Age-related changes in intestinal permeability and absorptive capacity in the domestic cat (*Felis catus*)

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Abstract

Ageing reduces fat and protein digestibility in the domestic cat which might have implications for their health and wellbeing. Reasons for the reduction in digestibility are unknown, but could be related to changes in the structure and/or function of the gastrointestinal tract (GIT), including increased permeability. Increased intestinal permeability may affect nutrient absorption, promote inflammatory responses, and allow pathogens to be absorbed. Currently, the literature that describes the relationship between intestinal permeability and age is conflicting, with limited studies undertaken in the domestic cat. This thesis compiled the first literature review of intestinal permeability and absorptive capacity, focusing on the lack of meaningful information and a standardised methodology regarding these GIT parameters in cats. It is hypothesised that intestinal permeability is negatively affected by age, and that interventions, such as probiotics, can reverse this.

In Chapter 2, the differential sugar absorption test (SAT), using lactulose, rhamnose, xylose, and 3-O-methylglucose (3-OMG) as markers of intestinal permeability and absorptive capacity, was developed using liquid chromatography-mass spectrometry (LC-MS).

By using this method in Chapter 3, a group of young cats were compared to a group of senior cats. The results supported the hypothesis that intestinal permeability increased in senior cats. There was no age-related change in absorptive capacity, but there was a trend where males tended to have higher absorptive capacity values than females.

To further understand GIT health in ageing cats and test possible interventions to strengthen the weakened intestinal barrier, probiotic yeast *Saccharomyces cerevisiae* var. *boulardii* (*S. boulardii*) was supplemented to midlife – senior aged cats in a crossover trial in Chapter 4. *S. boulardii* supplementation did not affect the GIT health parameters measured. Secondary analysis showed that with increasing age, the cats faced reduced fat digestion and increased intestinal permeability. This study highlighted the variation in data sets obtained in senior cats which should be accounted for in future research. Additionally, future research should focus on the mechanism by which altered intestinal permeability decreases nutrient digestibility to promote better nutrition for senior cats.

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"It's a dangerous business, going out your door. You step onto the road, and if you don't keep your feet, there's no knowing where you might be swept off to."

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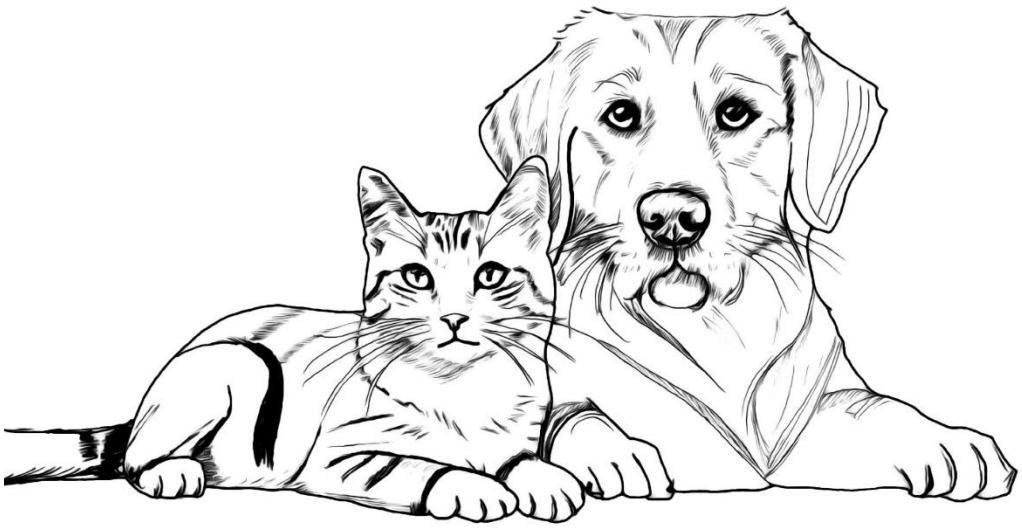
3-O-methylglucose	3-OMG
^{51}Chromium-labelled ethylenediamine tetra-acetic acid	^{51}Cr-EDTA
Alpha-1-antitrypsin	AA
Acetonitrile	ACN
Adenosine triphosphate	ATP
Advanced glycation end products	AGE
Amplicon sequence variant	ASV
Analysis of variance	ANOVA
Apparent total tract digestibility	ATTD
Association of American Feed Control Officials	AAFCO
Baseline	BL
Body weight	BW
Colony forming units	CFU
Chenodeoxycholic acid	CDCA
Chronic inflammatory enteropathy	CIE
Chronic kidney disease	CKD
Coefficient score	CS
Complete blood count/ haematology	CBC
Crude fat	CFat
Crude fibre	CF
Crude protein	CP
Cumulative Link Mixed Model	CLMM
Degrees of freedom	DF
Deoxyribonucleic acid	DNA
Dry matter	DM
Dry matter intake	DMI
Electrospray ionisation	ESI
Enzymatic assay	ENL
Enzyme-linked immunosorbent assay	ELISA
Red blood cell	RBC
Extraction recovery	ER
Extraction recovery percentage	ER %
False discovery rate	FDR
Female entire	FE
Female neutered	FN
Fluorescein isothiocyanate	FITC
Formic acid	FA
Fructooligosaccharide	FOS
F-statistic	F
Galactooligosaccharide	GOS
Gas chromatography-mass spectrometry	GC-MS

Gas-liquid chromatography	GLC
Gastrointestinal tract	GIT
Glucagon-like peptide	GLP
Gross energy	GE
Haematocrit	HCT
Haemoglobin	Hb
High-performance liquid chromatography	HPLC
Hydrophilic Interaction Liquid Chromatography	HILIC
Hydroxypropylmethylcellulose	HPMC
Ileal lipid binding protein	ILBP
Incremental area under the curve	I-AUC
Indole-3-propionate	IPA
Inflammatory bowel disease	IBD
Interferon	IFN
International Accreditation New Zealand	IANZ
Interquartile range	IQR
Intestinal fatty acid-binding protein	IFABP
Junctional adhesion molecule-A	JAMA
Lactulose: Mannitol	LM
Lactulose: Rhamnose ; intestinal permeability	LR
Limit of detection	LOD
Limit of quantitation	LOQ
Linear discriminant analysis	LDA
Linear discriminant analysis effect size	LEfSe
Lipopolysaccharide	LPS
Liquid chromatography-mass spectrometry	LC-MS
Lithocholic acid	LCA
Male neutered	MN
Mannan-oligosaccharides	MOS
Massey University Animal Ethics Committee	MUAEC
Mean corpuscular haemoglobin	MCH
Mean corpuscular haemoglobin concentration	MCHC
Mean corpuscular volume	MCV
Mean square	MS
Methanol	MeOH
Neutered: entire	N : E
Nonsteroidal anti-inflammatory drugs	NSAID
Normalised collision energy	NCE
Operational taxonomic unit	OTU
Parallel reaction monitoring	PRM
Partial least squares	PLS
Partial least squares discriminant analysis	PLS-DA
Placebo; control treatment	CTRL
Polyethylene glycol	PEG

Polymerase chain reaction	PCR
Polyunsaturated fatty acid	PUFA
Pooled quality control	PQC
Principal component analysis	PCA
Quantitative Insights Into Microbial Ecology	QIIME
Relative standard deviation	RSD
Reticulocyte haemoglobin equivalent	Retic Hb
Saccharomyces cerevisiae var. boulardii	SB
Senescence-accelerated prone mouse	SAMP
Short chain fatty acid	SCFA
Standard deviation	SD
Sugar absorption test	SAT
Sum of squares	SS
Technical quality control	TQC
Tight junction	TJ
Total dietary fibre	TDF
Total sum scaling	TSS
Transepithelial electrical resistance	TEER
Trimethylamine N-oxide	TMAO
Tumor necrosis factor	TNF
White blood cell	WBC
Xylose to 3-O-methylglucose ratio; absorptive capacity	XG
Zonula occluden	ZO

Chapter 1

Literature Review



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A published version of this paper is available in Appendix 1.

Chapter 1: Literature Review

1.1 Introduction

Current research on the effect that age has on the gastrointestinal tract (GIT) of companion animals, namely domestic cats and dogs, is currently lacking, contributing to a dearth of knowledge in this area. As cats and dogs live longer due to improved diet and veterinary care, there is a growing desire among owners to enhance the quality of life. Older cats can experience a decrease in total tract digestibility of fat and protein, whereas in dogs, age does not have a consistent impact on nutrient digestibility. The underlying reasons for these age-related changes are unclear, and may involve a decline in the integrity and function of the GIT, decreased immune responses such as inflammageing, both of which may be underpinned by changes in the GIT microbiome, and its metabolite profile. This review compiles the documented effects that age has on the GIT in cats and dogs, with a specific focus on GIT function, namely intestinal permeability and mucosal absorptive capacity. These are characteristics of the intestinal barrier that affect its ability to selectively absorb molecules whilst restricting pathogens and toxins and facilitating nutrient absorption. An increase in permeability and a decrease in absorptive capacity are both indicators of intestinal damage and impaired function. The influence of age on these processes remains unclear in cats, although it has been previously studied in dogs, revealing no discernible age-related differences. Additionally, ageing animals undergo immunosenescence, which can lead to inflammageing, a state of chronic inflammation that may contribute to gastrointestinal inflammation, exacerbating increased intestinal permeability. Furthermore, decreased microbial diversity and change of bacterial populations that come with age may also change the nutrient requirements and digestive capabilities of companion animals. In summary, the impacts of ageing on intestinal function are not well understood and therefore warrant more research in the future.

Pet cats and dogs are an integral part of the family. Like humans, their lifespans are increasing due to better diet and veterinary care (HealthforAnimals, 2022). Currently, it is estimated that 20 – 40 % of pets are classed as being late midlife (J. Bellows *et al*., 2016; Bellows *et al*., 2015). While the classification of age and associated category in the cat is relatively simple due to a relatively consistent body weight (BW) across different breeds, for dogs the classification is more complicated due to the effects of vastly different body sizes and life expectancy associated with different breeds. For the cat, lifespan is predicted largely by lean body mass; lean tissue and overall BW starts to decline after 12 years of age, lean tissue weight dropping to approximately one-third of the mass during adulthood (Perez-Camargo, 2003). Similarly, in dogs, ageing is also associated with a reduction of lean body mass (Fahey *et al*., 2008; Harper, 1998). Recently, Salt *et al*. (2023) categorised dog age based on the development of disease (Table 1). For example, small-breed

dogs could be classified as senior, two years later than large-breed dogs (Table 1.1). In this data set which consisted of over 4.4 million dogs and almost 2.0 million cats, the incidence of diseases increased from the senior life stage onwards, with a marked clustering of disease in the super-senior life stage (Salt *et al*., 2023). This thesis will utilise these age classifications unless otherwise stated.

Table 1.1. Age classifications for pet cats and dogs as summarised by (Salt *et al*., 2023).

Cat		**Dog**		
Youth	1-4 years	**Toy**	**Youth**	1-6 years
			Midlife	7-11 years
			Senior	12-13 years
			Super-Senior	≥ 14 years
Early Midlife	5-9 years	**Small**	**Youth**	1-6 years
			Midlife	7-11 years
			Senior	12-13 years
			Super-Senior	≥ 14 years
Late Midlife	10-11 years	**Medium**	**Youth**	1-5 years
			Midlife	6-9 years
			Senior	10-13 years
			Super-Senior	≥ 14 years
Senior	12-13 years	**Large**	**Youth**	1-5 years
			Midlife	6-9 years
			Senior	10-11 years
			Super-Senior	≥ 12 years
Super-Senior	≥ 14 years			

The consequences of ageing are difficult to establish and are complex for pet cats and dogs (McKenzie, 2022). However, observations in the cat suggest that after approximately 7 years of age, their ability to digest nutrients decreases (Fahey *et al*., 2008; Harper, 1998), suggesting a change in overall GIT function. However, while the role of ageing on the physiology of molecular, cellular, and tissue processes in companion animals has been reviewed relatively recently (McKenzie, 2022), there is little information relating to the effects of ageing on markers of GIT function in the cat and dog. Therefore, the aim of this review is to understand the effects of ageing on markers of GIT function in the cat and dog.

1.2 Gastrointestinal function

A healthy GIT is believed to be vital to overall health and wellbeing (Farré *et al*., 2020), primarily through the role it plays in the digestion and absorption of nutrients, aided in part by its population of microbes which provide a range of functions including fermentation of dietary ingredients. Additionally, the GIT is pivotal for the immune response of the host. The intestinal barrier is also a physical barrier between the host and its external environment.

1.2.1 Macronutrient digestibility

The digestibility of nutrients is affected by many parameters including, but not limited to, fibre content and type (Donadelli & Aldrich, 2020; Fekete *et al*., 2004; Harper & Siever-Kelly, 1997; Marx *et al*., 2022; Prola *et al*., 2010; Silvio *et al*., 2000), dietary format (Bermingham *et al*., 2013c; Kim *et al*., 2021; Tanprasertsuk *et al*., 2021), and heat processing (de-Oliveira *et al*., 2012; Hendriks *et al*., 1999b). Combined, this has made understanding the impacts of age on nutrient digestibility difficult to interpret due to different experimental designs incorporating different dietary regimes.

However, observations from colony cats indicate the percentage of cats with fat and protein digestibility below 80 % increases with age, starting in cats from 8 years of age and becomes more apparent in cats over 12 years of age (fat digestibility) and 14 years of age (protein digestibility) (Patil & Cupp, 2010). Research indicates that fat digestibility (Anantharaman-Barr *et al*., 1991; Harper, 1998; Peachey *et al*., 1999; Perez-Camargo, 2003; Teshima *et al*., 2010) and to a lesser extent protein digestibility (Bermingham *et al*., 2013b; Harper, 1998; Perez-Camargo, 2003; Teshima *et al*., 2010) decrease in senior and super-senior cats. Diet composition and processing may be a causal factor in sustaining high macronutrient digestibility in ageing cats. Quality and composition of ingredients seemed to have more of an effect on digestive efficiency than the proportions of macronutrients from said ingredients (Schauf *et al*., 2021). Animal-based proteins were better digested by cats of all ages than plant-based proteins, regardless of fat and fibre levels (Schauf *et al*., 2021). Raw or cooked beef diets resulted in higher apparent total tract digestibility (ATTD) of crude protein (CP), crude fat (CFat), and gross energy (GE) in dogs and cats when compared to an extruded kibble (Bermingham *et al*., 2017; Butowski *et al*., 2019; Crissey *et al*., 1997; Hamper *et al*., 2016; Kerr *et al*., 2012). Although there is a dearth of research in ageing cats with nutrient malabsorption, a complete, balanced diet with antioxidant, prebiotic, and polyunsaturated fatty acid (PUFA) supplementation was able to reduce BW loss and improve longevity in cats over the age of 7 (Cupp *et al*., 2006).

While data in the cat is relatively consistent, the age-related decline in macronutrient digestibility in the dog is more contradictory. For example, while many studies have observed no significant age-associated decline in nutrient digestibility in the dog (Harper, 1998; Larsen & Farcas, 2014; Schauf *et al*., 2021; Sheffy

et al., 1985; Taylor *et al*., 1995), other studies do indicate age-associated affects. For example, Schauf *et al*., 2021 found that while the digestibility of CFat was unaffected by age, midlife and senior dogs had increased digestibility of CP, and total dietary fibre (TDF) on a 2.1 % crude fibre (CF), 6.3 % TDF diet in comparison to adult dogs, but this effect was not observed when the dogs were fed diets with higher amounts of CF (19 % dry matter; DM) and TDF (29 % DM) (Schauf *et al*., 2021). Maria *et al*. (2017), however, observed reduced digestibility for CFat and CP in late midlife when supplemented with a fermentable as opposed to non-fermentable fibre (Maria *et al*., 2017). Results from Swanson *et al*. (2004), indicated no effect of age on protein and fat digestibility between 11-year old beagle dogs and puppies once they reached 1 year of age (Swanson *et al*., 2004). A more recent study has suggested that moisture content may also play a role in nutrient digestibility in ageing dogs, although this may be impacted by breed. Kim *et al*. (2021) observed no age-related decline in digestibility of macronutrients and amino acids, but nitrogen-free extract digestibility was higher in senior dogs fed dry food compared to adults on the same diet and CFat was also higher in senior small-breed dogs than adults on both wet and dry diets (Kim *et al*., 2021).

1.2.2 Micronutrient digestibility

There are relatively few studies that have investigated the impacts of age on mineral and vitamin digestibility in the cat and dog. There was no significant effect of age on mineral absorption in the cat (Teshima *et al*., 2010), however Schauf *et al*. (2021), demonstrated decreased calcium digestibility in early midlife to senior cats aged 7 – 13 years, compared to young cats aged 1 – 5 years. Contrastingly, in dogs, age was seen to increase calcium and phosphorous digestibility in lower fibre diets (Schauf *et al*., 2021). The impacts of age on vitamin digestibility are not well understood in the cat, however, reduced fat digestibility has been associated with lowered vitamin E concentrations in serum (Patil & Cupp, 2010). Vitamin E is a fat-soluble vitamin, and given that vitamins A, D, and K are also fat-soluble, it is possible that the digestibility of these vitamins will also decrease in ageing cats. In dogs, there is a paucity of studies outlining the impacts of age on vitamin digestibility.

1.2.3 Intestinal permeability

The intestinal barrier is composed of three main non-immune defences that protect the internal environment from external substances and microorganisms that could provoke illness or other undesirable health effects (Cummings *et al*., 2004). Firstly, the gastrointestinal microbiome on the mucosal layer is responsible for stimulating the immune system and preventing the growth and passage of pathogenic microorganisms. Secondly, the chemical barrier which uses digestive secretions, and antimicrobial peptides, brought in by the enterocytes and Paneth cells to prevent intrusion by bacteria from the external lumen (Cummings *et al*., 2004), and thirdly the physical barrier itself is composed of the continually proliferating epithelial layer that uses its high stem cell turnover rate to create enterocytes, goblet cells, and Paneth cells

that further contribute to protecting the epithelium from foreign substances and microorganisms (Cummings *et al*., 2004).

Permeability of the intestinal wall is a characteristic that allows for the selective absorption of nutrients while protecting against the uptake of pathogenic bacteria and other toxins (Bischoff *et al*., 2014). Passage through the physical barrier is regulated by tight junctions (TJ), and is further governed by the proteins found within these complexes. Occludin, claudin, and zonula occluden (ZO) proteins are essential to TJ stability and maintaining transport of water and electrolyte ions (Bischoff *et al*., 2014). Members of the claudin family, however, can have drastically different effects on the intestinal barrier. Claudin-1 maintains the integrity of TJ, whereas claudin-2 is responsible for forming additional channels leading to an increase in the number of molecules taken up through the barrier (Suzuki *et al*., 2011; Van Itallie *et al*., 2008). Molecules are taken up via paracellular or transcellular routes, by means of passive or active diffusion. It's important to highlight that these findings are derived from various animal models, and assume that these molecular mechanisms are widely preserved throughout the mammalian kingdom.

1.2.4 Absorptive capacity

Mucosal absorptive capacity, which characterises carrier-mediated active transport, can be used to assess the ability of the intestinal mucosa to absorb dietary carbohydrates and other sugars. It can be split into two parts, similarly to intestinal permeability; active and passive absorption (Wijtten *et al*., 2011). Active transport uses adenosine triphosphate (ATP) and specific transporters, like the SGLT1 transporter for glucose, as well as a passive transcellular route (Drozdowski & Thomson, 2006).

1.2.4.1 Transport pathways across the epithelium

Tight junction proteins regulate paracellular transport between epithelial cells for larger hydrophilic compounds and water, while transporters on the surface of the cell regulate transcellular transport for smaller hydrophilic or lipophilic compounds (Farré *et al*., 2020). Whereas paracellular transport is a means of passive diffusion, transcellular transport can involve both passive and active mechanisms. There are two different paracellular transport routes, named the “pore pathway” and the “leak pathway”. The pore pathway is regulated by claudin proteins that can transport high volumes of uncharged, small molecules. The leak pathway, regulated by occludin and ZO proteins, has a much greater diameter than the pore pathway and is non-charge selective, but operates at a lower capacity than the pore pathway. These pathways can become unregulated during epithelial damage and increase in capacity, with no sensitivity to size or charge, leading to increased transport of large, and potentially pathogenic or toxic, molecules across the epithelial barrier (Chanez-Paredes *et al*., 2021; Farré *et al*., 2020). Active transcellular transport is required for sugars, amino acids, and vitamins, and even larger molecules are transported across the epithelium in vesicles (Farré *et al*., 2020).

1.2.4.2 Methods to measure intestinal permeability and absorptive capacity

Intestinal permeability can be measured using a variety of methodologies, each with their own advantages and specificity. These methods include the use of biomarkers such as zonulin, D-Lactate and calprotectin, which have been summarised in Table 1.2. This review will however focus on *in vivo* methodology pertaining to the use of the differential sugar absorption test (SAT).

Methodologies commonly utilised to study intestinal permeability and absorptive capacity in the cat and dog are shown in Table 1.3. While the use of ^{51}Chromium-labelled ethylenediamine tetra-acetic acid (^{51}Cr-EDTA) is considered gold standard, it is radioactive and therefore not practical for clinical use (Bischoff *et al*., 2014; Frias *et al*., 2012). Therefore, a common method to determine intestinal permeability and mucosal absorptive capacity is the differential SAT. The differential SAT uses monosaccharides, polyalcohols and/or disaccharides as indicators of permeability and small intestinal function (Lostia *et al*., 2008). Monosaccharides, such as rhamnose or mannitol, allow researchers to measure the transcellular permeability of small molecules while disaccharides, such as lactulose, measure the paracellular permeability of large molecules through the intercellular junction complex. Transepithelial electrical resistance (TEER) is a method of measuring the ionic conductance of the intestinal barrier and is a reliable way to determine paracellular permeability and integrity (Srinivasan *et al*., 2015). Transepithelial electrical resistance is measured in ohms (Ω) with an inverse correlation to permeability; so a decrease in TEER is indicative of increased paracellular permeability (Li *et al*., 2008; Srinivasan *et al*., 2015). Comparing these studies and drawing solid conclusions is challenging because of the variations in methodology and the absence of standardisation among the SAT utilised in the pre-existing studies. The *ex vivo* and *in vitro* approach taken by Man *et al*. (2015) can accurately measure the permeability of specific sites within the GIT, but does not account for other factors that influence intestinal permeability, potentially simplifying the biological relevance (Galipeau & Verdu, 2016). A reoccurring issue with intestinal permeability and absorptive capacity work is the lack of standardisation between studies, making it challenging to make meaningful comparisons.

Non-metabolisable monosaccharides, xylose and 3-O-methylglucose (3-OMG), can also be used to quantify absorptive capacity of the small intestine (Rodríguez *et al*., 2009; Zhang *et al*., 2017). 3-O-methylglucose is the non-metabolisable, synthetic derivative of glucose, rendering it an adequate marker since nearly the full amount absorbed via active transport through SGLT1 (Csáky & Glenn, 1956; Fordtran *et al*., 1962). Xylose is thought to be measured passively as well as through carrier-mediated active transport (Wijtten *et al*., 2011).

Table 1.2. Biomarkers of intestinal permeability

Biomarker	Interpretation	Where Probe is Measured	Reference Range in Literature	Notes
Zonulin	Higher concentrations linked to increased intestinal permeability (Qi *et al*., 2017)	Serum (Dinesh *et al*., 2022), or plasma (Li *et al*., 2016)	Cats: Not established Dogs: 0.27 ng/ml, ranging from 0.05 to 3.67 ng/ml (Dinesh *et al*., 2022)	Unreliable to measure with ELISA (Kuzma *et al*., 2019)
D-Lactate	Higher concentrations linked to increased intestinal permeability (Bischoff *et al*., 2014)	Serum or plasma (Bischoff *et al*., 2014)	Cats: 0.04 – 0.87 mmol/L (median: 0.22 mmol/L) (Packer *et al*., 2012) Dogs: mean 0.31 ± 0.05 mmol/L (Venn *et al*., 2020)	Presence of bacterial overgrowth can skew results (Bischoff *et al*., 2014)
Diamine Oxidase	Higher concentrations linked to increased intestinal permeability (Yanxia Liu *et al*., 2012)	Serum (Yulan Liu *et al*., 2012) or plasma (Sun *et al*., 2008)	Cats: Not established Dogs: Not established	Inversely correlated with TJ proteins ZO-1 and occludin, positively correlated with D-Lactate (Yanxia Liu *et al*., 2012; Sun *et al*., 2008)
LPS and LPS-Binding Protein	Higher concentrations linked to increased intestinal permeability (Bischoff *et al*., 2014)	Serum (Tatucu-Babet *et al*., 2020)	Cats: Not established Dogs: Not established	Recommended to use serum from portal vein as opposed to peripheral blood (Galipeau & Verdu, 2016)
Calprotectin	Higher concentrations linked to intestinal inflammation (Cummings *et al*., 2004)	Serum (Heilmann *et al*., 2018) or faeces (Enderle *et al*., 2022)	Cats: 108.8 to 255.3 µg/L (serum) (Heilmann *et al*., 2018), 1.5 to 66.5 µg/g (faecal) (Heilmann *et al*., 2018), and <64 µg/g (faecal) (Enderle *et al*., 2022)	Not site-specific (Valentini *et al*., 2014) and serum measurements are not as accurate as faecal when

			Dogs: 219.4 µg/L (28.0 – 1123.0) (serum) (Heilmann *et al*., 2018), 2.9 – 109.8 µg/g (faecal) (Grellet *et al*., 2013), and Grellet *et al*. (2013) and <41 µg/g (Enderle *et al*., 2022)	diagnosing CIEs (Otoni *et al*., 2018)
Alpha-1 Antitrypsin	High concentrations linked to increased inflammation (Wang *et al*., 2015)	Serum and faeces (Bischoff *et al*., 2014)	Cats: 0.6 – 1.4 g/L (serum), and 0.04 – 1.9 µg/g (faeces) (Burke *et al*., 2012) Dogs: 1.42 ± 0.32 g/L (0.901 – 1.96) (serum) (Melgarejo *et al*., 1998), and 2.0 ± 1.82 µg/g (0.023 – 5.67) (faeces) (Melgarejo *et al*., 1998)	Unclear specificity (Burke *et al*., 2012). Cats, humans, dogs, and horses have comparable reference intervals of serum AAT, but faecal concentrations are significantly lower in cats than dogs (Burke *et al*., 2012)
IFABP	High concentrations linked to epithelial cell damage and increased intestinal permeability (Galipeau & Verdu, 2016; Van Wijck *et al*., 2012)	Serum, plasma, and urine (Bischoff *et al*., 2014)	Cats: Not established Dogs: 2.88 ± 0.13 ng/mL (serum IFABP-2) (Eregowda *et al*., 2020) and 2.58 ± 0.17 ng/mL (serum IFABP) (Gulersoy *et al*., 2023)	Increased concentrations in blood are associated with intestinal damage (Gulersoy *et al*., 2023) and positively correlated with LPS, LPS-binding protein, and zonulin during times of increased intestinal permeability (Ciccia *et al*., 2017)

Abbreviations: ELISA, enzyme-linked immunosorbent assay; TJ, tight junction; ZO, zonula occluden; LPS, lipopolysaccharide; CIE, chronic inflammatory enteropathy; AA, alpha-1 antitrypsin; IFABP, intestinal fatty acid-binding protein.

Table 1.3. Methodology used to determine intestinal permeability in the cat and dog.

Permeability Assay Probe	Method of Absorption	Location	Where Probe is Measured	Interpretation
Lactulose/ rhamnose or Lactulose/ mannitol	Paracellular diffusion / Transcellular diffusion (Craven *et al*., 2007; Lostia *et al*., 2008)	Small intestine (Lostia *et al*., 2008)	Urine (Kubica *et al*., 2012), plasma (Bruet *et al*., 2008), or serum (Rodríguez *et al*., 2009)	Lower LR or LM ratio indicates a healthy intestine (Craven *et al*., 2007)
Iohexol	Paracellular diffusion (Frias *et al*., 2012)	Evidence suggests iohexol is absorbed similarly to ^{51}Cr-EDTA (Frias *et al*., 2012)	Serum (Frias *et al*., 2012; Klenner *et al*., 2009; Rummell *et al*., 2022; Watson *et al*., 2019), or urine (Beneyto, 2013)	Lower recovery of iohexol indicates a healthy intestine (Frias *et al*., 2012)
^{51}Cr-EDTA	Paracellular diffusion (González-González *et al*., 2019)	Whole intestine (Galipeau & Verdu, 2016)	Serum, plasma (Beneyto, 2013), or urine	Lower recovery of ^{51}Cr-EDTA indicates a healthy intestine (Bischoff *et al*., 2014)
PEG	Paracellular (>300 Da) (Van Wijck *et al*., 2012)	Whole intestine (Van Wijck *et al*., 2012)	Urine (Van Wijck *et al*., 2012), blood (Galipeau & Verdu, 2016)	Lower recovery of larger PEG probes or lower recovery ratio of larger to smaller PEG probes indicate a healthy intestine (Van Wijck *et al*., 2012)
FITC-Labelled Dextran	Paracellular diffusion (González-González *et al*., 2019)	Whole intestine (Galipeau & Verdu, 2016)	Plasma (Galipeau & Verdu, 2016)	Low recovery of FITC – labelled dextran indicates a healthy intestine (Frias *et al*., 2012; Tan *et al*., 2015)

Xylose/ 3-O-methylglucose	Carrier-mediated active transport/ ATP-dependent mediated active transport (Rodríguez *et al*., 2009)	Duodenum and jejunum/ length of small intestine (Rodríguez *et al*., 2009)	Urine (Sørensen *et al*., 1993), plasma (Sørensen *et al*., 1997), or serum (Rodríguez *et al*., 2009)	Higher XG ratio indicates a healthy intestine (Craven *et al*., 2007)

Abbreviations: LR, lactulose to rhamnose ratio; LM, lactulose to mannitol ratio; Cr-EDTA, chromium-labelled ethylenediamine tetra-acetic acid; PEG; polyethylene glycol; FITC, fluorescein isothiocyanate; ATP, adenosine triphosphate; XG, xylose to 3-O-methylglucose ratio.

Xylose is more sensitive than 3-OMG, and the ratio of both markers provides a better indication of the condition of the intestinal barrier (Rodríguez *et al*., 2009; Zhang *et al*., 2017). Xylose is used as a marker for intestinal absorption because it possesses a lower affinity for sugar carriers than glucose and other hexoses, preventing high absorption and loss of the marker and therefore making it a more sensitive measure of intestinal function (Fordtran *et al*., 1962). A decrease in the xylose and 3-OMG ratio is indicative of intestinal damage due to its reduced absorptive capacity for these sugars (Rodríguez *et al*., 2009; Sørensen *et al*., 1997). In a rat model, 3-OMG has been found to be significantly correlated with intestinal surface area, as well as dietary fat and protein absorption, but not carbohydrates (Martin *et al*., 2003). Therefore, both sugars are needed to determine absorptive capacity of carbohydrates.

Intestinal permeability and absorptive measurements are reported in Table 1.4 for cats and Table 1.5 for dogs. Randell *et al*. (2001) found that cats had a significantly higher urinary lactulose to rhamnose (LR) recovery ratio than dogs, suggesting that cats have greater intestinal permeability than dogs (Randell *et al*., 2001). Tight junction complexes in cats are different to those of dogs which may explain their higher measurements of intestinal permeability. Lactulose and ^{51}Cr-EDTA are recovered at levels more than 4 times higher in cats than in dogs since their small intestine is more permeable to large molecules, however there are few differences between cats and dogs in rhamnose absorption, a smaller monosaccharide (Johnston *et al*., 2001). Comparable studies have conflicting results regarding xylose absorption between cats and dogs, with older studies stating that their measurements in cats were much lower than dogs (Johnston *et al*., 2001; Sherding *et al*., 1982), but another study found much less variation between individual cats and between cats and dogs (Randell *et al*., 2001). It's important to emphasise that earlier research exclusively assessed xylose absorption, a parameter highly influenced by the administered dosage, which varies across these studies and lacks standardisation. Reasons for the difference in intestinal permeability may be due to physiological differences between the species including, shorter small intestines, and therefore less surface area in the cat compared to the dog (Figure 1.1), which may lead to their ultimately higher permeability measurements (Johnston *et al*., 2001; Randell *et al*., 2001). For these reasons, results from one species may not be relevant if extrapolated to the other.

Table 1.4. Intestinal permeability and absorptive capacity values for cats. Data are expressed as mean ± standard deviation (range) unless otherwise stated. Some values have been calculated from given data or plots (the latter represented by the approximation symbol, ≈).

	Measurement	Breed	Sex	Age	Method	Reference
Intestinal permeability	0.03 ± 0.003	Not reported	Unknown	Unknown	Urinary LM ratio	(Bijlsma *et al*., 1995)
	GLM: 0.27 ± 0.21 (0.03 – 0.57)	Not reported	Mixed	5 – 12 years	Urinary LM ratio	(Papasouliotis *et al*., 1993)
	ENZ: 0.47 ± 0.38 (0.02 – 1.21)	Not reported	Mixed	5 – 12 years	Urinary LM ratio	(Papasouliotis *et al*., 1993)
	0.40 ± 0.20 (0.15 – 0.68)	Domestic shorthair	Female	2 – 4 years	Urinary LR ratio	(Johnston *et al*., 2001)
	0.52 ± 0.19 (0.3 – 0.98)	American Short hair and mixed breed domestic shorthair	Mixed	2 – 7 years	Urinary LR ratio	(Randell *et al*., 2001)
	0.53 ± 0.23	Specific pathogen-free	Male	"Adult"	Urinary LM ratio	(Marks *et al*., 1999)
	5.95 ± 2.21 % (2.66 – 8.93)	Domestic shorthair	Female	2 – 4 years	Urinary ^{51}Cr-EDTA recovery	(Johnston *et al*., 2001)
	12.62 ± 7.23 µg/mL (6.99 – 23.01)	Not reported	Mixed	6 weeks	Serum iohexol	(Watson *et al*., 2019)
Absorptive capacity	0.70 ± 0.08 (0.60 – 0.78)	Domestic shorthair	Female	2 – 4 years	Urinary XG ratio	(Johnston *et al*., 2001)
	31.89 ± 15.78 % (15.84 – 58.66)	Domestic shorthair	Female	2 – 4 years	Urinary xylose recovery	(Johnston *et al*., 2001)

26.0 ± 9.2 mg/dL	Unknown	Unknown	2 – 5 years	Plasma levels of xylose at 60 minutes	(Hawkins *et al*., 1986)
≈ 46.04 ± 30.45 (10.94 – 72.45) mg/dL	Unknown	Mixed	6 weeks	Serum D-xylose	(Watson *et al*., 2019)
43.4 ± 12.95 (27.2 – 68.9) mg/dL	Domestic shorthair and Siamese	Mixed	Unknown	Plasma levels of xylose at 60 minutes	(Emms *et al*., 1982)
42.6 ± 17.8 mg/dL	Domestic shorthair	Unknown	3 – 9 years	Plasma levels of xylose at 60 minutes	(Sherding *et al*., 1982)

Abbreviations: LR, lactulose to rhamnose ratio ; LM, lactulose to mannitol ratio; GLC, gas-liquid chromatography; ENZ, enzymatic assay; Cr-EDTA, Chromium-labelled ethylenediamine tetra-acetic acid. XG, xylose to 3-O-methylglucose ratio.

Table 1.5. Intestinal permeability and absorptive capacity values for dogs. Data are expressed as mean ± standard deviation (range) unless otherwise stated. Some values have been calculated from given data or plots (the latter represented by the approximation symbol, ≈).

	Measurement	**Breed**	**Sex**	**Age**	**Method**	**Reference**
Intestinal permeability	0.012 ± 0.001	Beagle	Unknown	"Adult"	Plasma LM ratio	(Tina *et al*., 2011)
	0.03 – 0.12	Unknown	Unknown	Unknown	Urinary LR ratio	(Rutgers *et al*., 1995)
	0.05 – 0.15	Beagle	Mixed	Unknown	Urinary LR ratio	(Steiner *et al*., 2001)
	0.08 ± 0.03 (0.03 – 0.12) 0.09 ± 0.04 (0.05 – 0.17)	Various breeds	Unknown	1+ years 1 – 8 years	Urinary LR ratio Plasma LR ratio	(Sørensen *et al*., 1997)
	0.10 ± 0.07 (0.03 – 0.18)	Irish Setter	Mixed	1 – 8 years	Urinary LR ratio	(Garden *et al*., 1997)
	0.11 ± 0.03 (0.07 – 0.14)	Golden Retriever	Mixed	2 – 7 years	Urinary LR ratio	(Randell *et al*., 2001)
	0.06 – 0.27* 0.05 – 0.18*	Alaskan sled dogs	Unknown	Racing age	Urinary LR ratio Serum LR ratio	(Davis *et al*., 2005)
	0.13 ± 0.04 (0.08 – 0.23)	Beagle	Male	"Adult"	Urinary LR ratio	(Beneyto, 2013)
	0.156 ± 0.03	Beagle	Mixed	8 months – 6.5 years	Plasma LM ratio	(Bruet *et al*., 2008)
	0.16 ± 0.04 (0.14 – 0.21) 0.25 ± 0.04 (0.19 – 0.29)	Miniature Poodle	Female	60 weeks 12 weeks	Urinary LR ratio	(Weber *et al*., 2002)
	0.19 ± 0.05 (0.13 – 0.26) 0.25 ± 0.03 (0.22 – 0.29)	Standard Schnauzer	Female	60 weeks 12 weeks	Urinary LR ratio	(Weber *et al*., 2002)
	0.20 ± 0.7 (0.08 – 0.34)	Mixed breed hounds	Mixed	2 – 7 years	Urinary LR ratio	(Randell *et al*., 2001)
	0.21 ± 0.16 (0.07 – 0.26)	Vizsla	Mixed	2 – 7 years	Urinary LR ratio	(Randell *et al*., 2001)
	0.25 ± 0.06 (0.17 – 0.32) 0.35 ± 0.06 (0.32 – 0.46)	Giant Schnauzer	Female	60 weeks 12 weeks	Urinary LR ratio	(Weber *et al*., 2002)
	0.27 ± 0.01 (0.19 – 0.34)	Greyhound	Mixed	2 – 7 years	Urinary LR ratio	(Randell *et al*., 2001)

	0.31 ± 0.08 (0.26 – 0.42) 0.30 ± 0.11 (0.16 – 0.43)	Great Dane	Female	60 weeks 22 weeks	Urinary LR ratio	(Weber *et al*., 2002)
Absorptive capacity	0.40 – 0.59	Beagle	Unknown	Unknown	Urinary XG ratio	(Steiner *et al*., 2001)
	0.56 ± 0.06 (0.45 – 0.65) 0.64 ± 0.05 (0.55 – 0.74)	Various breeds	Unknown	1+ years	Plasma XG ratio Urinary XG ratio	(Sørensen *et al*., 1997)
	0.57 ± 0.05 (0.52 – 0.65) 0.60 ± 0.03 (0.57 – 0.64)	Miniature Poodle	Female	60 weeks 12 weeks	Urinary XG ratio	(Weber *et al*., 2002)
	0.58 ± 0.03 (0.54 – 0.62) 0.60 ± 0.03 (0.57 – 0.62)	Giant Schnauzer	Female	60 weeks 12 weeks	Urinary XG ratio	(Weber *et al*., 2002)
	0.59 ± 0.03 (0.46 – 0.81)	Irish Setter	Mixed	1 – 12 years	Urinary XG ratio	(Garden *et al*., 1997)
	0.59 ± 0.06 (0.51 – 0.68) 0.64 ± 0.05 (0.56 – 0.66)	Standard Schnauzer	Female	60 weeks 12 weeks	Urinary XG ratio	(Weber *et al*., 2002)
	0.59 ± 0.04 (0.56 – 0.62) 0.65 ± 0.07 (0.53 – 0.71)	Great Dane	Female	60 weeks 22 weeks	Urinary XG ratio	(Weber *et al*., 2002)
	0.6 – 0.79	Unknown	Unknown	Unknown	Urinary XG ratio	(Rutgers *et al*., 1996)
	0.69 ± 0.05 (0.6 – 0.79) 34.2 ± 11.5 % (15.5 – 57.8)	Various breeds	Unknown	1+ years	Urinary XG ratio Urinary xylose recovery	(Sørensen *et al*., 1993)
	0.73 ± 0.09 (0.60 – 0.90)	Beagle	Male	"Adult"	Urinary XG ratio	(Beneyto, 2013)

Some data have been converted into the same measurement unit for better comparison (µg/mL) or calculated from given data. Abbreviations: LR, lactulose to rhamnose ratio; LM, lactulose to mannitol ratio; Cr-EDTA, Chromium-labelled ethylenediamine tetra-acetic acid; I-AUC, Incremental area under the curve; XG, xylose to 3-O-methylglucose ratio. The use of * denotes the median.

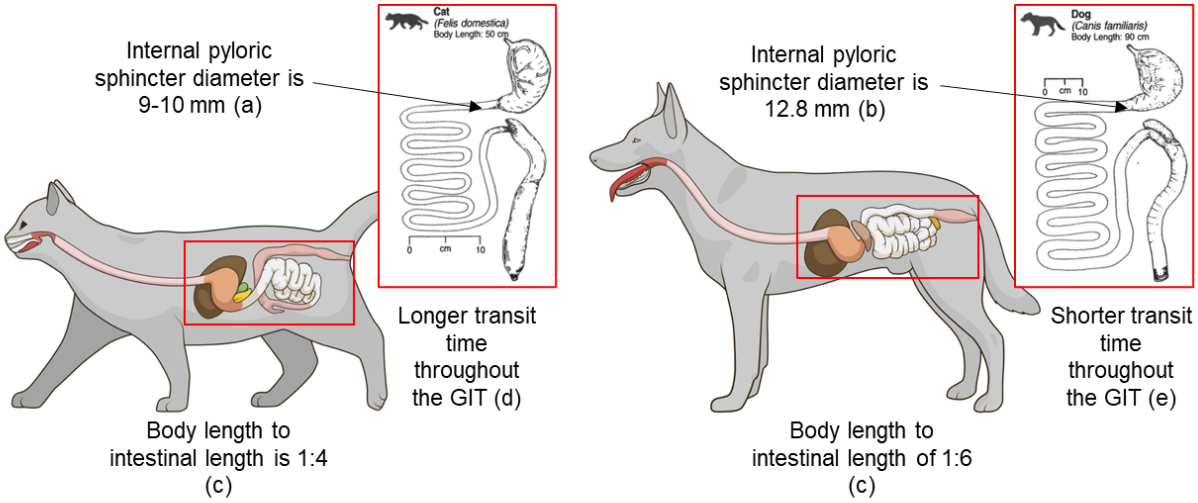


Figure 1.1. Adapted from (Stevens, 1977; Stevens & Hume, 1995). (a) (Lamoureux *et al*., 2019); (b) (Kim *et al*., 2018); (c) (Kararli, 1995). Cats have shorter intestinal length, and therefore less intestinal surface area than dogs, potentially a contributing factor to their greater permeability to large molecules (Johnston *et al*., 2001; Kararli, 1995; Randell *et al*., 2001). (d) Median gastrointestinal transit time in the cat was 1752 minutes (1105 – 5451) and 2795 minutes (926 – 6563) for pre- and post- feeding, respectively (Telles *et al*., 2021). (e) Median gastrointestinal transit time in the dog was 1070 minutes (244 - 1430) and 1708 minutes (539 - 2743) for pre- and post- feeding, respectively (Tolbert *et al*., 2022).

Two studies examined the pre- and post-feeding gastric emptying times of healthy young dogs (beagles) and early midlife cats. Although the difference was insignificant, gastric emptying times in domestic cats were consistently greater (longer) than in beagles (Telles *et al*., 2021; Tolbert *et al*., 2022). The same results have also been determined previously using radioscintigraphy, the gold standard method used in gastric emptying research (Husnik *et al*., 2017; Schmitz *et al*., 2016). Despite beagles being approximately three times heavier with an estimated 2.3 times longer GIT than the average cat (Kararli, 1995), gastric and intestinal transit times were both shorter in beagles than in cats, supporting the idea that cats have slower gastric emptying possibly due to their smaller body size and shorter intestines. The inverse relationship between gastric emptying time and body size has been established in an earlier study where large breed dogs had faster gastric emptying times than medium-sized dogs (Boillat *et al*., 2010). This study did not include any dogs below 19.6 kg BW, however, so no conclusion can be reached regarding small-breed dogs. An older study using dogs that ranged in BW from 3.5 – 59.1 kg provided contradicting results; using ^{13}C-octanoic breath testing, where longer gastric emptying times in larger dogs compared to smaller dogs were recorded (Bourreau *et al*., 2004). Additional research using radioscintigraphy to evaluate a larger variety of dog breeds and sizes is needed to reach a firm conclusion regarding the inverse relationship

between gastric emptying time and body size. It has been hypothesised that the diameter of the pyloric sphincter plays a role in determining the speed of gastric emptying, with beagles having a larger sphincter diameter reported as 12.8 mm (Kim *et al*., 2018) than cats which have a median diameter of 9 mm (Lamoureux *et al*., 2019). Shorter intestines, smaller surface area, and smaller pyloric sphincter diameter may all be contributing factors to the longer gastric emptying time in cats to allow for maximal breakdown and absorption of nutrients (Figure 1.1).

1.2.4.3 Effect of age on intestinal permeability and absorptive capacity

Intestinal permeability is strongly correlated with other aspects of health, although the mechanisms behind these relationships have not been fully identified. It is known that permeability of the intestinal barrier is closely associated with the immune system and gastrointestinal microbiome (Bischoff *et al*., 2014). In a group of senior women, 10 of the 48 tested had increased intestinal permeability and all were at risk of or experiencing malabsorption of nutrients. Further studies need to be completed to explore the connection between mucosal permeability and malnutrition, but it is believed that the increased permeability of the small intestine negatively impacts nutrient absorption, eventually leading to malnutrition in the elderly (Bolin *et al*., 2010). Although multiple human studies have found no differences in intestinal permeability between healthy seniors and young adults (Saltzman *et al*., 1995; Wilms *et al*., 2020), intestinal permeability increases with age in other species including rats, mice, and baboons (Katz *et al*., 1987; Ma *et al*., 1992; Thevaranjan *et al*., 2017; Tran & Greenwood-Van Meerveld, 2013). The limited research available in dogs does not support the claim that age has any effect on intestinal permeability or absorptive capacity, but changes in intestinal morphology have been documented (Garden *et al*., 1997; Kuzmuk *et al*., 2005; Weber *et al*., 2002).

There appears to be no differences in intestinal transit time and gastric emptying time between young and senior/super-senior cats (Papasouliotis *et al*., 1998; Peachey *et al*., 2000). Senior cats exhibited greater variation in intestinal transit time than younger cats, suggesting that they may take longer for complete emptying, however, this could been explained by age-related changes in intestinal morphology (Höhn *et al*., 1978; Peachey *et al*., 2000). The absorptive capacity of cats, as determined by a xylose sugar test, varies between studies, creating difficulty evaluating its relationship with age (Table 1.4) (Emms *et al*., 1982; Johnston *et al*., 2001; Sherding *et al*., 1982). It is hypothesised, however, to lose effectiveness with age, as seen in mice (Chen *et al*., 1990; Ferraris & Vinnakota, 1993).

Dog size affects intestinal permeability, being greater in large-breed dogs (Weber *et al*., 2002). Intestinal permeability is greater in 12 week - old puppies compared with 60 week - old (young) dogs (Weber *et al*., 2002), however these differences largely disappear after 12 weeks of age. In this study, higher faecal scores (more liquid stools) were recorded for the large-breed dogs and puppies, potentially a symptom of

greater permeability. In a study in Irish setter dogs, there were also no significant changes in intestinal permeability or mucosal absorptive capacity in dogs aged between 1 to 12 years (Garden *et al*., 1997). Randell *et al*. (2001) also published data that showed significantly differences in intestinal permeability measurements between dog breeds. Caution must be taken when comparing the permeability measurements of active animals, however, since the greyhounds involved in this study were exercising throughout the trial and exercise has past been shown to increase intestinal permeability in dogs (Davis *et al*., 2005; Randell *et al*., 2001).

Although intestinal functionality is seemingly unaffected by age, its morphology does appear to change. For example, senior dogs have lower duodenal villus area, jejunal villus height, and jejunal villus: crypt ratio and increased ileal villus width and greater colonic crypt depth compared to young dogs (Garden *et al*., 1997; Kuzmuk *et al*., 2005; Weber *et al*., 2002). Senior dogs also have more genes expressed in their colonic mucosa associated with inflammation, cell proliferation, stress, and cellular metabolism, but less genes expressed that are associated with apoptosis and cellular defence (Kil *et al*., 2010).

There is no clear relationship between age and absorptive capacity in dogs (Table 1.5). It is hypothesised that differences in ranges of absorptive capacity measurements could be due to the volume of sugar solution ingested as larger volumes may influence intestinal motility, differences between breeds, or subclinical enteropathy (Garden *et al*., 1997; Randell *et al*., 2001).

1.3 Immune system

The GIT is part of the innate immune system and therefore is a vital component of GIT function (Jan Bellows *et al*., 2016). Ageing animals experience immunosenescence - age-related changes in the immune system, that effectively lead to inflammageing, the chronic, sterile, low-grade inflammation that can become damaging to the body (Jan Bellows *et al*., 2016; Franceschi *et al*., 2018). Although there is a dearth of research in this area in the dog and cat, inflammageing and immunosenescence are believed to also occur in these animals specifically (Day, 2010; McKenzie, 2022).

In the cat, pro-inflammatory cytokines IL-1β, IL-6, and IL-12 p40 were significantly greater in animals between the ages of 8-10 years compared to the same cats at the ages of 3 – 5, while the anti-inflammatory cytokine, IL-10, levels were lower (Kipar *et al*., 2005). Late-midlife to super-senior cats exhibited lower numbers of CD4+ T-cells, CD8+ T-cells, and CD21+ B-cells (Campbell *et al*., 2006; Campbell, Rawlings, Heaton, *et al*., 2004; Campbell, Rawlings, Koelsch, *et al*., 2004) leading to a lower responses to T-cell mitogens concanavalin A and phytohaemagglutinin in comparison to young cats (Campbell *et al*., 2006). Cats over the age of 10 also exhibited lower natural killer cell, white blood cell (WBC), lymphocyte, and

eosinophil counts, with higher concentrations serum immunoglobulins IgA and IgM than young and early midlife cats (Campbell, Rawlings, Koelsch, *et al*., 2004).

Late-midlife to super-senior dogs are similar to cats, with older individuals having reduced WBC counts, erythrocytes, leukocytes, band neutrophils, T-, B-, and T-cytotoxic lymphocytes, CD5+, and CD4+ cells (Gomes *et al*., 2011; Maria *et al*., 2017; Pereira *et al*., 2019; Strasser *et al*., 2000). In German Shepherd dogs, researchers did not observe age-related changes in serum tumor necrosis factor (TNF), but did see lower concentrations of IL-2 in older dogs, which is responsible for regulation of WBCs, and an increase in pro-inflammatory IL-1 in older females (Strasser *et al*., 2000). In dogs, serum IgM and 8-hydroxy-2-deoxyguanosine concentrations increase with age, but C-reactive protein concentrations don't increase significantly until the last year of life (Alexander *et al*., 2018). Ageing dogs also seem to be worse at dealing with oxidative stress than their younger counterparts, which is shown by increased biomarkers of oxidative damage (Alexander *et al*., 2018).

1.3.1 Immune system and intestinal barrier function

It has been hypothesised that inflammation causes gaps within TJs, allowing for an influx of molecules across the intestinal barrier (Karper, 2011). While intestinal barrier function in the dog and cat have not been explored in great detail, in humans and rodents, pro-inflammatory cytokines TNF-α and interferon (IFN-γ) are associated with increased intestinal permeability (Bischoff *et al*., 2014; Keita & Söderholm, 2010; Kiesslich *et al*., 2007; Ordiz *et al*., 2018). TNF-α promotes systemic inflammation (Thevaranjan *et al*., 2017), and causes an increase in cell shedding in the small intestine of mice which weakens barrier function and forms epithelial gaps that increase cellular permeability (Kiesslich *et al*., 2007). Elevated TNF-α concentration is a factor behind age-related microbial dysbiosis which causes increases in intestinal permeability and leads to systemic inflammation (Thevaranjan *et al*., 2017). IFN-γ causes an increase in TJ permeability and influx of molecules such as mannitol or inulin (Madara & Stafford, 1989).

When incubated with IL-6, Caco-2 cells show increased expression of claudin-2 and a decrease in TEER, instigating greater uptake of cations (Suzuki *et al*., 2011). This is also shown in human ileal biopsies, where increased IL-6 with age upregulates claudin-2 expression and decreases TEER, characterising increased permeability (Man *et al*., 2015). IL-1β can modulate TJ barrier function and cause increased intestinal permeability which plays a role in intestinal inflammation (Kaminsky *et al*., 2021). IL-13 can also increase paracellular permeability by increasing claudin-2 expression (Ordiz *et al*., 2018). The upregulation of IL-1β and IL-6 in cats (Kipar *et al*., 2005), like humans, presents the possibility that barrier function may also weaken as a result of heightened inflammation.

1.4 Faecal microbiome

The gastrointestinal microbiome, made up of primarily bacteria, plays an essential role in maintaining and improving its host's metabolism and immune system while also producing vitamins, minerals, and other metabolites that have widespread effects throughout the body (Suchodolski, 2022). A healthy, diverse gastrointestinal microbiome is essential to the wellbeing of companion animals, but age-related changes may be the underlying cause of inefficiency of the GIT in ageing animals. Age-related changes to the gastrointestinal microbiome are not as well researched in companion animals compared to humans, but current research suggests there are relatively small changes over time (Suchodolski, 2022). Features of the microbiome are affected by many factors such as age, diet, health status, and environment, making it difficult to directly link the microbiome to gastrointestinal impairment.

The dominant phyla in cats across studies are Bacillota (formerly known as Firmicutes), Bacteroidota, and Fusobacteria (Butowski *et al*., 2022; Ganz *et al*., 2022; Masuoka *et al*., 2017a; Suchodolski, 2022), with Actinomycetota (formerly known as Actinobacteria) and Bacillota presenting as the main phyla in cats over the age of 8 (Jia *et al*., 2011). While the composition of the bacterial community, alpha or beta diversity, or relative abundance of the core taxa do not significantly change with age, a decrease in the core taxa's population with age suggests that some populations decrease while others increase to maintain the same relative abundance (Ganz *et al*., 2022). However, other studies have shown that ageing cats have less diverse microbiota. For instance, a study comparing the faecal microbiome and metabolites of young (2.48 years ± 0.164) and old (12.4 years ± 3.408) cats found reduced microbiota diversity in the older group (Tian *et al*., 2023). As cats age, the relative abundance of *Fusicatenibacter*, *Subdoligranulum*, *Enterococcus*, *Faecalibacterium*, and *Megasphaera* decreases, along with faecal bifidobacteria and lactobacilli, and the family Enterobacteriaceae (Bell *et al*., 2014; Bermingham *et al*., 2018; Ganz *et al*., 2022; Masuoka *et al*., 2017a; Patil & Cupp, 2010), while the levels of *Bacteroides* and *Collins*, as well as faecal *Clostridium perfringens* increases (Bermingham *et al*., 2018; Patil & Cupp, 2010; Tian *et al*., 2023). Interestingly, in a longitudinal study, as kittens fed kibble diets aged into early midlife, they had reduced populations of *Lactobacillus* and *Bifidobacterium*, as seen in older cats, but they also had increased *Prevotella* and *Megasphaera* (Bermingham *et al*., 2018). However, lactobacilli and bifidobacteria counts can be highly variable in cats, and make up only a small portion of the cat's faecal microbiome so they may play a much smaller role in feline gastrointestinal health when compared to humans (Jia *et al*., 2011; Masuoka *et al*., 2017a; Mitsuoka, 2014; Mitsuoka & Kaneuchi, 1977).

It is well accepted that the dominant phyla in dogs are the same as cats: Bacteroidota, Bacillota, and Fusobacteria, followed by Pseudomonadota (formerly known as Proteobacteria) and Actinomycetota (Butowski *et al*., 2022; Garrigues *et al*., 2022; Suchodolski, 2022), with major genera being *Fusobacterium,*

Bacteroides, Prevotella, Peptoclostridium, and *Alloprevotella* (Kubinyi *et al*., 2020). Microbial diversity seems to be reduced as dogs age due to declining populations of minor groups of bacteria, which agrees with the current literature in other species (Mizukami *et al*., 2019). The dominating Fusobacteria population is also lower in comparison to other phyla in dogs as they age (Kubinyi *et al*., 2020). Late midlife and senior dogs have smaller populations of *Bacteroides* than young dogs, but lactobacilli and bifidobacteria numbers were not found to be influenced by age in multiple studies (Gomes *et al*., 2011; Simpson *et al*., 2002). Other studies where late midlife and senior dogs had increased faecal counts of lactobacilli, clostridia, and bifidobacteria directly contradict these preliminary findings (Kearns *et al*., 2000; Maria *et al*., 2017; Suchodolski, 2022). In a study completed by Masuoka *et al*. (2017b), using beagles from 11 days to 17 years old, researchers found that lactobacilli and Enterobacteriaceae were less prevalent and occurred in smaller numbers in older animals (Masuoka *et al*., 2017b). The lack of bifidobacteria in the canine microbiome suggests that this genus is not as important to gastrointestinal health as it is in humans, although it has been found in the microbiome of dogs in multiple other studies, so a definitive conclusion cannot be drawn yet (Masuoka *et al*., 2017b). The study also indicated that lactobacilli could be an important genus due to its change in microbial populations during ageing, however this is heavily debated. These changes observed in older dogs are characterised by a decrease in microbial diversity, potentially changing the way that older dogs can respond to diseases and regulate absorption and use of macro and micronutrients (Ghosh *et al*., 2022; Mizukami *et al*., 2019; Suchodolski, 2022).

Some observed differences in the GIT microbiome may be related to diet, rather than age. Significant alterations in the microbiome of cats and dogs typically occur when transitioning to high-protein or high-fibre diets. However, adjustments in moisture levels, nutrient composition, and the addition of prebiotic fibres can also contribute to these changes, although they do not have as significant an impact as disease (Pilla & Suchodolski, 2021).

1.4.1 Faecal microbiome and intestinal permeability

Studies in mice indicate that there may be a link between gastrointestinal microbiota and intestinal permeability (Brun *et al*., 2007; Cani *et al*., 2008; Cani *et al*., 2009). During ageing, changes in the gastrointestinal microbiota, mucus layer, and epithelial integrity can induce translocation of luminal content to inner layers of the intestinal barrier which can lead to altered intestinal permeability (Bischoff *et al*., 2014). There is limited research and evidence to make the firm connection between the GIT microbiome and intestinal permeability, although increased intestinal permeability can be considered a characteristic of microbial dysbiosis (Conway & Duggal, 2021). When the composition and function of microbiota are altered beyond the point of maintaining the intricate balance between bacterial communities, the microbiome is said to be in dysbiosis (Bäckhed *et al*., 2012). During dysbiosis, the host is at increased risk

of disease or infection, providing a direct correlation between the microbiome and disease, inflammation, genetic markers, and overall gastrointestinal health (Bäckhed *et al*., 2012; Sung *et al*., 2022).

The relationship between intestinal bacteria and the intestinal barrier has been described as mutualistic, primarily being studied through the favourable effects of probiotics on intestinal physiology and inflammation of the mucosal barrier and adverse reactions to reductions in bacterial populations (Natividad & Verdu, 2013). Age-related changes in gastrointestinal microbiota, notably an increase in Gammaproteobacteria populations, have been associated with increased intestinal permeability in *Drosophila*. *Drosophila* show increased bacterial loads as they age, preceding intestinal barrier dysfunction and irregular TJ protein gene expression which creates opportunity to predict age-related changes to the intestinal barrier (Clark *et al*., 2015). Increased intestinal permeability and other signs of impaired intestinal function have been associated with microbiota changes caused by diet (Bermingham, *et al*., 2013a). Dysfunction of the GIT can coincide with disease, disorders, and other underlying causes that affect the immune system or alter the gastrointestinal microbiome leading to an increase in intestinal permeability and dysbiosis (Suchodolski, 2011). Surprisingly, cats administered with metronidazole antibiotic showed no significant difference to a control group in permeability and absorption through a differential sugar absorption test (Johnston *et al*., 2001). Johnston *et al*. (2001) hypothesised that similar to other species, antibiotic administration would reduce the growth of pathogenic bacteria and therefore decrease intestinal permeability. Acute or chronic diarrhoea in cats is associated with diminished microbiome diversity, reduced *Faecalibacterium* species, and increased populations of Pseudomonadota and Bacillota, specifically *Clostridium* (Suchodolski *et al*., 2015). Bacteroidaceae were specifically increased in acute diarrhoea compared to the increased population of *Lactobacillus* and decreased *Faecalibacterium* spp. in cats with chronic diarrhoea (Suchodolski *et al*., 2015).

Intestinal permeability also provides a link between the brain and the gastrointestinal microbiome. Psychological stress causes a spike in cortisol in the body which can cause the intestinal barrier to be more susceptible to the absorption of pathogens and other enteric microbiota, which then in turn causes a pro-inflammatory state and a cycle of dysbiosis (Dinan & Cryan, 2012). Other than inducing cognitive decline, young rats who underwent faecal microbiota transplantation with microbiota from aged rats had an increase in oxidative stress and expression of pro-inflammatory cytokines (Li *et al*., 2020).

1.5 Metabolome

Bacteria in the microbiome are responsible for producing metabolites in the body, collectively referred to as the metabolome. These metabolic products are vital parts of the immune system and mitigate disease (Rooks & Garrett, 2016). There have been few studies directly studying the relationship between the

metabolome and age in cats. Tian *et al*. (2023) found 23 faecal metabolite concentrations decreased in an older cat cohort compared to the younger one. These included metabolites in pathways pertaining to choline metabolism, glycerophosolipid metabolism, retrograde endocannabinoid signalling, and linoleic acid metabolism (Tian *et al*., 2023). Serum glucose, serum amyloid A, triglyceride concentration and blood urea nitrogen were higher while the malate dehydrogenase: lactate dehydrogenase ratio and albumin decreased in subsets of older cats compared to young cats (Mizorogi *et al*., 2020). Domestic cats have been shown to have lower faecal concentrations of butyrate, isobutyrate, valerate, isovalerate, hexanoate, and succinate with age (Bermingham *et al*., 2018). In contrast, butyrate, valerate, and isovalerate are found in greater concentrations in colonic aliquots of senior dogs (Kuzmuk *et al*., 2005). This, contradicts findings by Gomes *et al*. (2011) who found lower faecal butyrate, histamine, agmatine, and spermine in beagle dogs over the age of 10 compared to dogs at 4 years of age (Gomes *et al*., 2011). An increase in bacterial groups that are producers of short chain fatty acids (SCFAs), such as *Roseburia* and *Blautia*, can improve the function of the intestinal barrier indirectly, through production of butyrate (Alessandri *et al*., 2020). Butyrate concentrations in faecal matter is inversely associated with a decrease in intestinal permeability in dogs (Alessandri *et al*., 2020; Apper *et al*., 2020).

The metabolome of dogs is well-studied in comparison to the cat, and knowledge of it continues to expand with researchers dedicated to large, longitudinal studies of ageing pets. As part of the Dog Aging Project in the United States, the plasma metabolome was measured in a cohort of 784 dogs, revealing post translationally modified amino acids from protein catabolism and multiple acetyl carnitines in plasma accumulated with age, while hydroxyproline decreased with age (Harrison *et al*., 2024). The study also found that 39 % of the aqueous metabolome changed with age, an association that will continue to be expanded upon as the longitudinal study continues. In another large study of dogs (2069 dogs) conducted in Finland, 112 of 119 metabolites (94 %) measured in serum were significantly affected by age. This large study found that the majority of all cholesterols, triglycerides and lipoproteins were increased in older dogs, and albumin and creatine dropped below reference levels (Puurunen *et al*., 2022). Dietary restriction has been shown to increase lifespan in dogs (Kealy *et al*., 2002), although a similar study observed ageing having a greater effect on the metabolome. Dogs over the age of 9 years had higher measured concentrations of α-ketobutyrate, 2-propanol, lactate, alanine, succinate, creatinine, dimethylglycine, and trimethylamine and lower concentration of mixed glycoproteins in their urine than younger dogs. Diet-restricted dogs (fed 75 % of control animals) had increased lactate, succinate, and 1-methylnicotinamide concentrations in their urine at 9 years of age compared to control-fed dogs (Wang *et al*., 2007). These studies highlight notable changes in the metabolome of ageing dogs, suggesting that metabolism undergoes complex changes over time.

Metabolites as biomarkers of the integrity of the intestinal barrier is an emerging concept, with work in companion animals extremely limited. Current reviewed research suggests that butyrate has beneficial effects on the epithelial barrier, through upregulation of TJ proteins, regulation of mucin expression, and the reduction of inflammatory cytokines' effects on intestinal permeability. Indole metabolites also contribute to a healthy intestinal barrier by increasing anti-inflammatory cytokine concentrations through activating the aryl hydrocarbon receptor. Bile acids lithocholic acid (LCA) and chenodeoxycholic acid (CDCA) have opposing effects, with LCA cause the vitamin D receptor to upregulate TJ proteins and CDCA reducing TJ protein occludin concentrations. Medium- and long-chain fatty acids can cause inflammation in the body, reducing TJ protein concentration, but linoleic acid does not account for this, instead being shown to strengthen the intestinal barrier (Iyer & Corr, 2021).

1.6 Variables affecting intestinal permeability

1.6.1 Impact of nutrition

Higher dietary fat intake can cause mast cells in the intestinal mucosa to activate, leading to increased intestinal permeability (Ji *et al*., 2011). For example increased intestinal permeability has been observed in obese rodents consuming high amounts of fat (Cani *et al*., 2008). Mice and Fischer 344 rats also show age-related increases in intestinal permeability when maintained on a high fat diet, providing a link between age, diet, and permeability (40 % calories from fat (Mullin *et al*., 2002), 60 % calories from fat (Jennis *et al*., 2018)). Higher intestinal permeability seen during fat absorption is due to temporary intestinal epithelial damage, which compromises the intestinal barrier and allows larger molecules, such as lipopolysaccharides (LPS), to be absorbed (Kvietys *et al*., 1991; Moreira *et al*., 2012). Overall, a high fat diet and an increase in chylomicrons can lead to an increase in bile secretion, and changes in microbiota composition which can cause increased intestinal permeability or can directly cause intestinal barrier dysfunction (Suzuki, 2020). Increased permeability to molecules can mean greater absorption or translocation of LPS and eventually metabolic endotoxaemia (Jergens & Allenspach, 2016; Moreira *et al*., 2012).

The non-enzymatic browning reaction known as the Maillard reaction, a chemical reaction between amino acids and sugars, often occurs in pet food processing, specifically during canned diet manufacturing. This process can cause a significant loss of protein digestibility through loss of enzyme efficacy, or through creating advanced glycation end products (AGE) and cross-linked proteins (Tuohy *et al*., 2006). Advanced glycation end products generated by Maillard reactions (Rapin & Wiernsperger, 2010) promote TNF-α and other pro-inflammatory cytokines (Vlassara *et al*., 2002). After midlife, dogs have been found to have greater concentrations of AGE in tissues that can potentially cause pathological problems due to their involvement in oxidative stress and inflammation (Bras *et al*., 2007; Chiers *et al*., 2010; Shapiro *et al*.,

2008). Research in rodent models indicate that the formation of AGEs in the diet through the Maillard reaction could increase intestinal permeability (Snelson *et al*., 2021).

1.6.2 Impact of sex

Research suggests that sex can have an impact on intestinal permeability and the ability of the intestinal barrier to withstand challenges (Teixeira *et al*., 2014). Nevertheless, in human females during the follicular phase of the menstrual cycle, which is linked to hormonal fluctuations, only marginal differences in intestinal permeability were observed in comparison to males (Snipe & Costa, 2018). Oestradiol has been shown to effectively decrease colonic paracellular permeability in rats by increasing occludin and junctional adhesion molecule-A (JAMA), proteins associated with TJ function (Braniste *et al*., 2009). Rats in oestrus have lower permeability and improved barrier function than in dioestrus, demonstrated by an increase in TEER indicative of improved TJ function in the follicular phase, which diminishes during the following luteal phase (Braniste *et al*., 2009; Homma *et al*., 2005). These results can also be mimicked by supplementing male rats with oestradiol (Homma *et al*., 2005; Kaliannan *et al*., 2018). Microbial diversity may be another cause of disparity in intestinal permeability measurements between sexes. In comparison to healthy men, healthy women exhibited reduced intestinal permeability along with greater duodenal and faecal microbial diversity (Edogawa *et al*., 2018). Gastrointestinal microbiota diversity and intestinal barrier integrity appear to exhibit greater resistance and resilience when faced with challenges such as stress, inflammation, nonsteroidal anti-inflammatory drugs (NSAID), or ischemic stroke in females, suggesting that oestradiol and microbiome diversity have protective effects on the intestinal barrier (Ahnstedt *et al*., 2020; Edogawa *et al*., 2018; Homma *et al*., 2005). The positive relationship between oestrogen and TJ protein expression, along with the increased diversity of the female gastrointestinal microbiome, contributes to enhanced barrier integrity and reduced intestinal permeability in females. Therefore, when conducting intestinal permeability studies not focused on hormonal variations, it is advisable to carry them out during the follicular phase of the menstrual cycle to enable more accurate comparisons between sexes and among individual females.

1.6.3 Bile acids

One hypothesis that attempts to explain reduced digestive capacity in ageing humans is the lower secretion of bile enzymes with age (Harper, 1998). Secretion of bile enzymes can halve in ageing rats, potentially creating a contributing factor to lower digestibility of nutrients (Hall *et al*., 2005; Handler *et al*., 1994). Dietary fats require conjugated bile acids for their absorption and may also inhibit the growth of small intestinal bacteria (Jones *et al*., 2008). Conjugated bile acids also suppress inflammation by decreasing intestinal permeability to endotoxins, which creates a problem when there is decreased conjugation or increased deconjugation (Lorenzo-Zúñiga, 2003; Parlesak *et al*., 2007). Unconjugated bile acids have been

extensively studied for their role as modulators of intestinal permeability by promoting inflammatory conditions and disrupting TJs (Calzadilla *et al*., 2022). Furthermore, increased intestinal permeability may cause conjugated bile acids to be passively absorbed, even though the intestinal barrier is normally impermeable to them, along with decreased conjugation or increased deconjugation, lead to a diminished concentration of bile acids in the small intestine (Lorenzo-Zúñiga, 2003). A smaller concentration of bile acids may cause more bacterial growth and continue the cycle of increased deconjugation to ultimately cause bacterial translocation or endotoxaemia (Lorenzo-Zúñiga, 2003).

Undigested proteins reaching the terminal ileum, as a result of proteins binding to Maillard reaction products, can serve as a substrate for bacteria and can initiate bacterial overgrowth (Rodríguez-Romero *et al*., 2022). Bacterial overgrowth is an additional cause behind increased deconjugation of bile acids, leaving them in diminished concentrations in the small intestine and creating loss through excess excretion (Ensari, 2014). When bile acids are lost before they can be reabsorbed due to bacterial overgrowth, the host loses the ability to conserve taurine which is normally recycled back into the gall bladder to form more bile acids.

To the author's knowledge, there is no record of any studies investigating the relationship between ageing and taurine transport or bile acid conjugation in cats or dogs, despite the fact that this area had been proposed for future research over 20 years ago (Harper, 1998). This lack of data further underscores the pressing need for additional research and highlights a significant gap in our understanding. Since cats and dogs can only use taurine to conjugate with bile acids, age-related changes in taurine metabolism, pancreatic enzyme secretion, and bile acid secretion may be factors in decreased nutrient digestibility (Burkholder, 1999). Bile secretion is already associated with increased intestinal permeability, small intestinal microbiota, and endotoxaemia (Moreira *et al*., 2012). Cats and dogs, especially larger dog breeds due to their reduced taurine biosynthesis rate compared to smaller breeds, are unable to synthesise taurine and must obtain it from their diet in order to conjugate it with bile acids (Anantharaman-Barr *et al*., 1994; Backus *et al*., 1994).

Intestinal microbiota growth can cause bile acid turnover (Eyssen, 1973; Hickman *et al*., 1992), which may cause overall taurine loss in cats due to the rapid conjugation of bile acids with taurine (Backus *et al*., 1994; Hickman *et al*., 1992). Adding fermentable carbohydrates to the diet stimulates microbial growth which may simply expedite the rate at which taurine is lost (Backus *et al*., 1994). Furthermore, soluble fibres in the diet lead to higher amounts of bile acids excreted in faeces according to a meta-analysis study conducted by Pezzali *et al*. (2021). This meta-analysis also concluded that higher dietary fat and protein concentrations may also lead to greater amounts of bile acids excreted in faeces causing a need for more taurine in the diet to use for conjugation (Pezzali *et al*., 2021). Anantharaman-Barr *et al*. (1994) found markedly different outcomes in taurine uptake and bile acid conjugation between canned and extruded

commercial cat foods (Anantharaman-Barr *et al*., 1994). Cats fed canned food had more primary to secondary bile acid conversion and significantly more taurine degradation to urea. A dry diet was associated with significantly greater plasma taurine levels and lesser faecal bile acid and secondary bile acid. Canned foods must contain twice as much taurine than dry kibble in order to meet dietary requirements, but these differences seemingly are related to a change in the gastrointestinal microbiota that cause excess taurine degradation (Anantharaman-Barr *et al*., 1994).

1.6.4 Gastrointestinal health modulators

The use of prebiotics, probiotics, and postbiotics to maintain or treat the gastrointestinal microbiome is widely studied with many researchers focusing on different aspects of their beneficial effects. Prebiotics are nondigestible dietary fibre sources that are substrates for gastrointestinal bacteria to digest. Probiotics are living microorganisms that reside in the GIT but can be supplemented to make a beneficial alteration to the microflora. Postbiotics are the products of the probiotic microorganisms that have fermented the prebiotic fibres (Patel & Denning, 2013). Prebiotics, probiotics, and postbiotics are considered tools to maintain a healthy microbiome, altering the composition to provide favourable effects on the intestinal barrier, immune system, digestive system, and number of pathogenic bacteria in the GIT. Not only do these exert local beneficial effects on the microbiome, they can act systemically since the GIT is connected to many parts of the body (Żółkiewicz *et al*., 2020). The gastrointestinal microbiome is already a known factor behind the structure and function of the intestinal barrier, providing potential to use pre-, pro-, and postbiotics as interventions to combat cases of increased intestinal permeability.

1.6.4.1 Prebiotics

Prebiotics can possibly stabilise the intestinal barrier (Bischoff *et al*., 2014). They can also change the gastrointestinal microbiota in a way that stimulates the endocrine and endocannabinoid system in the intestine to support a decrease in intestinal permeability (Delzenne *et al*., 2011). This occurs through an increase in glucagon-like peptide one (GLP-1) and GLP-2 and increased expression of TJ proteins ZO-1 and occludin, which inhibit over-absorption of LPS (Delzenne *et al*., 2011).

In cats, prebiotic administration of a fructooligosaccharide (FOS) and inulin (another prebiotic source of FOS), significantly increased an unknown species of Veillonellaceae, significantly decreased an unknown species of Gammaproteobacteria, and in two feline subjects was associated with increased Lactobacillales (Garcia-Mazcorro *et al*., 2017). Fructooligosaccharide and FOS plus galactooligosaccharide (GOS) supplementation increased the amounts of SCFAs and beneficial microbes, and also increased faecal *Bifidobacterium* spp., butyrate, valerate, and improved other parameters of digestive health in cats, respectively (Barry *et al*., 2012; Barry *et al*., 2010; Kanakupt *et al*., 2011). Senior cats supplemented with dried chicory root, a source of inulin, along with antioxidants and PUFAs, appeared to have a healthier

gastrointestinal microbiome with a better *Bifidobacterium* and *Lactobacillus* to *C. perfringens* population, exhibited better gastrointestinal health, and lived significantly longer than a group on a complete, balanced, control diet, or a group fed antioxidants only to the control diet (Cupp *et al*., 2006). Chicory root predominantly consists of inulin, but also contains polyphenols, specifically flavonoids, which may also have beneficial effects on ageing cats (Cupp *et al*., 2006). As a whole, the feline microbiome is highly conserved and prebiotics have been considered to have little effect on the GIT metagenome (Barry *et al*., 2012), although effects varying considerably between individuals (Garcia-Mazcorro *et al*., 2017).

Inulin inclusion in the diet of healthy adult dogs significantly increased *Lactobacillus* and faecal bifidobacteria concentrations when supplemented to senior and geriatric dogs in the form of chicory root (Beloshapka *et al*., 2013; Grieshop *et al*., 2004). Mannan-oligosaccharides (MOS) supplemented to senior and geriatric dogs can also increase faecal bifidobacteria concentration as well as decreasing faecal *Escherichia coli* concentration (Grieshop *et al*., 2004). Lactobacilli and bifidobacteria populations significantly increased in healthy adult dogs treated with FOS who also experienced a reduction of *C. perfringens* (Swanson *et al*., 2002). Short-chain FOS supplementation to obese dogs increases faecal concentrations of butyrate which is a beneficial SCFA to the intestinal barrier (Apper *et al*., 2020; Peng *et al*., 2009). In healthy, adult sled dogs, concentrated brewer's yeast with a β-glucan dose of 7 mg/kg BW daily tended to improve intestinal permeability and decreased haptoglobin (Rummell *et al*., 2022).

1.6.4.2 Probiotics

Probiotics can alter the composition of the gastrointestinal microbiome; with some supporting the proliferation of beneficial bacteria and reducing the concentrations of pathogens. These beneficial bacteria, in turn, can increase epithelial cell regeneration, TJ protein expression, and a number of antimicrobial peptides which maintain or benefit the integrity of the intestinal barrier (Gou *et al*., 2022). Use of a probiotic combination of bifidobacteria, lactobacilli, and streptococci can restore normal colonic intestinal permeability in IL-10 deficient mice and even reduce it in control mice (Madsen *et al*., 2001). Supplementation of a lactobacilli probiotic lowers lactulose to mannitol ratio (LM) ratio in children with atopic dermatitis suggesting probiotics may stabilise intestinal barrier function, reduce small intestinal permeability, decrease systemic inflammation, and decrease gastrointestinal symptoms (Rosenfeldt *et al*., 2004). *Lactobacillus plantarum* had a positive effect on gene expression of TJ proteins occludin, ZO-1, ZO-2, and cingulin in Caco-2 cell monolayers, which may strengthen TJ integrity and improve overall barrier function (Anderson *et al*., 2010; Karczewski *et al*., 2010; Qin *et al*., 2009). *Lactobacillus rhamnosus* CNCM I-3690 acts as an anti-inflammatory commensal that also upregulates occludin and E-cadherin expression, improving intestinal barrier integrity and function in Caco-2 monolayer cells and an *in vivo* mouse model (Laval *et al*., 2015). *Lactobacillus acidophilus* was determined to be an effective probiotic at improving TJs

and overall intestinal barrier function during *in vitro* cell and *in vivo* mice trials, showing a significant increase in TEER compared to over 20 other *Lactobacillus* and *Bifidobacterium* probiotics (Al-Sadi *et al*., 2021). It is important to note that these results were very strain-specific and only one *L. acidophilus* strain effectively protects the barrier and prevents an increase in permeability to this extent. *Streptococcus thermophilus* and *L. acidophilus* probiotic mixes successfully stop TNF-α and IFN-γ from increasing intestinal permeability in human intestinal epithelial cells (Resta–Lenert & Barrett, 2006). A probiotic mixture of *S. thermophilus*, *Bifidobacterium* strains, and *Lactobacillus* strains supplemented to young senescence-accelerated prone mice (SAMP) who had not previously experienced chronic intestinal inflammation provided a preventative effect against increased paracellular permeability (Corridoni *et al*., 2012). This probiotic mixture increased TEER and occludin, while decreasing claudin-2 expression, but did not have the same effects in mice with the control parental strain, making researchers hypothesise that perhaps certain genes in SAMP mice allow for these beneficial effects in response to the probiotic mix. *Bifidobacterium lactis* given to preterm infants with increased intestinal permeability lowered the LM during a differential sugar absorption test, signifying a decrease in permeability (Stratiki *et al*., 2007).

In companion animal nutrition, the common probiotics recognised include *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* species (Wernimont *et al*., 2020). A recent study added commercial kefir to the diet of Angora cats and recorded significant increases in lactococci, lactobacilli, yeast, and total mesophilic aerobic bacteria, and a decrease in enterococci, without affecting other aspects of health including haematological parameters, most biochemical parameters, BW, body condition, and faecal scores (Kabakçi *et al*., 2022). Kefir is a fermented milk product, and therefore also contains milk oligosaccharides and SCFA that could have contributed to these outcomes (Kabakçi *et al*., 2022). Healthy cats supplemented with multiple strains of the probiotic *L. acidophilus* have shown an increase in faecal *L. acidophilus* and other *Lactobacillus* spp. and a reduction in faecal coliform, *Clostridium* spp. and *Enterococcus faecalis* (Fusi *et al*., 2019; Marshall-Jones *et al*., 2006).

The probiotic *B. animalis* AHC7 had success reducing total faecal Clostridia and *Clostridium difficile* concentrations, but no effect on *Bacteroides*, *E. coli*, Lactobacilli, or Bifidobacteria (O'Mahony *et al*., 2009). In a study completed by White *et al*. (2017), dogs with idiopathic inflammatory bowel disease (IBD) were assigned a standard therapy of diet and prednisone or therapy with a multi-strain probiotic containing *L. plantarum*, *S. thermophilus*, *Bifidobacterium breve*, *L. acidophilus*, *Bifidobacterium longum*, and *Bifidobacterium infantis*. Dogs receiving the probiotic had a higher population of mucosal *Lactobacillus* spp., and higher expression of E-cadherin, occludin, and zonulin indicating that the probiotic may have beneficial effects on the intestinal barrier (White *et al*., 2017).

1.6.4.3 Postbiotics

Postbiotics have been defined by the International Scientific Association of Probiotics and Prebiotics as an inactivated preparation of microorganisms or their components that provides health benefits to the host and is not limited to the GIT (Salminen *et al*., 2021). Postbiotic metabolites are an area of research interest in humans and rodents with little research in the field of companion animal nutrition, especially in regard to their effects on the intestinal barrier. However, one study in dogs supplemented with a *Lactobacillus* fermentation product, *Limosilactobacillus fermentum* and *L. delbrueckii*, did show a significant decrease in the concentration of circulating IgM compared to controls from baseline (Koziol *et al*., 2023).

In other species and cell cultures, postbiotics have promising effects on the intestinal barrier. *L. plantarum* RG14, previously mentioned as having beneficial probiotic effects on the intestinal barrier, exhibits similar effects administered as a postbiotic supernatant to weaned lambs. Lambs in the postbiotic group experienced increased TJ protein-1, claudin-1, and claudin-4 mRNA as well as an upregulation of IL-6 and a downregulation of IL-1β, IL-10, and TNF mRNA. These alterations point to a strengthening of the intestinal barrier and a stimulation of the immune system (Izuddin *et al*., 2019). Gao *et al*. (2019) studied the effects of the HM0539 protein from *L. rhamnosus* GG in neonatal rats and in an intestinal epithelial cell line. The postbiotic helped strengthen occludin and ZO-1 expression, and intestinal mucin secretion against LPS and TNF-α, overall reducing intestinal permeability (Gao *et al*., 2019). The SCFA butyrate has been extensively studied for its role as a postbiotic that improves intestinal barrier function. In Caco-2 cells, butyrate is shown to regulate claudin-2 expression, aid in the assembly of other TJ proteins, reduce uptake of mannitol, and improve the TEER of a membrane challenged by TNF-α, IFN-γ, and IL-13 (Huang *et al*., 2021; Mariadason *et al*., 1997; Peng *et al*., 2009). Indole-3-propionate (IPA) has several properties that contributed to improved intestinal health in *in vivo* trials. Overall, it improves intestinal homeostasis and barrier integrity by lowering TNF-α and increasing epithelial junctional complex protein encoding mRNA expression in mice (Alexeev *et al*., 2018; Venkatesh *et al*., 2014). In HCT-8 cells, indole downregulated pro-inflammatory factors TNF-α, NF-κB, and IL-8, while upregulating IL-10, additionally causing a reduction in inflammation and increased epithelial TJ integrity (Bansal *et al*., 2010). In mice, when intestinal permeability is increased due to a high fat diet, supplementation of IPA leads to a decrease of LPS in circulation and reduced intestinal permeability (Galligan, 2018; Jennis *et al*., 2018). Polyamines facilitate TJ protein expression and are a key component of sustaining intestinal barrier function, with reduced concentrations resulting an increase of permeability (Rao *et al*., 2020; Timmons *et al*., 2014).

1.7 Conclusions

Intestinal permeability and absorptive capacity have been well studied in humans, which has led to the term "leaky gut" becoming popularised in the 21st century. However, the implications and cause of this phenomenon remain relatively unresearched in companion animals, specifically cats. A more standardised methodology must be created to allow accurate comparison between new research and allow meaningful conclusions to be made.

There is little data available on specific age-related effects on inflammatory pathways in cats and dogs, leaving us with limited insights into how these processes manifest in companion animals. Additionally, the exploration of intestinal barrier function in these species remains relatively uncharted territory, although findings in humans and rodents suggest a potential link between pro-inflammatory cytokines and increased intestinal permeability. Furthermore, while promising biomarkers show potential for diagnosing intestinal inflammation, they often fail to pinpoint the underlying causes. Closing these knowledge gaps is essential to advancing the understanding of age-related health issues in dogs and cats and developing effective strategies for their care and well-being.

The gastrointestinal microbiome plays a crucial role in maintaining the metabolic and immune functions of its host, including companion animals. However, changes in the gastrointestinal microbiome may contribute to issues in the GIT of older animals. The age-related alterations in bacterial populations, such as decreased microbial diversity and changes in specific bacterial groups, are associated with potential shifts in intestinal permeability and digestive capacity. Despite limited research on age-related changes in the gastrointestinal microbiome of companion animals, it is suggested that these changes may influence how older animals respond to diseases and absorb nutrients. The relationship between the gastrointestinal microbiota and intestinal permeability is of growing interest, with emerging evidence suggesting that microbiota changes may contribute to increased permeability, especially in ageing individuals.

With a deeper understanding as to how age affects the many different digestive processes in the cat and dog, pet food formulations can begin to advance towards improved overall welfare and longevity.

1.8 Scientific aims

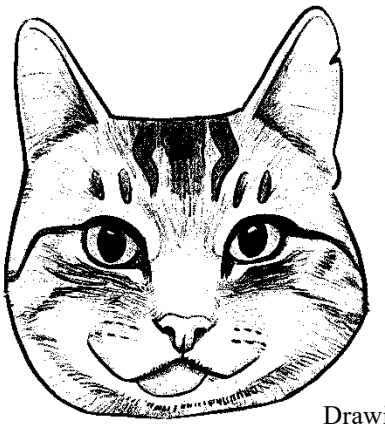
The goal of this thesis was to ultimately determine the relationship between intestinal permeability and absorptive capacity with age. The reason behind doing so was to assess how the gastrointestinal health of senior cats' changes and potentially find a reason behind their reduced digestive capacity. This initially required developing a method to quantify both measures in the cat using minimally invasive techniques with the expectation that this method would standardise the measurement of these two GIT health parameters in the future. Therefore, the first chapter of this thesis seeks to review the previous literature and give the background behind these two measurements and other variables that could impact GIT or more specifically, digestive health. Following that, Chapter 2 addresses the design and testing of a new method, based on previous research done in cats and other species. Once a method was developed that could effectively quantitate intestinal permeability and absorptive capacity in the cat, with a small error range, the main goal of the thesis could be investigated.

To answer the primary research question "*does intestinal permeability increase and absorptive capacity decrease with age*?", a simple comparative study using young and senior cats was completed in Chapter 3. The results of this study would determine the next scientific aim to continue to research GIT health in the ageing cat.

Informed by the results of Chapter 3, a study on a group of midlife to super-senior cats was carried out. The aim of this was to evaluate three things. Firstly, since intestinal permeability does increase in older cats, is it associated with reduced digestive function? Secondly, how are microbial metabolites influenced by increased intestinal permeability and age? And lastly, will a probiotic that helps improve the gut barrier in other species, have the same effects on ageing cats?

Chapter 2

Development and validation of an LC-MS/MS method for the quantification of oral-sugar probes in plasma to test small intestinal permeability and absorptive capacity in the domestic cat (*Felis catus*)



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Chapter 2: Development and validation of an LC-MS/MS method for the quantification of oral-sugar probes in plasma to test small intestinal permeability and absorptive capacity in the domestic cat (*Felis catus*)

2.0 Abstract

A novel method for quantifying the concentration of lactulose, rhamnose, xylose, and 3-OMG in cat plasma using LC-MS was developed. Domestic male cats (n = 13) were orally dosed with a solution containing the four sugars to test the permeability and absorptive capacity of their intestinal barrier. Plasma samples were taken 3 hours later and were prepared with acetonitrile (ACN), dried under N2, and reconstituted in 90 % ACN with 1 mM ammonium formate. Stable isotope labelled ^{13}C standards for each analyte were used as internal standards. Chromatographic separation was conducted using a Phenomenex Luna NH_2 column with a gradient elution system of deionised water and 90 % ACN with 1 mM ammonium formate at 300 µL/minutes for 13 minutes total analysis time. Recovery trials were conducted in triplicate over 3 days with RSD values (%) for each day ranging from 1.2 to 1.4 for lactulose, 5.4 – 6.0 for rhamnose, 3.3 – 5.5 for xylose, and 2.6 – 5.6 for 3-OMG. Inter-day variations for each analyte were not different ($p > 0.05$). Limit of detection and quantification were 0.2 and 0.7 µg/mL for lactulose, 0.8 and 2.4 µg/mL for rhamnose, 0.6 and 1.8 µg/mL for xylose, and 0.3 and 1.1 µg/mL for 3-OMG, respectively. Plasma sugar concentrations recovered from cats were above the limit of quantification and below the highest calibration standard, validating the use of this method to test intestinal permeability and absorptive capacity in cats.

2.1 Introduction

The intestinal barrier plays a critical role in maintenance of normal physiologic processes of the digestive system, maintaining homeostasis in the GIT. It enables the selective permeability of molecules, allowing for absorption of nutrients and water, but prohibiting the passage of pathogens and other potential toxins. These molecules can cross the barrier through different paracellular, transcellular, carrier-mediated active transport, and ATP-dependent mediated active transport pathways, depending on their size and charge. The paracellular route is mediated by tight junctions between epithelial cells which facilitate the transport of ions, water, and larger hydrophilic compounds, in contrast to the transcellular route which permits small hydrophilic and lipophilic compounds to pass through the plasma membrane of the epithelial cell. Active transport pathways are reserved for the larger nutrients and molecules that require energy to move across the epithelial barrier, a characteristic also known as the absorptive capacity (Farré *et al*., 2020).

Ageing domestic cats experience a reduced ability to digest nutrients, specifically fat and protein, with no clear cause (Perez-Camargo, 2003). The relationship between age and intestinal permeability has not been examined in cats, offering a potential new perspective for investigation of this phenomenon. There is conflicting evidence whether intestinal permeability increases with age in other species, where it seems to be supported in fruit flies, mice, rats, and baboons, but not in dogs and humans (Katz *et al*., 1987; Ma *et al*., 1992; Thevaranjan *et al*., 2017; Tran & Greenwood-Van Meerveld, 2013; Weber *et al*., 2002; Wilms *et al*., 2020). Exploring the potential relationship between age and intestinal permeability will enhance our fundamental understanding of the domestic cat's gastrointestinal system, enabling researchers to investigate interventions that could ultimately enhance the quality of life for senior cats.

A common method to quantify the permeability and absorptive capacity of the small intestine is the differential sugar absorption test (SAT). A differential SAT involves administering a monosaccharide and a disaccharide, such as rhamnose and lactulose respectively, to measure transcellular and paracellular transport, respectively (Fleming *et al*., 1996; Lostia *et al*., 2008; Randell *et al*., 2001; Steiner *et al*., 2002). Non-metabolisable monosaccharides, xylose and 3-OMG, can be orally administered as well to determine the mucosal absorptive capacity (Rodríguez *et al*., 2009; Zhang *et al*., 2017). Several studies have evaluated intestinal permeability in the cat, using varying SAT methodologies as the basis of investigation. These studies have all used different doses of disaccharides and monosaccharides and chose to analyse urine, which is harder to collect aseptically than plasma. Additionally, the sugar concentration in the urine was quantified using various methods including high-performance liquid chromatography (HPLC), gas-liquid chromatography (GLC), and enzymatic assay (ENL) (Bijlsma *et al*., 1995; Johnston *et al*., 2001; Marks *et al*., 1999; Papasouliotis *et al*., 1993; Randell *et al*., 2001). Emms *et al*. (1982) did however analyse the concentration of D-xylose in plasma to determine absorptive capacity in the cat using a method described

by Trinder (1975) involving phloroglucinol (Emms *et al*., 1982; Trinder, 1975). Unfortunately, while Emms *et al*. (1982) and Randell *et al*. (2001) both successfully orally syringed the sugar solution to cats without sedation, these two studies do not clearly explain the protocol, making it hard to duplicate, especially given the great differences in the dose of probes used between them.

The differential SAT can also be performed using serum and plasma samples, as shown in multiple studies in humans and dogs (Bruet *et al*., 2008; Fleming *et al*., 1996; Lukanc *et al*., 2017; Rodríguez *et al*., 2009; Tina *et al*., 2011). Obtaining sterile urine and free catch urine samples are difficult in cats since cystocentesis is invasive as well as challenging to do on a partially filled bladder and they do not urinate on command, thus blood-based intestinal permeability tests in cats may be easier to conduct. A pilot study using sugars lactulose, rhamnose, xylose, and 3-OMG in the domestic cat was undertaken to determine the feasibility of using this multi-sugar procedure in a larger-scale study. Due to the lack of replicable and reliable oral sugar probe and intestinal permeability studies completed in cats, this study was also required to develop and evaluate the methodology and the sensitivity of the proposed laboratory analysis of feline blood samples. The aim of this study was to develop a quantitative LC-MS/MS method measuring the oral sugar probes in plasma using stable isotope standards. Benefits of such a method include less invasive and more reliable sampling, and thus ethically less expensive means of evaluating intestinal permeability and absorptive capacity in cats.

2.2 Materials and methods

2.2.1 Ethics

The preliminary validation pilot study and subsequent research trial were both approved by the Massey University Animal Ethics Committee (MUAEC 22/69 and 23/14) which meets the requirements of the Animal Welfare Act ("Animal Welfare Act," 1999). Cats selected for each experiment continued to be provided with their normal commercially canned wet cat food (Heinz Wattie's Ltd., Hastings, New Zealand) which is complete and balanced according to Association of American Feed Control Officials (AAFCO) requirements for adult cats. They were fed *ad libitum* daily in their enclosures leading up to the trial and fasted in metabolic cages, as described by Hendriks *et al*. (1999a), for 12 hours before sugar probe ingestion with access to fresh water at all times.

2.2.2 Animal studies

This study was split into two parts. The first trial aimed to determine the feasibility and palatability of giving cats a single oral dose of sugar solution and was then followed by a second pilot trial whereby the detection of the sugar probes in plasma was verified.

In order to assess the feasibility of dosing adult cats a sugar solution containing 0.07 g/mL lactulose, 0.02 g/mL rhamnose, 0.07 g/mL xylose, and 0.02 g/mL 3-OMG (1160 mOsm) was mixed in purified water. Six, neutered, male adult (n=6) domestic shorthair cats aged 5.0 to 8.9 years (mean ± SD, 5.8 ± 1.8 years) and weighing from 3.7 to 5.2 kg (4.3 ± 0.6 kg) received a single oral dose of 2 mL/kg.

All cats tolerated the dosing protocol well and the full dose was administered. From this, the pilot study was undertaken. Following an overnight fast, 2 mL of blood was taken from 13 healthy male adult cats (mean age ± SD (range), 2.5 ± 0.8 (1.3-3.4) years) via jugular venepuncture (T_0). The cats were then dosed with 2 ml/kg of the sugar probe solution (0.07 g/mL lactulose, 0.02 g/mL rhamnose, 0.07 g/mL xylose, and 0.02 g/mL 3-OMG; osmolarity 1160 mOsm). After 3 hours, 2 mL blood samples were collected again from the opposite jugular vein, and the cats were permitted to return to their regular enclosures where they had free access to both food and water.

The dose of the sugar solution made of lactulose, rhamnose, xylose, and 3-OMG was extrapolated from previous studies in cats and dogs (Bruet *et al*., 2008; Emms *et al*., 1982; Randell *et al*., 2001; Rodríguez *et al*., 2009; Rutgers *et al*., 1995; Sørensen *et al*., 1993; Weber *et al*., 2002). The sugar solution had an osmolality of 1160 mOsm, which is under the threshold of 1500 mOsm when hypertonic solutions begin to increase permeability (Laker & Menzies, 1977). The final blood samples were collected from the cats 3 hours after oral dosing based on findings from multiple studies that blood collected at this time is the most consistent between samples (Bruet *et al*., 2008; Rodríguez *et al*., 2009; Sørensen *et al*., 1997).

2.2.3 Chemicals and standards

$^{13}C_{12}$ lactulose was purchased from Santacruz Biotechnology Inc (Santa Cruz, USA), $^{12}C_6$ 3-OMG from Toronto Research Chemicals (Toronto, Canada), $^{13}C_5$ xylose, $^{13}C_6$ 3-OMG, and $^{13}C_6$ rhamnose from Omicron Biochemicals Inc (South Bend, USA), ^{12}C lactulose, ^{12}C rhamnose, ^{12}C xylose, acetonitrile (HPLC grade), and ammonium formate (HPLC grade) from Sigma-Aldrich (Auckland, New Zealand). Ultrapure water was prepared with a Milli-Q® ultrapure water system (Merck, Auckland, New Zealand) with a resistivity of 18.2 MΩ.

A mixed internal standard solution containing the four ^{13}C labelled sugars was made from stock solutions of individual ^{13}C sugars in 90 % ACN to yield a final concentration of 50 µg/mL. Similarly, a mixed standard of unlabelled sugars was made from stock solutions of individual target sugars in 90 % ACN to yield a final concentration of 1 mg/mL. This mixed standard, along with the mixed internal standard solution, was used to make the calibration standards (in 90 % ACN) for calculating concentration and testing linearity (Table 2.1) and preparing fortified samples for recovery and repeatability experiments. A seven-

point calibration series was prepared containing 0, 1, 5, 10, 20, 50 or 100 µg/mL of the target unlabelled sugars, and 5 µg/mL of the ^{13}C labelled sugars.

Table 2.1. Parallel reaction monitoring inclusion list

Name	Mass (m/z)	Isolation width (m/z)	Adduct	Start	End	(N)CE
Xylose	149	2	[M-H]-	1.5	6	10
$^{13}C_5$ xylose	154	2	[M-H]-	1.5	6	10
Rhamnose	163	2	[M-H]-	1.5	6	10
$^{13}C_6$ rhamnose	169	2	[M-H]-	1.5	6	10
3-OMG	239	2	[M+HCO2]-	1.5	6	10
$^{13}C_6$ 3-OMG	245	2	[M+HCO2]-	1.5	6	10
Lactulose	387	2	[M+HCO2]-	6	11	10
$^{13}C_{12}$ lactulose	399	2	[M+HCO2]-	6	11	10

Abbreviations: 3-OMG, 3-O-methylglucose; NCE, normalised collision energy

2.2.4 Sample preparation

Whole blood was transferred to a K2 EDTA 5.4 mg vacutainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and spun at 3000 x *g* at 4 °C for 15 minutes. Plasma was then aliquoted into microcentrifuge tubes and frozen at −80 °C until analysis.

Plasma samples (200 µL) were thawed, then mixed with ice cold acetonitrile (ACN; 590 µL) and spiked with an internal standard mixture (10 µL) containing 50 µg/mL of each of the four ^{13}C labelled sugars. Samples were then vortexed thoroughly, incubated (−20 °C, 60 minutes), vortexed again, and centrifuged (14,000 x *g*, 10 minutes, 4 °C). Then, 100 µL of purified water was added to each sample, vortexed, and centrifuged (14,000 x *g*, 10 minutes, 4 °C) for a second time. A fixed volume of the supernatant (800 µL) was removed and then dried under nitrogen at 35 °C, and the dry extract was reconstituted in 100 µL 90 % ACN with 1 mM ammonium formate.

2.2.5 Preparation of fortified samples

Plasma samples collected before oral administration of sugar solution (T_0) were pooled into a composite sample and aliquots were spiked with a known amount of the target sugars, then subjected to the extraction protocol (as described in section 2.2.4). The experiment was conducted using no spike, low spike, and high spike plasma samples in triplicate. No spike samples were unaltered blank plasma matrix, low spike samples were 5 µL of mixed sugar standard (^{12}C) with 995 µL of blank plasma (concentration: 5 µg/mL), and high spike samples were 25 µL of mixed sugar standard (^{12}C) with 975 µL of blank plasma (concentration: 25 µg/mL). Blank plasma was unaltered T_0, fasted plasma samples that served as a control.

2.2.6 LC-MS/MS conditions

LC-MS/MS was carried out using a Thermo Scientific Accela HPLC system coupled to a Q Exactive Orbitrap mass spectrometer with electrospray ionisation (ESI) (Thermo Scientific, New Zealand). Samples were introduced to the HPLC system via a PAL autosampler (injection volume = 5 µL), and chromatography was performed using a Phenomenex Luna NH2 column (2 x 150 mm, 3 µm, Phenomenex, New Zealand) with a gradient elution of deionised water with 1 mM ammonium formate (eluent A) and 90 % ACN with 1 mM ammonium formate (eluent B) at a flow rate of 300 µL/minute. The gradient elution program was as follows: 100 % B (0 – 0.5 minutes), 100 – 85 % B (0.5 – 3.5 minutes), 85 % B (3.5 – 11 minutes), and a re-equilibration period at 100 % B for 3 minutes (11 – 13 minutes). The first 1.5 minutes of the chromatographic run was diverted to waste and data was acquired from 1.5 – 11 minutes. The mass spectrometer was operated in negative ionisation mode and data was acquired in full scan MS1 mode (50 – 500 m/z) and parallel reaction monitoring (PRM) mode monitoring the MS2 using dynamic first mass for all samples. The ESI source was operated with a spray voltage of 4.5 kV, capillary temperature of 350 °C, sheath gas flow of 20 L/ minute, aux gas flow of 10 L/ minute and sweep gas flow of 5 L/ minute. The PRM mode was operated with the inclusion list and relevant parameters in Table 2.1.

Data acquisition and processing was carried out using Thermo Xcalibur software (version 3.1.66.10; Thermo Fisher Scientific). Regression statistics and standard deviation of the equation were calculated using Microsoft Excel (Office 360, Microsoft Corporation, Redmond, Washington, United States).

2.2.7 Calculations

Concentration of sugars in the sample (Ci) was calculated by using the concentration measured by the instrument (Cf) and adjusting for factors introduced during sub-sampling (Sf) and during extraction such as the pre-concentration factor (Pf). The equation is shown in equation (1) below:

$$Ci = \frac{Cf * \frac{1}{Sf}}{Pf} = \frac{Cf * \frac{9}{8}}{2} = \frac{Cf * 9}{8 * 2} = \frac{9Cf}{16} \qquad (1)$$

2.3 Results

Quantification for xylose, 3-OMG, and lactulose was performed using MS1 accurate mass peak areas, including molecular ions and any reliable adduct ions to increase sensitivity. MS1 masses 161.0434 and 167.0634 were observed in authentic standards and target samples for ^{12}C 3-OMG and ^{13}C 3-OMG, respectively, and have been previously used to quantify lactulose (Kubica *et al*., 2012). ^{12}C rhamnose and ^{13}C rhamnose were solely characterised and quantified in MS2. Exact ions used for quantitation are shown

in Table 2.2 with a mass tolerance of 10.0 ppm. Identifications confirmed by monitoring the MS2 spectra for characteristic fragment ions (Figure. 2.1) and retention time compared to authentic standards.

Table 2.2. Ions measured for each analyte

Analyte	Ion	MS1 (m/z)	MS2 (m/z)
^{12}C xylose	$[M-H]^-$	149.0432	
	$[M+HCOO]^-$	195.0488	
	$[M+Cl]^-$	185.0202	
^{13}C xylose	$[M-H]^-$	154.0600	
	$[M+HCOO]^-$	200.0657	
	$[M+Cl]^-$	190.0370	
^{12}C 3-OMG		161.0434	
	$[M+HCOO]^-$	239.0751	
	$[M+Cl]^-$	229.0462	
^{13}C 3-OMG		167.0634	
	$[M+HCOO]^-$	245.0951	
	$[M+Cl]^-$	235.0666	
^{12}C lactulose	$[M-H]^-$	341.1061	
	$[M+HCOO]^-$	387.1112	
	$[M+Cl]^-$	377.0825	
^{13}C lactulose	$[M-H]^-$	353.1464	
	$[M+HCOO]^-$	399.1519	
	$[M+Cl]^-$	389.1230	
^{12}C rhamnose			59.0123
^{13}C rhamnose			61.0190

Abbreviations: 3-OMG, 3-O-methylglucose

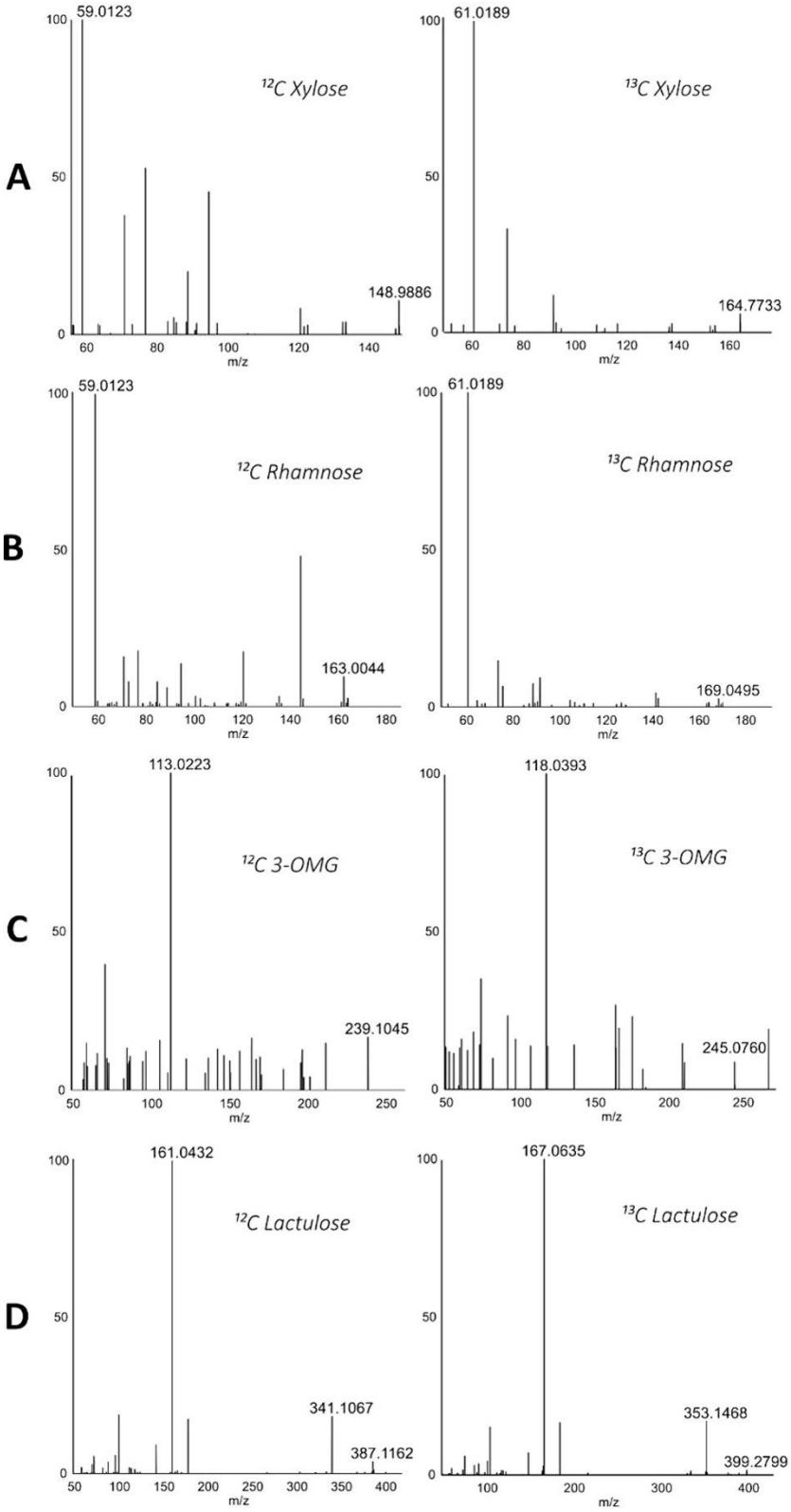

Figure 2.1. MS2 spectra for (A) xylose, $^{13}C_5$ xylose, (B) rhamnose, $^{13}C_6$ rhamnose, (C) 3-O-methylglucose, $^{13}C_6$ 3-O-methylglucose, and (D) lactulose, $^{13}C_{12}$ lactulose.

2.3.1 Tandem mass spectrometry detection

MS2 spectra of the four analytes along with their internal standards are presented in Figure 2.1. MS2 fragmentation patterns for ^{12}C xylose, rhamnose, 3-OMG, and lactulose are presented in Figure 2.2, including predicted fragment ions from the MetFrag tool (Ruttkies *et al*., 2016). It is important to note that ^{13}C standards follow the same fragmentation pattern, but exhibit a mass shift relative to the number of ^{13}C atoms present.

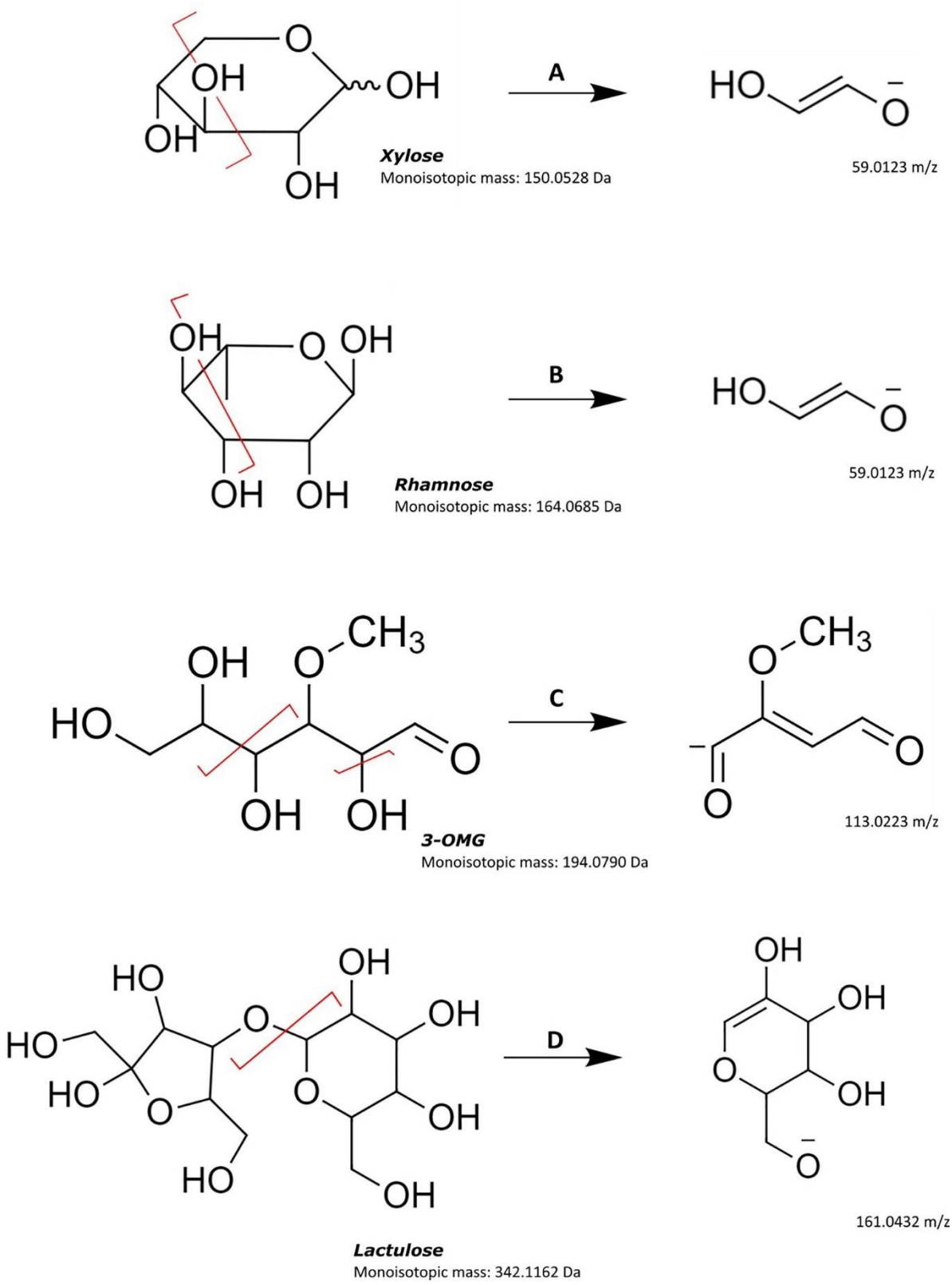

Figure. 2.2. (A) Xylose, (B) rhamnose, (C) 3-O-methylglucose (3-OMG), and (D) lactulose fragments used for identification and quantification. Fragments predicted using the in silico fragmenter tool, MetFrag (Ruttkies *et al*., 2016).

2.3.2 Chromatographic separation

Chromatographic separation of the sugars was successfully achieved between ≈3.5 and 9 minutes. While the peaks for both analyte and internal standard of xylose and rhamnose suffered some minor tailing, this did not affect linearity, recovery, or repeatability for these sugars (Table 2.3). Chromatograms of all 4 analytes and the 4 internal standards in the level 4 calibration standard mixture (concentration of ^{13}C internal standards at 5 µg/mL and target analytes at 20 µg/mL) are shown in Figure 2.3.

Table 2.3. Linearity, limit of detection and limit of quantitation

Analyte	Equation	R^2	LOD (µg/mL)	LOQ (µg/mL)
Lactulose	$y = 0.171145x - 0.0475757$	0.9997	0.2	0.7
Rhamnose	$y = 0.136588x + 0.0198997$	0.9990	0.8	2.4
Xylose	$y = 0.134708x + 0.0888231$	0.9991	0.6	1.8
3-OMG	$y = 0.155246x - 0.0730001$	0.9995	0.3	1.1

Abbreviations: LOD, limit of detection; LOQ, limit of quantitation; 3-OMG, 3-O-methylglucose

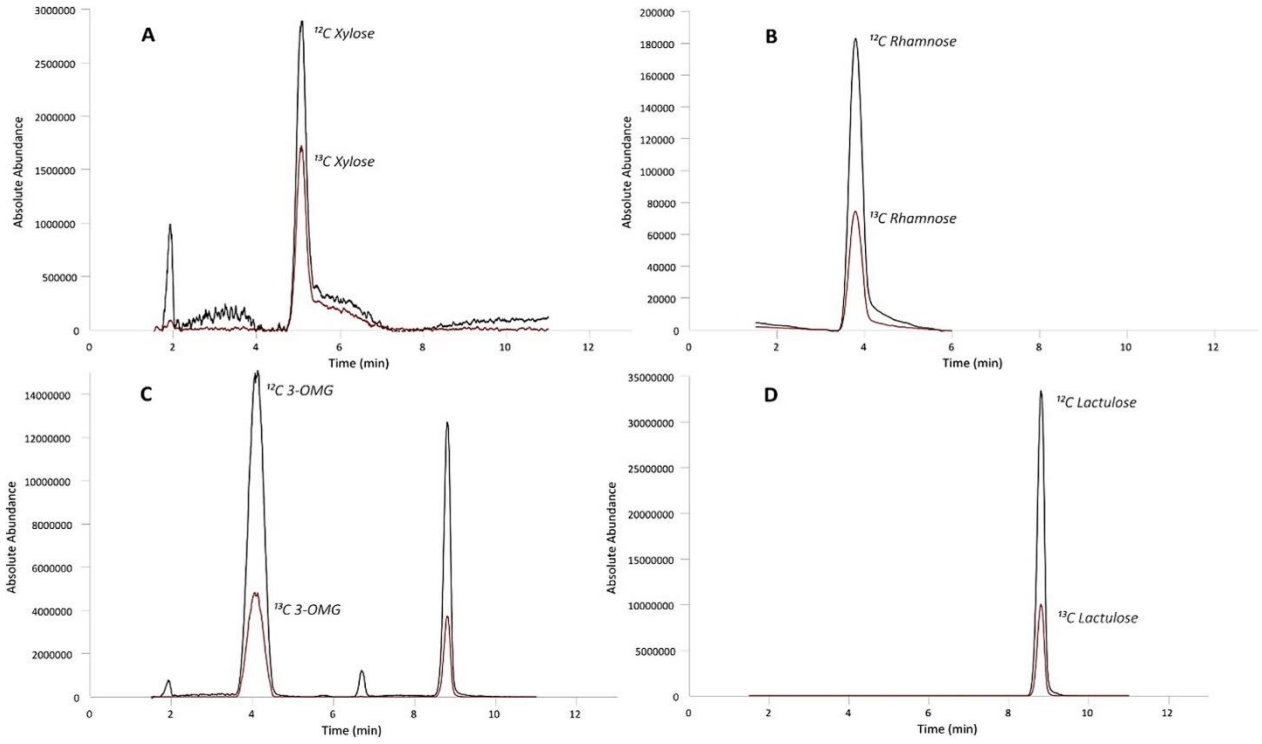

Figure 2.3. Chromatograms of analytes and internal standards, (A) xylose, (B) rhamnose, (C) 3-O-methylglucose (3-OMG), and (D) lactulose, in a calibration standard mixture containing 20 µg/mL detected by negative ionisation electrospray and MS/MS. Concentration of ^{13}C internal standards are 5 µg/mL.

2.3.3 Linearity, LOQ, and LOQ

Limit of Detection (LOD) and Limit of Quantitation (LOQ) for each sugar was calculated by using the standard deviation of the response of the blank (σ) and the slope (S) of the calibration curve. The LOD and LOQ were calculated from these values using equations (2) and (3) respectively. The linearity and sensitivity values for the method are summarised in Table 2.3.

$$LOD = 3.3 \times \frac{\sigma}{S} \qquad (2)$$

$$LOQ = 10 \times \frac{\sigma}{S} \qquad (3)$$

2.3.4 Accuracy, intermediate precision, and repeatability

Trueness, repeatability, and intermediate precision of the method were tested using the spiked samples at two concentration levels (low and high, 5 and 25 µg/mL respectively) and unfortified T_0 samples. The two spike levels and matrix blank were each analysed in triplicate within the same day and across 3 days. The difference in mean recovery was significantly different based on concentration ($p < 0.05$). Trueness and repeatability of this method met the acceptance criteria (RSD < 10 % and inter-day variation with $p > 0.05$) for the analysis of samples from the main study (Table 2.4). The mean and intermediate precision of the intestinal permeability (LR) and absorptive capacity (XG) was calculated for each spike level and the combined total (Table 2.5).

Table 2.4. Repeatability study, mean recovery, standard deviation, relative standard deviation, and significance of inter-day variation

Analyte	Day	Mean ER* (%)	SD	RSD	Inter-day Variation (ANOVA)
Lactulose	Day1	112.2	1.6	1.4	
	Day2	111.7	1.4	1.2	
	Day3	112.5	1.6	1.4	
	All Days	112.1	1.5	1.3	p = 0.66
Rhamnose	Day1	106.2	5.7	5.4	
	Day2	105.7	6.3	6.0	
	Day3	107.7	5.9	5.5	
	All Days	106.5	5.7	5.3	p = 0.84
Xylose	Day1	109.6	6.0	5.5	
	Day2	109.0	5.6	5.1	
	Day3	109.5	3.6	3.3	

	All Days	109.4	4.9	4.4	p = 0.97
3-OMG	Day1	97.7	5.5	5.6	
	Day2	97.5	5.3	5.4	
	Day3	99.7	2.6	2.6	
	All Days	98.3	4.5	4.6	p = 0.67

*Number of samples used to calculate the mean per day was 6 (3 samples from each spike level). Abbreviations: ER, extraction recovery; 3-OMG, 3-O-methylglucose.

Table 2.5. Mean and intermediate precision of intestinal permeability (LR) and absorptive capacity (XG)

Measure	Spike Level	Mean	RSD
LR	Low	1.1	4.4
	High	1.0	0.7
	Total	1.1	5.2
XG	Low	1.1	2.8
	High	1.1	3.2
	Total	1.1	2.9

Abbreviations: LR, lactulose: rhamnose; XG, xylose: 3-O-methylglucose.

Recovery was calculated based on the concentration measured in the spiked sample (C_s), blank sample (C_b), and the theoretical known concentration (C_a). C_s and C_b were calculated as described in section 2.2.7, and the extraction recovery percentage (ER %) was then estimated by equation (4):

$$ER\ \% = \left(\frac{C_s - C_b}{C_a}\right) * 100 \qquad (4)$$

2.4 Analysis of experimental samples

The concentrations of the oral-dosed sugars measured in plasma, normalised by dose administered to each cat, are displayed in Table 2.6. Equations used to calculate LR and XG ratios are shown in equations (5) and (6), respectively, using the concentration of the sugar quantified in the plasma and the amount of the sugar ingested by each cat in the sugar solution. Concentrations of analytes reported in the samples were adjusted based on the recoveries presented in Table 2.4. The concentration of all analytes in samples exceeded their respective LOQ and were within the calibration range (0 – 100 µg/mL), deeming this method fit for purpose. Urinary ratios have previously been observed as being directly comparable to plasma and serum ratios (Bruet *et al*., 2008; Fleming *et al*., 1996), but have never been compared in the domestic cat. The intestinal permeability values, the lactulose to rhamnose ratio, obtained in this study showed higher

permeability than previously documented urinary ratios. Earlier reported values varied between 0.03 and 0.53, with a median of approximately 0.44, which is lower than the present range of 0.38 – 0.78, with a median of 0.60 (Bijlsma *et al*., 1995; Johnston *et al*., 2001; Marks *et al*., 1999; Papasouliotis *et al*., 1993; Randell *et al*., 2001). The absorptive capacity values, the xylose to 3-OMG ratio, range from 0.48 to 0.69 in the present study, with a median of 0.50, which is lower than the only other reported xylose to 3-OMG ratio of 0.70 in a urine-based study by Johnston *et al*. (2001). The values obtained in this study could be different from the literature due to a variety of factors, including but not limited to, age, environment, neuter status, genetics, diet, and osmolarity of solution.

$$LR = \frac{(Lactulose\ Recovered)}{(Rhamnose\ Recovered)} = \frac{\left(\frac{Lactulose\ Concentration}{Lactulose\ Ingested}\right)}{\left(\frac{Rhamnose\ Concentration}{Rhamnose\ Ingested}\right)} \quad (5)$$

$$XG = \frac{(Xylose\ Recovered)}{(3-OMG\ Recovered)} = \frac{\left(\frac{Xylose\ Concentration}{Xylose\ Ingested}\right)}{\left(\frac{3-OMG\ Concentration}{3-OMG\ Ingested}\right)} \quad (6)$$

Table 2.6. Analyte concentration of real samples

Cat ID	**Dose (mL)**	**Lactulose (µg/mL)**	**Rhamnose (µg/mL)**	**Intestinal Permeability (LR)**	**Xylose (µg/mL)**	**3-OMG (µg/mL)**	**Absorptive Capacity (XG)**
1	10.2	8.42	3.92	0.61	53.87	26.09	0.59
2	9.8	31.36	14.97	0.60	83.09	34.99	0.68
3	7.8	7.08	3.64	0.56	48.87	27.40	0.51
4	8.7	56.76	23.05	0.70	86.61	46.06	0.54
5	7.7	35.69	14.42	0.71	75.30	35.87	0.60
6	9.2	8.65	5.06	0.49	48.61	27.03	0.51
7	8.9	17.64	6.43	0.78	47.21	25.31	0.53
8	9.1	22.97	16.18	0.41	92.35	49.29	0.54
9	6.7	5.54	3.79	0.42	42.50	17.53	0.69
10	8.6	17.26	11.05	0.45	51.96	30.70	0.48
11	8.5	10.32	4.41	0.67	51.67	27.22	0.54
12	8.3	26.28	11.20	0.67	59.97	32.66	0.52
13	6.8	15.06	11.44	0.38	82.89	42.43	0.56

Abbreviations: LR, lactulose: rhamnose; 3-OMG, 3-O-methylglucose; XG, xylose: 3-O-methylglucose

2.5 Discussion

The aim of this study was to develop a LC-MS/MS method for quantifying the intestinal permeability and absorptive capacity of cats using the simultaneous analysis of oral-dosed sugars in plasma 3 hours after dosing. Lactulose, rhamnose, xylose, and 3-OMG were all detected in plasma 3 hours following a single oral dose, indicating this method is highly suitable for future use examining impacts on intestinal permeability and absorptive capacity from factors such as diet and ageing in the domestic cat.

The extraction process was quick, and simple to complete. The use of stable isotope standards for each target sugar ensured high quantitative accuracy and precision (Yu *et al*., 2023). The selectivity of the high-resolution mass spectrometry and PRM and MS/MS fragmentation patterns provided the high degree of sensitivity and selectivity required to work in plasma as opposed to urine as is commonly used. Previous studies evaluating sugar probe concentrations in canine plasma used gas chromatography-mass spectrometry (GC-MS) due to its increased sensitivity (Bruet *et al*., 2008; Rodríguez *et al*., 2009). However, sugars are non-volatile compounds and require chemical derivatisation to be measured using GC–MS. This process not only adds complexity to the sample preparation, but may also cause unstable or incomplete derivatisation in the case of some compounds, generating multiple sugar peaks due to the presence of their isomers (Putri *et al*., 2022) and therefore incorrect concentration calculations. Derivatisation steps are often characterised for variability, which can be influenced by many factors, for example, by moisture (Putri *et al*., 2022). To overcome the challenges linked to GC–MS, the initial testing of six cats was completed to verify that the samples could be quantified within the detection limits of LC-MS.

Cats were fasted for 12 hours before the baseline venepuncture and administration of the oral sugar solution. While the fasting method clearly demonstrated no other sugars present at detectable levels in the plasma that could act as interfering peaks, if this was an issue then utilising MS/MS would help to differentiate between isobaric compounds based on their fragmentation patterns. The baseline samples produced no peaks within the retention times that were tested, which was not surprising since the cats were fasted. This also confirmed that these sugars are not endogenously produced at detectable levels within the cat.

Diarrhoea is a potential issue when consuming high levels of sugar, however this was not observed in any of the cats after ingestion of the hyperosmolar sugar solution. The sugar solution made in the current study was made to be hyperosmolar for multiple reasons. Firstly, the solution needed to be given in a dose that could be easily administered to the cats and therefore, needed to be concentrated to lower the volume of solution being orally syringed. Secondly, a previous study in dogs used a 1560 mOsm solution of lactulose and mannitol to successfully determine intestinal permeability (Tina *et al*., 2011), comparable to the dose

used in the current study. Lastly, hyperosmolar solutions have been said to provide a better discrimination between a healthy and a damaged small intestinal mucosal barrier (Corpeleijn *et al*., 2011; Gardner *et al*., 1991; Travis & Menzies, 1992; Uil *et al*., 2000). The reasoning behind this increase in lactulose permeation is unknown, however authors have speculated that a hyperosmolar solution may influence paracellular transport via tight junctions or cause solvent drag (Travis & Menzies, 1992; Uil *et al*., 2000). Although the dose used in this study did not reach the level of hyperosmolarity proposed to increase permeation of lactulose, the use of a hyperosmolar solution as opposed to an iso-osmolar one used in previous literature with cats may explain the higher lactulose recovery rates in comparison to previous urinary studies (Bijlsma *et al*., 1995; Johnston *et al*., 2001; Marks *et al*., 1999; Papasouliotis *et al*., 1993; Randell *et al*., 2001). The dose may therefore pose a challenge for directly comparing results to previous trials measuring feline intestinal permeability, but it paves the way for exciting opportunities and progress in future research within this area. This new LC-MS/MS method should be applied to a larger sample size which will help in determining a reference range for lactulose to rhamnose and xylose to 3-OMG ratio when measured in plasma using this dose.

2.6 Conclusion

The LC-MS/MS method developed here for measuring sugars used in intestinal permeability assays was shown to be sensitive, reliable, and reproducible for a small volume of plasma. It was successful in its ability to be minimally invasive, fast, selective, and sensitive for the accurate and precise quantification of four different test sugars in plasma using stable isotope internal standards. The development and application of this method is the first reported quantitative analysis of these sugars used for permeability assays in feline plasma. It proves to be a potential tool to analyse intestinal permeability and absorptive capacity in cats.

Chapter 3

Chapter 3: Age-dependent increase in small intestinal permeability and sex-dependent absorptive capacity in cats (*Felis catus*)

Drawing by Natalia González

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Statement of contributions available in Appendix 3.

Chapter 3: Age-dependent increase in small intestinal permeability and sex-dependent absorptive capacity in cats (*Felis catus*)

3.0 Abstract

Age-associated changes in intestinal permeability and function have not been studied in domestic cats, leaving a key factor in the relationship between age and digestive health in cats unexplored. Due to factors not currently understood, mature and senior cats may experience a loss of fat and protein digestibility, along with a loss of body weight (BW), impacting lifespan and quality of life. Therefore, to establish the relationship between age and intestinal health, intestinal permeability and absorptive capacity were quantified in young and senior cats using a differential sugar absorption test (SAT) on cat plasma. A solution containing four different sized sugars was orally administered to 36 healthy mixed-breed domestic shorthair cats (male (n=21) and female (n=15)) split into two groups by age, young 2.40 ± 0.758 (n=21) and senior 11.23 ± 1.896 (n=15) years (mean ± SD). Blood was collected prior to and again 3 hours after dosage and plasma was analysed using liquid chromatography mass-spectrometry (LC-MS). Intestinal permeability was higher ($p = 0.004$) in senior cats than young cats, and was not affected by sex ($p = 0.288$), sampling date ($p = 0.652$), or BW ($p = 0.951$). Absorptive capacity was higher ($p = 0.033$) in male cats than females, and was not affected by age class ($p = 0.440$), sampling date ($p = 0.580$), or BW ($p = 0.652$). In conclusion, intestinal permeability was higher in older cats and suggests age-related changes in intestinal barrier structure and function. These findings highlight the need to further consider increased intestinal permeability as a cause of reduced nutrient digestibility in older cats, offering a new target for interventions to enhance their health and well-being.

3.1 Introduction

The integrity and functionality of the intestinal barrier can be characterised by its permeability and absorptive capacity. A healthy gastrointestinal tract (GIT) epithelium maintains low permeability to maintain selective absorption of molecules, while limiting the entry of potentially harmful substances, such as pathogens and bacteria (Craven et al., 2007). Permeability of the intestinal barrier is managed by tight junctions (TJ), specialised intercellular structures between epithelial cells that control paracellular transport and help preserve the barrier's integrity and function (Bischoff et al., 2014). Tight junction proteins control the paracellular pathway, the transport of hydrophilic molecules and water between epithelial cells, while cell surface transporters control the transcellular pathway, the transport of hydrophilic or lipophilic molecules through the cells (Farré et al., 2020; Vanuytsel et al., 2021). Another characteristic of the intestinal barrier is its mucosal absorptive capacity. This measurement assesses the efficacy of carrier-mediated active transport pathways, primarily used to absorb dietary sugars and other substances from the digestive process (Rajan et al., 1961; Johnston et al., 2001).

The impacts of age on intestinal permeability have not been studied in cats. In rats, mice, and baboons, studies indicate an increase of intestinal permeability with age (Katz et al., 1987; Ma et al., 1992; Tran and Greenwood-Van Meerveld, 2013; Thevaranjan et al., 2017). On the other hand, contrasting findings in humans claim the absence of age-related effects (Wilms et al., 2020). Increased intestinal permeability, and thus a weakened mucosal barrier, has been linked to disease, and has been shown to promote inflammation, influence the gut microbiome, and overall weaken the protective effects of the mucosal barrier (Bischoff et al., 2014). Cats have increasing risk of decreased fat (Anantharaman-Barr et al., 1991; Harper, 1998; Peachey et al., 1999; Perez-Camargo, 2003; Fahey et al., 2008; Patil and Cupp, 2010; Teshima et al., 2010; Salas et al., 2014) and protein (Harper, 1998; Perez-Camargo, 2003; Patil and Cupp, 2010; Teshima et al., 2010) digestibility with age. Evaluating the relationship between age and intestinal permeability will improve basic knowledge of the GIT of the domestic cat and allow researchers to study the possible cause of reduced GIT function in senior cats.

One method of quantifying intestinal permeability *in vivo* is through the differential SAT. This method employs disaccharides and monosaccharides or polyalcohols to measure paracellular and transcellular permeability, respectively, of the small intestinal mucosal barrier (Lostia *et al*., 2008). Normally, the TJs that line the intestinal barrier do not allow a high concentration of disaccharides as opposed to monosaccharides to permeate through, creating a low ratio of disaccharides to monosaccharides, commonly LM or LR (Bischoff *et al*., 2014). When the intestinal barrier is compromised, the concentration of disaccharides able to pass through the barrier increases, therefore increasing the numerical value of the ratio. Absorptive capacity of the intestine can also be determined using a SAT with different sugars: Xylose and

3-OMG can be used to assess carrier-mediated active transport and ATP-dependent mediated active transport, respectively (Rodríguez *et al*., 2009). A healthy intestine will maintain a higher xylose to 3-OMG (X:G) ratio, signifying their ability to actively transport molecules across the membrane (Craven *et al*., 2007). A pilot study was conducted to establish a minimally invasive method to measure small intestinal permeability and absorptive capacity in the domestic cat, whereby the sugars ('sugar probes') lactulose, rhamnose, xylose, and 3-OMG were administered to 13 young adult male cats. The dose described in this study was sufficient to allow detection of the sugar probes in both plasma and serum at 180 minutes (Chapter 2; Patterson *et al*., 2024).

Advancing age is hypothesised to increase intestinal permeability (increasing LR ratio) and decrease absorptive capacity (decreasing XG ratio). Therefore, the objectives of the present study were to determine if there are any differences in intestinal permeability and absorptive capacity between young and late midlife to super-senior cats, defined previously (Salt *et al*., 2023), which are classified as youth and senior groups in this paper. A CBC was carried out to ensure the older cats were healthy and these were used as explanatory values in the analysis.

3.2 Materials and methods

3.2.1 Ethics

This study was approved by the Massey University Animal Ethics Committee (MUAEC 23/14), New Zealand which meets the requirements of the Animal Welfare Act ("Animal Welfare Act," 1999).

3.2.2 Animals, diets, and housing

Thirty-seven healthy male and female domestic shorthair cats from the Centre for Feline Nutrition at Massey University, Palmerston North, New Zealand were selected based on age and sex (Table 3.1). Due to the nature of the research colony, all male cats were neutered at approximately 6 months of age and the majority of female cats were entire (Table 3.1). One young, female cat did not give enough blood for a complete blood count (CBC), so her health was determined based on previous blood samples and a physical check. Data from one senior male cat was omitted due to a difficult time blood sampling and a resulting 216-minute gap in between T0 and T3 blood samples. Cats in this colony are monitored on a daily basis for behaviour and stool quality, weighed on a weekly basis, and are up to date with appropriate vaccinations. A CBC was taken to ensure the older cats were healthy and these were used as explanatory values in the analysis.

The sample size was determined by comparison previous related literature as there was no established clinically important difference in intestinal permeability and mucosal absorption between groups of cats. Two similar previous studies have used a sugar absorption test in cats; one using 12 cats, and the other 11.

Although previous companion animal research has not found sex as a confounding variable, it is also not thoroughly studied. Therefore, this study aimed to have 10 cats per age group per sex to gain further understanding of these potential sex differences and allowing more power to the age analysis but was limited by the number of animals who met the eligibility criteria, resulting in uneven groups and more young adult males.

Table 3.1. Cats selected for Chapter 3 trial

	Male senior	**Male youth**	**Female senior**	**Female youth**
n	8	13	7	8
Age (years)	11.53 ± 2.316	2.48 ± 0.845	10.88 ± 1.364	2.26 ± 0.616
Mean ± SD	(9.23 – 16.21)	(1.26 – 3.37)	(9.48 – 12.53)	(1.26 – 3.37)
(range)				
BW (g)	3934.82 ± 383.016	4238.79 ± 514.716	3112.36 ± 334.414	3193.89 ± 370.784
Mean ± SD	(3536.10 – 4415.30)	(3358.60 – 5079.90)	(2796.60 – 3745.80)	(2609.60 – 3540.50)
(range)				
N: E	8:0	13:0	3:4	0:8

Abbreviation: BW, body weight; N: E, neutered: entire

As part of normal husbandry, cats were fed *ad libitum* in their group enclosure (Figure 3.1 from (Smit *et al*., 2023)) with multiple flavours of a commercially available canned diet (Heinz Wattie's Ltd., Hastings, New Zealand) formulated to meet AAFCO requirements for adult cats and always had access to fresh water.

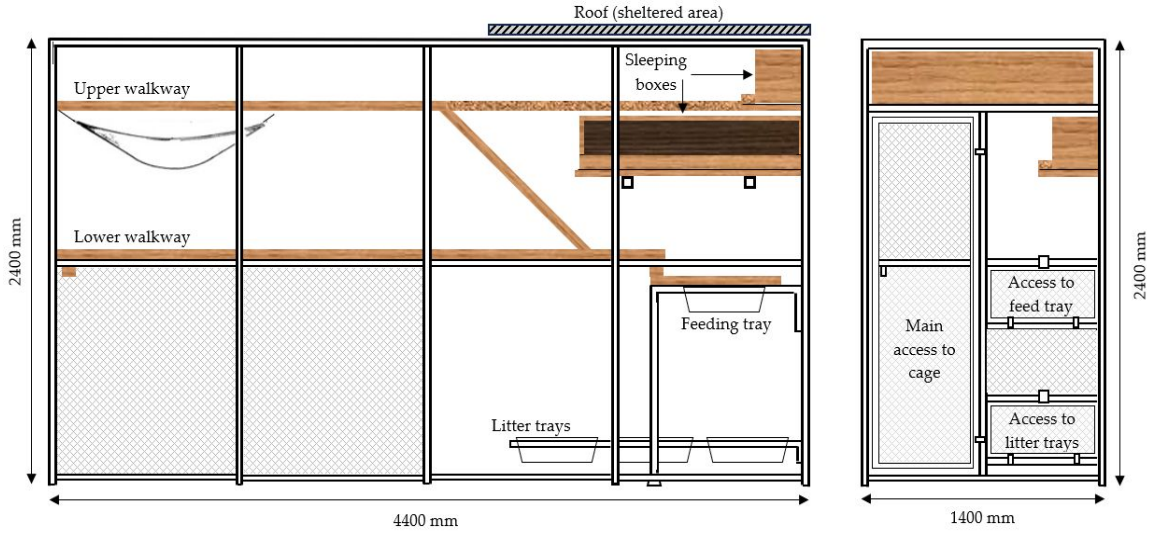


Figure 3.1. Colony cages at Massey University's Centre for Feline Nutrition, in mm. Groups range from 6 – 10 cats.

3.2.3 Sample collection

The cats underwent a 12-hour fasting period with only access to water before the initial blood sample was collected where they were placed in individual metabolic cages (measuring 80 x 80 x 110 cm) as previously described in (Hendriks *et al*., 1999a). This fasting period was implemented to confirm the cats' physical well-being through a CBC and to establish a sugar-free baseline. At the end of the 12-hour fast, cats were weighed, 2 mL of blood via jugular venepuncture was taken for a baseline sample (T_0) for sugar probe analysis and a CBC, and then cats were dosed with a sugar probe mix at 2 mL/kg BW. The probe mix was formulated to contain 0.07 g/mL lactulose, 0.02 g/mL rhamnose, 0.07 g/mL xylose, and 0.02 g/mL 3-OMG dissolved in purified water (Chapter 2; Patterson *et al*., 2024).

Cats were returned to their enclosure for 3 hours with no access to food and *ad libitum* access to water. After 3 hours, blood sampling was repeated on the opposite jugular vein, again taking 2 mL of blood (T_3). Once the final blood sample was taken, the cats were returned to their colony cages with access to their normal diet. Then, 1 mL of the T_0 blood was transferred to a 3.6 mL K3-EDTA tube (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for CBC. The remainder of the T_0 blood, and all the T_3 whole blood was transferred to a 5.4 mg K2-EDTA tube (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and spun at 3000 x g at 4 °C for 15 minutes. Plasma was then aliquoted into microcentrifuge tubes for LC-MS and frozen at -80 °C until analysis. Due to the number of animals, the sugar probe testing and blood sampling was conducted over 3 consecutive days.

3.2.4 Sample preparation and analysis

Haematology (CBC) values were analysed by IDEXX using a ProCyte Dx Haematology Analyser (IDEXX Laboratories Pty. Ltd., Tennant Drive, PO Box 325, Palmerston North 4440, New Zealand) the same day blood was collected. Plasma was analysed for reticulocyte haemoglobin equivalent (retic Hb), Red blood cell (RBC) count, haemoglobin (Hb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), platelet count, absolute reticulocyte count, WBC count, band neutrophil count, segmented neutrophil count, lymphocyte count, monocyte count, eosinophil count.

The sugar probes sample preparation and quantification was performed as previously described (Chapter 2; Patterson *et al*., 2024). Briefly, plasma samples (200 µL) were mixed with ice cold acetonitrile (ACN; 590 µL) and internal standard mixture (10 µL containing all four ^{13}C-labelled sugars), vortexed thoroughly, incubated (-20 °C, 60 minutes), vortexed again, and centrifuged (14000 x *g*, 10 minutes, 4 °C). Then, 100 µL of Milli-Q® water was added to each sample, vortexed, and centrifuged (14000 x *g*, 10 minutes, 4 °C) for a second time. A fixed volume of the supernatant (800 µL) was dried under nitrogen at 35 °C, and the dry extract was reconstituted in 100 µL of 90 % ACN with 1 mM ammonium formate.

3.2.5 Liquid chromatography- mass spectrometry

The LC-MS method used to measure the concentration of the sugars in the plasma has been previously described (Chapter 2; Patterson *et al*., 2024). Analyses were conducted over two consecutive days and for both days, a pooled sample of a homogenous mixture of an aliquot of each cat plasma sample was analysed as a pooled quality control (PCQ) and repeats of the mid-range calibration standard were analysed as a technical quality control (TQC). The LOD and LOQ were assessed using the same protocol as described in Chapter 2 (Patterson *et al*., 2024). Linearity and quality control measures are summarised in Table 3.2.

Table 3.2. Data collected from calibration curves

Analyte	Equation	R^2	$PQC_{RSD\ (\%)}$	$TQC_{RSD\ (\%)}$
Lactulose	y = 0.187873*x* – 0.11872	0.9989	Day 1 – 2.914	1.769
			Day 2 – 3.521	
Rhamnose	y = 0.130345*x* – 0.0315225	0.9997	Day 1 – 4.084	2.144
			Day 2 – 5.956	
Xylose	y = 0.0660358*x* – 0.00774623	0.9999	Day 1 – 0.855	1.334
			Day 2 – 1.147	
3-OMG	y = 0.159498*x* – 0.0839806	0.9997	Day 1 – 2.771	0.998
			Day 2 – 2.345	

Abbreviations: PQC, pooled sample quality control; TQC, technical quality control; 3-OMG, 3-O-methylglucose; RSD, relative standard deviation

3.2.6 Equations

Sugar concentrations were calculated based on equations provided in Chapter 2, then divided by the amount of sugar ingested to normalise for the dosage. Equations used to calculate LR and XG ratios are shown in equations (5) and (6), respectively, using the concentration of the sugar quantified in the plasma and the amount of the sugar ingested by each cat in the sugar solution.

$$L{:}\,R = \frac{(Lactulose\ Recovered)}{(Rhamnose\ Recovered)} = \frac{\left(\frac{Lactulose\ Concentration}{Lactulose\ Ingested}\right)}{\left(\frac{Rhamnose\ Concentration}{Rhamnose\ Ingested}\right)} \qquad (5)$$

$$X{:}\,G = \frac{(Xylose\ Recovered)}{(3-OMG\ Recovered)} = \frac{\left(\frac{Xylose\ Concentration}{Xylose\ Ingested}\right)}{\left(\frac{3-OMG\ Concentration}{3-OMG\ Ingested}\right)} \qquad (6)$$

3.2.7 Statistical analysis

Certain CBC data were omitted from the analysis as they were identified as outliers according to Chauvenet's criterion.

All statistical analyses were carried out using RStudio version 4.1.1. Correlations between haematology data and age as well as haematology data and intestinal permeability data were assessed using the Pearson correlation. Intestinal permeability and absorptive capacity data were determined to be normally distributed by performing a Shapiro-Wilk test and visually assessing density, histogram, and QQ plots for both data and residuals. The data was assessed for homogeneity using a Levene's test. One-way analyses of variance (ANOVAs) were used to determine the effects of age class on intestinal permeability and absorptive capacity while blocking for sex, sample date, and BW. After the ANOVA showed that intestinal permeability was only significantly affected by age, and absorptive capacity only significantly affected by sex, a Welch's t-test was used without the non-significant confounding factors. Welch's t-test was chosen to analyse significance as it has been shown to limit Type 1 errors and remains rather robust even in cases of violations of normality, homoscedasticity, and uneven sample sizes (Delacre *et al*., 2017). Results are presented as mean ± SD. Statistical significance was defined at $p < 0.05$ and trends at $p < 0.1$.

3.3 Results

3.3.1 Intestinal permeability and absorptive capacity

Ratios were calculated using concentrations of each sugar recovered from plasma, standardised by percentage of dose administered as stated in section 3.2.6 Equations. Intestinal permeability, represented as the LR ratio, was higher ($p = 0.004$) in senior cats (0.75 ± 0.160) than young cats (0.59 ± 0.139) (Figure 3.2), but not different ($p = 0.288$) between male (0.63 ± 0.161) and female (0.70 ± 0.170) cats. There were no significant effects observed for sampling date ($p = 0.652$) or BW ($p = 0.951$). Absorptive capacity, represented as the XG ratio, was not different ($p = 0.440$) between senior (0.56 ± 0.113) and young cats (0.54 ± 0.089). There were no significant effects observed for sample date ($p = 0.580$) or BW ($p = 0.652$). Sex, however, had a significant ($p = 0.033$) effect on the ratio of XG, being higher in male cats (0.58 ± 0.070) compared to female cats (0.50 ± 0.117). This effect was not significant within the senior group between the males (0.61 ± 0.075) and females (0.51 ± 0.130) ($p = 0.105$) or the young group between the males (0.56 ± 0.063) and females (0.50 ± 0.114) ($p = 0.173$).

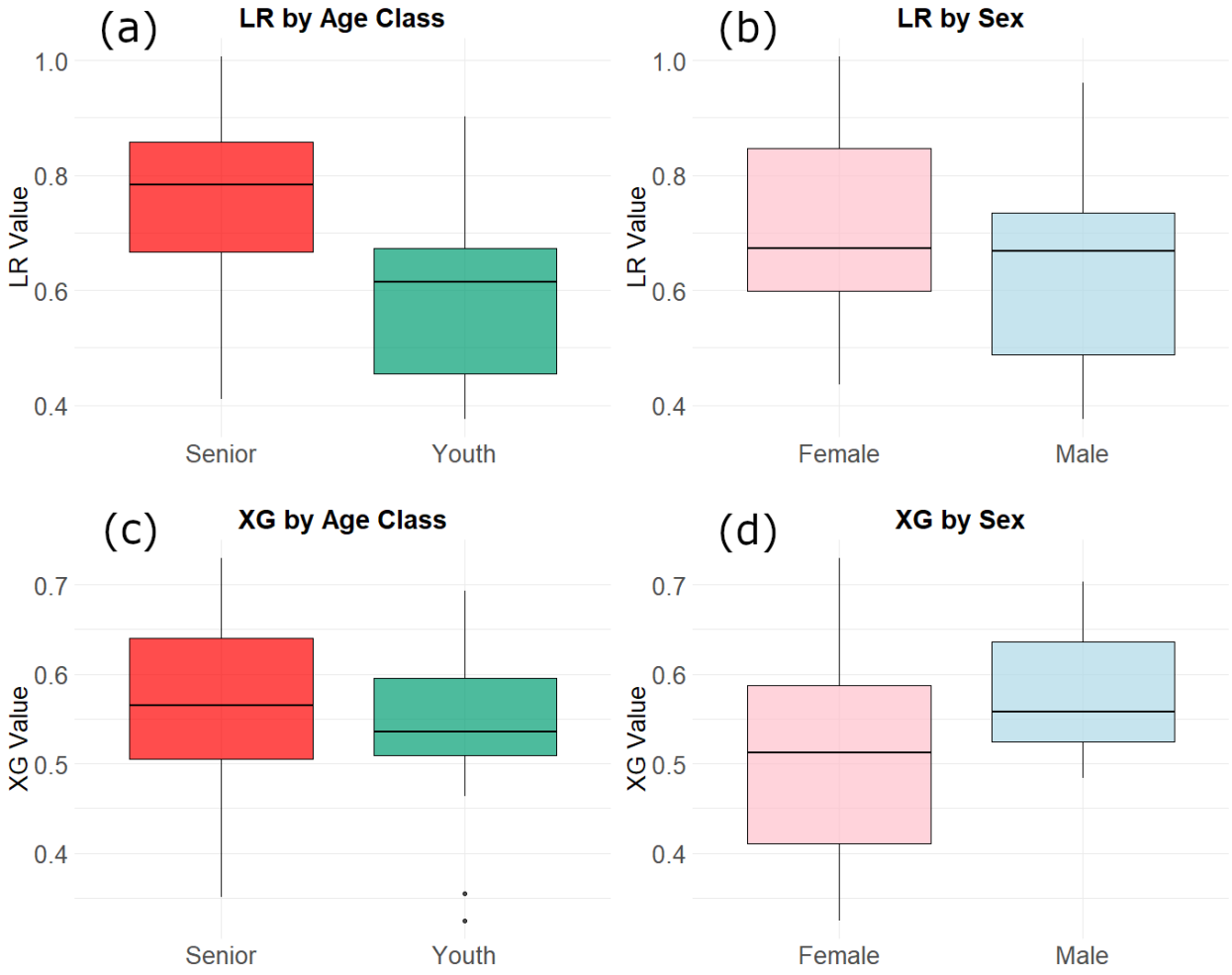


Figure 3.2. Ratios of lactulose to rhamnose (LR) and xylose to 3-O-methylglucose (XG) in plasma of domestic cats. (a) LR for senior (n = 15) and young (n = 21) cats (p = 0.004), (b) LR for female (n = 15) and male (n = 21) cats, (c) XG for senior (n = 15) and young (n = 21) cats, (d) XG for female (n = 15) and male (n = 21) cats (p = 0.033).

3.3.2 Complete blood count

Based on CBC analysis, all cats had values for each blood component within the normal range therefore the cats were considered to be healthy. Segmented neutrophil count (p = 0.033) was positively correlated with the senior age class, while haemoglobin (p = 0.001), HCT (p = 0.010), MCH (p = 0.011), platelets (p = 0.031), and lymphocyte count (p = 0.006) were negatively correlated with the senior age class. MCHC (p = 0.010) was positively correlated with LR, and there was a negative trend between MCV and LR (p = 0.055). There was a negative trend between RBC count and XG (p = 0.062) and a positive trend between MCV and XG (p = 0.081). See supplementary information for correlation figures (Figures S3.1-3.10 available in Appendix 4).

3.4 Discussion

To the authors' knowledge, this is the first study to use the SAT and discover intestinal permeability is greater, but absorptive capacity is similar in older cats when compared to young cats. Further, while permeability was similar, absorptive capacity was greater in male cats as compared to female cats when analysing the whole study population. This new knowledge of physiological changes associated with ageing in cats provides a new area to further research and potentially improve the health of older individuals.

The increased intestinal permeability in older cats observed in the current study supports the hypothesis that the feline GIT undergoes age-related alterations. This contrasts with a study conducted in dogs, which did not find an increase in intestinal permeability with age (Garden *et al*., 1997). While there is documented evidence of increased intestinal permeability in *Drosophila* (Clark *et al*., 2015), rats (Katz *et al*., 1987), mice (Thevaranjan *et al*., 2017), and baboons (Tran & Greenwood-Van Meerveld, 2013) using various methods, studies in humans have reported no such change (Saltzman *et al*., 1995; Wilms *et al*., 2020).

In the current study, age did not affect absorptive capacity in the cat, however absorptive capacity was higher in male compared to female cats. While there are no specific studies assessing absorptive capacity by sex in the cat, a study involving dogs with chronic inflammatory enteropathies (CIE) found no difference in absorptive capacity or intestinal permeability between sexes using urinary LR and XG ratios, which have been found comparable to plasma and serum ratios in multiple studies (Allenspach *et al*., 2006; Bruet *et al*., 2008; Fleming *et al*., 1996). Sex may also play a role in influencing the integrity and functionality of the small intestinal barrier. There is a positive correlation between oestradiol concentration and the expression of TJ proteins, with intestinal permeability decreasing during oestrus and rising again in the following luteal phase in rats (Braniste *et al*., 2009; Homma *et al*., 2005). Females also tend to possess a more diverse gastrointestinal microbiome that is linked with reduced intestinal permeability compared to males in humans (Edogawa *et al*., 2018). The combination of oestradiol and greater microbial diversity seem to have protective effects that enhances their ability to withstand and adapt to various challenges (Ahnstedt *et al*., 2020; Edogawa *et al*., 2018; Homma *et al*., 2005). In a 2014 study involving 4009 cats, female cats (15.0 years; interquartile range (IQR) 11.0–17.4) had a higher ($p < 0.001$) median longevity than male cats (13.0 years; IQR 7.6–16.0) (O'Neill *et al*., 2015). The authors of this study did not delve further into possible explanations behind this, allowing for speculation that microbial diversity could be a contributing factor. Hormonal fluctuations can be controlled for in research, which occurred in the present study, by sampling females during the follicular phase or having them neutered to prevent hormonal surges (Snipe & Costa, 2018).

However, one study has detected an age-related increase in intestinal permeability in humans through the presence of inflammatory and TJ protein biomarkers associated with mucosal barrier and TJ integrity and function (Man *et al*., 2015). The heightened inflammation typically observed in older animals, as indicated in this study by an increased segmented neutrophil count and decreased lymphocyte count, might be responsible for the increased intestinal permeability observed in the cats. The higher intestinal permeability observed in senior cats could be indicative of inflammation in the GIT, leading to infiltration of pathogens and toxins into the periphery. Senior cats over the age of ten have significantly lower RBC counts, Hb, HCT, WBC, lymphocyte counts, and eosinophil counts than younger adult cats (Campbell, Rawlings, Koelsch, *et al*., 2004; Czarnecki-Maulden *et al*., 2004) and generally agrees with the present study. A decrease in these parameters can be indicative of immunosenescence, the gradual decline of the efficacy of the immune system as a natural part of the ageing process (Franceschi *et al*., 2007). These findings suggest age-related changes in cats' immune system, which may impact their ability to respond to infections and immune challenges as they get older. The chronic, low-level inflammation that accompanies ageing in humans and other mammals, referred to as inflammageing, is also thought to manifest in cats, suggesting a potential link between inflammation and the disruption of TJ, leading to increased intestinal permeability during states of inflammation (Karper, 2011; McKenzie, 2022). Increased neutrophil counts have been seen with age in humans and mice, and are commonly associated with inflammation and observed to stimulate cellular senescence, further exacerbating systemic inflammation (Avondt *et al*., 2023). In humans, pro-inflammatory cytokines have been shown to be greater in the elderly along with neutrophils, while platelet, lymphocyte, and basophil counts are lower (Valiathan *et al*., 2016).

The dose used in the present study follows the validated method as used in Chapter 2. The solution was hyperosmolar to better discriminate between healthy and impaired intestinal barriers, however a hyperosmolar solution can also cause increased permeation of lactulose (Travis & Menzies, 1992; Uil *et al*., 2000). While the osmolarity of the solution administered did not reach the level of hyperosmolarity shown to enhance lactulose permeation (Travis & Menzies, 1992), the choice of a hyperosmolar solution, unlike the iso-osmolar approach used in prior cat studies (Bijlsma *et al*., 1995; Papasouliotis *et al*., 1993), might clarify the higher LR ratio observed in the present study. In a study with a sample size of two humans, urinary recovery of lactulose and mannitol were comparable from both hyperosmolar and iso-osmolar sugar solutions, suggesting that osmolarity does not impact the comparison between subjects (Bijlsma *et al*., 1995). This discrepancy, however, makes it challenging to directly compare the results to previous research on feline intestinal permeability. Nevertheless, it paves the way for further exploration in this field as the hyperosmolar dose is able to be given to most cats without sedation and by using low-stress handling techniques.

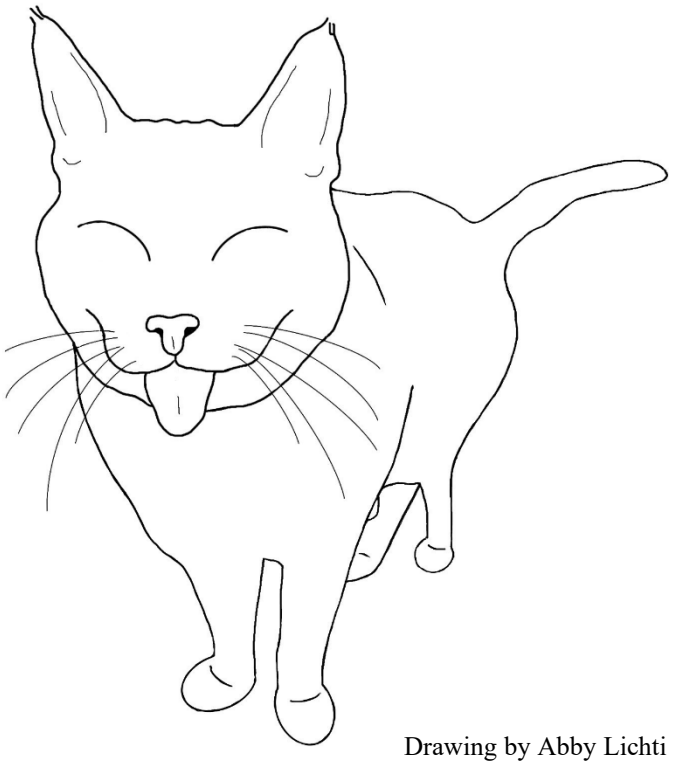
As one of the few intestinal permeability or absorptive capacity trials conducted in cats, and the only one to compare these measurements across age class and sex, these results offer a unique and comprehensive insight into feline gastrointestinal physiology clearly suggesting a reduction in intestinal permeability due to ageing and differences in intestinal absorption between males and females. Further research should focus on determining the relationship that intestinal permeability has with other aspects of ageing, such as reduced nutrient digestibility, immunosenescence, and loss of microbial diversity.

3.5 Conclusion

In conclusion, senior cats had significantly greater intestinal permeability values when compared to younger cats, suggesting age-related changes in their GIT. Using the statistical power of all the cats in the study, there was also a significant difference in absorptive capacity between males and females, indicating that hormonal makeup could influence nutrient absorption. The correlations of intestinal permeability with markers of inflammation suggest that inflammation may play a role in these changes, or be affected by them. Further exploration of biomarkers of intestinal health and pro-inflammatory cytokines may provide additional insight into the relationship between intestinal permeability and inflammation. This study significantly contributes to the pool of knowledge of feline gastrointestinal physiology, shedding light on age and sex-related differences in haematological parameters, intestinal permeability, and absorptive capacity.

Chapter 4

The effect of *Saccharomyces boulardii* on the gastrointestinal health of senior cats (*Felis catus*)



Drawing by Abby Lichti

Chapter 4: The effect of *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 on the gastrointestinal health of senior cats (*Felis catus*)

4.0 Abstract

Probiotic yeast *Saccharomyces cerevisiae* var. *boulardii* (*S. boulardii*) has been studied extensively for human use, effectively targeting gastrointestinal disorders and the intestinal barrier. More recently, it has been researched as a probiotic for cats and dogs. The domestic cat can experience age-related changes to its GIT including reduced digestive function and increased permeability of the small intestine. In order to investigate the effects of *S. boulardii* on the health of the GIT in older cats, a crossover trial was performed on 19 healthy mixed-breed domestic shorthair cats (male: n = 8; and female: n = 11) aged 10.0 ± 1.9 years split into two treatments over two periods. During period 1 of the trial, Group 1 (n = 9) was supplemented with 2.5 x 10^8 colony forming units (CFU) of *S. boulardii* and Group 2 (n = 10) was given a placebo capsule (control; CTRL). The first period was 31 days, followed by a 28 – day washout period, and treatments switched for period 2 (31 days). Supplementation with *S. boulardii* (0.92 ± 0.127) increased (p = 0.048) absorptive capacity (XG) compared to the control group (0.82 ± 0.12). Supplementation of *S. boulardii* had no effect on any other GIT parameter measured in this study. Based on secondary analyses, the ATTD of fat (p = 0.039) and energy (p = 0.094) were reduced with increasing age. Additionally, the ATTD of fat (p = 0.058), energy (p = 0.027), and DM (p = 0.023) were increased in Period 2. Intestinal permeability (LR), increased with increasing age (p = 0.034), and absorptive capacity tended (p = 0.060) to be higher in males (0.88 ± 0.145) than females (0.82 ± 0.129). Several plasma and faecal metabolites were correlated with increasing age and intestinal permeability. Trimethylamine N-oxide (TMAO) increased while CDCA decreased with increasing age and LR. While there was limited effects of *S. boulardii* in the current study, the secondary analyses based on age and intestinal permeability present a greater understanding of how the GIT changes in ageing cats.

4.1 Introduction

Chapter 3 identified that intestinal permeability increases with age in the domestic cat. Increased permeability of the intestinal barrier diminishes its effectiveness of selectively absorbing molecules and allows uptake of pathogens and other toxins which may increase the risk of inflammation and disease (Bischoff *et al*., 2014). It has been suggested that increased intestinal permeability may affect the composition of the gastrointestinal microbiome or conversely, the microbiome may also affect the intestinal barrier (Bischoff *et al*., 2014). Surprisingly, the relationship between the intestinal microbiome and intestinal permeability has not been well studied in mammals, however in some models, such as *Drosophila* (Clark *et al*., 2015), increased microbial dysbiosis has been linked to increased intestinal permeability. The relationship between intestinal permeability and microbial composition has not been studied in the cat, and while age-related changes to the microbiome have been identified, there is conflicting evidence on whether or not cats experience a loss in microbial diversity with age (Ganz *et al*., 2022; Tian *et al*., 2023).

Previous studies indicate that the ATTD of CFat (Anantharaman-Barr *et al*., 1991; Fahey *et al*., 2008; Harper, 1998; Patil & Cupp, 2010; Peachey *et al*., 1999; Perez-Camargo, 2003; Salas *et al*., 2014; Taylor *et al*., 1995; Teshima *et al*., 2010) and CP (Harper, 1998; Patil & Cupp, 2010; Perez-Camargo, 2003; Teshima *et al*., 2010) is lower in older cats. In healthy adult cats, the ATTD of CP has been positively linked to bacterial families Peptostreptococcaeae, Eubacteriaceae, Fusobacteriaceae, and Peptococcaceae (Bermingham *et al*., 2018). Therefore, it is of interest to investigate the relationship between intestinal permeability and digestibility, which may offer insights into age-related declines in the GIT and the role of the microbiome

One mechanism by which the faecal microbiome may impact intestinal permeability is through the faecal metabolome; this is defined as the metabolites produced by the gastrointestinal microbiome. Therefore, age-associated changes in the composition of gastrointestinal microbiome may affect the faecal metabolome. Microbial metabolites can activate cell receptors and alter both health and disease states through regulating immunity function (Chang, 2024). There is a scarcity of information on changes in microbial metabolites in the ageing cat. Furthermore, there has been no published research looking at the correlation between faecal metabolites and gastrointestinal health in the cat.

Probiotics are defined as live micro-organisms that can improve the health status of the pet (Wernimont *et al*., 2020). Typically, the health impacts of probiotics may occur through their effects on the gastrointestinal microbiome, as well as direct interactions with host cells, which can lead to improvements in the physical structure of the intestinal wall, reduction of pathogen load, and strengthening of the immune system (Yang & Wu, 2023). Another mechanism by which probiotics can exert these benefits is via

microbial metabolites. *Saccharomyces cerevisiae* var. *boulardii* (*S. boulardii*) is a probiotic yeast that has been studied for its effectiveness as a treatment in gastrointestinal diseases (Pais *et al*., 2020). *S. boulardii* has been widely studied in humans and seen as an effective treatment against gastrointestinal disorders and diarrhoea (Kelesidis & Pothoulakis, 2012; McFarland, 2010). *S. boulardii* also improved body condition score and faecal parameters, and increased faecal IgA, indicating improved intestinal health in adult dogs (Lonigro *et al*., 2025b), and has also been associated with decreased CIEs in dogs (D'Angelo *et al*., 2018). In adult cats, supplementation of a multi-strain probiotic containing *S. boulardii* and *Pediococcus acidilactici* increased the concentration of SCFA, faecal antioxidants, and faecal inflammatory markers (Li *et al*., 2023). *S. boulardii* altered the faecal microbiome, increasing Pediococcus and Bacillaceae, and decreasing the proportion of Bacillota, Bacteroidota (Li *et al*., 2023). In kittens, 4 weeks of *S. boulardii* supplementation altered biomarkers of intestinal barrier function, suggesting improved intestinal permeability (Zhang *et al*., 2023b). *S. boulardii* lowered faecal scores, improved concentrations of inflammatory biomarkers, and impacted the gastrointestinal microbiome by reducing the population of microbes considered harmful (Zhang *et al*., 2023b). However, the effect of *S. boulardii* supplementation on the intestinal function, including permeability in older cats is unknown.

Links between the microbiome and health outcomes in the ageing cat have not been established. Therefore, age-related changes to the composition or diversity of microbiome may be the cause of increased intestinal permeability and may identify a possible target to treat or protect the animal from a weakened barrier. One mechanism by which this can occur is via probiotic supplementation. Therefore, this study was undertaken to investigate the role of *S. boulardii* on gastrointestinal function in midlife to senior cats. It was hypothesised that *S. boulardii* would improve intestinal permeability in old cats and therefore improve CFat and CP ATTD. Futhermore, it was hyothesised that *S. boulardii* would alter the composition of the faecal microbiome, and its metabolite profile and that this could be a mechanism by which improvements in intestinal permeability and absorptive capacity occur.

4.2 Materials and methods

4.2.1 Ethics

The trial was approved by the Massey University Animal Ethics Committee (MUAEC 24/03) which meets the requirements of the Animal Welfare Act ("Animal Welfare Act," 1999).

4.2.2 Animals

Twenty healthy domestic shorthair cats (males: n = 9 and females: n = 11) from the Centre for Feline Nutrition at Massey University, Palmerston North, New Zealand were selected for the trial based on age and sex (Table 4.1) and randomly allocated into two dietary treatment groups. Due to the husbandry

practices at the research colony, all male cats were neutered at approximately 6 months of age and females were spayed on a case by case basis. Group 1 consisted of 5 male neutered (MN), 1 female neutered (FN), and 4 female entire (FE) cats, and Group 2 consisted of 4 MN, 3 FN, and 3 FE cats. 1 week into the start of the trial, 1 MN cat from Group 1 was removed due to unrelated health problems and their data was not used for any statistical analysis.

The sample size was determined with a power analysis using the detectable difference between the young and senior groups in Chapter 3. At 90 % power and a 5 % significance level, 10 cats were needed per treatment group.

One week prior to adaptation to the diets, cats were moved into their treatment groups, with each group spread across two to three pens. Husbandry continued as normal during the trial and washout period.

Table 4.1. Number, age, weight, and neuter status of selected cats at the beginning of the trial.

Period 1	**n**	**Age (years)**	**Weight (kg)**	**N : E**
Male	8	9.72 ± 1.98 (8.24 – 13.41)	4.16 ± 0.760 (3.26 – 5.35)	8 : 0
Female	11	10.20 ± 1.903 (8.24 – 13.41)	3.11 ± 0.307 (2.59 – 3.52)	4 : 7
Total	19	10.00 ± 1.900 (8.24 – 13.41)	3.55 ± 0.749 (2.59 – 5.35)	12 : 7

Abbreviation: N, neutered E, entire. Results are presented as mean ± SD (range).

4.2.3 Diet

Cats selected for the trial were adapted to a single batch of a commercially available complete and balanced canned diet (Chunky with Chicken flavour, Heinz Wattie's Ltd., Hastings, New Zealand; Table 4.2). The diet was from a single production run to minimise batch effects. The diet was fed *ad libitum* daily throughout the trial and each cat was given two "TEMPTATIONS™ Tasty Chicken Flavor" treats (Mars, Incorporated, 2024) daily (Table 4.3).

Table 4.2. Dry matter (DM) content and composition of the diet[1] (DM basis)

Component	**Period 1**	**Period 2**
	Diet	**Diet**
DM (g/100 g as is)	16.1	18.9
CP (g/100 g DM)	52.8	52.7
CFat (g/100 g DM)	26.2	27.9
CF (g/100 g DM)	1.5	1.5
Ash (g/100 g DM)	12.7	12.3
GE (kJ/g DM)	22.6	23.7

Abbreviations: DM, dry matter; CP, crude protein; CFat, crude fat; CF, crude fibre; GE, gross energy.

[1]Ingredients: Chicken by-product meal, ground corn, animal fat (preserved with mixed tocopherols), brewers rice, wheat flour, dried meat by-products, natural flavours, brewers dried yeast, potassium chloride, choline chloride, salt, taurine, DL-methionine, calcium carbonate, DL-alpha tocopherol acetate (source of vitamin E), zinc sulfate, mixed tocopherols (preservative), dried cheese, copper sulfate, vitamin A acetate, niacin supplement, vitamin B12 supplement, riboflavin supplement, manganese sulfate, thiamine mononitrate, D-calcium pantothenate, biotin, vitamin D3 supplement, potassium iodide, pyridoxine hydrochloride (vitamin B6), folic acid (Kraft Heinz, 2025).

Table 4.3. Guaranteed analysis of the "TEMPTATIONS™ Tasty Chicken Flavor" treats[1]

Component	Treat
DM (g/100 g as is) (max.)	90.0
CP (g/100 g DM) (min.)	28.0
CFat (g/100 g DM) (min.)	21.0
CF (g/100 g DM) (max.)	4.5

Abbreviations: CP, crude protein; CFat, crude fat; CF, crude fibre.

[1]Ingredients: Chicken by-product meal, ground corn, animal fat (preserved with mixed tocopherols), brewers rice, dried meat by-products, natural flavours, brewers dried yeast, potassium chloride, choline chloride, salt, DL-methionine, taurine, calcium carbonate, vitamin E supplement, zinc sulphate, ferrous sulphate, dried cheese, mixed tocopherols (preservative), copper sulphate, vitamin A supplement, citric acid (preservative), niacin supplement, vitamin B12 supplement, riboflavin supplement, manganese sulphate, thiamine mononitrate, D-calcium pantothenate, pyridoxine hydrochloride (vitamin B6), vitamin D3 supplement, biotin, potassium iodide, folic acid, rosemary extract (Mars Inc., 2025)

4.2.4 Treatment

Each group was supplemented daily with one vegetal capsule (hydroxypropylmethylcellulose (HPMC) caps clear, Suheung Co, South Korea) containing either 200 mg of maltodextrin (CTRL), or 2.5 x 10^8 CFU of *S. boulardii* CNCM I-1079 (in 200 mg maltodextrin). Capsules were provided by Lallemand (19 rue des Briquetiers, 31702 Blagnac, France). Cats in Group 1 received a probiotic capsule of *S. boulardii* in period 1 and a placebo (CTRL) in period 2, with cats in Group 2 receiving CTRL in period 1 and *S. boulardii* in period 2.

4.2.5 Study periods

A double-blinded, randomised, placebo-controlled, 2-period crossover study design with a 28-day washout was utilised for the trial (Figure 4.1). The washout was 4 weeks long to ensure no traces of the probiotic would carry over into the second period, and was based off observations in dogs from a previous study (D'Angelo *et al*., 2018). Each study period comprised a seven-day adaptation period to the study diet, handling, and baseline measurements and a 31-day treatment period. Period 1 occurred during months April – May, while Period 2 occurred during June – July in the North Island of New Zealand.

For the first 2 days of each study period, cats were adapted to individual cages and double litter trays which allow urine to drain through and faeces to be collected, as previously described by Hendriks *et al*. (1999a). During adaptation, litter was gradually removed from the tray and baseline individual feed intake was determined. A fresh faecal sample was then collected which was used for faecal microbiome and metabolomic analysis as described below. The cats were then fasted overnight before a differential SAT was completed the following day (see Chapter 2). Both groups began oral administration of treatment or control the next day while residing in individual cages to monitor individual feed intake. After 2 days they were returned to group housing where they remained until day 22 of treatment where they returned to individual cages with double litter trays to collect a second fresh faecal sample for microbiome and metabolomics. They remained in the individual cages for the sampling of ATTD of macronutrients, energy and DM, conducted over treatment days 24 – 28, using the AAFCO cat quantitative faecal collection protocol used previously (Bermingham *et al*., 2013b). A fresh faecal sample collected on treatment day 29 for faecal microbiome and metabolomic analysis. Cats were returned to group housing and after day 31 of treatment were fasted overnight and had blood drawn for a final SAT after which they began their washout period.

The BW of each cat was recorded weekly throughout both study periods and washout period and individual or group intakes were recorded daily.

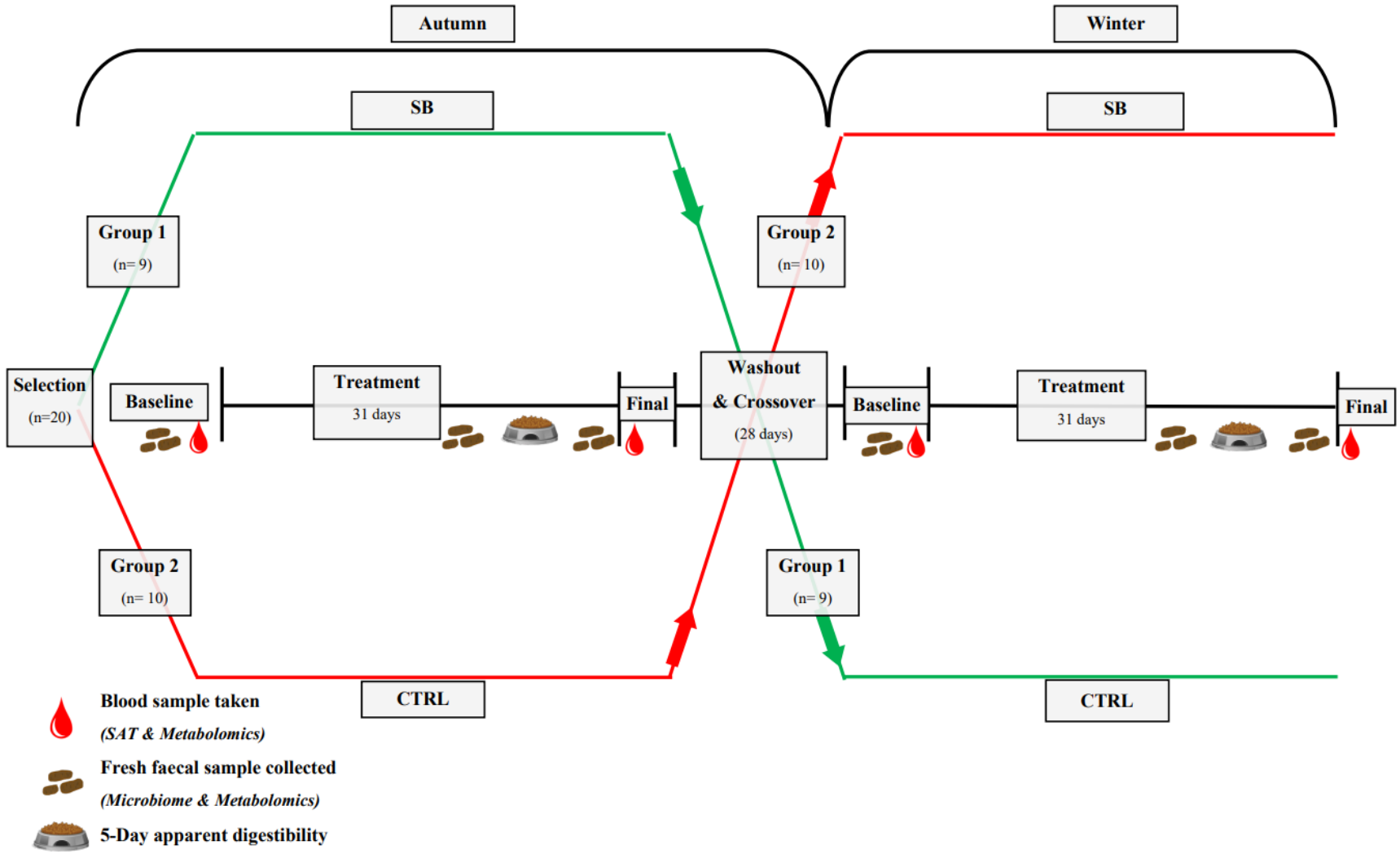


Figure 4.1. Diagram of the crossover study design. SB represents the daily treatment with *S. boulardii*, while CTRL represents the daily administration of the control capsule.

4.2.6 Plasma collection

The differential SAT was performed on each cat twice per study period. The baseline measurement was taken on day 0 (where day 1 was the first day of treatment), and day 32 (where day 31 was the last day of treatment). Plasma was collected from the cats using the methods previously described in Chapters 2 and 3. The baseline sample (T_0) was used for both the differential SAT and metabolomics analysis. The T_3 blood sample was used only for the differential SAT. Unlike Chapter 3, every cat completed the differential SAT and blood collection on 1 day, eliminating the potential effect of sampling day. Plasma was aliquoted into separate microcentrifuge tubes, T_0 samples for baseline SAT and metabolomics, and T_3 for post-sugar ingestion SAT. Additional back-up aliquots were also created so, plasma would only have to be thawed out once if re-analysis was required. Samples were frozen at -80 °C until analysis.

4.2.7 Faecal collection

4.2.7.1 Faecal microbiome

A fresh faecal sample was collected from the cats three times during each study period. The baseline sample was collected on day -2, the 3-week sample on day 22, and the final sample on day 29. Cats continued to receive the treatment until day 31, the day before the differential SAT, due to sample collection scheduling and logistics. While housing the cats in individual cages with double litter trays during these collection days, litter trays were checked every 10 minutes for faeces. If faeces were detected, a sample from the core of the pellet was collected, immediately frozen in liquid nitrogen for 10 minutes then stored at -80 °C until analysis. These faeces were visually scored using a 5-point scale which classified "bullet like" faeces as grade 1, and "entire liquid stool" as grade 5 (Moxham, 2001). These samples were used for both faecal microbiome and metabolomics analysis.

4.2.7.2 Apparent total tract digestibility

During days 24-28, the cats were housed in individual cages with double litter trays to measure diet ATTD. Total food intake and faecal output were measured each day, with the faeces again scored using the same 5-point scale (Moxham, 2001). Daily faecal output and a sample of canned food from each period was also weighed and stored at -20 °C before further analysis.

4.2.8 Sample preparation and analysis

4.2.8.1 Apparent total tract digestibility

Faecal and diet samples were freeze-dried, re-weighed, and ground before all samples from each cat per period were combined and submitted for macronutrient analysis. Both the homogenous diet and faecal samples were analysed in the Massey University Nutrition Laboratory, accredited under International Accreditation New Zealand (IANZ). Ash (AOAC 920.153, 923.03), DM (AOAC 925.10, 930.15), CP

(AOAC 968.06; Dumas method and using the standard nitrogen conversion factor of 6.25), CF (AOAC 962.09/978.10), and GE (using bomb calorimetry) were determined. Faecal CFat was determined using the Mojonnier method (AOAC 954.02) and dietary CFat using the Soxtec hexanes extraction method (AOC 2003.06).

4.2.8.2 Differential sugar absorption test

Whole blood was transferred to a K2 EDTA 5.4 mg vacutainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and spun at 3000 x *g* at 4 °C for 15 minutes. Plasma was then aliquoted into microcentrifuge tubes and frozen at −80 °C until analysis.

Analysis of the sugar probes in plasma was undertaken using the method described in Chapter 2 and (Patterson *et al*., 2024), with two modifications. Firstly, in order to ensure more accurate quantification, the internal standard solution was made from stock solutions of individual ^{13}C sugars using 40 µL of $^{13}C_6$ rhamnose, 40 µL of $^{13}C_5$ xylose, 20 µL of $^{13}C_6$ 3-OMG, and 500 µL of $^{13}C_{12}$ lactulose in 400 µL of 90 % ACN with 1 mM ammonium formate. Secondly, an eight-point calibration series was prepared containing 0, 1, 5, 10, 20, 50, 100, or 500 µg/mL of the target unlabelled sugars, and 10 µg/mL of the ^{13}C labelled sugars, and a final high calibration standard was added for xylose quantification as occasional sample values were not contained within the first 0 – 7 calibration sets.

4.2.8.3 Faecal microbiome

DNA was extracted from faecal samples using the NucleoSpin® Soil kits (November 2021/Rev. 10, Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Samples were thawed on ice before 200 ± 10 mg of each sample was aliquoted into a bead beating tube and vortexed with 700 µL of lysis buffer SL2. Next, 150 µL of the Enhancer SX was added to each tube to maximise DNA yield. Samples were put into a Mini-Beadbeater-96 (BioSpec Products, Bartlesville, OK, USA) for 5 minutes. Contaminants in the samples were precipitated by centrifuging samples for 2 minutes at 11 000 x *g*, adding 150 µL of lysis buffer SL3, vortexing for 5 seconds, keeping on ice for 5 minutes, then centrifuging again at 13 000 x *g* for 5 minutes. The lysate was filtered through a new Eppendorf tube with a silica membrane column (NucleoSpin® Inihbitor Removal Column) by taking 700 µL of the supernatant from the bead beating tubes and centrifuging it through the new filtered tube at 11 000 x *g* for 1 minute. The silica membrane column was then discarded. The collection tube containing the supernatant was cleared by adding 250 µL of binding buffer SB and vortexing for 5 seconds. To bind the DNA, 550 µL of sample was transferred into a new Eppendorf tube with a silica membrane column (NucleoSpin® Soil Column), centrifuged at 11 000 x *g* for 1 minute, the fluid at the bottom of the Eppendorf tube was discarded, and the rest of the sample was added back to the tube with the column and the process repeated, leaving only the empty Eppendorf tube with the silica membrane column. Following this, a four-step wash of the silica

membrane occurred, each step ending with fluid being discarded from the Eppendorf tube, and the same silica membrane column being reinserted. The first wash started with 500 µL of binding buffer SB added to the tube, centrifugation at 11 000 x *g* for 30 seconds, then the flow through fluid was discarded. During the second wash, 550 µL of wash buffer SW1 was added to the tube then discarded after being centrifuged at 11 000 x *g* for 30 seconds. Thirdly, 650 µL of wash buffer SW2 (containing ethanol) was added to the tube, vortexed for 2 seconds, centrifuged at 11 000 x *g* for 30 seconds, and the flow through fluid was discarded. This step was repeated exactly for the fourth wash. To dry the silica membrane, the column was placed back into the empty Eppendorf tube and centrifuged at 11 000 x *g* for 5 minutes. During the final elution of DNA step, the silica membrane column was placed into a new collection tube and 50 µL of elution buffer SE was added directly to the membrane and left at room temperature for 5 minutes. Tubes were centrifuged at 11 000 x *g* for 30 seconds and the silica membrane column was discarded, leaving only the final collection tube.

A NanoDrop (ND-1000 Spectrophotometer, ThermoScientific, New Zealand) was used to quantify the DNA concentration for sequencing, but also to determine if any samples needed to be re-analysed as samples were not accepted if the DNA concentration was less than 20 ng/µL.

DNA sequencing was performed using Qubit HS DNA reagent and normalised to 5 ng/µL using the EpMotion robot at Auckland Genomics (University of Auckland, Auckland, New Zealand). Illumina protocol "16S Metagenomic Sequencing Library Preparation: Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System-b" was used with forward primer sequence V3-341F: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGAGGCAGCAG and reverse primer sequence V4-806R: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVG GGTWTCTAAT. The polymerase chain reaction (PCR) (Platinum™ SuperFi II PCR Master Mix, Catalog number: 12368010, Thermofisher) was performed with the following conditions: 95 °C for 3 minutes for 1 cycle, then 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds for 25 cycles, next 72 °C for 5 minutes, and lastly a pause at 4 °C. AmpureXP beads at a ratio of 1:1 were used to purify the amplicons from each sample then eluted in 20 µl of nuclease free water. A Qubit HS DNA reagent was used to quantify DNA and an EpMotion robot normalised it to 5 ng/µl. The indexing PCR (Platinum™ SuperFi II PCR Master Mix, Catalog number: 12368010, Thermofisher) used 15 µl and Nexera XT primers, custom synthesised by IDT. Equimolar pools of sample were purified twice using AmpureXP beads at a 1:1 ratio and eluted in 40 µl of nuclease free water before finally being check for size using a bioanalyzer (Aglilent), which was adjusted to 4nM, and being run on an Illumina MiSeq machine using a 600 cycle V3 kit (2 x 300 bp) with 10 % phiX, a control library. A total of 25,402,648 paired reads passed the filtering process, generating 15.6 Gbp. Approximately 300,000 reads were generated per sample.

Both forward and reverse sequences were obtained for each DNA fragment, each 300 base pairs long. The raw sequencing data in Fastq files, which contained the sequence and quality for each base, were imported demultiplexed, quality filtered, and dereplicated using the DADA2 algorithm. This algorithm identified amplicon sequence variants (ASVs) which are more precise than operational taxonomic units (OTUs) for identifying microbial diversity which was the aim of this analysis. Since reverse reads gave a warning from 30 bp, analysis was carried out using only forward read. Sequences were aligned de novo using MAFFT and phylogeny construction using FastTree2. Refraction curves were used to choose the sampling depth of 69,128 sequences for alpha and beta diversity, with Shannon index and weighted and unweighted Unifrac distances. Taxonomy was assigned to the ASVs using the SILVA database (SILVA-138-V3V4-99-classifier), a reference database of 16S rRNA sequences, trained with a naive Bayes classifier for the primer set 341F to help assign taxonomic labels to each ASV based on its sequence.

4.2.8.4 Plasma and faecal metabolomics

Plasma and faecal samples were prepared and analysed in a random order, respectively, using the following protocol. Plasma and faecal samples were each run in a single analytical batch. This method of extracting polar and semi-polar compounds was previously described in (Garrigues *et al*., 2024). Faecal samples first had to be prepared as faecal water, using the frozen aliquots taken as fresh faecal samples during faecal microbiome collection days. Faeces were homogenised, then 1 g was aliquoted and mixed with 1 g of deionised water. The mixture was then vortexed for 30 seconds, sonicated for 2 minutes, vortexed for another 30 seconds, and centrifuged at 13,000 x *g* for 30 minutes at 4 °C. Then, 1 mL the resulting supernatant was collected and stored at -80 °C until metabolite extraction and analysis. On the day of extraction, 100 µL of plasma or faecal water from each sample was pooled into one vial to create PQC samples from pooled plasma and pooled faecal water which were then extracted and run throughout the analysis batch for quality control and data normalisation.

Samples were thawed from -80 °C on ice then vortexed before 100 µL of plasma or faecal water sample was aliquoted into an Eppendorf tube. Extraction blanks were also prepared using MilliQ water, prepared with a Milli-Q® ultrapure water system. 400 µL of cold methanol (MeOH) containing buffering agents HEPES, PIPES, and CHES standards, was added to each Eppendorf tube, before vortexing at low speed for 1 minute. Samples were incubated at -20 °C for 1 hour, and vortexed again for 30 seconds. Samples were then centrifuged at 4 °C for 15 minutes at 11 000 x *g*, and 450 µL of the upper phase aliquoted into 10 kDa centrifugal filters (VWR, Radnor, PA, USA) to then be centrifuged again at 4 °C for 45 minutes at 11 000 x *g*. The filtered solution was split into two identical Eppendorf tubes, 200 µL to be used for C18 analysis, and 200 µL to be used for Hydrophilic Interaction Liquid Chromatography (HILIC). The solvent was evaporated under a nitrogen flow and then reconstituted in 125 µL of solvent made up of 9 parts MilliQ

water to 1 part ACN with 0.1 % formic acid (FA) for C18 analysis and 1 part MilliQ water to 9 parts ACN with 0.1 % FA for HILIC analysis. Samples were vortexed for 1 minute and then the entire solution was transferred into 0.2 µm centrifugal filters (VWR, Radnor, PA, USA) and centrifuged at 4 °C for 15 minutes at 11 000 x *g*. The final filtered solution was aliquoted into glass vials for sample and pool analyses. Diluted pool replicas at 2, 4, 8, and 16 dilution factors were also created from the pooled sample and blank solvent.

LC-MS/MS was carried out using high performance liquid chromatography (Dionex Ultimate 3000, Thermo Scientific, Bremen, Germany) coupled to an Orbitrap Exploris 240 (Thermo Scientific) mass spectrometer. Samples were analysed in random order, with pool samples injected after every five biological samples at an injection volume of 5 µL. Chromatographic separation utilised both reverse, C18, and normal, HILIC, phases. For C18 separation, a Hypersil Gold C18 (100 mm x 2.1 mm x 1.9 µL) (Thermo Scientific) column was used with a gradient elution of MilliQ water with 0.1 % formic acid (eluent A) and acetonitrile with 0.1 % formic acid (eluent B) at a flow rate of 400 µL/minute. At oven temperature 40 °C, the gradient elution program used was as follows: 100 % A (0 – 1 minutes), 0 % of B in the isocratic elution on a linear gradient to 100 % B (1 – 11 minutes), held isocratic (11 – 13 minutes), 100 – 0 % B (13 – 14 minutes), and a re-equilibration period at 0 % B for 2 minutes (14 – 16 minutes For HILIC separation, a HILIC (Se-Quant, ZIC-HILIC Peek Coated 150 x 2.1 mm x 5 µm, Merck) column was used with a gradient elution of MilliQ water with 16 mM ammonium formate (eluent A) and acetonitrile with 0.1 % formic acid at a flow rate of 250 µL/ minute. At oven temperature 25 °C, the gradient elution program was as follows: 97 % of B (0 – 2 minutes), 97 – 70 % B (2 – 10 minutes), 70 – 10 % B (10 – 15 minutes), held isocratic (15 – 17 minutes), 10 – 97 % B (17 – 18 minutes), and a re-equilibration period of 97 % B (18 – 27 minutes). Data was acquired in both positive and negative ionisation mode using polarity ion switching mode (60 – 800 m/z) with a resolving power of 45000 FWHM for C18 and 60000 FWHM for HILIC (for 200 m/z). The ESI source was operated with spray voltage of ± 3.5 kV, capillary temperature of 320 °C, sheath gas flow of 30 L/ minute, auxiliary gas flow of 8 L/ minute, and sweep gas flow of 0 L/ minute.

4.2.9 Statistical analysis

4.2.9.1 Feed intake, body weight, and faecal health score

Statistical analyses were carried out using RStudio version 4.1.1. A linear-mixed effects model was employed using the lmer() function from the lmerTest package. Individual feed intake measurements were transformed for each cat from 14 different timepoints across the study into dry matter intake (DMI) per kg of BW as the dependent variable while the treatment groups (consisting of baselines, *S. boulardii* treatment, and CTRL) and period interaction was the fixed effect, and cat was a random effect to account for subject-level variability. The model's residuals were visually inspected for homogeneity of variance and normality.

The overall significance of the model was assessed using an ANOVA via the anova() function and the pairwise comparisons were completed using TukeyHSD () function.

A similar model was used for BW in RStudio version 4.1.1. A linear-mixed effects model was employed using the lmer() function from the lmerTest package. Body weight from the beginning and end of each period was the dependent variable while date and treatment were fixed effects, and cat was a random effect to account for subject-level variability. The model's residuals were visually inspected for homogeneity of variance and normality. The overall significance of the model was assessed using an ANOVA via the anova() function and the pairwise comparisons were completed using TukeyHSD() function.

Faecal score was evaluated using a Cumulative Link Mixed Model (CLMM) from the "ordinal" package in RStudio version 4.1.1 since it is an ordinal outcome variable that ranges from 1 – 5. The analysis used faecal score as a dependent variable and treatment, timepoint, age, and LR for fixed effects, and cat as a random effect. Results are presented as mean ± SD. Statistical significance was defined at $p < 0.05$ and trends at $p < 0.1$.

4.2.9.2 Apparent total tract digestibility

All statistical analyses were carried out using RStudio version 4.1.1. All data from one female cat in period 1 were identified as outliers using Chauvenet's Criterion and removed. Firstly, an ANOVA was conducted using aov() function and post-hoc pairwise tests were performed using the Tukey HSD method to identify significant differences between period, treatment, or their interaction. Summary statistics of number of observations, mean, and standard deviation were calculated using the dplyr package and superscripts assigned based on statistical significance at $p < 0.1$ based on the adjusted p-values.

Linear-mixed models, lmer(), from the lmerTest package were fitted for each digestibility parameter with fixed effects age, sex, neuter status, period, treatment, XG, and LR, and cat as a random effect. The summary of the model was evaluated and then a stepwise regression procedure, using step(), was performed to simplify the model by removing non-significant predictors. Crude fat was transformed to achieve normality using the Box-Cox procedure using the boxcox() function from the MASS package and the optimal transformation parameter, λ, was identified by maximising the likelihood function. Model diagnostics were performed by visualising residuals with diagnostic plots and conducting a Shapiro-Wilk test for normality to ensure the model's assumptions were met. Due to the nature of the linear stepwise model, cats were removed from this dataset if they did not have a complete set of data across all variables. Therefore, in total, four rows of data have been omitted from three females and one male because of outliers and absent LR and XG values. Results are presented as mean ± SD. Statistical significance was defined at $p < 0.05$ and trends at $p < 0.1$.

4.2.9.3 Differential sugar absorption test

Data was omitted from two female cats due to vomiting after the solution was administered, from period 2 baseline, and period 2 final timepoints. Outliers were identified and removed from the datasets using Chauvenet's Criterion. Statistical analyses were carried out using RStudio version 4.1.1.

A Welch's t-test was used to compare treatment with the overall change in intestinal permeability and absorptive capacity, determined by looking at the difference between final LR and XG ratios and baseline ratios for each treatment. This comparison was meaningful as there were no significant differences between any baseline measures between periods or groups as shown by a Welch's t-test. To determine which factors might influence the change in LR or XG during the trial, linear mixed-effect models were fitted including Period, Treatment, plus the interaction between the two, age, sex, and weight as fixed effects, and cat as the random effect. Model selection was carried out using ANOVA and stepwise regression, and final models were identified for both LR and XG changes. The simplified model for mean LR change contained only the period as a predictive factor, after removing all other predictors through the stepwise selection process. Additionally, the simplified model for mean XG change contained only the intercept, suggesting there are no predictors for the change in XG during either period. Residuals from these models were checked for normality with Shapiro-Wilk tests, and diagnostic plots were examined. Predicted means for specific factors were calculated using the predictmeans() function for pairwise comparisons.

Further analysis on the LR and XG values was completed again using a linear mixed-effects model. For LR, fixed effects were listed as date, treatment, the interaction between the two, age, sex, and weight, with cat as a random effect. A similar model was used for XG but values of XG were log-transformed to achieve normality. Residual diagnostics and pairwise predictions were again conducted, with the final models identified using the stepwise function, step(). Results are presented as mean ± SD. Statistical significance was defined at $p < 0.05$ and trends at $p < 0.1$.

4.2.9.4 Faecal microbiome

The sequencing data was quality checked, then processed using Quantitative Insights Into Microbial Ecology (QIIME2; version 2020.2) to analyse the data and taxonomic classification. The analysis was done with a forward read as reverse reads gave a warning from 30 bp, the Phred score was 20. Due to this, no species level identifications occurred as the Phred score limits accurate classification at this level, and to a lesser extent, the genus level. DADA2 amplicon sequencing was used for identification and correction of sequencing errors to accurately generate microbial sequence variants from the original raw data.

An OTU abundance table was uploaded to microbiomeanalyst.ca (Lu *et al*., 2023) using SILVA taxonomy labels, with the week 4 and 12 data (final timepoints of period 1 and 2) from one female cat

removed because they were identified as outliers. There were 524 singletons removed, 159,351 maximum counts per sample, and 69,121 minimum counts per sample. The number of processed samples was 82. In the data filtering step, a low count filter of minimum count of 20, and 20 % of prevalence in sample was selected with 0 % removed using low variance filter. This step removed a total of 276 low abundance features based on prevalence and 0 low variance features were removed based on IQR, leaving a remaining 137 features. Data was not rarefied or transformed, and total sum scaling (TSS) was used to normalise the data. Alpha diversity comparisons were carried out using statistical method Welch T-test/ANOVA with Posthoc pairwise comparisons and reported results based on the false discovery rate (FDR) p-values obtained from multi-testing adjustments based on the Benjamini-Hochberg procedure. Observed Species, Chao 1, and Shannon diversity measures were calculated to cover richness and evenness. Beta diversity was analysed using a Principal Coordinate Analysis (PCoA) with a Bray-Curtis index, followed by pairwise PERMANOVA.

4.2.9.5 Plasma and faecal metabolomics

MSDial version 5.4 was used to import, select, and annotate the metabolites from the plasma or faecal HILIC positive, HILIC negative, C18 positive, C18 negative analyses. The filters used were "Ref. matched", "Suggested", and "MS2 acquired". Three libraries, one in-house and two curated .MSP spectral libraries from the MS-Dial website (MSMS-Public-Neg-VS15 and MSMS-Public-Pos-VS15 from https://systemsomicslab.github.io/compms/msdial/main.html#MSP) were used to annotate the metabolites, and metabolites were selected based on retention time match (for metabolites in the house library), mass (m/z), absence in blanks, and representative vs. reference mass spectrum. Selected metabolites were then normalised using LOWESS method applied to the pooled quality control (QC) samples and subsequently exported as LOWESS normalised data.

SIMCA version 16.0.1 was used to evaluate all HILIC and C18 data for both plasma and faecal samples. Due to a sample preparation error, one plasma sample from period two baseline from a female cat was omitted and due to a lack of plasma, one sample from a male cat at the end of period 1 was omitted.

The selection process for both plasma and faecal metabolites began with summing the peak intensities of adducts of the same metabolites and then choosing which metabolites to retain if they appeared multiple times throughout the HILIC negative, HILIC positive, C18 negative, C18 positive streams. The metabolites were selected for being unique to one stream, or for having a lower CV and/or higher peak intensity than the other duplicates. This final datasheet with all plasma or all faecal metabolites was analysed in MetaboAnalyst (www.metaboanalyst.ca), using module "Statistical Analysis [one factor]", using data type peak intensities, reliability filter for RSDs greater than 25 %, variance filter of the IQR filtering out 10 %,

abundance filter at 0 %, with log transformation on plasma and faecal datasets. P-values were adjusted for FDR

To analyse the relationship between plasma metabolites with age and intestinal permeability, a partial least square (PLS) model was run in SIMCA version 16.0.1. The first model was run using age as the predictor (y value), containing 262 x variables, including plasma metabolites, LR, and XG. This PLS regression was then filtered by coefficient score (CS), excluding x variables that contributed least to the model, leaving the model with 89 x variables, including 88 plasma metabolites, XG, and LR. The process was repeated, using LR as the y value, and including all plasma metabolites, age, and XG as x variables. After CS filtering, the LR PLS regression model was also left with 89 x variables, including 88 plasma metabolites, XG, and age.

This process was repeated with faecal metabolites. The first model was run using age as a y value, containing 475 x variables, including faecal metabolites, LR, and XG. This PLS regression was then filtered by CS, excluding x variables that contributed the least to the model, leaving the model with 72 x variables, including 71 faecal metabolites and LR. The process was repeated, using LR as a y value, including all plasma metabolites, age, and XG as x variables. After CS filtering, the LR PLS regression model was left with 113 x variables, including 112 faecal metabolites and age.

The remaining x variables from the plasma metabolite models after CS filtering were then evaluated individually using the Pearson correlation coefficient to either age or LR in RStudio version 4.1.1.

4.3 Results

4.3.1 Feed intake, body weight, and faecal health score

Feed intake (DMI / kg BW) was significantly affected by *S. boulardii* supplementation ($p < 0.001$), period ($p < 0.001$), and the interaction between the two variables ($p = 0.028$; Figure 4.2). Feed intake during the baseline period before period 2 was higher ($p < 0.01$) than any other timepoint, and both groups in period 2 had significantly higher feed intake than cats dosed with *S. boulardii* in period 1 ($p < 0.05$).

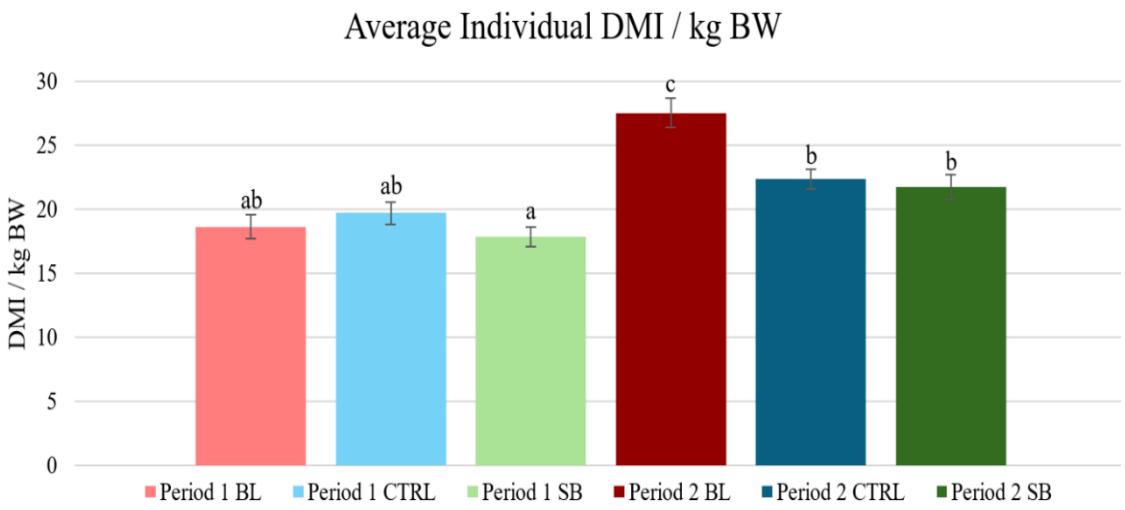


Figure 4.2. Effect of *S. boulardii* supplementation (2.5 x 10^8 CFU/day for 31 days) on dry matter intake (DM/kg BW) in midlife to senior cats in a crossover design. Results are presented as means ± SEM. Different superscripts denote significance at $p < 0.05$.

Abbreviations: DMI, dry matter intake (g); BW, body weight; BL, baseline; SB, *S. boulardii*; CTRL, control

Supplementation of *S. boulardii* did not significantly affect BW of cats ($p = 0.372$), but there was an effect of period ($p < 0.001$; Figure 4.3).

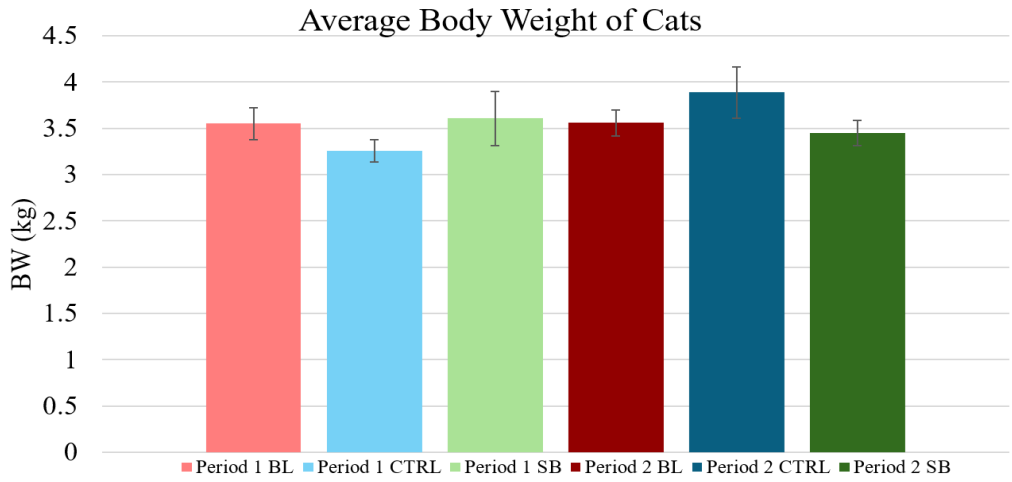


Figure 4.3. Effect of *S. boulardii* supplementation (2.5 x 10^8 CFU/day for 31 days) on average BW of midlife to senior cats in a crossover design. Results are presented as means ± SEM.

Abbreviations: BW, body weight; BL, baseline; SB, *S. boulardii*; CTRL, control

Faecal health scores were not significantly affected by *S. boulardii* supplementation ($p > 0.100$; Figure 4.4). However, secondary analysis showed that increasing age had a significant positive effect on the score,

resulting in looser stools (p = 0.014). Additionally, faecal health scores were lower at the end of period 1 (p = 0.009) compared to other timepoints in the trial.

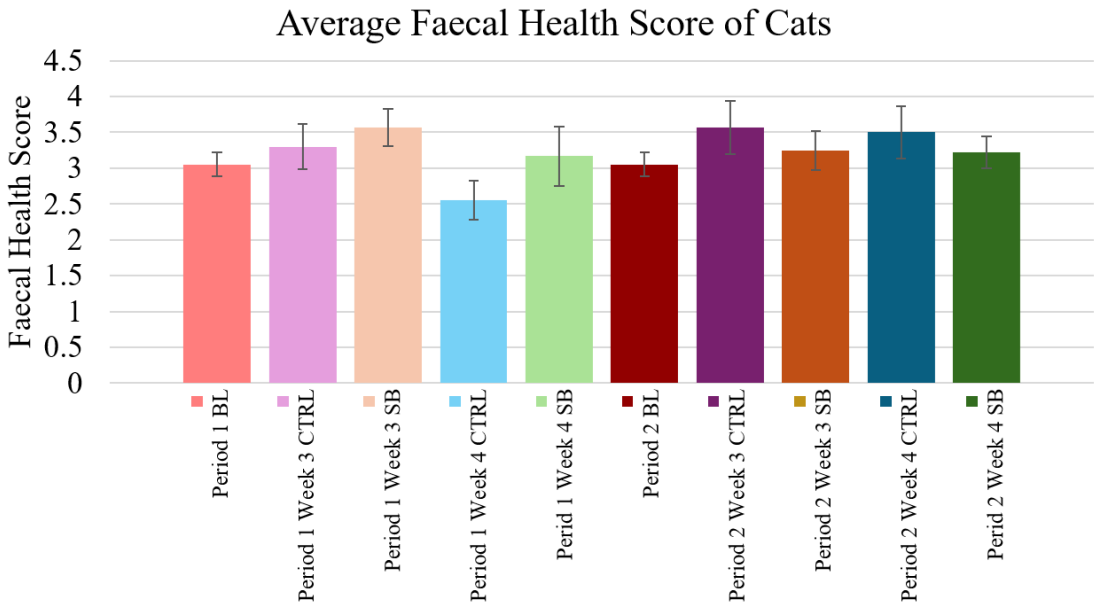


Figure 4.4. Effect of *S. boulardii* supplementation (2.5 x 10^8 CFU/day for 31 days) on average faecal health score of midlife to senior cats in a crossover design. Results are presented as means ± SEM. Cats in both groups, on average, had lower faecal health scores at the end of period 1 (week 4) (p = 0.009), but were not significantly different when separated based on treatments or through a pairwise comparison.

Abbreviations: BW, body weight; BL, baseline; SB, *S. boulardii*; CTRL, control

4.3.2 Apparent total tract digestibility

Apparent total tract digestibility was not significantly affected by *S. boulardii* supplementation (p > 0.1; Table 4.4).

Table 4.4. The ATTD of macronutrients across treatment and period of cats dosed with 2.5 x 10^8 CFU of *S. boulardii* for 24-28 days in a crossover design. Results presented as mean ± standard deviation.

	Period 1 CTRL ***n = 9***	**Period 1 SB** ***n = 9***	**Period 2 CTRL** ***n = 9***	**Period 2 SB** ***n = 10***
DM Digestibility (%)	68.8 ± 7.01	71.3 ± 9.59	75.0 ± 5.35	74.3 ± 5.35
Energy Digestibility (%)	71.8 ± 9.58	73.4 ± 11.25	77.4 ± 6.47	76.2 ± 8.00
CP Digestibility (%)	80.9 ± 4.30	82.1 ± 6.19	83.3 ± 3.09	83.7 ± 2.92
CFat Digestibility (%)	80.5 ± 16.10	81.2 ± 15.29	84.4 ± 11.04	83.2 ± 14.25

Abbreviations: ATTD, apparent total tract digestibility; SB, *S. boulardii*; CTRL, control; DM, dry matter; CP, crude protein; CFat, crude fat.

Based on post-hoc analysis, the ATTD of energy (p = 0.027), CFat (p = 0.058), and DM (p = 0.023) was higher in period 2 than in period 1 (Table 4.5). As age of the cat increased, the ATTD of energy (p = 0.094) and CFat (p = 0.039) decreased.

Table 4.5. P-values of post-hoc analysis of ATTD from a stepwise linear model of cats dosed with 2.5 x 10^8 CFU of *S. boulardii* for 24-28 days in a crossover design.

	DM	**Energy**	**CP**	**CFat**
Neuter Status	0.521	0.760	0.500	0.123
Period * Treatment	0.376	0.483	0.451	0.352
Sex	0.864	0.312	0.729	0.675
LR	0.155	0.198	0.261	0.872
XG	0.150	0.147	0.116	0.348
Treatment	0.302	0.329	0.189	0.208
Age	0.134	0.094	0.143	0.039
Period	0.023	0.027	0.127	0.058
Final Model	DM ~ Period + (1 | Cat)	Energy ~ Period + (1 | Cat)	CP ~ (1 | Cat)	CFat ~ Age + (1 | Cat)

Abbreviations: ATTD, apparent total tract digestibility; DM, dry matter; CP, crude protein; CFat, crude fat; LR, lactulose: rhamnose; XG, xylose: 3-O-methylglucose.

4.3.3 Intestinal permeability (LR) and absorptive capacity (XG)

There was no effect of *S. boulardii* supplementation (i.e. the change from baseline to day 31) on the LR value in cats (p = 0.729). When analysing the change in these intestinal measurements from baseline to the last day of treatment, only period was associated with a positive change in LR (p = 0.050; Table 4.6). Similarly, there was no effect of *S. boulardii* supplementation on the XG value (p = 0.421), however, day 31 values of XG were higher (p = 0.048) in cats dosed with *S. boulardii* (0.92 ± 0.127) compared to CTRL (0.82 ± 0.12) (Figure 4.5).

Table 4.6. The mean change of LR and XG of cats dosed with 2.5 x 10^8 CFU of *S. boulardii* from baseline to day 31 in a crossover design. Results presented as mean ± standard deviation.

	Period 1 CTRL *n = 9*	**Period 1 SB *n = 9***	**Period 2 CTRL *n = 9***	**Period 2 SB *n = 10***	**p- value of treatment**	**p-value of period**
LR Change	- 0.11 ± 0.098	- 0.02 ± 0.132	0.09 ± 0.214	0.02 ± 0.255	0.756	0.050
XG Change	0.03 ± 0.191	0.04 ± 0.169	0.002 ± 0.211	0.10 ± 0.200	0.417	0.799

Abbreviations: SB, *S. boulardii*; CTRL, control; LR, lactulose to rhamnose ratio; XG, xylose to 3-O-methylglucose ratio.

Secondary analysis indicated that XG tended (p = 0.060) to be higher in males (0.88 ± 0.145) than females (0.82 ± 0.129) (Figure 4.5).

Additionally, secondary analysis indicated that LR increased (p = 0.034) with increasing age (Figure 4.6).

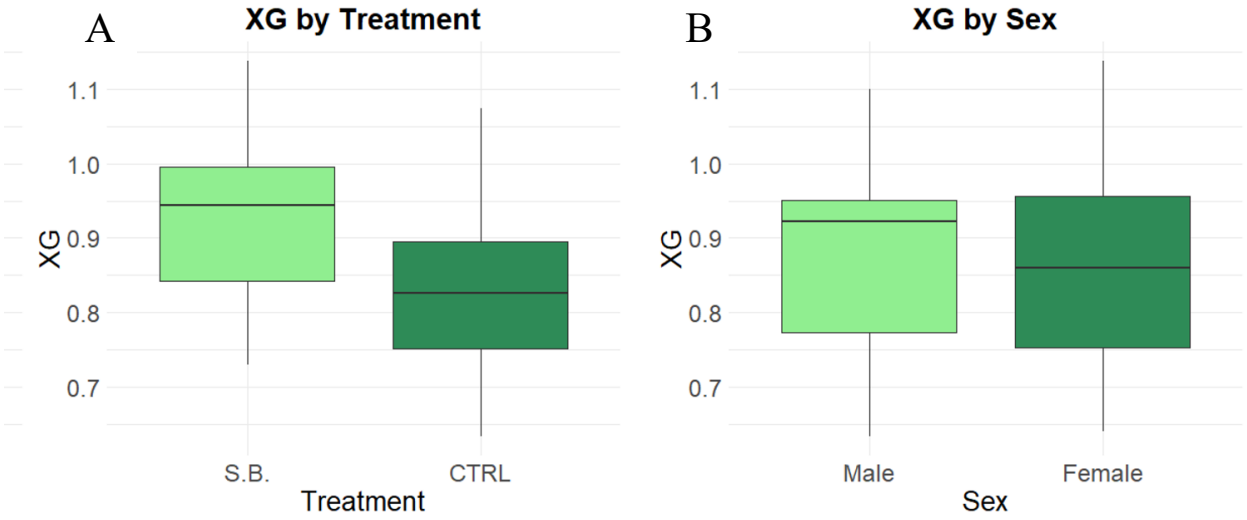


Figure 4.5. Treatment results and secondary analysis of the differential sugar absorption test (SAT) on midlife to senior cats in a crossover design. Cats supplemented with 2.5 x 10^8 CFU *S. boulardii* (SB) for 31 days had significantly higher absorptive capacity than those supplemented with the placebo (CTRL) (p = 0.048) (A). While not significant, the ratio of xylose to 3-O-methylglucose (XG), indicative of absorptive capacity, is higher in males than females (p = 0.060) (B).

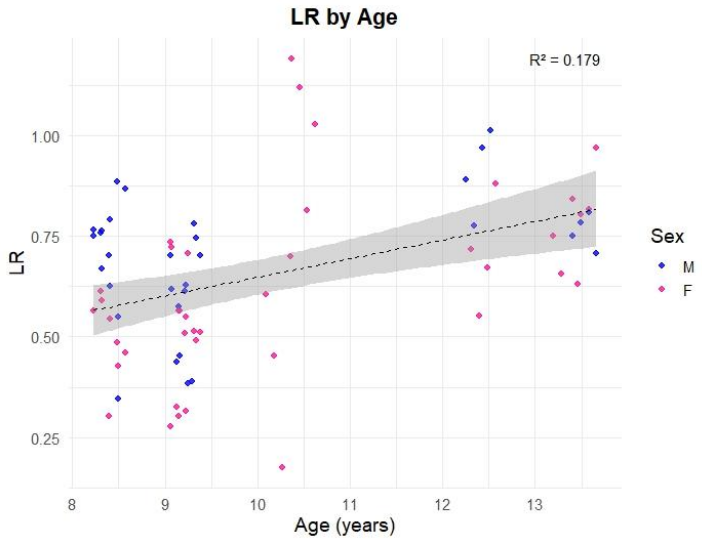


Figure 4.6. Treatment results and secondary analysis of the differential sugar absorption test (SAT) on midlife to senior cats in a crossover design. A linear regression model shows a significant ($p = 0.034$, $R^2 = 0.179$) increase in the ratio of lactulose to rhamnose (LR), indicative of intestinal permeability, with increasing age.

4.3.4 Faecal microbiome

There was no effect of supplementation of *S. boulardii* on the composition of the faecal microbiome. There were no differences in alpha diversity richness or evenness or beta diversity associated with supplementation of *S. boulardii* ($p > 0.05$). Irrespective of probiotic treatment, the faecal microbiome at the Phyla level was dominated by Bacillota (66.5 % of sequence reads), followed by Fusobacteriota (17.4 % of sequence reads), Bacteroidota (9.9 % of sequence reads), and to a lesser extent, Pseudomonadota (3.3 % of sequence reads) and Actinomycetota (2.5 % of sequence reads) (Figure 4.7). In terms of microbial families, the most represented family was Peptostreptococcaceae, followed by Fusobacteriotaceae, Lachnospiraceae, and Prevottellaceae. *Peptoclostridium*. *Fusobacterium*, *Clostridium sensu stricto*, *Blautia* and *Negatibacillus* were the dominant genera (Figure 4.8).

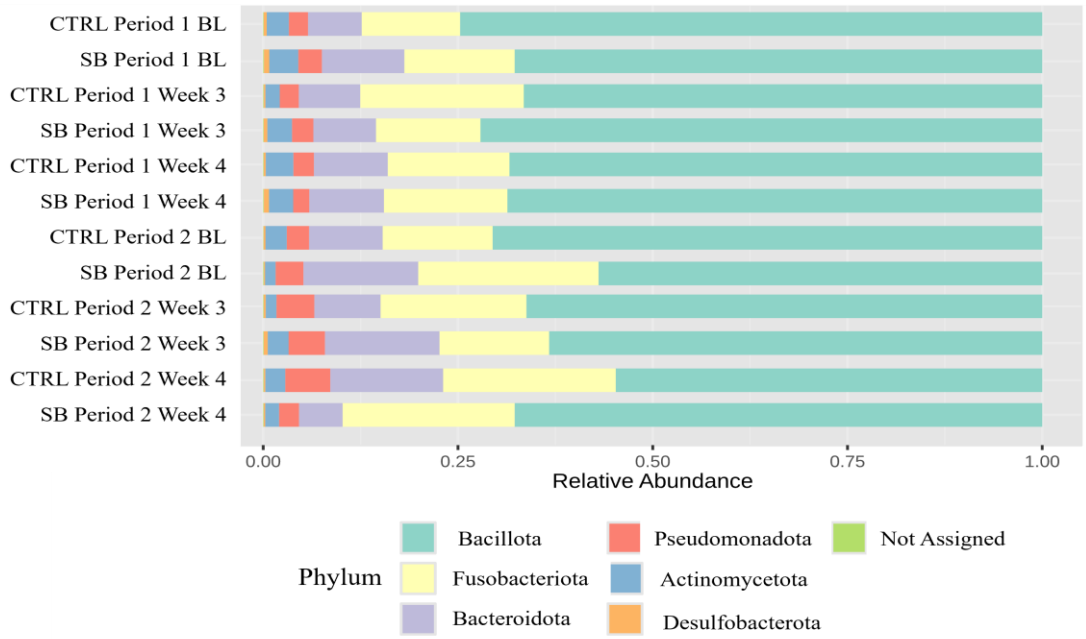


Figure 4.7. Effect of *S. boulardii* supplementation (2.5 x 10^8 CFU/day for 31 days) on relative phyla abundance of midlife to senior cats in a crossover design. There was no significant effect of treatment.

Abbreviations: CTRL, control; SB, *S. boulardii*; BL, baseline.

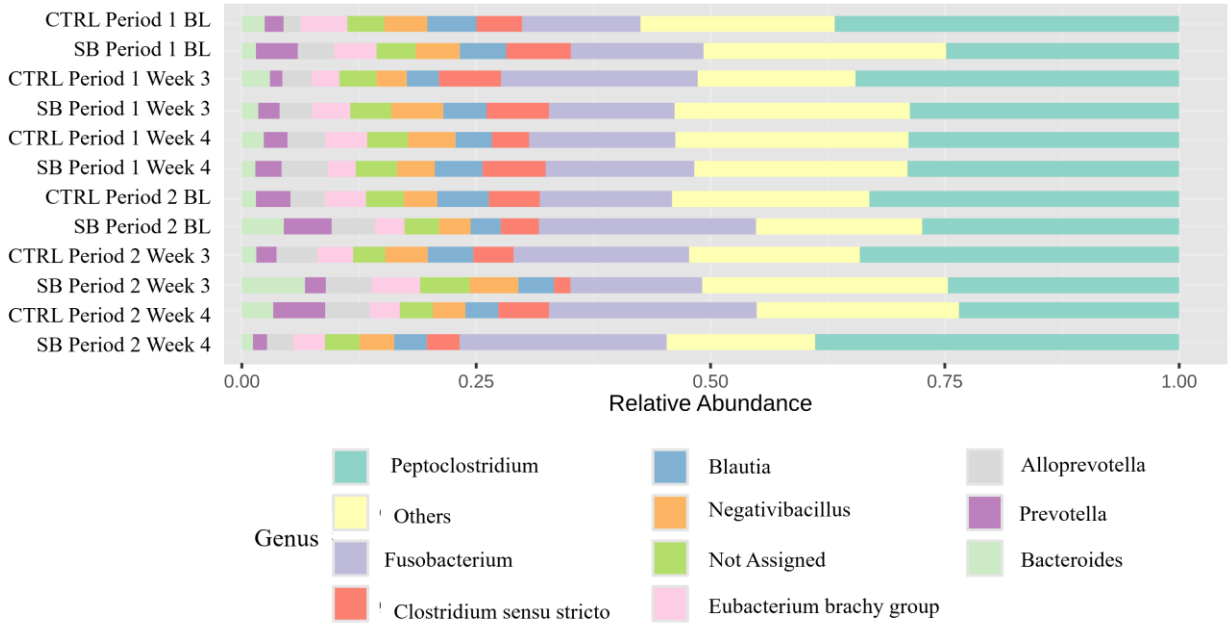


Figure 4.8. Effect of *S. boulardii* supplementation (2.5 x 10^8 CFU/day for 31 days) on relative genera abundance of midlife to senior cats in a crossover design. There was no significant effect of treatment.

Abbreviations: CTRL, control; SB, *S. boulardii*; BL, baseline.

4.3.5 Plasma and faecal metabolomics

There were 52 positive-mode and 100 negative-mode metabolites annotated in polar HILIC metabolite analysis of plasma samples, and 146 positive-mode and 128 negative-mode metabolites annotated in semi-polar C18 analysis of plasma samples. After samples were merged and metabolites selected for each stream based on uniqueness, lowest coefficient of variation, and highest intensity, 260 total plasma metabolites remained to be analysed. There were 97 positive-mode and 103 negative-mode metabolites annotated in polar HILIC metabolite analysis of faecal water samples, and 278 positive-mode and 170 negative-mode metabolites annotated in semi-polar C18 analysis of faecal water samples. After samples were merged and metabolites selected for each stream based on the same parameters as for plasma, 475 total faecal water metabolites remained to be analysed.

Multivariate analysis (principal components analysis; PCA and PLS-Discriminant Analysis; PLS-DA) analysis of the metabolomic data was conducted with no discernible differences between treatment in both the plasma and faecal water sample matrices, with a large amount of individual animal variance being the main factor observed (not shown). The PLS-DA models were not valid, confirmed by their low Q2 and insignificant p-values. Further analysis was done using univariate statistics and there was no effect ($p > 0.05$) of *S. boulardii* treatment on plasma or faecal metabolites.

Following the secondary approach for the age and LR analyses above, PLS regression was used to determine if there were plasma or faecal metabolites correlated with the age or LR. The PLS regressions completed on CS-filtered models of age and LR had smaller residuals than the models containing all variables, indicating that removing variables with low coefficient scores from the model improved the model's accuracy at predicting the fitted values (Anscombe and Tukey, 1963). All CS-filtered models had improved Q2 values that were closer to the R2Y than the non-CS-filtered models. Details about the models can be found in Table 4.7.

The CS-filtered PLS regressions were then mapped using PCA plots to visualise the regression selected components on the clustering of samples from the cats based on age or LR (Figure 4.9). The PCA(x) of plasma metabolites colour-coded by age (y) shows that the first two components explain 34.2 % of the total variation in the data, while the first two components of the plasma metabolite PCA(x) colour-coded by LR explain 35.6 % of the total variation. The PCA(x) of faecal metabolites colour-coded by age (y) shows that the first two components explain 27.6 % of the total variation in the data, while the first two components of the faecal metabolite PCA(x) colour-coded by LR explain 60.3 % of the total variation.

Table 4.7. PLS regression models of plasma and faecal metabolites, including variables XG and age or LR, with age and LR

	PLS Model	N (X)	R2X	R2Y	Q2		SS	DF	MS	F	p-value	SD
Plasma						**Total corr.**	73	73	1			1
	Y = age	262	0.356	0.939	0.871	**Regression**	63.128	6	10.521	71.409	3.595 e-27	3.244
						Residual	9.872	67	0.147			0.384
	Y = age (CS filtered)	89	0.383	0.955	0.921	**Total corr.**	73	73	1			1
						Regression	67.170	6	11.195	128.658	8.816 e-35	3.346
						Residual	5.830	67	0.087			0.295
	Y = LR	262	0.292	0.527	0.125	**Total corr.**	70	70	1			1
						Regression	4.823	4	1.206	1.221	0.310	1.098
						Residual	65.177	66	0.988			0.994
	Y = LR (CS filtered)	89	0.140	0.450	0.309	**Total corr.**	70	70	1			1
						Regression	21.620	2	10.810	15.194	3.510 e-06	3.288
						Residual	48.380	68	0.711			0.843
Faecal						**Total corr.**	112	112	1			1
	Y = age	475	0.473	0.923	0.569	**Regression**	55.569	10	5.557	10.044	1.469 e-11	2.357
						Residual	56.431	102	0.553			0.744

Y = age (CS filtered)	72	0.282	0.881	0.779	**Total corr.**	112	112	1			1
					Regression	87.137	6	14.523	61.914	2.063 e-32	3.811
					Residual	24.864	106	0.235			0.484
Y = LR	475	0.273	0.195	0.126	**Total corr.**	73	73	1			1
					Regression	9.233	2	4.617	5.140	0.008	2.149
					Residual	63.767	71	0.898			0.948
Y = LR (CS filtered)	113	0.501	0.214	0.171	**Total corr.**	73	73	1			1
					Regression	12.454	2	6.227	7.302	0.001	2.500
					Residual	60.546	71	0.853			0.923

Abbreviations: PLS, partial least squares; LR, lactulose: rhamnose; SS, sum of squares; DF, degrees of freedom; MS, mean square; F, F-statistic; SD, standard deviation; CS, coefficient score.

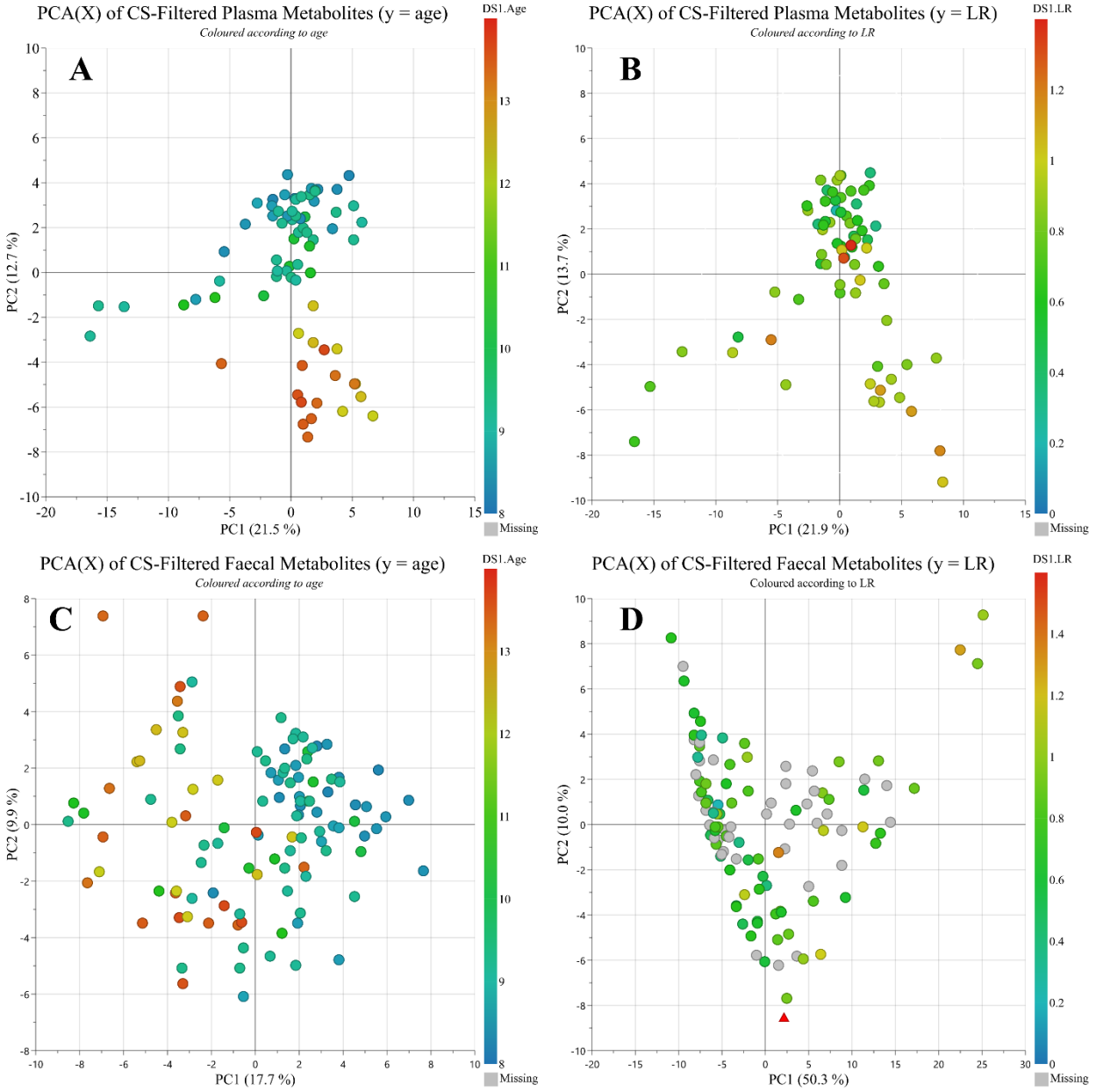


Figure 4.9. PLS regressions from Table 4.7 transformed into PCA(x) plots showing the distribution of samples along the first two principal components (PC1 and PC2). Plasma metabolites are very clearly separated by age (A), and LR, to a lesser extent (B) based on visual observation. Faecal metabolites are separated by age (C), and also LR, to a lesser extent (D). Triangle denotes that the LR value was an outlier (D).

Secondary analysis showed both age and LR affected the plasma and faecal metabolome (Table 4.8). For example, in plasma, TMAO increased with increasing age and LR value, along with docosatetraenoic acid, lysophosphatidylcholine, linoleic acid, oleic acid, and cis-vaccenic acid. Cis-vaccenic acid also had a weak, yet positive correlation with LR values in faecal samples. Bile acid CDCA decreased in plasma with

both increasing age and LR. Vitamins that were observed significantly changing with age were ascorbic acid and thiamine, increasing and decreasing, respectively, with increased age.

Table 4.8. Plasma and faecal metabolites of interest associated with age or LR in cats aged 8 – 14[1]

Group	**Metabolite**	**Plasma Metabolome & Age**		**Plasma Metabolome & LR**		**Faecal Metabolome & Age**		**Faecal Metabolome & LR**	
		R	***p-value***	***R***	***p-value***	***R***	***p-value***	***R***	***p-value***
Alkaloids and Derivatives									
	Trigonelline	0.47	< 0.001	-	-	-	-	-	-
Amino Acids and Derivatives									
	4-Guanidinobutyric acid	-	-	-	-	- 0.42	< 0.001	- 0.44	< 0.001
	Alanylaline	-	-	-	-	- 0.45	< 0.001	-	-
	Aspartylphenylalanine	0.49	< 0.001	-	-	-	-	-	-
	Isoleucul-isoleucine	-	-	-	-	0.50	< 0.001	-	-
	N-Acetylserine	0.50	< 0.001	0.32	0.006	-	-	-	-
	N-Methylglutamate	-	-	-	-	- 0.57	< 0.001	- 0.45	< 0.001
	Pyroglutamate	-	-	-	-	-	-	- 0.40	< 0.001
Benzoic acids									
	6-Dihydroxybenzoic acid	-	-	-	-	- 0.49	< 0.001	- 0.35	0.003
Bile Acids									
	Chendeoxycholic acid	- 0.40	< 0.001	- 0.32	0.006	-	-	-	-
Carbohydrates and Conjugates									
	Gluconic acid	0.62	< 0.001	-	-	-	-	-	-
	Glyceraldehyde	0.42	< 0.001	-	-	-	-	-	-
	Glycerate	-	-	-	-	0.55	< 0.001	0.38	< 0.001

Lipids, Fatty Acids, and Derivatives									
	cis-Vaccenic acid	0.43	< 0.001	0.33	0.004	-	-	0.28	0.014
	Dipalmitoylphosphatidylcholine	- 0.53	< 0.001	- 0.34	0.003	-	-	-	-
	Docosatetraenoic acid	0.53	< 0.001	-	-	-	-	-	-
	Lysophosphatidylcholine	0.40	< 0.001	-	-	-	-	-	-
	Linoleic acid	0.48	< 0.001	0.32	0.005	-	-	-	-
	Oleic acid	0.50	< 0.001	0.26	0.029	-	-	-	-
Nucleotides and Nucleosides									
	Nicotinic acid mononucleotide	-	-	-	-	0.46	< 0.001	-	-
	Uracil	0.47	< 0.001	-	-	-	-	-	-
Organonitrogen Compounds									
	Histamine	-	-	-	-	0.37	< 0.001	0.41	< 0.001
	L-Carnitine	-	-	-	-	0.41	< 0.001	-	-
	Trimethylamine N-oxide	0.64	< 0.001	0.29	0.012	-	-	-	-
Purines and Derivatives									
	8-Oxo-2'-deoxyadenosine	-	-	-	-	-	-	- 0.40	< 0.001
	Guanine	-	-	-	-	0.51	< 0.001	0.37	0.001
Vitamins									
	Ascorbic acid	0.61	< 0.001	-	-	-	-	-	-
	Thiamine	-	-	-	-	- 0.53	< 0.001	- 0.25	0.032

Abbreviations: LR; lactulose : rhamnose measurement of intestinal permeability. Groupings completed through the Human Metabolome Database (Wishart *et al*., 2022). The cutoff for metabolites to be considered significant was the R value ≥ 0.4 (or ≤ -0.4), with a raw p-value of ≤ 0.001 for at least one of the four parameters of interest. [1] Unknowns excluded or metabolite matches with no biological relevance (e.g. lidocaine from the local anaesthetic administered during blood draws).

4.4 Discussion

Treatment of cats with 2.5 x 10^8 CFU of *S. boulardii* was hypothesised to improve intestinal permeability in ageing cats and change the plasma and faecal metabolome based on its mechanisms of action. Statistical analysis showed no response of intestinal permeability measures to the treatment in the areas studied in the present trial, but absorptive capacity was higher in groups treated with *S. boulardii*. Subsequently, a secondary analysis was performed which showed significant effects of age even in midlife to senior cats. Age affected faecal score, ATTD, intestinal permeability measured by the LR ratio, and some metabolites in both the plasma and faecal metabolomes.

4.4.1 Feed intake, body weight, and faecal health scores

Treatment with *S. boulardii* did not affect feed intake, in contrast with previous studies in cats (Lonigro *et al*., 2025a; Zhang *et al*., 2023b). Body weight was not affected by *S. boulardii* in the current study, which was consistent with results from other studies in young cats (Lonigro *et al*., 2025a) and kittens (Zhang *et al*., 2023b). However, feed intake (DMI / kg BW) and BW were both were higher in period 2. Due to the experimental design, the trial was conducted over autumn and winter seasons. Older cats voluntarily ingest more food in late autumn and winter months (Bermingham *et al*., 2013b; Serisier *et al*., 2014), which may explain the observations in the current study. Furthermore, cats have been observed to show a seasonal rhythm in bodyweight, gaining BW in the months leading up to winter and then decreasing BW in spring (Bermingham *et al*., 2024), which may help explain the increase in BW over the trial period.

S. boulardii supplementation in kittens with mild diarrhoea improved faecal health score (Zhang *et al*., 2023b), with similar results observed in adult cats (Lonigro *et al*., 2025a). This contrasts with the results from the current study where no difference in faecal health score was observed with *S. boulardii* supplementation. This could be due to different dose rates used, as the dose rate in the present study was lower than that used by Zhang *et al*. (2023b) and Lonigro *et al*. (2025a). Additionally, secondary analysis indicated that increasing age was significantly correlated with higher faecal scores in the current study, with many of the more senior cats experiencing diarrhoea consistently across the collection dates. While older cats displayed higher faecal scores, the supplementation with *S. boulardii* did not lead to improvements in faecal health scores. This lack of benefit may be due to more pronounced digestive disturbances in older animals compared to kittens, which could involve more extensive intestinal damage or a diminished response to probiotic treatment. Severe diarrhoea is associated with cats over the age of 11, and regular diarrhoea with being in a multi-cat pen (German *et al*., 2015), although this data was taken from cats in adoption centres across the United Kingdom, a similar situation may well occur in breeding units like the Massey University Centre for Feline Nutrition, where cats have been born and raised. However, stress can

still influence faecal consistency, so although the cats are used to their husbandry conditions and have been adapted to extra handling, blood collections, syringe dosing, and individual caging are all factors that over time may cause more stress than their normal baseline.

Diet may also play a role on the effect of *S. boulardii* on faecal health scores. In previous studies, the cats were fed a dry commercial diet (Lonigro *et al*., 2025a; Zhang *et al*., 2023b), whereas the cats in the present study were fed a wet one. This may account for the lower faecal score as dry and wet diets have markedly different effects on gastrointestinal health, including microbiome (Bermingham *et al*., 2013c) potentially causing *S. boulardii* to produce firmer faeces on dry diets. There is no explainable reason why faecal scores would be lower at the end of the first period, since there was no significant change in feed intake, but perhaps could be related to the loss of BW or the variation in moisture levels of the diet (Table 4.2) seen at this time.

4.4.2 Apparent total tract digestibility

There was no effect of *S. boulardii* supplementation on ATTD of macronutrients. There is no data reported showing the effects of *S. boulardii* supplementation on ATTD in the cat.

Secondary analysis identified that the ATTD of CFat, and energy, to a lesser extent, decreases with age. This is consistent with previous results (Anantharaman-Barr *et al*., 1991; Fahey *et al*., 2008; Harper, 1998; Patil & Cupp, 2010; Peachey *et al*., 1999; Perez-Camargo, 2003; Salas *et al*., 2014; Taylor *et al*., 1995; Teshima *et al*., 2010). The ATTD of CFat in the current study was lower (c.82 %) than previously reported in young cats on a similar diet (c.94 %) (Bermingham *et al*., 2018), further supporting a reduction in ATTD associated with age. Given cats primarily rely on fat as a significant energy source (Villaverde & Fascetti, 2014), a decline in the cat's ability to fully digest fat would reduce the amount of energy they can extract from their food and may have nutritional implications for senior pets.

4.4.3 Intestinal permeability and absorptive capacity

S. boulardii treatment did not affect intestinal permeability, but was associated with increased absorptive capacity measurements at the end of each period. Previously, in kittens, *S. boulardii* was shown to significantly decrease markers of intestinal barrier function including D-lactate, diamine oxidase, lipopolysaccharide, and intestinal fatty acid binding protein, signifying an improved intestinal permeability compared to their control group. These kittens also had increased IL-10 concentrations, but decreased IL-1β, IL-6, and TNF-α concentrations, signifying a decrease in inflammation (Zhang *et al*., 2023b). Adult cats fed a multi-strain probiotic consisting of *S. boulardii* and *Pediococcus acidilactici*, had a lower concentration of faecal calprotectin, indicating that the probiotic was linked to lower intestinal inflammation (Li *et al*., 2023). Inflammatory markers of the intestinal barrier were not measured in the present study.

Most of the variability of absorptive capacity seems to be explained by the cat itself, suggesting that cats are unable to be compared directly against others and the predictive factor behind absorptive capacity has yet to be studied. In this case, a crossover design is beneficial as each animal acts as its own control, limiting variability across animals, but longer term studies are subject to the confounding factor of season. Absorptive capacity was not significantly associated with sex, but showed a tendency to be higher in males, demonstrating similar results to Chapter 3. Few studies have examined the impact of sex on absorptive capacity, however, one study in humans specifically reported no effect of sex in a xylose absorption test (Kendall & Nutter, 1970). Future research may need to consider sex as part of the experimental design.

4.4.4 Faecal microbiome

There was no effect of *S. boulardii* on the community composition of the faecal microbiome. This is similar to previous studies in the kitten (Zhang *et al*., 2023b) and young cats fed a multi-strain probiotic of *S. boulardii* and *Pediococcus acidilactici* (Li *et al*., 2023). The alpha and beta diversity and richness of the microbiome remained stable over the trial, which was similar to the other *S. boulardii* supplementation trials in cats (Li *et al*., 2023; Zhang *et al*., 2023b), and suggests that the probiotic does not have an impact on the overall composition or variety of microbial species present in the GIT.

The five most prevalent phyla present in the current study were Bacillota, Fusobacteriota, Bacteroidota and to a lesser extent, Pseudomonadota and Actinomycetota. This is consistent with the literature (Bermingham *et al*., 2018; Butowski *et al*., 2022; Ganz *et al*., 2022; Li *et al*., 2023; Masuoka *et al*., 2017a; Suchodolski, 2022; Zhang *et al*., 2023b). Consistent with the lack of differences between treatment groups in the present study, Li *et al*. (2023), found that both adult control-fed cats and those fed a multi-strain probiotic containing *S. boulardii* showed no significant variation in relative abundance at the genus level. The dominant genus in that study was *Peptoclostridium*, followed by *Blautia* and *Bacteroides* (Li *et al*., 2023).

Dietary format, as well as the CP and CFat content of the diet, have been evaluated for their effects on the microbiome. It has been observed that high-protein, low-carbohydrate extruded diets increase species diversity in comparison to moderate-protein extruded diets (Pilla & Suchodolski, 2021). Canned diets are even higher in CP, CFat, and moisture, with lower amounts of carbohydrates compared to dry food, which has been shown to increase species richness, and concentrations of Fusobacteria, Proteobacteria, *Clostridium*, *Blautia*, *Bacteroides*, and unidentified Peptostreptococcaceae, while decreasing *Lactobacillus*, *Megasphaera*, and *Olsenella* in young adult cats (Bermingham *et al*., 2013c).

Senior cats in other studies had high relative abundances of bacterial members Actinomycetota and Bacillota (Jia *et al*., 2011), made up of higher amounts of faecal *C. perfringens*, *Bacteroides*, and *Collins*

(Bermingham *et al*., 2018; Patil & Cupp, 2010; Tian *et al*., 2023), and reduced relative abundance or amount of *Faecalibacterium*, *Fusicatenibacter*, *Subdoligranulum*, *Enterococcus*, and *Megasphaera* (Bell *et al*., 2014; Bermingham *et al*., 2018; Ganz *et al*., 2022; Masuoka *et al*., 2017a; Patil & Cupp, 2010). In the present study, the most represented genera were *Peptoclostridium*, followed by *Fusobacterium*, *Clostridium sensu stricto*, *Blautia* and *Negatibacillus*.

4.4.5 Plasma and faecal metabolome

The plasma and faecal metabolomes were not affected by supplementation of *S. boulardii* in old cats. A previous study in kittens also showed that faecal SCFAs were not affected by supplementation with *S. boulardii* (Zhang *et al*., 2023b). Both plasma and faecal metabolites did differ between period, potentially due to the differences in intake or variation of moisture in the diet seen during the trial.

There are few metabolomic studies exploring metabolites in the cat, let alone the ageing cat. The secondary analysis indicated that there was an effect of age and LR in the current study. This analysis was completed on cats over the age of 8, already in early midlife stage, so while there were still trends in the metabolome with age, there is no capacity to presently compare the metabolome of young adult and old cats.

While there was a positive association of plasma trigonelline with age, the literature suggests that serum trigonelline concentrations in humans are reduced with sarcopenia and increased in multiple species with muscle strength (Membrez *et al*., 2024). Mean percentage BW decreases in cats typically after 12 years of age, with a tendency for old cats before this point to be classified as obese (Perez-Camargo, 2003). Since the oldest cats in this study were still only 13 years of age and maintaining their body condition score, it is possible that their trigonelline levels had not started to decrease yet, if it follows the same trend as in humans.

Circulating levels of plasma amino acids in older cats have not been reported, creating difficulties interpreting the increases of some (aspartylphenylalanine, isoleucul-isoleucine, N-acetylserine) amino acids with age and the decreases of others (4-guanidinobutyric acid, alanylaline, N-methylglutamate). Impaired uptake of amino acids can be associated with a loss of lean body mass, which occurs in sarcopenia, and potentially can explain loss of lean body mass in older cats as well as an increase in some circulating amino acids (Freeman, 2012).

Interestingly, as cats aged, even in this senior group, the primary bile acid CDCA significantly decreased. There are only a few studies that have investigated bile acids in cats (Rowe & Winston, 2024), and none that have tracked the changes of bile acids throughout life. In a study comparing kittens to adult cats up to 3 years of age, concentrations of CDCA, taurocholate, cholate, and taurochenodeoxycholate were reduced, and L-cysteine and taurocyamine levels were increased in the adult cats' serum (Liu *et al*., 2023).

These results could indicate that bile acid pools decrease throughout life or stabilise upon maturity and perhaps only degrade in old age. There is not enough research to accurately depict how microbiome-altering diets and treatments impact the bile acid pool in cats (Ephraim & Jewell, 2021). Dietary supplementation of CDCA reduced intestinal permeability in piglets (van der Meer *et al*., 2012) and has been shown to protect against LPS-induced increased permeability (Song *et al*., 2019). The protective effects of this bile acid would effectively be lost as it reduces in concentration, perhaps contributing to increased intestinal permeability. However, CDCA has also been recorded to increase IL-8 secretion, reduce transepithelial electrical resistance, and decrease occludin levels, and increase intestinal permeability (Calzadilla *et al*., 2022). Decreased secretion of bile acids has been previously hypothesised as the cause of reduced CFat digestibility in older cats (Burkholder, 1999; Sparkes, 2011). Since bile acids aid in dietary lipid digestion, the decrease of bile acid concentration in older cats gains more traction as a possible cause of reduced CFat digestibility.

Increased concentrations of multiple fatty acids were seen in ageing cats and in cats with high intestinal permeability, with many seen in both categories. In humans and rodent models, age is associated with an increase of system free fatty acids, plasma lipoproteins, and plasma triglycerides, possibly contributing to inflammageing (Chung, 2021; Pararasa *et al*., 2015). Elevated concentration of free fatty acids in the blood are attributed to adipose tissue dysfunction (Pararasa *et al*., 2015), but potentially may also be related to the reduction of intestinal fatty acid binding protein (IFABP) and ileal lipid binding protein (ILBP) (Woudstra *et al*., 2004). Dietary free fatty acids causing systemic inflammation can also increase intestinal permeability, much like a high-fat diet, providing further insight into the relationship between increased lipid metabolites and increased LR (Rohr *et al*., 2020).

The GIT-derived, organonitrogen compound TMAO, is metabolised from betaine, carnitine, L-carnitine and their metabolites including choline, and phosphatidylcholine (Zhang *et al*., 2023a). While TMAO is linked to ageing, disease, and senescence in humans, it firstly plays a role as a regulator of many functions, including tissue osmotic pressure, stabilising proteins, and regulating sterol and cholesterol metabolism (Zhang *et al*., 2023a). Trimethylamine N-oxide is now also known as a metabolite that exacerbates mitochondrial damage, thus increasing oxidative stress, and even increasing expression of pro-inflammatory cytokines (Zhang *et al*., 2023a). Several recent studies examining TMAO in cats have observed high correlations with it and chronic kidney disease (CKD) in cats (Li *et al*., 2024; Nealon *et al*., 2024; Summers *et al*., 2023; Summers *et al*., 2021; Van Mulders *et al*., 2025). Researchers have recorded large intra- and interindividual variability of TMAO in cats, as well as it having an increased concentration in a fasted state compared to a fed state (Summers *et al*., 2023; Summers *et al*., 2021). Colony cats have been shown to have higher concentration of serum TMAO with age, when split into two groups, under 12 years of age, and over,

corroborating the age-related increase seen in the present study (Li *et al*., 2024). Additionally, TMAO can induce permeability in endothelial cells by disrupting ZO-1 expression, potentially explaining the mechanism behind the positive correlation with LR seen in cats (Boini *et al*., 2017).

Increased ascorbic acid seen with age could also be related to a decrease in renal function, as seen by increased plasma concentration of ascorbic acid in dogs with CKD (Galler *et al*., 2012). Thiamine (Vitamin B_1) may potentially be reduced in the faecal metabolome of older cats and cats with increased LR, due to them experiencing malabsorption. As cats age, they are already at risk of having a reduced ability to digest fat-soluble vitamins, but other water-soluble B vitamins, such as thiamine, may also be at risk of malabsorption (Laflamme, 2005). These findings were previously noted for cats and dogs with subclinical diseases that caused mild malabsorption syndrome, but the association presented between thiamine and LR may suggest that increased permeability of the small intestines results in more thiamine passing the intestinal barrier and less of it being passed into the faeces.

The mechanisms of action of *S. boulardii* have been widely studied, but it has only recently started to be used in companion animal research. A review of these actions include antimicrobial activity, antitoxin effects, re-establishment of normal microbiota, decreasing intestinal permeability in Crohn's disease patients, stimulating the immune response, and promoting anti-inflammatory signals (Kelesidis & Pothoulakis, 2012). The limited treatment effects observed in the present study may be a result of low dose (McFarland, 2010), the age of the cats, or the wrong mechanism of action being targeted.

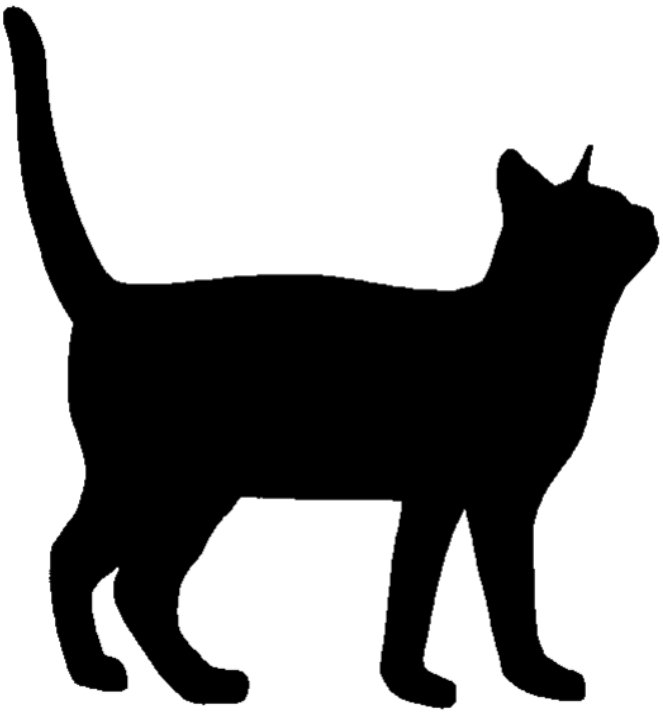
Overall, while the lack of research in companion animals prevents comparative conclusions to be drawn, the changes in the metabolome with increasing age and intestinal permeability point towards inflammatory states, reduced CFat digestion, and an increased risk of disease or mortality.

4.5 Conclusion

Supplementation of the probiotic yeast, *Saccharomyces cerevisiae* var. *boulardii*, did not result in major changes to the gastrointestinal health of colony-housed, domestic cats, aged between 8 – 14, but was associated with increased absorptive capacity after 31 days of supplementation. Secondary analysis was indicative of many age-related changes, even in this older cohort of cats, with ageing cats experiencing looser stools, and increased intestinal permeability. This older cohort also experienced lower CFat digestibility as seen in the literature, and therefore lower energy digestibility. There were also numerous metabolites significantly affected by age.

Chapter 5

General discussion



Drawing by Abby Lichti

Chapter 5: General discussion

5.1 Introduction

This thesis aimed to investigate the impact of ageing in cats on intestinal function (intestinal permeability and absorptive capacity) macronutrient ATTD and microbial bacteria and metabolites. In order to quantify intestinal permeability and absorptive capacity in the cat, a standardised method was developed in Chapter 2 and subsequently used to determine whether age affects intestinal permeability and absorptive capacity (Chapter 3). Finally, a study was conducted to understand whether probiotic intervention to a canned diet could improve intestinal permeability and absorptive capacity and also investigate whether the probiotic would affect the ATTD of macronutrients, the faecal microbiome and its metabolites with a secondary outcome of interest showing the relationship between age and these various endpoints (Chapter 4).

Results from this thesis showed that there is a clear age-related increase in intestinal permeability, but not absorptive capacity (Chapter 3). Probiotic (*S. boulardii*) intervention did not improve intestinal permeability but was associated with increased absorptive capacity (Chapter 4). Furthermore, *S. boulardii* supplementation did not alter the faecal microbiome (and its metabolites), nor the ATTD of macronutrients. Reasons for the lack of effect on key parameters are unknown, but the dose used in the current study (2.5 x 10^8 CFU/day) was lower than that previously used in kittens (2×10^{10} CFU/kg of food, based on approximate daily feed intake of 90 g, dose was approximately 1.8×10^9 CFU/day; (Zhang *et al*., 2023b)) and cats (5×10^9 CFU/kg of food, based on approximate daily feed intake of 330 g, dose was approximately 1.65×10^9 CFU/day; (Lonigro *et al*., 2025a)). The inherent variation observed in probiotic studies is well documented (El Jeni *et al*., 2024; López Martí *et al*., 2025) and coupled with a large degree of inter-animal variation observed in the current study as well as differences in dietary format, could explain the lack of effect in Chapter 4. Interestingly, secondary analysis of data generated in Chapter 4 identified a number of observations including the increase in intestinal permeability, reduced fat digestibility, and changes in plasma and faecal metabolites associated with age. Other key observations from the findings of this thesis indicate that the sex of the cat may also affect absorptive capacity, but neuter status is hypothesised to affect intestinal permeability due to hormones. These observations will be discussed below.

5.2 Sugar absorption test variability

The differential SAT methodology, used for measuring intestinal permeability and absorptive capacity is highly variability between studies (Beneyto, 2013; Bijlsma *et al*., 1995; Bruet *et al*., 2008; Davis *et al*.,

2005; Garden *et al*., 1997; Johnston *et al*., 2001; Marks *et al*., 1999; Papasouliotis *et al*., 1993; Randell *et al*., 2001; Rutgers *et al*., 1995; Sørensen *et al*., 1997; Steiner *et al*., 2001; Tina *et al*., 2011; Weber *et al*., 2002). The first research chapter (Chapter 2) was focused on the development of a single methodology to detect all four sugars in cat plasma, using a dose small enough to orally administer, on LC-MS. The quantified LR ratios obtained in plasma via this method are higher than the previously documented ratios in cat urine (Bijlsma *et al*., 1995; Johnston *et al*., 2001; Marks *et al*., 1999; Papasouliotis *et al*., 1993; Randell *et al*., 2001). This could be due to the hyperosmolarity of the solution which was needed in order to reduce the dose volume and clearly discriminate between a healthy and damaged small intestinal barrier (Uil *et al*., 2000). The XG ratio was lower in samples collected in Chapters 2 and 3, but higher in Chapter 4, when compared to Johnston *et al*. (2001), the only comparable study in cats, indicating that there could be other factors that influence the ratio. However, the validation of a simple, single run method to detect four sugar probes concurrently provides an invaluable tool future research, and the use of a standardised approach will reduce between study variability caused by the different methodologies used.

5.2.1 Intra-study variability

By comparing a cohort of young cats with a late midlife to super-senior cohort, the method developed in Chapter 2 showed that there is an age-related increase of small intestinal permeability, but not absorptive capacity in this population of colony cats. In both Chapters 3 and 4, there were age-related increases in intestinal permeability observed (Figure 5.1). By combining the datasets as shown in Figure 5.2, intestinal permeability, as measured by the LR ratio, was similar across both studies whereby the average absorptive capacity, as measured by XG, was vastly different between studies, being higher in the trial from Chapter 4. Additionally, The XG data from the five cats who participated in both studies were observed to be noticeably higher in Chapter 4 than Chapter 3, indicating that at the very least, it is not the variability between cats used in each study that is causing the higher values, and there must be another source of variation.

The xylose test alone has been deemed unhelpful and non-discriminatory in cats, potentially due to their low d-xylose uptake through transporter GLUT5 (Hall, 2013; Sherding *et al*., 1982). The ratio of xylose and 3-OMG is the proposed way to avoid confounding factors that cause issues with interpreting the xylose test (Hall, 2013; Johnston *et al*., 2001), however there was still a significantly difference in the recovered concentrations of xylose and 3-OMG between two studies using the same method. Three studies comparing the absorption of xylose and glucose between dogs and cats have been completed with the latest one reporting a similarity between the species (Johnston *et al*., 2001), contradicting the previous ones that presented lower concentrations in cats than dogs (Hawkins *et al*., 1986; Sherding *et al*., 1982). Therefore, although the variation of XG is smaller in each study than LR, shown by its lower RSD, there could

potentially be a confounding factor not accounted for that caused the XG values to increase significantly in Chapter 4. One possible reason for the difference between the samples could be the fact that they were taken a year apart, which could have allowed for significant changes to occur during that time.

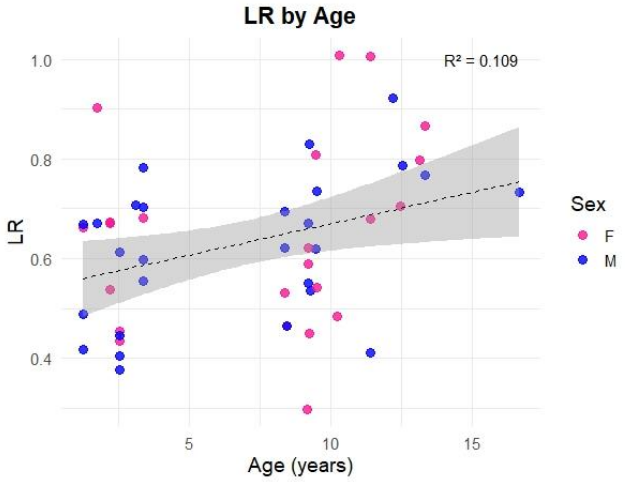


Figure 5.1. Intestinal permeability increases ($p < 0.05$, $R^2 = 0.109$) as cats age, as shown by a linear regression model between LR and age. Each data point represents a unique cat sampled in this thesis. For cats with multiple measurements, both LR and age values were averaged to produce a single representative point.

Figure 5.2. Ratios of lactulose to rhamnose (LR), and xylose to 3-O-methylglucose (XG) in plasma of domestic cats in Chapters 3 and 4. The LR values are comparable as there is not a significant difference between studies, but there is a large increase in XG values from Chapter 3 to Chapter 4, possibly due to unaccounted sources of variation.

5.3 Experimental design

Large intra- and inter-variability of parameters have been reported in cat studies previously (Andrews *et al*., 2015; Summers *et al*., 2021), so a crossover trial design was used in Chapter 4 to increase the statistical power by using each cat as their own control. One confounding factor seen in the data due to the crossover design was a period effect that affected most of the GIT health parameters measured. Overall, feed intake, BW, and the ATTD of energy, CFat, and DM were higher in period 2 than period 1. Although not studied further, period also had an effect on plasma and faecal metabolites, whereas treatment did not. Since cats show seasonal patterns in feed intake, energy requirements, and BW (Bermingham *et al*., 2013b), changes in nutrient digestibility could also be a confounding factor when assessing digestibility over time. Another potential cause of a period difference would be the diet given to the cats. Although efforts were made to minimise batch effects by using diets from a single production run, there was some variation within this as shown by the proximate analysis in Table 4.2, potentially causing differences in ATTD and the metabolome.

5.3.1 Post-hoc and secondary statistical analyses

Post-hoc and secondary analyses in probiotic studies involving cats and dogs has been used to uncover additional insights beyond the primary objectives of the research. For example, studies have explored the effects of probiotics on gastrointestinal health, immune response, and even chronic conditions like inflammatory bowel disease (IBD) in animals (López Martí *et al*., 2025). Another study used post-hoc analysis to help identify specific probiotic strains that were more effective in reducing symptoms in dogs with acute gastroenteritis (Fernández-Pinteño *et al*., 2023). However, post-hoc and secondary analyses has its limitations, including an increased risk of bias, whereby there is unintentional focus on patterns that confirm research expectations, reduced statistical power (e.g., multiple comparisons, which can increase the risk of false positives), and the lack of predefined hypotheses (Curran-Everett & Milgrom, 2013). Nevertheless, secondary analysis in Chapter 4 confirmed a number of key observations including the increase in intestinal permeability and reduced fat digestibility associated with age. More interestingly, it identified a number of key plasma and faecal metabolites that seemed to be associated with increasing age. These included markers of impaired protein turnover which may relate to sarcopenia, markers associated with fatty acid metabolism, which may relate back to the reduction in the ATTD of CFat, and other more novel markers associated with ageing such as TMAO. Additionally, it was observed that concentration of primary bile acid CDCA in plasma decreases in older cats. Chenodeoxycholic acid plays a key role in the primary pathway of bile acid production and aids in emulsifying fats for digestion (Chadaideh & Carmody, 2021; Rohr *et al*., 2020). This becomes more prevalent in the cat when considered with current research that indicates primary bile acids are essential for digesting and absorbing long-chain saturated fats that are found in domesticated meat products (Chadaideh & Carmody, 2021). Thus, the author hypothesises that due to the

vital role CDCA plays in saturated fat digestion, there is a possible correlation between the significant decrease of CDCA and loss of fat digestibility with increasing age. A more comprehensive understanding of the roles of these metabolites are of importance for future research.

The secondary analysis from Chapter 4 indicated that intestinal permeability significantly increased with age, corroborating the results obtained in Chapter 3 when comparing a young and old cohort of cats. As observed in Figure 5.1, the LR values start to deviate after the age of 10, indicating that intestinal permeability starts to increase during late-midlife. It has been hypothesised that physiological changes in the cat begin around 8 years of age when seasonal rhythms in bodyweight start to change (Bermingham *et al*., 2024), pro-inflammatory cytokines begin to increase (Kipar *et al*., 2005), anti-inflammatory cytokines begin to decrease (Kipar *et al*., 2005), and cats can start being referred to as middle-aged, although generally won't be classified as senior until 10 years of age, or even 12 years based on disease stratification (Ray *et al*., 2021; Salt *et al*., 2023).

5.4 Cat numbers

This thesis aimed to investigate the impact of age on a number of parameters associated with ageing. As identified by Salt *et al*. (2023), there are multiple age classifications for cats, defined from observations of the age clusters associated with different levels of disease severity. However, there was a lack of older cats present at the Massey University Centre for Feline Nutrition, creating a large range in ages for number of "senior" cats required statistically for the work. For example, the senior treatment group included in Chapters 3 and 4 contain cats that would be classified in the later part of early midlife (5 – 9 years of age), late midlife (10 – 11 years of age), and senior (12 – 13 years of age). Chapter 3 did have measurements from one super-senior cat (14 + years of age), but his data was unable to be used in Chapter 4. Due to the nature of working with older cats, he had experienced health problems unrelated to the trial and had to be euthanised, further reducing the power of the analysis. These age classifications and range in the age of cats selected for each trial may explain the observation in Chapter 4 that there appeared to be clustering of intestinal permeability results between 8 – 10 year old cats and 10 – 14 year old cats (Figure 5.1). If increasing age does cause an increase in disease severity (as indicated by Salt *et al*., 2023), this adds another potentially confounding effect to the results in this thesis. In general, the cats were deemed to be healthy, and while they did not have any known medical conditions or abnormalities, they may have been impacted by minor unknown factors that affect their gastrointestinal health. While a CBC was done in Chapter 3 to check the health status of the cats, it does not diagnose or provide a fully comprehensive examination into all aspects of health. Furthermore, while the cats were trained for blood draws and oral dosing, some cats became stressed during the procedure and either vomited or became agitated so that blood samples could not be collected.

It is important to note that genetics may play a factor in the results of this thesis as the cats were sourced from a breeding colony. So, in the comparison in Chapter 3, all young cats from came from one of two sires and senior cats came from one of three sires, creating many full- and half-sibling groups. The controlled nature of the study was also an advantage as the cats at the Massey University Centre for Feline Nutrition share the same environmental, and social conditions, minimising confounding factors and challenges to the intestinal barrier.

5.4.1 Sex effects on intestinal permeability and absorptive capacity

In this thesis an effect of sex on absorptive capacity was observed. Absorptive capacity tended to be higher in males compared to females in both Chapters 3 and 4, demonstrating that there is likely a hormonal influence on this GIT parameter that must be considered in future trials. There is only one published study that investigated the impacts of sex on intestinal permeability and absorptive capacity, with the authors finding no significant effect (Allenspach *et al*., 2006). There is no research that supports a hypothesis as to why absorptive capacity may be sex-dependent, but it is something that should be investigated in future studies using this method.

Related to sex is neuter status, which due to the husbandry practises of the colony, could not be controlled for in this thesis. Since oestrogen plays a role in protecting the intestinal barrier (Braniste *et al*., 2009), a comparison between neutered and entire cats is warranted.

5.5 Future Directions

The knowledge gap facing this area of feline research is immense, but there are specifics that should be addressed first and foremost, to better understand the gastrointestinal system and barrier function of the ageing cat. Firstly, based on the considerable variation and lack of change with age of the XG test, the intestinal permeability test could potentially be used without xylose or 3-OMG. The lack of these two sugars in the dose also allows researchers to give the cats a smaller dose of solution at the same osmolarity or add more water to the solution of lactulose and rhamnose to create an iso-osmolar dose. There could be potential benefits to each option, the former creating a less stressful dosing situation for the cats and possibly reduce vomiting by reducing the amount of liquid being dosed, and the latter possibly lowering LR ratio values and completely reducing the risk of increased paracellular permeability due to increased lactulose permeation. An iso-osmolar dose could conceivably lower the variation and create a tighter reference range, while the hyperosmolar dose theoretically marks a discernible difference between cats with a healthy intestine compared to ones who have higher intestinal permeability.

The differential SAT, as used multiple times throughout this thesis, should also be compared to immune biomarkers, as mentioned in Table 1.2. Not only would it strengthen the validity of this test, but using the

SAT as a reference could establish other biomarkers for intestinal permeability in the cat that could be used in a clinical setting, and possibly be less invasive, less expensive, and more time efficient. The SAT may also be a tool for discriminating or even diagnosing cats with CIEs, since these cats would have higher intestinal permeability and may be cause for outliers in this thesis, but when studied alone have greater biological meaning.

To further explore the metabolome of the ageing cat, a valuable next step would be to apply the methodology from Chapter 4 in a targeted metabolomics approach, focusing on quantifying age-associated metabolites such as bile acids, TMAO, and fatty acids. This approach could enhance understanding of the biological mechanisms underlying ageing, potentially validating the mechanisms driving age-related changes. Additionally, it could lay the groundwork for identifying potential therapeutic interventions aimed at mitigating age-related health decline.

The findings of this thesis highlight the capacity of the GIT to change with age in the domestic cat. The question presented at the beginning of this thesis regarding the relationship between reduced digestibility in ageing cats and intestinal permeability has been answered. There is no evident link between intestinal permeability and nutrient digestibility, even if they are both closely related to age. Even so, it still leaves the question of why ageing cats experience reduced nutrient digestibility and without this answer, there can be no targeted interventions to combat this condition. The author proposes future work to concentrate on bile acids through plasma and faecal metabolomics to further investigate the relationship between bile acids, age, and reduced fat digestibility.

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Appendices

Appendix 1.

Statement of contribution for published manuscript. Bermingham, E. N., Patterson, K., Shoveller, A.K., Fraser, K., Butowski, C.F, Thomas, D.G. (2024). "Nutritional needs and health outcomes of ageing cats and dogs: is it time for updated nutrient guidelines?" *Animal Frontiers* 14(3): 5-16.

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:	Keely Patterson
Name and title of main supervisor:	Assoc. Prof. D.G. Thomas
In which chapter is the manuscript/published work?	Chapter 1

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]
Emma Bermingham: Writing - original draft, review & editing, conceptualization. Keely Patterson: Writing – original draft, review & editing. Karl Fraser: Review & editing, funding acquisition. Anna Kate Shoveller: Review & editing. Christina Butowski: Writing - original draft, review & editing, visualization. David Thomas: Review & editing,

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Feature Article

Nutritional needs and health outcomes of ageing cats and dogs: is it time for updated nutrient guidelines?

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Implications

- While cats are classed as senior at 10 years of chronological age, physiological and health changes occur from 8 years of age and it appears that diet may influence the ageing process.
- Dogs are classed as senior at 12 years for smaller dogs and 10 years for larger breeds. Due to differences in longevity associated with breed size a definite age that dogs start to experience changes is difficult to establish.
- Despite our pets ageing, living in extreme cases to 30 + years, there are no explicit nutritional guidelines for feeding ageing animals. Increased scientific knowledge around the specific nutritional requirements of ageing cats and dogs is required.
- Many of the underlying physiological processes (e.g., immune function) and age-associated health conditions (e.g., cognitive decline) respond to nutritional intervention. This suggests that nutritional and regulatory guidelines, should consider recommendations for 'senior+' pets.
- Due to the unique nutritional requirements of cats and dogs, more specific knowledge around the mechanisms of ageing is required.

Key words: cognitive function, healthspan, inflammageing, lifespan, nutrients, sarcopenia, senior

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Introduction

In October 2023, Bobi, the oldest dog in the world, died. His age was estimated to be 31 years and 165 days. Creme Puff, the oldest cat in the world, who, in August 2005, passed away at the age of 38 years and 3 days. While certainly these two pets were at the extreme end of lifespan, it is undeniable that our pet cats and dogs are living longer lives; this has been attributed to improved veterinary care and diet formulation (Bellows et al., 2015, 2016). Indeed, it is currently estimated that 20% to 40% of pets are "old" being greater than 11 years of age (Bellows et al., 2015, 2016). However, information relating to how our pets age, and how we can improve not only their lifespan (i.e., chronological age), but healthspan (i.e., longer, *healthier* lifespan) is lacking. Therefore, the aim of this review is to summarize recent publications and current global initiatives investigating ageing and its processes in cats and dogs. Further, we aim to review the nutritional requirements of our ageing cats and dogs, including evidence for specific nutrients which have health benefits for common ailments associated with ageing.

Nutritional requirements of cats and dogs

There are several differences between the nutrient requirements of cats and dogs; these include specific amino acids (e.g., taurine is an essential amino acid for the cat, but not the dog), fatty acids, vitamins, and minerals. Additionally, there are differences in the minimum and maximum inclusion levels of these nutrients. Evolution of the two species is likely the cause of these differences, supporting the notion that cats are obligate carnivores (requiring animal-tissues to survive), whereas dogs are facultative carnivores—requiring these animal proteins and fats for optimal nutrition, but being able to survive via scavenging a range of food resources (Plantinga et al., 2011; Bosch et al., 2015). The nutrient requirements of cats and dogs are vastly different to those of their owners, and reflect an evolutionary adaptation to a diet with high levels of animal tissue. Therefore, cats are not small dogs, and nor are cats and dogs, small humans.

Despite the pet food industry existing since the 1950s, there is relatively little scientific literature pertaining to the specific nutrient requirements of cats and dogs; including energy requirements which underpin feeding guidelines and nutrient availability. The consequence of our sparse nutritional knowledge of the cat and dog means that when it comes to interpreting the requirements of the ageing cat and dog from experimental studies, we are heavily reliant on translating such findings from omnivores.

The global pet food industry generally adheres to two nutritional regulatory guidelines; namely the American Association of Feed Control Officials (AAFCO) and the European equivalent, European Pet food Industry Federation (FEDIAF). Both regulatory bodies update their guidelines frequently, and while they may make changes based on new information, they are largely based on data outlined by the "Nutrient Requirements of Dogs and Cats", last updated by the National Research Council (NRC) in 2006 (National Research Council, 2006) and therefore do not include data published since then. In addition, many of the nutritional requirements stipulated by AAFCO and FEDIAF are based on extruded-kibble formulations which are recognized as being less digestible than other formats (e.g., retorted or raw diets), or on semi-purified diets which increase the nutrient bioavailability and don't represent a typical pet food. This is increasingly important with the rapid shifts in pet food manufacturing, product formulations (i.e., extruded kibble, retorted can diets, air/freeze-dried, sous vide, etc.), and ingredients used.

How does health change in ageing cats and dogs?

Much of the scientific literature around ageing in the cat and dog stipulate that ageing is not a disease. Indeed, ageing has been defined as a natural 'series of life stages', whereas senescence is the deterioration of the health and quality of life (Case et al., 2011). The definition of life stages has typically followed a chronological ordering based on years of life, especially for the cat (Quimby et al., 2021). Typically, cats have been classed as 'senior' when they are older than 8 to 10 years of age (Ray et al., 2021). For the dog, body size and breed have a major influence on life stage, with larger dogs reaching 'old age' at an earlier chronological age than smaller dogs (Creevy et al., 2019). However, more recent work has re-classified life stages of the cat and dog based on diagnosis of disease (Salt et al., 2023), and suggested additional age classifications on this basis (Table 1).

Physiological decline in ageing cats and dogs

While it is understood that reduced ability of cats to digest nutrients and utilize energy stores may begin after 7 years of age (Bellows et al., 2015, 2016), more recent research suggests dietary format influences this. For example, cats fed ad libitum extruded or retorted diets were able to maintain a healthy weight range until approximately 8 years of age, at which point bodyweight declined for animals consuming the retorted diet (Figure 1). Research indicates that fat digestibility and to a lesser extent protein digestibility (Harper, 1998; Perez-Camargo, 2004; Teshima et al., 2010; Bermingham et al., 2013, 2018), decrease with age in the cat; however, the extent to which they are affected may be dependent on dietary format and nutrient content (Figure 2). Changes in nutrient digestibility in the ageing cat may be due to changes in intestinal morphology (Peachey et al., 2000), as there are limited impacts of ageing on intestinal transit time and gastric emptying time between young and senior/super-senior cats (Papasouliotis et al., 1998;

Table 1. Age classifications of cats and dogs with additional age classifications as suggested by Salt et al. (2023), indicated by an asterix

Cat		Dog		
Kitten	<1 year		Puppy	<1 year
Young/youth	1-4 years	Toy	Youth*	1-6 years
			Midlife*	7-11 years
			Senior	12-13 years
			Super-Senior*	≥ 14 years
Early midlife*	5-9 years	Small	Youth	1-6 years
			Midlife	7-11 years
			Senior	12-13 years
			Super-Senior	≥ 14 years
Late midlife*	10-11 years	Medium	Youth	1-5 years
			Midlife	6-9 years
			Senior	10-13 years
			Super-Senior	≥ 14 years
Senior	12-13 years	Large	Youth	1-5 years
			Midlife	6-9 years
			Senior	10-11 years
			Super-Senior	≥ 12 years
Super-senior*	≥ 14 years			

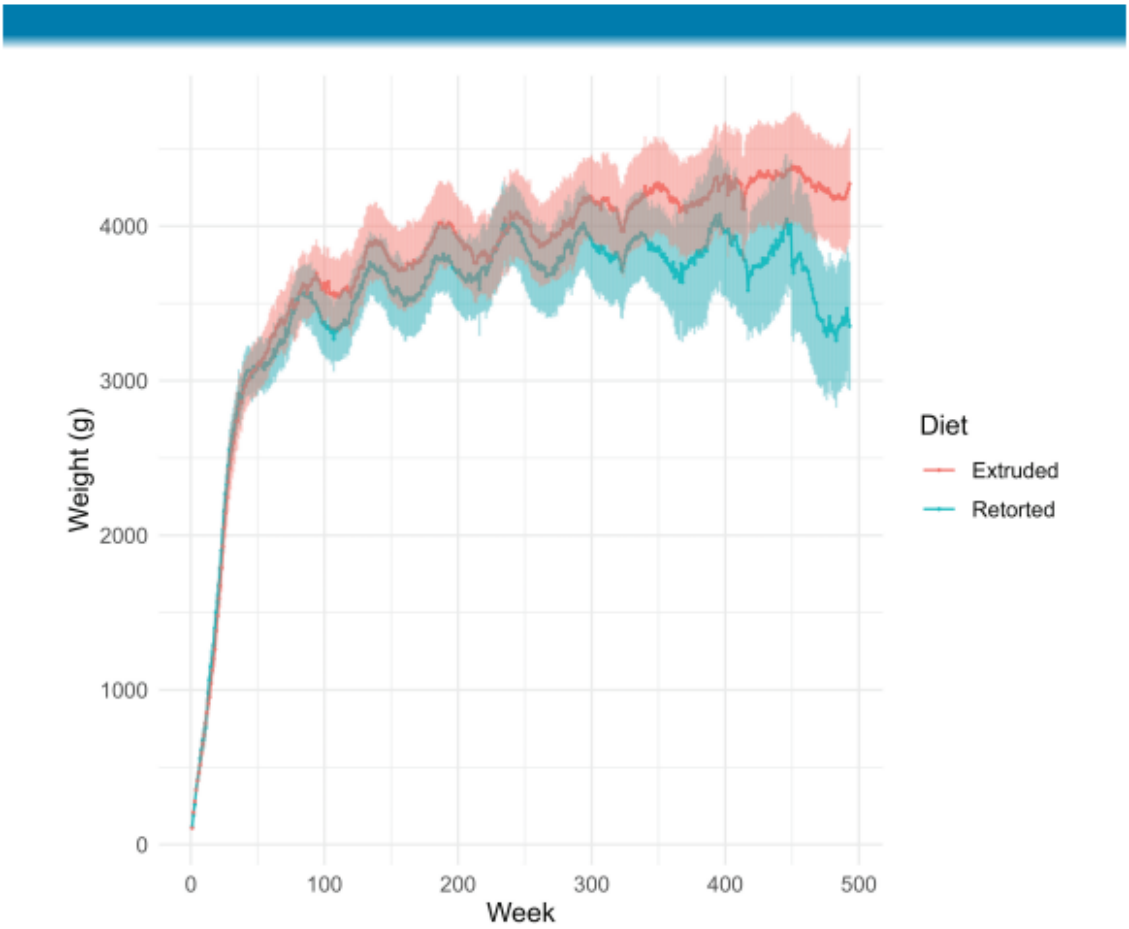

Figure 1. Weekly bodyweight in cats fed either an extruded kibble (n = 10) or retorted canned (n = 10) diets for 12 years. From this graph, we can see a) that there is a natural rhythm associated with bodyweight across a year; typically increasing in the months leading up to winter and decreasing in spring and b) there is very little difference between the bodyweights of cats fed either diet until approximately 8 years of age where the cats fed the extruded diet maintained their "winter weight".

Peachey et al., 2000). This suggests that there is a defined window in which the cat is likely to be undergoing significant changes in its metabolism and physiology which may present an opportunity for nutritional interventions.

Research suggests that dogs experience no change in intestinal permeability or absorptive capacity as they mature from adult to seniors and beyond, however changes in intestinal morphology have been documented (Garden et al., 1997; Weber et al., 2002; Kuzmuk et al., 2005). The impacts of age on nutrient digestibility are affected by both dietary format and ingredient composition and warrant further investigation (Sheffy et al., 1985; Taylor et al., 1995; Larsen and Farcas, 2014). For example, observations suggest that increasing the amount of total dietary fiber appears to mitigate the age-associated impacts on fat digestibility (Schauf et al., 2021). Additionally, it is often reported that ageing dogs require 20% to 30% more dietary protein to maintain muscle mass (Laflamme, 2005).

What is the role of the gut microbiome in ageing?

Microbial changes to the gastrointestinal tract in older dogs are characterized by a decrease in microbial diversity, potentially changing the way that older dogs can respond to diseases, regulate nutrient absorption, and energy and protein metabolic efficiency by peripheral tissues (Mizukami et al., 2019; Ghosh et al., 2022; Suchodolski, 2022). In the cat, microbial diversity has been observed to be relatively consistent with age (Figure 3a). However, both taxonomic composition differs with age (Figure 3b and 3C, respectively) with changes observed dependent on the diet consumed. Given that numerous bacterial

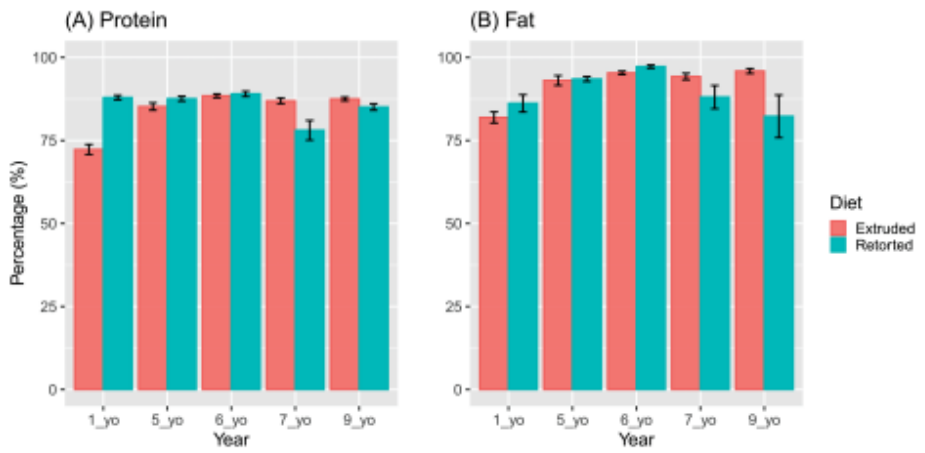

Figure 2. Apparent total tract digestibility of crude protein and fat in the domestic cat at 1, 5, 6, 7, and 9 years of age (yo) (from Bermingham et al 2018 and unpublished results).These results show that a) protein digestibility in young cats fluctuates with age and b) fat digestibility is affected by age, but there is no difference in cats fed retorted or extruded diets.

species are known (in omnivore models at least) to alter gut permeability (Ulluwishewa et al., 2015), and that gut microbiota composition (Bermingham et al., 2018) and its metabolic potential (Deusch et al., 2015) changes with age, it is likely that the microbiota play a critical role in health and wellbeing during ageing and is certainly an area of importance for our understanding ageing in the pet.

Age-associated conditions in cats and dogs

The mechanisms of ageing in the cat and dog have recently been reviewed (McKenzie, 2022). They identify that ageing is a complex inter-related process, which includes inflammageing, sarcopenia, insulin resistance, obesity, and cellular senescence (McKenzie, 2022). These processes accumulate to increase frailty, decrease tissue function, and increase disease risk and mortality. Research gaps associated with the mechanisms of ageing specifically in the cat and dog are indicated in Table 2. Interesting, despite inflammageing being touted as an underpinning condition for ageing cats and dogs, there is limited information in the two species to support the role of inflammageing associated with ageing diseases including sarcopenia and cognitive decline. Indeed, the authors concluded their comprehensive review by saying 'a great deal of additional research is needed to clarify the details of ageing mechanisms [in dogs and] cats'. Indeed, recent approaches such as the MARS PETCARE BIOBANK™ (Alexander et al., 2023), "Generation Pup" (Murray et al., 2021) and the Ageing Dog Project (Bray et al., 2023) may one day clarify these mechanisms.

Table 3 outlines observations from longitudinal studies published in the cat and dog. Earlier studies, tended to focus on outcomes related to lean body mass and diet, whereas more recent studies have assessed inflammageing (i.e., inflammatory markers) and changes to the fecal microbiome as a proxy for the gastrointestinal microbiome. In the cat, there are few studies assessing life span changes, but preliminary evidence suggests that diet appears to affect the way in which cats age (Table 3). A study using data obtained from veterinary visits from approximately 2 million cats and 4.4 million dogs, identified that the incidence of disease and age were interlinked (Salt et al., 2023). For example, younger cats were more likely to be 'healthy' or present for conditions such as fleas or parasite infections, whereas older cats were more likely to present with conditions such as 'underweight', periodontal disease, arthritis and renal failure. While trends in the dog were somewhat affected by body size (i.e., toy vs large breeds), typically younger dogs were classified as 'healthy' whereas older dogs typically presented with conditions such as 'underweight', 'geriatric pet' arthritis/osteoarthritis, heart failure, renal failure. Interestingly, these conditions appeared to occur at earlier chronological age in medium and large dog breeds. It is the hope of these authors that this data can be eventually used to target dietary interventions to improve the healthspan of our cats and dogs.

Nutrition for the ageing cat and dog

There are a number of excellent resources for veterinary practitioners that consider the nutritional management of senior cats and dogs, for example the American Animal Hospital Association (AAHA; Dhaliwal et al., 2023) or the American Association of Feline Practitioners (AAFP; Ray et al., 2021). However, from a pet food regulatory point of view, while the NRC 2006, AAFCO and FEDIAF outline nutritional guidelines for gestation/lactation/growth and adult life stages, none include specific requirements for senior pets, even at the macronutrient (i.e., protein and fat) level. FEDIAF released a statement of the nutrition of senior dogs in 2017 (FEDIAF, 2017), which called for the industry to pay particular attention to

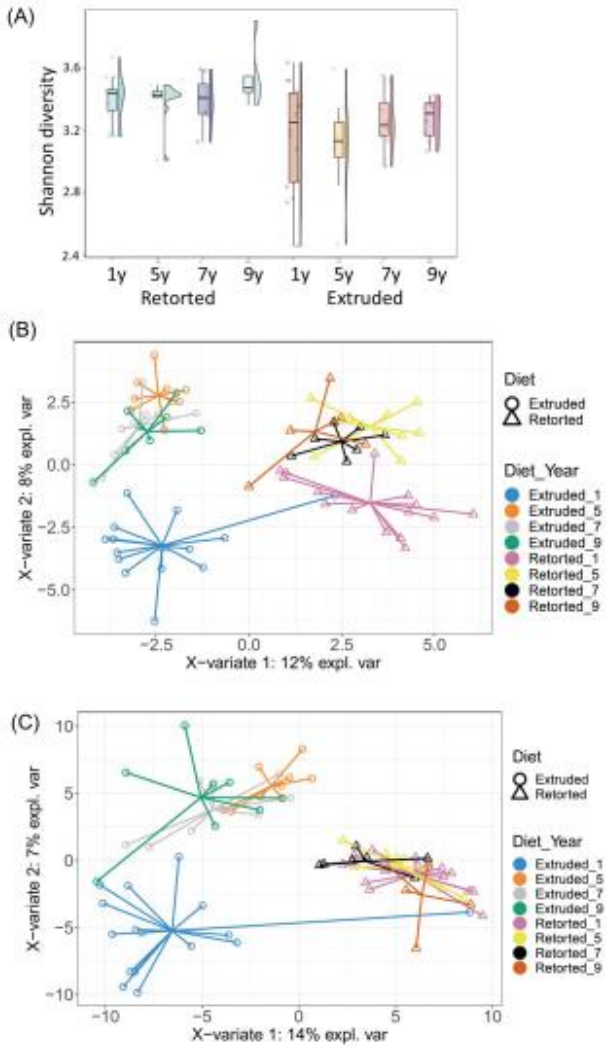

Figure 3. Shannon diversity indices (A) of cats (n = 16) fed either a retorted or extruded kibble for 1, 5, 7, and 9 years. Partial least squares discriminant analysis (PLSDA) of taxonomic (B) and function (C) composition of the fecal microbiome of cats (n = 16) fed either a retorted (circle) or extruded (triangle) diet. Samples of the same color and shape indicate samples from the same diet and year. From Bermingham et al 2018 and unpublished results. These graphs show that a) cats fed the retorted diet had higher microbial diversity compared to the cats fed the extruded diet irrespective of age, b) that young cats (triangle and circle) had a distinct microbial profile (i.e., they cluster differently) compared to older cats; this figure also shows that the cats fed the retorted diets had a different microbial population than cats fed the extruded diet and c) that the function of the microbiome is different between cats fed the extruded (triangle) and retorted (circle) diets; there is less separation associated with age with the exception of the young cats fed the extruded diets (pink triangle).

specific nutrients such as crude fiber (to maintain gut motility) vitamin E, zinc, selenium, and docosahexaenoic acid (DHA); however, it was unable to provide specific recommendations around the minimum requirements for senior dogs due to "the lack of information available". Additionally, it stated that the industry should work within the maximal levels of any particular nutrient stated by FEDIAF/AAFCO; this is especially important with nutrients such as eicosapentaenoic acid (EPA) and DHA which have safe upper limits identified for various health concerns. Finally, many of the ingredients which have been shown to have beneficial effects on the ageing pet (Table 4) have no labelling requirements—i.e., minimum or

Table 2. Research Gaps associated with ageing mechanisms in the cat and dog summarized from McKenzie (2022)

Area	Mechanism	Gap
Cellular and molecular ageing	DNA damage	No information pertaining to nutritional modulation of DNA damage in ageing cats and dogs
	Epigenetic clock	Little evidence understanding how epigenetic clocks represent biological ageing in cats
	Intercellular signalling	Limited information around inflammageing in the cat and dog and nutritional improvement of this process
Tissue	Sarcopenia/muscle	No evidence to support elevation of cytokines (inflammageing) associated with sarcopenia in ageing dogs
		Limited evidence to support role of inflammageing in sarcopenia in ageing cats
		No information pertaining to role of insulin in ageing of feline muscle cells
		Limited evidence to support function of satellite muscle cells in ageing cats and dogs
		No evidence to support loss of type II muscle cells in ageing cats
		No evidence to understanding the role of exercise interventions on longevity in the cat or dog
	Bone	Limited evidence to support changes in bone structure in ageing cats
		Limited evidence to support the role of bone marrow stem cells in bone ageing in dogs
		Limited evidence to support the role of Growth Hormone and Interleukin Growth Factor-1 (IGF-1) in the ageing cat
		No information pertaining to the role of inflammation in the loss of bone associated with age in the cat and dog
		No information pertaining to the role of insulin in age-related loss of bone in the cat and dog
		No information pertaining to the role of exercise in maintaining bone health and function in the ageing cat and dog
	Joint Disease	No information pertaining to the role of chronic inflammation on development of joint disease in the ageing cat
		No information pertaining to the role of exercise in preventing Osteoarthritis (OA) in the ageing cat and dog
	Brain	No information pertaining to the role on chronic inflammation on the ageing brain is unknown in the cat and dog
		No information pertaining to the role of cellular senescence in the ageing brain in the dog
		No information pertaining to the role of physical activity on the ageing brain in the cat and dog
		Specific ageing processes unknown in the ageing cat brain (e.g., cellular senescence, inflammageing)
	Adipose tissue	Limited evidence to support the role of adipose mass, distribution and function in the ageing cat
		Limited evidence to support the development of sarcopenic obesity in the ageing cat and dog
		Limited evidence to support the role of inflammageing in fat mass distribution and function in the cat and dog
		No information pertaining to the role of exercise on fat mass distribution and function in the ageing cat and dog

suggested inclusion levels are not regulated, making it difficult for pet owners to interpret whether 'senior' diets really provide additional benefits for their ageing pets. Indeed, recent research identified no nutritional differences (with the exception of crude fiber) in diets marketed for 'adults' or 'seniors' (Summers et al., 2020). However, it is apparent that nutrition, including water intake, can ameliorate the health conditions associated with age, or the underlying physiological process (Table 4). This suggests that current nutritional guidelines, including energy requirements which underpin feeding guidelines need dedicated scientific investigation to determine the nutritional needs for our senior and older cats and dogs.

There are a number of health concerns affecting senior pets, including hydration status and oral health (Salt et al., 2023), this review will summarize literature pertaining to sarcopenia, inflammation/inflammageing, and cognitive health.

Sarcopenia

Maintenance of lean body mass is a major predictor of lifespan in both the cat and dog, having the most profound impact on mobility. Sarcopenia is defined as the loss of skeletal muscle mass and function with ageing. Paradoxically, this loss of muscle mass often occurs in the presence of obesity, so-called sarcopenic obesity (Freeman, 2012; LaFlamme, 2016). Approximately 40% of 'old' cats and dogs (Mao et al., 2013) are obese, and 12% to 15% of these are considered to have extremely low levels of lean mass. Therefore, this is a very common condition in older companion animals, although its mechanism are not well understood (McKenzie, 2022). Sarcopenia results primarily from impaired protein synthesis, with a smaller contribution from increased protein degradation, which ultimately leads to atrophy of skeletal muscle fibers and reduced mitochondrial function (Greenlund and Nair, 2003). A long-term study showed that in the elderly cat, fortification of a high protein and high fat diet with linoleic acid reduced the loss of lean body mass (Cupp et al., 2007).

Inflammageing

Ageing is associated with a natural deterioration in health; more recently, the term inflammageing is used. This is the age-associated decline in immune function that occurs in most species. It is generally thought that inflammageing is influenced by the interactions between the host's immune response and gastrointestinal microbiome (Fransen et al., 2017; Franceschi et al., 2018), but this has not been investigated in the cat and dog directly, nor are the cellular mechanisms well defined in the cat and dog (McKenzie, 2022). Inflammageing is believed

Table 3. Outcomes of longitudinal studies in healthy cats and dogs

Species	Age at beginning of study; mean (range)	Length of study	Sample size	Breed	Diet	Observation	Reference
Cat	7–17 years; groups: 7–9, 10–12, 13 + years	7 years	90; 30 per age group	Mixed breed	Extruded diet with/without antioxidants, a prebiotic, and omega-3 and omega-6 fatty acids	Cats fed diet with nutritional additives had increased lifespan of one year, greater serum vitamin E, HCT, haemoglobin, and RBC count, slower loss of BW, lean body mass, and skin thickness with age, and a tendency to be more active	Cupp et al. (2008), Cupp and Kerr (2010)
Cat	8 weeks	11 years	21; 10–11 per group	Domestic shorthair	Extruded vs. retorted diet	ATTD, fecal microbiome (composition, function), plasma inflammatory markers, plasma lipidome and metabolome were affected by age and diet	Bermingham et al. (2018) and unpublished data
Dog	1.9–8.1 years	13 years; adulthood to end of life	80	Labrador retriever	Extruded diet with/without avocado extract (< 0.10 %)	Age related increase in inflammation, oxidative stress, and tissue damage similar to "inflammageing" in humans with diet having no effect	Alexander et al. (2018)
Dog	5.5 years (4.3–7.5)	Median 14 years; puppy to end of life	39	Labrador retriever	Extruded diet	Lean and fat mass may influence longevity in the dog	Penell et al. (2019)
Dog	8 weeks	Median 13 years; puppy to end of life	48	Labrador retriever	Extruded diet with/without 25 % caloric restriction	Caloric restricted dogs lived longer and had delayed signs of chronic disease with no effect on skeletal structure/function	Lawler et al. (2008)
Dog	8 weeks	14 years; puppy to end of life	48	Labrador retriever	Extruded diet with/without 25 % caloric restriction	Caloric restricted dogs had healthier hips; reduced occurrence and delayed onset of OA	Smith et al. (2006)
Dog	8 weeks	8 years	48	Labrador retriever	Extruded diet with/without 25 % caloric restriction	Caloric restricted dogs had less severe OA	Kealy et al. (2000)
Dog	0.2–5.9 years	~ 8 years	6084	Labrador retriever	N/A—observational cohort in homes	Gastrointestinal illnesses in dogs may be affected by, location, presence of other animals in the home, and characteristics of owners	(Pugh et al., 2017)
Dog	0.7–5.0 years	~5 years	~6,000	Labrador retriever	N/A—observational cohort in homes	Genetics may play a role in limber tail in addition to being a working dog in a higher latitude	Pugh et al. (2016)
Dog	≥ 8 weeks and < 2 years	N/A	2,764	Golden retriever	N/A—observational cohort in homes	Dogs who have undergone gonadectomy had increased risk of becoming overweight/obese and dogs who were gonadectomised before 6 months of age had increased risk of orthopaedic injury compared to intact dogs	Simpson et al. (2019)
Dog	8 weeks—2 years	~1 year	160	Labrador retrievers, golden retrievers, and Labrador × golden crosses	N/A—observational cohort in homes	Cognition at young adult life stage can be predicted from early development stages	Bray et al. (2021)

Abbreviations: HCT, haematocrit; RBC, red blood cell; BW, body weight; ATTD, apparent total-tract digestibility; OA, osteoarthritis.

to be the leading cause of morbidity and mortality in cats and dogs (Day, 2010); interestingly, many of the health conditions associated with ageing identified by Salt et al. (2023) such as osteoarthritis, renal failure and heart failure are underpinned by changes in inflammatory markers in both the cat and dog. Indeed, pro-inflammatory cytokines increase while anti-inflammatory cytokines decrease in the cat around the age of 8-10 years (Kipar et al., 2005). Nutrients such as β-carotene (a pre-cursor of vitamin A for dogs), vitamin E (tocopherol), and poly-unsaturated fatty acids (PUFA; typically supplied via fish/flax seed oil or algae) have been observed to improve immune status in cats and dogs (Table 4).

Cognitive function

Dogs, and to a lesser extent cats, have often been used as a model for cognitive decline in humans; from this perspective there are a relatively large number of studies that have investigated the impacts of dietary nutrients on markers of cognition, including stress and anxiety. Nutrients, typically in proprietary blends, such as dietary lipids (PUFA, medium-chain triglycerides, and phospholipids), antioxidants, B-vitamins, carnitine, and specific amino acids such as arginine have been observed to improve markers of cognitive function in the ageing dog and to a lesser extent, the cat (Table 4).

Table 4. Nutrients with proven efficacy in cats and dogs.

Species	Age (range)	Intervention Target	Nutrient	Marker	Reference
Dog	1.7–10.6 years	Immune status	β-Carotene (20 or 40 mg β -carotene/kg diet)	Improved immune response	(Massimino et al., 2003)
Dog	7–10 years	Immune status	α-Tocopherol acetate/vitamin E (101 mg/kg diet)	Improved immune response	(Hall et al., 2003)
Dog	1.7–5.4 years	Immune status	Omega-6:omega-3 diet of 5:1 (dose not stated)	Positive effect on immune response	(Kearns et al., 1999)
Dog	1–4 years	Immune status	EPA (1.75 g/kg diet), DHA (2.2 g/kg diet) [omega-6:omega-3 of 3.4:1], sunflower oil (0.6 g/kg diet), menhaden fish oil (7 g/kg at 1.65% oil DMB)	Anti-inflammatory effects	(LeBlanc et al., 2008)
Cat	1.5–10 years	Immune status	Vitamin E (225 mg/kg DM diet)	Improved immune function	(O'Brien et al., 2015)
Cat	2.0–11.0 years	Immune status	Omega-6:omega-3 of 4.77:1 using salmon oil (dose not stated)	Improved immune system	(Rutherfurd-Markwick et al., 2013)
Dog	3 years	Cognitive function (behavior)	*Punica granatum* (457 mg/kg diet), *Valeriana officinalis* (260 mg/kg diet), *Rosmarinus officinalis* (0.44 mg/kg diet), *Tilia* species (635 mg/kg diet), *Crataegus oxyacantha* (392 mg/kg diet), $_{L}$-Theanine (310 mg/kg diet), $_{L}$-Tryptophan (329 mg/kg diet)	Improved neuroendocrine parameters associated with behavioral disorders (e.g., stress, anxiety, aggression)	Sechi et al. (2017)
Dog	2–12.6 years	Cognitive function	$_{DL}$-α-Tocopherol (1000 ppm), $_{L}$-carnitine (250 ppm), $_{DL}$- α-LA (120 ppm), ascorbic acid 80 ppm, 1% inclusions of spinach flakes, tomato pomace, grape pomace, carrot granules and citrus pulp	Reduced cognitive dysfunction	Milgram et al. (2004)
Dog	2–12.5 years	Cognitive function	$_{DL}$-α-Tocopherol (1,050 ppm), $_{L}$-carnitine (260 ppm), $_{DL}$-α-LA (128 ppm), ascorbic acid (80 ppm), 1% inclusions of spinach flakes, tomato pomace, grape pomace, carrot granules and citrus pulp	Reduced cognitive dysfunction	Milgram et al. (2002)
Dog	7–9 years	Cognitive function	α-LA (11.0 mg/kg diet), acetyl-$_{L}$-carnitine (27.5 mg/kg diet)	Improved cognitive function demonstrated on two landmark discrimination tasks	Milgram et al. (2007)
Dogs	9–11.5 years	Cognitive function	Vitamin E (551 mg/kg diet), vitamin C (84.7 mg/kg diet), arginine (2.52 % as fed), thiamine (18.67 mg/kg diet), riboflavin (13.35 mg/kg diet), pantothenic acid (34.07 mg/kg diet), niacin (102.57 mg/kg diet), pyridoxine (11.05 mg/kg diet), cyanocobalamin (0.1 mg/kg diet), folic acid (3.94 mg/kg diet), EPA (0.24 % as fed), and DHA (0.21 % as fed)	Improved cognitive function (e.g., improved discrimination learning tasks)	Pan et al. (2018a)
Dogs	9–16 years	Cognitive function	6.5% MCT + Brain Protection Blend Vitamin E (552 mg/kg diet), vitamin C (151 mg/kg diet) arginine (1.79 % as fed), thiamine (58.7 mg/kg diet), riboflavin (26.5 mg/kg diet), pantothenic acid (77.3 mg/kg diet), niacin (225.76 mg/kg diet), pyridoxine (17.8 mg/kg diet), cyanocobalamin (0.175 mg/kg diet), folic acid (8.39 mg/kg diet), EPA (0.30 % as fed), selenium (0.681 mg/ kg diet), and DHA (0.23 % as fed)	Improved cognition scores (e.g., Senior Canine Behavior Questionnaire and a Canine Medical Health Questionnaire)	Pan et al. (2018b)
Dogs	6.8–8 years	Cognitive function	α-LA (30 mg (3 mg/kg BW)) and carnitine (60 mg (6 mg/kg BW))	Improved cognition (e.g., delayed recall aspect of delayed non-match to position (measuring short-term spatial memory) task)	Snigdha et al. (2016)
Dog	8–12 years	Cognitive Function	Vitamin E (800 IU or 210 mg/day (21 mg/kg BW/day)), vitamin C (16 mg/day (1.6 mg/kg BW/day)), carnitine (52 mg/day (5.2 mg/kg BW/day)), LA (26 mg/day (2.6 mg/kg BW/day))	Maintained cognition and reduced oxidative damage and Aβ pathology	Dowling and Head (2012)
Cat	5.5–8.7 years	Cognitive function	Vitamin E (550 mg/kg diet), vitamin C (80 mg/kg diet), arginine (2.3 % as fed), thiamine (55.0 mg/kg diet), riboflavin (30.9 mg/kg diet), pantothenic acid (55.4 mg/kg diet), pyridoxine (18 mg/kg diet), cyanocobalamin (0.09 mg/kg diet), folic acid (4.25 mg/kg diet), EPA (0.28 % as fed), and DHA (0.27 % as fed)	Improved cognitive function (e.g., egocentric learning, spatial memory)	Pan et al. (2013)
Cats	7–17 years	Longevity	Vitamin E (140.7 IU/1000 kcal), ß-carotene (5 mg/1000 kcal)	Increased longevity, and reduced disease incidence	Cupp et al. (2007)
Cats	7–17 years	Longevity	Vitamin E (140.7 IU/1000 kcal), ß-carotene (5 mg/1000 kcal), linoleic acid (21.3 % of dietary fat), chicory root (dose not stated)	Increased longevity, reduced disease incidence and improved intestinal health	Cupp et al. (2007)

Abbreviations: OA, osteoarthritis; BW, body weight; HA, hyaluronic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ETA, eicosatetraenoic acid; DMB, dry matter basis; DM, dry matter; LA, lipoic acid; MCT, medium chain triglycerides.

Conclusion

Our pet cats and dogs are living longer lives. As a consequence, more specific research into energy, macronutrient (protein, fat) and micronutrient requirements of our senior + pet cats and dog is required. Given that many of the conditions associated with ageing, or the underpinning physiological processes such as the immune system, respond to nutritional interventions in experimental settings suggests that ageing cats and dogs *do* have specific nutrient requirements. Therefore, research to better understand the energy and nutritional requirements is necessary and will hopefully enable the development of specific nutritional guidelines for our ageing cats and dogs.

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Conflict of Interest Statement

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Appendix 2.

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Development and validation of an LC-MS/MS method for the quantification of oral-sugar probes in plasma to test small intestinal permeability and absorptive capacity in the domestic cat (*Felis catus*)

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ABSTRACT

A novel method for quantifying the concentration of lactulose, rhamnose, xylose, and 3-O-methylglucose (3-OMG) in cat plasma using liquid chromatography-mass spectrometry (LC-MS) was developed. Domestic male cats (n = 13) were orally dosed with a solution containing the four sugars to test the permeability and absorptive capacity of their intestinal barrier. Plasma samples were taken 3 h later and were prepared with acetonitrile (ACN), dried under N_2, and reconstituted in 90 % ACN with 1 mM ammonium formate. Stable isotope labelled ^{13}C standards for each analyte were used as internal standards. Chromatographic separation was conducted using a Phenomenex Luna NH2 column with a gradient elution system of deionized water and 90 % ACN with 1 mM ammonium formate at 300 µL/min for 13 min total analysis time. Recovery trials were conducted in triplicate over three days with RSD values (%) for each day ranging from 1.2 to 1.4 for lactulose, 5.4 – 6.0 for rhamnose, 3.3 – 5.5 for xylose, and 2.6 – 5.6 for 3-OMG. Inter-day variations for each analyte were not different ($p > 0.05$). Limit of detection and quantification were 0.2 and 0.7 µg/mL for lactulose, 0.8 and 2.4 µg/mL for rhamnose, 0.6 and 1.8 µg/mL for xylose, and 0.3 and 1.1 µg/mL for 3-OMG, respectively. Plasma sugar concentrations recovered from cats were above the limit of quantification and below the highest calibration standard, validating the use of this method to test intestinal permeability and absorptive capacity in cats.

1. Introduction

The intestinal barrier plays a critical role in maintenance of normal physiologic processes of the digestive system, maintaining homeostasis in the gastrointestinal tract. It enables the selective permeability of molecules, allowing for absorption of nutrients and water, but prohibiting the passage of pathogens and other potential toxins. These molecules can cross the barrier through different paracellular, transcellular, carrier-mediated active transport, and ATP-dependent mediated active transport pathways, depending on their size and charge. The paracellular route is mediated by tight junctions between epithelial cells which facilitate the transport of ions, water, and larger hydrophilic compounds, in contrast to the transcellular route which permits small hydrophilic and lipophilic compounds to pass through the plasma membrane of the epithelial cell. Active transport pathways are reserved for the larger nutrients and molecules that require energy to move across the epithelial barrier, a characteristic also known as the absorptive capacity [1].

Abbreviations: 3-OMG, 3-O-methylglucose; SAT, sugar absorption test; AAFCO, Association of American Feed Control Officials; L:R, lactulose: rhamnose; X:G, xylose: 3-O-methylglucose.

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Ageing domestic cats experience a reduced ability to digest nutrients, specifically fat and protein, with no clear cause [2]. The relationship between age and intestinal permeability has not been examined in cats, offering a potential new perspective for investigation of this phenomenon. There is conflicting evidence whether intestinal permeability increases with age in other species, where it seems to be supported in fruit flies, mice, rats, and baboons, but not in dogs and humans [3–8]. Exploring the potential relationship between age and intestinal permeability will enhance our fundamental understanding of the domestic cat's gastrointestinal system, enabling researchers to investigate interventions that could ultimately enhance the quality of life for senior cats.

A common method to quantify the permeability and absorptive capacity of the small intestine is the differential sugar absorption test (SAT). A differential SAT involves administering a monosaccharide and a disaccharide, such as rhamnose and lactulose respectively, to measure transcellular and paracellular transport, respectively [9–12]. Non-metabolisable monosaccharides, xylose and 3-O-methylglucose (3-OMG), can be orally administered as well to determine the mucosal absorptive capacity [13,14]. Several studies have evaluated intestinal permeability in the cat, using varying SAT methodologies as the basis of investigation. These studies have all used different doses of disaccharides and monosaccharides and chose to analyse urine, which is harder to collect aseptically than plasma. Additionally, the sugar concentration in the urine was quantified using varying methods including high-performance liquid chromatography (HPLC), gas–liquid chromatography (GLC), and enzyme assay [11,15–18]. Emms *et al.* (1982) did however analyse the concentration of D-xylose in plasma to determine absorptive capacity in the cat using a method described by Trinder (1975) involving phloroglucinol [19,20]. Unfortunately, while Emms *et al.* (1982) and Randell *et al.* (2001) both successfully orally syringed the sugar solution to cats without sedation, these two studies do not clearly explain the protocol, making it hard to duplicate, especially given the great differences in the dose of probes used between them [11,19].

The differential SAT can also be performed using serum and plasma samples, as shown in multiple studies in humans and dogs [10,13,21–24]. Obtaining sterile urine and free catch urine samples are difficult in cats since cystocentesis is invasive as well as challenging to do on a partially filled bladder and they do not urinate on command, thus blood-based intestinal permeability tests in cats may be easier to conduct. A pilot study using sugars lactulose, rhamnose, xylose, and 3-OMG in the domestic cat was undertaken to determine the feasibility of using this multi-sugar procedure in a larger-scale study. Due to the lack of replicable and reliable oral sugar probe and intestinal permeability studies completed in cats, this study was also required to develop and evaluate the methodology and the sensitivity of the proposed laboratory analysis of feline blood samples. The aim of this study was to develop a quantitative LC-MS/MS method measuring the oral sugar probes in plasma using stable isotope standards. Benefits of such a method include less invasive and more reliable sampling, and thus ethically less expensive means of evaluating intestinal permeability and absorptive capacity in cats.

2. Materials and methods

2.1. Ethics

The preliminary validation pilot study and subsequent research trial were both approved by the Massey University Animal Ethics Committee (MUAEC 22/69 and 23/14) which meets the requirements of the Animal Welfare Act [25]. Cats selected for each experiment continued to be provided with their normal commercially retorted wet cat food (Heinz Wattie's Ltd., Hastings, New Zealand) which is complete and balanced according to Association of American Feed Control Officials (AAFCO) requirements for adult cats. They were fed *ad libitum* daily in their enclosures leading up to the trial and fasted in metabolic cages, as described by Hendriks *et al.* (1999) [26], for 12 h before sugar probe ingestion with access to fresh water at all times.

2.2. Animal studies

This study was split into two parts. The first trial aimed to determine the feasibility and palatability of giving cats a single oral dose of sugar solution and was then followed by a second pilot trial whereby the detection of the sugar probes in plasma was verified.

In order to assess the feasibility of dosing adult cats a sugar solution containing 0.07 g/mL lactulose, 0.02 g/mL rhamnose, 0.07 g/mL xylose, and 0.02 g/mL 3-OMG (1160 mOsm) was mixed in purified water. Six, neutered, male adult (n = 6) domestic shorthair cats aged 5.0 to 8.9 years (mean ± SD, 5.8 ± 1.8 years) and weighing from 3.7 to 5.2 kg (4.3 ± 0.6 kg) received a single oral dose of 2 mL/kg.

All cats tolerated the dosing protocol well and the full dose was administered. From this, the pilot study was undertaken. Following an overnight fast, 2 mL of blood was taken from 13 healthy male adult cats (mean age ± SD (range), 2.5 ± 0.8 (1.3–3.4) years) via jugular venepuncture (T_0). The cats were then dosed with 2 ml/kg of the sugar probe solution (0.07 g/mL lactulose, 0.02 g/mL rhamnose, 0.07 g/mL xylose, and 0.02 g/mL 3-OMG; osmolarity 1160 mOsm). After three hours, 2 mL blood samples were collected again from the opposite jugular vein, and the cats were permitted to return to their regular enclosures where they had free access to both food and water.

The dose of the sugar solution made of lactulose, rhamnose, xylose, and 3-OMG was extrapolated from previous studies in cats and dogs [8,11,13,19,21,27,28]. The sugar solution had an osmolality of 1160 mOsm, which is under the threshold of 1500 mOsm when hypertonic solutions begin to increase permeability [29]. The final blood samples were collected from the cats 3 h after oral dosing based on findings from multiple studies that blood collected at this time is the most consistent between samples [13,21,30].

2.3. Chemicals and standards

$^{13}C_{12}$ lactulose was purchased from Santacruz Biotechnology Inc (Santa Cruz, USA), $^{12}C_6$ 3-OMG from Toronto Research Chemicals (Toronto, Canada), $^{13}C_5$ xylose, $^{13}C_6$ 3-OMG, and $^{13}C_6$ rhamnose from Omicron Biochemicals Inc (South Bend, USA), ^{12}C lactulose, ^{12}C rhamnose, ^{12}C xylose, acetonitrile (HPLC grade), and ammonium formate (HPLC grade) from Sigma-Aldrich (Auckland, New Zealand). Ultrapure water was prepared with a Milli-Q® ultrapure water system (Merck, Auckland, New Zealand) with a resistivity of 18.2 MΩ.

A mixed internal standard solution containing the four ^{13}C labelled sugars was made from stock solutions of individual ^{13}C sugars in 90 % ACN to yield a final concentration of 50 μg/mL. Similarly, a mixed standard of unlabelled sugars was made from stock solutions of individual target sugars in 90 % ACN to yield a final concentration of 1 mg/mL. This mixed standard, along with the mixed internal standard solution, were used to make the calibration standards (in 90 % ACN) for calculating concentration and testing linearity (Table 1) and preparing fortified samples for recovery and repeatability experiments. A seven-point calibration series was prepared containing 0, 1, 5, 10, 20, 50 or 100 μg/mL of the target unlabelled sugars, and 5 μg/mL of the ^{13}C labelled sugars.

2.4. Sample preparation

Whole blood was transferred to a K2 EDTA 5.4 mg vacutainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and spun at 3000xg at 4 °C for 15 min. Plasma was then aliquoted into microcentrifuge tubes and frozen at −80 °C until analysis.

Plasma samples (200 μL) were thawed, then mixed with ice cold acetonitrile (ACN; 590 μL) and spiked with an internal standard mixture (10 μL) containing 50 μg/mL of each of the four ^{13}C labelled sugars.

Table 1
Parallel reaction monitoring inclusion list.

Name	Mass (m/z)	Isolation width (m/z)	Adduct	Start	End	(N) CE
Xylose	149	2	$[M-H]^-$	1.5	6	10
$^{13}C_5$ xylose	154	2	$[M-H]^-$	1.5	6	10
Rhamnose	163	2	$[M-H]^-$	1.5	6	10
$^{13}C_6$ rhamnose	169	2	$[M-H]^-$	1.5	6	10
3-OMG	239	2	$[M+HCO_2]^-$	1.5	6	10
$^{13}C_6$ 3-OMG	245	2	$[M+HCO_2]^-$	1.5	6	10
Lactulose	387	2	$[M+HCO_2]^-$	6	11	10
$^{13}C_{12}$ lactulose	399	2	$[M+HCO_2]^-$	6	11	10

Abbreviations: 3-OMG = 3-O-methylglucose; NCE = normalised collision energy.

Samples were then vortexed thoroughly, incubated (−20 °C, 60 min), vortexed again, and centrifuged (14,000xg, 10 min, 4 °C). Then, 100 μL of purified water was added to each sample, vortexed, and centrifuged (14,000xg, 10 min, 4 °C) for a second time. A fixed volume of the supernatant (800 μL) was removed and then dried under nitrogen at 35 °C, and the dry extract was reconstituted in 100 μL 90 % ACN with 1 mM ammonium formate.

2.5. Preparation of fortified samples

Plasma samples collected before oral administration of sugar solution (T_0) were pooled into a composite sample and aliquots were spiked with a known amount of the target sugars, then subjected to the extraction protocol (as described in section 2.4). The experiment was conducted using no spike, low spike, and high spike plasma samples in triplicate. No spike samples were unaltered blank plasma matrix, low spike samples were 5 μL of mixed sugar standard (^{12}C) with 995 μL of blank plasma (concentration: 5 μg/mL), and high spike samples were 25 μL of mixed sugar standard (^{12}C) with 975 μL of blank plasma (concentration: 25 μg/mL). Blank plasma was unaltered T_0, fasted plasma samples that served as a control.

2.6. LC-MS/MS conditions

LC-MS/MS was carried out using a Thermo Scientific Accela HPLC system coupled to a Q Exactive Orbitrap mass spectrometer with electrospray ionisation (ESI) (Thermo Scientific, New Zealand). Samples were introduced to the HPLC system via a PAL autosampler (injection volume = 5 μL), and chromatography was performed using a Phenomenex Luna NH2 column (2 x 150 mm, 3 μm, Phenomenex, New Zealand) with a gradient elution of deionized water with 1 mM ammonium formate (eluent A) and 90 % ACN with 1 mM ammonium formate (eluent B) at a flow rate of 300 μL/min. The gradient elution program was as follows: 100 % B (0 – 0.5 min), 100 – 85 % B (0.5 – 3.5 min), 85 % B (3.5 – 11 min), and a re-equilibration period at 100 % B for 3 min (11 – 13 min). The first 1.5 min of the chromatographic run was diverted to waste and data was acquired from 1.5 – 11 min. The mass spectrometer was operated in negative ionisation mode and data was acquired in full scan MS1 mode (50 – 500 *m/z*) and parallel reaction monitoring (PRM) mode monitoring the MS2 using dynamic first mass for all samples. The ESI source was operated with a spray voltage of 4.5 kV, capillary temperature of 350 °C, sheath gas flow of 20 L/min, aux gas flow of 10 L/min and sweep gas flow of 5 L/min. PRM mode was operated with the inclusion list and relevant parameters in Table 1.

Data acquisition and processing was carried out using Thermo Xcalibur software (version 3.1.66.10; Thermo Fisher Scientific). Regression statistics and standard deviation of the equation were calculated using Microsoft Excel (Office 360, Microsoft Corporation, Redmond, Washington, United States).

2.7. Calculations

Concentration of sugars in the sample (Ci) was calculated by using the concentration measured by the instrument (Cf) and adjusting for factors introduced during sub-sampling (Sf) and during extraction such as the pre-concentration factor (Pf). The equation is shown in equation (1) below:

$$Ci = \frac{Cf * \frac{1}{Sf}}{Pf} = \frac{Cf * \frac{9}{8}}{2} = \frac{Cf * 9}{8 * 2} = \frac{9Cf}{16} \quad (1)$$

3. Results

Quantification for xylose, 3-OMG, and lactulose was performed using MS1 accurate mass peak areas, including molecular ions and any reliable adduct ions to increase sensitivity. MS1 masses 161.0434 and 167.0634 were observed in authentic standards and target samples for ^{12}C 3-OMG and ^{13}C 3-OMG, respectively, and have been previously used to quantify lactulose [31]. ^{12}C rhamnose and ^{13}C rhamnose were solely characterised and quantified in MS2. Exact ions used for quantitation are shown in Table 2 with a mass tolerance of 10.0 ppm. Identifications confirmed by monitoring the MS2 spectra for characteristic fragment ions (Fig. 1) and retention time compared to authentic standards.

3.1. Tandem mass spectrometry detection

MS2 spectra of the four analytes along with their internal standards are presented in Fig. 1. MS2 fragmentation patterns for ^{12}C xylose, rhamnose, 3-OMG, and lactulose are presented in Fig. 2, including predicted fragment ions from the MetFrag tool [32]. It is important to note that ^{13}C standards follow the same fragmentation pattern, but exhibit a mass shift relative to the number of ^{13}C atoms present.

3.2. Chromatographic separation

Chromatographic separation of the sugars was successfully achieved between ≈3.5 and 9 min. While the peaks for both analyte and internal standard of xylose and rhamnose suffered some minor tailing, this did not affect linearity, recovery, or repeatability for these sugars (Table 3). Chromatograms of all 4 analytes and the 4 internal standards in the level

Table 2
Ions measured for each analyte.

Analyte	Ion	MS1 (m/z)	MS2 (m/z)
^{12}C xylose	$[M-H]^-$	149.0432	
	$[M+HCOO]^-$	195.0488	
	$[M+Cl]^-$	185.0202	
^{13}C xylose	$[M-H]^-$	154.0600	
	$[M+HCOO]^-$	200.0657	
	$[M+Cl]^-$	190.0370	
^{12}C 3-OMG		161.0434	
	$[M+HCOO]^-$	239.0751	
	$[M+Cl]^-$	229.0462	
^{13}C 3-OMG		167.0634	
	$[M+HCOO]^-$	245.0951	
	$[M+Cl]^-$	235.0666	
^{12}C lactulose	$[M-H]^-$	341.1061	
	$[M+HCOO]^-$	387.1112	
	$[M+Cl]^-$	377.0825	
^{13}C lactulose	$[M-H]^-$	353.1464	
	$[M+HCOO]^-$	399.1519	
	$[M+Cl]^-$	389.1230	
^{12}C rhamnose			59.0123
^{13}C rhamnose			61.0190

Abbreviations: 3-OMG = 3-O-methylglucose.

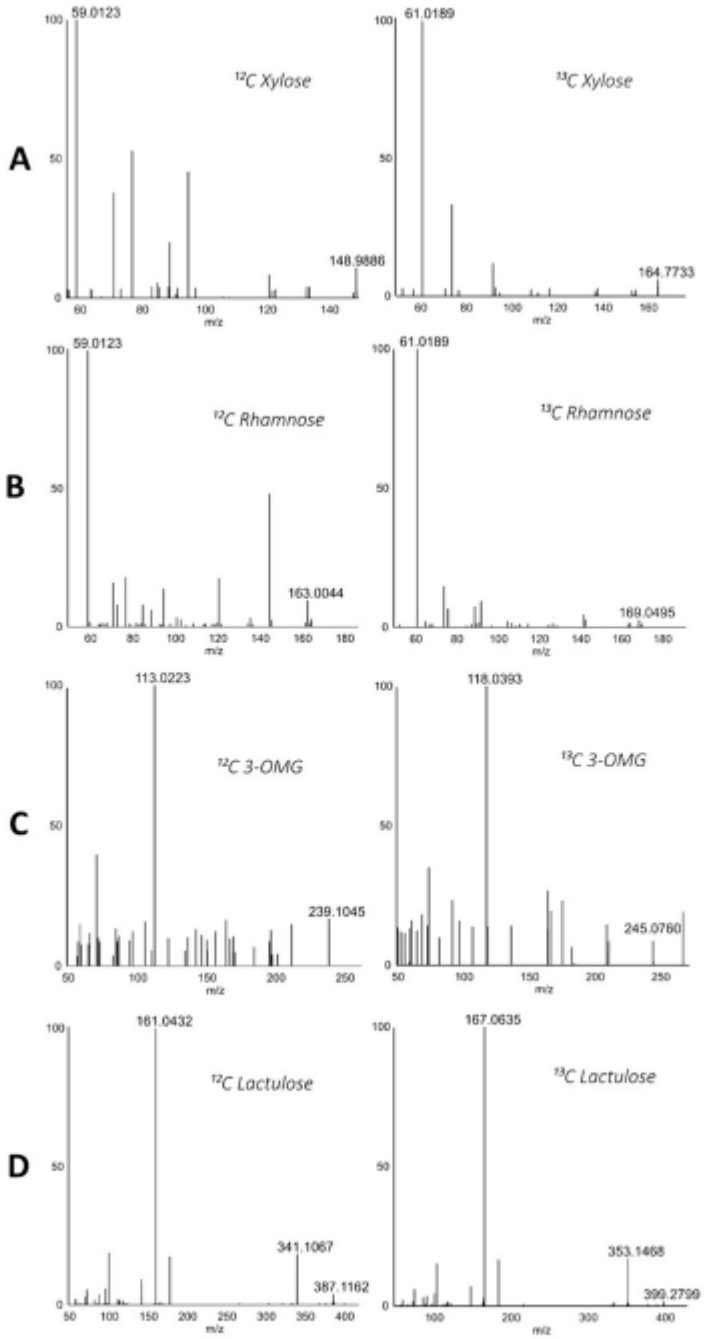

Fig. 1. MS2 spectra for (A) xylose, $^{13}C_5$ xylose, (B) rhamnose, $^{13}C_6$ rhamnose, (C) 3-O-methylglucose, $^{13}C_6$ 3-O-methylglucose, and (D) lactulose, $^{13}C_{12}$ lactulose.

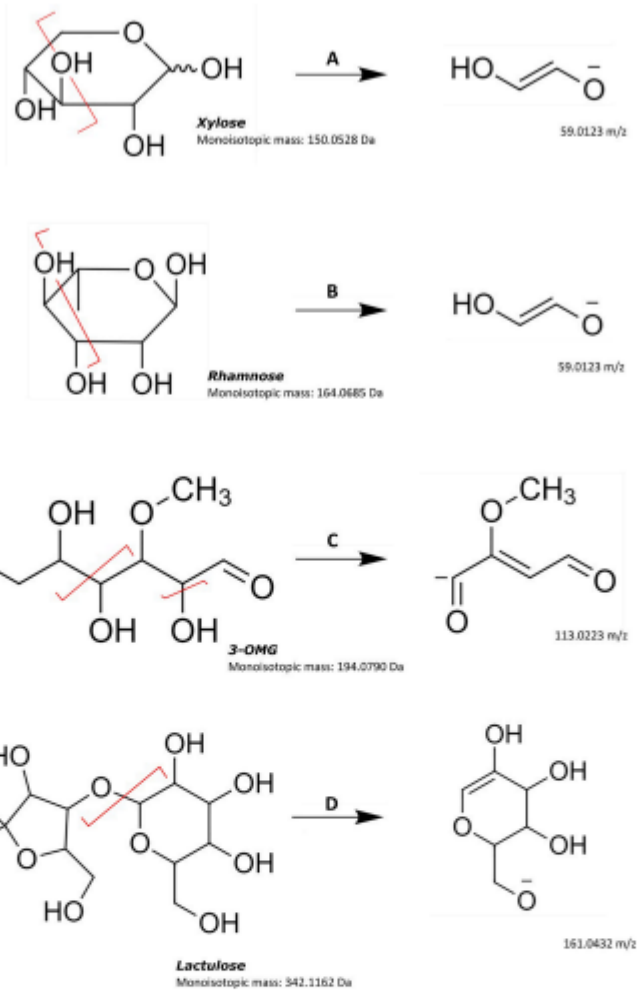

Fig. 2. (A) Xylose, (B) rhamnose, (C) 3-O-methylglucose (3-OMG), and (D) lactulose fragments used for identification and quantification. Fragments predicted using the in silico fragmenter tool, MetFrag [32].

Table 3
Linearity, limit of detection and limit of quantitation.

Analyte	Equation	R^2	LOD (μg/mL)	LOQ (μg/mL)
Lactulose	y = 0.171145x – 0.0475757	0.9997	0.2	0.7
Rhamnose	y = 0.136588x + 0.0198997	0.9990	0.8	2.4
Xylose	y = 0.134708x + 0.0888231	0.9991	0.6	1.8
3-OMG	y = 0.155246x – 0.0730001	0.9995	0.3	1.1

Abbreviations: LOD = limit of detection; LOQ = limit of quantitation; 3-OMG = 3-O-methylglucose.

4 calibration standard mixture (concentration of ^{13}C internal standards at 5 μg/mL and target analytes at 20 μg/mL) are shown in Fig. 3.

3.3. Linearity, LOD, and LOQ

Limit of Detection (LOD) and Limit of Quantitation (LOQ) for each sugar was calculated by using the standard deviation of the response of the blank (σ) and the slope (S) of the calibration curve. LOD and LOQ were calculated from these values using equations (2) and (3) respectively. The linearity and sensitivity values for the method are summarised in Table 3.

$$LOD = 3.3 \times \frac{\sigma}{S} \quad (2)$$

$$LOQ = 10 \times \frac{\sigma}{S} \quad (3)$$

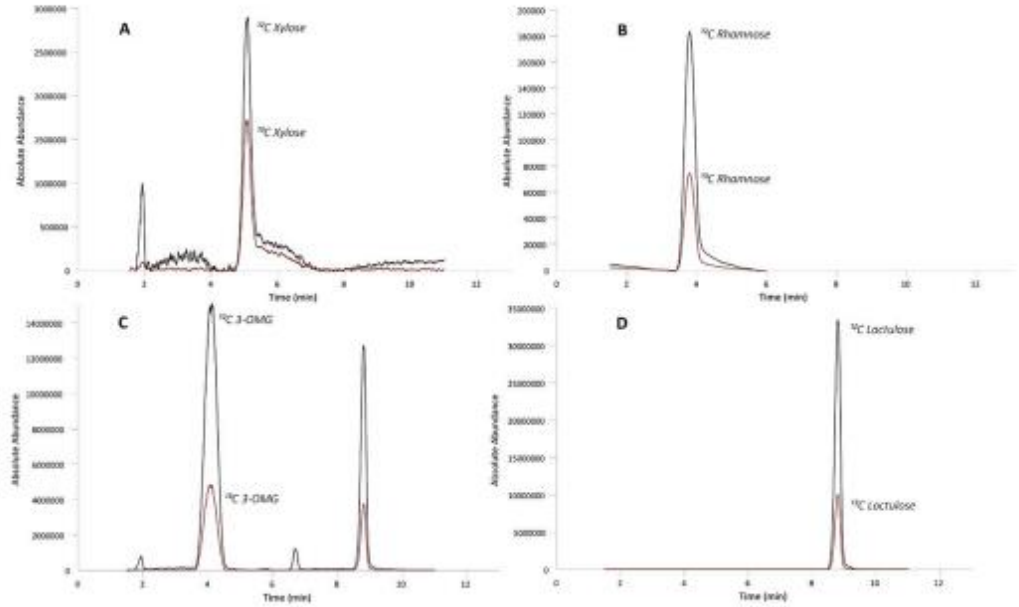

Fig. 3. Chromatograms of analytes and internal standards, (A) xylose, (B) rhamnose, (C) 3-O-methylglucose (3-OMG), and (D) lactulose, in a calibration standard mixture containing 20 μg/mL detected by negative ionisation electrospray and MS/MS. Concentration of ^{13}C internal standards are 5 μg/mL.

3.4. Accuracy, intermediate precision, and repeatability

Trueness, repeatability, and intermediate precision of the method were tested using the spiked samples at two concentration levels (low and high, 5 and 25 μg/mL respectively) and unfortified T_0 samples. The two spike levels and matrix blank were each analysed in triplicate within the same day and across three days. The difference in mean recovery was significantly different based on concentration ($p < 0.05$). Trueness and repeatability of this method met the acceptance criteria (RSD < 10 % and inter-day variation with $p > 0.05$) for the analysis of samples from the main study (Table 4). The mean and intermediate precision of the intestinal permeability (L:R) and absorptive capacity (X:G) was calculated for each spike level and the combined total (Table 5).

Table 4
Repeatability study, mean recovery, standard deviation, relative standard deviation, and significance of inter-day variation.

Analyte	Day	Mean ER* (%)	SD	RSD	Inter-day Variation (ANOVA)
Lactulose	Day1	112.2	1.6	1.4	
	Day2	111.7	1.4	1.2	
	Day3	112.5	1.6	1.4	
	All Days	112.1	1.5	1.3	p = 0.66
Rhamnose	Day1	106.2	5.7	5.4	
	Day2	105.7	6.3	6.0	
	Day3	107.7	5.9	5.5	
	All Days	106.5	5.7	5.3	p = 0.84
Xylose	Day1	109.6	6.0	5.5	
	Day2	109.0	5.6	5.1	
	Day3	109.5	3.6	3.3	
	All Days	109.4	4.9	4.4	p = 0.97
3-OMG	Day1	97.7	5.5	5.6	
	Day2	97.5	5.3	5.4	
	Day3	99.7	2.6	2.6	
	All Days	98.3	4.5	4.6	p = 0.67

*Number of samples used to calculate the mean per day was 6 (3 samples from each spike level) Abbreviations: ER – extraction recovery; 3-OMG – 3-O-methylglucose.

Table 5
Mean and intermediate precision of intestinal permeability (L:R) and absorptive capacity (X:G).

Measure	Spike Level	Mean	RSD
L:R	Low	1.1	4.4
	High	1.0	0.7
	Total	1.1	5.2
X:G	Low	1.1	2.8
	High	1.1	3.2
	Total	1.1	2.9

Abbreviations: L:R – lactulose: rhamnose; X:G – xylose: 3-O-methylglucose.

Recovery was calculated based on the concentration measured in the spiked sample (C_s), blank sample (C_b), and the theoretical known concentration (C_a). C_s and C_b were calculated as described in section 2.7, and the extraction recovery percentage (ER%) was then estimated by equation (4):

$$ER\% = \left(\frac{C_s - C_b}{C_a}\right) * 100 \quad (4)$$

4. Analysis of experimental samples

The concentrations of the oral-dosed sugars measured in plasma, normalised by dose administered to each cat, are displayed in Table 6. Equations used to calculate L:R and X:G ratios are shown in equations (4) and (5), respectively, using the concentration of the sugar quantified in the plasma and the amount of the sugar ingested by each cat in the sugar solution. Concentrations of analytes reported in the samples were adjusted based on the recoveries presented in Table 4. The concentration

Table 6
Analyte concentration of real samples.

Cat ID	Dose (mL)	Lactulose (μg/mL)	Rhamnose (μg/mL)	Intestinal Permeability (L:R)	Xylose (μg/mL)	3-OMG (μg/mL)	Absorptive Capacity (X:G)
1	10.2	8.42	3.92	0.61	53.87	26.09	0.59
2	9.8	31.36	14.97	0.60	83.09	34.99	0.68
3	7.8	7.08	3.64	0.56	48.87	27.40	0.51
4	8.7	56.76	23.05	0.70	86.61	46.06	0.54
5	7.7	35.69	14.42	0.71	75.30	35.87	0.60
6	9.2	8.65	5.06	0.49	48.61	27.03	0.51
7	8.9	17.64	6.43	0.78	47.21	25.31	0.53
8	9.1	22.97	16.18	0.41	92.35	49.29	0.54
9	6.7	5.54	3.79	0.42	42.50	17.53	0.69
10	8.6	17.26	11.05	0.45	51.96	30.70	0.48
11	8.5	10.32	4.41	0.67	51.67	27.22	0.54
12	8.3	26.28	11.20	0.67	59.97	32.66	0.52
13	6.8	15.06	11.44	0.38	82.89	42.43	0.56

Abbreviations: L:R = lactulose: rhamnose; 3-OMG = 3-O-methylglucose; X:G = xylose: 3-O-methylglucose.

of all analytes in samples exceeded their respective LOQ and were within the calibration range (0 – 100 μg/mL), deeming this method fit for purpose. Urinary ratios have previously been observed as being directly comparable to plasma and serum ratios [10,21], but have never been compared in the domestic cat. The intestinal permeability values, the lactulose to rhamnose ratio, obtained in this study showed higher permeability than previously documented urinary ratios. Earlier reported values varied between 0.03 and 0.53, with a median of approximately 0.44, which is lower than the present range of 0.38 – 0.78, with a median of 0.60 [11,15–18]. The absorptive capacity values, the xylose to 3-OMG ratio, range from 0.48 to 0.69 in the present study, with a median of 0.50, which is lower than the only other reported xylose to 3-OMG ratio of 0.70 in a urine-based study by Johnston *et al.* (2001). The values obtained in this study could be different from the literature due to a variety of factors, including but not limited to, age, environment, neuter status, genetics, diet, and osmolarity of solution.

$$L:R = \frac{(LactuloseRecovered)}{(RhamnoseRecovered)} = \frac{\left(\frac{LactuloseConcentration}{LactuloseIngested}\right)}{\left(\frac{RhamnoseConcentration}{RhamnoseIngested}\right)} \quad (4)$$

$$X:G = \frac{(XyloseRecovered)}{(3-OMGRecovered)} = \frac{\left(\frac{XyloseConcentration}{XyloseIngested}\right)}{\left(\frac{3-OMGConcentration}{3-OMGIngested}\right)} \quad (5)$$

5. Discussion

The aim of this study was to develop a LC-MS/MS method for quantifying the intestinal permeability and absorptive capacity of cats using the simultaneous analysis of oral-dosed sugars in plasma 3 h after dosing. Lactulose, rhamnose, xylose, and 3-OMG were all detected in plasma 3 h following a single oral dose, indicating this method is highly suitable for future use examining impacts on intestinal permeability and absorptive capacity from factors such as diet and ageing in the domestic cat.

The extraction process was quick, and simple to complete. The use of stable isotope standards for each target sugar ensured high quantitative accuracy and precision [33]. The selectivity of the high-resolution mass spectrometry and PRM and MS/MS fragmentation patterns provided the high degree of sensitivity and selectivity required to work in plasma as opposed to urine as is commonly used. Previous studies evaluating sugar probe concentrations in canine plasma used gas chromatography-mass spectrometry (GC–MS) due to its increased sensitivity [13,21]. However, sugars are non-volatile compounds and require chemical derivatisation to be measured using GC–MS. This process not only adds complexity to the sample preparation, but may also cause unstable or incomplete derivatisation in the case of some compounds, generating multiple sugar peaks due to the presence of their isomers [34] and therefore incorrect concentration calculations. Derivatisation steps are often characterised for variability, which can be influenced by many factors, for example, by moisture [34]. To overcome the challenges linked to GC–MS, the initial testing of six cats was completed to verify that the samples could be quantified within the detection limits of LC-MS.

Cats were fasted for 12 h before the baseline venepuncture and administration of the oral sugar solution. While the fasting method clearly demonstrated no other sugars present at detectable levels in the plasma that could act as interfering peaks, if this was an issue then utilising MS/MS would help to differentiate between isobaric compounds based on their fragmentation patterns. The baseline samples produced no peaks within the retention times that were tested, which was not surprising since the cats were fasted. This also confirmed that these sugars are not endogenously produced at detectable levels within the cat.

The dose of the sugar solution made of lactulose, rhamnose, xylose, and 3-OMG was extrapolated from previous studies in cats and dogs [8,11,13,19,21,27,28]. The sugar solution had an osmolality of 1160 mOsm, which is under the threshold of 1500 mOsm when hypertonic solutions begin to increase permeability [29]. The final blood samples were collected from the cats 3 h after oral dosing based on findings from multiple studies that blood collected at this time is the most consistent between samples [13,21,30].

Diarrhoea is a potential issue when consuming high levels of sugar, however this was not observed in any of the cats after ingestion of the hyperosmolar sugar solution. The sugar solution made in the current study was made to be hyperosmolar for multiple reasons. Firstly, the solution needed to be given in a dose that could be easily administered to the cats and therefore, needed to be concentrated to lower the volume of solution being orally syringed. Secondly, a previous study in dogs used a 1560 mOsm solution of lactulose and mannitol to successfully determine intestinal permeability [24], comparable to the dose used in the current study. Lastly, hyperosmolar solutions have been said to provide a better discrimination between a healthy and a damaged small intestinal mucosal barrier [35–38]. The reasoning behind this increase in lactulose permeation is unknown, however authors have speculated that a hyperosmolar solution may influence paracellular transport via tight junctions or cause solvent drag [35,36]. Although the dose used in this study did not reach the level of hyperosmolarity proposed to increase permeation of lactulose, the use of a hyperosmolar solution as opposed to an *iso*-osmolar one used in previous literature with cats may explain the higher lactulose recovery rates in comparison to previous urinary studies [11,15–18]. The dose may therefore pose a challenge for directly comparing results to previous trials measuring feline intestinal permeability, but it paves the way for exciting opportunities and progress in future research within this area. This new LC-MS/MS method should be applied to a larger sample size which will help in determining a

reference range for lactulose to rhamnose and xylose to 3-OMG ratio when measured in plasma using this dose.

6. Conclusions

The LC-MS/MS method developed here for measuring sugars used in intestinal permeability assays was shown to be sensitive, reliable, and reproducible for a small volume of plasma. It was successful in its ability to be minimally invasive, fast, selective, and sensitive for the accurate and precise quantification of four different test sugars in plasma using stable isotope internal standards. The development and application of this method is the first reported quantitative analysis of these sugars used for permeability assays in feline plasma. It proves to be a potential tool to analyse intestinal permeability and absorptive capacity in cats.

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Data availability statement

The data that support the findings of this study are available from the corresponding author, KP, upon reasonable request.

CRediT authorship contribution statement

Keely Patterson: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Karl Fraser:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Daniel Bernstein:** Writing – review & editing, Validation, Methodology, Data curation. **Emma N. Bermingham:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Karin Weidgraaf:** Resources, Investigation. **Anna Kate Shoveller:** Writing – review & editing, Supervision, Project administration, Conceptualization. **David Thomas:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix 3.

Statement of contribution for manuscript under review with *Journal of Animal Physiology and Animal Nutrition.*

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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:	Keely Patterson
Name and title of main supervisor:	Assoc. Prof. D.G. Thomas
In which chapter is the manuscript/published work?	Chapter 3

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]
Keely Patterson: Writing – original draft, review & editing, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Karl Fraser: Review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. Daniel Bernstein: Review & editing, Validation, Methodology, Data curation. Emma N. Bermingham: Review & editing, Supervision, Project administration, Methodology, Conceptualization. Karin Weidgraaf: Resources, Investigation. Anna Kate Shoveller: Review & editing, Supervision, Project administration, Conceptualization. David Thomas: Review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Please provide the full reference of the research output:

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Journal of Animal Physiology and Animal Nutrition

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Student's signature:	Keely Patterson	Main supervisor's signature:	David Thomas

This form should be placed at the beginning of each relevant thesis chapter.

[1] Refer to the Massey University Publishing and Authorship guidelines (OneMassey for staff, Stream for students) and/ or Contributor Roles Taxonomy (CRediT) guidelines for guidance.

Appendix 4.

Linear correlation plots from Chapter 3.

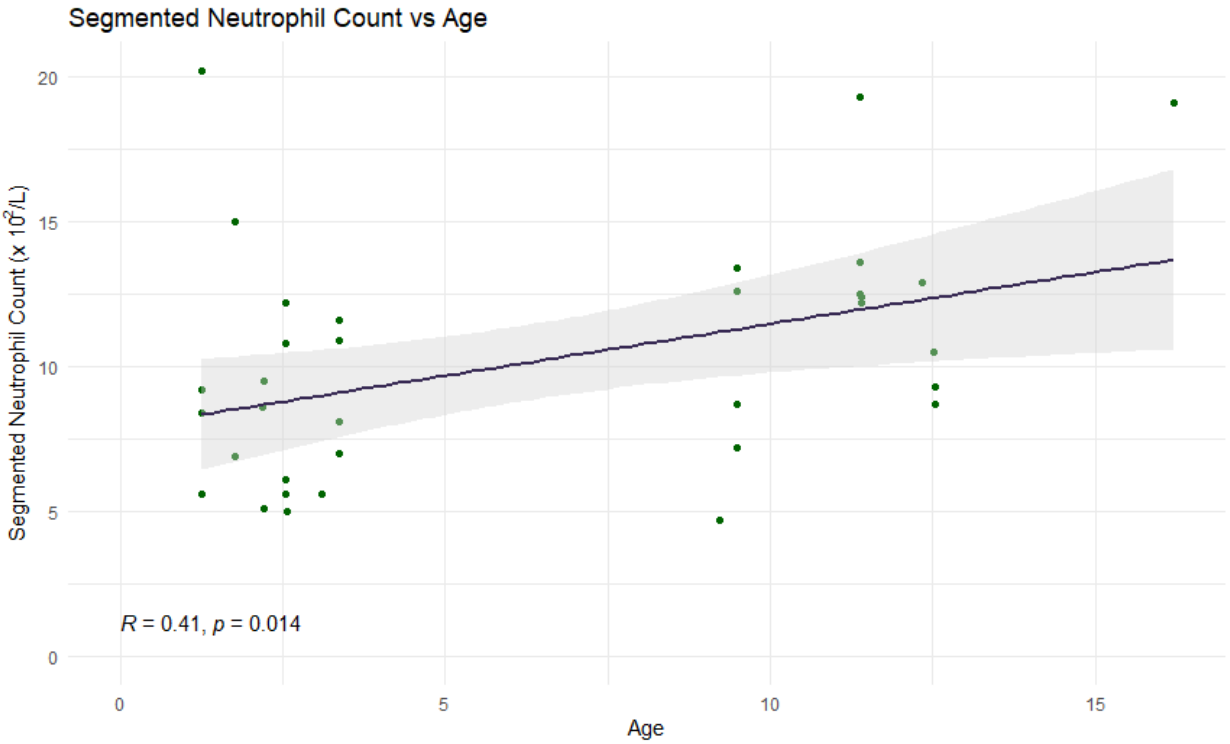

Figure S3.1. Segmented neutrophil count is positively correlated with the senior age class (p = 0.033) and positively correlated with continuous age (p = 0.014).

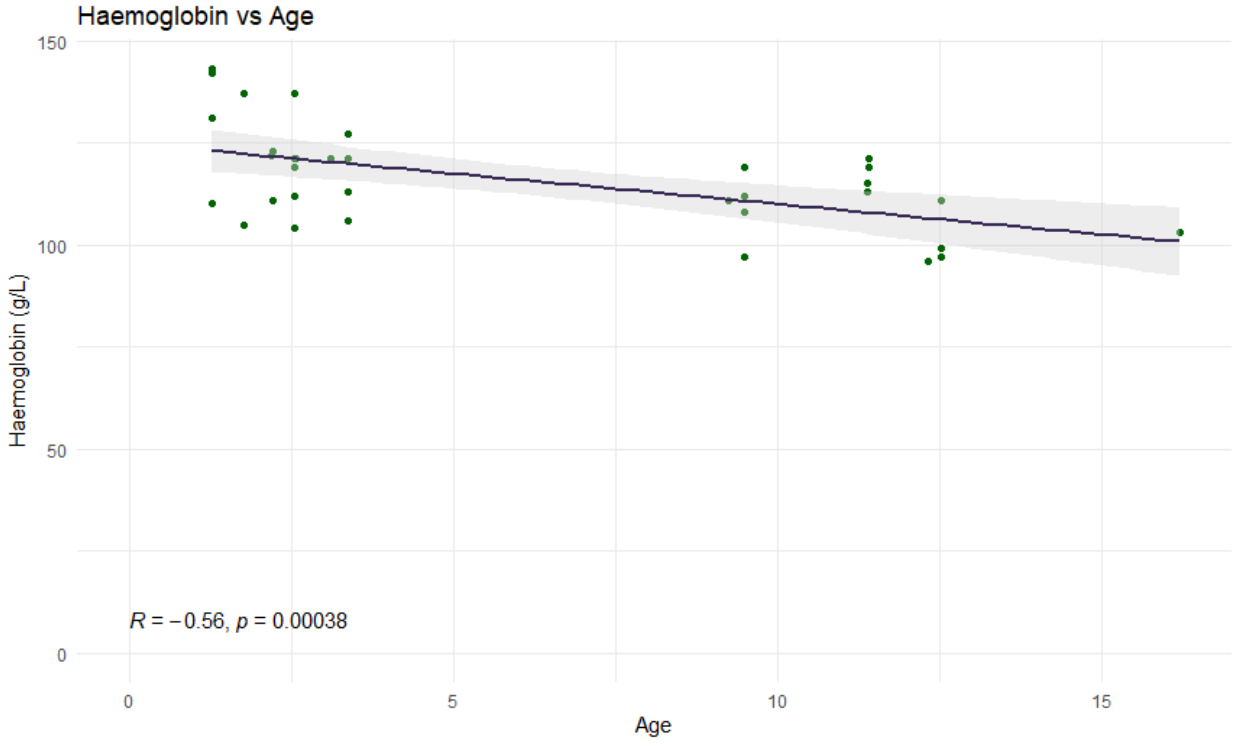

Figure S3.2. Haemoglobin is negatively correlated with the senior age class (p = 0.001) and negatively correlated with continuous age (p < 0.001).

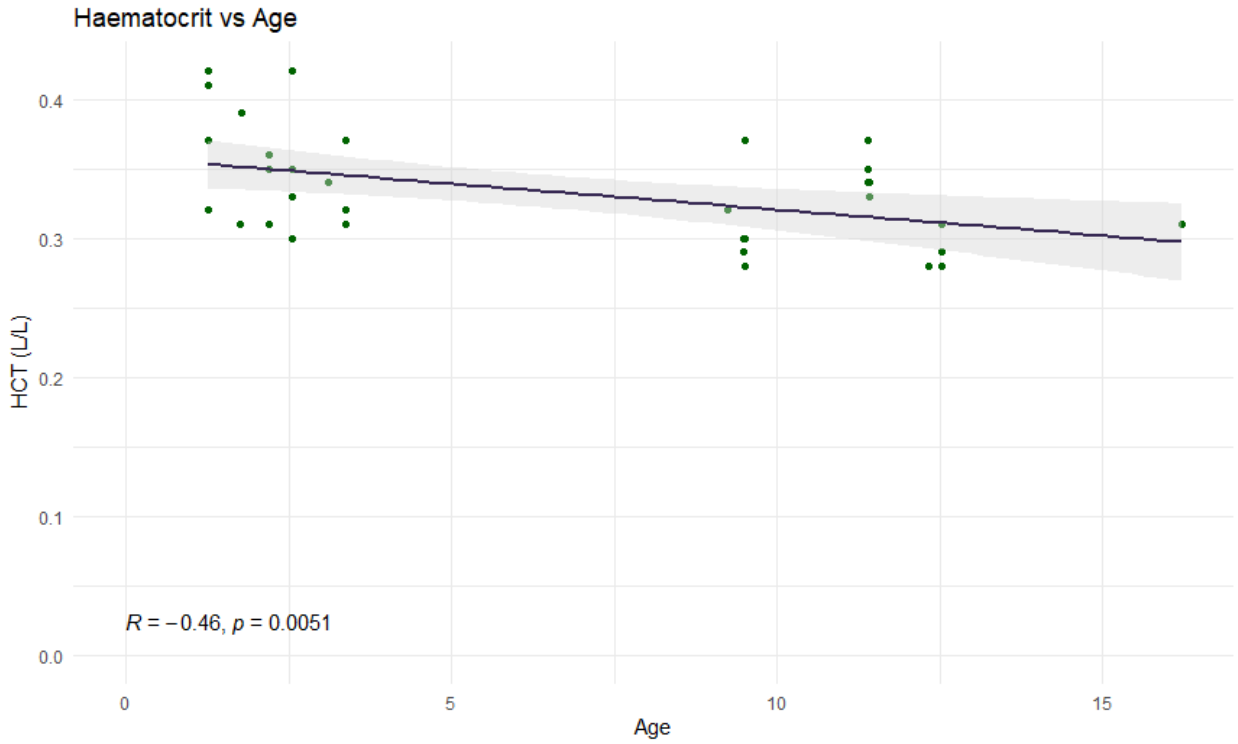

Figure S3.3. Haematocrit (HCT) is negatively correlated with the senior age class (p = 0.010) and negatively correlated with continuous age (p = 0.005).

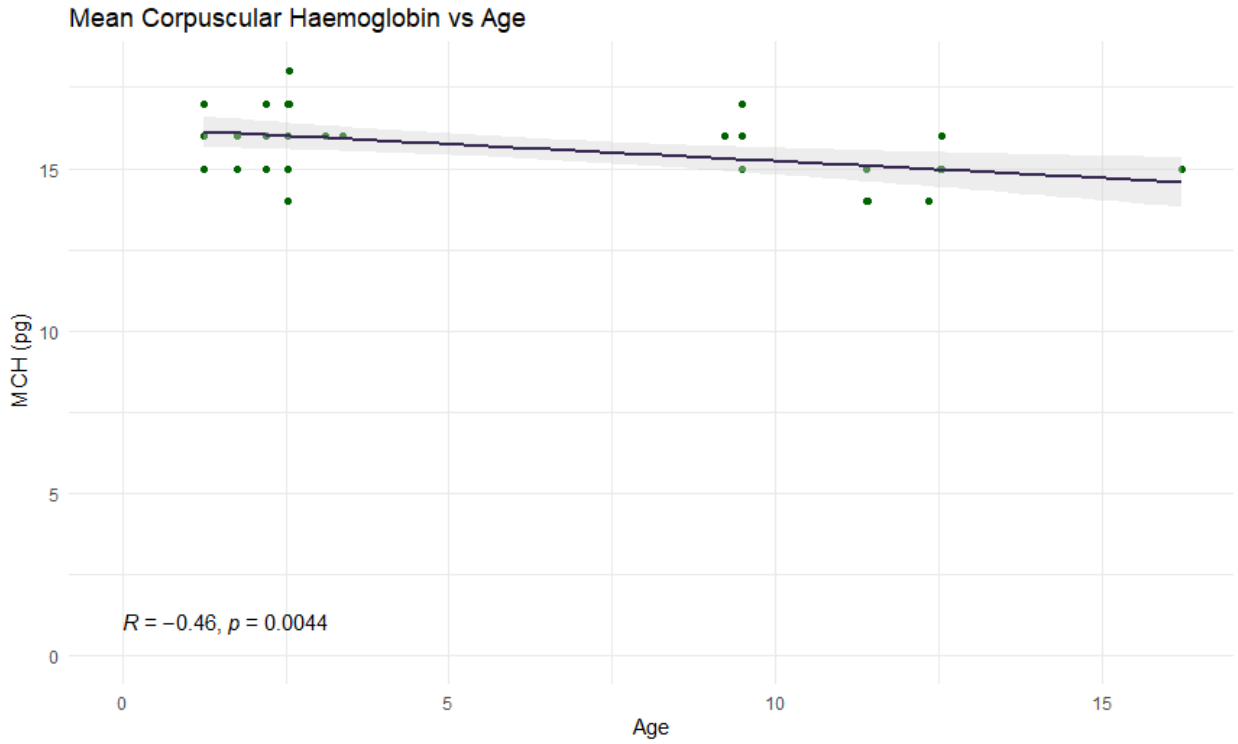

Figure S3.4. Mean corpuscular haemoglobin (MCH) is negatively correlated with the senior age class (p = 0.011) and negatively correlated with continuous age (p = 0.004).

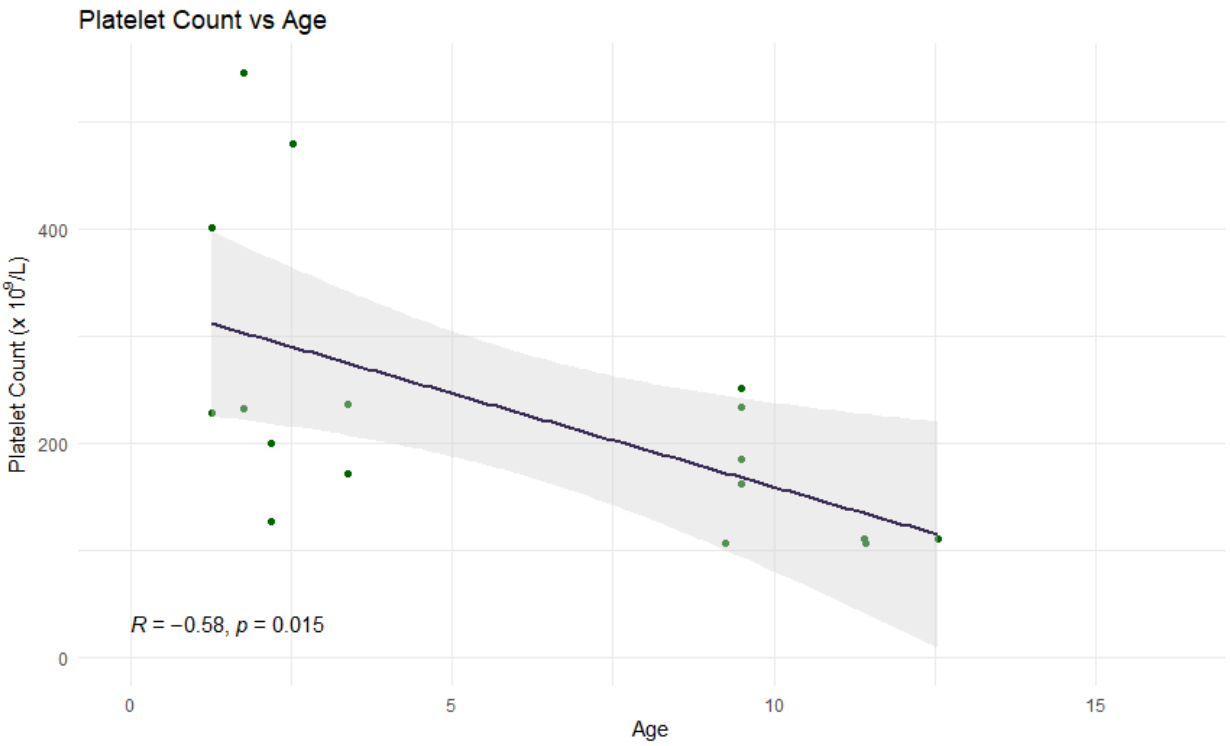

Figure S3.5. Platelet count is negatively correlated with the senior age class (p = 0.031) and negatively correlated with continuous age (p = 0.015).

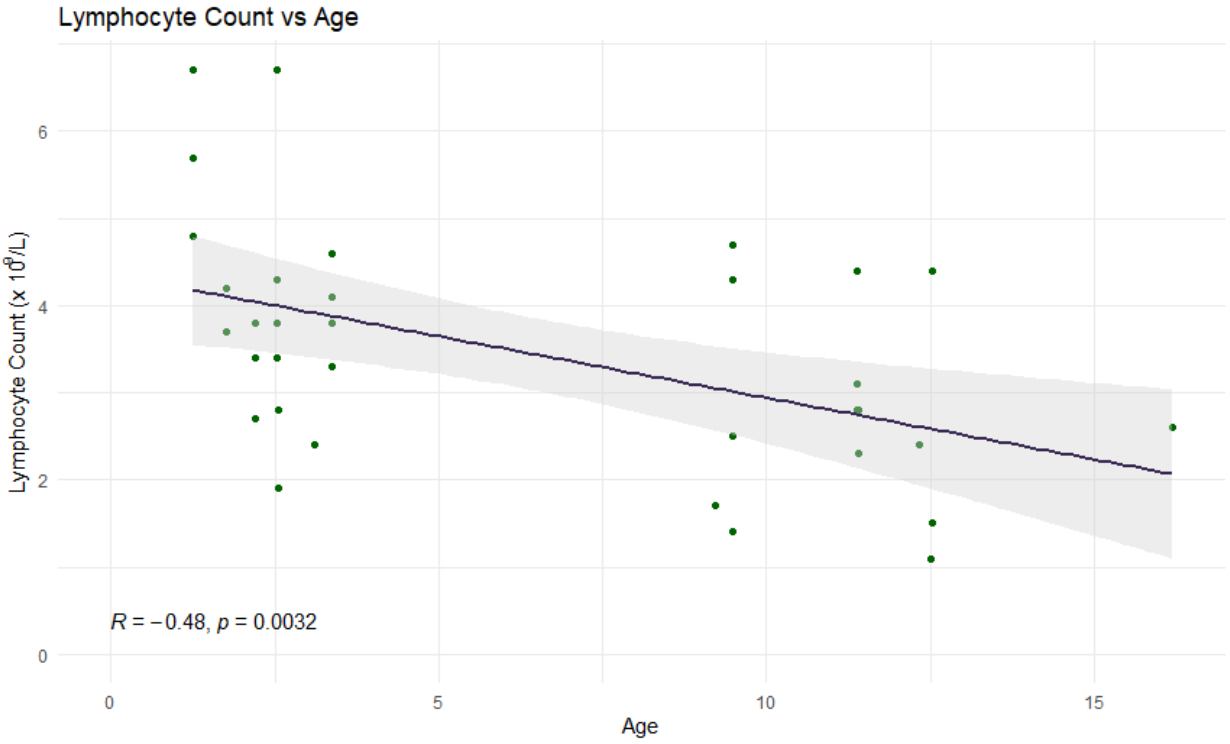

Figure S3.6. Lymphocyte count is negatively correlated with the senior age class (p = 0.006) and negatively correlated with continuous age (p = 0.003).

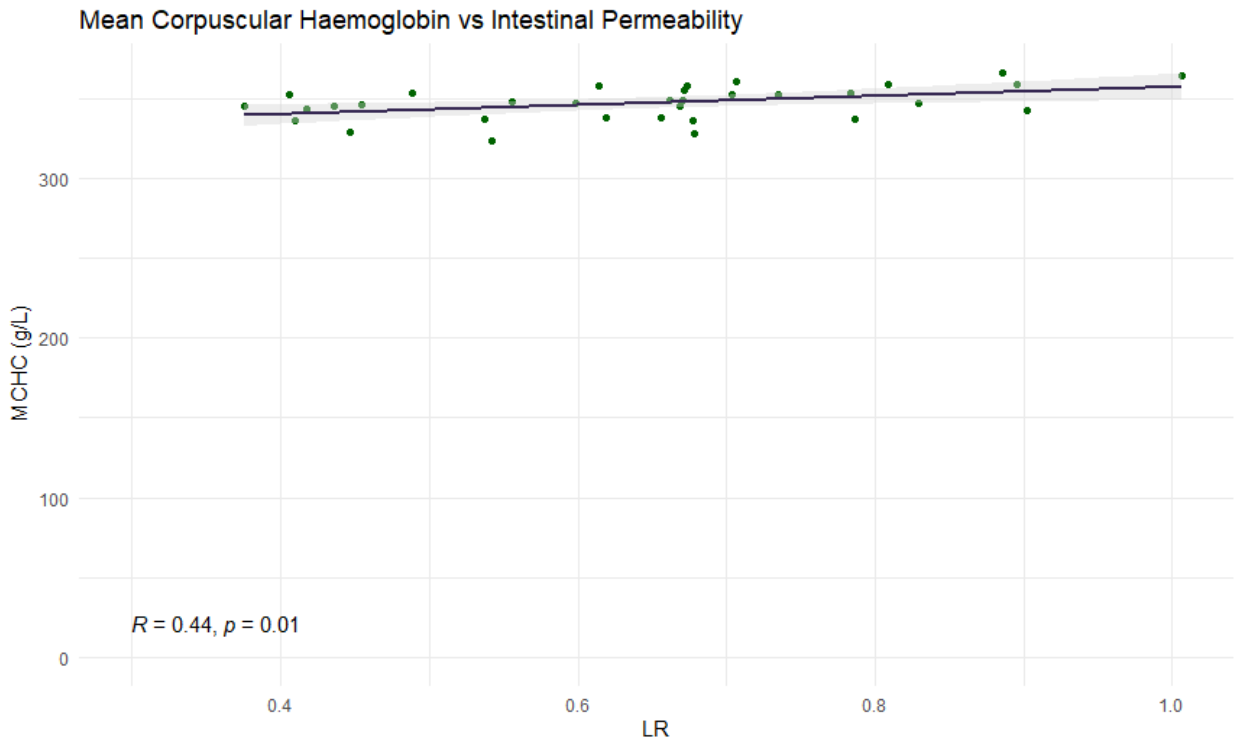

Figure S3.7. Mean corpuscular haemoglobin (MCHC) is positively correlated with intestinal permeability (LR) (p = 0.010)

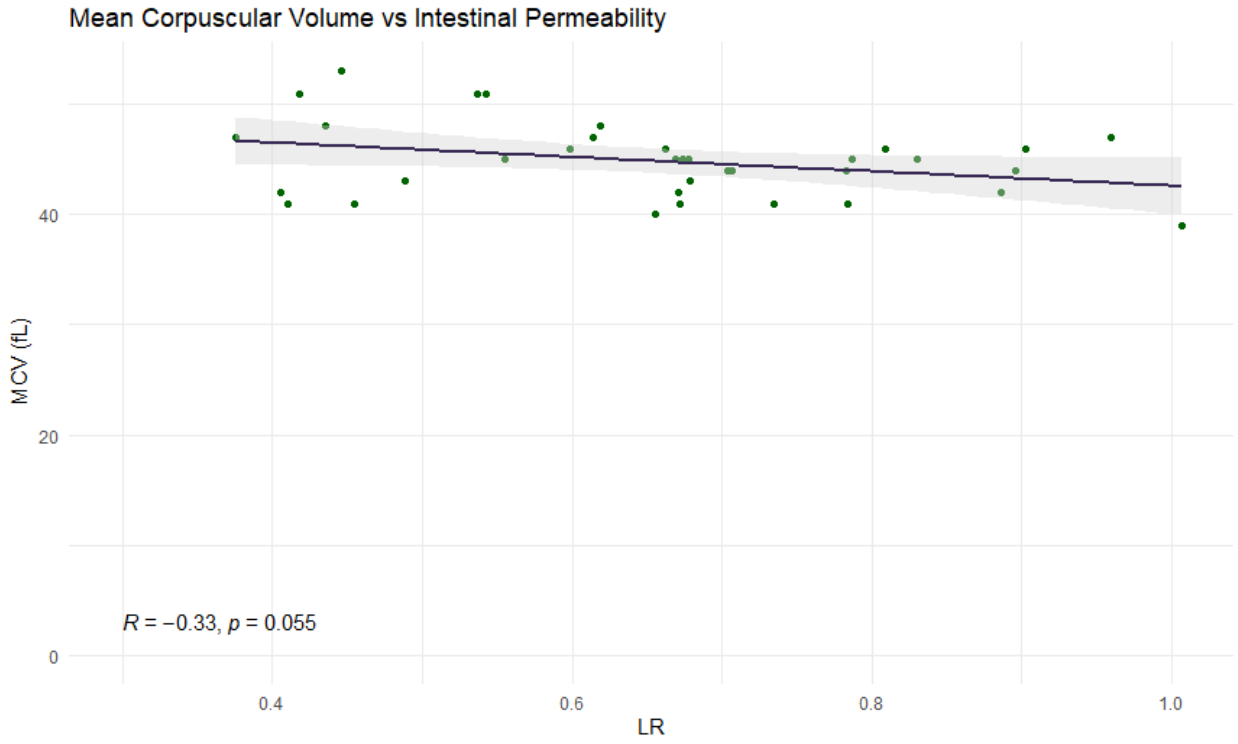

Figure S3.8. Mean corpuscular volume (MCV) is negatively correlated with intestinal permeability (LR) (p = 0.055)

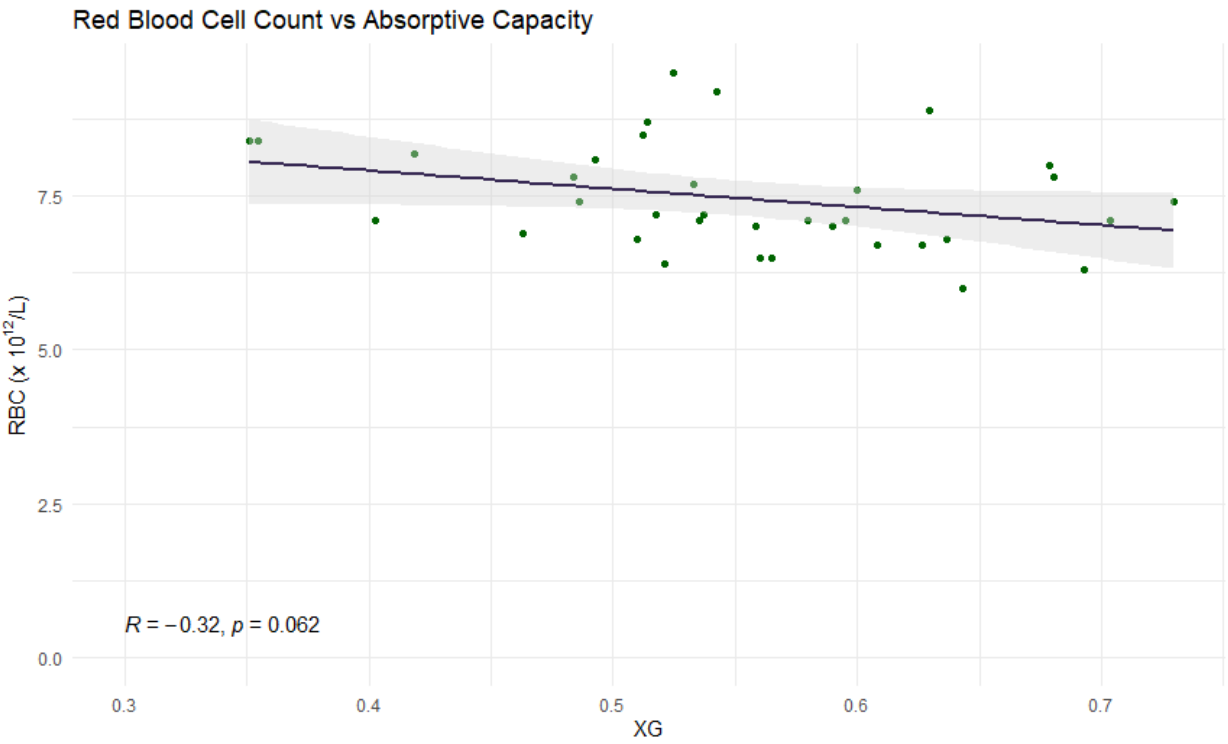

Figure S3.9. Red blood cell count (RBC) is negatively correlated with absorptive capacity (XG) (p = 0.062)

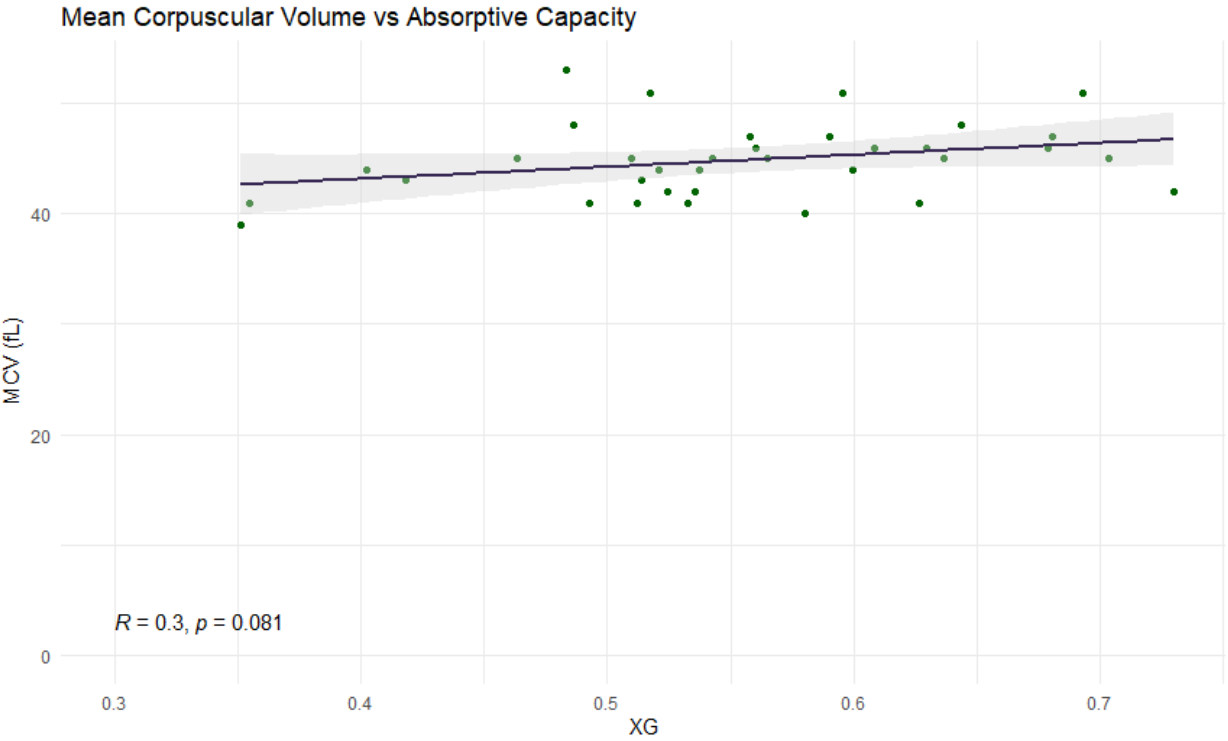

Figure S3.10. Mean corpuscular volume (MCV) is positively correlated with absorptive capacity (XG) (p = 0.081)