

Can diet composition affect behaviour in dogs?

Food for thought

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Abstract

The consumption of food goes beyond the basic provision of energy and essential nutrients for the maintenance of physical health. Studies in rats, pigs, and human subjects have shown that behaviour and mood can be influenced by specific nutrients consumed. The research described in this thesis aimed to evaluate the impact of dietary composition on two physiological systems involved in the regulation of canine behaviour. In other studies it has been shown that physical activity of pigs can be influenced by dietary fibre type, likely through sustaining satiety after a meal. It appears that the fermentable fibres can stimulate several mechanisms involved in sustaining satiety including the stimulation of the secretion of satiety-related metabolites by the gastrointestinal tract. Furthermore, hunger has been found to influence anxiety in rats. The current study evaluated the potential impact of dietary fibre types for effects on satiety and behaviour in dogs. Two *in vitro* fermentation studies were conducted to evaluate the microbial fermentation activity in the canine gastrointestinal tract and to screen the fermentability of various fibrous ingredients. Based on these *in vitro* fermentability data, two diets were formulated expected to differ in fibre fermentability *in vivo*. The difference in fibre fermentability between diets was confirmed in an *in vivo* study by evaluation of fibre degradation and concentrations of faecal short-chain fatty acids. In this latter study, the secretion of satiety-related hormones was found not to differ between treatment groups. Feeding dogs a high-fermentable fibre diet did result in a lower motivation to eat 6 hours after their morning meal and a lower activity in their home-kennel compared to dogs fed a low-fermentable fibre diet. Treatment groups did not differ in their responses to short-lasting challenges in a test arena conducted 5 to 7 hours after their morning meal. The second dietary strategy investigated was the use of the essential amino acid tryptophan, the precursor of the neurotransmitter serotonin in the brain. It has been shown that dietary tryptophan supplementation reduces anxiety in rats and increases resilience in dealing with stress in pigs. To investigate if similar effects would occur in dogs, a study was designed and conducted in mildly anxious privately-owned dogs fed diets differing in tryptophan content. Dogs were fed the study diet for 8 weeks using a randomised double-blinded, placebo-controlled design. Intake of the tryptophan supplemented diet increased plasma tryptophan concentrations by 37.4% and its ratio with large neutral amino acids by 31.2% compared to the control diet but the data reported by owners did not show a significant change in the behavioural of the dogs over time that could be attributed to the specific dietary treatment. More controlled behavioural tests conducted on a subset of dogs in both dietary treatment groups failed to show a significant difference of supplementation of the diet with tryptophan. In conclusion, the present work has shown that dietary fibre type can have an impact on canine behaviour through feeding motivation. The measured satiety-related metabolites were not affected by dietary fibre type indicating that other mechanisms were involved in sustaining satiety. Dietary supplementation of tryptophan had no effect on the behaviour of privately-owned dogs.

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Chapter 1

General introduction

Background

The consumption of food is known to have effects beyond the basic provision of energy and essential nutrients for maintaining physical health⁽¹⁾. Studies in rats, pigs, and human subjects showed that mood and behaviour can be influenced by the specific composition of the diet consumed.

The domestic dog (*Canis familiaris*) is believed to have evolved from the grey wolf (*Canis lupis*) as a separate species at least 15,000 years ago^(2,3). Like their wild ancestors, domestic dogs are considered to be carnivores in nature⁽⁴⁾. Unlike wolves, which consume predominantly meat-based diets⁽⁴⁾, companion dogs are most commonly fed dry extruded diets, nowadays consisting predominantly of grains and milling by-products and animal tissue by-products from the meat-packing, poultry-processing, and fish-canning industries⁽⁵⁾. The composition of such dry foods differs significantly from that of the dog's ancestor in terms of nutrients and their origin (animal- *vs.* plant-derived). Commercial dry dog foods may contain, for example, up to 50% carbohydrates (starch)⁽⁶⁾ whereas the consumed parts of prey animals are almost devoid of carbohydrates⁽⁷⁾. Although commercially available foods deviate in nutrient composition from ancestral canine diets, they are generally adequate in providing dogs with the nutrients required for maintenance, growth or reproduction. Commercial dog foods do vary in the content of essential nutrients and the presence or absence of certain non-essential dietary constituents, with possible effects on behaviour. For example, some of the essential dietary constituents present in canine foods are precursors for the synthesis of neurotransmitters in the brain and as such the dietary content of these components could have an impact on neurotransmission and, thereby, modulate behaviour. Furthermore, enrichment of diets with antioxidants and mitochondrial cofactors have been found to decrease the rate of age-related cognitive decline and associated behavioural changes in dogs^(8,9). Finally, the inclusion of soy-based ingredients in dog foods results in the presence of physiological active phytoestrogens⁽¹⁰⁾ known to influence anxiety-related behaviour in rats^(11,12) and impair social behaviour in monkeys⁽¹³⁾. Another reason to study the relation between nutrition and behaviour in dogs is that current nutritional guidelines used for formulating commercial dog foods are based on tests that do not take into account the dog's behaviour as a response criterion.

Objective and outline of the thesis

The main objective of the research described in this thesis is to evaluate the nutritional impact on canine behaviour. Firstly, the scientific literature is reviewed to investigate the current knowledge on the influence of dietary macronutrient composition on the behaviour of dogs and to explore potential useful dietary strategies reported in other mammals (Chapter 2). Two food strategies that are known to modify behaviour in at least some species are selected for further study in dogs. The first is the influence of the fermentability of dietary fibre on behaviour, likely through its effects on the duration of satiety after a meal. Two *in vitro* fermentation studies are conducted to evaluate the microbial fermentation activity in the

canine gastrointestinal tract and to screen the fermentability of various fibrous ingredients (Chapter 3). Based on the *in vitro* fermentability, fibrous ingredients are selected for further *in vivo* study. Two diets differing in the fermentability of the included fibrous ingredients are tested for satiating properties and behavioural effects in dogs, under standard laboratory conditions. Thus, it is investigated if differences in diet fermentability affect the secretion of satiety-related hormones, and the motivation of dogs to eat several hours after their morning study meals (Chapter 4). Behavioural effects of the different types of dietary fibre are studied by means of observing the behaviour of dogs in their home-kennel and their responses to short-lasting challenges in a test arena (Chapter 5). The second dietary strategy that is investigated involves supplementation of the essential amino acid tryptophan. Tryptophan is the dietary precursor of the neurotransmitter serotonin, which influences various parts of the brain involved in controlling emotional states and behaviour. A randomised double-blinded, placebo-controlled study is performed to evaluate the influence of dietary tryptophan supplementation on behaviour of mildly anxious privately-owned dogs (Chapter 6). Owners feed their dogs the diets and evaluate the dogs' behaviour in the home situation by means of filling in a questionnaire. A selection of dogs from the study population is observed for their responses during behavioural tests. In the final chapter of this thesis (Chapter 7), the most important findings are summarised and its practical relevance is discussed. Also, suggestions for further research are outlined and the influences and consequences of current feeding practices on behaviour in dogs are considered.

Hypotheses

The main hypotheses in this thesis are:

1. The *in vitro* microbial degradation of dietary fibres using faecal inoculum can be used for the screening of fermentation characteristics (kinetics and products) of fibrous ingredients (Chapter 3).
2. Microbial fibre degradation of dietary fibre along the canine gastrointestinal tract stimulates postprandial secretion of peptide tyrosine tyrosine (PYY) and glucagon-like-peptide-1 (GLP-1) and decrease the secretion of ghrelin in dogs (Chapter 4).
3. Inclusion of fermentable fibre in a diet lowers feeding motivation in dogs compared to that in dogs fed a low-fermentable fibre-diet (Chapter 4).
4. Dogs fed a diet containing high-fermentable fibre are less active in their home-kennel than dogs fed a low-fermentable fibre source (Chapter 5).
5. Anxiety-related behavioural responses to short-lasting challenges in a test arena are lower in dogs fed a diet containing high-fermentable fibre compared to dogs fed a low-fermentable fibre diet (Chapter 5).
6. Dogs fed a diet supplemented with tryptophan show less anxiety-related behaviour during everyday life (Chapter 6).
7. Anxiety-related behavioural responses during behavioural tests are lower in dogs fed a diet supplemented with tryptophan compared to dogs fed a control diet (Chapter 6).

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Chapter 2

Impact of nutrition on canine behaviour: current status and possible mechanisms

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Abstract | Each year, millions of dogs worldwide are abandoned by their owners, relinquished to animal shelters, and euthanized because of behaviour problems. Nutrition is rarely considered as one of the possible contributing factors of problem behaviour. This contribution presents an overview of current knowledge on the influence of nutrition on canine behaviour and explores the underlying mechanisms by which diet may affect behaviour in animals. Behaviour is regulated by neurotransmitters and hormones, and changes in the availability of their precursors may influence behaviour. Tryptophan, the precursor of serotonin, may affect the incidence of aggression, self-mutilation and stress resistance. The latter may also be influenced by dietary tyrosine, a precursor to catecholamines. As diet composition, nutrient availability and nutrient interactions affect the availability of these precursors in the brain, behaviour or stress resistance may be affected. PUFA, especially DHA, have an important role as structural constituents in brain development, and dietary supply of n-3 and n-6 PUFA could modify aspects of the dopaminergic and serotonergic system and, consequently, cognitive performance and behaviour. Finally, persistent feeding motivation between meals can increase stereotyped behaviour and aggression and decrease resting time. This feeding motivation may be altered by dietary fibre content and source. At present, few studies have been conducted to evaluate the role of nutrition in canine (problem) behaviour through the above mentioned mechanisms. Studies that explore this relationship may help to improve the welfare of dogs and their owners.

Introduction

The domestic dog (*Canis familiaris*) is believed to have evolved from the grey wolf (*Canis lupis*) as a separate species at least 15,000 years ago and it is thought to be the first animal species to be domesticated by humans^(1,2). At the present time, as a result of selective breeding, approximately 400 distinct dog breeds are recognised worldwide, representing a large variation in body size and weight, with the latter ranging from 1 to 90 kg. Initial functions of dogs such as hunting, shepherding and guarding have diminished gradually in importance in favour of the dog's role as a companion to humans⁽³⁾. Though most human–dog relationships are fulfilling, each year a large number of animals are abandoned by their owners or relinquished to animal shelters⁽⁴⁾. Aggression toward people and animals, running away, destructive behaviour, disobedience, house soiling and excessive barking are unwanted behaviours that make owners relinquish or abandon their dogs⁽⁵⁾. Although only 20% of the dogs in the US shelters are assigned by their owners for euthanasia⁽⁶⁾, a further 40% of dogs admitted are euthanized⁽⁷⁾. Of the sheltered dogs that are purchased by new owners, approximately 20% are returned to shelters^(6,7) and a large proportion of these animals are euthanized⁽⁴⁾. The number of dogs and cats euthanized annually in the USA is estimated to be between 5 and 17 million^(8,9), with 3–6 million as a result of behaviour problems⁽¹⁰⁾. Strategies that combat problem behaviours in dogs will greatly benefit animal welfare. The behaviour of individual dogs is controlled by numerous factors and from studies in humans it can be derived that nutrition plays a role also. For example, diets rich in vitamins and minerals may decrease anti-social behaviour in schoolchildren⁽¹¹⁾ and supplementation of vitamins, minerals and essential fatty acids decreased anti-social behaviour, including violence, of young adult prisoners⁽¹²⁾. Dietary effects on behaviour have been investigated for anti-social aspects, but also for behavioural changes related to ageing and, in this, dogs have been used as a model for humans. Dogs develop similar cognitive deficits and neuropathology as can be seen in ageing humans and elderly suffering from dementia⁽¹³⁾. Milgram and co-workers initiated a series of experiments with young and aged beagle dogs to study dietary interventions on age-related cognitive decline. Results showed that canine food enriched with antioxidants and mitochondrial cofactors decreased the rate of cognitive decline in aged beagle dogs under laboratory conditions and improved age-related behavioural changes in older pet dogs held in home situations (for reviews, see Roudebush *et al.*⁽¹⁴⁾ and Zicker⁽¹⁵⁾). These findings demonstrate clearly that canine behaviour can be influenced by dietary components.

The present review presents an overview of our current knowledge on the influence of dietary macronutrient composition on the behaviour of dogs and explores the underlying mechanisms by which diet may affect behaviour. Findings from food–behaviour studies in dogs and other mammals are integrated to assess in what way problem behaviour in dogs may be reduced through dietary means.

Effects of dietary amino acids and protein content on behaviour

After ingestion, proteins are enzymically degraded and absorbed in the small intestine mainly as tripeptides, dipeptides and free amino acids. After hydrolysis of the peptides in the enterocytes, the free amino acids are transported through the portal vein to the liver. Amino acids are important constituents required for the synthesis of enzymes and other proteins, and used as precursors for the synthesis of neurotransmitters and hormones⁽¹⁶⁾. For example, serotonin, catecholamines, acetylcholine and histamine are metabolites from tryptophan, tyrosine, choline and histidine, respectively⁽¹⁷⁾. These neurotransmitter precursors (except for choline) are amino acids and are natural dietary constituents. Behaviour results from signal detection, transmission and processing in the (central) nervous system, which is accomplished and modulated by chemical messengers such as neurotransmitters and hormones. Changes in neurotransmitter precursors such as tryptophan and tyrosine are, therefore, likely to influence behaviour. The amount and timing of food intake, diet composition and digestibility are all factors that determine the availability of different amino acids, i.e. precursors of chemical messengers. Consequently, such factors may influence behaviour. The effects of tryptophan and tyrosine on behaviour will be discussed as these could be relatively potent modulators; for similar reports on choline, histidine and threonine, we refer to Young⁽¹⁷⁾.

Findings and mechanisms in different mammals

Tryptophan

A diet high in tryptophan has been shown to reduce mouse killing by rats^(18,19), reduce aggression in vervet monkeys⁽²⁰⁾, enhance exploratory behaviour in female silver foxes⁽²¹⁾ and reduce self-injurious behaviour in rhesus monkeys⁽²²⁾. In contrast to the observed reductions in aggression in some experimental conditions, dietary supplementation of tryptophan has also been shown to increase territorial aggression in male mice⁽²³⁾. Dietary tryptophan may also influence the resistance or tolerance to stress and, therefore, change the behavioural stress response. Koopmans *et al.*⁽²⁴⁾ reported enhanced recovery after social stress as measured by lower plasma cortisol and noradrenaline concentrations in pigs fed a surplus of dietary tryptophan compared with pigs fed diets containing a 'normal' concentration of tryptophan. In addition, supplementation of dietary tryptophan reduced plasma cortisol concentrations during a stress-inducing mental arithmetic task in healthy stress-vulnerable humans⁽²⁵⁾. It was, therefore, suggested by Markus *et al.*⁽²⁵⁾ that tryptophan supplementation above normal dietary concentrations could improve the ability of an individual to cope with stress. The effects of dietary tryptophan on stress resistance involve different pathways. In rats a variety of stressors, such as immobilisation, foot shock, and hypothermia, increase brain tryptophan and serotonin turnover⁽²⁶⁻²⁹⁾. Depressed humans show decreased plasma tryptophan concentrations in comparison with normal subjects⁽³⁰⁾. It appears that initially stressors stimulate serotonin turnover, which over time may deplete serotonin (precursor) supplies and result in decreased serotonin (precursor) concentrations.

Quantitatively the most important pathway for tryptophan metabolism, after protein synthesis, is the kynurenine pathway which is responsible for over 90% of tryptophan catabolism⁽³¹⁾. In humans, normally 1% of the available tryptophan is converted to serotonin which is mainly present in the gastrointestinal tract⁽³²⁾. The first and rate-limiting step in the synthesis of serotonin is the hydroxylation of tryptophan to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase (Figure 1). Tryptophan hydroxylase is normally about half saturated with tryptophan⁽³³⁾. Consequently, an increase in tryptophan in the brain, which increases serotonin synthesis and serotonergic neurotransmission⁽³⁴⁾, can maximally double serotonin synthesis. The second step in the synthesis of serotonin is the decarboxylation of 5-hydroxytryptophan to serotonin which is stored in vesicles in the nerve terminal where it is held before release. When serotonin is released into the synaptic cleft, serotonin can bind to different subtype receptors (for reviews, see Barnes & Sharp⁽³⁵⁾ and Hoyer *et al.*⁽³⁶⁾). Via binding to these different receptors, serotonin can produce many different effects on post-synaptic cells influencing various parts of the brain involved in controlling a variety of physiological functions including hormone releases, cardiovascular functioning, pain, appetite, and in general mood and behaviour⁽³⁵⁻³⁷⁾.

Tryptophan transport across the blood–brain barrier and metabolism is in part affected by animal factors such as breed⁽³⁸⁾, sex^(21,39), social status^(40,41), age^(39,42), activity⁽⁴³⁾ and level of arousal⁽⁴⁴⁾. The availability of dietary tryptophan to the brain is largely dependent on the composition of the ingested diet. Tryptophan is found in nearly all protein-containing foods where it is found in a lower concentration compared with the other large neutral amino acids (LNAA) tyrosine, phenylalanine, leucine, isoleucine and valine⁽⁴⁵⁾. For access into the brain, tryptophan shares the same carrier as other LNAA for transport across the blood–brain barrier⁽³⁴⁾. Central tryptophan concentrations can either be increased by increasing plasma tryptophan or by lowering plasma concentrations of LNAA^(34,46). As tryptophan is normally present in only small concentrations in dietary protein compared with other LNAA, the consumption of a meal high in protein will decrease the ratio of tryptophan to other LNAA⁽⁴⁷⁾ and thereby potentially lower serotonin synthesis.

The fraction of unbound tryptophan as compared with that bound to albumin is another factor that may influence tryptophan availability to the brain⁽⁴⁸⁾. In mammals, approximately 80–90% of all tryptophan molecules in the blood are bound to serum albumin⁽⁴⁹⁾. It has been suggested that the majority of the albumin-bound tryptophan is available for passage across the blood–brain barrier^(46,50), but possibly the concentration of circulating free tryptophan may be especially important⁽⁴⁸⁾. According to Chaouloff⁽⁴⁸⁾, three factors affect circulating free and bound tryptophan concentrations: (i) the rate of lipolysis because blood non-esterified fatty acids displace tryptophan from its binding to albumin⁽⁵¹⁾; (ii) the activity of tryptophan 2,3-dioxygenase, the rate-limiting enzyme in tryptophan detoxication through the kynurenine pathway – activation (inactivation) of this enzyme decreases (increases) circulating blood tryptophan levels⁽⁵²⁾; (iii) uptake into peripheral and

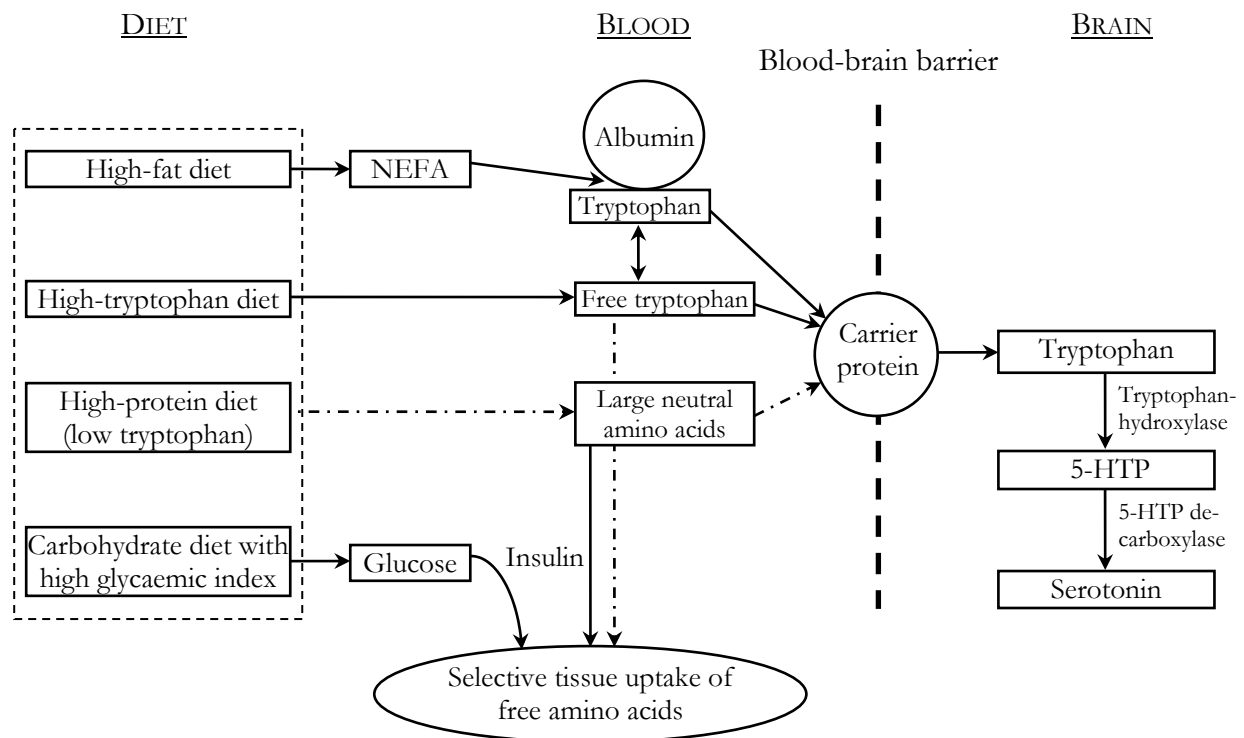


Figure 1 | Effects of dietary characteristics on tryptophan uptake by the central nervous system and synthesis of serotonin from brain tryptophan (adapted from Grimmett & Sillence⁽⁵³⁾ with modifications). (---), Factors that may ultimately decrease brain tryptophan; 5-HTP, 5-hydroxytryptophan; NEFA, non-esterified fatty acids.

central tissues. Carbohydrate-induced insulin rises facilitate the uptake of most LNAA into skeletal muscle, but not tryptophan bound to albumin^(54,55). Consequently, the ratio of tryptophan relative to LNAA increases. This results in a competitive advantage of tryptophan over LNAA for uptake at the blood–brain barrier. However, as little as 2–4% of the energy of a meal as protein seems to prevent this increased availability of tryptophan^(31,56).

Tyrosine

In rats, a high-tyrosine diet prevents adverse behavioural and neurochemical effects (for example, immobility during a swim test, depletion of brain noradrenaline) of various acute stressors including hypothermia⁽⁵⁷⁾, restraint and tail-shock⁽⁵⁸⁻⁶⁰⁾. Human studies also suggest beneficial effects of tyrosine under conditions of stress (for reviews, see Lieberman⁽⁶¹⁾ and Young⁽¹⁷⁾).

Tyrosine, which can be synthesised from phenylalanine, is the direct precursor for the catecholamines dopamine, noradrenaline and adrenaline⁽³²⁾. Dopamine can be synthesised from tyrosine in neurons in two steps. The first and rate-limiting step is the conversion of tyrosine to dihydroxyphenylalanine by the enzyme tyrosine hydroxylase. In rats, central tyrosine hydroxylase is approximately 75% saturated with tyrosine⁽³³⁾. In the second step,

dihydroxyphenylalanine is decarboxylated to dopamine which can be used as an end-product (neurotransmitter) in neurons or further converted to noradrenaline or adrenaline⁽⁶²⁾. Like tryptophan, tyrosine competes with other LNAA at the blood–brain barrier for entry into the brain⁽³⁴⁾ and is taken up into skeletal muscle under the influence of insulin^(54,55). In diets, tyrosine is typically available in much higher concentrations compared with tryptophan and high-protein meals will typically raise tyrosine concentrations in the brain, but will lower the concentration of tryptophan⁽⁶³⁾. Catecholamines play a key role in a variety of behavioural, neuroendocrine and cardiovascular responses during stress⁽⁶¹⁾. Increases in brain tyrosine have little or no effect on catecholamine synthesis⁽¹⁷⁾, but the situation may be different during stress when brain noradrenaline turnover increases and noradrenaline concentrations decrease^(58,64). An enhanced noradrenergic activity is part of a normal adaptive stress response⁽⁶⁵⁾. In stressed rats (tail-shock), ingestion of a high-tyrosine diet reversed the post-stress decline in brain noradrenaline and attenuated behaviour changes, i.e. decreased locomotion, standing on hind legs, hole-poking in a novel open field⁽⁵⁸⁾. This suggests that a high-tyrosine diet may be beneficial during severe stress, as it prevents depletion of the substrate required for catecholamine synthesis in times of high catecholaminergic activity and demand.

Findings in dogs

Studies on the effects of tryptophan or tyrosine on behaviour in dogs seem to be limited to one. DeNapoli *et al.*⁽⁶⁶⁾ formulated diets with high or low protein content (approximately 310 or 190 g crude protein/kg, respectively) and with or without tryptophan supplementation (1.45 g/kg) in order to provide varying tryptophan contents and tryptophan:LNAA ratios (Table 1). Each of the four diets was fed in random order for 1 week to 33 privately owned dogs that displayed a high territorial aggression, dominance aggression or hyperactivity. There was no effect of dietary protein or tryptophan content on the behavioural scores within each group of problem behaviour. However, when the groups of dogs were analysed as one study population a lower territorial aggression score was obtained for dogs fed the high-tryptophan diet compared with dogs fed the low-tryptophan diet, but only when fed a low-protein diet. In addition, dogs fed the high-protein diet without tryptophan supplementation showed a higher dominance aggression score compared with dogs on the other dietary treatments.

Three studies in literature have reported that low-protein diets decreased aggression in dogs, though these were not performed under controlled experimental conditions. In a study with seven aggressive golden retrievers held at in-home living conditions, incidences of aggression as reported by their owners immediately decreased after the introduction of a low-protein diet (15–18% of total energy)⁽⁶⁷⁾. Unfortunately, neither the composition of the experimental diet nor the composition(s) of the diet(s) before the dietary intervention were reported. The reduction in aggressive incidences, however, was only sustained in three dogs; two dogs deteriorated again in their behaviour and contact was lost with the remaining two

Table 1 | Effect of dietary protein and tryptophan (Trp) content on canine behaviour.

Authors	Dogs and design	Diets ¹	Results
Mugford ⁽⁶⁷⁾	Seven aggressive golden retrievers at in-home living situations. Measurements were reported.	15–18% protein of total dietary energy based on approximately 20% meat and 80% boiled rice	Seven dogs improved of which three sustained the improvement, two worsened, and contact was lost with two clients.
Dodman <i>et al.</i> ⁽⁶⁸⁾	Twelve territorial aggressive, dominance aggressive, fourteen control dogs (age > 1 year) diet (Latin square) at in-home living situations for 14 d. Each dog, their dogs for dominance aggression, fearfulness.	twelve aggressive, twelve hyperactive and fed each living in-home owners scored for territorial aggression, excitability and fearfulness. 1) 180 g protein/kg; 0.024:1 Trp:LNAA 2) 250 g protein/kg; 0.024:1 Trp:LNAA 3) 310 g protein/kg; 0.021:1 Trp:LNAA	a) Territorial aggressive dogs showed lower territorial aggression scores when fed diets 1 and 2 compared with diet 3. b) Seven territorial aggressive dogs were fearful and showed lower territorial aggression scores when fed diets 1 and 2 compared with diet 3; the remaining five territorial aggressive dogs tended to be dominant which was not affected by dietary treatment. c) No changes in behaviour scores of dogs within the dominance aggressive, hyperactive and control groups.
DeNapoli <i>et al.</i> ⁽⁶⁶⁾	Eleven territorial aggressive and dominance aggressive dogs (age > 1 year) at maintenance level for 7 d. Each dog, their dogs for dominance aggression, fearfulness and hyperactivity.	eleven aggressive, eleven hyperactive and fed each diet (at random) living in-home owners scored for territorial aggression, excitability, and hyperactivity. 1) 186 g protein/kg; 0.044:1 Trp:LNAA 2) 188 g protein/kg; 0.073:1 Trp:LNAA 3) 308 g protein/kg; 0.035:1 Trp:LNAA 4) 315 g protein/kg; 0.054:1 Trp:LNAA	a) No changes in behaviour within each behaviour group for any dietary treatment. b) When all dogs were combined, dominance aggression scores were higher for dogs fed diet 3 compared with dogs fed diets 1, 2 and 4. c) When all dogs were combined, territorial aggression scores were higher for dogs fed diet 1 compared with dogs fed diet 2.

LNAA, large neutral amino acids (tyrosine, phenylalanine, leucine, isoleucine, valine).

¹Values presented on DM basis.

clients. In another study, twelve dogs that exhibited either high territorial aggression, dominance aggression or hyperactivity and fourteen control dogs were fed each of three diets varying in protein content (180, 250 and 310 g crude protein/kg DM) for 2 weeks at in-home living situations⁽⁶⁹⁾. The low-protein diet and medium-protein diet decreased territorial aggression scores compared with the high-protein diet. No effects of dietary protein content in dogs with dominance aggression or hyperactivity were found. Additional behavioural analysis of the group of dogs demonstrating territorial aggression revealed that five of these dogs showed dominance-related territorial aggression, whereas the other seven dogs showed fear-related territorial aggression. In the latter dogs, territorial aggression decreased when fed the low-protein diet.

For adult dogs fed at maintenance, the minimal dietary tryptophan requirements are currently set at 0.0669 g/1000 kJ (0.28 g/1000 kcal) metabolisable energy (ME) with a tryptophan:LNAA ratio of 0.061:1 and for tyrosine and phenylalanine the minimal dietary requirements are 0.3537 g/1000 kJ (1.48 g/1000 kcal) ME⁽⁷⁰⁾. The Association of American Feed Control Officials (AAFCO)⁽⁷¹⁾ has minimum dietary requirements for these nutrients which are slightly higher (0.1099 and 0.4995 g/ 1000 kJ (0.46 and 2.09 g/1000 kcal) ME, respectively) in order to account for the lower digestibility and availability of nutrients in commercial canine foods compared with semi-synthetic diets. Nutritional guidelines for humans⁽¹²⁾ and dogs rarely take behaviour into account as a response criterion, something which has been criticised^(72,73). The minimum quantity of tryptophan in a commercial canine dry expanded diet that has passed a maintenance AAFCO feeding protocol has been reported to be 0.0502 g/1000 kJ (0.21 g/1000 kcal) ME⁽⁷⁰⁾. The criteria for passing an AAFCO maintenance feeding protocol however, do not take into account animal behaviour. It is unknown if the minimal amount of tryptophan in typical dog foods meets the requirements of the wide variety of dogs, for example, from emotionally stable to anxious individuals, under different conditions, for example, from stress-free to stressful. Both excessive intake and a deficiency of tryptophan are detrimental to the health of an animal⁽³¹⁾ and are likely to affect behaviour. In horses, a dose of 0.1 mg/kg body weight appears to be too low, causing mild excitation⁽³⁸⁾. In humans, the most common side effect of overfeeding precursors of neurotransmitters has been reported to be nausea⁽¹⁷⁾. There are currently no requirement estimates for the maximum amount of tryptophan in canine food and it remains to be determined how high-tryptophan diets affect the health of dogs and their behaviour in the long term.

Effects of dietary lipids on behaviour

Lipids have various functions, such as constituents of cellular membranes, precursors for chemical messengers (for example, steroid hormones) and their use as an energy source or stored in the body as adipose tissue. After adipose tissue, the central nervous system has the greatest concentration of lipids⁽⁷⁴⁾. The structural constituents in the grey matter of the

brain and retinal tissues in mammals are derived from dietary linoleic acid (18:2n-6) and α -linolenic acid (18:3n-3). Both are polyunsaturated fatty acids (PUFA) and can be metabolised to long-chain PUFA by sequential alternating enzymic desaturation and elongation. Linoleic acid can be metabolised to arachidonic acid (20:4n-6) which can be further metabolised to docosapentaenoic acid (22:5n-6). The enzymic desaturation and elongation of α -linolenic acid yields eicosapentaenoic acid (EPA) (20:5n-3) which can be further metabolised to docosahexaenoic acid (DHA) (22:6n-3)⁽⁷⁵⁾.

Findings and mechanisms in different mammals

There is ample scientific literature available in which the effects of both dietary deficiency and supplementation of PUFA on animal performance in cognitive or behavioural tests are evaluated (for reviews, see Wainwright⁽⁷⁶⁾ and McCann & Ames⁽⁷⁷⁾). For example, the learning ability of rodents decreased when fed n-3 fatty-acid-deficient diets^(78,79) and increased when fed DHA-supplemented diets⁽⁸⁰⁾ compared with rodents fed diets adequate in n-3 fatty acid concentrations. Other studies, however, did not find effects of dietary n-3 PUFA manipulation on learning performance as tested with a Morris water-maze in rats⁽⁸¹⁾ or mice⁽⁸²⁾. Dietary PUFA seem to affect animal cognition but can also cause behavioural changes. Rats fed n-3 PUFA-deficient diets showed increased aggression scores in a resident intruder test⁽⁸³⁾ and increased expression of stress-related behaviours during several stress tests⁽⁸⁴⁾ compared with male rats fed adequate amounts of n-3 PUFA. Similarly, anxiety was found to be increased in mice fed a diet deficient in n-3 PUFA⁽⁸⁵⁾, though others did not observe any effects of dietary PUFA on anxiety in mice⁽⁸⁶⁾ or rats⁽⁸⁷⁾. The dopaminergic and serotonergic systems in the brain are known to play important roles in learning, emotions, and impulse control^(37,88-92), which makes it tempting to assume that the effects of PUFA on behaviour run through these systems. Indeed, both systems are known to be influenced by PUFA. Rats deficient in n-3 PUFA compared with rats fed diets with α -linolenic acid showed a reduction in dopamine concentration in the frontal cortex⁽⁹³⁻⁹⁶⁾ and an increase in dopamine concentration in the nucleus accumbens⁽⁹⁵⁾ but no effects in the striatum^(93,94). In the frontal cortex of these animals the rate of dopamine synthesis and breakdown mediated by monoamine oxidase was not affected^(94,96) and the reduced concentrations may have been linked to the reduced dopaminergic storage pools^(96,97). Changes in dopamine concentrations were followed by changes in number of D2 receptors⁽⁹⁶⁾. n-3 PUFA-deficient rats had a lower number of D2 receptors in the frontal cortex^(93,94,96) but higher in the nucleus accumbens^(95,96). Rats fed diets supplemented with EPA and DHA had an increased dopamine concentration and D2 binding possibly as a result of a reduction in monoamine oxidase activity in the frontal cortex compared with rats fed adequate amounts of PUFA⁽⁸⁷⁾.

As for dopamine concentrations, frontal cortex serotonin concentrations were increased in rats fed diets supplemented with n-3 PUFA⁽⁸⁷⁾. In line with this, serotonin in the frontal cortex was reduced in piglets fed n-3 and n-6 PUFA-deficient formula for 18 d from birth compared with piglets fed formula supplemented with linoleic acid and α -linolenic acid

and/or arachidonic acid and DHA⁽⁹⁸⁾. The findings in the frontal cortex may not extrapolate to other brain areas. For example, in the hippocampus of 2-month-old rats fed an n-3 PUFA-deficient diet extracellular basal serotonin concentrations were increased⁽⁹⁹⁾. This was probably due to reduced storage pools⁽⁹⁹⁾, not due to decreased activity of monoamine oxidase⁽¹⁰⁰⁾. Such effects of n-3 PUFA deficiency on serotonin concentrations are not found in all studies (for example, Delion *et al.*^(93,94)).

In addition to the observed changes in the dopaminergic and serotonergic systems in different brain regions, physical properties (for example, fluidity, permeability) of cerebral membranes may also mediate dietary effects on cognition and behaviour⁽¹⁰¹⁾. For example, chronic dietary deficiency in n-3 PUFA resulted in low concentrations of n-3 PUFA in the rat brain^(78,79) whereas diets high in EPA and DHA resulted in high concentrations of EPA and DHA in the brain of rats^(87,102,103). In addition, dietary α -linolenic acid deficiency induces a more pronounced reduction in DHA concentrations in the frontal cortex than in the striatum and cerebellum^(74,93). Besides changes in brain PUFA compositions, dietary PUFA may alter properties of the neuronal membrane, such as the activity of membrane-bound enzymes, receptors and ion channels⁽¹⁰⁴⁾. These alterations may affect neurological functioning and may, therefore, also contribute to the observed changes in cognitive functioning and behaviour.

Findings in dogs

To the authors' knowledge, there are at this moment no scientific articles available regarding the influence of n-3 or n-6 PUFA deficiency or enrichment on canine behaviour or cognitive performance. Since DHA is essential for the development and function of the brain and retina⁽⁷⁵⁾, its supply may affect neurological development in puppies. For example, low dietary concentrations of DHA during the gestation or lactation of bitches and dry diets for puppies depressed their retinal sensitivity^(105,106). Although the immediate connection between the cellular effects of DHA and visual sharpness and cognitive abilities in receiving dietary DHA still needs more support⁽⁷⁵⁾, studies seem to emphasise the importance of DHA in the diet of bitches during gestation until weaning and the diet of puppies in order to ensure optimal neurological development. At present, there is no recommended allowance for DHA for both bitches in gestation and lactation or puppies, but the recommended allowance for α -linoleic acid is 3.35 g/ 1000 kJ (0.8 g/1000 kcal) ME⁽⁷⁰⁾. A diet high in α -linolenic acid fed from breeding throughout lactation increased α -linolenic acid concentration in milk but failed to do this for DHA^(107,108). In a recent study, puppies converted α -linolenic acid to DHA during the first month of weaning but little conversion of α -linolenic acid to DHA occurs after weaning⁽¹⁰⁸⁾. It seems that the capacity of puppies to synthesise DHA from dietary α -linolenic acid or other n-3 fatty acid precursors is active for only a short time during the neonatal period and is decreased thereafter. The amount of dietary α -linolenic acid for sufficient synthesis of DHA and the amount of DHA required for optimal neurological development in puppies still remain to be determined. Whether the provision of sufficient

DHA for optimal neurological development in dogs also results in changes in the dopaminergic and serotonergic systems and subsequent effects in cognitive abilities or behaviour in later life remains to be confirmed.

Concerning commercial dog food, it seems likely that in dogs deficiencies of PUFA are rare as long as fat oxidation during process and storage of the food is limited⁽¹⁰⁹⁾. Levels of PUFA, particularly the n-3 family, are nowadays higher in commercial dog food compared to foods of several years ago⁽¹¹⁰⁾. However, the amount and ratio between n-6 and n-3 fatty acids may differ considerably between commercially available diets. The n-6:n-3 fatty acid ratio of twelve commercial dry dog foods was found to differ considerably, ranging from 17:1 to 5:1⁽¹¹¹⁾.

Effects of dietary carbohydrates on behaviour

Feeding of mammals is a discontinuous process in which periods of food consumption are interspersed with periods of non-eating⁽¹¹²⁾. Food intake behaviours are controlled by feelings of hunger⁽¹¹³⁾ and satiety⁽¹¹²⁾, but may be modulated by psychological and social factors⁽¹¹⁴⁾. Numerous central and peripheral signal molecules are involved in the regulation of eating (for reviews, see Bray⁽¹¹⁵⁾, de Graaf *et al.*⁽¹¹⁶⁾ and Strader & Woods⁽¹¹⁷⁾). The rate and site of degradation of nutrients largely determines the postprandial physiological state of an animal and in this way the extent and duration of satiety and, therefore, behaviour. There is a wide variety of carbohydrates with different physical and chemical properties. These properties can affect the rate and site of degradation of these carbohydrates⁽¹¹⁸⁾. In single-stomached animals, degradable carbohydrates may be digested with endogenous enzymes in the first part of the gastrointestinal tract, or fermented by micro-organisms that colonise predominantly the last part of the gastrointestinal tract. Products derived from digestible carbohydrates are mainly monosaccharides. The digestion of starch and absorption of monosaccharides are primarily responsible for the fluctuations in the postprandial blood glucose concentrations that subsequently may modify tryptophan availability in the brain when protein intake is low (see section on Findings and mechanisms in different mammals: Tryptophan), and influence mood in at least humans (for a review, see Benton⁽¹¹⁹⁾). The indigestible carbohydrates are often referred to as dietary fibre, which contains non-starch polysaccharides, resistant starch and non-digestible oligosaccharides. The fermentation end-products of dietary fibre are volatile fatty acids (VFA; acetic, propionic and butyric acid), lactate, alcohol and the gases methane, hydrogen and carbon dioxide⁽¹²⁰⁾. Apart from the fermentability, other physical and chemical properties of dietary fibre include solubility, ability to bind water and affect viscosity, and possible interactions with the digestion and absorption of starch, protein and fat. In addition, the duration of satiety experienced by animals between meals may be affected by carbohydrates, which in turn may reduce the behavioural side effects of a high feed motivation.

Findings and mechanisms in different mammals

The effects of dietary carbohydrate sources (i.e. fibrous ingredients) on animal behaviour have been relatively well studied especially in pigs, where non-lactating sows were fed energy-restricted diets in order to prevent excessive lipid deposition and reduced reproduction performance. Commonly diets for sows are formulated to meet the daily nutrient requirements for maintenance and reproduction. However, the latter may not result in a sufficient level of satiety between meals and is believed to be an important reason for a persistent high feeding motivation throughout the day contributing to the development of stereotyped behaviour⁽¹²¹⁾. In order to reduce stereotyped behaviour in sows, diets high in fibrous ingredients (sugarbeet pulp, oat hulls, soybean hulls, wheat bran) can be fed^(122,123), resulting in an increased time of sows lying down⁽¹²⁴⁾, increased resting time, less time spent on foraging and aggression⁽¹²⁵⁾ and reduced posture changes 8 and 10 h after feeding⁽¹²⁶⁾. The latter authors compared sows fed a high and a low-fermentable carbohydrate diet (for further examples, see Meunier-Salaün *et al.*⁽¹²⁷⁾). The relationship between dietary fibre content and stereotyped behaviour has also been documented in horses. A large survey among trainers of race horses in Sweden revealed a negative correlation between the amount of roughage provided and the incidence of stereotyped behaviour (cribbing or wind-sucking, weaving, box-walking) or wood-chewing in horses⁽¹²⁸⁾. Wood-chewing may be related to a 'fibre deficiency' in the diet and represent attempts to increase dietary fibre intake⁽¹²⁸⁻¹³⁰⁾. The effect of fibrous ingredients on behaviour is not generic for all fibre sources; for example, solvent-extracted coconut meal and soybean hulls as a dietary fibre source do not appear to affect physical activity in pigs⁽¹³¹⁾, whereas sugarbeet pulp silage does⁽¹³²⁾. Since sows which are fed low amounts of feed were shown to be more active compared with sows fed large amounts of feed⁽¹³³⁾ it has been suggested that hunger is most likely the cause of the increased physical activity⁽¹³⁴⁾.

The variety in physical and chemical properties of different fibrous ingredients results in differences between these fibres in creating and maintaining satiety and preventing feelings of hunger. The biological mechanisms behind the satiating properties of dietary fibre are still not fully understood, but several dietary fibre characteristics seem to be important. First, fibres with a high water-binding capacity may increase the volume and weight of the gastric contents when liquids are available. The weight or volume may stimulate stretch receptors that can induce gastric signals of satiation^(116,135). Second, gastric emptying can be affected either directly by dietary fibres high in intra-gastric viscosity⁽¹³⁶⁾ or indirectly through the stimulation of the release of glucagon-like peptide-1 (GLP-1) (a potent inhibitor of gastric emptying⁽¹³⁷⁾). Stimulation of GLP-1 production can be mediated through carbohydrate fermentation in the distal part of the gastrointestinal track⁽¹³⁸⁾ or through the production of VFA (mainly acetate) which stimulates the release of peptide tyrosine tyrosine (PYY)⁽¹³⁹⁻¹⁴¹⁾. The effects of GLP-1 and PYY in delaying gastric emptying are referred to as the 'ileal brake' mechanism which results in a moderate and stable flow of nutrients from the stomach into the small intestine⁽¹¹⁶⁾. A decrease in postprandial gastric-emptying rate will, consequently,

prolong gastric distension and gastric signals of satiation^(135,142). This mechanism was studied by Moran *et al.*⁽¹⁴³⁾ in rhesus monkeys where intramuscular injections of PYY reduced gastric emptying and resulted in a decrease in food intake. In addition, there are indications that PYY in the brain reduces appetite in humans⁽¹⁴⁴⁾, although this is still a subject for debate⁽¹⁴⁵⁾. Third, fibrous dietary ingredients may increase small-intestinal transit time⁽¹⁴⁶⁾, possibly also by stimulation of PYY which is found to suppress intestinal motility⁽¹⁴⁷⁾. An increase in small-intestinal transit time: (i) prolongs contact between nutrients and intestinal receptors involved in maintaining satiety^(148,149) and postpones feelings of hunger⁽¹¹⁴⁾; (ii) results in the slowing down of starch digestion and subsequent absorption of glucose, thereby maintaining more stable postprandial glucose and insulin concentrations in the blood⁽¹⁵⁰⁾. A transient decline in blood glucose level preceded meal initiation in rats⁽¹⁵¹⁾ and humans^(152,153) and caused a delay in the decrease in blood glucose concentrations. This may prolong satiety and postpone hunger and meal initiation (for a review, see Campfield & Smith⁽¹⁵⁴⁾). Finally, fermentation of carbohydrates may yield VFA which leads to a higher level of satiety by (i) PYY-mediated reduction of gastric emptying rate⁽¹⁵⁵⁾ and (ii) becoming a source of energy (mainly acetate) at times when glucose supply from the small intestine is decreasing, which stimulates longer-term satiety^(120,135,156,157).

As suggested previously, hunger is most likely the cause for the observed behavioural effects seen in sows⁽¹³⁴⁾. Hunger or appetite is correlated with the peripheral concentration of ghrelin⁽¹⁵⁸⁾, a twenty-eight amino acid peptide synthesised predominantly in the stomach^(159,160). For example, a rise in blood ghrelin concentration is associated with meal initiation in humans⁽¹⁶¹⁾. Supplementation of short-chain oligofructose (average degree of polymerization of 4.5) in a diet for 3 weeks decreased energy intake and lowered ghrelin concentrations in rats compared with rats fed the control diet without fructan supplementation. However, rats fed a diet supplemented with long-chain oligofructose (average degree of polymerization of 25.0) showed a decrease in energy intake but not in ghrelin concentrations compared to rats fed the control diet⁽¹³⁸⁾. It is suggested that the lower blood ghrelin concentrations may contribute to a decrease in appetite during fasting⁽¹⁶²⁾. Whether these results were accompanied with changes in behaviour (for example, food-seeking behaviour) requires further investigation. Figure 2 shows the effects of dietary fibre on satiety.

Findings in dogs

'When we are considering how a dog is behaving, we really should be considering what is inside the stomach' (Mugford⁽⁶⁷⁾, p. 1046). Despite this statement, little additional research has been conducted on the association between canine behaviours and satiety or feeding motivation between meals. To the authors' knowledge, three studies have investigated the effects of dietary fibre on satiety and feeding motivation in dogs of which only one also studied canine behaviour and another measured *ad libitum* food intake of dogs fed diets varying in fibre source and content (Table 2). Butterwick & Markwell⁽¹⁶³⁾ fed overweight dogs

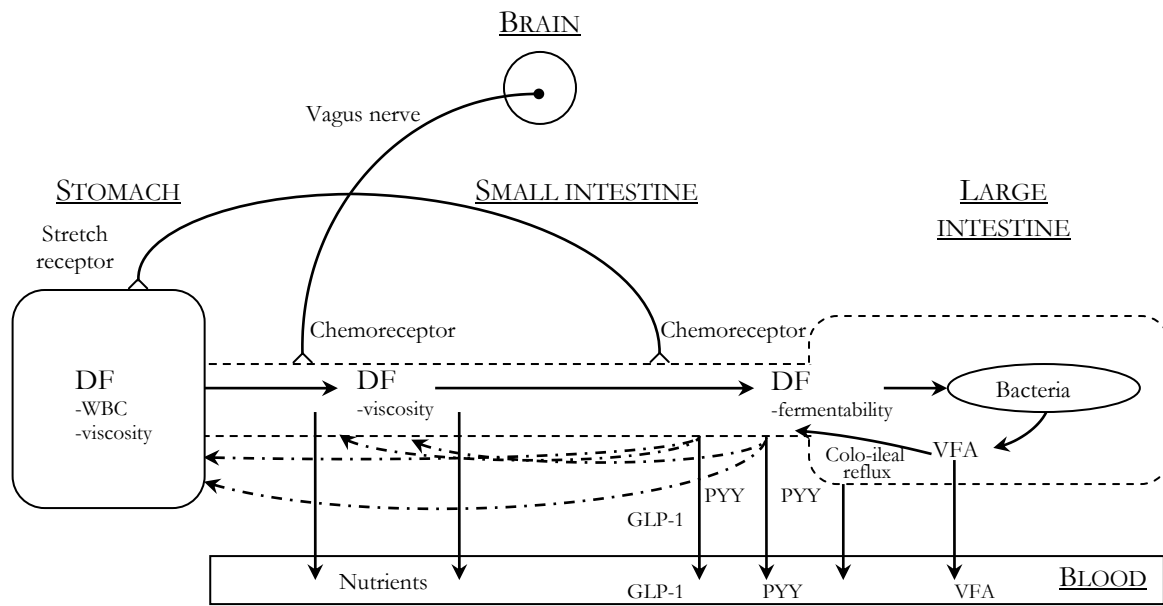


Figure 2 | Effects of dietary fibre (DF) on satiety. (---), Factors that may ultimately increase the residence time of digesta in the designated segments of the gastrointestinal tract; WBC, water-binding capacity; VFA, volatile fatty acids; GLP-1, glucagon-like peptide-1; PYY, peptide tyrosine tyrosine.

(>115% ideal body weight) six different moist diets varying in type and amount of fibre on an energy-restricted basis (45% restriction of calculated maintenance energy requirements; ME (kJ) = 461 x body weight (kg)^{0.75}). The four experimental high-fibre diets formulated to vary in soluble fibre (SF) and insoluble fibre (ISF), i.e. (g/kg DM) 40.8 SF and 13.6 ISF; 112.5 SF and 37.5 ISF; 35.7 SF and 202.4 ISF; 24.8 SF and 310.6 ISF, were compared with two dry control diets (36.5 SF and 14.6 ISF; 45.5 SF and 15.2 ISF). The authors found no differences in time spent at behaviours related to feeding motivation (i.e. cumulative time spent at feeding bowl and number of visits to bowl 30 min after feeding, intake of a meal (canned diet) provided 3 h after introduction of the test diets) between dogs fed the different diets. In contrast, Jewell & Toll⁽¹⁶⁴⁾ did find effects of fibre content on the satiety of dogs. Dogs with *ad libitum* access to dry diets with a medium or high crude fibre content (135.5 and 223.4 g/kg DM) decreased total ME intake compared with dogs that had *ad libitum* access to low-crude fibre diets (16.3 and 16.4 g/kg DM). When dogs were offered a subsequent meal, 30 min after the end of the last meal, energy and DM intake were lower in dogs fed the high-fibre diet compared with dogs consuming the low-fibre diet⁽¹⁶⁴⁾. Similarly, Jackson *et al.*⁽¹⁶⁵⁾ observed that a high-fibre content in dry diets reduced energy intakes in dogs. These authors fed dogs in the morning either a diet high in total dietary fibre (26.7 SF, 263.7 ISF g/kg as fed) or low in total dietary fibre (18.1 SF, 123.2 ISF g/kg as fed) followed 6 h later by *ad libitum* access to a diet containing 23.2 SF, 123.5 ISF g/kg as fed. Average energy intake over the day was lower (kJ/kg body weight) in the dogs fed the high-fibre diet in the morning

compared with the energy intake of the dogs fed the low-fibre diet in the morning (273 *vs.* 332 kJ (65.3 *vs.* 79.4 kcal)/kg body weight). The difference in average daily energy intake was the result of the energy intake in the morning since there were no significant differences observed in intake of the diet provided in the afternoon between the high-fibre (181 kJ (43.2 kcal)/kg body weight) and low-fibre (197 kJ (47.2 kcal)/kg body weight) groups. These latter two studies showed that high levels of fibrous dietary ingredients in dogs can increase satiety and reduce energy intake. This, however, was not confirmed in a study by Butterwick & Markwell⁽¹⁶³⁾. The latter may be due to the energy restriction and the large differences in DM content of diets between studies. Energy restriction will result in an increased feeding motivation in dogs to a level that nullifies the possible effects of fibre on satiety⁽¹⁶⁵⁾. DM content of the moist diets fed to dogs in the study of Butterwick & Markwell⁽¹⁶³⁾ ranged between 132 and 168 g/kg whereas Jewell & Toll⁽¹⁶⁴⁾ and Jackson *et al.*⁽¹⁶⁵⁾ fed dry diets with a DM content between 908 and 923 g/kg. On an energy basis, intake of a diet with a high DM content or high energy density will result in lower weight of the digesta in the stomach compared with a diet with similar nutrient composition but lower DM content. A low dietary DM content will therefore have a higher weight of digesta in the stomach and will stimulate stretch receptors which affect satiety in dogs⁽¹⁶⁶⁾. Finally, food intake in g DM/kg body weight was found to be lower in dogs with *ad libitum* access to a diet with 15 g short chain fructo-oligosaccharides/kg DM compared with dogs with *ad libitum* access to a diet with 60 g cellulose/kg DM⁽¹⁶⁷⁾. The authors suggested that satiety between diets was altered because of the differences in fermentability of the fibre sources included in the diets. Unfortunately, no measurements were made in this study to elucidate possible mechanisms underlying their observed difference in food intake.

The mechanisms behind the observed effects of dietary fibre on inducing and maintaining satiety in humans and pigs (see previous section) have in part been also observed in dogs. Stimulation of stretch receptors through infusion of liquids or filling a balloon with water placed in the stomach reduced sham feeding in dogs, indicating that stimulation of stretch receptors induces satiety in dogs⁽¹⁶⁶⁾. Gastric emptying was reduced in dogs as fibre (for example, psyllium, guar gum) content and viscosity of the meal increased⁽¹⁶⁸⁾, which will prolong gastric distension and gastric signals of satiation. In addition, a study of Bueno *et al.*⁽¹⁴⁶⁾ in which dogs were fed different fibre sources (wheat bran, cellulose, guar gum), both gastric emptying and intestinal transit time were affected with the effect depending on the fibre source included. A delay in gastric emptying and thus an increase in intestinal transit time by dietary fibre (alginate) results in more stable blood glucose concentrations as observed by Murray *et al.*⁽¹⁶⁹⁾. In dogs fed a diet with a high level of fermentable fibres (sugarbeet pulp, gum arabic and fructo-oligosaccharides), intestinal GLP-1 concentrations were found to be increased compared with dogs fed a diet with low-fermentable fibre (cellulose) levels⁽¹⁷⁰⁾. GLP-1 slows down gastric emptying⁽¹³⁷⁾ and intestinal transit⁽¹⁷¹⁾, which may result in prolonged gastric fill and delayed nutrient digestion and absorption. In dogs, the

'ileal brake' mechanism may also result from stimulation of the release of PYY by fatty acids sufficient to delay gastric emptying in dogs⁽¹⁷²⁾.

As reported above, fermentation of carbohydrates yields VFA⁽¹²⁰⁾, which may lead to prolongation of satiety by becoming a source of energy (mainly acetate) at times when glucose supply from the small intestine is decreasing^(120,135,156,157). Although dogs have a relatively small and simple large intestine, dogs are capable of fermenting a significant quantity of dietary non-digestible carbohydrates⁽¹⁷³⁾. Moreover, the faecal microflora of dogs were found to give similar *in vitro* organic matter disappearance results compared with the microflora from humans, pigs and horses⁽¹⁷⁴⁾. The latter indicates that differences between these species in carbohydrate fermentation capacity are probably dependent on factors other than the microbial population. The extent of fermentation in the gastrointestinal tract in an animal largely depends on the time available for microbial fermentation⁽¹⁷⁴⁻¹⁷⁶⁾. In dogs, a transit time through the total gastrointestinal tract between 20 and 35 h is considered normal⁽¹⁷⁷⁾. The large-intestinal transit of digesta can take up to 90% of the total gastrointestinal transit time^(178,179), presenting a considerable time for large-intestinal microflora to ferment undigested substrates entering from the ileum. The VFA produced can be used by the hindgut bacteria for protein synthesis, resulting in an increase in microbial mass, or absorbed in the large intestine. The contribution of large-intestinal VFA absorption towards the total energy maintenance requirements of dogs has been reported to be approximately 2–7%^(180,181). However, the latter authors did not provide information on the way these values were derived. In addition, the effect of production and absorption of acetate as an energy source for body tissues on postprandial satiety remains to be investigated. The work of Pouteau *et al.*^(182,183) on a method to evaluate acetate production and metabolism using stable isotopes may be the starting point for further exploration of the importance of carbohydrate fermentation in the gastrointestinal tract and satiety in dogs.

To our knowledge, there is no information available in the scientific literature regarding possible influences of dietary fibre on ghrelin concentrations and behaviour in dogs. However, when dogs are fed one scheduled meal per day, ghrelin concentrations increase before and decrease rapidly after the meal to remain relatively constant throughout the rest of the day⁽¹⁸⁴⁾, which may indicate little potency of ghrelin concentrations to affect canine behaviour throughout the day.

Nowadays, dry extruded diets for dogs may contain 30% or more carbohydrates of which starch is the major component. Moreover, the non-digestible carbohydrate fraction in diets can also make up a considerable proportion⁽¹⁷³⁾. As mentioned previously, fibres differing in physical and chemical properties have diverse physiological responses in animals. Nutrient digestion as well as transit time through the gastrointestinal tract may be influenced by the amount and source of fibre included in canine diets. In the case of a reduction in nutrient digestibility when fibres are included, it is necessary to increase the concentration of some nutrients in order to ensure that the nutrient requirements of the animals are met⁽¹⁸⁵⁾. Future canine research on the behavioural effects of dietary fibre should account for the fact

Table 2 | Effect of dietary fibre on food intake and canine behaviour.

Authors	Dogs and design	Diets ¹	Results
Jewell & Toll ⁽¹⁶⁴⁾	<p>Study 1, two groups of fifteen beagle dogs were assigned to one of two dry diets fed once per d at maintenance level for 14 d On day 7, one of two diets was provided 75 min after first meal. On day 14, the other diet was offered 75 min after first meal. After 14 d, the experimental design was repeated but each group of dogs received the other of the two diets Study 2, identical as study 1 but with different diets.</p>	<p>1) 16 g CF/kg (study 1) 2) 136 g CF/kg (study 1) 3) 16 g CF/kg (study 2) 4) 223 g CF/kg (study 2)</p>	<p>a) Energy intake of all dogs was lower than energy on offer. b) Dogs fed diets 2 and 4 had lower daily energy intake than dogs fed diets 1 and 3. c) Energy intake of the second meal 75 min after first meal was lower when dogs were fed diets 2 and 4 compared with dogs fed diets 1 and 3.</p>
Butterwick & Markwell ⁽¹⁶⁵⁾	<p>Six obese terrier dogs (>115% of ideal BW) were fed each of the six wet diets (6 x 6 Latin square) at 45% of maintenance level for 12 d. Number of visits to the bowl and cumulative time spent at the bowl were observed for 30 min from the start of the meal. On day 7 and 10, 8 and 11, or 9 and 12, dogs had <i>ad libitum</i> access to a wet diet that was provided 180 min after the first meal and food intake was measured.</p>	<p>1) 7 g CF/kg; 41 g SF/kg; 14 g ISF/kg 2) 13 g CF/kg; 113 g SF/kg; 38 g ISF/kg 3) 143 g CF/kg; 36 g SF/kg; 202 g ISF/kg 4) 149 g CF/kg; 25 g SF/kg; 311 g ISF/kg 5) 15 g CF/kg; 37 g SF/kg; 15 g SF/kg 6) 8 g CF/kg; 46 g SF/kg; 15 g ISF/kg</p>	<p>a) No differences between diets in daily energy intake. b) No differences between diets in observed behaviours. c) No differences between diets in food intake of the second meal 180 min after first meal.</p>
Jackson <i>et al.</i> ⁽¹⁶⁵⁾	<p>Two groups of fifteen miniature schnauzers and toy poodles were assigned to one of two dry diets fed in the morning at 50% of daily intake and had <i>ad libitum</i> access to a control diet in the afternoon (approximately 6 h later) for 8 d.</p>	<p>1) 95 g CF/kg; 27 g SF/kg; 264 g ISF/kg 2) 20 g CF/kg; 18 g SF/kg; 123 g ISF/kg 3) 21 g CF/kg; 23 g SF/kg; 124 g ISF/kg (control)</p>	<p>a) Dogs fed diet 1 had lower morning and daily energy intake/kg BW than dogs fed diet 2. b) There was no difference in food intake of diet 3 in the afternoon between dietary treatments.</p>
Howard <i>et al.</i> ⁽¹⁶⁷⁾	<p>Twenty-eight adult beagle dogs were stratified by BW and assigned at random to one of four dry diets with <i>ad libitum</i> access for 35 d.</p>	<p>1) 60 g cellulose/kg 2) 15 g FOS/kg 3) 60 g beet pulp/kg 4) 60 g beet pulp/kg; 20 g gum talha/kg; 15 g FOS/kg</p>	<p>a) Dogs fed diet 1 had lower morning and daily energy intake/kg BW than dogs fed diet 2. b) There was no difference in food intake of diet 3 in the afternoon between dietary treatments.</p>

CF, crude fibre; BW, body weight; SF, soluble fibre; ISF, insoluble fibre; FOS, fructo-oligosaccharides.

¹Values are presented on a DM basis except for the data of Jackson *et al.*⁽¹⁶⁵⁾, which are as-fed.

that different breeds may respond differently (in terms of satiety). Gastric emptying rate is inversely related to body weight in dogs of different sizes⁽¹⁸⁶⁾. Moreover, large-breed dogs have a longer large-intestinal transit time and increased apparent total dietary fibre digestibility⁽¹⁸⁷⁾, which may increase the production and the use of VFA but may increase gastrointestinal discomfort as a result of enhanced fermentation activity.

The degree of satiety in animals such as pigs has been shown to affect behaviour, including aggressive and stereotyped behaviour. Although likely, it is up till now unknown whether canine behaviour can be affected by degree of satiety and further research is required. Assuming that behaviours in dogs are more favourable during times of satiety than during times of hunger as observed in pigs (for example, aggression), specific dietary fibres through their potential to prolong satiety may assist in preventing unwanted canine behaviours.

Conclusions

The present contribution provides an overview of current knowledge on the influence of dietary macronutrient composition on canine behaviour. It can be concluded that little research has been conducted in this field although research in other species indicates that there is potential to modify behaviour in dogs through nutrition. There is evidence that dietary composition can modulate animal and human behaviour through different mechanisms. Dietary protein may contain the precursors tryptophan and tyrosine for the respective neurotransmitters serotonin and catecholamines. Since bioavailability of both tryptophan and tyrosine in the brain are dependent on the dietary protein content and amino acid composition, dietary composition may have an impact on the behaviour and wellbeing of dogs under specific circumstances (for example, stress). However, before application and extrapolation of the evidence found in mostly rodent laboratory studies into commercial canine diets is undertaken, research is required to identify the optimal and safe dietary inclusion level in combination with behavioural tests to study the magnitude of effects on (problem) canine behaviour. The n-3 PUFA have an important role in the development of the brain, and the supply of essential fatty acids such as DHA could affect aspects of the dopaminergic and serotonergic system and, consequently, cognitive performance and behaviour as observed in rodents. Most canine studies and dietary n-3 PUFA have been mainly focused on the effect of maternal intake of different dietary n-3 PUFA during gestation and lactation on n-3 PUFA in the milk and/or n-3 PUFA intake on retinal function of puppies. It would be of interest to examine the DHA required for optimal neurological development and whether this leads to alterations in cognitive abilities or behaviour later in life of dogs. In the literature, studies have been reported which show that, depending on the physical and chemical properties, certain dietary fibres induce satiation or prolongation of satiety after a meal. However, there have been no studies conducted in which the effect of dietary fibre on physiological satiety parameters, behaviour (for example, activity) and/or

feeding motivation were studied in dogs. If dietary fibre has short-term effects that result in prolongation of satiety and a reduction of hunger between meals, it may help to prevent unwanted canine behaviours and also promote long-term weight control.

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Chapter 3

Comparative *in vitro* fermentation activity in the canine distal gastrointestinal tract and fermentation kinetics of fibre sources

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Abstract | The current study aimed to evaluate the variation in fermentation activity along the distal canine gastrointestinal tract (GIT, Exp. 1). It also aimed to assess fermentation kinetics and end product profiles of 16 dietary fibres for dog foods using canine faecal inoculum (Exp. 2). For Exp. 1, digesta was collected from the distal ileum, proximal colon, transverse colon, and rectum of three adult dogs. Digesta per part of the GIT was pooled over three dogs, diluted (1:25, wt/vol), mixed, and filtered for the preparation of inoculum. A fructan, ground soy hulls, and native potato starch were used as substrates and incubated for cumulative gas production measurement as an indicator of the kinetics of fermentation. In addition, fermentation bottles with similar contents were incubated but were allowed to release their gas throughout incubation. Fermentation fluid was sampled at 4, 8, 12, 24, 48, and 72 h after initiation of incubation and short-chain fatty acids (SCFA) and NH₃ were measured. Results showed comparable maximal fermentation rates for rectal and proximal colonic inocula ($P>0.05$). Production of SCFA was lowest for the ileal and highest for the rectal inoculum ($P<0.05$). It is concluded that for *in vitro* studies faecal microbiota can be used as an inoculum source but may slightly overestimate *in vivo* fermentation. Experiment 2 evaluated the gas production, fermentation kinetics, and end product profiles at 8 and 72 h of incubation for citrus pectin, 3 fructans, gum arabic, 3 guar gums, pea fibre, peanut hulls, soy fibre, sugarbeet fibre, sugarbeet pectin, sugarbeet pulp, wheat fibre, and wheat middlings. Faeces of four adult dogs was used as inoculum source. Similar techniques were used as in Exp. 1 except for the dilution factor

used (1:10, wt/vol). Among substrates large variations in fermentation kinetics and end product profiles were noted. Sugarbeet pectin, the fructans, and the gums were rapidly fermentable indicated by a greater maximal rate of gas production (R_{\max}) compared to all other substrates ($P<0.05$) whereas peanut hulls and wheat fibre were poorly fermentable indicated by a lowest amount of gas produced ($P<0.05$). Sugarbeet fibre, sugarbeet pulp, soy fibre, and wheat middlings were moderately fermentable with a low R_{\max} . Citrus pectin and pea fibre showed a similar low R_{\max} but time at which this occurred was later compared to sugarbeet fibre, sugarbeet pulp, soy fibre, and wheat middlings ($P<0.05$). Results of this study can be used to formulate canine diets that stimulate dietary fibre fermentation along the distal GIT that may optimize GIT health and stimulate the level of satiety in dogs.

Introduction

Although dogs have a relatively simple large intestine, they have an active microbial community that is capable of fermenting a significant quantity of dietary fibre⁽¹⁾. This fermentation results in the production of mainly short-chain fatty acids (SCFA) that may affect host health. For example, acetate and propionate may stimulate the secretion of the satiety hormone peptide YY (PYY) by endocrine L-cells in the distal GIT⁽²⁾ and butyrate may protect against ulcerative colitis⁽³⁾.

The largest fermentation activity is found in the proximal colon and declines further down the gastrointestinal tract (GIT) when the availability of substrates decreases⁽⁴⁾. The presence of fermentable substrates affects microbial growth and as a result of changing substrate availability the microbial population changes in terms of species and numbers along the distal GIT^(5,6). In relation to large intestinal health, it is of interest to stimulate degradation of dietary fibre and production of SCFA along the entire large intestine. It is thought that the kinetics and extent of fermentation of fibre indicate where the product is likely to be fermented in the GIT⁽⁷⁾.

Although several *in vitro* studies have been conducted to evaluate the extent of fermentation of dietary fibres, little information is available regarding the fermentation kinetics of fibres for dogs. Moreover, the different *in vitro* studies used either ileal or faecal inoculum to characterize fermentation. The variation in the microbial community along the GIT questions whether inocula sources commonly used in literature are representative for the activity along the entire colonic region. The current study aimed to evaluate 1) the microbial fermentation activity in the canine lower GIT and 2) the fermentation kinetics of fibres for canine foods.

Materials and methods

The first study reported here was approved by and conformed to the requirements of the Ethical Review Committee for Animal Experiments of NOTOX B.V. ('s Hertogenbosch, The Netherlands).

Experiment 1

Substrates

Three different types of dietary fibre (i.e. non-digestible oligosaccharides, cell-wall constituents, and resistant starch) were selected as potentially fermentable fibre sources to study the activity of fractions of the microbial population present in various parts of the GIT⁽⁸⁾. Substrates were fructan (non-digestible oligosaccharides, Raftifeed IPS, Orafiti, Tienen, Belgium), soy hulls, and native potato starch (both obtained from Research Diet Services, Wijk bij Duurstede, The Netherlands). Soy hulls were ground over a 1 mm sieve. Fructan and potato starch samples were already provided in a powdered form. Each substrate was analysed for DM, ash, CP, and crude fat. Soy hulls were also analysed for NDF, ADF, and ADL. The analysed composition of each substrate is presented in Table 1.

Digesta collection

In addition, this was an opportunistic study; inocula were obtained from dogs that were used in earlier experiments and scheduled for euthanasia. Three two-year old, healthy Beagle dogs (Marshall Bioresources, NY, USA) with a mean BW \pm SEM of 9.6 ± 1.08 kg were housed individually and had free access to drinking water. Dogs were individually fed twice daily a commercially available dry extruded diet (Carocroc Super Premium Lamb & Rice Diet, Vobra Special Petfoods B.V., Veghel, The Netherlands) for one week before digesta sampling. Dogs were fed approximately 500 kJ energy/kg BW^{0.75} per day. The diet was composed of dried lamb meat, rice, corn, dried beet pulp, beef fat, yeast, lamb meat extracts, vegetable oils, lecithin, vitamins, and micronutrients containing (g/kg DM) 212 CP, 72 crude fat, 64 ash, 71 NDF, 39 ADF, and 14 ADL. Dogs were fed approximately 4 h before digesta sampling to ensure that sufficient digesta was available in the various intestinal segments for the preparation of the inoculum.

Dogs were anaesthetized with an intravenous injection of 0.003 mg/kg BW Fentanyl-Janssen (Janssen-Cilag B.V., Tilburg, The Netherlands) and 50 mg/kg BW Pentothal (Abbott B.V., Hoofddorp, The Netherlands). Subsequently, fatal incisions were made for exsanguination via the brachial and femoral arteries and veins in the groins of the front and hind legs. Immediately after euthanasia, the abdominal cavity was opened and four specific sections of the GIT of interest were clamped off to avoid mixing of digesta. Sections of interest were the terminal ileum (~15 cm proximal from the ileo-caecal junction), the proximal colon (~10 cm distal the ileo-caecal junction) including the caecum, the transverse colon (between the right and left colic flexures), and the rectum (~10 cm proximal from the anus). The GIT from stomach to anus was lifted out from the abdominal cavity and each section with digesta contents was separated from the GIT. All materials used for digesta collection and storage were pre-sterilized using 70% ethanol. Each section was placed on a glass plate and opened using a scalpel. The content of each section was transferred into sterile containers pre-filled with carbon dioxide using a sterile spoon. All digesta samples were

Table 1 | Analysed composition of the fibrous substrates used in Exp. 1 and 2.

Substrate ¹	DM, %	OM	CP	Crude fat	NDF	ADF	ADL
<i>Experiment 1</i>							
Fructan	96.5	100.0	0.2	0.1	ND ²	ND	ND
Soy hulls	85.6	94.9	11.6	1.5	64.7	50.7	1.8
Native potato starch	82.6	99.6	0.4	0.1	ND	ND	ND
<i>Experiment 2</i>							
Citrus pectin ³	93.9	97.2	6.8	0.8	ND	60.3	2.4
Fructan 1	95.8	99.8	0.1	0.3	ND	ND	ND
Fructan 2	96.8	100.0	0.0	0.3	ND	ND	ND
Fructan 3	97.0	100.0	0.0	0.3	ND	ND	ND
Gum arabic	92.5	96.2	9.4	0.4	ND	ND	ND
Guar gum1	87.1	98.9	5.1	0.6	ND	ND	ND
Guar gum2	88.9	99.1	5.2	0.5	ND	ND	ND
Guar gum3	89.6	96.0	4.3	0.5	ND	ND	ND
Pea fibre	95.5	97.4	5.5	0.2	68.8	64.1	0.3
Peanut hulls	90.7	97.1	7.7	1.9	80.1	71.2	31.7
Soy fibre	94.7	93.6	10.7	0.1	17.7	14.5	0.4
Sugarbeet fibre	92.0	94.2	9.6	0.3	33.1	24.8	2.1
Sugarbeet pectin	88.3	96.6	6.4	0.1	0.0	0.4	0.3
Sugarbeet pulp	89.2	92.6	8.9	0.4	46.8	24.6	2.0
Wheat fibre	91.0	97.5	6.6	1.6	78.3	27.5	1.9
Wheat middlings	87.2	94.0	18.1	3.5	37.6	11.7	3.4

¹Fructan, Raftifeed IPS, Orafti, Tienen, Belgium; soy hulls and native potato starch, both Research Diet Services, Wijk bij Duurstede, The Netherlands; citrus pectin, Herbacel AQ Plus type F, Herbafood Ingredients GmbH, Werder, Germany; fructan 1, Zopas, DP=4; fructan 2, Raftifeed IPS, 2<DP<60; fructan 3, Beneo HP, DP>22, all fructans from Orafti, Tienen, Belgium; gum arabic, TIC Pretested Gum Arabic FT Powder; guar gum 1, TIC Pretested Pre-Hydrated Guar Gum 8/24 Powder; guar gum 2, TIC Pretested Gum Guar SCM Powder; guar gum 3, TIC Pretested Nutriloid 010 Powder, all gums from TIC Gums, Inc. Belcamp, MD, USA; pea fibre, Exafine 250, Cosucra SA, Warcoing, Belgium; peanut hulls, supplied by anonymous company; soy fibre, Fibrim 1020 IP Non-GM, The Solae Company, Le Grand-Saconnex, Switzerland; sugarbeet fibre, Fibrex 595, Danisco Sugar AB, Malmö, Sweden; sugarbeet pectin, Pectin Classic RU 301, Herbstreith & Fox KG, Neuenbürg, Germany; unmolassed sugarbeet pulp and wheat middlings, both Research Diet Services, Wijk bij Duurstede, The Netherlands; wheat fibre, Xylo-Gold Moon wheat fibre, Meneba Feed Ingredients, Rotterdam, The Netherlands.

²ND = not determined.

³NDF-content of the product could not be determined due to gel formation during analysis.

stored on ice (0°C) and transported to the laboratory of the Animal Nutrition Group (Wageningen University, Wageningen, The Netherlands).

Fermentation procedures

Methods used conformed to the procedures described by Williams *et al.*⁽⁷⁾. Because in this study the variation among dogs was not of interest, digesta of the three dogs were quantitatively pooled to provide one representative inoculum for each GIT section for the specific diet⁽⁹⁾. Digesta was diluted 1:25 by wet weight in a 39°C anaerobic sterile physiological saline solution (9 g/l NaCl). The large dilution was necessary due to the limited

quantity of material obtained. The diluted mixture was then homogenized for 60 s using a hand-blender and filtered through a double layer of sterile cheesecloth (16 threads/cm in both directions). The resulting filtrate was used as the inoculum. All procedures were carried out under a constant stream of carbon dioxide to maintain strictly anaerobic conditions.

Two batches of fermentation bottles were prepared. The first batch was used to measure the *in vitro* cumulative gas production for 72 h using an Automated Pressure Evaluation System (APES; Davies *et al.*⁽¹⁰⁾). This technique measures the production of gas in the head-space of bottles using pressure sensitive switches and solenoid valves, which release a fixed amount of gas at a pre-determined pressure threshold. The cumulative amount of gas produced during incubation time can be used to describe the kinetics of fermentation. To measure the production of fermentation end products over time, a second batch of fermentation bottles was incubated from which bottles were removed at regular time intervals (4, 8, 12, 24, and 48 h of incubation).

For the APES, a 5-ml sample of the pooled inoculum was injected into each 100-ml fermentation bottle (four replicates per inoculum-substrate combination) and immediately attached to the APES. Each bottle contained approximately 0.5 g substrate and 84 ml of a medium as described by Williams *et al.*⁽⁷⁾. Incubation temperature was set at 39°C. Two blanks per inoculum containing only medium and inoculum were placed in the APES.

For measurement of fermentation end products, a 2.5-ml sample of the pooled inoculum was injected into 50-ml serum bottle fermentation vessel. For each time point, at 4, 8, 12, 24, and 48 h of incubation, four replicates per inoculum per substrate were used. Each vessel contained approximately 0.25 g substrate, 2.5 ml inoculum, and 42 ml of a medium. For each time point, two blanks per inoculum containing only medium and inoculum were prepared. Each fermentation bottle was equipped with a rubber stopper through which a needle (BD Microlance, 0.45 × 13 mm, BD, Drogheda, Ireland) was pierced to release gas and placed in the incubator at 39°C. After inoculation of all fermentation bottles, each inoculum was analyzed for DM and ash.

Cumulative gas production kinetics

The monophasic model described by Groot *et al.*⁽¹¹⁾ was fitted to the data for cumulative gas production [OM cumulative volume (OMCV) in ml of gas produced/g of OM weighed into the bottle] as follows: $OMCV = A / [1 + (C/t)^B]$, where A=asymptotic gas production, B=switching characteristic of the curve, C=time at which one-half of the asymptote had been reached, and t=time (h). The maximum rate of gas production (R_{max}) and the time at which it occurred (T_{max}) were calculated according to the following equations⁽¹²⁾: $R_{max} = \{A \times (C^B) \times B \times [T_{max}^{-(B-1)}]\} / \{1 + (C^B) \times [T_{max}^{(-B)}]\}^2$ and $T_{max} = C \times \{(B - 1)/(B + 1)\}^{(1/B)}$, respectively.

Chemical analyses

Dry matter and ash were determined by drying to a constant weight at 103°C and combusting at 550°C, respectively. Crude protein ($6.25 \times N$) was determined using the Kjeldahl-method (ISO, 2005) and crude fat was analyzed according to the Berntrop-method (ISO, 1999). Neutral detergent fibre was analyzed according to a modified method of Van Soest *et al.*⁽¹³⁾ with a heat stable amylase and expressed exclusive of residual ash, as described by Goelema *et al.*⁽¹⁴⁾, and ADF and ADL were determined according to Van Soest⁽¹⁵⁾.

Samples of fermentation liquids were analyzed for SCFA and NH₃. Short-chain fatty acids were determined using a gas chromatograph (Fisons HRGC Mega 2, Milan, Italy) equipped with a capillary column (Mega bore EC. 1000, internal diameter 0.53 mm, film thickness 1.0 µm, length 30 m, Alltech, Deerfield, IL, USA). The ratio of the split injection used was 1:10. A flame ionization detector was used to identify the components within the sample. Column temperature was 110°C and was increased at a rate of 18°C/min up to 200°C in which T₁=1 min and T₂=2 min. Helium was used as a carrier gas at a flow rate of 8 ml/min with a 10 minute run time for each sample. *Iso*-caproic acid was used as an internal standard. NH₃ concentration of each fermentation liquid was determined by deproteinisation of the supernatant using 10 g/l trichloroacetic acid. NH₃ and phenol were oxidized by sodium hypochlorite in the presence of sodium nitroprusside to form a blue complex. The intensity of the blue colour was measured colourimetrically at a wavelength of 623 nm.

Statistical analyses

Overall effects of inoculum and substrate on the fermentation kinetics were tested for significance using ANOVA by Proc GLM of SAS (SAS Inst. Inc., Cary, NC, USA). The statistical model used was $Y = \mu + I_i + S_j + (I \times S)_{ij} + \epsilon_{ijk}$, where Y=parameter to be tested, μ =mean, I_i =effect of inoculum i , S_j =effect of incubation substrate j , and ϵ_{ijk} =error term. For the fermentation end products, the statistical model also included incubation time as main effect and possible interactions of incubation time with inoculum and incubation substrate.

Experiment 2

Substrates

Selection of substrates was based on their (potential) use in canine foods. Substrates used were citrus pectin (Herbaccel AQ Plus type F, Herbafood Ingredients GmbH, Werder, Germany), fructans varying in chain-length expressed as degree of polymerization (DP) (fructan 1, Zopas, DP=4; fructan 2, Raftifeed IPS, 2<DP<60; fructan 3, Beneo HP, DP>22, all from Orafti, Tienen, Belgium), gums varying in botanical origin and viscosity (gum arabic, TIC Pretested Gum Arabic FT Powder, 0-300 centipoise (cP) of 30% solution; guar gum 1, TIC Pretested Pre-Hydrated Guar Gum 8/24 Powder, 4,000-6,500 cP of 1% solution; guar gum 2, TIC Pretested Gum Guar SCM Powder, 2,900-3,200 cP of 1% solution; guar gum 3, TIC Pretested Nutriloid 010 Powder, 0-200 cP of 2% solution, all from TIC Gums, Inc. Belcamp, MD, USA), pea fibre (Exafine 250, Cosucra SA, Warcoing, Belgium), peanut hulls

(supplied by a company wishing to remain anonymous), soy fibre (Fibrim 1020 IP Non-GM, The Solae Company, Le Grand-Saconnex, Switzerland), sugarbeet fibre (Fibrex 595, Danisco Sugar AB, Malmö, Sweden), sugarbeet pectin (Pectin Classic RU 301, Herbstreith & Fox KG, Neuenbürg, Germany), unmolassed sugarbeet pulp and wheat middlings (both Research Diet Services, Wijk bij Duurstede, The Netherlands), wheat fibre (Xylo-Gold Moon wheat fibre, Meneba Feed Ingredients, Rotterdam, The Netherlands). Substrates not already in a powder form were ground over a 1 mm sieve. Each substrate was analyzed for DM, ash, CP, and crude fat while all substrates, except for the fructans and gums, were analyzed for NDF, ADF, and ADL. The analyzed composition of substrates used is presented in Table 1.

Faeces collection

Four mature Labrador Retrievers with a mean BW \pm SEM of 36.3 ± 2.51 kg from a local dog breeder were used as faecal donors. Dogs were fed twice daily the identical dry diet at the same level as used in Exp. 1 for one week. The diet provided small amounts of fermentable fibre, and thus avoid selection of microbes that had adapted to the substrates being tested *in vitro*⁽¹⁶⁾. Immediately after defecation, faeces from each dog was collected and placed in a sterile plastic jar pre-filled with carbon dioxide. All faecal samples were transported in an thermal insulated container within 10 min to the laboratory of the Animal Nutrition Group (Wageningen University, Wageningen, The Netherlands).

Fermentation procedures

Faeces of the four dogs was pooled by weight and diluted 1:10 by wet weight in a 39°C anaerobic sterile physiological saline solution (9 g/l NaCl), and further processed as described in Exp. 1. Two batches of fermentation bottles were prepared. The first batch was used to measure the *in vitro* cumulative gas production for 72 h using the APES and end product profile according to the procedures of Exp. 1. As the concentrations of end products at 72 h may be more appropriate to describe the end product profile for slowly fermentable fibres⁽¹⁷⁾ a second batch of fermentation vessels was used to evaluate the SCFA production for rapidly fermentable substrates. A pilot experiment revealed that the most rapidly fermentable fibres reached R_{\max} at around 8 h of incubation. It was therefore decided to stop incubation after 8 h for measurement of SCFA concentrations. A 5-ml sample of the pooled inoculum was injected into 100-ml serum bottle fermentation vessel (three replicates per substrate) containing approximately 0.5 g substrate and 84 ml of a medium. Two blanks containing only medium and inoculum were prepared. Each fermentation bottle was equipped with a rubber stopper through which a needle (BD Microlance, 0.45×13 mm, BD, Drogheda, Ireland) was pierced to release gas and placed in the incubator at 39°C for 8 h. After incubation, all liquids were sampled and analyzed for SCFA and NH_3 . After inoculation of all fermentation bottles, inoculum was analyzed for DM and ash.

Cumulative gas production kinetics

The monophasic model was used to describe cumulative gas production data. Equations used to calculate model parameters, T_{\max} , and R_{\max} were the same as in Exp. 1.

Chemical analyses

Substrates were analyzed for composition using procedures described previously. Fermentation liquids were analyzed for SCFA and NH_3 according to the procedures described in Exp. 1.

Statistical analyses

Overall effect of substrate on the parameters for fermentation kinetics were tested for significance using a 1-way ANOVA by Proc GLM of SAS (SAS Inst. Inc., Cary, NC, USA). The statistical model was $Y = \mu + S_i + \epsilon_{ij}$, where Y =parameter to be tested, μ =mean, S_i =effect of substrate i , and ϵ_{ij} =error term. The same model was used to evaluate the formation of fermentation products on 8 and 72 h of incubation separately. For each parameter, differences among means of substrates were tested for significance by ANOVA using the Tukey multiple range test. Individual means were considered significantly different from one another when they exceeded the minimum significant difference calculated by Tukey multiple range test.

Results

Experiment 1

Cumulative gas production kinetics

Incubation with the fructan samples yielded more OMCV compared to incubation with soy hulls or potato starch samples (Table 2). The low amount of gas produced from potato starch resulted in only a few measuring points, insufficient for the statistical program to fit the curve and allow estimation of R_{\max} and T_{\max} . Soy hulls showed significantly lower R_{\max} and greater T_{\max} than incubation with the fructan ($P < 0.001$ and $P < 0.001$). There was no significant difference in OMCV among inocula sources ($P = 0.298$). However, R_{\max} and T_{\max} were different ($P = 0.028$ and $P < 0.001$, respectively). The rectal inoculum had greater R_{\max} than the transverse colonic inoculum ($P = 0.020$). Compared to the large intestinal inocula, the ileum inoculum showed greater T_{\max} ($P < 0.001$). Interactions between inoculum source and substrate were not significant for OMCV ($P = 0.245$), while interactions for R_{\max} and T_{\max} were significant ($P < 0.001$ and $P = 0.002$, respectively). Ileal inoculum showed fastest fermentation (i.e., higher R_{\max} and earlier time of T_{\max}) when incubated with soy hulls whereas incubation with fructans resulted in the slowest fermentation compared to the other inocula (data not shown).

Table 2 | Fermentation parameters according to the effects of inoculum source and substrate (Exp. 1).

Item	Fermentation parameters ¹					
	R _{max}	T _{max}	OMCV	A	B	C
<i>Inoculum source</i>						
Ileum	10.7 ^{ab}	14.4 ^a	127.7	180.0	5.11 ^a	18.82 ^a
Proximal colon	12.5 ^{ab}	10.0 ^b	141.0	181.9	2.61 ^b	12.00 ^{bc}
Transverse colon	8.3 ^b	9.3 ^b	133.0	173.4	2.09 ^b	14.20 ^c
Rectum	14.2 ^b	6.3 ^b	150.3	178.6	2.70 ^b	9.04 ^b
SE	1.4	0.8	8.8	12.0	0.30	0.98
P-value ²	0.028	<0.001	0.298	0.967	<0.001	<0.001
<i>Substrate</i>						
Fructan	15.7 ^a	13.4 ^a	292.2 ^a	296.2 ^a	2.91	18.17 ^a
Soy hulls	7.1 ^b	5.9 ^b	71.9 ^b	60.7 ^b	3.43	8.86 ^b
Potato starch	MP ³	MP	49.8 ^b	NC ⁴	NC	NC
SE	0.9	0.6	7.6	8.5	0.21	0.69
P-value	<0.001	<0.001	<0.001	<0.001	0.159	<0.001
<i>Interaction⁵</i>						
I × S	<0.001	0.002	0.245	0.989	<0.001	<0.001

^{a-c}Least squares means in the same column of each main effect that do not have common superscript differ, $P < 0.05$.

¹R_{max} = maximal rate of gas production (ml/h); T_{max} = time of occurrence of R_{max} (h); OMCV = OM cumulative volume (ml of gas produced/g OM incubated); A, B, and C are parameters of the monophasic model described by Groot *et al.*⁽¹¹⁾ with A = asymptotic gas production (ml of gas produced/g OM incubated), B = switching characteristic of the curve, and C = time at which one-half of the asymptote had been reached (h); measurements were based four replicates per inoculum per substrate; least square means with different superscripts.

²P-value for main effects.

³MP = not able to calculate R_{max} or T_{max} as described by Bauer *et al.*⁽¹²⁾ because of missing model parameters.

⁴NC = non-convergence to monophasic model described by Groot *et al.*⁽¹¹⁾.

⁵I = inoculum source; S = substrate.

End product profile

There was an effect ($P < 0.001$) of inoculum source, substrate, incubation time, and their interactions on fermentation end products (Table 3). The ileal inoculum produced the lowest amount of total SCFA per unit OM throughout the incubation ($P < 0.001$). For the large intestinal inocula, total SCFA production was significantly lower for the proximal and transverse colon compared to the rectum ($P < 0.001$ and $P < 0.001$, respectively). Incubation with the fructan samples resulted in the largest production whereas incubation with potato starch resulted in the lowest production of total SCFA per unit OM ($P < 0.001$). Furthermore, fermentation of soy hulls resulted in the highest NH₃ production compared to the fructans and potato starch. All interactions between inoculum source, substrate, and incubation time were significant for production of acetate, propionate, butyrate, total SCFA, and NH₃ ($P < 0.001$).

Table 3 | Concentration of fermentation products according to the effects of inoculum source, substrate, and incubation time (Exp. 1).

Item	Fermentation products ¹				
	Acetate	Propionate	Butyrate	Total SCFA	NH ₃
<i>Inoculum source</i>					
Ileum	1.25 ^a	0.35 ^a	0.22 ^a	1.83 ^a	2.02 ^a
Proximal colon	1.62 ^b	0.72 ^b	0.30 ^b	2.64 ^b	2.14 ^b
Transverse colon	1.70 ^b	0.72 ^b	0.35 ^c	2.77 ^b	2.18 ^c
Rectum	2.06 ^c	0.80 ^c	0.41 ^d	3.27 ^c	2.24 ^d
SE	0.03	0.02	0.01	0.04	0.01
<i>P</i> -value ²	<0.001	<0.001	<0.001	<0.001	<0.001
<i>Substrate</i>					
Fructan	2.11 ^a	1.09 ^a	0.40 ^a	3.60 ^a	1.37 ^a
Soy hulls	1.80 ^b	0.51 ^b	0.31 ^b	2.62 ^b	2.70 ^b
Potato starch	1.07 ^c	0.35 ^c	0.25 ^c	1.67 ^c	2.37 ^c
SE	0.03	0.01	0.01	0.03	0.01
<i>P</i> -value	<0.001	<0.001	<0.001	<0.001	<0.001
<i>Incubation time</i>					
4 h	0.55 ^a	0.14 ^a	0.12 ^a	0.80 ^a	1.82 ^a
8 h	0.85 ^b	0.27 ^b	0.19 ^b	1.32 ^b	1.99 ^b
12 h	1.27 ^c	0.42 ^c	0.27 ^c	1.97 ^c	2.13 ^c
24 h	1.81 ^d	0.71 ^d	0.34 ^d	2.86 ^d	2.21 ^d
48 h	2.47 ^e	1.08 ^e	0.42 ^e	3.98 ^e	2.36 ^e
72 h	2.99 ^f	1.26 ^f	0.58 ^f	4.83 ^f	2.39 ^e
SE	0.04	0.02	0.01	0.05	0.01
<i>P</i> -value	<0.001	<0.001	<0.001	<0.001	<0.001
<i>Interaction³</i>					
I × S	<0.001	<0.001	<0.001	<0.001	<0.001
I × T	<0.001	<0.001	<0.001	<0.001	<0.001
S × T	<0.001	<0.001	<0.001	<0.001	<0.001
I × S × T	<0.001	<0.001	<0.001	<0.001	<0.001

^{a-f}Least squares means in the same column of each main effect that do not have common superscript differ, $P < 0.05$.

¹Total SCFA = total short-chain fatty acids (acetate + propionate + butyrate); acetate, propionate, butyrate, total SCFA, and NH₃ are expressed as mmol/g of OM incubated; measurements were based on four replicates per inoculum per substrate per time point.

²*P*-value for main effects.

³I = inoculum source; S = substrate; T = incubation time.

Experiment 2

Cumulative gas production kinetics

Cumulative gas production per unit organic matter differed among substrates (Table 4). Peanut hulls and wheat fibre resulted in the lowest OMCV (respectively 36.0 and 34.6 ml/g OM) ($P < 0.05$). Except for wheat middlings (151.2 ml/g OM), all substrates resulted in OMCV values between 204.8 and 249.2 ml/g OM. The low gas production with wheat fibre and peanut hulls resulted in missing model parameters such that the statistical program was unable to fit the curve and allow estimation of the parameters for fermentation kinetics.

Table 4 | Fermentation parameters for each of the 16 substrates (Exp. 2).

Substrate ²	Fermentation parameters ¹					
	R _{max}	T _{max}	OMCV	A	B	C
Citrus pectin	10.0	26.2	242.6	280.3	4.00	29.77
Fructan 1	52.9	5.2	245.0	222.4	5.12	5.60
Fructan 2	68.1	4.8	277.6	262.6	5.22	5.21
Fructan 3	58.6	5.3	249.2	231.2	5.54	5.63
Gum arabic	34.2	32.3	266.3	245.2	17.95	32.53
Guar gum 1	89.4	8.8	246.7	231.6	13.67	8.91
Guar gum 2	78.7	8.7	276.4	281.4	9.81	8.83
Guar gum 3	49.5	8.2	214.6	218.5	8.17	8.53
Pea fibre	4.0	30.5	212.4	404.9	1.77	62.28
Peanut hulls	MP ³	MP	36.0	NC ⁴	0.49	NC
Soy fibre	7.3	13.0	219.6	263.4	1.95	23.25
Sugarbeet fibre	9.1	3.4	220.9	270.1	1.31	21.83
Sugarbeet pectin	80.6	5.1	271.1	251.3	6.65	5.34
Sugarbeet pulp	7.5	11.3	204.8	233.6	1.95	20.05
Wheat fibre	MP	MP	34.6	43.6	0.71	14.57
Wheat middlings	9.8	6.3	151.2	158.2	2.07	10.65
SE	3.7	1.4	21.1	29.8	0.83	2.53
MSD ⁵	19.4	7.1	113.8	158.7	4.46	13.43

¹R_{max} = maximal rate of gas production (ml/h); T_{max} = time of occurrence of R_{max} (h); OMCV = OM cumulative volume (ml of gas produced/g OM incubated); A, B, and C are parameters of the monophasic model described by Groot *et al.*⁽¹¹⁾ with A = asymptotic gas production (ml of gas produced/g OM incubated), B = switching characteristic of the curve, and C = time at which one-half of the asymptote had been reached (h); measurements were based four replicates per inoculum per substrate; least square means with different superscripts.

²Citrus pectin, Herbacel AQ Plus type F, Herbafood Ingredients GmbH, Werder, Germany; fructan 1, Zopas, DP=4; fructan 2, Raftifeed IPS, 2<DP<60; fructan 3, Beneo HP, DP>22, all fructans from Orafiti, Tienen, Belgium; gum arabic, TIC Pretasted Gum Arabic FT Powder; guar gum 1, TIC Pretasted Pre-Hydrated Guar Gum 8/24 Powder; guar gum 2, TIC Pretasted Gum Guar SCM Powder; guar gum 3, TIC Pretasted Nutriloid 010 Powder, all gums from TIC Gums, Inc. Belcamp, MD, USA; pea fibre, Exafine 250, Cosucra SA, Warcoing, Belgium; peanut hulls, supplied by a company wishing to remain anonymous; soy fibre, Fibrim 1020 IP Non-GM, The Solae Company, Le Grand-Saconnex, Switzerland; sugarbeet fibre, Fibrex 595, Danisco Sugar AB, Malmö, Sweden; sugarbeet pectin, Pectin Classic RU 301, Herbstreith & Fox KG, Neuenbürg, Germany; unmolassed sugarbeet pulp and wheat middlings, both Research Diet Services, Wijk bij Duurstede, The Netherlands; wheat fibre, Xylo-Gold Moon wheat fibre, Meneba Feed Ingredients, Rotterdam, The Netherlands.

³MP = not able to calculate R_{max} or T_{max} as described by Bauer *et al.*⁽¹²⁾ because of missing model parameters or B < 1.

⁴NC = non-convergence to monophasic model described by Groot *et al.*⁽¹¹⁾.

⁵MSD = minimum significant difference between any 2 mean values in the same column, P<0.05.

Sugarbeet pectin, the fructans, and the guar gums showed high R_{max} in combination with low T_{max} values. Among the four tested gums, incubation with gum arabic resulted in greatest T_{max} (P<0.05). Sugarbeet fibre, sugarbeet pulp, soy fibre, and wheat middlings were moderately fermentable indicated by a low R_{max} (smaller than 10 ml/h). Similarly, citrus pectin and pea fibre showed a low R_{max} but T_{max} was greater compared to sugarbeet fibre, sugarbeet pulp, soy fibre, and wheat middlings (P<0.05).

End product profile

At 8 h of incubation, sugarbeet pectin showed the largest production of acetate and total SCFA ($P<0.05$) (Table 5). The three sources of fructans produced greater amounts of propionate and butyrate compared to sugarbeet pectin ($P<0.05$). Fructans produced the second largest amount of total SCFA and the smallest amount of NH_3 compared to the other substrates ($P<0.05$). Among the four tested gums, large differences in total SCFA production were observed. The largest amount of total SCFA were produced by guar gum 1 and guar gum 3 ($P<0.05$). Fermentation of sugarbeet fibre resulted in a larger amount of acetate compared to sugarbeet pulp and citrus pectin ($P<0.05$). Finally, compared to wheat fibre, incubation with wheat middlings resulted in similar amounts of total SCFA but significantly larger amounts of butyrate ($P<0.05$).

At 72 h of incubation, the lowest SCFA production was found for peanut hulls and wheat fibre ($P<0.05$). Apart from incubation of wheat middlings, fermentation of the remaining substrates yielded SCFA values of approximately 10.5 mmol/g OM. The three fructans and guar gums resulted in the highest propionate production ($P<0.05$). Wheat fibre and peanut hulls showed smallest amounts of acetate and propionate values ($P<0.05$). Butyrate values were lowest for peanut hulls ($P<0.05$). Incubation with pea fibre and wheat middlings resulted in the greatest butyrate production. The largest amount of NH_3 production was found for wheat middlings ($P<0.05$) whereas the three fructans and gum arabic showed lowest NH_3 values compared to all other substrates except guar gum 2 ($P<0.05$).

Discussion

The present study is the first to provide insight in the fermentation activity of the microbial population along the canine distal GIT. Previous *in vitro* studies have investigated the fermentation characteristics of a wide range of fibre sources using canine ileal digesta⁽¹⁸⁻²⁰⁾ or faeces^(19,21-26) as the inoculum source. Studies using faeces as the inoculum source made a general assumption that the fermentation by faecal microbes was representative for fermentation in the lower GIT. However, the assumption has not been tested. Fermentation characteristics of a dietary fibre depends on the physical and chemical composition of the fibre, the species and numbers of microbial community present in the GIT, and on gastrointestinal transit time⁽²⁷⁾. Transit of digesta through the large intestine was found to be related to body size and varied between 9 h for Miniature Poodles and 39 h for Giant Schnauzers⁽²⁸⁾. In addition, there are community as well as numerical differences in the microbial population in the canine GIT. Suchodolski *et al.*⁽⁶⁾ reported similarities among the microbial populations in various parts residing in the canine GIT by comparing profiles of denaturing gradient gel electrophoresis (DGGE). The ileum and colon had a similarity of 38.8%, colon and rectum showed 57.9% similarity, and ileum and rectum had the smallest DGGE similarity of 32.8%. Numerically, the digesta in the ileum contains approximately 100

Table 5 | Concentration of fermentation products at 8 h and 72 h incubation for each of the 16 substrates (Exp. 2).

Substrate ²	Fermentation products ¹											
	Acetate		Propionate		Butyrate		Total SCFA		NH ₃			
	8 h	72 h	8 h	72 h	8 h	72 h	8 h	72 h	8 h	72 h		
Citrus pectin	1.59	7.82	0.38	1.34	0.26	0.93	2.23	10.09	2.60	2.60		
Fructan 1	4.57	6.30	3.07	3.97	0.49	0.74	8.13	11.01	0.75	1.80		
Fructan 2	4.72	6.54	2.88	3.86	0.51	0.74	8.11	11.14	0.66	1.69		
Fructan 3	4.43	6.63	2.58	3.61	0.48	0.77	7.49	11.01	0.89	1.71		
Gum arabic	1.31	7.75	0.30	2.26	0.23	0.67	1.85	10.68	2.66	1.94		
Guar gum 1	3.33	6.20	1.99	3.47	0.38	0.76	5.70	10.43	1.75	2.56		
Guar gum 2	2.20	5.35	1.14	3.65	0.36	0.72	3.69	9.72	2.02	2.09		
Guar gum 3	3.07	5.93	1.77	3.84	0.34	0.65	5.18	10.42	1.93	2.24		
Pea fibre	1.51	7.94	0.43	1.68	0.25	1.01	2.19	10.64	2.65	2.22		
Peanut hulls	1.25	2.04	0.36	0.56	0.26	0.30	1.86	2.90	2.74	3.39		
Soy fibre	2.14	6.81	0.78	2.99	0.28	0.79	3.20	10.59	2.69	2.56		
Sugarbeet fibre	3.41	7.20	1.09	2.10	0.32	0.68	4.82	9.98	2.37	2.69		
Sugarbeet pectin	7.57	8.40	1.74	2.20	0.38	0.52	9.69	11.12	1.93	2.88		
Sugarbeet pulp	1.99	7.61	0.56	1.94	0.33	0.85	2.88	10.40	2.74	2.82		
Wheat fibre	1.72	2.44	0.53	0.76	0.30	0.33	2.55	3.53	2.65	3.33		
Wheat middlings	2.32	5.00	0.79	2.03	0.44	0.99	3.55	8.01	3.06	4.03		
SE	0.16	0.28	0.11	0.08	0.01	0.04	0.25	0.34	0.01	0.00		
MSD	0.82	1.49	0.57	0.41	0.07	0.22	1.34	1.78	0.36	0.18		

¹Total SCFA = total short-chain fatty acids (acetate + propionate + butyrate); acetate, propionate, butyrate, total SCFA, and NH₃ are expressed as mmol/g of OM incubated; measurements were based on 4 replicates per inoculum per substrate per time point.

²Citrus pectin, Herbacel AQ Plus type F, Herbafood Ingredients GmbH, Werder, Germany; fructan 1, Zopas, DP=4; fructan 2, Raftifed IPS, 2<DP<60; fructan 3, Beneo HP, DP>22, all fructans from Orafiti, Tienen, Belgium; gum arabic, TIC Pretested Gum Arabic FT Powder; guar gum 1, TIC Pretested Pre-Hydrated Guar Gum 8/24 Powder; guar gum 2, TIC Pretested Gum Guar SCM Powder; guar gum 3, TIC Pretested Nutriloid 010 Powder, all gums from TIC Gums, Inc. Belcamp, MD, USA; pea fibre, Exafine 250, Cosucra SA, Warcoing, Belgium; peanut hulls, supplied by a company wishing to remain anonymous; soy fibre, Fibrim 1020 IP Non-GM, The Solae Company, Le Grand-Saconnex, Switzerland; sugarbeet fibre, Fibrex 595, Danisco Sugar AB, Malmö, Sweden; sugarbeet pectin, Pectin Classic RU 301, Herbstreith & Fox KG, Neuenbürg, Germany; unmolassed sugarbeet pulp and wheat middlings, both Research Diet Services, Wijk bij Duurstede, The Netherlands; wheat fibre, Xylo-Gold Moon wheat fibre, Meneba Feed Ingredients, Rotterdam, The Netherlands.

³MSD = minimum significant difference between any 2 mean values in the same column, $P<0.05$.

times less bacteria than the caecum⁽²⁹⁾ whereas the digesta in the caecum contains 250 times less total anaerobes compared to faeces⁽⁵⁾. The inoculum source used in *in vitro* studies reported in literature, however, was not obtained from the location in the canine GIT where most fermentation can be expected, i.e. the colon, and, thereby, may have over- or underestimated fermentability of dietary fibres.

Microbial fermentation activity in the present study differed among inoculum sources. In pigs, Williams *et al.*⁽³⁰⁾ found greater total SCFA production after 72 h of incubation with faecal inoculum compared to caecal inoculum. However, compared to large intestinal microbes, incubation of potato starch with small intestinal microbes resulted in higher *in vitro* gas and SCFA production values in pigs⁽³¹⁾. Bauer *et al.*⁽¹²⁾ evaluated the microbial activity of caecal, mid-colonic, and rectal inocula in pigs and found that the amount of total gas produced and R_{\max} were greatest using inocula from the rectum, intermediate for mid-colon and the lowest for the caecum. The latter authors indicated that faecal inoculum used for *in vitro* fermentation studies could actually overestimate the microbial activity of the caecum. The ranking of the substrates, however, remained the same, independent of large intestinal site from which the inoculum was obtained. This is in line with observations made here where an absolute difference in microbial activity among inoculum sources occurred. Large intestinal inocula showed slightly different fermentation kinetics but each large intestinal inoculum site gave a similar ranking of the substrates based on their SCFA production over time (i.e. SCFA yield was greatest for fructan, intermediate for soy hulls, and lowest for potato starch). The latter conclusion has important implications for *in vitro* fermentation studies to evaluate large intestinal fermentability of dietary fibre sources for use in dog foods. As the ranking remained the same for the large intestinal inocula, the use of faeces for inoculum preparation appears to be suitable for *in vitro* screening purposes. However, seeing that the total SCFA values for the proximal and transverse colonic inoculum were lower than the rectal inoculum, *in vitro* studies may be overestimate the extent of fermentation in these two sites when freshly voided faeces are used. The use of ileal digesta as an inoculum source is not recommended to simulate fermentation activity by the proximal colonic microbial population. The generally lower fermentation activity by the ileal microbial population found in the present study in combination with the rapid transit time of digesta in the ileum⁽²⁸⁾ does not make it likely that fermentation of fibre in the ileum is considerable in healthy dogs although fermentation may start there.

Fermentation of fructans with faecal inoculum resulted in SCFA production similar to values reported in other *in vitro* studies^(23,26). For soy hulls, however, total SCFA values in the current study (2.49 mmol/g OM at 12 h and 3.08 mmol/g OM at 24 h) were approximately 2 to 3 times greater than those observed by Sunvold *et al.*⁽²²⁾ and Vickers *et al.*⁽²⁶⁾ (1.02 and 0.84 mmol/g OM at 12 h, 1.40 and 1.56 mmol/g OM at 24 h, respectively). Potato starch has also been used as a substrate source but only with canine ileal digesta. Murray *et al.*⁽²⁰⁾ found a total SCFA content of 1.64 mmol/g OM after 5 h of incubation with ileal digesta which was considerably greater than the 0.64 mmol/g OM reported by Bednar *et al.*⁽¹⁹⁾ after 7.5 h of

incubation and the 0.38 and 0.37 mmol/g OM found in the present study at 4 and 8 h of incubation, respectively. There are no *in vitro* fermentation studies available in literature in which canine ileal or colonic digesta was used as inoculum source and incubated with fructan or soy hulls. Differences among studies in SCFA yield may be related to variation in chemical composition of substrates, differences in composition of microbial communities among donors as affected by e.g. genetics⁽³²⁾ or diet composition⁽²²⁾, and methodological variations such as medium⁽³³⁾ and dilution factor for inoculum preparation⁽¹²⁾.

The present study is the first to provide characteristics of fermentation kinetics of a wide variety of dietary fibres, some commonly used in commercial canine diets. The fermentation kinetics may provide information on the likely site in the GIT where the fibre source will be fermented⁽⁷⁾. Rapidly fermentable dietary fibre may be fermented more proximally in the GIT (ileum and proximal colon) whereas slower fermentable fibres are likely to reach also the more distal part of the GIT (e.g. distal colon). If fibres are very slowly fermentable, they will be voided in the faeces before fermentation is complete. The production of specific end-products of microbial degradation of dietary fibre at 8 h and 72 h may provide insight in its potential to influence the host animal. For example, butyrate, the preferred energy source of colonocytes, can play an important role in the metabolism and normal development of colonic epithelial cells. This has been implicated in protection against ulcerative colitis⁽³⁾. In addition, it should be noted that also protein and amino acids from both undigested protein and proteins originating from endogenous secretions and desquamated mucosal cells of the GIT may be metabolized by the microbial population. If fermentable carbohydrate sources become depleted due to microbial degradation, fermentation becomes proteolytic that may lead to the production of NH₃, branched-chain SCFA, amines, volatile phenols and indoles, some of which can be toxic⁽³⁴⁾. Thus, it is of interest to supply saccharolytic sources of energy (dietary fibre) and production of SCFA from this source along the entire large intestine. Furthermore, SCFA may play a role in host satiety and appetite through its stimulation of GIT satiety hormones. For example, infusion of SCFA in rats⁽³⁵⁾ and fatty acids in dogs⁽³⁶⁾ increased peripheral PYY concentrations. It is suggested that the SCFA (mainly acetate and propionate) activate a receptor (GPR43, Brown *et al.*⁽³⁷⁾; Le Poul *et al.*⁽³⁸⁾) expressed by endocrine L-cells, present predominantly in the canine distal GIT⁽³⁸⁾, which are consequently stimulated to release PYY⁽²⁾. Stimulation of the secretion of glucagon-like peptide-1 (GLP-1), another satiety hormone produced and secreted by the endocrine L-cells⁽³⁹⁾, was also found to be increased by the inclusion of fermentable fibres in diets of dogs⁽⁴⁰⁾. It should be noted, however, that other characteristics of dietary fibres such as ability to bind water and ability to affect viscosity of digesta may also contribute to the prolongation of satiety.

Cumulative gas production from the OM, R_{\max} , and T_{\max} as well as the end product profile at 8 and 72 h of incubation differed among substrates. According to the results of this experiment, sugarbeet pectin, the three fructans, the guar gums, and gum arabic can be characterized as rapidly fermentable (large R_{\max}). Soy fibre, pea fibre, sugarbeet fibre,

sugarbeet pulp, and wheat middlings were slowly fermentable. Wheat fibre and peanut hulls were poorly fermentable. For fermentation of gum arabic, it should be noted that this substrate showed significantly greater T_{\max} compared to the other rapidly fermentable substrates. It appeared that the substrate was poorly fermentable during the first part of incubation. The poor fermentability of gum arabic was also found in other studies as indicated by low SCFA production at 6, 12, and 24 h of incubation^(22,23). The large T_{\max} may be due to a low amount of inoculated faecal microbes capable of degrading the substrates. After selective growth of these microbes during incubation gum arabic appeared to be rapidly degradable indicated by the large R_{\max} (34.2 ml/h). The data here suggests that gum arabic may also be rapidly fermentable *in vivo* but after adaptation to the substrate.

There was no effect of DP of tested fructans on fermentation rate or end product profile ($P>0.05$). Vickers *et al.*⁽²⁶⁾ also did not find differences in SCFA production among fructans varying in DP. However, Roberfroid *et al.*⁽⁴¹⁾ reported that fructans with a DP<10 have a faster *in vitro* degradation rate compared to fructans with a longer chain-length. Concerning the end product profile, rats fed a diet with fructan with a low DP showed higher butyrate concentration, whereas rats fed a diet with fructans with a high DP give a higher concentration of propionate⁽⁴²⁾. Furthermore, rats fed fructan containing diets showed increased GLP-1 content and proglucagon mRNA in the proximal colon compared to controls with the short-chain inulin-type fructans (low DP) more potent to induce this effect⁽⁴³⁾. More *in vivo* studies are needed to demonstrate that the rate of degradation is actually different among fructans varying in DP.

Combined information about the fermentation kinetics and the individual SCFA produced at 8 and 72 h of a dietary fibre may be indicative of where the fibre is likely to be fermented in the GIT and where the end products (i.e. SCFA) may become available. As dietary inclusion level of rapidly fermentable fibres is limited and some fermentation of dietary fibre may start already in the ileum, the rapidly fermentable fibre source may become depleted resulting in low amounts of fermentable carbohydrates more distal in the colon. The fibre sources characterized as slowly fermentable *in vitro* are expected to also be slowly fermentable *in vivo* and will yield moderate amounts of SCFA along the distal GIT. Howard *et al.*⁽⁴⁴⁾ suggested that a combination of fibre sources could potentially be used to stimulate fermentation along the complete distal GIT in dogs. From the present study this can be achieved by combining sugarbeet pectin or fructans, which are rapidly fermentable and yield large amounts of acetate and propionate, with pea fibre or sugarbeet pulp, which are slower fermentable and yielded large amounts of butyrate. The latter combination of fibre sources may be optimal not only for GIT health but also to stimulate the secretion of PYY and GLP-1. The latter could prolong feelings of satiety after a meal in dogs.

The present study shows that faeces can be used as an inoculum source for *in vitro* fermentation studies but may slightly overestimate the actual fermentation processes occurring more proximally in the large intestine. The use of ileal digesta as an inoculum source is not recommended. The second experiment revealed a large variation in the amount

of fermentable components, the fermentation kinetics, and in the end product profile among tested fibre sources. Sugarbeet pectin, the three fructans, the guar gums, and gum arabic were rapidly fermentable whereas soy fibre, pea fibre, sugarbeet fibre, sugarbeet pulp, and wheat middlings were slowly fermentable. Wheat fibre and peanut hulls were poorly fermentable. Sugarbeet pectin and the fructans resulted in the great amounts of acetate and propionate while pea fibre, wheat middlings, and citrus pectin yielded the large amounts of butyrate. These results can be used to formulate canine diets to modulate the amount and site at which fermentation end products are generated to optimize not only GIT health but potentially also the level of satiety in dogs.

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Chapter 4

The effects of dietary fibre type on satiety-related hormones and voluntary food intake in dogs

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Abstract | Depending on the type and inclusion level, dietary fibre may increase and maintain satiety and postpone the onset of hunger. This 7-week study evaluated the effect of fibre fermentability on physiological satiety-related metabolites and voluntary food intake (VFI) in dogs. Sixteen healthy adult dogs were fed a low-fermentable fibre (LFF) diet containing 8.5% cellulose or a high-fermentable fibre (HFF) diet containing 8.5% sugarbeet pulp and 2% inulin. Large intestinal fibre degradation was evaluated by apparent faecal digestibility of nutrients and faecal short-chain fatty acids and ammonia concentrations. Postprandial blood samples were obtained to determine postprandial plasma glucose, insulin, total peptide tyrosine tyrosine (PYY), total glucagon-like peptide-1 (GLP-1), and total ghrelin concentrations. At the end of the study, dogs were given a single meal of a dry dog food to determine VFI. Dogs fed the HFF diet had a significant higher ($P<0.05$) large intestinal fibre degradation and production of SCFA compared to the dogs fed the LFF diet. HFF-fed dogs tended ($P=0.058$) to show a lower VFI at the end of the study. No treatment effects were found for postprandial plasma glucose, PYY, GLP-1, and ghrelin responses. The concentrations of these metabolites could not be related to the observed difference in VFI. The inclusion of fermentable fibre in canine diets may contribute to the prevention or mitigation of obesity through its effects on satiety. The underlying mechanisms require further investigation.

Introduction

Obesity is the most common nutritional disorder in companion animals nowadays⁽¹⁾. Studies conducted in different countries (e.g. England, Australia, United States of America) have estimated the incidence of overweight and obesity in the dog population between 22 and 40%⁽²⁾. The cause of overweight and obesity is a chronic energy intake that exceeds energy expenditure. Dietary fibre may aid in the mitigation and prevention of obesity as it may increase and maintain satiety and prevent feeling of hunger in dogs. The feeling of hunger may result in an increase in begging and scavenging behaviour⁽³⁾ which in turn may encourage owners to feed their pet more than the animal's physiological energy requirement⁽⁴⁾.

Several studies have evaluated the effect of dietary fibre on satiety in dogs. Jewell & Toll⁽⁴⁾ and Jackson *et al.*⁽⁵⁾ showed a reduced daily energy intake when dogs were fed high-fibre diets. In addition, voluntary food intake (VFI) of an additional meal 75 min after consumption of the morning meal was lower in dogs fed high fibre diets⁽⁴⁾. No effect of dietary fibre on VFI in dogs was found by Butterwick & Markwell⁽⁶⁾. However, the dogs in the latter study were overweight and supplied with approximately 45% of calculated maintenance energy requirements at target body weight (BW) to induce weight loss. This restriction in daily energy intake may have resulted in an increased feeding motivation to a level that nullified possible effects of dietary fibre on satiety⁽⁵⁾.

Several physical and chemical properties of dietary fibres may influence the duration of postprandial satiety. Fibre fermentability yielding SCFA may affect satiety through its actions on the production and secretion of gastrointestinal satiety hormones. Infusion of SCFA in the colon of rats⁽⁷⁾ and oleic acid in the colon of dogs⁽⁸⁾ increased peripheral peptide tyrosine tyrosine (PYY) concentrations. PYY can cross the blood-brain-barrier and act on the arcuate nucleus of the hypothalamus, stimulating neurons that create a sensation of satiety and inhibiting neurons that stimulate feeding behaviour⁽⁹⁾. Stimulation of the secretion of glucagon-like peptide-1 (GLP-1), a proglucagon-derived peptide secreted by the enteroendocrine L-cells present in the distal part of the gastrointestinal tract⁽¹⁰⁾, was increased by the inclusion of fermentable fibres in diets of dogs during an oral glucose tolerance test⁽¹¹⁾. Both PYY and GLP-1 contribute to the ileal-brake and increase gastric emptying time and small intestinal transit time⁽¹²⁾. This may prolong gastric distension and gastric signals of satiation⁽¹³⁾ and prolong contact between nutrients and small intestinal receptors involved in maintaining satiety⁽¹⁴⁾. A delay in gastric emptying will also delay starch digestion and subsequent absorption of glucose⁽¹⁵⁾ thereby maintaining more stable postprandial glucose and insulin concentrations in the blood⁽¹⁶⁾. Sows fed a diet high in sugarbeet pulp had stable postprandial glucose concentrations compared to sows fed a low-fibre diet that showed a drop in glucose concentration below basal levels. This was associated with an increase in physical activity possibly caused by feelings of hunger⁽¹⁷⁾. Fermentable fibres have also been found to affect peripheral ghrelin concentrations, a hormone correlated to hunger or appetite⁽¹⁸⁾. Rats fed diets supplemented with a short-chain oligofructose showed lower active

ghrelin plasma concentrations 8 h after the last meal compared to rats fed the diet without fructan supplementation⁽¹⁹⁾.

There is still little information available regarding the potency of various fermentable fibres to affect satiety in dogs. The aim of the current study was therefore to investigate whether an increase in dietary fibre fermentability prolongs the duration of postprandial satiety as measured by VFI and physiological satiety-related metabolites when included in diets of dogs.

Experimental methods

Animals

Sixteen (eight male and eight female) healthy adult beagle dogs aged between 2 and 6 years with an initial bodyweight (BW) between 7.2 and 11.4 kg were individually housed in indoor pens at the Laboratory of Animal Nutrition of Ghent University (Merelbeke, Belgium). Dietary treatments were equally distributed among pens. The dogs were assigned to one of two dietary treatments (low-fermentable fibre (LFF) or high-fermentable fibre (HFF)) according to BW and sex (blocking factors) resulting in a mean (SEM) BW of 9.7 (0.5) kg and 9.7 (0.4) kg for the high-fermentable fibre (HFF) group. All the dogs were weighed before the start of the experiment and thereafter every 2 weeks until the end of the experiment. Each dog was fed individually to meet its maintenance energy requirement estimated at 415 kJ metabolisable energy/kg BW^{0.75} ⁽²⁰⁾. The diets were fed twice daily in two equal portions at 08:30 and 18:30 after mixing with an equal amount of lukewarm water to increase palatability. Food intake was recorded during each meal throughout the entire experimental period and freshwater was provided *ad libitum*. All animal housing, care and experimental procedures were approved by and conformed to the requirements of the Ethical Committee of the Faculty of Veterinary Medicine of the Ghent University (Belgium, EC 2007/40).

Diets

The dogs were fed one of the two experimental diets formulated to be iso-nitrogenous and iso-energetic on a metabolisable energy basis, and iso-fibrous on a total dietary fibre (TDF) basis. Ingredient composition of both diets is shown in Table 1. The LFF diet contained cellulose as a fibre source, whereas the HFF diet contained a combination of sugarbeet pulp and inulin. Differences in fermentability between fibre sources used were based on the *in vitro* studies (Chapter 3 and Sunvold *et al.*⁽²¹⁾). The content of molasses in the sugarbeet pulp was estimated to be 5% and an identical amount of molasses was added to the LFF diet. TiO₂ (2 g per kg diet) was included as an inert digestibility marker⁽²²⁾.

Chemical analyses

Diets were analysed for DM, ash, starch, sugar, crude protein, crude fat, total dietary fibre (TDF), insoluble dietary fibre, neutral detergent fibre (NDF), acid-detergent fibre

Table 1 | Composition of the low-fermentable fibre (LFF) and high-fermentable fibre (HFF) diets.

Composition	LFF	HFF
<i>Ingredients (g/kg as is)¹</i>		
Wheat starch (pre-gelatinised)	468.8	463.0
Poultry meat meal (low-ash)	285.0	275.0
Poultry fat	135.0	135.0
Cellulose	85.0	0.0
Sugarbeet pulp (molassed)	0.0	85.0
Inulin	0.0	20.0
Premix ²	10.0	10.0
Digest	10.0	10.0
Molasses	4.3	0.0
Titanium(IV) oxide	2.0	2.0
<i>Nutrients (g/kg DM)</i>		
Ash	37.5	42.0
Starch	372.4	367.5
Sugar	13.6	41.6
Crude protein	274.1	262.2
Crude fat	191.4	191.2
TDF	123.7	93.9
IDF	110.9	74.7
SDF ³	12.8	19.2
NDF	139.4	99.5
ADF	93.9	41.7
ADL	11.1	10.8
NSP ⁴	111.0	95.1
<i>Energy content (kJ/100 g DM)</i>		
Gross energy	2294	2300

TDF, total dietary fibre; IDF, insoluble dietary fibre; SDF, soluble dietary fibre; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; NSP, non-starch polysaccharides.

¹Wheat starch, Pregel Wheat Alpha (Meneba, Weert, The Netherlands); poultry meat meal, Meat Meal 63 (Sonac, Lingen, Germany); poultry fat (Sonac, Lingen, Germany); cellulose, Arbocel BW40 (J. Rettenmaier Benelux, Zutphen, The Netherlands); sugarbeet pulp, molasses (Research Diet Services, Wijk bij Duurstede, The Netherlands); inulin, Beneo IPS (Orafti, Tienen, Belgium); premix (Twilmij B.V., Stroe, The Netherlands); digest, Luxus Digest N8008 (AFB International, Nuland, The Netherlands); titanium(IV) oxide (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands).

²The premix provided per kilogram of diet: Ca, 0.41 g; P, 0.07 g; Mg, 0.05 g; K, 0.1 g; Na, 0.01 g; Cl, 0.09 g; linoleic acid, 0.15 g; PUFA, 0.17 g; lysine, 0.05 g; methionine, 0.02 g; methionine+cysteine, 0.04; threonine, 0.04 g; tryptophan, 0.02 g; retinol, 5.25 mg; vitamin D₃, 50 µg; vitamin E, 100 mg; vitamin K₃, 2 mg; vitamin B₁, 10 mg; vitamin B₂, 10 mg; niacin, 50 mg; pantothenic acid, 25 mg; vitamin B₆, 7.5 mg; vitamin B₁₂, 50 µg; biotin, 300 µg; choline chloride, 475 mg; folic acid, 1.25 mg; vitamin C, 100 mg; Fe, 75 mg; Mn, 35 mg; Cu, 5 mg; Zn, 75 mg; I, 1.75 mg; Co, 2 mg; and Se, 0.2 mg.

³Calculated by subtracting the IDF content from the TDF content.

⁴Derived by subtracting the crude protein, crude fat, starch, and sugar content from the organic matter content⁽¹⁷⁾. As inulin was included in the analysed sugar content, the NSP content of the HFF diet is underestimated with approximately 18 g/kg DM (20 g/kg included in the diet with 90% pure inulin).

(ADF), acid-detergent lignin and Ti. DM and ash contents were determined by drying to a constant weight at 103°C and combustion at 550°C, respectively. The starch content was

analysed enzymatically⁽²³⁾, while reducing sugars were extracted from the feed samples using 40% ethanol and determined as described by Suárez *et al.*⁽²⁴⁾. Crude protein ($6.25 \times N$) was determined using the Kjeldahl method (ISO 5983-1, 2005) and crude fat was analysed according to the Berntop method (ISO 6492, 1999) with faecal samples being pre-digested with HCl. TDF and insoluble dietary fibre were analysed using the Association of Official Analytical Chemists methods^(25,26). The soluble dietary fibre content was calculated by subtracting the insoluble dietary fibre content from the TDF content. Note that the inulin would not be recovered in the TDF fraction⁽²⁷⁾. NDF was analysed in defatted diet samples (fat extraction with petroleum-ether) according to a modified method of Van Soest *et al.*⁽²⁸⁾ described by Goelema *et al.*⁽²⁹⁾. The ADF and acid-detergent lignin contents were determined according to Van Soest⁽³⁰⁾. Ti was analysed using a modified method based on the work by Short *et al.*⁽³¹⁾ and Myers *et al.*⁽³²⁾. The content of NSP was calculated by subtracting the starch, sugar, crude protein and crude fat content from the organic matter content⁽¹⁷⁾. As inulin was included in the analysed sugar content, the NSP content of the HFF diet is underestimated with approximately 18 g/kg DM (20 g/kg included in the diet with 90% pure inulin).

Apparent digestibility

After 10 d of adaptation to the experimental diets, a 3 d faecal collection was conducted for the determination of apparent digestibility of nutrients. On these days, all faeces produced by each dog were collected twice a day and weighed. The faeces were freeze-dried to a constant weight, pooled per dog and ground over a 1mm sieve in a Retsch mill (ZM100, Retsch B.V., Ochten, The Netherlands). Then the faeces of each dog were analysed for DM, ash, crude protein, crude fat, NDF, ADF, acid-detergent lignin and Ti, according to the procedures described previously. Starch and sugar were not analysed as these were assumed to be completely digested and absorbed. The NSP content of faeces was calculated by subtracting the crude protein and fat contents from the organic matter content. The apparent digestibility coefficient (ADC) for the nutrients was calculated using the following equations:

$$\text{Nutrient}_{\text{flow}} = \text{Nutrient}_f \times \text{Ti}_i / \text{Ti}_f$$

$$\text{ADC (\%)} = (\text{Nutrient}_i - \text{Nutrient}_{\text{flow}}) / \text{Nutrient}_i \times 100\%$$

where $\text{Nutrient}_{\text{flow}}$ is the nutrient flow (g/d), Nutrient_f is the nutrient content of faeces (g/kg DM), Ti_i is the titanium intake (g), Ti_f is titanium content of faeces (g/kg DM), and Nutrient_i is nutrient intake (g/d).

Faecal consistency and fermentation products

To evaluate colonic microbial fermentative activity for both dietary treatment groups, fresh faeces were collected from each dog during week 5 of the experiment within 15 min of defecation. Faeces consistency was scored using the following system⁽³³⁾: 1 = hard, dry pellets – small, hard mass; 2 = hard, formed, dry stool – remains firm and soft; 3 = soft, formed

moist – softer stool that retains shape; 4 = soft, unformed – stool assumes shape of container; and 5 = watery – liquid that can be poured. Directly after faecal scoring, faeces were collected and homogenised using two spoons whereafter samples were taken for SCFA, NH₃, and DM contents. All materials used for faeces collection and sampling were pre-sterilised using 70% ethanol. For the determination of faecal SCFA and NH₃ content, a sample of approximately 0.5-1.0 g was added to a 2-ml safe-lock tube (Eppendorf AG, Hamburg, Germany) containing 1.0 ml 0.1 N H₃PO₄ for SCFA analysis or 1.0 ml 10% TCA for NH₃ analysis. After the addition of faeces, the contents of each tube were mixed on a vortex for 3 s, weighed and stored at -20°C. For DM determination, approximately 1.5 g of faeces was added to a pre-weighed 2-ml safe-lock tube (Eppendorf AG), weighed and stored at -20°C. For determination of SCFA and NH₃, samples were thawed, mixed and centrifuged at 15 000 rpm for 5 min at 4°C (Centrifuge 5417R, Eppendorf AG). Concentrations of the SCFA (i.e. acetate, propionate, butyrate, *iso*-butyrate, valerate, and *iso*-valerate) in the supernatant were determined as described in Chapter 3. Branched-chain proportion was calculated as the percentage of branched-chain fatty acids (*iso*-butyrate, valerate, *iso*-valerate) of total SCFA⁽³⁴⁾. The faecal DM content was determined by freeze-drying to constant weight and used to calculate SCFA and NH₃ content in the original faeces.

Blood sampling and plasma analyses

Blood sampling was performed in week 6 of the experiment. Dogs were sedated using 0.02 ml/kg BW methadone hydrochloride (Mephenon®, Denolin, Bussels, Belgium) and a central venous catheter (18G/20 cm Leaderflex®; Vygon, Écouen, France) was placed in the jugular vein. The catheters were flushed with 1 ml heparinised saline (0.1 mg heparin/ml saline solution) directly after catheter placement and just before the sampling procedure. Furthermore, at time of placement of the catheter, 15 mg/kg BW Amoxyceilline (Clamoxyl LA®, GlaxoSmithKline N.V., Genval, Belgium) was administered subcutaneously. Blood samples (2.5-3.0 ml) were obtained from each dog 30 min prior to feeding and 20, 40, 60 and 90 min postprandial. Thereafter, blood was sampled from four dogs in each group at 120, 180, 240, 300, 360, 420, 480 and 540 min after feeding, while the other four dogs in each group were sampled at 150, 210, 270, 330, 390, 450, 510 and 570 min after feeding. The blood samples were collected in chilled collection tubes containing K₃EDTA as an anticoagulant. After gentle mixing of the contents, each collection tube was opened and 25 ml dipeptidyl peptidase-IV inhibitor (Linco Research, MI, USA) and 125 µl Trasylol® (1.4 mg aprotinin, Bayer AG, Leverkusen, Germany) were added. After gentle mixing of contents, tubes were temporarily stored on ice until centrifugation at 2500 × *g* for 15 min at 4°C. After centrifugation, plasma was removed and stored in safe-lock tubes (Eppendorf AG) at -20°C until analysis. Each blood sample was processed within 30 min after collection. Blood plasma was analysed for glucose, insulin, total PYY, total GLP-1, and total ghrelin concentration. Plasma glucose was analysed according to the hexokinase method using a commercial test kit (Human GmbH, Wiesbaden, Germany), while plasma insulin, total PYY and total ghrelin

were analysed using commercial RIA kits (human-specific insulin RIA kit, Linco Research; rat/mouse PYY RIA kit, Linco Research; and total ghrelin RIA kit, Linco Research, respectively). Plasma GLP-1 was analysed using an RIA specific for the C-terminal of the amidated GLP-1^(35,36). The intra-assay coefficients of variation for the assays were 7.1% for insulin, 6.2% for ghrelin, 14.8% for PYY and 6% for GLP-1. The values obtained at 120 and 150, 180 and 210, 240 and 270, 300 and 330, 360 and 390, 420 and 450, and 480 and 540 min postprandial were analysed together and are presented as time points 135, 195, 255, 315, 375, 435 and 495 min, respectively. The basal concentration was defined as the average of the level in the first and last samples (30 min before the morning feeding and 45 min before the evening feeding, respectively). For PYY, GLP-1 and ghrelin, the area under the curve (AUC) from basal until 195 min after the meal and the AUC from 195 to 495 min after the meal for each measured parameter were approximated using the trapezoidal summation. Trapezoids were calculated as the length of the base (interval time between consecutive samples in min) times the average of the heights of the two sides (concentrations of consecutive samples). The time intervals were selected based on a minimal oro-caecal transit time of approximately 2.7 h in Standard Schnauzers with a BW of 12.9 (SEM 2.1) kg⁽³⁷⁾. From this time onwards, the digesta arrives in the large intestine and fermentable dietary fibre becomes available for the microbial population and SCFA may be produced.

Voluntary food intake

At the end of the study (week 7), each dog was offered 1 kg of the dry extruded control diet that dogs previously experienced as palatable (Hill's Science Plan Canine Adult with Beef, Hill's Pet Nutrition Inc., Topeka, KS, USA). The dogs were allowed to eat for 20 min, after which food intake was recorded. The diet was offered to each dog at precisely 6 h after the morning feeding (14:30).

Statistical analyses

The dogs were randomly allocated to the two treatments according to the BW and sex. All data were analysed using the Statistical Analysis Systems statistical software package version 9.1 (SAS Institute, Cary, NC, USA). Differences in the ADC of nutrients, faecal characteristics (faecal score, DM, SCFA and NH₃) and plasma metabolites (the basal concentrations of glucose, insulin, PYY, GLP-1, and ghrelin and AUC (0-195 min and 195-495 min) of PYY, GLP-1, and ghrelin) between the dietary treatment groups were tested for significance using ANOVA by Proc GLM. The model used was $Y = \mu + D_i + \epsilon_{ij}$, where Y is the dependent variable, μ is the average intercept, D_i is the effect of diet i, and ϵ_{ij} is the error term. For the VFI data, BW loss (as the percentage of initial BW) tended to be significant ($P=0.098$) and was therefore included in the statistical model as a covariate. The effects of diet and time after feeding on plasma glucose, insulin, PYY, GLP-1 and ghrelin were tested for significance using ANOVA by Proc MIXED. The statistical model was $Y = \mu + D_i + T_j + (D \times T)_{ij} + \epsilon_{ijk}$, where Y is the dependent variable, μ is the average intercept, D_i is the

effect of diet i , T_j is the effect of time j , $(D \times T)_{ij}$ is the interaction between diet and time, and ε_{ijk} is the error term. The basal concentrations were significant ($P < 0.010$) and included in the model as covariate. The correlations between VFI and plasma glucose and hormone concentrations were calculated using the Proc CORR statement. Differences were considered to be significant at $P \leq 0.05$.

Results

All dogs remained healthy throughout the study, although a general decrease in the BW was observed for both groups (approximately 5% BW loss for each dietary treatment). No significant differences were found between the dietary treatments in the BW at the start and end of the experiment and BW loss ($P = 0.906$, $P = 0.909$, and $P = 0.927$, respectively; data not shown). One dog in the LFF treatment group lost substantial BW during the trial and showed very high concentrations of ghrelin compared with the other dogs. The obtained physiological and VFI data from this dog were therefore excluded from the statistical analyses.

Apparent digestibility

The dogs fed the HFF diet showed higher ADC for DM and organic matter ($P < 0.001$), whereas the LFF-fed dogs had a higher ADC for crude fat ($P < 0.001$) and tended to have a higher crude protein digestibility ($P = 0.099$; Table 2). The NSP digestibility was higher for the HFF diet compared to the LFF diet ($P < 0.001$). In addition, dogs fed the HFF diet showed higher ADC for NDF ($P < 0.001$) and ADF ($P = 0.002$) and tended to have a lower ADC for acid-detergent lignin ($P = 0.082$) compared to dogs fed the LFF diet. Finally, the ADC for energy was higher for the HFF-fed dogs compared to LFF-fed dogs ($P < 0.001$).

Table 2 | Apparent digestibility coefficient (ADC) for nutrients and energy in the low-fermentable fibre diet (LFF, $n=8$) or the high-fermentable diet (HFF, $n=8$) fed to dogs.

ADC (%)	LFF		HFF		P-value
	Mean	SEM	Mean	SEM	
DM	77.9	0.28	80.9	0.56	<0.001
OM	80.6	0.25	84.1	0.57	<0.001
Crude protein	77.3	0.81	75.4	0.68	0.099
Crude fat ¹	94.5	0.19	92.3	0.18	<0.001
NDF	37.0	0.59	62.3	2.14	<0.001
ADF	3.3	0.96	22.3	5.00	0.002
ADL	43.7	4.13	34.9	2.26	0.082
NSP	-2.8	0.69	20.6	4.96	<0.001
Gross energy	82.7	0.25	84.9	0.43	<0.001

SEM, standard error of the mean; OM, organic matter; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; NSP, non-starch polysaccharides.

¹Due to limited amount of faecal material available for analysis values presented were based on seven dogs for the LFF and six dogs for the HFF treatment.

Faecal consistency and fermentation products

Significant differences in the faecal characteristics between the treatment groups were observed (Table 3). The faecal DM content was lower for the dogs fed the HFF than the LFF ($P<0.001$) diet. Compared with the dogs fed the LFF diet, higher total SCFA, acetate and propionate concentrations were found for the dogs fed the HFF diet ($P<0.001$). Moreover, butyrate concentrations tended to be higher in the HFF dogs ($P=0.060$). The dogs fed the LFF diet showed a higher branched-chain ratio and NH_3 concentration in the faeces compared to the dogs fed the HFF diet ($P=0.002$ and $P=0.009$, respectively). No treatment effect was found for faecal consistency score ($P=0.590$).

Table 3 | Characteristics of the faeces of the dogs fed the low-fermentable fibre (LFF, $n=8$) diet and the high-fermentable (HFF, $n=8$) diet.

Parameter	LFF		HFF		P-value
	Mean	SEM	Mean	SEM	
DM (g/kg)	379.1	15.5	231.0	9.5	<0.001
Total SCFA (mmol/g DM)	0.26	0.02	0.54	0.04	<0.001
Acetate (mmol/g DM)	0.14	0.02	0.32	0.03	<0.001
Propionate (mmol/g DM)	0.06	0.01	0.14	0.01	<0.001
Butyrate (mmol/g DM)	0.03	0.00	0.05	0.01	0.060
BCP (%) ¹	8.51	0.87	4.40	0.68	0.002
NH_3 (mg/g DM)	2.73	0.24	3.45	0.53	0.240
NH_3 (mg/ml faecal water)	1.66	0.15	1.02	0.15	0.009
Faecal score (1-5)	2.44	0.11	2.50	0.00	0.590

SEM, standard error of the mean; SCFA, short-chain fatty acids; BCP, branched-chain proportion.

¹Calculated as the percentage of branched-chain fatty acids (*iso*-butyrate, valerate, *iso*-valerate) of total SCFA⁽³⁴⁾.

Table 4 | Plasma glucose, insulin, PYY, GLP-1, and ghrelin parameters in dogs fed a low-fermentable fibre diet (LFF, $n=7$) or a high-fermentable fibre diet (HFF, $n=8$).

Parameter	LFF		HFF		<i>P</i> -value
	Mean	SEM	Mean	SEM	
<i>Basal concentration</i>					
Glucose (mmol/l)	5.6	0.2	5.8	0.2	0.481
Insulin (μ U/ml)	10.5	1.8	10.2	1.7	0.918
PYY (pg/ml)	954	79	993	79	0.734
GLP-1 (pmol/l)	18.6	2.3	23.1	1.9	0.164
Ghrelin (pg/ml)	2308	492	2900	461	0.396
<i>AUC_{0-195 min}</i>					
PYY ((pg/ml)/min)	1114	56	1117	56	0.977
GLP-1 ((pmol/l)/min)	26.4	3.3	25.8	2.8	0.885
Ghrelin ((pg/ml)/min)	1852	338	2094	293	0.599
<i>AUC_{195-495 min}</i>					
PYY ((pg/ml)/min)	1060	47	1074	47	0.833
GLP-1 ((pmol/l)/min)	24.4	1.9	25.6	1.8	0.681
Ghrelin ((pg/ml)/min)	2200	473	2657	410	0.479

SEM, standard error of the mean; AUC, area under the curve.

Plasma metabolites

Plasma glucose, insulin, PYY, GLP-1, and ghrelin parameters for both the dietary groups are shown in Table 4. The basal concentrations of measured metabolites were not different between the treatments groups ($P>0.05$). For all the measured metabolites, postprandial concentrations changed after the meal ($P<0.01$), but concentrations were not affected by the dietary treatment ($P>0.10$ for diet and diet \times time-interaction, data not shown). No significant differences were found between treatment groups in AUC_{0-195 min} and AUC_{195-495 min} of PYY, GLP-1, and ghrelin ($P>0.10$).

Voluntary food intake

For each dog, the amount of food consumed at the end of the study was lower than the amount of food offered. The dogs fed the HFF diet tended to show a lower VFI compared with the dogs fed the LFF diet ($P=0.058$, Figure 1). No significant correlations were found between VFI and glucose, insulin, PYY, GLP-1 or ghrelin concentration in plasma at 6 h after the meal ($P>0.05$, data not shown).

Discussion

The present study evaluated the impact of dietary fibre fermentability on the duration of postprandial satiety as measured by the hormones involved in satiation and VFI in dogs. The selection of fibre sources was based on the *in vitro* fermentation studies^(21,22) that showed a low microbial degradability for cellulose and moderate and rapid fermentability for,

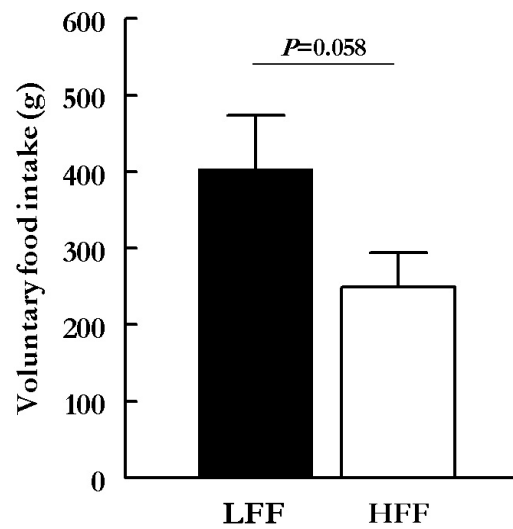


Figure 1 | Voluntary food intake of the low-fermentable fibre (LFF) and high-fermentable diet (HFF)-fed dogs. Dogs had *ad libitum* access for 20 min to the control diet that was presented 6 h following their morning meal (experimental diet). Values are means for seven dogs fed the LFF and eight dogs fed the HFF, with their standard errors represented by vertical bars.

respectively, sugarbeet pulp and inulin using the faeces from the dogs as inoculate. The difference in fibre degradability between the two diets was also shown in the present study. The dogs fed the HFF diet showed a higher ADC for NDF, ADF and NSP compared with the LFF-fed dogs, indicating a higher intestinal microbial degradability of those fibre sources used in the HFF diet. The higher microbial fibre degradation in the HFF-fed dogs resulted in a higher SCFA production, also reflected in a higher SCFA concentration in the faeces of these dogs. In the case of low availability of fermentable fibre (as with the LFF diet), the microbial population will probably resort to more proteolytic fermentation⁽³⁸⁾. This was observed in the present study where the LFF-fed dogs showed a higher faecal NH_3 concentration and BCP, both being the indicators of microbial protein degradation⁽³⁹⁾. Finally, the ADC for crude protein tended to be lower for the dogs on the HFF diet, which is in agreement with the similar studies evaluating fermentable dietary sources^(20,40,41). It is suggested that this decrease should not be attributed to a lower true protein digestibility⁽⁴⁰⁾, but is related to an increased microbial proliferation and to a higher faecal bacterial protein excretion^(20,41). From these summarised results, it can be concluded that compared with the LFF diet, the HFF diet resulted in higher large intestinal dietary fibre fermentation. This would consequently lead to higher SCFA concentrations in the large intestine.

The increased large intestinal fermentation was expected to have an impact on host satiety and appetite through its effect on the secretion of the gastrointestinal satiety-related hormones PYY, GLP-1 and ghrelin. Concerning the feelings of satiety and appetite, the dogs in the HFF treatment group tended to have a lower VFI compared with the LFF-fed dogs

($P=0.058$). This suggests that dogs fed the HFF diet were less motivated to consume food when freely available. The amount of food consumed was however not correlated with any of the measured physiological metabolites. The causal relationship between the postprandial satiety-related hormone concentrations and the feelings of satiety or hunger varies between studies. For example, several recent studies found an association in the human subjects of changes in self-reported hunger or satiety after a test meal with changes in the concentrations of postprandial PYY^(42,43), whereas Weickert *et al.*⁽⁴⁴⁾ reported blunted postprandial PYY and ghrelin responses in healthy women without alterations in hunger scores. Furthermore, Smeets *et al.*⁽⁴⁵⁾ found that a high-protein lunch increased satiety but without increasing the plasma GLP-1 response, whereas a lunch adequate in protein but with a high carbohydrate content resulted in lower satiety rating but with increased GLP-1 response. Based on these findings, it was therefore suggested that the concentrations in the satiety-related metabolites may be related to the nutrient-induced satiety without being directly and mathematically related to satiety⁽⁴⁶⁾. This relationship may also indicate differences in interactions with other hormones or central sensitivity for these hormones⁽⁴⁵⁾.

It can be questioned whether the dietary contrasts in the present study were sufficient to evoke differences in secretion of measured hormones. The HFF contained 85 g/kg as fed sugarbeet pulp and 20 g/kg as fed inulin that was slightly higher compared to the HFF diet used by Massimino *et al.*⁽¹¹⁾ (60 g/kg sugarbeet pulp, 20 g/kg gum arabic, 15 g/kg fructo-oligosaccharide on as fed basis). Dogs fed the latter diet showed enhanced GLP-1 production and plasma GLP-1 concentrations after an oral load of glucose compared to dogs fed a diet containing 70 g/kg as fed cellulose⁽¹¹⁾. The dietary contrast in the current experiment can therefore be considered to have the potential to affect at least GLP-1 production and secretion

The present study aimed to induce a contrast in large intestinal SCFA concentrations that would affect the secretion of PYY and GLP-1 by the enteroendocrine L-cells. These specialized cells are present predominantly in the canine distal gastrointestinal tract⁽⁴⁷⁾. It has been suggested that SCFA (mainly acetate and propionate) activate the GPR43-receptor expressed by the L-cells which are consequently stimulated to release PYY⁽⁴⁸⁾. Several studies reported increased PYY release after large intestinal infusion of SCFA in rats⁽⁷⁾ and oleic acid in dogs⁽⁸⁾. In addition, inclusion of fermentable fibre in a diet increased large intestinal PYY gene expression⁽⁴⁹⁾ and PYY concentrations in rats^(49,50). Gee and Johnson⁽⁵¹⁾ reported similar effects of a single meal of fermentable fibre on plasma PYY concentrations in rats, but in human subjects the observed effects were less. In addition to the production of PYY, L-cells produce GLP-1 derived from the precursor molecule proglucagon⁽¹⁰⁾. Several studies reported enhanced expression of the proglucagon gene by SCFA⁽⁵²⁾ or inclusion of fermentable fibre in the diet^(11,49). Moreover, fermentable fibre increased the number of L-cells in the proximal colon of rats⁽⁵³⁾. On the other hand, GLP-1 release was not stimulated after large intestinal SCFA infusion in rats⁽⁷⁾. Interactions between satiety-related hormones may have contributed to observed effects on satiety. For example, Neary *et al.*⁽⁵⁴⁾ observed

additive effects of PYY and GLP-1 in the inhibition of appetite and induction of satiety. Similarly, in obese rats a combination of intraperitoneal injection of amylin and PYY was found to reduce food intake more than amylin or PYY alone⁽⁵⁵⁾.

Other mechanisms underlying the feelings of satiety or hunger may also have contributed to the observed differences between the treatment groups. Although for both experimental diets postprandial glucose concentrations were equally stable and no large fall below basal glucose level was found in sows as observed by de Leeuw *et al.*⁽¹⁷⁾, small transient declines in blood glucose concentrations could still be present and different between treatments. A transient decline in blood glucose preceded meal initiation in rats⁽⁵⁶⁾ and a meal request in humans⁽⁵⁷⁾. Similar observations could only be performed when blood was sampled more frequently for glucose determination or continuous monitoring of blood glucose concentrations. Furthermore, the SCFA mainly produced in the HFF-fed dogs can be used as a source of energy (mainly acetate) at times when glucose supply from the small intestine is decreasing^(58,59). Bleiberg *et al.*⁽⁵⁸⁾ estimated that large intestinal acetate production could contribute in excess of 5% of the total energy needs of dogs. Whether the SCFA from large intestinal fermentation and used as a source of energy will lead to pronounced differences in the feelings of satiety remains to be investigated.

In conclusion, the present study showed that the dogs fed the HFF diet had an increased large intestinal fibre degradation and the production of SCFA than the dogs fed the LFF diet. The HFF-fed dogs consumed less food during a challenge meal, which may be related to increased feelings of satiety. Postprandial plasma PYY, GLP-1, ghrelin and glucose responses did not differ between the treatment groups and could not be linked to the observed lowered voluntary food consumption of the dogs fed the HFF diet. It is likely that other satiety-related hormones and/or mechanisms controlling the feelings of satiety or hunger may have been involved in the observed decrease in VFI in the present study. Finally, inclusion of fermentable fibre in canine diets may contribute to the prevention or mitigation of obesity through its effects on satiety.

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Chapter 5

Effect of dietary fibre type on physical activity and behaviour in dogs

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Abstract | Dog diets may differ in their effectiveness of maintaining satiety after a meal. Consequently, sensations of hunger, feeding motivation, physical activity, and sensitivity to environmental stressors may be increased. Dietary fibre may be effective in prolonging postprandial satiety depending on type and inclusion level. This study evaluated the effect of fibre fermentability on behaviour in dogs. Sixteen healthy adult dogs were housed individually and fed a low-fermentable fibre (LFF) diet containing 8.5% cellulose or a high-fermentable fibre (HFF) diet containing 8.5% sugarbeet pulp and 2% inulin. Dogs were fed 2 equal portions at 8:30 and 18:30 according to energy requirements. Behaviour of dogs in their home-cage was recorded and analysed by instantaneous scan sampling (2×24 h with 15 min intervals) and focal sampling continuous recordings (10 min per animal per h, from 9:00 until 18:00). Dogs were subjected to a behaviour test composed of the subtests open-field, sudden-silence, novel-object, and acoustic-startle. The behavioural responses of each dog were recorded. Scores for the scan and focal samples were expressed per clock hour and DIET×TIME effects were tested statistically using Residual Maximum Likelihood (REML). Data from the tests were examined using principal component analysis resulting in the compilation of two components. Data were tested statistically for DIET and DIET×SUBTEST effects using REML. Variables specific for the open-field and novel-object test were analysed using analysis of variance. For the scans, a significant DIET×TIME effect was found for resting. At night and in the morning, HFF-dogs rested more compared to LFF-dogs, but they rested less between 14:00 and

17:00. For the continuous recordings, the main findings were a tendency for DIET×TIME effect for time spent resting with a pattern consistent with that for the scans. The interaction was significant for inactive-alert (lie with head up or sitting) with HFF-fed dogs having lower values around 10:00-11:00 and higher values hereafter. Finally, time spent tail wagging was significantly higher for LFF-fed dogs just before the evening meal that may indicate higher level of arousal. For the behaviour tests, no significant DIET or DIET×SUBTEST effects were detected. It is concluded that compared to the LFF diet, the HFF diet increased inactivity in kennelled beagle dogs likely through the prolongation of postprandial satiety. This effect did not change the reaction to stressful events in kennelled laboratory dogs. Enhanced susceptibility to environmental stressors at times of hunger in sensitive companion dogs may occur but requires further study.

Introduction

The behaviour of privately owned dogs may be influenced by dietary effects on satiety. Typically, pet dogs are provided with sufficient nutrients and energy in their diets to meet their requirements, but they may be hungry during part of the day. Hunger and high feed motivation may have an undesirable effect on behaviour such as the facilitation of begging and scavenging behaviour⁽¹⁾. These behaviours encourage owners to feed their pet above their energy requirement⁽²⁾ result in overweight and obesity, i.e. the main nutritional disorder in companion animals nowadays⁽³⁾. In rats, hunger results in increased levels of anxiety^(4,5). If the same is true for pet dogs, hunger may contribute to the expression of anxiety-related problem behaviours, though this remains speculative as relatively little is known with regard to the relationship between satiety level and behaviour in dogs (Chapter 2).

Dietary fibres may be useful for the development of strategies to influence satiety as these can increase satiety and its duration in dogs⁽²⁾. The effectiveness of fibrous ingredients to stimulate and prolong satiety depends on its properties and inclusion level as they are resistant to digestion and absorption in the small intestine, but can be degraded by the microbial population in the large intestine of dogs. The fermentation of dietary fibre yields, apart from several gases, short-chain fatty acids (SCFA, mainly acetate, propionate, and butyrate)⁽⁶⁾. These SCFA may stimulate the production and secretion of several satiety-related hormones. One of these is peptide tyrosine tyrosine (PYY), a satiety-promoting hormone released by the enteroendocrine L-cells present in the distal part of the gastrointestinal tract in response to contact with SCFA⁽⁷⁾. Release of glucagon-like peptide-1 (GLP-1), another satiety-promoting hormone produced by the L-cells⁽⁸⁾, is increased in dogs fed fermentable fibre compared to dogs fed low-fermentable fibre⁽⁹⁾. In rats, diets supplemented with a fermentable fibre reduces plasma concentrations of ghrelin, a hormone that is associated with feelings of hunger or appetite in humans⁽¹⁰⁾, at 8 h after the last meal⁽¹¹⁾. Besides the effects of SCFA on satiety-related hormones, absorbed SCFA (mainly acetate) can also be utilised as an energy source, typically at times when the absorption of nutrients from the digestible fraction of the diet is decreasing or is completed⁽⁶⁾. Thus, different mechanisms have been suggested

to make fermentable dietary fibres prolong postprandial satiety in dogs and in this way affect their behaviour, though specially the latter needs yet to be validated.

This study is part of an experiment evaluating the use of fermentable fibre in diets for dogs to increase satiety. Here it is investigated if dietary inclusion of fermentable fibre, by increasing satiety, influences behaviour in dogs and is a candidate strategy to combat unwanted behaviour. Dietary effects on digestibility, satiety-related hormones, and voluntary food intake has been presented elsewhere (Chapter 4). Beagle dogs were fed one of two diets differing in dietary fibre fermentability and studied for their behaviour as recorded in their home-kennel and following short-lasting challenges in a test arena. It was expected that dogs fed fermentable fibre were less active in their home-kennel and less responsive in challenging situations.

Materials and methods

Animals and housing

Nine intact male and 7 intact female healthy adult beagle dogs of 2 to 6 years-of-age with body weights (BW) between 7.2 and 11.4 kg were housed individually in indoor kennels at the Laboratory of Animal Nutrition of Ghent University (Merelbeke, Belgium). Twelve kennels were 1.5 × 1.5 m and 4 kennels measured 1.2 × 1.6 m. Metal plate partitions between kennels prevented visual and physical contact, i.e. direct behavioural interactions between dogs. Food was provided twice daily in two equal portions at 8:30 and 18:30 and fresh water was available *ad libitum*. Dogs were submitted to weekly health checks and weighed every two weeks. Animal housing and experimental procedures were approved by the Ethical Committee of the Faculty of Veterinary Medicine of the Ghent University (Belgium, EC 2007/40).

Treatments

Dogs were fed one of two experimental diets for 8 weeks. The dogs' behaviour in their home-kennels was observed during the fifth week of feeding. During week 7, dogs were subjected to short-lasting challenges in a test arena. The diets differed in the fermentability of fibrous ingredients used. The low-fermentable fibre (LFF) diet contained cellulose as a fibre source whereas the high-fermentable fibre (HFF) diet contained a combination of sugarbeet pulp and inulin. Differences in fermentability of fibres were confirmed *in vitro* (Chapter 3 and Sunvold *et al.*⁽¹²⁾) and *in vivo* (Chapter 4). Diets were formulated to be iso-nitrogenous, iso-energetic on a digestible energy basis, and iso-fibrous on a total dietary fibre basis. Ingredient and nutrient compositions of both diets are shown in Table 1. Each dog was individually fed to meet its daily energy requirement, which was estimated at 415 kJ of metabolisable energy/kg BW^{0.75}. Diets were in mash form and fed after mixing with an equal amount of lukewarm water to increase palatability. Food intake was recorded during each meal throughout the entire experimental period.

Table 1 | Composition of the low-fermentable fibre (LFF) and high-fermentable fibre (HFF) diets.

Composition	LFF	HFF
<i>Ingredients (g/kg as is)¹</i>		
Wheat starch (pre-gelatinised)	468.8	463.0
Poultry meat meal (low-ash)	285.0	275.0
Poultry fat	135.0	135.0
Cellulose	85.0	0.0
Sugarbeet pulp (molassed)	0.0	85.0
Inulin	0.0	20.0
Premix ²	10.0	10.0
Digest	10.0	10.0
Molasses	4.3	0.0
Titanium(IV) oxide	2.0	2.0
<i>Nutrients (g/kg DM)³</i>		
Ash	37.5	42.0
Starch	372.4	367.5
Sugar	13.6	41.6
Crude protein	274.1	262.2
Crude fat	191.4	191.2
TDF	123.7	93.9
IDF	110.9	74.7
SDF	12.8	19.2
NSP	111.0	95.1
<i>Energy content (kJ/100 g DM)</i>		
Gross energy	2294	2300

¹Wheat starch, Pregel Wheat Alpha (Meneba, Weert, The Netherlands); poultry meat meal, Meat Meal 63 (Sonac, Lingen, Germany); poultry fat (Sonac, Lingen, Germany); sugarbeet pulp, molasses (Research Diet Services, Wijk bij Duurstede, The Netherlands); inulin, Beneo IPS (Orafti, Tienen, Belgium); cellulose, Arbocel BWW40 (J. Rettenmaier Benelux, Zutphen, The Netherlands); premix (Twilmij B.V., Stroe, The Netherlands); digest, Luxus Digest N8008 (AFB International, Nuland, The Netherlands); titanium(IV) oxide (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands).

²The premix provided per kilogram of diet: Ca, 0.41 g; P, 0.07 g; Mg, 0.05 g; K, 0.1 g; Na, 0.01 g; Cl, 0.09 g; linoleic acid, 0.15 g; PUFA, 0.17 g; lysine, 0.05 g; methionine, 0.02 g; methionine+cysteine, 0.04; threonine, 0.04 g; tryptophan, 0.02 g; vitamin A, 17,500 IU; vitamin D3, 2000 IU; vitamin E, 100 mg; vitamin K₃, 2 mg; vitamin B₁, 10 mg; vitamin B₂, 10 mg; niacin, 50 mg; pantothenic acid, 25 mg; vitamin B₆, 7.5 mg; vitamin B₁₂, 50 µg; biotin, 300 µg; choline chloride, 475 mg; folic acid, 1.25 mg; vitamin C, 100 mg; Fe, 75 mg; Mn, 35 mg; Cu, 5 mg; Zn, 75 mg; I, 1.75 mg; Co, 2 mg; and Se, 0.2 mg.

³TDF, total dietary fibre; IDF, insoluble dietary fibre; SDF, soluble dietary fibre derived by subtracting the IDF content from the TDF content; NSP, non-starch polysaccharides, derived by subtracting the crude protein, crude fat, starch, and sugar content from the organic matter content⁽¹³⁾. Inulin is not recovered in the TDF fraction⁽¹⁴⁾ resulting in an underestimation of the TDF content in the HFF diet of approximately 18 g/kg DM (20 g/kg included in the diet with 90% pure inulin). Similarly, the NSP content of the HFF diet is underestimated with approximately 18 g/kg DM as inulin was included in the analysed sugar content.

Dogs were randomly assigned to one of the two dietary treatments (LFF or HFF diet), with dietary groups being balanced for BW and gender (blocking factors). Dogs in LFF group ($n=8$) included 4 males and had a mean BW \pm SEM of 9.7 ± 0.5 kg. The HFF group ($n=8$) included 5 males and dogs weighed on average 9.8 ± 0.5 kg.

Behavioural observations

Twenty-four hours activity patterns

During week 5 of the experiment, on two non-consecutive days, the dogs' behaviour was continuously recorded on video for a period of 24 h and activity patterns were analyzed by instantaneous scan sampling using intervals of 15 min. Dogs were scanned for the locomotion states resting (lying with head rested), lying (lying with head up), sitting, standing, standing up and walking. Video analysis was conducted by one person who was ignorant of a dogs experimental treatment.

Interprandial behaviour

The behaviour of the dogs between meals was evaluated in more detail by focal sampling continuous recording, using again the video recordings collected. Per hour, bouts of 10 min were analyzed starting at 30 min after the morning meal (approximately 9:00) until 30 min before the evening meal (approximately 18:00). The total observation time per dog was 200 min. In addition to the behaviours described in section 'Twenty-four hours activity patterns', the following were recorded for the duration of occurrences: posture (high, neutral, and low), autogrooming, nosing, manipulations of the environment, and tail wagging. Behaviours scored for the frequency of occurrence were: body shaking, stretching, yawning, drinking, coprophagia, defecating, and urinating. The number of changes of locomotion states (CLS) were calculated as the sum of the frequency of occurrence of each locomotion state minus 1 (starting locomotion state). For a description of the behaviours see Beerda *et al.*⁽¹⁵⁾. As the position of the ears and legs were difficult to assess from video recordings, only the position of the tail was used as an indicator of posture. Behavioural observations were performed by one person who was ignorant of a dog's experimental treatment and recorded using the Observer 5.0 software package (Noldus Information Technology B.V., Wageningen, The Netherlands).

Behavioural responses

Seven weeks after the start of the study, the dogs were studied for anxiety levels as assessed in an open field test situation. The behaviour test was composed of the subtests 'open-field' (OF), 'sudden-silence' (SS), 'novel-object' (NO) and 'acoustic-startle' (AS). Subtests (for a description see below) were performed conjointly in the described order. Dogs were tested between 13:30 and 15:20. Prior to testing, the dogs were habituated to procedures. This involved the experimenter walking the dog during each of the 5 days preceding the test to the test location, which was approximately 50 m from the kennels. The dogs did not enter the test arena and as such dogs were habituated to procedures prior to testing (i.e. being taken out of the cage, walking to the test location), but the novelty of the test arena was maintained. One day before the first behavioural tests, urine collected from multiple dog kennels was mixed with water and dispersed over the floor and walls of the test arena after which the arena was cleaned again with water. In this way the smell of urine was

always present and possible influences of odour on behaviour were assumed to be similar for all dogs. After each test, the arena was cleaned again with water before the next test started.

Dogs were introduced into a test arena measuring 3 m × 3 m, with floor markings indicating 16 sections measuring 75 cm × 75 cm squares each. Dogs were left undisturbed for 5 min and observed for their behaviour (OF) with background white noise on at an intensity of 60 dB. The background noise was switched off leaving the dog in a silent test arena for 1 min (SS). Next, a standard size plastic shopping bag filled with paper was lowered from the ceiling in the centre of the test room almost touching the floor and the dogs' responses were recorded for 5 min (NO). Finally a short sound blast (~1 s, ~96 dB) was produced using a fog horn (Motip Dupli B.V., Wolvega, The Netherlands) whereafter behavioural responses were recorded for 1 min (AS).

Live observations, done by one observer, were performed behind an one-way screen to prevent the dogs reacting on the observer. A digital lightweight video-camera was mounted above the test arena enabling clear view of the dog's behaviour during the test. Live observations were computer aided as described above.

The dogs' behavioural responses during the test were measured by continuous recording of the behaviours described previously (see sections 'Twenty-four hours activity patterns' and 'Interprandial behaviour') as well as the following behaviours: vocalisations (barking; growling, howling, whining, and yelping), floor licking, panting, oral behaviours, paw lifting, trembling, crouching, and freezing. For a description of howling see Lund & Jørgensen⁽¹⁶⁾ and for the other behaviours see Beerda *et al.*⁽¹⁵⁾. For the OF, the number of line crossings and the total time spent in the outer sections (i.e. the 75 cm × 75 cm squares next to the walls) were recorded. The latency period for a dog to make contact to the novel object and the time spent within 1 m of it were recorded for the NO test.

Data processing

Twenty-four hours activity patterns

Four scan samples per hour were summed resulting in 24 observations per dog per day with behavioural parameter scores ranging from 0 to 4. The general activity levels of the dogs were of most interest and the locomotion states lying and sitting were pooled and labelled 'inactive-alert'. Standing, standing up, and walking were pooled and labelled as 'active'. Resting was maintained as a separate category.

Interprandial behaviour

Behaviours scored for duration of occurrence were expressed as percentage of the observation time (i.e. 10 min) resulting in 10 observations per dog per day with behavioural parameter scores ranging from 0 to 100%. Behaviours scored as frequencies were expressed as times per 10 min. For the purpose of data reduction the scores for body shaking, stretching, and yawning were summed and analysed as a single parameter. The posture of the dogs was recorded only when they were standing, standing up or walking and scores were

expressed as percentage of the summed time standing, standing up and walking. Tail wagging was treated in the same way. The behaviours drinking, coprophagia, defecating, and urinating were observed less than 15 times across dogs throughout the experiment and were, therefore, excluded from further analysis.

Behavioural responses

Again behaviours scored for duration of occurrence were expressed as percentage of the observation time (i.e. 1 or 5 min) and those scored as frequencies were expressed as time per 1 or 5 min. The locomotion states lying and sitting as well as standing and standing-up were summed and labelled 'inactive-alert' and 'standing', respectively. The number of behavioural parameters was reduced by pooling the scores for autogrooming and floor licking (hereafter referred to as 'licking'), whining and yelping ('vocalising'), urinating and defecating ('eliminating'). The behaviours resting, manipulations of the environment, high posture, body shaking, yawning, barking, growling, howling, and trembling were observed less than 15 times during the behaviour tests across dogs and excluded from further analysis. As dogs showed either a neutral or low posture during the tests, only percentage of observation time in low posture was analysed.

Statistical analyses

Behavioural parameters were analyzed with linear mixed models (LMMs) using Residual Maximum Likelihood (REML) in the statistical package ASReml⁽¹⁷⁾. For the non-normal distributed binary or count data, LMM takes the actual distribution into account and implements REML-type analyses (conform Pryce *et al.*⁽¹⁸⁾). The following statistical model was used for parameters of the 24-h activity patterns:

$$Y_{ijklm} = \mu + \text{GENDER}_i + \text{DIET}_j + f(\text{TIME}_{jk}) + \text{DAY}_l + \text{DOG}_m + \varepsilon_{ijklm}$$

where Y_{ijklm} is a measurement recorded for dog m with gender i , which was fed diet j , on day l at time point k . DAY (1 or 2) and DOG (1 to 16) make up the random component of the model, with GENDER (male, female), DIET (LFF or HFF) and TIME (0:00, 1:00, ..., 23:00), here fitted as a spline per diet group, representing the fixed component. For interprandial behaviour parameters the statistical model was similar except for the time frame: 9:00, 10:00, ..., 18:00.

Data reduction was established by means of principal components analysis (PCA, Jolliffe⁽¹⁹⁾) on behavioural response parameters, following similar procedures as described by van Reenen *et al.*⁽²⁰⁾. Principal components represent linear combinations of the behavioural scores and reflect the underlying correlation matrix, but correlations (represented by loadings) may become inflated due to effects of, for example, gender or dietary treatments. This was controlled for by first conducting a PCA on residuals (64 records) calculated by REML (GenStat version 10.2 Lawes Agricultural Trust, 2007), using a LMM with GENDER (male, female), SUBTEST (OF, SS, NO, AS) and DIET (LFF, HFF) in the fixed component and DOG ($n=16$) in the random component. A total of 15 parameters (inactive-alert, standing,

walking, low posture, nosing, tail wagging, freezing, panting, licking, eliminating, vocalising, oral behaviours, crouching, paw lifting, and CLS) were fed into the PCA analysis on residuals. For the calculation of principal component scores, which were derived from the scores for the multiple behavioural parameters corrected for their respective loadings on a given principal component, a second PCA was performed on the raw scores for only the behavioural parameters with loadings above 0.50 or below -0.50 on principal components in the first PCA (i.e. on residuals). The scores for PCA components and individual behavioural response parameters (those not included in one of the PCA components), were analysed with LMMs using Residual Maximum Likelihood (REML) in the statistical package ASReml⁽¹⁷⁾. The following statistical model was used:

$$Y_{ijkl} = \mu + \text{GENDER}_i + \text{DIET}_j + \text{SUBTEST}_k + \text{DOG}_l + \epsilon_{ijkl}$$

where Y_{ijkl} is a measurement recorded for dog l with gender i , which was fed diet j , at subtest k . DOG (1 to 16) makes up the random component of the model, with GENDER , DIET , and SUBTEST representing the fixed components. Variables specific for the OF subtest, i.e. time spent in the outside sections and number of line crossings, and the NO subtest, i.e. latency to contact and time spent within 1 m of the NO, were analysed using ANOVA in GenStat version 10.2 (Lawes Agricultural Trust, 2007). The statistical model used was:

$$Y_{ij} = \mu + \text{GENDER}_i + \text{DIET}_j + \epsilon_{ij}$$

where Y_{ij} is a measurement recorded for a dog with gender i , which was fed diet j . Differences were considered to be significant at $P \leq 0.05$.

Results

Animals

Dogs consumed all the food provided readily and remained healthy throughout the study. Both groups of dogs lost an average of 5% of their initial BW with no significant difference between dietary treatment groups (data not shown).

Behavioural observations

Twenty-four hours activity patterns

The amount of time that dogs were active (stood / walked), inactive-alert (lie down / sat) or resting changed during the day ($P < 0.001$ for each state, Figure 1). Only, for resting this diurnal rhythm differed between the dietary treatment groups ($P = 0.045$ for $\text{DIET} \times \text{TIME}$ effect). Dogs were active during a 2 to 4 h period around the morning meal at 8:30 and during a 3 to 4 h period before the evening meal at 18:30. Typically, they rested during the periods from 1:00 to 6:00 and from 10:00 to 11:00. The resting pattern of the HFF dogs differed from that of the LFF dogs in that they rested more between 0:00 and 7:00 (see least square means in Figure 1). Less clear-cut were the increased levels of resting in the HFF dogs as compared to the LFF dogs during the first 4 hours after the morning meal, and the decreased levels around 14:00-17:00. During the latter period the HFF dogs behaved

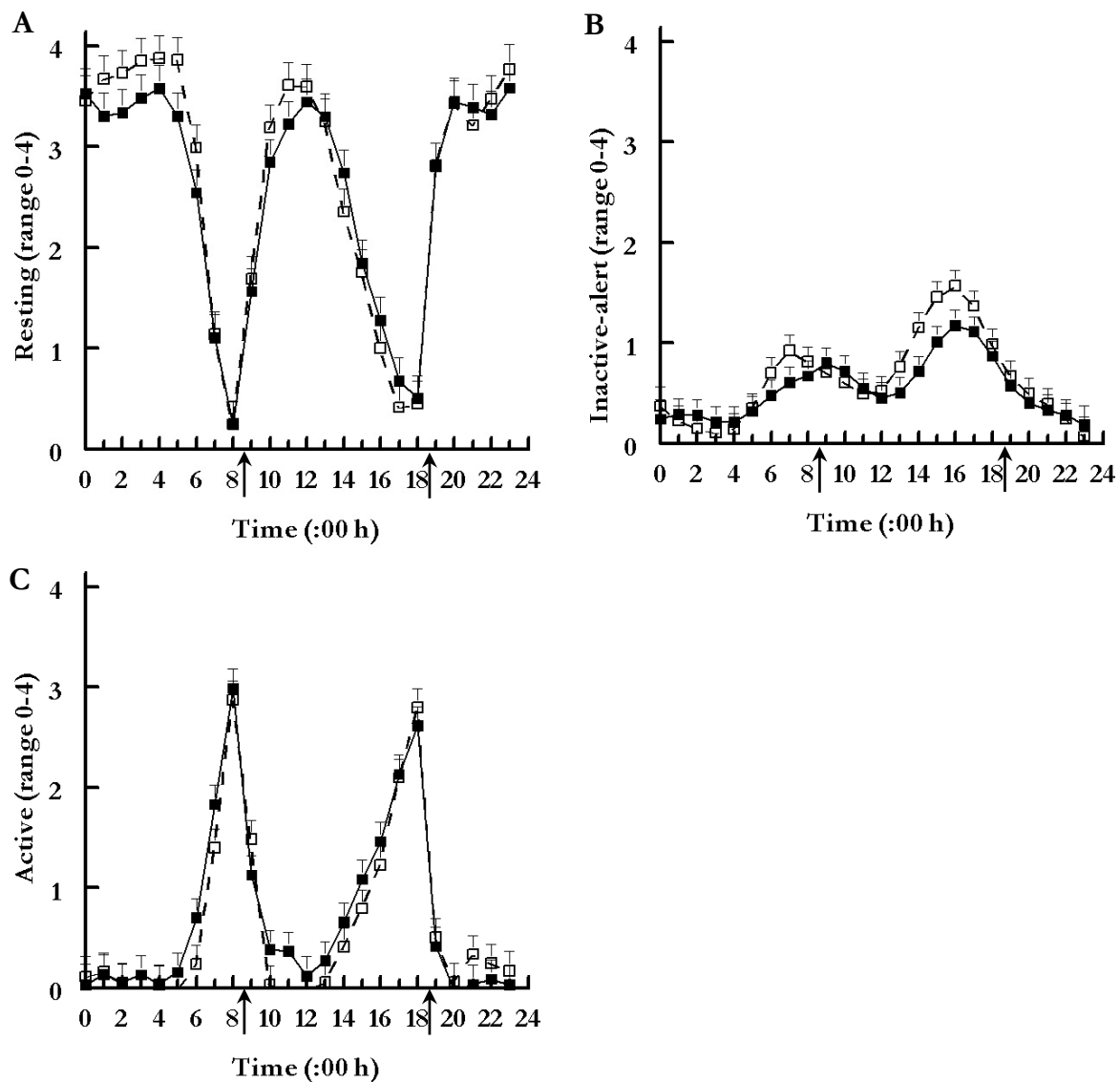


Figure 1 | Diurnal rhythms in resting (lie with head down, panel A), inactive-alert (lie with head up or sitting, panel B) and active (stand or walk, panel C). Least square means and standard errors by the statistical model represent the times a behaviour was observed per hour per dog, given 4 scans per hour. Results are presented separately for the dogs fed the low-fermentable fibre (■, $n=8$) and the high-fermentable fibre diet (□, $n=8$). The arrows represent feeding times (8:30 and 18:30). Changes during the day were significant for all three behaviours ($P<0.001$) and only for resting such changes were different between the dietary groups ($P=0.045$ for DIET×TIME effect).

relatively inactive, and together the results indicate that dogs in the HFF group showed relatively low activity levels, especially during the night. General activity levels were unaffected by gender.

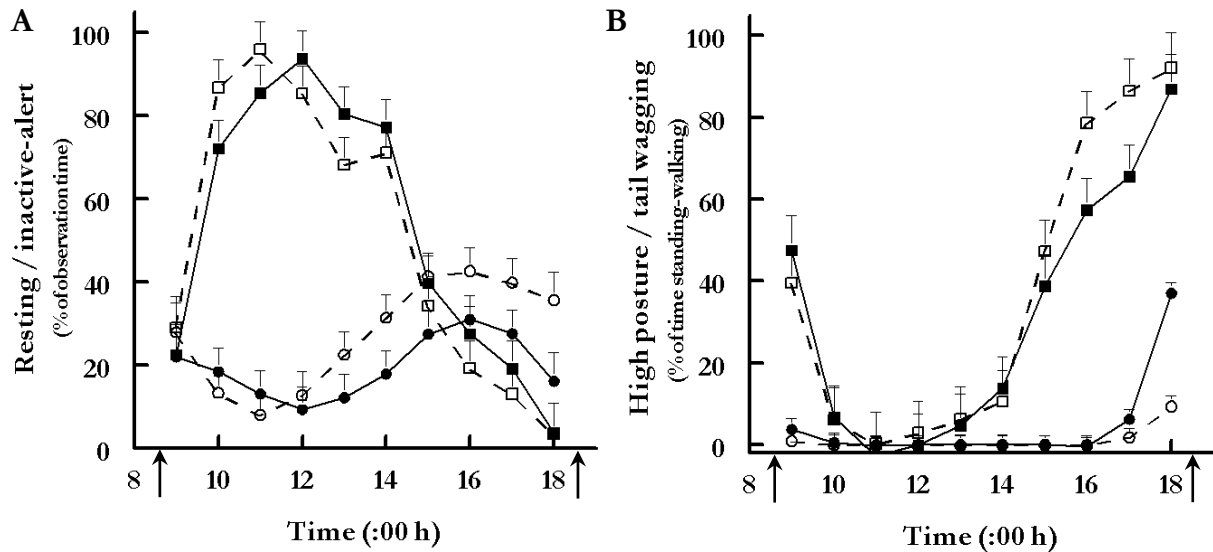


Figure 2 | Interprandial patterns in resting (lie with head down, panel A, ■, □), inactive-alert (lie with head up or sitting, panel A, ●, ○), high posture (panel B, ■, □), and tail wagging (panel B, ●, ○). Least square means and standard errors by the statistical model represent the time spent on a behaviour as percentage of observation time per hour per dog, given 10 min continuous behaviour recording per hour. Results are presented separately for dogs fed the low-fermentable fibre (■, ●, $n=8$) and the high-fermentable fibre (□, ○, $n=8$) diet. The arrows represent feeding times (8:30 and 18:30). Changes between the meals were significant for all four behaviours ($P<0.001$). For inactive-alert, high posture, and tail wagging such changes were different between the dietary groups (respectively $P=0.028$, $P=0.036$, and $P<0.001$ for DIET×TIME effect). Dietary groups tended to differ in time spent resting ($P=0.095$ for DIET×TIME effect). A tendency for the effect of dietary treatment was found for inactive-alert ($P=0.092$) and tail wagging ($P=0.051$).

Interprandial behaviour

Patterns in resting, inactive-alert, high posture, and tail wagging between meals are presented in Figure 2. The amount of time dogs rested during the day tended to vary differently for dogs in the LFF and HFF groups ($P=0.095$ for DIET×TIME effect), with results replicating those reported for 24h activity patterns. Typically, dogs rested from 10:00 to 13:00. Compared to the LFF dogs, the HFF dogs rested more during the period from 10:00 to 11:00 and less during the period from 12:00 to 13:00 and around 16:00. In a reversed way, such DIET×TIME effect ($P=0.028$) was found for inactive-alert (Figure 2). The time dogs were inactive-alert decreased up to 3 to 4 h after the morning meal followed by an increase until 16:00 and decrease hereafter. The HFF dogs were less inactive-alert around 10:00-11:00 than the LFF dogs but more thereafter. The postures of the dogs changed during the day, with differences between dietary groups ($P=0.036$ for DIET×TIME effect on high posture). High postures were observed most during the times dogs were active, i.e. after the morning meal until 11:00 and after 14:00. Differences between dietary groups were most pronounced at 16:00 and 17:00, with higher postures in HFF dogs than LFF dogs. Tail wagging occurred especially around one hour before the evening meal with less tail wagging

Table 2 | Least squares means and standard errors (SEM) for the behavioural parameters observed between morning and evening meals for dogs fed the low-fermentable fibre (LFF, $n=8$) and the high-fermentable fibre (HFF, $n=8$) diet.

Behaviours	LFF		HFF		<i>P</i> -value		
	Mean	SEM	Mean	SEM	DIET	TIME	DIET × TIME
<i>Locomotion</i> ¹							
Active	7.4	7.4	4.2	7.4	0.225	<0.001	0.628
CLS	6.1	10.1	7.5	10.1	0.463	<0.001	0.540
<i>Posture</i>							
Low	3.7	2.0	3.1	2.1	0.792	0.350	0.780
Neutral	19.2	4.8	19.0	4.9	0.581	0.660	0.926
<i>Other</i>							
Autogrooming	5.6	1.9	9.3	2.0	0.496	0.050	0.652
Nosing	0.4	0.2	0.2	0.2	0.416	0.001	0.722
Manipulations	2.0	0.6	0.3	0.6	0.099	0.867	0.849
Body shaking, stretching, yawning	0.5	0.2	0.5	0.2	0.957	0.996	0.866

¹Active consists out of standing, standing up, and walking; CLS, changes in locomotion states.

in HFF dogs than in LFF dogs ($P<0.001$ for DIET×TIME effect). Other behaviours did not show DIET×TIME or DIET effects and are presented in Table 2. TIME effects occurred in that dogs decreased their activity (including CLS and nosing) after the morning meal until 11:00. From 12:00 to 18:00 dogs became increasingly active. Dogs tended to groom themselves just after the morning meal (9:00-10:00) and in the afternoon (13:00-15:00). Gender did not significantly affect any of the behaviours measured between meals.

Behavioural responses

For the purpose of data reduction, behavioural parameters recorded during the behaviour tests were investigated for interrelationships using PCA. Two PCA components explained a substantial part of the variation (20 and 14%) in a dataset of 15 parameters. The first component grouped behaviours indicative of ‘restlessness-exploration’, namely walking (loading of 0.91), nosing (0.91), CLS (0.87) and, reversely related to these, inactive-alert (-0.85). The second component grouped oral behaviours (0.81), paw lifting (0.56) and, reversely related to these, standing (-0.60), and was interpreted as ‘anxiety’. Component scores were calculated from the dogs’ scores for the different behavioural parameters, weighing behavioural parameters relative to their loadings.

Least squares means for component scores (i.e. restlessness-exploration and anxiety) and behavioural parameters that did not fit these components are presented in Table 3. Effects of DIET or DIET×SUBTEST were in none of the cases significant. Also, SUBTEST specific parameters like distance to the novel object or latency to contact it were not significantly different between the groups.

Table 3 | Least squares means and standard errors (SEM) for the behavioural parameters recorded during the behaviour tests for dogs fed the low-fermentable fibre (LFF, $n=8$) and the high-fermentable fibre (HFF, $n=8$) diet.

Behaviours	LFF		HFF		DIET	<i>P</i> -value	
	Mean	SEM	Mean	SEM		SUBTEST	DIET × SUBTEST
Restlessness-exploration ¹	-0.04	0.35	-0.05	0.36	0.975	<0.001	0.869
Anxiety ²	0.00	0.27	0.05	0.27	0.909	<0.001	0.264
Low posture	48.2	10.6	52.2	10.7	0.818	<0.001	0.918
Tail wagging	13.1	5.7	5.3	5.8	0.358	0.042	0.642
Vocalising	0.6	0.8	1.9	0.9	0.311	0.162	0.599
Licking	1.1	0.6	1.6	0.6	0.575	0.904	0.103
Panting	1.6	3.1	6.2	3.1	0.306	0.250	0.136
Freezing	2.6	0.8	2.0	0.9	0.628	0.071	0.156
Crouching	0.3	0.1	0.4	0.1	0.882	<0.001	1.000
Eliminating	1.7	0.5	1.6	0.5	0.934	<0.001	0.996
# Line crossings ³	141.0	21.4	127.5	21.7	0.665	-	-
Outer sections ³	87.0	2.3	85.7	2.3	0.698	-	-
Inside meter ⁴	13.9	4.6	21.3	4.7	0.277	-	-
Latency contact ⁴	168.2	45.1	136.5	45.8	0.631	-	-

¹PCA component grouping walking, nosing, changes of locomotion states, and inactive-alert.

²PCA component grouping oral behaviours, paw lifting, and standing.

^{3,4}Apply to the open-field test (³) or novel-object test (⁴) and was analysed with ANOVA instead of REML.

Dogs behaved differently during the different phases of the behaviour test (OF, SS, NO, AS) as indicated by significant SUBTEST effects. In short, tail wagging typically occurred during the OF and SS tests whilst dogs tended to crouch in response to NO and AS and to freeze in response to SS and AS tests. Minor differences between sexes existed in that males showed higher frequencies of urination and defecation compared to females ($P<0.001$) and they spent relatively more time within 1 m from the novel object ($P=0.017$).

Discussion

Although dogs are generally provided with sufficient nutrients and energy for maintenance, they may be hungry for part of the day, resulting in a high feeding motivation with undesirable effects on behaviour. The aim of the present study was to evaluate the effects of diets differing fibre fermentability on behaviour in dogs. The HFF diet was formulated to prolong satiety, which was indicated by the reduced ($P=0.058$) food consumption of a freely available standard food 6 h after the morning meal in dogs fed HFF (mean uptake \pm SEM of 404 ± 70 g) as compared to those fed low-fermentable fibre (LFF, 250 ± 46 g) (Chapter 4). Beforehand, we assumed that prolonged satiety in dogs fed the HFF diet would lower spontaneous physical activity during parts of the diurnal cycle. Indeed, dogs fed the HFF diet rested more during the night and just before the morning feeding and were relatively inactive (more inactive-alert) in the afternoon compared to the LFF-fed dogs. The a

priori assumption that the dogs fed the HFF diet would be less anxious during the behaviour tests, was not confirmed in the present study.

The present findings associate reduced satiety with increased spontaneous activity, which is in line with earlier reports on energy restriction induced increases in spontaneous activity in dogs⁽²¹⁾, pigs⁽²²⁾, and rats⁽²³⁾. In rats, increased spontaneous activity has been demonstrated to occur after intracerebroventricular injection of the hunger-hormone ghrelin⁽²⁴⁾. Regarding the feeding of fermentable fibre, studies in sows and pigs have shown calming effects of diets containing fermentable fibre sources on physical activity compared to diets with low-fermentable or low amounts of fibre⁽²⁵⁾. Together, earlier and present findings support the idea that high levels of behavioural activity in dogs can in part be due to hunger.

During the one to two hour period before the evening meal, dogs on a HFF diet showed a higher posture and less tail wagging than those on a LFF diet. Relatively high postures may be interpreted as a sign of feeling well, i.e. when extrapolation from the finding that low postures are associated with acute and chronic stress^(15,26). Increased tail wagging in the LFF dogs was most likely due to relatively high levels of excitement / arousal⁽²⁶⁾ as related to the anticipation of food. Increased arousal before feeding was also noticed for dogs restricted in their energy intake⁽²¹⁾. The findings on posture and tail wagging provide further support for the assumption that the HFF dogs were more relaxed than the LFF dogs. In line with this, there were indications ($P < 0.10$) that dogs fed the LFF diet spent relatively more time on scratching the wooden resting-plate in their home cage (recorded as manipulation of the environment). Such behaviour could be characterised as stereotypic as it was shown repetitively, unvarying, and apparently without function. The low occurrence of repetitive behaviour in dogs fed HFF corresponds with reports on reduced stereotypic behaviour pigs fed fermentable fibres like sugarbeet pulp^(27,28).

Anxiety may be influenced by hunger or satiety. For example, the intracerebroventricular injection of ghrelin in mice and rats increases anxiety as measured with an elevated plus-maze^(4,5). Furthermore, pigs fed a diet high in sugarbeet pulp (high-satiating diet) seem to be less aroused in an OF test and less motivated to escape from the environment, suggesting reduced anxiety compared to pigs fed a diet high in starch (low-satiating diet)⁽²⁹⁾. To evaluate whether stress responses would be affected by differences between treatment groups, dogs were subjected to several behaviour tests. Dogs were tested around 6 h after the morning meal, approximating the time at which the voluntary food intake measurement was conducted (Chapter 4). The tests in the current experiment were used to study anxiety and provoke mild stress. The OF test is frequently used to study exploration and anxiety in rodents⁽³⁰⁾. Time spent in the outer sections was evaluated as animals that spent the greatest time in these sections are regarded as more anxious than those that prefer the central region⁽³¹⁾. The SS test has been used as a model for mild unexpected stress in rodent studies eliciting immediate behavioural arrest with orientational movements^(32,33). The NO and AS test procedures were similar to tests reported by Beerda *et al.*⁽¹⁵⁾ which induced acute stress in dogs. It was expected that dogs fed the HFF diet, which

appeared to experience less hunger, would show lowered behavioural stress responses during these tests compared to the more hungry LFF-fed dogs. No effects of dietary treatment or the interaction between treatment and test were found for any of the analysed variables. For some behavioural variables, the different tests were more or less effective in provoking a response from the dogs. It should be noted that the effect of a test may be confounded with habituation to the experimental room and carry-over effects may have occurred from one test to the next. The lack of treatment effect could be attributed to several possibilities. Firstly, hunger and increased feed motivation may not influence the susceptibility to stress and anxiety in dogs. Secondly, the test procedures used were too mild or too severe preventing variation in stress responses to occur between treatment groups. It is unlikely that the tests were too mild as several behavioural typical indicators of stress were exhibited by the dogs during the tests. The first component contained behavioural elements related to activity and paw lifting. High levels of walking, nosing and changing from one state of locomotion or sector to another may be interpreted as restlessness or avoidance behaviour and may be a sign of moderate stress response⁽¹⁵⁾. Furthermore, paw lifting was found to be an indicator of acute stress⁽¹⁵⁾. The second component was composed of oral behaviours, standing and standing up, and paw lifting. Oral behaviour has also been shown to be expressed during stressful events^(15,34). Standing, and standing up relate to immobility. Like avoidance behaviour, immobility may be a manifestation of fear in dogs⁽³⁵⁾. Furthermore, the only vocalisations recorded were whining and yelping which were suggested to indicate distress related to fear⁽¹⁶⁾. Low posture and crouching behaviour occurred mainly during the NO and AS tests which is in accordance to findings of Beerda *et al.*⁽¹⁵⁾. Some behavioural variables related to stress like yawning, body shaking, and trembling, however, were not observed or observed at a low frequency. Yawning has been associated with psychological tension or mild stress in primates⁽³⁶⁾. Body shaking could be interpreted as a sign of tension release or relief rather than stress⁽¹⁵⁾. It is possible that the tests were not sufficient in intensity to provoke dogs in performing these behaviours. Yet, both behaviours could be performed after leaving the test room rather than during the tests in the test arena. Trembling may not have been displayed because dogs did not experience the relatively high levels of stress that are typically associated with trembling. It is not likely that the tests were too severe in inducing stress as the intensity in the display of stress-related behaviours varied among dogs. Based on the observed behavioural variables, the tests appeared to be effective in provoking acute stress in the dogs. Thirdly, as some dogs in both dietary groups lost some BW during the study they experienced hunger. Although the HFF-fed dogs appeared less hungry as they consumed less food during the satiety test (Chapter 4), these dogs may also have experienced feelings of hunger sufficient to increase stress susceptibility to a similar level as for the LFF-fed dogs. Likewise, the contrasts between treatment groups in hunger or satiety could be not large enough to create differences in behavioural stress responses at the time of testing. The contrast in hunger at which differences in stress susceptibility occur remains to be investigated. To further explore the effects of hunger and satiety on stress susceptibility, it

would be important to include positive and negative controls in the study. One possibility to do this is to subject dogs to similar tests either shortly after a meal (fully satiated) or after a considerable time of fasting (hungry).

There were few differences observed in responsiveness during the tests between male and female dogs. Females spent less time inside a meter from the novel object (data not shown) but latency to contact was not affected by gender. The former is in line with results in other studies in which the stress response was more pronounced in female than in male dogs^(15,37). The frequency of urination and defecation was higher for males than for females. Most urination and defecation occurred during the OF test and gradually decreased in frequency with each subsequent test. It is suggested that urination during the OF test is primarily an index of territorial marking⁽³¹⁾. The motivation to mark the novel test room appeared to be stronger in male dogs than in female dogs which is in agreement with other studies^(15,38). It is therefore likely that this behaviour was the result of territorial marking rather than the expression of stress. Overall, it appeared that in the current study male and female dogs were equal in their responsiveness to the applied stressors.

This study indicated that satiety may be prolonged depending on the fermentability of dietary fibre sources used. Assuming that feelings of hunger are unpleasant, these findings may aid in the development of dietary strategies that optimally stimulate satiety in dogs. This is practically relevant as it is likely that satiated dogs will show less begging behaviour and scavenging, behaviours that encourage owners to feed their dog above energy requirements^(1,2). Furthermore, the present study did not show an effect on stress susceptibility in dogs. Extrapolation of this finding to field situations should be undertaken with caution. Dogs in the present study were kennelled laboratory beagle dogs. Under field conditions, depending on the dietary composition and feeding regime dogs may experience feelings of hunger more intensive and for longer periods of time, as well as be subjected to more severe stressful events and be more sensitive these events. As a result, diets high in satiating properties may prevent enhancement of sensitivity to stressful events for some companion dogs of the dog population.

Conclusion

Compared to the diet containing the low-fermentable fibre, fermentable dietary fibre enhanced inactivity in kennelled beagle dogs likely through its effects on increasing satiety. These differences in hunger or satiety were not associated with changes in the susceptibility to stressful events in kennelled laboratory dogs.

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Chapter 6

Dietary tryptophan supplementation in privately-owned mildly anxious dogs: a randomised double-blinded placebo-controlled behavioural study

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Abstract | Food composition has been reported to influence mood and behaviour in humans and animals and it could help to reduce unwanted behaviour in dogs. Anxiety-related behaviour is associated with the functioning of the central serotonergic system and here it was investigated if dietary supplementation with the serotonin precursor tryptophan (Trp) affects behaviour in privately-owned dogs. For 8 weeks, privately-owned dogs were fed a control diet ($n=66$) or a diet containing 2.6-fold more Trp than the control diet ($n=72$), using a randomised double-blinded, placebo-controlled approach. A third diet fortified with Trp, beet pulp, salmon oil, soy lecithin, and green tea extract was studied for its potential in 69 dogs. Owners reported about their dogs' behaviour in the home situation by filling out a web-based questionnaire before the onset of dietary treatment and after 4 and 8 weeks of feeding the diets. Thirty-four dogs fed the control diet and 39 dogs fed the Trp diet were subjected to behaviour tests before and after 8 weeks of dietary treatment. The test included open-field situations and owner-separation procedures and were set up to measure anxiousness. Blood was collected after 8 weeks from dogs in the control (15 dogs) and Trp (15) groups for evaluation of plasma amino acid concentrations. Dietary effects on behaviour were investigated for significance by means of testing interactions between diet and time, using Residual Maximum Likelihood. Intake of the Trp supplemented diet significantly increased plasma Trp concentrations by 37.4% and its ratio with large neutral amino acids by 31.2% compared to the control diet but owners did not report on behavioural changes that could be attributed to a specific dietary

treatment. Also, the dogs' responses in the behavioural tests, including those in saliva cortisol, were unaffected after 8 weeks of consuming the Trp supplemented food. A number of significant changes in both owner-reported assessments and behavioural responses did occur over time, possibly mirroring a placebo-effect and / or influences of a new diet regardless of its specific composition. It is concluded that intake of diets supplemented solely with Trp or in combination with beet pulp, salmon oil, soy lecithin, and green tea extract does not change (anxiety-related) behaviour in privately-owned dogs that do not show clear signs of abnormal behaviour. The influence of dietary Trp intake on behaviour of pathological anxious or chronically stressed dogs remains to be established.

Introduction

The intake of specific nutrients can have positive effects on the behaviour in humans and animals. Food or feed constituents demonstrated to influence behaviour in humans and animals include beet pulp as a source of fermentable fibre⁽¹⁾, salmon oil as a source of n-3 polyunsaturated fatty acids⁽²⁾, soy lecithin as a source of phosphatidylserine⁽³⁾, and green tea extract as a source of catechins⁽⁴⁾. One dietary component of particular importance is tryptophan (Trp), the precursor for the synthesis of the neurotransmitter serotonin (5-HT). 5-HT has widespread functions throughout the brain, playing a role in a variety of physiological functions, affective states, and behaviours⁽⁵⁻⁷⁾. The central serotonergic system has been linked to fear- and anxiety-related states and stress responsivity^(8,9). An increased intake of Trp has been shown to decrease quarrelsomeness in healthy human subjects⁽¹⁰⁾, reduce anxiety in rats⁽¹¹⁾, reduce fear in silver foxes⁽¹²⁾, and increase resilience in dealing with stress in pigs^(13,14). Also coping with mental stress was improved in healthy stress-vulnerable human subjects when dietary Trp intake was increased⁽¹⁵⁾.

The first and rate-limiting step in the synthesis of 5-HT is the hydroxylation of Trp to 5-hydroxytryptophan by the enzyme Trp hydroxylase, which is followed by the decarboxylation of 5-hydroxytryptophan to 5-HT. Trp hydroxylase is normally about half saturated with Trp⁽¹⁶⁾ indicating that 5-HT synthesis can be doubled via the route of raising central Trp concentrations. The transport of Trp across the blood-brain barrier depends on plasma concentrations of both Trp and other large neutral amino acids (LNAA; tyrosine, phenylalanine, leucine, isoleucine, and valine) as these compete for the same carrier mechanism⁽¹⁷⁾. Thus, variations in dietary intake of Trp and LNAA may influence 5-HT synthesis in the brain^(18,19). Dietary protein contains only small concentrations of Trp relative to other LNAA and likely the consumption of a meal high in protein decreases the ratio of Trp to other LNAA⁽²⁰⁾, thereby restricting central 5-HT synthesis.

In dogs, studies on the effects of Trp and protein intake on behaviour are limited to aggressive and hyperactive behaviour. Owners reported lowered fear-related territorial aggression in seven dogs when fed a low-protein diet for 2 weeks compared to dogs fed a high-protein diet with no further effects of dietary protein content on dominance aggression or hyperactivity⁽²¹⁾. DeNapoli *et al.*⁽²²⁾ studied the effects of feeding diets differing in protein

and Trp contents on behaviour in dogs diagnosed to show dominance aggression, territorial aggression, or hyperactivity. For none of the groups of dogs behavioural changes were noted by the dog-owners, but for the total study population, dietary Trp supplementation to a low-protein diet reduced scores for territorial aggression without affecting dominance aggression, fear, hyperactivity, and excitability. Supplementation of Trp to a high-protein diet reduced dominance aggression with no effects on other behaviours. Dietary Trp supplementation has thus been linked to aggression, in part as related to fear, anxiety-related behaviour, and stress susceptibility and it is likely that such effects run via increased central 5-HT synthesis. Trp supplementation reduces anxiety-related states, presumably as anxiolytics typically act strongly on the serotonergic system⁽²³⁾.

The main objective of the present study was to assess the effect of increased dietary Trp intake on behaviour in dogs studied in their home-situations and under controlled test conditions, focussing on anxiety-related behaviours. Also in this study a second modified diet that combined additional Trp with four other components that potentially could affect behaviour in a positive way (i.e. beet pulp, salmon oil, soy lecithin, and green tea extract), was used. The study was conducted as a randomised double-blinded, placebo-controlled design.

Materials and methods

All experimental procedures were approved by the Animal Care and Use Committee of Wageningen University and Research Centre. The period during which the study diets were fed to the dogs was from June to October 2008.

Recruitment and eligibility criteria

Candidate participants, which were recruited via advertisements in national media, completed a web-based questionnaire containing questions on general characteristics of the dog (e.g. breed, gender, neuter status, age, body weight, health status) and questions regarding the dog's behaviour as derived from the Canine Behavioural Assessment and Research Questionnaire (CBARQ, Hsu & Serpell⁽²⁴⁾). Dogs were excluded from the study if they suffered from a medical condition, (had) received behavioural therapy, were pregnant or younger than 6 months. Beforehand it was decided to include up to 300 dogs, which were selected for their relatively high anxiety scores (for details see section Behaviour questionnaire and Table 2). Owners of dogs that met the eligibility criteria were fully informed of the study procedures and were asked to sign an informed consent.

Treatment blinding and randomisation

Participating dogs were assigned a code after completion of the internet questionnaire and randomly assigned to the dietary treatment groups using the ARANDOMIZE procedure in GenStat version 11.1 (Lawes Agricultural Trust, 2007). Allocation of dogs to dietary treatments and labelling of diets was conducted by a person not involved in data collection.

Experimenters and dog-owners remained therefore blinded to the dietary treatments throughout the study.

Dietary treatments

The assigned study diets for individual dogs were distributed to pet shops across The Netherlands in 20-kg, white paper bags labelled with the name of the owner and a dog code. Owners collected the diets and were asked to feed the diet to the dogs for 8 weeks. The control-diet group (CDG) was provided with a high-quality commercially available dog food (Casa-Fera adult, Vobra Special Petfoods, Veghel, the Netherlands) containing rice, maize, barley, poultry meal, dried beet pulp, hydrolysed chicken protein, vitamins, dehydrated globin, salmon oil, poultry fat, herring meal, lamb meat, beef fat, yeast, chicken liver extract, egg powder, lecithin, L-lysine, linseed oil, potassium chloride, and DL-methionine (quantitative information remains confidential). The tryptophan-diet group (TDG) received the control diet supplemented with Trp at the expense of crude protein. The third diet differed in composition from the control diet in that it was fortified (FDG) with extra beet pulp (8% instead of 4% on as is basis), extra salmon oil (2% instead of 1%), extra soy lecithin (1% instead of 0.5%), and green tea extract (0.03%). Owners were provided with written instructions on how to switch to the new diet and on the amount to feed. Instructions included a request to provide the diets as the sole source of nutrition (no other foods or treats) to ensure that the main nutrient intake by the dogs was from the study diets. The analysed nutrient composition of the study diets are shown in Table 1.

Behaviour questionnaire

For the purpose of monitoring the dogs' behaviour in their home-situation, a questionnaire was made available on the internet for the owners. Questions were derived from the CBARQ and dealt with fear, anxiety, attachment, and excitability. Specific questions as listed by Hsu & Serpell⁽²⁴⁾ were used for expressing the degree of stranger-directed fear (3 questions), non-social fear (6), separation-related behaviour (8), attachment or attention seeking behaviour (6), excitability (6) and pain sensitivity (3). Questions on stranger-directed fear did not discriminate between responses to men or women. Answers were presented on a 5-point scale, awarded a value from 0 to 4, respectively indicating that a behaviour was not performed or excessively, and summed per aforementioned traits. Furthermore, the scores on all questions relating to fear and anxiety (24 in total) were summed and labelled 'aggregated anxiety'. Finally, questions on body weight, body condition and coat condition, with assessments again on a 5-point scale, were part of the questionnaire.

Behaviour tests

Dogs from the CDG and TDG with a relatively high initial score for aggregated anxiety (for an overview see Table 2), were selected and subjected to a behaviour test before the start of dietary treatment and at least 8 weeks thereafter. Two saliva samples were taken per dog,

Table 1 | Analysed nutrient composition of the study diets.

Nutrients (g/kg DM)	Control	Tryptophan	Fortified
Dry matter (g/kg)	960.8	902.9	905.0
Ash	53.2	48.4	59.3
NFE ¹	507.7	516.2	477.9
Crude protein (N × 6.25)	212.3	210.3	203.9
Crude fat	81.1	84.4	90.9
TDF	145.7	140.7	168.0
IDF	117.6	117.4	129.3
SDF ²	28.1	23.3	38.7
Gross energy (kJ/100 g DM)	2056	2060	2059
Isoleucine	7.34	7.03	6.91
Leucine	15.51	14.50	14.25
Phenylalanine	8.70	8.24	8.00
Tryptophan	2.18	5.70	4.21
Tyrosine	6.50	6.26	6.14
Valine	9.64	9.23	9.10
Molar tryptophan/LNAA ³	0.031	0.085	0.064
Linoleic acid	16.37	16.01	17.41
Arachidonic acid	0.25	0.24	0.28
Alpha-linolenic acid	4.14	4.00	3.42
Eicosapentaenoic acid	0.55	0.55	1.21
Docosahexaenoic acid	0.55	0.57	1.23

NFE, nitrogen-free extract; TDF, total dietary fibre; IDF, insoluble dietary fibre; SDF, soluble dietary fibre; LNAA, large neutral amino acids.

¹Calculated by subtracting ash, crude protein, crude fat, and TDF from 1000.

²Calculated content by subtracting IDF from TDF.

³Sum of valine, isoleucine, leucine, tyrosine, and phenylalanine.

just before the onset of the test and another immediately after the tests were done. Saliva was collected from each dog by gently rotating two cotton swaps in the dogs' cheek pouches. After 45-60 s, the swaps were stored in Salivettes tubes (Sarstedt AG & Co., Nümbrecht, Germany) on ice until the end of the day when the tubes were centrifuged at $3,000 \times g$ for 10 min at room temperature. The collected saliva was stored at -20°C until cortisol analysis.

The behaviour tests involved a 4-min lasting composite open-field test followed by an 11-min lasting owner-separation test. Dogs were introduced into an empty room measuring $4.5 \text{ m} \times 4.2 \text{ m} \times 3.0 \text{ m}$ ($L \times W \times H$) where background white noise was played at an intensity of 60 dB. The room consisted of solid walls, except for a one-way window of $90 \text{ cm} \times 195 \text{ m}$ ($W \times H$) at 95 cm height. Dogs were left alone and undisturbed for 1 min (novel environment phase) after which the background noise was switched off (sudden silence phase). After 1 min, a sound pulse of 1 s in duration and 90 dB in intensity was produced by a computer (startle phase) and 1 min after this a $30 \text{ cm} \times 25 \text{ cm}$ plastic tubing was dropped from the ceiling just touching the floor (novel object phase). One minute after the falling object, dogs were led out of the test arena to have a walk outside with their owner. Within 5 min the test room was furnished to resemble a Dutch living room and the owner-separation procedure was conducted, which consisted of three phases. Low-intensity background noise

Table 2 | Overview of the number of dogs and their scores for aggregated anxiety¹ included in the statistical analyses for each study group before the start of dietary treatment.

Measurement	Control				Tryptophan				Fortified			
	<i>n</i>	Mean	SEM	Range	<i>n</i>	Mean	SEM	Range	<i>n</i>	Mean	SEM	Range
Behaviour questionnaire	66	25.7	1.7	8-76	72	26.1	1.7	5-68	69	24.8	1.5	7-58
Behaviour tests	34	27.0	2.3	11-72	39	28.9	2.0	5-59	-	-	-	-
Blood collection	15	29.9	4.1	11-72	15	32.1	3.0	14-52	-	-	-	-

¹Scores were calculated based on 24 questions relating to fear and anxiety. Answers were presented on a 5-point scale, awarded a value from 0 to 4 indicating that a behaviour was not performed or excessively, respectively.

(60 dB) was played throughout the test to mask possible distracting noises from the environment. Both owner and the dogs were in the room for 3 min during which time the owner sat on a couch reading a magazine. This phase ‘together’ is labelled TO. Following this, the owner left the room in a manner as they practiced routinely at home. Dogs were left alone in the room for 5 min (alone, AL) after which the owner re-entered the room, greeted the dog, and started reading on the couch again. This final ‘reunion’ (RE) phase lasted for 3 min.

Throughout the behaviour tests dogs were continuously recorded on video and behaviours that are difficult to detect from video recordings were observed from behind the one-way window. Observations were performed by one of two observers which had a high inter-observer reliability as indicated by the Cohen’s kappa coefficients⁽²⁵⁾ with $\kappa=0.93$ for behaviour scored for duration and $\kappa=0.75$ for behaviours scored for frequency of occurrence. Observations and analyses of video-recordings were performed using the Observer 5.0 software package (Noldus Information Technology B.V., The Netherlands, Wageningen).

Behavioural parameters scored for duration were walking, standing, sitting, lying, high posture, neutral posture, low posture, very low posture, tail wagging, trembling, panting, and close to novel object (within 0.5 m). The latency for the dog to contact the novel object was recorded for the novel object phase. Parameters scored for frequency were the barking, howling (high frequency vocalisations varying in pitch), growling, whining, snout-licking, tongue out, paw lifting, sudden sniff (sudden, short sniffing of the floor), body shaking, autogrooming, yawning, stretching, freezing (interrupted walking followed by standing erect with the position of the head elevated), turn away head (turning away the head from object or person without losing eye-contact with it), crouching, standing against, door scratching (scratching the door or around it), manipulating environment, urinating, defecating, and making contact with novel object. For a description of those behaviours mentioned above which are not explained (in brackets) see Beerda *et al.*⁽²⁶⁾.

Blood sampling

After 8 weeks on the diet, a blood sample was obtained from a subset of dogs from the CDG ($n=16$) and TDG ($n=16$) while in their homes. Selection of the dogs used for blood collection was based on owners' consent and geographical distance from the research facilities. Blood (10 ml) was collected in EDTA-collection tubes via vena puncture in the Venus jugularis and centrifuged immediately at $10\,000 \times g$ for 10 min. Plasma was separated and stored on ice until the end of the collection day when plasma samples were stored at -80°C .

Chemical analyses

Diets were analysed for DM, ash, crude protein, crude fat, total dietary fibre (TDF), insoluble dietary fibre (IDF), and gross energy according to procedures described in Chapter 4. Trp was analysed by an in-house method of Ajinomoto Eurolysine (Amiens, France) from the abrogated standard AFNOR XP V18-114. Diet samples were hydrolysed under alkaline conditions with barium hydroxide and heated in an autoclave at 120°C for 16 h. The hydrolysates were acidified with chlorhydric acid at pH 3.0 in which Trp was analysed by high performance liquid chromatography (HPLC) (Knauer GmbH, Berlin, Germany) equipped with a Nova-Pak reverse-phase C18 column (Waters, Milford, MA, USA) and using fluorometric detection. All other amino acids were analysed according to directive 98/64/CE. For analysis of fatty acid composition, total fat in the diet was extracted with a chloroform/methanol mixture (2/1, vol/vol) according to the method of Folch *et al.*⁽²⁷⁾. An internal standard (C13:0) was added to the extracted fat before the mixture was saponified with 0.5 M methanolic sodium hydroxide and methylated with boronitride in methanol⁽²⁸⁾. Obtained fatty acid methyl esters were separated and analysed by gas chromatography (Trace GC Ultra, Interscience, Breda, The Netherlands) equipped with a Restek RT-2560 column (Restek, Bellefonte, PA, USA). Nitrogen-free extract was calculated by subtracting ash, crude protein, crude fat, and TDF from 1000. The soluble dietary fibre content was calculated by subtracting the IDF content from the TDF content.

For the analyses of plasma amino acid concentrations, plasma (1 ml) was deproteinised using 1 ml of 3% sulfosalicylic acid. After 15 min of mixing, the mixture was stored for 1 h at 4°C followed by centrifugation at $800 \times g$ for 15 min. Trp in the supernatant was quantified as described above. For the plasma concentrations of the other amino acids, 400 μl of the supernatant was diluted with 1000 μl of lithium buffer and quantified as described above.

Saliva cortisol concentrations were determined using a solid-phase ^{125}I -radio immunoassay (Coat-A-Count Cortisol, Diagnostic Products Corporation, Los Angeles, CA, USA). The performance deviated from the standard procedures in that calibrators were diluted in range of 1.38-138 nmol/l. Saliva samples (200 μl) were incubated for 2 h in a shaking water bath of 37°C .

Data processing

Low posture and very low posture were summed and labelled as 'low posture'. Tongue out and snout-licking were summed and labelled as 'oral behaviour'. The number of changes of locomotion states (CLS) and changes of posture states (CPS) were calculated as the sum of the frequency of occurrence of each state minus 1 (starting state).

Behaviours scored for duration of occurrence were expressed as percentage of observation time, i.e. 4 min for the open-field test and 3 min (OW), 5 min (AL) and 3 min (RE) for the subsequent phases of the owner-separation procedure. Those scored as frequencies were expressed as times per open-field test or owner-separation test phase. Behaviours occurring in less than 15% of the observations were not included for further statistical analyses, i.e. growling, howling, sudden sniff, body shaking, urinating, defecating, autogrooming, yawning, manipulating the environment, stretching, freezing, crouching, and looking away for the open-field test and growling, howling, sudden sniff, urinating, defecating, manipulating the environment, stretching, freezing, crouching, looking away, and trembling for the owner-separation procedure.

Data reduction was established by means of principal components analysis (PCA, Jolliffe⁽²⁹⁾) on remaining behavioural response parameters in 71 dogs for the open-field test and in 73 dogs for the owner-separation procedure, following similar procedures as described by van Reenen *et al.*⁽³⁰⁾. Briefly, PCA identifies parameters that co-vary (in the same or opposite direction) as indicated by relatively high absolute loadings, which like correlations range from -1 to +1, for a given component. For a major component, as indicated by a relatively high proportion of variance in the data set that is explained by it, scores are calculated based on a dog's scores for multiple parameters, using loadings as weighing factors. This means that component scores integrate different behaviours, weighing those with high absolute loadings the strongest.

For the open-field tests conducted before and after the start of the dietary treatment, a total of 21 parameters were analysed, namely walking, standing, standing against, sitting, lying, CLS, high posture, neutral posture, low posture, CPS, trembling, panting, tail wagging, oral behaviour, paw lifting, door scratching, barking, making contact with novel object, close to novel object, and latency to contact novel object. For the three owner-separation test phases, which were analysed separately, these (20 parameters) were walking, standing, standing against, sitting, lying, CLS, high posture, neutral posture, low posture, CPS, panting, tail wagging, oral behaviour, paw lifting, body shaking, door scratching, barking, whining, autogrooming, and yawning. Component scores were calculated by rerunning the PCA with only those parameters that loaded significantly (i.e. absolute values ≥ 0.5) on one of the main components. The resulting scores for PCA components and individual behavioural response parameters that were not part of a component, were analysed statistically as described below.

Statistical analyses

The parameters from the questionnaire and open-field test were analysed with linear mixed models (LMMs) using Residual Maximum Likelihood (REML) in the statistical package GenStat version 11.1 (Lawes Agricultural Trust, 2007). The following model was used:

$$Y_{ijk} = \mu + \text{DIET}_i + \text{WEEK}_j + (\text{DIET} \times \text{WEEK})_{ij} + \text{DOG}_k + \varepsilon_{ijk}$$

where Y_{ijk} is a measurement recorded in week j for dog k which was fed diet i . DOG makes up the random component of the model, with DIET (CDG, TDG, or FDG), WEEK (before start of dietary treatment, after 4 weeks of feeding, or after 8 weeks of feeding), and their interaction representing the fixed component. For the owner-separation procedure, PHASE of test (TO, AL, or RE) and its interactions with DIET and WEEK were also included in the model. Fitted values were plotted against residuals to check if dependent variables deviated strongly from normal distribution. If this seemed to be the case statistical outcomes on original data were compared to those on transformed data and discrepancies are reported. Changes in saliva cortisol concentrations were evaluated by the statistical methods described, but with the inclusion of SAMPLE (pre-test or post-test) and its interactions with DIET and WEEK in the fixed component of the model. Plasma amino acid concentrations were tested for DIET (CDG or TDG) effects using GLM. The time (h) between last meal and blood collection was included in the model as a covariate of a linear, quadratic, and cubic order to correct for differences between dogs in the course of postprandial digestion and absorption of nutrients after their meals. Differences were considered to be significant at $P \leq 0.05$.

Results

For a total of 378 dogs questionnaires were received of which 271 were selected to enter the study. For various reasons, 51 dogs did not start dietary treatment and 13 dogs were lost to follow up during the study. Diets were well accepted, but for five dogs with a maximum of two in one dietary group. The dogs' body weights and scores for skin and coat condition did not change significantly (results not shown) during the study in any of the dietary groups.

Behaviour questionnaire

The owner reported assessments of the dogs' behaviour ($n=207$) as summarised in seven different parameters are presented in Table 3. The scores for the behavioural parameters derived from the questionnaire entries were never affected significantly by dietary treatments as concluded from tests for DIET \times WEEK interactions (Table 3). Owners gave lower (read more favourable) scores in weeks 4 and / or 8 of the study as compared to the initial scores given in week 0 ($P < 0.05$ for WEEK), except for stranger-directed fear. For non-social fear, the owners awarded significantly higher scores in week 4 than in week 8. The

Table 3 | Overview of the owner-reported scores (least square means with standard errors (SEM)) for each behavioural parameter from the questionnaire for dogs fed the control diet ($n=66$), dogs fed the tryptophan diet ($n=72$), and dogs fed the fortified diet ($n=69$) for a minimum of 8 weeks.

Parameter ¹	Control		Tryptophan		Fortified		<i>P</i> -value ² DIET × WEEK
	Mean	SEM	Mean	SEM	Mean	SEM	
Stranger-directed fear (3)	2.3	0.4	2.5	0.4	2.9	0.4	0.886
Non-social fear (6)	6.8	0.7	6.3	0.6	6.3	0.6	0.105
Separation-related behaviour (8)	6.4	0.8	7.0	0.8	5.5	0.8	0.168
Attachment or attention seeking behaviour (6)	13.3	0.6	12.9	0.5	11.8	0.6	0.746
Excitability (6)	14.8	0.6	14.0	0.6	14.5	0.6	0.957
Pain sensitivity (3)	3.2	0.3	2.4	0.3	3.3	0.3	0.297
Aggregated anxiety (24)	22.6	1.7	22.6	1.6	22.5	1.6	0.884

¹Number of questions used to calculate each questionnaire parameter is indicated between brackets. Answers were presented on a 5-point scale, awarded a value from 0 to 4 indicating that a behaviour was not performed or excessively, respectively.

²*P*-values are based on questionnaire data obtained before the start of the dietary treatment and after 4 and ≥8 weeks of feeding the study diets.

random assignment of the dogs to dietary treatments holds a chance that dogs with a specific behavioural profile are overrepresented in one dietary group, which here would surface as a DIET effect. For the owner reported behavioural parameters such DIET effects were never significant.

Plasma amino acids

Thirty-two dogs were scheduled for blood sampling and for 30 this was done successfully. Of the two remaining dogs one was aggressive and the appointment for sampling the other dog was missed. Mean amino acid concentrations and Trp/LNAA-ratio for the CDG and TDG are presented in Table 4. Compared to plasma Trp concentrations of the CDG, the concentrations of the TDG were 37.4% higher ($P=0.020$). In line with this, plasma Trp to LNAA ratios were 31.2% higher in the latter group of dogs than in the former ($P=0.034$). Postprandial interval significantly effected both plasma Trp concentrations and plasma Trp/LNAA-ratios ($P<0.05$). None of the other amino acids that were measured were affected significantly by dietary treatment.

Behaviour tests

Thirty-four dogs from the CDG and 39 dogs from the TDG were observed for their behaviour during the open-field and owner-separation procedures, both before the start of the dietary treatment and after at least 8 weeks of feeding the experimental diets. For two dogs the open-field test was not performed according to procedures and these animals were

Table 4 | Amino acid concentrations ($\mu\text{mol/l}$) and tryptophan to large neutral amino acids (LNAA) ratio in the plasma (least square means and standard errors (SEM)) of dogs fed the control diet ($n=15$) and dogs fed the tryptophan diet ($n=15$) for a minimum of 8 weeks.

Parameter	Control		Tryptophan		<i>P</i> -value
	Mean	SEM	Mean	SEM	
Tryptophan	63.1	6.3	86.7	6.8	0.020
Isoleucine	61.8	3.5	68.2	3.8	0.240
Leucine	138.0	6.0	144.7	6.5	0.467
Phenylalanine	62.4	3.3	63.7	3.6	0.795
Tyrosine	53.0	3.4	61.0	3.7	0.131
Valine	190.2	8.0	201.4	8.6	0.355
Molar tryptophan/LNAA ¹	0.125	0.011	0.164	0.012	0.034

¹Sum of valine, isoleucine, leucine, tyrosine, and phenylalanine in moles.

excluded from analyses. Saliva samples were collected before and after the tests, though in 66% of the samplings too little saliva was collected for assaying on cortisol. Mean saliva cortisol concentrations, calculated from a total of 159 samples taken in 73 dogs, are shown in Figure 1. Cortisol concentrations were higher in saliva collected after the behaviour test than in saliva collected before it ($P=0.009$). Dogs had lower salivary cortisol concentrations the first time they were tested, i.e. before the start of dietary treatment, than the second time after at least 8 weeks of having been fed the experimental diet ($P<0.001$). There were no significant effects of dietary treatment on saliva cortisol concentrations as measured before or after the test ($P>0.05$ for DIET \times WEEK \times SAMPLE interaction).

For the purpose of data reduction, interrelationships between behavioural parameters that were recorded during the behaviour tests were investigated by PCA. The PCA analysis on open-field behaviours of 71 dogs resulted in one component that explained 20% of the variation in the data set, grouping eight of 21 behaviours. Behaviours included in this component seemed indicative of variation along the axis of ‘confident exploration’ to ‘timid immobility’ (hereafter referred to as confidence), namely CLS (loading of 0.70), latency to contact novel object (-0.67), making contact with novel object (0.63), walking (0.62), low posture (-0.57), close to novel object (0.57), neutral posture (0.56), and standing against (0.53). For the owner-separation procedure, here analysed for 219 records (i.e. 73 dogs times 3 subsequent test phases), one PCA component was identified that explained 17% of the variation in a dataset of 20 behaviours, grouping 5 behaviours that seemed indicative of variation along the axis of ‘aroused’ to ‘calm’ (hereafter referred to as arousal), namely CLS (loading of -0.76), walking (-0.64), tail wagging (-0.59), standing (-0.54), and CPS (-0.51).

The dogs’ behaviour during the open-field test, including confidence, is summarized in Table 5. There was no evidence for an effect of the Trp diet on open-field behaviour as indicated by non-significant DIET \times WEEK interactions. Significant effects of WEEK were observed. Compared to the first time of behavioural testing, the second time dogs showed less high posture, oral behaviours, and paw lifting ($P=0.015$, $P<0.001$, $P=0.010$, respectively)

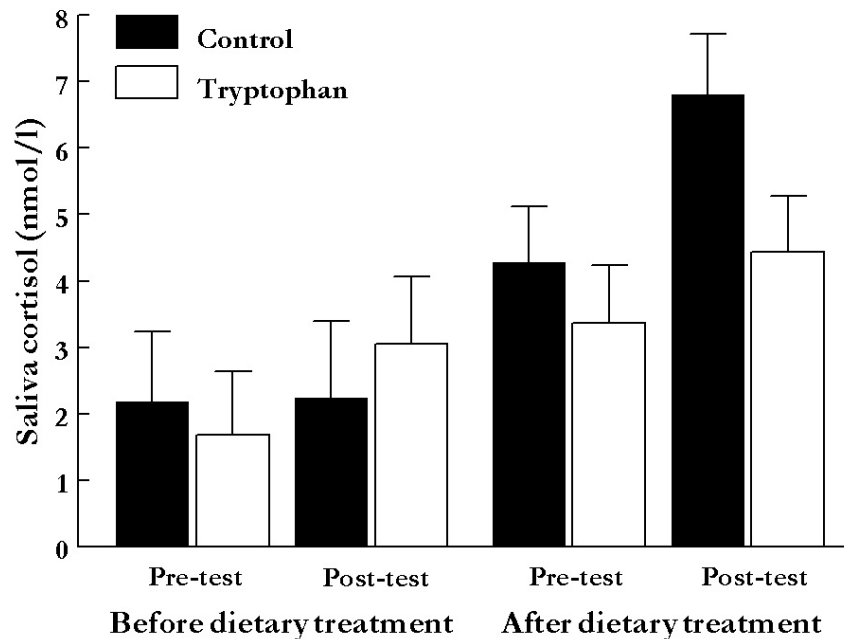


Figure 1 | Saliva cortisol concentrations (least square means with standard errors) for dogs in the control-diet group (CDG) and the tryptophan-diet group (TDG). Dogs were subjected to a behaviour test before the start of dietary treatment and again after minimally 8 weeks of feeding the study diets. Saliva was sampled before and after the behaviour test indicated as pre-test and post-test, respectively. Pre-test data was based on 29 and 31 dogs for the CDG and TDG, respectively, and post-test data was based on 32 and 36 dogs for the CDG and TDG, respectively. The behaviour test induced in a significant increase in saliva cortisol concentrations ($P=0.009$) with higher concentrations during the second behaviour test compared to first one ($P<0.001$). Dietary treatment did not affect saliva cortisol concentrations ($P=0.199$).

whereas they showed more confidence ($P<0.001$).

Table 6 provides an overview of the behaviours dogs showed during the three phases of the owner-separation procedure. One dog started to demolish the couch during the AL phase upon which the test was stopped. Effects of DIET×WEEK or PHASE×DIET×WEEK interactions were never significant, meaning that there were no influences of dietary treatment on the behaviour of the dogs. Weak indications for dietary effects were found for low posture ($P=0.087$ for DIET×WEEK), and body shaking ($P=0.098$ for DIET×WEEK). The second time of testing the dogs spent more time in a low posture compared to the first time ($P<0.001$ for WEEK) and this increase over time was more pronounced in dogs in the CDG (from 57.0 ± 5.6 to 70.0 ± 5.6) than those in the TDG (from 65.6 ± 5.2 to 70.4 ± 5.2). Similarly, the dogs that received the Trp diet showed a decrease in frequency of body shaking over time (from 0.3 ± 0.1 to 0.1 ± 0.1 times during the complete owner-separation procedure) as compared to an increase in dogs fed the control diet (from 0.2 ± 0.1 to 0.3 ± 0.1 times). Differences in the time spent showing a low posture during the first and second time of testing was mirrored, reversely, in neutral and high

Table 5 | Behavioural parameters recorded during the open-field test (least squares means and standard errors (SEM)) for dogs fed the control diet ($n=33$) and dogs fed the tryptophan diet ($n=38$) for a minimum of 8 weeks.

Parameter	Control		Tryptophan		<i>P</i> -value ¹ DIET × WEEK
	Mean	SEM	Mean	SEM	
<i>PCA component</i>					
Confidence ²	0.72	0.31	0.54	0.29	0.858
<i>Percentage of time of test</i>					
Standing	53.1	4.3	45.3	4.0	0.977
Sitting	15.2	3.8	29.0	3.6	0.312
Lying	15.3	3.1	7.6	2.9	0.156 ^a
High posture	2.5	2.6	3.6	2.4	0.374
Panting	37.8	5.8	39.6	5.4	0.893
Trembling	7.1	2.2	2.9	2.1	0.168
<i>Frequency per test</i>					
Whining	13.7	2.0	13.5	1.9	0.876
Barking	7.9	5.2	15.0	4.9	0.630 ^a
Oral behaviour	8.7	1.0	8.2	0.9	0.169
CPS ³	4.4	0.7	4.4	0.7	0.235
Door scratching	2.4	0.7	2.2	0.7	0.439
Paw lifting	0.5	0.3	0.5	0.3	0.686

¹Calculation of the *P*-value was based on open-field test conducted before the onset of dietary treatment and after 8 or more weeks of feeding the study diets.

²Grouping together number of changes of locomotion states, latency to contact novel object, making contact with novel object, walking, low posture, close to novel object, neutral posture, and standing against.

³CPS, number of changes of posture states.

^a*P*-values presented were derived from statistical analyses after log-transformation of data as residuals from the original data appeared non-normal distributed.

postures ($P=0.013$ and $P<0.001$, respectively, for WEEK). Neutral posture decreased in time from 32.8 ± 3.5 to 26.9 ± 3.5 and high posture decreased from 5.9 ± 1.4 to 2.0 ± 1.4 . The mean frequency of paw lifting was decreased at the second time of testing (from 0.4 ± 0.1 to 0.3 ± 0.1 , $P=0.035$ for WEEK) whereas for standing against this was increased (from 0.7 ± 0.2 to 1.3 ± 0.2 , $P=0.008$). Component scores for arousal tended to be higher (indicating more calmness) in dogs when they were tested the second time as compared to when they were tested the first time (from -0.11 ± 0.13 to 0.11 ± 0.13 , $P=0.052$ for WEEK). Obviously, the dogs behaved differently during the subsequent phases (OW, AL, and RE) of the owner-separation procedure, with $P<0.05$ for PHASE for all behaviours but panting and oral behaviours (see Table 6). Briefly, dogs tended to show relatively low levels of lying and high arousal during OW. During AL relatively high levels of paw lifting, oral behaviours, whining, barking, door scratching, standing against, sitting, and low posture and low levels of autogrooming, yawning, and neutral posture were observed. Behaviour typically expressed at

Table 6 | Behavioural parameters recorded during the three phases of the owner-separation procedure (least squares means and standard errors (SEM)) for dogs fed the control diet ($n=34$) and dogs fed the tryptophan diet ($n=39$) for a minimum of 8 weeks.

Parameter	Control		Tryptophan		<i>P</i> -value ¹	
	Mean	SEM	Mean	SEM	DIET × WEEK	PHASE × DIET × WEEK
<i>PCA component</i>						
Arousal ²	0.01	0.19	0.21	0.18	0.529	0.293
<i>Percentage of time of test phase</i>						
Lying	28.8	4.2	27.1	4.0	0.887	0.737
Sitting	16.2	3.6	23.5	3.4	0.455	0.080 ^a
Low posture	70.0	5.5	70.4	5.2	0.087	0.282
Neutral posture	28.5	5.1	25.3	4.7	0.347	0.113
High posture	1.5	2.1	2.5	2.0	0.302	0.834
Panting	72.9	4.0	78.5	3.8	0.638	0.935
<i>Frequency per test phase</i>						
Oral behaviour	11.4	1.0	11.7	1.0	0.557	0.873
Barking	9.0	3.4	7.3	3.2	0.773	0.878
Whining	6.1	1.1	4.8	1.0	0.943	0.711
Standing against	1.5	0.3	1.2	0.2	0.589	0.683
Door scratching	1.1	0.3	0.3	0.3	0.360	0.490
Yawning	0.4	0.1	0.6	0.1	0.443	0.218
Paw lifting	0.3	0.1	0.2	0.1	0.142	0.665
Body shaking	0.3	0.1	0.1	0.1	0.098	0.684
Autogrooming	0.1	0.1	0.3	0.1	0.289	0.879

¹Calculation of the *P*-values was based on owner-separation procedure conducted before the onset of dietary treatment and after 8 or more weeks of feeding the study diets.

²Grouping together number of changes of locomotion states, walking, tail wagging, standing, and number of changes of posture states.

³*P*-value presented was derived from statistical analyses after log transformation of data as residuals from the original data appeared non-normal distributed.

high levels during RE were body shaking, lying, and calmness (indicated by high component scores for arousal).

Discussion

Dietary Trp intake influences behaviour, at least in rodents⁽¹¹⁾, silver foxes⁽¹²⁾, pigs^(13,14), and humans⁽¹⁰⁾. A number of significant changes in both owner-reported assessments and behavioural responses did occur over time, possibly mirroring influences of a new diet regardless of its specific composition, placebo-like effects in the owners regarding the perception of their dogs' behaviour and / or habituation to the behavioural tests to which the dogs were exposed twice.

Beforehand it was assumed that effects of Trp supplementation on behaviour would most likely show as reduced expression of anxiety-related behaviours, which implies that

positive results were expected especially in anxious individuals. This assumption was based on findings in which increased Trp intake decreased anxiety in rats⁽¹¹⁾ and fear in silver foxes⁽¹²⁾. The study population and read out parameters were tailored to the aforementioned assumption and the recruitment and selection of the participating dogs was focussed towards relatively anxious dogs. Survey questions regarding the dogs' behaviour and the set-up of the behavioural tests were therefore directed mainly at assessing anxiety thereby excluding possibly effects of Trp supplementation on behaviours unrelated to anxiety, like aggression. A post-hoc statistical power analysis for the aggregated anxiety score indicated that the minimal significant difference between CDG and TDG that could be proven was 6.3 or 28% from the mean score of the CDG ($\alpha=0.05$, $\beta=0.1$, $n=60$ and $\sigma=11.4$ for CDG, $n=64$ and $\sigma=13.3$ for TDG). The absence of behavioural or mood effects of dietary fortification of normal well-fed individuals is in line with observations in humans. Dietary supplementation may have clear effects in individuals suffering from malnutrition (e.g. Gesch *et al.*⁽³¹⁾; Schoenthaler & Bier⁽³²⁾) but in well-nourished individuals the effects are often less clear⁽³³⁾.

In human subjects, individuals differ in their vulnerability to beneficial effects of dietary increases in the plasma Trp/LNAA-ratio^(15,34). It can be speculated that as the present study population contained a number of non-anxious dogs, the relative sensitivity of the population to the beneficial effects of Trp supplementation was low. However, additional analysis on 50% of the dogs with the highest aggregated anxiety scores showed no significant anxiousness-reducing effects of Trp supplementation, or feeding the fortified diet (data not shown).

An obvious reason for the lack of effects of Trp supplementation on the dogs' behaviour may have been an inappropriate dietary Trp content. The control diet (2.18 g Trp/kg DM or 0.68 g Trp/1000 kcal ME) was formulated to contain close to 0.46 g Trp/1000 kcal ME, the minimal allowance of Trp for adult dogs fed at maintenance⁽³⁵⁾. The Trp content in the supplemented diet was formulated to be 200% above that of the control diet. This was based on dosages of 100% to 300% additional Trp used in studies that reported behavioural effects in silver foxes⁽¹²⁾ and pigs^(13,14). Also, plasma Trp concentration, as well as Trp/LNAA-ratio, in the dogs on the Trp supplemented diet were increased compared to the dogs on the control diet. For rats, it has been suggested that only when plasma Trp/LNAA-ratios exceed normal ratios, and central Trp concentrations are at least twice normal concentrations, concentrations of 5-HT and that of its metabolite (5-hydroxyindolacetic acid or 5-HIAA)⁽³⁶⁾ are increased. This was also found in pigs where a two-fold increase in plasma Trp concentrations was accompanied by an increase in 5-HT by 11% and 5-HIAA by 52%⁽¹⁴⁾.

However, functional effects have been found in studies with smaller changes in plasma Trp concentrations and Trp/LNAA-ratios. Rats consuming an alpha-lactalbumin compared to a casein-based control diet had a 49% increase in plasma Trp concentration and a 40% increase in Trp/LNAA-ratio⁽³⁷⁾ and showed decreased anxiety during behaviour tests⁽¹¹⁾. Furthermore, in stress-vulnerable human subjects, an increase in Trp/LNAA-ratio of 42%

and 48% reduced saliva cortisol concentrations and improved cognitive performance and mood during experimental stress^(15,34). The present increase in plasma Trp (37.4%) and its ratio to LNAA (31.2%) may have been too low to result in functional changes in 5-HT neurotransmission, and, consequently, behaviour.

Scores for the questionnaire items (non-social fear, separation-related behaviour, attachment or attention seeking behaviour, excitability, pain sensitivity, and aggregated anxiety) were lower, thus more favourable, after the onset of any dietary treatment than before it. Besides the presence of a possible placebo-effect, basic aspects shared by the three diets may have played a role. Prior to the start of the study dogs were fed mainly dry diets varying from low-price, standard dog foods to the more expensive, super premium foods which are higher in quality (in terms of digestibility of nutrients). Dogs confined in a public animal shelter showed reduced signs of behavioural reactivity to novel situations when fed a high-quality diet for 8 weeks compared to dogs fed a standard diet⁽³⁸⁾. Switching from the original diet to experimental diet may have caused positive behavioural effects in a number of dogs. The more favourable questionnaire results following dietary treatments could be explained also by a change in the way owners perceived the (unchanged) behaviour of their dogs, for example caused by owners' expectations on the type of diet that they received. This was further investigated and after the study owners were asked which dietary treatment (control or experimental or no idea) their dog had received. Three-quarter of the owners responded and the proportion of owners that assumed to have received an experimental diet was 28% (out of 51 respondents), 29% (57) and 50% (50) of those that had received the control diet, Trp supplemented diet, and fortified diet, respectively. Statistical analyses replacing dietary treatment by 'expected dietary treatment', did not show a significant effects (data not shown) therefore bias due to the owners' expectations on the dietary treatment seems unlikely.

In the subset of dogs where anxiousness was measured during the open-field test and subsequent owner-separation tests, salivary cortisol concentrations after testing were on average 1.4 times higher than before testing ($P < 0.05$). The latter indicates that a number of dogs experienced some degree of stress^(26,39-41) which was confirmed by the behaviour of the dogs. During the 4-min open-field test, dogs panted and trembled about 40 and 5% of the time, respectively, and performed whining (~13 times), barking (~10), oral behaviours (~8), door scratching (~2) and, sometimes, paw lifting (~0.5), which in earlier studies have been linked to stress and fear-related states^(26,42,43).

The PCA analyses grouped together behaviours that mirrored activity / exploration (CLS, making contact with novel object or being close to it, walking, standing against and, in a reversed way, latency to contact novel object) and posture (neutral and, reversely, low). Integrated scores for this component seemed to discriminate confident dogs, as indicated by a neutral posture, that explored the novel environment actively from those that acted timidly by showing a low posture and being inactive. Since behaviours indicative of anxiety-related states were shown in abundance by the dogs during the open-field, the latter seems to have

been appropriate for measuring diet-induced changes in anxiousness. This was also shown by the modifying effects of the anxiolytic clomipramine on behavioural responses of dogs during novel object and startle tests⁽⁴⁴⁾. The same applies to the owner-separation tests during which dogs spent much time panting and showing a low posture (both typically over 70% of the observation time), and frequently performed whining (~5 times per test phase), barking (~8), and oral behaviours (~11). Door scratching, yawning, paw lifting and body shaking occurred, but to a lesser extent (less than about once per test phase). Body shaking by dogs was typically performed after the AL phase (i.e. during the RE phase) and can be interpreted as a sign of tension release or relief rather than stress⁽²⁶⁾. Yawning has been associated with psychological tension or mild stress in primates⁽⁴⁵⁾. Typically, dogs yawned during the OW and RE phases and less during the AL phase, suggesting that yawning may occur following previously experienced stress. The PCA analysis on behaviours that dogs showed during the owner-separation procedure grouped together CLS, walking, tail wagging, standing, and CPS. The parameters CLS, walking, and CPS indicate increased restlessness and activity. Tail wagging seems to be a sign of arousal⁽⁴¹⁾ and the integrated scores for the PCA component were assumed to reflect variation in this state.

To obtain an indication of the sensitivity of the behaviour tests, scores for confidence during the open-field test and the scores for arousal and low posture for the AL phase of the owner-separation tests were subjected to the post-hoc statistical power analysis as described above. The minimal difference between CDG and TDG for these parameters was between 19.6 and 31.5% from the mean score of the CDG.

There is evidence for an increased central synthesis and utilisation of 5-HT during stress. Neuronal depletion of 5-HT under such conditions may be prevented by increased Trp availability and activity of Trp hydroxylase^(8,46). Increasing brain Trp availability facilitates coping with enduring stress^(15,34,47) and Trp supplementation may only be useful therefore under stressful conditions that lead to relatively severe Trp depletion or in dogs with pathological low 5-HT neurotransmission. Accordingly, dogs subjected to chronic stress, e.g. dogs confined in animal shelters⁽⁴⁸⁾, may have increased Trp requirements and could benefit from increased dietary Trp intake.

Anxious dogs are sensitive for agents manipulating the central serotonergic system^(49,50), but dietary constituents are less potent to influence behaviour than that of drugs affecting similar functions⁽⁵¹⁾. The present study could not show beneficial effects of dietary Trp supplementation in privately-owned dogs. Similarly, supplementing a diet with Trp, beet pulp, salmon oil, soy lecithin, and green tea extract did also not result in changes in behaviour of dogs during their everyday life.

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Chapter 7

General discussion

Introduction

Studies in rats, pigs, and human subjects showed that behaviour and mood can be influenced by the specific composition of the diet consumed. Extrapolation of such findings to dogs could mean that their general well-being and their interactions with their social environment could be influenced by the consumed diet. Several mechanisms may underlie changes in behaviour following changes in nutrient intake (reviewed in Chapter 2) and two dietary strategies representing two of such mechanisms were selected for further investigation. This selection was based on a strategy's potential in other animals to affect behaviour and the likelihood that dietary effects on behaviour (if present) would become apparent within a relatively short period, i.e. within weeks instead of within several months or years. The first strategy was based on the impact of fermentation on general physical activity, supposedly as dietary fibre may influence behaviour through its impact on the duration of satiety. The second strategy was the use of dietary tryptophan to influence behaviour through its actions on the serotonergic system. In this general discussion the findings of the different studies are integrated, interpreted and discussed, including as related to the implications for practical nutrition in dogs and future research directions.

Dietary fibre, satiety, and behaviour

Satiety can be defined as the inhibition of food intake resulting from the consequences of food ingestion⁽¹⁾. The composition of the food ingested may affect the duration of satiety and, consequently, the timing of the onset of hunger or feeding motivation (Chapter 2). Increased feeding motivation is known to raise spontaneous physical activity in dogs⁽²⁾. Feeding motivation may be altered by dietary fibres, with effects depending on the fibre type and inclusion level. Fermentation of fibres along the distal gastrointestinal tract seems beneficial for the host's health, but also effective in the stimulation of satiety and the inactivity resulting from satiety. The fermentative activity of the microbial population residing in the distal gastrointestinal tract of dogs and fermentation characteristics (kinetics and end-products) of various fibrous substrates were studied in an *in vitro* system (Chapter 3). It was concluded that rectal inoculum had higher fermentative activity compared to proximal and transverse colonic inocula. Ranking of substrates based on their fermentation level and fermentation rate is not expected to be different between the large intestinal inocula from different sites. This suggests that faecal inoculum can be used for *in vitro* screening of fibrous ingredients. Based on this *in vitro* screening, ingredients were used in the formulation of diets with different *in vivo* large intestinal fermentation properties (Chapter 4). The low-fermentable fibre (LFF) and high-fermentable fibre (HFF) diet were fed to dogs and difference in fibre fermentability between diets was confirmed by an increase in total tract fibre degradation and faecal indicators of fermentation (Chapter 4). At the end of this study, dogs were tested for their motivation to eat. Furthermore, postprandial concentrations of satiety-related metabolites (i.e. peptide tyrosine tyrosine (PYY), glucagon-like peptide-1 (GLP-1), and ghrelin) and the behaviour of dogs in their home-kennel and in standardised challenging

situations were studied. Results of these studies showed that the dogs fed the HFF diet tended to have lower feeding motivation than dogs fed the LFF diet. There were no differences in the postprandial satiety-related plasma metabolite concentrations in dogs fed the HFF diet or LFF diet (Chapter 4). Concerning the behavioural aspect of the study (presented in Chapter 5), dogs that received the HFF diet showed increased inactivity during specific parts of the day compared to dogs that received the LFF diet. The challenging situations to which the dogs were exposed were an open-field test, a sudden-silence test, a novel-object test, and an acoustic-startle test. Responses to these tests were not affected by dietary fibre type. Together the results indicated that the HFF diet prolonged postprandial satiety as indicated by the lower feeding motivation and higher inactivity during the day.

Dietary fibre and satiety: fibre properties and satiety mechanisms involved

The mechanisms underlying these effects could not be revealed by the obtained data as plasma metabolite concentrations were similar for dogs fed different diets. In the following section other fibre properties and satiety mechanisms are considered that may also have contributed to the lowered feeding motivation of the dogs fed the HFF diet. Besides the fermentability of dietary fibre, ability to bind water and properties that affect viscosity may influence postprandial satiety^(3,4). Cellulose used in the LFF diet is considered to be a non-viscous component of plant fibre⁽⁵⁾. The HFF diet contained sugarbeet pulp and inulin. Sugarbeet pulp has a high water-binding capacity⁽⁴⁾ and contains soluble fibres (mainly pectin) accounting for approximately 20% of total dietary fibre^(5,6) that can increase digesta viscosity⁽⁷⁾. Inulin is highly soluble⁽⁸⁾ but will have little effect on the viscosity of digesta⁽⁹⁾. As indicated in Chapter 2, these differences in fibre properties between diets may have caused physiological effects contributing to the observed effects on satiety. In short, the high water-binding capacity of fibres can increase the volume and weight of the gastric contents when liquids are available. As a result this can stimulate the stretch receptors which are involved in gastric signalling of satiation^(10,11). In human male subjects, increasing the volume of a preload (a milk-based drink) from 300 ml to 600 ml with similar energy and nutrient contents decreased energy intake 4 h later⁽¹²⁾. The sugarbeet pulp can bind water and probably also gastric liquids. This will increase the volume of gastric contents and may have resulted also in such depressing effects on energy intake during the feeding motivation test. Fibres that increase digesta viscosity, may decrease gastric emptying rate⁽¹³⁾ and increase small intestinal transit time⁽¹⁴⁾. It is thought that this will prolong gastric signals of satiation^(11,15) and prolong contact between nutrients and intestinal receptors involved in maintaining satiety^(16,17). Furthermore, the combination of decreased gastric emptying rate and a reduction in the nutrient absorption efficiency of the small intestine⁽³⁾, will result in delayed postprandial nutrient absorption and more stable blood glucose concentrations (e.g. see de Leeuw *et al.*⁽¹⁸⁾). If the rate of gastric emptying and small intestinal passage and subsequent digestion and absorption of nutrients would have been slowed down due to the contrasts in properties of fibres used in the present study (i.e. ability to bind water and to affect viscosity), this should

have reflected in the postprandial blood glucose. Postprandial glucose and insulin curves did not differ between treatments and glucose concentrations were close to basal values at the time of the feeding motivation test, indicating that most of the diet consumed was digested and absorbed. Thus, the observed effects of the fibre types on satiety were likely determined predominantly by the differences in fermentation properties.

Postprandial plasma concentrations of total PYY, total GLP-1, and total ghrelin were measured as the secretion of these metabolites are found to be affected by dietary fibre type in humans, rats, and dogs⁽¹⁹⁻²¹⁾ and are related to satiety (PYY and GLP-1) and hunger (ghrelin)⁽²²⁾. The study aimed to evaluate possible satiety promoting effects of the HFF diet and to unravel the mechanism underlying it. None of the satiety-related metabolites that were measured differed in plasma concentrations between dogs fed LFF and those fed HFF, and possibly other metabolites played a role in the observed difference in satiety. In rats, feeding fermentable fibre resulted in a sustained release of pre-proglucagon derivatives (oxyntomodulin and glicentin) by the L-cells compared to rats fed a non-fermentable fibre⁽²¹⁾. In humans, intravenous infusion of oxyntomodulin acutely reduces hunger and the size of a single-meal food intake⁽²³⁾. However, it is rather unlikely that peripheral oxyntomodulin concentrations would have been higher in the HFF-fed dogs when the two other L-cell products (i.e. PYY and GLP-1) were not. Interactions between small (undetectable) increases in multiple satiety-related hormones may have contributed to observed effects on satiety. For example, Neary *et al.*⁽²⁴⁾ observed additive effects of PYY and GLP-1 in the inhibition of appetite and induction of satiety. Similarly, in obese rats a combination of intraperitoneal injection of amylin and PYY was found to reduce food intake more than amylin or PYY alone⁽²⁵⁾. An alternative explanation of the observed lower feeding motivation of the HFF-fed dogs could be that the large intestinal production of acetate was increased in these dogs and used as an extra source of energy. The amount of energy in the form of produced acetate derived from fibre in dogs has been estimated to be at least 5% of energy requirements⁽²⁶⁾. Based on the amount of fibre degraded, it is possible to estimate the amount of energy derived from microbial fermentation of the HFF diet. The apparent digestibility coefficient of the non-starch polysaccharides (NSP) for the HFF diet was $20.6 \pm 4.96\%$ (Table 2, Chapter 4). As inulin was not included in the dietary NSP content this value is underestimated by 18 g/kg (footnote Table 1, Chapter 4). Inulin is rapidly fermentable *in vitro* (Chapter 3) and it is therefore assumed that it was completely degraded at the end of the digestive tract. The NSP digestibility would increase to $33.2 \pm 3.61\%$. The metabolisable energy (ME) content of fermentable carbohydrates has been estimated to be 6.3 kJ/g⁽²⁷⁾. The amount of ME derived from the degraded fibre fraction in the HFF diet was: $11.31 \text{ g}/100 \text{ g DM} \times 33.2\% \times 6.3 \text{ kJ ME/g} = 23.7 \text{ kJ ME}/100 \text{ g DM}$. The ME content of the HFF diet estimated using the Atwater factors was 1664.1 kJ ME/100 g DM. The amount of ME derived from fermentation would be 1.4% of total ME in the HFF diet. Based on the large intestinal transit time of dogs similar in body size⁽²⁸⁾, this amount would have been spread out over a period of approximately 18 h with the majority becoming available at the beginning of this period⁽²⁹⁾. It

is suggested that acetate produced can be used as a source of energy at times when glucose supply from the small intestine is decreasing^(26,30) thereby possibly postponing the end of the post-absorptive phase after a meal. According to Bellisle⁽³¹⁾, post-absorptive signals maintain satiety during this phase until the fuel brought by the previous meal has been utilised and/or stored. In line with this, healthy human subjects consuming a meal containing partially indigestible (retrograded) corn starch reported higher blood acetate concentrations and higher satiety levels 10 h after consumption than when consuming a meal with digestible (pregelatinised) corn starch⁽³²⁾. Although the amount of energy derived from fermentation seems to be small, it is still unknown to what extent it contributes to the prolongation of satiety in dogs and much remains to be investigated regarding the relative contributions of the different properties of fibres in sustaining satiety in dogs.

Dietary fibre and behaviour

From earlier studies dealing with the effects of dietary fibres on behaviour in pigs it was concluded that fermentable fibre sources reduced physical activity for several hours after feeding whereas the influences of low fermentable fibres on activity were less conclusive⁽⁴⁾. In line with this, the dogs fed the HFF diet showed a lower feeding motivation and were more inactive compared to the LFF-fed dogs (Chapters 4 and 5), though the present effects in dogs were less pronounced than those reported in pigs. In part, this is explained by differences in dietary fibre inclusion levels and the fermentation capacity of the large intestine. Diets that were fed to the pigs contained, for example, 25% to 60% sugarbeet pulp⁽⁴⁾ whereas the study described in this thesis used 8.5% sugarbeet pulp and 2% inulin. Although the *in vitro* fermentation activity (i.e. production of SCFA) of faecal inoculum from pigs was comparable to that from dogs⁽⁶⁾, pigs have a longer large intestinal transit time than dogs. Depending on the inclusion level of wheat bran, large intestinal transit time in sows varied from 39.1 h to 62.7 h⁽³³⁾. The transit time in the large intestine in the dogs would have been approximately 18.5 h⁽²⁸⁾. The longer large intestinal transit time in pigs results in increased microbial fibre degradation as compared to that in dogs. In pigs, the combination of relatively large amounts of fibre in their diets and a high fermentative capacity likely contributes to marked effects of fermentable fibre on behaviour. No effects of dietary treatments were found on behavioural responses during the challenging situations. Several studies showed that hunger increases anxiety in rats⁽³⁴⁻³⁶⁾ and pigs⁽³⁷⁾ and increases nervousness in humans^(38,39), which makes it somewhat surprising that dogs on the HFF diet and LFF diet reacted similarly. The behaviour test was conducted around the same time the difference in feeding motivation was observed between both dietary treatment groups. It is possible that the difference in hunger was minimal and not strong enough to cause a divergence in anxiousness of the dogs during the test.

Suggestions for future research

The studies described in Chapter 4 and 5 aimed to gain insight in the effects of fibre type on satiety and behaviour in dogs. Feeding motivation was lower in dogs fed the HFF diet but the conducted measurements could not explain this observation. Furthermore, it is not known how long satiety was sustained after a meal and it is possible that the difference in feeding motivation was not maximal at time of testing. It is of importance to continue with studying the influence of dietary manipulation on feeding motivation in dogs. Hungry dogs may encourage their owners to feed them above energy requirements^(40,41) resulting in overweight dogs. Overweight and obesity can be accompanied with a range of serious medical conditions resulting in detrimental effects on the health and longevity of dogs⁽⁴²⁾ and its manifestation should therefore be prevented. The feeding motivation test (Chapter 4) was studied once at about 6 h after the morning meal but it could be that the maximal difference between dietary treatments was earlier or later. Multiple moments of testing for feeding motivation will provide additional insight on the influences of specific fibre properties on the duration of satiety. Methods such as operant conditioning can be used to assess feeding motivation in subtle way as demonstrated in pigs^(43,44). In dogs, operant conditioning techniques have been used successfully to study food preferences^(45,46). In 2005, a cognitive-based testing procedure for assessing satiety in the dog was developed by Chan *et al.*⁽⁴⁷⁾. Drawbacks of such tests are that it requires training of the dogs and, consequently, are relatively laborious compared to the feeding motivation test described in Chapter 4. On the positive side the work load the dogs need to perform to obtain a reward can be adjusted and quantified and such tests could aid future research on the satiety-inducing properties of diets for dogs.

Technologies manipulating the properties of isolated fibres (i.e. water-binding capacity, ability to increase viscosity, fermentability) make it possible to unravel the relative contributions of these properties on the duration of satiety. Sources like cellulose and (carboxy)methylcellulose are both unfermentable but may differ considerably in their ability to bind water and how they affect viscosity. Likewise, fructooligosaccharides and guar gums are similar in that they are rapidly fermentable (Chapter 3), but differ in their viscous properties. Sodium alginate, a soluble fermentable fibre isolated from the cell walls of brown algae, is also a gelling agent, but differs in gelling properties to other fibres such as guar gum in that it specifically gels in the stomach⁽⁴⁸⁾. The inclusion of singular and combinations types of fibres will increase the insight in the relative contributions of the properties of dietary fibres in maintaining satiety.

Implications for canine diets

Satiety in dogs can be prolonged by means of including extra fermentable fibre in the diets (Chapter 4). Depending on the type and inclusion level of fermentable fibres its consumption may also affect regulation of intestinal transit time, mineral absorption, nutrient digestibility, faecal characteristics, defecation frequency, intestinal morphology, intestinal

health, and intestinal microbial populations⁽⁴⁹⁻⁵¹⁾. These aspects will not be affected equally in different dogs. Dogs vary considerably in body sizes and in weights (1-90 kg) influencing aspects of their gastrointestinal physiology⁽⁵²⁻⁵⁶⁾. Previous studies showed little effect of body size on orocecal transit time^(28,54,56) but considerable effects on large intestinal transit times⁽²⁸⁾. Large intestinal transit time varies from 9.1 ± 1.1 h in Miniature Poodles to 39.4 ± 1.6 h for Giant Schnauzers⁽²⁸⁾. This increase in large intestinal transit time will give the microbial population more time for degrading fermentable fibres. If the aim is to stimulate microbial fibre degradation along the large intestine, the fermentation rate of dietary fibres (see Chapter 3) can be used for the selection of the most appropriate fibres for small and larger breeds of dogs. Depending on the inclusion level, fibre will have less pronounced fermentation effects in small breed dogs compared to larger breeds. If fermentation of dietary fibre contributes to the prolongation of satiety then inclusion of fermentable fibres in diets to stimulate satiety will have more potential in large breeds of dogs compared to the small breeds (with less capacity to degrade dietary fibres).

The ongoing growth of the prevalence of overweight and obesity in companion dogs emphasises that current feeding practices by dog-owners are often suboptimal. Commercial pet foods can be categorised into four basic types: dry, semi-moist, moist, and snacks. Dry pet foods comprise the largest segment of total pet foods sold and approximately 95% of the pet foods are extruded⁽⁵⁷⁾. The satiating properties of dry extruded dog foods are expected to be lower than wet dog foods. Dry extruded dog foods typically contain low amounts of moisture (5-10%) resulting in a high energy and nutrient density. Furthermore, protein content ranges between 15% to 34% on a dry matter (DM) basis⁽⁵⁸⁾. Moist dog foods are less energy and nutrient dense as they contain 70% to 80% moisture and generally contain more protein (26.9% to 62.6% crude protein on a DM basis, Nguyen *et al.*⁽⁵⁸⁾). Furthermore, these wet diets often contain gelling agents such as guar gums and carrageenans that can affect viscosity of the gastrointestinal contents and are fermentable. For dogs fed a dry diet, a smaller amount of the diet in terms of weight and volume is required to meet its energy and nutrient requirements compared to dogs fed a wet diet. Dogs consuming dry diets will therefore have less food in the stomach compared to dogs fed a similar amount of energy in the form of a wet diet. Although gastric distension is a signal for satiation in the dogs⁽⁵⁹⁾, it is unlikely that dogs are not satiated after a meal of dry extruded food, but the duration of satiety may be shorter than after a meal of wet food. Typically, dogs are offered one type of food and a fixed amount of it. This can lead to learned associations between a certain level of gastric content and the expected postingestive effects facilitating satiation and inhibiting further feeding motivation⁽³¹⁾. The duration of satiety will be influenced by the energy content of the consumed diet. The consumption of a large amount of energy in a small volume of food (high energy density) induces a short duration of digestion and thus a quicker return of hunger and feeding motivation, compared with consumption of the same amount of energy ingested in a large volume⁽³¹⁾. Accordingly, dogs consuming dry dog foods (a high energy-dense food), will have a shorter duration of satiety than dogs fed the same amount of energy

in a wet dog food (a low energy-dense food). The difference in protein content between dry and wet dog foods adds to the contrast in the satiating properties as protein is the most potent of the macronutrients in stimulating satiety⁽⁶⁰⁾. The low protein content of the dry diets will result in less stimulation of satiety compared to dogs digesting a wet diet with a high protein content. The aforementioned properties of dry dog foods make them less satiating than wet foods and dogs that are fed such foods may be more likely to experience hunger and high feeding motivation. Increased begging and scavenging may result⁽⁴⁰⁾ and such behaviour encourages owners to feed their dog more than the animal's energy requirement⁽⁴¹⁾, leading to an increase in body weight. A dietary strategy to *prevent* dogs becoming overweight is to include both more protein and fibre. A recent study showed that a combination of increased protein and fibre content in diets for overweight dogs promoted satiety⁽⁴⁰⁾. Selection of specific fibrous ingredients based on their potential physiological effects can further influence the duration of satiety, as shown in Chapter 4. Fibrous ingredients are commonly included in commercial dry diets. There is a large variation in type of fibres used and in total dietary fibre contents. For example, Nguyen *et al.*⁽⁵⁸⁾ reported that total dietary fibre contents in a variety of dry dogs foods ($n=15$) ranges from 3.3% to 39.1% on a DM basis with insoluble fibres ranging from 3.2% to 34.6% and soluble fibres ranging from 0.0% to 4.7% on a DM basis. Diets high in fibre are used for the management of overweight and obesity in dogs. These dogs are restricted in their energy-intake that is likely accompanied by feelings of hunger during parts of the day. The high-fibre diets contain typically high amounts of insoluble low-fermentable fibres. Besides adding bulk and thus diluting the energy content of the diet, these fibrous ingredients have little physiological effect on the prolongation of satiety. The inclusion of fermentable fibres could be more beneficial for the energy-restricted dogs as they add to the ability of fed diets to sustain satiety. Feeding frequency may limit or pronounce the contribution of fibre fermentation in satiety. Studies in human subjects showed that more hunger was experienced when feeding frequency was low^(61,62). Thus, the prolongation of satiety may especially be important if the interval between meals is large due to a low feeding frequency (e.g. once or twice a day; the most common feeding frequencies for companion dogs⁽⁶³⁾). The study of Achour *et al.*⁽³²⁾ suggested that the fermentation of fibre could prolong satiety in human subjects. If this effect would also be present in dogs, dogs fed once or twice a day may in particular benefit from the inclusion of fermentable fibres in diets as satiety is then sustained over a longer period. The prevention of feeding motivation may be particularly important for specific breeds of dogs fed dry extruded diets. Several breeds of dogs have a reputation for being able to consume large meals very rapidly and they become obese if allowed to feed *ad libitum*⁽⁶⁴⁾. Diets that decrease feeding motivation of (in particular these) dogs would help dog-owners managing the weight of their dogs. However, dog-owners may perceive side-effects of feeding diets high in fermentable fibres as negative, e.g. flatulence or increased resting times (Chapter 5).

Dietary tryptophan and behaviour

Tryptophan is the dietary precursor of the neurotransmitter serotonin, which is involved in mood, stress responses and, consequently, behaviour⁽⁶⁵⁻⁶⁷⁾. Tryptophan shares a carrier mechanism at the blood-brain barrier with the large neutral amino acids (LNAA; tyrosine, phenylalanine, leucine, isoleucine, and valine) and competes with these for access into the brain⁽⁶⁸⁾. Variations in dietary intake of tryptophan and LNAA can influence central serotonin synthesis^(69,70) and affect behaviour⁽⁷¹⁻⁷⁶⁾, mood^(77,78), and coping with stressors^(74,79-81). It has been demonstrated that anxiety in rats could be reduced by the dietary supplementation of tryptophan⁽⁷⁵⁾ and it is known that anxious dogs are sensitive for agents manipulating the central serotonergic system^(82,83). From literature (Chapter 2) it could not be determined if anxious dogs fed the minimal amount of tryptophan set by nutritional AAFCO⁽⁸⁴⁾ guidelines for maintenance diets will improve in behaviour when fed higher amounts of dietary tryptophan. The main aim of study described in Chapter 6 was to evaluate the influence of an increased tryptophan intake on behaviour of privately-owned dogs in home situations and under standardised test situations. For 8 weeks, privately-owned dogs were fed a control diet or a tryptophan supplemented diet, using a randomised double-blinded, placebo-controlled approach. Owner reported about their dogs' behaviour in the home situation by filling out a web-based questionnaire. A selection of dogs from study population fed the control and tryptophan supplemented diets were sampled for blood to evaluate plasma amino acid concentrations. The study showed that dogs fed the high-tryptophan diet had a higher plasma tryptophan concentration and tryptophan to LNAA ratio compared to dogs fed the control diet. Improvements in behaviour, i.e. less non-social fear, separation-related behaviour, attachment or attention seeking behaviour, excitability, pain sensitivity, and aggregated anxiety (for explanation of parameters see Chapter 6), were indicated by owners, but this was irrespective of the dietary treatment the dogs had received. A subset of dogs were observed in behaviour tests that were designed to measure anxiety. Again, there were no indications that feeding dogs increased amounts of dietary tryptophan changed their behaviour or, specifically, behavioural responses in stressful situations (as indicated by saliva cortisol concentrations). Owners did not report problems with their dogs consuming the high-tryptophan diet on a daily basis. In human subjects, there are few if any side effects of the intake of large amounts of tryptophan but if present they are generally mild with the most common being nausea and lightheadedness⁽⁸⁵⁾. For a 20 kg dog consuming 330 g of the tryptophan diet (according to the manufacturer's guideline), the tryptophan intake per day was approximately 1.7 g (~85 mg/kg BW). A single 5 g dose of tryptophan caused headache and nausea in human subjects⁽⁸⁶⁾ whereas no adverse effects were reported in another study in which 10 g of tryptophan (~140 mg/kg) was ingested⁽⁸⁷⁾. Tryptophan has a bitter taste that could possibly depress the palatability of the study diet^(88,89). The acceptance of the tryptophan supplemented diet seemed to be comparable to the control diet (Chapter 6) indicating that the 5.70 g tryptophan/kg DM was not too high with respect to decreasing palatability. The discussion in Chapter 6 covers several other aspects of the study, including

the power of the study and the lack of dietary treatment effects on behaviour. It was concluded that the present study could not confirm beneficial effects of tryptophan supplementation in privately-owned dogs possibly because the dogs were not pathological anxious or chronically stressed and in need of extra tryptophan. In the following it is explored if under specific conditions dietary tryptophan supplementation can influence behaviour in dog. The implications of the present findings for practical nutrition of dogs and directions of future research are addressed also.

Dietary tryptophan and behaviour

There are numerous studies that evaluated the effect of altered tryptophan availability on behaviour, mood, and responses to stressors in various animal species. Studies that experimentally decreased tryptophan availability by using the acute tryptophan depletion technique (administration of an amino acid mixture without tryptophan and high in LNAA) showed for example an increase in aggressive behaviour^(71,77,90) and a lowering of mood^(77,91,92). Although these studies provide insight in the effects of impaired serotonin neurotransmission, it is unlikely that such pronounced effects on tryptophan availability will occur in normal individuals consuming adequate diets. The focus in this thesis was therefore on the influence of tryptophan intake above minimum requirements set by nutritional guidelines. Tryptophan intake above requirements was effective in influencing behaviour or stress responses in rats⁽⁷⁵⁾, pigs^(74,76,79-81), silver foxes^(93,94), and human subjects^(72,73,78) indicating that increased tryptophan intake can have an impact on the behaviour of various animal species. As discussed in Chapter 6, it is of importance to consider the characteristics of the study population when assessing the potency of dietary tryptophan supplementation on behaviour. For the studies conducted in rats, pigs, and silver foxes, the rearing (e.g. socialisation, exposure to novel situations) of the experimental animals differed from the conditions in which companion dogs are raised and kept. In part, this explains differences in behaviour and, possibly, how it is influenced by dietary tryptophan supplementation as rearing conditions have pronounced effects on how an animal will react to challenges. For example, silver fox cubs exposed to handling from 2 to 8 weeks of age reduced fear and increased exploration during an open field test at 6 months of age⁽⁹⁵⁾. In pigs, enriching the rearing environment increased the amount of exploratory behaviour as well as reducing the animals' responsiveness to novel stimuli at the end of the rearing period⁽⁹⁶⁾. Thus, it appears that barren rearing conditions increase the sensitivity to challenges, possibly by an increased state of anxiety or chronic stress. It is also suggested that continuous housing of dogs in a shelter result in a sensitisation of the hypothalamic-pituitary-adrenal axis in response to standardised novel stressful test situations⁽⁹⁷⁾. This makes it likely that confined animals will be relatively susceptible to the benefits of increased tryptophan availability, in a similar way as observed in stress-vulnerable human subjects⁽⁷⁸⁾. Other studies also emphasise that only specific individuals (e.g. those with an history of aggression or depression) are susceptible to respond to treatments influencing tryptophan availability^(90,92,98-103) and it may be that only a

small subset of the privately-owned dog population will benefit from the supplementation of tryptophan to diets (see below).

Suggestions for future research

In the foregoing it has been argued that individual dogs with specific traits (e.g. anxiousness) or those experiencing negative states like chronic stress may show effects of elevated tryptophan on behaviour. Dogs confined to animal shelters experience high levels of stress due to separation from their former social environment, disruption of familiar routines, and uncontrollable and unpredictable environmental events⁽¹⁰⁴⁾. If such stress endures, the increased central serotonin turnover may result in a shortage of available tryptophan and serotonin, causing serotonergic activity to fall below functional needs⁽¹⁰⁵⁻¹⁰⁷⁾. Besides animal shelters, breeding stations and boarding kennels may also be perceived as stressful by dogs as the living conditions are often similar⁽¹⁰⁸⁾. It is of interest to investigate whether increased tryptophan intake supports dogs to cope with such demanding conditions. Hennessy *et al.*^(97,109) reported that dogs confined in a public animal shelter fed a diet high in digestible protein, fat, energy, and animal-derived ingredients decreased the activity of the hypothalamic-pituitary-adrenal axis (i.e. lower ACTH concentrations) and reduced signs of behavioural reactivity when subjected to standardised stressful test situations compared to dogs fed a diet lower mentioned dietary characteristics. These findings could be indicative that these dogs are susceptible to dietary treatments such as tryptophan supplementation. Dogs that are pathological anxious may be another group of dogs for which dietary tryptophan may be beneficial. Biochemical imbalances in the serotonergic system in the brain^(65,110,111) are assumed to play a role in anxiety-related behaviour disorders. Dogs showing anxiety-related behaviour disorders have been demonstrated to be sensitive for agents manipulating the central serotonergic system. The tricyclic antidepressant clomipramine, which inhibits the neuronal reuptake of serotonin, decreased signs of separation-anxiety if given in addition to behavioural therapy⁽⁸²⁾. Fluoxetine, a selective serotonin reuptake inhibitor, improved separation anxiety in dogs also without the addition of behavioural therapy⁽¹¹²⁾. Furthermore, fear and anxiety were mildly decreased in dogs during ground transport when clomipramine was administered⁽¹¹³⁾. Together, these studies support an important role for the serotonergic system in states of anxiety in dogs and, in theory, these dogs should be susceptible to treatments that manipulate serotonin neurotransmission. The impact of dietary tryptophan on behaviour will probably only be apparent in a specific population of dogs and this is first to be confirmed. Future studies on the impact of dietary tryptophan on behaviour could consider the effective dietary inclusion level of tryptophan, the duration of the minimum period of exposure needed to induce a beneficial behavioural effect, side effects, and whether the chronic consumption of a high-tryptophan diet leads to adaptations resulting in only a temporary improvement in behaviour. It appears that behavioural effects of increased tryptophan intake can occur rapidly and are lasting. For example, feeding a diet high in tryptophan for 3 to 12 days resulted in behavioural changes

and stress-reducing in pigs^(74,76,79-81) whereas effects on behaviour of silver foxes were noted after 13 and 20 weeks of feeding a high-tryptophan diet^(93,94).

Finally, it should be noted that the role of the serotonergic system in the modulation of mood and behaviour is complex^(65,114,115). It is known that there is a large family of serotonin receptors^(116,117) whose function are only beginning to be understood. Furthermore, besides being the precursor of serotonin, it has recently been demonstrated that the kynurenines, derived from the tryptophan metabolism via the kynurenine pathway, may also influence brain function and play a role in anxiety and behaviour^(118,119). Thus, the exact mechanisms by which tryptophan could exert effects on mood and behaviour appear not to run solely through the serotonergic system.

Implications for canine diets

The use of dietary intervention for treating anxiety-related behaviour disorders in dogs may not be obvious as most problems have their origin in actions by the owner, by the owner's habits, and by the environment which they provide for the dog⁽¹²⁰⁾. It is unlikely that elevating dietary tryptophan intake on itself will solve anxiety-related behaviour disorders in dogs and it needs to be confirmed if a high-tryptophan diets has a facilitating role in the treatment (e.g. in conjunction with behavioural therapy) of these disorders. Dogs in animal shelters, kennels, breeding stations, boarding houses may also benefit from high-tryptophan diets.

Does diet composition affect canine behaviour?

Commercial dog diets in Europe and North America are generally formulated to meet the nutritional guidelines defined by respectively FEDIAF⁽¹²¹⁾ or AAFCO⁽⁸⁴⁾. These nutritional guidelines provide minimal and maximal inclusion levels of nutrients required for maintaining physical health or growth and reproduction. Variations in digestibility and availability of nutrients in commercial canine foods are taken into account in these recommendations. Although it still needs to be established if these recommendations also fulfil the requirements of emotional unstable dogs and dogs under stressful conditions, it is likely that requirements are met in dogs fed commercial diets that meet the set recommendations.

Current feeding practices of companion dogs vary considerably. There is a wide variety of commercial dog foods available that differ in ingredients, nutrient compositions, and bioavailability of nutrients. Furthermore, dogs are often fed non-commercial diets⁽¹²²⁾ that contribute to their nutrient intake. These include table scraps, home-prepared diets, bones and raw food, and treats. These non-commercial diets may not be nutritionally adequate, which may become a problem if fed exclusively. In the United States and Australia, about 7% of the dogs received at least half of their diet from home-prepared diets. Streiff *et al.*⁽¹²²⁾ reported that several nutrients (Ca, vitamin E, K, Cu, Zn) in home-prepared diets were below

AAFCO⁽⁸⁴⁾ minimum recommendations for adult dogs whereas commercial dry diets were above the minimum recommendations. Unfortunately, no information was available about several other essential nutrients which makes it difficult to indicate if feeding home-prepared diets will impair behavioural functioning.

Although most commercial diets are nutritionally adequate, they may contain ingredients that are known to influence behaviour in other animal species. Dietary phytoestrogens present in soy-based ingredients were found to increase aggressive behaviour and decrease affiliative behaviour in male cynomolgus monkeys⁽¹²³⁾ and increase anxiety in male rats^(124,125). In an evaluation of commercial dog diets containing soy-based ingredients⁽¹²⁶⁾ the average amount of phytoestrogens (genistein + daidzein + glycitein) was 327 mg/kg DM (range of 0 to 1328 mg/kg DM) which is approximately the same concentrations used in the studies of Simon *et al.*⁽¹²³⁾ and approximately 5 times higher than that used in the study of Patisaul *et al.*⁽¹²⁵⁾. Whether phytoestrogens have similar behavioural and emotional effects in especially male dogs has not been established. Besides the routine use of soy-based ingredients in commercial dog diets, phytoestrogens may specifically be included in diets for the prevention of weight gain in future diets. It has been shown that diets containing phytoestrogens reduced weight gain and fat accumulation in neutered dogs fed above energy requirements for maintenance^(127,128). Unfortunately the dietary concentrations were not reported for these studies. The behavioural effects of phytoestrogens in other animal species and its wide spread use in diets for dogs emphasise the need for studies into the effects of dietary phytoestrogens on the behaviour of dogs.

Finally, many of the nutritional recommendations for dogs are now regarded as important while once they were considered unnecessary or excessive. New recommendations for optimal nutrition are becoming the norm. For example, increased levels of antioxidants as well as certain fibre sources and n-3 polyunsaturated fatty acids are now considered normal in dog food recipe design⁽¹²⁹⁾. It is not expected that the minimal and maximal inclusion levels will change majorly in future recommendations as these have been well established for most nutrients in the past. The current research focus is predominantly on physical health with optimal nutrition for dogs varying in breed, life stage, gender, and clinical conditions. Future recommendations for optimal nutrition may change for specific parts of the dog population that are, for example, prone to become obese, highly anxious, or subjected to chronic stress.

Final thoughts about food

Scientific examination of the relationship between nutrition and behaviour is a relatively new area of study, and as such, generally accepted standards do not exist⁽¹³⁰⁾. When considering the impact of nutrition on behaviour it should be noted that only subtle effects may be expected. Plant and animal products may contain components that have potent effects on the brain and are considered to be drugs, substances of abuse, or poisons⁽¹³¹⁾. Non-drugs constituents from plants or animals may have less potent effect on brain function and

behaviour. The relatively moderate effects of these dietary functional components on brain function and behaviour contributes to the many controversies in the field of research where nutritional effects on human behaviour are studied⁽¹³⁰⁾. It is highly likely that the latter will also occur in current and future studies assessing nutritional effects on canine behaviour.

The ability of researchers to assess potential nutritional effects on the behaviour of dogs depends on available methods that can be used to reliably measure behaviour. There are many validated behavioural tests for measuring performance (e.g. fear, anxiety, and motivation) in rats and mice. It is widely recognised that it is vital that tests are performed in a consistent and standardised manner as small changes in the procedure can result in an inappropriate test, a misinterpretation of the outcome of the test, or a change in the response of the animal⁽¹³²⁾. When evaluating behaviour as affected by a (dietary) treatment, it is also advised to perform a series of different tests for each behaviour in order to measure different aspects of a complex behaviour and obtain reliable information on the behaviour of the animal^(133,134). For canine studies aimed at evaluating responses to treatments using behaviour tests, these methodological aspects should also be considered. This is especially critical when effects of dietary treatments on behaviour are considered to be subtle⁽¹³¹⁾. An extensive review of canine behavioural tests used in studies has recently been performed by Diedrich & Giffroy⁽¹³⁵⁾. One of the main conclusions of their review was that behavioural testing in dogs is lacking standardisation in the way it is implemented. Besides standardisation, tests need to be validated to ensure reliable measurement of performance. In line with this, Ley *et al.*⁽⁸³⁾ used an anxiolytic agent (clomipramine) to evaluate the use of several types of behaviour testing procedures for the assessment of fear in dogs. The behaviour tests described in the present thesis were based on a number of studies⁽¹³⁶⁻¹³⁹⁾ and on experience with canine behaviour tests at the Department of Animal Science at Wageningen University. Obviously, development and validation of behaviour tests that are sensitive and accurate in measuring performance of dogs and are straightforward in their execution to allow replication by other researchers will greatly benefit future studies investigating the impact of nutrition on canine behaviour.

Main findings of the studies reported within this work

- 1a. Faeces can be used as an inoculum source for *in vitro* fermentation studies, but may slightly overestimate the actual fermentation processes occurring more proximally in the large intestine. The use of ileal digesta as an inoculum source is not recommended.
- 1b. Fibrous ingredients used in canine diets vary largely in concentration of fermentable components, the *in vitro* fermentation kinetics, and in the end product profile (i.e. short-chain fatty acids, ammonia).
- 2a. Dogs fed a diet containing fermentable fibres (sugarbeet pulp and inulin) showed higher dietary fibre degradation and faecal short-chain fatty acids concentrations compared to dogs fed a diet containing a low-fermentable fibre source (cellulose).
- 2b. Postprandial responses in plasma peptide tyrosine tyrosine (PYY), glucagon-like peptide-1 (GLP-1), and ghrelin were not influenced by dietary fibre fermentability.
3. Dogs fed a diet containing fermentable fibres had less feeding motivation 6 h after their morning meal compared to dogs fed a low-fermentable fibre diet.
4. A diet containing high-fermentable fibre decreased activity in kennelled dogs compared to a diet containing a low-fermentable fibre source.
5. Anxiety-related behavioural responses of dogs to short-lasting challenges in a test arena conducted 5 to 7 h after their morning meal were not affected by dietary fibre fermentability.
6. Dietary tryptophan supplementation did not result in (favourable) effects on anxiety-related behaviours in privately-owned dogs during everyday life.
7. Anxiety-related behavioural responses of dogs during behavioural tests were not influenced by dietary tryptophan content.

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Summary/Samenvatting

Summary

The consumption of food is known to have effects beyond the basic provision of energy and essential nutrients for maintaining physical health. Studies in rats, pigs, and human subjects showed that mood and behaviour can be influenced by the specific composition of the diet consumed. The main objective of the research described in this thesis was to evaluate the nutritional impact on behaviour in dogs. In Chapter 2, scientific literature is reviewed to investigate the current knowledge on the influence of nutrition on canine behaviour and explores the underlying mechanisms by which dietary composition may affect behaviour in animals. Behaviour is regulated by neurotransmitters and hormones, and changes in the availability of their precursors may influence behaviour. The essential amino acid tryptophan, the precursor of the neurotransmitter serotonin, may affect anxiety-related behaviour and stress resistance. The latter may also be influenced by dietary tyrosine, a precursor to catecholamines. As diet composition, nutrient availability, and nutrient interactions affect the availability of these precursors in the brain, behaviour and stress resistance may be affected through nutrition. It was noted that polyunsaturated fatty acids, especially docosahexaenoic acid, have an important role as structural constituents in brain development. Also, dietary supply of polyunsaturated fatty acids could modify aspects of the dopaminergic and serotonergic system and, consequently, cognitive performance and behaviour. Finally, behaviour, e.g. physical activity, can be influenced by feeding motivation between meals. This feeding motivation may be altered by dietary fibre type and content. At present, only few studies have been conducted to investigate the role of nutrition in (problem) behaviour of dogs. Two of the above mentioned food strategies that are known to modify animal behaviour were selected for further study for its effects in dogs: 1) the influence of the dietary fibre type on behaviour and 2) the affect of dietary tryptophan content on behaviour in dogs.

Fermentable dietary fibres are degraded by the microbial population in the distal canine gastrointestinal tract. This degradation process yields short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate and several gasses. The acetate and propionate can be used as an energy source by the host and they can also stimulate the production and secretion of satiety-related hormones by the L-cells (i.e. peptide tyrosine tyrosine [PYY] and glucagon-like peptide-1 [GLP-1]). Based on these effects of these SCFA, it was hypothesised that the inclusion of fermentable fibres in a diet will prolong the duration of satiety after a meal compared to a diet containing fibres that are non-fermentable. As it is shown that feeding motivation increases physical activity and anxiety-related behaviour in pigs and rats, it was expected that dietary fibre fermentability would affect behaviour in dogs. Two *in vitro* studies were conducted to evaluate the fermentation activity along the distal canine gastrointestinal tract and to screen the fermentation kinetics and end-product profiles of various dietary fibres for dog foods (Chapter 3). For the first fermentation study, digesta was collected from the distal ileum, proximal colon, transverse colon, and rectum of three adult dogs. An inoculum was prepared from the digesta of each part of the gastrointestinal tract and

incubated together with a fructan, ground soy hulls, or native potato starch after which the production of SCFA and gas production were measured. Results showed comparable rates of maximal gas production for rectal and proximal colonic inocula. SCFA production was lowest for the ileal inoculum and highest for the rectal inoculum. It was concluded that the faecal microbiota can be used as an inoculum source for *in vitro* fermentation studies but it may slightly overestimate *in vivo* large intestinal fermentation. The second *in vitro* study evaluated the gas production, fermentation kinetics, and end-product profiles at 8 and 72 h for incubation of faecal inoculum with citrus pectin, three fructans, gum arabic, three guar gums, pea fibre, peanut hulls, soy fibre, sugarbeet fibre, sugarbeet pectin, sugarbeet pulp, wheat fibre, or wheat middlings. Sugarbeet pectin, the fructans, and the gums showed a high maximal rate of gas production compared to all other substrates whereas peanut hulls and wheat fibre were poorly fermentable indicated by a low amount of gas produced. Sugarbeet fibre, sugarbeet pulp, soy fibre, and wheat middlings were moderately fermentable with a low rate. Citrus pectin and pea fibre showed a similar low maximal gas production rate but time at which this occurred was later compared to sugarbeet fibre, sugarbeet pulp, soy fibre, and wheat middlings. Based on their *in vitro* fermentation characteristics, sugarbeet pulp and a fructan (inulin) were selected for further *in vivo* study. In addition, based on other *in vitro* and *in vivo* studies, cellulose was also selected as this fibre source is low-fermentable. These types of dietary fibres were used in a study that evaluated the effect of dietary fibre type on satiety-related metabolites in dogs and their feeding motivation (both described in Chapter 4) and its effect on behaviour of dogs (Chapter 5). Sixteen healthy adult dogs were fed a diet containing low-fermentable fibres (LFF, 8.5% cellulose) or high-fermentable fibres (HFF, 8.5% sugarbeet pulp and 2% inulin). The dogs were fed two equal portions at 8:30 and 18:30 according to their energy requirements for maintenance. The higher large intestinal fibre degradation anticipated in dogs fed the HFF diet was confirmed by higher apparent faecal digestibility of the non-starch polysaccharides and higher SCFA and lower NH_3 concentrations in the faeces compared to the LFF-fed dogs. At the end of the study, dogs were given a single meal of a dry dog food 6 h after their morning meal to determine voluntary food intake as an indication of feeding motivation. HFF-fed dogs tended to consume less food than LFF-fed dogs. Dietary fibre type did not affect postprandial plasma glucose, PYY, GLP-1, and ghrelin responses. The concentrations of these metabolites could not be related to the voluntary food intake. It was concluded that fibre type can affect feeding motivation but the mechanisms that underlie this observation require further investigation. The behavioural effects of dietary fibre type were evaluated by means of observing the behaviour of dogs in their home-kennel and their behavioural responses to short-lasting challenges in a test arena. Results showed that at night and in the morning, HFF-dogs rested more compared to LFF-dogs, but they rested less in the afternoon. HFF-fed dogs showed lower values for inactive-alert (lie with head up or sitting) around 10:00-11:00 and higher values hereafter. Time spent tail wagging was higher for LFF-fed dogs just before the evening meal that may indicate higher level of arousal. No effects of dietary fibre type on behavioural

responses to the challenges were detected. In conclusion, the HFF diet increased inactivity in kennelled dogs, likely through sustaining satiety for a longer time than the LFF diet. Dietary fibre type did not affect the behavioural response to the challenges in these dogs.

The second strategy that was investigated involved the effect of tryptophan on behaviour in dogs. A randomised double-blinded placebo-controlled study was performed to evaluate the influence of dietary tryptophan supplementation on behaviour of mildly anxious privately-owned dogs (Chapter 6). For 8 weeks, dogs were fed a control diet or a diet containing more tryptophan than the control diet. A third diet fortified with tryptophan, beet pulp, salmon oil, soy lecithin, and green tea extract was also included in the study to evaluate its potential to influence behaviour. Owners reported about their dogs' behaviour in the home situation by filling out a web-based questionnaire before the onset of dietary treatment and again after 4 and 8 weeks of feeding the experimental diets. Results showed that owners indicated behavioural changes of their dogs, but these changes could not be attributed to a specific dietary treatment. A selection of dogs fed the control diet or the tryptophan diet were subjected to behaviour tests before and after 8 weeks of dietary treatment. The test included open-field situations and owner-separation procedures and were set up to measure anxiousness. Blood was collected after 8 weeks from dogs in the control and tryptophan groups for evaluation of plasma amino acid concentrations. Intake of the tryptophan supplemented diet increased plasma tryptophan concentrations and its ratio with large neutral amino acids compared to the control diet. The dogs' responses in the behavioural tests, including those in saliva cortisol, were not affected by dietary tryptophan content. A number of changes in both owner-reported assessments and behavioural responses did occur over time, possibly mirroring a placebo-effect and / or influences of the new diets regardless of its specific composition. It was concluded that intake of diets supplemented solely with tryptophan or in combination with beet pulp, salmon oil, soy lecithin, and green tea extract does not change (anxiety-related) behaviour in mildly anxious privately-owned dogs.

From the studies included in this thesis, it can be concluded that dietary fibre type can affect physical activity in kennelled dogs likely through its effect on satiety. Furthermore, increasing the dietary tryptophan content did not affect behaviour of mildly anxious privately-owned dogs.

Samenvatting

Het is bekend dat het effect van het eten van voedsel verder gaat dan alleen het leveren van energie en essentiële nutriënten die nodig zijn voor het onderhouden van de fysieke gezondheid. Studies met ratten, varkens en mensen hebben laten zien dat stemming en gedrag beïnvloed kunnen worden door de specifieke samenstelling van het geconsumeerde voeder of eten. Het doel van het onderzoek dat in dit proefschrift is beschreven was om de invloed van voersamenstelling op het gedrag bij honden te onderzoeken. Hoofdstuk 2 omvat een literatuurstudie naar de huidige kennis van het effect van voeding op het gedrag van honden en naar de mechanismen die hieraan ten grondslag liggen. Gedrag wordt gereguleerd door neurotransmitters en hormonen, en veranderingen in de beschikbaarheid van de precursors van deze stoffen kunnen gedrag beïnvloeden. Het essentiële aminozuur tryptofaan, de precursor van de neurotransmitter serotonine, kan gedrag gerelateerd aan gespannenheid en de gevoeligheid voor stress beïnvloeden. Dit laatste kan ook worden beïnvloed door tyrosine, een precursor van de catecholamines. Omdat de voersamenstelling, de beschikbaarheid van nutriënten en interacties tussen nutriënten effect hebben op de beschikbaarheid van deze precursors in de hersenen, kunnen gedrag en stressgevoeligheid beïnvloed worden door voeding. Meervoudig onverzadigde vetzuren, in het bijzonder docosahexaeenzuur, spelen een belangrijke rol als bouwstoffen die nodig zijn bij de ontwikkeling van de hersenen. De meervoudig onverzadigde vetzuren uit voedsel kunnen ook aspecten van het dopaminerge en het serotonerge systeem veranderen en daardoor het cognitief functioneren en gedrag beïnvloeden. Tenslotte kan gedrag, bijv. fysieke activiteit, beïnvloed worden door de eetmotivatie tussen twee maaltijden. De eetmotivatie kan beïnvloed worden door het gehalte en type vezels in de voeding. Er zijn op dit moment nog weinig studies verricht naar de rol van voeding in (probleem)gedrag bij honden. Twee van de bovenstaande voerstrategieën waarvan bekend is dat deze effect hebben op het gedrag bij dieren, zijn geselecteerd voor verdere studie naar de effecten op het gedrag van honden: 1) de invloed van type voedingsvezel en 2) het effect van het gehalte aan tryptofaan in een voeder.

Fermenteerbare voedingsvezels worden afgebroken door de microbiële populatie in het lagere gedeelte van het verteringskanaal. Dit afbraakproces levert kortketenige vetzuren op zoals azijnzuur, propionzuur en boterzuur en verschillende gassen. Azijnzuur en propionzuur kunnen door de gastheer als energiebron worden gebruikt en kunnen de aanmaak en afgifte van verzadigingshormonen door zogenaamde L-cellen (peptide tyrosine tyrosine [PYY] en glucagon-like peptide-1 [GLP-1]) stimuleren. Op basis van de effecten van deze kortketenige vetzuren werd de hypothese gevormd dat het toevoegen van fermenteerbare vezels aan een voeder de duur van verzadiging na een maaltijd verlengt ten opzichte van een voeder dat niet-fermenteerbare vezels bevat. Omdat het bekend is dat eetmotivatie fysieke activiteit en gespannenheid verhoogt bij varkens en ratten, werd er verwacht dat de fermenteerbaarheid van vezels ook effect heeft op het gedrag van honden. Twee *in vitro* studies zijn uitgevoerd om de fermentatieactiviteit in het distale gedeelte van het verteringskanaal te evalueren en om

de snelheid en eindproducten van de fermentatie van verschillende voedingsvezels te evalueren (Hoofdstuk 3). Voor de eerste fermentatiestudie werd digesta verzameld uit het distale ileum, proximale colon, transverse colon en het rectum van drie volwassen honden. Van de verzamelde digesta werd er per darmdeel een inoculum gemaakt dat geïncubeerd werd met een fructaan, met gemalen sojahuilen of met natief aardappelzetmeel. Tijdens de incubatie werd de productie van de kortketenige vetzuren en gas gemeten. De maximale snelheid van gasproductie van het rectale inoculum was vergelijkbaar met die van het proximale colon. Vetzuurproductie was het laagst voor het ileale inoculum en het hoogst voor het rectale inoculum. Geconcludeerd kan worden dat fecale microbiota zouden kunnen worden gebruikt als bron voor inoculum voor *in vitro* fermentatiestudies maar dat dit wel de *in vivo* fermentatie in de dikke darm licht kan overschatten. In de tweede *in vitro* studie werd de gasproductie, snelheid van fermentatie en werden fermentatie-eindproducten geëvalueerd op 8 en 72 uur voor incubatie met fecaal inoculum en citruspectine, drie fructanen, Arabische gom, drie guar gums, erwtenvezel, pindahullen, sojavezel, suikerbietenvezel, suikerbietenpectine, suikerbietenpulp, tarwevezel of tarwegries. Suikerbietenpectine, de fructanen en de gums bleken een hoge waarde te hebben voor de maximale snelheid van gasproductie vergeleken met alle andere substraten. Incubatie met pindadoppen en tarwevezel resulteerde in weinig gasproductie en waren dus slecht fermenteerbaar. Suikerbietenvezel, suikerbietenpulp, sojavezel en tarwegries werden goed gefermenteerd maar met een lage maximale snelheid van gasproductie. Citruspectine en erwtenvezel hadden een vergelijkbare lage maximale snelheid met suikerbietenvezel, suikerbietenpulp, sojavezel en tarwegries de maximale snelheid werd bereikt op een later tijdstip. Op basis van de *in vitro* fermentatie karakteristieken werden suikerbietenpulp en één van de fructanen (inuline) geselecteerd voor verdere studie *in vivo*. Voor de vervolgstudie werd, op basis van eerdere gepubliceerde *in vitro* en *in vivo* studies, ook cellulose geselecteerd als een laag fermenteerbare vezel. Deze vezeltypes werden gebruikt in een studie gericht op de effecten van vezeltype op metabolieten gerelateerd aan verzadiging en eetmotivatie van honden (Hoofdstuk 4) en op gedrag van honden (Hoofdstuk 5). Zestien volwassen honden kregen een voeder met laag fermenteerbare vezels (LFV, 8.5% cellulose) of hoog fermenteerbare vezels (HFV, 8.5% suikerbietenpulp en 2% inuline). De honden ontvingen hun voeder in twee gelijke porties om 8:30 en 18:30 naar hun energiebehoefte voor onderhoud. Vergeleken met de LFV-honden hadden de HFV-honden een hogere vezelafbraak in de dikke darm gezien de hogere schijnbare verteerbaarheid van de niet-zetmeel celwandpolysacchariden, fecale hogere kortketenige vetzuur- en lagere NH₃-concentraties. Aan het einde van de studie kregen de honden 6 uur na hun ochtendmaaltijd één enkele maaltijd commercieel droog hondenvoer om de vrijwillige voeropname vast te stellen als indicatie voor eetmotivatie. HFV-honden neigden minder te eten dan de LFV-honden. Type voedingsvezel had geen effect op de concentratie glucose, PYY, GLP-1 en ghreline in plasma na de maaltijd. De concentraties van deze metabolieten konden niet worden gerelateerd aan de vrijwillige voeropname. Er werd geconcludeerd dat vezeltype eetmotivatie kan beïnvloeden maar dat het begrijpen van het onderliggende mechanisme verdere studie vereist. De effecten van type voedingsvezel op

gedrag zijn bestudeerd door gedragsobservaties van de honden in hun kennel en tijdens kortdurende testen in een gedragsruimte. 's Nachts en in de ochtend rustten de HFV-honden meer vergeleken met de LFV-honden, maar ze rustten minder in de middag. HFV-honden lieten lagere waarden zien voor inactief-alert (liggen met de kop omhoog of zitten) rond 10:00-11:00 uur en hogere waarden daarna. De LFV-honden kwispelden meer net voor het avondmaal hetgeen een verhoogde opwindning kan betekenen. Effecten van vezeltype op de gedragsrespons zijn tijdens de testen niet waargenomen. Geconcludeerd kan worden dat bij deze honden in een kennel het HFV-voeder de activiteit verlaagde, waarschijnlijk doordat dit voeder een langer gevoel van verzadiging geeft dan het LFV voeder. Het type voedingsvezel had geen effect op de gedragsrespons bij deze honden.

De tweede onderzochte strategie was het effect van tryptofaan op het gedrag bij honden. Een gerandomiseerde, dubbelblinde en placebo-gecontroleerde studie werd uitgevoerd om het effect van suppletie van tryptofaan aan een voeder op het gedrag van enigszins gespannen of nerveuze particulier gehouden honden te onderzoeken (Hoofdstuk 6). Gedurende 8 weken werden de honden gevoerd met een controle voeder of een voeder met meer tryptofaan dan het controle voeder. Een derde voeder verrijkt met tryptofaan, bietenpulp, zalmolie, soja lecithine en groene thee-extract werd eveneens in de studie opgenomen om een potentieel effect op het gedrag te evalueren. Eigenaren rapporteerden over het gedrag van hun hond in de thuissituatie door een vragenlijst in te vullen op internet vóór aanvang van de proef en na 4 en 8 weken voeren van de experimentele voeders. Eigenaren rapporteerden geen gedragsveranderingen die door een specifieke voerbehandeling werd veroorzaakt. Een selectie van honden, gevoerd met de controle of tryptofaan voeders, werd blootgesteld aan gedragstesten vóór het begin en na 8 weken van de proef. De test bevatte een openveld situatie en een procedure waarbij de hond van de eigenaar werd gescheiden en was opgezet om nervositeit van de hond te meten. Daarnaast werd na 8 weken voeren bloed verzameld bij honden in de controle en tryptofaan groepen voor het vaststellen van aminozuur concentraties in het plasma. Het met tryptofaan gesuppleerde voeder verhoogde de tryptofaanconcentraties en de verhouding tussen tryptofaan en de grote neutrale aminozuren in plasma vergeleken met het controle voeder. De respons van honden tijdens de gedragstesten, waaronder het cortisolgehalte in het speeksel, werd niet beïnvloed door het tryptofaangehalte van het voeder. Enkele gedragsveranderingen gerapporteerd door de eigenaren en in de respons zijn tijdens de studie gevonden, waarschijnlijk veroorzaakt door een placebo-effect en/of de invloed van de nieuwe voeders onafhankelijk van de specifieke samenstelling. Er werd geconcludeerd dat de opname van een voeder gesuppleerd met alleen tryptofaan of in combinatie met bietenpulp, zalmolie, soja lecithine en groene thee extract geen gedragsverandering veroorzaakt in licht nerveuze particulier gehouden honden.

De studies beschreven in dit proefschrift leiden tot de conclusie dat het type voedingsvezel fysieke activiteit in honden in een kennel kan beïnvloeden, waarschijnlijk door het effect op verzadiging. Daarnaast heeft het verhogen van het tryptofaangehalte geen effect op het gedrag van licht nerveuze particulier gehouden honden.

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Guido

Wageningen, januari 2009

Curriculum Vitae

Guido Bosch werd geboren op 17 januari 1980 te Heerlen en groeide op in Kerkrade en later in Landgraaf. In 1998 behaalde hij zijn Atheneum diploma aan het Grotius College te Heerlen. In datzelfde jaar begon hij met de studie Zoötechniek aan de toenmalige Landbouwniversiteit Wageningen. Tijdens deze studie deed hij twee afstudeervakken, in de richtingen Diervoeding en Ethologie. In 2004 studeerde hij af in Zoötechniek met de specialisatie Diervoeding. In februari 2005 begon hij binnen de leerstoelgroep Diervoeding aan een promotietraject aangaande de invloed van voeding op gedrag van honden, mede mogelijk gemaakt door financiering van Wageningen Institute of Animal Sciences (WIAS). Het onderzoek dat hij uitvoerde werd in december 2008 afgerond en staat beschreven in dit proefschrift. Daarnaast is hij als secretaris actief geweest in het Bestuur van de WIAS Associated Ph.D. Students (WAPS). Sinds februari 2009 is hij werkzaam als onderzoeker bij de leerstoelgroep Diervoeding van Wageningen Universiteit.

Guido Bosch was born on 17 January 1980 in Heerlen and grew up in Kerkrade and later in Landgraaf. In 1998 he graduated from the secondary grammar school Grotius College in Heerlen. In the same year he started the study Animal Science at the former Agricultural University in Wageningen. During this study he completed two theses, concerning Animal Nutrition and Ethology. In 2004 he obtained his Master's diploma in Animal Science with animal nutrition as a specialisation. In February 2005 he started with a Ph.D. project within the Animal Nutrition Group about the influence of nutrition on behaviour in dogs, financed by the Wageningen Institute of Animal Sciences (WIAS). The research he conducted was finished in December 2008 and is described in this Ph.D. thesis. In addition, he was the secretary of the Board of the WIAS Associated Ph.D. Students (WAPS). Since February 2009 he has been working as a researcher at the Animal Nutrition Group of Wageningen University.

Publications

Refereed scientific journals

- Bosch G, Beerda B, Hendriks WH, van der Poel AFB & Verstegen MWA (2007). Impact of nutrition on canine behaviour: current status and possible mechanisms. *Nutrition Research Reviews*, **20**: 180-194.
- Bosch G, Pellikaan WF, Rutten PGP, van der Poel AFB, Verstegen MWA & Hendriks WH (2008). Comparative in vitro fermentation activity in the canine distal gastrointestinal tract and fermentation kinetics of fiber sources. *Journal of Animal Science*, **86**: 2979-2989.
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- Ange-van Heugten KD, van Heugten E, Timmer S, Bosch G, Elias A, Whisnant S, Swarts JJG, Ferket P & Verstegen MWA (2009). Fecal and Salivary Cortisol Concentrations in Woolly (*Lagothrix lagotricha* spp.) and Spider Monkeys (*Ateles* spp.). *International Journal of Zoology*, **2009**, Article ID 127852, 9 pages, doi:10.1155/2009/127852.
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- Bosch G, Khoa MA, Koene P, Bokkers EAM & van der Poel AFB. A liquid, acidified wheat by-product in diets for broilers: impact on feed intake, performance, leg condition and diet selection. Submitted to *British Poultry Science*.
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Bosch G, van der Poel AFB, Beerda B, van der Borg JAM & Verstegen MWA (2005). Introduction of the PhD-project: effects of diet composition (fermentable carbohydrates/tryptophan) on the behaviour in dogs. *Proceedings 30^{ste} Studiedag Nederlandstalige Voedingsonderzoekers*, April 8, Merelbeke, Belgium, pp 75-76.

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Ange-van Heugten KD, Verstegen MWA, Swarts JJG, Bosch G, Ferket P, Ruijs J & van Heugten E (2007). Fecal cortisol in two New World primate species supplemented with inulin-type fructans. *The 4th Crissey Zoological Nutrition Symposium*, December 7-8, Raleigh, NC, USA, pp 37-38.

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Bosch G, Beerda B, Hesta M, van der Poel AFB, Janssens GPJ & Hendriks WH (2009). Dietary fiber type affects behavior in kennel dogs. *Proceedings of the Nestlé Purina Nutrition Forum: Changing Paradigms in Nutrition*, October 2-4, St. Louis, MO, USA. Published in: *Compendium: Continuing Education for Veterinarians*, **31** (Supplement): pp 63.

Training and Supervision Plan		Graduate School WIAS	
<i>Name:</i>	Guido Bosch		
<i>Group:</i>	Animal Nutrition		
<i>Period:</i>	2005-2009		
<i>Supervisors:</i>	Dr. ir. A.F.B. van der Poel (daily), Dr. ir. B. Beerda Prof. dr. ir. W.H. Hendriks, Prof. dr. ir. M.W.A. Verstegen		
		Year	Credits¹
The Basic Package			
WIAS Course on Philosophy of Science and Ethics		2005	1.5
WIAS Introduction Course		2006	1.5
International Conferences			
Eurostarch, Groningen, The Netherlands		2006	0.6
Petfood Forum Europe, Utrecht, The Netherlands		2007	0.3
ESVCN, Leipzig, Germany		2007	1.0
Nestlé Purina Nutrition Forum, St. Louis, USA		2008	1.0
Seminars and Workshops			
WIAS Science Day (5x)		2005-9	1.5
Meeting of the NVO (Studiedag Nederlandstalige Voedingsonderzoekers; 3x)		2005,7,8	0.9
Themamiddag “Fermentatie bij vleesvarkens”		2005	0.2
Orafti Petfood Seminar		2005	0.2
9 th Annual Gut Day (Darmendag)		2007	0.2
WIAS Seminar “Personalities in animals”		2007	0.2
Platform Behaviour and Animal Welfare Seminar “Behavioural adaptation”		2007	0.2
Presentations			
Meeting of the NVO (3x)		2005,7,8	3.0
ESVCN		2007	1.0
Nestlé Purina Nutrition Forum		2008	1.0
In-depth Studies			
Graduiertenkurs der Gesellschaft für Tierzuchtwissenschaft		2005	1.8
WIAS Advanced Statistics Course: Design of Animal Experiments		2005	1.0
VLAG International Advanced Course: Regulation of Food Intake and its Implications for Nutrition and Obesity		2006	1.0
WBS Course: Advances in Feed Evaluation Science		2006	1.5
ANU International Postgraduate Course: Pet Nutrition & Health		2007	0.6
WIAS Course: Statistics for the Life Sciences		2008	2.0
Professional Skills Support Courses			
WIAS Course: Techniques for Writing and Presenting a Scientific Paper		2006	1.2
OWU Course: Supervising M.Sc. Thesis Work		2007	0.6
WGS Course: Career Assessment (Ph.D. Midterm Assessment)		2007	0.3
WGS Course: Personal Efficacy		2008	0.5
WGS Course: Project- and Time Management		2008	1.5
Didactic Skills Training			
Supervisor B.Sc. Course: Introduction to Animal Science (YLS-10806; 3x)		2005-7	1.5
Supervisor B.Sc. Theses and Internships (7 Students)		2006-8	7.0
Supervisor M.Sc. Theses (10 Students)		2005-8	17.0
Lecturer ANU International Postgraduate Course: Pet Nutrition & Health		2007	1.5
Lecturer M.Sc. Course: Companion Animals (YLS-34306/YAS-32306)		2008-9	0.8
Management Skills Training			
Organisation WIAS Science Day		2006	1.0
Secretary WIAS Associated Ph.D. Students Council		2007-8	4.0
Total			59

¹One ECTS (European Credit Transfer System) credit equals a study load of approximately 28 hours.

Colophon

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