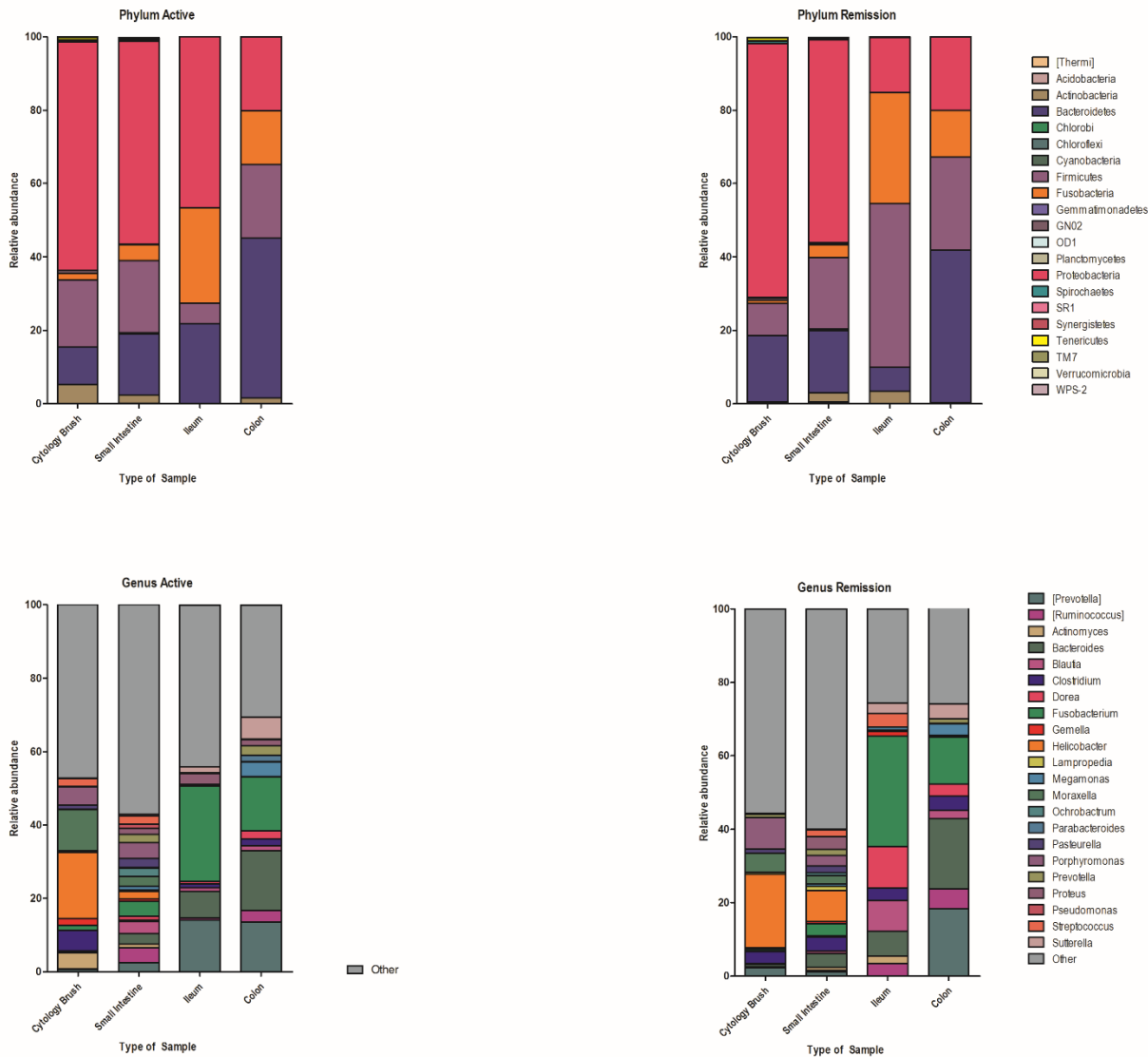
5067

5068

5069

5070

5071

5072

5073

5074 Figure 3.24: Relative abundance of the major phylogenetic levels in dogs with chronic enteropathy, active

5075 disease (right panel), Remission (left panel). Small_intestine (duodenum). Active N = 23 dogs. Cytology_Brush n

5076 =7; Small_intestine_biopsy n=17; Ileum n=2; Colon_biopsy n=14. Remission N= 20 dogs. Cytology_Brush n =7;

5077 Small_intestine_biopsy n=15; Ileum n=1; Colon_biopsy n=18.

5078

5079

5080 **3.3.3.3.2.3 Diversity Analysis**
5081
5082
5083   -   Alpha diversity
5084
5085

5086   When alpha diversity analysis was performed, contrary to expectations, the highest diversity was found in

5087   the cytology brush and small intestine, and the lowest found in the colon. When cytology brush and small

5088   intestine biopsy were compared, there was not a significant difference in alpha diversity. (Figure

5089   3.25).Likewise, there was not a significant difference when the different stages where compared (Figure
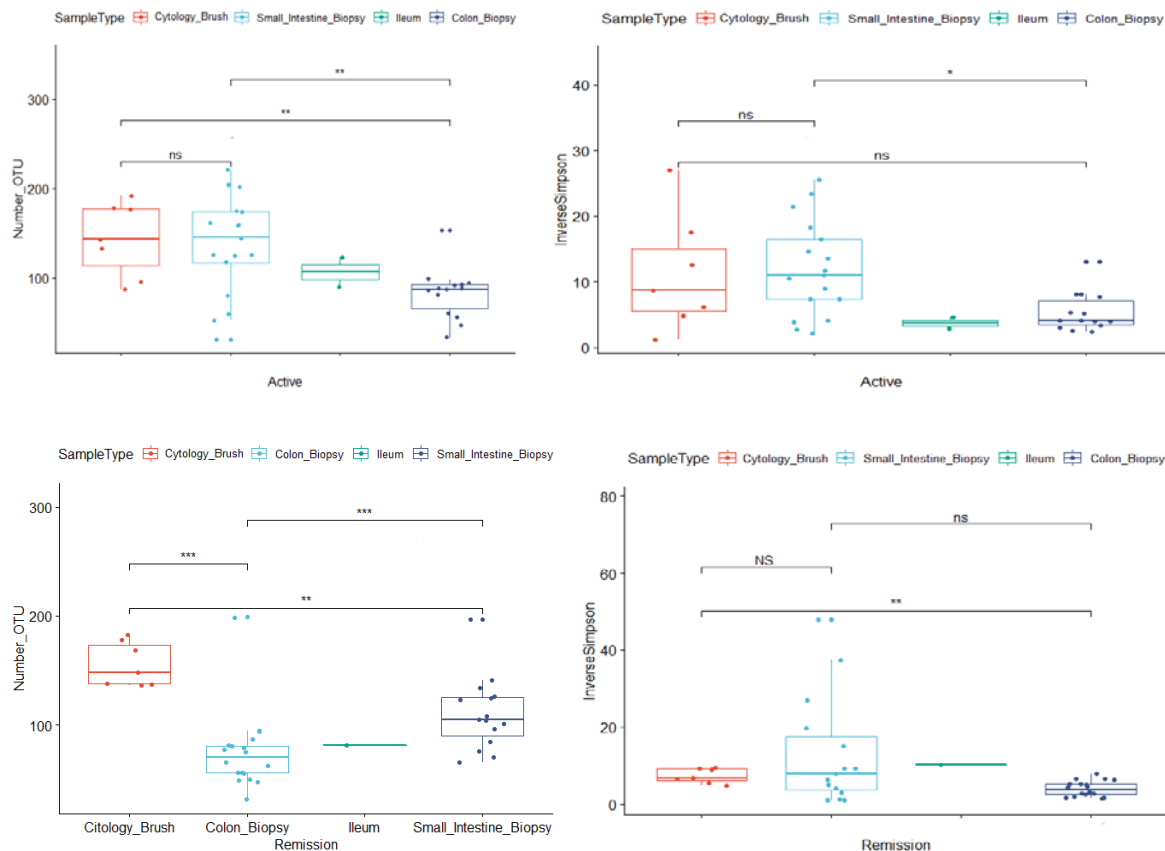
5090   3.26)

5091

5092

5093

5094

Figure 3.25: Alpha diversity analysis in dogs with chronic enteropathy in cytology brush and mucosal samples at different stages. Upper panel: Active disease, Lower Panel: Remission. Right panel: Number of OTUs (Observed), Left panel: Inversed Simpson's Index, Active N = 23 dogs. Cytology_Brush n =7; Small_intestine_biopsy n=17; Ileum n=2; Colon_biopsy n=14. Remission N= 20 dogs. Cytology_Brush n =7; Small_intestine_biopsy n=15; Ileum n=1; Colon_biopsy n=18.
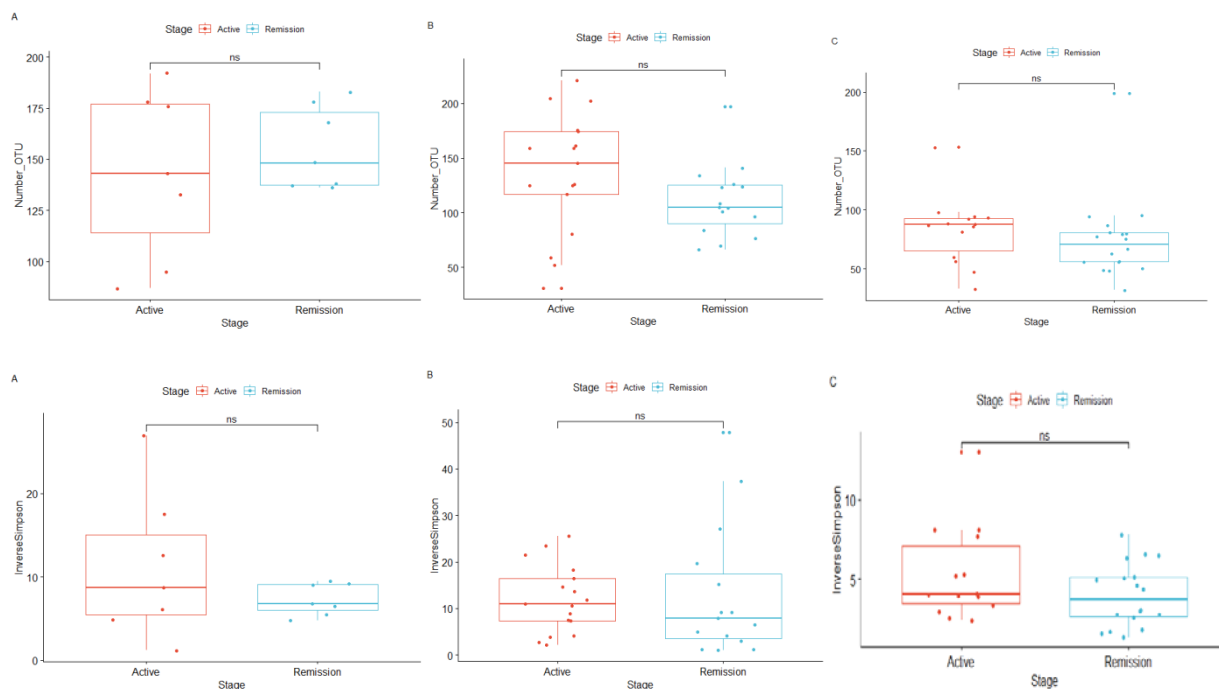
Figure 3.26: Alpha diversity analysis in dogs with chronic enteropathy in cytology brush and mucosal samples at different stages and per type of sample. Active disease versus remission.Upper panel: Number of OTUs (Observed), Lower Panel: Inversed Simpson's Index. A: Cytology Brush (Active n=7, Remission n=7); B: Small instestine biopsy (Active n=17, Remission n=15); C: Colon biopsy (Active n=14, Remission n=18).

5106

5107   -   Beta diversity

5108

5109   When samples were plotted using Unifrac analysis, and compared according to the stage of the disease

5110   and type of sample, samples of cytology brush and small intestine tended to cluster together, while the

5111   ileum and colon clustered together separately from the small intestine.   We did not find any difference

5112   between active or remission stages (Table 3.8, 3.9 and 3.10) (Figure 3.27). As expected, faecal samples

5113   clustered together with ileum and colon biopsies and there was not a difference during disease or remission

5114   (Figure 3.27).

5115

5116

5117

5118

5119

5120

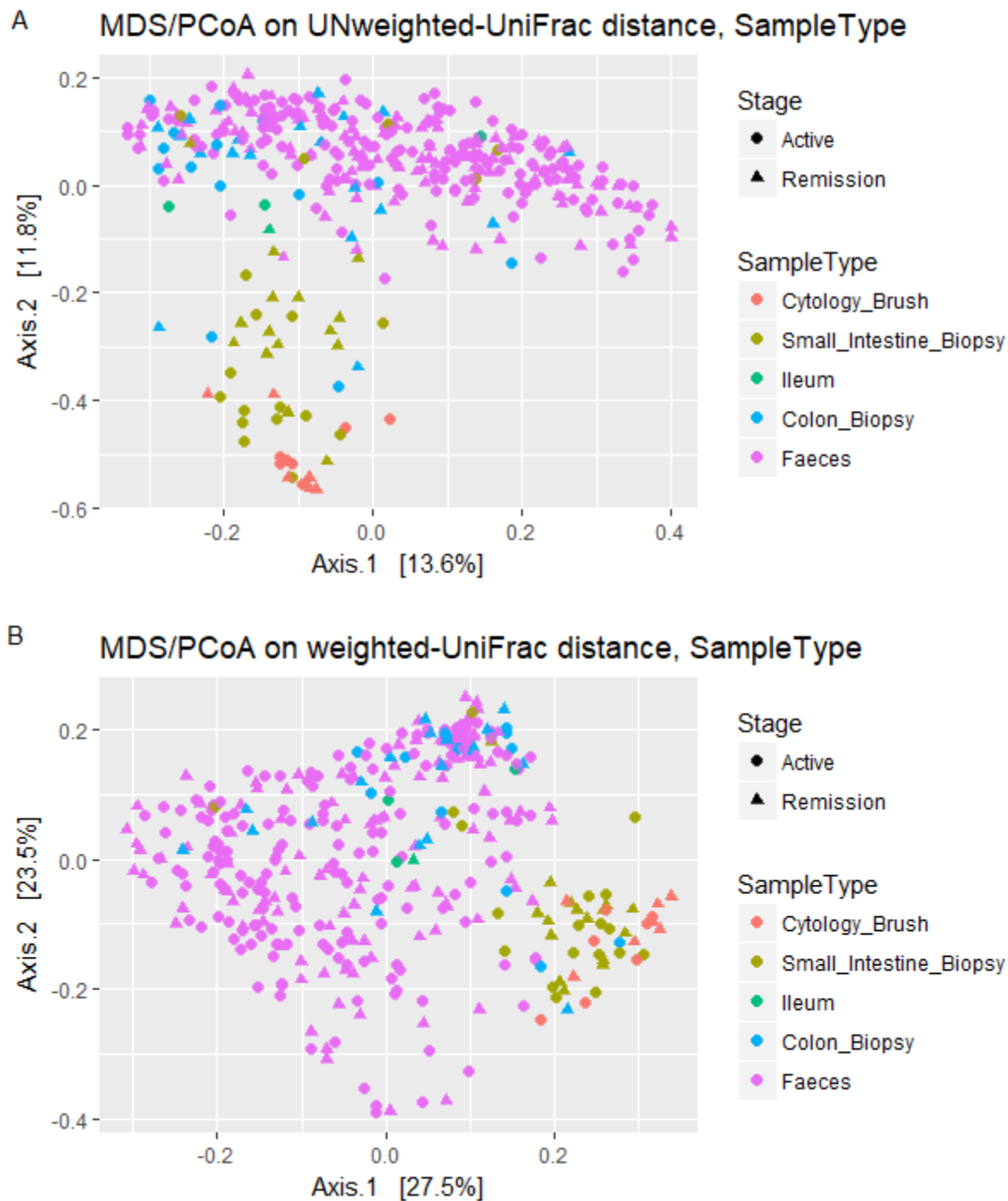5121

5122

Figure 3.27: Beta diversity analysis in dogs with chronic enteropathy, according to disease stage (Active disease versus Remission) and type of sample. A: Unweighted UniFrac and B: Weighted UniFrac analysis. Active N= 23 dogs. Cytology_Brush n=7; Small_intestine n=19; Ileum n=3 and Colon n=14. Remission N= 20 dogs. Cytology_Brush n=7; Small_intestine n=16; Ileum n=1 and Colon n=19.

5127   Table 3.8: Comparison of microbial communities (Beta- diversity) according to sample type and intestinal segment in

5128   dogs with chronic enteropathy, active disease. CEA: Active disease

Group comparison		R2	p-value
Cytology Brush CEA vs.	Small Intestine CEA	0.12073	0.004
	Ileum CEA	0.54141	0.008
	Colon CEA	0.41362	0.001
Small Intestine CEA vs.	Ileum CEA	0.09022	0.035
	Colon CEA	0.14386	0.001
Ileum CEA vs.	Colon CEA	0.06316	0.357

5129

5130

5131

5132

5133

5134   Table 3.9: Comparison of microbial communities (Beta- diversity) according to sample type and intestinal segment in

5135   dogs with chronic enteropathy, active disease. CER: Remission.

Group comparison		R^2	p-value
Cytology Brush CER vs.	Small Intestine CER	0.17389	0.001
	Colon CER	0.37833	0.001
Small Intestine CER vs.	Ileum CER	0.08095	0.161
	Colon CER	0.19926	0.001

5136

5137

5138

5139

5140

5141

5142   Table 3.10: Comparison of microbial communities (Beta- diversity) according to sample type and intestinal segment

5143   in dogs with chronic enteropathy according to stage CEA: Active disease versus CER: remission.

5144

Group comparison		R2	p-value
Cytology Brush CEA vs.	Cytology Brush CER	0.07589	0.456
Small Intestine CEA vs.	Small Intestine CER	0.03202	0.278
Colon CEA vs.	Colon CER	0.02841	0.535

5145

5146   3.3.3.3.2.4 Microbial differential abundance testing
5147
5148
5149   Populations of bacteria in the different segments (cytology brush of small intestine, small intestine and colon) were compared between different

5150   stages of the disease. We only found bacterial goups differentially enriched in cytology brushes. Four family groups were significantly enriched

5151   during active disease: Actinomycetaceae, Lachnospiraceae, Enterococcaceae and Carnobacteriaceae. (Figure 3.29).

5152

| OTUs	base Mean	log2Fold Change	lfcSE	stat	P value	P adj	Phylum	Class	Order	Family	Genus	Species
Otu130	217.1	4.909	1.144	4.289	3.71e-05	0.001	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Actinomyces	NA
Otu445	17.887	7.238	1.712	4.226	2.37e-05	0.001	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
Otu31	121.48	3.986	1.007	3.958	7.51e-05	0.003	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	NA
Otu517	105.08	4.620	1.246	3.707	0.0002	0.007	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Trichococcus	NA

5153

5154

5155

5156

5157
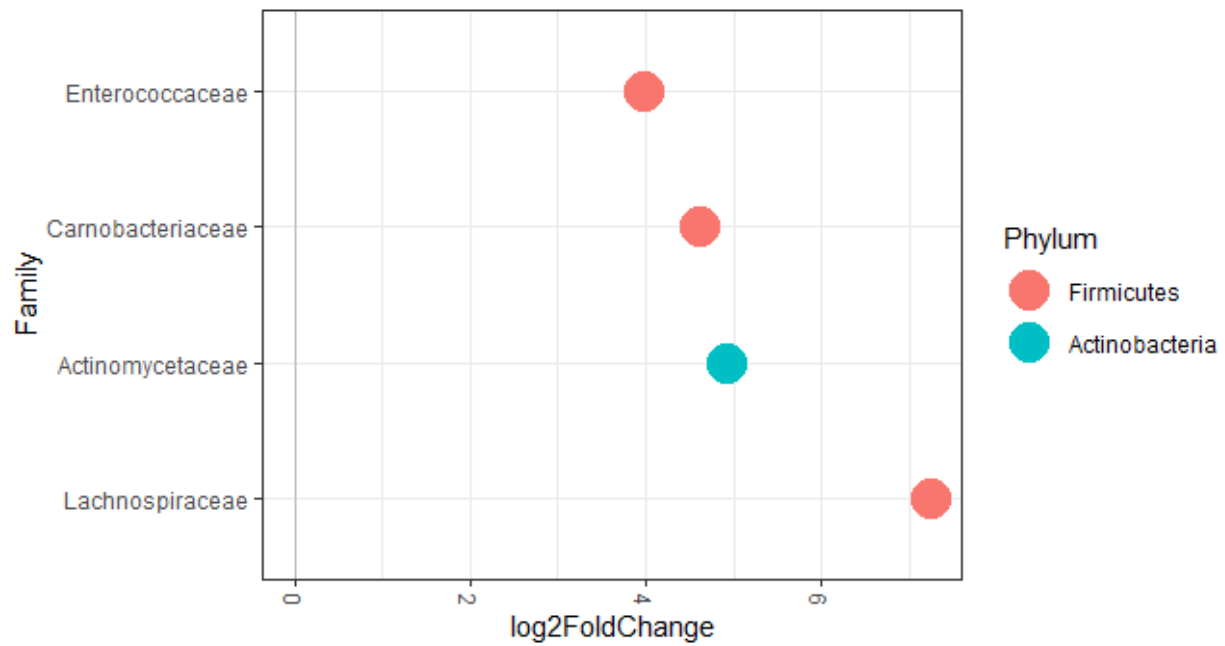
5158 Figure 3.29 Microbial differential Abundance Testing in the cytology brushes. Active disease versus

5159 Remission. DEseq2 method was used.

5160

5161   Interestingly, when dogs were evaluated individually for immunoglobulin coating, there was a clear

5162   distinction between active and remission periods with beta-diversity analysis, especially for IgA. Thus, it

5163   seems that bacteria that are altered during disease are highly variable among individuals (Figure 3.29).
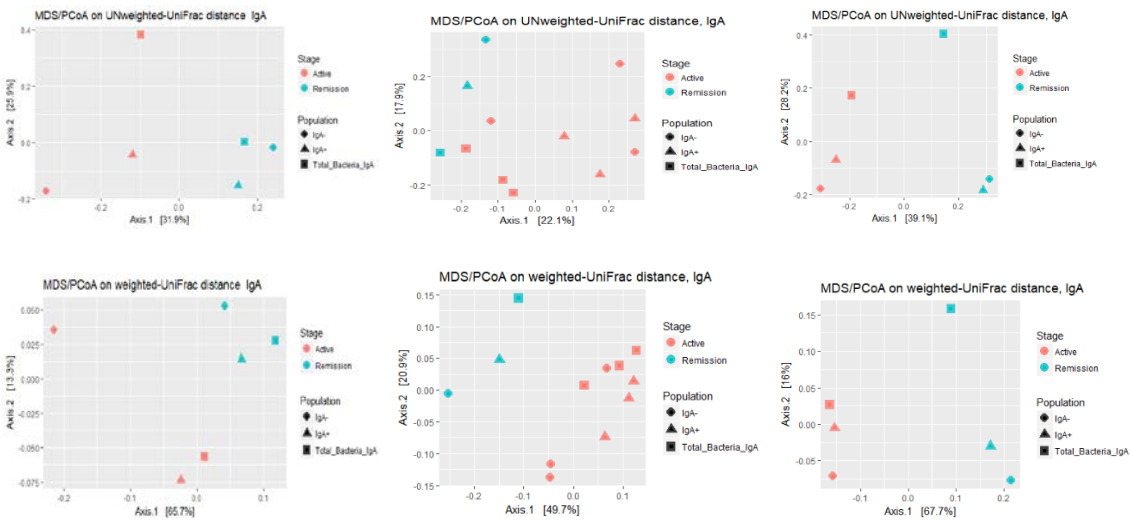
5164

5165

5166

5167

5168

5169

Figure 3.29: Beta diversity analysis of immunoglobulin A according to disease stage: active versus remission and type of sample. Unweighted (upper line) and Weighted UNIFRAC analysis (lower line). Three representative patients (CE dog 6, CE dog 7 and CE dog 19).

5173

5174

5175

5176 **3.3.4 Enrichment of immunoglobulin A and G in Chronic Enteropathies**
5177
5178
5179 Enrichment of bacteria with immunoglobulin A and G was assessed in every patient. This enrichment was
5180 calculated by dividing the fractional abundance of the bacteria at family level in the positive population
5181 between the fractional abundance in the negative population and then log transforming the value. Zero
5182 values means that the group was found in equal proportions in the positive and negative population;
5183 negative values indicate enrichments towards the negative population and positive values indicate
5184 enrichments towards the positive population. We focused our studies in the positive population as they
5185 have been reported to be the members with the strongest immune stimulatory properties. Additionally, we
5186 found a clear separation in the IgA+ population when samples were plotted using UNIFRAC analysis.
5187
5188 The phylogenetic family level was chosen, as many bacteria could not be classified at lower phylogenetic
5189 levels.
5190
5191 Every patient had its own profile, and we could not find a family that was consistently enriched in all
5192 patients. The most common families enriched in the IgA-positive population during active disease
5193 comprised Erysipelotrichaceae (60% of the patients) Lachnospiraceae (80%) and Clostridiaceae (50%).
5194
5195 Some of the bacteria remained enriched but at lower levels during the remission period, others were
5196 enriched towards the negative population and others were not enriched during the remission period (Figure
5197 3.30 A and Figure 3.31 A). Interestingly, When the IgG enrichment profile was assessed, different group of
5198 families were enriched compared to the IgA profile in many cases (Figure 3.30 B and 3.31 B).
5199
5200 When we evaluated patients at different time points during active disease, families enriched differed in
5201 every visit. Although, it was possible to distinguish a group of families, that were enriched only during
5202 active disease (Figure 3.32 and 3.33).
5203
5204 Thus, the bacterial coating profile is characterised by (1) being highly unstable during disease, so different
5205 members can stimulate the immune system over the course of the disease and by (2) being highly
5206 personalised.
5207

5208   Figure 3.30 A Relative enrichment of taxa in the IgA+ fraction. A. Average relative abundance of indicated taxa

5209   during active disease versus remission (Upper panel). B. Relative IgA enrichment, active (red) versus Remission

5210   (green) (Lower panel). Representative patient (CE dog 2).

5211

5212   Figure 3.30 B: Relative enrichment of taxa in the IgG+ fraction. A. Average relative abundance of indicated taxa

5213   during active disease versus remission (Upper panel). B. Relative IgA enrichment, active (red) versus Remission

5214   (green) (Lower panel). Representative patient (CE dog 2).
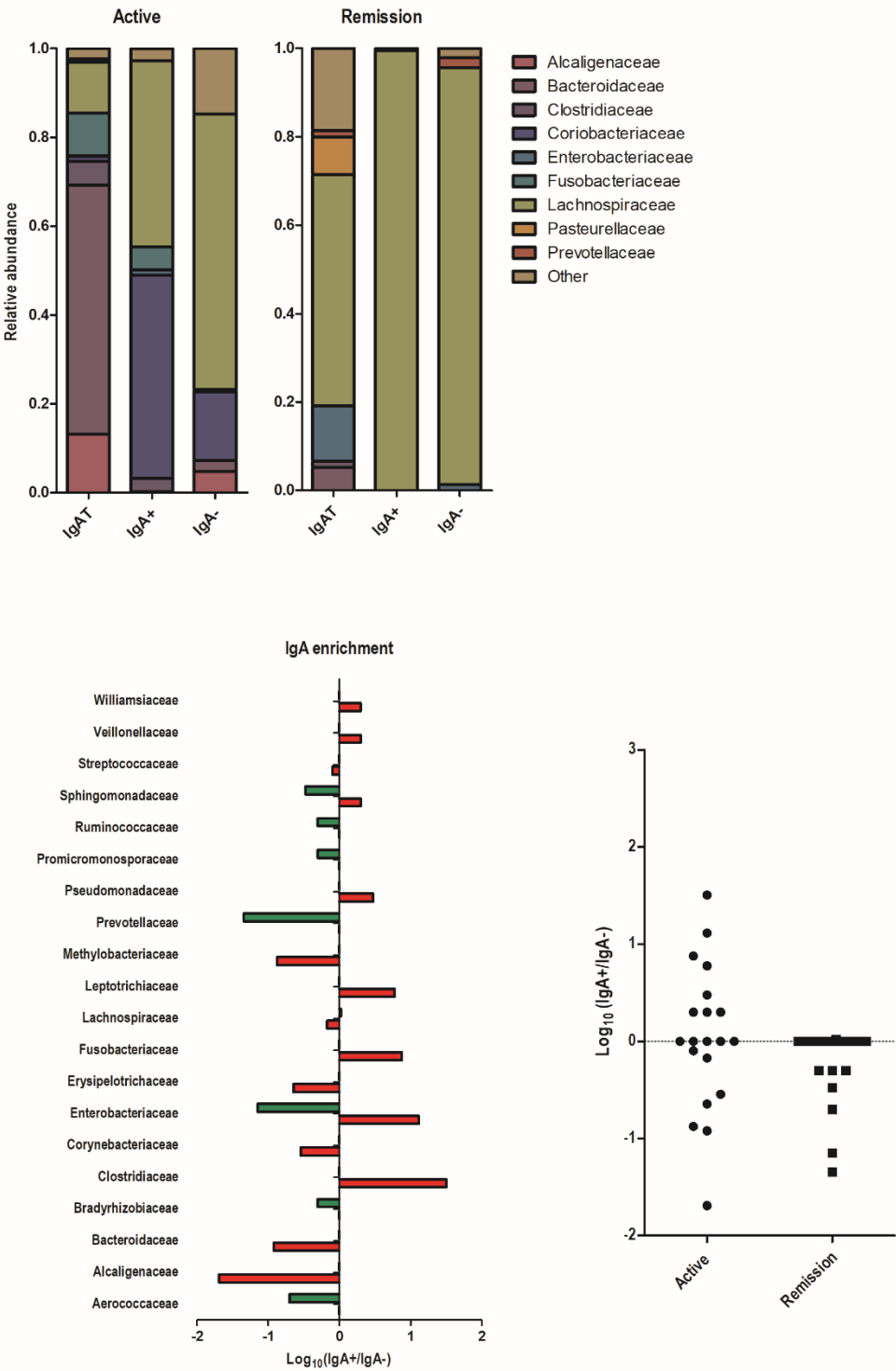
5215
5216

5217
5218

5219   Figure 3.31 A: Relative enrichment of taxa in the IgA+ fraction. A. Average relative abundance of indicated taxa

5220   during active disease versus remission (Upper panel). B. Relative IgA enrichment, active (red) versus Remission

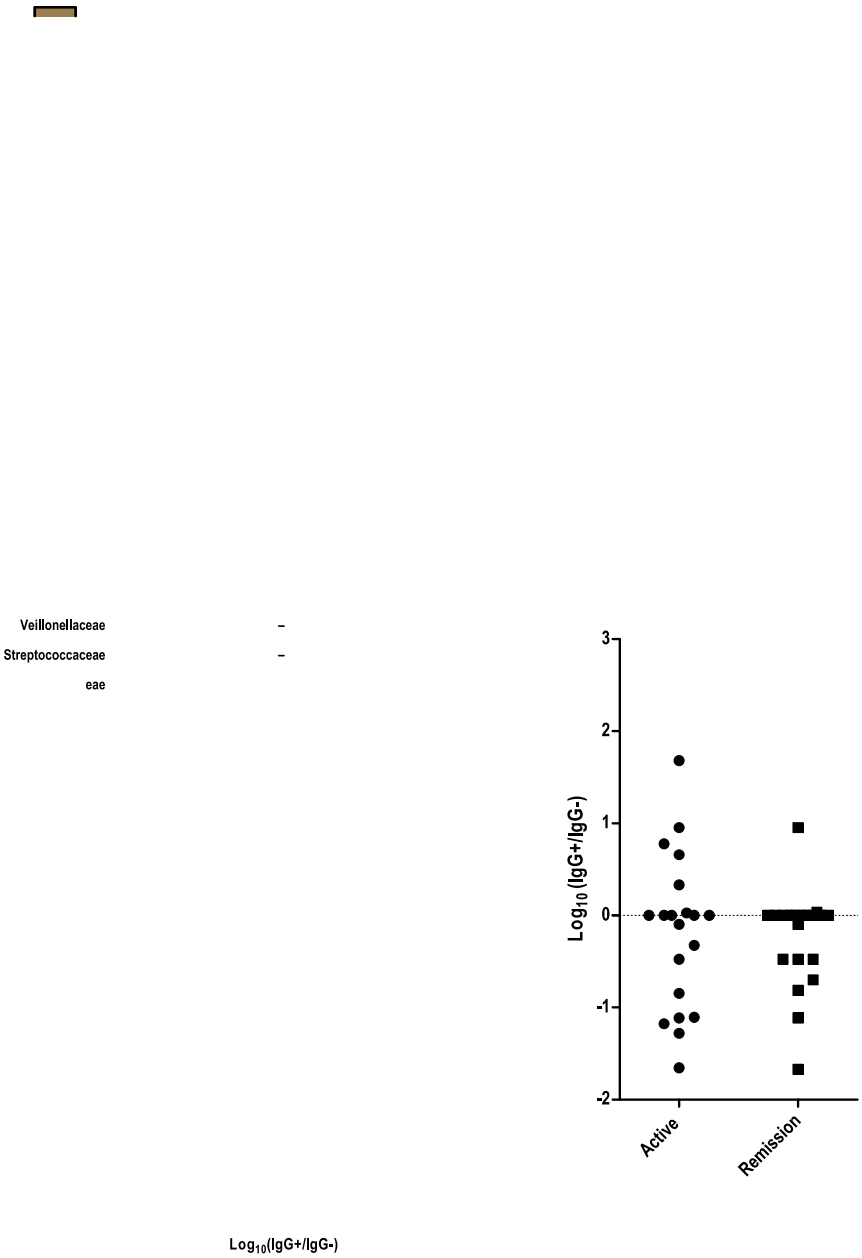5221   (green) (Lower panel). Representative patient (CE dog 17).

5222

5223   Figure 3.31 B: Relative enrichment of taxa in the IgG+ fraction. A. Average relative abundance of indicated taxa

5224   during active disease versus remission (Upper panel). B. Relative IgA enrichment, active (red) versus Remission

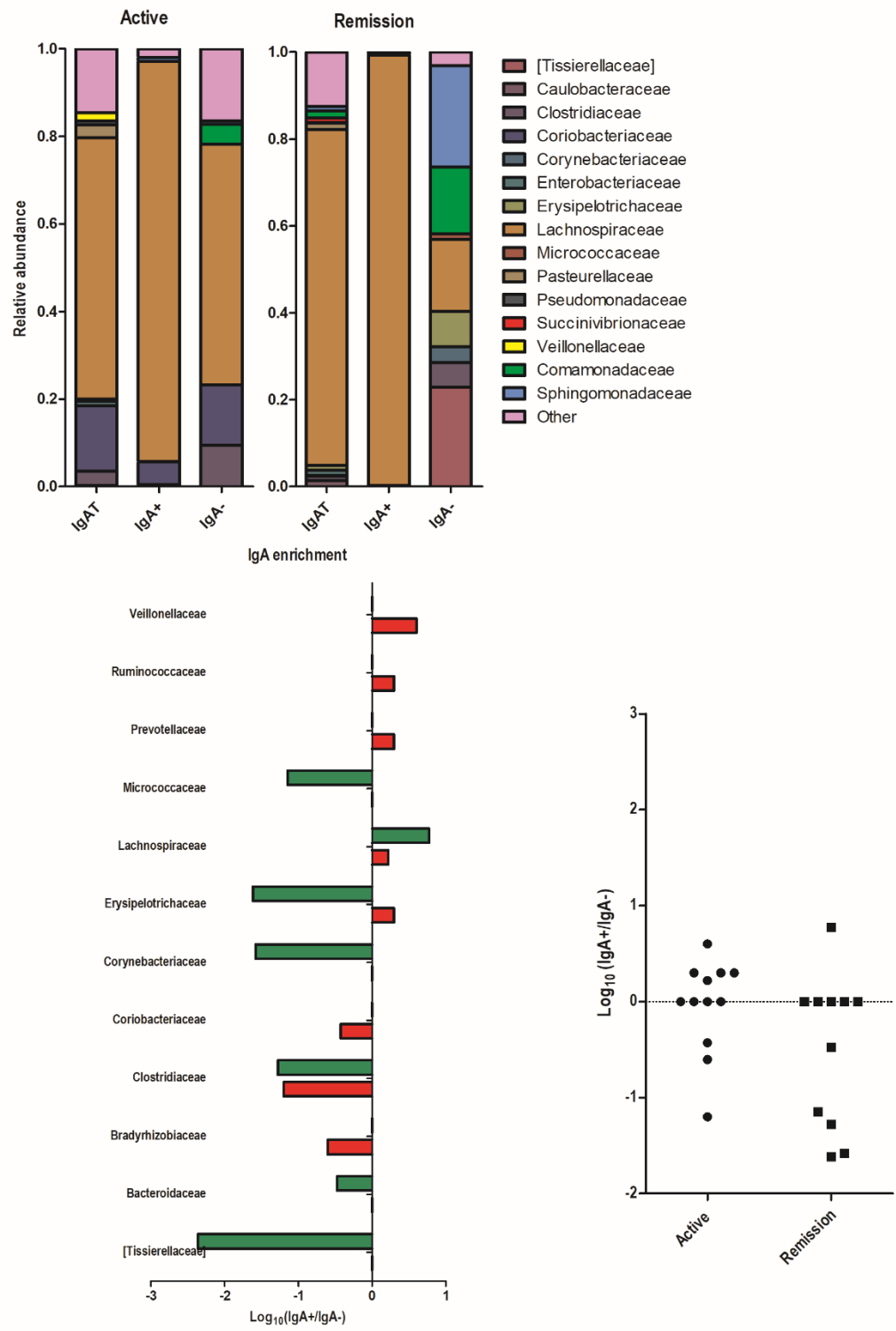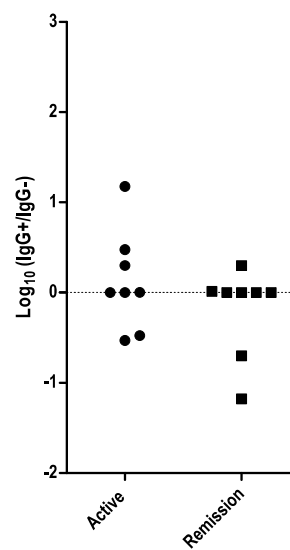5225   (green) (Lower panel). Representative patient (CE dog 17).

5226

5227
5228
5229
5230

5231   Figure 3.32 A. Average relative abundance of indicated taxa over time. Visit 1-4: Active disease, Remision, visit 5.

5232   Representative patient (CE dog 7). Immunoglobulin A.

ospiraceae

5233
5234
5235
5236

5237 Figure 3.32 B Relative enrichment of taxa in the IgA⁺ fraction over time. Visit 1-4: Active disease, visit 5.

5238 Representative patient (CE dog 7). Immunoglobulin A

5239

5240
5241
5242
5243
5244
5245
5246
5247
5248
5249

5250   Figure 3.33 A. Average relative abundance of indicated taxa over time. Visit 1-4: Active disease, visit 5 Remission.

5251   Representative patient (CE dog 7). Immunoglobulin G

5252
5253

er

5254
5255
5256
5257
5258
5259
5260
5261
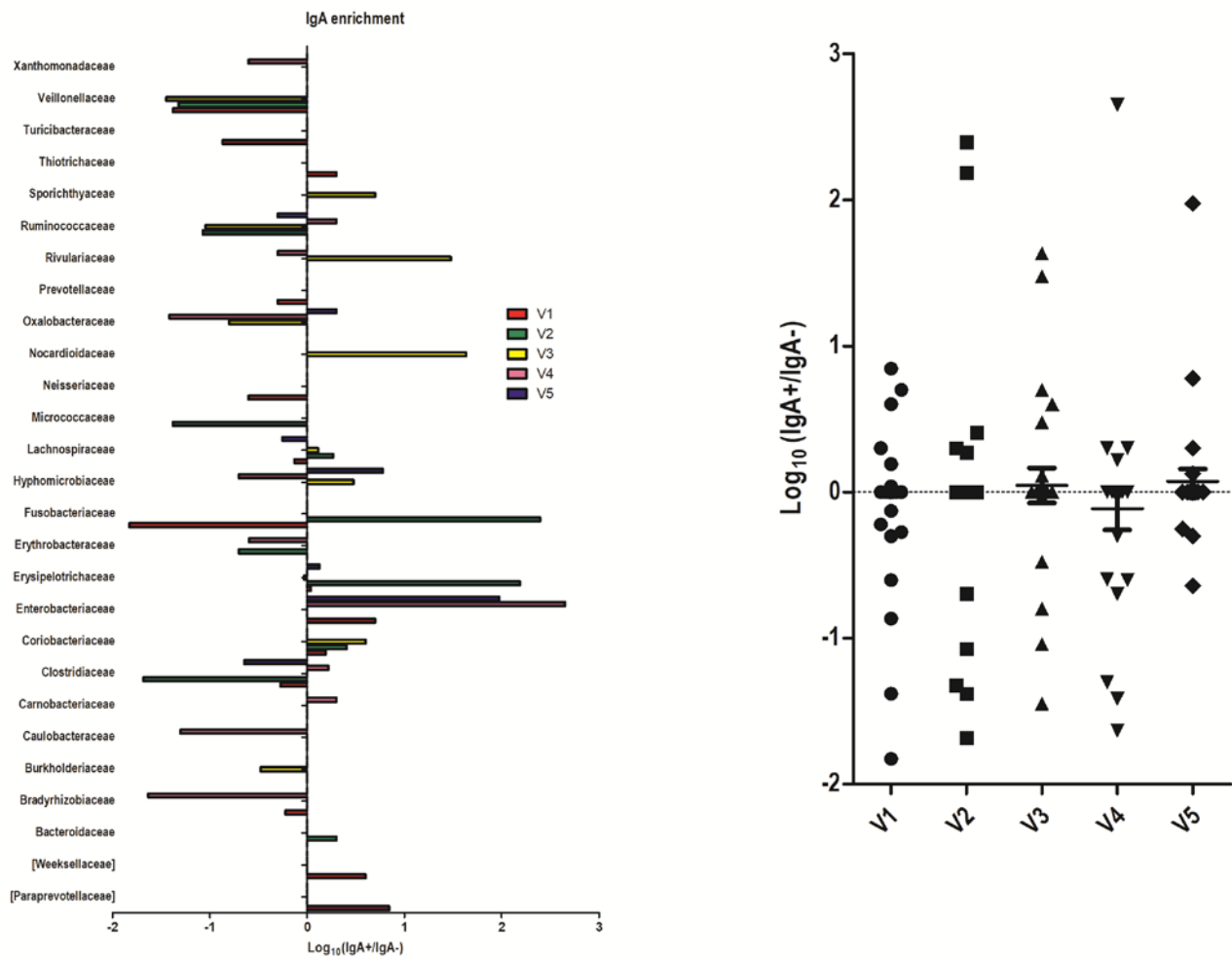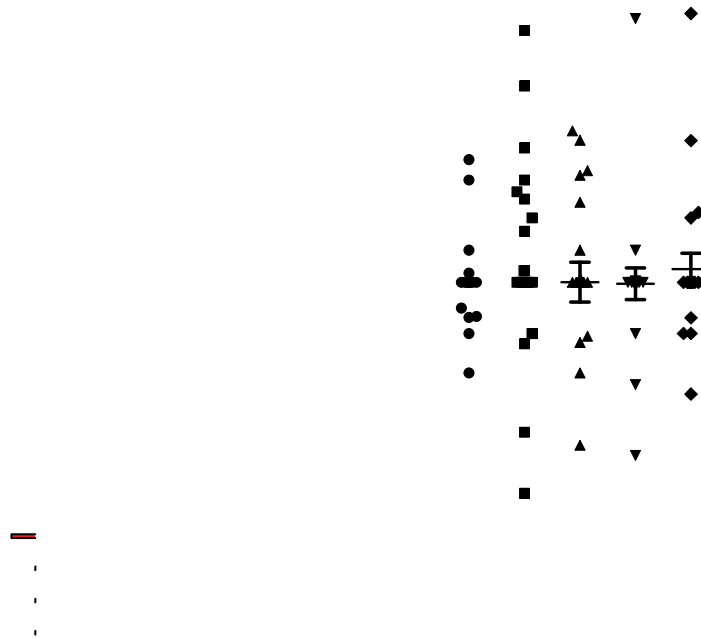
5262
5263
5264   Figure 3.33 B Relative enrichment of taxa in the IgG+ fraction over time. Visit 1-4: Active disease, visit 5 Remission.

5265   Representative patient (CE dog 7). Immunoglobulin G

5266

5267
5268
5269

5270
5271
5272
5273
5274
5275
5276
5277
5278
5279
5280
5281

5282   3.4. Discussion

5283

5284   Immunoglobulins constitute a crucial arm of the immune system that provide protection against pathogens

5285   and help maintain a homeostatic state in the intestine. IgA is the most abundant isotype secreted in the

5286   intestine. However, IgG and IgM are also important, especially during periods of disease or in animals with

5287   IgA-deficiency.

5288

5289   Here, we analysed the percentage of faecal bacteria coated with IgA and IgG in healthy dogs and dogs with

5290   CE during active disease (at the time of diagnosis) and shortly after clinical remission. As has been seen in

5291   people, only a small percentage of bacteria were coated with IgA or IgG in healthy dogs. However, when

5292   dogs with the disease were evaluated, it was found that the percentage of coated bacteria did not change

5293   in active disease compared to healthy dogs and in fact decreased and was significant lower during

5294   remission.

5295

5296   Interestingly, the pattern between IgA and IgG was similar, in both healthy and sick dogs. This is

5297   unexpected, as the main isotype in the gastrointestinal tract is IgA. Whereas, IgG is not actively transported

5298   into mucosal secretions, except during disease. Previous studies in dogs, assessing the amount of IgA, IgM

5299   and IgG in faecal samples of healthy dogs; found that the faecal concentrations of IgA, IgM and IgG were

5300   significantly correlated with each other. It is considered that this could be a reflection of the contribution of

5301   bile to the faecal immunoglobulin measurements as the concentrations of IgA and IgG have previously

5302   been found to be similar in canine bile (German *et al.*, 1998).

5303

5304   People with coeliac disease exhibit a similar profile to what we encountered, with proportions of IgA, IgM

5305   and IgG-coated bacteria significantly lower in affected individuals compared to healthy controls in both

5306   untreated and treated patients (De Palma *et al.*, 2010). This suggests that either low or high levels of Ig-

5307   coated bacteria can be indicative or predispose to disease.

5308

5309   In general, it is expected that during inflammation, levels of immunoglobulins increase in an attempt to

5310   maintain intestinal homeostasis. Conversely, the increase could be an indication of a break in tolerance

5311   with induction or exacerbation of inflammation secondarily, as has been suggested in people with IBD.

5312

5313 On the other hand, impaired production of IgA has been reported in patients with IBD and it is considered to

5314 be a predisposing factor, by promoting the growth of bacteria with inflammatory properties that can invade

5315 the mucosa or stimulate the immune system causing inflammation. In people and in dogs, studies has been

5316 discordant and have reported increase and deficiency of immunoglobulin during intestinal inflammation.

5317

5318 Several studies have identified deficiency for IgA in several breeds, and other studies have also found that

5319 dogs with chronic enteropathy have lower levels of intestinal IgA (German, Hall, *et al.,* 2000) (Maeda *et al.,*

5320 2013). Our results suggest that dogs with chronic enteropathy may be deficient in immunoglobulin

5321 secretion. During disease, dogs with CE respond against mucosal bacteria and reach Ig levels that are

5322 similar to those seen in healthy dogs. When the disease is controlled, levels of immunoglobulins return to

5323 normal levels, but still remain lower when compared to healthy dogs.

5324

5325 However, that reduction may not be persistent or may not be the cause of the initial disease onset. In one

5326 of our dogs we collected a sample five months after resolution of clinical signs and we found that at that

5327 time point, the dog exhibited a similar profile to that seen in healthy dogs. This could be an indication of

5328 subclinical disease, but the dog has remained clinical healthy for a further two years.

5329

5330 In people with long-term remission, the percentages of immunoglobulin-coated bacteria return to control

5331 values. Thus, initial clinical remission of IBD patients occurs before coating of bacteria returns to normal;

5332 which could be an indication of a stage of sub clinically active IBD (van der Waaij *et al.,* 2004). This could

5333 also happen in dogs but we need to analyse more long-term remission samples. If this is true, dogs with CE

5334 could have impairment in the immunoglobulin response rather than a deficiency in immunoglobulins. In

5335 dogs, it has been reported that the gut microbiota and serum metabolome undergo only minor

5336 normalization after 3 weeks (Minamoto et al., 2015) or after 8 weeks of therapy (Rossi et al., 2014), in

5337 dogs showing improvement of the clinical signs.

5338

5339 It would be interesting to include immunoglobulin M (IgM) for further analyses. Studies have shown that IgM

5340 can partially compensate for the lack of IgA. In people, it was recently found that bacteria recognized by

5341 SIgM (secretory IgM) were dually coated by SIgA and showed increased richness and diversity compared

5342 to IgA-only-coated or uncoated bacteria. Thus, SIgM can help IgA to anchor highly diverse commensal

5343 communities to mucus, suggesting that IgM may compensate IgA deficiency (Magri et al., 2017).

5344

5345   Next, we performed 16S rRNA sequencing of Ig-coated and uncoated faecal bacteria in healthy dogs and

5346   in dogs with active CE and shortly after remission. Recently, Palm *et al.*, using flow cytometry and 16S

5347   ribosomal ribonucleic acid (rRNA) sequencing, showing that high IgA coating selectively marks specific

5348   members of mice and human intestinal microbiota that can drive or exacerbate intestinal inflammation in a

5349   mouse model (Palm *et al.*, 2014).

5350

5351   The phyla Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes and Fusobacteria

5352   constitute almost 99% of the gut microbiota in dogs. The remaining 1% is represented by the

5353   phyla Tenericutes, Verrucomicrobia, TM7, Cyanobacteria, Chloroflexi, and a few unclassified bacterial

5354   lineages. The relative proportions of these groups vary along the GI tract. We analysed the immunoglobulin

5355   coating of bacteria in faecal samples from healthy dogs.

5356

5357   The same phylogenetics groups that have been reported in previous studies were found in healthy dogs. In

5358   a previous study, samples from these dogs were sequenced using faeces and DNA extraction. However,

5359   when we compared both studies, we found that the proportion of each phylogenetic group differed. At

5360   phylum level, faecal material was dominated by Bacteroidetes (56% versus 20% in faecal suspensions),

5361   whereas Firmicutes was the predominant group in faecal suspensions (62% versus 32%). This variability

5362   may be due to differences in laboratory methodologies such as sample handling and DNA extraction. It is

5363   well known that this can have a huge effect on the bioinformatics results (Boers, Jansen, & Hays, 2016). In

5364   particular, differences in cell lysis treatments for DNA extraction can decrease the recovery of intact DNA

5365   from Gram-negative bacteria associated with harsher lysis conditions (Bacteroidetes gram-negative) or of

5366   gram-positive bacteria associated with insufficient cell lysis (Firmicutes gram-positive) (Yuan, Cohen,

5367   Ravel, Abdo, & Forney, 2012).

5368

5369   When IgA and IgG profiles were compared, diversity differed between coated and uncoated bacteria, but

5370   not between positive or negative populations. Interestingly, some groups that were found in the sorted

5371   population were not found in the pre-sorted population (total bacteria). PCR bias towards the populations

5372   that are more frequently found in the sample. Thus, low frequency but important taxa could be missed and

5373   only found when they were sampled in higher proportion due to coating with immunoglobulins (D'Auria *et*

5374   *al.,* 2013). When we selected for IgA and IgG populations, we potentially overcame this bias because

5375   some groups of bacteria that only comprised a small amount of the total sequence were highly bound by

5376   IgA. We believe that using this method, allows us to identify physiologically important bacteria that may

5377   otherwise be missed by standard sequencing.

5378

5379   Although, the phylum Firmicutes predominated in all the samples evaluated; the phylum Bacteroidetes was

5380   present in a higher proportion in negative populations (17% versus 9%). At lower phylogenetic levels,

5381   divergence between negative and positive populations was more evident. At genus level [Ruminococcus]

5382   reached a proportion of 28% in positive samples, whereas in negative populations, only 2%. At genus

5383   level, the Ig-negative population was dominated by *Fusobacterium* and *Blautia*. Other genera groups that

5384   differed between positive and negative populations were [Prevotella] (6% vs. 0,7%). Fusobacterium (10%

5385   vs. 0,7%), Clostridium (1% versus 6%), Enterococcus (0,001% versus 2%) and Catenibacterium (2% vs.

5386   5%). A recent study has found that SIgA targets preferentially Firmicutes, Actinobacteria and

5387   Proteobacteria. Whereas Bacteroidetes, are largely underrepresented compared to total microbiota

5388   composition (Fadlallah et al., 2018). Another study found that SIgA coating has a preference for the

5389   Proteobacteria phylum, whereas most members of the Fimicutes and Bacteroidetes remain uncoated

5390   (Bunker et al., 2017).

5391

5392   Next, we evaluated the immunoglobulin coating of bacteria in dogs with chronic enteropathy. We

5393   hypothesized that there is a difference in diversity between active and remission periods and between

5394   healthy and sick dogs. However, we did not find a clear difference between stages of the disease, between

5395   health and disease or between the different types of CE. However, our observations were limited to faeces,

5396   and maybe a more severe dysbiosis could be present in the small intestine.

5397

5398   As it was seen in healthy dogs, populations diverged between positive and negative samples but not

5399   between the types of immunoglobulin present. This could suggest that maybe immunoglobulin coating

5400   doesn't play a significant role in the pathogenesis of chronic enteropathies in our population of dogs.

5401   However, when we evaluated each dog individually, immunoglobulin positive populations during active

5402   disease and remission clustered separately in PCoA plots using UNIFRAC analysis. There is a large

5403   amount interpersonal variation in the resident microbiota, so it is likely that what causes disease in one

5404   individual is different from what causes disease in another (Stephens & Round, 2014).

5405

5406   Studies in people have found that serum antibodies levels exhibit a considerable heterogeneity in microbial

5407   specificities among IBD patients; suggesting that rather a global loss of tolerance against intestinal

5408   bacteria, the response is individual and pathogen- specific (Landers *et al.,* 2002). This high variability

5409   together with the small number of dogs in our study could hinder evaluation of differences between healthy

5410   and sick dogs and between different stages of the disease. In these cases, the dog itself would constitute

5411   the best control and comparisons within the same dog would be more appropriate.

5412

5413   When we evaluated immunoglobulin enrichment individually, we found that every individual dog has a

5414   specific profile. Also, it was possible to find specific bacteria that were enriched during active disease

5415   compared to remission in each case. However, none of the families of bacteria was found to be enriched in

5416   all dogs. When we analysed microbial differential abundance in the IgA+ population between active and

5417   remission disease, only the genus *Suterella* (species could not be identified) was significantly enriched

5418   during active disease. Although *Sutterella spp.* have been suspected to play a part in the pathogenesis of

5419   inflammatory bowel disease (Mangin et al., 2004) (Gophna, Sommerfeld, Gophna, Doolittle, & Veldhuyzen

5420   van Zanten, 2006) (Lavelle et al., 2015. In other studies no difference in the prevalence of Sutterella spp.

5421   has been found between the IBD patients and the healthy subjects {Mukhopadhya, 2012 #515) (Hansen et

5422   al., 2013).

5423

5424   Increase in the amount of Proteobacteria may contribute to non-specific mucosal inflammation due to LPS

5425   as a potent stimulator and possibly predispose the host to a chronic inflammatory disease (Round &

5426   Mazmanian, 2009). *Suterella spp.* are considered, mildly pro-inflammatory, and some studies have

5427   indicated that they are unlikely to play a significant role in the development of gastrointestinal inflammation.

5428   Instead, members of this genus may help keep immune system responses at an appropriate level

5429   (Hiippala, Kainulainen, Kalliomaki, Arkkila, & Satokari, 2016).

5430

5431   *Sutterella* is a genus of bacteria that is normally found in the gastrointestinal tract of dogs. And although,

5432   increases in some members of the Proteobacteria phylum have also been associated with intestinal

5433   inflammation in dogs (Minamoto et al., 2015) (J. S. Suchodolski et al., 2012), *Suterella spp.* in particular,

5434   has been associated with non IBD-patients in a correlation network used to determine the dysbiosis index

5435   in dogs (Vazquez-Baeza et al., 2016).

5436

5437   Comparison between healthy and sick dogs, found an enrichment of the Clostridiaceae and

5438   Lachnospiraceae. Clostridiaceae is a highly diverse family, encompassing genera that are important in

5439   nutrient digestibility and immunomodulation; and those that are considered to be pathogenic (Rajilic-

5440   Stojanovic & de Vos, 2014). Studies in dogs have found that Clostridia is increased in dogs with

5441   haemorrhagic diarrhoea (J. S. Suchodolski et al., 2012) and acute diarrhoea (Guard et al., 2015),whereas

5442   in non-haemorrhagic diarrhoea,levels were found to be similar to those found in healthy dogs (J. S.

5443   Suchodolski et al., 2012). We could not identify the kind of species enriched in this case.

5444

5445   Lachnospiraceae is a family of bacteria normally found in the gut of healthy dogs (Jan S. Suchodolski,

5446   2013). One of its primary functions is to produce SCFAs that have important anti-inflammatory properties.

5447   Loss of this commensal microbiota (but not increase) has been linked to metabolic changes, for example

5448   alterations in immunomodulatory bacterial metabolites, such as short chain fatty acids and secondary bile

5449   acids in intestinal diseases (J. S. Suchodolski et al., 2012).

5450

5451   Several hypotheses could explain these findings. Enrichment with IgA can enhance or diminish bacterial

5452   fitness. It has been shown that enrichement can protect bacteria from destruction, increase their survival

5453   and promote symbiosis and intestinal homeostasis (Donaldson et al., 2018) (Fadlallah et al., 2018). Thus,

5454   this enrichment in groups associated with anti-inflammatory properties, could be a compensatory

5455   mechanism. However, it has been reported that this enrichment, is also associated with the facilitation of

5456   destruction and exclusion of bacteria (Cerutti & Rescigno, 2008) (Fadlallah et al., 2018). In this case, it

5457   could be destruction of potentially pathobionts (at least for the Clostridia group); or it could be a reflection of

5458   loss of tolerance towards commensals (Lachnospiraceae and Suterella), with subsequent depletion of

5459   beneficial bacteria. Or, alternatively, dogs with the disease could harbour bacteria with more immunogenic

5460   potential.

5461

5462   It is also important to note that there is an overlap in the dysbiosis patterns of many GI diseases. Many

5463   bacterial associations are not specific to individual diseases but rather respond to multiple disease states.

5464   Thus, the interpretation should be done carefully, as the alteration of these microbes may be indicative of a

5465   shared response to disease, rather than part of disease-specific differences (Duvallet, Gibbons, Gurry,

5466   Irizarry, & Alm, 2017).

5467

5468   Interestingly, when we evaluated sick dogs over time, we could see how unstable the gut microbiota is

5469   during disease. The enrichment profile changed visit after visit and thus, it would be more appropriate to

5470   collect samples at different time points, as transient enrichment of a group of bacteria, not necessarily

5471   would mean that particular group of bacteria could be responsible for the disease. Alternatively, they could

5472   be a reflection of the clinical status of the dog (e.g. worsening of the clinical signs or sub-clinical stage). The

5473   reduction of species diversity in people with UC is associated with temporal instability of the dominant taxa.

5474   Additionally, serial collection of faecal samples in remission and with stable medication during a year of

5475   follow-up, showed that only one-third of the dominant taxa was persistently detected over time. In contrast,

5476   studies in healthy individuals have showed a remarkable stability (intra-individual similarities indices ~80%)

5477   (Manichanh *et al.*, 2012) (Faith et al., 2013) and that for most species there was a single, persistently

5478   dominant strain, termed "single-strain stability" (Truong, Tett, Pasolli, Huttenhower, & Segata, 2017).

5479   Instability and dysbiosis have been correlated with a variety of immunological and metabolic diseases.

5480   Recent studies have shown that immunoglobulins, in particular IgA, are thought to influence microbiome

5481   stability, independently of diet. In fact, IgA deficiency in mice increases interindividual variability in the

5482   microbiome, alters microbiome composition, increases susceptibility to microbial translocation, reduces

5483   microbial fitness and decreases diversity (not reflected in faeces but in the small intestine) (Fagarasan et

5484   al., 2002). Thus, one possibility in dogs with CE is that low levels or impaired secretion of IgA, could have

5485   an impact on the disease process.

5486

5487   Treatment of CE in dogs consists in the administration of antibiotics in cases where the initial dietary

5488   approach does not work. It is well known that antibiotics can have a profound effect in the gut microbiota.

5489   Antibiotics have been proven to be efficacious in inducing and maintaining IBD remission in people so it

5490   can be suggested that modifying the pattern of intestinal bacteria may change intestinal disease status.

5491   Some antibiotics provide a so-called 'eubiotic' effect, by increasing abundance of beneficial bacteria

5492   (Gevers et al., 2017). However, it has been shown that they can enhance the dysbiosis or lead to the to the

5493   false impression on follow-up samples that the dysbiosis is persistent due to GI disease, whereas the

5494   changes may be attributable to antimicrobial treatment (J. S. Suchodolski, 2016).

5495

5496   Antibiotic therapy causes changes in the gut microbiota that are asymmetric, as some bacteria are more

5497   susceptible than others and fitness varies among microorganisms. Some individuals return to pre-treatment

5498   states, whereas other individuals have an alteration in the composition of the microbiota to alternative

5499   stable states. The magnitude of the disturbance, speed and extent of recovery depend on drug related

5500   factors such as class, pharmacokinetics, pharmacodynamics and range of action, as well as their dosage,

5501   duration and administration route; and on host-related factors such as the initial composition of the

5502   microbiota, age and lifestyle (Dethlefsen, Huse, Sogin, & Relman, 2008).

5503

5504   Some studies have analysed the effect of antibiotic on the gut microbiota in healthy dogs (J. S. Suchodolski

5505   et al., 2009) (Igarashi et al., 2014). However, it is not known what effects antibiotics have during periods of

5506   intestinal disease, where the bacterial composition and the microenvironment may be profoundly altered.

5507   Because of the nature of an ecosystem, the changes that are induced by an antibiotic on a set of

5508   organisms will affect directly or indirectly others. So, the initial microbial structure will shape the changes in

5509   microbiota during the antibiotic course

5510

5511   In our study, we used oxytetracycline. Studies using members of the tetracycline family in mice models of

5512   colitis, showed that tetracyclines not only affect the gut microbiota composition, but also have

5513   immunomodulatory properties that can secondarily change the gut microbiota. It has been observed a

5514   pronounced decrease in the proportion of reads of Actinobacteria was observed in antibiotic-treated colitic

5515   groups (J. Garrido-Mesa et al., 2018). In ARD, we observed a significant decrease in the proportions of

5516   members of the phylum Firmicutes (especially members of the families Lachnospiraceae,

5517   Ruminococcaceae and Clostridiaceae). Members of these families are attributed to have important anti-

5518   inflammatory properties. This suggest that although oxytetracyline cause relieve of clinical symptons,

5519   changes in the gut microbiota could lead to exacerbation of the dysbiosis, Long- term studies are needed

5520   to assess the magnitude of the change and future effects.

5521

5522   A big limitation of this study was the presence of bacterial DNA and contamination of the pre- sorting water.

5523   Although we took several measures to minimize this, it was not possible to prevent it. We excluded these

5524   groups from the analysis of our samples. However, some of these bacteria could be truly present in the

5525   sample and not due to contamination. Also, we focused our study only in the positive population. There is a

5526   possibility that the negative population could also exert a role in the pathogenesis of the disease.

5527

5528   Other technical limitations included staining techniques. Bacterial DNA content was detected using Syto-17.

5529   Although useful, other members of the SYTO family are reported to be better for bacterial staining such as

5530   SYTO 9. We could not use this dye, as its signal has the same Excitation/Emission spectrum than the one

5531   found with FITC. FITC was used for labelling the antibodies against immunoglobulins. In future studies,

5532   antibodies could be labelled with other fluorescent dye that has a different excitation/Emission spectrum

5533   than SYTO 9. Additionally, the background noise of FITC may in some cases be high, making the

5534   distinction between positive and negative populations more difficult. Another approach for improving the

5535   distinction between Ig+ and Ig- population could be the incorporation of Magnetic-activated cell sorting

5536   (MACS) before the bacteria sorting, to increase the purity of the positive population (A.Gonzalez, personal

5537   communication. August 2017).

5538

5539   We also evaluated the diversity of the gut microbiota in intestinal mucosal samples. It is considered that

5540   mucosal samples are superior to faecal samples in assessing the true intestinal microbiota (Cassmann *et*

5541   *al.,* 2016). Knowledge of host-microbiota interactions, in particular the role of attaching and invading

5542   bacteria, is important since an abnormal mucosal microbiota may interact more closely with the innate

5543   immune system to modulate gut health and disease (Allenspach et al., 2010).

5544

5545   We did not find a significant difference between pre- and post- treatment samples, or in the global bacterial

5546   composition. Previous studies in dogs have also found no difference at global level, and only differences in

5547   specific groups of bacteria (Cassmann *et al.,* 2016). Additionally, we sampled the duodenum using a

5548   cytology brush. Cytology brush has the advantage that it samples both the luminal and mucosa microbiota.

5549   As it was expected, the diversity profile between the small intestine and the cytology brush was very

5550   similar. Thus, cytology brush could constitute a good alternative for assessing the mucosal microbiota. We

5551   found some bacterial groups that were enriched in the cytology brushes during active disease compared to

5552   remission (Actinobacteria, Clostridia and Bacilli).

5553

5554   Previous studies have also reported the increase of some of these groups in mucosal samples of dogs with

5555   CE (not duodenum). In German shepherd dogs with chronic intestinal inflammation, mucosal-adherent

5556   microbiota were analysed in small intestinal brush samples and showed significant over-representation of

5557   Bacilli and Erysipelotrichi when compared to healthy Greyhounds (Allenspach et al., 2010). Also, in situ

5558   hybridization analysis (FISH) in ileal and colon mucosal biopsies from dogs with CE and healthy dogs,have

5559   found that dogs with CE harboured more ($P < 0.05$) mucosal bacteria belonging to the Clostridium-

5560   coccoides/Eubacterium rectale group, Bacteroides, Enterobacteriaceae, and Escherichia coli compared to

5561   healthy controls (Cassmann et al., 2016). In people, increase in the proportion of Actinobacteria in mucosal

5562   samples has been associated with IBD (Frank et al., 2007) (Gophna et al., 2006).

5563

5564   Thes significance of these changes and the role of these groups of bacteria are still unknown. These

5565   changes could be secondary to the inflammatory environment of the intestine or they could contribute to the

5566   inflammatory response as they could interact more closely with the mucosal immune system.

5567

5568   In our study, samples differed according to location but not stage. It has been shown that the microbiota

5569   diversity increases along the gastrointestinal tract, reaching its maximum complexity in the colon. However,

5570   we found the lowest alpha diversity in colon samples. A previous study found that the ileal and colonic

5571   mucosal is predominantly colonised by bacteria localised to the free and adherent mucus compartments

5572   (Cassmann *et al.*, 2016). As part of the protocol, all dogs receive routine colonic cleansing (enemas) prior

5573   to collection of ileal and colonic mucosal biopsies. It is possible that dogs cleansed by colonic electrolyte

5574   lavage and enemas, might have had disrupted mucus compartments, characterised by reduced bacterial

5575   populations. Thus, we have concluded that enemas performed before the endoscopy may have had an

5576   effect on the microbiota. However, studies in people with IBD did not observe any difference between

5577   patients prepared by oral electrolyte lavage or enema versus patients that did not receive colonic cleansing

5578   (Swidsinski *et al.*, 2007).

5579

5580   Some limitations of this study were the small number of samples available and the absence of a group of

5581   healthy control dogs. However, performing repeated gastrointestinal endoscopies under general

5582   anaesthesia in healthy dogs was not endorsed by our ethics committee.

5583

5584   Recently, a study has given some new insights into the dominant site of microbial IgA induction. Bunker *et*

5585   *al* found that IgA targets mainly bacteria residing in the small intestine and those that are positive in colon,

5586   are also positive in the duodenum. Every segment of the gastrointestinal tract can be affected in dogs with

5587   CE, however the duodenum is more frequently affected (Dandrieux, 2016). As such, Ig-coating in faeces

5588   could be a non-invasive method to evaluate the bacteria with high immune stimulatory properties that could

5589   be causing or exacerbating intestinal inflammation in the duodenum.

5590

5591   When we compared the IgA positive population with faeces and with the intestine, we saw that Ig-positive

5592   population clustered with faecal samples but not with intestine. This is not surprising as faecal samples are

5593   the reflection of what is happening in colon.

5594

5595   Other limitations in this study included different breed of dogs included in the study. It has been suggested

5596   that this could have an impact in the cytokine profile.In turn this will affect the gut microbiota characteristics

5597   (Peiravan et al., 2018). Also, different strains within a genus may have different associations with disease,

5598   which could not be detected in this study. Future studies, focusing on strain identification (such us strain-

5599   level metagenomics profiling), could give more clues about specific members of the microbiota involve in

5600   the pathogenesis of intestinal inflammation.

5601

5602   In summary, the gut microbiota during disease exhibits a high instability that is reflected in the

5603   immunoglobulin coating of bacteria with IgA and IgG and that individually differs between active and

5604   remission periods. Also, this study confirms the spatial variation between the luminal, mucosal and faecal

5605   communities during disease.

5606

5607

5608

5609

5610 **Chapter 4: Characterisation of thymic stromal lymphopoietin in the intestine of healthy dogs and**
5611 **dogs with chronic enteropathies**

5612

5613 **4.1 Introduction**

5614
5615

5616 The interaction between the host and the microbiota is a vital mechanism involved in intestinal homeostasis

5617 and inflammation. Under normal conditions, the immune system tolerates commensal bacteria and rapidly

5618 recognises and destroys pathogenic microorganisms. However, in chronic enteropathy, loss of tolerance or

5619 dysregulation of the immune system can cause an exaggerated response toward microbiota, food antigens

5620 or endogenous antigens that leads to chronic inflammation of the gastrointestinal tract (Fogle & Bissett,

5621 2007). Intestinal epithelial cells (IEC) comprise part of the mucosal epithelium, as part of the innate

5622 immune system; they are a fundamental cell population that not only provides a physical barrier to separate

5623 the luminal content from the underlying immune system but also produces factors that actively regulate

5624 intestinal immune responses (Rimoldi *et al.*, 2005).

5625

5626 Thymic stromal lymphopoietin (TSLP) is an IEC-derived cytokine that exerts dual immunoregulatory

5627 functions: it can promote or prevent inflammation (Fornasa *et al.*, 2015). Previously, it was believed that the

5628 primary effect was context-specific and dependent on the antigenic stimulus, route of exposure, site of the

5629 inflammatory lesion and TSLP concentration (Rimoldi et al., 2005). However, recently two transcript

5630 variants of TSLP have been identified in people (Bjerkan et al., 2015). The long isoform, or variant 1

5631 (lfTSLP); and the short isoform or variant 2 (sfTSLP) (Bjerkan *et al.,* 2015) (Fornasa *et al.*, 2015).

5632

5633 Under physiological conditions, sfTSLP is the predominant form and is constitutively expressed in the

5634 intestine, where it has a critical role in preserving immunotolerance (Bjerkan *et al.*, 2015; Fornasa *et al.*,

5635 2015). Conversely, lfTSLP is pathogenic and its expression is induced by inflammatory stimuli such as

5636 pathogenic bacteria in intestinal epithelial cells and cells of the immune system (Cullender *et al.*, 2013).

5637

5638 Under steady-state conditions, TSLP promotes a dendritic cell (DC) non-inflammatory phenotype, where

5639 they induce the production of T regulatory cells and gives them the ability to produce constitutively

5640 interleukin-6 (IL-6) and a proliferation-inducing ligand (APRIL) which drives the development of

5641 immunoglobulin A (IgA)-producing plasma cells (Sato *et al.,* 2003). Additionally, TSLP inhibits the

5642 expression of IL12/IL-23 P40 subunit in DCs, that causes an impairment in the ability of DCs, to promote

5643 antigen-specific T$_{H1}$ differentiation (Chung *et al.*, 2009).

5644

5645 Thus, the homeostasis of the gut is preserved through the continuous generation of non-inflammatory

5646 helper T cells and antibody responses that limit bacterial entrance and promote an environment tolerant to

5647 commensal bacteria.

5648

5649 Due to the fact, that TSLP exert several roles that are crucial for adequate intestinal function, it would be

5650 interesting to investigate the expression of this factor in the intestine of dogs during homeostasis and during

5651 pathologic conditions such as chronic enteropathies

5652

5653 Although TSLP is widely expressed in many tissues and involved in many cellular processes, the role of

5654 this cytokine in canine intestine has not been explored yet. The aim of this study is to characterise the

5655 expression of TSLP in the intestine of healthy dogs and dogs with chronic enteropathies during active

5656 disease and clinical remission.

5657

5658 Due to the fact, that TSLP exert several roles that are crucial for adequate intestinal function, it would be

5659 interesting to investigate the expression of this factor in the intestine of dogs during homeostasis and during

5660 pathologic conditions such as chronic enteropathies

5661

5662 **4.2 Methodology**

5663

5664 **4.2.1 Study dogs**

5665

5666 Dogs with signs of chronic gastrointestinal disease (> 3 weeks), including persistent and/or recurrent

5667 vomiting and/or diarrhoea and/or weight loss; presented at the veterinary hospital of the University of

5668 Melbourne were enrolled into the prospective study. A total of 8 dogs were enrolled. Dogs underwent a

5669 complete clinical evaluation by an internal medicine specialist. Dogs were evaluated for co-morbidities and

5670 extra-intestinal disease prior to inclusion by a combination of faecal analysis (faecal flotation and faecal

5671 cytology), blood testing (including canine pancreatic lipase immunoreactivity, cobalamin and canine trypsin-

5672 like immunoreactivity) and abdominal ultrasound. Dogs were not included in the trial if there was a history

5673   of dietary or medical therapy 3 weeks prior to analysis, or if hypoalbuminemia (albumin < 20 g/L) was

5674   present.

5675

5676   The disease activity was scored using the canine chronic enteropathy activity index (CCECAI). For this

5677   score, nine signs (attitude/activity, appetite, vomiting, stool consistency, stool frequency, weight loss,

5678   albumin levels, ascites/peripheral oedema and pruritus) are scored from 0 to 3 based on the magnitude of

5679   their alterations. The scores are added, yielding a total cumulative score. Five categories are defined:

5680   insignificant disease, 0–3; mild disease, 4– 5; moderate disease, 6–8; severe disease, 9–11; very severe

5681   disease >11(Allenspach *et al.*, 2007).

5682

5683   One patient withdrawal from the study and the second endoscopy could not be performed (Mouse). Two

5684   patients did not participate in the study but owners agreed to donate some samples for the study (Ruby and

5685   Buckley) (New study). According to the classification based on response to treatment, four dogs had diet-

5686   responsive enteropathy (DRE); four dogs had antibiotic-responsive enteropathy (ARE) and two dogs had

5687   steroid-responsive enteropathy (SRE) (Dandrieux, 2016) Detailed information about the patients can be

5688   found in table 4.1.

5689

5690   Samples from dogs of a previous study performed between 2012 and 2014 were included. Selection criteria

5691   were the same as specified above. A total of 11 patients were enrolled in this study. According to the

5692   classification based on response to treatment, six dogs had diet-responsive enteropathy (DRE); three dogs

5693   had antibiotic-responsive enteropathy (ARE) and one dog had steroid-responsive enteropathy (SRE)

5694   (Dandrieux, 2016). Detailed information about the patients can be found in table 4.1.

5695

5696   Healthy subjects: Samples from a previous study were used. Dogs were rescued working dogs that were

5697   rehomed at the end of that study. Additionally, skin biopsies of a healthy dog were used as a positive

5698   control for TSLP expression. Detailed information about the patients can be found in table 4.2.

5699

5700   All experimental procedures were approved from the Animal Ethic committee of University of Melbourne.

5701   (Animal Ethics Committee approval AEC # 1112072.2).

5702

5703   Owners gave written consent in which they agreed to participate in initial and follow-up diagnostic valuation.

5704   They could withdraw their animals from the trial at any point.

5705

5706   Table 4.1: Metadata information of dogs with chronic enteropathies

Patient	Breed	Age (y)	Type CE	Localisation	CCECAI-1	CCECAI-2	SampleV1 (Y/N)	SampleV2 (Y/N)	RNA preservation method	Study period
Dog1	Spoodle	5	SRE	Mixed	5	0	Y	Y	Stabilization solution	New
Dog2	Japanese Spitz	1.5	ARE	Mixed	8	2	Y	Y	Transition solution	Old
Dog3	Whippet	4	ARE	SI	6	NA	Y	N	Stabilization solution	New
Dog4	Border Collie	5	ARE	SI	Unknown	Unknown	N	Y	Stabilization solution	Old
Dog5	Golder Retriever	5	FRE	SI	1	1	Y	Y	Stabilization solution	New
Dog6	Staffordhire Bull Terrier	2	FRE	Mixed	11	2	Y	Y	Transition solution	Old
Dog7	Labrador Retriever	10	SRE	Mixed	7	0	N	Y	Stabilization solution	New
Dog8	Labrador Retriever		FRE	Mixed	7	4	Y	Y	Transition solution	New
Dog9	Basset Hound		ARE		5	0	Y	N	Transition solution	Old
Dog10	GSD	14	FRE	Mixed	6	0	Y	Y	Stabilization solution	New
Dog11	Labrador Retriever	2	ARE	SI	7	1	Y	Y	Transition solution	Old
Dog12	Chihuahua	9	FRE	Mixed	Unknown	Unknown	Y	Y	Stabilization solution	New

Dog13	GSD	1.5	ARE	Mixed	12	1	Y	Y	Transition solution	Old
Dog14	Maltese Cross	5	FRE	SI	6	0	Y	Y	Transition solution	Old
Dog15	GSD	3.5	ARE	SI	9	0	N	Y	Transition solution	Old
Dog16	Greyhound	2	ARE	Mixed	5	0	Y	Y	Stabilization solution	New
Dog17	Basset Hound	3	ARE	Mixed	5	0	Y	Y	Transition solution	Old
Dog18	Toy Poodle	2.5	ARE	LI	9	0	Y	N	Transition solution	Old
Dog19	Flat Coated Retriever	9	SRE	SI	Unknown	Unknown	Y	N	Stabilization solution	New
Dog20	Labrador Retriever	3.6	ARE	Mixed	Unknown	Unknown	Y	N	Stabilization solution	New
Dog21	Weimaraner	1.7	FRE	Mixed	4	0	Y	Y	Transition solution	Old

5707

5708

5709

5710

5711

5712   Table 4.2: Metadata information healthy dogs.

Dog	Breed	Age (years)	Diet	Neutering Status
Dog1	Mix-Breed	2	Advance Dry Food®	Male/Neutered
Dog2	Mix-Breed	3	Advance Dry Food®	Male/Neutered
Dog3	Mix-Breed	3	Advance Dry Food®	Male/Neutered
Dog4	Mix-Breed	7	Advance Dry Food®	Male/Neutered
Dog5	Mix-Breed	5	Advance Dry Food®	Male/Neutered
Dog6	Mix-Breed	6	Advance Dry Food®	Male/Neutered
Dog7	Mix-Breed	3	Advance Dry Food®	Male/Neutered
Dog8	Mix-Breed	3	Advance Dry Food®	Female/Spayed
Dog9	Mix-Breed	2	Advance Dry Food®	Female/Spayed
Dog10	Mix-Breed	1	Advance Dry Food®	Female/Spayed
Dog11	Mix-Breed	3	Advance Dry Food®	Female/Spayed
Dog12	Mix-Breed	2	Advance Dry Food®	Female/Spayed
Dog13	Mix-Breed	5	Advance Dry Food®	Female/Spayed

5713

5714

5715

4.2.2 Samples

Two pinch biopsies from each intestinal segment obtained during endoscopic examinations (stomach, duodenum, colon and if feasible also ileum) in dogs with chronic enteropathies prior to treatment trial and after treatment success (defined as a decrease in the clinical CE activity index of at least 75% for at least six weeks) were placed in an eppendorf tube containing RNAlater stabilisation solution (Ambion™), and then stored at -20°C. Biopsies from healthy dogs obtained in a previous study stored in RNAlater transition solution (Ambion™) at -80°C were also analysed. A total of 13 healthy dogs, 21 dogs with active CE and 16 dogs with CE in remission were analysed; 13 dogs had samples from both the active and remission period. As most of the samples from the old study were preserved in transition solution, whereas the samples from the new study were preserved in stabilization solution; one sample was placed in RNAlater stabilisation solution (Ambion™), and in RNAlater transition solution (brand) for checking of potentially differences in RNA expression due to different storage techniques but we did not find any difference (Dog1 V1 small intestine).

4.2.3 RNA isolation

Total RNA was isolated from the endoscopy biopsies (8-18 mg) using the RNeasy microarray tissue Mini kit (Qiagen™); tissue was removed from the RNAlater using sterile forceps and placed in a 2mL safe-lock microcentrifuge tube containing 500µL of lysis buffer. Samples were put on ice and then disrupted and homogenised with a homogeniser containing a sterile 3mm probe at 17 cycles per second during 2-5 minutes. Subsequent steps were performed as recommended by the manufacturer. The RNA was eluted in 60 µL of RNase-free water.

Assessment of RNA quality and quantity.

The quantity and quality of the RNA was assessed via spectrophotometry. UV absorption ratios 260:280 nm and 260:230 nm were assessed in a ND1000 spectrophotometer using 1µl of sample. Ratios A260:A230 greater than 1.7 and A260:A280 between1.8 to 2.0 were considered as appropriate. RNA was stored at -80°C.

5749 **4.2.4 cDNA isolation**
5750

5751 Synthesis of cDNA was carried out in 500 ng of RNA using the Quantinova Reverse™ transcription kit

5752 (Qiagen) and per manufacturer's instructions. Genomic DNA digestion was performed prior to cDNA

5753 synthesis as specified in the protocol. Reactions were done in duplicate. Additionally, one sample

5754 containing the QuantiNova™ Internal Control RNA (QN IC RNA) but not template was used as an internal

5755 amplification control to test successful reverse transcription/amplification; another sample with template but

5756 not reverse transcription enzyme was used to check for the presence of genomic DNA. cDNA was diluted

5757 1:10 in nuclease-free water and stored at -20°C.

5758

5759 **4.2.5 Real-Time PCR**
5760
5761

5762 TSLP primers were designed using the primer-BLAST program from NCBI (NCBI). Reference genes were

5763 the same as those ones described by Peter *et al,* 2007. For detailed information of the primers, refer to

5764 Table 4.3 Primers were synthesised by Geneworks® (Australia) and were reconstituted in nuclease-free

5765 water and stored at -20°C. Real-time PCR was performed using the Quantinova SYBR™ green PCR

5766 (Qiagen®) per manufacturer's instructions. Gene specific amplification was performed using 0.7 µM of each

5767 primer as recommended. Sample incubations were performed in a Rotor Gene Q™ cycler (Qiagen®) at

5768 95°C for 2 min and then 40 cycles of 95°C for 5 seconds and 60°C for 10 seconds during which the

5769 fluorescence data were collected. The melt curve was created by heating the samples from 65 to 95°C in

5770 0.5°C increments with a dwell time at each temperature of 3s during which time the fluorescent data were

5771 collected.

5772

5773 No template RNA and no RT samples using G3PDH primers for checking the absence of genomic DNA.

5774 RNAse-free water passed through the RNA isolation was assessed in a similar manner to control for

5775 sample contamination. A negative control of nuclease-free water and a positive control (skin) with a known

5776 threshold cycle value (Ct) were included with all sample runs to control for run-to-run variation. Ct value

5777 was calculated as the cycle when the fluorescence of the sample exceeded a threshold level corresponding

5778 to 10 standard deviations from the mean of the baseline fluorescence. PCR reactions were done in

5779 triplicate. A mean Ct value was calculated for each sample using these values.

5780

5781   **4.2.6 Assay Validation**

5782

5783

5784   To verify the specificity of the primers, real-time PCR products were purified using the MinElute™ PCR

5785   purification kit (Qiagen®). The protocol was performed according to manufacturer's instructions with a minor

5786   modification at the first step, where two samples of each gene were pooled to increase DNA yield. Purified

5787   product was sent for sequencing by capillary separation (Micromon, Monash University, Australia).

5788   Sequences obtained were analysed in Sequence scanner software 2™ (Thermo Fisher) and blasted in the

5789   NCBI database.

5790

5791   Three uL of PCR product was run in gel electrophoresis to check the presence of single bands products of

5792   appropriate size and melt curve analysis were performed to check the presence of a single peak.

5793

5794   Reaction efficient was calculated using a 6-fold serial dilution of skin RNA. A graph of threshold cycle

5795   versus log10 relative copy number of the sample from the dilution was produced by the Rotor gene

5796   software. The slope of this graph was used to determine reaction efficiency. Good efficiencies were

5797   considered with values of 90–100% ($-3.6 \geq$ slope ≥ -3.3) (table 4.4)

5798

5799   **4.2.7 Determination Reference genes**

5800

5801

5802   The identification of the most stably expressed genes and the minimum number of genes required for

5803   reliable normalisation were calculated using the program NormFinder™ (Department of Molecular

5804   Medicine, Aarhus University Hospital, Denmark) (Vandesompele *et al.,* 2002). Six reference genes were

5805   chosen as previously recommended by Peter *et al*, 2007 (Peters, Peeters, Helps, & Day, 2007). Ten

5806   samples per group were used (dogs with active disease, remission period and active disease). The optimal

5807   number of genes required for reliable normalisation; are calculated by the estimation of the intra-group

5808   variation and inter-group variation using the log-transformed Cq values (in this case the natural log). Having

5809   estimated both the intra- and inter-group variation, the program combines the two into a stability value,

5810   which intuitively adds the two sources of variation and thus represents a practical measure of the

5811   systematic error that will be introduced when using the investigated gene.

5812

5813   **4.2.8 Statistical Analysis**
5814
5815   For gene expression analysis of TSLP in healthy dogs and dogs with CE in active and remission periods
5816   the software REST2009® was used. REST2009 applies a mathematic model (livak method) that considers
5817   the different PCR efficiencies of the gene of interest and reference genes (Livak & Schmittgen, 2001).
5818   Samples from proximal small intestine were chosen as it has been reported that most of the patients with
5819   CE, exhibit clinical signs associated with the small intestine more frequently. The fold expression change
5820   was calculated using the $2^{-\Delta\Delta Ct}$ method, wherein each value is presented as an *n*-fold difference relative
5821   to the geometric mean of the two reference genes. To determine whether there is a significant difference
5822   between groups, Rest2009 uses randomisation techniques. The hypothesis test (P(H1) performs a large
5823   number of random reallocations of samples and controls between the groups. It then counts the number of
5824   times the relative expression of the randomly assigned group is greater than the sample data. P(H1) values
5825   < 0,05 were considered as significant.

5826
5827

5828   **4.3 Results**
5829
5830   The MIQE guideline was used as a reference for the processin and analysis of the results (Bustin et al.,
5831   2009). Validation of the reference genes using NormFinder™ found that YWHAZ was the most stable
5832   reference gene (Stability value 0.003). The other genes exhibited a stability of G3PDH:0.004, TBP: 0.011,
5833   HMBS: 0.004, RPL32:0.014 and SDHA: 0.004. The best combination of reference genes was SDHA and
5834   YWHAZ (stability value for best combination of two genes was 0.002). Therefore, SDHA and YWHAZ were
5835   used as reference genes for the relative quantification of TSLP gene expression in the intestinal tissues.

5836

5837   As previously reported (Klukowska-Rotzler *et al.*, 2013), skin from a healthy dog was used as a positive
5838   control for TSLP expression. We found that TSLP is also expressed in the stomach, proximal small
5839   intestine and colon of healthy dogs (Figure 4.1).

5840

5841   For gene expression analysis of TSLP in healthy dogs and dogs with CE in active and remission periods
5842   the software REST2009® was used. There was no significant difference in the TSLP expression in healthy
5843   dogs compared to dogs with active CE or dogs with remission CE or between dogs with active CE and
5844   dogs with CE in remission (Tables 4.5, 4.6, 4.7 and 4.8). Also, we did not find any difference among paired
5845   samples   (active   disease-remission).

5846

5847   Table 4.3: Reference genes and TSLP genes

Gene	Accession number	Forward Primer (5′ – 3′)	Reverse Primer (5′ – 3′)	Product length (base pairs)	Pseudogenes	Primer concentrations
G3PDH (Glyceraldehyde 3-phosphate dehydrogenase)	XM_014122594.1	TCA ACG GAT TTG GCC GTA TTG G	TGA AGG GGT CAT TGA TGG CG	90	YES	0.7 µM each
SDHA (*Canis lupus familiaris* succinate deshydrogenase complex, subunit A, flavoprotein (fp), transcript variant X2)	XM_535807.5	GCC TTG GAT CTC TTG ATG GA	TTC TTG GCT CTT ATG CGA TG	92	YES	0.7 µM each
RPL32 (*Canis lupus* ribosomal protein L32)	NM_001252169	TGG TTA CAG GAG CAA CAA GAA A	GCA CAT CAG CAG CAC TTC A	100	YES	0.7 µM each
TBP (*Canis lupus familiaris* TATA box binding protein, transcript variant X1)	XM849432.4	CTA TTT CTT GGT GTG CAT GAG G	CCT CGG CAT TCA GTC TTT TC	96	YES	0.7 µM each
HMBS (Hydroxymethylbilane synthase, transcript varian X5)	XM_014113375	TCA CCA TCG GAG CCA TCT	GTT CCC ACC ACG CTC TTC T	112	YES	0.7 µM each

HAZ (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein,zeta, transcript variant X2	XM_843951.5	CGA AGT TGC TGC TGG TGA	TTG CAT TTC CTT TTT GCT GA	94	YES	0.7 µM each
TSLP (Thymic stromal lymohopoietin)	XM_005618038.2	GCA GCG CCG ATA AAT AAT ACC	TAA GTG TGC GAC TTG TTC CC	90	YES	0.7 µM each

5848

5849

5850

5851

5852

5853

5854

5855   Table 4.4: Standard curve of reference genes and TSLP

Gene	R	R^2	M	B	Efficiency
HMBS	0.99985	0.99971	-3.621	28.866	0.89
YWHAZ	0.99944	0.99888	-3.558	25.435	0.91
TBP	0.99975	0.99949	-3.518	25.344	0.92
SDHA	0.99826	0.99652	-3.600	27.698	0.90
G3PDH	0.99605	0.99211	-3.422	26.767	0.96
RPL32	0.99849	0.99697	-3.501	23.877	0.93
TSLP	0.99553	0.99109	-3.551	28.547	0.91

5856

5857

5858

5859

5860   Table 4.5: Cq values of reference genes and TSLP at different disease stages

Sample SI	TSLP Cq	SDHA Cq	YWHAZ Cq	SDHA-YWHAZ cq	ΔCq value
Healthy	36.69±1.11	31.86±1.54	30.59±1.71	31.22±1.61	5.47±0.69
CE active	33.14±0.74	28.4±0.73	26.21±0.88	27.28±0.79	5.85±0.42
CE remission	32.1±0.65	27.31±0.75	25.00±0.93	26.16±0.82	5.99±0.34

5861

5862

5863

5864

5865   Table 4.6: 2^-ΔΔCt dogs with active disease compared to healthy dogs

ΔCt Value (CE active)	ΔCt Value (healthy)	Delta Delta Ct Value	Expression Fold Change	95% C.I Expression ratios	P(H1) Result
ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt		
5.85	5.47	0.38	0.768437591	0.151 - 6.355	0.540

5866

5867

5868

5869   Table 4.7: 2^-ΔΔCt dogs with disease in remission compared to healthy dogs

ΔCt Value (CE remission)	**Δ**Ct Value (Healthy)	Delta Delta Ct Value	Expression Fold Change	95% C.I Expression ratios	P(H1) Result
ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt		
5.99	5.47	0.52	0.697371833	0.119 - 2.499	0.149

5870

5871

5872

5873   Table 4.8: 2^-ΔΔCt dogs with active disease compared to dogs with disease in remission.

ΔCt Value (CE active)	ΔCt Value (CE remission)	Delta Delta Ct Value	Expression Fold Change	95% C.I Expression ratios	P(H1) Result
ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt		
5.85	5.99	-0.14	1.101905116	0.143 - 2.526	0.359

5874

5875

5876

5877
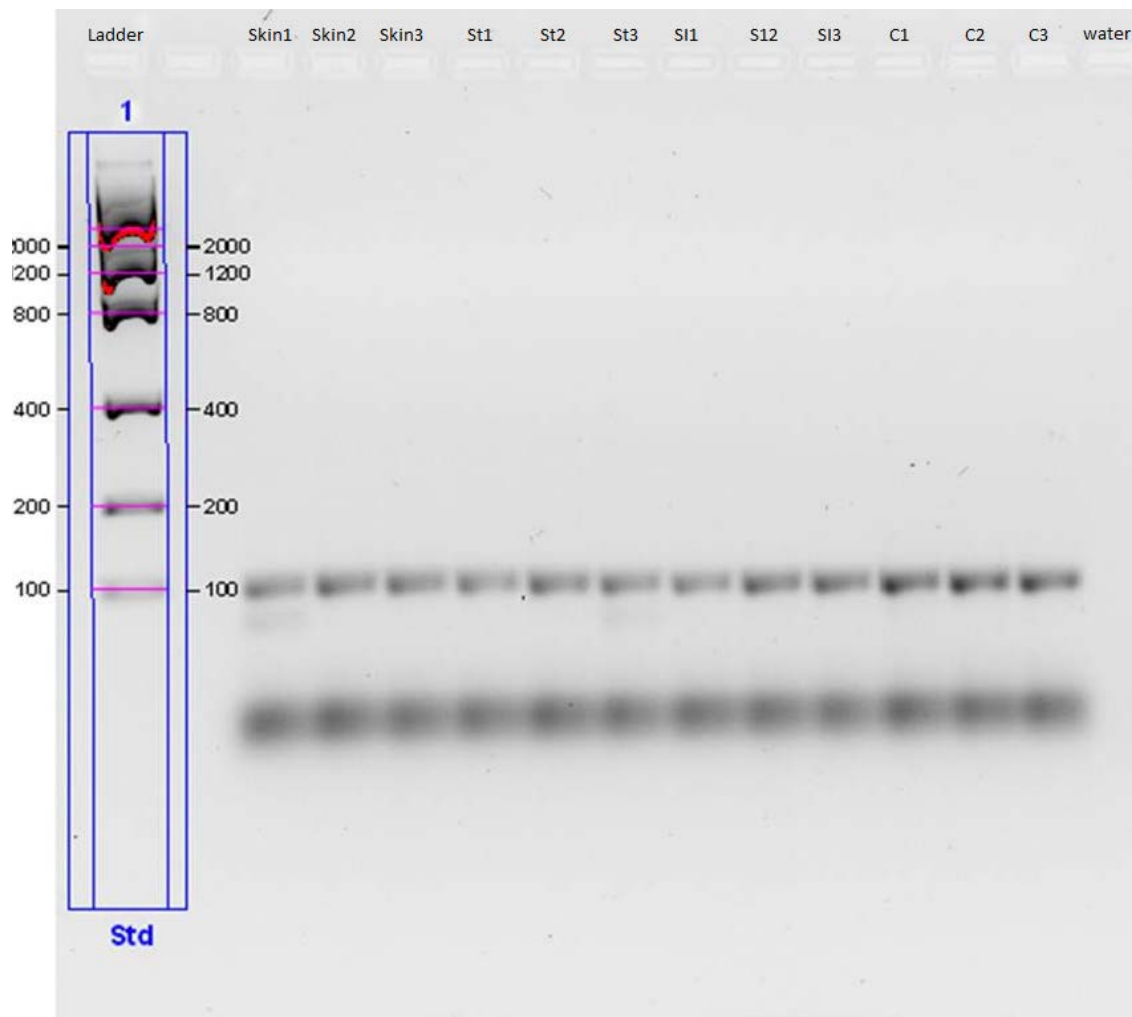
5878

5879　Figure 4.1: Expression of TSLP in health. Skin (positive control), St: stomach, SI: Small intestine and C:

5880　colon. n=3. PCR marker Sigma Aldrich.

5881

5882

5883

5884   **4.4 Discussion**
5885
5886
5887   TSLP is a cytokine with an important role in the regulation of inflammatory processes in the intestine that
5888   creates a bridge between DCs and IECs, which is vital for maintaining intestinal homeostasis. Until
5889   recently, the expression of TSLP had been only studied in the skin, where it has been implicated in the
5890   pathogenesis of atopic dermatitis and is considered a pro-inflammatory factor. However, it has not been
5891   possible to elucidate whether the increase in TSLP has causative effects or whether it is a consequence of
5892   the inflammatory process in allergic conditions. By using PCR, we assessed the expression of TSLP in the
5893   gastrointestinal tract of dogs, and found that TSLP is constitutively expressed in the stomach, intestine and
5894   colon of healthy dogs.

5895

5896   In humans, TSLP has a dual role in the intestine. On one hand, it is one of the key factors for conditioning
5897   DCs to produce T_{reg} cells by inducing Foxp3 expression in naïve T cells and to promote the synthesis of
5898   IgA; thus, promoting an anti-inflammatory and tolerogenic environment under physiologic conditions. On
5899   the other hand, it promotes inflammation under pathological conditions and protects the host against
5900   invasive pathogens (Tsilingiri *et al.*, 2017). As a deregulation of the immune system and a pro-inflammatory
5901   environment has been associated with chronic enteropathies, we investigated the mRNA expression of
5902   TSLP in dogs with active disease and dogs during remission and compared them to the mRNA expression
5903   in healthy dogs. We did not find any significant difference in the expression of TSLP in the small intestine
5904   among any of the groups.

5905

5906   Recently, Osada *et al* 2017 evaluated the mRNA expression of *IL-25, -33* and *TSLP* in the duodenal and
5907   colonic mucosa of dogs with antibiotic-responsive enteropathy (ARE), diet-responsive enteropathy (DRE)
5908   and steroid-responsive enteropathy, and did not find any difference in TSLP expression among groups and
5909   compared to healthy dogs. This finding is supported by our study, where we also evaluated active disease
5910   versus remission (Osada *et al.*, 2017).

5911

5912   Several reasons could explain our findings. Although 80% of the dogs with CE manifest more clinical signs
5913   compatible con small intestinal disease and not with large intestine disease; some dogs have a more
5914   severe histopathologic changes in the colon. Even if the correct anatomical location is assessed; the multi-
5915   focal variable expression of inflammation, across the intestine; means that individual samples could not

5916   reflect what it is happening globally. We evaluated the expression of TSLP only in the small intestine, so

5917   we cannot exclude that TSLP expression pattern could be different in colon. Expression of TSLP varies

5918   throughout the length of the gastrointestinal tract, with its highest expression in the large intestine,

5919   indicating differential regulation depending on the microenvironment (Rimoldi *et al.*, 2005). The large

5920   intestine differs from the small intestine, not only in cell types but also in the population of commensal

5921   bacteria. As bacteria-cell contact regulates TSLP expression, it is possible that differences and alterations

5922   in the abundance or composition of bacterial flora along the GIT may influence TSLP mRNA expression.

5923

5924   TSLP has been implicated mainly in the upregulation of T_{H2} responses and dampening of T_{H1} and T_{H17}

5925   responses. Although several studies have investigated cytokine and chemokine expression in dogs with

5926   chronic enteropathies; there is no clear T_{H1}, T_{H2} or T_{H17} polarisation (Kolodziejska-Sawerska *et al.*, 2013)

5927   (Heilmann & Suchodolski, 2015). Results have been varied with breed, disease stage and severity,

5928   intestinal region affected, employed methodology as well as other confounding factors such as therapy and

5929   demography. Accordingly, dogs with a T_{H2} phenotype could exhibit more TSLP expression, whereas dogs

5930   with more T_{H1} responses could exhibit lower expression of TSLP. We did not assess the expression of

5931   cytokines related to any of these pathways so we cannot exclude that possibility.

5932

5933   It has been shown that colonic epithelial cells from people with CD who exhibit a strong T_{H1} response have

5934   a lower expression of TSLP; whereas people with UC, where a T_{H2} response predominates, have a higher

5935   expression of the TSLP gene (Fornasa *et al.*, 2015) (Rimoldi *et al.*, 2005).

5936

5937   In people, results assessing TSLP expression have been discordant with variable primers used, dosage

5938   and pathology. Previous studies have shown that there are bacterial strain-specific effects on TSLP

5939   secretion. This could mean that the dysbiosis observed in dogs with chronic enteropathies could potentially

5940   impact the balance of TSLP secretion or pattern of expression. In vitro studies using intestinal explants and

5941   specific bacterial strains could help to elucidate this process in dogs.

5942

5943   Recently analysis of the human TSLP locus indicates that there are two variants of the TSLP. The short

5944   isoform, that is considered anti-inflammatory and the long isoform that is considered pro-inflammatory.

5945   Studies using primers targeting specific isoforms have found that under normal conditions only the short

5946   isoform is expressed and under pathologic conditions, the expression of the short isoform is lost or the

5947   expression of the long isoform is increased favouring mucosal inflammation. Older studies in people used

5948   primers that were targeting both isoforms and that could be one of the reasons of discordant result among

5949   studies.

5950

5951   Although the presence of isoforms has not been described in dogs, alignment of the coding sequence of

5952   canine TSLP mRNA (GenBank accession number XM_005618038.2) with the orthologous human

5953   sequence (GenBank accession number NM_033035.4), the nucleotide identity was 70% and covered the

5954   signal peptide and full-length mature human protein. Additionally, the predicted amino acid sequence

5955   deduced from the canine TSLP cDNA (accession number: I1VWC7) shares 60.8% identity with human

5956   (accession number NP_149024.1) TSLP protein, opening the possibility of the presence of isoforms in dogs

5957   (Klukowska-Rotzler *et al.,* 2013).

5958

5959   In mice, even though there are no open reading frames for sfTSLP, this protein might still be generated by

5960   protease cleavage from lfTSLP. One study tested the human sfTSLP in mice, and it could be seen that

5961   human sfTSLP could protect the mice from endotoxin shock and DSS colitis, suggesting not only an anti-

5962   inflammatory role *in vivo* but the possibility of a presence of a sfTSLP in mice.

5963

5964   The transcriptome comprises all kind of transcripts and their quantity, including mRNAs, non-coding RNAs

5965   and small RNAs, and gives information about their start sites 5′ and 3′ ends, splicing patterns and other

5966   post-transcriptional modifications. This technique could be used to assess the presence of isoforms in the

5967   dog and could serve to monitor the change of expression of a specific isoform for a particular physiological

5968   stage or condition (Yau, Leong, Zeng, & Wasinger, 2013).

5969

5970   Also, there is a possibility that TSLP function is not affected during CE in dogs; or is redundant and other

5971   cytokines present in the gastrointestinal tract can exert the same functions. One study in mice, showed

5972   that TSLPR$^{-/-}$ mice have normal numbers of circulating CD4$^+$CD25$^+$FoxP3$^+$ T$_{reg}$ indicating that TSLP is not

5973   essential for the development of natural T$_{reg}$. However, studies conducted using a chimeric mouse model,

5974   where developing T cells are deprived of IL-7 and TSLP receptor signalling; exhibited a defect in T$_{reg}$

5975   development in the thymus but were not required for survival of mature peripheral T$_{reg}$ cells. Thus, although

5976   it seems that TSLP is not required for adaptive T$_{reg}$ development in the periphery, TSLP could be able to

5977   modulate these processes in cases of altered availability (Mazzucchelli *et al.,* 2008).

5978

5979 However, TSLP could still be useful during disease where a bacterial component is implicated now that

5980 studies *in vitro* have shown that TSLP fragments can retain their antimicrobial activities. (Bjerkan *et al.*,

5981 2015) (Sonesson *et al.*, 2011).

5982

5983 Some limitations of this study include the small number of samples, different storage conditions and

5984 technical limitations. Although quantitative PCR (qPCR) is a very useful technique for assessing mRNA

5985 levels; the mRNA expression of a gene, does not guarantee that the gene is exercising actions and protein

5986 levels should be analysed as well. On the other hand, although spectrophotometer analysis can give an

5987 indication of the RNA quality, other methods such as gel-electrophoresis and fluorometric quantification

5988 give a more precise estimation of RNA quality. After finishing the study, we had the opportunity to check

5989 some samples using the bioanalyzer® and we found that the quality differed among samples, which

5990 potentially could influence the amount of TSLP expressed in qPCR.

5991

5992

5993

5994   Chapter 5: Supplementary material
5995
5996   5.1 Processing of 16S rRNA amplicon sequences
5997
5998
5999   1. *Creating and checking the mapping file:*
6000
6001   For using QIIME you need to start with three specific input files: 1) your sample metadata
6002   mapping file which contains the per-sample barcode sequences and other technical information;
6003   2) a fastq file containing your amplicon sequence reads and 3) a corresponding fastq file
6004   containing the barcode reads for each amplicon sequence.
6005
6006   •   This step is also useful for the purpose of thinking about experimental design and hypothesis
6007   testing. The mapping file for QIIME:
6008   •   Includes information about your sequencing files and their associated metadata.
6009   •   Should be a tab-delimited text file.
6010   •   Must include the columns SampleID, BarcodeSequence, LinkerSequence (primers) and
6011   Description for each sample.
6012   •   Must have SampleIDs that refer to the sequence headers used in the FASTA files (symbol #).
6013   •   Can have other columns of metadata as needed.
6014   •   Must have the description column as the last column.
6015
6016   Example:

#SampleID	BarcodeSequence	LinkerPrimerSequence	ReversePrimer	SampleNumber	personID	Stool	Method	HoursToNeg80Storage	Description
2001	CTCTCTATTCGCCTTA	GGACTACNVGGGTWTCTAAT	GTGYCAGCMGCCGCGGTAA	106	55	1	D	25.5	106.1rep1
2002	TTCACGCATCCTCTAC	GGACTACNVGGGTWTCTAAT	GTGYCAGCMGCCGCGGTAA	106	55	1	D	25.5	106.1rep2
2003	AAGGAGTATTAGGCAT	GGACTACNVGGGTWTCTAAT	GTGYCAGCMGCCGCGGTAA	106	55	1	D	25.5	106.1rep3

6017
6018
6019
6020   Check if the mapping file has errors.
6021
6022   ```
validate_mapping_file.py -m Mapping_file_Leaky_Gut_Study.csv
```
6023
6024   2.  *Extracting the barcodes:*
6025
6026   Based on the mapping file, this QIIME script trimms the barcode from all the sequences:
6027
6028   ```
extract_barcodes.py -f ../1-Raw_data_and_quality/MISEQ2128_S1_L001_R1_001.fastq -r ../1-
Raw_data_and_quality/MISEQ2128_S1_L001_R2_001.fastq -c barcode_paired_end -o
./bar_exed_sep_ends -l 8 -L 8 -m Mapping_file_Leaky_Gut_Study.csv --
attempt_read_reorientation
```
6032
6033   -f → Input fastq filepath. This file is considered read 1.
6034   -r → Input fastq filepath. This file is considered read 2. [default: None]
6035   -c → Specify the input type. barcode_paired_end: Input is a pair of fastq files (–fastq1 and –
6036   fastq2) that each begin with a barcode sequence. The barcode for fastq1 will be written first,
6037   followed by the barcode from fastq2.
6038   -o → Directory prefix for output files [default: .]

6039   -l → Specify the length, in base pairs, of barcode 1. This applies to the –fastq1 file and all
6040   options specified by –input_type [default: 6]
6041   -L → Specify the length, in base pairs, of barcode 2. This applies to the –fastq2 file and options
6042   "barcode_paired_end", "barcode_paired_stitched", and "barcode_in_label" for the –input_type
6043   [default: 6]
6044   -m → Filepath of mapping file. NOTE: Must contain a header line indicating SampleID in the
6045   first column and BarcodeSequence in the second, LinkerPrimerSequence in the third and a
6046   ReversePrimer   column   before   the   final   Description   column.   Needed   for   –
6047   attempt_read_orientation option. [default: None]
6048   --attempt_read_reorientation → Will attempt to search for the forward and reverse primer in the
6049   read and adjust the sequence orientation to match the orientation of the forward primer.
6050
6051   Resulting files:
6052
6053   `barcodes.fastq  barcodes_not_oriented.fastq  reads1.fastq  reads2.fastq`
6054
6055   **Note:** If the resulting file reads1.fastq has in the header a 1, the order of the primers in the
6056   mapping file is correct.
6057
6058   3.   *Merging paired-end reads:*
6059
6060   **PEAR** is an ultrafast, memory-efficient and highly accurate pair-end read merger. It is fully
6061   parallelized and can run with as low as just a few kilobytes of memory. PEAR evaluates all
6062   possible paired-end read overlaps and without requiring the target fragment size as input. In
6063   addition, it implements a statistical test for minimizing false-positive results. Together with a
6064   highly optimized implementation, it can merge millions of paired end reads within a couple of
6065   minutes on a standard desktop computer.
6066   `pear -f bar_exed_sep_ends/reads1.fastq -r bar_exed_sep_ends/reads2.fastq  -v 100 -m 600 -n`
6067   `80 -j 24 -o bar_exed`
6068
6069   were:
6070   -f → forward reads (pair 1)
6071   -r → reverse reads (pair 2)
6072   -v → min overlap
6073   -m → max assembled length
6074   -n → min assemble length
6075   -j → number of threads
6076
6077   **Note:** PEAR essentially add the 2 quality scores if the calls agree (with mathematical
6078   justification). However, this breaks the conventions of the phred score in FASTQ. It doesn't
6079   break FASTQ completely - values up to ASCII 126 = Phred+33 93 - can be used, but they are
6080   unconventional!   But   this   is   something   QIIME   1.9.0's   `split_libraries_fastq.py`   (for
6081   demultiplexing step below) cannot handle and therefore **QIIME 1.8.0 must be used for this**
6082   **step**.
6083
6084   4.   *Discard sequences in barcodes.fastq that are not in sequences file:*
6085

```
6086   python
6087   /home/users/allstaff/schulze.a/Papenfuss_lab/projects/metagenomics/ENDIA/ENDIA_QC/analysis_
6088   tools/trim_fastq_to_matching.py -f bar_exed.assembled.fastq -m
6089   bar_exed_sep_ends/barcodes.fastq -o MISEQ2128.barcodematched.fastq
```

6090

6091   **Note:** This is a script that was written by Jocelyn (`trim_fastq_to_matching.py`). The path to the
6092   directory is written there.

6093

6094   *5. Demultiplexing fastq sequencing data:*

6095

6096   To run the qiime 1.8.0 version of split_libraries_fastq use
```
6097   /usr/local/bioinfsoftware/python/python-2.7.3/bin/split_libraries_fastq.py
```

6098

6099   You may need to first run:

6100
```
6101   module load python/2.7.3
6102
6103   and
6104
6105   mkdir ~/tmp
6106
6107   lamboot
6108
6109   /usr/local/bioinfsoftware/python/python-2.7.3/bin/split_libraries_fastq.py -i
6110   ./MISEQ2128.barcodematched.fastq -b ./bar_exed_sep_ends/barcodes.fastqtrimmed -m
6111   Mapping_file.csv --barcode_type 16 -p 0.90 --phred_offset 33 -q 29 -o labelled_hiqual/ -v
```

6112

6113   -i → The sequence read fastq files (comma-separated if more than one)
6114   -b → The barcode read fastq files (comma-separated if more than one) [default: None]
6115   -m → Metadata mapping files (comma-separated if more than one) [default: None]. NOTE: Must
6116   contain a header line indicating SampleID in the first column and BarcodeSequence in the
6117   second, LinkerPrimerSequence in the third.
6118   --barcode_type → The type of barcode used. This can be an integer, e.g. for length 6 barcodes
6119   -p → Min number of consecutive high quality base calls to include a read (per single end read) as
6120   a fraction of the input read length [default: 0.75]
6121   -q → The maximum unacceptable Phred quality score (e.g., for Q20 and better, specify -q 19)
6122   [default: 3]
6123   -o → Directory to store output files

6124

6125   Results:

6126
```
6127   histograms.txt  seqs.fna  split_library_log.txt
```

6128

6129   Your demultiplexed sequences (separated by sample) are in the seqs.fna file. The histogram
6130   shows the length distribution of your reads and the split_library_log.txt shows the number of
6131   reads per sample/library.

6132

6133   **Note:** Before continuing you should start a new session (close the terminal a ssh again into your
6134   account), so qiime 1.9.0 is used for the following steps.

6135

6136   *6. Obtain the reverse complement:*

6137
6138   `adjust_seq_orientation.py -i ./labelled_hiqual/seqs.fna`
6139
6140 Results:
6141 **`seqs_rc.fna`**
6142
6143 It might be necessary to run this command to ensure sequences are in the correct orientation.
6144
6145   *7. <u>Remove the amplicon primer: this trims 16S rRNA primers as well as Illumina universal*
6146   *sequencing primers:</u>*
6147
6148 `python`
6149 `/home/users/allstaff/schulze.a/Papenfuss_lab/projects/metagenomics/ENDIA/ENDIA_QC/analysis_`
6150 `tools/trim_fasta_amplicons.py -i ./seqs_rc.fna -d Forward -o `**`seqsNAmp.fna`**
6151
6152 Verbose results:
6153
6154 `seqDirection: Forward  regionV: V4`
6155 `primer1: GTG[CT]CAGC[AC]GCCGCGGTAA , primer2: ATTAGA[AT]ACCC[CGT].GTAGTCC`
6156 `Forward primers found: 6482499 Counts`
6157 `Number of bases trimmed from sequence start when forward primer not found: 41`
6158 `Reverse primers found: 6497133 Counts`
6159 `Number of bases trimmed from sequence end when reverse primer not found: 44`
6160 `Both primers found: 6280188`
6161
6162 **<u>Note:</u>** This is a script that was written by Jocelyn (`trim_fasta_amplicons.py`). The path to the
6163 directory is written there.
6164
6165   8. <u>*Align sequences and cut the alignment (MOTHUR):*</u>
6166
6167 This is something implemented in <u>MOTHUR</u> that for some reason is not done in QIIME. The
6168 reason to do this is to keep only sequences from the same region of the 16S rRNA gene and to
6169 have all the reads with the exactly same length (meaning exactly the same region!). For this we
6170 use the silva database from MOTHUR:
6171
6172 Run mothur: `mothur` (enter)
6173
6174   a) Align to silva.bacteria database (you should copy this to your directory in which you run
6175   mothur).
6176
6177 `align.seqs(fasta=`**`seqsNAmp.fna`**`,          reference=silva.bacteria.fasta,          flip=t,`
6178 `processors=24)`
6179
6180 Results:
6181
6182 `Output File Names:`
6183 **`seqsNAmp.align`**
6184 `seqsNAmp.align.report`
6185 `seqsNAmp.flip.accnos`
6186
6187   b) Check in which bases are most of the sequences aligned:
6188
6189 `summary.seqs(fasta=seqsNAmp.align, processors=24)`

6190
6191   Example results:
6192   Start   End   NBases   Ambigs   Polymer NumSeqs
6193   Minimum: 1044   1056   2   0   2   1
6194   2.5%-tile: 13862   23444   252   0   3   167533
6195   25%-tile: 13862   23444   253   0   4   1675326
6196   Median: 13862   23444   253   0   4   3350651
6197   75%-tile: 13862   23444   253   0   4   5025976
6198   97.5%-tile: 13862   23444   253   0   6   6533768
6199   Maximum: 43115   43116   276   0   51   6701300
6200   Mean: 13866.7 23443.3 252.802 0   4.15174
6201   # of Seqs: 6701300
6202
6203   Output File Names:
6204   seqs_rcNAamp.summary
6205
6206   So what does this mean? You'll see that the bulk of the sequences start at position 13'862 and
6207   end at position 23,444. Some sequences in the example start at position 1044 or 43115 and end at
6208   1056 or 43116. These deviants from the mode positions are likely due to an insertion or deletion
6209   at the terminal ends of the alignments. Sometimes you'll see sequences that start and end at the
6210   same position indicating a very poor alignment, which is generally due to non-specific
6211   amplification.
6212
6213   **Note:** It can happen that if you have two different sequencing datasets together, one is in the
6214   reverse position and one in the forward. In that case the alignment is going to show like two
6215   groups   of   sequences   aligned   in   different   regions   using   Jocelyn's   script:
6216   adjust_seq_orientation.py (described above)
6217
6218   c) Run screen.seqs:
6219
6220   To make sure that everything overlaps the same region we'll run screen.seqs to get sequences
6221   that start at or before position 13862 and end at or after position 23444 (which is based on the
6222   summary results). We'll also set the maximum homopolymer length to 8 since there's nothing in
6223   the database with a stretch of 9 or more of the same base in a row.
6224
6225   screen.seqs(fasta=seqsNAmp.align, start=13862, end=23444, maxhomop=8, processors=24)
6226
6227   Results:
6228
6229   Output File Names:
6230   **seqsNAmp.good.align**
6231   seqsNAmp.bad.accnos
6232
6233   d) Make sure that our sequences only overlap the specific region:
6234
6235   We filter the sequences to remove the overhangs at both ends. Since we've done paired-end
6236   sequencing, this shouldn't be much of an issue. In addition, there are many columns in the
6237   alignment that only contain gap characters (i.e. "-"). These can be pulled out without losing any
6238   information. We'll do all this with:
6239
6240   filter.seqs(fasta=seqsNAmp.good.align, vertical=T, trump=.)
6241

6242 Example results:

6243
6244 `Length of filtered alignment: 462`
6245 `Number of columns removed: 49538`
6246 `Length of the original alignment: 50000`
6247 `Number of sequences used to construct filter: 6'478'106`

6248
6249 This means that our initial alignment was 50000 columns wide and that we were able to remove
6250 49538 terminal gap characters using trump=. and vertical gap characters using vertical=T. The
6251 final alignment length is 462 columns (or bases).

6252
6253
6254 `Output File Names:`
6255 `seqsNAmp.filter`
6256 **`seqsNAmp.good.filter.fasta`**

6257
6258   e) Get rid of the "-"→ convert alignment to a fasta file for further analysis in QIIME:

6259
6260 Out of mothur using a perl on-liner:

6261
6262 `perl -pe 's/-//g' seqsNAmp.good.filter.fasta > seqsNAmp.good.filter_MOTHUR.fna`

6263
6264 Results:
6265 **`seqsNAmp.good.filter_MOTHUR.fna`**

6266
6267 **Note:** Make sure to record the number of sequences that remained after all this process.
6268 Once you are satisfied, you are ready to move on to OTU picking.

6269
6270   9. *OTU picking or clustering of sequences into OTUs using UPARSE from USEARCH (not*
6271   *in QIIME) at 97% sequence identity*

6272
6273   a) Deduplicate the sequences (it is like clustering at 100% sequence identity, but it keeps the
6274   abundance information in the header).

6275
6276 The input sequences to cluster_otus must be a set of unique sequences sorted in order of
6277 decreasing abundance with size annotations in the labels. The derep_fulllength command can be
6278 used to find the unique sequences and add the size annotations. The input to derep_fulllength
6279 should be the reads after any quality filtering or length trimming:

6280
6281 `usearch -derep_fulllength seqsNAmp.good.filter_MOTHUR.fna -fastaout`
6282 **`seqsNAmp.good.filter_MOTHUR_unique.fna`** `-sizeout -minseqlength 64 -threads 20`

6283
6284 When you have to many samples, usearch won't be able to run this step, you can use VSEARCH
6285 instead and then keep using Usearch:

6286
6287 `vsearch --derep_full seqsNAmp.good.filter_MOTHUR.fna --output`
6288 **`seqsNAmp.good.filter_MOTHUR_unique_VSEARCH.fna`** `--log=log --sizeout --minseqlength 64`

6289
6290
6291   b) Make the reference (chimeras are filtered in this step):

6292

```
usearch -cluster_otus seqsNAmp.good.filter_MOTHUR_unique_VSEARCH.fna -minsize 2 -otus
otus_mc2.fa -relabel Otu
```

Example of verbose results:

```
01:47 66Mb    100.0% 849 OTUs, 14740 chimeras
```

minsize 2 → means that it will discard singleton sequences. In other words, for a sequence to stay it has to appear identically at least 2 times. This is the minimum you have to filter to avoid keeping erroneous sequences.

Notice you have as results your sequences clustered into 849 OTUs. 14740 sequences from your total were detected as chimeras.

c) Change the header of the **original** file (non-deuniqued):

```
perl -pe 'if($_=~/>.+(_\d+)/) {$_=~s/(_\d+)//g}' seqsNAmp.good.filter_MOTHUR.fna >
seqsNAmp.good.filter_MOTHUR_renamed.fna
```

d) Make OTUs:

```
usearch -usearch_global seqsNAmp.good.filter_MOTHUR_renamed.fna -db otus_mc2.fa -strand
plus -id 0.97 -otutabout otutab_mc2.txt
```

Example of verbose results:

```
00:39 159Mb   100.0% Searching, 77.1% matched
4726252 / 6144699 mapped to OTUs (76.9%)
```

10. Assign taxonomy to the uniqued sequences.

The Greengenes database of 16S sequences is the database of reference 16S sequences used to assign the taxonomy. A Qiime python script is used for this with the file `97_otus.fasta` that functions as a reference FASTA file of all sequences with known taxonomy

```
parallel_assign_taxonomy_uclust.py -i otus_mc2.fa -o tax_otus_mc2 -O 20 -t
/usr/local/bioinfsoftware/qiime/qiime_v2-1.8.0/qiime-deploy/qiime_software/gg_otus-13_8-
release/taxonomy/97_otu_taxonomy.txt -r /usr/local/bioinfsoftware/qiime/qiime_v2-
1.8.0/qiime-deploy/qiime_software/gg_otus-13_8-release/rep_set/97_otus.fasta
```

Resulting files:

```
otus_mc2_tax_assignments.log  otus_mc2_tax_assignments.txt
```

11. Make a biom file and add the taxonomic and metadata information:

a) Convert txt table to biom file

To convert a tab-delimited table to a JSON biom format. The biom format is designed to be a general-use format for representing biological sample by observation contingency tables (http://biom-format.org/index.html).

```
biom convert -i otutab_mc2.txt -o otutab_mc2.biom --table-type="OTU table" --to-json
```

6347
6348   b) Add the specific header to file with taxonomies:
6349
6350 `nano head`
6351 `#OTUID  taxonomy        confidence`
6352
6353 `cat head  otus_mc2_tax_assignments.txt > `**`otus_mc2_tax_assignments_C.txt`**
6354
6355
6356   c) Add the taxonomy to the biom file:
6357
6358 `biom add-metadata --sc-separated taxonomy --observation-header OTUID,taxonomy --`
6359 `observation-metadata-fp `**`tax_otus_mc2`**`/otus_mc2_tax_assignments_C.txt -i otutab_mc2.biom -o`
6360 **`otutab_mc2_tax.biom`**
6361
6362   d) Add metadata:
6363
6364 `biom add-metadata -i otutab_mc2_tax.biom -o `**`otutab_mc2_AllMeta.biom`**` --sample-metadata-fp`
6365 `Mapping_file.csv`
6366
6367 **Note:** if you have an error it would probably be related to not having the same samples in the
6368 sequences and in the mapping file.
6369
6370   *12. Obtain a phylogenetic tree:*
6371
6372   a) Align sequences from the reference using Mothur
6373
6374 `align.seqs(fasta=`**`otus_mc2.fa`**`, reference=silva.bacteria.fasta, flip=t, processors=24)`
6375
6376 `Output File Names:`
6377 **`otus_mc2.align`**
6378 `otus_mc2.align.report`
6379
6380   b) Make sure that your sequences only overlap the specific region:
6381
6382 `filter.seqs(fasta=otus_mc2.align, vertical=T, trump=.)`
6383
6384 `Length of filtered alignment: 302`
6385 `Number of columns removed: 49698`
6386 `Length of the original alignment: 50000`
6387 `Number of sequences used to construct filter: 849`
6388
6389 `Output File Names:`
6390 `otus_mc88.filter`
6391 **`otus_mc88.filter.fasta`**
6392
6393   c) Make the phylogenetic tree using qiime:
6394
6395 `make_phylogeny.py -i otus_mc2.filter.fasta -o fasttree_mc2`
6396
6397 Result:
6398 **`fasttree_mc2.tre`**
6399
6400   13. *Normalize .biom table using the CSS normalization in QIIME* (from "Robust methods for
6401   differential abundance analysis in marker genes surveys" to do PCoA plots):
6402
6403 `normalize_table.py -i otutab_mc2_AllMeta.biom -a CSS —o`
6404 **`CSS_normalized_otutab_mc2_AllMeta.biom`**

6405
6406   **Note:** The phylogenetic tree is added in phyloseq

6407   5.2 Supplementary figures
6408
6409

6410

6411
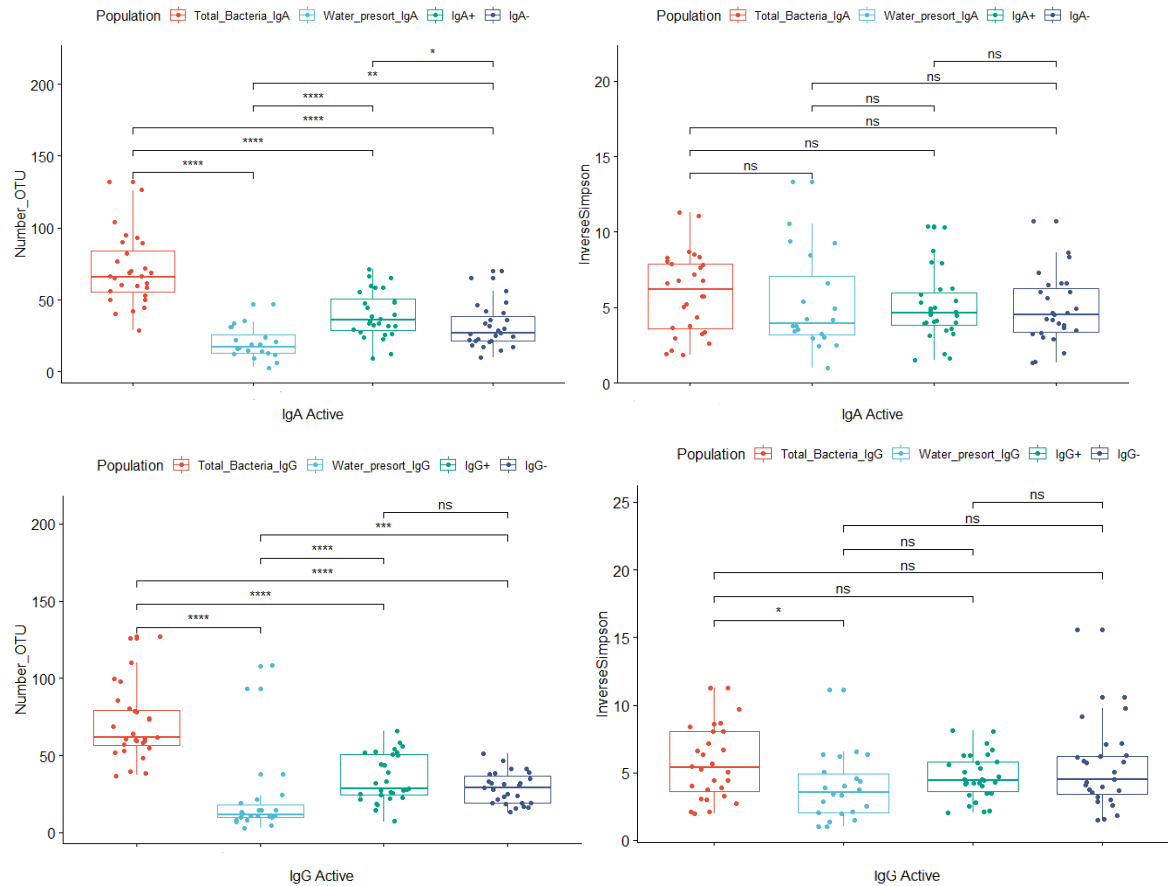
6412 Supplementary figure 1: Alpha diversity analysis of Immunoglobulins in dog with Chronic Enteropathy during active

6413 disease. Upper panel: Immunoglobulin A (IgA); Lower panel Immunoglobulin G (IgG); N: 18 IgA: Total n =28, Pre-

6414 sorting water n =20, IgA+ = 28, IgA- =27. IgG: Total n =28, Pre-sorting water n =23, IgG+ = 30, IgG- =27. Right

6415 panel: Number of OTUs (Observed), Left panel: Inversed Simpson's Index.

6416

6417

6418
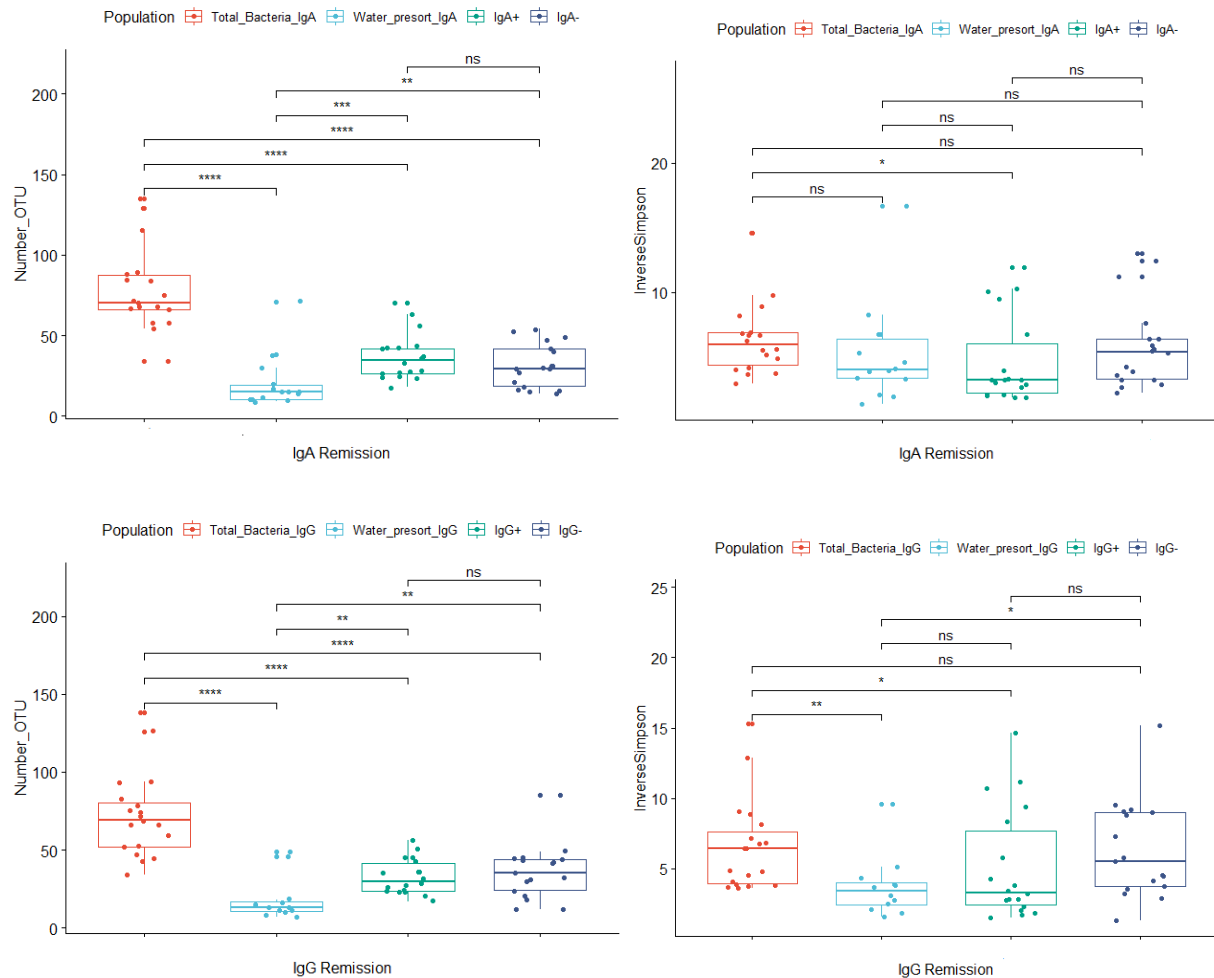
6419 Supplementary figure 2: Alpha diversity analysis of Immunoglobulins in dog with Chronic Enteropathy during

6420 remission disease. Upper panel: Immunoglobulin A (IgA); Lower panel Immunoglobulin G (IgG); N: 18 IgA: Total n

6421 =18, Pre-sorting water n =14, IgA+ = 18, IgA- =18. IgG: Total n =19, Pre-sorting water n =12, IgG+ = 18, IgG- =17.

6422 Right panel: Number of OTUs (Observed), Left panel: Inversed Simpson's Index.

6423

6424   **Chapter 6: General discussion**
6425
6426
6427   This study emphasises that there are individual variations in the GI microbiome of dogs, which may be
6428   missed by single time-point analysis. Therefore, when studying the microbiome in a group of dogs,
6429   collection of multiple samples over time will be of most benefit. Although our study had relatively small
6430   numbers, the conclusions were strengthened, and substantially more information was obtained by
6431   analysing multiple samples from individual dogs.
6432
6433   This project used new analytical tools and sample processing that allowed us to find some new information
6434   useful in the field of veterinary medicine. The advantages of using flow cytometry are to characterise
6435   bacteria that interact with the intestinal mucosa, allowing us to cut out the 'noise' and bias of standard
6436   microbiome analysis.
6437
6438   In chapter 2, we assessed the microbiome of two litters from birth, and compared this to the microbiome of
6439   mothers. We confirmed that the GI tract of puppies has commensal bacterial population when born. We
6440   were only able to analyse one meconium sample, but this too had a bacterial population, similar to that
6441   found in human infants (predominant phyla Proteobacteria). The adult-type microbiome started to develop
6442   post-weaning (predominant phyla Bacteroidetes, Firmicutes, and Fusobacteria), becoming stable at higher
6443   phylogenetic levels when the dogs were fed an adult diet.
6444
6445   We also determined that the maternal microbiome appears to alter prior to birth, with predominantly
6446   Firmicutes phyla (approaching 95% total bacteria). Although additional studies collecting more samples
6447   from different dogs and at different points during pregnancy are needed; this change could be hormonally
6448   driven. We postulate that this shift to a predominant Firmicutes pattern could be a mechanism by which the
6449   mothers maximise energy production from food. Additionally, we found that the puppy microbiome appears
6450   to influence the maternal microbiome (of both oral and faecal microbiota), rather than the expected
6451   opposite. This may be due to the mother cleaning the puppies. The route of delivery did not influence the
6452   gut microbiota of puppies in this study. However, all of our puppies have similar genetic background (i.e.
6453   Labradors) and environmental conditions, so this may not be applicable to other breeds or environments.
6454

6455   In adults dogs, the gut microbiota appeared to be highly stable over time at higher phylogenetic levels

6456   (Firmicutes, Bacteroidetes, Fusobacteria), even in senior dogs, provided that they maintained a good health

6457   status. When we analysed at lower phylogenetic levels (family and genus); the gut microbiota does change

6458   over time, although the variation was higher between individuals that within the same individual. The

6459   change at family and genus level within individuals was highly variable, which may be due to different

6460   environmental conditions for each dog (privately owned pets) and incidental colonisation. Limitations with

6461   this aspect of the study include the number of healthy dogs in the older age categories, and in some dogs

6462   only limited samples were collected. Complicating the interpretation of these findings are the multiple

6463   breeds and variable environmental exposure. However, although the latter may be considered a weakness,

6464   this reflects the difficulty in interpreting single time-point microbiome analysis in dogs.

6465

6466   In Chapter 3, we showed that different sets of bacteria were coated with IgA and IgG in health and disease

6467   (chronic enteropathy: active and remission). These profiles overall appeared similar (regardless of health

6468   status, or the IG involved), however when analysed at individual dog level, there were dramatic changes

6469   between the bacteria coated in active disease compared to remission.

6470

6471   In healthy dogs, IgA predominantly coated bacteria from the phyla Firmicutes, followed by Bacteroidetes. At

6472   lower phylogenetic levels, there was a wide range of bacterial groups at genus/family level that were similar

6473   between IgA and IgG.

6474

6475   In dogs with active chronic enteropathy, at the phyla level the coating appears similar to healthy dogs for

6476   both IgA and IgG. At the family level it was not possible to identify a predominant bacteria coated by either

6477   IgA or IgG. In dogs with CE that were in remission, again overall the coating distribution was similar to that

6478   of healthy dogs and dogs with active disease.

6479

6480   However, when individual dogs were analysed, profound differences in bacteria coated with IgA (but not

6481   IgG) were observed when comparing samples obtained during active versus remission disease. The

6482   change was not predictable, and the shift in some dogs was towards a lower number of bacteria coated

6483   with IgA when clinically improving. In that sub-set of dogs, we hypothesize that this indicates a reduction in

6484   the local inflammatory response to the GI microbiome; whether the microbiome shift drove the reduction in

6485   inflammation or vice versa cannot be determined. The lack of change in IgG coating is not surprising,

6486   based on the fact that the inflammation in CE is localised in the intestinal mucosa.

6487

6488   One of the major contributors to this finding could well be that we were analysing heterogeneous groups of

6489   conditions: there may be different etiologies, genetic background and environmental factors contributing to

6490   the disease (CE) in each individual dog. Additionally, the time period for which we obtained remission

6491   samples may be insufficient to gain a complete understanding of the 'healthy' microbiome in each individual

6492   dog. This is because the microbiome may be unstable until remission is achieved for many months.

6493   Unfortunately, the study could not follow all dogs for this length of time.   The methodology used in this

6494   study was novel in veterinary gastroenterology. We experienced some technical issues when we performed

6495   flow cytometry (likely due to the stain used) and contamination of the sheath fluid despite extreme care

6496   being taken to avoid this. Refinement of these technical issues may improve the utility of this analytical

6497   methodology. In veterinary medicine, because there is heterogeneity in CE cases, using IgA coating

6498   analysis may be beneficial to follow up individual dogs, to assess response and to try and further elucidate

6499   an understanding of the disease process in each dog.

6500   Future studies could also determine whether the intensity of the staining (dim versus bright) could be

6501   correlated with bacterial populations bound by low- versus high- affinity IgA, respectively and whether those

6502   distinct populations could be associated with health or disease. On one hand, IgA exludes and eliminates

6503   pathobionts, but on the other hand, IgA protects commensals by agglutination and by localizing these

6504   bacteria in a favorable environment (Fadlallah et al., 2018) (Donaldson et al., 2018).

6505

6506   In chapter 4, we could determine that TSLP is expressed in the intestine during health and disease (CE).

6507   Unfortunately, TSLP expression was not helpful in distinguishing active disease versus remission, and

6508   indeed did not differ between sick and healthy dogs. This may be due to TSLP simply not playing a role in

6509   canine CE, as no specific $T_{H1}/T_{H2}/T_{H17}$ profile has been identified in dogs. Other explanations for our

6510   findings could be different isoforms expressed in dogs, as occurs in people. Technical issues such as

6511   sample location, preparation and processing may also contribute.

6512

6513   The next steps into unraveling the role of the microbiota could include a more detailed study of the maternal

6514   microbiota during pregnancy, not only at the level of the faecal microbiota, but also at the level of skin, oral,

6515   and vaginal microbiota; and how these influence on puppy microbiome. Additionally, the study during the

6516 post- partum period at different time points could help to assess the dynamics, stability and the time that it

6517 takes to return to normal levels and the influence of the puppy microbiome on its profile.

6518

6519 In puppies, it would be very useful to assess the development and establishment of the gut microbiota in

6520 different breeds and born via different delivery routes. Prenatally, it would be very useful to investigate the

6521 presence of microbiota in placenta and confirm the presence of bacteria in meconium and their possible

6522 role during health and disease.

6523

6524 In regards to IgA and IgG-coating, the next steps should include more longitudinal studies followed over

6525 time and over longer periods; to determine whether the changes could be correlated to changes in

6526 treatment. It would also be clinically useful to determine whether this methodology could be used to monitor

6527 when an individual dog may be coming out of remission (prior to the development of overt clinical signs).

6528 Additionally, it would be very interesting to determine whether the IgA and IgG coated bacteria have

6529 different metabolic functions or impact on host metabolome, and to determine the genetic programming of

6530 those bacteria.

6531

6532 Also, it is well known that community structure is better defined in terms of functional diversity rather than

6533 taxonomic diversity alone. Thus, the combination of diversity with functional studies such as

6534 metatranscriptomics, metagenomics and metabolomics could offer a better understanding of the

6535 significance of the changes in the microbiota in health and disease.

6536

6537 Finally, for TSLP, metatranscriptomic studies have the potential to identify possible isoforms of TSLP that

6538 could impact the role of this gene in inflammation.

6539

6540 In summary, we have used new methodology to deepen our understanding of the microbiome in dogs in

6541 health and disease. Using determination of faecal coating is a useful technique to evaluate bacteria that

6542 directly interact with the host mucosal immune system and that can potentially be useful for deepening into

6543 the role of the relationship of the immune system with the microbiota.

6544

6545

6546

6547

6548 Bibliography

6549

6550

6551 Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., & Versalovic, J. (2014). The
6552   placenta harbors a unique microbiome. *Sci Transl Med, 6*(237), 237ra265.
6553   doi:10.1126/scitranslmed.3008599
6554 Aarti Kathrani, * Arthur House,1 Brian Catchpole,2 Angela Murphy,3 Alex German,4 Dirk
6555   Werling,2 and Karin Allenspach. (210). Polymorphisms in the Tlr4 and Tlr5 Gene Are
6556   Significantly Associated with Inflammatory Bowel Disease in German Shepherd Dogs.
6557   *PLoS One, 5*(12).
6558 Abeles, A. M., Pillinger, M. H., & Abramson, S. B. (2015). 23 - Inflammation and its mediators
6559   A2 - Hochberg, Marc C. In A. J. Silman, J. S. Smolen, M. E. Weinblatt, & M. H.
6560   Weisman (Eds.), *Rheumatology (Sixth Edition)* (pp. 169-182). Philadelphia: Content
6561   Repository Only!
6562 Abrahamsson, T. R., Wu, R. Y., & Jenmalm, M. C. (2015). Gut microbiota and allergy: the
6563   importance of the pregnancy period. *Pediatr Res, 77*(1-2), 214-219.
6564   doi:10.1038/pr.2014.165
6565 Aceti, A., Gori, D., Barone, G., Callegari, M. L., Di Mauro, A., Fantini, M. P., . . . on behalf of
6566   the Italian Society of, N. (2015). Probiotics for prevention of necrotizing enterocolitis in
6567   preterm infants: systematic review and meta-analysis. *Italian Journal of Pediatrics, 41*,
6568   89. doi:10.1186/s13052-015-0199-2
6569 Albenberg, L. G., & Wu, G. D. (2014). Diet and the intestinal microbiome: associations,
6570   functions, and implications for health and disease. *Gastroenterology, 146*(6), 1564-1572.
6571   doi:10.1053/j.gastro.2014.01.058
6572 Allakhverdi, Z., Comeau, M. R., Jessup, H. K., Yoon, B. R., Brewer, A., Chartier, S., . . .
6573   Delespesse, G. (2007). Thymic stromal lymphopoietin is released by human epithelial
6574   cells in response to microbes, trauma, or inflammation and potently activates mast cells. *J
6575   Exp Med, 204*(2), 253-258. doi:10.1084/jem.20062211
6576 Allenspach, K. (2011). Clinical immunology and immunopathology of the canine and feline
6577   intestine. *Vet Clin North Am Small Anim Pract, 41*(2), 345-360.
6578   doi:10.1016/j.cvsm.2011.01.004
6579 Allenspach, K., House, A., Smith, K., McNeill, F. M., Hendricks, A., Elson-Riggins, J., . . .
6580   Suchodolski, J. S. (2010). Evaluation of mucosal bacteria and histopathology, clinical
6581   disease activity and expression of Toll-like receptors in German shepherd dogs with
6582   chronic enteropathies. *Vet Microbiol, 146*(3-4), 326-335.
6583   doi:10.1016/j.vetmic.2010.05.025
6584 Allenspach, K., Wieland, B., Grone, A., & Gaschen, F. (2007). Chronic enteropathies in dogs:
6585   evaluation of risk factors for negative outcome. *J Vet Intern Med, 21*(4), 700-708.
6586 Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance.
6587   *Austral Ecology, 26*(1), 32-46. doi:doi:10.1111/j.1442-9993.2001.01070.pp.x
6588 Arboleya, S., Suarez, M., Fernandez, N., Mantecon, L., Solis, G., Gueimonde, M., & de Los
6589   Reyes-Gavilan, C. G. (2018). C-section and the Neonatal Gut Microbiome Acquisition:
6590   Consequences for Future Health. *Ann Nutr Metab, 73 Suppl 3*, 17-23.
6591   doi:10.1159/000490843

Ardissone, A. N., de la Cruz, D. M., Davis-Richardson, A. G., Rechcigl, K. T., Li, N., Drew, J. C., . . . Neu, J. (2014). Meconium microbiome analysis identifies bacteria correlated with premature birth. *PLoS One, 9*(3), e90784. doi:10.1371/journal.pone.0090784

Arima, K., Watanabe, N., Hanabuchi, S., Chang, M., Sun, S. C., & Liu, Y. J. (2010). Distinct signal codes generate dendritic cell functional plasticity. *Sci Signal, 3*(105), ra4. doi:10.1126/scisignal.2000567

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., . . . Bork, P. (2011). Enterotypes of the human gut microbiome. *Nature, 473*(7346), 174-180. doi:10.1038/nature09944

Aubry, C., Michon, C., Chain, F., Chvatchenko, Y., Goffin, L., Zimmerli, S. C., . . . Chatel, J. M. (2015). Protective effect of TSLP delivered at the gut mucosa level by recombinant lactic acid bacteria in DSS-induced colitis mouse model. *Microb Cell Fact, 14*(1), 176. doi:10.1186/s12934-015-0367-5

Aubry, C., Michon, C., Chain, F., Chvatchko, Y., Goffin, L., Zimmerli, S. C., . . . Chatel, J. M. (2016). Erratum to: Protective effect of TSLP delivered at the gut mucosa level by recombinant lactic acid bacteria in DSS-induced colitis mouse model. *Microb Cell Fact, 15*(1), 188. doi:10.1186/s12934-016-0592-6

Backhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., . . . Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A, 101*(44), 15718-15723. doi:10.1073/pnas.0407076101

Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., & Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science, 307*(5717), 1915-1920. doi:10.1126/science.1104816

Backhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., . . . Wang, J. (2015). Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host Microbe, 17*(6), 852. doi:10.1016/j.chom.2015.05.012

Bager, P., Wohlfahrt, J., & Westergaard, T. (2008). Caesarean delivery and risk of atopy and allergic disease: meta-analyses. *Clin Exp Allergy, 38*(4), 634-642. doi:10.1111/j.1365-2222.2008.02939.x

Basler, M., Pilhofer, M., Henderson, G. P., Jensen, G. J., & Mekalanos, J. J. (2012). Type VI secretion requires a dynamic contractile phage tail-like structure. *Nature, 483*(7388), 182-186. doi:10.1038/nature10846

Batt, R. M., Barnes, A., Rutgers, H. C., & Carter, S. D. (1991). Relative IgA deficiency and small intestinal bacterial overgrowth in German shepherd dogs. *Res Vet Sci, 50*(1), 106-111.

Belkaid, Y., & Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell, 157*(1), 121-141. doi:10.1016/j.cell.2014.03.011

Bell, J. A., Kopper, J. J., Turnbull, J. A., Barbu, N. I., Murphy, A. J., & Mansfield, L. S. (2008). Ecological characterization of the colonic microbiota of normal and diarrheic dogs. *Interdiscip Perspect Infect Dis, 2008*, 149694. doi:10.1155/2008/149694

Benno, Y., Nakao, H., Uchida, K., & Mitsuoka, T. (1992). Impact of the advances in age on the gastrointestinal microflora of beagle dogs. *J Vet Med Sci, 54*(4), 703-706.

Biagi, E., Candela, M., Fairweather-Tait, S., Franceschi, C., & Brigidi, P. (2012). Aging of the human metaorganism: the microbial counterpart. *Age (Dordr), 34*(1), 247-267. doi:10.1007/s11357-011-9217-5

Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., Pini, E., . . . De Vos, W. (2010). Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. *PLoS One, 5*(5), e10667. doi:10.1371/journal.pone.0010667

Biagi, E., Rampelli, S., Turroni, S., Quercia, S., Candela, M., & Brigidi, P. (2017). The gut microbiota of centenarians: Signatures of longevity in the gut microbiota profile. *Mech Ageing Dev, 165*(Pt B), 180-184. doi:10.1016/j.mad.2016.12.013

Biancheri, P., Di Sabatino, A., Rescigno, M., Giuffrida, P., Fornasa, G., Tsilingiri, K., . . . Corazza, G. R. (2015). Abnormal thymic stromal lymphopoietin expression in the duodenal mucosa of patients with coeliac disease. *Gut*. doi:10.1136/gutjnl-2014-308876

Bjerkan, L., Schreurs, O., Engen, S. A., Jahnsen, F. L., Baekkevold, E. S., Blix, I. J., & Schenck, K. (2015). The short form of TSLP is constitutively translated in human keratinocytes and has characteristics of an antimicrobial peptide. *Mucosal Immunol, 8*(1), 49-56. doi:10.1038/mi.2014.41

Black, H. E. (1988). The effects of steroids upon the gastrointestinal tract. *Toxicol Pathol, 16*(2), 213-222. doi:10.1177/019262338801600214

Boers, S. A., Jansen, R., & Hays, J. P. (2016). Suddenly everyone is a microbiota specialist. *Clin Microbiol Infect, 22*(7), 581-582. doi:10.1016/j.cmi.2016.05.002

Boivin, M. A., Ye, D., Kennedy, J. C., Al-Sadi, R., Shepela, C., & Ma, T. Y. (2007). Mechanism of glucocorticoid regulation of the intestinal tight junction barrier. *Am J Physiol Gastrointest Liver Physiol, 292*(2), G590-598. doi:10.1152/ajpgi.00252.2006

Borgo, P. V., Rodrigues, V. A., Feitosa, A. C., Xavier, K. C., & Avila-Campos, M. J. (2014). Association between periodontal condition and subgingival microbiota in women during pregnancy: a longitudinal study. *J Appl Oral Sci, 22*(6), 528-533. doi:10.1590/1678-775720140164

Brandtzaeg, P. (2006). The changing immunological paradigm in coeliac disease. *Immunol Lett, 105*(2), 127-139. doi:10.1016/j.imlet.2006.03.004

Brandtzaeg, P. (2010). Update on mucosal immunoglobulin A in gastrointestinal disease. *Curr Opin Gastroenterol, 26*(6), 554-563. doi:10.1097/MOG.0b013e32833dccf8

Brandtzaeg, P., Carlsen, H. S., & Halstensen, T. S. (2006). The B-cell system in inflammatory bowel disease. *Adv Exp Med Biol, 579*, 149-167. doi:10.1007/0-387-33778-4_10

Breitbart, M., Haynes, M., Kelley, S., Angly, F., Edwards, R. A., Felts, B., . . . Rohwer, F. (2008). Viral diversity and dynamics in an infant gut. *Res Microbiol, 159*(5), 367-373. doi:10.1016/j.resmic.2008.04.006

Buddington, R. K. (2003). Postnatal changes in bacterial populations in the gastrointestinal tract of dogs. *Am J Vet Res, 64*(5), 646-651.

Buffie, C. G., & Pamer, E. G. (2013). Microbiota-mediated colonization resistance against intestinal pathogens. *Nat Rev Immunol, 13*(11), 790-801. doi:10.1038/nri3535

Bunker, J. J., Erickson, S. A., Flynn, T. M., Henry, C., Koval, J. C., Meisel, M., . . . Bendelac, A. (2017). Natural polyreactive IgA antibodies coat the intestinal microbiota. *Science, 358*(6361). doi:10.1126/science.aan6619

Bunker, J. J., Flynn, T. M., Koval, J. C., Shaw, D. G., Meisel, M., McDonald, B. D., . . . Bendelac, A. (2015). Innate and Adaptive Humoral Responses Coat Distinct Commensal Bacteria with Immunoglobulin A. *Immunity, 43*(3), 541-553. doi:10.1016/j.immuni.2015.08.007

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., . . . Wittwer, C. T. (2009). The MIQE guidelines: minimum information for publication of quantitative

real-time PCR experiments. *Clin Chem, 55*(4), 611-622.
doi:10.1373/clinchem.2008.112797

Calder, P. C. (2005). Polyunsaturated fatty acids and inflammation. *Biochem Soc Trans, 33*(Pt 2), 423-427. doi:10.1042/bst0330423

Campbell, K. L. N., C.; Zuckermann, F.A. (1991). Immunoglobulin A deficiency in the dog. *Canine Practice, 16*(4), 7-11.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., . . . Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods, 7*(5), 335-336. doi:10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Costello, E. K., Berg-Lyons, D., Gonzalez, A., Stombaugh, J., . . . Knight, R. (2011). Moving pictures of the human microbiome. *Genome Biol, 12*(5), R50. doi:10.1186/gb-2011-12-5-r50

Cassmann, E., White, R., Atherly, T., Wang, C., Sun, Y., Khoda, S., . . . Jergens, A. (2016). Alterations of the Ileal and Colonic Mucosal Microbiota in Canine Chronic Enteropathies. *PLoS One, 11*(2), e0147321. doi:10.1371/journal.pone.0147321

Cerquetella, M., Spaterna, A., Laus, F., Tesei, B., Rossi, G., Antonelli, E., . . . Bassotti, G. (2010). Inflammatory bowel disease in the dog: differences and similarities with humans. *World J Gastroenterol, 16*(9), 1050-1056.

Cerutti, A., Chen, K., & Chorny, A. (2011). Immunoglobulin responses at the mucosal interface. *Annu Rev Immunol, 29*, 273-293. doi:10.1146/annurev-immunol-031210-101317

Cerutti, A., & Rescigno, M. (2008). The biology of intestinal immunoglobulin A responses. *Immunity, 28*(6), 740-750. doi:10.1016/j.immuni.2008.05.001

Ci, X., Chu, X., Chen, C., Li, X., Yan, S., Wang, X., . . . Deng, X. (2011). Oxytetracycline attenuates allergic airway inflammation in mice via inhibition of the NF-kappaB pathway. *J Clin Immunol, 31*(2), 216-227. doi:10.1007/s10875-010-9481-7

Claesson, M. J., Cusack, S., O'Sullivan, O., Greene-Diniz, R., de Weerd, H., Flannery, E., . . . O'Toole, P. W. (2011). Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proc Natl Acad Sci U S A, 108 Suppl 1*, 4586-4591. doi:10.1073/pnas.1000097107

Cohn, I., Jr., & Heneghan, J. B. (1991). Germfree animals and technics in surgical research. *Am J Surg, 161*(2), 279-283.

Collado, M. C., Cernada, M., Bauerl, C., Vento, M., & Perez-Martinez, G. (2012). Microbial ecology and host-microbiota interactions during early life stages. *Gut Microbes, 3*(4), 352-365. doi:10.4161/gmic.21215

Collado, M. C., Isolauri, E., Laitinen, K., & Salminen, S. (2008). Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. *Am J Clin Nutr, 88*(4), 894-899.

Collado, M. C., Rautava, S., Aakko, J., Isolauri, E., & Salminen, S. (2016). Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Sci Rep, 6*, 23129. doi:10.1038/srep23129

consortium, T. h. m. p. (2012). Structure, function and diversity of the healthy human microbiome. *Nature, 486*(7402), 207-214. doi:10.1038/nature11234

Costello, E. K., Lauber, C. L., Hamady, M., Fierer, N., Gordon, J. I., & Knight, R. (2009). Bacterial community variation in human body habitats across space and time. *Science, 326*(5960), 1694-1697. doi:10.1126/science.1177486

Cox, L. M., Yamanishi, S., Sohn, J., Alekseyenko, A. V., Leung, J. M., Cho, I., . . . Blaser, M. J. (2014). Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. *Cell, 158*(4), 705-721. doi:10.1016/j.cell.2014.05.052

Crabbe, P. A., Nash, D. R., Bazin, H., Eyssen, H., & Heremans, J. F. (1970). Immunohistochemical observations on lymphoid tissues from conventional and germ-free mice. *Lab Invest, 22*(5), 448-457.

Craven, M., Mansfield, C. S., & Simpson, K. W. (2011). Granulomatous colitis of boxer dogs. *Vet Clin North Am Small Anim Pract, 41*(2), 433-445. doi:10.1016/j.cvsm.2011.01.003

Craven M, M. J., Gao C. (2010). *Genome-wide association scan reveals NFC2 polymorphism in boxers with adherent invasive E.coli associated granulomatous colitis: a potential model of chronic granulomatous disease*. Paper presented at the DDW.

Cullender, T. C., Chassaing, B., Janzon, A., Kumar, K., Muller, C. E., Werner, J. J., . . . Ley, R. E. (2013). Innate and adaptive immunity interact to quench microbiome flagellar motility in the gut. *Cell Host Microbe, 14*(5), 571-581. doi:10.1016/j.chom.2013.10.009

Cunningham-Rundles, C. (2001). Physiology of IgA and IgA deficiency. *J Clin Immunol, 21*(5), 303-309.

Czinn, S. J., Cai, A., & Nedrud, J. G. (1993). Protection of germ-free mice from infection by Helicobacter felis after active oral or passive IgA immunization. *Vaccine, 11*(6), 637-642.

Chaban, B., Links, M. G., & Hill, J. E. (2012). A molecular enrichment strategy based on cpn60 for detection of epsilon-proteobacteria in the dog fecal microbiome. *Microb Ecol, 63*(2), 348-357. doi:10.1007/s00248-011-9931-7

Chung, J. Y., Sung, E. J., Cho, C. G., Seo, K. W., Lee, J. S., Bhang, D. H., . . . Kim, C. J. (2009). Effect of recombinant lactobacillus expressing canine GM-CSF on immune function in dogs. *J Microbiol Biotechnol, 19*(11), 1401-1407.

D'Auria, G., Peris-Bondia, F., Dzunkova, M., Mira, A., Collado, M. C., Latorre, A., & Moya, A. (2013). Active and secreted IgA-coated bacterial fractions from the human gut reveal an under-represented microbiota core. *Sci Rep, 3*, 3515. doi:10.1038/srep03515

Dandrieux, J. R. (2016). Inflammatory bowel disease versus chronic enteropathy in dogs: are they one and the same? *J Small Anim Pract, 57*(11), 589-599. doi:10.1111/jsap.12588

Day, M. J. (2012). *Clinical immunology of the dog and the cat*: CRC Press.

De Filippo, C., Di Paola, M., Ramazzotti, M., Albanese, D., Pieraccini, G., Banci, E., . . . Lionetti, P. (2017). Diet, Environments, and Gut Microbiota. A Preliminary Investigation in Children Living in Rural and Urban Burkina Faso and Italy. *Front Microbiol, 8*, 1979. doi:10.3389/fmicb.2017.01979

De La Cochetiere, M. F., Durand, T., Lepage, P., Bourreille, A., Galmiche, J. P., & Dore, J. (2005). Resilience of the dominant human fecal microbiota upon short-course antibiotic challenge. *J Clin Microbiol, 43*(11), 5588-5592. doi:10.1128/jcm.43.11.5588-5592.2005

De Palma, G., Nadal, I., Medina, M., Donat, E., Ribes-Koninckx, C., Calabuig, M., & Sanz, Y. (2010). Intestinal dysbiosis and reduced immunoglobulin-coated bacteria associated with coeliac disease in children. *BMC Microbiol, 10*, 63. doi:10.1186/1471-2180-10-63

DeBey, M. C. (2010). Primary immunodeficiencies of dogs and cats. *Vet Clin North Am Small Anim Pract, 40*(3), 425-438. doi:10.1016/j.cvsm.2010.01.001

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res, 54*(9), 2325-2340. doi:10.1194/jlr.R036012

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., . . . Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol, 72*(7), 5069-5072. doi:10.1128/aem.03006-05

Dethlefsen, L., Huse, S., Sogin, M. L., & Relman, D. A. (2008). The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol, 6*(11), e280. doi:10.1371/journal.pbio.0060280

Dicksved, J., Floistrup, H., Bergstrom, A., Rosenquist, M., Pershagen, G., Scheynius, A., . . . Jansson, J. K. (2007). Molecular fingerprinting of the fecal microbiota of children raised according to different lifestyles. *Appl Environ Microbiol, 73*(7), 2284-2289. doi:10.1128/aem.02223-06

DiGiulio, D. B. (2012). Diversity of microbes in amniotic fluid. *Semin Fetal Neonatal Med, 17*(1), 2-11. doi:10.1016/j.siny.2011.10.001

DiGiulio, D. B., Callahan, B. J., McMurdie, P. J., Costello, E. K., Lyell, D. J., Robaczewska, A., . . . Relman, D. A. (2015). Temporal and spatial variation of the human microbiota during pregnancy. *Proc Natl Acad Sci U S A, 112*(35), 11060-11065. doi:10.1073/pnas.1502875112

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., & Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci U S A, 107*(26), 11971-11975. doi:10.1073/pnas.1002601107

Dominguez-Bello, M. G., De Jesus-Laboy, K. M., Shen, N., Cox, L. M., Amir, A., Gonzalez, A., . . . Clemente, J. C. (2016). Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer. *Nat Med, 22*(3), 250-253. doi:10.1038/nm.4039

Donaldson, G. P., Ladinsky, M. S., Yu, K. B., Sanders, J. G., Yoo, B. B., Chou, W. C., . . . Mazmanian, S. K. (2018). Gut microbiota utilize immunoglobulin A for mucosal colonization. *Science, 360*(6390), 795-800. doi:10.1126/science.aaq0926

Donaldson, G. P., Lee, S. M., & Mazmanian, S. K. (2016). Gut biogeography of the bacterial microbiota. *Nat Rev Microbiol, 14*(1), 20-32. doi:10.1038/nrmicro3552

Donohoe, D. R., Holley, D., Collins, L. B., Montgomery, S. A., Whitmore, A. C., Hillhouse, A., . . . Bultman, S. J. (2014). A gnotobiotic mouse model demonstrates that dietary fiber protects against colorectal tumorigenesis in a microbiota- and butyrate-dependent manner. *Cancer Discov, 4*(12), 1387-1397. doi:10.1158/2159-8290.cd-14-0501

Duchmann, R., Kaiser, I., Hermann, E., Mayet, W., Ewe, K., & Meyer zum Buschenfelde, K. H. (1995). Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *Clin Exp Immunol, 102*(3), 448-455.

Duncan, S. H., Barcenilla, A., Stewart, C. S., Pryde, S. E., & Flint, H. J. (2002). Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. *Appl Environ Microbiol, 68*(10), 5186-5190.

Duvallet, C., Gibbons, S. M., Gurry, T., Irizarry, R. A., & Alm, E. J. (2017). Meta-analysis of gut microbiome studies identifies disease-specific and shared responses. *Nat Commun, 8*(1), 1784. doi:10.1038/s41467-017-01973-8

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., . . . Relman, D. A. (2005). Diversity of the human intestinal microbial flora. *Science, 308*(5728), 1635-1638. doi:10.1126/science.1110591

Eckmann, L., & Stappenbeck, T. S. (2015). IgG "detoxes" the intestinal mucosa. *Cell Host Microbe, 17*(5), 538-539. doi:10.1016/j.chom.2015.05.001

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics, 26*(19), 2460-2461. doi:10.1093/bioinformatics/btq461

Elinav, E., Strowig, T., Kau, A. L., Henao-Mejia, J., Thaiss, C. A., Booth, C. J., . . . Flavell, R. A. (2011). NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell, 145*(5), 745-757. doi:10.1016/j.cell.2011.04.022

Elliott, D. R., Wilson, M., Buckley, C. M., & Spratt, D. A. (2005). Cultivable oral microbiota of domestic dogs. *J Clin Microbiol, 43*(11), 5470-5476. doi:10.1128/jcm.43.11.5470-5476.2005

Endt, K., Stecher, B., Chaffron, S., Slack, E., Tchitchek, N., Benecke, A., . . . Hardt, W. D. (2010). The microbiota mediates pathogen clearance from the gut lumen after non-typhoidal Salmonella diarrhea. *PLoS Pathog, 6*(9), e1001097. doi:10.1371/journal.ppat.1001097

Eri, R. D., Adams, R. J., Tran, T. V., Tong, H., Das, I., Roche, D. K., . . . McGuckin, M. A. (2011). An intestinal epithelial defect conferring ER stress results in inflammation involving both innate and adaptive immunity. *Mucosal Immunol, 4*(3), 354-364. doi:10.1038/mi.2010.74

Fadlallah, J., El Kafsi, H., Sterlin, D., Juste, C., Parizot, C., Dorgham, K., . . . Gorochov, G. (2018). Microbial ecology perturbation in human IgA deficiency. *Sci Transl Med, 10*(439). doi:10.1126/scitranslmed.aan1217

Fagarasan, S., Muramatsu, M., Suzuki, K., Nagaoka, H., Hiai, H., & Honjo, T. (2002). Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. *Science, 298*(5597), 1424-1427. doi:10.1126/science.1077336

Faith, J. J., Guruge, J. L., Charbonneau, M., Subramanian, S., Seedorf, H., Goodman, A. L., . . . Gordon, J. I. (2013). The long-term stability of the human gut microbiota. *Science, 341*(6141), 1237439. doi:10.1126/science.1237439

Fallani, M., Young, D., Scott, J., Norin, E., Amarri, S., Adam, R., . . . Dore, J. (2010). Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *J Pediatr Gastroenterol Nutr, 51*(1), 77-84. doi:10.1097/MPG.0b013e3181d1b11e

Farrell, R. J., & Kelleher, D. (2003). Glucocorticoid resistance in inflammatory bowel disease. *J Endocrinol, 178*(3), 339-346.

Feldman E, N. R. (2004). *Canine and Feline Endocrinology and Reproduction*: Elsevier Health Sciences.

Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Gorfer, V., Fedi, S., . . . Segata, N. (2018). Mother-to-Infant Microbial Transmission from Different Body Sites Shapes the Developing Infant Gut Microbiome. *Cell Host Microbe, 24*(1), 133-145.e135. doi:10.1016/j.chom.2018.06.005

Finlay, C. M., Walsh, K. P., & Mills, K. H. (2014). Induction of regulatory cells by helminth parasites: exploitation for the treatment of inflammatory diseases. *Immunol Rev, 259*(1), 206-230. doi:10.1111/imr.12164

Fischer, A., Gluth, M., Weege, F., Pape, U. F., Wiedenmann, B., Baumgart, D. C., & Theuring, F. (2014). Glucocorticoids regulate barrier function and claudin expression in intestinal epithelial cells via MKP-1. *Am J Physiol Gastrointest Liver Physiol, 306*(3), G218-228. doi:10.1152/ajpgi.00095.2013

6864 Flancman, R., Singh, A., & Weese, J. S. (2018). Evaluation of the impact of dental prophylaxis
6865   on the oral microbiota of dogs. *PLoS One, 13*(6), e0199676.
6866   doi:10.1371/journal.pone.0199676
6867 Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P., & Forano, E. (2012). Microbial degradation of
6868   complex carbohydrates in the gut. *Gut Microbes, 3*(4), 289-306. doi:10.4161/gmic.19897
6869 Fogle, J. E., & Bissett, S. A. (2007). Mucosal immunity and chronic idiopathic enteropathies in
6870   dogs. *Compend Contin Educ Vet, 29*(5), 290-302; quiz 306.
6871 Forbes, S. J., Bumpus, T., McCarthy, E. A., Corthesy, B., & Mantis, N. J. (2011). Transient
6872   suppression of Shigella flexneri type 3 secretion by a protective O-antigen-specific
6873   monoclonal IgA. *MBio, 2*(3), e00042-00011. doi:10.1128/mBio.00042-11
6874 Fornasa, G., Tsilingiri, K., Caprioli, F., Botti, F., Mapelli, M., Meller, S., . . . Rescigno, M.
6875   (2015). Dichotomy of short and long thymic stromal lymphopoietin isoforms in
6876   inflammatory disorders of the bowel and skin. *J Allergy Clin Immunol, 136*(2), 413-422.
6877   doi:10.1016/j.jaci.2015.04.011
6878 Fragiadakis, G. K., Smits, S. A., Sonnenburg, E. D., Van Treuren, W., Reid, G., Knight, R., . . .
6879   Sonnenburg, J. L. (2018). Links between environment, diet, and the hunter-gatherer
6880   microbiome. *Gut Microbes*, 1-12. doi:10.1080/19490976.2018.1494103
6881 Francavilla, R., Cristofori, F., Tripaldi, M. E., & Indrio, F. (2018). Intervention for Dysbiosis in
6882   Children Born by C-Section. *Ann Nutr Metab, 73 Suppl 3*, 33-39. doi:10.1159/000490847
6883 Francino, M. P. (2018). Birth Mode-Related Differences in Gut Microbiota Colonization and
6884   Immune System Development. *Ann Nutr Metab, 73 Suppl 3*, 12-16.
6885   doi:10.1159/000490842
6886 Frank, D. N., St Amand, A. L., Feldman, R. A., Boedeker, E. C., Harpaz, N., & Pace, N. R.
6887   (2007). Molecular-phylogenetic characterization of microbial community imbalances in
6888   human inflammatory bowel diseases. *Proc Natl Acad Sci U S A, 104*(34), 13780-13785.
6889   doi:10.1073/pnas.0706625104
6890 Frese, S. A., & Mills, D. A. (2015). Birth of the infant gut microbiome: moms deliver twice! *Cell
6891   Host Microbe, 17*(5), 543-544. doi:10.1016/j.chom.2015.04.014
6892 Friend, S. L., Hosier, S., Nelson, A., Foxworthe, D., Williams, D. E., & Farr, A. (1994). A
6893   thymic stromal cell line supports in vitro development of surface IgM+ B cells and
6894   produces a novel growth factor affecting B and T lineage cells. *Exp Hematol, 22*(3), 321-
6895   328.
6896 Fujiwara, N., Tsuruda, K., Iwamoto, Y., Kato, F., Odaki, T., Yamane, N., . . . Noguchi, M.
6897   (2017). Significant increase of oral bacteria in the early pregnancy period in Japanese
6898   women. *J Investig Clin Dent, 8*(1). doi:10.1111/jicd.12189
6899 Garcia-Alvarez, L., & Oteo, J. A. (2010). [Nonantimicrobial effects of tetracyclines]. *Rev Esp
6900   Quimioter, 23*(1), 4-11.
6901 Garcia-Mazcorro, J. F., Dowd, S. E., Poulsen, J., Steiner, J. M., & Suchodolski, J. S. (2012).
6902   Abundance and short-term temporal variability of fecal microbiota in healthy dogs.
6903   *Microbiologyopen, 1*(3), 340-347. doi:10.1002/mbo3.36
6904 Garrido-Mesa, J., Algieri, F., Rodríguez-Nogales, A., Vezza, T., Utrilla, M. P., Garcia, F., . . .
6905   Gálvez, J. (2018). Immunomodulatory tetracyclines ameliorate DNBS-colitis: Impact on
6906   microRNA expression and microbiota composition. *Biochemical Pharmacology, 155*,
6907   524-536. doi:https://doi.org/10.1016/j.bcp.2018.07.044
6908 Garrido-Mesa, N., Camuesco, D., Arribas, B., Comalada, M., Bailon, E., Cueto-Sola, M., . . .
6909   Galvez, J. (2011). The intestinal anti-inflammatory effect of minocycline in experimental

6910   colitis involves both its immunomodulatory and antimicrobial properties. *Pharmacol Res,*
6911   *63*(4), 308-319. doi:10.1016/j.phrs.2010.12.011
6912 German, A. J., Hall, E. J., & Day, M. J. (1998). Measurement of IgG, IgM and IgA
6913   concentrations in canine serum, saliva, tears and bile. *Vet Immunol Immunopathol, 64*(2),
6914   107-121.
6915 German, A. J., Hall, E. J., & Day, M. J. (2000). Relative deficiency in IgA production by
6916   duodenal explants from German shepherd dogs with small intestinal disease. *Vet*
6917   *Immunol Immunopathol, 76*(1-2), 25-43.
6918 German, A. J., Helps, C. R., Hall, E. J., & Day, M. J. (2000). Cytokine mRNA expression in
6919   mucosal biopsies from German shepherd dogs with small intestinal enteropathies. *Dig*
6920   *Dis Sci, 45*(1), 7-17.
6921 Gevers, D., Kugathasan, S., Knights, D., Kostic, A. D., Knight, R., & Xavier, R. J. (2017). A
6922   Microbiome Foundation for the Study of Crohn's Disease. *Cell Host Microbe, 21*(3), 301-
6923   304. doi:10.1016/j.chom.2017.02.012
6924 Giguere, J., Presscott, J., & Baggot, J. (2006). *Antimicrobial therapy in veterinary medicine*:
6925   Blackwell Publishing.
6926 Gomez de Aguero, M., Ganal-Vonarburg, S. C., Fuhrer, T., Rupp, S., Uchimura, Y., Li, H., . . .
6927   Macpherson, A. J. (2016). The maternal microbiota drives early postnatal innate immune
6928   development. *Science, 351*(6279), 1296-1302. doi:10.1126/science.aad2571
6929 Gonzalez-Ortiz, G., Castillejos, L., Mallo, J. J., Angels Calvo-Torras, M., & Dolores Baucells,
6930   M. (2013). Effects of dietary supplementation of Bacillus amyloliquefaciens CECT 5940
6931   and Enterococcus faecium CECT 4515 in adult healthy dogs. *Arch Anim Nutr, 67*(5),
6932   406-415. doi:10.1080/1745039x.2013.830517
6933 Gonzalez-Perez, G., Hicks, A. L., Tekieli, T. M., Radens, C. M., Williams, B. L., & Lamouse-
6934   Smith, E. S. (2016). Maternal Antibiotic Treatment Impacts Development of the Neonatal
6935   Intestinal Microbiome and Antiviral Immunity. *J Immunol, 196*(9), 3768-3779.
6936   doi:10.4049/jimmunol.1502322
6937 Gophna, U., Sommerfeld, K., Gophna, S., Doolittle, W. F., & Veldhuyzen van Zanten, S. J.
6938   (2006). Differences between tissue-associated intestinal microfloras of patients with
6939   Crohn's disease and ulcerative colitis. *J Clin Microbiol, 44*(11), 4136-4141.
6940   doi:10.1128/jcm.01004-06
6941 Griffin, N. W., Ahern, P. P., Cheng, J., Heath, A. C., Ilkayeva, O., Newgard, C. B., . . . Gordon,
6942   J. I. (2017). Prior Dietary Practices and Connections to a Human Gut Microbial
6943   Metacommunity Alter Responses to Diet Interventions. *Cell Host Microbe, 21*(1), 84-96.
6944   doi:10.1016/j.chom.2016.12.006
6945 Grzeskowiak, L., Collado, M. C., Mangani, C., Maleta, K., Laitinen, K., Ashorn, P., . . .
6946   Salminen, S. (2012). Distinct gut microbiota in southeastern African and northern
6947   European infants. *J Pediatr Gastroenterol Nutr, 54*(6), 812-816.
6948   doi:10.1097/MPG.0b013e318249039c
6949 Grzeskowiak, L., Sales Teixeira, T. F., Bigonha, S. M., Lobo, G., Salminen, S., & Ferreira, C. L.
6950   (2015). Gut Bifidobacterium microbiota in one-month-old Brazilian newborns. *Anaerobe,*
6951   *35*(Pt B), 54-58. doi:10.1016/j.anaerobe.2015.07.004
6952 Guard, B. C., Barr, J. W., Reddivari, L., Klemashevich, C., Jayaraman, A., Steiner, J. M., . . .
6953   Suchodolski, J. S. (2015). Characterization of microbial dysbiosis and metabolomic
6954   changes in dogs with acute diarrhea. *PLoS One, 10*(5), e0127259.
6955   doi:10.1371/journal.pone.0127259

Guard, B. C., Mila, H., Steiner, J. M., Mariani, C., Suchodolski, J. S., & Chastant-Maillard, S. (2017). Characterization of the fecal microbiome during neonatal and early pediatric development in puppies. *PLoS One, 12*(4), e0175718. doi:10.1371/journal.pone.0175718

Gupta, S. (2017). Microbiome: Puppy power. *Nature, 543*, S48. doi:10.1038/543S48a

Gutzeit, C., Magri, G., & Cerutti, A. (2014). Intestinal IgA production and its role in host-microbe interaction. *Immunol Rev, 260*(1), 76-85. doi:10.1111/imr.12189

Hall, E. J. (2011). Antibiotic-responsive diarrhea in small animals. *Vet Clin North Am Small Anim Pract, 41*(2), 273-286. doi:10.1016/j.cvsm.2010.12.004

Hall, M. W., Singh, N., Ng, K. F., Lam, D. K., Goldberg, M. B., Tenenbaum, H. C., . . . Senadheera, D. B. (2017). Inter-personal diversity and temporal dynamics of dental, tongue, and salivary microbiota in the healthy oral cavity. *NPJ Biofilms and Microbiomes, 3*, 2. doi:10.1038/s41522-016-0011-0

Handl, S., Dowd, S. E., Garcia-Mazcorro, J. F., Steiner, J. M., & Suchodolski, J. S. (2011). Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. *FEMS Microbiol Ecol, 76*(2), 301-310. doi:10.1111/j.1574-6941.2011.01058.x

Hang, I., Heilmann, R. M., Grutzner, N., Suchodolski, J. S., Steiner, J. M., Atroshi, F., . . . Spillmann, T. (2013). Impact of diets with a high content of greaves-meal protein or carbohydrates on faecal characteristics, volatile fatty acids and faecal calprotectin concentrations in healthy dogs. *BMC Vet Res, 9*, 201. doi:10.1186/1746-6148-9-201

Hansen, R., Berry, S. H., Mukhopadhya, I., Thomson, J. M., Saunders, K. A., Nicholl, C. E., . . . Hold, G. L. (2013). The microaerophilic microbiota of de-novo paediatric inflammatory bowel disease: the BISCUIT study. *PLoS One, 8*(3), e58825. doi:10.1371/journal.pone.0058825

Harmsen, H. J., Pouwels, S. D., Funke, A., Bos, N. A., & Dijkstra, G. (2012). Crohn's disease patients have more IgG-binding fecal bacteria than controls. *Clin Vaccine Immunol, 19*(4), 515-521. doi:10.1128/cvi.05517-11

Harmsen, H. J., Wildeboer-Veloo, A. C., Raangs, G. C., Wagendorp, A. A., Klijn, N., Bindels, J. G., & Welling, G. W. (2000). Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr, 30*(1), 61-67.

Haro, C., Garcia-Carpintero, S., Alcala-Diaz, J. F., Gomez-Delgado, F., Delgado-Lista, J., Perez-Martinez, P., . . . Perez-Jimenez, F. (2016). The gut microbial community in metabolic syndrome patients is modified by diet. *J Nutr Biochem, 27*, 27-31. doi:10.1016/j.jnutbio.2015.08.011

Heilmann, R. M., & Suchodolski, J. S. (2015). Is inflammatory bowel disease in dogs and cats associated with a Th1 or Th2 polarization? *Vet Immunol Immunopathol, 168*(3-4), 131-134. doi:10.1016/j.vetimm.2015.10.008

Hiippala, K., Kainulainen, V., Kalliomaki, M., Arkkila, P., & Satokari, R. (2016). Mucosal Prevalence and Interactions with the Epithelium Indicate Commensalism of Sutterella spp. *Front Microbiol, 7*, 1706. doi:10.3389/fmicb.2016.01706

Hooda, S., Minamoto, Y., Suchodolski, J. S., & Swanson, K. S. (2012). Current state of knowledge: the canine gastrointestinal microbiome. *Anim Health Res Rev, 13*(1), 78-88. doi:10.1017/s1466252312000059

Hooper, L. V., & Macpherson, A. J. (2010). Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol, 10*(3), 159-169. doi:10.1038/nri2710

7002 Hu, J., Nomura, Y., Bashir, A., Fernandez-Hernandez, H., Itzkowitz, S., Pei, Z., . . . Peter, I.
7003   (2013). Diversified Microbiota of Meconium Is Affected by Maternal Diabetes Status.
7004   *PLoS One, 8*(11), e78257. doi:10.1371/journal.pone.0078257
7005 Igarashi, H., Maeda, S., Ohno, K., Horigome, A., Odamaki, T., & Tsujimoto, H. (2014). Effect
7006   of oral administration of metronidazole or prednisolone on fecal microbiota in dogs.
7007   *PLoS One, 9*(9), e107909. doi:10.1371/journal.pone.0107909
7008 invivogen. http://www.invivogen.com.
7009 Isaiah, A., Hoffmann, A. R., Kelley, R., Mundell, P., Steiner, J. M., & Suchodolski, J. S. (2017).
7010   Characterization of the nasal and oral microbiota of detection dogs. *PLoS One, 12*(9),
7011   e0184899. doi:10.1371/journal.pone.0184899
7012 Jackson, M. A., Jeffery, I. B., Beaumont, M., Bell, J. T., Clark, A. G., Ley, R. E., . . . Steves, C.
7013   J. (2016). Signatures of early frailty in the gut microbiota. *Genome Med, 8*(1), 8.
7014   doi:10.1186/s13073-016-0262-7
7015 Jakobsson, H. E., Abrahamsson, T. R., Jenmalm, M. C., Harris, K., Quince, C., Jernberg, C., . . .
7016   Andersson, A. F. (2014). Decreased gut microbiota diversity, delayed Bacteroidetes
7017   colonisation and reduced Th1 responses in infants delivered by caesarean section. *Gut,*
7018   *63*(4), 559-566. doi:10.1136/gutjnl-2012-303249
7019 Jandhyala, S. M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., & Reddy, D. N.
7020   (2015). Role of the normal gut microbiota. *World Journal of Gastroenterology : WJG,*
7021   *21*(29), 8787-8803. doi:10.3748/wjg.v21.i29.8787
7022 Jergens, A. E., Gamet, Y., Moore, F. M., Niyo, Y., Tsao, C., & Smith, B. (1999). Colonic
7023   lymphocyte and plasma cell populations in dogs with lymphocytic-plasmacytic colitis.
7024   *Am J Vet Res, 60*(4), 515-520.
7025 Jergens, A. E., Moore, F. M., Kaiser, M. S., Haynes, J. S., & Kinyon, J. M. (1996).
7026   Morphometric evaluation of immunoglobulin A-containing and immunoglobulin G-
7027   containing cells and T cells in duodenal mucosa from healthy dogs and from dogs with
7028   inflammatory bowel disease or nonspecific gastroenteritis. *Am J Vet Res, 57*(5), 697-704.
7029 Jia, J., Frantz, N., Khoo, C., Gibson, G. R., Rastall, R. A., & McCartney, A. L. (2010).
7030   Investigation of the faecal microbiota associated with canine chronic diarrhoea. *FEMS*
7031   *Microbiol Ecol, 71*(2), 304-312. doi:10.1111/j.1574-6941.2009.00812.x
7032 Jimenez, E., Fernandez, L., Marin, M. L., Martin, R., Odriozola, J. M., Nueno-Palop, C., . . .
7033   Rodriguez, J. M. (2005). Isolation of commensal bacteria from umbilical cord blood of
7034   healthy neonates born by cesarean section. *Curr Microbiol, 51*(4), 270-274.
7035   doi:10.1007/s00284-005-0020-3
7036 Johansen, F. E., Pekna, M., Norderhaug, I. N., Haneberg, B., Hietala, M. A., Krajci, P., . . .
7037   Brandtzaeg, P. (1999). Absence of epithelial immunoglobulin A transport, with increased
7038   mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient
7039   mice. *J Exp Med, 190*(7), 915-922.
7040 Jorgensen, G. H., Gardulf, A., Sigurdsson, M. I., Sigurdardottir, S. T., Thorsteinsdottir, I.,
7041   Gudmundsson, S., . . . Ludviksson, B. R. (2013). Clinical symptoms in adults with
7042   selective IgA deficiency: a case-control study. *J Clin Immunol, 33*(4), 742-747.
7043   doi:10.1007/s10875-012-9858-x
7044 Kalenyak, K., Isaiah, A., Heilmann, R. M., Suchodolski, J. S., & Burgener, I. A. (2018).
7045   Comparison of the intestinal mucosal microbiota in dogs diagnosed with idiopathic
7046   inflammatory bowel disease and dogs with food-responsive diarrhea before and after
7047   treatment. *FEMS Microbiol Ecol, 94*(2). doi:10.1093/femsec/fix173

7048 Kalliomaki, M., Collado, M. C., Salminen, S., & Isolauri, E. (2008). Early differences in fecal
7049   microbiota composition in children may predict overweight. *Am J Clin Nutr, 87*(3), 534-
7050   538.
7051 Kamada, N., Sakamoto, K., Seo, S. U., Zeng, M. Y., Kim, Y. G., Cascalho, M., . . . Nunez, G.
7052   (2015). Humoral Immunity in the Gut Selectively Targets Phenotypically Virulent
7053   Attaching-and-Effacing Bacteria for Intraluminal Elimination. *Cell Host Microbe, 17*(5),
7054   617-627. doi:10.1016/j.chom.2015.04.001
7055 Kathrani, A., House, A., Catchpole, B., Murphy, A., German, A., Werling, D., & Allenspach, K.
7056   (2010). Polymorphisms in the TLR4 and TLR5 gene are significantly associated with
7057   inflammatory bowel disease in German shepherd dogs. *PLoS One, 5*(12), e15740.
7058   doi:10.1371/journal.pone.0015740
7059 Kathrani, A., House, A., Catchpole, B., Murphy, A., Werling, D., & Allenspach, K. (2011).
7060   Breed-independent toll-like receptor 5 polymorphisms show association with canine
7061   inflammatory bowel disease. *Tissue Antigens, 78*(2), 94-101. doi:10.1111/j.1399-
7062   0039.2011.01707.x
7063 Kau, A. L., Planer, J. D., Liu, J., Rao, S., Yatsunenko, T., Trehan, I., . . . Gordon, J. I. (2015).
7064   Functional characterization of IgA-targeted bacterial taxa from undernourished Malawian
7065   children that produce diet-dependent enteropathy. *Sci Transl Med, 7*(276), 276ra224.
7066   doi:10.1126/scitranslmed.aaa4877
7067 Kerr, K. R., Forster, G., Dowd, S. E., Ryan, E. P., & Swanson, K. S. (2013). Effects of dietary
7068   cooked navy bean on the fecal microbiome of healthy companion dogs. *PLoS One, 8*(9),
7069   e74998. doi:10.1371/journal.pone.0074998
7070 Kett, K., & Brandtzaeg, P. (1987). Local IgA subclass alterations in ulcerative colitis and
7071   Crohn's disease of the colon. *Gut, 28*(8), 1013-1021.
7072 Khan, M. J., Gerasimidis, K., Edwards, C. A., & Shaikh, M. G. (2016). Role of Gut Microbiota
7073   in the Aetiology of Obesity: Proposed Mechanisms and Review of the Literature. *Journal
7074   of Obesity, 2016*, 7353642. doi:10.1155/2016/7353642
7075 Kim, S., & Jazwinski, S. M. (2015). Quantitative measures of healthy aging and biological age.
7076   *Healthy Aging Res, 4*. doi:10.12715/har.2015.4.26
7077 Kim, S., & Jazwinski, S. M. (2018). The Gut Microbiota and Healthy Aging: A Mini-Review.
7078   *Gerontology*, 1-8. doi:10.1159/000490615
7079 Klukowska-Rotzler, J., Chervet, L., Muller, E. J., Roosje, P., Marti, E., & Janda, J. (2013).
7080   Expression of thymic stromal lymphopoietin in canine atopic dermatitis. *Vet Dermatol,
7081   24*(1), 54-59.e13-54. doi:10.1111/j.1365-3164.2012.01096.x
7082 Koboziev, I., Reinoso Webb, C., Furr, K. L., & Grisham, M. B. (2014). Role of the enteric
7083   microbiota in intestinal homeostasis and inflammation. *Free Radic Biol Med, 68*, 122-
7084   133. doi:10.1016/j.freeradbiomed.2013.11.008
7085 Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., . . . Ley, R. E.
7086   (2011). Succession of microbial consortia in the developing infant gut microbiome. *Proc
7087   Natl Acad Sci U S A, 108 Suppl 1*, 4578-4585. doi:10.1073/pnas.1000081107
7088 Kolodziejska-Sawerska, A., Rychlik, A., Depta, A., Wdowiak, M., Nowicki, M., & Kander, M.
7089   (2013). Cytokines in canine inflammatory bowel disease. *Pol J Vet Sci, 16*(1), 165-171.
7090 Konstantinov, S. R., van der Woude, C. J., & Peppelenbosch, M. P. (2013). Do pregnancy-
7091   related changes in the microbiome stimulate innate immunity? *Trends Mol Med, 19*(8),
7092   454-459. doi:10.1016/j.molmed.2013.06.002

Koren, O., Goodrich, J. K., Cullender, T. C., Spor, A., Laitinen, K., Backhed, H. K., . . . Ley, R. E. (2012). Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell, 150*(3), 470-480. doi:10.1016/j.cell.2012.07.008

Koren, O., Knights, D., Gonzalez, A., Waldron, L., Segata, N., Knight, R., . . . Ley, R. E. (2013). A guide to enterotypes across the human body: meta-analysis of microbial community structures in human microbiome datasets. *PLoS Comput Biol, 9*(1), e1002863. doi:10.1371/journal.pcbi.1002863

Koskinen, S. (1996). Long-term follow-up of health in blood donors with primary selective IgA deficiency. *J Clin Immunol, 16*(3), 165-170.

Kostic, A. D., Gevers, D., Siljander, H., Vatanen, T., Hyotylainen, T., Hamalainen, A. M., . . . Xavier, R. J. (2015). The dynamics of the human infant gut microbiome in development and in progression toward type 1 diabetes. *Cell Host Microbe, 17*(2), 260-273. doi:10.1016/j.chom.2015.01.001

Kostic, A. D., Xavier, R. J., & Gevers, D. (2014). The microbiome in inflammatory bowel disease: current status and the future ahead. *Gastroenterology, 146*(6), 1489-1499. doi:10.1053/j.gastro.2014.02.009

La Rosa, P. S., Warner, B. B., Zhou, Y., Weinstock, G. M., Sodergren, E., Hall-Moore, C. M., . . . Tarr, P. I. (2014). Patterned progression of bacterial populations in the premature infant gut. *Proc Natl Acad Sci U S A, 111*(34), 12522-12527. doi:10.1073/pnas.1409497111

Laing, B., Barnett, M. P. G., Marlow, G., Nasef, N. A., & Ferguson, L. R. (2018). An update on the role of gut microbiota in chronic inflammatory diseases, and potential therapeutic targets. *Expert Rev Gastroenterol Hepatol*, 1-15. doi:10.1080/17474124.2018.1505497

Landers, C. J., Cohavy, O., Misra, R., Yang, H., Lin, Y. C., Braun, J., & Targan, S. R. (2002). Selected loss of tolerance evidenced by Crohn's disease-associated immune responses to auto- and microbial antigens. *Gastroenterology, 123*(3), 689-699.

Langdon, A., Crook, N., & Dantas, G. (2016). The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation. *Genome Med, 8*(1), 39. doi:10.1186/s13073-016-0294-z

Lauder, A. P., Roche, A. M., Sherrill-Mix, S., Bailey, A., Laughlin, A. L., Bittinger, K., . . . Bushman, F. D. (2016). Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota. *Microbiome, 4*(1), 29. doi:10.1186/s40168-016-0172-3

Lavelle, A., Lennon, G., O'Sullivan, O., Docherty, N., Balfe, A., Maguire, A., . . . O'Connell, P. R. (2015). Spatial variation of the colonic microbiota in patients with ulcerative colitis and control volunteers. *Gut, 64*(10), 1553-1561. doi:10.1136/gutjnl-2014-307873

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., . . . Gordon, J. I. (2008). Evolution of mammals and their gut microbes. *Science, 320*(5883), 1647-1651. doi:10.1126/science.1155725

Li, H., Limenitakis, J. P., Fuhrer, T., Geuking, M. B., Lawson, M. A., Wyss, M., . . . Macpherson, A. J. (2015). The outer mucus layer hosts a distinct intestinal microbial niche. *Nature Communications, 6*, 8292. doi:10.1038/ncomms9292

Li, J., Jia, H., Cai, X., Zhong, H., Feng, Q., Sunagawa, S., . . . Wang, J. (2014). An integrated catalog of reference genes in the human gut microbiome. *Nat Biotechnol, 32*(8), 834-841. doi:10.1038/nbt.2942

7137 Lim, E. S., Zhou, Y., Zhao, G., Bauer, I. K., Droit, L., Ndao, I. M., . . . Holtz, L. R. (2015). Early
7138   life dynamics of the human gut virome and bacterial microbiome in infants. *Nat Med,*
7139   *21*(10), 1228-1234. doi:10.1038/nm.3950
7140 Littler, R. M., Batt, R. M., & Lloyd, D. H. (2006). Total and relative deficiency of gut mucosal
7141   IgA in German shepherd dogs demonstrated by faecal analysis. *Vet Rec, 158*(10), 334-
7142   341.
7143 Liu, Y. J. (2009). TSLP in epithelial cell and dendritic cell cross talk. *Adv Immunol, 101*, 1-25.
7144   doi:10.1016/s0065-2776(08)01001-8
7145 Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-
7146   time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods, 25*(4), 402-408.
7147   doi:10.1006/meth.2001.1262
7148 Loewen, K., Monchka, B., Mahmud, S. M., t Jong, G., & Azad, M. B. (2018). Prenatal antibiotic
7149   exposure and childhood asthma: a population-based study. *Eur Respir J, 52*(1).
7150   doi:10.1183/13993003.02070-2017
7151 Lotz, M., Gutle, D., Walther, S., Menard, S., Bogdan, C., & Hornef, M. W. (2006). Postnatal
7152   acquisition of endotoxin tolerance in intestinal epithelial cells. *J Exp Med, 203*(4), 973-
7153   984. doi:10.1084/jem.20050625
7154 Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and
7155   dispersion for RNA-seq data with DESeq2. *Genome Biol, 15*(12), 550.
7156   doi:10.1186/s13059-014-0550-8
7157 Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity,
7158   stability and resilience of the human gut microbiota. *Nature, 489*(7415), 220-230.
7159   doi:10.1038/nature11550
7160 Macfarlane, S., & Macfarlane, G. T. (2003). Regulation of short-chain fatty acid production.
7161   *Proc Nutr Soc, 62*(1), 67-72. doi:10.1079/pns2002207
7162 Macpherson, A. J., & McCoy, K. D. (2015). Independence Day for IgA. *Immunity, 43*(3), 416-
7163   418. doi:10.1016/j.immuni.2015.08.024
7164 Maeda, S., Ohno, K., Fujiwara-Igarashi, A., Tomiyasu, H., Fujino, Y., & Tsujimoto, H. (2014).
7165   Methylation of TNFRSF13B and TNFRSF13C in duodenal mucosa in canine
7166   inflammatory bowel disease and its association with decreased mucosal IgA expression.
7167   *Vet Immunol Immunopathol, 160*(1-2), 97-106. doi:10.1016/j.vetimm.2014.04.005
7168 Maeda, S., Ohno, K., Uchida, K., Nakashima, K., Fukushima, K., Tsukamoto, A., . . . Tsujimoto,
7169   H. (2013). Decreased immunoglobulin A concentrations in feces, duodenum, and
7170   peripheral blood mononuclear cells of dogs with inflammatory bowel disease. *J Vet
7171   Intern Med, 27*(1), 47-55. doi:10.1111/jvim.12023
7172 Magri, G., Comerma, L., Pybus, M., Sintes, J., Llige, D., Segura-Garzon, D., . . . Cerutti, A.
7173   (2017). Human Secretory IgM Emerges from Plasma Cells Clonally Related to Gut
7174   Memory B Cells and Targets Highly Diverse Commensals. *Immunity, 47*(1), 118-
7175   134.e118. doi:10.1016/j.immuni.2017.06.013
7176 Mandigers, P. J., Biourge, V., van den Ingh, T. S., Ankringa, N., & German, A. J. (2010). A
7177   randomized, open-label, positively-controlled field trial of a hydrolyzed protein diet in
7178   dogs with chronic small bowel enteropathy. *J Vet Intern Med, 24*(6), 1350-1357.
7179   doi:10.1111/j.1939-1676.2010.0632.x
7180 Mangin, I., Bonnet, R., Seksik, P., Rigottier-Gois, L., Sutren, M., Bouhnik, Y., . . . Dore, J.
7181   (2004). Molecular inventory of faecal microflora in patients with Crohn's disease. *FEMS
7182   Microbiol Ecol, 50*(1), 25-36. doi:10.1016/j.femsec.2004.05.005

Manichanh, C., Borruel, N., Casellas, F., & Guarner, F. (2012). The gut microbiota in IBD. *Nat Rev Gastroenterol Hepatol, 9*(10), 599-608. doi:10.1038/nrgastro.2012.152

Manrique, P., Bolduc, B., Walk, S. T., van der Oost, J., de Vos, W. M., & Young, M. J. (2016). Healthy human gut phageome. *Proc Natl Acad Sci U S A, 113*(37), 10400-10405. doi:10.1073/pnas.1601060113

Mariat, D., Firmesse, O., Levenez, F., Guimaraes, V., Sokol, H., Dore, J., . . . Furet, J. P. (2009). The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol, 9*, 123. doi:10.1186/1471-2180-9-123

Marks, S. L., Laflamme, D. P., & McAloose, D. (2002). Dietary trial using a commercial hypoallergenic diet containing hydrolyzed protein for dogs with inflammatory bowel disease. *Vet Ther, 3*(2), 109-118.

Marques, T. M., Wall, R., Ross, R. P., Fitzgerald, G. F., Ryan, C. A., & Stanton, C. (2010). Programming infant gut microbiota: influence of dietary and environmental factors. *Curr Opin Biotechnol, 21*(2), 149-156. doi:10.1016/j.copbio.2010.03.020

Martin, R., Heilig, H. G., Zoetendal, E. G., Jimenez, E., Fernandez, L., Smidt, H., & Rodriguez, J. M. (2007). Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. *Res Microbiol, 158*(1), 31-37. doi:10.1016/j.resmic.2006.11.004

Martinez, I., Stegen, J. C., Maldonado-Gomez, M. X., Eren, A. M., Siba, P. M., Greenhill, A. R., & Walter, J. (2015). The gut microbiota of rural papua new guineans: composition, diversity patterns, and ecological processes. *Cell Rep, 11*(4), 527-538. doi:10.1016/j.celrep.2015.03.049

Massacand, J. C., Stettler, R. C., Meier, R., Humphreys, N. E., Grencis, R. K., Marsland, B. J., & Harris, N. L. (2009). Helminth products bypass the need for TSLP in Th2 immune responses by directly modulating dendritic cell function. *Proc Natl Acad Sci U S A, 106*(33), 13968-13973. doi:10.1073/pnas.0906367106

Mathewson, N. D., Jenq, R., Mathew, A. V., Koenigsknecht, M., Hanash, A., Toubai, T., . . . Reddy, P. (2016). Gut microbiome-derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. *Nat Immunol, 17*(5), 505-513. doi:10.1038/ni.3400

Mazmanian, S. K., Round, J. L., & Kasper, D. L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature, 453*(7195), 620-625. doi:10.1038/nature07008

Mazzucchelli, R., Hixon, J. A., Spolski, R., Chen, X., Li, W. Q., Hall, V. L., . . . Durum, S. K. (2008). Development of regulatory T cells requires IL-7Ralpha stimulation by IL-7 or TSLP. *Blood, 112*(8), 3283-3292. doi:10.1182/blood-2008-02-137414

McConnell, E. L., Basit, A. W., & Murdan, S. (2008). Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments. *J Pharm Pharmacol, 60*(1), 63-70. doi:10.1211/jpp.60.1.0008

McMurdie, P. J., & Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One, 8*(4), e61217. doi:10.1371/journal.pone.0061217

Mentula, S., Harmoinen, J., Heikkila, M., Westermarck, E., Rautio, M., Huovinen, P., & Kononen, E. (2005). Comparison between cultured small-intestinal and fecal microbiotas in beagle dogs. *Appl Environ Microbiol, 71*(8), 4169-4175. doi:10.1128/aem.71.8.4169-4175.2005

Miller, W. H., Jr., Wellington, J. R., & Scott, D. W. (1992). Dermatologic disorders of Chinese Shar Peis: 58 cases (1981-1989). *J Am Vet Med Assoc, 200*(7), 986-990.

7229 Million, M., Lagier, J. C., Yahav, D., & Paul, M. (2013). Gut bacterial microbiota and obesity.
7230   *Clin Microbiol Infect, 19*(4), 305-313. doi:10.1111/1469-0691.12172
7231 Minamoto, Y., Otoni, C. C., Steelman, S. M., Buyukleblebici, O., Steiner, J. M., Jergens, A. E.,
7232   & Suchodolski, J. S. (2015). Alteration of the fecal microbiota and serum metabolite
7233   profiles in dogs with idiopathic inflammatory bowel disease. *Gut Microbes, 6*(1), 33-47.
7234   doi:10.1080/19490976.2014.997612
7235 Moon, C. D., Young, W., Maclean, P. H., Cookson, A. L., & Bermingham, E. N. (2018).
7236   Metagenomic insights into the roles of Proteobacteria in the gastrointestinal microbiomes
7237   of healthy dogs and cats. *Microbiologyopen*, e00677. doi:10.1002/mbo3.677
7238 Mor, G., & Cardenas, I. (2010). The immune system in pregnancy: a unique complexity. *Am J
7239   Reprod Immunol, 63*(6), 425-433. doi:10.1111/j.1600-0897.2010.00836.x
7240 Moreno, P. S., Wagner, J., Mansfield, C. S., Stevens, M., Gilkerson, J. R., & Kirkwood, C. D.
7241   (2017). Characterisation of the canine faecal virome in healthy dogs and dogs with acute
7242   diarrhoea using shotgun metagenomics. *PLoS One, 12*(6), e0178433.
7243   doi:10.1371/journal.pone.0178433
7244 Moroff, S. D., Hurvitz, A. I., Peterson, M. E., Saunders, L., & Noone, K. E. (1986). IgA
7245   deficiency in shar-pei dogs. *Vet Immunol Immunopathol, 13*(3), 181-188.
7246 Mshvildadze, M., & Neu, J. (2010). The infant intestinal microbiome: friend or foe? *Early Hum
7247   Dev, 86 Suppl 1*, 67-71. doi:10.1016/j.earlhumdev.2010.01.018
7248 Mulder, S. J., & Mulder-Bos, G. C. (2006). Most probable origin of coeliac disease is low
7249   immune globulin A in the intestine caused by malfunction of Peyer's patches. *Med
7250   Hypotheses, 66*(4), 757-762. doi:10.1016/j.mehy.2005.09.050
7251 Nakajima, A., Vogelzang, A., Maruya, M., Miyajima, M., Murata, M., Son, A., . . . Suzuki, K.
7252   (2018). IgA regulates the composition and metabolic function of gut microbiota by
7253   promoting symbiosis between bacteria. *J Exp Med, 215*(8), 2019-2034.
7254   doi:10.1084/jem.20180427
7255 NCBI. https://blast.ncbi.nlm.nih.gov/Blast.cgi.
7256 Newbern, D., & Freemark, M. (2011). Placental hormones and the control of maternal
7257   metabolism and fetal growth. *Curr Opin Endocrinol Diabetes Obes, 18*(6), 409-416.
7258   doi:10.1097/MED.0b013e32834c800d
7259 Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., & Pettersson, S.
7260   (2012). Host-gut microbiota metabolic interactions. *Science, 336*(6086), 1262-1267.
7261   doi:10.1126/science.1223813
7262 Noti, M., Wojno, E. D., Kim, B. S., Siracusa, M. C., Giacomin, P. R., Nair, M. G., . . . Artis, D.
7263   (2013). Thymic stromal lymphopoietin-elicited basophil responses promote eosinophilic
7264   esophagitis. *Nat Med, 19*(8), 1005-1013. doi:10.1038/nm.3281
7265 Nuriel-Ohayon, M., Neuman, H., & Koren, O. (2016). Microbial Changes during Pregnancy,
7266   Birth, and Infancy. *Front Microbiol, 7*, 1031. doi:10.3389/fmicb.2016.01031
7267 Oh, C., Lee, K., Cheong, Y., Lee, S.-W., Park, S.-Y., Song, C.-S., . . . Lee, J.-B. (2015).
7268   Comparison of the Oral Microbiomes of Canines and Their Owners Using Next-
7269   Generation Sequencing. *PLoS One, 10*(7), e0131468. doi:10.1371/journal.pone.0131468
7270 Olsson, M., Frankowiack, M., Tengvall, K., Roosje, P., Fall, T., Ivansson, E., . . . Hammarstrom,
7271   L. (2014). The dog as a genetic model for immunoglobulin A (IgA) deficiency:
7272   identification of several breeds with low serum IgA concentrations. *Vet Immunol
7273   Immunopathol, 160*(3-4), 255-259. doi:10.1016/j.vetimm.2014.05.010

Omori, M., Maeda, S., Igarashi, H., Ohno, K., Sakai, K., Yonezawa, T., . . . Matsuki, N. (2017). Fecal microbiome in dogs with inflammatory bowel disease and intestinal lymphoma. *J Vet Med Sci, 79*(11), 1840-1847. doi:10.1292/jvms.17-0045

Omori, M., & Ziegler, S. (2007). Induction of IL-4 expression in CD4(+) T cells by thymic stromal lymphopoietin. *J Immunol, 178*(3), 1396-1404.

Onderdonk, A. B., Hecht, J. L., McElrath, T. F., Delaney, M. L., Allred, E. N., & Leviton, A. (2008). Colonization of second-trimester placenta parenchyma. *Am J Obstet Gynecol, 199*(1), 52.e51-52.e10. doi:10.1016/j.ajog.2007.11.068

Osada, H., Ogawa, M., Hasegawa, A., Nagai, M., Shirai, J., Sasaki, K., . . . Ohmori, K. (2017). Expression of epithelial cell-derived cytokine genes in the duodenal and colonic mucosae of dogs with chronic enteropathy. *J Vet Med Sci, 79*(2), 393-397. doi:10.1292/jvms.16-0451

Pabst, O. (2012). New concepts in the generation and functions of IgA. *Nat Rev Immunol, 12*(12), 821-832. doi:10.1038/nri3322

Pacheco, A. R., Barile, D., Underwood, M. A., & Mills, D. A. (2015). The impact of the milk glycobiome on the neonate gut microbiota. *Annu Rev Anim Biosci, 3*, 419-445. doi:10.1146/annurev-animal-022114-111112

Palm, N. W., de Zoete, M. R., Cullen, T. W., Barry, N. A., Stefanowski, J., Hao, L., . . . Flavell, R. A. (2014). Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell, 158*(5), 1000-1010. doi:10.1016/j.cell.2014.08.006

Palmer, C., Bik, E. M., DiGiulio, D. B., Relman, D. A., & Brown, P. O. (2007). Development of the human infant intestinal microbiota. *PLoS Biol, 5*(7), e177. doi:10.1371/journal.pbio.0050177

Parthasarathy, G., Chen, J., Chen, X., Chia, N., O'Connor, H. M., Wolf, P. G., . . . Bharucha, A. E. (2016). Relationship Between Microbiota of the Colonic Mucosa vs Feces and Symptoms, Colonic Transit, and Methane Production in Female Patients With Chronic Constipation. *Gastroenterology, 150*(2), 367-379.e361. doi:10.1053/j.gastro.2015.10.005

Peiravan, A., Allenspach, K., Boag, A. M., Soutter, F., Holder, A., Catchpole, B., . . . Procoli, F. (2016). Single nucleotide polymorphisms in major histocompatibility class II haplotypes are associated with potential resistance to inflammatory bowel disease in German shepherd dogs. *Vet Immunol Immunopathol, 182*, 101-105. doi:10.1016/j.vetimm.2016.10.012

Peiravan, A., Bertolini, F., Rothschild, M. F., Simpson, K. W., Jergens, A. E., Allenspach, K., & Werling, D. (2018). Genome-wide association studies of inflammatory bowel disease in German shepherd dogs. *PLoS One, 13*(7), e0200685. doi:10.1371/journal.pone.0200685

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., . . . Stobberingh, E. E. (2006). Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics, 118*(2), 511-521. doi:10.1542/peds.2005-2824

Perez, P. F., Dore, J., Leclerc, M., Levenez, F., Benyacoub, J., Serrant, P., . . . Donnet-Hughes, A. (2007). Bacterial imprinting of the neonatal immune system: lessons from maternal cells? *Pediatrics, 119*(3), e724-732. doi:10.1542/peds.2006-1649

Peters, I. R., Calvert, E. L., Hall, E. J., & Day, M. J. (2004). Measurement of immunoglobulin concentrations in the feces of healthy dogs. *Clin Diagn Lab Immunol, 11*(5), 841-848. doi:10.1128/cdli.11.5.841-848.2004

Peters, I. R., Peeters, D., Helps, C. R., & Day, M. J. (2007). Development and application of multiple internal reference (housekeeper) gene assays for accurate normalisation of

7320   canine gene expression studies. *Vet Immunol Immunopathol, 117*(1-2), 55-66.
7321   doi:10.1016/j.vetimm.2007.01.011
7322 Picchianti-Diamanti, A., Rosado, M. M., & D'Amelio, R. (2017). Infectious Agents and
7323   Inflammation: The Role of Microbiota in Autoimmune Arthritis. *Front Microbiol, 8*,
7324   2696. doi:10.3389/fmicb.2017.02696
7325 Plumb, D. (2011). *Veterinary drug handbook*
7326 Wiley Blackwell.
7327 Price, M. N., Dehal, P. S., & Arkin, A. P. (2009). FastTree: computing large minimum evolution
7328   trees with profiles instead of a distance matrix. *Mol Biol Evol, 26*(7), 1641-1650.
7329   doi:10.1093/molbev/msp077
7330 Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., . . . Wang, J. (2010). A
7331   human gut microbial gene catalogue established by metagenomic sequencing. *Nature,*
7332   *464*(7285), 59-65. doi:10.1038/nature08821
7333 Rajilic-Stojanovic, M., & de Vos, W. M. (2014). The first 1000 cultured species of the human
7334   gastrointestinal microbiota. *FEMS Microbiol Rev, 38*(5), 996-1047. doi:10.1111/1574-
7335   6976.12075
7336 Rajilic-Stojanovic, M., Heilig, H. G., Tims, S., Zoetendal, E. G., & de Vos, W. M. (2012). Long-
7337   term monitoring of the human intestinal microbiota composition. *Environ Microbiol*.
7338   doi:10.1111/1462-2920.12023
7339 Rakoff-Nahoum, S. (2016). Another Reason to Thank Mom: Gestational Effects of Microbiota
7340   Metabolites. *Cell Host Microbe, 19*(4), 425-427. doi:10.1016/j.chom.2016.03.012
7341 Rea, M. C., Sit, C. S., Clayton, E., O'Connor, P. M., Whittal, R. M., Zheng, J., . . . Hill, C.
7342   (2010). Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum
7343   of activity against Clostridium difficile. *Proc Natl Acad Sci U S A, 107*(20), 9352-9357.
7344   doi:10.1073/pnas.0913554107
7345 Reardon, C., Lechmann, M., Brustle, A., Gareau, M. G., Shuman, N., Philpott, D., . . . Mak, T.
7346   W. (2011). Thymic stromal lymphopoetin-induced expression of the endogenous
7347   inhibitory enzyme SLPI mediates recovery from colonic inflammation. *Immunity, 35*(2),
7348   223-235. doi:10.1016/j.immuni.2011.05.015
7349 Rimoldi, M., Chieppa, M., Salucci, V., Avogadri, F., Sonzogni, A., Sampietro, G. M., . . .
7350   Rescigno, M. (2005). Intestinal immune homeostasis is regulated by the crosstalk
7351   between epithelial cells and dendritic cells. *Nat Immunol, 6*(5), 507-514.
7352   doi:10.1038/ni1192
7353 Rodriguez, J. M. (2014). The origin of human milk bacteria: is there a bacterial entero-mammary
7354   pathway during late pregnancy and lactation? *Adv Nutr, 5*(6), 779-784.
7355   doi:10.3945/an.114.007229
7356 Roger, L. C., Costabile, A., Holland, D. T., Hoyles, L., & McCartney, A. L. (2010). Examination
7357   of faecal Bifidobacterium populations in breast- and formula-fed infants during the first
7358   18 months of life. *Microbiology, 156*(Pt 11), 3329-3341. doi:10.1099/mic.0.043224-0
7359 Rossi, G., Pengo, G., Caldin, M., Palumbo Piccionello, A., Steiner, J. M., Cohen, N. D., . . .
7360   Suchodolski, J. S. (2014). Comparison of microbiological, histological, and
7361   immunomodulatory parameters in response to treatment with either combination therapy
7362   with prednisone and metronidazole or probiotic VSL#3 strains in dogs with idiopathic
7363   inflammatory bowel disease. *PLoS One, 9*(4), e94699. doi:10.1371/journal.pone.0094699

7364 Round, J. L., & Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune
7365   responses during health and disease. *Nat Rev Immunol, 9*(5), 313-323.
7366   doi:10.1038/nri2515

7367 Saenz, S. A., Taylor, B. C., & Artis, D. (2008). Welcome to the neighborhood: epithelial cell-
7368   derived cytokines license innate and adaptive immune responses at mucosal sites.
7369   *Immunol Rev, 226*, 172-190. doi:10.1111/j.1600-065X.2008.00713.x

7370 Salter, S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., . . . Walker,
7371   A. W. (2014). Reagent and laboratory contamination can critically impact sequence-
7372   based microbiome analyses. *BMC Biol, 12*, 87. doi:10.1186/s12915-014-0087-z

7373 Samuel, B. S., Shaito, A., Motoike, T., Rey, F. E., Backhed, F., Manchester, J. K., . . . Gordon, J.
7374   I. (2008). Effects of the gut microbiota on host adiposity are modulated by the short-chain
7375   fatty-acid binding G protein-coupled receptor, Gpr41. *Proc Natl Acad Sci U S A, 105*(43),
7376   16767-16772. doi:10.1073/pnas.0808567105

7377 Sato, A., Hashiguchi, M., Toda, E., Iwasaki, A., Hachimura, S., & Kaminogawa, S. (2003).
7378   CD11b+ Peyer's patch dendritic cells secrete IL-6 and induce IgA secretion from naive B
7379   cells. *J Immunol, 171*(7), 3684-3690.

7380 Schirmer, M., Smeekens, S. P., Vlamakis, H., Jaeger, M., Oosting, M., Franzosa, E. A., . . .
7381   Xavier, R. J. (2016). Linking the Human Gut Microbiome to Inflammatory Cytokine
7382   Production Capacity. *Cell, 167*(4), 1125-1136.e1128. doi:10.1016/j.cell.2016.10.020

7383 Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., . . . Weber,
7384   C. F. (2009). Introducing mothur: open-source, platform-independent, community-
7385   supported software for describing and comparing microbial communities. *Appl Environ
7386   Microbiol, 75*(23), 7537-7541. doi:10.1128/aem.01541-09

7387 Sears, C. L., & Garrett, W. S. (2014). Microbes, microbiota, and colon cancer. *Cell Host
7388   Microbe, 15*(3), 317-328. doi:10.1016/j.chom.2014.02.007

7389 Sepp, E., Julge, K., Vasar, M., Naaber, P., Bjorksten, B., & Mikelsaar, M. (1997). Intestinal
7390   microflora of Estonian and Swedish infants. *Acta Paediatr, 86*(9), 956-961.

7391 Sherrill, J. D., Gao, P. S., Stucke, E. M., Blanchard, C., Collins, M. H., Putnam, P. E., . . .
7392   Rothenberg, M. E. (2010). Variants of thymic stromal lymphopoietin and its receptor
7393   associate with eosinophilic esophagitis. *J Allergy Clin Immunol, 126*(1), 160-165.e163.
7394   doi:10.1016/j.jaci.2010.04.037

7395 Shin, N. R., Whon, T. W., & Bae, J. W. (2015). Proteobacteria: microbial signature of dysbiosis
7396   in gut microbiota. *Trends Biotechnol, 33*(9), 496-503. doi:10.1016/j.tibtech.2015.06.011

7397 Sierro, F., Dubois, B., Coste, A., Kaiserlian, D., Kraehenbuhl, J. P., & Sirard, J. C. (2001).
7398   Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of
7399   dendritic cells. *Proc Natl Acad Sci U S A, 98*(24), 13722-13727.
7400   doi:10.1073/pnas.241308598

7401 Simpson, J. M., Martineau, B., Jones, W. E., Ballam, J. M., & Mackie, R. I. (2002).
7402   Characterization of fecal bacterial populations in canines: effects of age, breed and
7403   dietary fiber. *Microb Ecol, 44*(2), 186-197. doi:10.1007/s00248-002-0001-z

7404 Simpson, K. W., & Jergens, A. E. (2011). Pitfalls and progress in the diagnosis and management
7405   of canine inflammatory bowel disease. *Vet Clin North Am Small Anim Pract, 41*(2), 381-
7406   398. doi:10.1016/j.cvsm.2011.02.003

7407 Slack, E., Balmer, M. L., Fritz, J. H., & Hapfelmeier, S. (2012). Functional flexibility of
7408   intestinal IgA - broadening the fine line. *Front Immunol, 3*, 100.
7409   doi:10.3389/fimmu.2012.00100

7410 Sonesson, A., Kasetty, G., Olin, A. I., Malmsten, M., Morgelin, M., Sorensen, O. E., &
7411   Schmidtchen, A. (2011). Thymic stromal lymphopoietin exerts antimicrobial activities.
7412   *Exp Dermatol, 20*(12), 1004-1010. doi:10.1111/j.1600-0625.2011.01391.x
7413 Song, S. J., Lauber, C., Costello, E. K., Lozupone, C. A., Humphrey, G., Berg-Lyons, D., . . .
7414   Knight, R. (2013). Cohabiting family members share microbiota with one another and
7415   with their dogs. *Elife, 2*, e00458. doi:10.7554/eLife.00458
7416 Soory, M. (2008). A role for non-antimicrobial actions of tetracyclines in combating oxidative
7417   stress in periodontal and metabolic diseases: a literature review. *Open Dent J, 2*, 5-12.
7418   doi:10.2174/1874210600802010005
7419 Spadoni, I., Iliev, I. D., Rossi, G., & Rescigno, M. (2012). Dendritic cells produce TSLP that
7420   limits the differentiation of Th17 cells, fosters Treg development, and protects against
7421   colitis. *Mucosal Immunol, 5*(2), 184-193. doi:10.1038/mi.2011.64
7422 Spiro, S. (2007). Regulators of bacterial responses to nitric oxide. *FEMS Microbiol Rev, 31*(2),
7423   193-211. doi:10.1111/j.1574-6976.2006.00061.x
7424 Stephens, W. Z., & Round, J. L. (2014). IgA targets the troublemakers. *Cell Host Microbe,*
7425   *16*(3), 265-267. doi:10.1016/j.chom.2014.08.012
7426 Stout, M. J., Conlon, B., Landeau, M., Lee, I., Bower, C., Zhao, Q., . . . Mysorekar, I. U. (2013).
7427   Identification of intracellular bacteria in the basal plate of the human placenta in term and
7428   preterm gestations. *Am J Obstet Gynecol, 208*(3), 226.e221-227.
7429   doi:10.1016/j.ajog.2013.01.018
7430 Strachan, D. P. (1989). Hay fever, hygiene, and household size. *Bmj, 299*(6710), 1259-1260.
7431 Suchodolski, J. S. (2011). Companion animals symposium: microbes and gastrointestinal health
7432   of dogs and cats. *J Anim Sci, 89*(5), 1520-1530. doi:10.2527/jas.2010-3377
7433 Suchodolski, J. S. (2013). Gastrointestinal Microbiota. In Elsevier (Ed.), *Canine and Feline*
7434   *Gastroenterology*.
7435 Suchodolski, J. S. (2016). Diagnosis and interpretation of intestinal dysbiosis in dogs and cats.
7436   *Vet J, 215*, 30-37. doi:10.1016/j.tvjl.2016.04.011
7437 Suchodolski, J. S., Camacho, J., & Steiner, J. M. (2008). Analysis of bacterial diversity in the
7438   canine duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis.
7439   *FEMS Microbiol Ecol, 66*(3), 567-578. doi:10.1111/j.1574-6941.2008.00521.x
7440 Suchodolski, J. S., Dowd, S. E., Westermarck, E., Steiner, J. M., Wolcott, R. D., Spillmann, T.,
7441   & Harmoinen, J. A. (2009). The effect of the macrolide antibiotic tylosin on microbial
7442   diversity in the canine small intestine as demonstrated by massive parallel 16S rRNA
7443   gene sequencing. *BMC Microbiol, 9*, 210. doi:10.1186/1471-2180-9-210
7444 Suchodolski, J. S., Markel, M. E., Garcia-Mazcorro, J. F., Unterer, S., Heilmann, R. M., Dowd,
7445   S. E., . . . Toresson, L. (2012). The fecal microbiome in dogs with acute diarrhea and
7446   idiopathic inflammatory bowel disease. *PLoS One, 7*(12), e51907.
7447   doi:10.1371/journal.pone.0051907
7448 Suchodolski, J. S., Ruaux, C. G., Steiner, J. M., Fetz, K., & Williams, D. A. (2004). Application
7449   of molecular fingerprinting for qualitative assessment of small-intestinal bacterial
7450   diversity in dogs. *J Clin Microbiol, 42*(10), 4702-4708. doi:10.1128/jcm.42.10.4702-
7451   4708.2004
7452 Suchodolski, J. S., Ruaux, C. G., Steiner, J. M., Fetz, K., & Williams, D. A. (2005). Assessment
7453   of the qualitative variation in bacterial microflora among compartments of the intestinal
7454   tract of dogs by use of a molecular fingerprinting technique. *Am J Vet Res, 66*(9), 1556-
7455   1562.

Suchodolski JS, S. C., Buono A, Lees GE, Dowd SE, Steiner JM. (2013). *Postnatal Development of the Canine Fecal Microbiome*. Paper presented at the ACVIM 2013.

Suchodolski, J. S., Xenoulis, P. G., Paddock, C. G., Steiner, J. M., & Jergens, A. E. (2010). Molecular analysis of the bacterial microbiota in duodenal biopsies from dogs with idiopathic inflammatory bowel disease. *Vet Microbiol, 142*(3-4), 394-400. doi:10.1016/j.vetmic.2009.11.002

Sunvold, G. D., Hussein, H. S., Fahey, G. C., Jr., Merchen, N. R., & Reinhart, G. A. (1995). In vitro fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inoculum from cats, dogs, horses, humans, and pigs and ruminal fluid from cattle. *J Anim Sci, 73*(12), 3639-3648.

Suzuki, K., Meek, B., Doi, Y., Muramatsu, M., Chiba, T., Honjo, T., & Fagarasan, S. (2004). Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc Natl Acad Sci U S A, 101*(7), 1981-1986. doi:10.1073/pnas.0307317101

Swanson, K. S., Dowd, S. E., Suchodolski, J. S., Middelbos, I. S., Vester, B. M., Barry, K. A., ... Fahey, G. C., Jr. (2011). Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. *Isme j, 5*(4), 639-649. doi:10.1038/ismej.2010.162

Swidsinski, A., Loening-Baucke, V., Theissig, F., Engelhardt, H., Bengmark, S., Koch, S., . . . Dorffel, Y. (2007). Comparative study of the intestinal mucus barrier in normal and inflamed colon. *Gut, 56*(3), 343-350. doi:10.1136/gut.2006.098160

Szabo, C., Ransy, C., Modis, K., Andriamihaja, M., Murghes, B., Coletta, C., . . . Bouillaud, F. (2014). Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms. *Br J Pharmacol, 171*(8), 2099-2122. doi:10.1111/bph.12369

Thompson-Chagoyan, O. C., Maldonado, J., & Gil, A. (2005). Aetiology of inflammatory bowel disease (IBD): role of intestinal microbiota and gut-associated lymphoid tissue immune response. *Clin Nutr, 24*(3), 339-352. doi:10.1016/j.clnu.2005.02.009

Thursby, E., & Juge, N. (2017). Introduction to the human gut microbiota. *Biochem J, 474*(11), 1823-1836. doi:10.1042/bcj20160510

Tirosh, O., Levy, E., & Reifen, R. (2007). High selenium diet protects against TNBS-induced acute inflammation, mitochondrial dysfunction, and secondary necrosis in rat colon. *Nutrition, 23*(11-12), 878-886. doi:10.1016/j.nut.2007.08.019

Truong, D. T., Tett, A., Pasolli, E., Huttenhower, C., & Segata, N. (2017). Microbial strain-level population structure and genetic diversity from metagenomes. *Genome Research, 27*(4), 626-638. doi:10.1101/gr.216242.116

Tsilingiri, K., Fornasa, G., & Rescigno, M. (2017). Thymic Stromal Lymphopoietin: To Cut a Long Story Short. *Cell Mol Gastroenterol Hepatol, 3*(2), 174-182. doi:10.1016/j.jcmgh.2017.01.005

Vaishampayan, P. A., Kuehl, J. V., Froula, J. L., Morgan, J. L., Ochman, H., & Francino, M. P. (2010). Comparative metagenomics and population dynamics of the gut microbiota in mother and infant. *Genome Biol Evol, 2*, 53-66. doi:10.1093/gbe/evp057

Valles, Y., Artacho, A., Pascual-Garcia, A., Ferrus, M. L., Gosalbes, M. J., Abellan, J. J., & Francino, M. P. (2014). Microbial succession in the gut: directional trends of taxonomic and functional change in a birth cohort of Spanish infants. *PLoS Genet, 10*(6), e1004406. doi:10.1371/journal.pgen.1004406

Van den Abbeele, P., Belzer, C., Goossens, M., Kleerebezem, M., De Vos, W. M., Thas, O., . . . Van de Wiele, T. (2013). Butyrate-producing Clostridium cluster XIVa species specifically colonize mucins in an in vitro gut model. *Isme j, 7*(5), 949-961. doi:10.1038/ismej.2012.158

van der Waaij LA, K. F., Jansen PLM, et al. (1997). IBD patients have a very high percentage of colonic anaerobic bacteria that are in vivo coated with IgA, IgG or IgM, irrespective of clinical activity. *Gut, 41*(suppl 3).

van der Waaij, L. A., Kroese, F. G., Visser, A., Nelis, G. F., Westerveld, B. D., Jansen, P. L., & Hunter, J. O. (2004). Immunoglobulin coating of faecal bacteria in inflammatory bowel disease. *Eur J Gastroenterol Hepatol, 16*(7), 669-674.

Van der Waaij LA, L. P., Mesander G, van der Waaij D. (1996). In vivo IgA coating of anaerobic bacteria in human faeces. *Gut, 38*(3), 348-354.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol, 3*(7), Research0034.

Vazquez-Baeza, Y., Hyde, E. R., Suchodolski, J. S., & Knight, R. (2016). Dog and human inflammatory bowel disease rely on overlapping yet distinct dysbiosis networks. *Nat Microbiol, 1*, 16177. doi:10.1038/nmicrobiol.2016.177

Viladomiu, M., Kivolowitz, C., Abdulhamid, A., Dogan, B., Victorio, D., Castellanos, J. G., . . . Longman, R. S. (2017). IgA-coated E. coli enriched in Crohn's disease spondyloarthritis promote TH17-dependent inflammation. *Sci Transl Med, 9*(376). doi:10.1126/scitranslmed.aaf9655

Viviano, K. R. (2013). Update on immununosuppressive therapies for dogs and cats. *Vet Clin North Am Small Anim Pract, 43*(5), 1149-1170. doi:10.1016/j.cvsm.2013.04.009

Walker, D., Knuchel-Takano, A., McCutchan, A., Chang, Y. M., Downes, C., Miller, S., . . . Garden, O. A. (2013). A comprehensive pathological survey of duodenal biopsies from dogs with diet-responsive chronic enteropathy. *J Vet Intern Med, 27*(4), 862-874. doi:10.1111/jvim.12093

Walsh, C. J., Guinane, C. M., O'Toole, P. W., & Cotter, P. D. (2014). Beneficial modulation of the gut microbiota. *FEBS Lett, 588*(22), 4120-4130. doi:10.1016/j.febslet.2014.03.035

Wang, H. B., Wang, P. Y., Wang, X., Wan, Y. L., & Liu, Y. C. (2012). Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein Claudin-1 transcription. *Dig Dis Sci, 57*(12), 3126-3135. doi:10.1007/s10620-012-2259-4

Wassenaar, T. M., & Panigrahi, P. (2014). Is a foetus developing in a sterile environment? *Lett Appl Microbiol, 59*(6), 572-579. doi:10.1111/lam.12334

Weinstock, G. M. (2011). The volatile microbiome. *Genome Biol, 12*(5), 114. doi:10.1186/gb-2011-12-5-114

Wells, J. M., Rossi, O., Meijerink, M., & van Baarlen, P. (2011). Epithelial crosstalk at the microbiota-mucosal interface. *Proc Natl Acad Sci U S A, 108 Suppl 1*, 4607-4614. doi:10.1073/pnas.1000092107

Willard, M. D., Simpson, R. B., Delles, E. K., Cohen, N. D., Fossum, T. W., Kolp, D., & Reinhart, G. (1994). Effects of dietary supplementation of fructo-oligosaccharides on small intestinal bacterial overgrowth in dogs. *Am J Vet Res, 55*(5), 654-659.

Wright, E. K., Kamm, M. A., Teo, S. M., Inouye, M., Wagner, J., & Kirkwood, C. D. (2015). Recent advances in characterizing the gastrointestinal microbiome in Crohn's disease: a

7546   systematic review. *Inflamm Bowel Dis, 21*(6), 1219-1228.
7547   doi:10.1097/mib.0000000000000382

7548 Xenoulis, P. G., Palculict, B., Allenspach, K., Steiner, J. M., Van House, A. M., & Suchodolski,
7549   J. S. (2008). Molecular-phylogenetic characterization of microbial communities
7550   imbalances in the small intestine of dogs with inflammatory bowel disease. *FEMS*
7551   *Microbiol Ecol, 66*(3), 579-589. doi:10.1111/j.1574-6941.2008.00556.x

7552 Xia, H., He, J.-R., Lu, J.-H., Shen, S.-Y., Kuang, Y.-S., Li, S.-H., . . . Qiu, X. 300: Difference in
7553   phylogenetic composition and function of gut microbiota between pregnant women and
7554   non-pregnant adults. *American Journal of Obstetrics & Gynecology, 216*(1), S184-S185.
7555   doi:10.1016/j.ajog.2016.11.558

7556 Xiong, N., & Hu, S. (2015). Regulation of intestinal IgA responses. *Cell Mol Life Sci, 72*(14),
7557   2645-2655. doi:10.1007/s00018-015-1892-4

7558 Xiong, Y., Miyamoto, N., Shibata, K., Valasek, M. A., Motoike, T., Kedzierski, R. M., &
7559   Yanagisawa, M. (2004). Short-chain fatty acids stimulate leptin production in adipocytes
7560   through the G protein-coupled receptor GPR41. *Proc Natl Acad Sci U S A, 101*(4), 1045-
7561   1050. doi:10.1073/pnas.2637002100

7562 Yau, Y., Leong, R. W., Zeng, M., & Wasinger, V. C. (2013). Proteomics and metabolomics in
7563   inflammatory bowel disease. *J Gastroenterol Hepatol, 28*(7), 1076-1086.
7564   doi:10.1111/jgh.12193

7565 Yoshida, M., Claypool, S. M., Wagner, J. S., Mizoguchi, E., Mizoguchi, A., Roopenian, D. C., . .
7566   . Blumberg, R. S. (2004). Human neonatal Fc receptor mediates transport of IgG into
7567   luminal secretions for delivery of antigens to mucosal dendritic cells. *Immunity, 20*(6),
7568   769-783. doi:10.1016/j.immuni.2004.05.007

7569 Yoshida, M., Kobayashi, K., Kuo, T. T., Bry, L., Glickman, J. N., Claypool, S. M., . . .
7570   Blumberg, R. S. (2006). Neonatal Fc receptor for IgG regulates mucosal immune
7571   responses to luminal bacteria. *J Clin Invest, 116*(8), 2142-2151. doi:10.1172/jci27821

7572 Yuan, S., Cohen, D. B., Ravel, J., Abdo, Z., & Forney, L. J. (2012). Evaluation of methods for
7573   the extraction and purification of DNA from the human microbiome. *PLoS One, 7*(3),
7574   e33865. doi:10.1371/journal.pone.0033865

7575 Zaph, C., Troy, A. E., Taylor, B. C., Berman-Booty, L. D., Guild, K. J., Du, Y., . . . Artis, D.
7576   (2007). Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune
7577   homeostasis. *Nature, 446*(7135), 552-556. doi:10.1038/nature05590

7578 Zhang, J., Kobert, K., Flouri, T., & Stamatakis, A. (2014). PEAR: a fast and accurate Illumina
7579   Paired-End reAd mergeR. *Bioinformatics, 30*(5), 614-620.
7580   doi:10.1093/bioinformatics/btt593

7581 Zhong, J., Sharma, J., Raju, R., Palapetta, S. M., Prasad, T. S., Huang, T. C., . . . Pandey, A.
7582   (2014). TSLP signaling pathway map: a platform for analysis of TSLP-mediated
7583   signaling. *Database (Oxford), 2014*, bau007. doi:10.1093/database/bau007

7584 Zhu, X., Wang, J., Reyes-Gibby, C., & Shete, S. (2017). Processing and Analyzing Human
7585   Microbiome Data. *Methods Mol Biol, 1666*, 649-677. doi:10.1007/978-1-4939-7274-
7586   6_31

7587 Ziegler, S. F., & Artis, D. (2010). Sensing the outside world: TSLP regulates barrier immunity.
7588   *Nat Immunol, 11*(4), 289-293. doi:10.1038/ni.1852

7589 Ziegler, S. F., Roan, F., Bell, B. D., Stoklasek, T. A., Kitajima, M., & Han, H. (2013). The
7590   biology of thymic stromal lymphopoietin (TSLP). *Adv Pharmacol, 66*, 129-155.
7591   doi:10.1016/b978-0-12-404717-4.00004-4

7592

7593