Further development of a cell culture based approach to model the diet-derived impacts on the faecal microbiome and potential host health in the domestic dog

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy (Ph.D.)

in

Animal Science

at Massey University Palmerston North, New Zealand

Francis David Phimister

2023

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

Note for Examiners of Doctoral Theses
Explanation of COVID-19 Impacts

The Doctoral Research Committee recognises the impacts of Covid-19 on research, particularly for doctoral candidates, and we appreciate the efforts made by supervisors and candidates to ensure timely completion of the doctoral thesis. We know that in some cases this has meant the project has needed to be changed in some way, including its final presentation. For students whose work has been impacted, we invite supervisors to provide a note for examiners explaining the circumstances.

Instructions for Supervisors:

The note is designed to enable you to communicate to examiners your desire for them to take account of certain factors in their assessment of a thesis to address delays and disruptions experienced by a thesis student as a result of the Covid-19 pandemic.

The attached form should be used to provide **an explanation** to the examiners on what to consider in their evaluation. It should detail how the project was altered or how the final product of the thesis has been affected as a result of the disruption. Statements should be clear and succinct for the benefit of the examiners and in fairness to the student and others in the student cohort.

The form should be signed by the student, the supervisor and the Head of Academic Unit, or nominee, and included in the information that is sent out with the thesis.

For doctoral candidates, the completed form should be inserted into the front of the thesis before the abstract by the candidate when submitting their digital thesis for examination in the Student Portal. At the completion of the examination, the amended form which excludes any confidential comments to the examiners, should be included in the appendices.

Please be sure to indicate whether a student has received a suspension of studies due to Covid-19 and/or an extension, as it is important to note if students have already had some special consideration.

Approved by DRC 10/Feb/2021
DRC 21/02/03

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

Note for Examiners
Explanation of COVID-19 Impacts

Thank you for taking the time to examine this thesis, which has been undertaken during the Covid-19 pandemic. The New Zealand Government's response to Covid-19 includes a system of Alert Levels which have impacted upon researchers. Our University's pandemic plan applied the Government's expectations to our research environment to ensure the health and safety of our researchers, however, research was impacted by restrictions and disruptions, as outlined below.

For a six-week period from March 26 to April 27 2020, New Zealand was placed under very strict lockdown conditions (Level 4 – Lockdown), with students and staff unable to physically access University facilities, unless they were involved in essential research related to Covid-19. All field work ceased and data collection with humans was restricted to online methods, if appropriate. The restrictions were partially lifted on April 27, but students and staff were not generally allowed back into University facilities until May 13.

Ongoing disruptions have also been encountered for some students due to uncertainties over the potential for future Covid-19-related restrictions on activities, and a Covid-19 cluster outbreak based in Auckland in New Zealand on 12 August 2020 led to the imposition of rolling Level 2 (Reduce) and Level 3 (Restrict) conditions until 23 September 2020. Auckland campus based students remained on Level 2 until 7 October 2020. This Alert Level system continues to be utilised throughout 2021.

These changing Alert Levels have meant that some research students had experimental, clinical, laboratory, field work, and/or data collection or analysis interrupted, and consequently may have had to adjust their research plans. For some students, the impacts of Covid-19 stretched far beyond the lockdown period in April/May 2020, as they may have had to significantly revise their research plans.

Overseas travel is not permitted by the University and restrictions have been placed on the New Zealand borders which are closed to non-New Zealand citizens and permanent residents. This meant that international students who were based offshore at the time of lockdown, were unable to return to New Zealand. A small number of offshore students were provided permission to return to New Zealand in early 2021. Many students have also suffered from anxiety and stress-related issues, and have had financial impacts, meaning their research progress has been significantly delayed.

This form, as completed by the supervisor and student, outlines the extent that the research has been affected by Covid-19 conditions.

Approved by DRC 10/Feb/2021
DRC 21/02/03

Please consider the factors listed below in your assessment of the work.

This statement has been prepared by the candidate's supervisor in consultation with the student and has been endorsed by the relevant Head of Academic Unit.

Student Name:	Francis David Phimister	ID Number:	[redacted]
Supervisor Name:	David Thomas	Date:	26/01/2023

Thesis title:

Further development of a cell culture based approach to model the diet-derived impact

Considerations to be taken into account. Note: This statement will remain in the final copy of the thesis which will be available from the Massey University Library following the examination process.
[*Enter key considerations here for the examiners. This can include but is not limited to change of scope, scale, topic, focus; limitations in relation to data collection, access to necessary literature or archival materials, laboratories, field sites; disruptions as a result of lockdown and various alert levels, medical or health considerations etc*]

This PhD project was severely impacted and limited by the Covid-19 pandemic. Project work was halted due to the unavailability of raw materials to conduct cell culture work. Due to the unavailability of the raw materials, the project scope was changed from developing an apically anaerobic model of the canine intestine to performing the cell culture experiments in aerobic conditions. This was as the apical anaerobic apparatus had been custom-designed to fit a particular type of cell culture insert - alternatives could not maintain the balance between aerobic and anaerobic environments.

Additionally, the lockdown occurred during a planned facility move (March 2020), which then caused delays to restarting laboratory work (work begun again in October 2020).

Finally, due to the restrictions on travel into the country, the Luminex work in Chapter Five was delayed by 6 months (samples were ready in January 2022, couldn't be tested until July 2022). The Luminex required an engineer to resolve technical issues with the equipment prior to the assays being conducted, however, the technician was based in Australia and couldn't come into New Zealand until the appropriate border restrictions had been lifted.

Approved by DRC 10/Feb/2021
DRC 21/02/03

Confidential for Examiners Only: [*Please enter any other considerations which are confidential for examiners only and should not be placed in the final thesis version submitted to Library following the examination process]*

Signed, confirming this is a fair reflection of the impact of Covid-19 on this research.

Student	Francis Phimister	Digitally signed by Francis Phimister Date: 2023.01.26 09:19:02 +13'00'
Supervisor	David Thomas	Digitally signed by David Thomas DN: cn=David Thomas, c=NZ, o=Massey University, ou=School of Agriculture & Environment, email=d.g.thomas@massey.ac.nz Date: 2023.01.26 14:31:12 +13'00'
Head of Academic Unit (or nominee)	Timothy J Wester	Digitally signed by Timothy J Wester DN: cn=Timothy J Wester, c=NZ, o=Massey University, ou=School of Agriculture & Environment, email=t.j.wester@massey.ac.nz Date: 2023.01.26 14:34:43 +13'00'

Approved by DRC 10/Feb/2021
DRC 21/02/03

ABSTRACT

Globally, diets that promote and optimise health and a 'healthy microbiome' are becoming increasingly popular for pets. However, the impacts of novel diet ingredients and formulations on the health of the host and their microbiome require testing to ensure there are no unforseen detrimental effects. However, there are currently a very limited number of canine-specific intestinal cell lines and the capacity to utilise these cells to model host-food interactions is limited.

Thus, this doctoral project aimed to develop an *in vitro* model of the canine intestine, using a previously established canine intestinal epithelial cell (cIEC) line. This could then be used to characterise the canine response to dietary challenges to the gut microbiota. As there is limited research that has assessed the interactions of the gut microbiota with the canine intestine, the initial step of this project was to evaluate the current knowledge of the intestinal inflammatory response to bacterial ligands and diet-derived metabolites (Chapter Two). This literature review indicated that prior to investigating the interactions between bacteria and the canine intestine an evaluation of the canine intestinal response to these challenge compounds was required.

Building on the knowledge base established in Chapter Two, key microbes associated with health in the dog required identification. Thus, this thesis provided the first meta-analysis of the available literature on the relationship of dietary nutrients and their impact on the gut microbiota in the dog (Chapter Three). The hypothesis of this meta-analysis was that dietary protein and dietary fats would have singificant impacts on the faecal microbiota of the dog and additionally, that this analysis would reveal bacterial genera associated with these dietary macronutrients.In the meta-analysis the novel discovery was made that despite its low relative abundance,*Sharpea* was the genera most associated with causing the shifts in microbial profiles in response to changes in both crude protein, and crude fats, thus confirming the hypotheses.

Early results indicated that the methods required to further refine the existing cIEC line as an *in vitro* model of the canine intestine were sub-optimal and required further development. These method developments are detailed in Chapter Four. Experiments in this chapter assessed methods to

promote cell growth and differentiation in a cellZscope system, which automatically performed barrier integrity assessments, whilst inside a temperature-controlled incubator. The inclusion of 150 nM hydrocortisonein cell culture media during the initial 48 hours of cellular differentiation, and subsequent removal of hydrocortisone after this period successfully enabled the cIEC to differentiate and form confluent monolayers in the cellZscope system. These methods were intended to be used going forwards in an apically anaerobic system that would more closely resemble conditions seen *in vivo* and would have allowed the simultaneous culture of the oxygen-requiring cIEC and anaerobic bacteria. Work utilising this model was stopped due to complications that arose from the Covid-19 pandemic, but work conducted and experiments that were planned are explored in Chapter Six.

Utilising the refined methods from Chapter Four, the inflammatory response of the cIEC to butyrate and bacterial lipopolysaccharides (LPS) were characterised (Chapter Five). This was performed to address gaps in the literature highlighted in Chapter Two.It was hypothesised that the stimulation with bacterial LPS would cause a pro-inflammatory response, whilst the stimulation with butyrate would cause an anti-inflammatory response. Furthermore, it was also hypothesised that the stimulation of the cIEC with both LPS and butyrate would cause the butyrate to reduce the pro-inflammatory response, and the LPS to reduce the anti-inflammatory response. It was observed that LPS induced a pro-inflammatory response in the cIEC, which butyrate was able to mitigate in most instances.

Overall, the methods developed and refined in this project will be able to be utilised in future experiments utilising these cells, such asevaulating new pet food ingredients for beneficial effects and exploring how changes in the gut microbiome impact gut health in the dog. It can take the knowledge established in Chapter Three to further investigate the impacts of the bacterial genera on the health of the dog. Futhermore, it can utilise the immune responses observed in Chapter Five to better understand the relationship between inflammation and diet in the dog. Future work can build on the knowledge discovered and presented in this thesis to fully understand the impact of diet changes on the health of the dog, and further define the microbial profile of the ‘healthy microbiome’ for the dog.

ACKNOWLEDGEMENTS

This PhD by and large wouldn't have been possible without the constant support of my supervisors for putting up with and answering my constant (often stupid and/or pointless) questions. Both Dr Emma Bermingham and Dr Rachel Anderson somehow managed to cope with my constant barrage of confusion and not only managed to entertain them, but also helped guide and steer me towards thinking and functioning like an actual scientist, for which I'm endlessly grateful. Special thanks go to Associate Professor David Thomas for not only constantly being on board to entertain my questions and questionable ideas, but for going out of his way to meet me and answer questions back before I'd even begun this PhD, during his holiday in the UK, no less. Additionally, I'm grateful for him and his wife Dr Renee Corner-Thomas offering me a place to live until I got settled over in this new country. More thanks, to Dr Michelle Farquhar, who came back off maternity leave to a sudden PhD student and was still on board to answer questions and offer me guidance, despite the time zone difference and late nights required.

In a similar vein, I would also like to extend my thanks to Dr Kevin Hughes and Dr Zoe Marshall-Jones, both formally of Waltham Petcare, who also took time out of their schedules to help me through this journey. Thanks to Kevin and the other members of Waltham who took the time out of their day before this project begun to introduce the project to me and the work that had been undertaken prior to the stage where I began. I'd like to thank all three centres for contributing to funding this PhD project and awarding me a scholarship so I could move across the world and study. Given the way that the UK and NZ both handled Covid-19 in separate ways, it was certainly a timely and beneficial move!

Throughout this project there have been many people helping in different capacities, and both in small and large manners. I have included specifics in each chapter… it takes a village to raise a child, and apparently it also takes a village to help one PhD student! Outside of my supervisory panel, the main help and person to thank is Dr Eva Maier, who took me under her wing, commiserated with me throughout the many failed experiments and shared many stories and insights from experiments

going wrong. When we joked that my project was the sequel disaster movie to her own PhD, I don't think either of us appreciated just how true that would become.

Similarly, I'd like to thank Dr Alicia Barnett and Dr Dulantha Ulluwishewa for their help with anything and everything cell culture related, and Ms Allison McCarthy and Ms Linda McVann for helping me navigate the turbid waters that are New Zealand's biosecurity controls. Additionally, I'd like to thank Ms Denise Martin for her invaluable organising and herding cats behind the scenes to make our lives as simple as possible. Additionally, thanks for the unsung saints that are the technicians for never complaining when another Monday arrived and I'd apologetically explain how I've used up yet more supplies in the lab over the weekend, and to all the cell culture users in general who dealt with my need to usurp two incubators just for myself.

My gratitude also goes out to the other students who shared our office space for the many hours of crazy stories, excuses for procrastination, entertainment during the tough period and the many times of sleep-deprived, highly caffeinated chaos. There were many times where we all needed to be the boot up someone else's rear end, the voice of reason, or just the person to vent to as things went sideways. Somehow, we've pulled through one by one and reached the peak of what often felt like a never-ending struggle. Here's hoping there are no more 3 a.m. work related woes to stop us from sleeping.

More thanks to Dr Diana Cabrera for the many random coffee conversations and entertaining stories, Dr Heike Schwendel for being the blunt kick up the backside I often needed, and to all my friends and chosen family who nodded along and pretended like they understood my word vomit, which was usually followed with the question of "so do you get to play with dogs all day then?"

The last person left to thank is the one that's been there beside me for the whole journey. My (begrudgingly admitted) better half, who has supported me throughout all the highs and lows and stayed by my side, despite the way the comfort eating ruined my waistline and the stresses ruined my hairline. It seems like no time at all has passed since I came in from work and dropped the news that I'd applied for a PhD all the way over in New Zealand. We'd laughed at how ridiculous such a drastic

change had sounded, and how farfetched the idea of it happening seemed to be. Now, one small global pandemic later, there's no one else I'd have rather in my corner.

TABLE OF CONTENTS

.

TABLE OF FIGURES

.

TABLE OF TABLES

TABLE OF EQUATIONS

TABLE OF APPENDICES

LIST OF ABBREVIATIONS

Abbreviatio	Definition
3 R's	Reduction, Refinement and Replacement of laboratory use of animals
AAFCO	American Feed Control Officials
BARF	Bones and Raw Food
BCFA	Branched chain fatty acids
BCS	Body Condition Score
BDM	Base Differentiation Medium
BW	Body Weight
CE	Chronic enteropathy
CHO	Carbohydrate
cIEC	Canine Intestinal Epithelial Cell
CLDN	Claudin
CP	Crude Protein
DAMP	Damage-Associated Molecular Pattern
DECC	Dual Environment Co-Culture
DF	Dietary Fat
DM	Dry Matter
DMEM	Dulbecco's Modified Eagle Media
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbant Assay
ENA	European Nucleotide Archive
FBS	Foetal Bovine Serum
FEDIAF	*Fédération Européenne de l'Industrie des Aliments pour Animaux Familiers*
FITC	Fluorescein isothiocyanate
FRE	Food Responsive Diarrhoea
FZD	Frizzled class receptor
GIT	Gastrointestinal Tract
GPR	G-Protein Coupled Receptor
HC	hydrocortisone
HEPES	Hydroxyethylpiperazine-N-2-ethane sulphonic acid
HIF	Hypoxia-Inducible Factor
IBD	Inflammatory Bowel Disease
IEB	Intestinal Epithelial Barrier
IEC	Intestinal Epithelial Cell
IFN	Interferon
IKK	Inhibitor of nuclear factor-κB (IκB) kinase
IL	Interleukin
IP-10	Interferon gamma-induced protein 10 (also referred to as chemokine (C-X-C motif) ligand 10)
IQ	Interquartile
JASM	Junctional Adhesion Molecules
KC	Keratinocyte-derived chemokine
KRAS	Kirsten rat sarcoma viral oncogene homolog
LPS	Lipopolysaccharides

LRR	Leucine-Rice Repeat
LTA	Lipoteichoic Acid
MAMP	Microbe Associated Molecular Pattern
MAPK	Mitogen-acitvated protein kinase
MCP-1	Monocyte chemoattractant protein 1 (also referred to as chemokine (C-C motif) ligand 2, CCL2)
MDCK	Madin-Derby Canine Kidney
MER	Maintenance Energy Requirements
mM	mili-Molar
MyD	Myeloid Differentiation protein
NCBI	National Centre for Biotechnology Information
NF-κB	Nuclear Factor kappa B
NGS	Next generation sequencing
NLR	Nucleotide-inding ligomerization domain-Like Receptors
NLRP3	NLR family pyrin domain containing 3
NOD2	Nucleotide-binding Oligomerization Domain–containing protein 2
NRC	National Research Council
OCLDN	Occludin
Opti-MEM	Opti-Minimal Essential Medium I Reduced Serum Media
PBS	Phosphate-Buffered Saline
PCoA	Principal Coordinate Analysis
PenStrep	Penicillin-Streptomycin
PLS-DA	Partial Least Squares-Discriminant Analysis
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-Analyses
PRR	Pattern Recognition Receptor
RAGE	Receptor of Advanced Glycation End products
RDM	Remastered Differentiation Medium
RIPK2	RICK protein kinase
RNA	Ribonucleic Acid
SCFA	Short-Chained Fatty Acid
SEM	Standard Error of Mean
TAK	Transforming growth factor β-Activated Kinase
TEER	Transepithelial Electrical Resistance
Th (1/2)	T helper type (1/2) cells
TIR	Toll-interleukin-1 receptor
TIRAP	Myeloid Differentiation adaptor-like
TJ	Tight Junction
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TRIF	Toll–interluekin-1 receptor domain-containing adaptor inducing interferon-β
ZO	Zonula Occludens

CHAPTER ONE

GENERAL INTRODUCTION AND THESIS AIMS

1.1. BACKGROUND

The ownership of domestic dogs (*Canis familaris)* is increasingly popular worldwide. In New Zealand alone, 34% of households own at least one dog, with approximately 850,000 pet dogs across the country (Companion Animals in New Zealand, 2020). 78% of dog owners see their pet dog as a member of the family (Companion Animals in New Zealand, 2020). This atittude is driving pet owners towards diets that optimise health. This coincides with the pet food industry's continual research into potential health and nutritional benefits from new diets and ingredients (Chen et al., 2012; Schleicher et al., 2019). In particular, understanding how dietary intervention can promote health and a healthy gastrointestinal tract (GIT) microbiota in the dog is a key research area (Wernimont et al., 2020b; Satyaraj et al., 2021).

However, there are concerns with the sustainability of the current resource demands for pet food, and the capacity to meet future resource demands (Bosch et al., 2016; Okin, 2017; Lisenko et al., 2018). Furthermore, the rearing costs associated with producing animal proteins for pet food account for an estimated 25-30% of the global environmental impacts from animal production (Okin, 2017). The increased demand for health-beneficial pet foods and alternative, sustainable proteins for consumption has also increased the need to ensure that these novel ingredients and/or pet food formulations are fit for purpose (Bosch et al., 2016; Lisenko et al., 2018; Schleicher et al., 2019).

All nutritionally complete pet food (including those marketed as a "whole diet" or as "complete and balanced") must undergo scientific trials to verify these claims prior to release (Ministry for Primary Industries, 2018). These trials can be animal feed based, can be analysed in a laboratory, or can be formulated using software (Ministry for Primary Industries, 2018). Worldwide, a strategy has been formed to help move towards robust, scientifically accurate and non-animal models (Council of the European Union, 2010; Ferdowsian and Beck, 2011; Doke and Dhawale, 2015). This is referred to as the the Reduction, Refinement and Replacement of laboratory use of animals (i.e., methods that reduce the number of animals being used, refine the experimental procedures to minimise pain, suffering or distress of animals, and replace the use of animals where possible; 3Rs) (Council of the

European Union, 2010; Ferdowsian and Beck, 2011; Doke and Dhawale, 2015). However, no models have yet been established to move away from animal feeding trials.

The testing of novel ingredients and food formulations can be performed with cell culture. Intestinal epithelial cells (IECs) are grown until they form a monolayer. This is then used as a model of the intestine (Juan et al., 2006). Immune cytokines and cellular junctional complexes expressed by the IECs are then measured in response to challenges to infer impacts to host health. This has been routinely employed to model the effects of food products and food constituents (e.g., plant polyphenols) on intestinal health in humans (Hashimoto et al., 1994; Juan et al., 2006; Anderson et al., 2018; Cai et al., 2018). The effects of compounds that are produced from the GIT microbiota-assisted fermentation of dietary nutrients (e.g., short-chain fatty acids (SCFA), tryptophan, etc) on intestinal health have also been assessed (Fusunyan et al., 1998; Asarat et al., 2015). Additionally, the response of the IEC monolayer to live and dead bacteria was investigated to understand the host interactions with "good" bacteria (Ulluwishewa et al., 2015; Maier et al., 2018). However, human-specific cell culture has built up a picture of the normal, healthy cellular response over a number of years. The Caco-2 cell line in particular was established and has been studied since 1977 (Lea, 2015). In comparison, IECs have been used to model the dog intestine since only 2005/2006 (Weng et al., 2005; Swerdlow et al., 2006). Thus, the knowledge and understanding of intestinal health in the dog is lacking when compared to human research.

This doctoral project aimed to create an *in vitro* model of the dog intestine as an alternative to using laboratory animals. This would then be used to investigate the interactions between the IECs and specific bacteria present in the GIT. To begin, the current knowledge and limitations in the literature regarding the three main subject areas of this project were assessed. These subject areas were:

1) The nutritional requirements of the domestic dog.
2) The diet-based impacts on the GIT microbiota of the domestic dog.
3) Intestinal health and the interactions between the IECs and the GIT microbiota in the domestc dog.

The identified limitations and current knowledge were then used to form the research pathway of this doctoral project.

1.2. NUTRITIONAL REQUIREMENTS OF THE DOMESTIC DOG

The domestic dog, like all animals, requires a nutritionally balanced diet to aid in the maintenance and promotion of overall health (FEDIAF, 2018). A balanced nutritional diet meets the maintenance energy requirements (MER) to support energy equilibrium and maintain body weight whilst accounting for spontaneous activity and exercise (Mullis et al., 2015). However, this can vary for each animal and is dependent on factors such as husbandry, activity level, breed, pregnancy, and life stage (puppy or adult) (Dobenecker et al., 2013; Bermingham et al., 2014; Mullis et al., 2015). Imbalanced intakes of essential dietary requirements results in malnutrition caused by either undernutrition or overnutrition (Remillard et al., 2001). Undernutrition can result in lower than ideal body weight (BW) and a lower body condition score (BCS). The BCS is a determination of health on a numerical scale of 1 – 9 (emaciated – several obese, respectively), where ideal weight sits at 4 or 5 (Remillard et al., 2001; Molina et al., 2018). Serious nutrient deficiencies caused by undernutrition can slow the lymphocyte response in dogs (Langweiler et al., 1981). In puppies nutrient deficienies can lead to skeletal deformation and hyperparathyroidism (Tal et al., 2018). Currently an estimated minimum of 25% of dogs that are admitted to veterinary services as suffering from undernutrition (Chandler and Gunn-Moore, 2004; Molina et al., 2018). Overnutrition (i.e., an energy supply in excess of the MER) can lead to increased BW, a BCS over the ideal score, and an increased risk of obesity (German et al., 2010; Tropf et al., 2017). The prevalence of obesity from overnutrition in dogs in some countries can be as high as 44.4% (McGreevy et al., 2005; Mao et al., 2013; Montoya-Alonso et al., 2017). Obesity in the dog can also cause an oversaturation of macrophages and a subsequent increase in pro-inflammatory cellular responses (German et al., 2010; Tropf et al., 2017). In puppies, overnutrition can also cause deformations in skeletal growth (Meyer and Zentek, 1992).

Creating and feeding a nutritionally balanced diet is therefore vital for the continued health of the dog at all life stages. Additionally, further research needs to be undertaken to explore how dietary

changes and nutrient imbalance can impact the health of the domestic dog. When creating and evaluating diets it is important to remember that though the dog is commonly classed as an omnivore (Bosch et al., 2015), this classification was based on dietary differences when compared to the domestic cat, a true obligate carnivore (Bosch et al., 2015). The dog retains carnivorous traits, such as a shorter and thicker-walled GIT (Bosch et al., 2015; Moon et al., 2018) and a heightened (compared to humans) capability to recycle and re-use fats (Bauer, 2004; Xenoulis and Steiner, 2010). Additionally, it also has an increased capability for carbohydrate digestion compared to obligate carnivores, owing to its domestication (Batchelor et al., 2011; Axelsson et al., 2013). Thus, the dog can be considered a facultative carnivore (Bermingham et al., 2018; Zafalon et al., 2020), or a 'carno-omnivore' (Batchelor et al., 2011).

Most commercially manufactured pet foods and research diets are designed to meet guidelines established by the National Research Council (NRC), the Association of American Feed Control Officials (AAFCO) and/or the European Pet Food Industry Federation (*Fédération européenne de l'industrie des aliments pour animaux familiers*; FEDIAF) (Butterwick et al., 2011). These dietary requirements can vary depending on life stage, and during pregnancy and lactation state in bitches (National Research Council, 2006; FEDIAF, 2018; AAFCO, 2019). Additionally, they are further influenced by current BW and BCS (Fascetti, 2010; Kathrani, 2016). The guidelines prescribe minimum requirements (or maximum/safe upper limits if the nutrient could cause toxicity/death of the animal) for growth/pregnancy or maintenance (National Research Council, 2006; FEDIAF, 2018; AAFCO, 2019). In general, these guidelines recommend relatively high levels of protein and moderate levels of fat (National Research Council, 2006; FEDIAF, 2018; AAFCO, 2019). (See Table 1.1).

***Table 1.1** – Association of American Feed Control Officials (AAFCO) and European Pet Food Industry Federation (FEDIAF) recommended minimum essential dietary nutrient levels for the domestic dog, expressed as a percentage of diet dry matter (% DM).*

Nutrient	**Growth & Reproduction Minimum (% DM)**	**Adult Maintenance Minimum (% DM)**

	FEDIAF	AAFCO	FEDIAF	AAFCO
Crude Protein	25.0	22.5	18.0	18.0
Arginine	0.82	1.00	0.52	0.51
Histidine	0.39	0.04	0.23	0.19
Isoleucine	0.65	0.71	0.46	0.38
Leucine	1.29	1.29	0.82	0.68
Lysine	0.88	0.90	0.42	0.63
Methionine	0.35	0.35	0.40	0.33
Methionine-cystine	0.70	0.70	0.76	0.65
Phenylalanine	0.50	0.83	0.54	0.45
Phenylalanine-Tyrosine	1.30	1.30	0.89	0.75
Threonine	0.81	1.04	0.52	0.48
Tryptophan	0.23	0.20	0.17	0.16
Valine[†]	0.68	0.68	0.59	0.49
Crude Fat	8.50	8.50	5.50	5.50
Linoleic acid*	1.30	1.30	1.32	1.10
alpha-Linolenic acid*	0.08	0.08	NS	NS
Eicosapentaenoic+ Docosahexaenoic acid*	0.05	0.05	NS	NS
Arachidonic acid	0.30	NS	NS	NS

* Maximum recommended ratio of 30:1 of (linoleic + arachidonic):(alpha-linolenic + eicosapentaenoic + docosahexaenoic)
NS = not specified

Essential dietary components outlined in Table 1.1 (such as arginine and taurine) cannot be synthesised at a sufficient level for required metabolic processes. Instead these are acquired from the diet (Dodd et al., 2018; Kaplan et al., 2018). Fats are used as an energy source preferentially over carbohydrates (CHO) in the dog (Hill, 2010). As depicted in Table 1.1, the dog has no nutritional

requirements for CHO. Dogs are able to use *de novo* gluconeogenesis as a means of obtaining glucose when the body has no starch to metabolise (Laflamme et al., 2014).

1.3. DIET AND THE DOG GIT MICROBIOTA

The gut microbiota in the dog are a diverse and complex collection of microorganisms that assist in many host processes. These processes include digestion and the maintenance and promotion of health (Moon et al., 2018; Valdes et al., 2018). The commensal bacteria sequences identified in the dog so far often fall into one of five bacterial phyla; Bacteroidetes, Fusobacteria, Firmicutes, Proteobacteria and Actinobacteria (Honneffer et al., 2017). Generally, these communities are reflective of the environment and functions of the area of the GIT they inhabit (Suchodolski et al., 2008; Honneffer et al., 2017; Pilla and Suchodolski, 2020). Obligate anaerobic bacteria, for example, are found predominantly in the anaerobic large intestine. These are predominantly Firmicutes or are capable of fermenting dietary fibres (Suchodolski et al., 2008; Panasevich et al., 2015; Honneffer et al., 2017). In contrast, the oxygenated small intestine houses aerobic and facultative anaerobes. These are mostly protein metabolising bacteria, which commonly belong to Proteobacteria (Suchodolski et al., 2008; Honneffer et al., 2017; Moon et al., 2018). These bacterial communities will change in a manner dependent on dietary nutritional composition (Wernimont et al., 2020a).

Generally, experimental diets are classed as a 'type' (i.e., raw food, etc) rather than classifications by dietary content (i.e., by protein/fat/fibre content). The impacts of these diet types on the faecal microbiome are summarised in Table 1.2.

Table 1.2 - *Changes in relative abundances of the dog faecal microbiota to different diet "types"*

Diet		**Microbial Response**	
Type	**Summary**	**Increase**	**Decrease**
Bones and Raw Food	High levels of raw meat, offal and bones Low levels of dietary fibre Negligible / no carbohydrate content (CHO)	Fusobacteria *Fusobacterium* *Clostridium* Lactobacillales *Allobaculum* Enterobacteriaceae Proteobacteria Actinobacteria *Bifidobacterium pseudolongum*	*Faecalibacterium* spp Ruminococcaceae Erysipelotrichaceae *Prevotella* *Sutterella* *Bifidobacteriaceae* Bacteroidetes
High Protein, Low Carbohydrate	High levels of protein Low or no CHO Includes 'natural diets' that meet these criteria but do not fall into any other categories in this table (ie using cooked animal or plant protein)	Fusobacteria Fusobacteriales Fusobacteriacea *Clostridium perfringens* *Clostridium* spp. *Eubacterium dolichum* *Clostridiaceae* *Dorea* *Erysipelotrichaceae* *Roseburia* *Blautia wexlerae* *Clostridium hiranonis* *Ruminococcus gnavus* *Slackia*	*Coprobacillus* *Clostridiales* *Lactobacillales* *Faecalibacterium* *Clostridium saccharogumia* *Prevotella* *Solobacterium* Bacteroidetes *Bacteroidales*
Increased Dietary Fibre	Diets with increased fibre, including those with the addition of prebiotic fibres to the diet	*Roseburia* *Lachnospira* Prevotellacea Selenomonadaceae *Faecalibacterium* *Lactobacillus*	*Fusobacterium* Fusobacteriaceae spp *Coprobacillus* *Prevotella* *Clostridium* *Kaistobacter*

		Enterococcus Sutterellaceae *Succinviibrio* *Bifidobacterium* *Bifidobacterium pseudolongum* *Slackia* *Collinsella stercoris*	Enterobacteriaceae Clostridiaceae Ruminococcaceae *Fecalibacterium prausnitzii* *Bacteroides uniformis*
Low Protein, High Carbohydrate	Diets where the percentage of CHO is higher than that of the protein	*Clostridium* spp. *Faecalibacterium* *Clostridium butyricum* *Bacteroides uniformis*	*Lactobacillales* Prevotellaceae *Bacteroidales*
Raw Red Meat	High levels of animal protein and fat Very low levels of CHO	*Fusobacterium* *Clostridium* *Lactobacillus* Veillonellaceae Streptococcaceae Enterobacteriaceae Proteobacteria *Collinsella* *Slackia* Coriobacteriaceae	*Faecalibacterium* *Prevotella* Peptostreptococcus *LactobacillusParalactobacillus* Bacteriodes

References used for the table: (Hang et al., 2012; Kerr et al., 2013; Panasevich et al., 2015; Sabbioni et al., 2016; Bermingham et al., 2017; Herstad et al., 2017; Kim et al., 2017; Li et al., 2017; Sandri et al., 2017; Xu et al., 2017; Alexander et al., 2018; Schauf et al., 2018; Schmidt et al., 2018; Alessandri et al., 2019; Jackson and Jewell, 2019; Kim et al., 2019; Lin et al., 2019).

However, there are difficulties in assessing the impacts of diet on the microbiota of the dog. As seen in Table 1.2, different studies report detected microbes at different taxonomic levels. Additionally, each method of classifying a diet (i.e., by dietary format, by dietary macronutrient content, etc.) comes with their own sets of challenges. For example, two studies both classed diets as "raw meat based" (Bermingham et al., 2017; Sandri et al., 2020). However, one was almost completely carbohydrate-free (0.6% content by dry matter analysis; % DM) (Bermingham et al., 2017). In comparison, the other study contained a carbohydrate content of 42-43% DM (Sandri et al., 2020). In this instance, the carbohydrate content was higher than that of commercial kibble used in another study (34.6% DM) (Bermingham et al., 2017). With the multitude of factors present in the diet that can impact the microbiota, it is currently nigh impossible to account for all of these in a single experiment or review of the literature.

As highlighted in Section 1.2, proteins and fats are the essential dietary macronutrients for the dog. The impacts of diet on the microbiome of the dog have been reviewed and summarised previously (Pilla and Suchodolski, 2020; Wernimont et al., 2020b). To better contribute to the existing knowledge, and to perform a study that would allign with the principals of the 3R's, it was apparently that a meta-analysis of the literature would be a more beneficial approach. This could interrogate the existing data, evaluate the impacts of dietary proteins and fats on the dog microbiota, and potentially generate new insights in the relationship of diet and the microbiota in the dog.

1.4. THE DOG INTESTINE

The intestine is lined with a barrier composed of a single layer of IECs. (See Figure 1.1). This barrier enables the absorption of nutrients and water whilst blocking absorption of unwanted pathogens (Anderson et al., 2018; Cai et al., 2018). Owing to the near-anaerobic environmental conditions of the GIT (Muir et al., 2014; Friedman et al., 2018), the IECs perform their roles in a state of physiological hypoxia (Zheng et al., 2015). IECs require oxygen to survive and their nutrient absorbing capacity is diminished by a decreased supply of oxygen (Ward et al., 2014). When this

barrier is compromised, the intestinal submucosa becomes vulnerable to invasion from bacterial pathogens in the gut lumen (Walsh-Reitz et al., 2005).

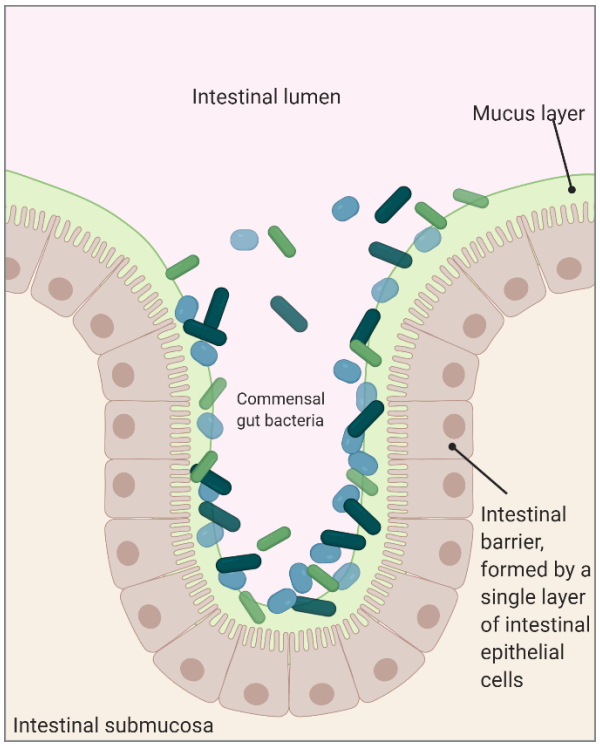


Figure 1.1 *– A simplified view of the intestinal barrier. Created with BioRender.com.*

Increased permeability is seen as a hallmark of various forms of intestinal disorders. When modelling the intestine using in vitro cell culture methods, decreases in barrier integrity are interpreted as detrimental effects. (Walsh-Reitz et al., 2005). In contrast, increases in barrier integrity are considered a beneficial response (Walsh-Reitz et al., 2005). For example, the epithelial permeability is increased in inflammatory bowel disease IBD. This is caused by a loss of barrier function proteins and an increase in pro-inflammatory cytokine production (Edelblum and Turner, 2009). However, there are currently few studies that utilise canine-specific primary cells, or cell lines derived from primary cells to investigate IEC functionality (Weng et al., 2005; Golaz et al., 2007; Farquhar et al., 2018; Reineking et al., 2018a; Ambrosini et al., 2020). Thus, this creates a limitation in undestanding how the dog intestine may respond to microbial challenges.

1.4.1. THE GIT MICROBIOTA AND INTESTINAL DEFENCES IN THE DOMESTIC DOG

There are no studies in the literature that have assessed the response of dog IECs to direct bacterial stimulation. The outer wall of bacteria contains peptidoglycans, lipoteichoic acid (LTA) and lipopolysaccharides (LPS) (Silhavy et al., 2010). Hereafter these are referred to as bacterial ligands. These can be used to simulate bacterial challenges, however, only two studies have performed this using dog IECs (Swerdlow et al., 2006; Farquhar et al., 2018). Comparatively, there is a wealth of information available for direct and simulated bacterial challenges on human IECs and this is reviewed and evaluated frequently (Valdes et al., 2018; Xue et al., 2020; Zheng et al., 2020). However, difficulties arise here in the extrapolation of these results from an omnivorous model to a more carnivorous one. For example, *Peptostreptococcus* and *Streptococcus* are associated with amino acid metabolism in humans (Allison and Macfarlane, 1989; Dai et al., 2011). However, in the dog these decreased in relative abundance after a high protein diet (Bermingham et al., 2017; Mori et al., 2019). This suggests that processes that require *Peptostreptococcus* and *Streptococcus* in humans are either not needed or utilise other microbes in the dog.

Similarly, though *Clostridium difficile* is a health concern in humans, in dogs this forms part of their normal GIT microbiota (Stone et al., 2019). However, in a study by Stone et. al., they found that the barrier integrity of canine epithelial cells and human Caco-2 cells was similarly impacted by *C. difficile* toxins (Stone et al., 2019). Furthermore, this study suggested that secondary bile acids synthesised by *Clostridum hiranonis,* which also forms part of the normal GIT microbiota in dogs, help to prevent the effects of *C. difficile* toxicity in dogs (Stone et al., 2019).

Currently there is a dominance of inflammatory bowel disease (IBD)-focused studies and how their microbial changes impact health in dogs. These studies are used to define bacteria as “good” or “bad” for the dog, based on their relationship with IBD (Suchodolski et al., 2012b; Pilla and Suchodolski, 2020; Wernimont et al., 2020a). For example, increased relative abundances of faecal Proteobacteria are associated with IBD (Suchodolski et al., 2012a; Minamoto et al., 2015; Kalenyak et

al., 2017). Thus, Proteobacteria would be inferred as a "bad" bacteria for gut health. However, in human IBD events, arginine metabolism is crucial for resolving intestinal inflammation (Sugihara et al., 2019). Proteobacteria are involved in the metabolism, biosynthesis and transport of arginine (Leyn et al., 2016). Thus, it would appear that Proteobacteria are involved in restoring gut homeostasis. Therefore, care must be taken when using IBD studies to infer postive relationships of the GIT microbiota and intestinal health.

Some studies that evaluate the changes in IBD do not consider, or present, the dietary information of the dogs (Kalenyak et al., 2017; Omori et al., 2017). Thus, the impacts of diet on their results are not usually considered. As an example, the *Clostridiaceae* members *Clostridia* and *Clostridium perfringens* increase in relation to IBD severity (Suchodolski et al., 2012a; Minamoto et al., 2015). However, *Clostridiaceae* levels positively correlate with dietary protein content (Bermingham et al., 2017). Additionally, *Clostridiaceae* are suggested to have a role in protein metabolism in the dog (Lubbs et al., 2009; Vital et al., 2014a). Interestingly, challenges of macrophages with bacteria from healthy dogs and those with IBD showed an inherent difference in immune response, with bacteria from healthy dogs resulting in less proinflammatory tumour necrosis factor (TNF)-α and greater anti-inflammatory interleukin (IL)-10 (Soontararak et al., 2019). The conclusion drawn in this experiment was that gut bacteria in dogs with IBD are inherently capable of stimulating a greater pro-inflammatory response than that of healthy dogs. There are no other studies to confirm or refute these conclusions. However. it does add an extra layer to the challenges and difficulties of understanding the relationship of the GIT microbiota with the healthy host.

Based on the literature available, it became apparent that before reviewing the literature on the interactions between bacteria and the intestine, the interactions between bacterial ligands and IEC needed to be evaluated first. This would then allow for more hypotheses to be drawn with more scientific confidence.

1.5. RESEARCH GOAL OF THE THESIS

This doctoral project aimed to develop a model of the dog intestine that was more reflective of conditions seen *in vivo*. To achieve this, the project aimed to utilise a previously established canine intestinal epithelial cell (cIEC) line (Weng et al., 2005; Farquhar et al., 2018) and characterise its response to direct bacterial stimulation. This approach aimed to perform this in a physiologically appropriate *in vitro* model capable of sustaining an apically anaerobic environment and a basally aerobic environment that would allow for the dual culture of the cIEC with anaerobic bacteria. Thus, this goal would work towards a new alternative to laboratory animal testing and therefore the goals of the 3 R's.

Based on the assessment of the current knowledge and limitations across the subject areas of the doctoral project, the project work was divided into the following chapters:

- Chapter Two was a review of the literature. Based on the assessment of the current knowledge, it was determined that the IEC responses to bacterial ligands and SCFA would need to be reviewed in-depth. Thus, the literature review explored the fundamentalphysiological and immune responses of the intestine to bacterial ligands and SCFAs in other species. The similarities and differences in the phsyiology of the dog intestine compared to other species was evaluated. This could then be used to formulate hypotheses as to the effects of direct bacterial stimulation on dog IECs.
- Chapter Three was a meta-analysis of the existing literature on the relationship between dietary protein, dietary fat, and the faecal microbiota in the domestic dog. This would further contribute to the existing literature in a manner that aligned with the 3 R's. Additionally, it would be used to determine which bacteria would be of interest to characterise the intestinal response towards in the project. This would also be able to characterise "good" bacteria for the dog in a non-IBD based assessment.
- Chapter Four was the method development conducted to adapt the existing methodology for cIEC differentiation for use in a cellZscope system. The cellZscope is

a commercially avaiable automated system designed to measure cellular barrier integrity whilst inside an incubator. This allows for the measurement to be conducted in temperatures reflective of *in vivo*. These methods were utilised throughout the project. Method refinements completed were appropriate for use in the automated systems, and in traditional cell culture.

- Chapter Five simulated a bacterial challenge to the cIEC by stimulating the cIEC with lipopolysaccharides (LPS), butyrate, or a combination of LPS and butyrate. The changes in gene expression and protein concentrations of the cIEC's cellular junction proteins and immunological pro- and anti-inflammatory responses were assessed. This was performed using methods refined in Chapter Four and hypotheses were generated using the knowledge gained in Chapter Two.
- Chapter Six was the initial work conducted towards adapting the cIEC to the apically anaerobic dual culture system. This utilised the methods refined and developed in Chapter Four. Additionally, it detailed planned experiments cut short due to the Covid-19 pandemic and discusses future opportunities using the apically anaerobic model based on planned experiments and results obtained.
- An overall discussion of the thesis is presented in Chapter Seven. This identifies key findings and presents recommendations for future research.

1.6. IMPACTS OF COVID-19

This PhD project was severely impacted and limited by the Covid-19 pandemic during 2020-2021. The initial aim of the project was to expand on the work conducted in Chapters Two – Five with the use of an apically anaerobic co-culture system. This would have allowed for the simultaneous culture of the oxygen-requiring cIEC and anaerobic bacteria. This would have used bacteria of interest highlighted from the meta-analysis in Chapter Three and the methods developed in Chapter Four. Results obtained in Chapter Five could have then been used to compare responses in traditional, aerobic culture to responses in an environment that more closely resembled *in vivo* conditions.

However, because of the pandemic, raw materials for the culture of the cIEC were unable to be produced and imported into the country. The apical anaerobic model used for the work had been custom built and used consumables that became unavailable. Thus, no alternatives could be used that were able to maintain the barrier between the aerobic and anaerobic environments. The pandemic occurred 18 months into the PhD project. By this point work had already begun on testing the methods used in Chapter Four. Additionally, during the PhD there was a planned move of the AgResearch laboratory facilities, from the Grasslands campus to the new Te Ohu Rangahau Kai building. Laboratories were shut down in March 2020 in preparation for the move. However, the pandemic delayed the opening of the new facility and laboratory work was only able to begin again in October 2020. Therefore, it was decided to refocus on the aerobic model, as seen in Chapter Five. However, as it formed a significant part of the initial project workflow, Chapter Six details the initial work conducted in the dual environment co-culture (DECC) system and discusses the planned work that would have been conducted in this system.

CHAPTER TWO

LITERATURE REVIEW

2.1. ABSTRACT

Diet changes are one of the main drivers of changes in the composition and abundances of the gastrointestinal microbiota in all species, including the domestic dog. Many studies have investigated the impacts of these bacteria on the host response in both *in vitro* and *in vivo* models. Bacterial ligands are the activators of the immune response and drive the subsequent alterations to the host intestinal defence response. In addition, short-chain fatty acids, produced by the fermentation of dietary products by the microbiota of the gastrointestinal tract, also contribute to maintaining and modulating these innate responses. By understanding the relationship between bacterial ligands, short-chained fatty acids and the defence systems of the intestine, new insights may be gleaned as to how these defences operate in the domestic dog, and how these may differ from responses in other species.

2.2. INTRODUCTION

The relationship between diet and health in the domestic dog is largely dependent on the relationship between diet and the microbiota of the GIT (Bresciani et al., 2018; Allaway et al., 2020; Atherly et al., 2020). Understanding how these bacteria interact with the GIT can help to understand how the diet-based modulation of the GIT microbiota can impact health (Rose et al., 2021).

Intestinal and overall host health are directly impacted by the ability of the intestinal barrier to keep out unwanted and opportunistic organisms (Ahn et al., 2016; Assimakopoulos et al., 2018). This barrier is formed from a single layer of IECs that maintain and defend host health physically through inter-cellular tight junctions (TJs) that control permeability (Robinson et al., 2015; Chelakkot et al., 2018). The IECs maintain intestinal defences by expressing pattern recognition receptors (PRRs). These membrane-bound PRRs include toll-like receptors (TLRs) and C-type lectin receptors (Zhang et al., 2010; Iliev et al., 2012; Maiolini et al., 2012). Receptors on the PRRs detect signature molecular patterns associated with pathogens (microbe associated molecular patterns; MAMPs) or those released by injured or dying cells (damage associated molecular patterns; DAMPs) (Swerdlow et al., 2006; House et al., 2008; Zhang et al., 2010; Mercier et al., 2012). These then trigger a cytokine

and chemokine response (Rahman et al., 2009; Fukata and Arditi, 2013). The PRRs are also capable of promoting the activation and response efficiency of T cells (Rahman et al., 2009; Fukata and Arditi, 2013). Meanwhile, activation of cytoplasmic PRRs such as nucleotide-binding oligomerization domain-like receptors (NLRs) triggers the activation of inflammatory caspases and nuclear factor kappa B (NF-κB) that form part of the immune response (Proell et al., 2008; Schmitz et al., 2015b).

The GIT microbiota are a mixed population of Gram-negative and Gram-positive bacteria. These have an outer shell which contain bacterial ligands (i.e., peptidoglycans, LTA and LPS) (Silhavy et al., 2010). The fermentation of dietary products by the GIT microbiota give rise to short chain fatty acids (SCFA) such as acetate, butyrate, and propionate. These can contribute to, and alter, the defences in the GIT (Peng et al., 2007; Bansal et al., 2010; Vinolo et al., 2011). Breaches in these defences can lead to GIT-wide inflammation and diseases such as IBD (Ahn et al., 2016). Aberrant alteration to the GIT microbiota, also known as dysbiosis, can also result in inflammation and diseases (Suchodolski et al., 2012a).

The importance of the relationship between diet and pet health is an ever-growing area of research (Kim et al., 2017). Few studies have evaluated the dog IEC response to stimulation with bacteria or bacterial ligands. Therefore, the aim of this literature review was to analyse the relationship between IECs and bacterial ligands and SCFAs. Firstly, current knowledge on the intestine and IECs was outlined. Current studies evaluating the potential use of dog IECs were then summarised. The cellular responses to bacterial ligands and SCFA were then evaluated, and the current literature was used to highlight potential differences in cellular responses that would occur in the dog. The data reviewed would then drive future hypotheses for experiments performed in Chapter Five.

2.3. THE PHYSICAL GUT BARRIER

The intestinal epithelial barrier (IEB) acts as a multi-faceted barrier against unwanted microbes, a selectively semi-permeable filter for water, nutrients and waste material, and a fundamental contributor to the innate immune response (Lee, 2015; Reineking et al., 2018a; Le et al., 2021b). This

barrier, illustrated in Figure 2.2, is formed from a single layer of IECs whose permeability is maintained and facilitated by TJs (Peterson and Artis, 2014).

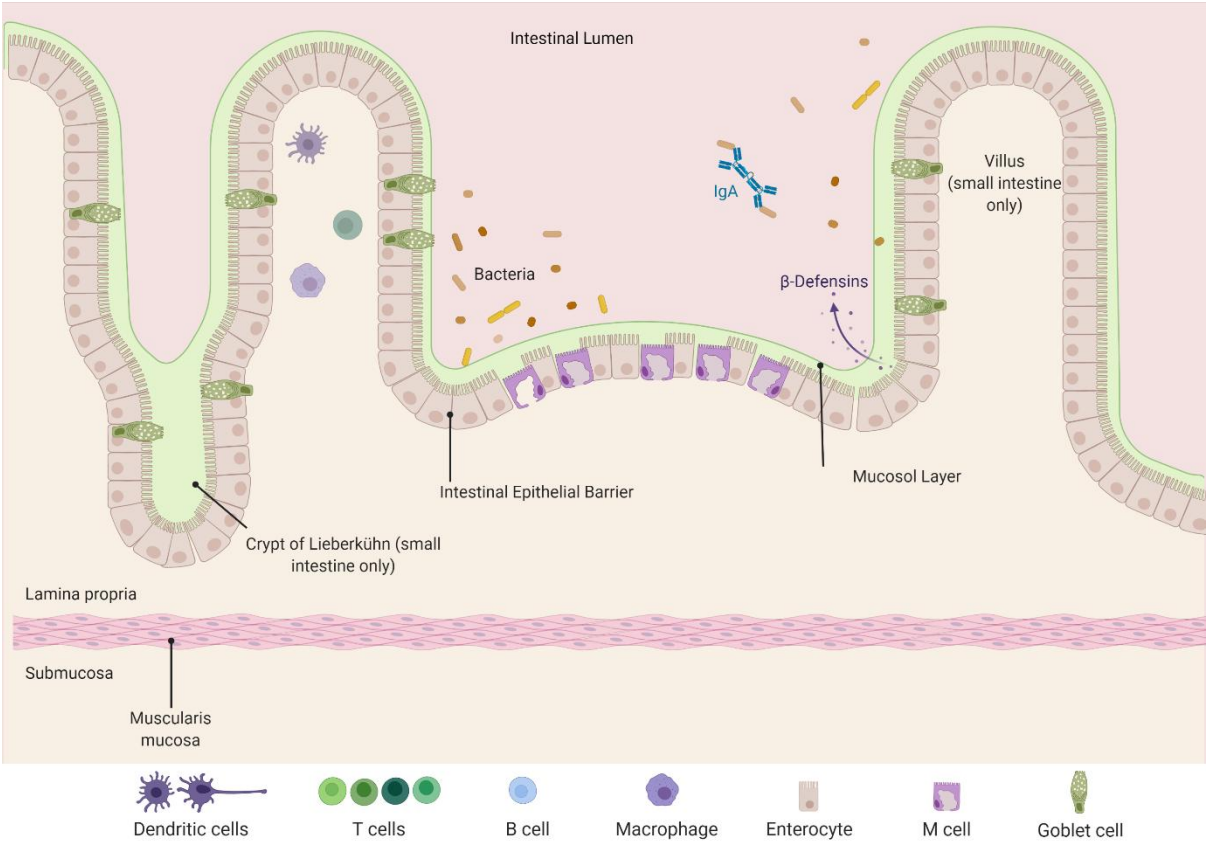


Figure 2.2*– A simplified view of the intestinal epithelial barrier in the canine small intestine.The intestinal epithelial barrier is comprised of a cell barrier that is a single cell in thickness. These cells, in the dog, consist of a mixture of enterocytes, M cells and goblet cells, which form from differentiated stem cells that travel up the villi from the crypts of Lieberkühn. Goblet cells are responsible for secreting the MUC2 protein that produces the mucosal layer that aids in separating the bacteria present in the intestinal lumen from the immune cells, such as T cells and macrophages. Enterocytes also secrete anti-microbial proteins into the mucosal layer, such as B-defensins, which aid in the immune response against antagonistic bacteria. Secretory IgA, meanwhile, can remove the non-commensal bacteria present in the intestinal lumen. Figure created with Biorender.*

Pluripotent stem cells reside in the crypts of Lieberkühn of the IEB. These renew the entire epithelial barrier by travelling up the villi (which are not present in the colon) after differentiation. In healthy conditions, they continue to travel upwards and eventually are shed into the gut lumen and die from anoikis (Peterson and Artis, 2014; Creff et al., 2021). Interestingly, the villi of the dog are

tongue-shaped, in contrast to the finger-shaped villi commonly known to characterise the human small intestine (Kararli, 1995). The impacts of this structural difference have not been assessed in the literature, although these villi shapes are also seen in chickens (Yamauchi et al., 2010) and the duodenum of young (7 week old), weaning piglets (Wiese et al., 2003).

2.3.1. CLASSES AND ROLES OF INTESTINAL EPITHELIAL CELLS

The process by which the IEB is completely replenished has a turnover rate of between three to five days (Peterson and Artis, 2014; Creff et al., 2021). The differentiated stem cells become one of the five main types of IEC that comprise the IEB in the domestic dog. Most cells (an estimated 80%) are absorptive enterocytes. The remainder is comprised of M cells, secretory IECs (enteroendocrine cells and goblet cells), and a small percentage of Tuft cells (Peterson and Artis, 2014; Creff et al., 2021). Unlike humans and rodents, dogs do not have Paneth cells. Therefore, dogs lack the gene that allows for expression of α-defensins (Ouellette, 2011; Kingsbury et al., 2017). However, the frizzled class receptor (FZD) 5 in the dog is theorised to compliment these missing pathways by secreting antimicrobial peptides such as beta-defensin 103 and cathelicidin, and the pro-inflammatory cytokine interleukin (IL)-17 (Chandra et al., 2019).

Enterocytes are responsible for the absorption of iron, regulated by the amino acid hepcidin (Fuqua et al., 2012). Additionally they are responsble for the absorption of vitamin D (Reboul, 2015), vitamin A, and carotenoids by lipid transport (Reboul, 2013). Enterocyte absorption of fructose occurs via the GLUT2 transporter (Steenson et al., 2017). In general, nutrient molecules enter the intestinal cell cytoplasm via epithelial transporters, whilst macromolecules enter via endocytosis (Snoeck et al., 2005). Because of the mode of absorption used by enterocytes, factors that affect the production of fatty acid binding proteins (Rodriguez Sawicki et al., 2017), bacterial infections such as salmonella (Broz, 2014) and viral infections like rotavirus (Rollo et al., 1999; Ramig, 2004) have detrimental effects on the enterocyte's absorptive abilities. Due to their roles in both nutrient absorption and innate immunity, intestinal enterocytes have been used as a cell culture model to better understand intestinal function. In response to inflammatory signals they secrete cytokines and

chemokines and are capable of recruiting and activating immune cells, such as T cells (Gronert et al., 1998; Chougule et al., 2012). However, they lack the mucin-producing functionalities of goblet cells (Peterson and Artis, 2014; Okumura and Takeda, 2016; Creff et al., 2021).

Enteroendocrine cells are the largest population of hormone producing cells in the body, despite only making up only 1% of the cells lining the intestinal lumen (Palazzo et al., 2007; van der Flier and Clevers, 2009). Enteroendocrine cells produce gastrointestinal hormones which act as satiety signals within seconds of nutrient uptake by the intestine (van der Flier and Clevers, 2009). The enteroendocrine cells also express TLR4, 5 and 9 (Garrett et al., 2010), suggesting that these cells play a role in inflammatory responses of the host (Palazzo et al., 2007).

Goblet cells are responsible for the production of the mucus layer(s) via the secretion of mucins, such as the gel-forming MUC2 and MUC5AC proteins (Nagata et al., 2022). When released from the goblet cell, mucin will unfold and expand over a thousand-fold to form staggered, net-like sheets that bond together via disulphide bonds (Ambort et al., 2012; Johansson and Hansson, 2014). In the colon MUC2 is the predominantly secreted mucin, and forms two mucus layers over the IEB. In the small intestine there is only one mucus layer (Nagata et al., 2022). This adds another layer of protection to separate the IEB from the intestinal lumen and separates the microbial contents of the gut lumen from the immune cells in the lamina propria (Peterson and Artis, 2014; Okumura and Takeda, 2016; Creff et al., 2021). In the dog, visual identification of MUC2 is difficult due to the lack of specific canine MUC2 antibodies for immunohistochemistry, and because the rat and human-based antibodies do not cross react (Weng et al., 2005; Chandra et al., 2019). However, these proteins are detectable in the dog when analysing gene expressions (Nagata et al., 2022). Similar to results seen in humans with ulcerative colitis, in dogs it appears that MUC5AC expression levels are altered in dogs with colorectal polyps, whilst MUC2 expression levels are unchanged (Nagata et al., 2022).

Tuft cells, whilst usually the most uncommon of the IECs, increase in abundance during the course of a parasitic infection (von Moltke et al., 2015; Gerbe et al., 2016; Howitt et al., 2016). Additionally, they regulate a type-2 immune response (von Moltke et al., 2015; Gerbe et al., 2016;

Howitt et al., 2016). This response is promoted by T helper type 2 cells (Th2) which are lymphocytes that secrete IL-4, IL-5, IL-9, IL-10, and IL-13 (Spellberg and Edwards, 2001; Gerbe et al., 2016). In comparison, T helper type 1 (Th1) cells secrete IL-2, interferon-γ (IFN- γ), and lymphotoxin-α (Spellberg and Edwards, 2001). Though these responses have not been characterised in canine tuft cells yet, they are being extrapolated to form hypotheses as canine-origin tuft cells are beginning to be isolated and investigated (Chandra et al., 2019).

2.3.2. JUNCTIONAL PROTEINS AND COMPLEXES BETWEEN INTESTINAL EPITHELIAL CELLS

Tight junctions (TJ) occur between IECs (Anderson et al., 2012). These form selectively permeable connections that allow for the absorption of nutrients and diffusion of water, immune cells and macromolecules via paracellular pathways. Additionally, they simultaneously act as a barrier to opportunistic organisms and unwanted pathogens (Lee, 2015; Robinson et al., 2015; Chelakkot et al., 2018). Over fifty proteins contribute to the formation of TJ, including the scaffolding protein zonula occludens-1 (ZO-1), and transmembrane proteins such as claudin (CLDN), occludin (OCLDN) and junctional adhesion molecules (JAM) (Ulluwishewa et al., 2011; Zhao et al., 2011; Fredriksson et al., 2015) (see Figure 2.3).

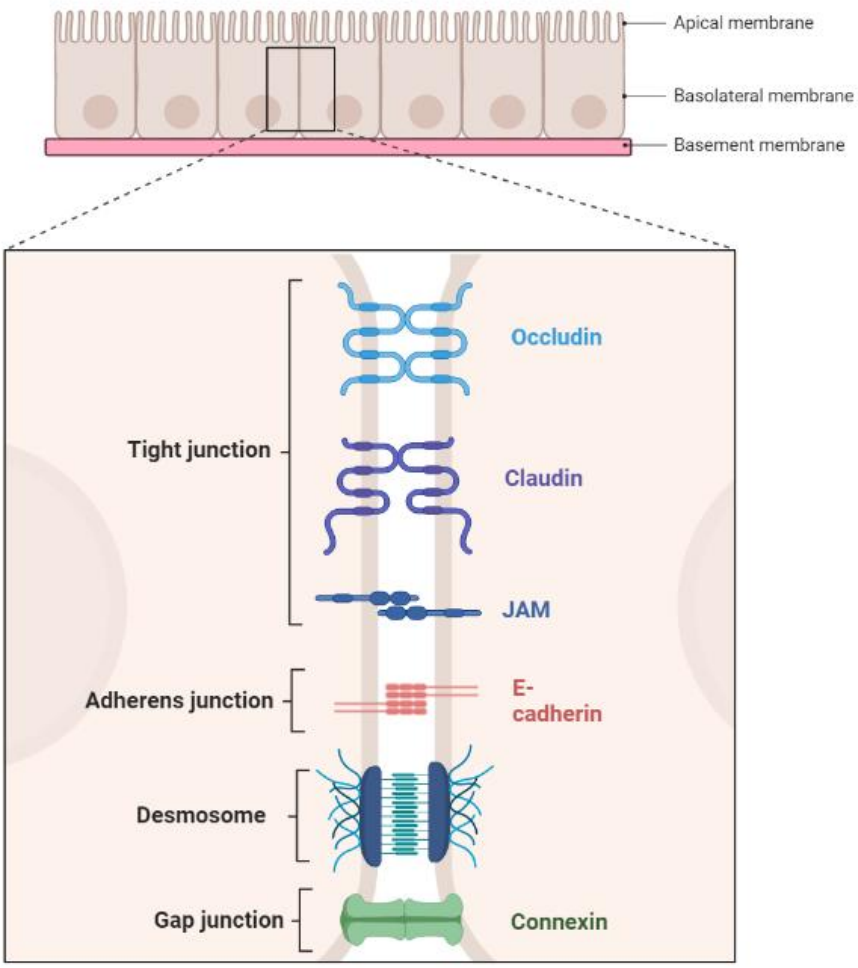


Figure 2.3 *– Types of tight junction proteins and their locations between intestinal epithelial cells. JAM, junctional adhesion molecule.Created with Biorender.*

Claudins are a major component of TJ strands (Nakamura et al., 2019). These polymerise to form strand networks that pair with adjacent cells to create charge-selective barriers (Itallie et al., 2019). Claudins form basally and travel up the cell to their localisation area, although inhibition of mitogen-activated protein kinase can reduce the activation and basal expression of these TJ proteins (Kinugasa et al., 2000; Itallie et al., 2019). To date there have been 27 different subtypes identified in mammals (26 in humans) that differ in expression between organs and host development stage (Nakamura et al., 2019). However, studies in the expression and regulation of these in the dog are limited (Ahn et al., 2016). Claudins localise at different parts of the cellular membrane (Itallie et al., 2019). This localisation is dependent on their relationship with, ZO proteins (Itallie et al., 2019).

However, not all CLDNs are related to strengthening TJs. For example, CLDN-1, CLDN-3 and CLDN-4 are associated with the tightening of TJs (Markov et al., 2010; Milatz et al., 2010; de Souza et al., 2013; Cong et al., 2015). However, CLDN-2 is a pore-forming, cation-selective protein that can increase permeability of the TJ in dogs (Furuse et al., 2001; Amasheh et al., 2002), humans (Luettig et al., 2015) and mice (Corridoni et al., 2012).

In addition to TJs, adherens junctions, gap proteins and desmosomes are also formed in the gaps between the columnar epithelial cells (Anderson et al., 2012). E-cadherin, which is one of the adherens junctions, is needed to trigger TJ formation. Suppresion or inhibition of E-cadherin can weaken the TJ complexes that maintain barrier stability (Cereijido et al., 2000). Desmosomes are responsible for the adhesion of adjacent cells (Kowalczyk and Green, 2013). Meanwhile, gap junctions are formed by the head-to-head docking of connexons and allow the diffusion of ions and small molecules between adjacent cells (Goodenough and Paul, 2009).

2.3.3. MEASUREMENT AND QUANTIFICATION OF BARRIER INTEGRITY *IN VITRO*

The formation of TJs result in either 'tight' or 'leaky' barriers (Anderson and Van Itallie, 2009; Srinivasan et al., 2015). These barriers allow Na+ ions to diffuse (Anderson and Van Itallie, 2009). This creates a situation where the transepithelial electrical resistance (TEER) can be measured across the epithelial monolayer (Zucco et al., 2005; Srinivasan et al., 2015). An electrode above the cell monolayer applies an alternating current, which is then measured by a basal electrode and can be used to calculate the ohmic resistance (Srinivasan et al., 2015) (see Figure 2.4).

Through this assessment of the ionic conductivity of the paracellular pathway, TEER measurements provide a way of quantifying the permeability and integrity of a cellular monolayer (Zucco et al., 2005; Srinivasan et al., 2015). These values are usually reported in units of Ωcm^2, calculated as the ohmic resistance of the cell monolayer/tissue surface (Ω), multiplied by the area (cm^2) (Srinivasan et al., 2015). Increases in TEER means a decrease in barrier permeability, and is

interpreted as a beneficial effect, and decreases in TEER are considered detrimental, indicating increased barrier permeability (Srinivasan et al., 2015). Changes in TEER can generally be observed in tandem with changes to TJ expression levels. For example, increases in CLDN-1, CLDN-3, CLDN-4, OCLDN, and ZO-1 are observed in relation to increased TEER, with concurrent decreases in CLDN-2 (Sheth et al., 2007; Fujita et al., 2012; Yan and Ajuwon, 2017; He et al., 2020; Wu et al., 2020).

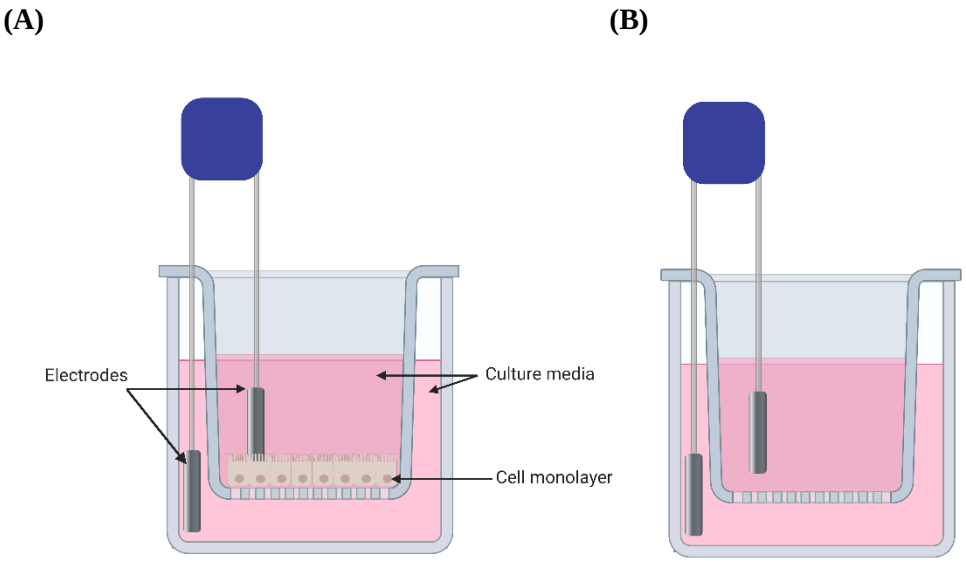


Figure 2.4*- Transepithelial electrical resistance measurement using chopstick electrodes. The ohmic resistance of the cell monolayer is obtained (A) and a blank measurement performed on the semipermeable membrane only (B), is subtracted from this to give the ohmic resistance of the cells. Figure created in BioRender.*

Tracer compounds such as biotin, fluorescent dextrans and enzymatic markers can also be used to assess the permeability of TJ *in vitro*, although these compounds can affect the cellular transport processes (Srinivasan et al., 2015). These compounds measure the paracellular flux (i.e., the transfer of substances through the intercellular space between the cells). Fluorescein isothiocyanate (FITC) dextran is one such compound used. FITC dextran with a weight of 4 kDa is added to the apical side of a cell monolayer and the media on the basal side of the cells is collected. The wavelengths of the basal media are measured (with excitation at 492 nm and emission at 520 nm), thereby allowing quantification of the paracellular flux (He et al., 2020).

2.3.4. CURRENT INTESTINAL EPITHELIAL CELL MODELS FOR THE DOG

Currently there are no immortalised intestinal epithelial cell lines from dogs that are available commercially. Biopsies from dogs have been utilised to study responses of the canine intestine to different challenges, although not all of these studies attempt to cultivate and culture successive passages of cells (Burgener et al., 2008; Allenspach et al., 2010; McMahon et al., 2010; Ohta et al., 2011; Mercier et al., 2012; Suchodolski et al., 2012a; Schmitz et al., 2015b; Atherly et al., 2020). These analyses are constrained by the inability to continuously culture these cells for long periods of time before viability is lost, stem cells overpopulate the culture, or morphological changes render them unable to be utilised for target investigations (Golaz et al., 2007; Reineking et al., 2018a). There are several studies that have investigated the capability of culturing intestinal epithelial cells from dogs as models for diet and immune responses, which are presented in Table 2.3.

Table 2.3 *- Publications that utilise dog intestinal epithelial cells (IECs) for the attempted establishment of re-usable cell lines.*

Region of Intestine IEC isolated from	Health status	Experimental Results	Reference
Jejunum	Healthy beagles (n not specified)	• Successful culture of confluent monolayers. • Cells grew microvilli in culture. • Cells could be cultured for a minimum of 6 passages. • CLDN-3, CLDN-4 and OCLDN were detected by Western Blot in the primary, first and second passages. • CLDN-2 and CLDN-5 not detected in any passages. • Cells did not respond to antibodies against mucin.	(Weng et al., 2005)
Duodenum	Healthy neonatal puppies (n = 14).	• Successful culture of confluent monolayers. • Cells were viable for study for two passages (an average of two weeks). • Alkaline phosphatase activity decreased over time from day 2 to day 8. • Sucrase activity did not change over time.	(Golaz et al., 2007)
Duodenum and colon	Healthy beagles (n = 4).	• All tight junction protein analysis performed by immunoblot. • Strong expression of CLDN-3, CLDN-5, E-cadherin, and B-catenin in duodenum. • Weak expression of CLDN-1 and CLDN-7 in duodenum. • CLDN-2, CLDN-4 and CLDN-8 not detected in the duodenum. • Strong expression of CLDN-2, CLDN-3 E-cadherin and B-catenin in colon. • Weak expression of CLDN-4 and CLDN-7 in colon. • CLDN-8 not detected in the colon. • CLDN-7 was located along the basolateral membrane in all instances.	(Ohta et al., 2011)

Region of Intestine IEC isolated from	Health status	Experimental Results	Reference
		• CLDN-3, E-cadherin and B-catenin localised primarily to the apical junction complex in the duodenum and colon.	
Jejunum and colon	Collected from euthanised dogs of various health conditions, though none had presented symptoms of gastrointestinal illnesses (n = 21).	• Isolated cells grew microvilli in culture. • Jejunal cells lost viability after a week. • Both jejunum and colonic cells expressed TLR5 and TLR9 intracellularly (determined by flow cytometry). • Both cell types expressed MHC II	(Reineking et al., 2018a)
Duodenum	Healthy neonatal puppies (n = 14). Used the same cells isolated in (Golaz et al., 2007).	• Infected cells with *N. caninum.* • Parasites invaded the IEC. • No analysis of tight junctions or cytokine responses performed.	(Hemphill et al., 2009)
Intestinal Tumours	Cancer cells (n = 57).	• Cells stained with hepatocyte paraffin 1. • No other analyses were performed.	(Ramos-Vara and Miller, 2002)
Duodenum, Ileum, Jejunum, and Colon.	Jejunum from healthy dogs (n = 28), all others taken from biopsies from dogs with IBD (n = 12).	• Successful generation and culture of canine intestinal organoids. • Cells were viable for up to twenty passages. • No major morphological differences in organoids between healthy and dogs with inflammatory bowel disease. • Microvilli, desmosomes, mucin droplets, and a luminal space grown in cultured jejunal organoids. • FZD5 expressed in crypts and villi. • CBD103, CATH, IL-17 identified in organoids. • Alkaline phosphatase and Neurogenin-3 expressed in cultured tissues. • Tuft cells cultured from isolated organoids.	(Chandra et al., 2019)
Colon	Healthy puppies (n not specified).	• Successful culture of colonic epithelial cells (CEC). • RT-PCR showed expression of TLR2, TLR4 and NOD2 in the	(Swerdlow et al., 2006)

Region of Intestine IEC isolated from	Health status	Experimental Results	Reference
		CEC. • TLR4 was upregulated after stimulation with 100 ng/mL LPS. • TLR2 and NOD2 were upregulated after stimulation with 1 μg/mL peptidoglycan.	
Jejunum	Healthy beagles (n not specified). Used the same cells as isolated in (Weng et al., 2005).	• Cells differentiated into enterocyte-like cells. • NF-κB nuclear translocation was induced by exposure to LPS, PMA and raw food ingredients. • Expression of CLDN-3, CLDN-4, ZO-1, OCLDN, TLR4, TLR5 and E-cadherin observed in differentiated cells. • Barrier function, as assessed by TEER, was greater in differentiated monolayers, compared to cells in the growth phase.	(Farquhar et al., 2018)
Colon	Healthy dogs (n = 3)	• Cells grew microvilli in culture. • Alkaline phosphatase and Neurogenin-3 expressed in cultured tissues. • Goblet cells were grown in cultured tissues. • ZO-1 and E-cadherin staining was observed. • Barrier integrity, assessed by TEER, was stable for up to 14 days.	(Ambrosini et al., 2020)

Abbreviations: CLDN = Claudin; OCLDN = Occludin; ZO = Zonula Occludens; TEER = Transepithelial electrical resistance; TLR = toll-like receptor; MHC II = major histocompatibility complex class II; FZD5 = frizzled 5 protein; CBD103 = beta-defensin 103; CATH = cathelicidin; IL = interleukin; RT-PCR = real-time polymerase chain reaction; NF-κB = nuclear factor kappa B; LPS = lipopolysaccharide; PMA = phorbol 12-myristate 13-acetate; N. caninum = Neospora caninum.

2.4. THE IMMUNOLOGICAL BARRIER

PRR receptors and inflammatory cytokines that are expressed by IECs contribute to the maintenance of intestinal homeostasis (Ridyard et al., 2002; Ridyard et al., 2007; Jergens et al., 2009; Kainulainen et al., 2015). Upon detecting the relevant MAMP or DAMP, the PRRs then trigger a cytokine and chemokine response (Swerdlow et al., 2006; House et al., 2008; Rahman et al., 2009; Zhang et al., 2010; Mercier et al., 2012; Fukata and Arditi, 2013). Cytokines are rapidly synthesised glycoproteins that can modulate the intestinal inflammatory and immune responses (Jergens et al., 2009; Kolodziejska-Sawerska et al., 2013). The inflammatory response in the intestine is mediated by Th1 and Th2 lymphocyte responses, which induce pro- and anti-inflammatory cytokines, respectively (Ridyard et al., 2002; Jergens et al., 2009). Chemokines are cytokines that activate in response to chemical stimuli. These use chemical signalling to attract and coordinate leukocytes to form the immune inflammatory response (Zimmerman et al., 2008; Maeda et al., 2012).

2.4.1. PATTERN RECOGNITION RECEPTORS

Pattern recognition receptors all recognise specific molecular patterns. These so regardless of their life-cycle stage, and are independent of immunologic memory (Akira et al., 2006). The PRRs in the dog include TLRs, nucleotide-binding oligomerization domain–containing protein (NOD) 2 and the receptor of advanced glycation end products (RAGE) (House et al., 2008; Heilmann and Allenspach, 2017).

The TLRs are the most well-studied of the PRRs, with ten separate TLRs identified in the dog thus far (Cuscó et al., 2014). Several studies have identified that dog IECs, like those in other species, can express TLRs (Weng et al., 2005; Swerdlow et al., 2006; Burgener et al., 2008; Allenspach et al., 2010; Bailey et al., 2015; Farquhar et al., 2018; Reineking et al., 2018a). In addition, immune cells such as macrophages, dendritic cells and T cells are also capable of expressing TLRs (Akira et al., 2006). The cellular location of these TLRs can vary; whilst TLR1, -5, -6 and -10 are expressed on cell surfaces and TLR3, -7, -8 and -9 are expressed intracellularly (Cuscó et al., 2014; Heilmann and

Allenspach, 2017). TLR2 and -4 are expressed in both of these locations (Cuscó et al., 2014). These locations relate to the molecular patterns these PRRs recognise. Bacterial LTA, LPS, flagellin are recognised by TLRs -2, -4 and -5, respectively, whilst viral ribonucleic acid (RNA) and bacterial deoxyribonucleic acid (DNA) are recognised by TLRs -3, -7, -8 and -9 (Rahman et al., 2009). The way these TLRs interact with each other can also alter their inflammatory response. For example, when TLR2 forms heterodimers with TLR1, it causes an anti-inflammatory IL-10 response, whereas when TLR2 heterodimeris with TLR6 it causes a pro-inflammatory response (Kang et al., 2009; Hug et al., 2018).

A leucine-rich repeat (LRR) motif contained in the TLRs acts as a ligand binding domain, an anchoring transmembrane domain, and a Toll–IL-1 receptor (TIR) that initiates the signal transduction (Rahman et al., 2009; Heilmann and Allenspach, 2017). These receptors recruit and form homodimers with adaptor proteins that contain TIR domains, such as myeloid differentiation protein (MyD) 88 (Rahman et al., 2009). The MyD88 signalling cascade is activated by TLRs 1-10, except for TLR3, which activates the Toll–IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) dependant signalling pathway instead (Rahman et al., 2009; Hu et al., 2015) (see Figure 2.5). TLR 4 is unique in that it is able to activate both of these immune signalling pathways (Hu et al., 2015). TLR2 is capable of activating the MyD88 cascade without the MyD88 adaptor-like (TIRAP) protein (Kenny et al., 2009).

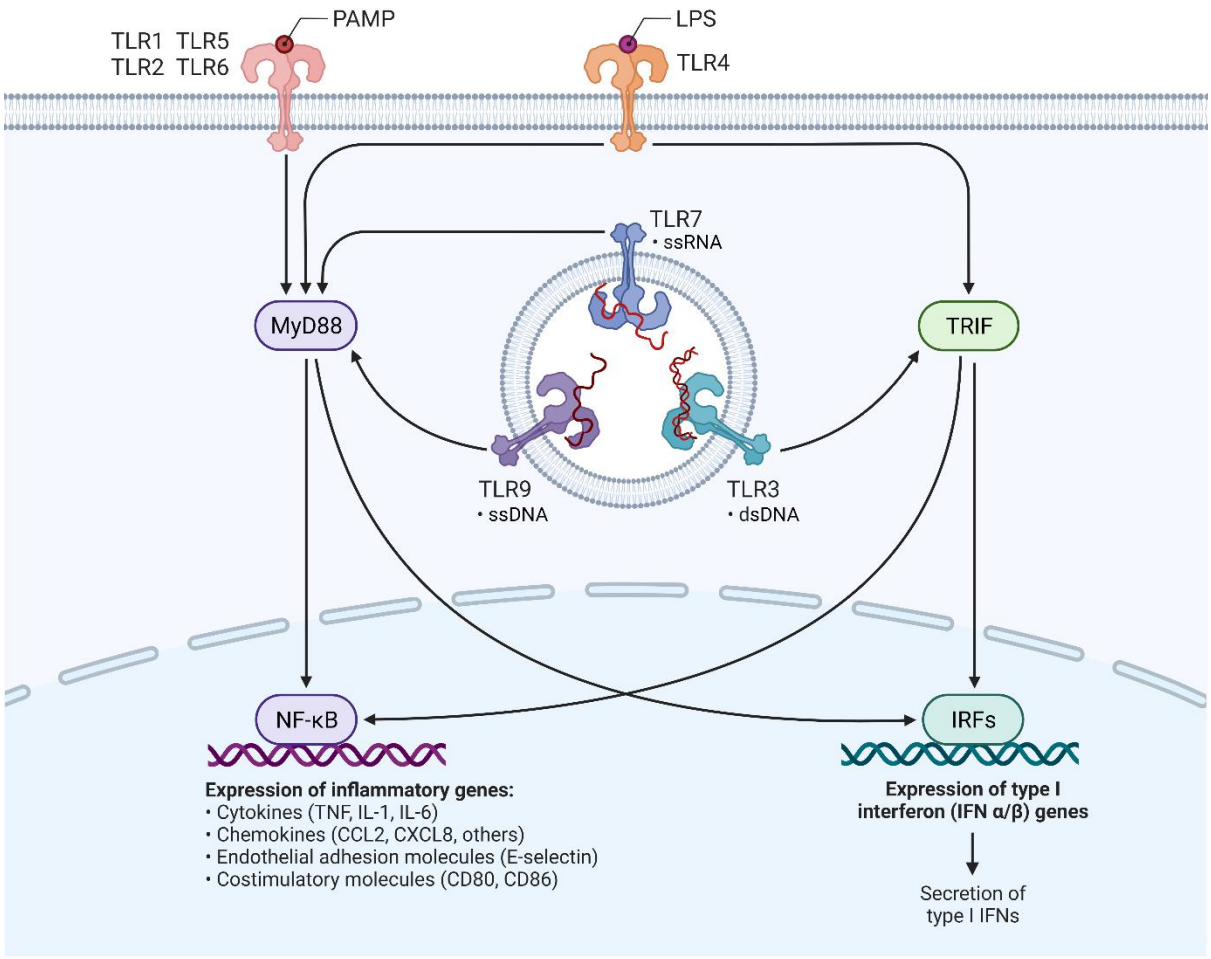


Figure 2.5 – *Toll-like receptor activation pathways.Abbreviations, in clockwise order from the top left; TLR = Toll-like receptor; PAMP = pathogen associated molecular pattern; LPS = lipopolysaccharide; TRIF = Toll–IL-1 receptor domain-containing adaptor inducing IFN-β; IRF = interferon regulatory factors; IFN = interferon; NF-κB = nuclear factor kappa B; TNF = tumour necrosis factor; IL = interleukin; CCL = chemokine (C-C motif) ligand; CXCL = chemokine (C-X-C motif) ligand; CD = cluster of differentiation; MyD = Myeloid differentiation protein. Figure created with Biorender.*

The intracellular PRR NOD2 comprises a LRR domain, two caspase recruitment domains that initiate the downstream pathways, and a nucleotide-binding oligomerisation domain (Heilmann and Allenspach, 2017). NOD2 recognises muramyl dipeptides in bacterial peptidoglycans (Akira et al., 2006; Heilmann and Allenspach, 2017). The downstream pathways activated by the caspase recruitment domains of NOD2 (see Figure 2.6) then recruit and activate RICK protein kinase (RIPK2)

(Akira et al., 2006). The NOD2-activated RIPK2 is then able to activate transforming growth factor β-activated kinase (TAK) 1. This subsequently induces and causes the nuclear translocation of NF-κB by the mitogen-activated protein kinase (MAPK) pathway, or the inhibitor of nuclear factor-κB (IκB) kinase (IKK) complex (Caruso et al., 2014). In mice and humans this also leads to the activation of caspase-1, resulting in the maturation of IL-1β through the cleavage of pro-IL-1β (Akira et al., 2006). However, dogs lack the genes required for caspase-1 production (Digby et al., 2021).

The activation and nuclear translocation of NF-κB is also induced by the RAGE signalling pathways (Heilmann and Allenspach, 2017) (see Figure 2.6). RAGE expression is also upregulated during intestinal inflammation (Body-Malapel et al., 2019). There are 5 domains in this PRR; a variable domain responsible for ligand binding, two constant domains, a transmembrane anchor and a cytoplasmic domain that initiates signalling pathways (Heilmann and Allenspach, 2017). The ligand binding domain has an affinity for a large range of molecules such as advanced glycation end products, β-amyloid peptide, and S100 calgranulins (Body-Malapel et al., 2019). Whilst RAGE usually activates MAPK via the Kirsten rat sarcoma viral oncogene homolog (KRAS) pathway, it can also activate MAPK by interacting with MyD88 and TIRAP to signal through the MyD88 dependent pathway (Heilmann and Allenspach, 2017).

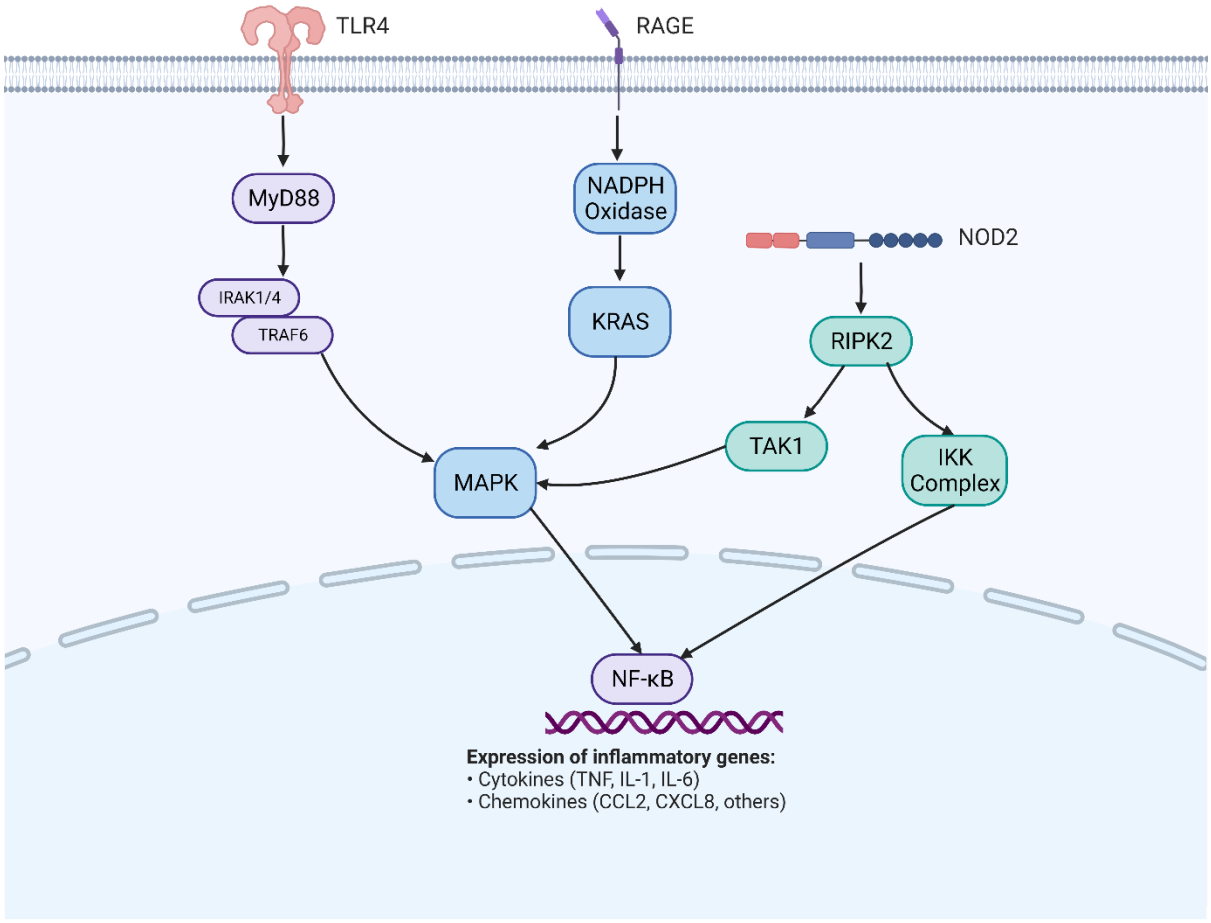


***Figure 2.6** – NF-κB activation pathways from TLR, RAGE and NOD2 signalling cascades.Figure adapted from (Caruso et al., 2014; Ott et al., 2014; Heilmann and Allenspach, 2017). Abbreviations, from left to right; TLR = toll-like receptor; MyD = Myeloid differentiation protein; IRAK = interleukin-1 receptor-associated kinases; TRAF = tumour necrosis factor receptor–associated factor; MAPK = mitogen-activated protein kinase; RAGE = receptor of advanced glycation end products; NADPH =nicotinamide adenine dinucleotide phosphate; KRAS =Kirsten rat sarcoma viral oncogene homolog; NF-κB = nuclear factor kappa B; TAK = transforming growth factor-β-activated kinase 1; RIPK = RICK protein kinase; NOD = nucleotide binding oligomerization domain containing protein; IKK = inhibitor of NF-κB kinase. Created with BioRender.*

2.4.2. Interactions of The Immunological Barrier with the Physical Gut Barrier

The immunological stimulation of TJ complexes in the dog IEC has not been directly assessed. However, cell models from other species have shown that exposure to proinflammatory cytokines

during inflammation can cause increased paracellular permeability by disrupting the TJ structure and composition, and thereby also impacts the TEER of the cellular monolayer (Bansal et al., 2010; Capaldo et al., 2014). The proinflammatory cytokines also can induce redistributions and mislocalisations of junctional proteins (Ozaki et al., 1999; Prasad et al., 2005). These changes can be seen through direct challenges with these cytokines, or through the activation of these proinflammatory cytokines via the TLR2 and TLR4 signalling cascades (Hanson et al., 2011; Nighot et al., 2017; Stephens and von der Weid, 2020). Additionally, continuous activation of T cells increases the permeability of Caco-2 monolayers (Le et al., 2021b). Though many studies have assessed the impacts of IBD and immune expressions in the dog GIT (A. Garden et al., 1999; German et al., 2000; Luckschander et al., 2010; Kathrani et al., 2019; Konstantinidis et al., 2021) it remains difficult to determine if these changes are resulting from illness or direct immune stimulation. Thus, extrapolation from other models is essential until a dog-specific cell line is characterised in these ways.

The relationship of pro-inflammatory cytokines with the IEB has also been studied in human-origin cell lines. Direct challenges of proinflammatory cytokines IFN-γ and TNF-α to human SKCO15, Caco-2 BBE, T84, and HeLA cell lines increased the motility of CLDN-4, thereby impairing IEC function and decreasing TEER (Ma et al., 2004; Prasad et al., 2005; Capaldo et al., 2014). Together, IFN-γ and TNF-α also caused JAM-A and CLDN-5 to mislocalise, redistributed OCLDN and ZO-1, and decreased the membrane raft association of OCLDN and JAM needed for cell localisation and T-cell activation (Ozaki et al., 1999; Ma et al., 2004; Prasad et al., 2005; Li et al., 2008a; Li et al., 2008b). However, IFN-γ and TNF-α together do not increase the expression of the pore-forming CLDN-2 (Prasad et al., 2005; Capaldo et al., 2014). The impacts on TJ permeability through these activations requires NF-κB activation (Ma et al., 2004).

Other cytokines also impact the physical gut barrier. For example, treatment of T84 cells with IL-13 increases the expression of CLDN-2 (Prasad et al., 2005). Anti-inflammatory IL-10 reduces the expression levels of CLDN-2 (Zheng et al., 2017). Thus, when changes in immune expressions are

observed, it remains important to consider how these will subsequently impact IEB permeability by potential alterations in TJ complexes and functionality.

2.5. BACTERIAL LIGANDS OF THE GUT MICROBIOTA

The gut microbiota in the dog are a diverse and complex collection of microorganisms that assist in many host processes, such as digestion and maintenance and promotion of health (Valdes et al., 2018; Alessandri et al., 2019). The populations and abundances of these Gram-negative and Gram-positive organisms can be significantly impacted by diet changes (Allaway et al., 2020). These microbiota assist in nutrient absorption, immune responses, and maintenance of the IEB homeostasis (Blake and Suchodolski, 2016). Additionally, these bacteria assist in the fermentation of dietary fibres in the colon, giving rise to SCFA such as butyrate (Jackson and Jewell, 2016; Nogueira et al., 2019). This in turn is used by colonocytes to maintain the anaerobic environment of the colon (Litvak et al., 2018b). However, the presence of oxygen is toxic to the obligate anaerobic bacteria present in the GIT (Lu et al., 2018), though toxicity is different for each microbe i.e., some anaerobic bacteria suffer terminal oxygen toxicity faster than others (Lu and Imlay, 2021).

Gram-positive and negative bacteria are defined by their capacity to be stained by Gram staining. Gram-positive bacteria stain violet or purple from a crystal violet-iodine complex and a safranin counter stain, and Gram-negative bacteria stain a pink colour as they do not retain the complex stain (Moyes et al., 2009). These results occur because of differences in the cell wall between Gram-positive and negative bacteria (Moyes et al., 2009), and due to the individual components in, and attached to, the cell wall (Figure 2.7). These components are linked to the different impacts Gram-positive and negative bacteria have on the host (Nocera et al., 2021).

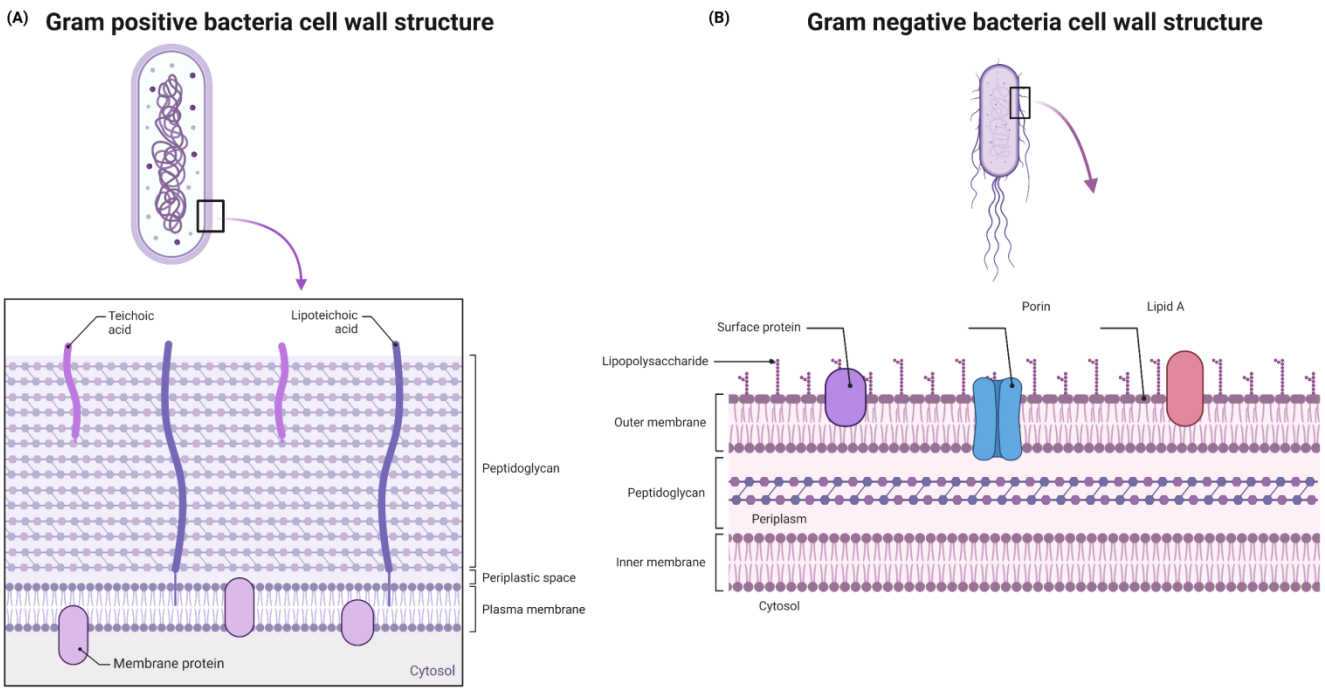


***Figure 2.7** – Structural composition of Gram-positive and Gram-negative bacteria. Created with BioRender.*

Gram-positive bacteria have thicker peptidoglycan walls compared to Gram-negative bacteria, with an outer lipid bilayer over their peptidoglycan layers, which helps them survive in environments such as the gut (Silhavy et al., 2010). Anchored to the peptidoglycan layer are teichoic acids. LTAs are amphiphilic glycopolymers that are linked via a lipid anchor to the plasma membrane of the bacterium (Neuhaus and Baddiley, 2003). The chemical structure of these polymers can vary between bacterial species (Morath et al., 2002; Draing et al., 2006). There are five main types of LTAs, referred to as type I-V, which have different polymers of varying complexity linked to the lipid anchor (Percy and Gründling, 2014). Both LTA and LPS are negatively charged (Morath et al., 2002), and are considered counterparts to each other (Van Amersfoort et al., 2003).

Gram-negative bacteria have a high resistance to antibiotics, predominantly due to their outer membrane (Breijyeh et al., 2020). This outer membrane forms a permeability barrier by downregulating or replacing porins that hydrophilic antibiotics will diffuse through (Blair et al., 2015). LPS are bound and expressed in this outer layer, are potent endotoxins that assist in the

establishment of the permeability barrier in the outer membrane (Galloway and Raetz, 1990; Nikaido, 2003). Additionally, they can induce inflammation in the host and can cause severe conditions such as sepsis (Tivers et al., 2015; Li et al., 2021b). They are formed from three structural domains; lipid A, a core polysaccharide, and a repeating oligosaccharide (see Figure 2.8). Lipid A is the biologically active component. This binds to TLR 4 and activates the immune response and is a hydrophobic acylated β-1‘-6-linked glucosamine disaccharide that is usually well-conserved (Raetz and Whitfield, 2002; Kalynych et al., 2014; Tivers et al., 2015; Bertani and Ruiz, 2018). The core oligosaccharide is a non-repeating oligosaccharide linked to the glucosamines of lipid A (Raetz and Whitfield, 2002). The repeating oligosaccharide outer core is linked to the highly variable O antigen, which can differ between species, and is not synthesised by some Gram-negative bacteria (Kalynych et al., 2014; Tivers et al., 2015; Bertani and Ruiz, 2018). These LPS molecules without the O antigen are denoted as “rough” LPS, whereas the LPS molecules with the O antigen are referred to as “smooth” LPS (Raetz and Whitfield, 2002).

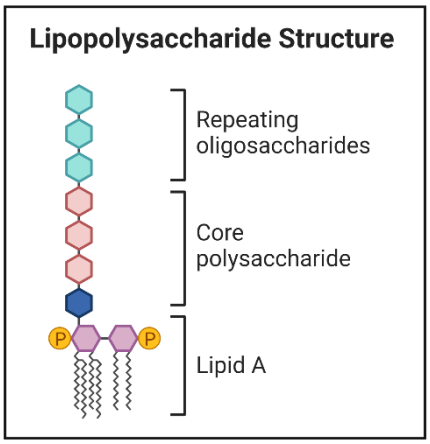


***Figure 2.8** – Structure of lipopolysaccharide (LPS) molecule. Created with BioRender.*

2.5.1. RELATIONSHIP BETWEEN THE PHYSICAL GUT BARRIER AND BACTERIAL LIGANDS

The relationship between the physical gut barrier and bacterial challenges has been investigated and reviewed in detail in humans and rodents. However, these studies rarely investigate if the changes

result from the bacterial ligands (Paradis et al., 2021). There is no literature that assesses the impacts of LPS or LTA stimulation on the TJ expressions or the TEER of dog IECs. This is a clear knowledge gap that requires addressing. Thus, these ligand-induced alterations must be extrapolated from other species.

LPS has a notable impact on the physical gut barrier. At high concentrations (50 µg/mL) LPS induces cell death in Caco-2 cells that results from caspase-3, -8, and -9 induced apoptosis (Yu et al., 2005). LPS levels of 0 – 1 ng/mL are considered physiologically relevant, whilst LPS levels of 2 – 10 ng/mL are considered relevant for modelling infection or illness (Nighot et al., 2017). Subsequently, the LPS concentrations used in the literature are generally low. Treatment of Caco-2 cells with 10 µg/mL LPS reduced TEER and the mRNA and protein expressions of ZO-1 and OCLDN compared to controls (He et al., 2020). In another study that treated Caco-2 cells with 1 µg/mL LPS, the LPS treatment caused reduced TEER and decreases in protein expressions of ZO-1 and OCLDN compared to controls (Wei et al., 2022). IPEC-J2 cells, which are porcine IECs, had reduced TEER and decreased OCLDN and CLDN-1 protein expression after treatment with 1 µg/mL LPS, compared to control cells (Wu et al., 2020). This study also showed that the treatment of the IECs with 1 µg/mL LPS damaged CLDN-1 structures (Wu et al., 2020). 10 µg/mL LPS was also found to suppress the mRNA levels of ZO-1, OCLDN and JAM in rat endothelial cells (Singh et al., 2007). Future investigations that profile the dog IEC response to LPS should initially measure TEER and characterise the expressions of ZO-1, OCLDN and CLDN-1 to determine if there are any differences in expected responses between species. Additionally, any studies that use LPS at concentrations above 50 µg/mL should measure the expressions of caspase-3, -8, or -9 to determine if LPS stimulation has induced apoptosis.

Most studies that challenge IEC with LTA assess the immune response rather than investigating alterations to TEER and TJ expressions. One study found that stimulation of the rodent small intestinal cell line IEC-6 with 5 µg/mL of LTA caused a rapid decline in TEER when compared to naïve controls (Meng et al., 2013). Another study stimulated rat endothelial cells with 25 µg/mL LTA and found LTA suppressed mRNA levels of ZO-1, OCLDN and JAM in comparison to controls

(Singh et al., 2007). Treatment of bovine endothelial cells with 10 and 30 µg/mL of LTA caused dose-dependent declines in TEER (i.e., 30 µg/mL of LTA reduced TEER more than 10 µg/mL of LTA did) (Boveri et al., 2006). Future investigations that profile the IEC response to LTA, in the dog and in other species, should determine the impacts LTA has on TEER and TJ expression. This will begin to address this knowledge gap in the literature.

However, when investigating the the responses of IEC, it is important to remember that the permeability of IEC TJsare influenced by the immune response. For example, IFN-γ decreases the expression and motility of CLDN-4 and increases the expression of the pore-forming CLDN-2 (Capaldo et al., 2014). Similarly, T cell activation regulates the IEB by increasing mRNA expressions of CLDN-1, -2, -4, and ZO-1 (Le et al., 2021a). However, extended activation of T cells resulted in increased barrier permeability from the upregulation of CLDN-2 mRNA (Le et al., 2021a). Therefore, it seems a logical conclusion that understanding the immune response to bacterial stimulation will help further form hypotheses and understandings for how the TJs and IEB reacts and responds to challenges from bacterial ligands.

2.5.2. RELATIONSHIP BETWEEN THE IMMUNOLOGICAL BARRIER AND BACTERIAL LIGANDS

The acyl chain of LTA is recognised by TLR2, and detection by the TLR2-TLR6 heterodimer leads to the subsequent inducement of the pro-inflammatory immune response (Morath et al., 2002; Kang et al., 2009; Villéger et al., 2014). This signalling has been found to occur preferentially at the plasma membrane, and in the same study CD14 and CD36 were found to enhance the TLR2-mediated activation of NF-kB (Nilsen et al., 2008). However, LTA can also activate the TLR4-dependent MyD88 inflammation cascade, and the TRAM downstream pathway (Sacre et al., 2007). In addition, LTAs can have different magnitudes of immune responses. A study investigating responses induced by two different strains of *Streptococcus pneumoniae* (serotype 2 and serotype 4) found serotype 4 exposure caused a significantly higher response of TNF-α, IL-1, IL-8, IL10 and IFN-γ in human blood than serotype 2 (Draing et al., 2006).

The literature is relatively sparse on the effects of LTA on dog IECs, however, the impacts of LTA on circulating immune expressions in the blood have been studied. One study that stimulated canine blood phagocytes with LTAs was able to demonstrate a TLR2-induced expression of IL-8 but did not investigate the expression levels of any other inflammatory markers (Bazzocchi et al., 2005). Another study compared IL-6, IL-10 and TNF-α from LTA-stimulated blood samples taken 6 weeks apart and found no difference in the immune response (Axiak-Bechtel et al., 2014). A further study also investigating the response of these immune markers found higher cytokine expression of IL-6 and TNF in dogs with diabetes, compared to healthy dogs (DeClue et al., 2012). These studies do allow for hypotheses to be drawn as to how LTA may impact TLR2-activated immune pathways in the dog IEC. Healthy canine IECs have been shown to express low levels of TLR2 (Swerdlow et al., 2006). This is also the case in human and mice intestinal cells (Cario et al., 2000). The expression levels of TLR2 across the canine GIT has also been explored (Burgener et al., 2008). With this knowledge base, future studies that investigate the response to LTA in dog cells would be a worthwhile area of investigation. This would be able to compare the TLR2 response to LTAs to other stimuli of TLR2 and possibly highlight LTA-specific responses.

The impacts of LTA on IEC have been assessed in other spoecies. Stimulation of Caco-2 cells with 10 µg/mL LTA caused increases in mRNA expression of IL-8 (Claes et al., 2012). Interestingly, it appears that whilst LTA stimulation alone causes an increase in IL-8 and decreases in intestinal permeability, LTA mediates the inflammatory response to other compounds when co-stimulating cells. For example, 10 and 100 µg/mL LTA reduced the protein expression of IL-8 in HT-29 cells that was induced by TNF-α stimulation (Kim et al., 2012). In another study, 10 and 30 µg/mL of LTA reduced protein and mRNA expression of IL-8 induced by Pam2CSK4, a TLR2/TLR6 agonist (Noh et al., 2015). This study further showed that this inhibition is caused by LTA suppressing TLR2 signalling pathways (Noh et al., 2015). This suggests a possible cross-competition for TLR2, or that LTAs are beneficial in the presence of other TLR2 stimulators. Studies that profile the response of dog IECs, or in other species, should investigate the impacts of LTA alone, and in combination with other pro-inflammatory stimuli to determine if there are beneficial impacts to LTA stimulation.

After extracellular detection of the lipid A LPS component by TLR4-MD2 complexes, LPS is subsequently transferred to this complex by LPS binding proteins and CD14 (Hailman et al., 1994; Ulevitch and Tobias, 1995; Ryu et al., 2017). The LPS is bound the MD2 portion of the TLR4-MD2 complex, which activates the MyD88 and TRIF-dependent downstream signalling cascades (Ryu et al., 2017). Variations in the lipid A component can result in different levels of immune responses (i.e., higher or lower levels of immune cytokines being produced), and it has been seen that the activation of the MyD88 or TRIF pathways can depend on the bacterial species (Needham et al., 2013).

LPS stimulation causes a pro-inflammatory response in dog IECs. TLR4, IL-7, and IL-8 protein expression was upregulated in dog colonic epithelial cells after stimulation with 0.1 μg/mL LPS (Swerdlow et al., 2006). Stimulation of dog IEC with 15 μg/mL LPS caused NF-κB activation (Farquhar et al., 2018). However, other species models have investigated the impacts of LPS stimulation on IEC further. IL-6 mRNA expression was increased in Caco-2 cells stimulated with 1 μg/mL LPS (Wu et al., 2020). Stimulation of Caco-2 cells with 1 ng/mL LPS induced protein expression of IL-4, IL-6, IL-8, IL-10, TNF-α and MCP-1 (Stephens and von der Weid, 2020). 0.3 ng/mL of LPS caused increases in protein abundances of TLR-4 and CD-14 in Caco-2 cells (Guo et al., 2013). The impacts of LPS on plasma and serum immune cytokines in the dog have been profiled in sepsis studies. These, in combination with existing studies on dog IEC and those performed with Caco-2 cells can be used to form hypotheses on how LPS treatment will impacts other immune cytokines not yet profiled in the dog IEC. 10 ng/mL LPS caused increased protein abundances of IL-6, IL-10 and TNF-α in blood (Dandrieux et al., 2019). In plasma, these cytokines were also raised after stimulation with 10 μg/kg LPS (Yu et al., 2012). IL-6 and TNF-α are pro-inflammatory markers that are activated from the MyD88 cascade (Tan and Kagan, 2014; Page et al., 2022). However, IL-10 is an anti-inflammatory cytokine that dampens the MyD88 signalling response (Chang et al., 2009). The *in vivo* study (Yu et al., 2012) showed that the TNF-α levels peaked at 3 hours post-dosage, 3 hours before the IL-10 and IL-6 levels peaked. As TNF-α induces IL-6 production via the mitogen-activated protein (MAP) kinase p38 pathway in mice (De Cesaris et al., 1998) it seems that this pathway functions in the same manner in the dog as in other species. Future studies using dog IEC

should characterise the response of IL-4, -6, IL-10, TNF-α, and MCP-1 after IL-8 stimulation. This will close the knowledge gap between dog IECs and knowledge in Caco-2 cells.

However, there are some differences in the immune cascades between species. The detection of LPS activates the NLR family pyrin domain containing 3 (NLRP3) inflammasome and subsequent caspase-1 and caspase 4/5 responses in humans (Feng et al., 2018b). Like other members of carnivora, dogs have an absence of individual caspase-1 and caspase-4/-5/-11 genes, although they form a caspase-1/-4 fusion protein (Digby et al., 2021). This combination protein lacks the LPS-sensing capabilities seen in caspase-4 in humans but appears capable of mediating caspase-1 and -4 functions without the intermediatory inflammasome activation, allowing for the IL-1β cleavage of LPS detection to occur in dogs (Devant et al., 2021; Digby et al., 2021). It has been suggested in dogs that the antimicrobial properties of carnivorous diets may compensate for the missing immune pathways (Digby et al., 2021) though this has not yet been investigated in the literature. Future studies should consider how these alternative immune pathways may cause different responses to stimuli.

2.6. MICROBE-DERIVED METABOLITES

The SCFA acetate, butyrate and propionate are the end-products of the bacterial fermentation of undigested dietary fibres and dietary proteins (Nery et al., 2012; Hang et al., 2013; Sandri et al., 2017) (see Figure 2.9). Acetate has been suggested to help in the regulation of body weight and insulin sensitivity (Hernández et al., 2019) and in hypoxic conditions it is used as a source of acetyl-CoA for cell growth, proliferation, and to induce fatty acid synthesis (Kamphorst et al., 2014; Gao et al., 2016). Propionate can increase satiety by increasing leptin production (Xiong et al., 2004). It has been postulated by Arora et al (2011) to be involved in cholesterol and obesity management, though the evidence for this is not yet fully conclusive (Arora et al., 2011). Butyrate, meanwhile, is the most well-studied of the SCFA and plays a role in the inflammatory response of the intestine. It is also commonly recognised as an energy source for colonocytes, though the importance of this is often not acknowledged (Apper et al., 2020; Ephraim and Jewell, 2020; Rossi et al., 2020). Colonocytes are responsible for maintaining the anaerobic environment of the colon, and when they are injured, or

lack a supply of butyrate, they can no longer maintain the hypoxic conditions (Litvak et al., 2018a). Without these anaerobic conditions, dysbiosis, caused by an influx of facultative anaerobic bacteria, can occur (Shin et al., 2015; Litvak et al., 2018a). This domino effect can then alter luminal oxygen content, create an inflammation response which further compromises the GIT microbiota homeostasis (Reese et al., 2018).

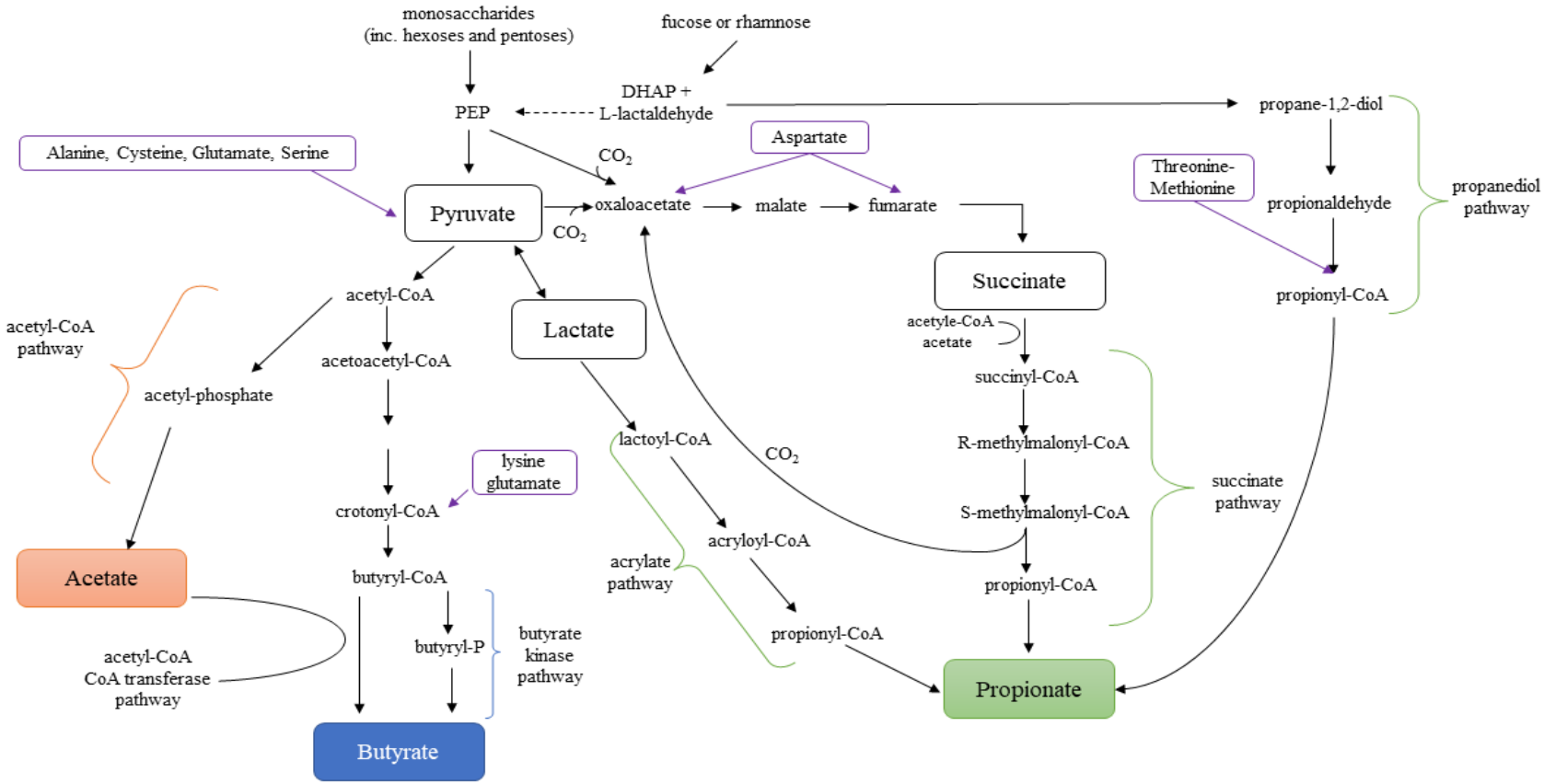


Figure 2.9 *- Carbohydrate and amino acid fermentation pathways to produce organic acids.Organic acids are denoted in coloured boxes, orange for acetate, blue for butyrate and green for propionate. The formation pathways are highlighted with brackets of the same colour. Purple boxes and arrows denote the amino acid fermentation pathways. Adapted from (Reichardt et al., 2014; Richards et al., 2016; Esquivel-Elizondo et al., 2017; Louis and Flint, 2017).* CO_2 *=carbon dioxide; CoA = co-enzyme A; DHAP = dihydroxyacetone phosphate; PEP = phosphoenolpyruvate.*

Branched chain fatty acids (BCFA) arise because of fermentation of the branched amino acids valine, leucine, and isoleucine which occur independently of carbohydrate metabolism (Beck, 2005; Heimann et al., 2016; Donadelli and Aldrich, 2019). In the diet, BCFA have been suggested as markers of colonic protein fermentation and low nutrient availability (Macfarlane et al., 1992; Calabrò et al., 2013). Some diet studies in dogs have investigated the relationship between faecal BCFA concentrations, diet changes and health (Bermingham et al., 2017; Nogueira et al., 2019). However, in these studies the dogs remained healthy so the role of BCFA in relation to health in the dog remains unclear. A recent review has highlighted the lack of knowledge of the role for diet-derived BCFAs in humans (Taormina et al., 2020), however, and this is unfortunately also true for dogs.

Indole, alongside pyruvate and ammonia, is produced by the degradation of tryptophan by tryptophanase (Chimerel et al., 2014; Li et al., 2021a). Owing to its production from protein fermentation, indole is associated with smellier faeces and so a decrease in faecal indole following dietary treatment is viewed as a beneficial effect (Mori et al., 2019; Nogueira et al., 2019). There is no data assessing the impact of indole directly on the canine intestine, although a study in dogs with IBD found that faecal indole levels were decreased in dogs with the condition when compared to healthy controls (Honneffer et al., 2015), which suggests that indole may confer beneficial effects in the dog.

2.6.1. RELATIONSHIP BETWEEN THE PHYSICAL GUT BARRIER AND SCFA

The impacts of SCFA have been studied extensively in *in vitro* models, although physiological concentrations of SCFA are generally higher than are used in cell culture. The *in vivo* GIT concentrations of SCFA in dogs, depending on diet, average around 70 mM acetate, 25 mM propionate, 10 mM butyrate (Kamath et al., 1987). In humans, average concentrations of 60 mM for acetate and 20 mM for butyrate are observed (Íñiguez-Gutiérrez et al., 2020). In comparison, concentrations used in cell culture can be as low as 0.01 mM (Feng et al., 2018b). However, most studies that investigate the impacts of SCFA in the dog are performed *in vivo* and focus on faecal characteristics or the impacts on the faecal microbiota (Gagné et al., 2013; Alexander et al., 2018).

SCFAs have been shown to reduce the expression of pore-forming TJs. In one study using Caco-2 cells, 0.5 acetate, 0.01 butyrate, and 0.01 mM propionate were all capable of decreasing the expression of CLDN-2 (Feng et al., 2018b). Additionally, the SCFA treatment was able to alleviate LPS-induced morphological changes to ZO-1 and OCLDN (Feng et al., 2018b). Another study, also using Caco-2 cells, showed that CLDN-3 expression was increased 24 hours after treatment with 1, 1.5, and 2 mM butyrate, and 48 hours after treatment with 1.5 mM butyrate, compared to an untreated control (Feng et al., 2018a). Similarly, treatment with 1 mM butyrate in porcine IEC found increased expression of CLDN-3 after 4 hours of treatment, and increased CLDN-4 after 8 hours of treatment (Yan and Ajuwon, 2017).

The impacts of indole on TJ expression has also been investigated. In human HCT-8 IEC, treatment with 1 mM of indole decreased the expression of CLDN-2, and increased the expression of ZO-1, ZO-3, and JAM (Bansal et al., 2010). Another study using a mixed culture of Caco-2 and HT29 cells, found that treatment of cells with 0.1 mM indole-3-propionic acid resulted in increased expression of CLDN-1, OCLDN, ZO-1 (Li et al., 2021a). Additionally, it also found that MUC2 and MUC4 production was increased by indole stimulation (Li et al., 2021a).

Additionally, the impacts of SCFA on the TEER of cellular monolayers has been investigated in various models. One study investigated the response to butyrate in an isolated dog IEC line, and found 2 mM of butyrate resulted in increases in TEER (Farquhar et al., 2018). This is also seen in human Caco-2 cells (Elamin et al., 2013). Most work investigating the IEB response to SCFA is performed in non-dog models.

Barrier integrity reacts paradoxically to SCFA treatments. In one study using Caco-2 cells, treatment with 0.5 mM/L acetate and 0.01 mM/L butyrate caused increases in TEER (Feng et al., 2018b). Another study found that low concentrations of SCFA (2 mM butyrate, 4 mM propionate, and 8 mM acetate) significantly improved TEER in Caco-2 cells (Elamin et al., 2013). In contrast, higher concentrations (10 and 20 mM butyrate, 20 and 40 mM propionate, and 40 mM acetate) did not (Elamin et al., 2013). Similarly in T84 cells, treatment with 5 mM butyrate increased TEER, whilst 20

mM of acetate and propionate caused no change in TEER (Zheng et al., 2017). This paradoxical effect of SCFA where low doses of butyrate increased TEER (Peng et al., 2007; Elamin et al., 2013; Feng et al., 2018b), and high doses caused significant reduction in TEER, suggests an upper limit of tolerance (Peng et al., 2007). This paradox has also been observed with acetate, where 8 mM increased TEER, whilst 80 mM decreased it (Elamin et al., 2013).

Low concentrations of indole appear to positively impact barrier integrity. In HCT-8 IEC, treatment with 1 mM of indole caused increased TEER (Bansal et al., 2010), as did treatment in a mixed culture of Caco-2 and HT29 cells with 0.1 mM indole-3-propionic acid (Li et al., 2021a). The impacts of indole on the TEER of dog IEC have not yet been investigated, though given indole's fermentation from protein and the dog's requirements of dietary protein, it is worth exploring.

2.6.2. Relationship between the immunological Barrier and SCFA

The impacts of SCFA on the immunological barrier are often assessed by means of their ability to alleviate responses induced by LPS challenges (Asarat et al., 2015; Li et al., 2021a). No published studies have investigated the effects of SCFA on the immune response in dog IECs.

SCFA treatment can reduce the pro-inflammatory response caused by bacterial ligands. In human IEC (HT-29 and T4056) challenged with LPS and with analysis performed by mRNA quantification and ELISA, a combination of acetate, propionate, and butyrate (total concentration 20 mM) was able to downregulate the proinflammatory IL-8 response to LPS stimulation (Asarat et al., 2015). Individually, acetate and butyrate were also able to downregulate the IL-8 expression compared to cells challenged only with LPS, but propionate alone had no effect (Asarat et al., 2015). In porcine IEC, 1 mM butyrate downregulated TLR4 expression (Yan and Ajuwon, 2017). In a study using Caco-2 cells, 0.5 mM acetate, 0.01 mM butyrate, and 0.01 mM propionate reduced the NLR family pyrin domain containing 3 (NLRP3) immune response (Feng et al., 2018b). However, the

NLRP3 response in dogs is limited compared to humans (Digby et al., 2021), and it is currently unknown if SCFA in the dog would interact with this immune cascade in a similar way.

Indole also a similar capacity to reduce pro-inflammatory responses. In the human HCT-8 IEC, treatment with 1mM of indole increased anti-inflammatory IL-10 expression, whilst simultaneously decreasing expression of pro-inflammatory IL-8 (Bansal et al., 2010). Additionally, the indole treatment decreased the tumour necrosis factor alpha (TNF-α) activation of NF-κB (Bansal et al., 2010). Treatment of a mixed culture of Caco-2 and HT29 cells with 0.1 mM indole-3-propionic acid was able to reduce the LPS-induced upregulation of IL-6, IL-8 and TNF-α mRNA expression (Li et al., 2021a).

Though most studies are performed *in vitro, in vivo* methods have also been utilised to investigate the SCFA induced immune response in mice. T cell activation occurs by SCFA activation of G-protein coupled receptor (GPR)41 and GPR 43 (Kim et al., 2013). Activation of GPR43 by treating with 10 mM acetate, 0.5mM propionate and 0.5mM butyrate induced the production of IL-10 from Th1 cells in mice (Sun et al., 2018), This was quantified by flow cytometry and enzyme-linked immunosorbant assay (ELISA), although SCFA treatment had no effect on interferon gamma (IFN-y) (Sun et al., 2018). This is a contrast to the usual increased IFN-y signalling normally seen following butyrate stimulation (Luu et al., 2018).

BCFA appear to cause reductions to pro-inflammatory responses, similar to those observed with SCFA and indole. A combination of BCFA administered in 20% w/w doses resulted in increased mRNA expression of IL-10 in the ileum of mice pups (Ran-Ressler et al., 2011a). BCFA were seen to suppress the pro-inflammatory response and promote the expression of anti-inflammatory IL-10 in cell culture conditions and a neonatal rat model, respectively (Ran-Ressler et al., 2011b; Yan et al., 2017; Yan et al., 2018). However, they were associated with impaired protein metabolism and muscle atrophy in cats (Summers et al., 2020).

2.7. LITERATURE SUMMARY AND THESIS AIMS

As summarised in Chapter One, the goal of the thesis was to characterise the dog IEC response to bacterial challenges and the first objective was to conduct a literature review of the intestinal response to bacterial ligand and SCFA stimulation. Following the review of the literature the following knowledge gaps and additional project objectives were identified:

1) No studies have profiled the dog IEC response to a bacterial challenge. The aim of Chapter Three was to determine bacteria of interest that would be used later in the project. This would ensure chosen bacteria had relevant diet and health implications for the dog.
2) The characterisation of dog IECs to LPS challenges is lacking, compared to Caco-2 cells. Performing these characterisations was a fundamental step required before direct bacterial challenges could be performed. The aim of this work (Chapter Five) was to build an extensive profile of these interactions that would close this knowledge gap.
3) SCFA also modulate the immune response. The impacts of these should be assessed indidivually, and in combination with pro-inflammatory stimuli such as LPS. The aim of this work (Chapter Five) was to profile this response alone, and in combination with LPS. This would more form a more physiologically relevant model that would also be able to determine how dietary modulation of GIT bacteria and SCFA would impact health.
4) LTAs appear to modulate the immune response when co-stimulating with pro-inflammatory compounds. IECs should be stimulated with LTA alone, and in combination with LPS. This would compliment existing LPS-stimulation work and simulation interactions with Gram-positive bacteria.

CHAPTER THREE

THE IMPACTS OF DIETARY PROTEIN AND DIETARY FAT CONTENT ON THE FAECAL MICROBIOTA OF DOMESTIC DOGS: A META-ANALYSIS

**Selected Material from this chapter will be submitted as:*

The Impacts of Dietary Protein and Fat Content on the Faecal Microbiota of Domestic Dogs: A Meta-Analysis, [Journal, Year.] Content from this paper has been adjusted to meet the Thesis submission requirements.

3.1 ABSTRACT

The interactions between diet and the microbiota of the gastrointestinal tract (GIT) may impact health of the domestic dog and are therefore of interest to the scientific community. The relationship that dietary protein and fat content have with the GIT microbiota in the dog is of key interest, given the oftentimes negative connotations associated with protein or fat-influenced changes to the GIT microbiota in humans. While many studies have been undertaken to understand the relationship between diet and the composition of the microbiome in the dog, a meta-analysis, whereby statical analysis of multiple studies are undertaken, can provide greater insights to that which a single study can provide. Therefore, this meta-analysis was conducted to determine the impact of dietary protein and fat on the faecal microbiota in healthy dogs, hypothesising that there would be a significant difference in the profile of the faecal microbiome depending on the macronutrient being investigated. Published literature was assessed for inclusion in the meta-analysis, in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement. 16 publications met the criteria for inclusion in the meta-analysis. Faecal microbial composition from these 16 publications were combined resulting in a total of 314 dogs in the final dataset. Diets were classed according to crude protein and dietary fat (crude fat and/or ether extract) following a low, moderate, high and supra categorisation for both macronutrients. *Prevotellacaea*_Ga6A1_groupand *Enterococcus* were associated with dietary protein, whilst *Allobaculum* and *Clostridium_sensu_stricto_13* were associated with dietary fat. Furthermore, it was observed that *Sharpea* was primarily responsible for the separation of the microbiome for both crude protein and crude fat, despite its low relative abundance in the faecal microbiome of the dog. Thus, this study has shown that while the faecal microbiota profiles separate based on dietary composition, the key bacteria associated with these differences may be present in relatively low abundances and that understanding the roles these relatively low abundant genera perform is an important direction for future research.

3.2 INTRODUCTION

Nutritionally balanced diets are essential for the maintenance and promotion of health and a functioning immune system in all animals (FEDIAF, 2018; Cotter et al., 2019; Pedrinelli et al., 2019). The nutritional needs and their relationship to health in the domestic dog are continually being re-assessed and improved upon (Bontempo, 2005; Di Cerbo et al., 2017). The minimal dietary requirements are established and maintained by the National Research Council (NRC) (National Research Council, 2006), the Association of American Feed Control Officials (AAFCO) (AAFCO, 2019) and/or the European Pet Food Industry Federation (*Fédération européenne de l'industrie des aliments pour animaux familiers*; FEDIAF) (FEDIAF, 2018). Imbalanced intakes of essential dietary requirements results in malnutrition caused by either under- or overnutrition (Remillard et al., 2001). The immune system of the dog is then impacted; slowed in the case of undernutrition, or in overnutrition there is a constant state of pro-inflammation (Langweiler et al., 1981; German et al., 2010; Tropf et al., 2017).

The impacts of dietary change on health in the domestic dog are tied into the relationship between diet and the microbiota of the gastrointestinal tract (GIT) (Bresciani et al., 2018; Allaway et al., 2020; Atherly et al., 2020). As with other species. changes to the domestic dog's diet can result in swift shifts in the GIT microbiota (Allaway et al., 2020). Many varieties of dietary impacts on the dog GIT microbiota have been explored. These include raw meat or 'natural diets' – such as bone and raw food (BARF)-based diets (Bermingham et al., 2017; Schmidt et al., 2018; Sandri et al., 2019; Sandri et al., 2020) – and altered nutritional profiles (i.e. above or meeting the AAFCO/FEDIAF/NRC recommended dietary minimums) of crude protein (CP) (Li et al., 2017; Ephraim et al., 2020; Bermingham et al., In Press) and crude fat/ether extract (hereafter referred to as dietary fat; DF) (Schauf et al., 2018; Moinard et al., 2020; Bermingham et al., In Press). In addition, diets with varying levels of carbohydrate (CHO) (Hang et al., 2012; Li et al., 2017; Schauf et al., 2018) and dietary fibre (Biagi et al., 2010; Kerr et al., 2013; Panasevich et al., 2015; Jackson and Jewell, 2016; Jackson and Jewell, 2019; Nogueira et al., 2019; Bermudez Sanchez et al., 2020; Sandri et al., 2020) content have

been explored. Additionally, the effects of insect (Jarett et al., 2019) and non-animal protein sources (Kerr et al., 2013; Bresciani et al., 2018) have also been investigated and reported.

However, some of the nutritional profiles from published diets were extremely diverse whilst still being classed as the same diet 'type'. For example, two studies both classed diets as "raw meat based" (Bermingham et al., 2017; Sandri et al., 2020), yet one was almost completely CHO-free (0.6% content by dry matter; % DM) (Bermingham et al., 2017), whilst the other contained 42-43% DM CHO (Sandri et al., 2020). This suggests that a systematic review and/or meta-analysis of the impacts of the dietary macronutrient content on the faecal microbiota in the dog may provide more clarity into the impacts of dietary macronutrients on the faecal microbiome of the dog.

Meta-analyses are a statistical tool used to examine the results of multiple studies to minimise the bias that can be introduced in separate, smaller studies, whilst also allowing for the possibility of challenging a larger overall dataset with new variables (Phillips, 2005). These meta-analyses are being used increasingly across medical and nutritional research (Haidich, 2010; Kelley and Kelley, 2019). Additionally, they are considered the strongest form of data with the fewest bias (Haidich, 2010). There are currently no published meta-analysis studies on the effects of nutrition in the dog. In contrast, nutritional meta-analyses have been successfully used to compared the effects of alternative diets (Manheimer et al., 2015), obesity (Katz et al., 2008), diabetes (Møller et al., 2017), and the impacts of diet and lifestyle on the gut microbiota in humans (Mancabelli et al., 2017).

The objective of this meta-analysis was to evaluate the impacts of the CP and DF content of the diet on the faecal microbiota of dogs. The hypothesis for this study was that the faecal microbiota would be significantly altered based on the CP or DF content of the diets. Furthermore, it was hypothesised that performing a meta-analysis of the data would generate novel insights into the relationship of diet and the faecal microbiota. For ease of comparison with AAFCO (AAFCO, 2019), FEDIAF (FEDIAF, 2018) and the NRC (National Research Council, 2006) all diet content is standardised to, and reported as % DM.

3.3 MATERIALS AND METHODS

3.3.1 STUDY PROTOCOL

This study was a meta-analysis of publications investigating dietary impacts on the gut microbiota in the domestic dog, and has been reported in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement (Moher et al., 2009). The PRISMA 2020 checklist is included as Appendix 2 The project was conceptualised in March 2020 and the protocol was agreed upon in advance before proceeding.

3.3.2 INFORMATION SOURCES AND SEARCHES

A search of the electronic scientific literature to identify publications that analysed the faecal microbiota of dogs and provided dietary information was conducted. Online resources searched included OVID databases (Medline, BIOSIS, Food science and technology abstracts (FSTA), CAB Abstracts), Scopus and PubMed. The search terms initially used were broad; "dog", "diet", "faecal", "gut" and "microbiome", to assess the number of studies that could be included and if a meta-analysis was a viable endeavour. The search terms "gut" and "faecal" were found to produce similar results, wherein faecal also produced more *in vitro* based publications, although both were dropped for the use of "diet", which gathered more publications with a focus on dietary changes and thus, the dietary profiles were more frequently included in the publication.

The initial search was conducted from April – June 2020, and a full search of all the databases with these keywords was performed in July 2020. Subsequently the search terms were expanded to include the keywords and terms as seen in Table 3.4, and the databases were re-assessed quarterly between July 2020 and June 2022 for new publications.

Table 3.4 - *Search terms used to identify publications for potential inclusion in the meta-analysis. Search terms were performed using a series of similar, interchangeable variable terms (Variable Terms), that were entered in a specific order (Variable Term Position). Final Search Terms denotes the specific search phrases used to collate publications for the analysis, which combined all possible iterations of Variable Terms.*

Variable Term Position	Variable Terms	Final Search Terms
1	a) "Canine", b) "Canine excluding dental" c) "Dog"	• "Canine"" Diet" " Microbiome" • "Canine"" Diet" " Microbiota" • "Canine"" Diet" " Microflora" • "Canine"" Diet" " Microbial" • "Canine"" Diet" " Bacterial Community" • "Canine"" Feeding Trial" " Microbiome" • "Canine"" Feeding Trial" " Microbiota" • "Canine"" Feeding Trial" " Microflora" • "Canine"" Feeding Trial" " Microbial" • "Canine"" Feeding Trial" " Bacterial Community" • "Canine"" Intervention" " Microbiome" • "Canine"" Intervention" " Microbiota" • "Canine"" Intervention" " Microflora" • "Canine"" Intervention" " Microbial" • "Canine"" Intervention" " Bacterial Community" • "Canine"" Diet" " Microbiome" • "Canine excluding dental"" Diet" " Microbiome" • "Canine excluding dental"" Diet" " Microbiota" • "Canine excluding dental"" Diet" " Microflora" • "Canine excluding dental"" Diet" " Microbial" • "Canine excluding dental"" Diet" " Bacterial Community" • "Canine excluding dental"" Feeding Trial" " Microbiome" • "Canine excluding dental"" Feeding Trial" " Microbiota" • "Canine excluding dental"" Feeding Trial" " Microflora" • "Canine excluding dental"" Feeding Trial" " Microbial" • "Canine excluding dental"" Feeding Trial" " Bacterial Community" • "Canine excluding dental"" Intervention" " Microbiome" • "Canine excluding dental"" Intervention" " Microbiota" • "Canine excluding dental"" Intervention" " Microflora" • "Canine excluding dental"" Intervention" " Microbial" • "Canine excluding dental"" Intervention" " Bacterial Community" • "Canine excluding dental"" Diet" " Microbiome" • "Dog"" Diet" " Microbiome" • "Dog"" Diet" " Microbiota" • "Dog"" Diet" " Microflora" • "Dog"" Diet" " Microbial"
2	d) "Diet" e) "Feeding Trial" f) "Intervention"	
3	g) "Microbiome" h) "Microbiota" i) "Microflora" j) "Microbial" k) "Bacterial Community"	

		• "Dog"" Diet" " Bacterial Community" • "Dog"" Feeding Trial" " Microbiome" • "Dog"" Feeding Trial" " Microbiota" • "Dog"" Feeding Trial" " Microflora" • "Dog"" Feeding Trial" " Microbial" • "Dog"" Feeding Trial" " Bacterial Community" • "Dog"" Intervention" " Microbiome" • "Dog"" Intervention" " Microbiota" • "Dog"" Intervention" " Microflora" • "Dog"" Intervention" " Microbial" • "Dog"" Intervention" " Bacterial Community" • "Dog"" Diet" " Microbiome"

3.3.2.1 Eligibility Criteria

A ‘publication’ was defined as a stand-alone piece of published work. Publications were analysed for eligibility as per criteria summarised in Table 3.5.

Table 3.5 - *Inclusion and exclusion criteria for publication eligibility in meta-analysis.*

Variable	Criteria for Inclusion	Criteria for Exclusion
Publication type	• Experimental publication that involved the use of domestic dogs.	• Non-experimental data publication (i.e., review, book chapter, meta-analysis). • *In vitro* experiments. • Computer modelling data. • Any experiments that did not use domestic dogs.
Publication date	• Any publication from 2010 (inclusive) onwards.	• Any study prior to 2009 (including those in 2009).
Animal Health	• Healthy Animals. • Non-inflammatory bowel disease or gastrointestinal-associated illnesses. • Non-obese (body condition score; BCS < 6). • Non-underweight (BCS > 3).	• Dogs with inflammatory bowel disease. • Dogs with other gastrointestinal-associated illnesses (e.g., food responsive diarrhoea, chronic enteropathy). • Obese animals (BCS > 6) • Underweight animals (BCS < 3).

Diet information	• Dietary information provided had to include macronutrient content for at least 4 of:
	  ○ Crude Protein,
	  ○ Crude Fat/ Ether Extract (Dietary Fat)
	  ○ Ash,
	  ○ Crude Fibre,
	  ○ Nitrogen-Free Extract/Carbohydrate
	• If at least 4 of the above macronutrients were not provided in the publication, clarification was sought from the contact author. If this was provided, the
		• Incomplete diet information after author contact.
		• Unclear diet information that was not able to be clarified from authors.
		• Diet content did not meet the minimum levels of crude protein or dietary fat required.

Variable	Criteria for Inclusion	Criteria for Exclusion
Diet information	information was included in the meta-analysis. • Diet information was available upon request or detailed the commercial brand of food used, where this information could be attained from a company website. • Diet information had to be either provided, or able to be calculated as a percentage of the dry matter content (% DM). • Diet content had to meet the minimum levels of crude protein (18.00 % DM) and dietary fat (5.00% DM) prescribed by the National Research Council (NRC) (National Research Council, 2006), the Association of American Feed Control Officials (AAFCO) (AAFCO, 2019) and the European Pet Food Industry Federation (*Fédération européenne de l'industrie des aliments pour animaux familiers*; FEDIAF) (FEDIAF, 2018).	
Faecal Microbiota Analysis	• Use of next generation sequencing (NGS) on the 16S rRNA. ○ Including MiSeq/HiSeq, 454 pyrosequencing and/or Shotgun sequencing. • Data deposited in a publicly accessible database (e.g.,National Centre for Biotechnology Information (NCBI), Mendeley, European Nucleotide Archive	• No Faecal Microbiota analysis performed. • No NGS performed. • Data not publicly deposited and/or not available from authors on request.

Variable	Criteria for Inclusion	Criteria for Exclusion
Faecal Microbiota Analysis	(ENA)) or available from authors on request.	
Use of Antibiotics and Pre/probiotics	• No animals that received antibiotics, prebiotics or probiotics during their dietary intervention periods were included.	• Animals that received antibiotics and/or pre/probiotics during dietary interventions were excluded.
Other Variables	• All neuter statuses were included. • All husbandry conditions were included (i.e., pets, kennel dogs, etc.). • All feeding trial designs were included. • Healthy control dog data was used from studies that investigated the effects in unhealthy dogs (i.e., the entire publication was not discarded).	• Animals removed from studies for any reason were not included in the analysis. • Withheld food experiments were not included. • In publications that investigated the response of the gut microbiota to vaccinations, the data from dogs that received the vaccine being investigated were not included.

Studies that examined the effects of antibiotics on the gut microbiota, or those that investigated the response to vaccinations received during the study period were not included. There was no exclusion based on age, breed, gender, or neuter status. Studies where dietary macronutrient profiles were incomplete but missing values could be calculated (e.g., missing one of; CP, DF, CHO/nitrogen free extract (NFE), inorganic matter/ash, crude fibre) were included. Studies that had CP or DF below the required minimums established by NRC/FEDIAF/AAFCO were excluded. Data from obese or overweight dogs was not included. Data from dogs suffering from any non-gut related illness (e.g., skin problems) were not immediately excluded, though these results were flagged.

Any data from dogs with forms of intestinal disorders such as inflammatory bowel disease (IBD), chronic enteropathy (CE), food responsive diarrhoea (FRE) and those removed from studies for any reasons were not included in the final data analysis. Food intake was not analysed or included as a factor. Husbandry status (e.g., kennel, pet, working) and social housing (e.g., individual, paired, pets with and without other pets) were not used as limiters and all information provided was included in data tabulation.

Studies were included so long as they performed faecal analysis via Next-Gen Sequencing (NGS) (i.e., 454 pyrosequencing, (M/H)iSeq and/or Shotgun Sequencing), and there was no exclusion based on the variable region of the 16S rRNA analysed. Any studies that did not perform NGS analysis were not included, so that all analyses were conducted with similar technologies. The methods of extraction of nucleic acids and 16S rRNA analysis were all included with data tabulation. All published data included in the analysis came from animals that were returned to their respective husbandry arrangements after the study concluded, although this was not a limiting factor in the inclusion or exclusion of data.

3.3.2.2 Study Selection and Data Collection

All publications identified from the electronic searches were reviewed and assessed for eligibility in a standardised, unblinded manner. This was performed quarterly between July 2020 and July 2022. Access rights from both Massey University and AgResearch were used to access the

material, and all eligible publications were able to be acquired through this process. All variables listed within each publication were tabulated into a computer spreadsheet (Microsoft Excel version Office 365, Microsoft, Redmond, USA). All decisions regarding the exclusion and inclusion of the identified publications were reviewed and confirmed by consensus of the supervisory team before data collection began.

A total of 315 publications were initially assessed. Of these, 168 papers were deemed ineligible based on exclusion criteria as outlined in Table 3.5. The remaining 147 papers were then evaluated for eligibility in the analysis. 77 of these papers were deemed ineligible, for reasons highlighted in Figure 3.10. 48 authors were contacted to request data access.

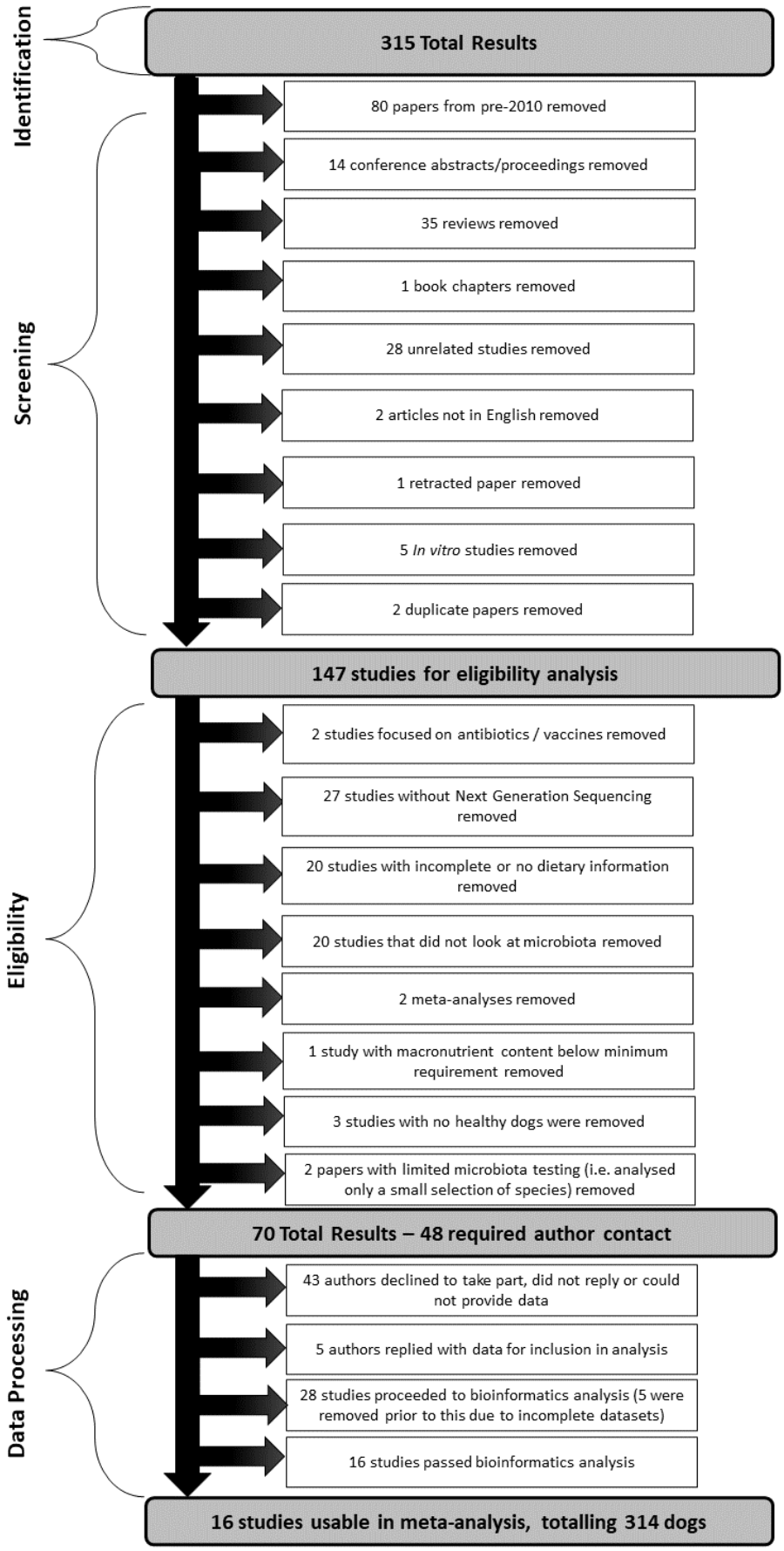


***Figure 3.10** – Meta-analysis publication inclusion/exclusion workflow.*

Each search was performed quarterly (i.e., every three months). At the end of each search period, if there were any publications that required author contact for data, diet information or clarification, the contact authors for all relevant publications were contacted on the same day. Author contact was conducted using a template email (see Appendix 1) that was agreed upon in advance by the supervisory team and that highlighted any potential conflicts of interest for contacted authors that may arise from Waltham Petcare Science Institute co-funding the PhD project. If there was no response after two weeks, contact authors were re-contacted, or the primary author was contacted if they were not the contact author, using the same template email. If no response was obtained from either author, or the request was refused, the study was deemed as unavailable. Any publications that were obtained were included in the data analysis. A full list of the publications (excluding the 80 pre-2010 papers), their inclusion or exclusion status, and the rationale for their exlcusion are included as Appendix 3.

A total of 23 publications had complete diet information and had deposited their data in publicly accessible archives (e.g., European Nucleotide Archive (ENA), Mendeley, National Centre for Biotechnology Information (NCBI)) and met all other inclusion criteria. An additional 5 publications were included from authors that responded to data queries, which provided a final amount of 28 publications for analysis, consisting of 74 dietary treatments. These were then assigned unique ID numbers and given to a biostatistician who downloaded all the data from these studies in bulk and analysed them as per Section 3.3.4.

3.3.3 DIET CLASSIFICATIONS

Diet groupings were performed before the bioinformatics analysis in Section 3.3.4. Therefore, diet groupings were decided upon from the 28 papers and 74 dietary treatments deemed eligible for analysis. The means, standard error of mean (SEM) and interquartile ranges of the CP and DF content from the dietary treatments are presented as below in Table 3.6.

Table 3.6 – *Summary of the crude protein and dietary fat contents of the diets included in the meta-analysis.*

	Crude Protein (% dry matter; % DM)	***Dietary Fat (% DM)***
Mean	30.32%	21.15%
Standard error of mean	1.09%	1.64%
1st interquartile	24.99%	14.00%
Median	27.78%	16.00%
3rd interquartile	31.49%	21.68%
Minimum Macronutrient Content	19.00%	6.63%
Maximum Macronutrient Content	76.30%	64.13%

The AAFCO recommended minimum CP and Fat contents for adult dogs are 18.00% and 5.00%, respectively. As the studies analysed used a range of CP and DF levels, the ranges used to assign dietary treatment groups (i.e., Low, Medium, and High) in the current study were established by using the interquartile ranges. For example, the 1st interquartile (IQ) range was used to determine the cut off for the Low group, rounded to the nearest 5%. The Moderate group was defined as between the 1st and 3rd IQ ranges, both rounded to the nearest 5%. The High group was defined as between the 3rd IQ range, rounded to the nearest 5%, and half of this value again as the upper limit (i.e., 30% was the lower limit for the High CP group, with (30 x 1.5 = 45) calculated as the upper limit. Anything above the upper limit of the High group was classified as Supra. The ranges for inclusion in these groups are shown in Table 3.7.

Table 3.7 – *Crude protein and dietary fat diet classifications for the meta-analysis.*

	Macronutrient Content (% dry matter basis)	
Diet Classification	***Crude Protein***	***Dietary Fat***
Low	≤ 25	≤ 15
Moderate	25 < x < 30	15 < x < 20
High	30 < x < 45	20 < x < 30
Supra	≥ 45	≥ 30

3.3.4 BIOINFORMATICS ANALYSIS

The reads produced by the sequencing instrument were paired using the program FLASH2 v2.2.00 (Magoč and Salzberg, 2011). Paired reads were then quality trimmed using Trimmomatic v0.38 (Bolger et al., 2014). The trimmed reads were reformatted as fasta, and the read headers were modified to include the sample name. All reads were compiled into a single file, and Mothur v1.45.2 (Schloss et al., 2009) was used to remove reads with homopolymers longer than 10 nucleotides (nt) and to collapse the reads into unique representatives. The collapsed reads were clustered using Swarm v2 (Mahé et al., 2014). The clustered reads were filtered based on their abundance, keeping representatives that were (i) present in one sample with a relative abundance of >0.1%, (ii) present in >2% of the samples with a relative abundance of >0.01%, or (iii) present in 5% of the samples at any abundance level. The selected representatives were annotated using QIIME 2 v2017.4 (Caporaso et al., 2010) with the SILVA database v138 (Quast et al., 2013). The annotated tables were then used for downstream statistical analysis.

Whole Genome Shotgun Sequencing (WGS) sequencing reads derived from ribosomal DNA was extracted using metaxa version 2.1.3 (Bengtsson-Palme et al., 2015) and aligned to the silva 138 ribosomal RNA database (Quast et al., 2013) using the "assign_taxonomy.py" script from qiime version 1.9 (Caporaso et al., 2010).

3.3.5 STATISTICAL ANALYSES

The data was loaded into R (Version 4.1.1, RStudio, Boston, MA, USA). The studies were evaluated for suitability by viewing the rarefaction curve produced by the *vegan* R package version 2.5-7 (Dixon, 2003). This plot compares genera identified with the number of reads sampled in each sample. Proportions of genera were first converted into pseudocounts based on the lowest proportion greater than 0 in the entire study and dividing 1 by it, giving a factor to multiply all the proportions by to resurrect relative counts. All studies were found to have sufficient counts. Non-parametric two-

sample Kolmogorov-Smirnov tests were performed to assess significance between protein and fat classifications.

Principal Coordinate Analysis (PCoA) was performed using the *pcoa* function in the *ape* R package version 5.5 (Paradis and Schliep, 2018) on the Bray-Curtis dissimilarity matrices derived using the *vegdist* function of the *vegan* R package version 2.5-7 (Dixon, 2003) from the proportions of the genera from each of the studies. The points were coloured by the *level* of protein in the diet. Partial Least Squares-Discriminant Analysis (PLS-DA) was performed on the proportions of genera using either the fat level or protein level as a response using the *opls* function in the *ropls* R package version 1.24.0 (Thévenot et al., 2015). The counts per taxa were converted into relative abundances (percentages), so all samples added up to an identical 100%. With PLS-DA the relative abundances were then standardised (sample mean = 0, sample standard deviation = 1, also called autoscaling) as part of the default settings. RandomForest analysis was also performed on the proportions of genera using either the fat level or protein level as a response using the *randomForest* function in the *RandomForest* R package version 4.6-14. (Liaw and Wiener, 2007). Normalisation of data was not required for RandomForest analysis (Lam et al., 2020) and so data analysis was performed on the relative abundances provided. Finally, permutation analysis of variances (ANOVAs) were performed on the proportions of genera using either the fat level or protein level as a response with the *aovp* function from the *lmPerm* R package version 2.1.0.

Genstat (19th Edition, VSN International, Hemel Hempstead, UK) was used to perform one-way ANOVA on the diet data to assess for differences between defined diet groupings, with Tukey's posthoc analysis to determine differences between groups. Finally, the composition of the faecal microbiome were analysed in R by the use of Kruskal–Wallis ANOVA with P values corrected for false discovery rate. Post-hoc analysis was performed by Fisher's least significant difference test using the LSD.test() function from the *agricolae* package. Results are presented as means with their corresponding SEM. In all cases $p < 0.05$ is considered significant and $p < 0.10$ a trend.

3.4 RESULTS

3.4.1 METADATA

3.4.1.1 Publication Meta-data

After the literature search and author contact, a total of 28 studies were eligible for inclusion. However, after data analysis, the data of only 16 of these 28 publications passed the bioinformatics analysis outlined in Section 3.3.4. Thus, a final total of 16 studies were included in the meta-analysis, producing a dataset that comprised of 314 dogs. Their information is included below in Table 3.8. A full list of final studies and their meta-data are included as Appendix 4. The breeds of the dogs from the analysed publications are included as Appendix 5.

Table 3.8 – *Meta-data of publications analysed in meta-analysis.*

ID	***Reference***	***Paper Title****	***Year of Publication***	***Number of dogs***
ID9	(Bermingham et al., In Press)	Effects of commercial high protein and fat diets on faecal organic acids and microbial composition in the dog.	Internal Data[¥]	15
ID18	(Scarsella et al., 2020)[§]	Interplay between neuroendocrine biomarkers and gut microbiota in dogs supplemented with grape proanthocyanidins: results of dietary intervention study	2020	8
ID19	(Sandri et al., 2020)[§]	Effect of different starch sources in a raw meat-based diet on fecal microbiome in dogs housed in a shelter	2020	27
ID23	(Kirchoff et al., 2019)	The gut microbiome correlates with conspecific aggression in a small population of rescued dogs (*Canis familiaris*)	2019	31
ID24	(Pilla et al., 2019)	Administration of a synbiotic containing enterococcus faecium does not significantly alter fecal microbiota richness or diversity in dogs with and without food-responsive chronic enteropathy	2019	8
ID25	(Sandri et al., 2019)[§]	Substitution of a commercial diet with raw meat complemented with vegetable foods containing chickpeas or peas affects faecal microbiome in healthy dogs	2019	8
ID27	(Jarett et al., 2019)	Diets with and without edible cricket support a similar level of diversity in the gut microbiome of dogs	2019	32
ID28	(Bresciani et al., 2018)	Effect of an extruded animal protein-free diet on fecal microbiota of dogs with food-responsive enteropathy	2018	14
ID29	(Coelho et al., 2018)	Similarity of the dog and human gut microbiomes in gene content and	2018	32

ID	***Reference***	***Paper Title****	***Year of Publication***	***Number of dogs***
		response to diet		
ID30	(Schauf et al., 2018)	Effect of dietary fat to starch content on fecal microbiota composition and activity in dogs	2018	12
ID32	(Li et al., 2017)	Effects of the dietary protein and carbohydrate ratio on gut microbiomes in dogs of different body conditions	2017	32
ID38	(Kilburn et al., 2020)	High-fat diets led to OTU-level shifts in fecal samples of healthy adult dogs	2020	8
ID39	(Beloshapka et al., 2020)	Graded dietary resistant starch concentrations on apparent total tract macronutrient digestibility and fecal fermentative end products and microbial populations of healthy adult dogs	2021	7
ID43	(Moinard et al., 2020)	Effects of high-fat diet at two energetic levels on fecal microbiota, colonic barrier, and metabolic parameters in dogs	2020	24
ID44	(Martínez-López et al., 2021)	Effect of sequentially fed high protein, hydrolyzed protein, and high fiber diets on the fecal microbiota of healthy dogs: a cross-over study	2021	46
ID45	(Eisenhauer et al., 2019)	Effects of Brewer's spent grain and carrot pomace on digestibility, fecal microbiota, and fecal and urinary metabolites in dogs fed low-or high-protein diets	2019	10

* Publication titles are presented as their submitted titles and may follow different English spelling rules (i.e., American English vs New Zealand English)

¥ This internal AgResearch dataset is from as as-yet unpublished study.

§ Studies originated from the same laboratory

3.4.1.2 Diet Meta-data

Diet information from the final included studies is included below as Table 3.9, including their diet group classification, as outlined by Table 3.7. For the purposes of this meta-analysis, the dietary treatments for each publication were annotated as Diet 1, Diet 2, etc., for each publication.

Table 3.9 – *Diet meta-data from publications analysed in the meta-analysis.*

		Reported macronutrient composition		***Dietary treatment classification***	
Paper ID	***Diet***	***Crude Protein (% Dry Matter)***	***Dietary Fat (% Dry Matter)***	***Protein****	***Fat[§]***
ID9	Diet 1	39.10%	19.80%	High	Moderate
	Diet 2	24.70%	12.30%	Moderate	Low
ID18	Diet 1	29.11%	17.33%	Moderate	Moderate
ID19	Diet 1	29.69%	21.68%	Moderate	High
	Diet 2	28.89%	21.74%	Moderate	High
	Diet 3	30.05%	22.80%	High	High
ID23	Diet 1	27.78%	15.56%	Moderate	Moderate
	Diet 2	32.22%	19.44%	High	Moderate
ID24	Diet 1	22.70%	10.81%	Low	Low
ID25	Diet 1	27.20%	19.20%	Moderate	Moderate
	Diet 2	26.00%	19.00%	Moderate	Moderate
	Diet 1	23.90%	15.20%	Low	Moderate
ID27	Diet 1	26.20%	14.20%	Moderate	Low
	Diet 2	27.40%	16.00%	Moderate	Moderate
	Diet 3	26.70%	14.70%	Moderate	Low
	Diet 4	25.80%	14.10%	Moderate	Low
ID28	Diet 1	21.97%	13.60%	Low	Low
ID29	Diet 1	27.30%	17.51%	Moderate	Moderate
	Diet 2	53.92%	18.44%	Supra	Moderate
ID29	Diet 3	29.97%	18.16%	Moderate	Moderate
ID30	Diet 1	28.49%	10.92%	Moderate	Low
	Diet 2	32.50%	23.19%	High	High
ID32	Diet 1	53.30%	15.11%	Supra	Moderate
	Diet 2	27.62%	15.68%	Moderate	Moderate

		Reported macronutrient composition		***Dietary treatment classification***	
Paper ID	***Diet***	***Crude Protein (% Dry Matter)***	***Dietary Fat (% Dry Matter)***	***Protein****	***Fat[§]***
	Diet 3	30.53%	17.19%	High	High
ID38	Diet 1	46.88%	32.05%	Supra	Supra
	Diet 2	42.72%	37.15%	High	Supra
	Diet 3	40.02%	41.89%	High	Supra
	Diet 4	38.19%	46.49%	High	Supra
ID39	Diet 1	37.73%	13.21%	High	Low
ID43	Diet 1	30.55%	34.52%	High	Supra
ID44	Diet 1	21.10%	6.63%	Low	Low
	Diet 2	19.00%	14.40%	Low	Low
	Diet 3	19.20%	8.70%	Low	Low
ID45	Diet 1	21.30%	10.30%	Low	Low
	Diet 2	40.90%	10.90%	High	Low
	Diet 3	21.60%	10.10%	Moderate	Low
	Diet 4	39.90%	10.50%	High	Low
	Diet 5	21.90%	9.86%	Moderate	Low

* Low protein was classed as < 25 % Crude Protein content by Dry matter (%DM) analysis. Moderate protein was between 25 and 30 % DM. High protein was between 30 and 45% DM. Any reported crude protein content higher than 45% DM was classed as Supra protein.

* Low fat was classed as < 15 % DM dietary fat content. Moderate fat was between 15 and 20 % DM. High fat was between 20 and 30% DM. Any reported dietary fat content higher than 30% DM was classed as Supra fat.

3.4.2 IMPACTS OF DIETARY PROTEIN ON THE FAECAL MICROBIOME

3.4.2.1 Diet classification

Of the diets investigated, 7 were classed as low protein, 17 were moderate protein, 12 were high protein and 3 were supra protein diets (Figure 3.11). There was a significant overall difference in CP content between protein classifications ($F(3,35) = 77.40$, $p < 0.001$), and protein content was different between all protein classifications (i.e., the CP content in the Low Protein classification was significantly different to the Moderate, High and Supra Protein classifications, etc., $p < 0.001$, represented in Figure 3.11 by different letters).

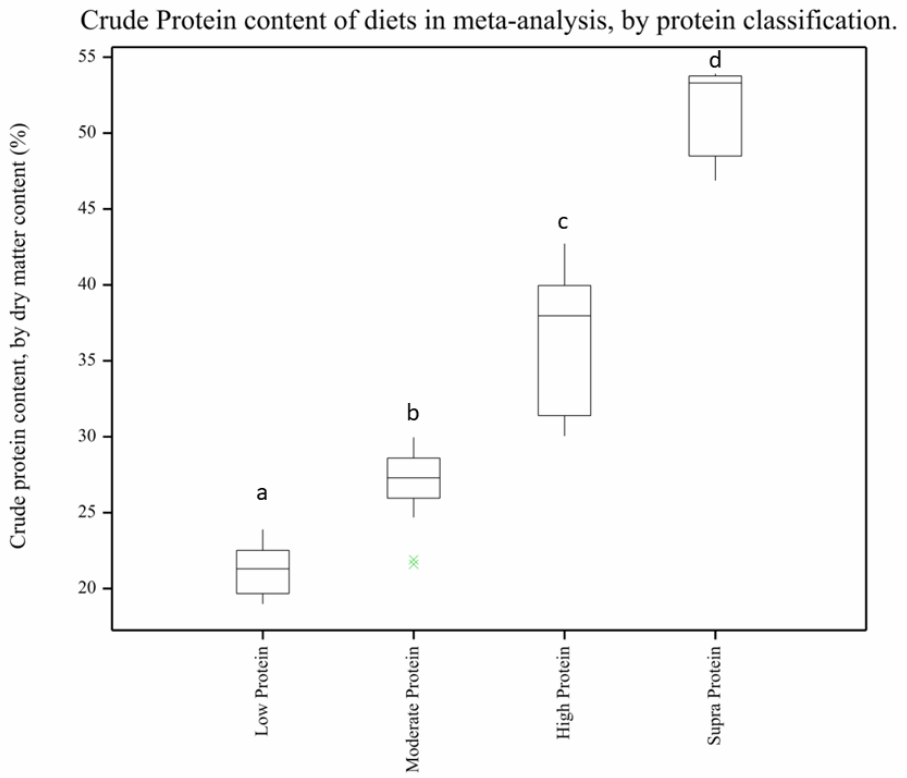


Figure 3.11 *- Crude Protein content of diets by protein classification.There were 39 dietary treatments across the protein classifications. Different letters above the boxplots denote a significant difference in crude protein content between classifications ($p < 0.001$).Green crosses denote outliers. Boxes represent the interquartile range between the first and third quartiles. Thick black line inside the box denotes the median.*

3.4.2.2 Impacts of Crude Protein on the Diversity of the Faecal Microbiota

The CP content of the diets significantly impacted ($p < 0.001$) the Shannon index (alpha diversity) of the faecal microbiota in the dog (Figure 3.12). There was also a significant difference between all individual protein classifications ($p < 0.05$). The greatest alpha diversity value was observed in the Moderate Protein classification and the least diverse was in the Supra Protein classification.

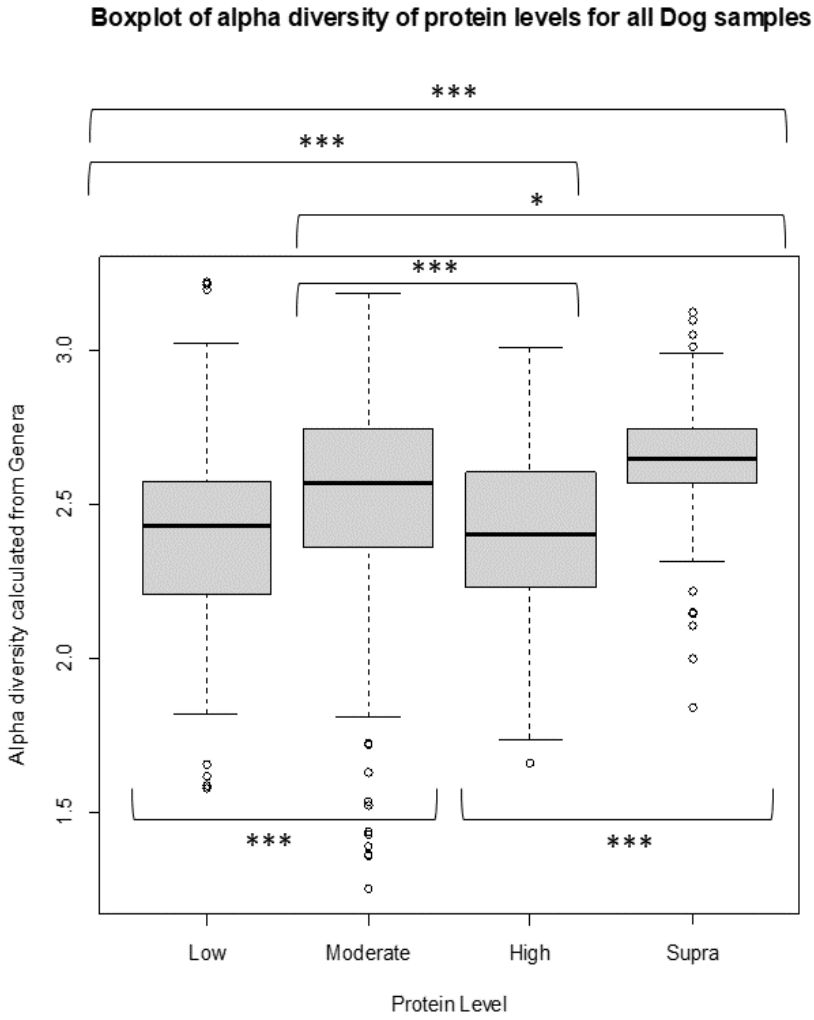


Figure 3.12 *–Shannon index alpha diversity of bacterial genera of the canine faecal microbiota, grouped by protein classification, n = 314.Statistical differences between protein classifications are denoted by an asterisk*

*(*), where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Circles crosses denote outliers. Boxes represent the interquartile range between the first and third quartiles. Thick black line inside the box denotes the median.*

The total richness of the canine faecal microbiota was assessed using the Chao1 index (Figure 3.13), wherein there was a significant impact on bacterial richness caused by dietary CP content ($p < 0.001$). The bacterial richness was highest in the Supra Protein classification ($p < 0.001$ compared to Low and High Protein, $p < 0.05$ compared to Moderate Protein). The bacterial richness was lowest in the Low Protein classification ($p < 0.001$ compared to all other protein classifications). Bacterial richness was lower in the High Protein classification in comparison to the Moderate Protein classification ($p < 0.001$). The Moderate Protein classification had the largest spread of richness of all protein classifications i.e., it had the furthest outliers.

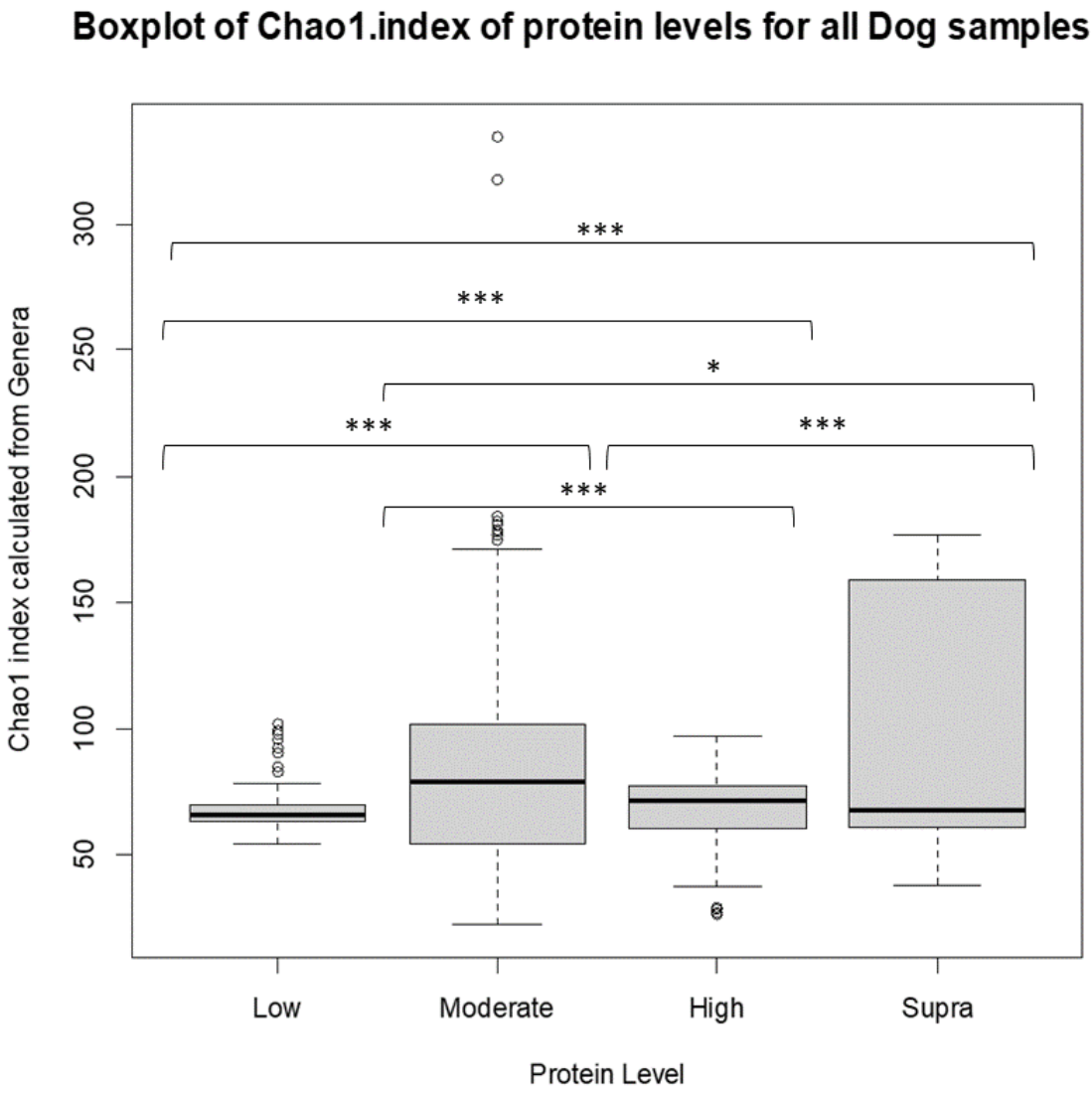


Figure 3.13 *-Chao 1 index of bacterial genera of the canine faecal microbiota, grouped by protein classification, n = 314.Statistical differences between protein classifications are denoted by an asterisk (*), where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Circles crosses denote outliers. Boxes represent the interquartile range between the first and third quartiles. Thick black line inside the box denotes the median.*

3.4.2.3 Impacts of Dietary Protein on Bacterial Genera in the faecal microbiota

The impacts of dietary CP content on the faecal microbiota was assessed, and are presented in Table 3.10. These are the mean abundances of each bacterial genera for each dietary protein classification. These are listed alphabetically, by bacterial phylum. The Diet Effect *p* value denotes if there was a significant difference in bacterial genera between dietary protein classifications ($p < 0.05$).

Peptoclostridium, *Bacteroides*, *Fusobacterium*, *Blautia* and *Streptococcus* were the dominant bacterial Genera in the Low Protein classification (relative abundances of 11.445% ± 0.825%, 10.469% ± 0.881%, 9.306% ± 0.747%, 8.455% ± 0.641% and 6.148% ± 1.020% of sequence reads, respectively). Additionally, *Peptoclostridium*, *Bacteroides*, *Fusobacterium*, *Blautia* and *Streptococcus* were significantly affected ($p < 0.001$) by CP content. *Fusobacterium* (16.345% ± 0.511% of sequence reads), *Bacteroides* (10.521% ± 0.411% of sequence reads), *Peptoclostridium* (8.418% ± 0.353% of sequence reads), *Prevotella* (7.839% ± 0.539% of sequence reads)and *Blautia* (5.518% ± 0.254% of sequence reads) were the most abundant bacterial Genera in the Moderate Protein classification and were all significantly affected by CP content ($p < 0.001$).

For the High Protein classification, the dominant bacterial genera in terms of relative abundance were *Fusobacterium* (20.012% ± 0.737% of sequence reads), *Bacteroides* (11.047% ± 0.509% of sequence reads), *Peptoclostridium* (9.880% ± 0.573% of sequence reads), *uncultured* (7.782% ± 0.483% of sequence reads) and *Allobaculum* (5.162% ± 0.454% of sequence reads). All were significantly affected by dietary CP content ($p < 0.001$). Finally, for the Supra Protein classification, the dominant bacterial genera were *Fusobacterium* (14.943% ± 0.853% of sequence reads), *Bacteroides* (13.915% ± 0.944% of sequence reads), *Prevotella* (10.556% ± 1.142% of sequence reads), *Peptoclostridium* (8.932% ± 1.201% of sequence reads) and *Alloprevotella* (7.333% ± 0.859% of sequence reads), which were all significantly affected by dietary CP content ($p < 0.001$).

Table 3.10 - *Mean relative abundances of bacterial genera, expressed as % of sequence reads for each Dietary Protein classification.Dietary data are from 314 dogs across 16 publications. Results are presented to three decimal places as means with their corresponding SEM, and with p values as determined by the Kruskal-wallis one-way ANOVA and corrected for false discovery rate. Dietary groupings are assigned by Fisher's least significant difference post-hoc analysis, where different letters following the relative abundances denote significant differences (p <0.05). Bacterial genera are listed alphabetically, by Phyla.*

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Protein	***Moderate Protein***	***High Protein***	***Supra Protein***	
Actinobacteria	*Actinomyces*	-*	0.000 ± 0.000[§]	0.000 ± 0.000	0.000 ± 0.000	<0.001
	Adlercreutzia	0.038 ± 0.007[a]	0.018 ± 0.001[b]	0.036 ± 0.003[a]	0.016 ± 0.003[b]	<0.001
	Atopobiaceae_ge	-	0.000 ± 0.000	-	0.000 ± 0.000	0.018
	Atopobium	-	0.014 ± 0.006	-	0.000 ± 0.000	<0.001
	Bifidobacterium	1.324 ± 0.281[a]	0.656 ± 0.130[b]	0.455 ± 0.063[b]	0.261 ± 0.076[b]	<0.001
	Collinsella	2.993 ± 0.33[a]	1.326 ± 0.073[b]	0.707 ± 0.064[b]	0.397 ± 0.050[b]	<0.001
	Coriobacteriaceae_UCG.002	0.050 ± 0.015[a]	0.013 ± 0.003[b]	0.067 ± 0.010[a]	0.039 ± 0.013[ab]	<0.001
	Coriobacterium	-	0.000 ± 0.000[b]	-	0.000 ± 0.000[a]	<0.001
	Corynebacterium	0.008 ± 0.005	0.000 ± 0.000	0.019 ± 0.018	0.001 ± 0.000	<0.001
	Cutibacterium	-	0.000 ± 0.000	-	0.000 ± 0.000	<0.001
	Denitrobacterium	0.002 ± 0.000[a]	0.001 ± 0.000[b]	0.000 ± 0.000[b]	0.000 ± 0.000[b]	<0.001
	Enorma	-	0.000 ± 0.000[b]	-	0.000 ± 0.000[a]	<0.001
	Libanicoccus	-	0.000 ± 0.000	-	0.001 ± 0.000	<0.001
	Olsenella	0.118 ± 0.030[a]	0.024 ± 0.006[b]	0.072 ± 0.016[ab]	0.034 ± 0.006[ab]	0.001
	Parvibacter	0.000 ± 0.000	0.000 ± 0.000	-	0.000 ± 0.000	<0.001
	Pseudarthrobacter	-	0.000 ± 0.000	0.001 ± 0.000	-	<0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Protein	***Moderate Protein***	***High Protein***	***Supra Protein***	
	Senegalimassilia	-	0.000 ± 0.000[b]	-	0.001 ± 0.000[a]	<0.001
	Slackia	0.059 ± 0.008[b]	0.198 ± 0.013[a]	0.052 ± 0.006[b]	0.054 ± 0.007[b]	<0.001
	Tetrasphaera	-	0.001 ± 0.000	-	0.000 ± 0.000	<0.001
	Trueperella	-	0.000 ± 0.000	0.065 ± 0.065	0.000 ± 0.000	<0.001
Bacteroidetes	*Alistipes*	0.005 ± 0.002[*a*]	0.001 ± 0.000[*b*]	0.004 ± 0.000[*a*]	0.006 ± 0.002[*a*]	<0.001
	Alloprevotella	1.515 ± 0.200[*c*]	3.959 ± 0.165[*b*]	4.227 ± 0.334[*b*]	7.333 ± 0.858[*a*]	<0.001
	Bacteroides	10.468 ± 0.881[*b*]	10.52 ± 0.410[*b*]	11.046 ± 0.508[*b*]	13.914 ± 0.943[*a*]	0.001
	Barnesiella	-	0.002 ± 0.000[*b*]	-	0.004 ± 0.002[*a*]	<0.001
	Butyricimonas	-	0.000 ± 0.000[*b*]	-	0.000 ± 0.000[*a*]	<0.001
	Muribaculaceae_ge	0.36 ± 0.086[*b*]	0.631 ± 0.088[*b*]	2.319 ± 0.275[*a*]	2.217 ± 0.481[*a*]	<0.001
	Myroides	-	-	0.025 ± 0.025	-	0.186
	Odoribacter	0.003 ± 0.000[*b*]	0.013 ± 0.001[*a*]	0.007 ± 0.001[*b*]	0.019 ± 0.003[*a*]	<0.001
	Parabacteroides	0.074 ± 0.028[*c*]	0.131 ± 0.010[*b*]	0.207 ± 0.018[*a*]	0.229 ± 0.034[*a*]	<0.001
	Paraprevotella	0.015 ± 0.003	0.005 ± 0.000	0.006 ± 0.001	0.009 ± 0.003	0.231
	Porphyromonas	-	0.000 ± 0.000	0.006 ± 0.006	0.001 ± 0.000	<0.001
	Prevotella	1.348 ± 0.241[*c*]	7.838 ± 0.539[*a*]	4.835 ± 0.494[*b*]	10.556 ± 1.141[*a*]	<0.001
	Prevotellaceae_Ga6A1_group	0.575 ± 0.104[*c*]	2.447 ± 0.159[*a*]	1.684 ± 0.196[*b*]	2.298 ± 0.568[*ab*]	<0.001
	Prevotellaceae_NK3B31_group	-	0.000 ± 0.000[*b*]	0.009 ± 0.005[*b*]	0.080 ± 0.076[*a*]	<0.001
	Prevotellaceae_UCG.001	0.000 ± 0.000[*bc*]	0.001 ± 0.000[*b*]	0.000 ± 0.000[*c*]	0.002 ± 0.000[*a*]	<0.001
	Prevotellaceae_UCG.003	0.001 ± 0.001	0.002 ± 0.000	0.028 ± 0.020	0.057 ± 0.050	<0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Protein	***Moderate Protein***	***High Protein***	***Supra Protein***	
	Rikenellaceae_RC9_gut_group	0.062 ± 0.011^{b}	0.066 ± 0.006^{b}	0.089 ± 0.013^{b}	0.175 ± 0.032^{a}	<0.001
Deferribacteres	*Mucispirillum*	0.006 ± 0.002^{b}	0.028 ± 0.003^{a}	0.015 ± 0.003^{b}	0.015 ± 0.003^{ab}	<0.001
Euryarchaeota	*Methanobrevibacter*	-	0.001 ± 0.000	0.016 ± 0.013	0.001 ± 0.001	<0.001
	Methanosphaera	0.008 ± 0.002^{b}	0.000 ± 0.000^{b}	0.109 ± 0.026^{a}	0.006 ± 0.003^{b}	<0.001
Firmicutes	*Acetanaerobacterium*	0.000 ± 0.000	0.000 ± 0.000	-	0.000 ± 0.000	<0.001
	Acidaminococcus	-	0.001 ± 0.000	0.006 ± 0.006	0.003 ± 0.001	<0.001
	Agathobacter	-	0.000 ± 0.000^{b}	-	0.003 ± 0.001^{a}	<0.001
	Allisonella	0.020 ± 0.007^{a}	0.015 ± 0.001^{ab}	0.010 ± 0.001^{b}	0.026 ± 0.004^{a}	<0.001
	Allobaculum	2.411 ± 0.458^{b}	1.610 ± 0.163^{b}	5.162 ± 0.454^{a}	2.571 ± 0.342^{b}	<0.001
	Amnipila	-	0.000 ± 0.000	0.020 ± 0.020	0.000 ± 0.000	<0.001
	Anaerofilum	0.059 ± 0.02^{a}	0.003 ± 0.000^{c}	0.016 ± 0.002^{b}	0.016 ± 0.004^{bc}	<0.001
	Anaerofustis	-	0.018 ± 0.004^{b}	0.003 ± 0.000^{b}	0.186 ± 0.061^{a}	<0.001
	Anaerospora	-	0.027 ± 0.013	0.000 ± 0.000	0.000 ± 0.000	<0.001
	Anaerostignum	-	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	<0.001
	Anaerostipes	0.004 ± 0.001	0.002 ± 0.001	-	0.003 ± 0.000	<0.001
	Anaerotruncus	-	0.000 ± 0.000^{b}	0.000 ± 0.000^{c}	0.000 ± 0.000^{a}	<0.001
	Anaerovibrio	-	0.004 ± 0.002	0.006 ± 0.002	0.011 ± 0.005	<0.001
	Anaerovoracaceae_ge	0.407 ± 0.077	0.330 ± 0.027	0.383 ± 0.035	0.378 ± 0.058	<0.001
	Angelakisella	-	0.000 ± 0.000^{b}	0.000 ± 0.000^{a}	0.000 ± 0.000^{a}	<0.001
	Bacillus	0.085 ± 0.046^{a}	0.004 ± 0.000^{b}	-	0.002 ± 0.000^{b}	<0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Protein	***Moderate Protein***	***High Protein***	***Supra Protein***	
Firmicutes	*Blautia*	8.455 ± 0.641^{a}	5.517 ± 0.254^{b}	4.517 ± 0.301^{c}	3.074 ± 0.295^{c}	<0.001
	Butyricicoccus	0.08 ± 0.007^{bc}	0.150 ± 0.008^{a}	0.061 ± 0.004^{c}	0.11 ± 0.009^{ab}	<0.001
	Candidatus_Arthromitus	0.004 ± 0.003^{ab}	0.007 ± 0.001^{a}	0.001 ± 0.000^{b}	0.000 ± 0.000^{b}	<0.001
	Candidatus_Soleaferrea	-	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	0.003 ± 0.001^{a}	<0.001
	Candidatus_Stoquefichus	0.015 ± 0.004^{b}	0.035 ± 0.004^{b}	0.069 ± 0.008^{a}	0.030 ± 0.014^{b}	<0.001
	Caproiciproducens	-	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	<0.001
	Carnobacterium	0.000 ± 0.000^{b}	0.025 ± 0.005^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	<0.001
	Catellicoccus	-	0.001 ± 0.000	-	-	<0.001
	Catenibacterium	0.876 ± 0.211^{bc}	0.957 ± 0.081^{b}	0.456 ± 0.083^{c}	1.697 ± 1.010^{a}	<0.001
	Catenisphaera	0.010 ± 0.005^{b}	0.067 ± 0.009^{b}	0.131 ± 0.033^{a}	0.051 ± 0.018^{b}	<0.001
	Cellulosilyticum	0.031 ± 0.013^{a}	0.039 ± 0.007^{a}	0.057 ± 0.009^{a}	0.062 ± 0.021^{a}	0.002
	CHKCI001	0.021 ± 0.008^{a}	0.000 ± 0.000^{b}	0.004 ± 0.001^{b}	0.000 ± 0.000^{b}	<0.001
	Christensenellaceae_ge	0.001 ± 0.001	0.000 ± 0.000	0.001 ± 0.000	0.000 ± 0.000	0.219
	Christensenellaceae_R.7_group	0.001 ± 0.000^{b}	0.012 ± 0.001^{a}	0.003 ± 0.000^{b}	0.015 ± 0.003^{a}	<0.001
	Clostridioides	0.026 ± 0.019^{b}	0.015 ± 0.002^{b}	0.003 ± 0.000^{b}	0.117 ± 0.037^{a}	<0.001
	Clostridium_sensu_stricto_1	2.518 ± 0.430	2.196 ± 0.172	1.705 ± 0.199	0.951 ± 0.224	0.084
	Clostridium_sensu_stricto_13	0.007 ± 0.003^{b}	0.042 ± 0.009^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	<0.001
	Clostridium_sensu_stricto_18	-	0.009 ± 0.001^{b}	-	0.019 ± 0.005^{a}	<0.001
	Clostridium_sensu_stricto_2	-	0.005 ± 0.002	-	0.000 ± 0.000	<0.001
	Clostridium_sensu_stricto_7	-	0.211 ± 0.065	-	0.000 ± 0.000	<0.001
	Colidextribacter	0.005 ± 0.002^{b}	0.012 ± 0.001^{a}	0.005 ± 0.001^{b}	0.020 ± 0.003^{a}	<0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Protein	***Moderate Protein***	***High Protein***	***Supra Protein***	
Firmicutes	*Coprobacillus*	-	0.003 ± 0.000^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{ab}	<0.001
	Coprococcus	0.000 ± 0.000^{c}	0.011 ± 0.001^{b}	0.005 ± 0.001^{bc}	0.045 ± 0.012^{a}	<0.001
	Defluviitaleaceae_UCG.011	-	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	<0.001
	Dialister	0.006 ± 0.004^{a}	0.000 ± 0.000^{b}	0.002 ± 0.002^{ab}	0.005 ± 0.005^{ab}	<0.001
	Dorea	0.000 ± 0.000^{c}	0.011 ± 0.002^{b}	0.000 ± 0.000^{c}	0.065 ± 0.019^{a}	<0.001
	Dubosiella	0.247 ± 0.058^{b}	0.196 ± 0.047^{b}	0.451 ± 0.049^{a}	0.256 ± 0.078^{ab}	<0.001
	Enterococcus	1.984 ± 0.483^{a}	0.312 ± 0.145^{b}	0.014 ± 0.004^{b}	0.012 ± 0.004^{b}	<0.001
	Epulopiscium	0.081 ± 0.018	0.056 ± 0.013	0.053 ± 0.016	0.006 ± 0.002	0.304
	Erysipelatoclostridium	0.217 ± 0.036	0.157 ± 0.014	0.147 ± 0.016	0.094 ± 0.015	0.306
	Erysipelotrichaceae_ge	0.014 ± 0.011^{ab}	0.014 ± 0.004^{a}	0.000 ± 0.000^{b}	0.012 ± 0.004^{ab}	<0.001
	Erysipelotrichaceae_UCG.003	0.497 ± 0.127^{a}	0.165 ± 0.028^{b}	0.064 ± 0.011^{b}	0.041 ± 0.013^{b}	<0.001
	Eubacterium	0.028 ± 0.006	0.021 ± 0.002	0.027 ± 0.003	0.026 ± 0.007	<0.001
	Faecalibacterium	1.927 ± 0.287	2.155 ± 0.124	2.359 ± 0.171	2.159 ± 0.284	0.002
	Faecalibaculum	0.06 ± 0.013^{b}	0.054 ± 0.011^{b}	0.107 ± 0.011^{a}	0.086 ± 0.026^{ab}	<0.001
	Faecalicoccus	-	0.000 ± 0.000^{a}	-	0.000 ± 0.000^{a}	<0.001
	Faecalitalea	0.079 ± 0.019^{a}	0.033 ± 0.005^{b}	0.039 ± 0.009^{b}	0.028 ± 0.016^{b}	<0.001
	Family_XIII_AD3011_group	0.001 ± 0.000	0.004 ± 0.000	0.012 ± 0.011	0.016 ± 0.004	<0.001
	Family_XIII_UCG.001	0.000 ± 0.000^{bc}	0.000 ± 0.000^{b}	-	0.004 ± 0.001^{a}	<0.001
	Flavonifractor	0.010 ± 0.004^{a}	0.000 ± 0.000^{b}	-	0.001 ± 0.000^{b}	<0.001
	Fournierella	0.040 ± 0.01^{c}	0.114 ± 0.007^{b}	0.134 ± 0.010^{b}	0.224 ± 0.027^{a}	<0.001
	Fusibacter	-	0.004 ± 0.001	0.007 ± 0.002	0.004 ± 0.001	<0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Protein	***Moderate Protein***	***High Protein***	***Supra Protein***	
Firmicutes	*Fusicatenibacter*	0.008 ± 0.003^{a}	0.000 ± 0.000^{b}	0.001 ± 0.000^{b}	0.003 ± 0.001^{b}	<0.001
	GCA.900066575	0.016 ± 0.004^{b}	0.057 ± 0.006^{a}	0.024 ± 0.005^{b}	0.043 ± 0.012^{ab}	<0.001
	Granulicatella	-	0.000 ± 0.000	-	0.000 ± 0.000	0.011
	Hathewaya	-	0.021 ± 0.005	-	0.000 ± 0.000	<0.001
	Holdemanella	0.905 ± 0.148	0.661 ± 0.063	0.469 ± 0.076	0.389 ± 0.078	0.806
	Holdemania	-	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	<0.001
	Howardella	0.006 ± 0.000^{b}	0.012 ± 0.000^{a}	0.004 ± 0.000^{b}	0.008 ± 0.001^{ab}	<0.001
	Hungateiclostridium	-	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	<0.001
	Hungatella	0.000 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.000 ± 0.000	0.045
	Hydrogenoanaerobacterium	-	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	<0.001
	Ileibacterium	0.010 ± 0.003^{b}	0.154 ± 0.047^{b}	0.326 ± 0.078^{a}	0.250 ± 0.101^{ab}	<0.001
	Intestinibacter	0.021 ± 0.003^{c}	0.088 ± 0.009^{b}	0.030 ± 0.007^{c}	0.197 ± 0.058^{a}	<0.001
	Intestinimonas	0.001 ± 0.000^{b}	0.006 ± 0.000^{a}	0.005 ± 0.000^{a}	0.007 ± 0.001^{a}	<0.001
	Lachnoclostridium	1.467 ± 0.327^{a}	0.469 ± 0.024^{b}	0.483 ± 0.039^{b}	0.605 ± 0.071^{b}	<0.001
	Lachnospira	0.288 ± 0.095^{a}	0.060 ± 0.009^{c}	0.159 ± 0.036^{b}	0.042 ± 0.011^{c}	<0.001
	Lachnospiraceae_AC2044_group	-	0.013 ± 0.003^{a}	0.001 ± 0.000^{b}	0.011 ± 0.003^{ab}	<0.001
	Lachnospiraceae_FCS020_group	0.005 ± 0.001	0.003 ± 0.000	0.003 ± 0.000	0.004 ± 0.001	<0.001
	Lachnospiraceae_ge	5.498 ± 0.475^{a}	2.635 ± 0.12^{b}	2.475 ± 0.136^{b}	1.877 ± 0.256^{b}	<0.001
	Lachnospiraceae_NC2004_group	0.000 ± 0.000^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	<0.001
	Lachnospiraceae_ND3007_group	-	0.000 ± 0.000^{b}	0.002 ± 0.000^{b}	0.011 ± 0.005^{a}	<0.001
	Lachnospiraceae_NK3A20_group	-	0.000 ± 0.000^{b}	-	0.003 ± 0.000^{a}	<0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Protein	***Moderate Protein***	***High Protein***	***Supra Protein***	
Firmicutes	*Lachnospiraceae_NK4A136_group*	0.189 ± 0.035^{bc}	0.239 ± 0.015^{b}	0.177 ± 0.016^{c}	0.36 ± 0.055^{a}	<0.001
	Lachnospiraceae_UCG.003	0.001 ± 0.000^{b}	0.008 ± 0.002^{a}	0.001 ± 0.000^{b}	0.000 ± 0.000^{b}	0.006
	Lachnospiraceae_UCG.004	0.000 ± 0.000^{c}	0.003 ± 0.000^{b}	0.001 ± 0.000^{c}	0.009 ± 0.002^{a}	<0.001
	Lachnospiraceae_UCG.010	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	<0.001
	Lachnospiraceae_XPB1014_group	-	0.000 ± 0.000^{b}	0.000 ± 0.000^{bc}	0.003 ± 0.000^{a}	<0.001
	Lactobacillus	1.604 ± 0.517^{b}	3.465 ± 0.372^{a}	4.265 ± 0.449^{a}	2.695 ± 0.721^{ab}	<0.001
	Lactococcus	0.021 ± 0.014^{ab}	0.027 ± 0.006^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{ab}	<0.001
	Leuconostoc	-	0.004 ± 0.000^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	<0.001
	Marvinbryantia	0.598 ± 0.175^{a}	0.090 ± 0.010^{b}	0.085 ± 0.011^{b}	0.016 ± 0.002^{b}	<0.001
	Megamonas	3.108 ± 0.517^{b}	4.518 ± 0.270^{a}	0.934 ± 0.117^{c}	2.266 ± 0.346^{bc}	<0.001
	Megasphaera	0.146 ± 0.075	0.043 ± 0.016	0.055 ± 0.032	0.205 ± 0.188	<0.001
	Mitsuokella	-	0.000 ± 0.000	0.002 ± 0.002^{a}	0.002 ± 0.002	<0.001
	Mogibacterium	-	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	0.001 ± 0.000^{a}	<0.001
	Monoglobus	0.000 ± 0.000^{b}	0.002 ± 0.000^{a}	0.000 ± 0.000^{b}	0.001 ± 0.000^{ab}	<0.001
	Negativibacillus	0.152 ± 0.035	0.172 ± 0.014	0.163 ± 0.019	0.182 ± 0.028	<0.001
	NK4A214_group	0.000 ± 0.000^{c}	0.004 ± 0.000^{b}	0.006 ± 0.001^{b}	0.013 ± 0.004^{a}	<0.001
	Oribacterium	0.017 ± 0.003^{b}	0.042 ± 0.003^{a}	0.034 ± 0.003^{a}	0.051 ± 0.009^{a}	<0.001
	Oscillibacter	0.002 ± 0.000^{c}	0.013 ± 0.001^{b}	0.005 ± 0.000^{c}	0.028 ± 0.005^{a}	<0.001
	Oscillospira	0.003 ± 0.000^{c}	0.017 ± 0.002^{a}	0.005 ± 0.001^{bc}	0.017 ± 0.006^{ab}	<0.001
	Oscillospiraceae_ge	-	0.000 ± 0.000	-	0.000 ± 0.000	<0.001
	Paeniclostridium	-	0.001 ± 0.000	0.001 ± 0.000	0.004 ± 0.001	<0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Protein	***Moderate Protein***	***High Protein***	***Supra Protein***	
Firmicutes	*Paludicola*	0.000 ± 0.000^{bc}	0.001 ± 0.000^{b}	0.000 ± 0.000^{c}	0.005 ± 0.001^{a}	<0.001
	Papillibacter	-	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	0.002
	Paraclostridium	0.061 ± 0.019^{b}	0.283 ± 0.064^{a}	0.162 ± 0.033^{ab}	0.048 ± 0.013^{b}	<0.001
	Peptoclostridium	11.445 ± 0.825^{a}	8.417 ± 0.353^{b}	9.879 ± 0.572^{a}	8.932 ± 1.200^{ab}	0.027
	Peptococcus	0.229 ± 0.036^{c}	0.836 ± 0.058^{a}	0.467 ± 0.037^{b}	0.274 ± 0.029^{bc}	<0.001
	Peptoniphilus	-	0.636 ± 0.130^{a}	-	0.001 ± 0.000^{b}	<0.001
	Peptostreptococcaceae_ge	0.008 ± 0.003^{ab}	0.016 ± 0.002^{a}	0.001 ± 0.000^{b}	0.005 ± 0.001^{ab}	<0.001
	Peptostreptococcus	0.064 ± 0.044^{b}	1.072 ± 0.150^{a}	0.198 ± 0.045^{b}	0.346 ± 0.094^{b}	<0.001
	Phascolarctobacterium	0.464 ± 0.058^{c}	1.821 ± 0.100^{a}	1.064 ± 0.079^{b}	1.503 ± 0.118^{ab}	<0.001
	Phocea	0.003 ± 0.001	0.005 ± 0.000	0.005 ± 0.000	0.006 ± 0.001	<0.001
	Pseudoflavonifractor	-	0.000 ± 0.000	-	-	0.414
	Pygmaiobacter	0.077 ± 0.013^{b}	0.137 ± 0.012^{a}	0.045 ± 0.007^{b}	0.027 ± 0.005^{b}	<0.001
	Robinsoniella	0.068 ± 0.016^{a}	0.001 ± 0.000^{c}	0.027 ± 0.008^{b}	0.008 ± 0.002^{bc}	<0.001
	Romboutsia	0.776 ± 0.231	0.628 ± 0.063	0.822 ± 0.079	0.701 ± 0.212	<0.001
	Roseburia	0.043 ± 0.012	0.026 ± 0.003	0.037 ± 0.010	0.053 ± 0.012	<0.001
	Ruminococcus	0.002 ± 0.001^{ab}	0.002 ± 0.000^{b}	0.009 ± 0.004^{a}	0.016 ± 0.007^{a}	<0.001
	Sarcina	0.490 ± 0.122^{a}	0.283 ± 0.109^{a}	0.000 ± 0.000^{b}	0.001 ± 0.000^{ab}	<0.001
	Sellimonas	0.000 ± 0.000^{c}	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	<0.001
	Sharpea	-	0.002 ± 0.000^{c}	0.050 ± 0.006^{a}	0.020 ± 0.005^{b}	<0.001
	Shuttleworthia	-	0.011 ± 0.002^{b}	0.000 ± 0.000^{c}	0.070 ± 0.019^{a}	<0.001
	Solobacterium	-	0.002 ± 0.001^{ab}	0.001 ± 0.001^{b}	0.011 ± 0.008^{a}	<0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Protein	***Moderate Protein***	***High Protein***	***Supra Protein***	
	Sporosarcina	-	0.001 ± 0.000^{a}	-	0.000 ± 0.000^{b}	<0.001
	Staphylococcus	0.01 ± 0.005^{a}	0.001 ± 0.000^{b}	0.007 ± 0.004^{ab}	0.004 ± 0.003^{ab}	<0.001
	Streptococcus	6.147 ± 1.019^{a}	2.183 ± 0.277^{b}	0.607 ± 0.186^{c}	1.558 ± 0.933^{bc}	<0.001
	Subdoligranulum	0.059 ± 0.045^{a}	0.005 ± 0.000^{b}	0.002 ± 0.001^{b}	0.027 ± 0.007^{ab}	<0.001
	Terrisporobacter	0.94 ± 0.198^{a}	0.122 ± 0.013^{b}	0.212 ± 0.021^{b}	0.172 ± 0.038^{b}	<0.001
	Turicibacter	1.201 ± 0.212^{b}	2.007 ± 0.163^{a}	1.008 ± 0.134^{b}	0.603 ± 0.093^{b}	<0.001
	Tuzzerella	0.000 ± 0.000^{c}	0.003 ± 0.000^{b}	0.004 ± 0.001^{b}	0.011 ± 0.005^{a}	<0.001
	Tyzzerella	0.315 ± 0.082^{a}	0.113 ± 0.008^{b}	0.022 ± 0.004^{c}	0.009 ± 0.002^{c}	<0.001
	Vagococcus	-	0.007 ± 0.001^{a}	-	0.000 ± 0.000^{b}	<0.001
	Weissella	0.019 ± 0.008^{a}	0.007 ± 0.003^{ab}	0.001 ± 0.001^{b}	0.000 ± 0.000^{b}	<0.001
Fusobacteria	*Cetobacterium*	0.175 ± 0.117^{b}	0.316 ± 0.068^{b}	0.565 ± 0.106^{a}	0.368 ± 0.148^{ab}	<0.001
	Fusobacterium	9.305 ± 0.747^{c}	16.344 ± 0.511^{b}	20.012 ± 0.737^{a}	14.942 ± 0.852^{b}	<0.001
	Oceanivirga	-	0.000 ± 0.000	0.031 ± 0.03	0.000 ± 0.000	<0.001
	Streptobacillus	-	0.000 ± 0.000	0.096 ± 0.095	0.000 ± 0.000	<0.001
Lentisphaerae	*Victivallis*	0.000 ± 0.000^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{ab}	-	0.059
Proteobacteria	*Acinetobacter*	-	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	<0.001
	Actinobacillus	-	0.000 ± 0.000	0.060 ± 0.058	0.001 ± 0.000	<0.001
	Aeromonas	-	0.000 ± 0.000^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{ab}	<0.001
	Aestuariibacter	0.052 ± 0.009^{a}	0.005 ± 0.000^{b}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	<0.001
	Anaerobiospirillum	0.202 ± 0.042^{c}	1.372 ± 0.083^{a}	0.96 ± 0.078^{b}	1.295 ± 0.205^{ab}	<0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Protein	***Moderate Protein***	***High Protein***	***Supra Protein***	
Proteobacteria	*Bilophila*	-	0.000 ± 0.000	-	0.000 ± 0.000	0.155
	Bosea	-	-	0.018 ± 0.013	0.000 ± 0.000	<0.001
	Campylobacter	0.056 ± 0.021^{a}	0.005 ± 0.001^{b}	0.004 ± 0.002^{b}	0.020 ± 0.016^{b}	<0.001
	Citrobacter	-	0.005 ± 0.001	-	0.006 ± 0.002	<0.001
	Cupriavidus	-	0.009 ± 0.001^{a}	0.001 ± 0.001^{b}	0.003 ± 0.001^{ab}	<0.001
	Desulfovibrio	0.000 ± 0.000	0.024 ± 0.010	-	0.000 ± 0.000	<0.001
	Enterobacter	0.499 ± 0.148^{a}	0.009 ± 0.004^{b}	0.003 ± 0.002^{b}	0.007 ± 0.003^{b}	<0.001
	Escherichia.Shigella	5.104 ± 0.835^{a}	1.344 ± 0.157^{b}	0.297 ± 0.086^{c}	0.218 ± 0.063^{c}	<0.001
	Hafnia.Obesumbacterium	-	0.002 ± 0.000	-	-	<0.001
	Helicobacter	0.37 ± 0.116^{a}	0.073 ± 0.011^{b}	0.061 ± 0.011^{b}	0.067 ± 0.036^{b}	<0.001
	Histophilus	-	-	0.070 ± 0.069^{a}	0.001 ± 0.000^{a}	<0.001
	Kosakonia	-	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.113
	Mailhella	-	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	<0.001
	Parasutterella	0.85 ± 0.172^{b}	1.072 ± 0.091^{b}	1.854 ± 0.168^{a}	2.076 ± 0.379^{a}	<0.001
	Plesiomonas	0.024 ± 0.019	0.058 ± 0.032	0.000 ± 0.000	0.000 ± 0.000	<0.001
	Pseudomonas	0.002 ± 0.000	0.017 ± 0.006	0.003 ± 0.002	0.001 ± 0.000	<0.001
	Sphingobium	-	0.000 ± 0.000^{b}	0.374 ± 0.25^{a}	0.063 ± 0.049^{ab}	<0.001
	Succinivibrio	0.099 ± 0.056^{ab}	0.122 ± 0.023^{a}	0.034 ± 0.01^{ab}	0.130 ± 0.037^{a}	<0.001
	Succinivibrionaceae_UCG.001	-	0.012 ± 0.010	0.046 ± 0.02	0.001 ± 0.000	<0.001
	Sutterella	0.733 ± 0.087^{b}	1.477 ± 0.068^{a}	0.857 ± 0.061^{b}	1.434 ± 0.140^{a}	<0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Protein	***Moderate Protein***	***High Protein***	***Supra Protein***	
Spirochaetes	*Leptospira*	-	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	<0.001
Tenericutes	*Anaeroplasma*	0.005 ± 0.001^{b}	0.035 ± 0.004^{a}	0.013 ± 0.001^{b}	0.042 ± 0.009^{a}	<0.001
	Mycoplasma	-	0.000 ± 0.000	0.013 ± 0.013	0.000 ± 0.000	<0.001
Undefined[¥]	*Chloroplast_ge*	-	0.000 ± 0.000^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{ab}	<0.001
	Clostridia_UCG.014_ge	0.115 ± 0.034	0.085 ± 0.015	0.053 ± 0.033	0.023 ± 0.008	<0.001
	Dojkabacteria_ge	-	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	<0.001
	DTU089	-	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	<0.001
	Erysipelatoclostridiaceae_ge	-	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	<0.001
	Gastranaerophilales_ge	-	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	0.000 ± 0.000^{a}	<0.001
	Incertae_Sedis	0.013 ± 0.002^{a}	0.005 ± 0.000^{b}	0.002 ± 0.000^{c}	0.003 ± 0.000^{bc}	<0.001
	Mitochondria_ge	0.035 ± 0.033^{a}	0.001 ± 0.000^{b}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	<0.001
	Oscillospirales_ge	0.000 ± 0.000	0.005 ± 0.001	0.002 ± 0.001	0.007 ± 0.004	<0.001
	RF39_ge	-	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	0.002 ± 0.002^{a}	<0.001
	S5.A14a	-	0.021 ± 0.003	0.028 ± 0.003	0.019 ± 0.009	<0.001
	T34_ge	-	0.001 ± 0.000	-	0.001 ± 0.000	<0.001
	UBA1819	0.002 ± 0.001^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	0.002 ± 0.000^{a}	<0.001
	UCG.003	-	0.000 ± 0.000	-	0.000 ± 0.000	<0.001
	UCG.004	0.014 ± 0.005^{a}	0.004 ± 0.000^{b}	0.008 ± 0.002^{ab}	0.012 ± 0.002^{ab}	<0.001
	UCG.005	0.061 ± 0.012^{b}	0.191 ± 0.011^{a}	0.169 ± 0.010^{a}	0.166 ± 0.020^{a}	<0.001
	UCG.008	-	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	0.002 ± 0.001^{a}	<0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Protein	***Moderate Protein***	***High Protein***	***Supra Protein***	
Undefined	*UCG.009*	0.001 ± 0.000^{a}	0.000 ± 0.000^{bc}	0.000 ± 0.000^{c}	0.001 ± 0.000^{ab}	<0.001
	uncultured	5.857 ± 0.685^{b}	2.918 ± 0.099^{c}	7.782 ± 0.482^{a}	4.007 ± 0.360^{c}	<0.001
	uncultured_ge	0.000 ± 0.000^{c}	0.010 ± 0.001^{b}	0.000 ± 0.000^{c}	0.027 ± 0.006^{a}	<0.001
	X44314	0.000 ± 0.000^{bc}	0.000 ± 0.000^{c}	0.001 ± 0.000^{ab}	0.003 ± 0.001^{a}	<0.001
	ZOR0006	-	0.004 ± 0.000^{b}	0.000 ± 0.000^{c}	0.016 ± 0.008^{a}	<0.001

* Results marked by a '-' denote that this genera was not detected.
§Results denoted as '0.000 ± 0.000' were detected at abundances that round out to 0 at three decimal places but were still detected.
¥Undefined Phyla are bacterial genera that have not yet been assigned a taxonomic phyla that they belong to.

3.4.2.4 Separation between community profiles was seen based on dietary protein classification

A principal coordinate analysis of Bray-Curtis dissimilarity was performed on the faecal microbiota results, based on protein classifications, seen in Figure 3.14. These showed that there was some separation seen in the community profiles based on dietary protein classification.

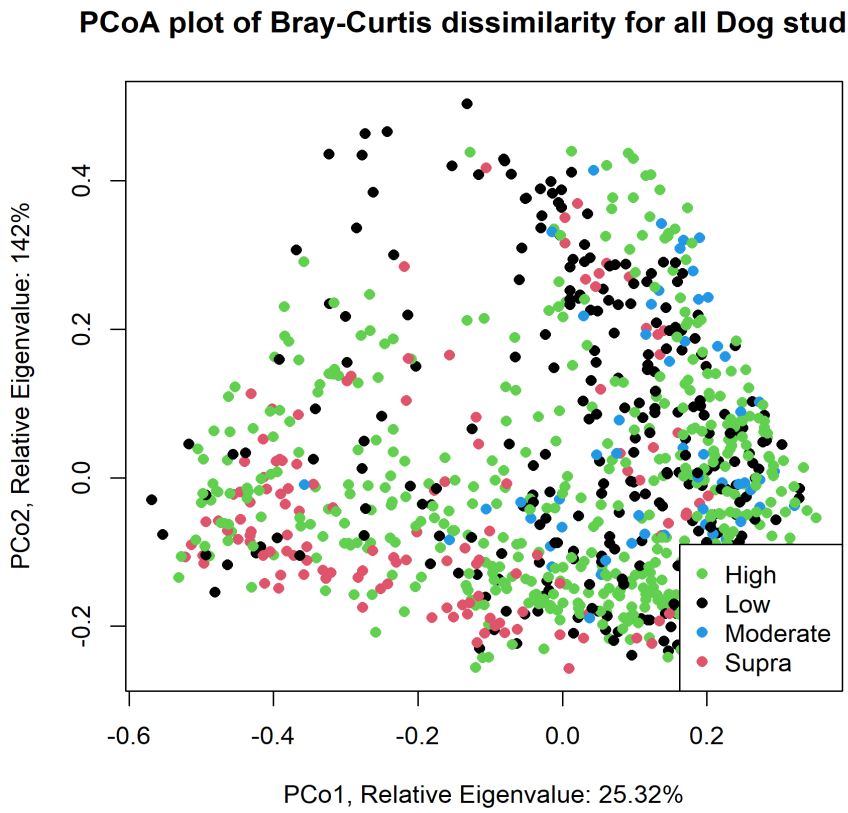


Figure 3.14 *-Principal coordinate analysis of Bray-Curtis dissimilarity for all dog protein classifications, n = 314.Each protein classification is presented with a different colour.*

A partial least squares-discriminant analysis (PLS-DA) was then performed on the dataset, Figure 3.15. These show that there was separation observed based on dietary protein classification (Figure 3.15A). The top PLS-DA loadings from these data (Figure 3.15B) predicated that *Allobaculum, Adlercreutzia, Faecalibaculum* and *Duosiella* would be positively impacted by changes in CP content, i.e., their relative abundances would increase relative to increased CP content, whilst

Genera such as *Peptostreptococcus* and *Colidextribacter* would be negatively impacted by changes in CP content.

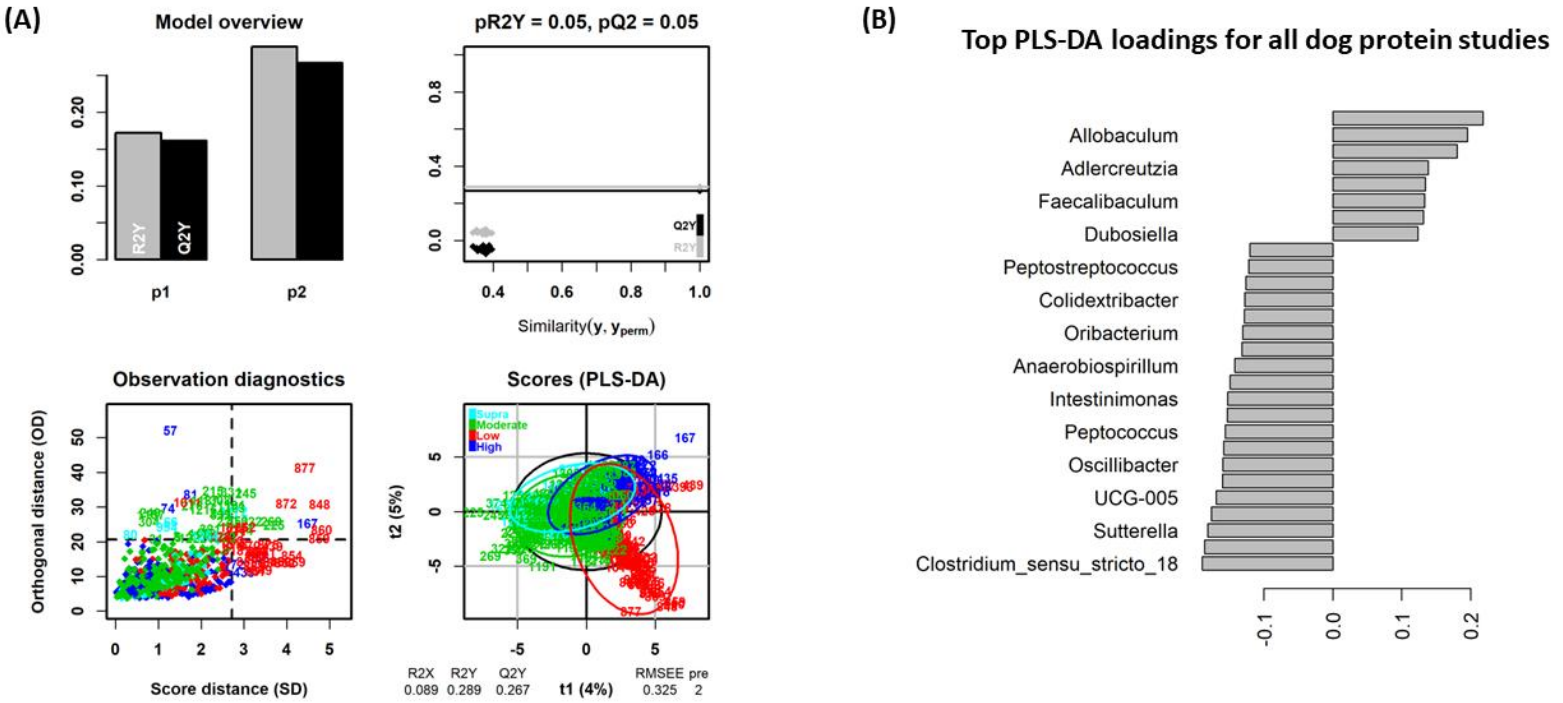


Figure 3.15 *- Partial least squares-discriminant analysis (PLS-DA) for dog studies by protein classification.(A) details the descriptive modelling of the PLS-DA, whilst (B) details the Genera predicted to be most impacted by dietary protein classification.*

The data was then assessed using random forest analysis to understand what bacterial genera were potentially driving separation between the protein classifcations. As shown in Figure 3.16, *Sharpea* was observed to drive the separation of community profiles for the dietary protein classification, followed by *Prevotellaceae_Ga6A1_group,Enterococcus,* and *Enterobacter*. All four of these genera were significantly affected by protein classification ($p < 0.001$ in all instances; Table 3.10).

Sharpea was not detected in the Low Protein classification. However, in all other protein classifications it had a low relative abundance (0.002% ± 0.000%, 0.050% ± 0.006% and 0.020% ± 0.005% of sequence reads for the Moderate, High and Supra Protein classifications, respectively). *Prevotellaceae*_Ga6A1_group had the largest overall relative abundances of these four genera, with relative abundances of 0.575% ± 0.104%, 2.447% ± 0.159%, 1.684% ± 0.196% and 2.298% ± 0.568% of sequence reads in the Low, Moderate, High and Supra Protein classifications, respectively. *Enterococcus* had the highest relative abundance in the Low Protein classification; 1.984% ± 0.483% of sequence reads, and decreased through the Moderate, High and Supra Protein classifications, going from 0.312% ± 0.145% of sequence reads in the Moderate Protein classification to 0.014% ± 0.004% of sequence reads in the High Protein classification, and finally 0.012% ± 0.004% of sequence reads in the Supra Protein. Meanwhile, *Enterobacter* had a relative abundance of < 0.01% of sequence reads in the Moderate, High and Supra Protein classifications, although in the Low Protein classification its relative abundance was 0.499% ± 0.148% of sequence reads.

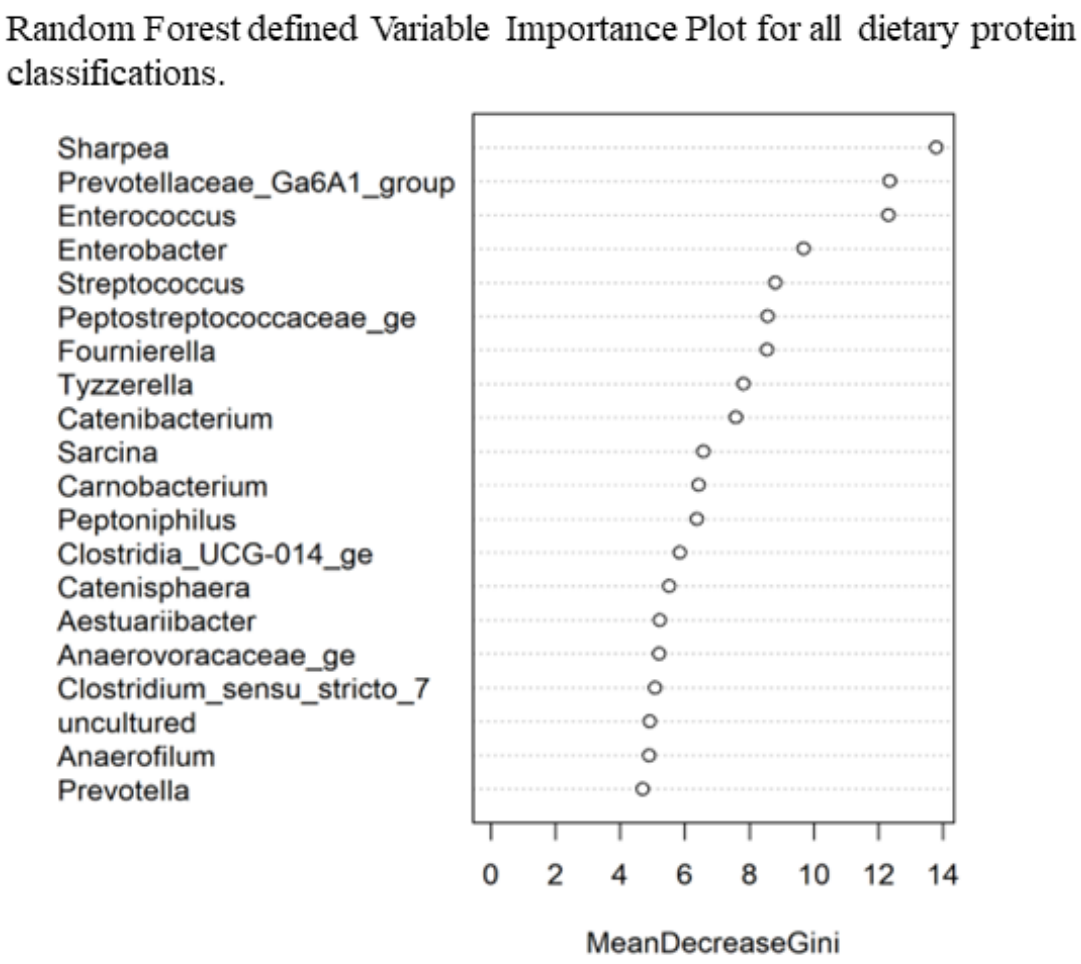


Figure 3.16 – *Bacterial genera in the faecal microbiota of the domestic dog associated with driving separations in diversity and richness of the faecal microbiome in relation to dietary crude protein content.Determined by random forest analysis, with a total number of dogs (n) of 314.*

3.4.3 IMPACTS OF DIETARY FAT ON THE FAECAL MICROBIOME

3.4.3.1 Diet classification

Of the diets investigated, 16 were classed as low fat, 13 were moderate fat, 5 were high fat and 5 were supra fat diets (Figure 3.17). There was a significant overall difference in DF content between fat classifications ($F(3,35) = 119.41$, $p < 0.001$). Fat content was different between all fat classifications (i.e., the DF content in the Low Fat classification was significantly different to the Moderate, High and Supra Fat classifications, etc., $p < 0.01$, represented in Figure 3.17 by different letters), bar Moderate Fat and High Fat. There was no significant difference in the DF content between these two classifications ($p > 0.05$).

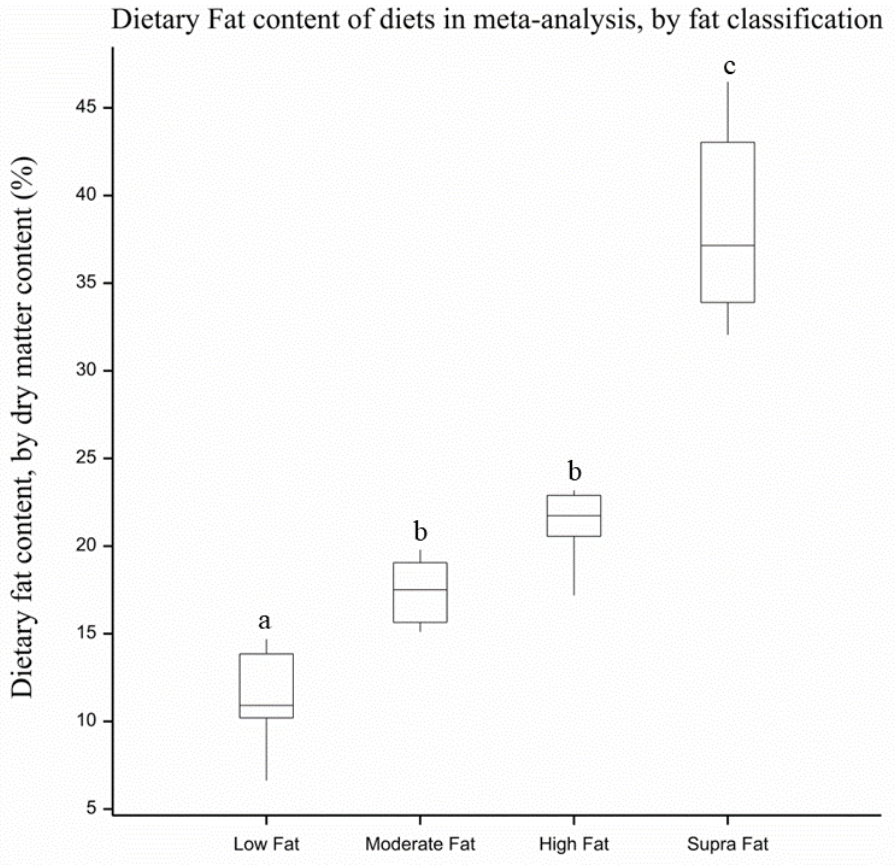


Figure 3.17– *Dietary fat content of diets by fat classification.Different letters above the boxplots denote a significant difference in dietary fat content between classifications (p < 0.01). Boxes represent the interquartile range between the first and third quartiles. Thick black line inside the box denotes the median.*

3.4.3.2 Impacts of Dietary Fat on the Diversity of the Faecal Microbiota

The Shannon index of the dog faecal microbiota was assessed (Figure 3.18), wherein there was a significant impact on bacterial diversity caused by dietary DF content ($p < 0.001$). There was a significant difference between all fat classifications ($p< 0.001$). The Low Fat classification had the highest diversity ($p < 0.001$ in comparison to all other classifications), and were the least diverse in the High Fat classification ($p< 0.001$ in comparison to all other classifications). The Low Fat classification had the largest spread of bacterial diversity across all classifications i.e., it had the furthest outliers.

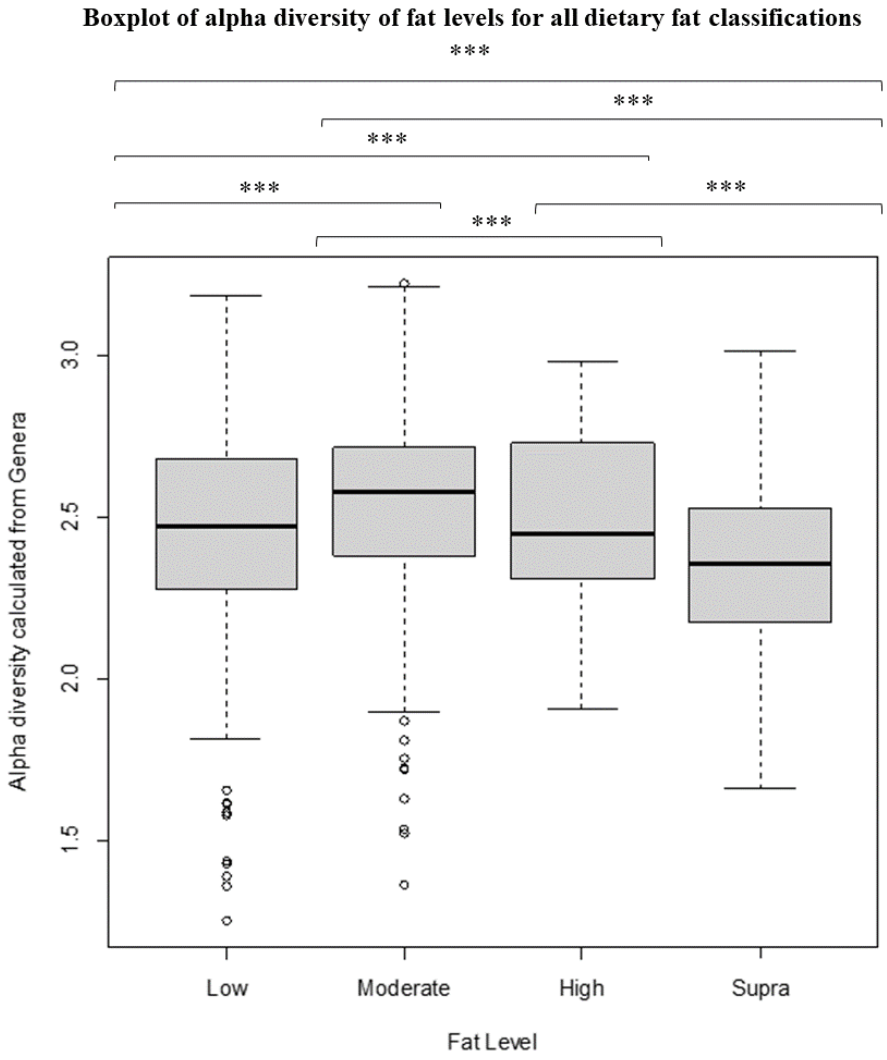


Figure 3.18 – *Shannon index (alpha diversit)y of bacterial genera of the canine faecal microbiota, grouped by fat classification, n = 314.Statistical differences between protein classifications are denoted by an asterisk (*), where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Circles denote outliers. Boxes represent the interquartile range between the first and third quartiles. Thick black line inside the box denotes the median.*

The total richness of the canine faecal microbiota was assessed by Chao1 index (Figure 3.19), wherein Kruksal-Wallis chi-squared showed there was a significant impact on bacterial richness caused by dietary CP content ($p < 0.001$). The bacterial richness was highest in the Low Fat classification, ($p< 0.001$ in all instances). The bacterial richness was lowest in the Supra Fat classification ($p< 0.001$ in all instances). The Moderate Fat classification had the largest spread of richness across all classifications i.e., it had the furthest outliers.

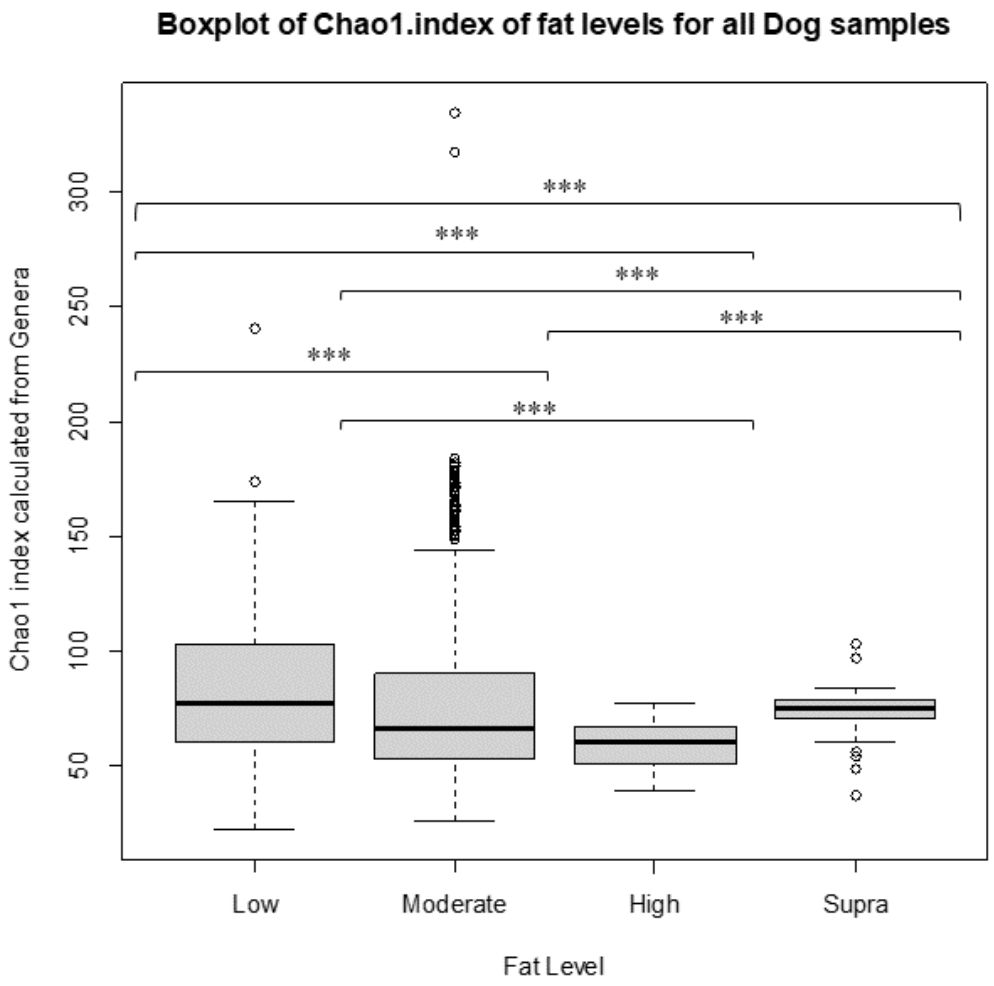


Figure 3.19 - *Chao 1 index of bacterial genera of the canine faecal microbiota, grouped by protein classification, n = 314.Statistical differences between fat classifications are denoted by an asterisk (*), where * p < 0.05, ** p < 0.01 and *** p < 0.001. Circles denote outliers. Boxes represent the interquartile range between the first and third quartiles. Thick black line inside the box denotes the median.*

3.4.3.3 Impacts of Dietary Fat on Bacterial Genera in the faecal microbiota

The impacts of DF on the faecal microbiota were assessed, and are presented in Table 3.11. The mean abundances of each bacterial genera for each dietary fat classification. These are listed

alphabetically, by bacterial Phyla. The Diet Effect p value denotes if there was a significant difference in bacterial genera between dietary protein classifications ($p< 0.05$).

Fusobacterium, *Bacteroides*, *Peptoclostridium*, *Blautia* and *Megamonas* were the dominant bacterial Genera in the Low Fat classification (relative abundances of 16.284% ± 0.548%, 11.276% ± 0.457%, 8.231% ± 0.358%, 5.540% ± 0.250% and 4.936% ± 0.314% of sequence reads, respectively). The relative abundances of *Fusobacterium, Bacteroides, Peptoclostridium, Blautia* and *Megamonas* were significantly affected by DF content ($p< 0.001$ in all instances). *Fusobacterium* (14.669% ± 0.539% of sequence reads), *Bacteroides* (11.513% ± 0.487% of sequence reads), *Prevotella* (11.200% ± 0.638% of sequence reads), *Peptoclostridium* (9.389% ± 0.485% of sequence reads)and *Alloprevotella* (5.193% ± 0.230% of sequence reads) were the most abundant bacterial Genera in the Moderate Fat classification and were all significantly affected by DF content ($p <0.001$for all).

For the High Fat classification, the dominant bacterial genera in terms of relative abundance were *Peptoclostridium* (15.761% ± 1.328% of sequence reads), *Blautia* (14.618% ± 1.249% of sequence reads), *Fusobacterium* (9.166% ± 1.124% of sequence reads), *Bacteroides* (7.013% ± 1.141% of sequence reads) and *Lactobacillus* (5.317% ± 1.142% of sequence reads). All were significantly affected by dietary DF content ($p< 0.001$ in all instances). Finally, for the Supra Fat classification, the dominant bacterial genera were *Fusobacterium* (22.765% ± 1.100% of sequence reads), *Bacteroides* (10.017% ± 0.644% of sequence reads), *Peptoclostridium* (9.407% ± 0.828% of sequence reads), *uncultured* (9.081% ± 0.643% of sequence reads) and *Allobacullum* (6.688% ± 0.692% of sequence reads), which were all significantly affected by dietary DF content ($p< 0.001$ in all instances).

Table 3.11 – *Mean relative abundances of bacterial genera, expressed as % of sequence reads for each Dietary Fat classification.Dietary data are from 314 dogs across 16 publications. Results are presented to three decimal places as means with their corresponding SEM, and with p values as determined by the Kruskal-wallis one-way ANOVA and corrected for false discovery rate. Dietary groupings are assigned by Fisher's least significant difference post-hoc analysis, where different letters following the relative abundances denote significant differences (p <0.05). Bacterial genera are listed alphabetically, by Phyla.*

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Fat	***Moderate Fat***	***High Fat***	***Supra Fat***	
Actinobacteria	*Actinomyces*	-*	0.000 ± 0.000[§]	0.004 ± 0.001	-	< 0.001
	Adlercreutzia	-	0.000 ± 0.000	-	-	< 0.001
	Atopobiaceae_ge	0.000 ± 0.000^{b}	0.002 ± 0.000^{ab}	-	0.012 ± 0.010^{a}	< 0.001
	Atopobium	-	0.000 ± 0.000	-	-	< 0.001
	Bifidobacterium	-	0.000 ± 0.000^{b}	-	0.107 ± 0.104^{a}	< 0.001
	Collinsella	-	0.000 ± 0.000^{b}	0.004 ± 0.001^{a}	-	< 0.001
	Coriobacteriaceae_UCG.002	0.021 ± 0.001^{b}	0.027 ± 0.003^{b}	0.019 ± 0.004^{b}	0.042 ± 0.004^{a}	< 0.001
	Coriobacterium	-	0.001 ± 0.000	-	-	< 0.001
	Corynebacterium	0.022 ± 0.003^{a}	0.000 ± 0.000^{b}	-	-	0.001
	Cutibacterium	-	0.001 ± 0.000	-	-	< 0.001
	Denitrobacterium	0.002 ± 0.000	0.003 ± 0.000	-	0.003 ± 0.000	< 0.001
	Enorma	0.015 ± 0.002^{ab}	0.021 ± 0.002^{a}	0.002 ± 0.001^{c}	0.008 ± 0.002^{bc}	< 0.001
	Libanicoccus	1.576 ± 0.205^{c}	2.748 ± 0.252^{b}	1.876 ± 0.182^{bc}	6.687 ± 0.692^{a}	< 0.001
	Olsenella	3.410 ± 0.199^{b}	5.192 ± 0.230^{a}	1.816 ± 0.286^{c}	3.517 ± 0.498^{b}	< 0.001
	Parvibacter	-	0.000 ± 0.000^{b}	-	0.036 ± 0.036^{a}	< 0.001
	Pseudarthrobacter	1.126 ± 0.084^{a}	1.200 ± 0.094^{a}	0.673 ± 0.133^{b}	0.916 ± 0.095^{ab}	< 0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Fat	***Moderate Fat***	***High Fat***	***Supra Fat***	
Actinobacteria	*Senegalimassilia*	0.025 ± 0.006^{a}	0.011 ± 0.001^{b}	0.006 ± 0.001^{b}	0.002 ± 0.000^{b}	< 0.001
	Slackia	-	0.063 ± 0.013^{a}	-	0.005 ± 0.000^{b}	< 0.001
	Tetrasphaera	0.023 ± 0.003^{b}	0.041 ± 0.005^{a}	0.008 ± 0.004^{bc}	0.005 ± 0.000^{c}	< 0.001
	Trueperella	-	0.043 ± 0.020	-	-	< 0.001
Bacteroidetes	*Alistipes*	0.000 ± 0.000^{b}	0.000 ± 0.000^{a}	-	-	< 0.001
	Alloprevotella	0.001 ± 0.000^{b}	0.004 ± 0.001^{a}	-	-	< 0.001
	Bacteroides	-	0.000 ± 0.000^{b}	0.001 ± 0^{a}	-	< 0.001
	Barnesiella	0.001 ± 0.000^{b}	0.010 ± 0.003^{a}	0.004 ± 0.004^{ab}	0.005 ± 0.003^{ab}	< 0.001
	Butyricimonas	0.391 ± 0.034^{b}	0.264 ± 0.023^{c}	0.648 ± 0.130^{a}	0.342 ± 0.040^{bc}	< 0.001
	Muribaculaceae_ge	-	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	< 0.001
	Myroides	-	0.000 ± 0.000	-	-	0.017
	Odoribacter	0.016 ± 0.007	0.000 ± 0.000	-	-	< 0.001
	Parabacteroides	0.029 ± 0.014	0.002 ± 0.000	-	-	< 0.001
	Paraprevotella	11.276 ± 0.457^{a}	11.512 ± 0.486^{a}	7.013 ± 1.140^{b}	10.016 ± 0.643^{a}	< 0.001
	Porphyromonas	-	0.004 ± 0.000	-	-	< 0.001
	Prevotella	1.105 ± 0.170^{a}	0.207 ± 0.028^{b}	0.051 ± 0.035^{b}	0.617 ± 0.101^{b}	< 0.001
	Prevotellaceae_Ga6A1_group	-	0.000 ± 0.000	-	-	< 0.001
	Prevotellaceae_NK3B31_group	5.539 ± 0.249^{b}	4.488 ± 0.246^{c}	14.618 ± 1.249^{a}	3.652 ± 0.293^{c}	< 0.001
	Prevotellaceae_UCG.001	-	0.000 ± 0.000^{b}	-	0.032 ± 0.024^{a}	< 0.001
	Prevotellaceae_UCG.003	0.147 ± 0.008^{a}	0.096 ± 0.005^{b}	0.129 ± 0.035^{ab}	0.038 ± 0.003^{c}	< 0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Fat	***Moderate Fat***	***High Fat***	***Supra Fat***	
	Rikenellaceae_RC9_gut_group	-	0.000 ± 0.000	-	-	< 0.001
Deferribacteres	*Mucispirillum*	0.020 ± 0.006	0.007 ± 0.002	-	0.008 ± 0.006	< 0.001
Euryarchaeota	*Methanobrevibacter*	0.004 ± 0.001^{ab}	0.007 ± 0.002^{a}	-	0.000 ± 0.000^{b}	< 0.001
	Methanosphaera	-	0.001 ± 0.000	-	0.000 ± 0.000	< 0.001
Firmicutes	*Acetanaerobacterium*	0.015 ± 0.001^{d}	0.042 ± 0.006^{c}	0.148 ± 0.026^{a}	0.071 ± 0.009^{b}	< 0.001
	Acidaminococcus	-	0.000 ± 0.000	-	-	< 0.001
	Agathobacter	0.014 ± 0.003^{b}	0.002 ± 0.001^{b}	0.086 ± 0.033^{a}	-	< 0.001
	Allisonella	-	0.003 ± 0.001	-	-	< 0.001
	Allobaculum	0.702 ± 0.086^{b}	0.956 ± 0.096^{b}	2.095 ± 0.37^{a}	0.502 ± 0.361^{b}	< 0.001
	Amnipila	0.021 ± 0.002^{c}	0.056 ± 0.009^{bc}	0.482 ± 0.144^{a}	0.109 ± 0.019^{b}	< 0.001
	Anaerofilum	0.030 ± 0.007^{b}	0.059 ± 0.009^{a}	0.010 ± 0.006^{b}	0.070 ± 0.013^{a}	< 0.001
	Anaerofustis	0.304 ± 0.079^{b}	0.265 ± 0.063^{b}	-	0.916 ± 0.172^{a}	< 0.001
	Anaerospora	0.008 ± 0.002^{a}	0.002 ± 0.000^{b}	-	-	< 0.001
	Anaerostignum	-	0.001 ± 0.000^{a}	-	0.000 ± 0.000^{b}	< 0.001
	Anaerostipes	0.001 ± 0.000	0.000 ± 0.000	-	-	< 0.001
	Anaerotruncus	0.010 ± 0.001^{a}	0.009 ± 0.001^{a}	-	0.003 ± 0^{b}	< 0.001
	Anaerovibrio	0.005 ± 0.001^{a}	0.002 ± 0.000^{b}	-	-	< 0.001
	Anaerovoracaceae_ge	0.114 ± 0.019^{a}	0.039 ± 0.006^{b}	0.005 ± 0.001^{b}	0.074 ± 0.059^{ab}	< 0.001
	Angelakisella	0.009 ± 0.005^{b}	0.042 ± 0.008^{a}	0.007 ± 0.003^{b}	0.004 ± 0.000^{b}	< 0.001
	Bacillus	2.251 ± 0.194^{b}	1.251 ± 0.176^{c}	4.383 ± 0.701^{a}	1.979 ± 0.231^{b}	< 0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Fat	***Moderate Fat***	***High Fat***	***Supra Fat***	
Firmicutes	*Blautia*	0.051 ± 0.010^{a}	0.000 ± 0.000^{b}	-	-	< 0.001
	Butyricicoccus	0.007 ± 0.001	0.008 ± 0.001	-	-	< 0.001
	Candidatus_Arthromitus	0.006 ± 0.002^{a}	0.000 ± 0.000^{b}	-	-	< 0.001
	Candidatus_Soleaferrea	0.241 ± 0.075^{a}	0.000 ± 0.000^{b}	-	-	< 0.001
	Candidatus_Stoquefichus	0.012 ± 0.001^{a}	0.013 ± 0.001^{a}	0.002 ± 0.000^{b}	0.001 ± 0.000^{b}	< 0.001
	Caproiciproducens	1.716 ± 0.119^{b}	0.788 ± 0.076^{c}	2.871 ± 0.315^{a}	0.67 ± 0.071^{c}	< 0.001
	Carnobacterium	-	0.005 ± 0.001	-	-	< 0.001
	Catellicoccus	0.001 ± 0.000^{b}	0.024 ± 0.003^{a}	0.022 ± 0.002^{a}	0.000 ± 0.000^{b}	< 0.001
	Catenibacterium	0.033 ± 0.006^{b}	0.029 ± 0.005^{b}	-	0.069 ± 0.015^{a}	< 0.001
	Catenisphaera	-	0.000 ± 0.000	-	-	< 0.001
	Cellulosilyticum	0.002 ± 0.001^{b}	0.000 ± 0.000^{b}	-	0.034 ± 0.032^{a}	< 0.001
	CHKCI001	0.001 ± 0.000^{b}	0.015 ± 0.003^{a}	-	-	< 0.001
	Christensenellaceae_ge	-	0.000 ± 0.000	-	-	0.123
	Christensenellaceae_R.7_group	-	0.000 ± 0.000	-	-	< 0.001
	Clostridioides	0.000 ± 0.000	0.001 ± 0.000	0.002 ± 0.001	-	< 0.001
	Clostridium_sensu_stricto_1	0.027 ± 0.012^{a}	0.001 ± 0.000^{b}	-	-	< 0.001
	Clostridium_sensu_stricto_13	0.001 ± 0.001^{ab}	0.000 ± 0.000^{b}	-	0.006 ± 0.004^{a}	< 0.001
	Clostridium_sensu_stricto_18	-	0.000 ± 0.000	-	-	< 0.001
	Clostridium_sensu_stricto_2	-	0.029 ± 0.005^{a}	0.004 ± 0.000^{b}	-	< 0.001
	Clostridium_sensu_stricto_7	-	0.000 ± 0.000	-	-	< 0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Fat	***Moderate Fat***	***High Fat***	***Supra Fat***	
Firmicutes	*Colidextribacter*	0.147 ± 0.026^{b}	0.409 ± 0.076^{a}	0.083 ± 0.045^{b}	0.474 ± 0.064^{a}	< 0.001
	Coprobacillus	-	0.000 ± 0.000	-	-	< 0.001
	Coprococcus	0.155 ± 0.047^{a}	0.013 ± 0.007^{b}	0.002 ± 0.001^{b}	0.004 ± 0.003^{b}	< 0.001
	Defluviitaleaceae_UCG.011	0.646 ± 0.155	0.434 ± 0.229	0.146 ± 0.065	-	< 0.001
	Dialister	0.101 ± 0.017^{a}	0.020 ± 0.011^{b}	-	0.024 ± 0.003^{b}	< 0.001
	Dorea	0.000 ± 0.000^{b}	0.000 ± 0.000^{a}	0.000 ± 0.000^{a}	-	< 0.001
	Dubosiella	0.094 ± 0.012^{c}	0.208 ± 0.019^{b}	0.515 ± 0.069^{a}	0.096 ± 0.014^{c}	< 0.001
	Enterococcus	0.000 ± 0.000^{b}	0.021 ± 0.005^{a}	0.045 ± 0.034^{a}	-	< 0.001
	Epulopiscium	0.176 ± 0.04^{a}	0.227 ± 0.042^{a}	0.281 ± 0.083^{a}	0.023 ± 0.01^{b}	< 0.001
	Erysipelatoclostridium	2.828 ± 0.316^{a}	0.362 ± 0.069^{b}	0.978 ± 0.358^{b}	0.268 ± 0.088^{b}	< 0.001
	Erysipelotrichaceae_ge	0.028 ± 0.002	0.018 ± 0.002	0.031 ± 0.015	0.022 ± 0.004	< 0.001
	Erysipelotrichaceae_UCG.003	2.247 ± 0.156^{a}	2.219 ± 0.128^{a}	1.155 ± 0.214^{b}	2.352 ± 0.242^{a}	< 0.001
	Eubacterium	0.039 ± 0.006^{b}	0.103 ± 0.018^{a}	0.004 ± 0.001^{b}	0.131 ± 0.014^{a}	< 0.001
	Faecalibacterium	-	0.000 ± 0.000	-	-	< 0.001
	Faecalibaculum	0.043 ± 0.007	0.031 ± 0.006	0.047 ± 0.023	0.052 ± 0.016	< 0.001
	Faecalicoccus	0.000 ± 0.000^{b}	0.009 ± 0.001^{ab}	-	0.022 ± 0.019^{a}	< 0.001
	Faecalitalea	-	0.002 ± 0.000	-	-	0.003
	Family_XIII_AD3011_group	0.003 ± 0.001	0.000 ± 0.000	-	-	< 0.001
	Family_XIII_UCG.001	0.094 ± 0.008^{b}	0.173 ± 0.01^{a}	0.06 ± 0.011^{b}	0.087 ± 0.007^{b}	< 0.001
	Flavonifractor	0.004 ± 0.001^{b}	0.002 ± 0.000^{b}	0.033 ± 0.010^{a}	-	< 0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Fat	***Moderate Fat***	***High Fat***	***Supra Fat***	
	Fournierella	0.003 ± 0.000	0.001 ± 0.000	-	-	< 0.001
	Fusibacter	16.283 ± 0.548^{b}	14.669 ± 0.531^{b}	9.165 ± 1.124^{c}	22.765 ± 1.099^{a}	< 0.001
	Fusicatenibacter	-	0.000 ± 0.000^{b}	-	0.000 ± 0.000^{a}	< 0.001
	GCA.900066575	0.009 ± 0.001^{c}	0.055 ± 0.006^{b}	0.282 ± 0.027^{a}	0.002 ± 0.000^{c}	< 0.001
	Granulicatella	-	0.000 ± 0.000	-	-	< 0.001
	Hathewaya	0.002 ± 0.000^{a}	0.000 ± 0.000^{b}	0.001 ± 0.000^{ab}	-	< 0.001
	Holdemanella	0.024 ± 0.006^{a}	0.000 ± 0.000^{b}	-	-	< 0.001
	Holdemania	0.139 ± 0.036	0.104 ± 0.019	0.038 ± 0.011	0.071 ± 0.013	< 0.001
	Howardella	-	-	-	0.126 ± 0.124	< 0.001
	Hungateiclostridium	0.467 ± 0.055^{c}	0.903 ± 0.089^{b}	1.325 ± 0.317^{a}	0.219 ± 0.035^{d}	< 0.001
	Hungatella	-	0.000 ± 0.000	-	-	< 0.001
	Hydrogenoanaerobacterium	0.010 ± 0.000^{ab}	0.009 ± 0.000^{b}	0.013 ± 0.003^{a}	0.001 ± 0.000^{c}	< 0.001
	Ileibacterium	-	0.000 ± 0.000	-	-	< 0.001
	Intestinibacter	0.002 ± 0.000^{a}	0.000 ± 0.000^{b}	-	0.002 ± 0.000^{a}	< 0.001
	Intestinimonas	-	0.000 ± 0.000	-	-	< 0.001
	Lachnoclostridium	0.008 ± 0.002^{c}	0.297 ± 0.077^{b}	-	0.552 ± 0.135^{a}	< 0.001
	Lachnospira	0.007 ± 0.000^{a}	0.003 ± 0.000^{b}	0.007 ± 0.001^{a}	0.002 ± 0.000^{b}	< 0.001
	Lachnospiraceae_AC2044_group	0.006 ± 0.001^{c}	0.124 ± 0.015^{b}	0.395 ± 0.035^{a}	0.000 ± 0.000^{c}	< 0.001
	Lachnospiraceae_FCS020_group	0.006 ± 0.000^{a}	0.005 ± 0.000^{b}	-	0.003 ± 0.000^{c}	< 0.001
	Lachnospiraceae_ge	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	-	< 0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Fat	***Moderate Fat***	***High Fat***	***Supra Fat***	
Firmicutes	*Lachnospiraceae_NC2004_group*	0.717 ± 0.104^{b}	0.496 ± 0.030^{bc}	1.145 ± 0.146^{a}	0.379 ± 0.036^{c}	< 0.001
	Lachnospiraceae_ND3007_group	0.179 ± 0.038^{a}	0.076 ± 0.011^{b}	0.062 ± 0.019^{b}	0.065 ± 0.01^{b}	< 0.001
	Lachnospiraceae_NK3A20_group	-	0.023 ± 0.005^{a}	0.002 ± 0.000^{b}	0.000 ± 0.000^{b}	< 0.001
	Lachnospiraceae_NK4A136_group	0.003 ± 0.000^{b}	0.003 ± 0.001^{b}	0.008 ± 0.002^{a}	-	< 0.001
	Lachnospiraceae_UCG.003	3.717 ± 0.191^{b}	1.705 ± 0.102^{d}	4.685 ± 0.419^{a}	2.539 ± 0.172^{c}	< 0.001
	Lachnospiraceae_UCG.004	0.000 ± 0.000	0.000 ± 0.000	-	-	< 0.001
	Lachnospiraceae_UCG.010	-	0.003 ± 0.001^{a}	-	0.003 ± 0.001^{a}	< 0.001
	Lachnospiraceae_XPB1014_group	-	0.002 ± 0.000	-	-	< 0.001
	Lactobacillus	0.248 ± 0.02^{a}	0.254 ± 0.019^{a}	0.154 ± 0.018^{b}	0.109 ± 0.008^{b}	< 0.001
	Lactococcus	-	0.011 ± 0.003^{b}	0.022 ± 0.007^{a}	-	< 0.001
	Leuconostoc	-	0.005 ± 0.000^{a}	0.006 ± 0.001^{a}	0.001 ± 0.001^{b}	< 0.001
	Marvinbryantia	0.000 ± 0.000	0.000 ± 0.000	-	-	< 0.001
	Megamonas	0.000 ± 0.000^{b}	0.001 ± 0.000^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	< 0.001
	Megasphaera	1.802 ± 0.269^{b}	4.905 ± 0.537^{a}	5.317 ± 1.142^{a}	4.02 ± 0.569^{a}	< 0.001
	Mitsuokella	0.02 ± 0.004^{b}	0.01 ± 0.006^{b}	0.072 ± 0.036^{a}	-	0.005
	Mogibacterium	-	0.000 ± 0.000	-	-	< 0.001
	Monoglobus	0.003 ± 0.000^{b}	0.000 ± 0.000^{c}	0.009 ± 0.005^{a}	-	< 0.001
	Negativibacillus	-	0.001 ± 0.000	-	-	< 0.001
	NK4A214_group	-	0.000 ± 0.000	-	-	< 0.001
	Oribacterium	0.281 ± 0.056^{a}	0.022 ± 0.002^{b}	0.044 ± 0.006^{b}	0.112 ± 0.015^{b}	< 0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Fat	***Moderate Fat***	***High Fat***	***Supra Fat***	
Firmicutes	*Oscillibacter*	4.936 ± 0.314^{a}	2.261 ± 0.187^{b}	2.321 ± 0.627^{b}	0.331 ± 0.062^{c}	< 0.001
	Oscillospira	0.03 ± 0.013^{b}	0.102 ± 0.038^{ab}	-	0.144 ± 0.085^{a}	< 0.001
	Oscillospiraceae_ge	0.001 ± 0.000^{b}	-	-	0.029 ± 0.024^{a}	< 0.001
	Paeniclostridium	0.008 ± 0.002^{b}	0.002 ± 0.000^{b}	-	0.178 ± 0.046^{a}	< 0.001
	Paludicola	0.011 ± 0.010	0.002 ± 0.000	-	-	< 0.001
	Papillibacter	-	0.000 ± 0.000^{b}	-	0.005 ± 0.004^{a}	< 0.001
	Paraclostridium	-	0.000 ± 0.000^{a}	-	0.000 ± 0.000^{b}	< 0.001
	Peptoclostridium	0.002 ± 0.000^{a}	0.000 ± 0.000^{b}	-	-	< 0.001
	Peptococcus	0.022 ± 0.003^{ab}	0.027 ± 0.003^{a}	0.007 ± 0.003^{b}	0.011 ± 0.004^{b}	< 0.001
	Peptoniphilus	0.249 ± 0.035^{c}	1.575 ± 0.167^{b}	0.249 ± 0.092^{c}	3.309 ± 0.459^{a}	< 0.001
	Peptostreptococcaceae_ge	-	0.000 ± 0.000^{b}	-	0.023 ± 0.023^{a}	< 0.001
	Peptostreptococcus	-	-	-	0.045 ± 0.045	< 0.001
	Phascolarctobacterium	0.213 ± 0.019^{a}	0.163 ± 0.014^{b}	0.079 ± 0.025^{c}	0.086 ± 0.012^{c}	< 0.001
	Phocea	0.002 ± 0.000^{b}	0.006 ± 0.000^{a}	-	0.009 ± 0.003^{a}	< 0.001
	Pseudoflavonifractor	-	0.000 ± 0.000^{b}	-	0.055 ± 0.054^{a}	0.097
	Pygmaiobacter	0.011 ± 0.001^{b}	0.016 ± 0.001^{a}	-	0.004 ± 0.000^{c}	< 0.001
	Robinsoniella	0.088 ± 0.015^{a}	0.027 ± 0.003^{b}	-	0.021 ± 0.003^{b}	< 0.001
	Romboutsia	0.042 ± 0.003^{a}	0.038 ± 0.003^{ab}	0.010 ± 0.002^{c}	0.029 ± 0.003^{b}	< 0.001
	Roseburia	0.008 ± 0.001^{b}	0.019 ± 0.001^{a}	0.001 ± 0.000^{c}	0.002 ± 0.001^{c}	< 0.001
	Ruminococcus	0.017 ± 0.002^{a}	0.010 ± 0.001^{b}	-	0.004 ± 0.002^{b}	< 0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Fat	***Moderate Fat***	***High Fat***	***Supra Fat***	
Firmicutes	*Sarcina*	0.000 ± 0.000^{a}	0.000 ± 0.000^{b}	-	-	< 0.001
	Sellimonas	0.004 ± 0.001	0.004 ± 0.000	-	0.003 ± 0.002	< 0.001
	Sharpea	0.001 ± 0.000^{b}	0.001 ± 0.000^{b}	0.005 ± 0.003^{a}	-	< 0.001
	Shuttleworthia	0.000 ± 0.000^{b}	0.003 ± 0.000^{a}	-	-	< 0.001
	Solobacterium	-	0.000 ± 0.000	-	-	< 0.001
	Sporosarcina	0.108 ± 0.013^{b}	0.201 ± 0.015^{a}	0.034 ± 0.018^{c}	0.216 ± 0.024^{a}	< 0.001
	Staphylococcus	0.118 ± 0.014^{b}	0.155 ± 0.031^{b}	1.115 ± 0.472^{a}	0.165 ± 0.045^{b}	< 0.001
	Streptococcus	0.008 ± 0.001^{a}	0.009 ± 0.001^{a}	-	0.004 ± 0.000^{b}	< 0.001
	Subdoligranulum	1.078 ± 0.095^{c}	1.493 ± 0.14^{b}	0.381 ± 0.124^{d}	2.069 ± 0.249^{a}	< 0.001
	Terrisporobacter	-	0.001 ± 0.000	-	-	< 0.001
	Turicibacter	8.23 ± 0.358^{b}	9.388 ± 0.484^{b}	15.76 ± 1.327^{a}	9.406 ± 0.827^{b}	< 0.001
	Tuzzerella	0.800 ± 0.065^{a}	0.366 ± 0.029^{c}	0.742 ± 0.12^{ab}	0.525 ± 0.051^{bc}	< 0.001
	Tyzzerella	0.726 ± 0.148^{a}	0.000 ± 0.000^{b}	-	-	< 0.001
	Vagococcus	0.011 ± 0.001^{a}	0.014 ± 0.004^{a}	-	0.001 ± 0.000^{b}	< 0.001
	Weissella	1.053 ± 0.164^{a}	0.399 ± 0.079^{b}	0.147 ± 0.147^{b}	0.154 ± 0.028^{b}	< 0.001
Fusobacteria	*Cetobacterium*	1.778 ± 0.104^{a}	1.314 ± 0.088^{b}	1.111 ± 0.219^{bc}	0.614 ± 0.067^{c}	< 0.001
	Fusobacterium	0.005 ± 0.000^{a}	0.007 ± 0.000^{a}	-	0.002 ± 0.000^{b}	< 0.001
	Oceanivirga	0.073 ± 0.037	0.001 ± 0.000	-	-	< 0.001
	Streptobacillus	-	0.001 ± 0.000	-	0.011 ± 0.011	< 0.001
Lentisphaerae	*Victivallis*	4.708 ± 0.489^{b}	11.199 ± 0.637^{a}	4.668 ± 0.828^{b}	1.233 ± 0.251^{c}	0.0753

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Fat	***Moderate Fat***	***High Fat***	***Supra Fat***	
Proteobacteria	*Acinetobacter*	2.425 ± 0.171^{a}	1.589 ± 0.122^{b}	0.455 ± 0.103^{c}	2.003 ± 0.377^{ab}	< 0.001
	Actinobacillus	-	0.001 ± 0.000^{b}	-	0.044 ± 0.029^{a}	< 0.001
	Aeromonas	-	0.002 ± 0.000^{a}	-	0.000 ± 0.000^{b}	< 0.001
	Aestuariibacter	0.000 ± 0.000^{b}	0.005 ± 0.000^{b}	-	0.068 ± 0.040^{a}	< 0.001
	Anaerobiospirillum	0.001 ± 0.000^{b}	0.000 ± 0.000^{c}	-	0.002 ± 0.000^{a}	0.547
	Bilophila	-	0.000 ± 0.000	-	-	0.0972
	Bosea	0.020 ± 0.007^{a}	0.000 ± 0.000^{b}	-	0.005 ± 0.004^{ab}	< 0.001
	Campylobacter	0.153 ± 0.013^{a}	0.046 ± 0.006^{bc}	-	0.074 ± 0.011^{b}	< 0.001
	Citrobacter	-	0.000 ± 0.000^{b}	-	0.001 ± 0.001^{a}	< 0.001
	Cupriavidus	0.048 ± 0.004^{c}	0.126 ± 0.011^{a}	0.028 ± 0.009^{c}	0.087 ± 0.021^{b}	< 0.001
	Desulfovibrio	0.038 ± 0.007^{a}	0.003 ± 0.000^{b}	-	-	< 0.001
	Enterobacter	0.812 ± 0.098^{b}	0.300 ± 0.033^{c}	0.305 ± 0.078^{c}	1.392 ± 0.142^{a}	< 0.001
	Escherichia.Shigella	0.028 ± 0.005^{a}	0.033 ± 0.004^{a}	0.042 ± 0.021^{a}	0.045 ± 0.017^{a}	< 0.001
	Hafnia.Obesumbacterium	0.004 ± 0.002	0.004 ± 0.000	-	0.008 ± 0.004^{a}	< 0.001
	Helicobacter	0.021 ± 0.003^{b}	0.006 ± 0.000^{c}	-	0.053 ± 0.006^{a}	< 0.001
	Histophilus	0.154 ± 0.039^{b}	0.437 ± 0.173^{a}	0.044 ± 0.028^{b}	-	< 0.001
Proteobacteria	*Kosakonia*	-	0.000 ± 0.000	-	-	< 0.001
	Mailhella	-	0.000 ± 0.000	-	-	0.002
	Parasutterella	-	0.016 ± 0.002^{b}	-	0.071 ± 0.010^{a}	< 0.001
	Plesiomonas	-	0.030 ± 0.005^{a}	-	0.000 ± 0.000^{b}	< 0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Fat	***Moderate Fat***	***High Fat***	***Supra Fat***	
	Pseudomonas	0.178 ± 0.014^{b}	0.074 ± 0.007^{c}	0.298 ± 0.032^{a}	0.027 ± 0.003^{d}	< 0.001
	Sphingobium	-	0.005 ± 0.001^{b}	0.015 ± 0.01^{a}	-	< 0.001
	Succinivibrio	-	0.000 ± 0.000^{b}	-	0.688 ± 0.443^{a}	< 0.001
	Succinivibrionaceae_UCG.001	0.001 ± 0.000^{a}	0.000 ± 0.000^{b}	-	-	< 0.001
	Sutterella	0.003 ± 0.001^{b}	0.001 ± 0.000^{b}	-	0.014 ± 0.008^{a}	< 0.001
Spirochaetes	*Leptospira*	-	0.000 ± 0.000^{b}	-	0.171 ± 0.169^{a}	0.002
Tenericutes	*Anaeroplasma*	2.579 ± 0.373^{a}	2.695 ± 0.420^{a}	3.054 ± 0.618^{a}	0.064 ± 0.031^{b}	< 0.001
	Mycoplasma	0.017 ± 0.014	0.013 ± 0.001	-	0.005 ± 0.003	< 0.001
Undefined[¥]	*Chloroplast_ge*	0.082 ± 0.023^{b}	0.164 ± 0.032^{a}	0.025 ± 0.006^{b}	0.015 ± 0.004^{b}	< 0.001
	Clostridia_UCG.014_ge	0.013 ± 0.011^{b}	0.000 ± 0.000^{b}	0.011 ± 0.005^{b}	0.078 ± 0.051^{a}	< 0.001
	Dojkabacteria_ge	1.341 ± 0.067^{a}	1.360 ± 0.081^{a}	0.359 ± 0.068^{c}	0.783 ± 0.082^{b}	0.002
	DTU089	0.001 ± 0.000^{b}	0.001 ± 0.000^{a}	-	-	< 0.001
	Erysipelatoclostridiaceae_ge	0.375 ± 0.065^{a}	0.119 ± 0.014^{b}	0.191 ± 0.052^{ab}	0.278 ± 0.032^{ab}	< 0.001
	Gastranaerophilales_ge	0.001 ± 0.000^{a}	0.000 ± 0.000^{b}	-	-	< 0.001
	Incertae_Sedis	-	0.000 ± 0.000^{b}	-	0.116 ± 0.116^{a}	< 0.001
	Mitochondria_ge	1.597 ± 0.154^{a}	1.870 ± 0.187^{a}	1.179 ± 0.292^{ab}	0.774 ± 0.172^{b}	< 0.001
	Oscillospirales_ge	0.002 ± 0.000^{b}	0.007 ± 0.001^{a}	0.004 ± 0.001^{ab}	-	< 0.001
	RF39_ge	0.178 ± 0.026^{a}	0.062 ± 0.007^{b}	0.107 ± 0.019^{ab}	0.011 ± 0.003^{b}	< 0.001
	S5.A14a	0.000 ± 0.000	0.001 ± 0.000	-	-	< 0.001
	T34_ge	-	0.000 ± 0.000	-	-	< 0.001

Phyla	***Genera***	***Mean Relative Abundance ± SEM***				***Diet Effect p value***
		Low Fat	***Moderate Fat***	***High Fat***	***Supra Fat***	
	UBA1819	0.007 ± 0.002^{ab}	0.010 ± 0.001^{a}	-	0.001 ± 0.001^{b}	< 0.001
	UCG.003	0.165 ± 0.012	0.151 ± 0.010	0.167 ± 0.021	0.191 ± 0.016	< 0.001
	UCG.004	-	0.001 ± 0.000	-	0.001 ± 0.000	< 0.001
	UCG.005	-	0.000 ± 0.000^{a}	-	0.000 ± 0.000^{b}	< 0.001
	UCG.008	4.264 ± 0.3^{b}	3.570 ± 0.163^{b}	3.884 ± 0.439^{b}	9.081 ± 0.642^{a}	< 0.001
	UCG.009	0.000 ± 0.000^{b}	0.020 ± 0.002^{a}	0.004 ± 0.001^{b}	0.000 ± 0.000^{b}	< 0.001
	uncultured	0.007 ± 0.001^{a}	0.001 ± 0.000^{b}	-	-	< 0.001
	uncultured_ge	0.000 ± 0.000	0.000 ± 0.000	-	-	< 0.001
	X44314	0.005 ± 0.002	0.011 ± 0.005	0.006 ± 0.004	-	< 0.001
	ZOR0006	-	0.002 ± 0.000	-	-	< 0.001

* Results marked by a '-' denote that this genera was not detected.
§Results denoted as '0.000 ± 0.000' were detected at abundances that round out to 0 at three decimal places, but were still detected.
¥Undefined Phyla are bacterial genera that have not yet been assigned a taxonomic phyla that they belong to.

3.4.3.4 Separation between community profiles was seen based on dietary Fat classification

A PCoA of Bray-Curtis dissimilarity was performed on the faecal microbiota results, based on dietary fat classifications, seen in Figure 3.20. These showed that there was some separation seen in the community profiles based on dietary protein classification.

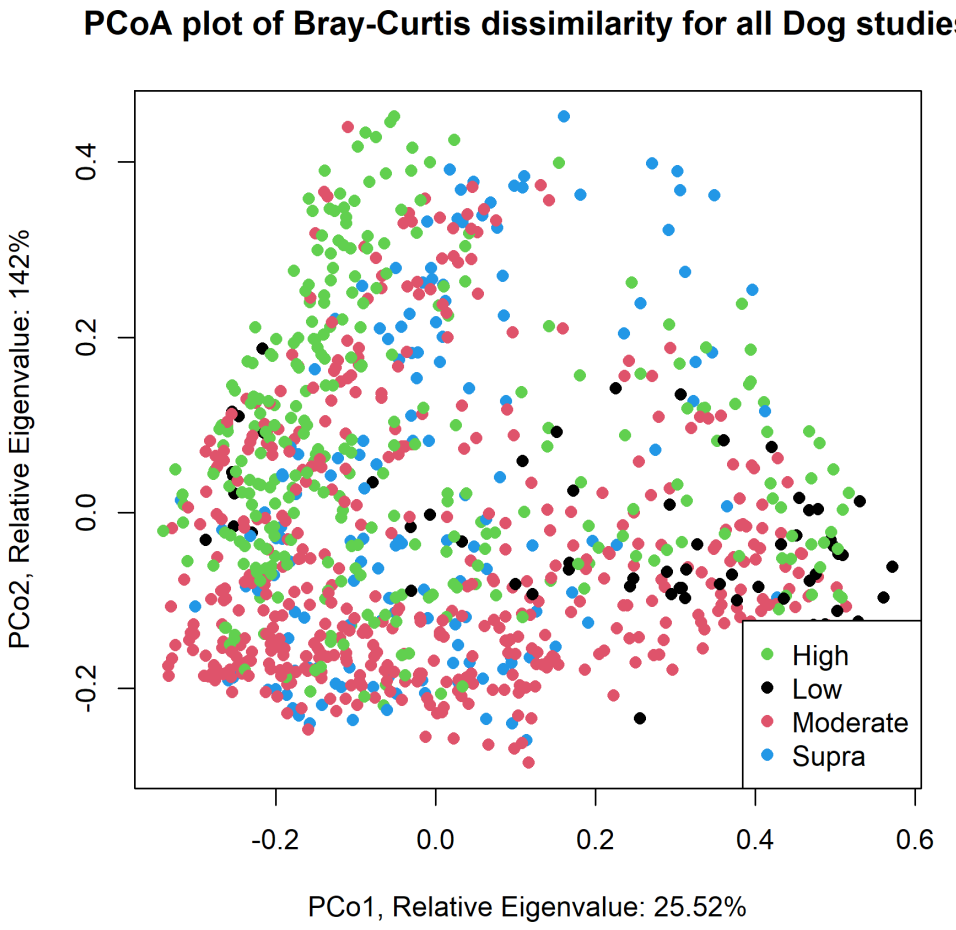


Figure 3.20 *- Principal coordinate analysis of Bray-Curtis dissimilarity for all dog fat classifications, n = 314.Each fat classification is presented with a different colour.*

A partial least squares-discriminant analysis (PLS-DA) was then performed on the dataset, Figure 3.21. These show that there was separation observed based on dietary fat classification (Figure 3.21A). The top PLS-DA loadings from these data (Figure 3.21B) predicated that *Shuttleworthia,*

Dorea, and *Clostridiodes* would be positively impacted by changes in DF content i.e., their relative abundances would increase relative to increased DF content, whilst Genera such as *Aestuariibacter* and *Monoglobus* would be negatively impacted by changes in DF content.

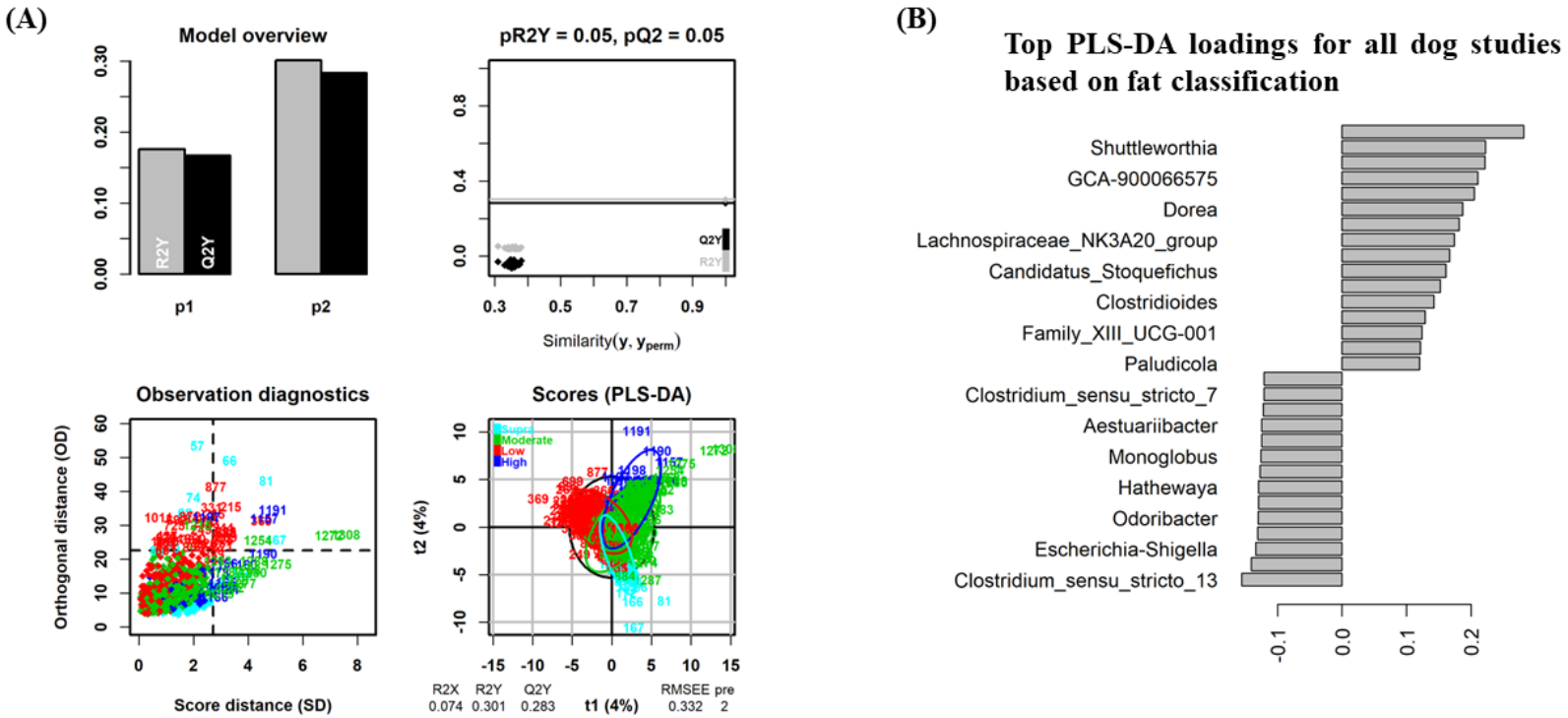


Figure 3.21 *- Partial least squares-discriminant analysis (PLS-DA) for dog studies by fat classification.(A) details the descriptive modelling of the PLS-DA, whilst (B) details the Genera predicted to be most impacted by dietary fat classification.*

The data was then assessed using random forest analysis to understand what bacterial general were potentially driving separation between the protein classifications. As shown in Figure 3.22, *Sharpea* was observed to drive the separation of community profiles for the dietary fat classification, followed by *Allobaculum,Clostridium_sensu_stricto_13,* and *Intestnibacter*. All four of these genera were significantly affected by fat classification ($p< 0.001$ in all instances; Table 3.11).

In a manner resembling the dietary protein analysis, *Sharpea* was determined to be the most influential on the separation of community profiles between dietary fat groups, despite its low relative abundance (<0.075% of sequence reads in all instances). In a similar manner, *Clostridium_sensu_stricto_13* had low relative abundances, with the highest being in the Low Fat group (0.051% ± 0.010% of sequence reads). *Allobaculum* was the most dominant bacterial Genera of these four, with relative abundances of 1.576% ± 0.205%, 2.748% ± 0.252%, 1.876% ± 0.182% and 6.687% ± 0.692% of sequence reads in the Low, Moderate, High and Supra classifications, respectively. Meanwhile, *Intestnibacter* reached its peak relative abundance in the High Fat classification (0.395% ± 0.035% of sequence reads) and decreased through the Moderate (0.124% ± 0.015% of sequence reads) and Low Fat (0.006% ± 0.001% of sequence reads) classifications.

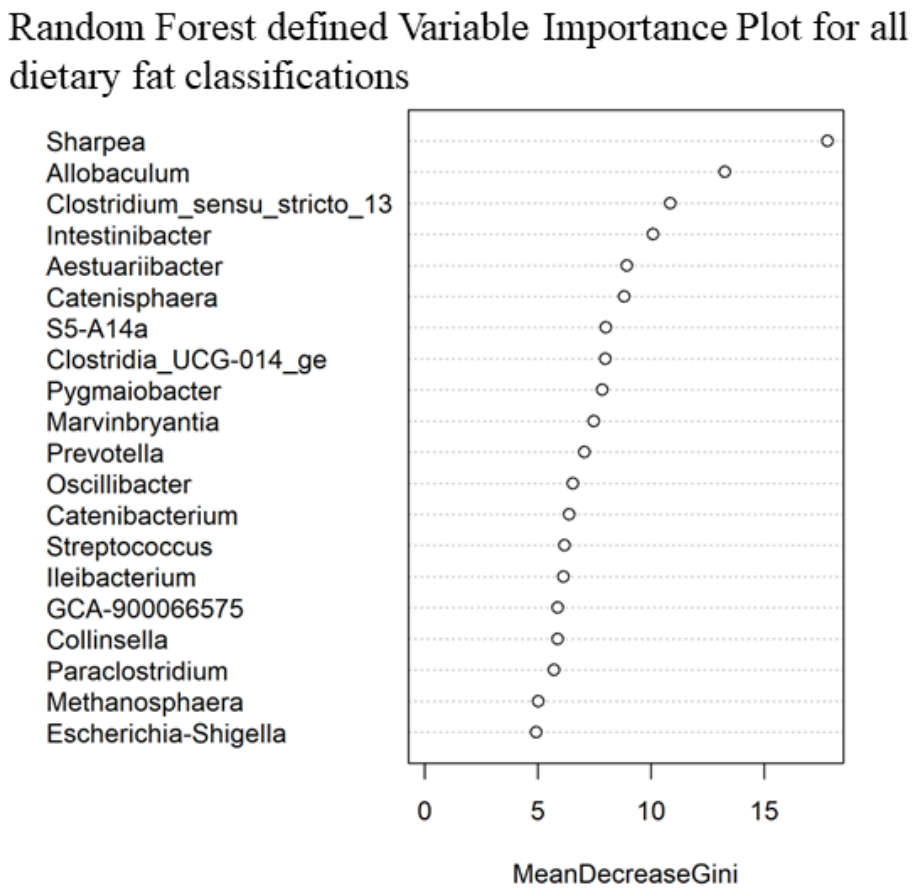


Figure 3.22 - *Bacterial genera in the faecal microbiota of the domestic dog associated with driving separations in diversity and richness of the faecal microbiome when assessed by dietary fat classifications.Data determined by random forest analysis, with a total number of dogs (n) of 314.*

3.4.4 CORRELATIONS OF DIETARY PROTEIN AND DIETARY FAT CONTENT WITH BACTERIAL GENERA

The correlations of the bacterial genera with the CP and DF content were assessed and are presented in Table 3.12. These are listed alphabetically, by bacterial Phyla.

Alloprevotella, Anaerofustis and *Robinsoniella* had the greatest positive association with CP content (i.e., relative abundances of bacterial genera would increase in relation to increased CP content and decrease in relation to decreased CP content. Correlations were 0.343, 0.275, and 0.268, respectively. $P < 0.001$ in all instances). The greatest negative associations with CP content (i.e., those

genera that would decrease in relation to increased protein, or vice versa) were found in *Aestuariibacter*, *Blautia* and *Collinsella* (correlation scores of -0.201, -0.208, and -0.283, respectively. $P < 0.001$ in all instances).

Regarding DF content, *Pseudarthrobacter*, *Allobaculum* and *Sharpea* had the greatest positive association with DF content (correlations scores of 0.396, 0.372, and 0.332, respectively. $P < 0.001$ in all instances). The greatest negative associations with DF content were found in *Aestuariibacter*, *Prevotella* and *Megamonas* (correlations scores of -0.214, -0.281, and -0.291, respectively. $P< 0.001$ in all instances).

Table 3.12 - *Pearson's Correlations between bacterial genera and dietary protein or dietary fat content.Correlations between 0 and 1 that are statistically significant (p < 0.05) denote a positive correlation between the bacterial genus and the dietary macronutrient (i.e., the genus increases in relative abundance in relation to dietary macronutrient content or decreases in relative abundance in relation to decreased macronutrient content). Correlations between 0 and -1 that are statistically significant denote a negative association between the bacterial genus and the dietary macronutrient (i.e., the bacterial genus decreases in relative abundance in relation to increases in dietary macronutrient content, and vice versa). Statistically significant p values are bolded. Dietary data are from 314 dogs across 16 publications, n = 701. Bacterial genera are listed alphabetically, by Phyla.*

Phyla	**Genus**	**Dietary Protein**		**Dietary Fat**	
		Pearson's correlation	***p value***	**Pearson's correlation**	***p value***
Actinobacteria	Actinomyces	0.030	0.434	0.014	0.720
	Adlercreutzia	-0.028	0.466	**0.173**	**< .001**
	Atopobiaceae_ge	-0.016	0.663	-0.029	0.437
	Atopobium	0.046	0.224	-0.040	0.288
	Bifidobacterium	**-0.113**	**0.003**	-0.014	0.713
	Collinsella	**-0.283**	**< .001**	**-0.183**	**< .001**
	Coriobacteriaceae_UCG.002	N/A*	N/A	N/A	N/A
	Coriobacterium	**0.197**	**< .001**	-0.047	0.211
	Corynebacterium	0.043	0.252	0.092	0.014
	Cutibacterium	0.053	0.165	-0.036	0.341
	Denitrobacterium	-0.057	0.131	-0.049	0.191
	Enorma	**0.187**	**< .001**	-0.052	0.169
	Libanicoccus	0.040	0.295	-0.043	0.259
	Olsenella	**0.113**	**0.003**	**0.081**	**0.033**
	Parvibacter	0.029	0.436	-0.073	0.053
	Pseudarthrobacter	0.004	0.917	**0.396**	**< .001**

Phyla	Genus	Dietary Protein		Dietary Fat	
		Pearson's correlation	***p value***	**Pearson's correlation**	***p value***
	Senegalimassilia	**0.201**	**< .001**	-0.063	0.093
	Slackia	-0.038	0.314	-0.046	0.227
	Tetrasphaera	0.056	0.136	-0.015	0.688
	Trueperella	0.039	0.298	**0.115**	**0.002**
Bacteroidetes	Alistipes	**0.172**	**< .001**	**0.080**	**0.033**
	Alloprevotella	**0.343**	**< .001**	-0.005	0.892
	Bacteroides	**0.103**	**0.007**	-0.078	0.038
	Barnesiella	**0.103**	**0.006**	-0.049	0.194
	Butyricimonas	**0.121**	**0.001**	-0.055	0.142
	Muribaculaceae_ge	**0.128**	**< .001**	**0.264**	**< .001**
	Myroides	0.050	0.187	**0.096**	**0.011**
	Odoribacter	**0.194**	**< .001**	-0.056	0.140
	Parabacteroides	**0.126**	**< .001**	**0.102**	**0.007**
	Paraprevotella	0.068	0.073	-0.005	0.892
Bacteroidetes	Porphyromonas	0.043	0.253	**0.112**	**0.003**
	Prevotella	**0.079**	**0.036**	**-0.281**	**< .001**
	Prevotellaceae_Ga6A1_group	**0.083**	**0.028**	0.020	0.595
	Prevotellaceae_NK3B31_group	**0.110**	**0.004**	**0.105**	**0.005**
	Prevotellaceae_UCG.001	N/A	N/A	N/A	N/A

Phyla	**Genus**	**Dietary Protein**		**Dietary Fat**	
		Pearson's correlation	***p value***	**Pearson's correlation**	***p value***
	Prevotellaceae_UCG.003	N/A	N/A	N/A	N/A
	Rikenellaceae_RC9_gut_group	**0.208**	**< .001**	**0.084**	**0.027**
Deferribacteres	Mucispirillum	-0.041	0.274	**-0.084**	**0.027**
Eurarchaeota	Methanobrevibacter	0.048	0.208	**0.134**	**< .001**
	Methanosphaera	0.008	0.840	**0.229**	**< .001**
Firmicutes	Acetanaerobacterium	-0.036	0.335	-0.033	0.382
	Acidaminococcus	0.050	0.182	**0.116**	**0.002**
	Agathobacter	**0.179**	**< .001**	-0.057	0.131
	Allisonella	0.036	0.344	-0.070	0.063
	Allobaculum	**0.076**	**0.043**	**0.372**	**< .001**
	Amnipila	0.049	0.197	**0.094**	**0.012**
	Anaerofilum	**0.147**	**< .001**	-0.061	0.109
	Anaerofustis	**0.275**	**< .001**	-0.028	0.459
	Anaerospora	-0.027	0.472	-0.042	0.262
	Anaerostignum	**0.076**	**0.044**	-0.069	0.067
	Anaerostipes	-0.014	0.708	-0.056	0.139
	Anaerotruncus	**0.095**	**0.012**	-0.010	0.797
	Anaerovibrio	0.055	0.149	0.011	0.780
	Anaerovoracaceae_ge	**0.135**	**< .001**	**0.179**	**< .001**

Phyla	Genus	Dietary Protein		Dietary Fat	
		Pearson's correlation	***p value***	**Pearson's correlation**	***p value***
Firmicutes	Angelakisella	**0.188**	**< .001**	**0.210**	**< .001**
	Bacillus	**-0.069**	**0.066**	**-0.076**	**0.043**
	Blautia	**-0.208**	**< .001**	**-0.115**	**0.002**
	Butyricicoccus	0.057	0.135	**-0.102**	**0.007**
	Candidatus_Arthromitus	-0.063	0.096	-0.071	0.062
	Candidatus_Soleaferrea	**0.225**	**< .001**	0.038	0.319
	Candidatus_Stoquefichus	0.024	0.525	**0.168**	**< .001**
	Caproiciproducens	**0.123**	**0.001**	-0.032	0.393
	Carnobacterium	-0.020	0.591	0.014	0.713
	Catellicoccus	-0.038	0.311	-0.059	0.119
	Catenibacterium	-0.009	0.814	**-0.093**	**0.014**
	Catenisphaera	0.020	0.600	**0.109**	**0.004**
	Cellulosilyticum	0.070	0.065	**0.101**	**0.007**
	CHKCI001	-0.044	0.249	-0.056	0.139
	Christensenellaceae_ge	0.017	0.657	-0.018	0.643
	Christensenellaceae_R.7_group	N/A	N/A	N/A	N/A
	Clostridioides	**0.167**	**< .001**	-0.063	0.095
	Clostridium_sensu_stricto_1	**-0.083**	**0.029**	**0.075**	**0.046**
	Clostridium_sensu_stricto_13	-0.062	0.102	-0.072	0.058
	Clostridium_sensu_stricto_18	**0.249**	**< .001**	-0.054	0.156

Phyla	**Genus**	**Dietary Protein**		**Dietary Fat**	
		Pearson's correlation	***p value***	**Pearson's correlation**	***p value***
Firmicutes	Clostridium_sensu_stricto_2	0.068	0.070	-0.014	0.706
	Clostridium_sensu_stricto_7	0.014	0.716	-0.029	0.450
	Colidextribacter	**0.183**	**< .001**	**-0.102**	**0.007**
	Coprobacillus	-0.040	0.285	-0.071	0.060
	Coprococcus	**0.243**	**< .001**	-0.052	0.167
	Defluviitaleaceae_UCG.011	N/A	N/A	N/A	N/A
	Dialister	0.030	0.423	0.066	0.082
	Dorea	**0.229**	**< .001**	-0.049	0.198
	Dubosiella	0.008	0.836	**0.115**	**0.002**
	Enterococcus	**-0.115**	**0.002**	**-0.128**	**< .001**
	Epulopiscium	-0.039	0.300	0.000	0.998
	Erysipelatoclostridium	**-0.132**	**< .001**	**-0.101**	**0.007**
	Erysipelotrichaceae_ge	-0.011	0.774	-0.025	0.507
	Erysipelotrichaceae_UCG.003	N/A	N/A	N/A	N/A
	Eubacterium	**0.087**	**0.022**	**0.110**	**0.004**
	Faecalibacterium	-0.044	0.242	-0.045	0.237
	Faecalibaculum	0.043	0.251	**0.151**	**< .001**
	Faecalicoccus	0.003	0.932	-0.024	0.530
	Faecalitalea	**-0.095**	**0.012**	-0.049	0.198
	Family_XIII_AD3011_group	**0.078**	**0.038**	**0.094**	**0.013**
	Family_XIII_UCG.001	N/A	N/A	N/A	N/A

Phyla	Genus	Dietary Protein		Dietary Fat	
		Pearson's correlation	***p value***	**Pearson's correlation**	***p value***
Firmicutes	Flavonifractor	**-0.080**	**0.034**	**-0.097**	**0.010**
	Fournierella	**0.231**	**< .001**	-0.062	0.100
	Fusibacter	0.031	0.406	-0.059	0.116
	Fusicatenibacter	-0.003	0.935	**-0.065**	**0.087**
	GCA.900066575	N/A	N/A	N/A	N/A
	Granulicatella	-0.025	0.511	-0.042	0.264
	Hathewaya	0.061	0.105	-0.030	0.429
	Holdemanella	**-0.118**	**0.002**	**-0.164**	**< .001**
	Holdemania	**0.095**	**0.012**	-0.046	0.219
	Howardella	0.037	0.326	**-0.093**	**0.013**
	Hungateiclostridium	**0.245**	**< .001**	-0.026	0.495
	Hungatella	-0.012	0.759	0.075	0.047
	Hydrogenoanaerobacterium	**0.227**	**< .001**	-0.028	0.456
	Ileibacterium	0.034	0.363	**0.128**	**< .001**
	Intestinibacter	**0.150**	**< .001**	-0.046	0.220
	Intestinimonas	**0.180**	**< .001**	-0.037	0.334
	Lachnoclostridium	**-0.085**	**0.025**	**-0.109**	**0.004**
	Lachnospira	0.012	0.744	0.040	0.286
	Lachnospiraceae_AC2044_group	-0.001	0.987	-0.070	0.062
	Lachnospiraceae_FCS020_group	-0.006	0.883	**-0.100**	**0.008**

Phyla	Genus	Dietary Protein		Dietary Fat	
		Pearson's correlation	***p value***	**Pearson's correlation**	***p value***
Firmicutes	Lachnospiraceae_ge	**-0.179**	**< .001**	-0.067	0.075
	Lachnospiraceae_NC2004_group	**0.158**	**< .001**	-0.037	0.329
	Lachnospiraceae_ND3007_group	**0.204**	**< .001**	0.044	0.242
	Lachnospiraceae_NK3A20_group	**0.178**	**< .001**	-0.061	0.104
	Lachnospiraceae_NK4A136_group	**0.078**	**0.038**	**-0.150**	**< .001**
	Lachnospiraceae_UCG.003	N/A	N/A	N/A	N/A
	Lachnospiraceae_UCG.004	N/A	N/A	N/A	N/A
	Lachnospiraceae_UCG.010	N/A	N/A	N/A	N/A
	Lachnospiraceae_XPB1014_group	**0.238**	**< .001**	-0.025	0.515
	Lactobacillus	-0.009	0.809	0.023	0.545
	Lactococcus	-0.046	0.228	-0.019	0.614
	Leuconostoc	-0.007	0.843	0.011	0.772
	Marvinbryantia	**-0.123**	**0.001**	**-0.077**	**0.042**
	Megamonas	**-0.142**	**< .001**	**-0.291**	**< .001**
	Megasphaera	0.033	0.382	0.057	0.132
	Mitsuokella	0.058	0.124	**0.125**	**< .001**
	Mogibacterium	**0.238**	**< .001**	0.044	0.243
	Monoglobus	**0.204**	**< .001**	-0.055	0.144
	Negativibacillus	**0.209**	**< .001**	0.002	0.953
Firmicutes	NK4A214_group	**0.183**	**< .001**	**0.167**	**< .001**

Phyla	**Genus**	**Dietary Protein**		**Dietary Fat**	
		Pearson's correlation	***p value***	**Pearson's correlation**	***p value***
	Oribacterium	**0.237**	**< .001**	**0.094**	**0.013**
	Oscillibacter	**0.248**	**< .001**	**-0.076**	**0.044**
	Oscillospira	**0.150**	**< .001**	-0.002	0.961
	Oscillospiraceae_ge	**0.128**	**< .001**	-0.048	0.208
	Paeniclostridium	0.070	0.065	-0.062	0.102
	Paludicola	**0.242**	**< .001**	-0.069	0.068
	Papillibacter	**0.110**	**0.003**	-0.030	0.421
	Paraclostridium	0.002	0.952	0.061	0.105
	Peptoclostridium	-0.017	0.662	0.015	0.684
	Peptococcus	0.051	0.174	**0.237**	**< .001**
	Peptoniphilus	**0.237**	**< .001**	-0.052	0.169
	Peptostreptococcaceae_ge	-0.060	0.111	**-0.102**	**0.007**
	Peptostreptococcus	0.044	0.247	-0.006	0.872
	Phascolarctobacterium	0.052	0.172	**-0.186**	**< .001**
	Phocea	**0.135**	**< .001**	-0.052	0.173
	Pseudoflavonifractor	-0.012	0.749	-0.016	0.676
	Pygmaiobacter	**-0.153**	**< .001**	**-0.092**	**0.014**
	Robinsoniella	**0.268**	**< .001**	-0.050	0.187
	Romboutsia	-0.006	0.869	**0.197**	**< .001**
	Roseburia	0.064	0.091	0.057	0.130

Phyla	**Genus**	**Dietary Protein**		**Dietary Fat**	
		Pearson's correlation	***p value***	**Pearson's correlation**	***p value***
	Ruminococcus	**0.171**	**< .001**	**0.134**	**< .001**
	Sarcina	**-0.078**	**0.038**	**-0.088**	**0.020**
	Sellimonas	0.064	0.089	-0.047	0.214
	Sharpea	**0.076**	**0.044**	**0.332**	**< .001**
	Shuttleworthia	**0.261**	**< .001**	-0.050	0.189
	Solobacterium	0.068	0.071	-0.023	0.545
	Sporosarcina	**0.091**	**0.016**	-0.037	0.322
	Staphylococcus	0.029	0.444	**0.118**	**0.002**
	Streptococcus	**-0.147**	**< .001**	**-0.186**	**< .001**
	Subdoligranulum	-0.020	0.606	-0.046	0.227
	Terrisporobacter	**-0.116**	**0.002**	**-0.077**	**0.041**
	Turicibacter	**-0.125**	**< .001**	**-0.087**	**0.022**
	Tuzzerella	**0.104**	**0.006**	**-0.081**	**0.032**
	Tyzzerella	**-0.172**	**< .001**	**-0.177**	**< .001**
	Vagococcus	-0.020	0.591	-0.026	0.494
	Weissella	-0.065	0.086	**-0.077**	**0.042**
Fusobacteria	Cetobacterium	0.031	0.420	**0.179**	**< .001**
	Fusobacterium	**0.113**	**0.003**	**0.294**	**< .001**
	Oceanivirga	0.041	0.283	**0.116**	**0.002**

Phyla	Genus	Dietary Protein		Dietary Fat	
		Pearson's correlation	***p value***	**Pearson's correlation**	***p value***
	Streptobacillus	0.040	0.292	**0.115**	**0.002**
Lentisphaerae	Victivallis	-0.008	0.827	-0.014	0.702
Proteobacteria	Acinetobacter	**0.141**	**< .001**	-0.041	0.274
	Actinobacillus	0.041	0.274	**0.117**	**0.002**
	Aeromonas	-0.054	0.154	**-0.106**	**0.005**
	Aestuariibacter	**-0.201**	**< .001**	**-0.214**	**< .001**
	Anaerobiospirillum	0.049	0.191	-0.045	0.235
	Bilophila	0.021	0.578	-0.017	0.658
	Bosea	0.056	0.137	**0.144**	**< .001**
	Campylobacter	-0.065	0.088	-0.067	0.078
	Citrobacter	**0.172**	**< .001**	-0.028	0.453
	Cupriavidus	-0.033	0.389	**-0.095**	**0.012**
	Desulfovibrio	-0.021	0.572	-0.024	0.533
	Enterobacter	**-0.125**	**< .001**	**-0.135**	**< .001**
	Escherichia.Shigella	N/A	N/A	N/A	N/A
Proteobacteria	Hafnia.Obesumbacterium	N/A	N/A	N/A	N/A
	Helicobacter	**-0.103**	**0.006**	**-0.093**	**0.014**
	Histophilus	0.040	0.286	**0.116**	**0.002**
	Kosakonia	0.004	0.913	-0.040	0.294
	Mailhella	**0.100**	**0.008**	-0.020	0.597

Phyla	**Genus**	**Dietary Protein**		**Dietary Fat**	
		Pearson's correlation	***p value***	**Pearson's correlation**	***p value***
	Parasutterella	**0.170**	**< .001**	**0.183**	**< .001**
	Plesiomonas	-0.049	0.196	-0.053	0.162
	Pseudomonas	0.060	0.111	**0.100**	**0.008**
	Sphingobium	0.069	0.068	**0.161**	**< .001**
	Succinivibrio	-0.011	0.767	**-0.077**	**0.041**
	Succinivibrionaceae_UCG.001	N/A	N/A	N/A	N/A
	Sutterella	0.006	0.864	**-0.194**	**< .001**
Spirochaetes	Leptospira	**0.116**	**0.002**	-0.026	0.497
Tenericutes	Anaeroplasma	0.041	0.284	**-0.146**	**< .001**
	Mycoplasma	0.050	0.186	**0.096**	**0.011**

* Results with an N/A denotes that there were not enough separate results to perform the correlation analysis.

3.5 DISCUSSION

This study shows that the composition of the faecal microbiota of the dog is affected by both CP and DF content of the diet. Furthermore, the random forest analysis performed in this meta-analysis suggests that the relative abundance of *Sharpea* was responsible for driving the separation between dietary classifications in both CP and DF.

In the current study the relative abundance of *Sharpea* was significantly affected by both CP and DF content ($p < 0.001$ for both) and was highest in the microbiome of dogs in the High Protein and Supra Fat classifications. Despite significant differences in the relative abundance between dietary classifications, overall *Sharpea* was present in relatively low abundances (< 0.1% of sequence reads in all instances). *Sharpea* had one of the greatest positive associations with DF content of all bacterial genera (Pearson's correlation score of 0.332, $p < 0.001$), and was also positively associated with CP content (Pearson's correlation score of 0.076, $p = 0.044$). *Sharpea* is a lactate and acetate producer in ruminants (Kumar et al., 2018) and its relative abundance decreased in piglets with diarrhoea (Yang et al., 2017). However, in the dog, the role of *Sharpea* is unknown, possibly owing to its low relative abundance in samples. In the studies included in this meta-analysis, the presence of *Sharpea* was not reported in the body of the publication and included only in the full results deposition. Overall, only 6/16 of the studies detected *Sharpea*. The presence of *Sharpea* in the faecal microbiota of dogs has only been reported in one publication, wherein its role was not investigated further than reporting its presence (Sturgeon, 2014). Though there is an increasing interest in the functionality of the relatively low abundant constituents (the so-called "dark matter") of the human microbiome (Cena et al., 2021), this is still an unexplored field in the dog. This highlights the potential of techniques such as meta-analysis in increasing our understanding of the role of relatively low abundant genera in the dog.

Aside from *Sharpea*, *Prevotellacaea*_Ga6A1_group and *Enterococcus* appear to be pivotal in driving the differences observed in the microbial profiles between dietary protein classifications in healthy dogs. This study shows that that the relative abundance of *Prevotellacaea*_Ga6A1_group was

significantly impacted by CP content, and the relative abundances were the highest in the Moderate Protein (25-30 %DM) classification (relative abundance of 2.447 ± 0.159), and the lowest in the Low Protein (<25 %DM) classification (relative abundance of 0.575 ± 0.104). There was also a positive correlation between CP content and relative abundances of *Prevotellacaea*_Ga6A1_group (Pearson's correlation score of 0.083, p = 0.028). Prevotellacaea_Ga6A1_group are referenced as SCFA producers (Zhang et al., 2021b), though the specific SCFA are not elaborated on further. Although the research involving this Genera in dogs is limited, *Prevotellacaea*_Ga6A1_group relative abundances decreased in association with weight loss in overweight dogs fed high protein (42.0% DM) diets (Phungviwatnikul et al., 2021). In mice it was found that probiotic dietary application of bifidobacteria resulted in decreases of *Prevotellacaea*_Ga6A1_group (Gryaznova et al., 2022). Interestingly, in the high-protein dog diet, *Bifidobacterium* relative abundances increased as *Prevotellacaea*_Ga6A1_group decreased, although this possible correlation was not investigated further (Phungviwatnikul et al., 2021). This suggests, though tenuously, that *Prevotellacaea*_Ga6A1_group and *Bifidobacterium* may occupy the same niche of SCFA production, and *Prevotellacaea*_Ga6A1_group are outcompeted by bifidobacteria, although further research is needed in this area to understand this relationship further.

Enterococcus relative abundances decreased as CP content increased (Pearson's correlation score of -0.115, p = 0.002), consistent with results seen elsewhere (Pinna et al., 2016; Phungviwatnikul et al., 2021). *Enterococcus spp.*, have been used as probiotics in dogs (Strompfová et al., 2004; Schmitz et al., 2015a; Schmitz et al., 2015b; Pilla et al., 2019; Hanifeh et al., 2021), and are mainly acetate producers (Wu et al., 2021). In addition, enterococcialso produce bile salt hydrolases (Phungviwatnikul et al., 2021), which catalyse the deconjugation of glyco-conjugated and tauro-conjugated bile acids, thereby performing a role in fat metabolism (Ren et al., 2011; Xu et al., 2019). However, in this study *Enterococcus* relative abundances also decreased as DF content increased. The results obtained here suggest that in healthy dogs *Enterococcus* may perform an important role in instances of low protein and/or fats.

The findings in the current study indicated that as well as *Sharpea*, *Allobaculum* and *Clostridum_sensu_stricto_13* were also important for the distinction between the faecal microbiota composition associated with dietary fat, and were all significantly affected by DF content.

The relative abundance of *Allobaculum* had the highest relative abundance in the Supra Fat classification (6.688% ± 0.692% of sequence reads), though interestingly there was no difference in relative abundances between the High Fat (1.876% ± 0.183% of sequence reads) classification and both the Low (1.577% ± 0.205% of sequence reads) and Moderate Fat (15-20% DM; relative abundance of 2.746% ± 0.252% of sequence reads) classifications. *Allobaculum* was one of the genera with the greatest positive correlations with DF content (Pearson's correlation score of 0.372, $p < 0.001$). *Allobaculum* is a butyrate producer (Greetham et al., 2004) and has been observed to decrease in the faeces of dogs fed animal protein-free diets (Bresciani et al., 2018). In one study the relative abundances were increased in overweight dogs fed high protein and high fat diets (Phungviwatnikul et al., 2021), however, in another study where dogs were fed low fat and moderate protein, the relative abundances of *Allobaculum* were higher in healthy dogs, compared to overweight dogs and those on weight loss programmes (Macedo et al., 2022). Additionally, its relative abundancedecreased in a completely CHO-free, meat-based diet, when compared to a commercial kibble diet (Bermingham et al., 2017). As *Allobaculum* hydrolyses mono- and disaccharides rather than starch (Greetham et al., 2004), diets high in starch or low in mono- and disaccharides – or both – may be responsible for these alterations to the abundances of *Allobaculum.* Starch content was reported as 345g/kg as fed in the animal protein-free diet (Bresciani et al., 2018), but was not assessed in the raw-meat based diet (Bermingham et al., 2017), the studies using overweight dogs (Phungviwatnikul et al., 2021; Macedo et al., 2022), and was not assessed in this meta-analysis, so this hypothesis cannot be effectively challenged. Future investigations could interrogate the effects of dietary fats and starch content on *Allobaculum* to detail their impacts on the faecal microbiota of the dog.

This study showed that the relative abundances of *Clostridum_sensu_stricto_13* were highest in the Low Fat (<15% DM; mean relative abundance 0.051% ± 0.01% of sequence reads) classification and was not detected in the High (20-30 % DM) and Supra Fat (>30% DM) classifications. In this

study there was a negative correlation of DF content with *Clostridum_sensu_stricto_13* that trended towards significance (Pearson's correlation score of -0.072, p = 0.058). The literature relating to *Clostridum_sensu_stricto_13* in the dog is sparse, though it is part of the normal faecal microbiome in dogs and cats (Alessandri et al., 2020). Additionally, altering dietary fibre type between cellulose, beet pulp and miscanthus grass fibre was found to cause changes in the relative abundances of *Clostridum_sensu_stricto_13* in the dog (Finet et al., 2022), although this has not been investigated further.

Typically, *Fusobacterium*, *Bacteroides*, and *Peptoclostridium* were the dominant genera observed in the microbiome of the dog, irrespective of the level of dietary classification. This is consistent with individual studies (Alessandri et al., 2019; Phungviwatnikul et al., 2021; Xu et al., 2021; You and Kim, 2021; Finet et al., 2022). The relative abundances of *Alloprevotella, Blautia, Faecalibacterium, Lactobacillus, Megamonas* and *Prevotella* also dominate the community profile of the healthy dog, albeit in a diet-associated manner, consistent with other studies (Martínez-López et al., 2021; Phungviwatnikul et al., 2021; You and Kim, 2021; Finet et al., 2022).

Fusobacterium, despite its association with negative health connotations in humans (Brennan and Garrett, 2019; Lee et al., 2022), is a main component of the gut microbiota of the domestic dog (Vázquez-Baeza et al., 2016; Alessandri et al., 2019). The data included in this analysis were all from healthy dogs, which further implies its role as a healthy commensal for the dog. Fusobacteria, and by extension, *Fusobacterium*, are butyrate producers, utilising lysine degradation pathways to produce butyrate from protein sources (Vital et al., 2014b; Louis and Flint, 2017). This may explain why the relative abundance of *Fusobacterium* increases in association with increased dietary protein in in healthy dogs. Interestingly, the response of *Fusobacterium* to dietary protein appears to have a U-shaped response, increasing from Low to High Protein, and then dropping back down again at Supra Protein (> 45 %DM). This suggests that at very high levels of dietary protein, they may be out competed by other protein utilisers, or that the environmental conditions at Supra Protein levels no longer favour *Fusobacterium*. In terms of its response to dietary fat, the relative abundance of Fusobacterium was lowest in high fat classification (9.166% of sequence reads) but increased to

approx. 22% of sequence reads in the Supra Fat classification. This may be a confounding effect of the high levels of dietary protein in the supra-fat diets (39.67 %DM) although interestingly, though it was positively associated with both CP and DF content, it had a greater positive correlation with DF content than it did CP content (Pearson's correlation scores of 0.113 for CP, $p = 0.003$, and score of 0.294, $p < 0.001$ for DF). This suggests further exploration may be needed to untangle the relationship of *Fusobacterium* with dietary protein and fat contents.

Peptoclostridium also comprises a large component of the commensal population of the faecal microbiome of healthy dogs (Alessandri et al., 2019; Phungviwatnikul et al., 2021; Xu et al., 2021; You and Kim, 2021; Finet et al., 2022). *Peptoclostridium* is also a butyrate producer, however rather than protein it instead ferments saccharides such as fructose, glucose, and xylose (Galperin et al., 2016; Pereira et al., 2016) to form butyrate. The relative abundance of *Peptoclostridium* has been shown to decrease in relation to diets containing higher protein content in both healthy and obese dogs (Phungviwatnikul et al., 2021; Xu et al., 2021). In this study *Peptoclostridium* relative abundance was the highest at 11.445% in the Low Protein classification, consistent with these results (Phungviwatnikul et al., 2021; Xu et al., 2021), however this study also suggests it is relatively stable in terms of protein supply, ranging from 8.417-9.879% sequence reads across the other, higher protein dietary classifications. In terms of DF, the relative abundance of *Peptoclostridium* was highest in the High Fat classification at 15.761%; and again, was relatively stable across all other DF classifications, ranging from 8.23 – 9.41% sequence reads. Additionally, in this study *Peptoclostridium* had no significant correlation with either CP or DF content (*p* values of 0.662 and 0.684, respectively). Based on the results in this study, it is difficult to tease out the impacts of CP and DF alone on Peptoclostridium, and it may be influenced by another macronutrient not assessed in this analysis.

In this study *Bacteroides* were relatively stable in the CP treatments (abundances were relatively stable at 10.520 – 11.046% of reads across Low, Moderate and High Protein), and were significantly higher than all other protein classifications in the Supra Protein (13.914% of reads). Similarly, *Bacteroides* abundances were also relatively stable across the Low, High and Supra Fat classifications (10.017 – 11.513% of reads), although in contrast to pattern in the CP, they were

significantly lower in the High Fat classification (7.013% of reads). In this study *Bacteroides* had a positive association with CP content (Pearson's correlation score of 0.103, $p = 0.007$) and a negative association with DF content (Pearson's correlation score of -0.078, $p = 0.038$). *Bacteroides* are saccharotic bacteria that are also producers of acetate and propionate (Rios-Covian et al., 2015; Nogal et al., 2021). *Bacteroides* are found to increase in response to beef-based protein diets, in comparison to those using chicken as a protein source in dogs (Herstad et al., 2017; Do et al., 2021). However, another study that compared beef and chicken-based protein diets showed that at the species level, beef protein resulted in increased faecal *Bacteroides vulgatus* and decreased *Bacteroides coprocola* in comparison to dogs fed chicken protein (Beloshapka et al., 2013). Bacteroides are also seen to be impacted by protein sources in rats (Zhu et al., 2016) and humans (Shi et al., 2021). Of note, though it was seen in rats that plant-based protein diets resulted in increased faecal *Bacteroides* in comparison to the animal-protein diets, this has not been seen in dogs (Kerr et al., 2013; Bresciani et al., 2018). This could be due to the nature of the dog as a facultative carnivore in comparison to the omnivorous rat, although further research is warranted to understand this relationship further.

A major limitation throughout the course of this study was the unavailability of necessary information for complete diet-microbiota analysis. Of the pool of potential publications to include in this study, 20 publications were discarded as the published diet information was incomplete or not included. There were studies discarded because of human error in the data deposition, and even in the final 16 publications included in the final assessment, not all publications included dog metadata such as age, breed, or neuter status. Several papers published average weights across a variety of different breeds of dogs and two of the 16 papers included in the analysis did not specify which region of the 16S rRNA gene they analysed in their experiment. In a similar vein, the dietary groups assigned in this meta-analysis used were not significantly different for their macronutrient of interest across all groups (i.e., the DF content between the Moderate Fat and High Fat classifications were not different, $p > 0.05$), possibly because of the small pool of publications that were used. In future meta-analyses the methods used to obtain and determine dietary groups can be refined to ensure these differences are significant, thereby allowing stronger conclusions to be drawn from the results. In future research,

there needs to be better accountability for data and more transparency to allow studies such as these to be conducted, but also to further the knowledge of gut health in the dog.

To further improve on this meta-analysis, which can be utilised in experiments going forwards, the impact of batch effects (i.e., how much of the data clustered due to sequecing batch in comparison to actual clustering from diet composition) can be assessed by analysing the beta diversity of the faecal microbiota. Additionally, publication bias and sensitivity analyses were not performed. In this experiment 3 of the 16 final publications were from the same laboratory. Performing an assessment of publication bias in future experiments will provide further clarity and confidence in data obtained. Similarly, though the methods of extraction and region of the 16S rRNA that was sequenced in the included publications were noted, these were not assessed as additional variables. The data in this experiment can be utilised going forwards to assess the impacts of these variables on the faecal microbiota, whilst accounting for any changes due to dietary protein and fat content.

Due to limitations in current knowledge and the lack of reported information, the effects of age, breed, neuter status, and gender on the microbiota could not be assessed. Future meta-analyses could investigate these as additional variables and further contribute to the scientific understanding in this field.

Overall, this meta-analysis has demonstrated the opportunities for further, in-depth investigations by using the existing literature to highlight overlooked or unnoticed trends. It has highlighted that whilst attentions are often drawn to the largest abundances and biggest changers, the bacteria present in low relative abundances are overlooked. Future research would benefit from investigating the overlooked genera of *Clostridium_senso_stricto_13, Sharpea,* and *Prevotellacaea*_Ga6A1_group and investigating their potential impacts on the faecal microbiota of the domestic dog.

3.6 CONCLUSIONS

Despite the limitations imposed on the study by the availability and accessibility of data, these results have provided more insights into the relationship of dietary protein and dietary fat with the gut microbiota in domestic dogs. It has highlighted that whilst there are bacterial genera that are highly abundant and associated with the bacterial fermentation of dietary compounds, the main 'drivers' behind the differences observed are surprisingly those on the low end of relative abundances and are sparsely reported on in the literature for dogs. This work has demonstrated that there are many feasible new opportunities to explore the relationship of diet and the gut microbiota that would add significant value to existing knowledge. As interest and experimental approaches continue to grow in this field, this work has also highlighted the importance of reporting all facets of data, to better understand and evaluate the literature, and to give rise to further opportunities for studies like these to be conducted.

The next step in the PhD workflow was to refine the methodologies to enable the co-culture of bacteria with the cIEC. Chapter Four details these method refinements.

3.7 ACKNOWLEDGEMENTS FOR CHAPTER THREE

For the work in this chapter I would like to acknowledge Joy Dick for performing the initial publication gathering from all databases, Nina Butowski for her assistance in the initial collection and organisation of the publications. In addition, I would also like to acknowledge Paul Maclean and Ruy Jauregui for their biostatistics magic in Section 3.3.4 and the PLS-DAs, and Wayne Young for being an invaluable font of knowledge when helping me with both R and the microbiome analysis.

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Francis David Phimister
Name and title of main supervisor:	Associate Professor David Thomas
In which chapter is the manuscript/published work?	Chapter Three
What percentage of the manuscript/published work was contributed by the student?	70%

Describe the contribution that the student has made to the manuscript/published work:

In the meta-analysis Francis performed the publication analyses for the inclusion/exclusion in the study, the generation of all relevant tables and figures, the statistical assessment of differences in bacterial genera based on dietary protein and fat, and wrote the discussion. He also drafted the manuscript for the meta-analysis publication and is responsible for corrections post-author comments.

Please select one of the following three options:

- ○ The manuscript/published work is published or in press
  Please provide the full reference of the research output:
- ○ The manuscript is currently under review for publication
  Please provide the name of the journal:
- ◉ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Student's signature:	Francis Phimister Digitally signed by Francis Phimister Date: 2023.01.28 09:24:35 +13'00'	Main supervisor's signature:	David Thomas Digitally signed by David Thomas DN: cn=David Thomas, c=NZ, o=Massey University, ou=School of Agriculture & Environment, email=d.g.thomas@massey.ac.nz Date: 2023.01.29 16:34:37 +13'00'

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

CHAPTER FOUR

METHOD REFINEMENT OF DIFFERENTIATION CONDITIONS FOR AN IMMORTALISED CANINE INTESTINAL EPITHELIAL CELL LINE*

**Selected material from this chapter and Chapter Six was presented as:*

'A Co-Culture Cell System to Model the Canine Intestine' Oral Presentation, Australian and New Zealand Laboratory Animal Association (ANZLAA) Winter Conference, Palmerston North, New Zealand, July 2019.

Waltham Internal Event Poster, Poster Presentation, Waltham internal review, Waltham-on-the-Wolds, United Kingdom, January 2020.

'The Development of a Novel Co-Culture System to Model Diet-Based Influences on the Canine Intestinal Epithelium' Accepted abstract, Global Animal Nutrition Summit, Guelph, Canada, August 2020.

'The development of an in vitro model of the canine intestine to evaluate and understand the diet-based influences on the health of the domestic dog.' Poster Presentation, AgResearch Science Hot Week, Palmerston North, New Zealand, September 2021.

4.1 ABSTRACT

Modelling the intestinal epithelial barrier *in vitro* allows for the investigation of the host response to dietary compounds and bacterial challenges. Currently, knowledge in the dog is extrapolated from other species, from results obtained using Madin-Derby Canine Kidney (MDCK) cells, or from primary cells. Each of these comes with their own drawbacks as to their suitability for use in furthering knowledge of the canine gut. To address this, an investigation was undertaken to use an existing canine intestinal epithelial cell (cIEC) line and adapt previously used protocols to successfully culture the cIEC in a cellZscope system. In this system the integrity of the cIEC barrier was quantified by automatically recording the transepithelial electrical resistance (TEER) every 30 minutes. Initial experiments revealed current methods were not compatible with the cellZscope. Therefore, method development was undertaken, wherein the effects of time, temperature, and media compositions on the TEER of the cIEC monolayer was investigated. The combined effects of an extended period of differentiation in the presence of hydrocortisone significantly improved the stability of the TEER over time. The refined protocol for the differentiation of the cIEC has laid the foundations to further investigate the intestinal barrier and the host response to dietary compounds or bacterial challenges in the domestic dog.

4.2 BACKGROUND

Intestinal enterocytes are often employed as a cell culture model of the IEB. They secrete cytokines and chemokines in response to inflammatory signals and are capable of recruiting and activating immune cells, such as T cells (Gronert et al., 1998; Chougule et al., 2012). The human colorectal adenocarcinoma cells (Caco-2) are capable of forming enterocyte-like cells upon differentiation and are considered the gold standard *in vitro* model of the human intestine (Darling et al., 2020). In stark contrast however, the most well-studied canine-specific cells – which are often employed to 'simulate' intestinal conditions – are MDCK cells. These originate from immature kidney cells (Ambrosini et al., 2020). However, MDCKs are seen as morphologically comparable to

Caco-2 cells due to the capacity of MDCKs to form polarized columnar monolayers with brush borders and TJs (Le Ferrec et al., 2001). Both the Caco-2 and MDCK cell lines benefit from being immortalised cell lines. This allows them to continually be grown and proliferate in culture conditions. Primary cultures are more physiologically relevant models. However, they reach a point in culture conditions where they no longer proliferate. This then requires repeated sample collections to ensure a continual supply (Rusu et al., 2005; Golaz et al., 2007; Chougule et al., 2012; Costa and Ahluwalia, 2019).

The cell culture model most often employed to simulate *in vivo* intestinalconditions involves culturing cell layers on porous filters. This allows for the nutrient supply from the basal compartment to stimulate cell differentiation, and can be seen in Figure 4.23. This promotes a polarised cell layer with closer morphological and functional similarity to *in vivo* processes (Chen et al., 2015). These *in vitro* methods have been employed in investigations into enterocyte functions across multiple different species and indirect enterocyte models. Said models have investigated TJ permeability (Artursson, 1990; Yamashita et al., 2000; Weng et al., 2005), the inflammatory response to bacterial challenges (Van De Walle et al., 2010), cellular differentiation (Jumarie and Malo, 1991), and intestinal absorption (Angelis and Turco, 2011).

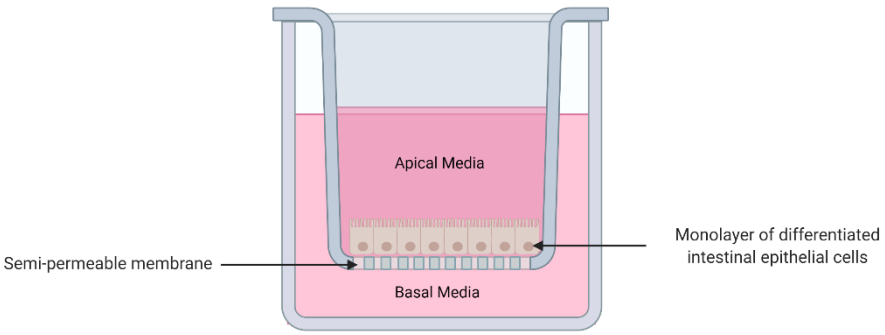


Figure 4.23 *- An overview of the inserts used for the traditional methods of cell culture. The basal media is used to simulate the lamina propria, whilst the apical media simulates the intestinal lumen. The semi-permeable*

membrane on the bottom of the inserts allows for the diffusion of nutrients from the basal media into the monolayer of differentiated cells. In this model challenges can be applied apically and/or basally to simulate and characterise a response to intestinal food products or infection, respectively. In this model the media can be collected, replaced, and analysed to understand the patterns of secretion of various cytokines, exosomal RNA and waste products from the cells' utilisation of nutrients in the media. Figure created with BioRender.

Studies which investigate healthy canine IECs are limited in number and have currently all been performed using this methodology (Weng et al., 2005; Golaz et al., 2007; Ohta et al., 2011; Farquhar et al., 2018; Reineking et al., 2018a; Chandra et al., 2019). Of these studies, there are three sets of canine IECs that have been explored relatively in depth. In one set, canine organoids were collected and cultured from the jejunum of healthy dogs (Chandra et al., 2019; Ambrosini et al., 2020). These have been used to grow different classes of IECs, including goblet cells and tuft cells, and were viable in culture for up to twenty passages (Chandra et al., 2019; Ambrosini et al., 2020). Another set of studies collected duodenum samples and successively grew these in culture; however, they were only viable for two passages (Golaz et al., 2007; Hemphill et al., 2009). Finally, the third set of studies used cells that were isolated from the jejunum of healthy beagles and immortalised using simian virus 40 (SV40) (Weng et al., 2005; Farquhar et al., 2018). These grew in culture and formed brush borders and basal infoldings that are utilised for the active transport of fluids and ions (Weng et al., 2005). The TJ protein expression of these cells was assessed via immunohistochemistry (Weng et al., 2005; Farquhar et al., 2018). Subsequent work successfully differentiated the cells, wherein they formed enterocyte-like phenotypes (Farquhar et al., 2018). Additionally, the barrier integrity – quantified using TEER – was stronger during differentiation (Farquhar et al., 2018). Furthermore, these cells could also continue to be cultured without any losses in viability (Farquhar et al., 2018).

TEER readings are usually measured at room temperature (Feighery et al., 2008; Farquhar et al., 2018). Temperature is a well-known factor that influences TEER (Srinivasan et al., 2015; Felix et al., 2021). A mathematical model has been developed to correct TEER values taken at temperatures that are not comparable to those seen *in vivo* (Blume et al., 2010). However, commercial systems

such as the cellZscope (nanoAnalytics GmbH, Munster, Germany) can perform the TEER assays automatically, non-invasively, and in incubators set to *in vivo* comparable temperatures (Maherally et al., 2018). This system has been well-utilised across a wide variety of cell types and models to assess cellular permeability (nanoAnalytics, 2022).

To begin to better characterise and model the canine IEB, the aim was to use the previously immortalised (Weng et al., 2005) canine IEC (cIEC) line that differentiates into enterocyte-like cells (Farquhar et al., 2018). The intention was to identify the appropriate conditions to model them in the cellZscopetoprovide a robust, physiologically relevant model of the canine intestinal epithelium. It was hypothesised that cIEC could be successfully cultured in the cellZscope and that the conditions required to achieve a period of intestinal epithelial barrier stability future experiments could be defined. TEER was used as a measurement of intestinal barrier integrity and permeability as the differentiation protocols for cIEC were adapted and refined. Previous work on this cell line had recorded TEER values of 600-800 Ωcm^2 after 48 hours of differentiation (Farquhar et al., 2018). The work in this chapter was conducted to establish conditions to challenge the integrity of the cIEC monolayer and identify a period of stable TEER for future experiments. Additionally, a target was to improve TEER in comparison to what had been previously attained (i.e., TEER readings above 800 Ωcm^2). However, TEER that aligned with previous work (Farquhar et al., 2018) would still be considered a success. Therefore, criteria for successful culture in the cellZscope were defined as:

1) TEER readings above or equal to 800 Ωcm^2 after 48 hours of differentiation.
2) Consecutive hours of stable TEER readings after 48 hours of differentiation, defined as 8 hours with no statistical difference ($p > 0.1$) in the change in TEER (see Equation 2) between timepoints.

If the culture methods passed the first criterion for successful culture, they were then analysed against the second criterion for success. If they did not pass the first criterion, the experiment was rejected as unsuccessful.

4.3 MATERIALS AND METHODS

4.3.1 REAGENTS AND GENERAL CONSUMABLES

Opti-Minimal Essential Medium I Reduced Serum Media (Opti-MEM), foetal bovine serum (FBS; gamma-irradiated triple-filtered), L-glutamine, GlutaMAX Supplement (GlutaMAX), N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES), phosphate-buffered saline (PBS), TrypLE™, trypan blue, Dulbecco's Modified Eagle Media (DMEM) and Countess Cell Counting Chamber Slides were all purchased from ThermoFisher (Carlsbad, California, USA). Epidermal Growth Factor (EGF), insulin, hydrocortisone, and 0.22 µM syringe filters were purchased from Sigma Aldrich (St Louis, Missouri, USA). Transwell® inserts (24-well, 6.5 mm, polyester, 0.4 µm pore size) were initially purchased from Corning (Corning, New York, USA), but were later replaced with CellQart inserts (24-well, 6.4 mm, polyester, 0.4 µm pore size; Sabeu GmbH & Co., Northeim, Germany). Cell culture flasks and Falcon tubes were obtained from Corning Incorporated (Corning, New York, USA).

Prior to commencement, cIEC were shipped from Waltham Petcare Science Institute, UK, to AgResearch, NZ, following all local and international import and export regulations. Stock vials of cIEC were maintained at -130°C in vapor phase liquid nitrogen storage cell dewars. The import permits for the cIEC are included as Appendix 6 and Appendix 7.

Several preparations of media were used during the method refinement to differentiate the cIEC in the cellZscope system. They are detailed individually in the first instance, and a quick-reference table is provided in Table 4.13 to explain the composition differences between these various media. All media preparations were filter sterilised using 0.22 µM syringe filters.

Table 4.13 - *Compositions of cell culture media used with the canine intestinal epithelial cells (cIEC).*

Name of cell media preparation	***Composition****	***Notes***
Growth Medium	Opti-MEM cell culture media supplemented with 4% FBS, 2 mM L-glutamine, 2 mM GlutaMAX, 10 mM HEPES, 20 ng/mL EGF, 10 µg/mL insulin, and 150 nM hydrocortisone.	No changes were made from previous methods (Farquhar et al., 2018).
Base Differentiation Medium (BDM)	DMEM supplemented with 7% FBS, 2 mM L-glutamine, 2 mM GlutaMAX and 10 mM HEPES.	Initial media used for differentiation of cIEC. This was later used during the experiments assessing transepithelial electrical resistance across cIEC monolayers.
Remastered Differentiation Medium (RDM)	DMEM supplemented with 7% FBS, 2 mM L-glutamine, 2 mM GlutaMAX, 10 mM HEPES and 150 nM hydrocortisone.	Final media composition used during the differentiation of cIEC monolayers.

*Opti-MEM, Opti-minimal essential medium I reduced serum media; FBS, foetal bovine serum; GlutaMAX, GlutaMAX Supplement; HEPES, hydroxyethylpiperazine-N-2-ethane sulphonic acid; EGF, epidermal growth factor; DMEM, Dulbecco's Modified Eagle Media.

4.3.2 CELL CULTURING CONDITIONS

Cells were used in experiments between passage 27 and 37. cIECs were maintained in cell culture flasks and grown in conditions previously published (Farquhar et al., 2018), with the modification of seeding at a fixed density of 1.0 x 10^4 cells/cm^2. The cells were propagated in Opti-MEM cell culture media supplemented with 4% FBS, 2 mM L-glutamine, 2 mM GlutaMAX Supplement, 10 mM HEPES, 20 ng/mL EGF, 10 µg/mL insulin, and 150 nM hydrocortisone (HC). This media composition is henceforth referred to as Growth Medium. Incubation occurred in a cell culture incubator (HERAcell 150i CO_2 incubator, Thermo Scientific, ThermoFisher, Waltham, Massachusetts, USA). The incubator was reserved solely for the use of growing the cIEC and was kept at 32°C, 6% v/v CO_2.Growth Medium in the cell culture flasks was replaced twice a week.

Cells were subcultured at approximately 80% confluence at a fixed seeding density of 1.0 x 10^4 cells/cm^2. In a sterile class II biosafety cabinet, cell culture media was removed with a sterile stripette and discarded in Virkon. Cells were washed briefly with PBS, with 1.0 mL used for T25 flasks, 5.0 mL for T125 and 7.0 mL for T175. Depending on flask size, 5.0 mL or 7.0 mL of pre-heated TrypLE™ was added (5.0 mL for T25 and T75 flasks, and 7.0 mL for T175) to cover the bottom of the flask. TrypLE™ was used instead of trypsin because it does not alter cell immune expressions (Tsuji et al., 2017). The flask containing the TryPLE and cells was then incubated at 32°C, 6% v/v CO_2 in a humidified atmosphere for 15-20 minutes, until the cells were dissociated from the flask bottom. After 15-20 minutes cells were removed from the incubator, ethanol sprayed into the biosafety cabinet and a volume of pre-heated Growth Medium equal to that of the TryPLE™ was added. This solution was mixed by gently pipetting up and down, then all liquid was removed and placed into a sterile 15 mL Falcon tube.

The cell suspension was centrifuged (Heraeus Megafuge 8, Thermo Scientific, ThermoFisher, Waltham, Massachusetts, USA) for 5 minutes at 300 x *g*. The supernatant was removed, and the cell pellet was re-suspended in 1.0 mL of preheated growth media. 50 µL of this re-suspended pellet was removed and mixed in a 1:1 ratio with trypan blue. As trypan blue can penetrate through the damaged

membrane of dead cells but cannot pass through the membranes of live cells, only dead cells appear stained blue under a microscope, whilst live ones remain colourless (Fang and Trewyn, 2012). 10 µL of this suspension was applied to each well of a Countess Cell Counting Chamber Slide (Countess, Invitrogen, Corporation, Carlsbad, California, USA). The total number of cells, live and dead, were then counted using a Countess Automated Cell Counter (Countess, Invitrogen, Corporation, Carlsbad, California, USA). The Countess system performed cell counts and viability measurements whilst automatically correcting for the use of trypan blue. All cell counts were recorded, and the average value of the cell counts for each flask was used for further calculations.

4.3.3 DIFFERENTIATION CONDITIONS AND BARRIER INTEGRITY ASSESSMENT ASSAYS

After cell counts were performed, cIEC were seeded on 24-well inserts at a density of 2.7 x 10^5 cells/cm^2 in Growth Medium. 260 µL of cIEC suspension in Growth Medium was gently aliquoted dropwise into the apical chamber. 810 µL of media was aliquoted into the basal compartment of the inserts. The seeded inserts were then cultured for 24 hours at 32°C, 6% v/v CO_2 in a humidified atmosphere. After 24 hours the Growth Medium was removed and the cIEC were replenished with the Base Differentiation Medium (BDM). This BDM consisted of DMEM supplemented with 7% FBS, 2 mM L-glutamine, 2 mM GlutaMAX and 10 mM HEPES. The cIEC were then randomly repositioned in the 24-well plate and cultured for 48 hours at 39°C, 6% v/v CO_2 in a cell culture incubator (Sanyo MCO-18AC CO_2 incubator, Marshall Scientific Hampton, New Hampshire, USA) that was reserved solely for cIEC use.

4.3.3.1 Manual Measurement of TEER

The resistance across the cIEC layer after 48 hours in differentiation media was measured using an EndOhm TEER cup (World Precision Instruments, Sarasota, Florida, USA) connected to an EVOM voltohmmeter (World Precision Instruments, Sarasota, Florida, USA). 1.0 mL of BDM that was preheated to 39°C was added to the TEER cup. The inserts containing the differentiated cIEC

monolayers were transferred from the insert plate to the TEER cup using sterile tweezers. The TEER cup lid that contained the top electrode was placed on top of the TEER cup. The resistance was measured with the voltohmeter and converted to TEER (Ω and Ωcm^2, respectively) with the use of the Equation 1. TEER readings were performed without equilibration to room temperature, i.e., readings were performed with media kept as close to 39°C as possible, to better simulate temperatures *in vivo*.

Equation 1: *Calculation of TEER from raw Ohmic resistance*

$$TEER\ (\Omega cm^2) = Resistance\ (\Omega) \times surface\ area\ of\ insert\ membrane\ (cm^2)^*$$

**The Corning Transwells® and CellQart inserts had surface areas of 0.30 and 0.33 cm^2, respectively.*

4.3.3.2 Automated TEER Measurement

Automated TEER measurement was performed in the cellZscope apparatus. Each component of the cellZscope that was autoclavable was sterilised at least 48 hours before use. The parts that were not autoclavable were sterilised with 70% ethanol before use. Following autoclaving or sterilisation with 70% ethanol, the cellZscope was assembled in a sterile class II biosafety cabinet the night before TEER measurement.

After 24 hours in Growth Medium, the Growth Medium in the inserts was removed and replaced with 260 µL of BDM. Each basal well in the cellZscope was filled with 810 µL of BDM. The inserts were placed in random positions in the cellZscope. The cellZscope apparatus was then incubated at 39°C, 6% v/v CO_2 in a humidified atmosphere. The cellZscope unit was connected to a cellZscope controller and monitoring PC via pre-existing modifications to the incubator. The TEER was measured *in situ* by the cellZscope software (version 4.3.1, nanoAnalytics GmbH, Munster, Germany) every 30 minutes. Initially this was to be captured over a 72-hour period, to build a data profile of the cIEC during differentiation.

4.3.3.3 Refinement of Differentiation Conditions

During the experiments, the differentiation conditions of the cIEC had to be refined to achieve the most appropriate conditions for their differentiation in the cellZscope. It was planned for future

analyses to be performed after 48 hours of differentiation, as per previous methods (Farquhar et al., 2018). One of the initial aims was to capture the entire differentiation period by placing the inserts containing cIEC into the cellZscope immediately after change from Growth Medium into BDM. However, initially the cIEC did not maintain TEER past the initial 24-hour period of differentiation. To ascertain if it was the media composition that needed changing, times in Growth Medium or another factor, variables as defined by Table 4.14 were investigated as part of the refinement of these conditions.

Table 4.14 - *Factors analysed as potentially causing impacts to transepithelial electrical resistance (TEER) of differentiating canine intestinal epithelial cell (cIEC) monolayers whilst in a cellZscope system for the automated measurement of TEER.*

Variable	***Comments****
Media change timepoint	Media was changed at 24, 36, 48 and 72 hours after initial placement of inserts in the cellZscope system.
Presence of antibiotics	Included 0.5 µg/µL of Penicillin-Streptomycin (PenStrep; ThermoFisher, Carlsbad, California, USA) in the Base Differentiation Medium (BDM; DMEM supplemented with 7% FBS, 2 mM L-glutamine, 2 mM GlutaMAX and 10 mM HEPES).
Time in Growth Medium[¥] in inserts before being placed into cellZscope system	Attempted 48 hours of growth time instead of the usual 24 hours.
Seeding density of cIEC in inserts	Attempted to seed the cIEC at higher densities in the inserts.
Pre-warming cellZscopes	Ordinarily, cellZscopes would be assembled and left in the sterile biosafety cabinet until inserts containing cIEC were added and then the cellZscope would be transferred to the incubator. Instead, the cellZscopes were assembled and then pre-warmed to the required 39°C. Afterwards, they were removed from the incubator, the cIEC were added and then the cellZscope was transferred immediately back into the incubator.
Gradual increase in temperature	As cells were transferred from 32°C in the growth phase to 39°C during differentiation, a gradual increase in temperature from 32°C to 39°C in the first hour of differentiation was tried to test the hypothesis that the sudden change in temperature was responsible for the decline in TEER.
Addition of insulin and/or hydrocortisone to differentiation media	When the cIEC were first isolated and immortalised, it was discovered that the increase of hydrocortisone in the TEER-measuring solution led to an increase in TEER, and insulin in the differentiation media showed similar results (Weng et al., 2005). The concentrations of insulin and hydrocortisone used in the method optimisations were the same as those used in the Growth Medium (10 µg/mL and 150 nM, respectively).
Initial differentiation outside of the	The inserts were allowed to differentiate in the inserts for a period of 24- or 48- hours before being placed

Variable	***Comments****
cellZscope system	into the cellZscope system. The inserts were incubated at 39°C.
Removal of hydrocortisone for experimental period	The presence of hydrocortisone can lower the expression of toll-like receptor 4, a pattern recognition receptor involved in the immune response (Meng et al., 2015; Zouboulis et al., 2021). Planned experiments involved evaluating the response of this PRR to specific challenges. Therefore, to ensure there were no potential changes induced from the presence of hydrocortisone during challenges, it was deemed necessary to investigate if the cIEC would have periods of stable barrier integrity without hydrocortisone present after differentiation.

* DMEM, Dulbecco's Modified Eagle Media; FBS, foetal bovine serum; GlutaMAX, GlutaMAX Supplement; HEPES, hydroxyethylpiperazine-N-2-ethane sulphonic acid; EGF, epidermal growth factor.

¥ Growth Medium; Opti-minimal essential medium I reduced serum media (Opti-MEM) supplemented with 4% FBS, 2 mM L-glutamine, 2 mM GlutaMAX, 10 mM HEPES, 20 ng/mL epidermal growth factor (EGF), 10 µg/mL insulin, and 150 nM hydrocortisone.

To evaluate if the TEER readings met the second criteria for successful culture in the cellZscope, data was expressed as a percentage of change in TEER over time. The TEER measurement taken before BDM was changed into fresh culture media was classed as the initial TEER. As the initial TEER for each insert was different, this expression of change in TEER over time was calculated for each well using the following equation:

Equation 2: *Calculation of the change in TEER, expressed as a percentage of the original TEER reading.*

$$\text{Change in TEER (\%)} = \frac{(TEER_{current} - TEER_{initial})}{TEER_{initial}} \times 100.$$

4.4 STATISTICAL ANALYSES

The statistical analyses performed using RStudio (Version 1.2.5001, RStudio, Boston, MA, USA). The effect of treatment on change in TEER over time was compared using a repeated measures mixed-effects model to account for the fact that the same monolayers were measured over time. Models were fitted by the maximum-likelihood (ML) method using the *nlme* package (Pinheiro J, 2022) in R. The statistical model included the effect of treatment, time, and their interaction as fixed effects, and the Transwell inserts nested within blocks (where one run of an experiment was considered a block) as a random effect. Post-hoc pairwise comparisons were performed via the Bonferroni correction, which were applied using estimated marginal means using the *emmeans* package (Lenth, 2022). The false discovery rate (q) was applied to the tests of the marginal means, with differences considered significant when $q < 0.05$.

Data visualisation was performed using Genstat (19th Edition, VSN International, Hemel Hempstead, UK). Statistical significance is reported as a p value < 0.05, whilst $p < 0.1$ was considered a trend. All data are presented as mean ± SEM unless otherwise noted. All experiments were performed in triplicate with a minimum of three replicates per experiment, unless otherwise noted.

4.5 RESULTS

4.5.1 INITIAL DIFFERENTIATION CONDITIONS DID NOT HAVE TEER READINGS ABOVE 800 ΩCM^2 AFTER 48 HOURS OF DIFFERENTIATION

The first experiment assessed the TEER of differentiating cIEC over time in the cellZscope with no changes to the previous methods. The results are presented as Figure 4.24. TEER changed significantly over time ($p < 0.001$). However, at 48 hours of differentiation, the mean TEER of the cIEC monolayer was 769.94 ± 74.78 Ωcm^2 and continued to decline sharply. The first criterion of successful culture in the cellZscope was to have a TEER above 800 Ωcm^2 after 48 hours of culture. This experiment failed to meet this criterion. Thus, it was deemed necessary to refine the existing methods to achieve successful culture of the cIEC in the cellZscope.

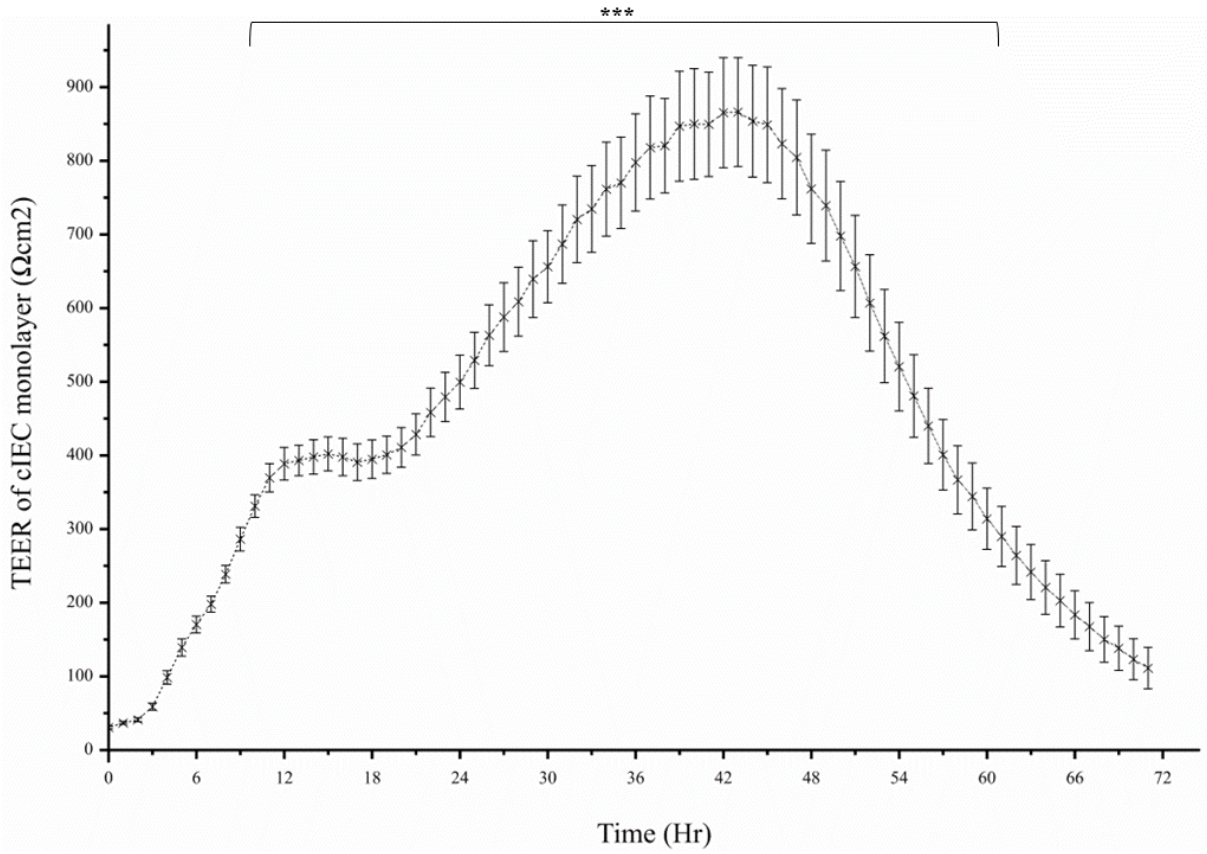


Figure 4.24 *– Mean (± SEM) transepithelial electrical resistance (TEER) from canine intestinal epithelial cells (cIECs) differentiated in the cellZscope.N = 6. Significant differences to the TEER at 0 hours are represented by *, where *** $p < 0.001$.*

4.5.2 INITIAL METHOD REFINEMENTS DID NOT HAVE TEER READINGS ABOVE 800 ΩCM2 AFTER 48 HOURS OF DIFFERENTIATION

The subsequent experiment assessed the impacts of changing the methods of growth or differentiation on the cIEC, in comparison to a control group where methods were not changed. Method alterations were an inclusion of 0.5 µg/µL of Penicillin-Streptomycin (PenStrep) in the differentiation media (Antibiotics), cIEC that were incubated in growth conditions for 48 hours instead of 24 hours (Extended Growth) and cIEC that were differentiated in a cellZscope that had been preheated to 39°C (Preheated). The effects of the different, refined methods (treatment) over time on the TEER of the cIEC monolayer was assessed and the results are shown in Figure 4.25. The effects of treatment over time were shown to significantly change TEER ($p< 0.001$). However, all

treatments in this experiment failed to meet the first criterion for success, i.e., the TEER after 48 hours of culture was below 800 Ωcm^2.

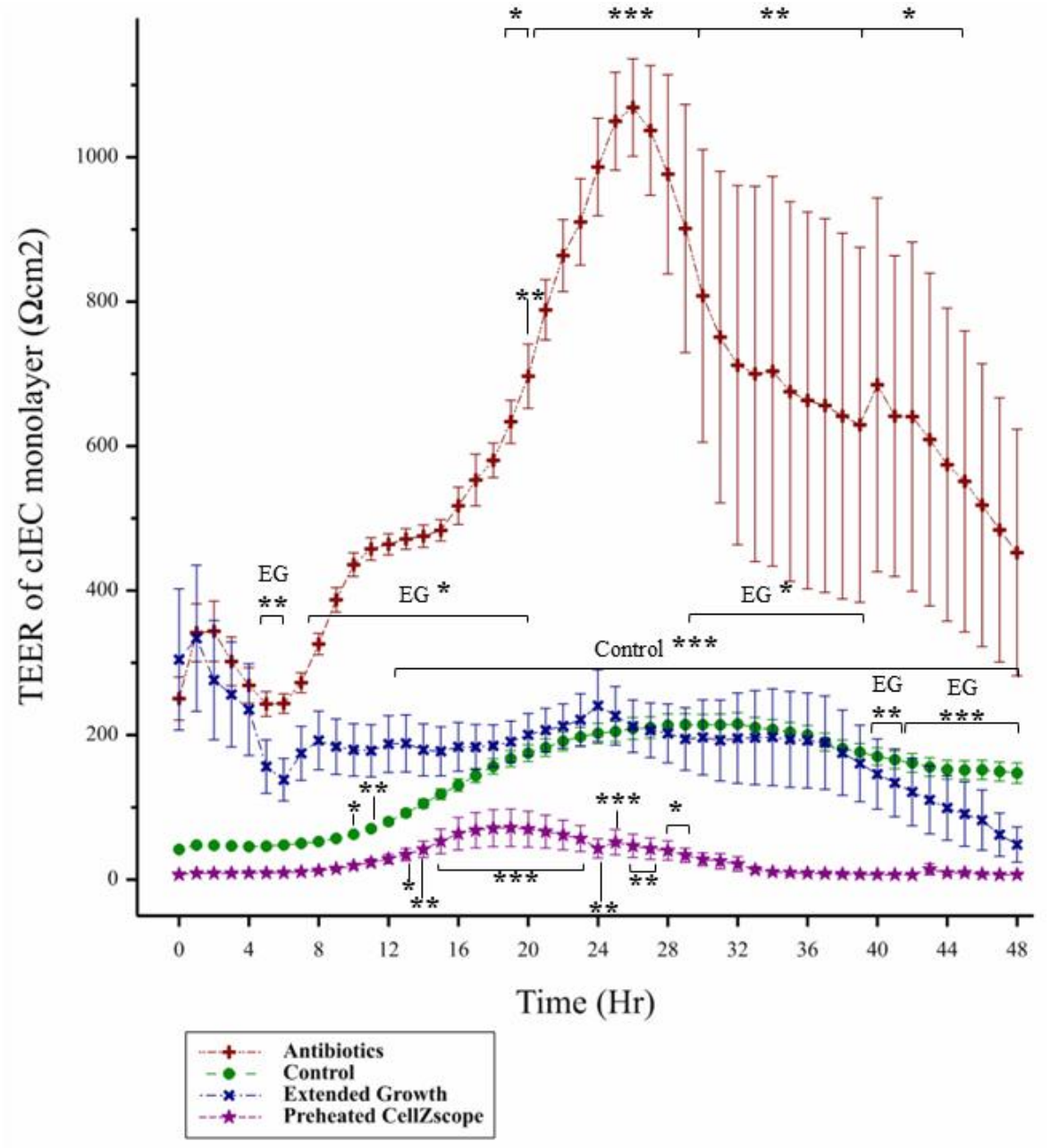


Figure 4.25- *Mean (± SEM) transepithelial electrical resistance (TEER) from canine Intestinal Epithelial cells (cIECs) under different method alterations.Method alterations were an inclusion of 0.5 µg/µL of Penicillin-Streptomycin in the differentiation media (Antibiotics), cIEC that were incubated in growth conditions for 48 hours instead of 24 hours (Extended Growth / EG) and cIEC that were differentiated in a cellZscope that had been preheated to 39°C. These were run in parallel to cIEC with no alterations in the methodologies (Control).*

*For each treatment, n > 9. Significant differences to the TEER at Time 0 for that treatment are represented by *, where * p < 0.05, ** p < 0.01 and *** p < 0.001.*

4.5.3 cIEC DIFFERENTIATED OUTSIDE THE CELLZSCOPE IN THE PRESENCE OF HYDROCORTISONE HAD STABLE TEER READINGS ABOVE 800 ΩCM2 AFTER 48 HOURS OF DIFFERENTIATION

The subsequent experiment analysed the impacts of differentiating the cIEC for 24 hours outside the cellZscope before transfer into the cellZscope, and the inclusion of extra components in the BDM. The effects of these changes on the TEER over time are displayed in Figure 4.26. There was a significant impact on TEER caused by treatment over time ($p< 0.001$). However, none of these treatments had TEER readings above 800 Ωcm^2 after 48 hours of culture. Thus, they did not meet the first criterion of successful culture in the cellZscope.

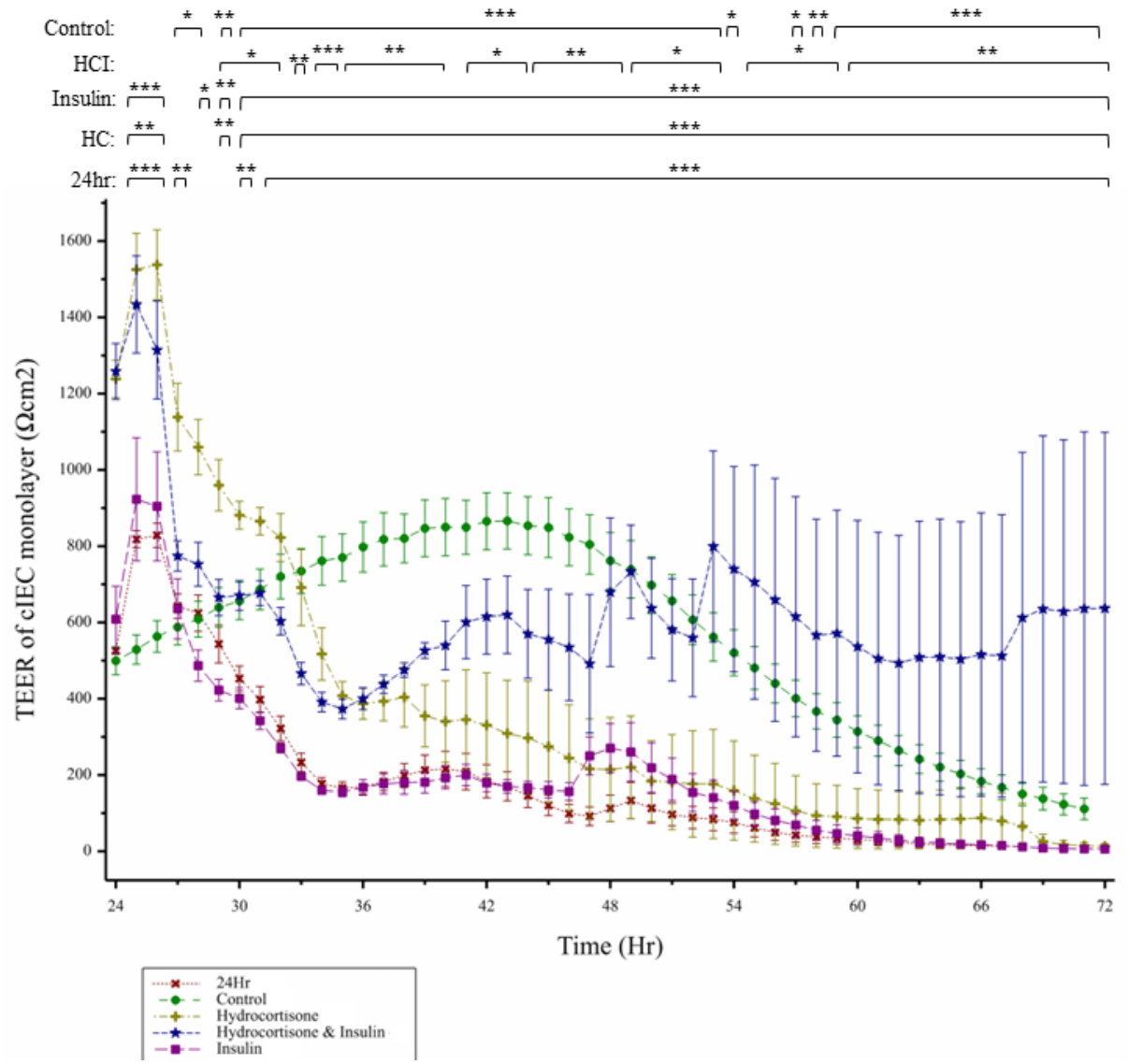


Figure 4.26 *- Mean (± SEM) transepithelial electrical resistance (TEER) from canine Intestinal Epithelial cells (cIECs) under different method alterations.All alterations were differentiated in the inserts, outside of the cellZscope for 24 hour before being placed into the cellZscope and TEER monitored (24Hr). Additionally, the differentiation media during this time was altered to contain 150 ng/mL hydrocortisone (Hydrocortisone / HC),10 µg/mL insulin (Insulin), or both hydrocortisone and insulin at these concentrations (Hydrocortisone and Insulin / HCl). These were run in parallel to cIEC with no alterations in the methodologies (Control). N = 5 for Insulin, n = 6 for all other treatments. Significant differences to the TEER at time 0 are represented by *, where * p < 0.05, ** p < 0.01 and *** p < 0.001.*

The subsequent experiment analysed the impacts of differentiating the cIEC for 48 hours outside the cellZscope before transfer into the cellZscope, and the inclusion of extra components in

the BDM. As the unchanged methods had repeatedly fallen outside the success criteria, they were not included in this experiment. The effects of these changes on the TEER over time are presented in Figure 4.27. There was a significant impact on TEER caused by treatment over time (p = 0.005). Differentiating cIEC outside of the cellZscope with the inclusion of 150 ng/mL HC in the BDM resulted in TEER readings that were above 800 Ωcm^2 for the entire period of TEER assessment. Additionally, the TEER after 64 and up to 72 hours was no different to the TEER at 48 hours ($p > 0.1$). The TEER of cIEC differentiated outside of the cellZscope with 150 ng/mL HC and 10 µg/mL insulin resulted in TEER readings above 800 Ωcm^2 from 48 hours until 62 hours of differentiation. Both of these treatments resulted in greater TEER compared to other treatments ($p< 0.05$). Thus, the first criterion of successful culture of the cIEC in the cellZscope (TEER readings ≥ 800 Ωcm^2 after 48 hours of differentiation) was achieved by these treatments.

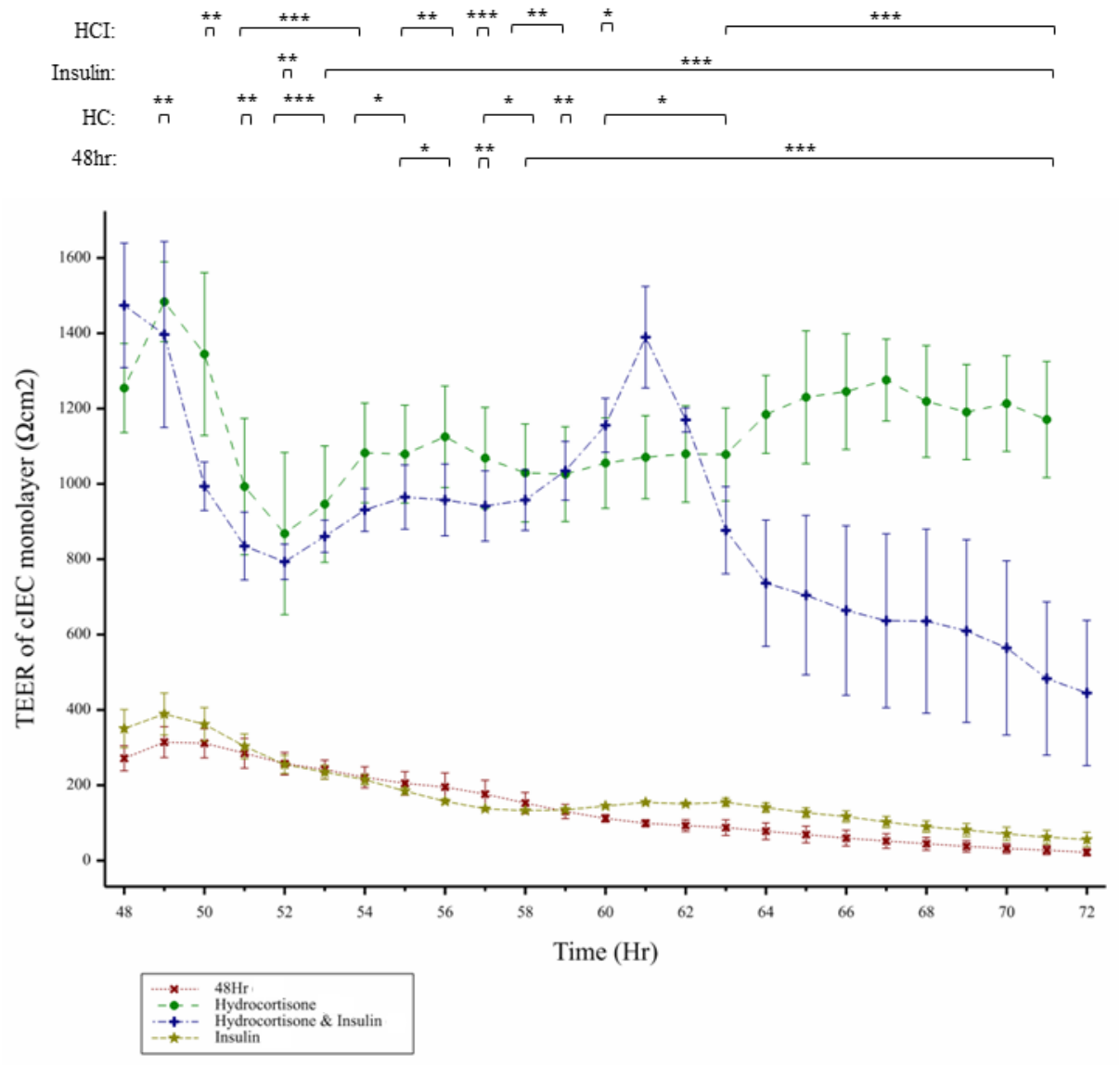


Figure 4.27 *- Mean (± SEM) transepithelial electrical resistance (TEER) from canine Intestinal Epithelial cells (cIECs) under different method alterations.All cells were differentiated in the inserts, outside of the cellZscope for 48 hours before being placed into the cellZscope and TEER monitored (48Hr). Additionally, the differentiation media during this time was altered to contain 150 ng/mL hydrocortisone (Hydrocortisone / HC),10 µg/mL insulin (Insulin), or both hydrocortisone and insulin at these concentrations (Hydrocortisone and Insulin / HCI). N = 6 for all treatments. Significant differences to the TEER at time 0 are represented by *, where * p < 0.05, ** p < 0.01 and *** p < 0.001.*

As two treatments passed the first criterion for successful culture of cIEC in the cellZscope, the impact of these treatments over time on the change in TEER was assessed. This would determine if they passed the second criterion for successful culture. This is presented in Figure 4.28. There was a

significant change in TEER over time caused by these treatments ($p < 0.001$). The second criteria for successful culture of the cIEC in the cellZscopes was a period of 8 hours where there was no difference in the change in TEER ($p > 0.1$). In the cIEC differentiated for 48 hours with HC in the BDM, two time periods were identified that met this criteria: between 55 and 64 hours, and between 57 and 66 hours of differentiation. In the cIEC differentiated for 48 hours with HC and insulin in the BDM, there was one time period that met this criteria: between 51 and 60 hours of differentiation.

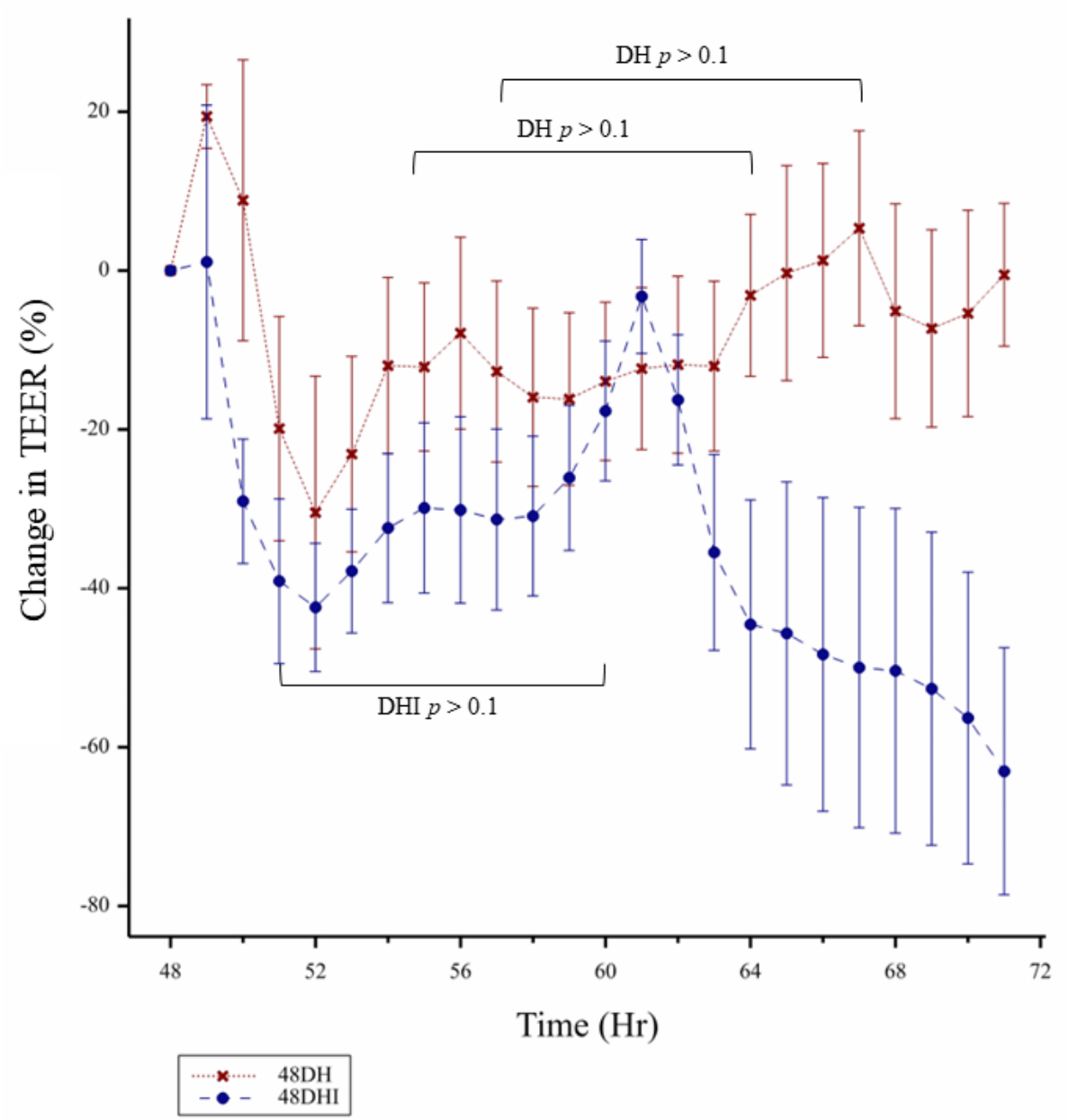


Figure 4.28 – *Mean (± SEM) change in transepithelial electrical resistance (TEER) from canine Intestinal Epithelial cells (cIECs) differentiated for 48 hours in the presence of hydrocortisone (48DH) or hydrocortisone*

and insulin (48DHI) before TEER analysis.Change in TEER defined as a percentage change compared to the initial TEER value for the same treatment, taken at 48 hours. N = 6 for each treatment. The experimental aim was to identify blocks of time where TEER did not change (p > 0.1). These periods are identified, with the treatment group they belong to.

Both treatments met the second criteria for successful culture of the cIEC in the cellZscope. Because there were two timeframes with stable TEER identified, the differentiation of the cIEC for 48 hours with hydrocortisone in the BDM was selected for use going forwards. Thus, the new differentiation conditions were defined as 48 hours of differentiation at 39°C in DMEM supplemented with 7% FBS, 2 mM L-glutamine, 2 mM GlutaMAX, 10 mM HEPES and 150 nM hydrocortisone. Hereafter this is referred to as the remastered differentiation medium (RDM).

4.5.4 REMOVAL OF HYDROCORTISONE DURING TEER ANALYSIS DID NOT ALTER TEER

Because hydrocortisone can alter PRR expression in cells, a final experiment was conducted to investigate the potential of performing the TEER analysis in BDM rather than RDM. The rationale was that this would remove this as a possible complication or interference for experiments in Chapter Five. Success was defined as there being no significant difference ($p > 0.1$) between treatment groups.

The impacts of having the cIEC in BDM or RDM during TEER analysis was assessed, outlined in Figure 4.29. There was no difference ($p = 0.301$) on the TEER results resulting from the different differentiation media compositions. Additionally, the TEER of the cIEC in BDM remained above 800 Ωcm^2 until 71.5 hours of differentiation. The TEER of cIEC in RDM remained above 800 Ωcm^2 at all timepoints TEER was measured. Thus, criteria one was also achieved in this experiment.

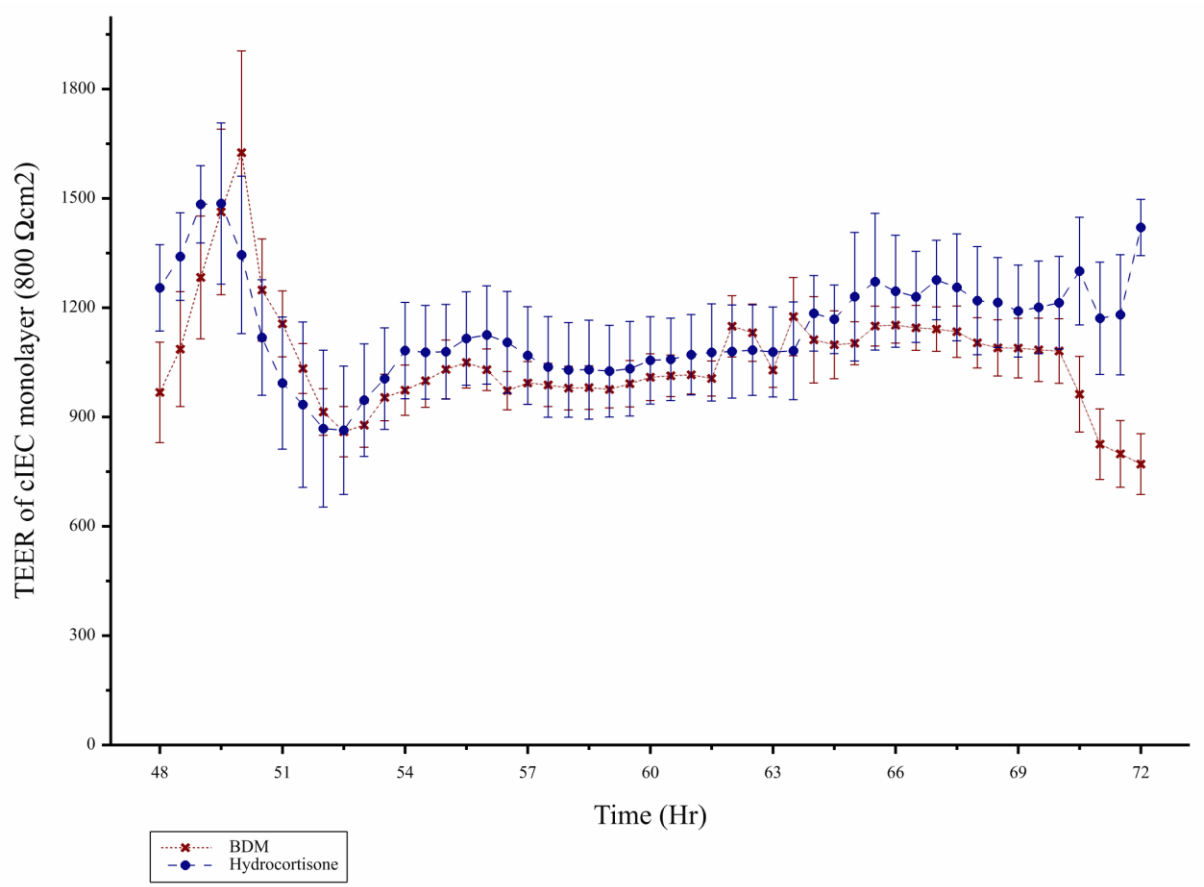


Figure 4.29- *Mean (± SEM) transepithelial electrical resistance (TEER) from canine Intestinal Epithelial cells (cIECs) in base differentiation media (BDM) during TEER analysis, or with the addition of 150 ng/mL hydrocortisone to BDM (Hydrocortisone).N (BDM) = 5, n (hydrocortisone) = 6. There were no significant differences between treatments.*

Subsequently, the effects of the treatment over time on the change in TEER was assessed to see if it passed criteria two for successful culture of the cIEC in the cellZscope, seen in Figure 4.30. The second criteria was that there had to be period of 8 hours or more where there was no difference in the change in TEER ($p > 0.1$). There was no impact of treatment over time on the change in TEER ($p = 0.198$).

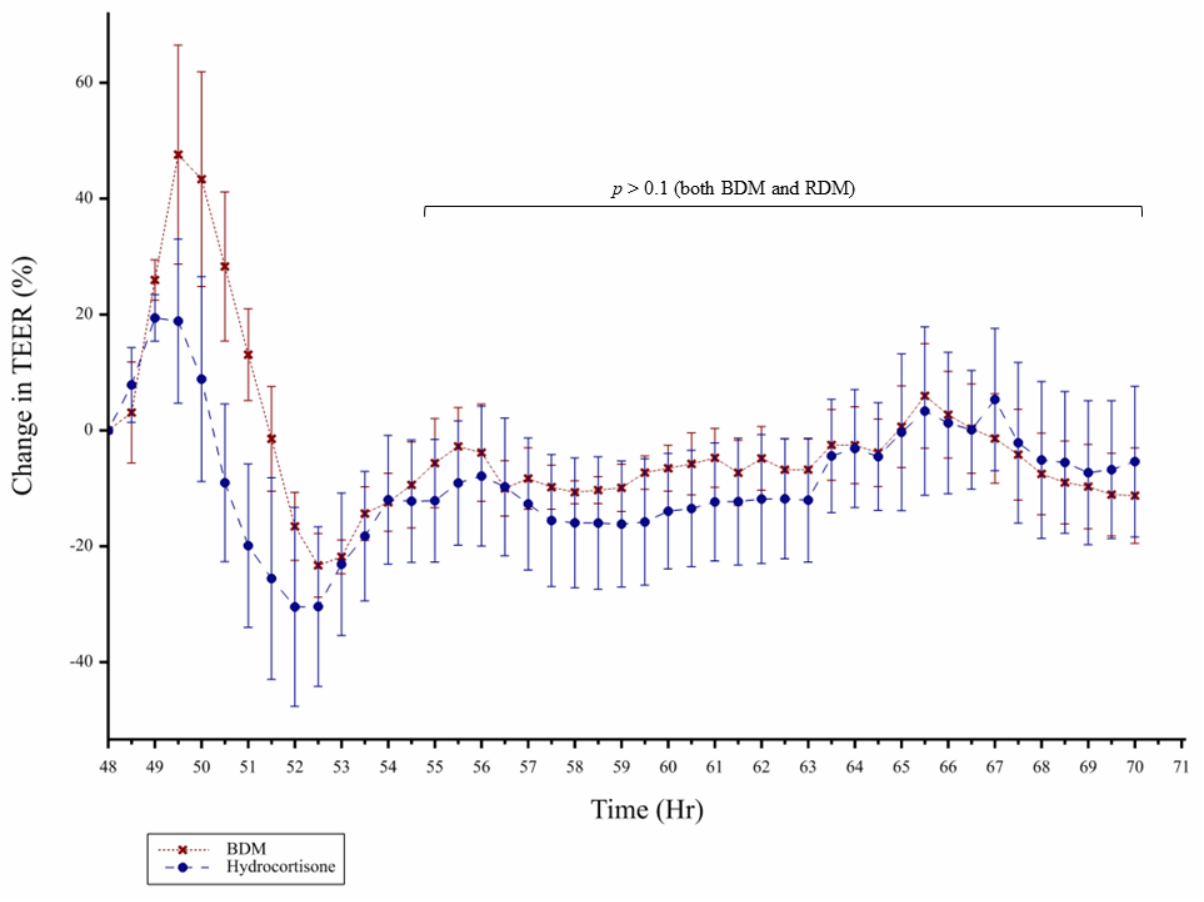


Figure 4.30 *- Mean (± SEM) change in transepithelial electrical resistance (TEER) from canine Intestinal Epithelial cells (cIECs) in base differentiation media (BDM) during TEER analysis, or with the addition of 150 ng/mL hydrocortisone to BDM (hydrocortisone).Change in TEER defined as a percentage change compared to the initial TEER value for the same treatment, taken at 48 hours. N (BDM) = 5, n (hydrocortisone) = 6. There were no significant differences between treatments.*

There was no difference ($p > 0.1$) in the change in TEER between 54 hours and 70.5 hours of differentiation for BDM and RDM. The second criterion of sucessful culture was achieved. Therefore, the updated differentiation conditions were defined as 48 hours of differentiation at 39°C in RDM, whereafter RDM would be removed and replace with BDM.

4.5.5 Refined Methods had Stable TEER readings above 800 Ωcm2 after 48 hours of differentiation when assessed manually

Initial project plans were to continue monitoring exclusively in the automated system. However, due to supply issues that arose from the Covid-19 pandemic, it became necessary for TEER collection to be conducted manually. Because of this, a final experiment was performed using the differentiation conditions refined in the cellZscope, with TEER values collected manually. The TEER changed significantly over time ($p < 0.001$). (See Figure 4.31). All timepoints bar 54 and 96 hours of differentiation had TEER above 800 Ωcm^2.

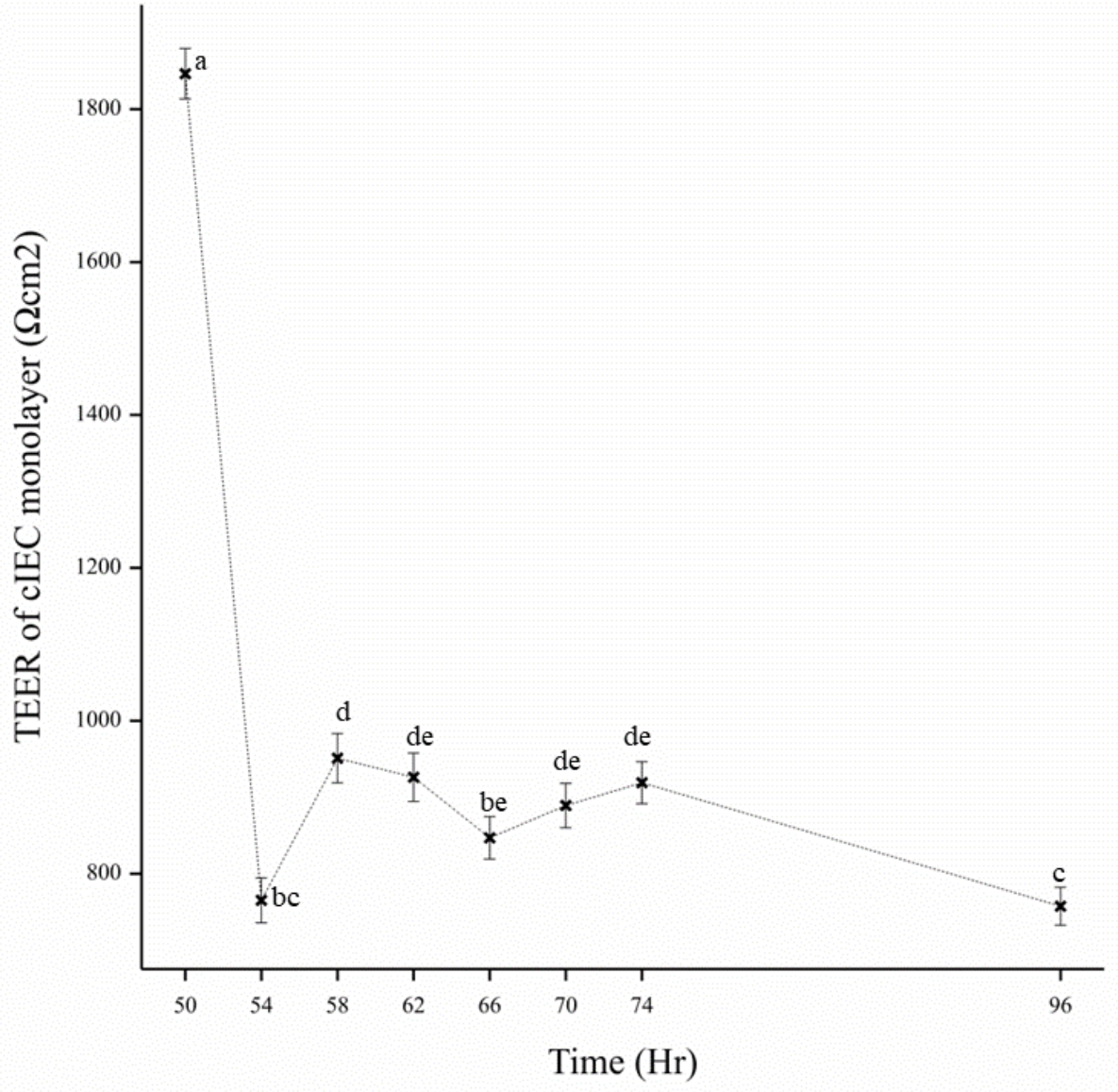


***Figure 4.31** – Mean (± SEM) transepithelial electrical resistance (TEER) from canine Intestinal Epithelial cells (cIECs).TEER measured manually, n = 8. Timepoints that do not share a common letter denotes a significant ($p < 0.05$) difference in TEER.*

Finally, the manually collected TEER readings were assessed to investigate if there was a period of stable TEER, which can be seen in Figure 4.32. TEER changed significantly over time ($p < 0.001$). There was no difference in the change in TEER between 62 and 74 hours of differentiation ($p > 0.1$).

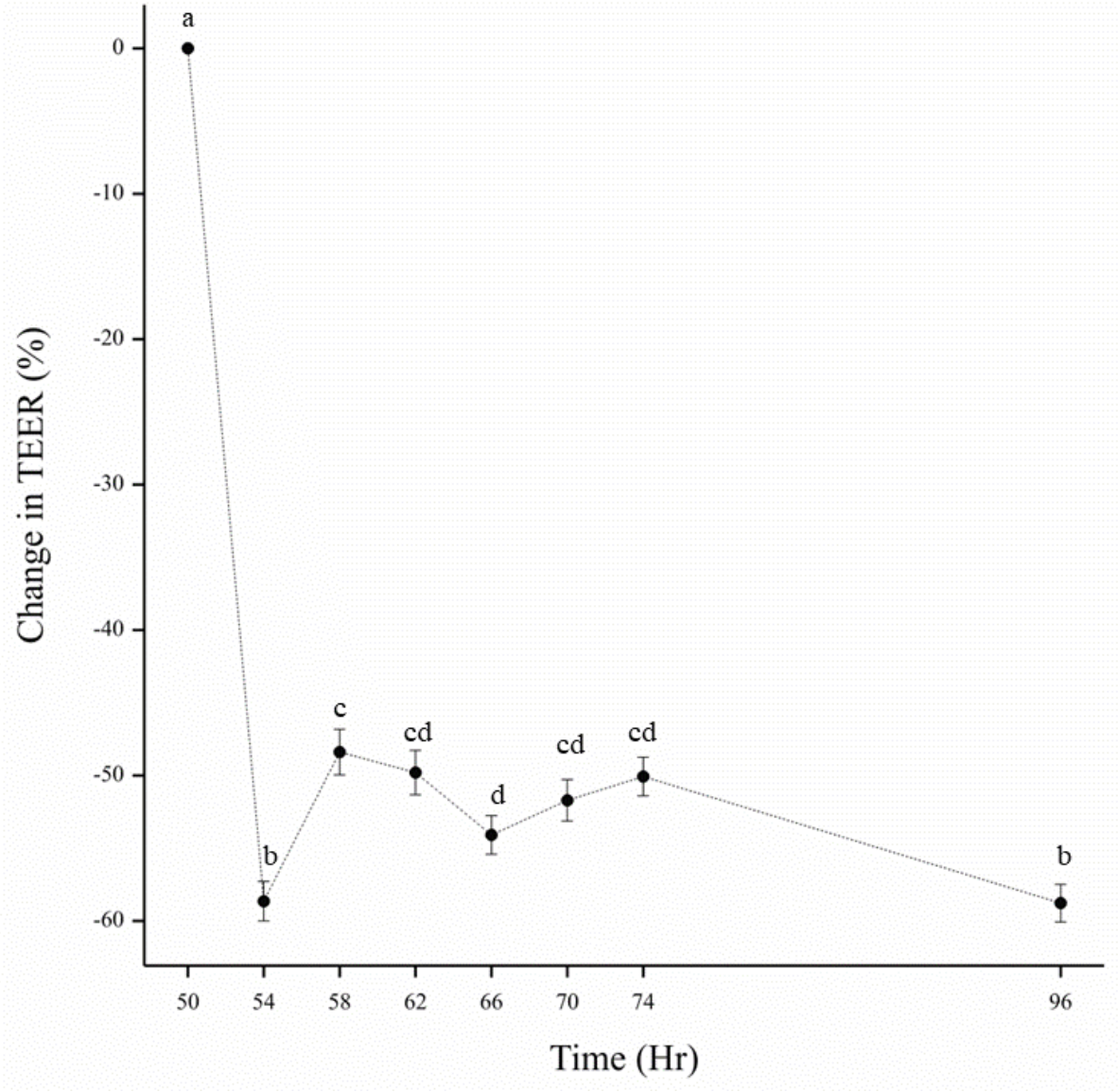


Figure 4.32- *Mean (± SEM) change in transepithelial electrical resistance (TEER) from canine Intestinal Epithelial cells (cIECs) TEER measured manually, n = 8.Timepoints that do not share a common letter denotes a significant ($p < 0.05$) difference in the change in TEER.*

As there was no significant difference in the change in TEER between 62 – 74 hours of differentiation, the TEER was therefore able to be considered stable. Thus, it was decided that further experiments investigating the impacts of different challenges on the cIEC barrier stability, Chapter 5, would be conducted during 58 and 74 hours of cIEC differentiation. Therefore, for both automated and manual collection of TEER, the refined methods of differentiating cIEC at 39°C, 6% v/v CO_2 for 48 hours in RDM, followed by a change into fresh BDM could be used.

4.6 DISCUSSION

This chapter showed that the cIEC could be successfully cultured in the cellZscope. During these experiments, it was hypothesised that cIEC could be successfully cultured in the cellZscope, and that the conditions required to achieve a period of intestinal epithelial barrier stability for future experiments could be defined. The criteria for these hypotheses were: a TEER of above 800 Ωcm^2 and a period of eight hours of more where there was no statistically different change in TEER between timepoints. Both criteria were achieved by a refinement of the differentiation conditions of the cIEC. These refined differentiation conditions were then used to establish and demonstrate periods of stable TEER that could then be used for future experiments.

The inclusion of HC in the RDM resulted in increased TEER, consistent with other dog and human intestinal epithelial cell models in the literature (Weng et al., 2005; Fischer et al., 2014). Additionally, these effects of HC on TEER have also been observed in human brain microvascular endothelial cells, suggesting that these impacts of HC are not intestine-specific (Furihata et al., 2015). Hydrocortisone can lower the expression of TLR4 and impacts the gene expression of TEER-reducing G-protein signalling and IL-17 signalling pathways, thereby potentially suppressing increases to barrier permeability (Lu et al., 2011; Meng et al., 2015; Setiadi et al., 2019; Byrne et al., 2020; Zouboulis et al., 2021). Additionally, the HC-induced suppressive effects on the pro-inflammatory immune response respond in a dose-dependent manner in both *in vitro* and *in vivo* experiments (Keh et al., 2003; Wang et al., 2004; Olnes et al., 2016; Salimiyan, 2022). In other cell culture models, the presence of HC has extended cell viability but impaired growth (Rouiller et al., 2012). These impacts were not assessed in these experiments and could be of interest in future experiments. Furthermore, low concentrations of HC were found to increase the concentrations of IL-6 over time in human adipose-derived mesenchymal stromal/stem cells, compared to control cells (Salimiyan, 2022). HC was found to be a critical component of the media composition during the initial differentiation of the cIEC in these experiments. Future experiments could characterise the cIEC during the initial 48 hours of differentiation in either BDM or RDM to assess if there are any altered immune profiles in the

cIEC from the addition of HC. This would better allow for hypotheses to be formed as to the potential longer-term impacts of the inclusion of HC in the RDM.

Cell culture by nature has been demonstrated to be difficult to reproduce between laboratories, even when following published protocols (Baker, 2016; Liu et al., 2019). Similarly, each cell line can require a specific blend of micronutrients to ensure viability, thereby increasing the number of inherent variables to work with (Arigony et al., 2013). With this consideration it is unsurprising that adapting the cIEC to the cellZscope required further method development to ensure the cIEC could be differentiated and analysed successfully. TEER values can be influenced by a variety of factors, such as cell medium composition, passage number of cells, the position of, and the electrodes used for TEER analysis and the maturity of the tight junction proteins (Weng et al., 2005; Srinivasan et al., 2015; Felix et al., 2021). Additional measurements of the integrity of the cIEC barrier, such as the use of 4 kDA fluorescein isothiocyanate (FITC)-dextran to assess permeability, would have been beneficial to include in the overall assessment of the cIEC's performance.

The study could have also benefitted from investigating the impacts of the RDM outside of the cellZscope, by collecting TEER from cIEC run inside and out of the cellZscope in parallel. Though this would have enabled evaluation of the cellZscope itself as a variable, this theoretical investigation would have been difficult to gather TEER values in a perfectly comparable method. This is because the cellZscope was able to remain at 39°C throughout the experiment, whilst the manual readings would have to be removed from the incubator, thereby introducing temperature fluctuation as a variable. During the initial cellZscope validation it was noticed that there was a consistent increase in TEER when the cells were removed from, and then placed at 39°C (data not shown). Caco-2 and HT-29 cells (both human origin) have demonstrated that heat stress, as assessed by heat shock protein expression, caused decreases in TEER, though no decrease in tight junction expressions associated with intestinal permeability (Lian et al., 2021). Measuring expression of heat shock proteins could determine if these are influencing TEER results.

One of the limitations of this study was that the manual measurements for Section 4.5.5 could not be recorded taken over the entire 48-hour period. For logistical reasons it was not possible to conduct this with a sole operator. Had there been no stability in the TEER between 50-74 hours, the barrier stability between 74 and 96 hours would have been investigated. As it stands it is an interesting possible future direction of exploration – in these refined conditions, the TEER of the cIEC is not significantly different at 96 hours as it is to 54 hours. Furthermore, the TEER values recorded at these timepoints (765.28 ± 29.40 and 757.53 ± 24.84 Ωcm^2, respectively) are similar to the values reported previously in these cells (~800 Ωcm^2 after 48 hours of cIEC differentiation) (Farquhar et al., 2018) and have documented a 24-hour improvement in the differentiation of the cIEC. Explored further, this could open pathways into investigating the physiological effects of differentiation of the cIEC over time – for example, investigating the maturity of tight junctions, or the paracellular flux over time. Additionally, the work conducted in this chapter was limited by the initial project goals of establishing the cIEC in the cellZscope. By the time that the method refinement had highlighted a new protocol to follow for the differentiation of cIEC in the cellZscopes, the project was unable to continue using the cellZscopes. This does highlight how future work using the cellZscope needs to focus on more than TEER results to critique and evaluate the model as a robust *in vitro* model of the canine intestine. This includes the use of FITC-dextrans to assess permeability, and cell viability assays. However, for the purposes of this project, this method refinement was usable going forwards to perform assays with manual TEER collection. By investigating responses in the cIEC using these refined protocols, it opens up the potential to compare results captured manually to those captured with automatic methods, thereby future proofing the modelling capabilities of these cells.

4.7 CONCLUSIONS

The work in this chapter refined the differentiation conditions of the cIEC and produced stable TEER in an automated TEER collection system, and in traditional manual methods. Thus, overall it has established a new protocol for the differentiation of cIEC that is used going forwards in this project, and can be used for any future work involving the cIEC.

The next step in the PhD workflow was to use these refined methods to challenge the cIEC with bacterial ligands and SCFA. Chapter Five details the impacts of the stimulation of cIEC with LPS, butyrate and a combination of the two.

4.8 ACKNOWLEDGEMENTS FOR CHAPTER FOUR

In addition to those people highlighted in the acknowledgements section of this thesis, for this chapter in particular I would also like to acknowledge the work of Peter Green and Rina Hannaford of AgResearch for the design of the R code used for results analysis, Melanie van Gendt and Hilary Dewhurst of AgResearch and Emma McCluskey of Waltham Petcare Science Institute for their expertise during the initial method optimisation.

CHAPTER FIVE

CHARACTERISATION OF THE INFLAMMATORY RESPONSE OF cIEC CHALLENGED WITH LPS AND/OR BUTYRATE*

**Selected Material from this chapter will be submitted for publication.*

5.1 ABSTRACT

Changes to diet can alter the GIT microbiota and the metabolites formed from the bacterial fermentation of dietary nutrients. Investigating the IEC response (i.e., the changes to cellular barrier integrity and the activation of immune cascades) to these bacteria and fermentive products can help understand how diet changes can impact host health. No studies have profiled the response of the dog IEC to bacterial challenges. By stimulating the cIEC with 250µg/mL LPS, 1 mM of butyrate, or a combination of LPS and butyrate compared to untreated controls, the work in this chapter sought to understand how diet-based modulation of the GIT microbiota may impact host health in the dog. It was hypothesised that LPS would cause a pro-inflammatory response, reduce concentrations of TJs associated with barrier function, and weaken barrier integrity. Butyrate was hypothesised to cause an anti-inflammatory response, strengthen concentrations of TJs associated with barrier function, and strengthen barrier integrity. Furthermore, it was also hypothesised that butyrate would mitigate the pro-inflammatory effects of LPS. LPS treatment reduced TEER over time and caused increases in the protein abundance and gene expression of IL-8 and CCL2 compared to untreated controls. Additionally, LPS treatment caused increases in the gene expression of interferon gamma-induced protein (IP)10, and increased protein abundances of keratinocyte chemotactic (KC)-like in apical and basal cell media compared to untreated cIEC. Butyrate treatment caused reduced protein expression of CCL2 in apical cell culture media and increased protein expression of IP-10. Additionally, butyrate reduced the LPS-induced IL-8 and KC-like increases to protein abundances in basal cell culture media compared to untreated canine IEC. Furthermore, butyrate was found to reduce and restore LPS-induced intestinal barrier permeability. However, butyrate and LPS in combination caused an increase in CCL2 gene expression compared to treatment with butyrate and LPS alone. Thus, this study has shown that butyrate can mitigate the LPS-induced inflammatory response in the canine intestine. Finally, this study also demonstrated for the first time that IP-10 RNA can be detected when the cytokine concentrations of IP-10 are undetectable.

5.2 INTRODUCTION

The IEB is a vanguard for health in all animals (Ahn et al., 2016; Assimakopoulos et al., 2018). TJs, the selectively permeable connections between the monolayer of IECs that form this barrier, simultaneously protect against unwanted pathogens and control the absorption of nutrients, immune cells and macromolecular via paracellular pathways (Robinson et al., 2015; Vancamelbeke and Vermeire, 2017; Chelakkot et al., 2018). These IECs express PRRs that recognise signature molecular patterns associated with pathogens or those released by injured or dying cells (Swerdlow et al., 2006; House et al., 2008; Zhang et al., 2010). Upon detecting these molecular patterns, the PRRs activate an immune cascade that triggers the production of cytokines and chemokines, and promotes the activation and response efficiency of T cells (Rahman et al., 2009; Fukata and Arditi, 2013).

The GIT microbiota assist the IECs in nutrient absorption, immune responses, and maintenance of the IEB homeostasis (Belkaid and Hand, 2014; Blake and Suchodolski, 2016; Foster et al., 2017). The GIT microbiota are a collection of Gram-positive and Gram-negative bacteria whose populations can be significantly altered by dietary changes (Allaway et al., 2020). LPS, which are found on Gram-negative bacteria, have consistently reduced the expression levels of TJ proteins including zonula occludin (ZO)-1 (Sheth et al., 2007; He et al., 2020), occludin (OCLDN) (He et al., 2020; Wu et al., 2020), and claudin (CLDN)-1 (Fujita et al., 2012; Wu et al., 2020). Additionally, LPS activates the PRR toll-like receptor (TLR)-4 and its subsequent downstream immune signalling cascades (Ryu et al., 2017).

SCFAs are produced by the fermentation of dietary products by the GIT microbiota (Peng et al., 2007; Bansal et al., 2010; Vinolo et al., 2011). SCFAs, including butyrate, acetate, and propionate, can also modulate the IEC immune response and intestinal homeostasis (Nery et al., 2012; Hang et al., 2013; Sandri et al., 2017). SCFAs activate T-cells and the production of cytokines through the G-protein coupled receptor (GPR) 41 & GPR 43 (Kim et al., 2013). SCFAs can downregulate the LPS-induced pro-inflammatory cytokine response of IECs, and can alleviate the LPS-induced morphological changes to ZO-1 and OCLDN (Feng et al., 2018b). Low levels of butyrate have been

shown to increase barrier integrity in cells, as assessed by TEER (Mariadason et al., 2001; Peng et al., 2009; Elamin et al., 2013; Feng et al., 2018b), whilst high levels have been seen to paradoxically decrease TEER (Peng et al., 2007).

Intestinal enterocytes have been used as a cell culture model to better understand intestinal function (Snoeck et al., 2005; Peterson and Artis, 2014). There are several studies that have investigated intestinal epithelial cells from dogs as models for diet and immune responses (Ramos-Vara and Miller, 2002; Weng et al., 2005; Golaz et al., 2007; Hemphill et al., 2009; Ohta et al., 2011; Farquhar et al., 2018; Reineking et al., 2018a; Reineking et al., 2018b; Chandra et al., 2019). However, in all of these studies, cell profiling was performed on either one or two specific cytokine or TJs, or one subset of PRRs.

The objective of this chapter was to use the cell culture methods refined and established in Chapter Four to simulate SCFA and bacterial interactions with the canine intestine. cIEC were treated with LPS, butyrate or a combination of the two. Untreated cIEC were ran in parallel as a control. It was hypothesised that LPS stimulation would cause increases in gene expression of pro-inflammatory cytokines such as IL-8, reduce protein concentrations of TJs associated with barrier integrity such as CLDN-1 and reduce TEER over time. Butyrate stimulation was hypothesised to would increase the gene expressions of anti-inflammatory cytokines such as IL-10, reduce protein concentrations of pore-forming TJs such as CLDN-2 and improve TEER over time. It was also hypothesised that the combination treatment of butyrate and LPS would mitigate the effects of each other; i.e., the presence of butyrate would reduce the pro-inflammatory response of the LPS, and the LPS would reduce the anti-inflammatory response of the butyrate.

5.3 MATERIALS AND METHODS

5.3.1 REAGENTS AND GENERAL CONSUMABLES

The reagents used for culture of cells were the same as those in Chapter Four, Section 4.3.1. In addition to those reagents, *Escherichia coli* and sodium butyrate were purchased from Sigma Aldrich (St Louis, Missouri, USA). Canine IL-8 and IL-10 ELISAs were obtained from Invitrogen (Thermofisher, Waltham, Massachusetts, USA). QIAGEN RNeasy Micro kits and RLT lysis buffer were obtained from QIAGEN (Hilden, Germany).

5.3.2 CELL CULTURING CONDITIONS

The cell culture conditions used were the same as outlined in Chapter Four, Section 4.3.2. Cell counts were also performed as outlined in Section 4.3.2.

Cell counts were established from cIEC in cell culture inserts. The apical and basal media was removed and 50 µL of TryPLE™ that had been preheated to 39°C was added into the apical compartment of the insert. These were placed back into the incubator for 5-10 minutes, or until the cells had dissociated from the bottom of the insert. 50 µL of preheated TEER Medium was then added to the apical well of the insert. This cell suspension was then centrifuged for 2 minutes at 300 x g. The supernatant was removed, and the cell pellet was re-suspended in 30 µl of preheated TEER Medium. 10 µL of a 1:1 mixture of re-suspended cell pellet and trypan blue was applied to each well of a Countess Cell Counting Chamber Slide (Countess, Invitrogen, Corporation, Carlsbad, California, USA). The total number of cells; live and dead, were then counted using a Countess Automated Cell Counter (Countess, Invitrogen, Corporation, Carlsbad, California, USA), which performed cell counts and viability measurements whilst automatically correcting for the use of trypan blue. All cell counts were recorded, and the average value of the cell counts for each flask was used for further calculations.

5.3.3 DIFFERENTIATION CONDITIONS AND BARRIER INTEGRITY ASSESSMENT ASSAYS

The differentiation conditions used were identical to those as outlined in Chapter Four, Section 4.3.3.

5.3.3.1 Measurement of TEER

The resistance across the cIEC layer was measured using an EndOhm TEER cup (World Precision Instruments, Sarasota, Florida, USA) connected to an EVOM voltohmmeter (World Precision Instruments, Sarasota, Florida, USA), after 48 hours in differentiation media, as outlined in Chapter 4, Section 4.3.1. Briefly, 1.0 mL of TEER Medium (DMEM supplemented with 7% FBS, 2 mM L-glutamine, 2 mM GlutaMAX, 10 mM HEPES) preheated to 39°C was added to the TEER cup. The inserts containing the differentiated cIEC monolayers were transferred from the insert plate to the TEER cup using sterile tweezers. The TEER cup lid that contained the top electrode was placed on top of the TEER cup and the resistance measured with the voltohmmeter and converted to TEER (Ω and Ωcm^2, respectively) using Equation 1, Chapter Four, Section 4.3.3.1. TEER readings were performed without equilibration to room temperature, i.e., readings were performed with media kept as close to 39°C as possible, to better simulate temperatures *in vivo*.

To evaluate the impacts of the LPS and butyrate challenges on TEER, data were expressed as a percentage of change in TEER over time. The TEER measurement from just before treatments were added, was subsequently classed as the initial TEER, which was different for each well. Change in TEER over time was calculated for each well using Equation 2, Chapter Four, Section 4.3.3.3.

5.3.3.2 Preparation of LPS and Sodium Butyrate

In a sterile class II biosafety cabinet, 1 mg freeze-dried LPS was resuspended in 1.0 mL of fresh TEER Medium to form a 1 mg/mL working stock solution. This was further diluted in TEER

Medium as appropriate. The LPS-TEER Medium solution was then preheated to 39°C for thirty minutes prior to experimental challenge. All required volumes of LPS-TEER Medium were made up fresh on the experimental day (i.e., no surplus was made and stored between experiments).

Sodium butyrate was prepared in 50 mL of fresh TEER Medium to form the appropriate concentrations. The mass of the sodium butyrate required was calculated using an online molarity calculator by GraphPad, (https://www.graphpad.com/quickcalcs/molarityform/). The calculated weight of sodium butyrate was weighed out using a balance (Mettler Toledo AG204, Mettler Toledo, Columbus, Ohio, USA) and added to a 50 mL Falcon tube. Subsequently, in a sterile class II biosafety cabinet, 50 mL of fresh TEER Medium was added to the sodium butyrate. This was then aliquoted into 5 mL volumes, which were stored at 4°C. Required volumes of the butyrate-TEER Medium were preheated to 39°C for thirty minutes prior to experimental challenge. Aliquots were made up fresh at the start of each experimental week and kept for seven days.

For the LPS-sodium butyrate challenge, LPS was prepared as above, but using TEER Medium that contained sodium butyrate, which was prepared as above. All required volumes of LPS/butyrate-TEER Medium were made up fresh on the experimental day (i.e., no surplus was made and stored between experiments).

5.3.4 ELISA METHODOLOGIES

Kits were stored at -20°C when not in use, as per manufacturer instructions. Kit components were allowed to thaw at room termperature for an hour prior to use. The standards were made up in fresh TEER Medium to account for any potential impact the phenol red in the media could have on the wavelength readings. Due to the limited volume of apical cell culture media, all samples were run at a 1:2 dilution in the kit Assay Diluent. A ‘sample’ was defined as a combination of the cIEC treatment and the location of the cell culture media. For example, the apical media from cIEC treated with LPS would be Sample 1, whilst the basal media from the same cIEC treated with LPS would be Sample 2. Samples were ran as one bioligical replicates per plate. ELISAs were performed as outlined

by kit manufacturers and followed the same basic outline. In brief, standards were prepared at manufacturer-specified concentrations in TEER Medium. The blank was TEER Medium. Standards were run in duplicate. 100 µL of standards or diluted sample was added to the appropriate wells. The ELISA plate was then covered with adhesive film and incubated for 180 minutes at room temperature.

Plates were then washed by discarding the solution and filling each well with 300 µL of wash buffer using a multichannel pipette. The wash solution in the wells was then discarded and this wash step was repeated three more times. At the end of washing, the plate was blotted against clean paper towels. 100 µL of the kit biotin conjugate was then added and the plate was recovered with adhesive film. The plate was then incubated at room temperature for another 60 minutes before it was washed four times as above.

100 µL of streptavidin- horseradish peroxidase (HRP) was then added to each well, and the plate was re-sealed and incubated for a further 45 minutes at room temperature. The plate was then washed another four times, as above, before each well was filled with 100 µL of 3,3',5,5'-Tetramethylbenzidine (TMB) Substrate. The plate was then placed in a drawer in the dark and was incubated for a further 30 minutes at room temperature. Finally, 50 µL of stop solution was added to each well and the plate was read at 450nm on a FlexStation 3 plate reader (Molecular Devices, San Jose, California, USA) using Softmax Pro (v5.4.1, Molecular Devices, San Jose, California, USA). Wavelengths were corrected against the average reading of the blank and a standard curve was generated using Microsoft Excel (Microsoft, Redmond, Washington, USA). Sample concentrations were then calculated using the generated standard curves and corrected for the dilution factor.

5.3.5 REFINEMENT OF LPS AND BUTYRATE DOSES

The experimental timeframe (i.e., how long the cIEC were cultured in media with LPS and/or butyrate) and the optimum concentrations of LPS and butyrate had to be determined. Dose responses to LPS and butyrate were quantified by change in TEER (Equation 2, Chapter Four, Section 4.3.3.3) and cytokine concentrations of IL-8 (Section 5.3.4) in apical cell media. Concentrations of LPS

assessed were 15.125, 31.25, 62.5, 125 and 250 μg/mL. The serial dilution was performed to determine if the cIEC had a dose-dependent response to LPS, as is seen in Caco-2 cells, where lower doses have less impact on TEER (He et al., 2020). LPS stimulation was performed twice using three biological replicates, with unchallenged cIEC ran in tandem as a negative control. Concentrations of butyrate assessed were 1, 5 and 10 mM. Low (1 mM), medium (5 mM), and high (10 mM) doses of butyrate were used to determine if the cIEC also had the paradoxical effects observed in other cells (where low doses have positive impacts on TEER, and high doses have negative impacts on TEER) (Elamin et al., 2013). Butyrate stimulation was performed twice using three biological replicates, with unchallenged cIEC ran in tandem as a negative control.

5.3.6 DEHYDRATION AND CONCENTRATION OF CIEC RNA FOR NANODROP AND NANOSTRING ANALYSIS

Canine intestinal epithelial cells were collected 8 hours post-treatment following the methods given for inserts in Section 5.3.2. However, the cell suspension was resuspended in 30 μL of Qiagen RLT buffer instead of TEER Medium. cIEC were treated with LPS, butyrate, or a combination of the two. Untreated controls were also ran in parallel and collected at the same timepoint. These were then labelled and frozen at -80°C until further use. The apical and basal media was collected and stored at -80°C until analysis on the Luminex platform.

The lysed cIEC were subsequently prepared for Nanostring analysis using a QIAGEN Micro Kit, following manufacturer instructions. In brief, samples were first thawed and homogenised with 350 μL of QIAGEN Buffer RLT. An equal amount of 70% ethanol was added to the sample and then this solution was transferred into a RNeasy MiniElute spin column. The spin column containing the lysed cIEC solution was then spun in a centrifuge (Eppendorf centrifuge 5425, Eppendorf, Hamburg, Germany) for 15 seconds at 8000 x *g*. The flow-through was discarded and 350 μL of QIAGEN Buffer RW1 was added to the spin column. This was then spun in a centrifuge for 15 seconds at 8000 x *g*.

After discarding the flow-through again, 500 µL of QIAGEN Buffer RPE was added to the spin column, and it was spun again for 15 seconds at 8000 x *g*. 500 µL of 80% ethanol was then added and the spin column was then spun at 8000 x *g* for 2 minutes. Subsequently, the flow-through was discarded again and the spin column was spun for 5 minutes at 8000 x *g* with the spin column lid slightly open to fully dry out the sample. Finally, the last flow-through was discarded and after placing the spin column in a fresh collection tube, 30 µL of RNase-free water was added into directly onto the centre of the spin column membrane. This was then spun for 1 minute at 8000 x *g* to elute the RNA. Another 30 µL of RNase-free water was added directly onto the centre of the spin column membrane and it was spun again for another 1 minute at 8000 x *g* to elute the RNA.

Samples were read individually on the Nanodrop (Nanodrop ND-1000 spectrophotometer, Thermo Scientific, ThermoFisher, Waltham, MA, USA), using the Nanodrop ND-1000 spectrophotometer software on an attached computer. The Nanodrop sensor was blanked by dispensing 1.0 µL of RNase-free water onto the sensor. Samples were then analysed, and their RNA content recorded.

Samples were then frozen at -80°C until Nanostring analysis was performed. Gene targets for the Nanostring analysis are listed in Table 5.15. Gene targets of TJs, PRRs and cytokines were based on information obtained during Chapter Two. The genes selected were a mixture of TJs (such as CLDNs), chemokines (such as C-C motif chemokine ligand 2; CCL2), pro-inflammatory cytokines (such as IL-8), anti-inflammatory cytokines (such as IL-10), and regulators of T cells (such as Forkhead box P3). Other genes, such as caspase 4 and programmed cell death 1 were selected due to their relation with cell destruction, i.e., these could be used to ascertain if treatment was influencing cell death. Housekeeping genes were determined from highly abundant mRNA expressions previously observed in the canine intestine (Cho et al., 2013).

Table 5.15 *- List of gene targets for Nanostring analysis.*

Gene	Description	National Centre for Biotechnology Information (NCBI) Gene ID
Housekeeping Genes		
SLC5A1	Solute carrier family 5 member 1	492299
SLC3A1	Solute carrier family 3 member 1	403700
FABP1	Fatty acid binding protein 1	403619
FABP2	Fatty acid binding protein 2	119867213
ABCB1	ATP binding cassette subfamily B member 1	403879
Target Genes		
AJAP1	Adherens junctions associated protein 1	607839
CASP4	Caspase 4, apoptosis-related cysteine peptidase	403724
CBD103	Beta-defensin 103	100170103
CCL2	C-C motif chemokine ligand 2	403981
CCL3	C-C motif chemokine ligand 3	448787
CCL4	C-C motif chemokine ligand 4	448786
CCL7	C-C motif chemokine ligand 7	491148
CD4	CD4 molecule	403931
CD14	CD14 molecule	607076
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	442858
CLDN1	Claudin 1	608207
CLDN10	Claudin 10	476963
CLDN11	Claudin 11	488160
CLDN12	Claudin 12	608397
CLDN14	Claudin 14	487751
CLDN15	Claudin 15	608226
CLDN16	Claudin 16	608218
CLDN17	Claudin 17	487720
CLDN18	Claudin 18	477079
CLDN19	Claudin 19	607005
CLDN2	Claudin 2	403649
CLDN3	Claudin 3	403648

Gene	Description	National Centre for Biotechnology Information (NCBI) Gene ID
CLDN4	Claudin 4	100856416
CLDN5	Claudin 5	100684266
CLDN6	Claudin 6	490048
CLDN7	Claudin 7	489466
CLDN8	Claudin 8	478401
CLDN9	Claudin 9	490049
CRP	C-reactive protein	488629
CXCL8	Chemokine (C-X-C motif) ligand 8	403850
CXCL10	C-X-C motif chemokine ligand 10 (IP-10)	478432
FFAR2	Free fatty acid receptor 2 (G-protein receptor (GPR)43)	484580
FFAR3	Free fatty acid receptor 3 (GPR41)	612659
FOXP3	Forkhead box P3	491876
GJA1	Gap junction protein alpha 1	403418
GJA5	Gap junction protein alpha 5	483155
HCAR2	Hydroxycarboxylic acid receptor 2 (GPR109-A)	486253
HSP90B1	Heat shock protein 90 beta family member 1	404019
IFNG	Interferon gamma	403801
IL1B	Interleukin-1 beta	403974
IL10	Interleukin-10	403628
IL12A	Interleukin-12A	403977
IL13	Interleukin-13	442990
IL18	Interleukin-18	403796
IL2	Interleukin-2	403989
IL4	Interleukin-4	403785
IL6	Interleukin-6	403985
IRAK1	Interleukin-1 receptor-associated kinase 1	492247
IRAK2	Interleukin-1 receptor-associated kinase 2	484657
IRAK3	Interleukin-1 receptor-associated kinase 3	481150
IRAK4	Interleukin-1 receptor-associated kinase 4	486601
LOC485869	Lipopolysaccharide binding protein	485869
LYZ	Lysozyme	474442

Gene	Description	National Centre for Biotechnology Information (NCBI) Gene ID
MUC1	Mucin 1, cell surface associated	448784
MUC2	Mucin 2, oligomeric mucus/gel-forming	119864303
MYD88	Myeloid differentiation primary response 88	477024
NFKB1	Nuclear factor kappa B subunit 1	442859
NOD1	Nucleotide-binding oligomerization domain containing 1	482382
NOD2	Nucleotide-binding oligomerization domain containing 2	487286
OCLN	Occludin	403844
PDCD1	Programmed cell death 1	486213
PRKCA	Protein kinase C alpha	490904
SAA1	Serum amyloid A1	6288
STAT3	Signal transducer and activator of transcription 3	490967
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein	609544
TLR1	Toll-like receptor 1	488834
TLR10	Toll-like receptor 10	100379585
TLR2	Toll-like receptor 2	448807
TLR3	Toll-like receptor 3	482905
TLR4	Toll-like receptor 4	403417
TLR5	Toll-like receptor 5	488605
TLR6	Toll-like receptor 6	111089957
TLR7	Toll-like receptor 7	491743
TLR8	Toll-like receptor 8	100684828
TLR9	Toll-like receptor 9	403502
TJP1	Tight junction protein 1 (zonula occludin (ZO)-1)	403752
TJP2	Tight junction protein 2 (ZO-2)	403854
TNF	Tumour necrosis factor (TNF)	403922
TRAF6	TNF receptor-associated factor 6	100688110

The Nanostring analysis was performed by the Hopkirk Research Institute. Hybridization buffer, Probe A, and Probe B were from nCounter ElementsTM TagSet (NanoString Technologies, Seattle, Washington, USA). Proteinase K was obtained from ThermoFisher (Carlsbad, California,

USA). RNAase-free water was from Qiagen (Hilden, Germany). In brief, a master mix was made by adding 70 µL of hybridization buffer and 7 µL of the Probe A Working Pool to the tube containing the TagSet. After inverting to mix, 7 µL of the Probe B Working Pool and 75 µL RNAse-free water were added. This was then inverted to mix again. 2.1 µL of 20 mg/mL Proteinase K was added and the master mix inverted to mix again. 1.5 µL of each sample was added to a microtube along with 13.5 µL of the mastermix. These were then placed in a thermal cycler (SensoQuest, Germany) at 67°C for 16 hours. Subsequently, samples were transferred to a nanoString sample cartridge and loaded into a PrepStation (Bio-Strategy, Auckland, NZ). After the PrepStation finished sample extraction, the sample cartridge was transferred to a digital analzer (Bio-Strategy, Auckland, NZ) and gene expressions quantified.

5.3.7 LUMINEX ANALYSIS

Luminex analysis was performed by the Massey Nutrition Laboratory using the MILLIPLEX® Canine Cytokine/Chemokine Magnetic Bead Panel - Immunology Multiplex Assay (Merck, Rahway, New Jersey, USA).

5.3.8 STATISTICAL ANALYSIS

The effect of treatment on change in TEER over time was compared using the statisical analysis as described in Section 4.4.

The effect of treatment on IL-8 concentrations was compared using the same methodology, with each ELISA plate considered a block. A repeated measures mixed-effects model was used based on the assumption that individual samples in the same treatment group were representative of the entire group. Pairwise comparisons were applied using estimated marginal means using the *emmeans* package (Lenth, 2022). The false discovery rate (q) was applied to the tests of the marginal means, with differences considered significant when $q < 0.05$.

Genstat (19th Edition, VSN International, Hemel Hempstead, UK) was used for MANOVAs to assess the differences in treatments and sample locations for Luminex results. MANOVAs were also performed on the nanostring RNA counts. Bonferroni post-hoc corrections were performed on statistically significant data in both cases. Data visualisation was also performed using Genstat.

Principal Coordinate Analysis (PCoA) was performed using the *pcoa* function in the *ape* R package version 5.5 (Paradis and Schliep, 2018) on the Bray-Curtis dissimilarity matricies derived using the *vegdist* function of the *vegan* R package version 2.5-7 (Dixon, 2003) from the proportions of the RNA counts of each treatment. The points were coloured by the treatment of the cIEC.

A p value < 0.05 was considered statistically significant in all cases, and a p value < 0.1 considered a trend. All data are presented as mean ± SEM unless otherwise noted.

5.4 Results

5.4.1 Treatment Conditions were determined as stimulation with 250 µg/mL LPS and/or 1 mM butyrate for 8 hours

The dose response experiments performed were successfully used to determine the concentrations of LPS and butyrate to use for experimental challenges. The timeframe for these challenges (i.e., how long the cIEC were cultured in media with LPS, butyrate, or a combination of the two) was also determined. These experiments results are summarised in Table 5.16 . The figures for these experiments are included as appendices as outlined in Table 5.16. Additionally, cell viability, assessed by cell counts as per Section 5.3.2, and IL-10 ELISAs were also performed to determine LPS and butyrate doses. However, there was no significant interaction of treatment over time on the viability of the cIEC (p = 0.66). The apical media from treated cIEC was also assessed in an IL-10 ELISA. However, there was no detectable IL-10 in the apical media of any sample. The mean R^2 value for the IL-10 ELISAs was 0.771 with a standard deviation of 0.123 (data not shown). Thus, these were determined as unsuitable methods for dose refinement.

Table 5.16 - *Summary of dose refinement experiments for LPS and butyrate treatement of cIEC.*

Appendix Reference	Experimental Overview	Experimental Results	Experimental Conclusion
Appendix 88	Serial dilution of LPS (concentrations of 15.125, 31.25, 62.5, 125, or 250 μg/mL) compared to an unchallenged cIEC (control). The change in TEER over time was used to determine the impacts of LPS dose on cIEC barrier integrity. N = 6 (three biological replicates, performed twice).	Interactions of treatment over time were significant ($p< 0.001$). All LPS treatments reduced TEER over time. All LPS treatments caused greater reductions in TEER compared to the untreated control. 250 μg/mL caused a greater reduction in TEER than 15.125 μg/mL. No differences in TEER occurred between other treatments (i.e., change in TEER between 31.25, 62.5, and 125 μg/mL were not significantly different to each other).	Dose response observed between the lowest dose (15.125 μg/mL) and highest dose (250 μg/mL) of LPS. Insufficient evidence from experiment to determine optimum LPS dose or experimental timeframe. Change in TEER would need to be analysed in parallel with IL-8 response to determine optimum LPS dose.
Appendix 9	Initial test using IL-8 ELISA. Apical media from the cIEC treated with 15.125, 62.5 and 250 μg/mL was collected at 0, 2, 4, 6, and 8 hours post-LPS treatment. Ran alongside apical media collected from unchallenged cIEC collected at the same times. N = 2 (biological replicates).	Interactions of treatment over time were significant ($p< 0.001$). Treatment with 62.5 and 250 μg/mLof LPS caused increased IL-8 expression after 6 hours of treatment. cIEC treated with 15.125 μg/mL LPS had no change in IL-8 expression levels, and were no different to untreated controls.	IL-8 protein abundance appeared to only increase 6 hours post-treatment. Timeframe for experimental challenge was determined as either 6- or 8-hours. Subsequent ELISA was to be performed to determine which doses of LPS were of interest at these timepoints.
Appendix 10	Further IL-8 ELISA. Apical media from all LPS treatments (15.125, 31.25, 62.5, 125, and 250 μg/mL) was collected at 0, 2, 4, 6, and 8 hours post-LPS	Interactions of treatment over time were significant ($p< 0.001$). All LPS treatments had increased IL-8 protein abundance at all timepoints, compared to the	IL-8 expression was heighest at 8 hours post-LPS treatment. The lowest and highest doses of LPS (15.125 and 250 μg/mL, respectively)

Appendix Reference	Experimental Overview	Experimental Results	Experimental Conclusion
	treatment. Ran alongside apical media collected from unchallenged cIEC collected at the same times. N = 6 (two bioligcal replicates, performed three times. Samples used in tests in Appendix x were also used in this assay).	unchallenged control. At 4- and 6-hours post-treatment, there was no difference in the IL-8 protein abundance from any LPS treatment. At 8-hours post-treatment, the IL-8 expression was heighed in the 250 μg/mL treatment. There was no difference in the IL-8 expression of all other LPS treatments at 8-hours post-treatment. IL-8 expression in cIEC treated with 15.125 μg/mL was increased compared at 8-hours post-treatment compared to 4 hours-post-treament. There was no change in IL-8 expression over time from cIEC treated with 31.25, 62.5 or 125 μg/mL LPS.	appeared the most suitable for the experimental model and were used in further testing. Experimental timeframe determined as 8-hours treatment with challenge compound.
Appendix 11	cIEC were stimulated with low (1 mM), medium (5 mM) and high (10 mM) concentrations of butyrate. Ran in parallel with unchallenged cIEC (control). The change in TEER over time was used to determine the impacts of butyrate on cIEC barrier integrity. N = 6 (three biological replicates, performed twice).	Interactions of treatment over time were significant (p< 0.001). 5 mM and 10 mM butyrate reduced TEER over time. 1 mM butyrate had greater TEER at 8 hours post-treatment than the control cIEC. Additionally, 1 mM butyrate caused an intial decrease in TEER that recovered over time.	Butyrate has paradoxical effects on cIEC barrier integrity, similar to other IEC. 1 mM butyrate determined as optimum treatment dose.
Appendix 12	cIEC treatment groups were: 15.125 μg/mL LPS, 250 μg/mL LPS, 1 mM butyrate, a combination of 15.125	Interactions of treatment over time were significant (p< 0.001). Butyrate treatment alleviated the	Co-stimulation of cIEC with butyrate and LPS proved successful. However, these

Appendix Reference	Experimental Overview	Experimental Results	Experimental Conclusion
	μg/mL LPS and 1 mM butyrate, and a combination of 250 μg/mL LPS and 1 mM butyrate. These were ran in parallel with control cIEC. The change in TEER over time was used to determine the impacts of butyrate on cIEC barrier integrity. N = 6 (three biological replicates, performed twice).	decreases in TEER caused by LPS treatment after 8 hours of treatment (i.e., the TEER in the LPS-butyrate treatments was higher compared to LPS treatment alone). LPS treatment signficiantly reduced TEER compared to all other treatments. 250 μg/mL LPS caused a greater reduction in TEER than 15.125 μg/mL LPS did. However, there was no difference in the TEER between the two combination treatment groups at 8 hours post-treatment.	results could not be used to determine the final LPS dose.
Appendix 13	IL-8 ELISA on apical media. cIEC treatment groups were: 15.125 μg/mL LPS, 250 μg/mL LPS, 1 mM butyrate, a combination of 15.125 μg/mL LPS and 1 mM butyrate, and a combination of 250 μg/mL LPS and 1 mM butyrate. Apical media was collected at 0, 2, 4, 6, and 8 hours post-LPS treatment. Ran alongside apical media collected from unchallenged cIEC collected at the same times. N = 6 (two bioligcal replicates, performed three times).	Interactions of treatment over time were significant ($p < 0.001$). IL-8 expression was increased 6 hours post-treatment in cIEC treated with 250 μg/mL LPS alone, or in combination with 1 mM butyrate. There was no change in IL-8 expression from cIEC treated with 15.125 μg/mL alone or in combination with butyrate. There was also no change in IL-8 expression in control cIEC and those treated with 1 mM butyrate alone.	250 μg/mL LPS was determined as the finalised dose for LPS treatment.

Abbreviations: LPS, lipopolysaccharides; cIEC, canine intestinal epithelial cell; TEER, trans-epithelial electrical resistance; IL-, interleukin; ELISA, enzyme-linked immunosorbant assay.

Thus, the finalised experimental conditions for LPS and butyrate treatment of cIEC were determined as: treatment with either 250 μg/mL LPS, 1 mM butyrate or a combination of the two. These were ran alongside untreated cIEC as controls. Samples were then collected for analysis 8 hours post-treatment.

5.4.2 Assessment of LPS and Butyrate Stimulation on the Physical and Immune Response of cIEC

Treatments used for the final dose response challenge were 1mM of sodium butyrate, 250 μg/mL LPS, and a combined treatment of both 1mM sodium butyrate and 250 μg/mL LPS. Untreated cIEC were performed in parallel as controls. TEER was recorded for these samples from treatment addition (Time 0) and then every 2 hours until 8 hours post-challenge when the cells were lysed for Nanostring analysis, and the apical and basal media collected for Luminex analysis.

5.4.2.1 The presence of butyrate alleviated the LPS-induced decreases to TEER.

The change in TEER for these samples was evaluated and can be seen in Figure 5.33. The interactions of treatment over time significantly changed TEER ($p < 0.001$). At 8 hours post-treatment, cIEC treated with LPS alone had the greatest reduction in TEER compared to all other treatments ($p < 0.01$ in all instances). The combination treatment of butyrate and LPS demonstrated that butyrate alleviated the LPS-induced decrease to TEER ($p < 0.01$). However, the TEER was still lower than that of the untreated and butyrate-treated cIEC ($p < 0.01$ for both). Though butyrate treatment caused an initial decrease in TEER, after 8 hours of treatment the TEER had fully recovered (i.e., TEER at 8 hours was not different to the TEER at Time 0; $p > 0.1$). The TEER of the untreated cIEC did not change over time ($p > 0.1$ in all instances).

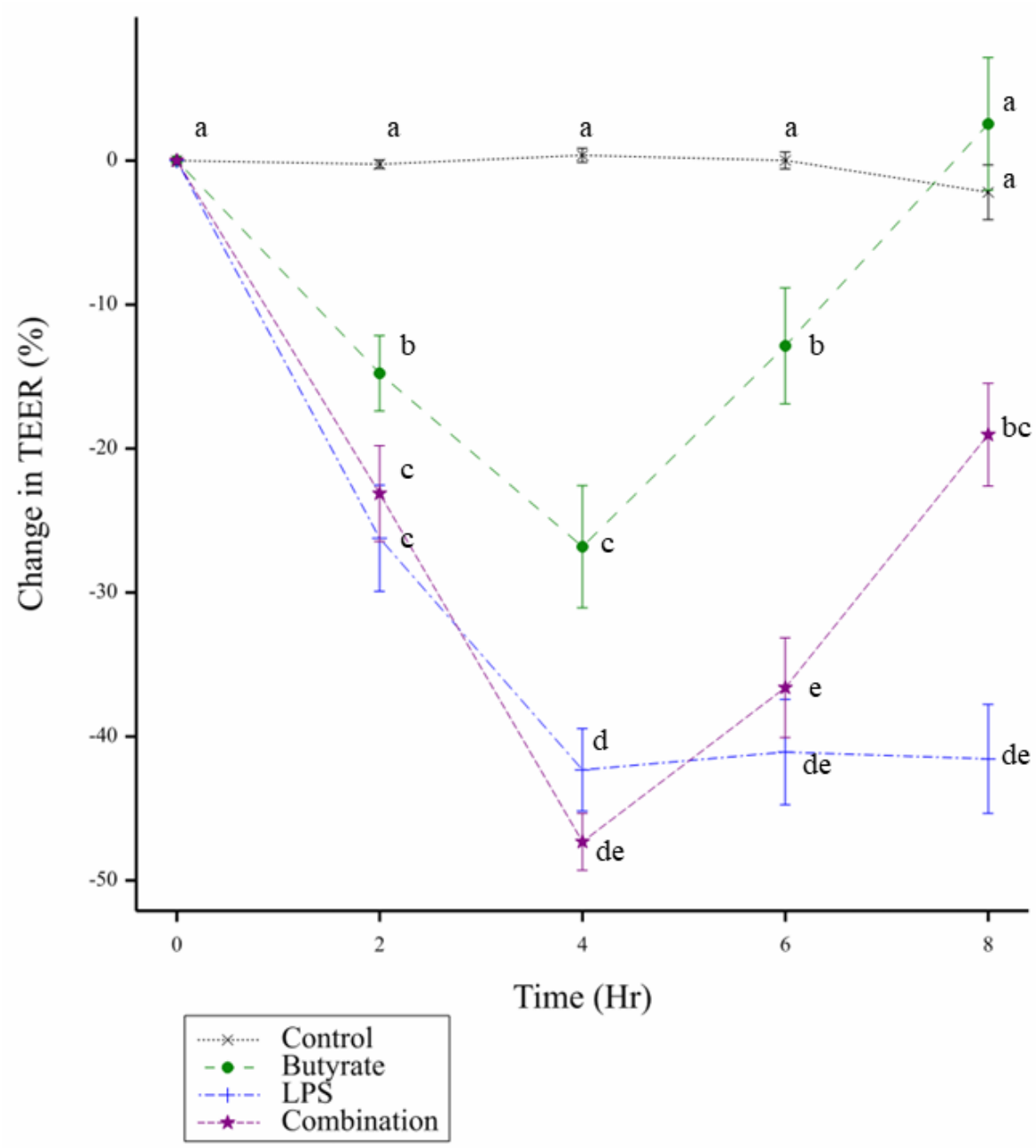


Figure 5.33 – *Mean (± SEM) change in TEER from canine Intestinal Epithelial cells (cIECs) challenged with different treatments.Change in TEER was defined as a percentage change compared to the initial, time 0 value for the same treatment. Treatments were 1 mM of sodium butyrate (Butyrate), 250 µg/mL lipopolysaccharides (LPS), both 1 mM sodium butyrate and 250 µg/mL LPS (Combination), and untreated cIECs (Control). N = 6 for each treatment. Timepoints that do not share a common letter denotes a significant ($p < 0.05$) difference in the change in TEER from the effects of treatment and time. Timepoints denote the time passed since the challenge compounds were applied to the cIEC.*

5.4.2.2 Stimulation of cIEC increased protein expression of IL-8, IP10, KC-like and MCP-1/CCL2

The apical and basal media from the cIEC were collected at 8 hours post-treatment and assessed for cytokine and chemokine concentrations. The effects of the treatment and the sample location (i.e., apical or basal) were assessed (see Table 5.17). The concentrations of keratinocyte-derived chemokine (KC)-like ($p < 0.001$), IP10 ($p < 0.001$), IL-8 ($p < 0.001$) and Monocyte Chemoattractant Protein (MCP)-1 (also referred to as chemokine (C-C motif) ligand 2, CCL2; $p < 0.001$) were all signficantly impacted by the effects of treatment and sample location. There was also a trend observed in the impacts of treatment and sample location on the concentration of IL-18 ($p = 0.064$). For the cytokines with significant differences between treatments (IL-8, IP10, KC-like and MCP-1/CCL2), the data is also displayed in graph form in Figure 5.34A, Figure 5.2B, Figure 5.2C, and Figure 5.2D, respectively.

Table 5.17 – *Cytokine and chemokine concentrations in pg/mL of canine intestinal epithelial cell samples collected 8 hours after a challenge with 1 mM sodium butyrate (Butyrate), 250 µg/mL lipopolysaccharides (LPS) or both 1 mM sodium butyrate and 250 µg/mL lipopolysaccharides (Combination). n = 48. Samples are comprised of apical media (n = 24) and basal media (n = 24). n = 6 for each treatment. Cytokine concentrations are presented as means ± SEM. Results that do not share the same superscript letter denotes a significant difference in cytokine concentration (p < 0.05). Significant p values and associated proteins are listed in bold.*

	Location of cell media, and treatment of the cells								
Cytokine Concentration (pg/mL)*	***Apical Cell Media***				***Basal Cell Media***				***Significance of Treatment and Location (p value)***
	Control	*Butyrate*	*LPS*	*Combination*	*Control*	*Butyrate*	*LPS*	*Combination*	
GM-CSF	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.11	0.00 ± 0.00	0.70 ± 0.46	0.00 ± 0.00	1.28 ± 0.99	0.00 ± 0.00	0.364
IFN-γ	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.40 ± 0.40	0.00 ± 0.00	1.00 ± 0.64	0.00 ± 0.00	0.213
KC-like	6.59 ± 0.31^{a}	3.43 ± 0.12^{a}	47.05 ± 2.44^{b}	48.61 ± 3.82^{b}	2.65 ± 0.24^{a}	1.05 ± 0.67^{a}	49.81 ± 5.82^{b}	0.00 ± 0.00^{a}	**< 0.001**
IP-10	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	2.45 ± 0.77^{b}	0.00 ± 0.00^{a}	4.59 ± 0.11^{c}	**< 0.001**
IL-2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.34 ± 4.34	0.00 ± 0.00	8.81 ± 4.72	0.00 ± 0.00	0.177
IL-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.67 ± 2.60	0.00 ± 0.00	6.19 ± 3.28	0.00 ± 0.00	0.135
IL-7	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	5.22 ± 5.22	0.00 ± 0.00	12.05 ± 6.77	0.00 ± 0.00	0.166
IL-8	63.11 ± 22.63^{a}	0.00 ± 0.00^{a}	481.63 ± 25.16^{b}	413.96 ± 17.97^{b}	28.66 ± 28.66^{a}	0.00 ± 0.00^{a}	303.98 ± 23.96^{c}	163.5 ± 51.33^{d}	**< 0.001**
IL-10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	N/A$^{¥}$
IL-15	0.22 ± 0.22	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	5.03 ± 4.46	0.00 ± 0.00	6.55 ± 5.11	0.00 ± 0.00	0.414

	Location of cell media, and treatment of the cells								
Cytokine Concentration	***Apical Cell Media***				***Basal Cell Media***				***Significance of Treatment and***
IL-18	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.81 ± 0.67	0.00 ± 0.00	2.23 ± 1.11	0.00 ± 0.00	0.064
MCP-1/CCL2	1338.79 ± 36.53[a]	855.16 ± 86.77[b]	1529.6 ± 44.8[a]	1427.14 ± 17.31[a]	350.87 ± 27.57[c]	613.6 ± 25.57[d]	1111.63 ± 29.11[e]	1119.5 ± 19.84[e]	< **0.001**
TNF-α	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.56 ± 1.56	0.00 ± 0.00	3.82 ± 2.56	0.00 ± 0.00	0.241

* GM-CSF, granulocyte-macrophages colony stimulating factor; IFN, interferon; KC, keratinocyte-derived chemokine; IP, interferon gamma-induced protein; IL, interleukin; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein (also referred to as chemokine (C-C motif) ligand 2, CCL2)); TNF, tumour necrosis factor.

¥ Results could not be assessed for significance as there was no detectable target cytokine content in any sample.

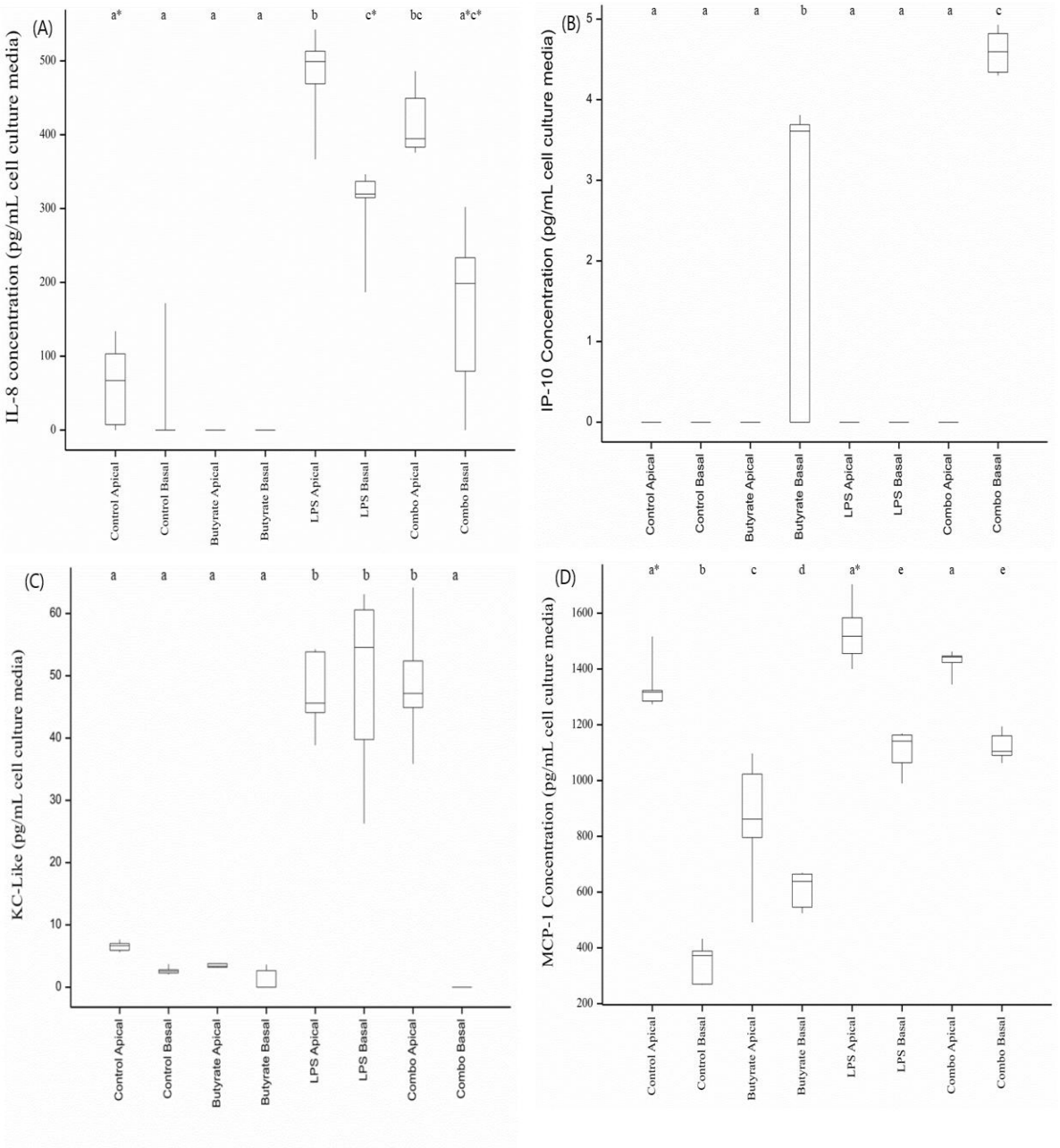


***Figure 5.34** – Cytokine concentrations of interleukin (IL)-8, interferon gamma-induced protein (IP-10), keratinocyte-derived chemokine (KC)-like, and monocyte chemoattractant protein-1 (MCP-1/CCL2).Cytokine concentrations detected in the cell culture media of canine Intestinal Epithelial cells (cIECs) collected 8 hours post-challenge. cIEC were challenged with 1 mM of sodium butyrate (Butyrate Apical & Butyrate Basal), 250 µg/mL lipopolysaccharides (LPS Apical & LPS Basal), or both 1 mM sodium butyrate and 250 µg/mL LPS (Combo Apical & Combo basal). Untreated cIECs were ran in parallel as negative controls (Control Apical & Control Basal). Apical and basal media was tested in duplicate, n = 6 for each treatment. Results that do not share a common letter denotes a significant ($p < 0.001$) difference in protein concentrations. Common letters marked with a * indicate that there was a significant ($p < 0.01$) difference between other results with common letters followed by a *. Boxes represent the interquartile range between the first and third quartiles. Thick black line inside the box denotes the median.*

5.4.2.3 Samples for Nanostring analysis were combined based on Nanodrop quanitification of RNA content

RNA content of the samples was assessed before Nanostring analysis was performed (see Table 5.18). Based on the genetic content, samples were combined as outlined in Table 5.18. These combined samples were then subsequently assessed for RNA content by Nanostring analysis.

Table 5.18 – *Gene content of canine intestinal epithelial cells collected 8 hours post-challenge with different treatments, as determined by nanodrop.Estimated RNA yields calculated based on final volume of sample for Nanostring analysis. Treatments were 1 mM of sodium butyrate (Butyrate), 250 µg/mL lipopolysaccharides (LPS), both 1 mM sodium butyrate and 250 µg/mL LPS (Combination), and untreated cIECs (Control).*

Sample ID	***Genetic content (ng/µL)***	***Total RNA yield after preparation***	***Combined Sample IDs***	***Estimated RNA yield of combined sample***
Control 1	4.6	276	Control 1+4	510
Control 2	7.9	474	Control 2+6	654
Control 3	13.8	828	Control 3+5	918
Control 4	3.9	234	LPS 1+3	258
Control 5	1.5	90	LPS 2+6	384
Control 6	3	180	LPS 4+5	372
LPS 1	2.1	126	Butyrate 1+3	201
LPS 2	3.5	210	Butyrate 2+4	216
LPS 3	2.2	132	Butyrate 5+6	240
LPS 4	4.2	252	Combination 1+5	258
LPS 5	2	120	Combination 2+4	264
LPS 6	2.9	174	Combination 3+6	234
Butyrate 1	1.2	69		
Butyrate 2	1.5	90		
Butyrate 3	2.2	132		
Butyrate 4	2.1	126		
Butyrate 5	2	120		
Butyrate 6	2	120		
Combination 1	2.1	126		
Combination 2	3	180		
Combination 3	2.2	132		
Combination 4	1.4	84		
Combination 5	2.2	132		
Combination 6	1.7	102		

5.4.2.4 Gene Expressions of CCL2/MCP-1, IL-8 and IP10 were increased from stimulation of cIEC

The effects of the treatment of the cIEC on gene content were assessed (see Table 5.19). Treatment with sodium butyrate, lipopolysaccharides or a combination of both, caused a significant impact in the gene expression levels of CCL2/MCP-1 ($p < 0.001$), IP10 ($p < 0.007$) and IL-8 ($p< 0.003$). There was a trend towards significance caused by treatment in the gene expression levels of CLDN-18 ($p = 0.061$) and TLR-1 ($p = 0.069$). Where gene expression was significantly different between treatments (CCL2/MCP-1, IL-8, and IP10), the data is also displayed in graph form in Figure 5.35A, Figure 5.3B, and Figure 5.3C, respectively.

Table 5.19 – *RNA counts from Nanostring analysis of canine intestinal epithelial cells collected 8 hours post-challenge with different treatments.Each result is presented as mean ± SEM. Each treatment comprised n = 3, and the impacts of treatment presented as the p value. Results that do not share the same superscript letter are significantly different in RNA counts (p < 0.05). Significant p values and associated gene targets are listed in bold.*

Gene Target	***Treatment***				***p value***
	Control	***Butyrate***	***LPS***	***Combination****	
Adherens junctions associated protein 1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A[§]
Caspase 4	8.0 ± 4.1	8.7 ± 4.4	0.0 ± 0.0	0.0 ± 0.0	0.124
Beta-defensin 103	3.5 ± 3.5	5.6 ± 5.6	0.0 ± 0.0	0.0 ± 0.0	0.579
C-C motif chemokine ligand 2	53.4 ± 11.1[a]	92.8 ± 6.2[a]	140.5 ± 29.6[b]	256.7 ± 27.7[c]	**<0.001**
C-C motif chemokine ligand 3	9.9 ± 5.4	10.6 ± 5.4	6.2 ± 6.2	19.2 ± 2.3	0.381
C-C motif chemokine ligand 4	4.0 ± 4.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.441
C-C motif chemokine ligand 7	14.7 ± 3.4	17.2 ± 2.9	19.7 ± 3.4	20.9 ± 1.3	0.484
Cluster of differentiation 4 molecule	4.0 ± 4.0	0.0 ± 0.0	3.8 ± 3.8	3.7 ± 3.7	0.801
Cluster of differentiation 14 molecule	0.0 ± 0.0	4.2 ± 4.2	0.0 ± 0.0	0.0 ± 0.0	0.441
Cadherin 1	86.2 ± 22.2	52.5 ± 5.5	73.3 ± 29.6	75.9 ± 7.7	0.666
Claudin 1	4.9 ± 4.9	4.0 ± 4.0	3.8 ± 3.8	16.9 ± 4.5	0.178
Claudin 10	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Claudin 11	13.4 ± 1.2	8.7 ± 4.4	10.3 ± 5.8	8.6 ± 8.6	0.923
Claudin 12	4.0 ± 4.0	3.9 ± 3.9	7.7 ± 3.8	4.8 ± 4.8	0.908
Claudin 14	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Claudin 15	31.0 ± 6.2	24.2 ± 5.1	27.2 ± 7.6	24.8 ± 3.3	0.839
Claudin 16	3.5 ± 3.5	7.4 ± 3.7	5.2 ± 5.2	8.6 ± 4.4	0.844
Claudin 17	0.0 ± 0.0	0.0 ± 0.0	11.0 ± 6.2	9.7 ± 4.9	0.154
Claudin 18	0.0 ± 0.0	7.3 ± 3.6	4.3 ± 4.3	13.2 ± 1.0	0.061
Claudin 19	33.2 ± 4.9	43.9 ± 6.9	32.5 ± 4.5	35.9 ± 8.8	0.620
Claudin 2	12.0 ± 0.6	12.2 ± 6.3	13.6 ± 2.5	15.8 ± 2.3	0.866
Claudin 3	37.3 ± 10.1	28.9 ± 5.7	27.8 ± 5.6	39.0 ± 6.5	0.618
Claudin 4	5.3 ± 5.3	14.0 ± 1.8	12.8 ± 0.7	9.1 ± 4.5	0.396

Gene Target	***Treatment***				***p value***
Claudin 5	28.5 ± 2.1	26.4 ± 3.7	29.1 ± 4.1	36.2 ± 7.8	0.559
Claudin 6	32.0 ± 8.3	28.2 ± 2.8	32.7 ± 6.6	41.4 ± 7.2	0.564
Claudin 7	74.4 ± 17.1	51.8 ± 6.5	55.8 ± 19.4	73.8 ± 2.8	0.540
Claudin 8	18.5 ± 5.5	11.0 ± 5.5	19.4 ± 0.4	20.7 ± 3.4	0.428
Claudin 9	13.9 ± 2.5	13.5 ± 1.4	12.2 ± 6.1	12.8 ± 0.9	0.985
C-reactive protein	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.5 ± 4.5	0.441
Chemokine (C-X-C motif) ligand 8 (IL-8)	31.5 ± 13.7[a]	14.9 ± 1.8[a]	125.0 ± 28.3[b]	90.3 ± 15.8[b]	**0.007**
C-X-C motif chemokine ligand 10 (IP-10)	15.9 ± 3.2[a]	22.0 ± 1.0[a]	65.6 ± 17.5[b]	83.0 ± 8.8[b]	**0.003**
Free fatty acid receptor 2 (GPR43)	0.0 ± 0.0	0.0 ± 0.0	3.8 ± 3.8	0.0 ± 0.0	0.441
Free fatty acid receptor 3 (GPR41)	3.6 ± 3.6	0.0 ± 0.0	7.9 ± 4.0	4.3 ± 4.3	0.491
Forkhead box P3	0.0 ± 0.0	3.7 ± 3.7	0.0 ± 0.0	0.0 ± 0.0	0.441
Gap junction protein alpha 1	15.9 ± 8.1	11.4 ± 5.8	6.7 ± 6.7	12.9 ± 6.4	0.813
Gap junction protein alpha 5	4.4 ± 4.4	4.0 ± 4.0	0.0 ± 0.0	0.0 ± 0.0	0.595
Hydroxycarboxylic acid receptor 2 (GPR109-A)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Heat shock protein 90 beta family member 1	51.1 ± 10.1	31.2 ± 3.6	43.1 ± 13.9	44.5 ± 6.2	0.535
Interferon gamma	22.0 ± 6.1	30.7 ± 7.1	18.9 ± 0.8	20.9 ± 6.7	0.525
Interleukin-1 beta	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Interleukin-10	18.2 ± 4.3	9.6 ± 4.8	5.2 ± 5.2	16.3 ± 8.2	0.425
Interleukin-12A	16.5 ± 2.9	11.5 ± 5.9	9.4 ± 4.7	14.4 ± 1.0	0.638
Interleukin-13	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Interleukin-18	4.0 ± 4.0	11.0 ± 5.5	0.0 ± 0.0	8.5 ± 4.2	0.295
Interleukin-2	0.0 ± 0.0	3.6 ± 3.6	0.0 ± 0.0	3.7 ± 3.7	0.595
Interleukin-4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Interleukin-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Interleukin-1 receptor-associated kinase 1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Interleukin-1 receptor-associated kinase 2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.7 ± 3.7	0.441
Interleukin-1 receptor-associated kinase 3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A

Gene Target	***Treatment***				***p value***
Interleukin-1 receptor-associated kinase 4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Lipopolysaccharide binding protein	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Lysozyme	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Mucin 1, cell surface associated	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Mucin 2, oligomeric mucus/gel-forming	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Myeloid differentiation primary response 88	5.3 ± 5.3	0.0 ± 0.0	10.3 ± 5.4	4.3 ± 4.3	0.469
Nuclear factor kappa B subunit 1	17.6 ± 2.0	18.5 ± 4.2	17.8 ± 4.1	17.2 ± 3.5	0.995
Nucleotide-binding oligomerization domain containing 1	3.8 ± 3.8	3.7 ± 3.7	0.0 ± 0.0	13.0 ± 6.7	0.254
Nucleotide-binding oligomerization domain containing 2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Occludin	47.0 ± 12.9	23.7 ± 0.3	31.8 ± 11.5	37.6 ± 10.1	0.458
Programmed cell death 1	13.6 ± 7.1	18.0 ± 5.2	14.7 ± 0.6	15.8 ± 2.6	0.914
Protein kinase C alpha	4.9 ± 4.9	0.0 ± 0.0	3.8 ± 3.8	5.7 ± 5.7	0.784
Serum amyloid A1	11.9 ± 6.0	6.3 ± 6.3	15.2 ± 0.4	8.1 ± 4.1	0.587
Signal transducer and activator of transcription 3	13.9 ± 6.9	5.6 ± 5.6	10.0 ± 5.1	14.5 ± 1.8	0.626
Toll-interleukin 1 receptor (TIR) domain containing adaptor protein	0.0 ± 0.0	3.7 ± 3.7	0.0 ± 0.0	3.8 ± 3.8	0.596
Toll-like receptor 1	21.9 ± 4.9	20.0 ± 3.4	15.8 ± 2.4	14.8 ± 7.5	0.712
Toll-like receptor 10	27.3 ± 5.0	25.2 ± 2.4	22.4 ± 6.0	37.8 ± 8.3	0.335
Toll-like receptor 2	13.9 ± 0.5	4.5 ± 4.5	0.0 ± 0.0	4.3 ± 4.3	0.069
Toll-like receptor 3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Toll-like receptor 4	4.9 ± 4.9	7.0 ± 7.0	6.7 ± 6.7	0.0 ± 0.0	0.787
Toll-like receptor 5	13.1 ± 1.5	8.6 ± 4.3	9.1 ± 4.7	11.3 ± 5.8	0.880
Toll-like receptor 6	16.5 ± 2.2	20.4 ± 4.1	15.5 ± 8.7	12.8 ± 6.5	0.839
Toll-like receptor 7	11.7 ± 6.6	0.0 ± 0.0	11.1 ± 5.9	13.3 ± 1.4	0.218
Toll-like receptor 8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.7 ± 3.7	0.441
Toll-like receptor 9	4.0 ± 4.0	5.1 ± 5.1	0.0 ± 0.0	5.4 ± 5.4	0.791
Tight junction protein 1 (zonula occludin (ZO)-1)	0.0 ± 0.0	4.2 ± 4.2	4.3 ± 4.3	3.7 ± 3.7	0.800
Tight junction protein 2 (ZO-2)	8.0 ± 4.1	4.9 ± 4.9	4.7 ± 4.7	4.3 ± 4.3	0.932

Gene Target	***Treatment***				***p value***
Tumour necrosis factor (TNF)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
TNF receptor-associated factor 6	9.7 ± 4.9	9.6 ± 5.0	10.7 ± 5.3	18.4 ± 2.6	0.515

* Combination; butyrate and lipopolysaccharide (LPS)-treated cells.

§ A value of N/A denotes that results could not be assessed for significance as there was not any gene response from the target gene across all treatments.

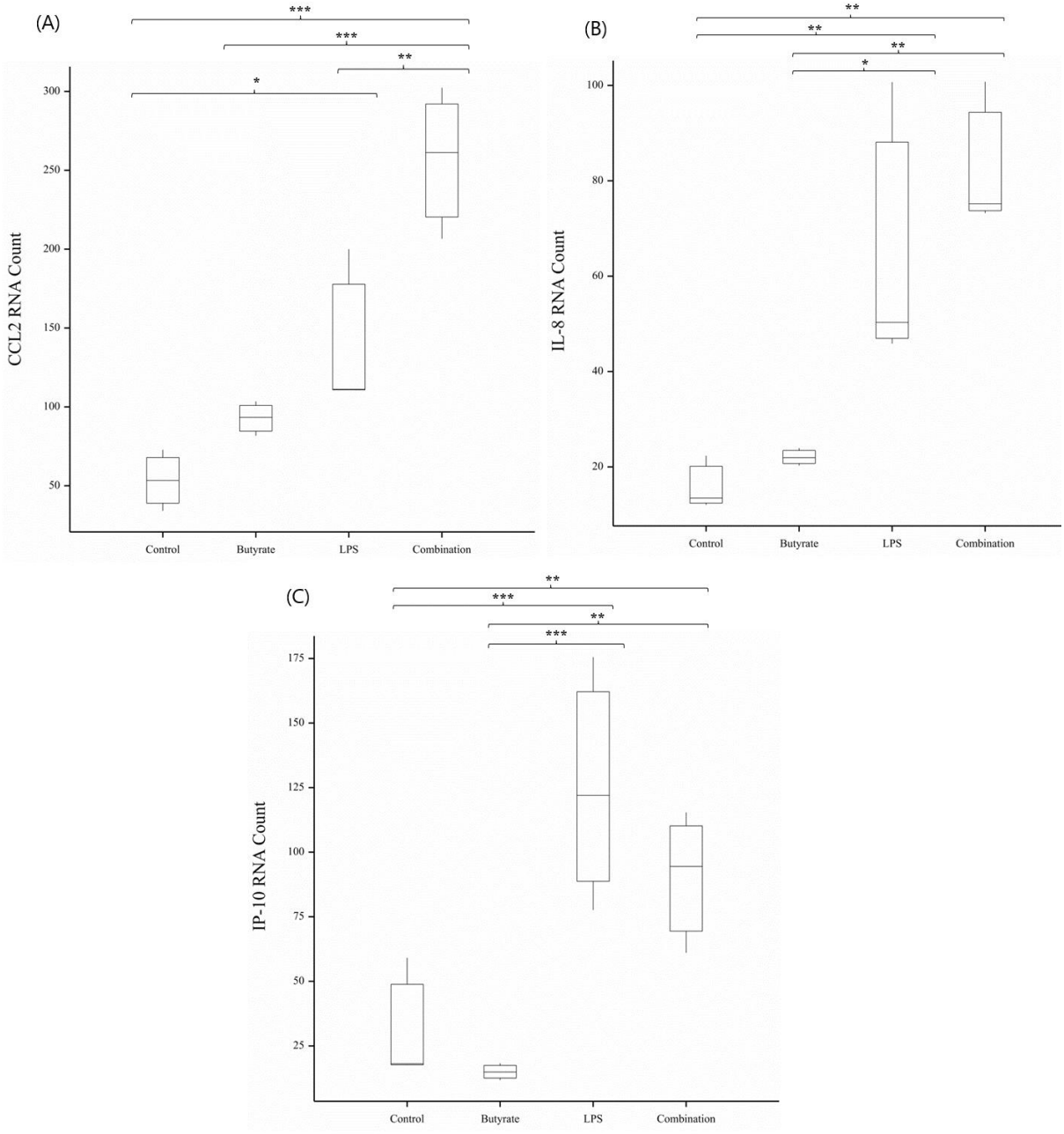


Figure 5.35 – *RNA counts of chemokine (C-C motif) ligand 2, interleukin (IL)-8, and interferon gamma-induced protein (IP)-10 in canine Intestinal Epithelial cells (cIECs).cIECs were collected 8 hours post-challenge with 1mM of sodium butyrate (Butyrate), 250 µg/mL lipopolysaccharides (LPS), or both 1mM sodium butyrate and 250 µg/mL LPS (Combination). Treated cIEC were ran in parallel with untreated cIECs (Control). N = 3 for each treatment. Significant differences are represented by *, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Boxes represent the interquartile range between the first and third quartiles. Thick black line inside the box denotes the median.*

A principal coordinates of analysis test was also performed on the Nanostring results (see Figure 5.36). The results clustered into two distinct groups; the untreated controls and cIEC treated

with butyrate (circled in black), and cIEC treated with LPS alone and those treated with both LPS and butyrate (circled in blue).

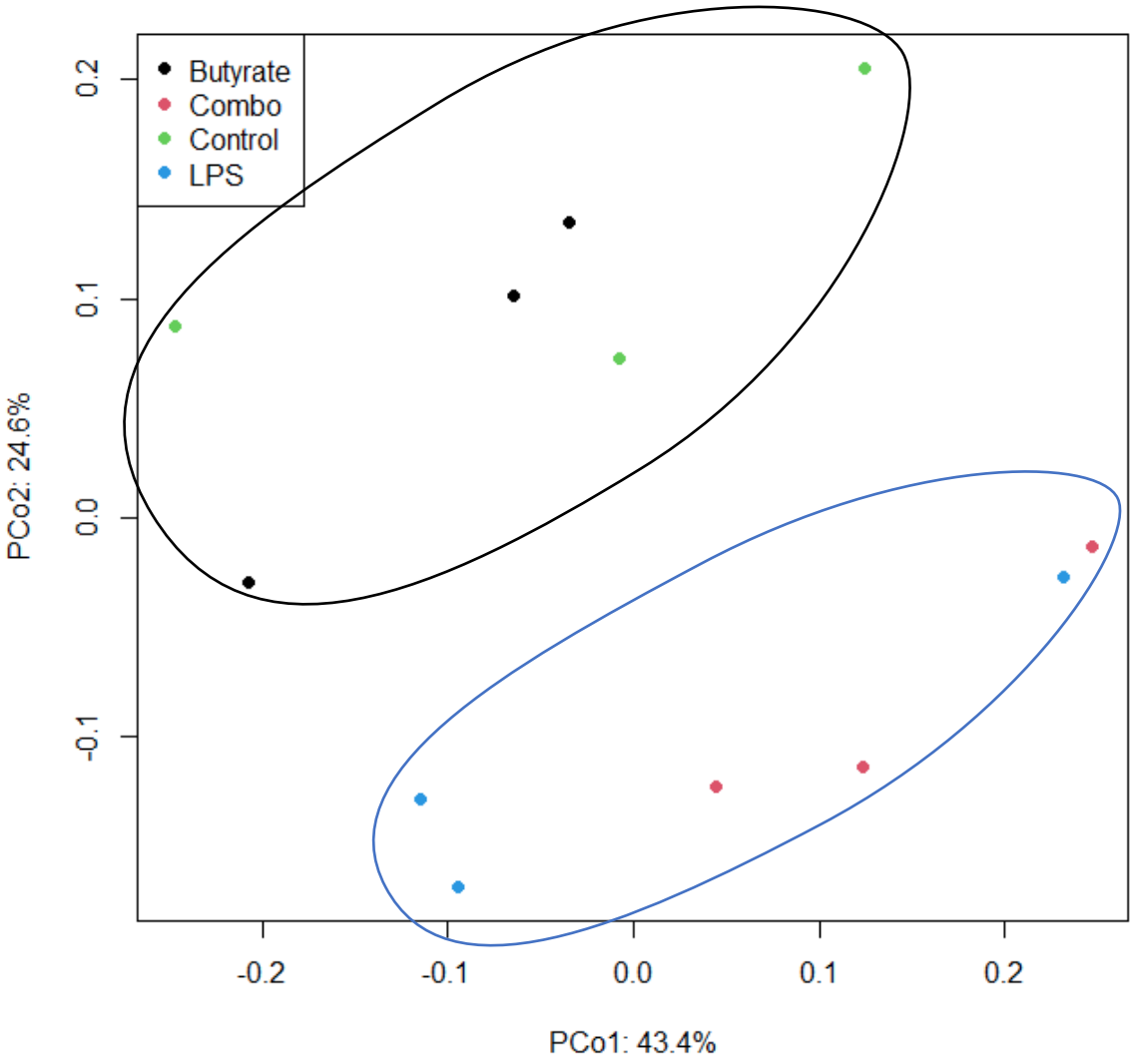


Figure 5.36 *– Scaled Bray-Curtis dissimilarity principal coordinates of analysis for the RNA counts of canine intestinal epithelial cells 8 hours post-challenge with 1mM of sodium butyrate (Butyrate), 250 µg/mL lipopolysaccharides (LPS), both 1mM sodium butyrate and 250 µg/mL LPS (Combo), and untreated cIECs (Control).X and Y axis show principal component 1 and principal component 2 that explain 43.4% and 24.6% of the total variance, respectively. N = 79 data points.*

Biplots from these results were also plotted (see Figure 5.37). In the biplot the vectors (in this instance, the detected genes) can be visually assessed for correlations. If they are close, they are positively correlated (such as CDH1 and CLDN7). Those that meet at 90° generally have no

correlation (such as CDH1 and CXCL10/IP10). Angles larger than 90° are negatively correlated (such as CCL2 and CDH1). The biplot showed that the clustering of results was driven by the RNA counts of CCL2, IL-8 (CXCL8) and IP-10 (CXCL10) in the LPS and butyrate combined treatment. Gene expression of CDH1, CLDN-7 and occludin appeared to be correlated and loosely clustered with the control cIEC.

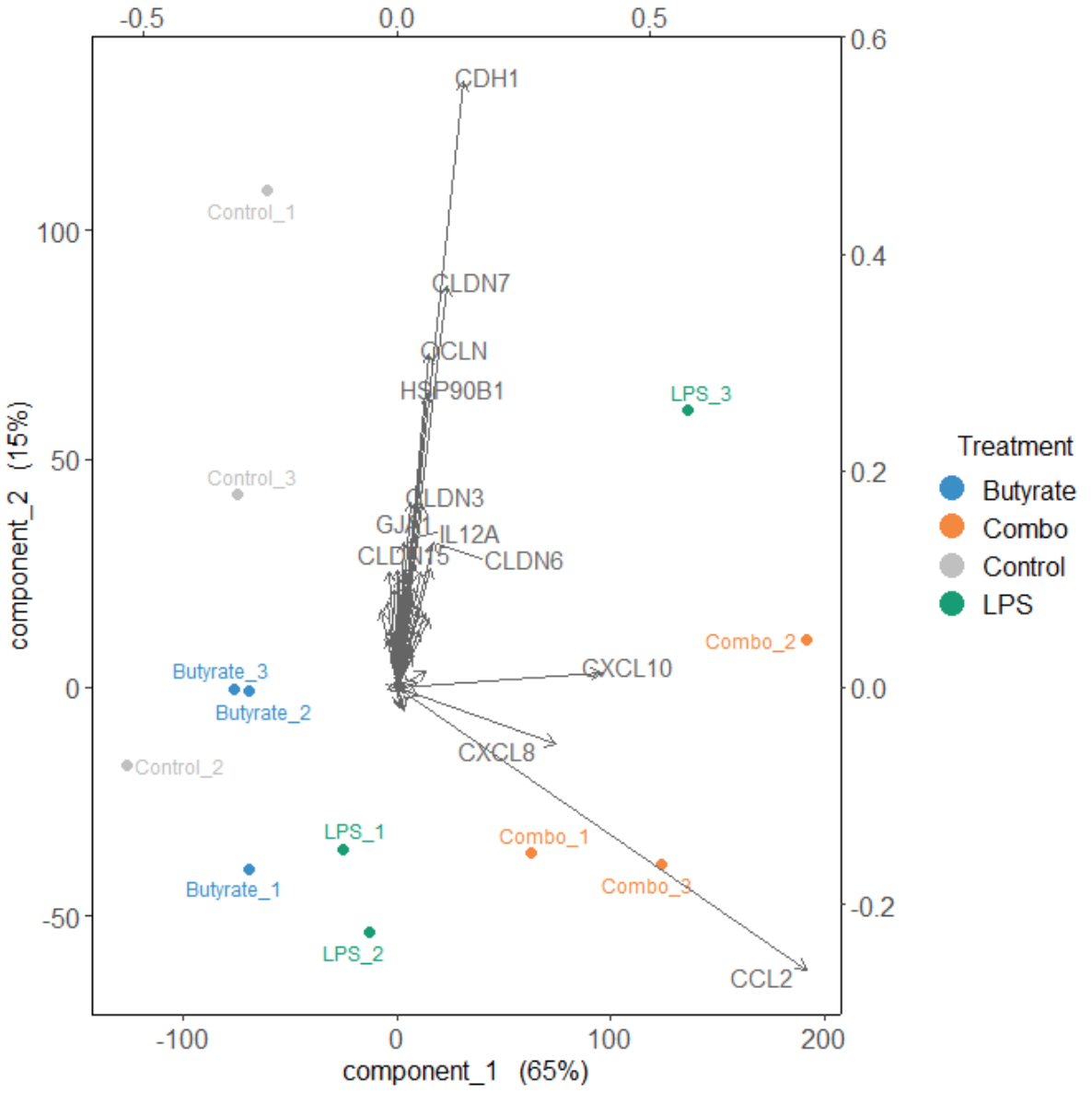


***Figure 5.37** – Scaled biplotfor the RNA counts of canine intestinal epithelial cells 8 hours post-challengee with 1mM of sodium butyrate (Butyrate), 250 µg/mL lipopolysaccharides (LPS), both 1mM sodium butyrate and 250 µg/mL LPS (Combo), and untreated cIECs (Control).X and Y axis show principal component 1 and principal component 2 that explain 65% and 15% of the total variance, respectively. N = 79 data points.*

5.5 DISCUSSION

This chapter successfully assessed the impacts of LPS and butyrate on the immune cascades and TJs in the canine gut in an *in vitro* model for the first time. Additionally it is the first study that has assessed both protein abundances and gene expressions of the immune cascades and TJs. It was hypothesised that LPS would cause a pro-inflammatory response, reduce gene expressions of TJs associated with barrier function, and weaken barrier integrity. LPS treatment caused increases in IL-8 cytokine and gene expression, CCL2 and IP10 gene expression, and in KC-like and CCL2 cytokine concentrations in apical cell media. Additionally, LPS consistently reduced TEER over time. However, LPS treatment did not affect the gene expression of any TJs. Thus, the hypothesis for LPS stimulation was proven (there was an increased pro-inflammatory response and weakened barrier integrity), and disproven (there was no reduction in TJ gene expressions). Butyrate was hypothesised to cause an anti-inflammatory response, increase gene expressions of TJs associated with barrier function, and strengthen barrier integrity. Work in this chapter also showed that the butyrate treatment reduced the cytokine concentration of CCL2 in apical cell culture media, which proved the first part of this hypothesis, although the remained of the butyrate hypothesis was disproven (there was no increase in TJ gene expression from butyrate treatment, and no overall improvement in TEER from butyrate treatment). Finally, it was also hypothesised that butyrate would mitigate the pro-inflammatory effects of LPS. Butyrate was found to reduce LPS-induced IL-8 and KC-like cytokine concentrations in basal cell culture media. Additionally, butyrate was found to reduce and restore LPS-induced intestinal barrier permeability. However, butyrate and LPS together caused an increase in CCL2 expression compared to treatment with butyrate and LPS alone. Thus, the hypothesis for the capacity of butyrate to reduce the effects of LPS pro-inflammation were simultaneously proven and disproven.

This study showed that pro-inflammatory IL-8 gene expression and cytokine content were increased in cIEC challenged with LPS. This is consistent with cell culture studies in other species (Angrisano et al., 2010; Kainulainen et al., 2015). Interestingly, though butyrate can increase IL-8

mRNA expression in human HT-29 and Caco-2 cells (Fusunyan et al., 1998; Asarat et al., 2015), there was no increase in IL-8 RNA expression or cytokine concentration compared to untreated controls in this experiment. Similarly, butyrate reduces LPS-induced IL-8 cytokine concentrations in human endothelial cells (Li et al., 2018) but in this experiment, butyrate only reduced LPS-induced IL-8 cytokine concentrations in the basal cell media. Apical secretions of IL-8 are suggested to demonstrate luminal autocrine functionality (Rossi et al., 2013). Basal secretions of IL-8 recruit neutrophils to sites of infection and injury (Rossi et al., 2013). The decreased IL-8 concentrations in basal media from butyrate-stimulated cIEC seen here suggests butyrate reduces the pro-inflammatory pathways in the host. These results show that the cIEC respond similarly to IEC from other species, however there are some differences which could be species derived. Additional research using other IEC from dogs and other species are required to confirm the source of these differences.

CCL2 is an inflammatory chemokine that attracts and activates macrophages and basophils (Carson et al., 2017) via TLR4 signalling (Martin-Vaquero et al., 2014). This chapter showed that CCL2 gene expression and concentration in apical cell media was increased in cIEC treated with LPS, consistent with results seen in human monocytes (Akhter et al., 2018). Butyrate was unable to reduce the LPS-induced CCL2 cytokine production in this experiment, although together they induced the greatest RNA expression. There are currently no publications assessing CCL2 expression in IECs from dogs, although CCL2 concentrations have been assessed in canine cancer cells (Regan et al., 2022) and in the spinal fluid of dogs with cervical spondylomyelopathy (Martin-Vaquero et al., 2014). Interestingly, though butyrate treatment of the cIEC decreased cytokine concentration of CCL2 in the apical media compared to the untreated control, there was no difference observed in the RNA counts between the two treatments. Increased levels of CCL2 have been observed in healthy dogs in comparison to those with cervical spondylomyelopathy (Martin-Vaquero et al., 2014), however this has not been assessed elsewhere in intestinal cells. Canine cancer cells had high expression levels of CCL2 (Regan et al., 2022), and dogs with multicentric lymphoma had higher CCL2 serum cytokine concentrations compared to healthy controls (Calvalido et al., 2016). The cIEC line used in the current work were immortalised using simian virus (SV)40 (Weng et al., 2005), which can cause cancers in

humans and other animals (Vilchez and Butel, 2004). The high levels of CCL2 cytokine in the apical media of untreated control cIEC could therefore be an artifact of their immortalisation with SV40. However, there is no literature to prove or disprove this hypothesis. Additional research using other IEC from dogs that are either immortalised using a different methodology or are not immortalised are required to understand the levels of CCL2 expression in healthy cells. This will also help to further understand the relationship of CCL2 with butyrate and LPS in the dog.

IP-10 is secreted in response to IFN-γ and chemotactically attracts T cells, monocytes and dendritic cells (Chen et al., 2020). In this study, IP-10 RNA expression was increased in cIEC treated with LPS, however there was no cytokine protein expression detected. In comparison, IP-10 cytokine concentrations were found to increase in human blood monocytes (Di Lorenzo et al., 2020) and rat microglia (Mayer et al., 2016) in response to LPS. In dogs, no studies have found differences in IP-10 cytokine concentrations in serum (Calvalido et al., 2016; Galán et al., 2018), plasma (Mazrier et al., 2022), or spinal fluid (Taylor et al., 2014) between healthy and unhealthy individuals. The results from this experiment show that only the basal cell culture media had any detectable IP-10 cytokine expression, and this was from cIEC treated with butyrate alone or LPS and butyrate. Importantly, in this experiment RNA expression from butyrate treated cIEC was no different to untreated cIEC. Additionally, IP-10 RNA expression was increased in cIEC treated with LPS compared to all other treatments, and in cIEC treated with LPS and butyrate compared to untreated controls and cIEC treated with butyrate. Despite its activation by IFN-γ, there was no difference in IFN-γ cytokine or RNA expression across any treatments, which suggests that there may be an alternative means of activation of IP-10 in the dog. However, with the current results in this study and the existing literature, it is difficult to say with confidence what this alternative activation could be.

KC-Like (also called CXCL-1) levels were increased in the apical and basal cell culture media of cIEC treated with LPS, and in the apical media of cIEC treated with LPS and butyrate. There was no change in relation to the untreated control amongst all other treatments and cell media location. KC-Like is suggested to be a potential biomarker for sepsis in dogs (Karlsson et al., 2016; Goggs and Letendre, 2019). LPS-induced sepsis has been modelled in human Caco-2 cells (Ling et al., 2016; Wei

et al., 2022). Thus, the results obtained here suggest that sepsis modelling could be a possible use of the cIEC.

In this study the TEER of cIEC monolayers was significantly altered over time by LPS treatment, in agreement with IEC models from other species and proving the study hypothesis (Hanson et al., 2011; Stephens and von der Weid, 2020). The paradoxical effect of butyrate, wherein low concentrations improve TEER or maintain TEER stability, and high concentrations reduce it (Gibson et al., 1999; Peng et al., 2007; Elamin et al., 2013) was also observed here. Though in this study the butyrate treatment did not cause an improvement to TEER over time, the time window in this study was 8 hours – in Caco-2 cells the improvement from butyrate treatment was seen after 24 hours, and up to 96 hours (Peng et al., 2007). The pattern of response in TEER to the butyrate challenge of cIEC suggests that, over a longer period, this may be observed in these cells too. However, the initial dose response screening (Appendix) did not follow this trend, suggesting the impacts of butyrate may be more dynamic in the cIEC. Additionally, this study showed that butyrate was able to reduce and restore LPS-induced disruptions to intestinal barrier permeability, which is also seen elsewhere (Yan and Ajuwon, 2017). However, in other studies changes in TEER are generally observed in tandem with changes to TJ mRNA and protein abundances, wherein CLDN-2 expressions tend to decrease and increases are seen in CLDN-1, CLDN-3, CLDN-4, OCLDN, and ZO-1 (Sheth et al., 2007; Fujita et al., 2012; Yan and Ajuwon, 2017; He et al., 2020; Wu et al., 2020). Similarly, the increases in TLR2 and TLR4 to mitigate LPS-induced changes on TEER (Hanson et al., 2011; Stephens and von der Weid, 2020) were also not observed in this study. There was detectable gene expression from these targets across treatments in this study, however none were altered by treatment. Further investigations with other dog IEC will determine if these results are inherent to the canine intestine, or if this is a trait unique to these cIEC.

IL-10 was consistently not detected in any of the work in this chapter. IL-10 is one of the key anti-inflammatory cytokines involved in the immune response (Couper et al., 2008). Other studies have detected IL-10 in the colonic mucosa of dogs (Peters et al., 2005; Tamura et al., 2014). The work in this chapter suggests that the cIEC do not mount an anti-inflammatory IL-10 response to expected

stimuli. Future investigations using the cIEC as a model of inflammation need to consider this before making assumptions about the inflammatory response in the canine intestine.

The TEER analysis and treatment of the cIEC was all performed under aerobic conditions. This was one of the major limitations of this work – it could not model the low-oxygen conditions across the GIT. Because of the near-anaerobic environmental conditions of the GIT (Muir et al., 2014; Friedman et al., 2018), the IECs perform their roles in a state of physiological hypoxia (Zheng et al., 2015). The impact of Covid-19 on the PhD programme introduced time constraints on the study which meant that planned work challenging cIEC with lipoteichoic acid, bacterial flagellin, acetate and propionate could not be completed. Future work building on the concepts established here could begin by looking at these other SCFA and bacterial ligands to build a better picture of how the GIT microbiota and diet-derived SCFA impact the intestinal health of the dog. Additionally, the use of another dog IEC to compare these results against would further shed light on potential species-derived differences in intestinal responses.

5.6 CONCLUSIONS

This research has provided insights into the relationship of the healthy canine intestine with LPS and butyrate. It has investigated healthy canine intestinal cells and demonstrated that whilst the dog IEC react in a similar manner to IEC from other species, there are differences in expected responses that may derive from species differences. In doing so, this work has profiled the IEC of the dog in further detail and established parameters of healthy canine IEC. Furthermore, it has further profiled and evaluated the capabilities of the cIEC to be used as an *in vitro* alternative to animal studies, in accordance with the 3R's, and its capacity to be used as an *in vitro* model of the inflammatory response in the canine intestine. As interest continues to grow in this field, this work has also highlighted the potential of using the cIEC for the study of sepsis, in addition to exploring the relationship between the GIT microbiota, SCFA and host health. Finally, it lays the foundations for further opportunities and explorations in this field.

5.7 ACKNOWLEDGEMENTS FOR CHAPTER FIVE

For the work in this chapter I would like to acknowledge Neville Haack and Axel Heiser (AgResearch) for their assistance with selecting gene targets for, and the running of, the Nanostring experiments. In addition, I would also like to acknowledge Felicity Jackson of the Massey Nutrition Laboratory for her organisation of the Luminex testing, and Rina Hannaford (AgResearch) for being an invaluable font of knowledge for the R analysis. Additonally, I would like to acknowledge Paul Maclean (AgResearch) for his assistance with the PCoA and Bi-Plots, and answering my endless stats-related questions so promptly.

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Francis David Phimister
Name and title of main supervisor:	Associate Professor David Thomas
In which chapter is the manuscript/published work?	Chapter Five
What percentage of the manuscript/published work was contributed by the student?	70%

Describe the contribution that the student has made to the manuscript/published work:

Francis was responsible for all cell culture based experiments in this chapter. He performed all experiments and collected all samples for further testing. He performed the included transepithelial electrical resistance analyses, ELISAs, and performed the statistical assessments of these data, and the data obtained from the Nanostring and Luminex assays. Additionally, he has drafted the manuscript for publication, and is responsible for re-drafting post-author comments.

Please select one of the following three options:

○	The manuscript/published work is published or in press Please provide the full reference of the research output:
○	The manuscript is currently under review for publication Please provide the name of the journal:
◉	It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Student's signature:	Francis Phimister Digitally signed by Francis Phimister Date: 2023.01.28 09:27:31 +13'00'	Main supervisor's signature:	David Thomas Digitally signed by David Thomas DN: cn=David Thomas, c=NZ, o=Massey University, ou=School of Agriculture & Environment, email=d.g.thomas@massey.ac.nz Date: 2023.01.29 16:34:17 +13'00'

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

CHAPTER SIX

INITIAL ADAPTATION OF THE CIECS TO THE APICAL ANAEROBIC MODEL

**Selected Material from this chapter and Chapter Four was presented as:*

'A Co-Culture Cell System to Model the Canine Intestine' Oral Presentation, Australian and New Zealand Laboratory Animal Association (ANZLAA) Winter Conference, Palmerston North, New Zealand, July 2019.

Waltham Internal Event Poster, Poster Presentation, Waltham internal review, Waltham-on-the-Wolds, United Kingdom, January 2020.

'The Development of a Novel Co-Culture System to Model Diet-Based Influences on the Canine Intestinal Epithelium' Accepted abstract, Global Animal Nutrition Summit, Guelph, Canada, August 2020.

'The development of an in vitro model of the canine intestine to evaluate and understand the diet-based influences on the health of the domestic dog.' Poster Presentation, AgResearch Science Hot Week, Palmerston North, New Zealand, September 2021.

6.1 ABSTRACT

Cell culture models of the intestinal barrier are traditionally performed in aerobic conditions. However, *in vivo* the GIT lumen is either completely, or very nearly anaerobic in nature. Aerobic models limit our capacity to understand the effects these low oxygen and/or hypoxic conditions have on the intestinal barrier. However, dual environment co-culture (DECC) systems have been developed that allow for the culture of oxygen requiring intestinal epithelial cells in an apically anaerobic environment. These more closely model conditions *in vivo* and have demonstrated previously unseen alterations in cellular expressions in the differently oxygenated environments. The work in this chapter follows on from method development refined and established in Chapter Four. It was hypothesised that the refined methodologies could be used to successfully culture the cIEC line in a DECC system. TEER was used as a means of quantifying barrier integrity and determining successful culture. It was then hypothesised that the cIEC-DECC model could then be used to investigate and characterise the canine host response to bacterial challenges in a physiologically relevant model. However, the work in this chapter was stopped due to supply issues that arose due to the Covid-19 pandemic. Consequently, the DECC model was no longer used for this PhD. Overall, this chapter describes the experiments used to ascertain the potential of the cIEC-DECC model. This chapter also includes in the discussion details of the planned work that could not be completed that would have further tested and evaluated the capabilities of the cIEC-DECC model.

6.2 BACKGROUND

The GIT maintains opposingly oxygenated environments to sustain the oxygen-requiring IECs and the GIT microbiota that are susceptible to oxygen toxicity (Ward et al., 2014; Lu et al., 2018). The IECs that comprise the intestinal barrier perform their roles in a state of physiological hypoxia (Zheng et al., 2015). A constant supply of oxygen from the lamina propria though the IECs maintains their survival and stops hypoxia-induced diminishment of their nutrient absorbing capacity (Ward et al., 2014). The anaerobic environment of the lumen sustains the obligate anaerobic GIT microbiota (Lu and Imlay, 2021). Some of these bacteria assist in the fermentation of dietary fibres in the colon,

giving rise to SCFAs such as butyrate (Jackson and Jewell, 2016; Nogueira et al., 2019). Butyrate in turn is used by colonocytes to maintain the anaerobic environment of the colon (Litvak et al., 2018b). As discussed in Chapter Four, the traditionally employed method of modelling the IEB *in vitro* involves culturing cell layers onto porous filters, but this is performed aerobically (Chen et al., 2015). Therefore, traditional aerobic culture only allows testing of non-viable obligate anaerobic bacteria with IECs, limiting the capability to accurately model *in vivo* conditions and processes (Maier et al., 2014).

Organ-on-a-chip models have been used to simulate anaerobic intestinal conditions as an *in vitro* model of the human GIT (Jalili-Firoozinezhad et al., 2019; Shin et al., 2019). A dual-environment model of the gut was also developed and utilised recently to investigate the effects of anaerobic bacteria on IECs, whilst keeping both cell lines alive (Zhang et al., 2021a). This system, denoted a GuMi (Gut Microbiome) Physiome Platform by the authors, was notable for its capability to refresh the oxygen content of the basal medium, and its capability of creating liquid flow across the apical layer, further modelling intestinal conditions *in vivo*(Zhang et al., 2021a).

Researchers at AgResearch had previously designed and utilised a dual-environment co-culture (DECC) system that was based off the cellZscope technology; capable of monitoring TEER periodically (Ulluwishewa et al., 2015; Maier et al., 2017; Maier et al., 2018). The DECC could be placed in an anaerobic environment wherein pressure seals kept the oxygenated basal cell medium separate from the apical anaerobic environment, which was also used for modelling the human GIT (Ulluwishewa et al., 2015; Maier et al., 2017; Maier et al., 2018), Figure 6.38. In contrast, there is currently no published literature that models the canine IEB using a system capable of maintaining anaerobic and aerobic conditions.

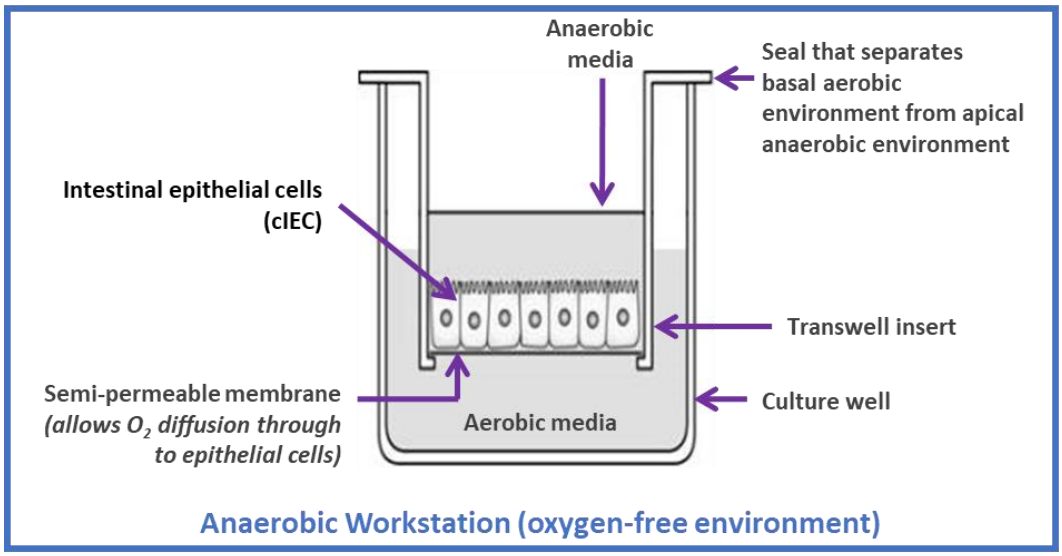


Figure 6.38 *– Dual environment co-culture (DECC) system overview.Throughout the experiment the DECC was housed in an anaerobic workstation. The viability of the canine intestinal epithelial cells (cIECs) were maintained by the diffusion of oxygen in the basal media through the semi-permeable membrane of the inserts. The aerobic nature of the basal media was maintained by seals that separate the two opposing environments. Figure adapted from (Maier, 2017).*

To address this, the initial aim of the PhD project was to adapt the cIEC to work in the DECC system previously employed by researchers at AgResearch, to provide a robust, physiologically relevant model of the canine IEB that could be employed to model host-bacterial interactions. It was hypothesised that cIEC could be successfully cultured in the DECC and that the conditions required to achieve a period of intestinal epithelial barrier stability future experiments could be defined. Transepithelial electrical resistance (TEER) was used as a measurement of intestinal barrier integrity and permeability as the growth and differentiation protocols for cIEC were adapted and refined from traditional, aerobic cell culture to a DECC system that maintained an apically anaerobic environment.

As the work in this chapter built on from that conducted in Chapter Four, the criteria utilised in that chapter was employed here. However, due to the design of the DECC, wherein basal oxygen does not replenish as it does in an aerobic environment, cells in this system die of hypoxia, thereby limiting experimental time in this model (Maier, 2017). Thus, a period of at least four consecutive hours of stable TEER was deemed a minimum requirement for successful culture of the cIEC in the DECC.

Previous work utilising the DECC with Caco-2 cells demonstrated stable TEER values of 600 Ωcm^2 during the experimental period (Maier, 2017). Under aerobic conditions, the cIEC had demonstrated greater TEER than Caco-2 cells (Farquhar et al., 2018), thus the previously attained TEER values of 600 Ωcm^2 were hypothesised to be an achievable target. Therefore, criteria for successful culture in the DECC were defined as:

3) TEER readings above 600 Ωcm^2 after 48 hours of differentiation.
4) A minimum of four consecutive hours of stable TEER readings after 48 hours of differentiation, defined as > 4 hours with no statistical difference ($p > 0.1$) in the change in TEER between timepoints.

As with the criteria for success in Chapter Four, if the culture methods passed the first criterion for successful culture, they were then analysed against the second criterion for success. If they did not pass the first criterion, the experiment was rejected as unsuccessful.

The study was impacted and limited by the Covid-19 pandemic during 2020-2021, whereby the Transwells® required for experiments were unable to be produced. The DECC had been custom built for the use of these inserts, so the seal separating the anaerobic apical media from the aerobic basal media could not be maintained with alternative inserts. This chapter details the work undertaken, building on from Chapter Four, to work in the DECC system, and provides an overview of the planned work that was unable to be completed because of the pandemic.

6.3 MATERIALS AND METHODS

6.3.1 REAGENTS

The reagents used for culture of cells were the same as those in Chapter 4, Section 4.1. In addition to those reagents, LPS from *Escherichia coli* was purchased from Sigma Aldrich (St Louis, Missouri, USA).

6.3.2 CELL CULTURING CONDITIONS

The cell culture conditions used were the same as outlined in Chapter 4, Section 4.3.2. Cell counts were also performed as outlined in Chapter 4, Section 4.3.2

6.3.3 DIFFERENTIATION CONDITIONS AND BARRIER INTEGRITY ASSESSMENT ASSAYS

Differentiation conditions used are identical to those outlined in Chapter 4, Section 4.3.3. Experiments were performed in blocks with four biological replicates per treatment, per block.

6.3.3.1 Automated TEER Collection in the DECC

Within 24 hours, and at least 12 hours prior to assays being performed, a 50 mL falcon tube containing base differentiation medium (BDM; DMEM supplemented with 7% FBS, 2 mM L-glutamine, 2 mM GlutaMAX, and 10 mM HEPES) was preconditioned to the anaerobic atmosphere. Automated assessment of TEER was performed as previously described (Maier, 2017; Maier et al., 2017; Maier et al., 2018). In summary, under aerobic conditions the basal wells of the DECC were filled with 3.0 mL of BDM. Additionally, and under aerobic conditions, the remastered differentiation medium (RDM; DMEM supplemented with 7% FBS, 2 mM L-glutamine, 2 mM GlutaMAX, 10 mM HEPES, and 150 nM hydrocortisone) was removed from the apical side of Transwells and replaced with 260µl of fresh BDM. The Transwell inserts containing the cell monolayers and 260 µl of TEER media were carefully inserted into the DECC, and the probe-containing lid was secured gently into place. The DECC was transferred into the anaerobic workstation (Model A85, Concept Plus, Ruskinn Technology Ltd, Bridgend, UK) and incubated at 39°C in an atmosphere of 10% CO_2, 10% H_2, and 80% N_2.

A modification to the workstation allowed for the DECC to be connected to a computer running the cellZscope software (version 4.3.1, nanoAnalytics GmbH, Munster, Germany) whilst maintaining the anaerobic environment of the workstation. The TEER across each cIEC cell monolayer was measured three times; the initial reading was taken when the DECC was first connected to the

monitoring laptop, and then subsequently was recorded once every thirty minutes. Thirty-minute readings were utilised as this was the shortest time available in the software that allowed enough time for the cellZscope software to read all 24 wells before the next round of reading began. The third TEER reading, which was taken after 60 minutes in the anaerobic workstation, was used as $TEER_{initial}$, as per Equation 2.

The aerobic, apical medium was then removed and replaced with 260 µL of the pre-conditioned anaerobic BDM. The TEER measurements were resumed and recorded every 30 minutes. As the initial TEER for each insert was different, the effect on TEER over time was expressed as the change in TEER compared to the baseline TEER for each insert, using Equation 2 (see Section 4.3.3.3).

6.3.4 PREPARATION OF LIPOPOLYSACCHARIDE CHALLENGE

In a sterile class II biosafety cabinet, 1 mg of freeze-dried LPS was resuspended in 1.0 mL of fresh BDM to form a 1 mg/mL working stock solution. The LPS-BDM solution was then preheated to 39°C for 30 minutes prior to experimental challenge. The LPS-BDM solution was diluted down further in BDM to form a serial dilution of LPS, which was then used to challenge the cIEC and ascertain dose responses, similar to experiments conducted in Chapter Five, Section 5.4.1.1. When the apical, aerobic BDM in the DECC was removed after 1 hour of TEER readings, 260 µL of the appropriate LPS challenge was aliquoted into the apical well, and the DECC continued to be set up for TEER readings as per Section 0. Concentrations used were 500, 250, 125, 62.5 and 31.25 µg/mL LPS. Untreated cIEC were ran in parallel as a negative control. Four biological replicates were used and tested once.

6.3.5 STATISTICAL ANALYSES

The statistical methods used were identical to those outlined in Chapter 4.3.4. Statistical significance is reported as a p value < 0.05, whilst $p < 0.1$ was considered a trend. All data are presented as mean ± SEM unless otherwise noted.

6.4 RESULTS

6.4.1 TEER WAS ABOVE 600 ΩCM2 AFTER 48 HOURS OF DIFFERENTIATION AND WAS STABLE FOR 4.5 HOURS

The first experiment was to continue work performed in Chapter Four, Section 4.4.4 and evaluate the TEER of the cIEC in BDM and RDM over time, Figure 6.39. There was a significant ($p < 0.001$) difference in TEER for both treatments over time. The TEER of the cIEC in BDM remained above 600 Ωcm^2 until 57 hours of differentiation. The TEER of cIEC in RDM remained above 600 Ωcm^2 until 51 hours of differentiation. Thus, criteria one was achieved in this experiment.

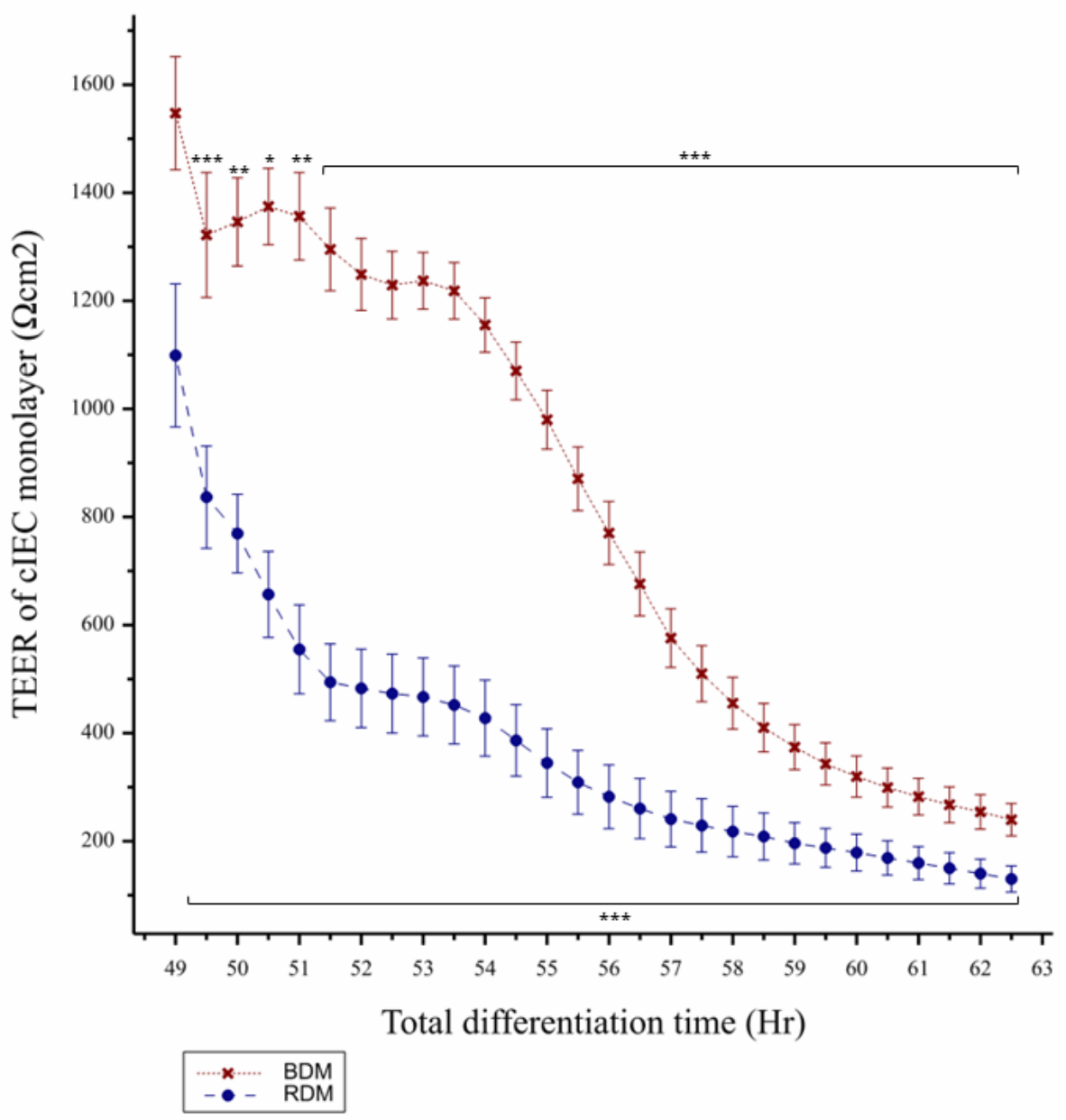


Figure 6.39 – *Mean (± SEM) transepithelial electrical resistance (TEER) from canine Intestinal Epithelial cells (cIECs) in base differentiation media (BDM) during TEER analysis, or with the addition of 150 ng/mL hydrocortisone to BDM (RDM).TEER results measured automatically whilst in an apically anaerobic environment. N (BDM) = 20, n (RDM) = 22. Significant differences to the TEER at 49 hours of differentiation (Time 0) are represented by *, where * p < 0.05, ** p < 0.01 and *** p < 0.001.*

As the treatment media passed the first criteria for successful culture of cIEC in the DECC, the impact of these treatments on the change in TEER over time was assessed and is presented as Figure 6.40. There was a significant (p< 0.001) difference between treatments over time. The second

criteria for successful culture of the cIEC in the DECC was a period of at least 4 hours where there was no difference in the change in TEER ($p > 0.1$). There was no significant difference ($p > 0.1$) in the change in TEER between 49.5 and 54 hours in the BDM. The RDM did not achieve a period of stability as defined.

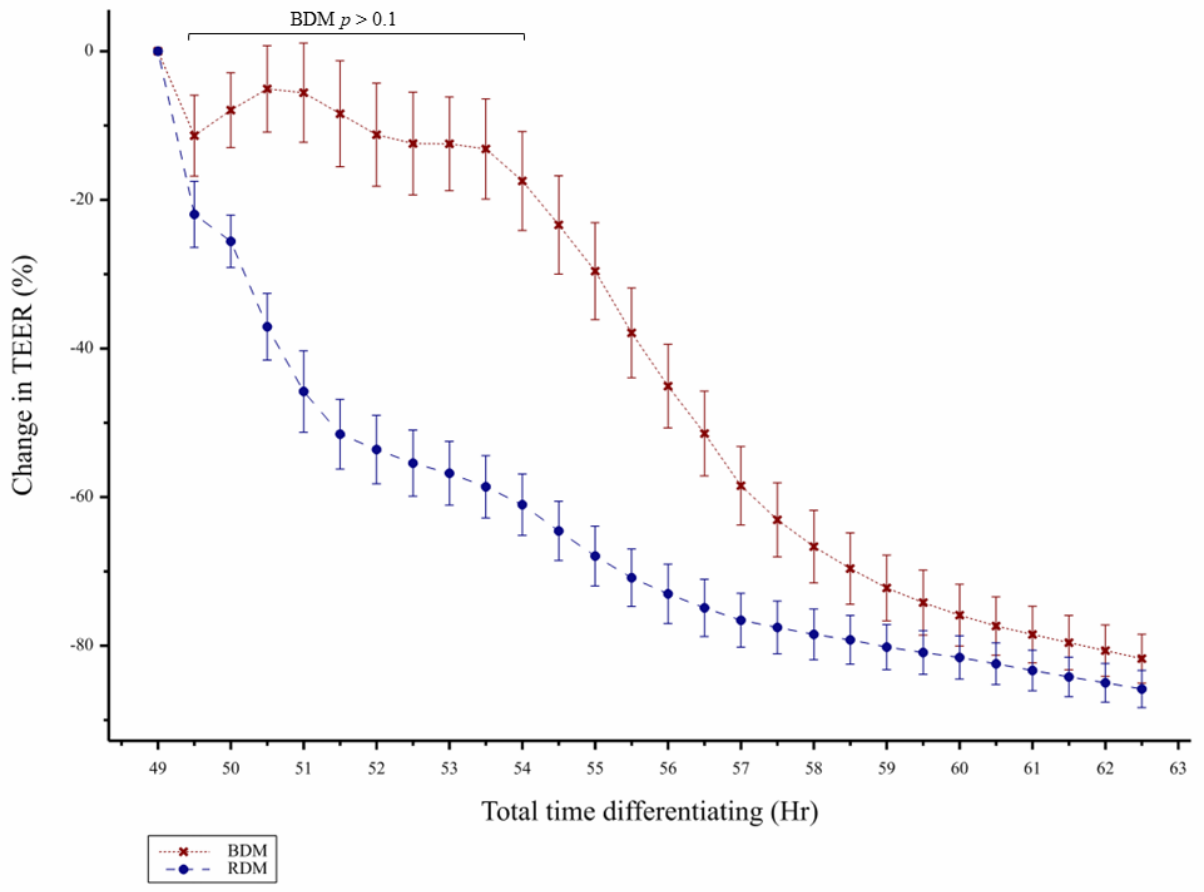


Figure 6.40 *– Mean (± SEM) change in transepithelial electrical resistance (TEER) from canine Intestinal Epithelial cells (cIECs) in base differentiation media (BDM) during TEER analysis, or with the addition of 150 ng/mL hydrocortisone to BDM (RDM).Change in TEER defined as a percentage change compared to the initial TEER value for the same treatment, taken at 49 hours. N (BDM) = 20, n (RDM) = 22. The experimental aim was to identify blocks of time where TEER did not change (p > 0.1). This period is identified, with the treatment group it belongs to.*

The refined methods from Chapter Four (differentiation of cIEC at 39°C, 6% v/v CO_2 for 48 hours in RDM, followed by a change into fresh BDM) met the second criteria for successful culture of the cIEC in the DECC. Thus, these methods were utilised going forwards for work with the DECC.

6.4.2 PRELIMINARY DATA SUGGESTED A DOSE DEPENDENT DECREASE IN TEER FOLLOWING LPS TREATMENT IN THE DECC

An LPS dose response experiment was performed to evaluate the impacts of LPS on the TEER over time in the DECC, Figure 6.41. Only one experiment was performed before the project had to be stopped. In this experiment, however, the effects of treatment over time on the TEER of differentiating cIEC trended towards significance (p = 0.086).

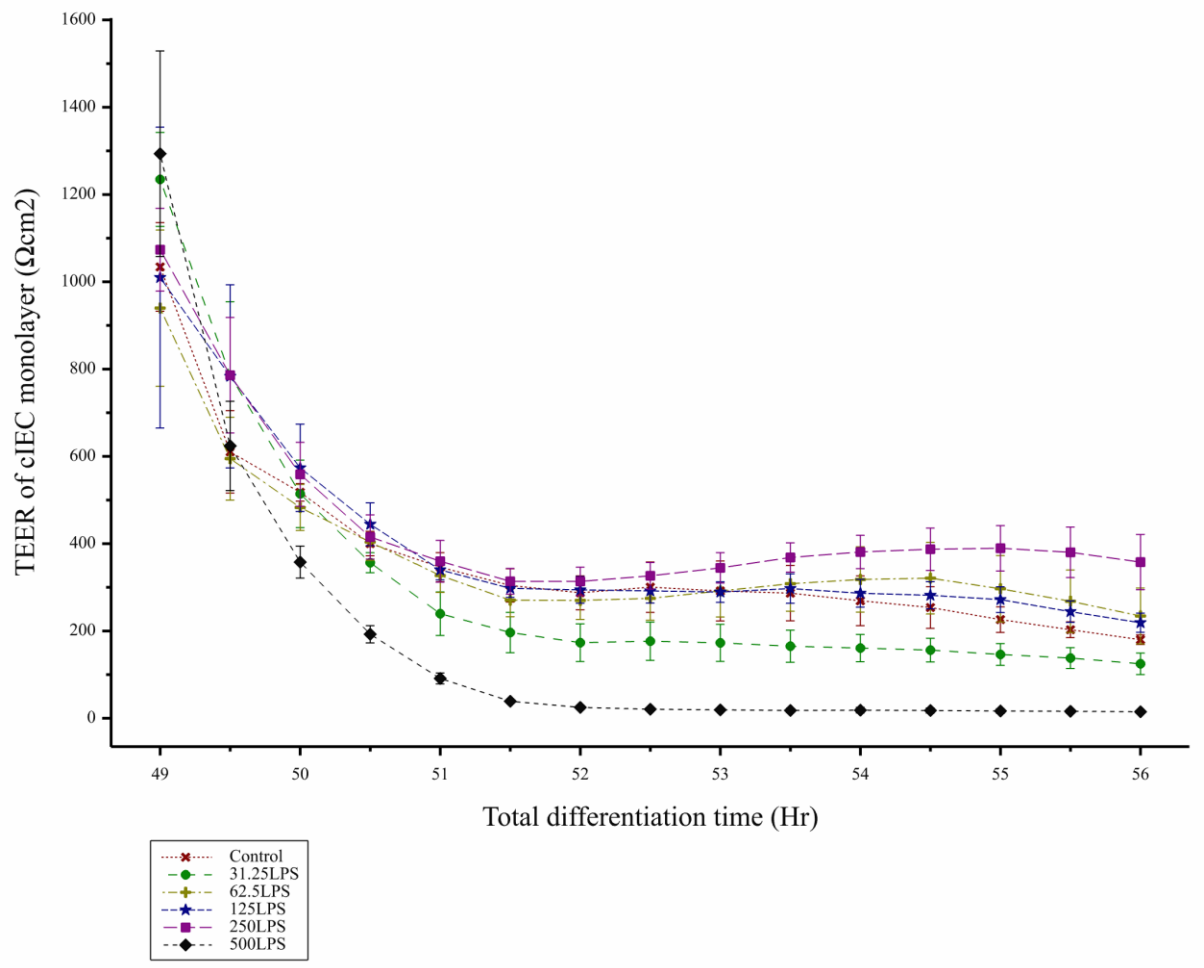


***Figure 6.41** – Mean (± SEM) transepithelial electrical resistance (TEER) from canine Intestinal Epithelial cells (cIECs) challenged with increasing doses of lipopolysaccharides (LPS).The cIEC were challenged with either 31.25, 62.5, 125, 250, or 500 µg/mL of LPS (31.25LPS, 62.5LPS, 125LPS, 250LPS and 500LPS, respectively), and were run in parallel with untreated cIEC (Control). N = 4 for 31.25LPS, n = 3 for all other treatments.*

The impacts of LPS treatment over time on the change in TEER were then assessed and are presented in Figure 6.42. The treatment over time caused a significant impact on the change in TEER (p = 0.014). The TEER at all timepoints was significantly changed compared to their initial reading at 49 hours (p< 0.001 in all instances). Treatment with 500 µg/mL LPS caused the greatest change in TEER compared to all other treatments (p< 0.001 in all instances). The cIEC treated with 31.25 µg/mL had the second greatest negative change in TEER across the experiment (p< 0.001 in all instances), although after 52 hours of differentiation (which was 3 hours post-treatment), there was no difference (p > 0.1) in the change in TEER between the untreated cIEC and those treated with 31.25 µg/mL LPS. Similarly, there was no difference in the change in TEER in the cIEC treated with 62.5, 125 and 250 µg/mL after 52 hours of differentiation (3 hours post-treatment; p > 0.1).

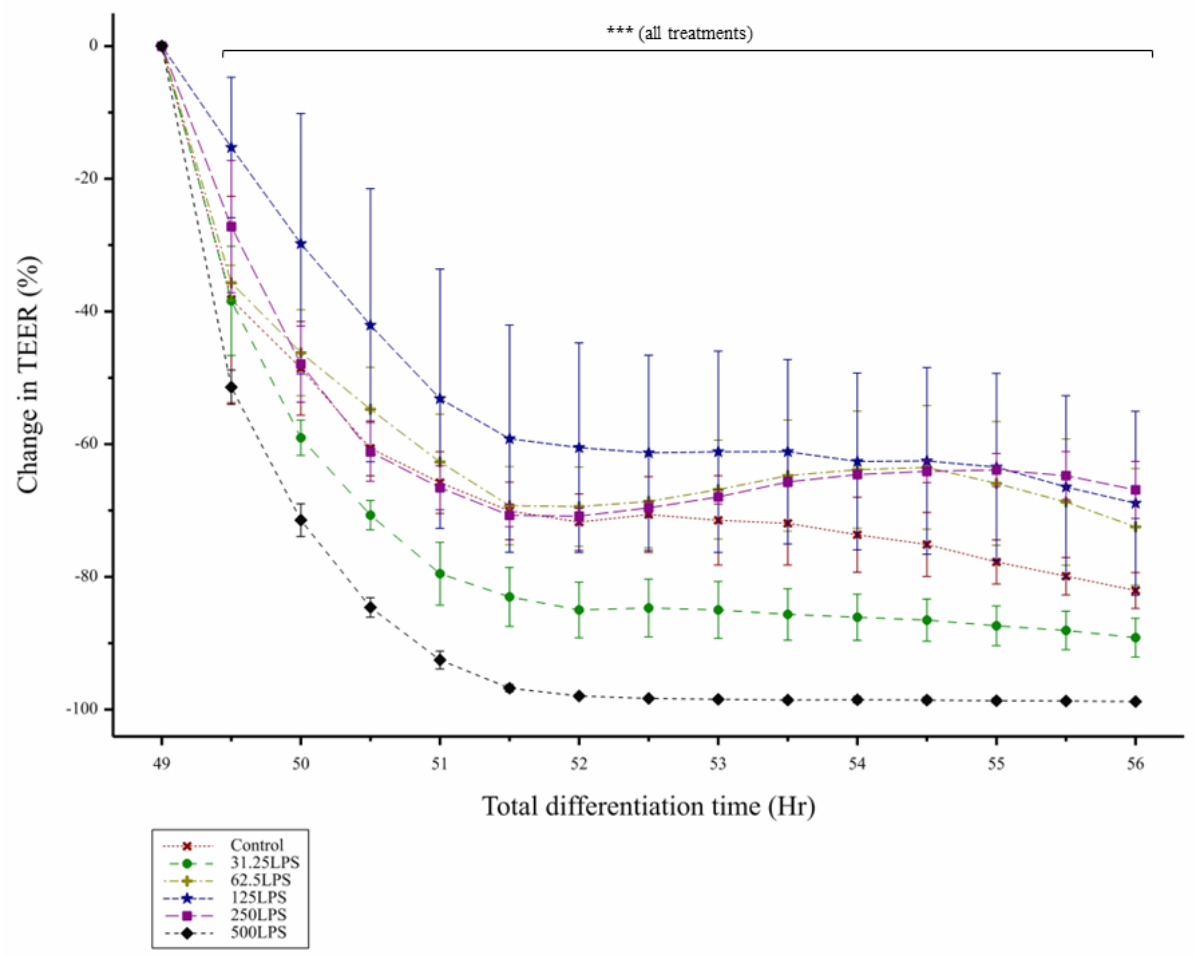


Figure 6.42 *– Mean (± SEM) change in transepithelial electrical resistance (TEER) from canine intestinal epithelial cells (cIECs) challenged with increasing doses of lipopolysaccharides (LPS).Change in TEER defined*

*as a percentage change compared to the initial TEER value for the same treatment, taken at 49 hours The cIEC were challenged with either 31.25, 62.5, 125, 250, or 500 µg/mL of LPS (31.25LPS, 62.5LPS, 125LPS, 250LPS and 500LPS, respectively), and were run in parallel with untreated cIEC (Control). N = 4 for 31.25LPS, n = 3 for all other treatments. Significant differences to the TEER at 0 hours are represented by *, where *** p < 0.001.*

6.5 DISCUSSION

This study showed that the cIEC could be successfully cultured in the DECC. Building from work in Chapter Four, it was hypothesised that the cIEC could be successfully cultured in the DECC. The criteria for this hypothesis were: a TEER of above 600 Ωcm^2 and a period of at least four hours or more where there was no statistically different change in TEER between timepoints. TEER remained above 600 Ωcm^2 until 57 hours of differentiation, and there was a period of stable TEER between 49.5 and 54 hours of differentiation. Thus, the hypothesis was successfully proven. This was then used to begin characterising the host response to bacterial challenges, although this work was cut short by supply issues caused by the Covid-19 pandemic. Whilst the work in this chapter did demonstrate that the stable period of TEER was achieved, the small window of time that the TEER is stable for may not be enough. Future work in challenging this period of stability with bacterial ligands and SCFA, furthering on from work in Chapter Five is a worthwhile endeavour, though alternatives to the DECC may also be worth consideration.

The work undertaken with LPS in this chapter needs further data before it can be compared with that undertaken in Chapter Five. The reduction of TEER in cIEC treated with 31.25 µg/mL LPS was similar to the reduction caused by highest dose of LPS, and does appear similar to results observed in Chapter Five. However, there was no change in TEER observed from the effects of treatment over time observed in this chapter, possibly due to insufficient power in the sample size. Comparatively, there was a significant impact on the change in TEER over time caused by treatments, where it appeared that the change in TEER caused by LPS treatment of cIEC with 62.5, 125 and 250

μg/mL was not as great as that observed in untreated cIEC. As these results are from a single experiment, however, it remains difficult to interpret these findings.

Planned work would have characterised the unchallenged cIEC in the DECC compared to an aerobic environment. This would have determined if there were any alterations to gene expression caused by the apically anaerobic environment as has been seen previously in Caco-2 cells (Ulluwishewa et al., 2015). Furthermore, planned work would have continued the groundwork set out in Chapter Five, expanding on LPS and butyrate testing, and characterising the response of the cIEC to bacterial flagellin, acetate, and propionate. This would have built a profile of the cIEC's response to bacterial ligands and SCFAs, allowing the proposed final investigations characterising the cIEC's response to live bacterial challenges to confidently discern the source of these impacts. Learnings obtained from Chapter Three's meta-analysis would have been used to define the bacteria of interest, such as *Sharpea*. Future work using the cIEC-DECC model can use results obtained in Chapter Five to compare the differences (if any) in response to LPS in aerobic and apically anaerobic environments. In addition, future work will benefit from following the planned work pathways discussed here.

One major limitation of this DECC system is that there is no way to replenish the oxygen in the basal media throughout the course of the experiment, unlike the GuMi model utilised by Zhang et. al.,(Zhang et al., 2021a). A lack of oxygen, leading to hypoxia, results in the expression of hypoxia-inducible factor (HIF)-1 and HIF-2 alpha (Schönenberger and Kovacs, 2015; Lee et al., 2019). The impacts of these are still being studied in non-canine models, although these HIF bind to claudin-1, thereby removing it from the maintenance of intracellular permeability and causing an overall decrease in permeability and therefore increasing TEER (Saeedi et al., 2015b; Saeedi et al., 2015a; Masterson et al., 2019). Similarly, induction of hypoxia has been seen to result in decreased TEER in Caco-2 and HT-29 cells (Lian et al., 2021), as well as brain capillary endothelial cells (Yamagata et al., 2004). Though cell viability was assumed at specific timepoints during these experiments via the collected TEER data, the impacts of hypoxia were not investigated in depth. Planned experiments would have evaluated the viability of the cIEC via trypan blue and neutral red staining to evaluate the

impacts of hypoxia. Another planned avenue of exploration was to observe if the effects of hypoxia on tight junction expression in the cIEC model were similar to published literature (i.e., decreases in CLDN-1 relative to increasing HIF-1). The goal of this would have been to establish an understanding of HIF-1 expression in the cIEC-DECC model and ultimately determine a timepoint in which HIF-1/CLDN-1 expression demonstrated a point of terminal hypoxia – i.e., the cellular expressions from that point onwards would have been considered to have been predominantly resulting from hypoxia, rather than any challenges or treatments applied to the cells.

On a similar note, hypoxia-induced apoptosis of cells has been found to be linked to CLDN-5; wherein CLDN-5 has been found to be redistributed to mitigate cellular apoptosis and resulting impacts to the blood-brain barrier (Yang et al., 2021; Yu et al., 2021). The intention with this would have been to use immunohistochemistry (IHC) to stain the cIEC for the HIF-1 and CLDN-5 proteins. This investigation would has assessed if CLDN-5 proteins were redistributed over time in the DECC and determined the relationship of this (if any) with the HIF-1/CLDN-1 expression levels over time. Additionally, it would have determined if there was a noticeable decrease in TEER, similar to what has been observed the zebra fish models (Yang et al., 2021; Yu et al., 2021), and in human brain capillary endothelial cells (Yamagata et al., 2004). Together these would have allowed for an in-depth analysis of the robustness of the cIEC-DECC model and are a worthwhile beginning path of exploration for future experiments.

Hypoxia-induced exosome secretions were found to increase insulin resistance and alter the gut microbiota in mice (Moreno-Indias et al., 2015; Khalyfa et al., 2021). In this study barrier permeability was also found to be increased, although tight junction proteins were not quantified (Khalyfa et al., 2021). The future work of the cIEC-DECC model was to investigate the interactions between live, anaerobic bacteria and the healthy canine intestinal barrier. Though these plans were cut short, there is increased interest in the literature regarding the relationship of hypoxia, HIF-1, and the gut microbiota, which has been reviewed in depth by Pral et. al., (Pral et al., 2021). The increased knowledge affords the opportunity to critique and evaluate the DECC model's project pathways going forwards. For robust data, it would be beneficial to perform side-by-side characterisation of the cIEC

in aerobic and the apical anaerobic conditions on exosomal production and tight junction expressions. This would allow for a knowledge base to be established wherein the impacts of hypoxia alone would be demonstrated and be accounted for in future analyses, so that evaluations of the host-microbiome interactions could be investigated and interpreted with greater confidence.

Ultimately, going forwards, the DECC as a model for the intestine is lacking key features, especially when compared to models such as the GuMi, which can replenish basal oxygen and simulating intestinal liquid flow. However, the DECC has 24 wells for samples, whereas the GuMi only has six. Thus, the DECC is a higher throughput model and is more useful for comparing the effects of treatment on cells. Considering the impacts to planned work, it has afforded time to reflect and consider the optimum analyses to be undertaken for future work using the cIEC-DECC model, and the DECC itself. Though the DECC allows for continual monitoring of TEER whilst the system is running, its inability to replenish oxygen means that after an as-yet undetermined timepoint, cells utilised in this model have succumbed to hypoxia. Therefore, any results produced after this point are unable to be used with any confidence. Going forwards in this model, the first step should be to establish a timeline of viability in these cells. The expression of HIF-1 can be utilised to build a timeline of cell viability and demonstrate a timepoint in which hypoxic-toxicity has rendered any further results untrustworthy. Until this time, the DECC model as it stands is an upgrade to aerobic culture, but fundamentally needs improvements to be a competitive *in vitro* model of the intestine.

6.6 CONCLUSIONS

The cIEC were successfully cultured in the DECC, and a preliminary investigation on the impacts of LPS on the cIEC in apically anaerobic conditions was able to be performed. The work that was achieved has formed an essential steppingstone into furthering current knowledge of gut health in the domestic dog. By combining the work begun in this chapter, the experimental procedures that were unable to be completed, as well as those identified as essential challenges and critiques of the cIEC-DECC model, future research can be performed in a robust *in vitro* model of the canine intestine that can be interpreted with confidence.

6.7 ACKNOWLEDGEMENTS FOR CHAPTER SIX

In addition to those people highlighted in the acknowledgements section of this thesis, for work relating to this chapter in particular I would also like to acknowledge the work of Peter Green and Rina Hannaford of AgResearch for the design of the R code used for results analysis, and Scott Sevier and Tore Rayner of AgResearch for their technical expertise and troubleshooting with the DECC.

CHAPTER SEVEN

GENERAL DISCUSSION AND FUTURE DIRECTIONS

7.1 General Discussion

The relationship between diet and the microbiota of the GIT in the domestic dog influences the relationship between diet and host health (Bresciani et al., 2018; Allaway et al., 2020; Atherly et al., 2020). Increased scientific exploration into the health benefits of pet food ingredients and changing pet owners' attitudes are driving the pet food industry towards optimising pet health through nutrition (Chen et al., 2012; Boya et al., 2015; Di Cerbo et al., 2017; Schleicher et al., 2019). Assessing the health benefits of these new diet formulations often requires feeding trials (Ministry for Primary Industries, 2018). However, the worldwide implementation of the Reduction, Refinement and Replacement of laboratory use of animals (3Rs) is moving towards the production of, and use of robust, scientifically accurate non-animal models (2010; Ferdowsian and Beck, 2011; Doke and Dhawale, 2015). Whilst cell culture has been routinely employed as an alternative method to model the effects of food products and dietary constituents on intestinal health and the microbiota of the GIT in humans (Hashimoto et al., 1994; Gossé et al., 2005; Juan et al., 2006; Anderson et al., 2018; Cai et al., 2018), this model has not yet been explored in-depth in a species specific model in the dog. Thus, this thesis aimed to utilise a previously characterised cIEC line (Weng et al., 2005; Farquhar et al., 2018) to model the impacts of dietary-included changes to the GIT microbiota on the intestinal, and host health of the domestic dog. The aim was to co-culture bacteria with the cIEC and characterise changes to the intestinal epithelial barrier and the immune response in an apically anaerobic environment. This first required a detailed understanding of the interactions between microbial ligands and microbial-derived compounds on the innate intestinal defences.

Chapter Two provided an in-depth review of the literature available on the interactions between the intestinal epithelial barrier and the innate immune response. It then reviewed the interactions of these two defence systems with microbial ligands and SCFAs, which are derived in the GIT from bacterial fermentation of proteins and fibres (Nery et al., 2012; Hang et al., 2013; Sandri et al., 2017). There was limited literature available that assessed these intestinal interactions in healthy dogs, and no literature that assessed the response of the dog IECs to bacterial challenges. Therefore, the review

drew on published studies in other models, and highlighted differences in the dog innate response and how these may differ. As an example, dogs have an absence of individual caspase-1 and caspase-4/-5/-11 genes, (Digby et al., 2021), which in humans and mice are activated as part of the immune cascade upon detection of bacterial lipopolysaccharides (LPS). Instead, dogs form a caspase-1/-4 fusion protein that causes the IL-1β cleavage of LPS detection without the activation of inflammasomes that occurs in humans(Devant et al., 2021; Digby et al., 2021). Similarly, the relationship of SCFA and the intestinal response in healthy dogs are not well-explored in the published literature. The effects of a low concentration (2 mM) of butyrate on the canine intestine increases barrier strength (Farquhar et al., 2018), which is also seen in human Caco-2 cells (Elamin et al., 2013). In other species, butyrate is able to modulate the pro-inflammatory response of the intestine, capable of down-regulating pro-inflammatory cytokines (Asarat et al., 2015). Overall, there was very limited information available on these interactions in the dog. Therefore, it became apparent that these interactions would need modelling as part of the doctoral project. The response of gene expressions associated with immune pathways in the dog, such as pro-inflammatory chemokines and cytokines, and the impacts on TJ expressions in dog IECs caused by bacterial ligands and/or SCFA stimulation was identified as a clear knowledge gap. An investigation determining these responses was deemed as a fundamental requirement for this doctoral project, and as fundamental for increasing knowledge of intestinal health in the dog.

To determine which bacteria would potentially be co-cultured with the cIEC, the impacts of diet on the faecal microbiota was assessed. To further contribute to existing knowledge, and to be performed using methods in line with the 3Rs, a meta-analysis was conducted. This enabled a more in-depth analysis than a literature review would provide, and generated new knowledge without performing additional animal trials. Thus, Chapter Three provided the first comprehensive meta-analysis of the literature to explore the impacts of dietary protein and dietary fat on the faecal microbiome of the domestic dog. A novel finding was that *Sharpea* was the genera most associated with causing the shifts in microbial profiles in response to changes in both crude protein, and crude fats, despite its low relative abundance. In addition, *Prevotellacaea*_Ga6A1_groupand *Enterococcus*

were associated with dietary protein levels, whilst *Allobaculum* and *Clostridium_sensu_stricto_13* were associated with dietary fats. The relationship of *Enterococcus* with dietary protein content was consistent with other studies (Pinna et al., 2016; Phungviwatnikul et al., 2021). The findings for *Sharpea* are novel and currently cannot be challenged or confirmed based on the available literature. *Prevotellacaea*_Ga6A1_group and *Allobaculum* have been reported to change in overweight dogs fed high protein diets (Phungviwatnikul et al., 2021), though have not been reported as affected by diet in healthy dogs. Similarly, a relationship of *Clostridum_sensu_stricto_13* with dietary protein or fat has not been reported in the literature.

The results obtained from this meta-analysis suggested that the bacteria most impacted by dietary changes in the dog were Gram-positive bacteria. Of the bacteria most affected by changes to dietary protein or fat content (and *Sharpea*, which was most responsible for driving shifts in microbial profile in response to diet changes), *Prevotellacaea*_Ga6A1_group is the only Gram-negative bacteria. Therefore, the cIEC were to be characterised in response to both LPS and lipoteichoic acids (LTAs), thereby simulating both Gram-positive and Gram-negative bacterial interactions. This would be complemented by characterisation of the cIEC in response to the SCFAs acetate, butyrate, and propionate. This would address the knowledge gaps identified in Chapter Two.

Chapter Four developed methods to be used for eventual characterisation of the cIEC in an apically anaerobic model. These method refinements defined a time in which barrier integrity was stable and challenges to this integrity could be conducted. This was achieved by the inclusion of hydrocortisone in the cell media during the initial 48 hours of cellular differentiation. The increases in barrier integrity caused by the inclusion of hydrocortisone were similar to those seen in other studies (Weng et al., 2005; Fischer et al., 2014; Furihata et al., 2015). The refined methods were able to be used with manual and automated methods of capturing TEER.

Chapter Five challenged the cIEC with LPS, butyrate, and a combination of the two. These were ran in parallel with untreated cIEC as a negative control. These were performed under aerobic conditions. Due to time constraints because of Covid-19, there was not enough time left to profile the cIEC in response to LPS, LTA and the three SCFAs. There was a pro-inflammatory response to LPS

seen in the cIEC, with increased IL-8 gene expression and cytokine content, consistent with other cell culture studies in other species (Angrisano et al., 2010; Kainulainen et al., 2015). Similarly, CCL2 gene expression and concentration in apical cell media was increased in cIEC treated with LPS, consistent with results seen in human monocytes (Akhter et al., 2018). LPS was also found to cause changes to KC-Like levels in the cIEC cell culture media. It has been suggested that KC-Like is a biomarker for sepsis in the dog (Karlsson et al., 2016; Goggs and Letendre, 2019), which implies a potential capability of using the cIEC to model sepsis in the dog.

The paradoxical effect of butyrate, wherein low concentrations improve TEER or maintain TEER stability, and high concentrations reduce it (Gibson et al., 1999; Peng et al., 2007; Elamin et al., 2013) was also observed in Chapter Five. Additionally, this study showed that butyrate was able to reduce and restore LPS-induced disruptions to intestinal barrier permeability, as has been seen elsewhere (Yan and Ajuwon, 2017). Interestingly, the cIEC had no detectable gene expression for GPR109-A, a G-protein receptor specifically for butyrate. GPR109-A has been quantified in dog plasma (Carballo-Jane et al., 2007). GPR 41 and GPR 43 gene expressions in the cIEC did not increase from butyrate stimulation. Dogs express the genes for these GPRs (Haitina et al., 2009). This suggests that rather than alternative pathways or mechanisms that may be in action, it could be a limitation of the cIEC that they do not express GPR109-A. No other studies have assessed the response of GPRs in dog IECs, so this cannot be challenged effecively at this moment in time.

Chapter Five also showed that the cIEC had no detectable gene expressions for several tight junctions and immune cytokines utilised in inflammatory responses, such as adherens junctions associated protein 1, IL-1β, NOD2, TNF and TLR3, amongst others, despite having high TEER values. This highlights potential areas where the cIEC would not be an appropriate model for some challenges. However, the lack of expression of these genes could also highlight alternative mechanims used by dog IECs to respond to bacterial challenges and mount immune responses. Additionally, the results in Chapter Five highlight opportunities for the utilisation of the cIEC as a model of food-derived impacts on host health, and potentially other applications for modelling health in the dog, such as sepsis modelling.

The PhD aimed to culture and characterise the cIEC in an apically anaerobic model. The method development for this apically anaerobic model was detailed in Chapter Six. A period of stable barrier integrity had been established, and initial experiments had shown that the cIEC had a possible dose-dependent response to LPS. However, the impacts of the Covid-19 pandemic stopped the work in the apically anaerobic model. Thus, the co-culture of bacteria with the cIEC could not be attempted. However, this PhD project did contribute novel findings from the meta-analysis and the stimulation of cIEC with LPS and butyrate. Additionally, it defined conditions for the cIEC to be successfully cultured in an apically anaerobic model. Each of these results are knowledge contributions that will further expand and develop the understanding of intestinal health in the dog and will be continually relevant in future research.

7.2 FUTURE DIRECTIONS

- The meta-analysis in Chapter Three indicated that low abundance bacteria may have large impacts on the microbiome. This highlighted that the increasing interest in the functionality of the relatively low abundant constituents (the so-called "dark matter") of the human microbiome (Cena et al., 2021) can be applied to the dog, and is a worthwhile future direction.
- The meta-analysis in Chapter Three also demonstrated that future diet-microbiome studies in the dog would benefit from better data clarity. One of the major limitations was the unavailability of necessary information for complete diet-microbiota analysis, which included poor or incomplete diet information, and errors in uploads of data to storage providers. It is worth considering the potential application of a standardisation of methods and result deposition for diet-microbiota to enable meta-analyses to become a more accessible method to further our understanding of diet and the microbiome in the dog.

- The method development in Chapter Four profiled the barrier integrity via the use of TEER. This could be further improved using tracer compounds such as FITC-Dextrans to assess barrier permeability.
- Similarly, the methods used in Chapter Four could be applied in more physiologically relevant conditions, such as in a cellZscope at 39°C, to better model the conditions *in vivo*. By utilising the method development established in Chapter Four, and continuing the experiments begun in Chapter Six, future work would be able to successfully model the canine intestine in low-oxygen conditions that better represent conditions *in vivo*.
- Chapter Five was able to detect IP-10 changes in RNA expression but had no detectable cytokine expression. Other studies in the dog have also not been able to detect IP-10 cytokine expression. These results suggest that for IP-10 in particular, the RNA expression is a preferable means of analysis compared to cytokine profiling. Future studies that intend on analysing this would benefit from analysing the RNA expression instead of cytokine profiling.
- The work from Chapter Five could also be expanded upon, by investigating the response of the cIEC to lipoteichoic acid, bacterial flagellin, acetate and propionate. Future work building on the concepts established in this thesis could begin by looking at these other SCFA and bacterial ligands to build a better picture of how the GIT microbiota and diet-derived SCFA impact the intestinal health of the dog.
- Chapter Five also indicated that the cIEC may be used as a model to study sepsis in the dog. By investigating the potential of utilising KC-Like as a sepsis biomarker, this would enable gram-negative infections to be studied and sepsis treatment in the dog to be modelled in a manner that complements the 3R's.

The results of this thesis indicate that there are many opportunities to further investigate the functionality of the innate intestinal defences in the dog in comparison to other models. Differences in intestinal physiology and immune responses may result in altered responses to diet changes. These, in combination with the dog's innate dietary requirements, mean that results in other species may not

always be true for the dog. Furthermore, the methodologies used in this thesis that compliment the 3R's are highly applicable for the future directions of diet and health-based research in the dog.

REFERENCES

A. Garden, O., C. M. Elwood, M. Desport, and R. M. Batt. 1999. In situ hybridization as a technique for the immunological investigation of canine intestine: jejunal expression of IFNγ and IL10 in Irish setters and beagles. Veterinary Immunology and Immunopathology 70(1):1-17. doi: https://doi.org/10.1016/S0165-2427(99)00036-7

AAFCO. 2019. Association of American Feed Control Officials 2019 Official Publication. AAFCO, Illinois.

Ahn, C., D.-H. Shin, D. Lee, S.-M. Kang, J.-H. Seok, H. Y. Kang, and E.-B. Jeung. 2016. Expression of claudins, occludin, junction adhesion molecule A and zona occludens 1 in canine organs. Mol Med Rep 14(4):3697-3703. doi: 10.3892/mmr.2016.5725

Akhter, N., A. Hasan, S. Shenouda, A. Wilson, S. Kochumon, S. Ali, J. Tuomilehto, S. Sindhu, and R. Ahmad. 2018. TLR4/MyD88 -mediated CCL2 production by lipopolysaccharide (endotoxin): Implications for metabolic inflammation. J Diabetes Metab Disord 17(1):77-84. doi: 10.1007/s40200-018-0341-y

Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen Recognition and Innate Immunity. Cell 124(4):783-801. doi: https://doi.org/10.1016/j.cell.2006.02.015

Alessandri, G., C. Milani, L. Mancabelli, G. Longhi, R. Anzalone, G. A. Lugli, S. Duranti, F. Turroni, M. C. Ossiprandi, D. van Sinderen, and M. Ventura. 2020. Deciphering the Bifidobacterial Populations within the Canine and Feline Gut Microbiota. Appl Environ Microbiol 86(7)doi: 10.1128/aem.02875-19

Alessandri, G., C. Milani, L. Mancabelli, M. Mangifesta, G. A. Lugli, A. Viappiani, S. Duranti, F. Turroni, M. C. Ossiprandi, D. van Sinderen, and M. Ventura. 2019. Metagenomic dissection of the canine gut microbiota: insights into taxonomic, metabolic and nutritional features. Environmental Microbiology 21(4):1331-1343. doi: 10.1111/1462-2920.14540

Alexander, C., T.-W. L. Cross, S. Devendran, F. Neumer, S. Theis, J. M. Ridlon, J. S. Suchodolski, M. R. C. de Godoy, and K. S. Swanson. 2018. Effects of prebiotic inulin-type fructans on blood metabolite and hormone concentrations and faecal microbiota and metabolites in overweight dogs. British Journal of Nutrition 120(6):711-720. doi: 10.1017/S0007114518001952

Allaway, D., R. Haydock, Z. N. Lonsdale, O. D. Deusch, C. O'Flynn, and K. R. Hughes. 2020. Rapid Reconstitution of the Fecal Microbiome after Extended Diet-Induced Changes Indicates a Stable Gut Microbiome in Healthy Adult Dogs. Appl Environ Microbiol 86(13):e00562-00520. doi: 10.1128/aem.00562-20

Allenspach, K., A. House, K. Smith, F. M. McNeill, A. Hendricks, J. Elson-Riggins, A. Riddle, J. M. Steiner, D. Werling, O. A. Garden, B. Catchpole, and J. S. Suchodolski. 2010. Evaluation of mucosal bacteria and histopathology, clinical disease activity and expression of Toll-like receptors in German shepherd dogs with chronic enteropathies. Veterinary Microbiology 146(3):326-335. doi: https://doi.org/10.1016/j.vetmic.2010.05.025

Allison, C., and G. T. Macfarlane. 1989. Influence of pH, nutrient availability, and growth rate on amine production by Bacteroides fragilis and Clostridium perfringens. Appl Environ Microbiol 55(11):2894-2898.

Amasheh, S., N. Meiri, A. H. Gitter, T. Schöneberg, J. Mankertz, J. D. Schulzke, and M. Fromm. 2002. Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. Journal of Cell Science 115(24):4969-4976. doi: 10.1242/jcs.00165 %J Journal of Cell Science

Ambort, D., M. E. V. Johansson, J. K. Gustafsson, H. E. Nilsson, A. Ermund, B. R. Johansson, P. J. B. Koeck, H. Hebert, and G. C. Hansson. 2012. Calcium and pH-dependent packing and release of the gel-forming MUC2 mucin. Proc Natl Acad Sci U S A 109(15):5645-5650. (Article) doi: 10.1073/pnas.1120269109

Ambrosini, Y. M., Y. Park, A. E. Jergens, W. Shin, S. Min, T. Atherly, D. C. Borcherding, J. Jang, K. Allenspach, J. P. Mochel, and H. J. Kim. 2020. Recapitulation of the accessible interface of biopsy-derived canine intestinal organoids to study epithelial-luminal interactions. PLOS ONE 15(4):e0231423. doi: 10.1371/journal.pone.0231423

Anderson, J. M., and C. M. Van Itallie. 2009. Physiology and function of the tight junction. Cold Spring Harb Perspect Biol 1(2):a002584-a002584. doi: 10.1101/cshperspect.a002584

Anderson, R., J. Dalziel, P. Gopal, S. Bassett, A. Ellis, and N. Roy. 2012. The Role of Intestinal Barrier Function in Early Life in the Development of Colitis. In: Dr.Fukata (ed.) Colitis. InTech.

Anderson, R. C., A. K. H. MacGibbon, N. Haggarty, K. M. Armstrong, and N. C. Roy. 2018. Bovine dairy complex lipids improve in vitro measures of small intestinal epithelial barrier integrity. PLoS One 13(1):e0190839. doi: 10.1371/journal.pone.0190839

Angelis, I. D., and L. Turco. 2011. Caco-2 Cells as a Model for Intestinal Absorption. Current Protocols in Toxicology 47(1):20.26.21-20.26.15. doi: https://doi.org/10.1002/0471140856.tx2006s47

Angrisano, T., R. Pero, S. Peluso, S. Keller, S. Sacchetti, C. B. Bruni, L. Chiariotti, and F. Lembo. 2010. LPS-induced IL-8 activation in human intestinal epithelial cells is accompanied by specific histone H3 acetylation and methylation changes. BMC Microbiol 10(1):172. doi: 10.1186/1471-2180-10-172

Apper, E., L. Privet, B. Taminiau, C. Le Bourgot, L. Svilar, J.-C. Martin, and M. Diez. 2020. Relationships between Gut Microbiota, Metabolome, Body Weight, and Glucose Homeostasis of Obese Dogs Fed with Diets Differing in Prebiotic and Protein Content. Microorganisms 8(4):513.

Arigony, A. L. V., I. M. de Oliveira, M. Machado, D. L. Bordin, L. Bergter, D. Prá, and J. A. P. Henriques. 2013. The influence of micronutrients in cell culture: a reflection on viability and genomic stability. Biomed Res Int 2013:597282-597282. doi: 10.1155/2013/597282

Arora, T., R. Sharma, and G. Frost. 2011. Propionate. Anti-obesity and satiety enhancing factor? Appetite 56(2):511-515. doi: https://doi.org/10.1016/j.appet.2011.01.016

Artursson, P. 1990. Epithelial transport of drugs in cell culture. I: A model for studying the passive diffusion of drugs over intestinal absorbtive (Caco-2) cells. Journal of Pharmaceutical Sciences 79(6):476-482. doi: https://doi.org/10.1002/jps.2600790604

Asarat, M., T. Vasiljevic, V. Apostolopoulos, and O. Donkor. 2015. Short-Chain Fatty Acids Regulate Secretion of IL-8 from Human Intestinal Epithelial Cell Lines in vitro. Immunological Investigations 44(7):678-693. doi: 10.3109/08820139.2015.1085389

Assimakopoulos, S. F., C. Triantos, I. Maroulis, and C. Gogos. 2018. The Role of the Gut Barrier Function in Health and Disease. Gastroenterology Res 11(4):261-263. doi: 10.14740/gr1053w

Atherly, T., G. Rossi, R. White, Y.-J. Seo, C. Wang, M. Ackermann, M. Breuer, K. Allenspach, J. P. Mochel, and A. E. Jergens. 2020. Glucocorticoid and dietary effects on mucosal microbiota in canine inflammatory bowel disease. PLOS ONE 14(12):e0226780. doi: 10.1371/journal.pone.0226780

Axelsson, E., A. Ratnakumar, M.-L. Arendt, K. Maqbool, M. T. Webster, M. Perloski, O. Liberg, J. M. Arnemo, Å. Hedhammar, and K. Lindblad-Toh. 2013. The genomic signature of dog domestication reveals adaptation to a starch-rich diet. Nature 495:360. doi: 10.1038/nature11837

Axiak-Bechtel, S., B. Fowler, D. H. Yu, J. Amorim, K. Tsuruta, and A. DeClue. 2014. Chemotherapy and remission status do not alter pre-existing innate immune dysfunction in dogs with lymphoma. Research in Veterinary Science 97(2):230-237. doi: https://doi.org/10.1016/j.rvsc.2014.07.009

Bailey, K. L., D. J. Romberger, D. M. Katafiasz, A. J. Heires, J. H. Sisson, T. A. Wyatt, and E. L. Burnham. 2015. TLR2 and TLR4 Expression and Inflammatory Cytokines are Altered in the Airway Epithelium of Those with Alcohol Use Disorders. Alcoholism, clinical and experimental research 39(9):1691-1697. doi: 10.1111/acer.12803

Baker, M. 2016. 1,500 scientists lift the lid on reproducibility. Nature 533(7604):452-454. doi: 10.1038/533452a

Bansal, T., R. C. Alaniz, T. K. Wood, and A. Jayaraman. 2010. The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. Proc Natl Acad Sci U S A 107(1):228-233. doi: 10.1073/pnas.0906112107

Batchelor, D. J., M. Al-Rammahi, A. W. Moran, J. G. Brand, X. Li, M. Haskins, A. J. German, and S. P. Shirazi-Beechey. 2011. Sodium/glucose cotransporter-1, sweet receptor, and

disaccharidase expression in the intestine of the domestic dog and cat: two species of different dietary habit. Am J Physiol Regul Integr Comp Physiol 300(1):R67-R75. doi: 10.1152/ajpregu.00262.2010

Bauer, J. E. 2004. Lipoprotein-mediated transport of dietary and synthesized lipids and lipid abnormalities of dogs and cats. Journal of the American Veterinary Medical Association 224(5):668-675. doi: 10.2460/javma.2004.224.668

Bazzocchi, C., M. Mortarino, S. Comazzi, C. Bandi, A. Franceschi, and C. Genchi. 2005. Expression and function of Toll-like receptor 2 in canine blood phagocytes. Veterinary Immunology and Immunopathology 104(1):15-19. doi: https://doi.org/10.1016/j.vetimm.2004.08.023

Beck, H. C. 2005. Branched-chain fatty acid biosynthesis in a branched-chain amino acid aminotransferase mutant of Staphylococcus carnosus. FEMS Microbiology Letters 243(1):37-44. doi: 10.1016/j.femsle.2004.11.041

Belkaid, Y., and Timothy W. Hand. 2014. Role of the Microbiota in Immunity and Inflammation. Cell 157(1):121-141. doi: https://doi.org/10.1016/j.cell.2014.03.011

Beloshapka, A. N., T.-W. L. Cross, and K. S. Swanson. 2020. Graded dietary resistant starch concentrations on apparent total tract macronutrient digestibility and fecal fermentative end products and microbial populations of healthy adult dogs. Journal of Animal Science 99(1)doi: 10.1093/jas/skaa409

Beloshapka, A. N., S. E. Dowd, J. S. Suchodolski, J. M. Steiner, L. Duclos, and K. S. Swanson. 2013. Fecal microbial communities of healthy adult dogs fed raw meat-based diets with or without inulin or yeast cell wall extracts as assessed by 454 pyrosequencing. FEMS Microbiology Ecology 84(3):532-541. doi: 10.1111/1574-6941.12081

Bengtsson-Palme, J., M. Hartmann, K. M. Eriksson, C. Pal, K. Thorell, D. G. Larsson, and R. H. Nilsson. 2015. METAXA2: improved identification and taxonomic classification of small and large subunit rRNA in metagenomic data. Mol Ecol Resour 15(6):1403-1414. doi: 10.1111/1755-0998.12399

Bermingham, E. N., P. Maclean, D. G. Thomas, N. J. Cave, and W. Young. 2017. Key bacterial families (Clostridiaceae, Erysipelotrichaceae and Bacteroidaceae) are related to the digestion of protein and energy in dogs. PeerJ 5:e3019. doi: 10.7717/peerj.3019

Bermingham, E. N., D. G. Thomas, N. J. Cave, P. J. Morris, R. F. Butterwick, and A. J. German. 2014. Energy Requirements of Adult Dogs: A Meta-Analysis. PLOS ONE 9(10):e109681. doi: 10.1371/journal.pone.0109681

Bermingham, E. N., D. G. Thomas, P. H. Maclean, and W. Young. In Press. Effects of commercial high protein and fat diets on faecal organic acids and microbial composition in the dog. .

Bermingham, E. N., W. Young, C. F. Butowski, C. D. Moon, P. H. Maclean, D. Rosendale, N. J. Cave, and D. G. Thomas. 2018. The Fecal Microbiota in the Domestic Cat (Felis catus) Is Influenced by Interactions Between Age and Diet; A Five Year Longitudinal Study. Front Microbiol 9(1231)(Original Research) doi: 10.3389/fmicb.2018.01231

Bermudez Sanchez, S., R. Pilla, B. Sarawichitr, A. Gramenzi, F. Marsilio, J. M. Steiner, J. A. Lidbury, G. R. T. Woods, A. J. German, and J. S. Suchodolski. 2020. Fecal microbiota in client-owned obese dogs changes after weight loss with a high-fiber-high-protein diet. PeerJ 8:e9706. doi: 10.7717/peerj.9706

Bertani, B., and N. Ruiz. 2018. Function and Biogenesis of Lipopolysaccharides. EcoSal Plus 8(1)doi: 10.1128/ecosalplus.ESP-0001-2018

Biagi, G., I. Cipollini, M. Grandi, and G. Zaghini. 2010. Influence of some potential prebiotics and fibre-rich foodstuffs on composition and activity of canine intestinal microbiota. Animal Feed Science and Technology 159(1):50-58. doi: https://doi.org/10.1016/j.anifeedsci.2010.04.012

Blair, J. M. A., M. A. Webber, A. J. Baylay, D. O. Ogbolu, and L. J. V. Piddock. 2015. Molecular mechanisms of antibiotic resistance. Nature Reviews Microbiology 13(1):42-51. doi: 10.1038/nrmicro3380

Blake, A. B., and J. S. Suchodolski. 2016. Importance of gut microbiota for the health and disease of dogs and cats. Animal Frontiers 6(3):37-42. doi: 10.2527/af.2016-0032

Blume, L. F., M. Denker, F. Gieseler, and T. Kunze. 2010. Temperature corrected transepithelial electrical resistance (TEER) measurement to quantify rapid changes in paracellular permeability. Pharmazie 65(1):19-24.

Body-Malapel, M., M. Djouina, C. Waxin, A. Langlois, C. Gower-Rousseau, P. Zerbib, A. M. Schmidt, P. Desreumaux, E. Boulanger, and C. Vignal. 2019. The RAGE signaling pathway is involved in intestinal inflammation and represents a promising therapeutic target for Inflammatory Bowel Diseases. Mucosal Immunology 12(2):468-478. doi: 10.1038/s41385-018-0119-z

Bolger, A. M., M. Lohse, and B. Usadel. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30(15):2114-2120. doi: 10.1093/bioinformatics/btu170

Bontempo, V. 2005. Nutrition and Health of Dogs and Cats: Evolution of Petfood. Veterinary Research Communications 29(2):45-50. doi: 10.1007/s11259-005-0010-8

Bosch, G., E. A. Hagen-Plantinga, and W. H. Hendriks. 2015. Dietary nutrient profiles of wild wolves: insights for optimal dog nutrition? British Journal of Nutrition 113(S1):S40-S54. doi: 10.1017/S0007114514002311

Bosch, G., J. J. M. Vervoort, and W. H. Hendriks. 2016. In vitro digestibility and fermentability of selected insects for dog foods. Animal Feed Science and Technology 221:174-184. doi: https://doi.org/10.1016/j.anifeedsci.2016.08.018

Boveri, M., A. Kinsner, V. Berezowski, A. M. Lenfant, C. Draing, R. Cecchelli, M. P. Dehouck, T. Hartung, P. Prieto, and A. Bal-Price. 2006. Highly purified lipoteichoic acid from gram-positive bacteria induces in vitro blood–brain barrier disruption through glia activation: Role of pro-inflammatory cytokines and nitric oxide. Neuroscience 137(4):1193-1209. doi: https://doi.org/10.1016/j.neuroscience.2005.10.011

Boya, U. O., M. J. Dotson, and E. M. Hyatt. 2015. A comparison of dog food choice criteria across dog owner segments: an exploratory study. International Journal of Consumer Studies 39(1):74-82. doi: https://doi.org/10.1111/ijcs.12145

Breijyeh, Z., B. Jubeh, and R. Karaman. 2020. Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It. Molecules 25(6)doi: 10.3390/molecules25061340

Brennan, C. A., and W. S. Garrett. 2019. Fusobacterium nucleatum - symbiont, opportunist and oncobacterium. Nat Rev Microbiol 17(3):156-166. doi: 10.1038/s41579-018-0129-6

Bresciani, F., Y. Minamoto, J. S. Suchodolski, G. Galiazzo, C. G. Vecchiato, C. Pinna, G. Biagi, and M. Pietra. 2018. Effect of an extruded animal protein-free diet on fecal microbiota of dogs with food-responsive enteropathy. Journal of Veterinary Internal Medicine 0(0):1-8. doi: doi:10.1111/jvim.15227

Broz, P. 2014. Getting Rid of the Bad Apple: Inflammasome-Induced Extrusion of Salmonella-Infected Enterocytes. Cell Host & Microbe 16(2):153-155. doi: https://doi.org/10.1016/j.chom.2014.07.010

Burgener, I. A., A. König, K. Allenspach, S. N. Sauter, J. Boisclair, M. G. Doherr, and T. W. Jungi. 2008. Upregulation of Toll-Like Receptors in Chronic Enteropathies in Dogs. Journal of Veterinary Internal Medicine 22(3):553-560. doi: doi:10.1111/j.1939-1676.2008.0093.x

Butterwick, R. F., J. W. Erdman, R. C. Hill, A. J. Lewis, and C. T. Whittemore. 2011. Challenges in developing nutrient guidelines for companion animals. British Journal of Nutrition 106(S1):S24-S31. doi: 10.1017/S000711451100184X

Byrne, E. M., M. Llorian-Salvador, A. Margariti, M. Chen, and H. Xu. 2020. Interleukin-17A mediates Blood-Retinal Barrier breakdown in vitro and in vivo via the JAK/STAT pathway. Investigative Ophthalmology & Visual Science 61(7):41-41.

Cai, T., Y. Qi, A. Jergens, M. Wannemuehler, T. A. Barrett, and Q. Wang. 2018. Effects of six common dietary nutrients on murine intestinal organoid growth. PLOS ONE 13(2):e0191517. doi: 10.1371/journal.pone.0191517

Calabrò, S., A. C. Carciofi, N. Musco, R. Tudisco, M. O. S. Gomes, and M. I. Cutrignelli. 2013. Fermentation Characteristics of Several Carbohydrate Sources for Dog Diets Using the In Vitro Gas Production Technique. Italian Journal of Animal Science 12(1):e4. doi: 10.4081/ijas.2013.e4

Calvalido, J., G. A. Wood, A. J. Mutsaers, D. Wood, W. Sears, and J. P. Woods. 2016. Comparison of serum cytokine levels between dogs with multicentric lymphoma and healthy dogs. Vet Immunol Immunopathol 182:106-114. doi: 10.1016/j.vetimm.2016.10.009

Capaldo, C. T., A. E. Farkas, R. S. Hilgarth, S. M. Krug, M. F. Wolf, J. K. Benedik, M. Fromm, M. Koval, C. Parkos, and A. Nusrat. 2014. Proinflammatory cytokine-induced tight junction remodeling through dynamic self-assembly of claudins. Mol Biol Cell 25(18):2710-2719. doi: 10.1091/mbc.E14-02-0773

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7(5):335-336. doi: 10.1038/nmeth.f.303

Carballo-Jane, E., L. S. Gerckens, S. Luell, A. S. Parlapiano, M. Wolff, S. L. Colletti, J. R. Tata, A. K. P. Taggart, M. G. Waters, J. G. Richman, M. E. McCann, and M. J. Forrest. 2007. Comparison of rat and dog models of vasodilatation and lipolysis for the calculation of a therapeutic index for GPR109A agonists. Journal of Pharmacological and Toxicological Methods 56(3):308-316. doi: https://doi.org/10.1016/j.vascn.2007.05.007

Cario, E., I. M. Rosenberg, S. L. Brandwein, P. L. Beck, H.-C. Reinecker, and D. K. Podolsky. 2000. Lipopolysaccharide Activates Distinct Signaling Pathways in Intestinal Epithelial Cell Lines Expressing Toll-Like Receptors. The Journal of Immunology 164(2):966-972. doi: 10.4049/jimmunol.164.2.966

Carson, W. F. t., S. E. Salter-Green, M. M. Scola, A. Joshi, K. A. Gallagher, and S. L. Kunkel. 2017. Enhancement of macrophage inflammatory responses by CCL2 is correlated with increased miR-9 expression and downregulation of the ERK1/2 phosphatase Dusp6. Cell Immunol 314:63-72. doi: 10.1016/j.cellimm.2017.02.005

Caruso, R., N. Warner, N. Inohara, and G. Núñez. 2014. NOD1 and NOD2: signaling, host defense, and inflammatory disease. Immunity 41(6):898-908. doi: 10.1016/j.immuni.2014.12.010

Cena, J. A. d., J. Zhang, D. Deng, N. Damé-Teixeira, and T. Do. 2021. Low-Abundant Microorganisms: The Human Microbiome's Dark Matter, a Scoping Review. Frontiers in Cellular and Infection Microbiology 11(Review) doi: 10.3389/fcimb.2021.689197

Cereijido, M., L. Shoshani, and R. G. Contreras. 2000. Molecular Physiology and Pathophysiology of Tight Junctions I. Biogenesis of tight junctions and epithelial polarity. American Journal of Physiology-Gastrointestinal and Liver Physiology 279(3):G477-G482. doi: 10.1152/ajpgi.2000.279.3.G477

Chandler, M. L., and D. A. Gunn-Moore. 2004. Nutritional Status of Canine and Feline Patients Admitted to a Referral Veterinary Internal Medicine Service. The Journal of Nutrition 134(8):2050S-2052S. doi: 10.1093/jn/134.8.2050S

Chandra, L., D. C. Borcherding, D. Kingsbury, T. Atherly, Y. M. Ambrosini, A. Bourgois-Mochel, W. Yuan, M. Kimber, Y. Qi, Q. Wang, M. Wannemuehler, N. M. Ellinwood, E. Snella, M. Martin, M. Skala, D. Meyerholz, M. Estes, M. E. Fernandez-Zapico, A. E. Jergens, J. P. Mochel, and K. Allenspach. 2019. Derivation of adult canine intestinal organoids for translational research in gastroenterology. BMC Biology 17(1):33. doi: 10.1186/s12915-019-0652-6

Chang, J., S. L. Kunkel, and C. H. Chang. 2009. Negative regulation of MyD88-dependent signaling by IL-10 in dendritic cells. Proc Natl Acad Sci U S A 106(43):18327-18332. doi: 10.1073/pnas.0905815106

Chelakkot, C., J. Ghim, and S. H. Ryu. 2018. Mechanisms regulating intestinal barrier integrity and its pathological implications. Experimental & Molecular Medicine 50(8):103. doi: 10.1038/s12276-018-0126-x

Chen, A., K.-p. Hung, and N. Peng. 2012. A cluster analysis examination of pet owners' consumption values and behavior – segmenting owners strategically. Journal of Targeting, Measurement and Analysis for Marketing 20(2):117-132. doi: 10.1057/jt.2012.10

Chen, S., R. Einspanier, and J. Schoen. 2015. Transepithelial electrical resistance (TEER): a functional parameter to monitor the quality of oviduct epithelial cells cultured on filter supports. Histochem Cell Biol 144(5):509-515. doi: 10.1007/s00418-015-1351-1

Chen, Y., J. Wang, C. Liu, L. Su, D. Zhang, J. Fan, Y. Yang, M. Xiao, J. Xie, Y. Xu, Y. Li, and S. Zhang. 2020. IP-10 and MCP-1 as biomarkers associated with disease severity of COVID-19. Molecular Medicine 26(1):97. doi: 10.1186/s10020-020-00230-x

Chimerel, C., E. Emery, David K. Summers, U. Keyser, Fiona M. Gribble, and F. Reimann. 2014. Bacterial Metabolite Indole Modulates Incretin Secretion from Intestinal Enteroendocrine L Cells. Cell Reports 9(4):1202-1208. doi: https://doi.org/10.1016/j.celrep.2014.10.032

Cho, S. M., S. W. Park, N. H. Kim, J. A. Park, H. Yi, H. J. Cho, K. H. Park, I. Hwang, and H. C. Shin. 2013. Expression of intestinal transporter genes in beagle dogs. Exp Ther Med 5(1):308-314. doi: 10.3892/etm.2012.777

Chougule, P., G. Herlenius, N. M. Hernandez, P. B. Patil, B. Xu, and S. Sumitran-Holgersson. 2012. Isolation and characterization of human primary enterocytes from small intestine using a novel method. Scandinavian journal of gastroenterology 47(11):1334-1343. doi: 10.3109/00365521.2012.708940

Claes, I. J. J., M. E. Segers, T. L. A. Verhoeven, M. Dusselier, B. F. Sels, S. C. J. De Keersmaecker, J. Vanderleyden, and S. Lebeer. 2012. Lipoteichoic acid is an important microbe-associated molecular pattern of Lactobacillus rhamnosus GG. Microbial Cell Factories 11(1):161. doi: 10.1186/1475-2859-11-161

Coelho, L. P., J. R. Kultima, P. I. Costea, C. Fournier, Y. Pan, G. Czarnecki-Maulden, M. R. Hayward, S. K. Forslund, T. S. B. Schmidt, P. Descombes, J. R. Jackson, Q. Li, and P. Bork. 2018. Similarity of the dog and human gut microbiomes in gene content and response to diet. Microbiome 6(1):72. (journal article) doi: 10.1186/s40168-018-0450-3

Companion Animals in New Zealand. 2020. Companion Animals in New Zealand, Auckland, New Zealand.

Cong, X., Y. Zhang, J. Li, M. Mei, C. Ding, R.-L. Xiang, L.-W. Zhang, Y. Wang, L.-L. Wu, and G.-Y. Yu. 2015. Claudin-4 is required for modulation of paracellular permeability by muscarinic acetylcholine receptor in epithelial cells. Journal of Cell Science 128(12):2271-2286. doi: 10.1242/jcs.165878

Corridoni, D., L. Pastorelli, B. Mattioli, S. Locovei, D. Ishikawa, K. O. Arseneau, M. Chieppa, F. Cominelli, and T. T. Pizarro. 2012. Probiotic Bacteria Regulate Intestinal Epithelial Permeability in Experimental Ileitis by a TNF-Dependent Mechanism. PLOS ONE 7(7):e42067. doi: 10.1371/journal.pone.0042067

Costa, J., and A. Ahluwalia. 2019. Advances and Current Challenges in Intestinal in vitro Model Engineering: A Digest. Frontiers in Bioengineering and Biotechnology 7(Review) doi: 10.3389/fbioe.2019.00144

Cotter, S. C., C. E. Reavey, Y. Tummala, J. L. Randall, R. Holdbrook, F. Ponton, S. J. Simpson, J. A. Smith, and K. Wilson. 2019. Diet modulates the relationship between immune gene expression and functional immune responses. Insect Biochemistry and Molecular Biology 109:128-141. doi: https://doi.org/10.1016/j.ibmb.2019.04.009

Council of the European Union. 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance 2010/63/EU No. OJ L 276. p 33–79, EU.

Couper, K. N., D. G. Blount, and E. M. Riley. 2008. IL-10: The Master Regulator of Immunity to Infection. The Journal of Immunology 180(9):5771-5777. doi: 10.4049/jimmunol.180.9.5771

Creff, J., L. Malaquin, and A. Besson. 2021. In vitro models of intestinal epithelium: Toward bioengineered systems. Journal of Tissue Engineering 12:2041731420985202. doi: 10.1177/2041731420985202

Cuscó, A., A. Sánchez, L. Altet, L. Ferrer, and O. Francino. 2014. Non-synonymous genetic variation in exonic regions of canine Toll-like receptors. Canine Genet Epidemiol 1:11-11. doi: 10.1186/2052-6687-1-11

Dai, Z. L., G. Wu, and W. Y. Zhu. 2011. Amino acid metabolism in intestinal bacteria: links between gut ecology and host health. Front Biosci (Landmark Ed) 16:1768-1786. doi: 10.2741/3820

Dandrieux, J. R. S., L. Narayanan, S. Firestone, T. M. Archer, and C. S. Mansfield. 2019. Effect of immunosuppressive drugs on cytokine production in canine whole blood stimulated with lipopolysaccharide or a combination of ionomycin and phorbol 12-myristate 13-acetate. Veterinary Medicine and Science 5(2):199-205. doi: 10.1002/vms3.143

Darling, N. J., C. L. Mobbs, A. L. González-Hau, M. Freer, and S. Przyborski. 2020. Bioengineering Novel in vitro Co-culture Models That Represent the Human Intestinal Mucosa With Improved Caco-2 Structure and Barrier Function. Frontiers in Bioengineering and Biotechnology 8(Original Research) doi: 10.3389/fbioe.2020.00992

De Cesaris, P., D. Starace, A. Riccioli, F. Padula, A. Filippini, and E. Ziparo. 1998. Tumor Necrosis Factor-α Induces Interleukin-6 Production and Integrin Ligand Expression by Distinct Transduction Pathways*. Journal of Biological Chemistry 273(13):7566-7571. doi: https://doi.org/10.1074/jbc.273.13.7566

de Souza, W. F., N. Fortunato-Miranda, B. K. Robbs, W. M. de Araujo, J. C. de-Freitas-Junior, L. G. Bastos, J. P. B. Viola, and J. A. Morgado-Díaz. 2013. Claudin-3 Overexpression Increases the Malignant Potential of Colorectal Cancer Cells: Roles of ERK1/2 and PI3K-Akt as Modulators of EGFR signaling. PLOS ONE 8(9):e74994. doi: 10.1371/journal.pone.0074994

DeClue, A. E., J. Nickell, C. H. Chang, and A. Honaker. 2012. Upregulation of proinflammatory cytokine production in response to bacterial pathogen-associated molecular patterns in dogs with diabetes mellitus undergoing insulin therapy. J Diabetes Sci Technol 6(3):496-502. doi: 10.1177/193229681200600303

Devant, P., A. Cao, and J. C. Kagan. 2021. Evolution-inspired redesign of the LPS receptor caspase-4 into an interleukin-1β–converting enzyme. Science Immunology 6(62):eabh3567. doi: doi:10.1126/sciimmunol.abh3567

Di Cerbo, A., J. C. Morales-Medina, B. Palmieri, F. Pezzuto, R. Cocco, G. Flores, and T. Iannitti. 2017. Functional foods in pet nutrition: Focus on dogs and cats. Research in Veterinary Science 112:161-166. doi: https://doi.org/10.1016/j.rvsc.2017.03.020

Di Lorenzo, F., M. D. Pither, M. Martufi, I. Scarinci, J. Guzmán-Caldentey, E. Łakomiec, W. Jachymek, S. C. M. Bruijns, S. M. Santamaría, J. S. Frick, Y. van Kooyk, F. Chiodo, A. Silipo, M. L. Bernardini, and A. Molinaro. 2020. Pairing Bacteroides vulgatus LPS Structure with Its Immunomodulatory Effects on Human Cellular Models. ACS Cent Sci 6(9):1602-1616. doi: 10.1021/acscentsci.0c00791

Digby, Z., P. Tourlomousis, J. Rooney, J. P. Boyle, B. Bibo-Verdugo, R. J. Pickering, S. J. Webster, T. P. Monie, L. J. Hopkins, N. Kayagaki, G. S. Salvesen, S. Warming, L. Weinert, and C. E. Bryant. 2021. Evolutionary loss of inflammasomes in the Carnivora and implications for the carriage of zoonotic infections. Cell Reports 36(8):109614. doi: https://doi.org/10.1016/j.celrep.2021.109614

Dixon, P. 2003. VEGAN, a package of R functions for community ecology. Journal of Vegetation Science 14(6):927-930. doi: https://doi.org/10.1111/j.1654-1103.2003.tb02228.x

Do, S., T. Phungviwatnikul, M. R. C. de Godoy, and K. S. Swanson. 2021. Nutrient digestibility and fecal characteristics, microbiota, and metabolites in dogs fed human-grade foods. J Anim Sci 99(2)doi: 10.1093/jas/skab028

Dobenecker, B., V. Endres, and E. Kienzle. 2013. Energy requirements of puppies of two different breeds for ideal growth from weaning to 28 weeks of age. Journal of Animal Physiology and Animal Nutrition 97(1):190-196. doi: https://doi.org/10.1111/j.1439-0396.2011.01257.x

Dodd, S. A. S., J. L. Adolphe, and A. Verbrugghe. 2018. Plant-based diets for dogs. Journal of the American Veterinary Medical Association 253(11):1425-1432. doi: 10.2460/javma.253.11.1425

Doke, S. K., and S. C. Dhawale. 2015. Alternatives to animal testing: A review. Saudi Pharmaceutical Journal 23(3):223-229. doi: https://doi.org/10.1016/j.jsps.2013.11.002

Donadelli, R. A., and C. G. Aldrich. 2019. The effects on nutrient utilization and stool quality of Beagle dogs fed diets with beet pulp, cellulose, and Miscanthus grass12. Journal of animal science 97(10):4134-4139. doi: 10.1093/jas/skz265

Draing, C., M. Pfitzenmaier, S. Zummo, G. Mancuso, A. Geyer, T. Hartung, and S. von Aulock. 2006. Comparison of Lipoteichoic Acid from Different Serotypes of Streptococcus pneumoniae*. Journal of Biological Chemistry 281(45):33849-33859. doi: https://doi.org/10.1074/jbc.M602676200

Edelblum, K. L., and J. R. Turner. 2009. The tight junction in inflammatory disease: communication breakdown. Current Opinion in Pharmacology 9(6):715-720. doi: https://doi.org/10.1016/j.coph.2009.06.022

Eisenhauer, L., W. Vahjen, T. Dadi, B. Kohn, and J. Zentek. 2019. Effects of Brewer's spent grain and carrot pomace on digestibility, fecal microbiota, and fecal and urinary metabolites in dogs fed low- or high-protein diets1. Journal of Animal Science 97(10):4124-4133. doi: 10.1093/jas/skz264

Elamin, E. E., A. A. Masclee, J. Dekker, H.-J. Pieters, and D. M. Jonkers. 2013. Short-Chain Fatty Acids Activate AMP-Activated Protein Kinase and Ameliorate Ethanol-Induced Intestinal Barrier Dysfunction in Caco-2 Cell Monolayers. The Journal of Nutrition 143(12):1872-1881. doi: 10.3945/jn.113.179549

Ephraim, E., C.-Y. Cochrane, and D. E. Jewell. 2020. Varying Protein Levels Influence Metabolomics and the Gut Microbiome in Healthy Adult Dogs. Toxins (Basel) 12(8):517. doi: 10.3390/toxins12080517

Ephraim, E., and D. E. Jewell. 2020. Effect of Added Dietary Betaine and Soluble Fiber on Metabolites and Fecal Microbiome in Dogs with Early Renal Disease. Metabolites 10(9):370.

Esquivel-Elizondo, S., Z. E. Ilhan, E. I. Garcia-Peña, and R. Krajmalnik-Brown. 2017. Insights into Butyrate Production in a Controlled Fermentation System via Gene Predictions. mSystems 2(4):e00051-00017. doi: doi:10.1128/mSystems.00051-17

Fang, I. J., and B. G. Trewyn. 2012. Chapter three - Application of Mesoporous Silica Nanoparticles in Intracellular Delivery of Molecules and Proteins. In: N. Düzgüneş, editor, Methods in Enzymology No. 508. Academic Press. p. 41-59.

Farquhar, M. J., E. McCluskey, R. Staunton, K. R. Hughes, and J. C. Coltherd. 2018. Characterisation of a canine epithelial cell line for modelling the intestinal barrier. Altern Lab Anim 46(3):115-132. (Article) doi: 10.1177/026119291804600304

Fascetti, A. J. 2010. Nutritional management and disease prevention in healthy dogs and cats. Revista Brasileira de Zootecnia 39:42-51.

FEDIAF. 2018. Nutritional Guidelines for Complete and Complementary Pet Food for Cats and Dogs., Fédération Européenne de l'Industrie des Aliments pour Animaux Familiers.

Feighery, L. M., S. W. Cochrane, T. Quinn, A. W. Baird, D. O'Toole, S.-E. Owens, D. O'Donoghue, R. J. Mrsny, and D. J. Brayden. 2008. Myosin Light Chain Kinase Inhibition: Correction of Increased Intestinal Epithelial Permeability In Vitro. Pharmaceutical Research 25(6):1377-1386. doi: 10.1007/s11095-007-9527-6

Felix, K., S. Tobias, H. Jan, S. Nicolas, and M. Michael. 2021. Measurements of transepithelial electrical resistance (TEER) are affected by junctional length in immature epithelial monolayers. Histochemistry and Cell Biology 156(6):609-616. doi: 10.1007/s00418-021-02026-4

Feng, W., Y. Wu, G. Chen, S. Fu, B. Li, B. Huang, D. Wang, W. Wang, and J. Liu. 2018a. Sodium Butyrate Attenuates Diarrhea in Weaned Piglets and Promotes Tight Junction Protein Expression in Colon in a GPR109A-Dependent Manner. Cellular Physiology and Biochemistry 47(4):1617-1629. doi: 10.1159/000490981

Feng, Y., Y. Wang, P. Wang, Y. Huang, and F. Wang. 2018b. Short-Chain Fatty Acids Manifest Stimulative and Protective Effects on Intestinal Barrier Function Through the Inhibition of NLRP3 Inflammasome and Autophagy. Cellular Physiology and Biochemistry 49(1):190-205. doi: 10.1159/000492853

Ferdowsian, H. R., and N. Beck. 2011. Ethical and Scientific Considerations Regarding Animal Testing and Research. PLOS ONE 6(9):e24059. doi: 10.1371/journal.pone.0024059

Finet, S., F. He, L. V. Clark, and M. R. C. de Godoy. 2022. Functional properties of miscanthus fiber and prebiotic blends in extruded canine diets. Journal of Animal Science 100(4)doi: 10.1093/jas/skac078

Fischer, A., M. Gluth, F. Weege, U.-F. Pape, B. Wiedenmann, D. C. Baumgart, and F. Theuring. 2014. Glucocorticoids regulate barrier function and claudin expression in intestinal epithelial cells via MKP-1. American Journal of Physiology-Gastrointestinal and Liver Physiology 306(3):G218-G228. doi: 10.1152/ajpgi.00095.2013

Foster, K. R., J. Schluter, K. Z. Coyte, and S. Rakoff-Nahoum. 2017. The evolution of the host microbiome as an ecosystem on a leash. Nature 548:43. (Perspective) doi: 10.1038/nature23292

Fredriksson, K., C. M. Van Itallie, A. Aponte, M. Gucek, A. J. Tietgens, and J. M. Anderson. 2015. Proteomic analysis of proteins surrounding occludin and claudin-4 reveals their proximity to signaling and trafficking networks. PloS one 10(3):e0117074-e0117074. doi: 10.1371/journal.pone.0117074

Friedman, E. S., K. Bittinger, T. V. Esipova, L. Hou, L. Chau, J. Jiang, C. Mesaros, P. J. Lund, X. Liang, G. A. FitzGerald, M. Goulian, D. Lee, B. A. Garcia, I. A. Blair, S. A. Vinogradov, and G. D. Wu. 2018. Microbes vs. chemistry in the origin of the anaerobic gut lumen. Proceedings of the National Academy of Sciences 115(16):4170-4175. doi: 10.1073/pnas.1718635115

Fujita, T., J. D. Firth, M. Kittaka, D. Ekuni, H. Kurihara, and E. E. Putnins. 2012. Loss of claudin-1 in lipopolysaccharide-treated periodontal epithelium. J Periodontal Res 47(2):222-227. doi: 10.1111/j.1600-0765.2011.01424.x

Fukata, M., and M. Arditi. 2013. The role of pattern recognition receptors in intestinal inflammation. Mucosal immunology 6(3):451-463. doi: 10.1038/mi.2013.13

Fuqua, B. K., C. D. Vulpe, and G. J. Anderson. 2012. Intestinal iron absorption. Journal of Trace Elements in Medicine and Biology 26(2):115-119. doi: https://doi.org/10.1016/j.jtemb.2012.03.015

Furihata, T., S. Kawamatsu, R. Ito, K. Saito, S. Suzuki, S. Kishida, Y. Saito, A. Kamiichi, and K. Chiba. 2015. Hydrocortisone enhances the barrier properties of HBMEC/ciβ, a brain microvascular endothelial cell line, through mesenchymal-to-endothelial transition-like effects. Fluids Barriers CNS 12:7. doi: 10.1186/s12987-015-0003-0

Furuse, M., K. Furuse, H. Sasaki, and S. Tsukita. 2001. Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. J Cell Biol 153(2):263-272.

Fusunyan, R. D., J. J. Quinn, Y. Ohno, R. P. MacDermott, and I. R. Sanderson. 1998. Butyrate Enhances Interleukin (IL)-8 Secretion by Intestinal Epithelial Cells in Response to IL-1β and Lipopolysaccharide. Pediatric Research 43(1):84-90. doi: 10.1203/00006450-199801000-00013

Gagné, J. W., J. J. Wakshlag, K. W. Simpson, S. E. Dowd, S. Latchman, D. A. Brown, K. Brown, K. S. Swanson, and G. C. Fahey. 2013. Effects of a synbiotic on fecal quality, short-chain fatty acid concentrations, and the microbiome of healthy sled dogs. BMC Veterinary Research 9(1):246. doi: 10.1186/1746-6148-9-246

Galán, A., I. Mayer, R. B. Rafaj, K. Bendelja, V. Sušić, J. J. Cerón, and V. Mrljak. 2018. MCP-1, KC-like and IL-8 as critical mediators of pathogenesis caused by Babesia canis. PLoS One 13(1):e0190474. doi: 10.1371/journal.pone.0190474

Galloway, S. M., and C. R. Raetz. 1990. A mutant of Escherichia coli defective in the first step of endotoxin biosynthesis. J Biol Chem 265(11):6394-6402.

Galperin, M. Y., V. Brover, I. Tolstoy, and N. Yutin. 2016. Phylogenomic analysis of the family Peptostreptococcaceae (Clostridium cluster XI) and proposal for reclassification of Clostridium litorale (Fendrich et al. 1991) and Eubacterium acidaminophilum (Zindel et al. 1989) as Peptoclostridium litorale gen. nov. comb. nov. and Peptoclostridium acidaminophilum comb. nov. International Journal of Systematic and Evolutionary Microbiology 66(12):5506-5513. doi: https://doi.org/10.1099/ijsem.0.001548

Gao, X., S.-H. Lin, F. Ren, J.-T. Li, J.-J. Chen, C.-B. Yao, H.-B. Yang, S.-X. Jiang, G.-Q. Yan, D. Wang, Y. Wang, Y. Liu, Z. Cai, Y.-Y. Xu, J. Chen, W. Yu, P.-Y. Yang, and Q.-Y. Lei. 2016. Acetate functions as an epigenetic metabolite to promote lipid synthesis under hypoxia. Nature communications 7:11960-11960. doi: 10.1038/ncomms11960

Garrett, W. S., J. I. Gordon, and L. H. Glimcher. 2010. Homeostasis and Inflammation in the Intestine. Cell 140(6):859-870. doi: https://doi.org/10.1016/j.cell.2010.01.023

Gerbe, F., E. Sidot, D. J. Smyth, M. Ohmoto, I. Matsumoto, V. Dardalhon, P. Cesses, L. Garnier, M. Pouzolles, B. Brulin, M. Bruschi, Y. Harcus, V. S. Zimmermann, N. Taylor, R. M. Maizels, and P. Jay. 2016. Intestinal epithelial tuft cells initiate type 2 mucosal immunity to helminth parasites. Nature 529:226. doi: 10.1038/nature16527

https://www.nature.com/articles/nature16527#supplementary-information

German, A. J., C. R. Helps, E. J. Hall, and M. J. Day. 2000. Cytokine mRNA expression in mucosal biopsies from German shepherd dogs with small intestinal enteropathies. Dig Dis Sci 45(1):7-17. doi: 10.1023/a:1005436721798

German, A. J., V. H. Ryan, A. C. German, I. S. Wood, and P. Trayhurn. 2010. Obesity, its associated disorders and the role of inflammatory adipokines in companion animals. The Veterinary Journal 185(1):4-9. doi: https://doi.org/10.1016/j.tvjl.2010.04.004

Gibson, P. R., O. Rosella, A. J. Wilson, J. M. Mariadason, K. Rickard, K. Byron, and D. H. Barkla. 1999. Colonic epithelial cell activation and the paradoxical effects of butyrate. Carcinogenesis 20(4):539-544. doi: 10.1093/carcin/20.4.539

Goggs, R., and J.-A. Letendre. 2019. Evaluation of the host cytokine response in dogs with sepsis and noninfectious systemic inflammatory response syndrome. Journal of Veterinary Emergency and Critical Care 29(6):593-603. doi: https://doi.org/10.1111/vec.12903

Golaz, J. L., N. Vonlaufen, A. Hemphill, and I. A. Burgener. 2007. Establishment and characterization of a primary canine duodenal epithelial cell culture. In Vitro Cell Dev Biol Anim 43(5-6):176-185. (journal article) doi: 10.1007/s11626-007-9034-4

Goodenough, D. A., and D. L. Paul. 2009. Gap junctions. Cold Spring Harb Perspect Biol 1(1):a002576-a002576. doi: 10.1101/cshperspect.a002576

Gossé, F., S. Guyot, S. Roussi, A. Lobstein, B. Fischer, N. Seiler, and F. Raul. 2005. Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. Carcinogenesis 26(7):1291-1295. doi: 10.1093/carcin/bgi074

Greetham, H. L., G. R. Gibson, C. Giffard, H. Hippe, B. Merkhoffer, U. Steiner, E. Falsen, and M. D. Collins. 2004. Allobaculum stercoricanis gen. nov., sp. nov., isolated from canine feces. Anaerobe 10(5):301-307. doi: https://doi.org/10.1016/j.anaerobe.2004.06.004

Gronert, K., A. Gewirtz, J. L. Madara, and C. N. Serhan. 1998. Identification of a human enterocyte lipoxin A4 receptor that is regulated by interleukin (IL)-13 and interferon gamma and inhibits tumor necrosis factor alpha-induced IL-8 release. J Exp Med 187(8):1285-1294. doi: 10.1084/jem.187.8.1285

Gryaznova, M., Y. Dvoretskaya, I. Burakova, M. Syromyatnikov, E. Popov, A. Kokina, E. Mikhaylov, and V. Popov. 2022. Dynamics of Changes in the Gut Microbiota of Healthy Mice Fed with Lactic Acid Bacteria and Bifidobacteria. Microorganisms 10(5)doi: 10.3390/microorganisms10051020

Guo, S., R. Al-Sadi, H. M. Said, and T. Y. Ma. 2013. Lipopolysaccharide Causes an Increase in Intestinal Tight Junction Permeability in Vitro and in Vivo by Inducing Enterocyte Membrane Expression and Localization of TLR-4 and CD14. The American Journal of Pathology 182(2):375-387. doi: https://doi.org/10.1016/j.ajpath.2012.10.014

Haidich, A. B. 2010. Meta-analysis in medical research. Hippokratia 14(Suppl 1):29-37.

Hailman, E., H. S. Lichenstein, M. M. Wurfel, D. S. Miller, D. A. Johnson, M. Kelley, L. A. Busse, M. M. Zukowski, and S. D. Wright. 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J Exp Med 179(1):269-277. doi: 10.1084/jem.179.1.269

Haitina, T., R. Fredriksson, S. M. Foord, H. B. Schiöth, and D. E. Gloriam. 2009. The G protein-coupled receptor subset of the dog genome is more similar to that in humans than rodents. BMC Genomics 10:24. doi: 10.1186/1471-2164-10-24

Hang, I., R. M. Heilmann, N. Grützner, J. S. Suchodolski, J. M. Steiner, F. Atroshi, S. Sankari, A. Kettunen, W. M. de Vos, J. Zentek, and T. Spillmann. 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 Veterinary Research 9(1):201. doi: 10.1186/1746-6148-9-201

Hang, I., T. Rinttila, J. Zentek, A. Kettunen, S. Alaja, J. Apajalahti, J. Harmoinen, W. M. de Vos, and T. Spillmann. 2012. Effect of high contents of dietary animal-derived protein or carbohydrates on canine faecal microbiota. BMC Veterinary Research 8(1):90. (Article) doi: 10.1186/1746-6148-8-90

Hanifeh, M., T. Spillmann, M. Huhtinen, Y. S. Sclivagnotis, T. Grönthal, and U. Hynönen. 2021. Ex-Vivo Adhesion of Enterococcus faecalis and Enterococcus faecium to the Intestinal Mucosa of Healthy Beagles. Animals 11(11):3283.

Hanson, P. J., A. P. Moran, and K. Butler. 2011. Paracellular permeability is increased by basal lipopolysaccharide in a primary culture of colonic epithelial cells; an effect prevented by an activator of Toll-like receptor-2. Innate Immun 17(3):269-282. doi: 10.1177/1753425910367813

Hashimoto, K., N. Matsunaga, and M. Shimizu. 1994. Effect of Vegetable Extracts on the Transepithelial Permeability of the Human Intestinal Caco-2 Cell Monolayer. Bioscience, Biotechnology, and Biochemistry 58(7):1345-1346. doi: 10.1271/bbb.58.1345

He, S., Y. Guo, J. Zhao, X. Xu, N. Wang, and Q. Liu. 2020. Ferulic Acid Ameliorates Lipopolysaccharide-Induced Barrier Dysfunction via MicroRNA-200c-3p-Mediated Activation of PI3K/AKT Pathway in Caco-2 Cells. Frontiers in Pharmacology 11(Original Research) doi: 10.3389/fphar.2020.00376

Heilmann, R. M., and K. Allenspach. 2017. Pattern-recognition receptors: signaling pathways and dysregulation in canine chronic enteropathies—brief review. Journal of Veterinary Diagnostic Investigation 29(6):781-787. doi: 10.1177/1040638717728545

Heimann, E., M. Nyman, A.-K. Pålbrink, K. Lindkvist-Petersson, and E. Degerman. 2016. Branched short-chain fatty acids modulate glucose and lipid metabolism in primary adipocytes. Adipocyte 5(4):359-368. doi: 10.1080/21623945.2016.1252011

Hemphill, A., N. Vonlaufen, J. L. Golaz, and I. A. Burgener. 2009. Infection of Primary Canine Duodenal Epithelial Cell Cultures with Neospora caninum. Journal of Parasitology 95(2):372-380, 379.

Hernández, M. A. G., E. E. Canfora, J. W. E. Jocken, and E. E. Blaak. 2019. The Short-Chain Fatty Acid Acetate in Body Weight Control and Insulin Sensitivity. Nutrients 11(8):1943. doi: 10.3390/nu11081943

Herstad, K. M. V., K. Gajardo, A. M. Bakke, L. Moe, J. Ludvigsen, K. Rudi, I. Rud, M. Sekelja, and E. Skancke. 2017. A diet change from dry food to beef induces reversible changes on the faecal microbiota in healthy, adult client-owned dogs. BMC Veterinary Research 13(1):147. (journal article) doi: 10.1186/s12917-017-1073-9

Hill, S. R. 2010. Changing the metabolism of dogs (Canis familiaris) and cats (Felis catus) at rest and during exercise by manipulation of dietary macronutrients : a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Nutritional Science, at Massey University, Palmerston North, New Zealand. Doctoral, Massey University.

Honneffer, J., B. Guard, J. M. Steiner, and J. S. Suchodolski. 2015. Mo1805 Untargeted Metabolomics Reveals Disruption Within Bile Acid, Cholesterol, and Tryptophan Metabolic Pathways in Dogs With Idiopathic Inflammatory Bowel Disease. Gastroenterology 148(4, Supplement 1):S-715. doi: https://doi.org/10.1016/S0016-5085(15)32435-5

Honneffer, J. B., J. M. Steiner, J. A. Lidbury, and J. S. Suchodolski. 2017. Variation of the microbiota and metabolome along the canine gastrointestinal tract. Metabolomics 13(3):26. doi: 10.1007/s11306-017-1165-3

House, A. K., S. P. Gregory, and B. Catchpole. 2008. Pattern-recognition receptor mRNA expression and function in canine monocyte/macrophages and relevance to canine anal furunuclosis. Veterinary Immunology and Immunopathology 124(3):230-240. doi: https://doi.org/10.1016/j.vetimm.2008.03.012

Howitt, M. R., S. Lavoie, M. Michaud, A. M. Blum, S. V. Tran, J. V. Weinstock, C. A. Gallini, K. Redding, R. F. Margolskee, L. C. Osborne, D. Artis, and W. S. Garrett. 2016. Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut. Science 351(6279):1329-1333. doi: 10.1126/science.aaf1648

Hu, W., A. Jain, Y. Gao, I. M. Dozmorov, R. Mandraju, E. K. Wakeland, and C. Pasare. 2015. Differential outcome of TRIF-mediated signaling in TLR4 and TLR3 induced DC maturation. Proceedings of the National Academy of Sciences 112(45):13994-13999. doi: 10.1073/pnas.1510760112

Hug, H., M. H. Mohajeri, and G. La Fata. 2018. Toll-Like Receptors: Regulators of the Immune Response in the Human Gut. Nutrients 10(2)doi: 10.3390/nu10020203

Iliev, I. D., V. A. Funari, K. D. Taylor, Q. Nguyen, C. N. Reyes, S. P. Strom, J. Brown, C. A. Becker, P. R. Fleshner, M. Dubinsky, J. I. Rotter, H. L. Wang, D. P. B. McGovern, G. D. Brown, and D. M. Underhill. 2012. Interactions Between Commensal Fungi and the C-Type Lectin Receptor Dectin-1 Influence Colitis. Science 336(6086):1314-1317. doi: 10.1126/science.1221789

Íñiguez-Gutiérrez, L., L. A. Godínez-Méndez, M. Fafutis-Morris, J. R. Padilla-Arellano, A. Corona-Rivera, M. R. Bueno-Topete, Ó. A. Rojas-Rejón, and V. Delgado-Rizo. 2020. Physiological concentrations of short-chain fatty acids induce the formation of neutrophil extracellular traps in vitro. International Journal of Immunopathology and Pharmacology 34:2058738420958949. doi: 10.1177/2058738420958949

Itallie, C. M. V., K. F. Lidman, A. J. Tietgens, and J. M. Anderson. 2019. Newly synthesized claudins but not occludin are added to the basal side of the tight junction. Mol Biol Cell 30(12):1406-1424. doi: 10.1091/mbc.E19-01-0008

Jackson, M. I., and D. E. Jewell. 2016. Soluble and Insoluble Fiber Differentially Impact Canine Fecal Microbiome and Circulating Metabolome. The FASEB Journal 30(1_supplement):124.126-124.126. doi: 10.1096/fasebj.30.1_supplement.124.6

Jackson, M. I., and D. E. Jewell. 2019. Balance of saccharolysis and proteolysis underpins improvements in stool quality induced by adding a fiber bundle containing bound polyphenols to either hydrolyzed meat or grain-rich foods. Gut Microbes 10(3):298-320. doi: 10.1080/19490976.2018.1526580

Jalili-Firoozinezhad, S., F. S. Gazzaniga, E. L. Calamari, D. M. Camacho, C. W. Fadel, A. Bein, B. Swenor, B. Nestor, M. J. Cronce, A. Tovaglieri, O. Levy, K. E. Gregory, D. T. Breault, J. M. S. Cabral, D. L. Kasper, R. Novak, and D. E. Ingber. 2019. A complex human gut microbiome cultured in an anaerobic intestine-on-a-chip. Nat Biomed Eng 3(7):520-531. doi: 10.1038/s41551-019-0397-0

Jarett, J. K., A. Carlson, M. Rossoni Serao, J. Strickland, L. Serfilippi, and H. H. Ganz. 2019. Diets with and without edible cricket support a similar level of diversity in the gut microbiome of dogs. PeerJ 7:e7661-e7661. doi: 10.7717/peerj.7661

Jergens, A. E., I. M. Sonea, A. M. O'Connor, L. K. Kauffman, S. D. Grozdanic, M. R. Ackermann, and R. B. Evans. 2009. Intestinal cytokine mRNA expression in canine inflammatory bowel disease: a meta-analysis with critical appraisal. Comp Med 59(2):153-162.

Johansson, Malin E. V., and Gunnar C. Hansson. 2014. Is the Intestinal Goblet Cell a Major Immune Cell? Cell Host & Microbe 15(3):251-252. doi: https://doi.org/10.1016/j.chom.2014.02.014

Juan, M. E. l., U. Wenzel, V. Ruiz-Gutierrez, H. Daniel, and J. M. Planas. 2006. Olive Fruit Extracts Inhibit Proliferation and Induce Apoptosis in HT-29 Human Colon Cancer Cells. The Journal of Nutrition 136(10):2553-2557. doi: 10.1093/jn/136.10.2553

Jumarie, C., and C. Malo. 1991. Caco-2 cells cultured in serum-free medium as a model for the study of enterocytic differentiation in vitro. Journal of Cellular Physiology 149(1):24-33. doi: https://doi.org/10.1002/jcp.1041490105

Kainulainen, V., Y. Tang, T. Spillmann, S. Kilpinen, J. Reunanen, P. E. Saris, and R. J. B. M. Satokari. 2015. The canine isolate Lactobacillus acidophilus LAB20 adheres to intestinal epithelium and attenuates LPS-induced IL-8 secretion of enterocytes in vitro. BMC Microbiol 15(1):4. (journal article) doi: 10.1186/s12866-014-0337-9

Kalenyak, K., A. Isaiah, R. M. Heilmann, J. S. Suchodolski, and I. A. Burgener. 2017. Comparison of the intestinal mucosal microbiota in dogs diagnosed with idiopathic inflammatory bowel disease and dogs with food-responsive diarrhea before and after treatment. FEMS Microbiology Ecology 94(2)doi: 10.1093/femsec/fix173

Kalynych, S., R. Morona, and M. Cygler. 2014. Progress in understanding the assembly process of bacterial O-antigen. FEMS Microbiol Rev 38(5):1048-1065. doi: 10.1111/1574-6976.12070

Kamath, P. S., M. T. Hoepfner, and S. F. Phillips. 1987. Short-chain fatty acids stimulate motility of the canine ileum. American Journal of Physiology-Gastrointestinal and Liver Physiology 253(4):G427-G433. doi: 10.1152/ajpgi.1987.253.4.G427

Kamphorst, J. J., M. K. Chung, J. Fan, and J. D. Rabinowitz. 2014. Quantitative analysis of acetyl-CoA production in hypoxic cancer cells reveals substantial contribution from acetate. Cancer Metab 2:23-23. doi: 10.1186/2049-3002-2-23

Kang, J. Y., X. Nan, M. S. Jin, S.-J. Youn, Y. H. Ryu, S. Mah, S. H. Han, H. Lee, S.-G. Paik, and J.-O. Lee. 2009. Recognition of Lipopeptide Patterns by Toll-like Receptor 2-Toll-like Receptor 6 Heterodimer. Immunity 31(6):873-884. doi: https://doi.org/10.1016/j.immuni.2009.09.018

Kaplan, J. L., J. A. Stern, A. J. Fascetti, J. A. Larsen, H. Skolnik, G. D. Peddle, R. D. Kienle, A. Waxman, M. Cocchiaro, C. T. Gunther-Harrington, T. Klose, K. LaFauci, B. Lefbom, M. Machen Lamy, R. Malakoff, S. Nishimura, M. Oldach, S. Rosenthal, C. Stauthammer, L. O'Sullivan, L. C. Visser, R. William, and E. Ontiveros. 2018. Taurine deficiency and dilated cardiomyopathy in golden retrievers fed commercial diets. PLOS ONE 13(12):e0209112. doi: 10.1371/journal.pone.0209112

Kararli, T. T. 1995. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. Biopharm Drug Dispos 16(5):351-380. doi: 10.1002/bdd.2510160502

Karlsson, I., R. Hagman, A. Johannisson, L. Wang, F. Södersten, and S. Wernersson. 2016. Multiplex cytokine analyses in dogs with pyometra suggest involvement of KC-like chemokine in canine bacterial sepsis. Veterinary Immunology and Immunopathology 170:41-46. doi: https://doi.org/10.1016/j.vetimm.2016.01.005

Kathrani, A. 2016. Nutritional support in the intensive care unit. In Practice 38(Suppl 4):18-24. doi: 10.1136/inp.i5414

Kathrani, A., V. Lezcano, E. J. Hall, A. E. Jergens, Y.-J. Seo, J. P. Mochel, T. Atherly, and K. Allenspach. 2019. Interleukin-13 and interleukin-33 mRNA are underexpressed in the duodenal mucosa of German Shepherd dogs with chronic enteropathy. Journal of Veterinary Internal Medicine 0(0):1-9. doi: 10.1111/jvim.15544

Katz, D. L., M. O'Connell, V. Y. Njike, M. C. Yeh, and H. Nawaz. 2008. Strategies for the prevention and control of obesity in the school setting: systematic review and meta-analysis. International Journal of Obesity 32(12):1780-1789. doi: 10.1038/ijo.2008.158

Keh, D., T. Boehnke, S. Weber-Cartens, C. Schulz, O. Ahlers, S. Bercker, H.-D. Volk, W.-D. Doecke, K. J. Falke, and H. Gerlach. 2003. Immunologic and Hemodynamic Effects of "Low-Dose" Hydrocortisone in Septic Shock. American Journal of Respiratory and Critical Care Medicine 167(4):512-520. doi: 10.1164/rccm.200205-446OC

Kelley, G. A., and K. S. Kelley. 2019. Systematic reviews and meta-analysis in nutrition research. British Journal of Nutrition 122(11):1279-1294. doi: 10.1017/S0007114519002241

Kenny, E. F., S. Talbot, M. Gong, D. T. Golenbock, C. E. Bryant, and L. A. J. O'Neill. 2009. MyD88 Adaptor-Like Is Not Essential for TLR2 Signaling and Inhibits Signaling by TLR3. The Journal of Immunology 183(6):3642-3651. doi: 10.4049/jimmunol.0901140

Kerr, K. R., G. Forster, S. E. Dowd, E. P. Ryan, and K. S. Swanson. 2013. Effects of Dietary Cooked Navy Bean on the Fecal Microbiome of Healthy Companion Dogs. PLoS ONE 8(9):e74998. doi: 10.1371/journal.pone.0074998

Khalyfa, A., A. Ericsson, Z. Qiao, I. Almendros, R. Farré, and D. Gozal. 2021. Circulating exosomes and gut microbiome induced insulin resistance in mice exposed to intermittent hypoxia: Effects of physical activity. EBioMedicine 64:103208-103208. doi: 10.1016/j.ebiom.2021.103208

Kilburn, L. R., L. R. Koester, S. Schmitz-Esser, N. V. L. Serão, and M. C. Rossoni Serão. 2020. High-Fat Diets Led to OTU-Level Shifts in Fecal Samples of Healthy Adult Dogs. Front Microbiol 11(3103)(Original Research) doi: 10.3389/fmicb.2020.564160

Kim, D.-H., D. Jeong, I.-B. Kang, H.-W. Lim, Y. Cho, and K.-H. Seo. 2019. Modulation of the intestinal microbiota of dogs by kefir as a functional dairy product. Journal of Dairy Science 102(5):3903-3911. doi: https://doi.org/10.3168/jds.2018-15639

Kim, H., B. J. Jung, J. H. Jung, J. Y. Kim, S. K. Chung, and D. K. Chung. 2012. Lactobacillus plantarum lipoteichoic acid alleviates TNF-α-induced inflammation in the HT-29 intestinal epithelial cell line. Molecules and Cells 33(5):479-486. doi: 10.1007/s10059-012-2266-5

Kim, J., J.-U. An, W. Kim, S. Lee, and S. Cho. 2017. Differences in the gut microbiota of dogs (Canis lupus familiaris) fed a natural diet or a commercial feed revealed by the Illumina MiSeq platform. Gut pathogens 9:68-68. doi: 10.1186/s13099-017-0218-5

Kim, M. H., S. G. Kang, J. H. Park, M. Yanagisawa, and C. H. Kim. 2013. Short-Chain Fatty Acids Activate GPR41 and GPR43 on Intestinal Epithelial Cells to Promote Inflammatory

Responses in Mice. Gastroenterology 145(2):396-406.e310. doi: https://doi.org/10.1053/j.gastro.2013.04.056
Kingsbury, D. D., L. Sun, Y. Qi, J. Fredericks, Q. Wang, M. J. Wannemuehler, A. Jergens, and K. Allenspach. 2017. Optimizing the Development and Characterization of Canine Small Intestinal Crypt Organoids as a Research Model. Gastroenterology 152(5):S353. doi: 10.1016/S0016-5085(17)31441-5
Kinugasa, T., T. Sakaguchi, X. Gu, and H. C. Reinecker. 2000. Claudins regulate the intestinal barrier in response to immune mediators. Gastroenterology 118(6):1001-1011. doi: 10.1016/S0016-5085(00)70351-9
Kirchoff, N. S., M. A. R. Udell, and T. J. Sharpton. 2019. The gut microbiome correlates with conspecific aggression in a small population of rescued dogs (Canis familiaris). PeerJ 7:e6103-e6103. doi: 10.7717/peerj.6103
Kolodziejska-Sawerska, A., A. Rychlik, A. Depta, M. Wdowiak, M. Nowicki, and M. Kander. 2013. Cytokines in canine inflammatory bowel disease. Pol J Vet Sci 16(1):165-171.
Konstantinidis, A. O., K. K. Adamama-Moraitou, D. Pardali, C. I. Dovas, G. D. Brellou, T. Papadopoulos, A. E. Jergens, K. Allenspach, and T. S. Rallis. 2021. Colonic mucosal and cytobrush sample cytokine mRNA expression in canine inflammatory bowel disease and their correlation with disease activity, endoscopic and histopathologic score. PLOS ONE 16(1):e0245713. doi: 10.1371/journal.pone.0245713
Kowalczyk, A. P., and K. J. Green. 2013. Structure, function, and regulation of desmosomes. Prog Mol Biol Transl Sci 116:95-118. doi: 10.1016/B978-0-12-394311-8.00005-4
Kumar, S., B. P. Treloar, K. H. Teh, C. M. McKenzie, G. Henderson, G. T. Attwood, S. M. Waters, M. L. Patchett, and P. H. Janssen. 2018. Sharpea and Kandleria are lactic acid producing rumen bacteria that do not change their fermentation products when co-cultured with a methanogen. Anaerobe 54:31-38. doi: https://doi.org/10.1016/j.anaerobe.2018.07.008
Laflamme, D., O. Izquierdo, L. Eirmann, and S. Binder. 2014. Myths and Misperceptions About Ingredients Used in Commercial Pet Foods. Veterinary Clinics of North America: Small Animal Practice 44(4):689-698. doi: https://doi.org/10.1016/j.cvsm.2014.03.002
Lam, K.-L., W.-Y. Cheng, Y. Su, X. Li, X. Wu, K.-H. Wong, H.-S. Kwan, and P. C.-K. Cheung. 2020. Use of random forest analysis to quantify the importance of the structural characteristics of beta-glucans for prebiotic development. Food Hydrocolloids 108:106001. doi: https://doi.org/10.1016/j.foodhyd.2020.106001
Langweiler, M., R. D. Schultz, and B. E. Sheffy. 1981. Effect of vitamin E deficiency on the proliferative response of canine lymphocytes. Am J Vet Res 42(10):1681-1685.
Le Ferrec, E., C. Chesne, P. Artusson, D. Brayden, G. Fabre, P. Gires, F. Guillou, M. Rousset, W. Rubas, and M. L. Scarino. 2001. In vitro models of the intestinal barrier: The report and recommendations of ECVAM workshop 461,2. ATLA Alternatives to Laboratory Animals 29(6):649-668. (Conference Paper)
Le, N., C. Mazahery, K. Nguyen, and A. D. Levine. 2021a. Regulation of Intestinal Epithelial Barrier and Immune Function by Activated T Cells. Cellular and Molecular Gastroenterology and Hepatology 11(1):55-76. doi: https://doi.org/10.1016/j.jcmgh.2020.07.004
Le, N., C. Mazahery, K. Nguyen, and A. D. Levine. 2021b. Regulation of Intestinal Epithelial Barrier and Immune Function by Activated T Cells. Cell Mol Gastroenterol Hepatol 11(1):55-76. doi: 10.1016/j.jcmgh.2020.07.004
Lea, T. 2015. Caco-2 Cell Line. In: K. Verhoeckx, P. Cotter, I. Lopez-Exposito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka and H. Wichers, editors, The Impact of Food Bioactives on Health: in vitro and ex vivo models. Springer International Publishing, Cham (CH). p. 103-111.
Lee, J. W., J. Ko, C. Ju, and H. K. Eltzschig. 2019. Hypoxia signaling in human diseases and therapeutic targets. Experimental & Molecular Medicine 51(6):1-13. doi: 10.1038/s12276-019-0235-1
Lee, S. H. 2015. Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. Intest Res 13(1):11-18. doi: 10.5217/ir.2015.13.1.11
Lee, S. J., Y. J. Baek, J. N. Kim, K. H. Lee, E. H. Lee, J. S. Yeom, J. Y. Choi, N. S. Ku, J. Y. Ahn, J. H. Kim, and S. J. Jeong. 2022. Increasing Fusobacterium infections with Fusobacterium

varium, an emerging pathogen. PLOS ONE 17(4):e0266610. doi: 10.1371/journal.pone.0266610
Lenth, R. 2022. emmeans: Estimated Marginal Means, aka Least-Squares Means R package version 3.1-158.
Leyn, S. A., I. A. Suvorova, A. E. Kazakov, D. A. Ravcheev, V. V. Stepanova, P. S. Novichkov, and D. A. Rodionov. 2016. Comparative genomics and evolution of transcriptional regulons in Proteobacteria. Microbial Genomics 2(7)doi: https://doi.org/10.1099/mgen.0.000061
Li, J., L. Zhang, T. Wu, Y. Li, X. Zhou, and Z. Ruan. 2021a. Indole-3-propionic Acid Improved the Intestinal Barrier by Enhancing Epithelial Barrier and Mucus Barrier. Journal of Agricultural and Food Chemistry 69(5):1487-1495. doi: 10.1021/acs.jafc.0c05205
Li, M., B. van Esch, P. A. J. Henricks, G. Folkerts, and J. Garssen. 2018. The Anti-inflammatory Effects of Short Chain Fatty Acids on Lipopolysaccharide- or Tumor Necrosis Factor α-Stimulated Endothelial Cells via Activation of GPR41/43 and Inhibition of HDACs. Front Pharmacol 9:533. doi: 10.3389/fphar.2018.00533
Li, Q., C. L. Lauber, G. Czarnecki-Maulden, Y. Pan, and S. S. Hannah. 2017. Effects of the Dietary Protein and Carbohydrate Ratio on Gut Microbiomes in Dogs of Different Body Conditions. mBio 8(1):e01703-01716. doi: 10.1128/mBio.01703-16
Li, Q., Q. Zhang, M. Wang, S. Zhao, J. Ma, N. Luo, N. Li, Y. Li, G. Xu, and J. Li. 2008a. Interferon-gamma and tumor necrosis factor-alpha disrupt epithelial barrier function by altering lipid composition in membrane microdomains of tight junction. Clin Immunol 126(1):67-80. doi: 10.1016/j.clim.2007.08.017
Li, Q., Q. Zhang, M. Wang, S. Zhao, G. Xu, and J. Li. 2008b. n-3 polyunsaturated fatty acids prevent disruption of epithelial barrier function induced by proinflammatory cytokines. Mol Immunol 45(5):1356-1365. doi: 10.1016/j.molimm.2007.09.003
Li, R. H. L., C. Hommel, and N. Nguyen. 2021b. Lipopolysaccharide-Activated Canine Platelets Upregulate High Mobility Group Box-1 via Toll-Like Receptor 4. Front Vet Sci 8(Original Research) doi: 10.3389/fvets.2021.674678
Lian, P., S. Braber, S. Varasteh, H. J. Wichers, and G. Folkerts. 2021. Hypoxia and heat stress affect epithelial integrity in a Caco-2/HT-29 co-culture. Scientific Reports 11(1):13186. doi: 10.1038/s41598-021-92574-5
Liaw, A., and M. C. Wiener. 2007. Classification and Regression by randomForest
Lin, C.-Y., C. Alexander, A. J. Steelman, C. M. Warzecha, M. R. C. de Godoy, and K. S. Swanson. 2019. Effects of a Saccharomyces cerevisiae fermentation product on fecal characteristics, nutrient digestibility, fecal fermentative end-products, fecal microbial populations, immune function, and diet palatability in adult dogs1. Journal of Animal Science 97(4):1586-1599. doi: 10.1093/jas/skz064
Ling, X., P. Linglong, D. Weixia, and W. Hong. 2016. Protective Effects of Bifidobacterium on Intestinal Barrier Function in LPS-Induced Enterocyte Barrier Injury of Caco-2 Monolayers and in a Rat NEC Model. PLoS One 11(8):e0161635. doi: 10.1371/journal.pone.0161635
Lisenko, K., M. de Godoy, M. Oliveira, T. Silva, T. Fontes, D. Costa, R. Lacerda, L. Ferreira, T. Gonçalves, M. Zangeronimo, and F. Saad. 2018. PSXIII-26 Compositional analysis and effects of dietary supplementation of insect meals on nutrient digestibility and gut health of adult dogs. Journal of Animal Science 96(suppl_3):158-159. doi: 10.1093/jas/sky404.346
Litvak, Y., M. X. Byndloss, and A. J. Bäumler. 2018a. Colonocyte metabolism shapes the gut microbiota. Science 362(6418):eaat9076. doi: 10.1126/science.aat9076
Litvak, Y., M. X. Byndloss, and A. J. Bäumler. 2018b. Colonocyte metabolism shapes the gut microbiota. Science (New York, N.Y.) 362(6418):eaat9076. doi: 10.1126/science.aat9076
Liu, Y., Y. Mi, T. Mueller, S. Kreibich, E. G. Williams, A. Van Drogen, C. Borel, M. Frank, P.-L. Germain, I. Bludau, M. Mehnert, M. Seifert, M. Emmenlauer, I. Sorg, F. Bezrukov, F. S. Bena, H. Zhou, C. Dehio, G. Testa, J. Saez-Rodriguez, S. E. Antonarakis, W.-D. Hardt, and R. Aebersold. 2019. Multi-omic measurements of heterogeneity in HeLa cells across laboratories. Nature Biotechnology 37(3):314-322. doi: 10.1038/s41587-019-0037-y
Louis, P., and H. J. Flint. 2017. Formation of propionate and butyrate by the human colonic microbiota. Environmental Microbiology 19(1):29-41. doi: https://doi.org/10.1111/1462-2920.13589

Lu, L., T. Li, G. Williams, E. Petit, M. Borowsky, and W. A. Walker. 2011. Hydrocortisone induces changes in gene expression and differentiation in immature human enterocytes. Am J Physiol Gastrointest Liver Physiol 300(3):G425-432. doi: 10.1152/ajpgi.00011.2010

Lu, Z., and J. A. Imlay. 2021. When anaerobes encounter oxygen: mechanisms of oxygen toxicity, tolerance and defence. Nature Reviews Microbiology 19(12):774-785. doi: 10.1038/s41579-021-00583-y

Lu, Z., R. Sethu, and J. A. Imlay. 2018. Endogenous superoxide is a key effector of the oxygen sensitivity of a model obligate anaerobe. Proceedings of the National Academy of Sciences 115(14):E3266-E3275. doi: 10.1073/pnas.1800120115

Lubbs, D. C., B. M. Vester, N. D. Fastinger, and K. S. Swanson. 2009. Dietary protein concentration affects intestinal microbiota of adult cats: a study using DGGE and qPCR to evaluate differences in microbial populations in the feline gastrointestinal tract. 93(1):113-121. doi: doi:10.1111/j.1439-0396.2007.00788.x

Luckschander, N., J. A. Hall, F. Gaschen, U. Forster, N. Wenzlow, P. Hermann, K. Allenspach, D. Dobbelaere, I. A. Burgener, and M. Welle. 2010. Activation of nuclear factor-κB in dogs with chronic enteropathies. Veterinary Immunology and Immunopathology 133(2):228-236. doi: https://doi.org/10.1016/j.vetimm.2009.08.014

Luettig, J., R. Rosenthal, C. Barmeyer, and J. D. Schulzke. 2015. Claudin-2 as a mediator of leaky gut barrier during intestinal inflammation. Tissue Barriers 3(1-2):e977176-e977176. doi: 10.4161/21688370.2014.977176

Luu, M., K. Weigand, F. Wedi, C. Breidenbend, H. Leister, S. Pautz, T. Adhikary, and A. Visekruna. 2018. Regulation of the effector function of CD8+ T cells by gut microbiota-derived metabolite butyrate. Scientific Reports 8(1):14430. doi: 10.1038/s41598-018-32860-x

Ma, T. Y., G. K. Iwamoto, N. T. Hoa, V. Akotia, A. Pedram, M. A. Boivin, and H. M. Said. 2004. TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. Am J Physiol Gastrointest Liver Physiol 286(3):G367-376. doi: 10.1152/ajpgi.00173.2003

Macedo, H. T., M. F. Rentas, T. H. A. Vendramini, M. V. Macegoza, A. R. Amaral, J. T. Jeremias, J. C. de Carvalho Balieiro, K. Pfrimer, E. Ferriolli, C. F. F. Pontieri, and M. A. Brunetto. 2022. Weight-loss in obese dogs promotes important shifts in fecal microbiota profile to the extent of resembling microbiota of lean dogs. Animal Microbiome 4(1):6. doi: 10.1186/s42523-021-00160-x

Macfarlane, G. T., G. R. Gibson, E. Beatty, and J. H. Cummings. 1992. Estimation of short-chain fatty acid production from protein by human intestinal bacteria based on branched-chain fatty acid measurements. FEMS Microbiology Letters 101(2):81-88. doi: 10.1111/j.1574-6968.1992.tb05764.x

Maeda, S., K. Ohno, K. Nakamura, K. Uchida, K. Nakashima, K. Fukushima, M. Nakajima, Y. Goto-Koshino, Y. Fujino, and H. Tsujimoto. 2012. Increased expression of fractalkine and its receptor CX3CR1 in canine inflammatory bowel disease and their possible role in recruitment of intraepithelial lymphocytes. Veterinary Immunology and Immunopathology 148(3):226-235. doi: https://doi.org/10.1016/j.vetimm.2012.04.021

Magoč, T., and S. L. Salzberg. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27(21):2957-2963. doi: 10.1093/bioinformatics/btr507

Mahé, F., T. Rognes, C. Quince, C. de Vargas, and M. Dunthorn. 2014. Swarm: robust and fast clustering method for amplicon-based studies. PeerJ 2:e593-e593. doi: 10.7717/peerj.593

Maherally, Z., H. L. Fillmore, S. L. Tan, S. F. Tan, S. A. Jassam, F. I. Quack, K. E. Hatherell, and G. J. Pilkington. 2018. Real-time acquisition of transendothelial electrical resistance in an all-human, in vitro, 3-dimensional, blood-brain barrier model exemplifies tight-junction integrity. FASEB J 32(1):168-182. doi: 10.1096/fj.201700162R

Maier, E. 2017. Effect of Faecalibacterium prausnitzii on intestinal barrier function and immune homeostasis : a dissertation presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Nutritional Science, Massey University, Manawatū, New Zealand. Doctoral, Massey University.

Maier, E., R. C. Anderson, E. Altermann, and N. C. Roy. 2018. Live Faecalibacterium prausnitzii induces greater TLR2 and TLR2/6 activation than the dead bacterium in an apical anaerobic co-culture system. Cell Microbiol 20(2):e12805. doi: 10.1111/cmi.12805

Maier, E., R. C. Anderson, and N. C. Roy. 2014. Understanding how commensal obligate anaerobic bacteria regulate immune functions in the large intestine. Nutrients 7(1):45-73. (Review) doi: 10.3390/nu7010045

Maier, E., R. C. Anderson, and N. C. Roy. 2017. Live Faecalibacterium prausnitzii Does Not Enhance Epithelial Barrier Integrity in an Apical Anaerobic Co-Culture Model of the Large Intestine. Nutrients 9(12):1349. doi: 10.3390/nu9121349

Maiolini, A., R. Carlson, and A. Tipold. 2012. Toll-like receptors 4 and 9 are responsible for the maintenance of the inflammatory reaction in canine steroid-responsive meningitis-arteritis, a large animal model for neutrophilic meningitis. Journal of Neuroinflammation 9(1):226. doi: 10.1186/1742-2094-9-226

Mancabelli, L., C. Milani, G. A. Lugli, F. Turroni, C. Ferrario, D. van Sinderen, and M. Ventura. 2017. Meta-analysis of the human gut microbiome from urbanized and pre-agricultural populations. Environmental Microbiology 19(4):1379-1390. doi: https://doi.org/10.1111/1462-2920.13692

Manheimer, E. W., E. J. van Zuuren, Z. Fedorowicz, and H. Pijl. 2015. Paleolithic nutrition for metabolic syndrome: systematic review and meta-analysis. The American Journal of Clinical Nutrition 102(4):922-932. doi: 10.3945/ajcn.115.113613

Mao, J., Z. Xia, J. Chen, and J. Yu. 2013. Prevalence and risk factors for canine obesity surveyed in veterinary practices in Beijing, China. Preventive Veterinary Medicine 112(3):438-442. doi: https://doi.org/10.1016/j.prevetmed.2013.08.012

Mariadason, J. M., A. Velcich, A. J. Wilson, L. H. Augenlicht, and P. R. Gibson. 2001. Resistance to butyrate-induced cell differentiation and apoptosis during spontaneous Caco-2 cell differentiation. Gastroenterology 120(4):889-899. doi: https://doi.org/10.1053/gast.2001.22472

Markov, A. G., A. Veshnyakova, M. Fromm, M. Amasheh, and S. Amasheh. 2010. Segmental expression of claudin proteins correlates with tight junction barrier properties in rat intestine. Journal of Comparative Physiology B 180(4):591-598. (journal article) doi: 10.1007/s00360-009-0440-7

Martin-Vaquero, P., R. C. da Costa, S. A. Moore, A. C. Gross, and T. D. Eubank. 2014. Cytokine concentrations in the cerebrospinal fluid of great danes with cervical spondylomyelopathy. J Vet Intern Med 28(4):1268-1274. doi: 10.1111/jvim.12388

Martínez-López, L. M., A. Pepper, R. Pilla, A. P. Woodward, J. S. Suchodolski, and C. Mansfield. 2021. Effect of sequentially fed high protein, hydrolyzed protein, and high fiber diets on the fecal microbiota of healthy dogs: a cross-over study. Animal microbiome 3(1):42-42. doi: 10.1186/s42523-021-00101-8

Masterson, J. C., K. A. Biette, J. A. Hammer, N. Nguyen, K. E. Capocelli, B. J. Saeedi, R. F. Harris, S. D. Fernando, L. B. Hosford, C. J. Kelly, E. L. Campbell, S. F. Ehrentraut, F. N. Ahmed, H. Nakagawa, J. J. Lee, E. N. McNamee, L. E. Glover, S. P. Colgan, and G. T. Furuta. 2019. Epithelial HIF-1α/claudin-1 axis regulates barrier dysfunction in eosinophilic esophagitis. The Journal of Clinical Investigation 129(8):3224-3235. doi: 10.1172/JCI126744

Mayer, A. M., J. Murphy, D. MacAdam, C. Osterbauer, I. Baseer, M. L. Hall, D. Feher, and P. Williams. 2016. Classical and Alternative Activation of Cyanobacterium Oscillatoria sp. Lipopolysaccharide-Treated Rat Microglia in vitro. Toxicol Sci 149(2):484-495. doi: 10.1093/toxsci/kfv251

Mazrier, H., L. J. Vogelnest, R. M. Taylor, and P. Williamson. 2022. Altered plasma cytokines in dogs with atopic dermatitis. Veterinary Dermatology 33(2):131-e138. doi: https://doi.org/10.1111/vde.13044

McGreevy, P. D., P. C. Thomson, C. Pride, A. Fawcett, T. Grassi, and B. Jones. 2005. Prevalence of obesity in dogs examined by Australian veterinary practices and the risk factors involved. Veterinary Record 156(22):695-702. doi: 10.1136/vr.156.22.695

McMahon, L. A., A. K. House, B. Catchpole, J. Elson-Riggins, A. Riddle, K. Smith, D. Werling, I. A. Burgener, and K. Allenspach. 2010. Expression of Toll-like receptor 2 in duodenal biopsies

from dogs with inflammatory bowel disease is associated with severity of disease. Veterinary Immunology and Immunopathology 135(1):158-163. doi: https://doi.org/10.1016/j.vetimm.2009.11.012

Meng, D., W. Zhu, H. N. Shi, L. Lu, V. Wijendran, W. Xu, and W. A. Walker. 2015. Toll-like receptor-4 in human and mouse colonic epithelium is developmentally regulated: a possible role in necrotizing enterocolitis. Pediatric Research 77(3):416-424. doi: 10.1038/pr.2014.207

Meng, J., H. Yu, J. Ma, J. Wang, S. Banerjee, R. Charboneau, R. A. Barke, and S. Roy. 2013. Morphine Induces Bacterial Translocation in Mice by Compromising Intestinal Barrier Function in a TLR-Dependent Manner. PLOS ONE 8(1):e54040. doi: 10.1371/journal.pone.0054040

Mercier, E., I. R. Peters, M. J. Day, C. Clercx, and D. Peeters. 2012. Toll- and NOD-like receptor mRNA expression in canine sino-nasal aspergillosis and idiopathic lymphoplasmacytic rhinitis. Veterinary Immunology and Immunopathology 145(3):618-624. doi: https://doi.org/10.1016/j.vetimm.2012.01.009

Meyer, H., and J. Zentek. 1992. Über den Einfluß einer unterschiedlichen Energieversorgung wachsender Doggen auf Körpermasse und Skelettentwicklung. Journal of Veterinary Medicine Series A 39(1 - 10):130-141. doi: https://doi.org/10.1111/j.1439-0442.1992.tb00165.x

Milatz, S., S. M. Krug, R. Rosenthal, D. Günzel, D. Müller, J.-D. Schulzke, S. Amasheh, and M. Fromm. 2010. Claudin-3 acts as a sealing component of the tight junction for ions of either charge and uncharged solutes. Biochimica et Biophysica Acta (BBA) - Biomembranes 1798(11):2048-2057. doi: https://doi.org/10.1016/j.bbamem.2010.07.014

Minamoto, Y., C. C. Otoni, S. M. Steelman, O. Büyükleblebici, J. M. Steiner, A. E. Jergens, and J. S. Suchodolski. 2015. Alteration of the fecal microbiota and serum metabolite profiles in dogs with idiopathic inflammatory bowel disease. Gut Microbes 6(1):33-47. (Article) doi: 10.1080/19490976.2014.997612

Ministry for Primary Industries. 2018. Further Processing and Manufacturing of Petfood. Operational Code: Petfood Processing. p 33, Wellington.

Moher, D., A. Liberati, J. Tetzlaff, D. G. Altman, and P. G. The. 2009. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLOS Medicine 6(7):e1000097. doi: 10.1371/journal.pmed.1000097

Moinard, A., C. Payen, K. Ouguerram, A. André, J. Hernandez, A. Drut, V. C. Biourge, J. S. Suchodolski, J. Flanagan, P. Nguyen, and V. Leray. 2020. Effects of High-Fat Diet at Two Energetic Levels on Fecal Microbiota, Colonic Barrier, and Metabolic Parameters in Dogs. Front Vet Sci 7(699)(Original Research) doi: 10.3389/fvets.2020.566282

Molina, J., M. Hervera, E. G. Manzanilla, C. Torrente, and C. Villaverde. 2018. Evaluation of the Prevalence and Risk Factors for Undernutrition in Hospitalized Dogs. Front Vet Sci 5:205-205. doi: 10.3389/fvets.2018.00205

Møller, G., H. K. Andersen, and O. Snorgaard. 2017. A systematic review and meta-analysis of nutrition therapy compared with dietary advice in patients with type 2 diabetes. The American Journal of Clinical Nutrition 106(6):1394-1400. doi: 10.3945/ajcn.116.139626

Montoya-Alonso, J. A., I. Bautista-Castaño, C. Peña, L. Suárez, M. C. Juste, and A. Tvarijonaviciute. 2017. Prevalence of Canine Obesity, Obesity-Related Metabolic Dysfunction, and Relationship with Owner Obesity in an Obesogenic Region of Spain. Front Vet Sci 4(59)(Original Research) doi: 10.3389/fvets.2017.00059

Moon, C. D., W. Young, P. H. Maclean, A. L. Cookson, and E. N. Bermingham. 2018. Metagenomic insights into the roles of Proteobacteria in the gastrointestinal microbiomes of healthy dogs and cats. MicrobiologyOpen 7(5)(Review) doi: 10.1002/mbo3.677

Morath, S., A. Stadelmaier, A. Geyer, R. R. Schmidt, and T. Hartung. 2002. Synthetic lipoteichoic acid from Staphylococcus aureus is a potent stimulus of cytokine release. J Exp Med 195(12):1635-1640. doi: 10.1084/jem.20020322

Moreno-Indias, I., M. Torres, J. M. Montserrat, L. Sanchez-Alcoholado, F. Cardona, F. J. Tinahones, D. Gozal, V. A. Poroyko, D. Navajas, M. I. Queipo-Ortuño, and R. Farré. 2015. Intermittent hypoxia alters gut microbiota diversity in a mouse model of sleep apnoea. European Respiratory Journal 45(4):1055-1065. doi: 10.1183/09031936.00184314

Mori, A., A. Goto, R. Kibe, H. Oda, Y. Kataoka, and T. Sako. 2019. Comparison of the effects of four commercially available prescription diet regimens on the fecal microbiome in healthy dogs. Journal of Veterinary Medical Science 81(12):1783-1790. doi: 10.1292/jvms.19-0055

Moyes, R. B., J. Reynolds, and D. P. Breakwell. 2009. Differential Staining of Bacteria: Gram Stain. Current Protocols in Microbiology 15(1):A.3C.1-A.3C.8. doi: https://doi.org/10.1002/9780471729259.mca03cs15

Muir, W. W., C. L. del Rio, Y. Ueyama, B. L. Youngblood, R. S. George, C. W. Rausch, B. S. H. Lau, and R. L. Hamlin. 2014. Dose-Dependent Hemodynamic, Biochemical, and Tissue Oxygen Effects of OC99 following Severe Oxygen Debt Produced by Hemorrhagic Shock in Dogs. Critical Care Research and Practice 2014:10. doi: 10.1155/2014/864237

Mullis, R. A., A. L. Witzel, and J. Price. 2015. Maintenance energy requirements of odor detection, explosive detection and human detection working dogs. PeerJ 3:e767-e767. doi: 10.7717/peerj.767

Nagata, N., H. Ohta, N. Yokoyama, Y. B. Teoh, N. Sasaki, K. Nakamura, and M. Takiguchi. 2022. Characterization of mucin gene expression and goblet cell proportion in inflammatory colorectal polyps in miniature dachshunds. J Vet Med Sci 84(6):872-876. doi: 10.1292/jvms.22-0076

Nakamura, S., K. Irie, H. Tanaka, K. Nishikawa, H. Suzuki, Y. Saitoh, A. Tamura, S. Tsukita, and Y. Fujiyoshi. 2019. Morphologic determinant of tight junctions revealed by claudin-3 structures. Nature Communications 10(1):816. doi: 10.1038/s41467-019-08760-7

nanoAnalytics. 2022. cellZscope-related peer-reviewed publications. https://www.nanoanalytics.com/en/products/cellzscope/publications.html (Accessed 06/12/2022.

National Research Council. 2006. Nutrient Requirements of Dogs and Cats. The National Academies Press, Washington, DC.

Needham, B. D., S. M. Carroll, D. K. Giles, G. Georgiou, M. Whiteley, and M. S. Trent. 2013. Modulating the innate immune response by combinatorial engineering of endotoxin. Proceedings of the National Academy of Sciences 110(4):1464-1469. doi: doi:10.1073/pnas.1218080110

Nery, J., R. Goudez, V. Biourge, C. Tournier, V. Leray, L. Martin, C. Thorin, P. Nguyen, and H. Dumon. 2012. Influence of dietary protein content and source on colonic fermentative activity in dogs differing in body size and digestive tolerance1. Journal of Animal Science 90(8):2570-2580. doi: 10.2527/jas.2011-4112

Neuhaus, F. C., and J. Baddiley. 2003. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev 67(4):686-723. doi: 10.1128/mmbr.67.4.686-723.2003

Nighot, M., R. Al-Sadi, S. Guo, M. Rawat, P. Nighot, M. D. Watterson, and T. Y. Ma. 2017. Lipopolysaccharide-Induced Increase in Intestinal Epithelial Tight Permeability Is Mediated by Toll-Like Receptor 4/Myeloid Differentiation Primary Response 88 (MyD88) Activation of Myosin Light Chain Kinase Expression. Am J Pathol 187(12):2698-2710. doi: 10.1016/j.ajpath.2017.08.005

Nikaido, H. 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67(4):593-656. doi: 10.1128/mmbr.67.4.593-656.2003

Nilsen, N. J., S. Deininger, U. Nonstad, F. Skjeldal, H. Husebye, D. Rodionov, S. von Aulock, T. Hartung, E. Lien, O. Bakke, and T. Espevik. 2008. Cellular trafficking of lipoteichoic acid and Toll-like receptor 2 in relation to signaling: role of CD14 and CD36. J Leukoc Biol 84(1):280-291. doi: 10.1189/jlb.0907656

Nocera, F. P., M. Ambrosio, F. Fiorito, L. Cortese, and L. De Martino. 2021. On Gram-Positive- and Gram-Negative-Bacteria-Associated Canine and Feline Skin Infections: A 4-Year Retrospective Study of the University Veterinary Microbiology Diagnostic Laboratory of Naples, Italy. Animals (Basel) 11(6)doi: 10.3390/ani11061603

Nogal, A., A. M. Valdes, and C. Menni. 2021. The role of short-chain fatty acids in the interplay between gut microbiota and diet in cardio-metabolic health. Gut Microbes 13(1):1-24. doi: 10.1080/19490976.2021.1897212

Nogueira, J. P. D. S., F. He, H. F. Mangian, P. M. Oba, and M. R. C. De Godoy. 2019. Dietary supplementation of a fiber-prebiotic and saccharin-eugenol blend in extruded diets fed to dogs. Journal of Animal Science 97(11):4519-4531. doi: 10.1093/jas/skz293

Noh, S. Y., S.-S. Kang, C.-H. Yun, and S. H. Han. 2015. Lipoteichoic acid from Lactobacillus plantarum inhibits Pam2CSK4-induced IL-8 production in human intestinal epithelial cells. Molecular Immunology 64(1):183-189. doi: https://doi.org/10.1016/j.molimm.2014.11.014

Ohta, H., T. Yamaguchi, B. K. Rajapakshage, M. Murakami, N. Sasaki, K. Nakamura, S. J. Hwang, M. Yamasaki, and M. Takiguchi. 2011. Expression and subcellular localization of apical junction proteins in canine duodenal and colonic mucosa. Am J Vet Res 72(8):1046-1051. doi: 10.2460/ajvr.72.8.1046

Okin, G. S. 2017. Environmental impacts of food consumption by dogs and cats. PloS one 12(8):e0181301-e0181301. doi: 10.1371/journal.pone.0181301

Okumura, R., and K. Takeda. 2016. Maintenance of gut homeostasis by the mucosal immune system. Proc Jpn Acad Ser B Phys Biol Sci 92(9):423-435. doi: 10.2183/pjab.92.423

Olnes, M. J., Y. Kotliarov, A. Biancotto, F. Cheung, J. Chen, R. Shi, H. Zhou, E. Wang, J. S. Tsang, and R. Nussenblatt. 2016. Corrigendum: Effects of Systemically Administered Hydrocortisone on the Human Immunome. Sci Rep 6:25215. doi: 10.1038/srep25215

Omori, M., S. Maeda, H. Igarashi, K. Ohno, K. Sakai, T. Yonezawa, A. Horigome, T. Odamaki, and N. Matsuki. 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

Ott, C., K. Jacobs, E. Haucke, A. Navarrete Santos, T. Grune, and A. Simm. 2014. Role of advanced glycation end products in cellular signaling. Redox Biology 2:411-429. doi: https://doi.org/10.1016/j.redox.2013.12.016

Ouellette, A. J. 2011. Paneth cell α-defensins in enteric innate immunity. Cell Mol Life Sci 68(13):2215-2229. doi: 10.1007/s00018-011-0714-6

Ozaki, H., K. Ishii, H. Horiuchi, H. Arai, T. Kawamoto, K. Okawa, A. Iwamatsu, and T. Kita. 1999. Cutting Edge: Combined Treatment of TNF-α and IFN-γ Causes Redistribution of Junctional Adhesion Molecule in Human Endothelial Cells. The Journal of Immunology 163(2):553-557.

Page, M. J., D. B. Kell, and E. Pretorius. 2022. The Role of Lipopolysaccharide-Induced Cell Signalling in Chronic Inflammation. Chronic Stress 6:24705470221076390. doi: 10.1177/24705470221076390

Palazzo, M., A. Balsari, A. Rossini, S. Selleri, C. Calcaterra, S. Gariboldi, L. Zanobbio, F. Arnaboldi, Y. F. Shirai, G. Serrao, and C. Rumio. 2007. Activation of Enteroendocrine Cells via TLRs Induces Hormone, Chemokine, and Defensin Secretion. The Journal of Immunology 178(7):4296-4303. doi: 10.4049/jimmunol.178.7.4296

Panasevich, M. R., K. R. Kerr, R. N. Dilger, G. C. Fahey, L. Guérin-Deremaux, G. L. Lynch, D. Wils, J. S. Suchodolski, J. M. Steer, S. E. Dowd, and K. S. Swanson. 2015. Modulation of the faecal microbiome of healthy adult dogs by inclusion of potato fibre in the diet. British Journal of Nutrition 113(1):125-133. doi: 10.1017/S0007114514003274

Paradis, E., and K. Schliep. 2018. ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. Bioinformatics 35(3):526-528. doi: 10.1093/bioinformatics/bty633

Paradis, T., H. Bègue, L. Basmaciyan, F. Dalle, and F. Bon. 2021. Tight Junctions as a Key for Pathogens Invasion in Intestinal Epithelial Cells. Int J Mol Sci 22(5)doi: 10.3390/ijms22052506

Pedrinelli, V., R. V. A. Zafalon, R. B. A. Rodrigues, M. P. Perini, R. M. C. Conti, T. H. A. Vendramini, J. C. de Carvalho Balieiro, and M. A. Brunetto. 2019. Concentrations of macronutrients, minerals and heavy metals in home-prepared diets for adult dogs and cats. Scientific Reports 9(1):13058. doi: 10.1038/s41598-019-49087-z

Peng, L., Z. He, W. Chen, I. R. Holzman, and J. Lin. 2007. Effects of Butyrate on Intestinal Barrier Function in a Caco-2 Cell Monolayer Model of Intestinal Barrier. Pediatric Research 61:37. (Article) doi: 10.1203/01.pdr.0000250014.92242.f3

Peng, L., Z.-R. Li, R. S. Green, I. R. Holzman, and J. Lin. 2009. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase

in Caco-2 cell monolayers. The Journal of nutrition 139(9):1619-1625. doi: 10.3945/jn.109.104638
Percy, M. G., and A. Gründling. 2014. Lipoteichoic Acid Synthesis and Function in Gram-Positive Bacteria. Annual Review of Microbiology 68(1):81-100. doi: 10.1146/annurev-micro-091213-112949
Pereira, F. L., C. A. Oliveira Júnior, R. O. S. Silva, F. A. Dorella, A. F. Carvalho, G. M. F. Almeida, C. A. G. Leal, F. C. F. Lobato, and H. C. P. Figueiredo. 2016. Complete genome sequence of Peptoclostridium difficile strain Z31. Gut Pathogens 8(1):11. doi: 10.1186/s13099-016-0095-3
Peters, I. R., C. R. Helps, E. L. Calvert, E. J. Hall, and M. J. Day. 2005. Cytokine mRNA quantification in histologically normal canine duodenal mucosa by real-time RT-PCR. Veterinary Immunology and Immunopathology 103(1):101-111. doi: https://doi.org/10.1016/j.vetimm.2004.08.020
Peterson, L. W., and D. Artis. 2014. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nature Reviews Immunology 14:141. (Review Article) doi: 10.1038/nri3608

https://www.nature.com/articles/nri3608#supplementary-information
Phillips, C. J. C. 2005. Meta-analysis - a systematic and quantitative review of animal experiments to maximise the information derived. Animal Welfare 14(4):333-338.
Phungviwatnikul, T., A. H. Lee, S. E. Belchik, J. S. Suchodolski, and K. S. Swanson. 2021. Weight loss and high-protein, high-fiber diet consumption impact blood metabolite profiles, body composition, voluntary physical activity, fecal microbiota, and fecal metabolites of adult dogs. Journal of Animal Science 100(2)doi: 10.1093/jas/skab379
Pilla, R., B. C. Guard, J. M. Steiner, F. P. Gaschen, E. Olson, D. Werling, K. Allenspach, S. Salavati Schmitz, and J. S. Suchodolski. 2019. Administration of a Synbiotic Containing Enterococcus faecium Does Not Significantly Alter Fecal Microbiota Richness or Diversity in Dogs With and Without Food-Responsive Chronic Enteropathy. Front Vet Sci 6:277-277. doi: 10.3389/fvets.2019.00277
Pilla, R., and J. S. Suchodolski. 2020. The Role of the Canine Gut Microbiome and Metabolome in Health and Gastrointestinal Disease. Front Vet Sci 6(498)(Review) doi: 10.3389/fvets.2019.00498
Pinheiro J, B. D., R Core Team. 2022. nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-158.
Pinna, C., C. G. Vecchiato, G. Zaghini, M. Grandi, E. Nannoni, C. Stefanelli, and G. Biagi. 2016. In vitro influence of dietary protein and fructooligosaccharides on metabolism of canine fecal microbiota. BMC Vet Res 12:53. doi: 10.1186/s12917-016-0672-1
Pral, L. P., J. L. Fachi, R. O. Corrêa, M. Colonna, and M. A. R. Vinolo. 2021. Hypoxia and HIF-1 as key regulators of gut microbiota and host interactions. Trends in Immunology 42(7):604-621. doi: https://doi.org/10.1016/j.it.2021.05.004
Prasad, S., R. Mingrino, K. Kaukinen, K. L. Hayes, R. M. Powell, T. T. MacDonald, and J. E. Collins. 2005. Inflammatory processes have differential effects on claudins 2, 3 and 4 in colonic epithelial cells. Laboratory Investigation 85(9):1139-1162. doi: 10.1038/labinvest.3700316
Proell, M., S. J. Riedl, J. H. Fritz, A. M. Rojas, and R. Schwarzenbacher. 2008. The Nod-Like Receptor (NLR) Family: A Tale of Similarities and Differences. PLOS ONE 3(4):e2119. doi: 10.1371/journal.pone.0002119
Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41(Database issue):D590-D596. doi: 10.1093/nar/gks1219
Raetz, C. R., and C. Whitfield. 2002. Lipopolysaccharide endotoxins. Annu Rev Biochem 71:635-700. doi: 10.1146/annurev.biochem.71.110601.135414
Rahman, A. H., D. K. Taylor, and L. A. Turka. 2009. The contribution of direct TLR signaling to T cell responses. Immunol Res 45(1):25-36. doi: 10.1007/s12026-009-8113-x
Ramig, R. F. 2004. Pathogenesis of intestinal and systemic rotavirus infection. Journal of Virology 78(19):10213-10220. doi: 10.1128/JVI.78.19.10213-10220.2004

Ramos-Vara, J. A., and M. A. Miller. 2002. Immunohistochemical characterization of canine intestinal epithelial and mesenchymal tumours with a monoclonal antibody to hepatocyte paraffin 1 (Hep Par 1). The Histochemical Journal 34(8):397-401. doi: 10.1023/A:1023683404230

Ran-Ressler, R. R., L. Khailova, K. M. Arganbright, C. K. Adkins-Rieck, Z. E. Jouni, O. Koren, R. E. Ley, J. T. Brenna, and B. Dvorak. 2011a. Branched Chain Fatty Acids Reduce the Incidence of Necrotizing Enterocolitis and Alter Gastrointestinal Microbial Ecology in a Neonatal Rat Model. PLOS ONE 6(12):e29032. doi: 10.1371/journal.pone.0029032

Ran-Ressler, R. R., L. Khailova, K. M. Arganbright, C. K. Adkins-Rieck, Z. E. Jouni, O. Koren, R. E. Ley, J. T. Brenna, and B. Dvorak. 2011b. Branched chain fatty acids reduce the incidence of necrotizing enterocolitis and alter gastrointestinal microbial ecology in a neonatal rat model. PloS one 6(12):e29032-e29032. doi: 10.1371/journal.pone.0029032

Reboul, E. 2013. Absorption of vitamin A and carotenoids by the enterocyte: focus on transport proteins. Nutrients 5(9):3563-3581. doi: 10.3390/nu5093563

Reboul, E. 2015. Intestinal absorption of vitamin D: from the meal to the enterocyte. Food & Function 6(2):356-362. (10.1039/C4FO00579A) doi: 10.1039/C4FO00579A

Reese, A. T., E. H. Cho, B. Klitzman, S. P. Nichols, N. A. Wisniewski, M. M. Villa, H. K. Durand, S. Jiang, F. S. Midani, S. N. Nimmagadda, T. M. O'Connell, J. P. Wright, M. A. Deshusses, and L. A. David. 2018. Antibiotic-induced changes in the microbiota disrupt redox dynamics in the gut. eLife 7:e35987. doi: 10.7554/eLife.35987

Regan, D. P., L. Chow, S. Das, L. Haines, E. Palmer, J. N. Kurihara, J. W. Coy, A. Mathias, D. H. Thamm, D. L. Gustafson, and S. W. Dow. 2022. Losartan Blocks Osteosarcoma-Elicited Monocyte Recruitment, and Combined With the Kinase Inhibitor Toceranib, Exerts Significant Clinical Benefit in Canine Metastatic Osteosarcoma. Clin Cancer Res 28(4):662-676. doi: 10.1158/1078-0432.Ccr-21-2105

Reichardt, N., S. H. Duncan, P. Young, A. Belenguer, C. McWilliam Leitch, K. P. Scott, H. J. Flint, and P. Louis. 2014. Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. ISME J 8(6):1323-1335. doi: 10.1038/ismej.2014.14

Reineking, W., J. Junginger, R. Mischke, and M. Hewicker-Trautwein. 2018a. Canine primary jejunal and colonic epithelial cells predominantly express TLR5 and TLR9 but do not change TLR expression pattern after stimulation with certain Toll-like receptor ligands. Vet Immunol Immunopathol 206:16-24. doi: 10.1016/j.vetimm.2018.11.003

Reineking, W., J. Junginger, R. Mischke, and M. Hewicker-Trautwein. 2018b. Canine primary jejunal and colonic epithelial cells predominantly express TLR5 and TLR9 but do not change TLR expression pattern after stimulation with certain Toll-like receptor ligands. Veterinary Immunology and Immunopathology 206:16-24. doi: https://doi.org/10.1016/j.vetimm.2018.11.003

Remillard, R. L., D. E. Darden, K. E. Michel, S. L. Marks, C. A. Buffington, and P. R. Bunnell. 2001. An investigation of the relationship between caloric intake and outcome in hospitalized dogs. Vet Ther 2(4):301-310.

Ren, J., K. Sun, Z. Wu, J. Yao, and B. Guo. 2011. All 4 Bile Salt Hydrolase Proteins Are Responsible for the Hydrolysis Activity in Lactobacillus plantarum ST-III. Journal of Food Science 76(9):M622-M628. doi: https://doi.org/10.1111/j.1750-3841.2011.02431.x

Richards, L. B., M. Li, B. C. A. M. van Esch, J. Garssen, and G. Folkerts. 2016. The effects of short-chain fatty acids on the cardiovascular system. PharmaNutrition 4(2):68-111. doi: https://doi.org/10.1016/j.phanu.2016.02.001

Ridyard, A. E., J. K. Brown, S. M. Rhind, R. W. Else, J. W. Simpson, and H. R. P. Miller. 2007. Apical Junction Complex Protein Expression in the Canine Colon:Differential Expression of Claudin-2 in the Colonic Mucosa in Dogs With Idiopathic Colitis. Journal of Histochemistry & Cytochemistry 55(10):1049-1058. doi: 10.1369/jhc.7A7211.2007

Ridyard, A. E., T. J. Nuttall, R. W. Else, J. W. Simpson, and H. R. P. Miller. 2002. Evaluation of Th1, Th2 and immunosuppressive cytokine mRNA expression within the colonic mucosa of dogs with idiopathic lymphocytic–plasmacytic colitis. Veterinary Immunology and Immunopathology 86(3):205-214. doi: https://doi.org/10.1016/S0165-2427(02)00039-9

Rios-Covian, D., B. Sánchez, N. Salazar, N. Martínez, B. Redruello, M. Gueimonde, and C. G. de los Reyes-Gavilán. 2015. Different metabolic features of Bacteroides fragilis growing in the presence of glucose and exopolysaccharides of bifidobacteria. Front Microbiol 6(Original Research) doi: 10.3389/fmicb.2015.00825

Robinson, K., Z. Deng, Y. Hou, and G. Zhang. 2015. Regulation of the Intestinal Barrier Function by Host Defense Peptides. Front Vet Sci 2(57)(Review) doi: 10.3389/fvets.2015.00057

Rodriguez Sawicki, L., N. M. Bottasso Arias, N. Scaglia, L. J. Falomir Lockhart, G. R. Franchini, J. Storch, and B. Córsico. 2017. FABP1 knockdown in human enterocytes impairs proliferation and alters lipid metabolism. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1862(12):1587-1594. doi: https://doi.org/10.1016/j.bbalip.2017.09.006

Rollo, E. E., K. P. Kumar, N. C. Reich, J. Cohen, J. Angel, H. B. Greenberg, R. Sheth, J. Anderson, B. Oh, S. J. Hempson, E. R. Mackow, and R. D. Shaw. 1999. The Epithelial Cell Response to Rotavirus Infection. The Journal of Immunology 163(8):4442-4452.

Rose, E. C., J. Odle, A. T. Blikslager, and A. L. Ziegler. 2021. Probiotics, Prebiotics and Epithelial Tight Junctions: A Promising Approach to Modulate Intestinal Barrier Function. Int J Mol Sci 22(13)doi: 10.3390/ijms22136729

Rossi, G., M. Cerquetella, A. Gavazza, L. Galosi, S. Berardi, S. Mangiaterra, S. Mari, J. S. Suchodolski, J. A. Lidbury, J. M. Steiner, and G. Pengo. 2020. Rapid Resolution of Large Bowel Diarrhea after the Administration of a Combination of a High-Fiber Diet and a Probiotic Mixture in 30 Dogs. Veterinary Sciences 7(1):21.

Rossi, O., J. Karczewski, E. H. Stolte, R. J. M. Brummer, M. A. van Nieuwenhoven, M. Meijerink, J. R. J. van Neerven, S. C. D. van Ijzendoorn, P. van Baarlen, and J. M. Wells. 2013. Vectorial secretion of interleukin-8 mediates autocrine signalling in intestinal epithelial cells via apically located CXCR1. BMC Research Notes 6(1):431. doi: 10.1186/1756-0500-6-431

Rouiller, Y., A. Périlleux, M. Marsaut, M. Stettler, M.-N. Vesin, and H. Broly. 2012. Effect of hydrocortisone on the production and glycosylation of an Fc-fusion protein in CHO cell cultures. Biotechnology Progress 28(3):803-813. doi: https://doi.org/10.1002/btpr.1530

Rusu, D., S. Loret, O. Peulen, J. Mainil, and G. Dandrifosse. 2005. Immunochemical, biomolecular and biochemical characterization of bovine epithelial intestinal primocultures. BMC Cell Biol 6:42-42. doi: 10.1186/1471-2121-6-42

Ryu, J.-K., S. J. Kim, S.-H. Rah, J. I. Kang, H. E. Jung, D. Lee, H. K. Lee, J.-O. Lee, B. S. Park, T.-Y. Yoon, and H. M. Kim. 2017. Reconstruction of LPS Transfer Cascade Reveals Structural Determinants within LBP, CD14, and TLR4-MD2 for Efficient LPS Recognition and Transfer. Immunity 46(1):38-50. doi: 10.1016/j.immuni.2016.11.007

Sabbioni, A., C. Ferrario, C. Milani, L. Mancabelli, E. Riccardi, F. Di Ianni, V. Beretti, P. Superchi, and M. C. Ossiprandi. 2016. Modulation of the Bifidobacterial Communities of the Dog Microbiota by Zeolite. Front Microbiol 7(1491)(Original Research) doi: 10.3389/fmicb.2016.01491

Sacre, S. M., A. M. C. Lundberg, E. Andreakos, C. Taylor, M. Feldmann, and B. M. Foxwell. 2007. Selective Use of TRAM in Lipopolysaccharide (LPS) and Lipoteichoic Acid (LTA) Induced NF-κB Activation and Cytokine Production in Primary Human Cells: TRAM Is an Adaptor for LPS and LTA Signaling. The Journal of Immunology 178(4):2148-2154. doi: 10.4049/jimmunol.178.4.2148

Saeedi, B. J., D. J. Kao, D. A. Kitzenberg, E. Dobrinskikh, K. D. Schwisow, J. C. Masterson, A. A. Kendrick, C. J. Kelly, A. J. Bayless, D. J. Kominsky, E. L. Campbell, K. A. Kuhn, G. T. Furuta, S. P. Colgan, and L. E. Glover. 2015a. HIF-dependent regulation of claudin-1 is central to intestinal epithelial tight junction integrity. Mol Biol Cell 26(12):2252-2262. doi: 10.1091/mbc.E14-07-1194

Saeedi, B. J., D. J. Kao, D. A. Kitzenberg, E. Dobrinskikh, K. D. Schwisow, J. C. Masterson, A. A. Kendrick, C. J. Kelly, A. J. Bayless, D. J. Kominsky, E. L. Campbell, K. A. Kuhn, G. T. Furuta, S. P. Colgan, and L. E. Glover. 2015b. HIF-dependent regulation of claudin-1 is central to intestinal epithelial tight junction integrity. Mol Biol Cell 26(12):2252-2262. doi: 10.1091/mbc.E14-07-1194

Salimiyan, S. M., M; Aliakbari, S; Kazemi, R; Amini, AA; Rahmani MR. 2022. Hydrocortisone Long-term Treatment Effect on Immunomodulatory Properties of Human Adipose-Derived

Mesenchymal Stromal/Stem Cells. Journal of Interferon & Cytokine Research 42(2):72-81. doi: 10.1089/jir.2021.0120

Sandri, M., S. Dal Monego, G. Conte, S. Sgorlon, and B. Stefanon. 2017. Raw meat based diet influences faecal microbiome and end products of fermentation in healthy dogs. BMC Veterinary Research 13(1)(Article) doi: 10.1186/s12917-017-0981-z

Sandri, M., S. Sgorlon, G. Conte, A. Serra, S. Dal Monego, and B. Stefanon. 2019. Substitution of a commercial diet with raw meat complemented with vegetable foods containing chickpeas or peas affects faecal microbiome in healthy dogs. Italian Journal of Animal Science 18(1):1205-1214. doi: 10.1080/1828051X.2019.1645624

Sandri, M., S. Sgorlon, E. Scarsella, and B. Stefanon. 2020. Effect of different starch sources in a raw meat-based diet on fecal microbiome in dogs housed in a shelter. Animal Nutrition doi: https://doi.org/10.1016/j.aninu.2020.03.003

Satyaraj, E., A. Reynolds, R. Engler, J. Labuda, and P. Sun. 2021. Supplementation of Diets With Spirulina Influences Immune and Gut Function in Dogs. Front Nutr 8:667072-667072. doi: 10.3389/fnut.2021.667072

Scarsella, E., M. Cintio, L. Iacumin, F. Ginaldi, and B. Stefanon. 2020. Interplay between Neuroendocrine Biomarkers and Gut Microbiota in Dogs Supplemented with Grape Proanthocyanidins: Results of Dietary Intervention Study. Animals (Basel) 10(3):531. doi: 10.3390/ani10030531

Schauf, S., G. de la Fuente, C. J. Newbold, A. Salas-Mani, C. Torre, L. Abecia, and C. Castrillo. 2018. Effect of dietary fat to starch content on fecal microbiota composition and activity in dogs1. Journal of Animal Science 96(9):3684-3698. doi: 10.1093/jas/sky264

Schleicher, M., S. B. Cash, and L. M. Freeman. 2019. Determinants of pet food purchasing decisions. Can Vet J 60(6):644-650.

Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn, and C. F. Weber. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75(23):7537-7541. doi: 10.1128/aem.01541-09

Schmidt, M., S. Unterer, J. S. Suchodolski, J. B. Honneffer, B. C. Guard, J. A. Lidbury, J. M. Steiner, J. Fritz, and P. Kölle. 2018. The fecal microbiome and metabolome differs between dogs fed Bones and Raw Food (BARF) diets and dogs fed commercial diets. PLoS ONE 13(8):e0201279. doi: 10.1371/journal.pone.0201279

Schmitz, S., B. Glanemann, O. A. Garden, H. Brooks, Y. M. Chang, D. Werling, and K. Allenspach. 2015a. A Prospective, Randomized, Blinded, Placebo-Controlled Pilot Study on the Effect of Enterococcus faecium on Clinical Activity and Intestinal Gene Expression in Canine Food-Responsive Chronic Enteropathy. Journal of Veterinary Internal Medicine 29(2):533-543. doi: https://doi.org/10.1111/jvim.12563

Schmitz, S., D. Werling, and K. Allenspach. 2015b. Effects of ex-vivo and in-vivo treatment with probiotics on the inflammasome in dogs with chronic enteropathy. PloS one 10(3):e0120779-e0120779. doi: 10.1371/journal.pone.0120779

Schönenberger, M. J., and W. J. Kovacs. 2015. Hypoxia signaling pathways: modulators of oxygen-related organelles. Frontiers in cell and developmental biology 3:42-42. doi: 10.3389/fcell.2015.00042

Setiadi, A. F., A. R. Abbas, S. Jeet, K. Wong, A. Bischof, I. Peng, J. Lee, M. Bremer, E. L. Eggers, J. DeVoss, T. Staton, A. Herman, H. C. von Büdingen, and M. J. Townsend. 2019. IL-17A is associated with the breakdown of the blood-brain barrier in relapsing-remitting multiple sclerosis. Journal of Neuroimmunology 332:147-154. doi: https://doi.org/10.1016/j.jneuroim.2019.04.011

Sheth, P., N. D. Santos, A. Seth, N. F. LaRusso, and R. K. Rao. 2007. Lipopolysaccharide disrupts tight junctions in cholangiocyte monolayers by a c-Src-, TLR4-, and LBP-dependent mechanism. American Journal of Physiology-Gastrointestinal and Liver Physiology 293(1):G308-G318. doi: 10.1152/ajpgi.00582.2006

Shi, J., D. Zhao, F. Zhao, C. Wang, G. Zamaratskaia, and C. Li. 2021. Chicken-eaters and pork-eaters have different gut microbiota and tryptophan metabolites. Scientific Reports 11(1):11934. doi: 10.1038/s41598-021-91429-3

Shin, N.-R., T. W. Whon, and J.-W. Bae. 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends in Biotechnology 33(9):496-503. doi: https://doi.org/10.1016/j.tibtech.2015.06.011

Shin, W., A. Wu, M. W. Massidda, C. Foster, N. Thomas, D.-W. Lee, H. Koh, Y. Ju, J. Kim, and H. J. Kim. 2019. A Robust Longitudinal Co-culture of Obligate Anaerobic Gut Microbiome With Human Intestinal Epithelium in an Anoxic-Oxic Interface-on-a-Chip. Frontiers in Bioengineering and Biotechnology 7(Original Research) doi: 10.3389/fbioe.2019.00013

Silhavy, T. J., D. Kahne, and S. Walker. 2010. The bacterial cell envelope. Cold Spring Harb Perspect Biol 2(5):a000414. doi: 10.1101/cshperspect.a000414

Singh, A. K., Y. Jiang, and S. Gupta. 2007. Effects of Bacterial Toxins on Endothelial Tight Junction In Vitro: A Mechanism-Based Investigation. Toxicology Mechanisms and Methods 17(6):331-347. doi: 10.1080/15376510601077029

Snoeck, V., B. Goddeeris, and E. Cox. 2005. The role of enterocytes in the intestinal barrier function and antigen uptake. Microb. Infect. 7(7):997-1004. doi: https://doi.org/10.1016/j.micinf.2005.04.003

Soontararak, S., L. Chow, V. Johnson, J. Coy, C. Webb, S. Wennogle, and S. Dow. 2019. Humoral immune responses against gut bacteria in dogs with inflammatory bowel disease. PLOS ONE 14(8):e0220522. doi: 10.1371/journal.pone.0220522

Spellberg, B., and J. J. E. Edwards. 2001. Type 1/Type 2 Immunity in Infectious Diseases. Clinical Infectious Diseases 32(1):76-102. doi: 10.1086/317537

Srinivasan, B., A. R. Kolli, M. B. Esch, H. E. Abaci, M. L. Shuler, and J. J. Hickman. 2015. TEER Measurement Techniques for In Vitro Barrier Model Systems. Journal of Laboratory Automation 20(2):107-126. doi: 10.1177/2211068214561025

Steenson, S., A. M. Umpleby, J. A. Lovegrove, K. G. Jackson, and B. A. Fielding. 2017. Role of the Enterocyte in Fructose-Induced Hypertriglyceridaemia. Nutrients 9(4):349. doi: 10.3390/nu9040349

Stephens, M., and P.-Y. von der Weid. 2020. Lipopolysaccharides modulate intestinal epithelial permeability and inflammation in a species-specific manner. Gut Microbes 11(3):421-432. doi: 10.1080/19490976.2019.1629235

Stone, N. E., A. E. Nunnally, V. Jimenez, E. K. Cope, J. W. Sahl, K. Sheridan, H. M. Hornstra, J. Vinocur, E. W. Settles, K. C. Headley, C. H. D. Williamson, J. R. Rideout, E. Bolyen, J. G. Caporaso, J. Terriquez, F. P. Monroy, J. D. Busch, P. Keim, and D. M. Wagner. 2019. Domestic canines do not display evidence of gut microbial dysbiosis in the presence of Clostridioides (Clostridium) difficile, despite cellular susceptibility to its toxins. Anaerobe 58:53-72. doi: https://doi.org/10.1016/j.anaerobe.2019.03.017

Strompfová, V., A. Lauková, and A. C. Ouwehand. 2004. Selection of enterococci for potential canine probiotic additives. Veterinary Microbiology 100(1):107-114. doi: https://doi.org/10.1016/j.vetmic.2004.02.002

Sturgeon, A. 2014. Analysis of the Oral and Fecal Microbiota of Companion Animals Using Next-Generation Sequencing of the 16S rRNA Gene. MSc, University of Guelph.

Suchodolski, J. S., J. Camacho, and J. M. Steiner. 2008. Analysis of bacterial diversity in the canine duodenum, jejunum, ileum, and colon by comparative16S rRNAgene analysis. FEMS Microbiol Ecol 66doi: 10.1111/j.1574-6941.2008.00521.x

Suchodolski, J. S., S. E. Dowd, V. Wilke, J. M. Steiner, and A. E. Jergens. 2012a. 16S rRNA gene pyrosequencing reveals bacterial dysbiosis in the Duodenum of dogs with idiopathic inflammatory bowel disease. PLoS ONE 7(6)(Article) doi: 10.1371/journal.pone.0039333

Suchodolski, J. S., M. E. Markel, J. F. Garcia-Mazcorro, S. Unterer, R. M. Heilmann, S. E. Dowd, P. Kachroo, I. Ivanov, Y. Minamoto, E. M. Dillman, J. M. Steiner, A. K. Cook, and L. Toresson. 2012b. The Fecal Microbiome in Dogs with Acute Diarrhea and Idiopathic Inflammatory Bowel Disease. PLoS ONE 7(12)(Article) doi: 10.1371/journal.pone.0051907

Sugihara, K., T. L. Morhardt, and N. Kamada. 2019. The Role of Dietary Nutrients in Inflammatory Bowel Disease. Front Immunol 9(3183)(Review) doi: 10.3389/fimmu.2018.03183

Summers, S., J. M. Quimby, R. K. Phillips, J. Stockman, A. Isaiah, J. A. Lidbury, J. M. Steiner, and J. Suchodolski. 2020. Preliminary evaluation of fecal fatty acid concentrations in cats with chronic kidney disease and correlation with indoxyl sulfate and p-cresol sulfate. Journal of Veterinary Internal Medicine 34(1):206-215. doi: https://doi.org/10.1111/jvim.15634

Sun, M., W. Wu, L. Chen, W. Yang, X. Huang, C. Ma, F. Chen, Y. Xiao, Y. Zhao, C. Ma, S. Yao, V. H. Carpio, S. M. Dann, Q. Zhao, Z. Liu, and Y. Cong. 2018. Microbiota-derived short-chain fatty acids promote Th1 cell IL-10 production to maintain intestinal homeostasis. Nature Communications 9(1):3555. doi: 10.1038/s41467-018-05901-2

Swerdlow, M. P., D. R. Kennedy, J. S. Kennedy, R. J. Washabau, P. S. Henthorn, P. F. Moore, S. R. Carding, and P. J. Felsburg. 2006. Expression and function of TLR2, TLR4, and Nod2 in primary canine colonic epithelial cells. Veterinary Immunology and Immunopathology 114(3):313-319. doi: https://doi.org/10.1016/j.vetimm.2006.09.003

Tal, M., J. M. Parr, S. MacKenzie, and A. Verbrugghe. 2018. Dietary imbalances in a large breed puppy, leading to compression fractures, vitamin D deficiency, and suspected nutritional secondary hyperparathyroidism. Can Vet J 59(1):36-42.

Tamura, Y., H. Ohta, N. Yokoyama, S. Y. Lim, T. Osuga, K. Morishita, K. Nakamura, M. Yamasaki, and M. Takiguchi. 2014. Evaluation of selected cytokine gene expression in colonic mucosa from dogs with idiopathic lymphocytic-plasmacytic colitis. J Vet Med Sci 76(10):1407-1410. doi: 10.1292/jvms.13-0635

Tan, Y., and Jonathan C. Kagan. 2014. A Cross-Disciplinary Perspective on the Innate Immune Responses to Bacterial Lipopolysaccharide. Molecular Cell 54(2):212-223. doi: 10.1016/j.molcel.2014.03.012

Taormina, V. M., A. L. Unger, M. R. Schiksnis, M. Torres-Gonzalez, and J. Kraft. 2020. Branched-Chain Fatty Acids-An Underexplored Class of Dairy-Derived Fatty Acids. Nutrients 12(9):2875. doi: 10.3390/nu12092875

Taylor, A. R., C. J. Welsh, C. Young, E. Spoor, S. C. Kerwin, J. F. Griffin, G. J. Levine, N. D. Cohen, and J. M. Levine. 2014. Cerebrospinal fluid inflammatory cytokines and chemokines in naturally occurring canine spinal cord injury. J Neurotrauma 31(18):1561-1569. doi: 10.1089/neu.2014.3405

Thévenot, E. A., A. Roux, Y. Xu, E. Ezan, and C. Junot. 2015. Analysis of the Human Adult Urinary Metabolome Variations with Age, Body Mass Index, and Gender by Implementing a Comprehensive Workflow for Univariate and OPLS Statistical Analyses. J Proteome Res 14(8):3322-3335. doi: 10.1021/acs.jproteome.5b00354

Tivers, M. S., V. J. Lipscomb, K. C. Smith, C. P. D. Wheeler-Jones, and A. K. House. 2015. Lipopolysaccharide and toll-like receptor 4 in dogs with congenital portosystemic shunts. The Veterinary Journal 206(3):404-413. doi: https://doi.org/10.1016/j.tvjl.2015.07.006

Tropf, M., O. L. Nelson, P. M. Lee, and H. Y. Weng. 2017. Cardiac and Metabolic Variables in Obese Dogs. Journal of veterinary internal medicine 31(4):1000-1007. doi: 10.1111/jvim.14775

Tsuji, K., M. Ojima, K. Otabe, M. Horie, H. Koga, I. Sekiya, and T. Muneta. 2017. Effects of Different Cell-Detaching Methods on the Viability and Cell Surface Antigen Expression of Synovial Mesenchymal Stem Cells. Cell Transplant 26(6):1089-1102. doi: 10.3727/096368917X694831

Ulevitch, R. J., and P. S. Tobias. 1995. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu Rev Immunol 13:437-457. doi: 10.1146/annurev.iy.13.040195.002253

Ulluwishewa, D., R. C. Anderson, N. C. Roy, W. C. McNabb, P. J. Moughan, and J. M. Wells. 2011. Regulation of Tight Junction Permeability by Intestinal Bacteria and Dietary Components. The Journal of Nutrition 141(5):769-776. doi: 10.3945/jn.110.135657 %J The Journal of Nutrition

Ulluwishewa, D., R. C. Anderson, W. Young, W. C. McNabb, P. van Baarlen, P. J. Moughan, J. M. Wells, and N. C. Roy. 2015. Live Faecalibacterium prausnitzii in an apical anaerobic model of the intestinal epithelial barrier. Cell Microbiol 17(2):226-240. doi: 10.1111/cmi.12360

Valdes, A. M., J. Walter, E. Segal, and T. D. Spector. 2018. Role of the gut microbiota in nutrition and health. BMJ 361:k2179. doi: 10.1136/bmj.k2179

Van Amersfoort, E. S., T. J. Van Berkel, and J. Kuiper. 2003. Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. Clin Microbiol Rev 16(3):379-414. doi: 10.1128/cmr.16.3.379-414.2003

Van De Walle, J., A. Hendrickx, B. Romier, Y. Larondelle, and Y.-J. Schneider. 2010. Inflammatory parameters in Caco-2 cells: Effect of stimuli nature, concentration, combination and cell differentiation. Toxicology in Vitro 24(5):1441-1449. doi: https://doi.org/10.1016/j.tiv.2010.04.002

van der Flier, L. G., and H. Clevers. 2009. Stem Cells, Self-Renewal, and Differentiation in the Intestinal Epithelium. Annual Review of Physiology 71(1):241-260. doi: 10.1146/annurev.physiol.010908.163145

Vancamelbeke, M., and S. Vermeire. 2017. The intestinal barrier: a fundamental role in health and disease. Expert review of gastroenterology & hepatology 11(9):821-834. doi: 10.1080/17474124.2017.1343143

Vázquez-Baeza, Y., E. R. Hyde, J. S. Suchodolski, and R. Knight. 2016. Dog and human inflammatory bowel disease rely on overlapping yet distinct dysbiosis networks. Nature Microbiology 1:16177. (Letter) doi: 10.1038/nmicrobiol.2016.177

https://www.nature.com/articles/nmicrobiol2016177#supplementary-information

Vilchez, R. A., and J. S. Butel. 2004. Emergent human pathogen simian virus 40 and its role in cancer. Clin Microbiol Rev 17(3):495-508, table of contents. doi: 10.1128/cmr.17.3.495-508.2004

Villéger, R., N. Saad, K. Grenier, X. Falourd, L. Foucat, M. C. Urdaci, P. Bressollier, and T. S. Ouk. 2014. Characterization of lipoteichoic acid structures from three probiotic Bacillus strains: involvement of D-alanine in their biological activity. Antonie Van Leeuwenhoek 106(4):693-706. doi: 10.1007/s10482-014-0239-8

Vinolo, M. A. R., H. G. Rodrigues, E. Hatanaka, F. T. Sato, S. C. Sampaio, and R. Curi. 2011. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. The Journal of Nutritional Biochemistry 22(9):849-855. doi: https://doi.org/10.1016/j.jnutbio.2010.07.009

Vital, M., J. Gao, M. Rizzo, T. Harrison, and J. M. Tiedje. 2014a. Diet is a major factor governing the fecal butyrate-producing community structure across Mammalia, Aves and Reptilia. ISME J 9(4):832-843. doi: 10.1038/ismej.2014.179

Vital, M., A. C. Howe, and J. M. Tiedje. 2014b. Revealing the Bacterial Butyrate Synthesis Pathways by Analyzing (Meta)genomic Data. Microbiome 5(2):e00889-00814. doi: 10.1128/mBio.00889-14 %J mBio

von Moltke, J., M. Ji, H.-E. Liang, and R. M. Locksley. 2015. Tuft-cell-derived IL-25 regulates an intestinal ILC2–epithelial response circuit. Nature 529:221. doi: 10.1038/nature16161

https://www.nature.com/articles/nature16161#supplementary-information

Walsh-Reitz, M. M., E. F. Huang, M. W. Musch, E. B. Chang, T. E. Martin, S. Kartha, and F. G. Toback. 2005. AMP-18 protects barrier function of colonic epithelial cells: role of tight junction proteins. Am J Physiol Gastrointest Liver Physiol 289(1):G163-171. doi: 10.1152/ajpgi.00013.2005

Wang, J., D. Elewaut, I. Hoffman, E. M. Veys, and G. Verbruggen. 2004. Physiological levels of hydrocortisone maintain an optimal chondrocyte extracellular matrix metabolism. Annals of the Rheumatic Diseases 63(1):61-66. doi: 10.1136/ard.2002.005298

Ward, J. B., S. J. Keely, and S. J. Keely. 2014. Oxygen in the regulation of intestinal epithelial transport. J Physiol 592(12):2473-2489. doi: 10.1113/jphysiol.2013.270249

Wei, C. X., J. H. Wu, Y. H. Huang, X. Z. Wang, and J. Y. Li. 2022. Lactobacillus plantarum improves LPS-induced Caco2 cell line intestinal barrier damage via cyclic AMP-PKA signaling. PLoS One 17(5):e0267831. doi: 10.1371/journal.pone.0267831

Weng, X. H., K. W. Beyenbach, and A. Quaroni. 2005. Cultured monolayers of the dog jejunum with the structural and functional properties resembling the normal epithelium. Am J Physiol Gastrointest Liver Physiol 288(4):G705-717. doi: 10.1152/ajpgi.00518.2003

Wernimont, S. M., J. Radosevich, M. I. Jackson, E. Ephraim, D. V. Badri, J. M. MacLeay, D. E. Jewell, and J. S. Suchodolski. 2020a. The Effects of Nutrition on the Gastrointestinal Microbiome of Cats and Dogs: Impact on Health and Disease. Front Microbiol 11(1266)(Review) doi: 10.3389/fmicb.2020.01266

Wernimont, S. M., J. Radosevich, M. I. Jackson, E. Ephraim, D. V. Badri, J. M. MacLeay, D. E. Jewell, and J. S. Suchodolski. 2020b. The Effects of Nutrition on the Gastrointestinal Microbiome of Cats and Dogs: Impact on Health and Disease. Front Microbiol 11:1266-1266. doi: 10.3389/fmicb.2020.01266

Wiese, F., O. Simon, and K. D. Weyrauch. 2003. Morphology of the Small Intestine of Weaned Piglets and a Novel Method for Morphometric Evaluation†. Anatomia, Histologia, Embryologia 32(2):102-109. doi: https://doi.org/10.1046/j.1439-0264.2003.00430.x

Wu, J., C. He, J. Bu, Y. Luo, S. Yang, C. Ye, S. Yu, B. He, Y. Yin, and X. Yang. 2020. Betaine attenuates LPS-induced downregulation of Occludin and Claudin-1 and restores intestinal barrier function. BMC Vet Res 16(1):75. doi: 10.1186/s12917-020-02298-3

Wu, Y., H. Xu, X. Tu, and Z. Gao. 2021. The Role of Short-Chain Fatty Acids of Gut Microbiota Origin in Hypertension. Front Microbiol 12(Review) doi: 10.3389/fmicb.2021.730809

Xenoulis, P. G., and J. M. Steiner. 2010. Lipid metabolism and hyperlipidemia in dogs. The Veterinary Journal 183(1):12-21. doi: https://doi.org/10.1016/j.tvjl.2008.10.011

Xiong, Y., N. Miyamoto, K. Shibata, M. A. Valasek, T. Motoike, R. M. Kedzierski, and M. Yanagisawa. 2004. Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc Natl Acad Sci U S A 101(4):1045-1050. doi: 10.1073/pnas.2637002100

Xu, F., X.-J. Hu, W. Singh, W. Geng, I. G. Tikhonova, and J. Lin. 2019. The complex structure of bile salt hydrolase from Lactobacillus salivarius reveals the structural basis of substrate specificity. Scientific Reports 9(1):12438. doi: 10.1038/s41598-019-48850-6

Xu, J., A. A. M. J. Becker, Y. Luo, W. Zhang, B. Ge, C. Leng, G. Wang, L. Ding, J. Wang, X. Fu, and G. P. J. Janssens. 2021. The Fecal Microbiota of Dogs Switching to a Raw Diet Only Partially Converges to That of Wolves. Front Microbiol 12(Original Research) doi: 10.3389/fmicb.2021.701439

Xu, J., A. Verbrugghe, M. Lourenço, A. Cools, D. J. X. Liu, T. Van de Wiele, M. Marzorati, V. Eeckhaut, F. Van Immerseel, L. Vanhaecke, M. Campos, and M. J. B. V. R. Hesta. 2017. The response of canine faecal microbiota to increased dietary protein is influenced by body condition. Vet Res 13(1):374. (journal article) doi: 10.1186/s12917-017-1276-0

Xue, J., K. M. Ajuwon, and R. Fang. 2020. Mechanistic insight into the gut microbiome and its interaction with host immunity and inflammation. Animal Nutrition 6(4):421-428. doi: https://doi.org/10.1016/j.aninu.2020.05.007

Yamagata, K., M. Tagami, F. Takenaga, Y. Yamori, and S. Itoh. 2004. Hypoxia-induced changes in tight junction permeability of brain capillary endothelial cells are associated with IL-1beta and nitric oxide. Neurobiology of Disease 17(3):491-499. doi: https://doi.org/10.1016/j.nbd.2004.08.001

Yamashita, S., T. Furubayashi, M. Kataoka, T. Sakane, H. Sezaki, and H. Tokuda. 2000. Optimized conditions for prediction of intestinal drug permeability using Caco-2 cells. European Journal of Pharmaceutical Sciences 10(3):195-204. doi: https://doi.org/10.1016/S0928-0987(00)00076-2

Yamauchi, K.-E., T. Incharoen, and K. Yamauchi. 2010. The relationship between intestinal histology and function as shown by compensatory enlargement of remnant villi after midgut resection in chickens. The Anatomical Record 293(12):2071-2079. doi: https://doi.org/10.1002/ar.21268

Yan, H., and K. M. Ajuwon. 2017. Butyrate modifies intestinal barrier function in IPEC-J2 cells through a selective upregulation of tight junction proteins and activation of the Akt signaling pathway. PLoS One 12(6):e0179586. doi: 10.1371/journal.pone.0179586

Yan, Y., Z. Wang, J. Greenwald, K. S. D. Kothapalli, H. G. Park, R. Liu, E. Mendralla, P. Lawrence, X. Wang, and J. T. Brenna. 2017. BCFA suppresses LPS induced IL-8 mRNA expression in human intestinal epithelial cells. Prostaglandins, Leukotrienes and Essential Fatty Acids 116:27-31. doi: https://doi.org/10.1016/j.plefa.2016.12.001

Yan, Y., Z. Wang, D. Wang, P. Lawrence, X. Wang, K. S. D. Kothapalli, J. Greenwald, R. Liu, H. G. Park, and J. T. Brenna. 2018. BCFA-enriched vernix-monoacylglycerol reduces LPS-induced inflammatory markers in human enterocytes in vitro. Pediatric Research 83(4):874-879. doi: 10.1038/pr.2017.297

Yang, Q., X. Huang, S. Zhao, W. Sun, Z. Yan, P. Wang, S. Li, W. Huang, S. Zhang, L. Liu, and S. Gun. 2017. Structure and Function of the Fecal Microbiota in Diarrheic Neonatal Piglets. Front Microbiol 8(Original Research) doi: 10.3389/fmicb.2017.00502

Yang, Z., P. Lin, B. Chen, X. Zhang, W. Xiao, S. Wu, C. Huang, D. Feng, W. Zhang, and J. Zhang. 2021. Autophagy alleviates hypoxia-induced blood-brain barrier injury via regulation of CLDN5 (claudin 5). Autophagy 17(10):3048-3067. doi: 10.1080/15548627.2020.1851897

You, I., and M. J. Kim. 2021. Comparison of Gut Microbiota of 96 Healthy Dogs by Individual Traits: Breed, Age, and Body Condition Score. Animals (Basel) 11(8)doi: 10.3390/ani11082432

Yu, D. H., B. Kim, and J. Park. 2012. Pathophysiologic and immunologic changes in a canine endotoxemia over a period of 24 hours. J Vet Med Sci 74(5):537-544. doi: 10.1292/jvms.11-0321

Yu, L. C. H., A. N. Flynn, J. R. Turner, and A. G. Buret. 2005. SGLT-1-mediated glucose uptake protects intestinal epithelial cells against LPS-induced apoptosis and barrier defects: a novel cellular rescue mechanism? The FASEB Journal 19(13):1822-1835. doi: https://doi.org/10.1096/fj.05-4226com

Yu, P., Y. Li, G. Zhong, W. Li, B. Chen, and J. Zhang. 2021. Claudin-5 Affects Endothelial Autophagy in Response to Early Hypoxia. Frontiers in Physiology 12(Original Research) doi: 10.3389/fphys.2021.737474

Zafalon, R. V. A., L. W. Risolia, T. H. A. Vendramini, R. B. Ayres Rodrigues, V. Pedrinelli, F. A. Teixeira, M. F. Rentas, M. P. Perini, I. C. Alvarenga, and M. A. Brunetto. 2020. Nutritional inadequacies in commercial vegan foods for dogs and cats. PLOS ONE 15(1):e0227046. doi: 10.1371/journal.pone.0227046

Zhang, J., Y.-J. Huang, J. Y. Yoon, J. Kemmitt, C. Wright, K. Schneider, P. Sphabmixay, V. Hernandez-Gordillo, S. J. Holcomb, B. Bhushan, G. Rohatgi, K. Benton, D. Carpenter, J. C. Kester, G. Eng, D. T. Breault, O. Yilmaz, M. Taketani, C. A. Voigt, R. L. Carrier, D. L. Trumper, and L. G. Griffith. 2021a. Primary Human Colonic Mucosal Barrier Crosstalk with Super Oxygen-Sensitive Faecalibacterium prausnitzii in Continuous Culture. Med 2(1):74-98.e79. doi: https://doi.org/10.1016/j.medj.2020.07.001

Zhang, Q., C. M. Zmasek, and A. Godzik. 2010. Domain architecture evolution of pattern-recognition receptors. Immunogenetics 62(5):263-272. doi: 10.1007/s00251-010-0428-1

Zhang, X., C. Li, W. Cao, and Z. Zhang. 2021b. Alterations of Gastric Microbiota in Gastric Cancer and Precancerous Stages. Frontiers in Cellular and Infection Microbiology 11(Original Research) doi: 10.3389/fcimb.2021.559148

Zhao, Y., G. Qin, Z. Sun, D. Che, N. Bao, and X. Zhang. 2011. Effects of Soybean Agglutinin on Intestinal Barrier Permeability and Tight Junction Protein Expression in Weaned Piglets. International Journal of Molecular Sciences 12(12):8502-8512.

Zheng, D., T. Liwinski, and E. Elinav. 2020. Interaction between microbiota and immunity in health and disease. Cell Res 30(6):492-506. doi: 10.1038/s41422-020-0332-7

Zheng, L., C. J. Kelly, K. D. Battista, R. Schaefer, J. M. Lanis, E. E. Alexeev, R. X. Wang, J. C. Onyiah, D. J. Kominsky, and S. P. Colgan. 2017. Microbial-Derived Butyrate Promotes Epithelial Barrier Function through IL-10 Receptor–Dependent Repression of Claudin-2. The Journal of Immunology:ji1700105. doi: 10.4049/jimmunol.1700105

Zheng, L., C. J. Kelly, and S. P. Colgan. 2015. Physiologic hypoxia and oxygen homeostasis in the healthy intestine. A Review in the Theme: Cellular Responses to Hypoxia. Am J Physiol Cell Physiol 309(6):C350-C360. doi: 10.1152/ajpcell.00191.2015

Zhu, Y., X. Lin, H. Li, Y. Li, X. Shi, F. Zhao, X. Xu, C. Li, and G. Zhou. 2016. Intake of Meat Proteins Substantially Increased the Relative Abundance of Genus Lactobacillus in Rat Feces. PLoS One 11(4):e0152678. doi: 10.1371/journal.pone.0152678

Zimmerman, N. P., R. A. Vongsa, M. K. Wendt, and M. B. Dwinell. 2008. Chemokines and chemokine receptors in mucosal homeostasis at the intestinal epithelial barrier in

inflammatory bowel disease. Inflammatory bowel diseases 14(7):1000-1011. doi: 10.1002/ibd.20480

Zouboulis, C. C., M. K. Oeff, N. Hiroi, E. Makrantonaki, and S. R. Bornstein. 2021. Involvement of Pattern Recognition Receptors in the Direct Influence of Bacterial Components and Standard Antiacne Compounds on Human Sebaceous Gland Cells. Skin Pharmacology and Physiology 34(1):19-29. doi: 10.1159/000513259

Zucco, F., A. F. Batto, G. Bises, J. Chambaz, A. Chiusolo, R. Consalvo, H. Cross, G. Dal Negro, I. de Angelis, G. Fabre, F. Guillou, S. Hoffman, L. Laplanche, E. Morel, M. Pinçon-Raymond, P. Prieto, L. Turco, G. Ranaldi, M. Rousset, Y. Sambuy, M. L. Scarino, F. Torreilles, and A. Stammati. 2005. An inter-laboratory study to evaluate the effects of medium composition on the differentiation and barrier function of Caco-2 cell lines. Altern Lab Anim 33(6):603-618. doi: 10.1177/026119290503300618

APPENDICES

Appendix 1 *- Draft email for author contact to request data for the meta-analysis*

Dear [name]

I am a PhD student at AgResearch in New Zealand researching the effects of diet on the gut microbiota of dogs and as part of my research, I am conducting a metadata analysis on the available literature with a focus on how the macronutrient composition of diet affects the gut microbiota. In the interest of full transparency, I have a supervisor working for, and I am partly funded by Mars Petcare.

According to the criteria of my search terms, your publication [name, DOI] is one of the papers that can be used in my analysis. However, I am in need of further information to include this in my study. Would it be possible for you to provide me with [information required]?

Thanks in advance for any assistance you are able to provide.

Kind Regards,

Francis

Francis Phimister
PhD Student
Food & Bio-based Products
T +64 6 351 8618
Based at Grasslands Campus agresearch.co.nz

agresearch
āta mātai, mātai whetū

Appendix 2 - *PRISMA 2020 Checklist for the meta-analysis*

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Section 3.3.1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Section 3.1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Section 3.2
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Section 3.2
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Section 3.3.2.1
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Section 3.3.2
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Section 3.3.2 Table 3.4
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Section 3.3.2.1 Table 3.5
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Section 3.3.2.2.
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Table 3.5
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Table 3.5
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Section 3.3.2.2
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Section 3.3.4
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Section 3.3.2.2
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Section 3.3.4
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Section 3.3.2.2
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Sections 3.3.2.2.,

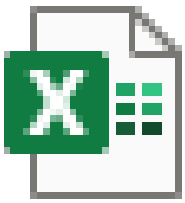
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Sections 3.3.4, 3.3.5
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	Sections 3.3.4, 3.3.5
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Section 3.5
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Sections 3.3.4, 3.3.5
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Figure 3.10
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Section 3.3.2.2.
Study characteristics	17	Cite each included study and present its characteristics.	Table 3.8
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Section 3.5
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Table 3.9, Figures 3.14, 3.20
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	N/A
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Sections 3.4.2, 3.4.3
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	N/A
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	Figures 3.15, 3.21
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	N/A – missing results not included
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Figures 3.16, 3.22
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Section 3.5
	23b	Discuss any limitations of the evidence included in the review.	Section 3.5
	23c	Discuss any limitations of the review processes used.	Section 3.5
	23d	Discuss implications of the results for practice, policy, and future research.	Section 3.5
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	N/A
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	N/A

	24c	Describe and explain any amendments to information provided at registration or in the protocol.	N/A
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	N/A
Competing interests	26	Declare any competing interests of review authors.	N/A
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	N/A

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71.doi: 10.1136/bmj.n71

For more information, visit:http://www.prisma-statement.org/

Appendix 3 - *Inclusion/Exclusion status of publications assessed for inclusion in the meta-analysis*



Appendix%20D%20
-%20Full%20list%20(

Appendix 4 - *Meta-data of publications included in the meta-analysis*

Appendix - Meta-Analysis Meta-

Appendix 5 - *Breeds of dogs included in the meta-analysis*

Any 'mongrel' or mixed breed is re-classed into mixed breed	***Paper ID***																
Breed Type	**ID 9**	**ID18**	**ID19**	**ID23**	**ID24**	**ID25**	**ID27**	**ID28**	**ID29**	**ID30**	**ID32**	**ID39**	**ID45**	**ID38**	**ID43**	**ID44**	**Total Number of Dogs**
American Pit Bull Terrier		1															1
American Staffordshire Terrier		1															1
Australian Shepherd Dog								1									1
Beagle							32	1	16	12	16		10	8	24		119
Bernese Mountain Dog								1									1
Border Collie		1				8		1									10
Bull Terrier					1												1
Dachshund					1												1
English Setter								1									1
Foxhound																46	46
Golden Retriever								4									4
Harrier Hound	15																15
Hungarian Vizsla					1												1
Labrador Retriever									16		16						32
Mastiff					2												2
Mixed Breed		4			3			4				7					18
Newfoundland								1									1
Not Specified			27														27
Pitbull Terrier		1		31													32

Appendix 6 - *Import permit for the cIEC (for 2017-2018)*

Ministry for Primary Industries
Manatō Ahu Matua

PERMIT TO IMPORT BIOLOGICAL PRODUCTS OF ANIMAL ORIGIN

This permit is issued under The Biosecurity Act, 1993. Any queries, please contact Animal Imports & Exports Group, MPI, PO Box 2526, Wellington. (Phone 64 4 8940100, Facsimile 64 4 8940733)

Authorising Officer : Katherine Hall on 30 Nov 2017

for Director General, Ministry for Primary Industries, acting under delegated authority

Permit for : AgResearch Limited, Grasslands
Tennent Drive
Palmerston North
New Zealand
Attention : Allison McCarthy
allison.mccarthy@agresearch.co.nz

Permit No : 2017066881
Replaces Permit No : 2017065320
Expiry Date : 30 Nov 2018
No of Consignments : Multiple
Import Purpose : Research

End Users : I 517 Rachel Anderson A McCarthy 2.5.18

Client Ref's : 1004462

Shipped From : All Countries

Description of Items	Supplier	Quantity	Measure
Mammalian primary cell lines from rabbit, cow, sheep, goat, horse, pig, rat, mouse, cat and dog		Unlimited	Units

Import Health Standard
CELLCULIC.ALL, 31 Jan 2010, CEL - Cell Cultures from All Countries

Post Entry Type : Transitional Fac

Standard Reference : 154.03.02 (Facilities for Microorganisms and Cell Cultures: 2007a)

Transitional Facility : AgResearch - Grasslands (P430) **Operator :** Tom Richardson
Alan Johns Building & Ulyatt-Reid Facili
Tennent Drive
Palmerston North

Ref : 2014

Growing and Protecting New Zealand

Permit No: 2017066881 Page 1 of 3

Appendix 7 - *Import permit for the cIEC (for 2018-2019)*

Ministry for Primary Industries
Manatū Ahu Matua

PERMIT TO IMPORT BIOLOGICAL PRODUCTS OF ANIMAL ORIGIN

This permit is issued under The Biosecurity Act, 1993. Any queries please contact Animal Imports & Exports Group, MPI, PO Box 2526, Wellington, (Phone 64 4 8940100, Freemail 64 4 8940733)

Authorising Officer : Katherine Hall on 13 Nov 2018

for Director General, Ministry for Primary Industries, acting under delegated authority

Permit for : AgResearch Limited, Grasslands
Tennent Drive
Palmerston North
New Zealand
Attention : Allison McCarthy
allison.mccarthy@agresearch.co.nz

Permit No : 2018070612
Replaces Permit No : 2017066881
Expiry Date : 13 Nov 2019
No of Consignments : Multiple
Import Purpose : Research

End Users : I 569 Rachel Anderson ARMcCarthy 2.05.19

Shipped From : All Countries

Description of Items	Supplier	Quantity	Measure
Mammalian primary cell lines from rabbit, cow, sheep, goat, horse, pig, rat, mouse, cat and dog		Unlimited	Units

Import Health Standard
CELLCULIC.ALL, 31 Jan 2010, CbL - Cell Cultures from All Countries

Post Entry Type : Transitional Fac

Standard Reference : 151.03.02 (Facilities for Microorganisms and Cell Cultures; 2007a)

Transitional Facility : AgResearch - Grasslands
Alan Johns Building and
Ulyatt-Reid Faci
Palmerston North

Operator : Tom Richardson

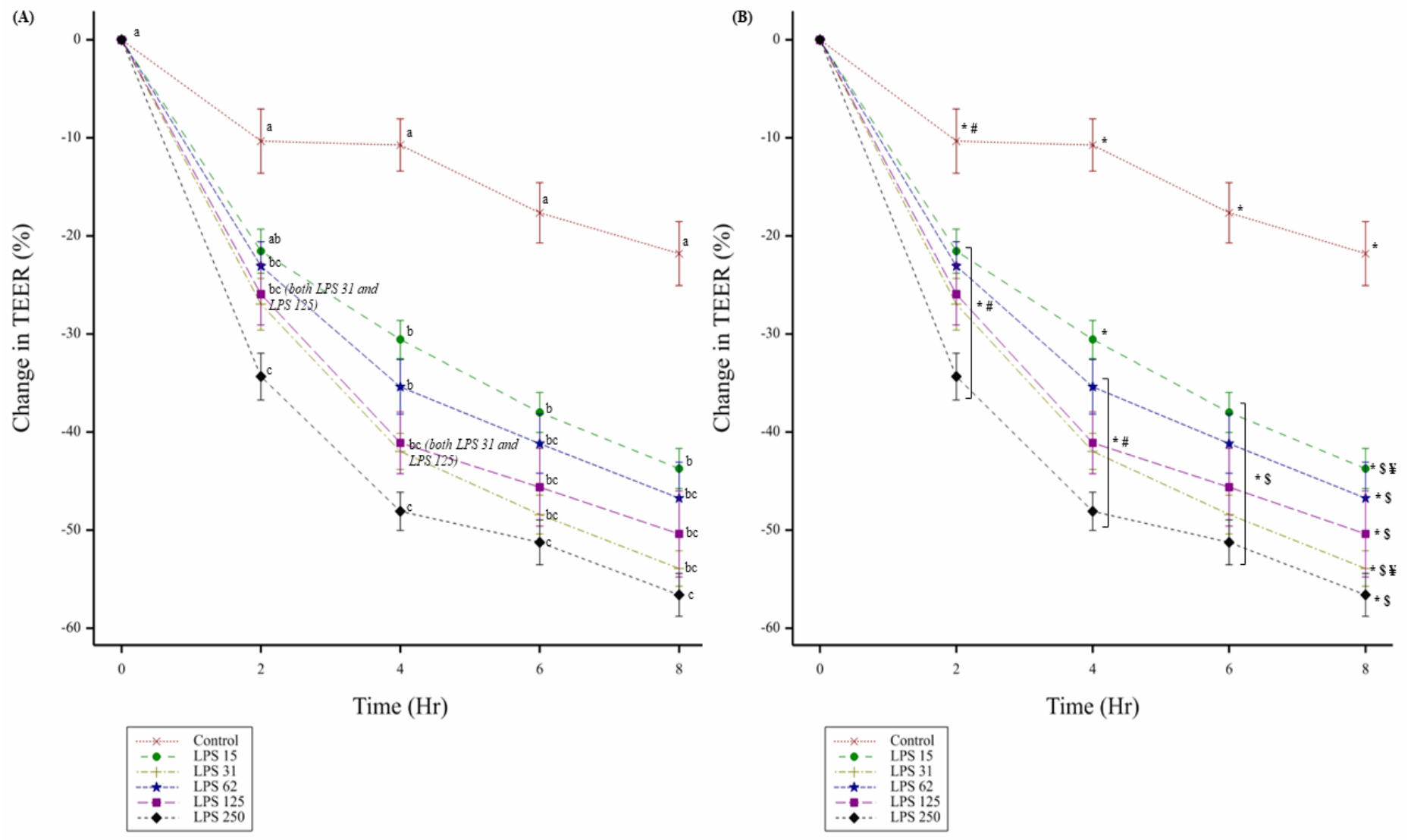
Ref : 2014

Special Conditions : You must comply with the import health standard(s) listed in this permit. For further information contact MPI at the top of the permit or via the MPI website;

Growing and Protecting New Zealand

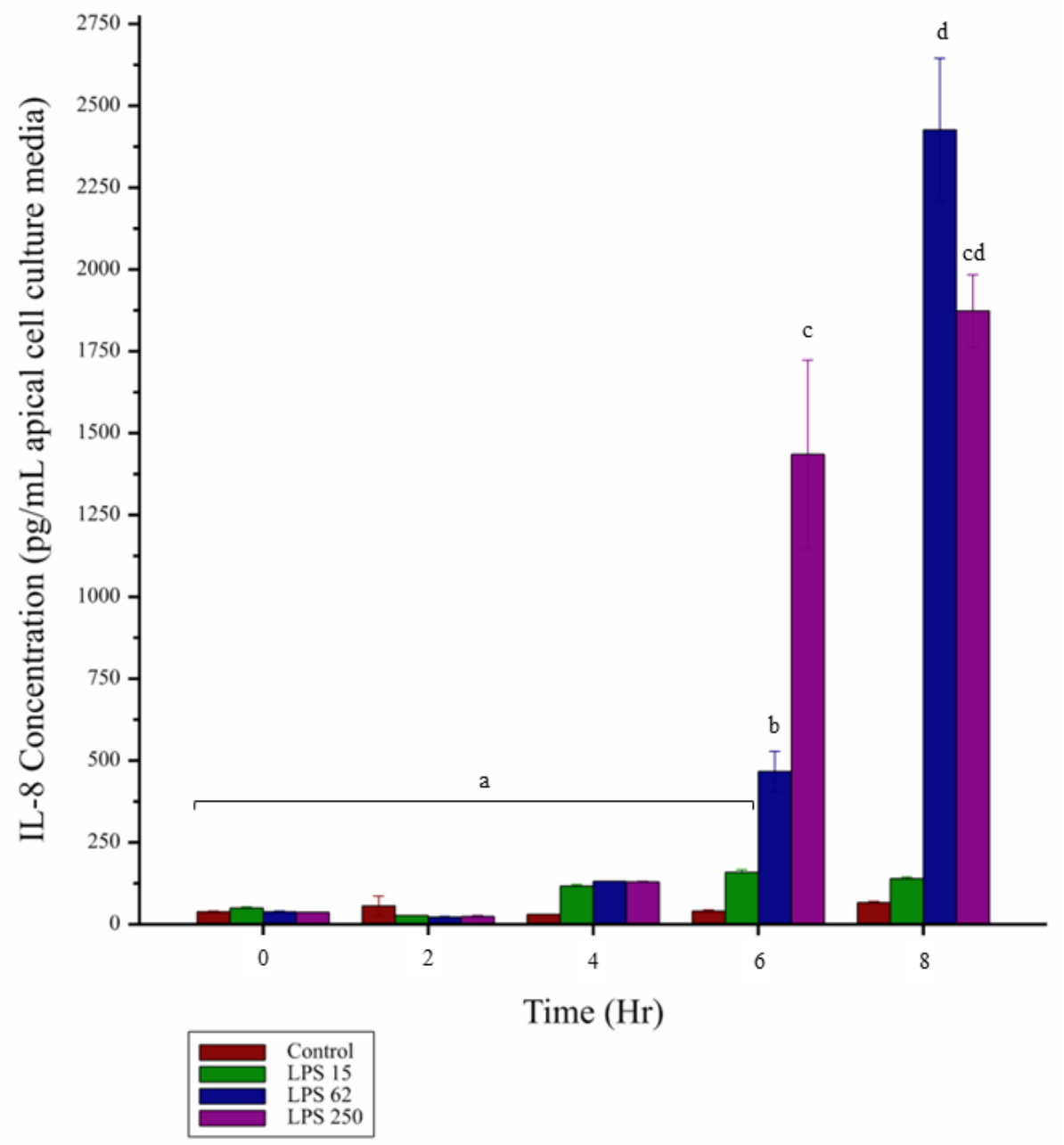
Permit No: 2018070612 Page 1 of 2

Appendix 8 – *Initial LPS Dose Refinement*



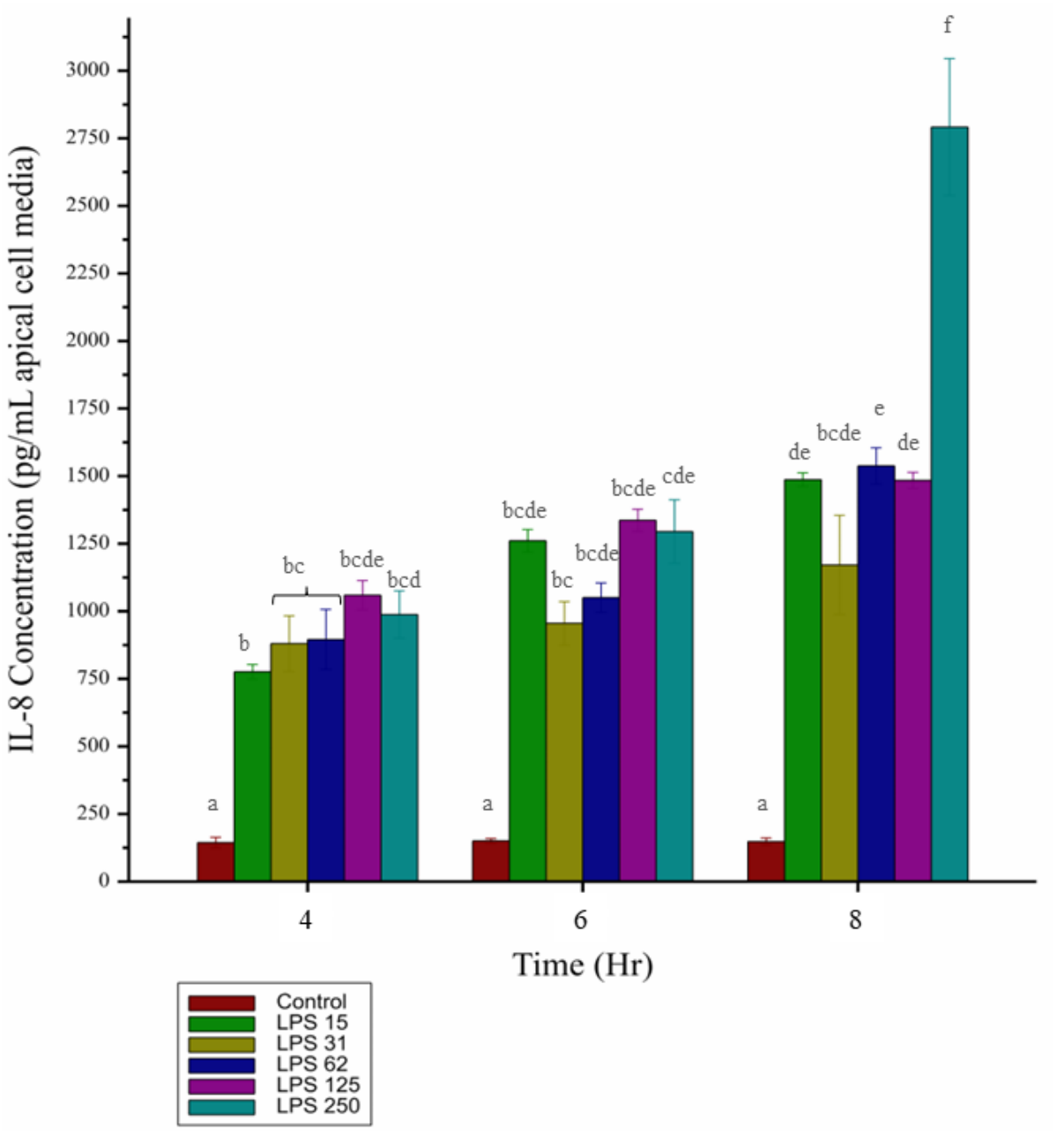
*Mean (± SEM) change in TEERfrom canine intestinal epithelial cells challenged with increasing doses of lipopolysaccharides (LPS). Change in TEER defined as a percentage change compared to the initial, time 0 value for the same treatment. The cIEC were challenged with either 15.125, 31.25, 62.5, 125 or 250 μg/mL of LPS (LPS 15, LPS 31, LPS 62, LPS 125 and LPS 250, respectively), and were run in parallel with untreated cIEC (Control). N = 6 for each treatment. In (A) the impact of treatments at defined timepoints is illustrated. Treatments at each timepoint that do not share a common letter are significantly ($p < 0.05$) different in the change in TEER. In (B), the effects of the individual treatments over time are presented, i.e., the change in TEER in the same treatment. In this, significant ($p < 0.05$) differences to the TEER at Time 0 are indicated by * and significant ($p < 0.05$) differences to the previous timepoint in the same treatment are indicated by #. For the 6- and 8-hour measurements, a significant ($p < 0.05$) difference to the change in TEER at 2 hours for the same treatment is represented by $. Finally, in the 8-hour measurements, a significant difference to the change in TEER at the 4-hour measurement for the same treatment is represented by ¥. Timepoints denote the time passed since the challenge compounds were applied to the cIEC.*

Appendix 9 *– Initial IL-8 ELISA*



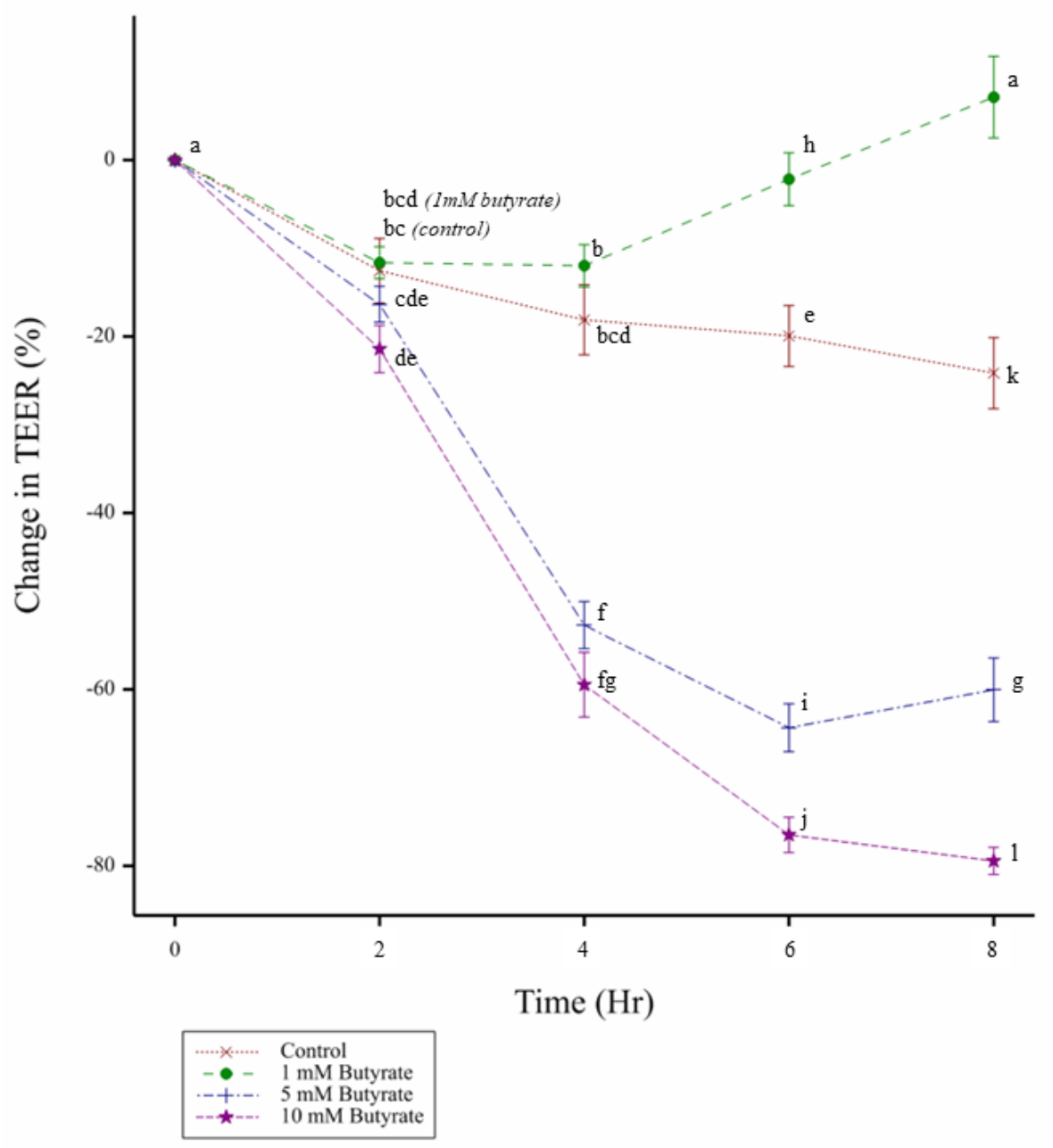
Initial test of interleukin (IL)-8 ELISA with lipopolysaccharide (LPS)-challenged canine intestinal epithelial cells (cIECs). The cIEC were challenged with either 15, 62.5 or 250 µg/mL of LPS (LPS 15, LPS 62, and LPS 250, respectively), and were run in parallel with untreated cIEC (CTRL). N = 2 for each treatment. Treatments within the same timepoint that do not share a common letter are significantly ($p < 0.05$) different in IL-8 concentration. Timepoints denote the time passed since the challenge compounds were applied to the cIEC.

Appendix 10 *– IL-8 ELISA of LPS-stimulated cIEC collected at 4, 6 and 8 hours post-treatment*



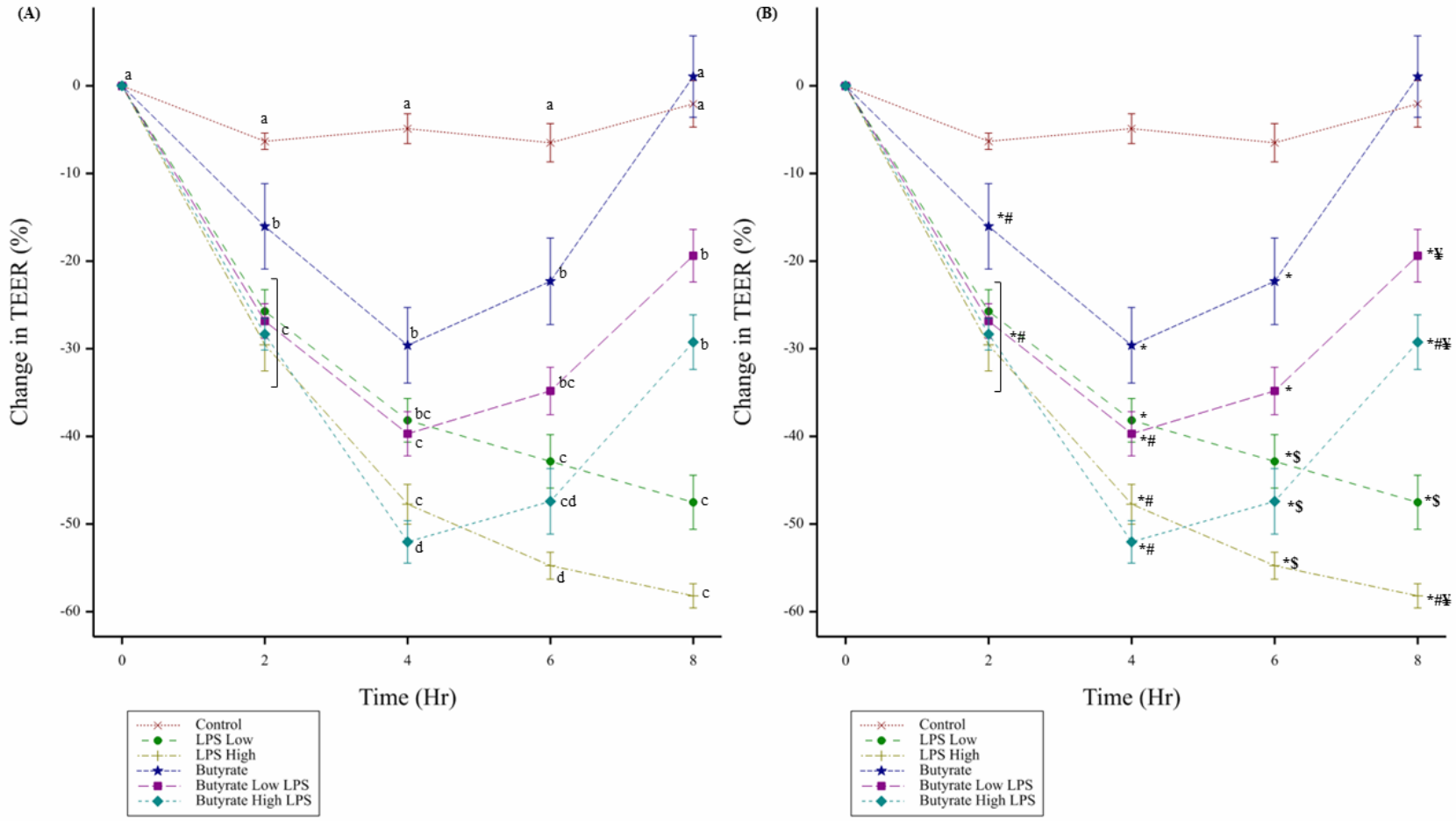
Mean (± SEM) interleukin (IL)-8 concentrations in pg/mL from canine intestinal epithelial cells (cIEC) challenged with increasing doses of lipopolysaccharides (LPS). The cIEC were challenged with either 15.125, 31.25, 62.5, 125 or 250 μg/mL of LPS (LPS 15, LPS 31, LPS 62, LPS 125 and LPS 250, respectively), and were run in parallel with untreated cIEC (CTRL). N = 6 for each treatment. Treatments within the same timepoint that do not share a common letter are significantly ($p < 0.05$) different in IL-8 concentration. Timepoints denote the time passed since the challenge compounds were applied to the cIEC.

Appendix 11 *– Change in TEER analysis to determine concentration of butyrate used to stimulate cIEC*



Mean (± SEM) change in TEER from canine Intestinal Epithelial cells (cIECs) challenged with different treatments. Change in TEER defined as a percentage change compared to the initial, time 0 value for the same treatment. Treatments were 1 mM, 5 mM or 10 mM of sodium butyrate (1 mM Butyrate, 5 mM Butyrate and 10 mM Butyrate, respectively), and untreated cIECs (Control). N = 6 for each treatment. Timepoints that do not share a common letter denotes a significant ($p < 0.05$) difference in the change in TEER. Timepoints denote the time passed since the challenge compounds were applied to the cIEC.

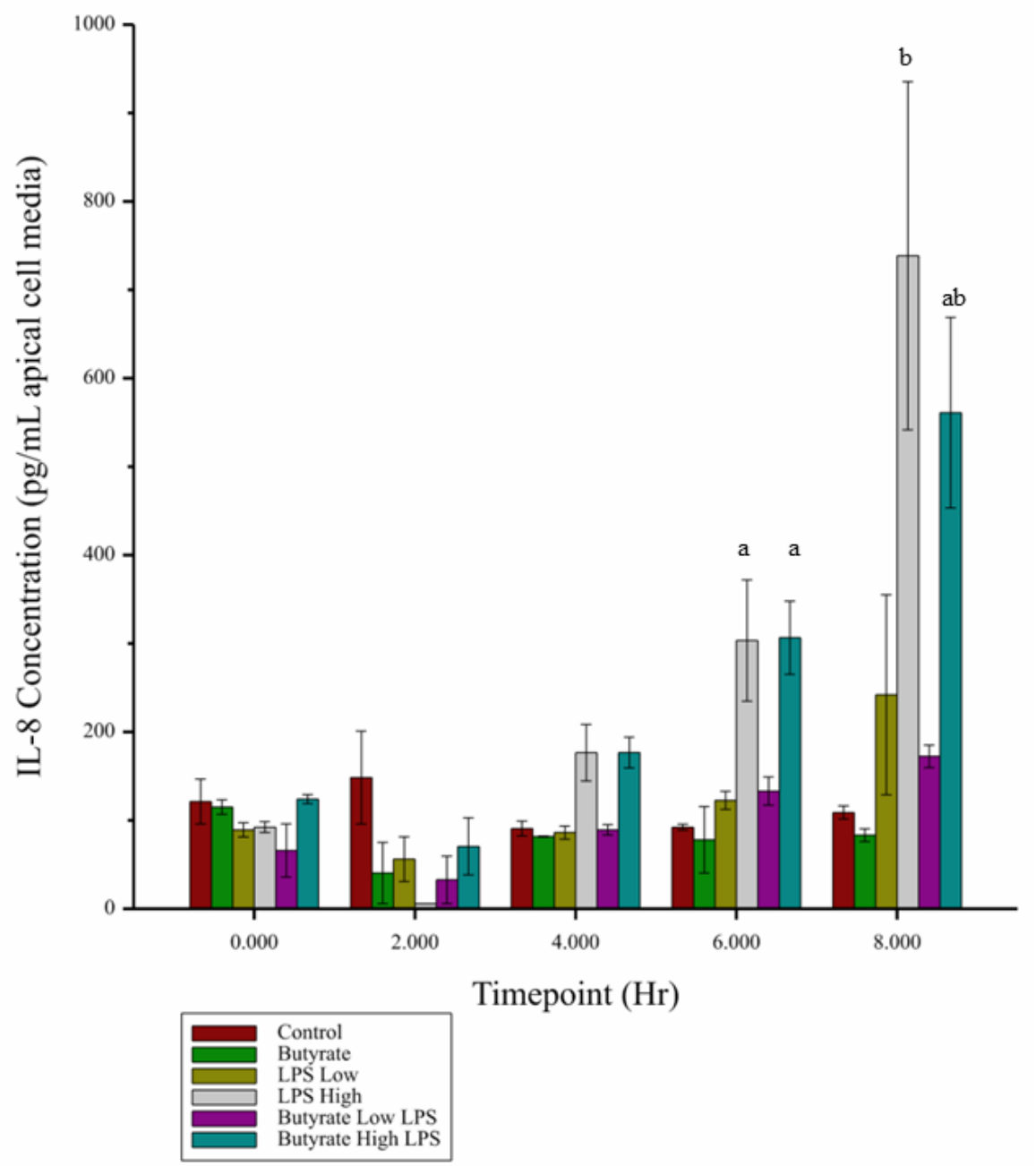
Appendix 12 – *Change in TEER analysis to determine concentration of butyrate used to stimulate cIEC*



Mean (± SEM) change in TEER from canine Intestinal Epithelial cells (cIECs) challenged with different treatments. Change in TEER defined as a percentage change compared to the initial, time 0 value for the same treatment. Treatments were 1 mM of sodium butyrate (Butyrate), 15.125 or 250 µg/mL lipopolysaccharides (LPS Low and LPS High), both 1 mM sodium butyrate and either 15.125 or 250 µg/mL LPS (Butyrate Low LPS and Butyrate High LPS, respectively), and untreated cIECs (Control). N = 6

*for each treatment. In (A) the impact of treatments at defined timepoints is illustrated. Treatments at each timepoint that do not share a common letter are significantly ($p < 0.05$) different in the change in TEER. In (B), the effects of the individual treatments over time are presented, i.e., the change in TEER in the same treatment. In this, significant ($p < 0.05$) differences to the TEER at Time 0 are indicated by * and significant ($p < 0.05$) differences to the previous timepoint in the same treatment are indicated by #. For the 6- and 8-hour measurements, a significant ($p < 0.05$) difference to the change in TEER at 2 hours for the same treatment is represented by $. Finally, in the 8-hour measurements, a significant difference to the change in TEER at the 4-hour measurement for the same treatment is represented by ¥. Timepoints denote the time passed since the challenge compounds were applied to the cIEC.*

Appendix 13 *– IL-8 ELISA of cIEC stimulated with LPS, butyrate, or a combination of the two*



Mean (± SEM) interleukin-8 concentrations in pg/mL from canine intestinal epithelial cells challenged with different treatments. Treatments were 1 mM of sodium butyrate (Butyrate), 15.125 or 250 µg/mL lipopolysaccharides (LPS Low and LPS High, respectively), and 1 mM sodium butyrate with either 15.125 or 250 µg/mL LPS (Butyrate Low LPS and Butyrate High LPS, respectively. Challenged cIEC were ran in parallel with untreated cIECs (CTRL). N = 3 for each treatment other than LPS High at timepoint 2, where n = 2. Treatments that do not share common letters denote a significant ($p < 0.05$) difference. All treatments marked by a letter are significantly ($p < 0.05$) different to treatments not marked with a letter. Timepoints denote the time passed since the challenge compounds were applied to the cIEC.