Metabolic effects of various carbohydrates and fibre in dog food

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Metabola effekter av olika typer av kolhydrater och fibrer i hundfoder

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Abstract

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The lack of scientific studies regarding dog nutrition highlights the need for future studies, especially when we know the importance of food in relation to health. The variety of ingredients in commercial dog food is huge. However, a common denominator in dry dog food is often the high amount of cereals, consisting usually 30–60% grains of the energy source in dry matter. There is an interest to study how different types of grains might impact the different types of metabolic pathways since the content of dietary fibres and bioactive compounds differ between grains. Dietary fibres have many beneficial health effects such as maintaining healthy gut microbiota and have shown a protective effect against diseases, mainly in human studies. Now we need metabolomic studies to provide wider knowledge about which different metabolites and metabolic pathways the different types of grains can stimulate in dogs. In this study, the metabolic response was analysed in 17 dogs after four weeks of feeding with three experimental feeds composed of different grain sources; whole grain wheat, peeled oats, and whole grain rye. Fasting and postprandial urine samples were used to generate metabolic profiles by Nuclear Magnetic Resonance spectroscopy (NMR). The multivariate analyses showed a significant differentiation in metabolic profiles between the diet with whole grain wheat and whole grain rye. A total of ten metabolites could be linked to this differentiation. The concentration of the ten metabolites was calculated in both fasting and postprandial samples to analyse if a difference could be found between the three experimental feeds. The metabolite methylguanidine showed a significant difference in concentration between the feed with whole grain wheat and peeled oat in postprandial urine. Additionally, the metabolite tyramine differed significantly postprandial between the feed with whole grain wheat and whole grain rye. The concentration of some identified metabolites differed between fasting and postprandial samples and most of these metabolites were linked to a significant shift in concentration after feed intake for the diet of whole grain wheat. In this study, only healthy dogs were included and no specific metabolic pathways or risk factors for metabolic diseases could be proven in connection to the differentiation between grains. However, the result showed variety in metabolic profiles after feed intake of different grains and now further studies are needed to look deeper into the relationship between metabolic differentiation of the grains and the activation of metabolic pathways.

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Abbreviations

ANOVA	Analysis of variance
BCS	Body condition score
DM	Dry matter
IQR	Interquartile range
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
PLS-DA	Partial least squares discriminate analysis
SCFAs	Short-chain fatty acids
SLU	Swedish University of Agricultural Sciences
VIP	Variable importance for projection

1. Introduction

More and more people want to have a dog in their lives today. In Sweden, there was a record in number of dogs registered in 2021 (Svenska kennelklubben 2022), indicating a positive trend in getting a dog leading to a growing dog population. We use the dog for many different purposes, such as companion dogs, competition dogs, hunting dogs, police dogs, and service dogs in the Armed Forces and hospitals. Due to the growing interest in dogs, the commercial pet food market has increased and developed feeds for dogs' individual needs. A recent review article confirmed that genes, conformation, behavior, and environmental inputs such as nutrition, fitness, and healthcare are key factors behind a healthy and well-performing dog (Zoran 2021). Nutrient proportions in dogs' diets must be adjusted for individual energy needs. Energy requirements must be fulfilled, and at the same time, the energy content needs to differ depending on breed, age, size, and working type (sprinting work as a police dog or long-term activity as a hunting dog) (Zoran 2021). If the energy content in the diet is too high relative to physical activity, the dog is at risk of becoming overweight (Zoran 2021). The fact that dogs are becoming overweight or obese has proven to be an increasing problem (German 2006). Approximately 30%–40% of dogs worldwide, regardless of breed, are classified as overweight and around 5%–20% as obese (McGreevy et al. 2005; Lund et al. 2006; Courcier et al. 2010). Corresponding to a summary by the World Health Organization (WHO), 30–80 % of adult humans in European countries are overweight (World Health Organization 2014). The diet composition is a risk factor for getting overweight, and the diet is also linked to non-communicable diseases (NCDs) in humans, including cardiovascular diseases, cancer, respiratory diseases, and diabetes (World Health Organization 2014). Likewise, similar diseases have been reported to associate with overweight and obesity in dogs (German 2006). Moreover, dogs are predisposed to abnormalities in circulating lipid profiles, urinary disorders, orthopedic disorders, and reproductive disorders (German 2006). These diseases entail a risk of decreasing the lifespan and longevity of dogs (German 2006).

The interest in how different carbohydrate sources influence the metabolism and intestinal health of dogs has increased. Moreover, there is an increased interest in studying the effect of the intestinal microbiota linked to disease development and the effect of dietary fibres since microbes have a huge impact on health, and

microbiota in the intestinal tract have a primary influence on the digestion of dietary fibres (Deng & Swanson 2015). The health of the gut is linked to the whole body's health since it is estimated that 70% of the immune cells are found in association to the gastrointestinal tract (Sanderson 2021). A variety of beneficial bacteria in the gut is necessary for a functioning system, and dietary fibre from undigested carbohydrates promotes the growth and reproduction of favourable bacteria (Sanderson 2021). Fermentation of dietary fibre produces short-chain fatty acids (SCFAs) that function as substrates in various metabolic pathways (Sanderson 2021). The SCFAs have many beneficial health effects with a primary role in the maintenance of healthy gut microbiota (Slavin 2004; National Research Council 2006). SCFAs have been shown to have a protective effect against diseases such as Cardiovascular disease (CVD), congenital heart defect (CHD), and diabetes mellitus which can be explained by their relation to lower serum cholesterol, improving glucose response, reducing insulin response and the effect on satiety and body weight (Slavin 2004; Frølich et al. 2013).

In comparison with human research, few studies have examined the effect of microbiota on health linked to various environmental factors such as diet in companion dogs (Deng & Swanson 2015). Previous studies had indicated the gut microbiota to be highly influenced by dietary changes (Deng & Swanson 2015). The amount of cereals is commonly high in dog food, consisting of about 30–60% of the energy source in dry matter (DM) (Kore et al. 2009; Roberts et al. 2018). There is a variation in the content of dietary fibres and bioactive compounds in different cereals (Slavin 2004). The content of dietary fibre has been shown to be highest in whole grain rye, near 20% on a DM basis, compared to whole grain wheat containing approximately 14% and dehulled oat containing about 10% (Frølich et al. 2013). Additionally, rye has been shown to have the highest concentration of the major bioactive compounds such as phytic acid, tocols, phenolic acids, and phytosterols, compared with the grains; wheat and oat (Frølich et al. 2013). The differences linked to dietary fibres and bioactive compounds have been of interest to study in human research considering positive health effects linked to the function of the gastrointestinal tract and the antioxidant activity (Wikström & Fredriksson 2017). Thus, there is an effort to deepen knowledge of how the composition of different carbohydrates can affect the dog's metabolism through metabolomic studies. Metabolomics is a research area that focuses on identifying metabolites in biological systems from cells, tissues, biofluids, and foods linked to humans or animals and provides information that can determine the metabolic state of an organism (Markley et al. 2017; Gowda & Raftery 2019). One technique to generate metabolomics data is Nuclear Magnetic Resonance spectroscopy (NMR) (Markley et al. 2017). This technique has previously been used to analyse metabolite profiles of urine in dogs and one of the findings was differences in the metabolomes between lean and overweight dogs in postprandial urine (Söder et al. 2017).

1.1 Aims of the thesis

This master's thesis aimed to study whether different types of grains in dog food impact the dog's metabolism. The grain sources included in the study were whole grain wheat, peeled oats, and whole grain rye. As the content of dietary fibres and other bioactive compound differs between these grain types it is important to understand if and how the content differences in these grains might impact different metabolic pathways. Metabolite profiles were generated from urine using NMR to study which different metabolites and metabolic pathways the different types of grains can stimulate. Multivariate data analysis was used for the evaluation. The research questions were:

- Does the type of grain in the dog food have any significance for the dog's metabolic response to the food?
- Can any connection be found between metabolic pathways for different types of grain and risk factors for metabolic diseases?

2. Literature review

2.1 Content differences in grains

Commercial dog foods are based on different carbohydrate sources with a huge variation in their format, macronutrient composition, ingredients, and fibre content (National Research Council 2006; Deng & Swanson 2015; Roberts et al. 2018). Cereal grains are commonly used with a variable amount depending on the type of diet (National Research Council 2006). The amount of fibre also varies in dog food which can be related to the fact that the food is adapted to the dogs' ability to utilize fibre in different life stages where energy needs differ (National Research Council 2006). Another reason for a variation of carbohydrates and fibres is the consequence of dry and wet feed processes requiring different compositions (Roberts et al. 2018). Dry diets usually contain 40%–60% of carbohydrates, 16%–38% protein, and 6%–18% fat on a DM basis (Roberts et al. 2018). In contrast, wet diet/raw diets commonly contain no or low levels (<10%) of carbohydrates and content of around 45% – 50% of protein and fat (Roberts et al. 2018). It is important to have in mind that the effects of processing methods on the digestion of different carbohydrates sources and fibre is still not completely understood (Kore et al. 2009). Dry dog food in the composition of kibbles is the most popular on the market (Kazimierska et al. 2021).

According to human consumption, wheat, maize and rice are defined as the major cereal grains while oats, rye, barley, triticale, sorghum, and millet are defined as minor grains (Slavin 2004). In the Scandinavian countries wheat, oat and rye are the most common whole grain cereal for humans to consume (Frølich et al. 2013). The structure of whole grains is similar, containing germ, endosperm and bran (Slavin 2004). The germ contains the plant embryo, endosperm, which provides food for the growing seedling, and the hard outer layer called bran (Slavin 2004). The endosperm consists of approximately 50% – 75% starch and 8% – 18% protein. The hulls of cereals are mainly made of cellulose and therefore diets with whole grains contain more cellulose (Kempe et al. 2008). Whole grains are a source of many different nutrients such as B vitamins, minerals, basic amino acids (for example, arginine and lysine), and bioactive compounds, such as phytic acid,

tocols, alkylresorcinols, phenolic acids and phytosterols (Slavin 2004; Frølich et al. 2013). Additionally, whole grains have a high concentration of fermentable carbohydrates, including dietary fibre and resistant starch. These nutrients and bioactive components are linked to positive health effects and disease prevention (Slavin 2004; Frølich et al. 2013). The bioactive compounds are important for many physiological functions and dietary fibres have an essential role of transport these compounds through the gastrointestinal tract where they can be activated (Frølich et al. 2013). The amount of fibre has been shown to vary in different grains. Whole grains, oats, rye and barley contain about one-third of soluble fibre and the rest amount is insoluble fibre, while wheat has a lower amount of soluble fibre when compared to most grains (Slavin 2004). Whole grain also contains other compounds such as antioxidants, phytic acid, lectins, phenolic compounds, amylase inhibitors, and saponins (Slavin 2004). The amount of lipids varies in different grain sources, where whole-grain wheat contains about 3%, which is lower than grain of oats which contain about 7.5% (Slavin 2004). Oats have been shown to contain a higher protein amount compared to other cereals such as barley, wheat, corn, and rice (Kempe et al. 2008). A higher proportion of amylose had also been measured in oats (26%) compared to wheat and barley (13–20%) (Kempe et al. 2008). The dietary fibre content and other nutrient composition differ significantly between whole grain wheat, whole grain rye, and dehulled oats, which are presented in Table 1 (Frølich et al. 2013). The total amount of dietary fibre is highest in whole grain rye, near 20% on a DM basis, compared to whole grain wheat containing approximately 14% and dehulled oat containing about 10% (Frølich et al. 2013). Rye has also the highest content of arabinoxylan compared to wheat and oat, which is explained by a thicker proportion of cell walls in the starchy endosperm (Frølich et al. 2013). Another difference between the grains is the content of *β*-glucan, where oat has a remarkably higher proportion than wheat and rye (Frølich et al. 2013). According to a summary, rye was shown overall to be the grain with the highest concentration of the bioactive compounds phytic acid, tocols, phenolic acids, phytosterols (Frølich et al. 2013) (Table 2).

Table 1. Dietary fibre content and composition of whole grain wheat, whole grain rye, and dehulled oats. Results are given as % of dry matter.

Component	Wheat	Rye	Oat
Total DF[a]	13.5	19.9	10.2
Arabinoxylan	5.6	8.9	2.0
Cellulose	2.5	2.9	1.3
β-glucan	0.8	1.5	5.0
Fructan	1.3	4.1	0.2
Lignin	0.8	1.1	1.4

[a]Dietary fibre

Values are adapted from a review by Frølich et al. (2013)

Table 2. Content of major bioactive compounds of whole grain wheat, whole grain rye, and dehulled oats (µg per g dry matter)

Component	Wheat	Rye	Oat
Phytic acid	390–1350	540–1460	420–1160
Tocols	28–80	44–67	16–36
Phenolic acids	326–1171	491–1082	351–873
Phytosterols	670–960	1098–1420	618–682

Values are adapted from a review by Frølich et al. (2013)

The metabolic effects of the inclusion of digestible and nondigestible carbohydrates in dog food is a rather novel area. Therefore, more information is needed to set up recommendations, including minimum and maximum requirements. However, according to previous studies, no adverse effects of the dietary level of carbohydrate sources in dog feed had been estimated (National Research Council 2006).

2.2 Whole Grains impact on Weight Management and health benefits

Whole grain has been stated to play an important role in weight management and reduce the risk of developing type 2 diabetes (Frølich et al. 2013). Evaluating the dog's body composition and documenting weight regularly is of great importance for preventing obesity and following up the weight loss for overweight dogs. Body Condition Score (BCS) is a validated tool to evaluate the dog's body composition, which creates a more standardized approach with clear guidelines for veterinarians and animal owners (Laflamme 1997). A linear relationship has been estimated between the percentage of body fat measured by X-ray absorptiometry (DEXA) and the BCS nine-point system. The association showed that body fat increased by approximately 5% for each increased unit of the BCS scale. This indicates that BCS is a reasonable estimate for accurate body composition, which can be described as predictability (Laflamme 1997). The BCS scale is often used in research to make it possible to follow up on the weight loss or weight gain of individuals throughout a study. One example from dog research is the use of BCS to divide dogs into groups; lean and overweight, to analyse metabolic differences linked to lipid metabolism after a high-fat mixed meal (Söder et al. 2016, 2017, 2019a; b)

Several studies have demonstrated that a feed composition containing dietary fibre could affect health status and has been linked to disease prevention (Frølich et al. 2013). One of the beneficial health effects is based on dietary fibre provision of short-chain fatty acids (SCFAs) (Slavin 2004; National Research Council 2006; Tazoe et al. 2008). The production of the SCFAs initiates when dietary fibres are fermented by the intestinal microbiota in the colon (Slavin 2004; Tazoe et al. 2008).

Acetate, butyrate, and propionate are the main SCFAs (Slavin 2004). These SCFAs are involved in the maintenance of healthy gut microbiota and have been related to lowered serum cholesterol, improved glucose response and decreased risk of cancer in humans and dogs (Slavin 2004; National Research Council 2006; Frølich et al. 2013). Furthermore, whole grain has shown to reduce the risk of CVD and promote gastrointestinal health (Frølich et al. 2013).

Other compounds in whole grain that contribute to positive health effects and have been linked to a decreased risk of congenital heart defect (CHD) in human research are antioxidants, phytic acid, lectins, phenolic compounds, amylase inhibitors, and saponins (Slavin 2004). Moreover, whole grains have also been shown to decrease intestinal transit time and this could influence the glucose and insulin response (Slavin 2004). The reduced insulin response of whole grains has additionally shown beneficial effects on satiety and body weight. Dietary magnesium (Mg), fibre and vitamin E are involved in insulin metabolism and these compounds can be found in whole grains. The blood glucose and blood insulin have shown a reduced response when the diet consists of cereal fibres and this is linked to a reduced risk of diabetes mellitus (Slavin 2004). Despite the good health effects of grain fibre, a gluten-free and grain-free diet for both humans and dogs are a highly debated subject. Grain-free dog food has been increasingly common, but its advantages and disadvantages are discussed. The domestic dog has developed the ability to digest, absorb and metabolise dietary carbohydrates, a difference from the domestic cat, which is an obligate carnivore (Deng & Swanson 2015). Gluten is composed of different classes of proteins derived from cereals such as wheat, barley, and rye (Meineri et al. 2020). A diet with low gluten has been shown to be linked with a significant decrease in body weight, changes in the intestinal microbiome, and changes in the urine metabolome in humans (Hansen et al. 2018). According to Meineri et al. (2020), there is currently no regulation and limit value to control the gluten content in dog food labelled “grain free”.

Satiety is an important factor when creating strategies for weight management. According to a study by Weber et al. (2007), the satiety effect was significantly better for the diet including both high protein and high fibre content compared with diet including high protein or high fibre content alone. However, the satiety effect seems to be greater for fibre, since the diet with high fibre content showed better satiety effect compared with the high protein diet (Weber et al. 2007). The reason behind this satiety effect could be linked to SCFA receptors located in mucosal enteroendocrine cells along the gastrointestinal tract (Tazoe et al. 2008). The receptors GPR41 and GPR43 control the intestinal muscle activity and regulate energy balance by influencing the production of hormones like peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) (Tazoe et al. 2008).

2.3 Diet and metabolic response

A study by Bosch et al. (2009) analysed the effects of dietary fibres on metabolism in dogs. The aim was to identify if satiety-related hormones and food intake differed depending on fibre type, low-fermentable fibre (LFF) diet containing 8.5% cellulose or a high-fermentable fibre (HFF) diet containing 8.5% sugarbeet pulp and 2% inulin. The digestibility coefficient (ADC) for the nutrient was significantly higher in the HFF diet for dry matter (DM), organic matter and NDF, while crude fat was significantly higher in the LFF diet with nearly a significantly higher crude protein digestibility. In summary, the ADC for energy was significantly higher in the HFF diet compared with the LFF diet. The HFF diet tended to reduce the feed intake at the end of the study. Additionally, a higher total SCFA (acetate and propionate) concentration was shown for the dogs fed with the HFF diet. The plasma glucose, insulin, PYY, GLP-1, and ghrelin were measured for the dogs with the HFF diet and LFF diet, but no significant difference was found between the dietary groups.

A previous study has looked into the relationship between gut microbiota and the response of glucose metabolism after 3-day consumption of barley kernel-based bread in humans (Kovatcheva-Datchary et al. 2015). The result showed a difference in the glucose response between individuals. Some individuals got a metabolic response to the diet, and some just got a low response or no response at all. Variation in response to carbohydrates can be explained by the diversity of gut microbiota involved in the fermentation of complex carbohydrates into metabolites such as short-chain fatty acids. The individuals that got a response had a higher *Prevotella/Bacteroides* ratio than non-responders. In addition, the results from Kovatcheva-Datchary et al. (2015) could indicate a relationship between gut microbiota involving *Prevotella* species and how well an individual can digest carbohydrates. A fibre-rich diet compared with a refined carbohydrate diet has been shown to improve glucose metabolism in mice (Kovatcheva-Datchary et al. 2015). A study in dogs has found a higher relative abundance of *Prevotella* in fecal microbiota as a response after a diet composed of 50% rye inclusion (by DM basis) compared to a diet composed of 50% wheat (by DM basis) (Palmqvist et al. 2022). The findings from Kovatcheva-Datchary et al. (2015) and Palmqvist et al. (2022) indicates that a fibre-rich diet, such as the inclusion of the cereal rye, increases microbial *Prevotella*. In addition, it seems that the higher abundance of *Prevotella* affects the fermentation pattern where the acetic acid increases and this had a favorable impact on stable glucose metabolism (Kovatcheva-Datchary et al. 2015; Palmqvist et al. 2022).

Given that there seem to be differences in the metabolic response between individuals, there had been an interest in studying whether the metabolic response

after feed intake can differ between lean or overweight individuals. A previous study by Söder et al. (2017) showed that metabolomic profiles varied between lean and overweight dogs after a high-fat mixed meal. This was studied by the NMR technique in 3-hours postprandial urine (Söder et al. 2017). One of the findings was that metabolic profiles varied between fasting and 3-hours postprandial urine (Söder et al. 2017). A total of nine phospholipids were shown to have an increased response to food intake in plasma (Söder et al. 2019b). Further, parameters for lipid metabolism were studied to analyse differences before and after feed intake in dogs with variation in BCS, classified as lean or overweight (Söder et al. 2017, 2019a; b). One of the main findings was that the concentration of taurine was lower for overweight dogs in the postprandial urinary excretion, which could indicate an alteration in lipid metabolism (Söder et al. 2017). Another parameter documented by Söder et al. (2019a; b) was a difference in plasma free carnitine and acetylcarnitine concentration in plasma between lean and overweight dogs. The overweight group had a lower concentration, which may indicate decreased or insufficiency of fatty oxidation and thereby relate to spontaneous adiposity and altered lipid metabolism (Söder et al. 2019a; b).

2.4 Metabolomics analysis techniques

Metabolomics analysis is a complex research area since many different factors influence the metabolic state of an organism, such as its genome, transcriptome, proteome, epigenome, microbiome, and exposome (environment) (Deng & Swanson 2015; Markley et al. 2017). Metabolomics focus on identifying metabolites in biological systems from cells, tissues, biofluids, and foods linked to humans or animals (Gowda & Raftery 2019). Nuclear Magnetic Resonance spectroscopy (NMR) is a robust and useful approach, providing information that can determine the metabolic state of an organism (Markley et al. 2017; Gowda & Raftery 2019). The technique makes it possible to elucidate mechanisms of metabolite transformations and explore the compartmentalization of metabolic pathways (Markley et al. 2017). The metabolites that are determined in metabolomics research is small molecules usually <1000 Dalton (Da) in molecular weight, for example, carboxylic acids, amines, alcohols, amino acids, certain carbohydrates and lipids (Gowda & Raftery 2019). NMR has previously been used to understand biological phenotypes, and identify disease biomarkers (Gowda & Raftery 2019), but also for instance, to elucidate the biochemical effects of rye- and wheat-based diets in pigs as a model for humans (Bertram et al. 2006). Urine sample is a biofluid commonly used for NMR analysis to identify and determine low-molecular-mass metabolites and study how the metabolome can be affected by the external stimulus, such as medications, diet, or exercise regimen (Percival et al. 2020).

Multivariate analysis techniques, such as principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) can be used to further analyze the output from NMR data. PCA is an analysis technique that creates a two-dimension loading score, where the first axis presents the principal component that accounts for the maximum of the total variance (Izquierdo-García et al. 2011). The second principal component accounts for the maximum residual variance. More principal components can be created until the total variance is fully explained. Samples that cluster together relate to each other (Izquierdo-García et al. 2011). PLS-DA, is a statistical model that combines the basics of PCA, canonical correlation analysis, and multiple regression analysis (Bi et al. 2021; Izquierdo-García et al. 2011). The model explains those components in the spectral region with a significant variation and maximum covariance with the class information vector (Izquierdo-García et al. 2011).

2.4.1 Biological sample types for metabolomics

Urine, fecal water and serum or plasma are biofluids that are commonly used in metabolic research for humans and animals (Claus & Swann 2013). The advantages of the use of these biofluids are that sampling is non-invasive (fecal water and urine samples) and there is an ability to collect several samples over time (Claus & Swann 2013). The urine consists of endogenous waste products, including urea and bile pigments (Sjaastad, Ø.V., Hove, K. & Sand, O. 2016). The composition of metabolic end products from the urine gives an overall metabolic historical overview of the individual (Claus & Swann 2013). Both endogenous and environmental factors such as gut microbiota and diet affect the urine composition (Claus & Swann 2013). An essential function of the kidney is the metabolism of protein, and the conservation of substances, such as glucose, amino acids, and proteins, before the excretion of the waste (Sjaastad, Ø.V., Hove, K. & Sand, O. 2016). The liver helps to convert waste products to water-soluble compounds that can excrete via the urine (Sjaastad, Ø.V., Hove, K. & Sand, O. 2016). Another function in connection with metabolism is the role of the kidney in the state of prolonged starvation. In that physical state, the kidney and the liver produce glucose from non-carbohydrate sources (gluconeogenesis) (Sjaastad, Ø.V., Hove, K. & Sand, O. 2016). Fecal water is the optimal sample type when detecting gut microbiota and the fecal metabolome also gives rich information from dietary inputs and biochemical events from the gastrointestinal tract (Claus & Swann 2013). Collecting blood samples is more invasive, however, the advantage is that blood gives metabolic information linked to the physical state of the individual at the time of sampling (Claus & Swann 2013).

2.4.2 Creatinine

Urine is commonly used in metabolomics since it is an easy body fluid to collect and the collection can be performed non-invasive (Tonomura et al. 2015). However, urine concentrations vary between individuals due to different water intake and other physiological traits and state (Lermen et al. 2019). Correcting the urine volume is one way to adjust for the individual difference in urine concentration when detecting biomarkers, however, the method using urinary creatinine is more generally used (Tonomura et al. 2015).

Body creatine is mainly stored in muscle cells, where skeletal muscle contains about 95% of total body creatine, and creatinine is a product of creatine catabolism (Braun et al. 2003; Tonomura et al. 2015). The conversion from creatine to creatinine occurs at an almost constate rate and is estimated to affect about 2% of the stored creatine daily (Braun et al. 2003). Creatinine concentration in the urine has been considered stable and largely unaffected by the diet (Braun et al. 2003). Therefore, it has commonly been used to correct urine concentration in urine-based studies (Tonomura et al. 2015). However, it has been stated in a study by Yamamoto et al. (2019) that the urine creatinine concentration can be affected by the different amounts of protein in the diet, where a low protein diet significantly increased the creatinine concentration in the urine compared with a standard protein diet and a high protein diet. This is probably because creatinine originates mainly from biosynthesis from the amino acids glycine, arginine, and methionine (Braun et al. 2003). Body index mass (BMI), age, and sex have been shown to influence the creatinine concentration in humans (Alessio et al. 1985; Lermen et al. 2019). Males had a higher concentration in the urine compared with women (Alessio et al. 1985). The creatinine concentration can fluctuate for the same individual and large variations from day to day have been shown in human urine (Alessio et al. 1985). Another parameter to use to adjust urine samples is Urine Specific Gravity (USG) (Alessio et al. 1985).

Sampling 24-hour urine gives an overall picture of the total excretion of urine, since the metabolite concentration in urine varies during the day. In human research, 24 hours sampling of urine generally is used (Scher et al. 2007). However, it is time-consuming and can be difficult to implement (Scher et al. 2007). It has been shown that 24-hour sampling and fasting spot sampling in the morning gives a comparable concentration of metabolites in the urine (Ishibashi et al. 2021). Morning samples or spot samples can therefore be an alternative, but the concentration of the urine samples needs to be adjusted to correct for the bias with diurnal variation and thereby get a representative result of the metabolites excreted (Tonomura et al. 2015). Urine creatinine concentration is commonly used in research as dilution correction of the urine samples to obtain reliable quantification of metabolites and

minimize the error of variance in the data due to differences in hydration state (Calafat et al. 2005; Li et al. 2015; Urbancova et al. 2017). The data will be more comparable to other studies when adjustment is made by creatinine levels in the urine samples (Seifert et al. 2000). Determination of creatinine in urine is a widely used measurement to indicate renal function by glomerular filtration rate (GFR) (Tonomura et al. 2015).

3. Materials and Methods

3.1 General study design

This master thesis is part of a research project approved by the Regional ethical committee in Uppsala (SLU.huv.2019.4.1-14). The primary purpose of the research project is to contribute knowledge about how different carbohydrates and fibres in dog food affect gut health, metabolism, appetite regulation, and levels of various hormones. A cross-over design was applied to study the impact of specifically prepared feeds with different carbohydrate sources. The study consisted of a balanced dog diet with three different grains: whole wheat, peeled oat, and whole-grain rye. Privately owned dogs were fed with each of the three feeds in a treatment period of four weeks. One feed at a time and in a random order for the dogs. The dog owners were blinded in which order. Feces-, blood-, and urine samples were collected at the end of every feed period. Urine samples were collected at fasting and 4 hours postprandial. Blood samples were collected in a series from feeding up to 4 hours postprandial and feces were collected one to two days before the urine and the blood sampling. All the treatment periods and urine collection were done in 2019. The analytic part of the research project that will be presented in this master thesis was started in spring 2022. A schematic illustration of the study workflow is shown in (Figure 1).

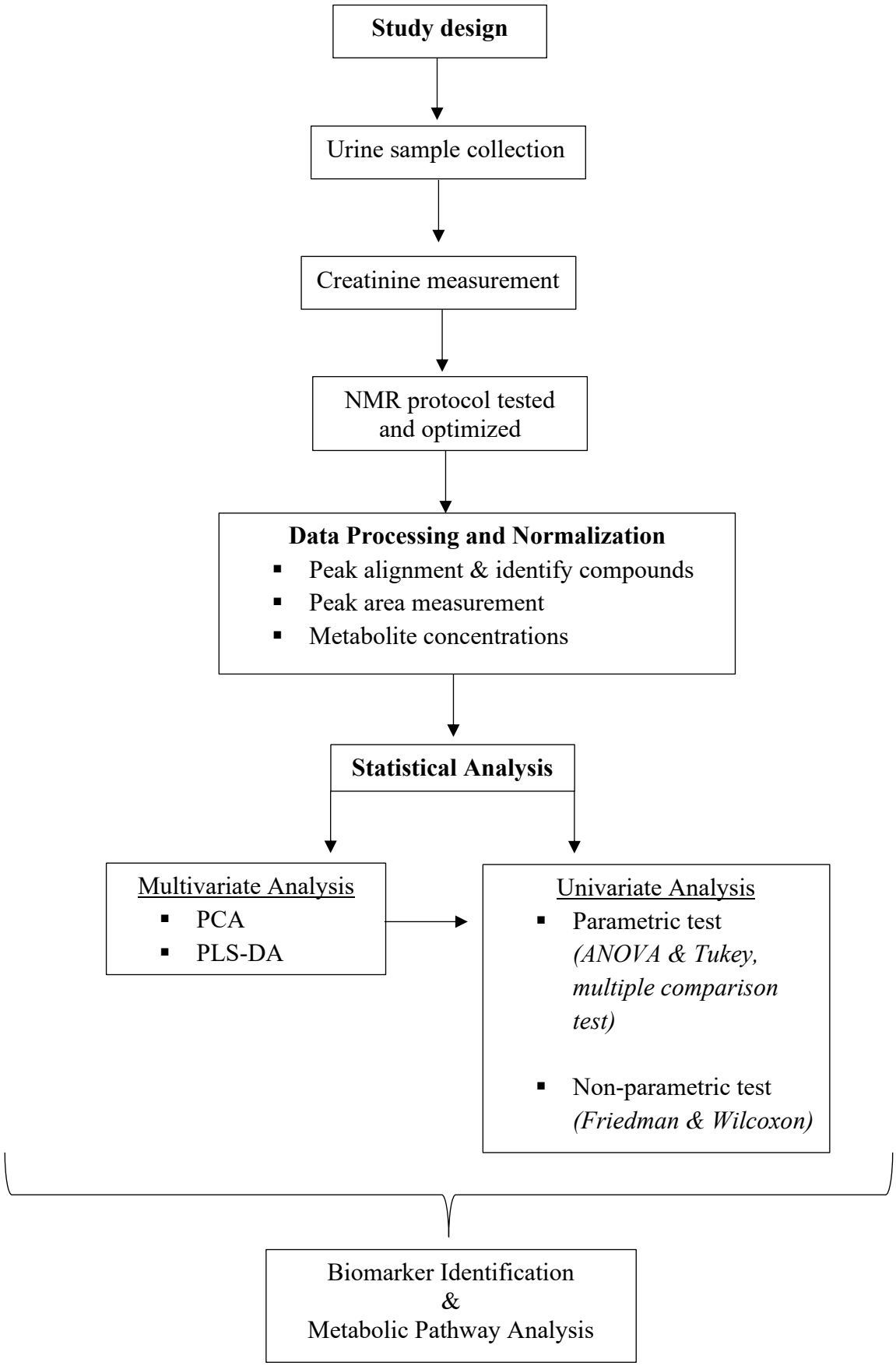

Figure 1. A schematic diagram of the study workflow

3.2 Animals

Seventeen privately owned dogs of different breeds, ages, weights, and BCS were included in this study. The dogs were recruited mainly through inquiries to students and staff at SLU but also through information sheets available in the waiting room at SLU University Animal Hospital (UDS). All the dogs recruited to the study underwent a health examination consisting of an owner interview for the health status of their dog and a routine physical examination by a veterinarian. The owners collected urine samples, and urine analysis was performed, including protein: creatinine ratio at the Clinical Chemistry Laboratory of UDS. To be included in the study, dogs had to be >1-year-old. They were not permitted to receive antibiotics or any medical or hormonal treatment (such as corticosteroids) for three months preceding or during the study. A recommendation was that the dog should be dewormed two weeks before starting with the control feed. High-performing dogs that received a lot of reward candy during training were not included in the study as it was not desirable for the dog to ingest too large amounts of anything other than the food to be tested. There was also a limit to how small the dogs could be to participate in the study to ensure that the serial blood sampling would not lead to an excessive loss of the dog's blood volume. During the experiment, all the dogs lived in their home environment, and the owners collected urine samples at the end of each feed period. The dogs were classified between 4 – 6 on a 9–point BCS scale.

A summary of animal information such as sex, breed, age, weight, and BCS is shown in

Table 3 Most of the dogs were intact females (7), some neutered males (5), some neutered females (4), and just one intact male. Four dogs were of mixed breeds, 2 dogs were of the breed Lagotto Romagnolo, one unknown and one each of another 10 breeds. The median age was 7 years (interquartile range, IQR 5 – 10 years), median body weight was 15,60 kg (IQR 10,60 – 26,40 kg), and median BCS 5 (IQR 5 – 6).

Table 3. Animal information; Individual ID, sex, breed, age, weight, BCS (n=17)

Ind. ID	Sex	Breed	Age	Weight	BCS
1	Intact female	Lagotto romagnolo	12	11.8	4
2	Intact female	Lagotto romagnolo	7	11.7	5
3	Neutered male	Mixed breed	10	42.7	5
4	Neutered male	Mixed breed	5	29.1	4
5	Neutered male	Mixed breed	7	8.7	6
6	Neutered female	English Whippet	5	14.5	5
7	Intact female	Border Collie	7	15.6	5
8	Intact female	Samoyed dog	6	30.3	5
9	Neutered male	Lhasa Apso	11	8.7	6
10	Intact female	Mixed breed	9	31.4	6
11	Neutered female	Labrador Retriever	5	20.1	5
12	Intact male	Kooikerhondje	3	9.8	5
13	Intact female	Medium Poodle	10	7.6	5
14	Neutered male	Australian Kelpie	6	19.2	5
15	Intact female	Xoloitzculintle	1	10.6	5
16	Neutered female	Unknown	10	20.7	6
17	Neutered female	Weimaraner	9	26.4	6

3.3 Experimental feeds and feeding

Feed changes must be done gradually to avoid stomach and intestinal disturbances. Therefore, the dog owners were instructed to mix the new feed into the older feed with increasing proportions for a couple of days. All dogs had a habituation period of seven days, where they gradually were introduced to the control feed at the start of the study. The feed with wheat as a grain source served as the control feed since wheat is commonly used in commercial dog food while oat and rye are less common. The dogs were fed with the experimental feeds two weeks after the habituation period. Each feed was fed for four weeks, including a gradual adaptation to the feed for over four to five days. The dogs were fed at their usual feeding hours and were given the amount of food that met their energy needs. One basis of the study design was that the absolute majority of the energy intake came from the tested feeds. Rewards were allowed but under specific guidelines. The guidelines were to perform documentation over what kind of rewards the dog got and approximately how much. The dog should also get roughly the same amount

of rewards during all feeding periods, but during the three last days before sampling, the dogs were not given any rewards.

The feed composition and energy content were produced to be as similar as possible between the different feeds to be able to compare the effect of different types of grains. In Table 4, the proximate analysis is shown on a wet matter basis. A summary of the diet composition is shown in Table 5. Each diet consisted of 25% of the grain; whole-grain wheat, peeled oat, or whole-grain rye on a dry matter basis. Corn, rice, and lignocellulose were other carbohydrate sources included in the diet in the same amount in all feeds, apart from a slight difference in rice percentage. The primary protein source was chicken, and some pig fat was added.

Table 4. Proximate analysis in percentage (%) of provided samples of the whole-grain wheat diet, peeled oat diet, whole-grain rye diet on a wet matter basis

Proximate analysis			
components	*whole-grain wheat diet*	*peeled oat diet*	*whole-grain rye diet*
Crude protein	28.60	28.40	27.70
EG-Fat	12.90	16.49	15.67
Crude fibre	1.78	1.45	1.65
Ash (550°C)	6.71	6.42	6.87
resistant starch	0.21	0.18	0.26
non-resistant starch	38.06	34.00	34.52
total starch	38.27	34.17	34.79
DM (103°C)	93.88	93.51	93.90
MJ/kg	19.60	20.26	20.19

Table 5. Composition in percentage (%) of provided samples of the whole-grain wheat diet, peeled oat diet, whole-grain rye diet on a dry matter basis

Composition			
Ingredients (%)	*whole-grain wheat diet*	*peeled oat diet*	*whole-grain rye diet*
whole-grain wheat	25.00	—	—
peeled oat	—	25.00	—
whole-grain rye	—	—	25.00
corn	15.00	15.00	15.00
rice	11.70	10.82	12.50
lignocellulose	1.50	1.50	1.50
chicken (dried)	29.70	30.39	29.90
chicken (fresh)	5.00	5.00	5.00
chicken broth	3.00	3.00	3.00
pig fat	7.49	7.63	6.43
premix	1.66	1.66	1.66

3.4 Urine sampling

The samples were collected between June and December 2019. The collection of the urine was not a part of this master thesis, and all samples were available at the start. The dog owners were informed of how to perform the urine collection and instructed to practice urine collection before starting the study. Sampling equipment and tubes were given to the dog owners. If the sample could not be left to the staff within an hour, it would be stored in the refrigerator. After that, all samples were aliquoted and stored at -80 °C until analysis. Each dog in the study left two urine samples after each feeding period, one fasting urine sample in the morning and one urine sample approximately four hours after breakfast feeding. The day before urine sampling, the dog owner was instructed not to provide any feed to the dog after 19 o'clock until breakfast feeding the following day.

All collected urine samples were analyzed for Urine Specific Gravity (USG) and pH. A digital refractometer (Pocket refractometer; Atago) was used to measure USG, and the pH was measured by pH paper with a pH scale of 0 – 14 and increment of 1 (MColorpHast; Merck). The USG and pH of the urine were done after the collection of the urine, and the data were available when this part of the research project started in January 2022.

3.5 Urine Creatinine measurement

Creatinine concentrations were used to adjust each of the urine samples to an even range to minimize the error variance for different urine concentrations in the NMR analysis.

Creatinine concentrations were measured using a urinary creatine ELISA kit from Arbor Assays (Michigan, USA). A total of 97 urine samples were thawed on ice and, then diluted 1:20 (10 µl urine sample and 190 µl Milli-Q water). A creatinine standard was prepared according to the protocol by diluting the standard stock solution in seven different concentrations (20, 10, 5, 2.5, 1.25, 0.625, 0.3125 (mg/dL)). The creatine standard was used to generate a standard curve, which is the frame of reference for calculating the creatinine concentration. The volume of 50 µl of the standards and samples were pipetted into clear 96-well microtiter plates. All standards and samples were analyzed in duplicate samples. The color generating the reaction (DetectX® Creatinine Reagent) was pipetted into each well (100 µl). The microtiter plate was incubated for 30 minutes at 18-25ºC, and after that, detection of the color intensity was evaluated in a microtiter reader measuring 490nm (reference 590nm). Optical density (O.D.) was measured in relation to the standard curve, and a mean was calculated for each sample from the duplicate

measures. Nine samples got an O.D. value above the highest value on the standard curve and therefore these samples needed to be analyzed again with a further dilution. The samples were diluted at 1:40 instead of 1:20, resulting in an O.D. value within the standard curve range. The creatinine concentration in the urine samples was calculated using the online tool from MyAssays (www.myassays.com/arbor-assays-creatinine-urinary-detection-kit.assay). The sample concentrations obtained were then multiplied by the dilution factor to reflect the concentration in the urine samples.

3.6 Nuclear Magnetic Resonance spectroscopy

The preparations for NMR analysis was performed at the Department of Animal Nutrition and Management at SLU, and the samples were then brought to the Department of Molecular Sciences to be analyzed in a Bruker Avance II 600 MHz spectrometer equipped with QCI H-C/P/N-D cryoprobe and a SampleJet sample changer with cooling system (Bruker Biospin AG, Fjällanden, Switzerland). All measurements were made at 25°C, and the NMR spectrums were created by a one-dimensional noesy sequence with water presaturation, including 128 scans with a relaxation delay of 4 seconds for each sample. The protocol for NMR preparation was tested before all samples were run, and, in that way, optimization was possible for the final run. No compensation was made due to creatinine concentration in the test runs. The protocol was based on a previous study using an NMR spectrometer at SLU to analyze metabolomics in human urine (Noorbakhsh et al. 2019). The urine samples were first thawed on ice and then centrifuged at 10.000g, 4°C for 15 min to precipitate any particles in the samples. Each NMR tube consisted of 310 µl urine supernatant, 65 µl Milli Q water, 150 µl sodium phosphate buffer (0.4 M) to stabilize urinary pH at 7.0, 60 µl Deuterium oxide (D2O), and 15 µl of the internal standard Trimethlsilylpropanoic acid (TSP; 20 mM). All components were pipetted into an Eppendorf tube for each sample and were mixed using a vortex for approximately 5 seconds before a total of 560 µl of the solution was aliquoted to an NMR tube.

The protocol for the preparation of the urine samples was evaluated before the final run in the NMR. Firstly, the protocol was evaluated with six samples from different dogs to check if the sample preparation generated NMR spectrums of good quality. Based on this evaluation, the protocol was optimized. Secondly, the protocol was evaluated with nine samples, where changes were made to a higher concentration of TSP from 5.8mM to 20mM and different urine dilutions with Milli Q water. The optimization obtained NMR spectra of a good quantification quality, and the urine samples were adjusted to an even creatinine level of 3 – 6.7 conc. mmol/L for the

final run with all the 97 urine samples. The pH in the NMR tubes of each sample was measured.

The software TopSpin 4.0.6, supplied by Bruker Biospin AG, was used for pre-processing the spectrums to reduce the variance that might interfere with data analysis. Calibration of the spectral axis was made by baseline correction, first around the TSP at 0.0 ppm and then the whole spectrum. The spectral region containing the water peak was excluded and spectra were saved from 9.5 ppm to 0.5 ppm with a so-called "bucket" width of 0.01. Another software, Chenomx profiler, was then used to align the spectra and identify compounds of targeted signals by the spectral reference library. The peak of a targeted compound was adjusted to fit the signal intensity, and thereby was the concentration calculated.

3.7 Statistical Analyses

PAleontological STatistics (PAST) software (version 4.07) (Hammer et al. 2001) was used to summarize the data and to perform statistical analysis. All the figures were made in Excel.

Descriptive statistics were performed to get an overview of creatinine concentrations. An Anderson-Darling test was performed to study the normal distribution of the data. The creatinine concentration met assumptions of normal distribution. Therefore, a parametric test (two-sample t-test) was used to compare the distribution in fasting urine and after food intake. An One-way ANOVA several-sample test analysed the dogs' creatinine concentrations concerning the experimental feeds. Spearman's rank correlation was used to analyse the correlation between creatinine concentration and USG. Results were considered to be significant when $p<0.05$.

The data acquired from the NMR analysis were further analysed using multivariate techniques, i.e. PCA and PLS-DA, including all 17 dogs (a total of 97 urine samples). The software SIMCA (version 14) was used for the multivariate analyses. PCA was first performed to visualize the correlation among the samples and to evaluate which exposures that impacted the metabolic profiles. The significance of the observed clustering was analysed using PLS-DA. A permutation test was used to determine the reliability of the multivariate model and the goodness of separation between feeds. The objective was thereafter to identify which regions in the spectrums that were related to the differentiation. The regions with the largest and most important difference from the PLS-DA were then characterized by variable importance for projection (VIP). Regions with VIP>0.5 were considered to have a

discriminative separation between the feeds and were significant. The spectral region of significant variation according to VIP was visualized in software TopSpin 4.0.6, where the spectrums of different experimental feeds overlapped. The software Chenomx profiler was then used to identify metabolites of targeted signals of significance by using the spectral reference library. The pH interval covering all values in the urine samples was added as a setting for identification in the Chenomx profiler. In that way, the software library could get representative matches with metabolites within the corresponding range of pH values. The peak of a targeted compound was adjusted to fit the signal intensity and thereby calculating the concentrations. The concentrations were adjusted to a creatinine ratio to get a comparable value independent of how much water intake the dog had and how concentrated the urine was at sampling. Creatinine was a targeted metabolite identified to study the agreement between the creatinine measurement with the ELISA kit from Arbor Assays (Michigan, USA) and the concentrations generated from the NMR spectrums by Pearson's correlation test.

The concentrations of the identified metabolites were tested for normal distribution by the Anderson-Darling test. If the metabolites meet the assumptions for normal distribution, the parametric test repeated measures analysis of variance (ANOVA) was performed to analyse if the metabolites' concentration differed significantly between the experimental feeds, both for fasting and postprandial urine samples, respectively. If the data distribution for a metabolite did not meet the assumptions for normal distribution, the Friedman non-parametric test was used instead. The Tukey, multiple comparison test, was used to investigate which experimental feed differed for normally distributed data, and Wilcoxon pairwise non-parametric test was used for data that did not meet the assumptions for normal distribution.

Paired sample *t*-tests were used to compare metabolite concentrations normally distributed between fasting and postprandial time points (including all 97 urine samples). If the data distribution for a metabolite did not meet the assumptions for normal distribution, the Wilcoxon non-parametric test was used instead. The analysis to study the differentiation of the metabolites between time points was done separately for each experimental diet.

4. Results

4.1 Creatinine concentration

The descriptive statistics resulted in a median of 26.95 mmol/L (IQR 17.65 – 32.88 mmol/L) for creatinine concentration (Figure 2) and a median of 1.043 (IQR 1.032 – 1.051) for USG. Urine creatinine concentration was not significantly different between fasting and after food intake. Neither could a significant difference be found between creatinine concentration and the three experimental feeds. A significant correlation (Pearson correlation coefficient, r = 0.86, p-value <2.2e-16) was found between USG and creatinine.

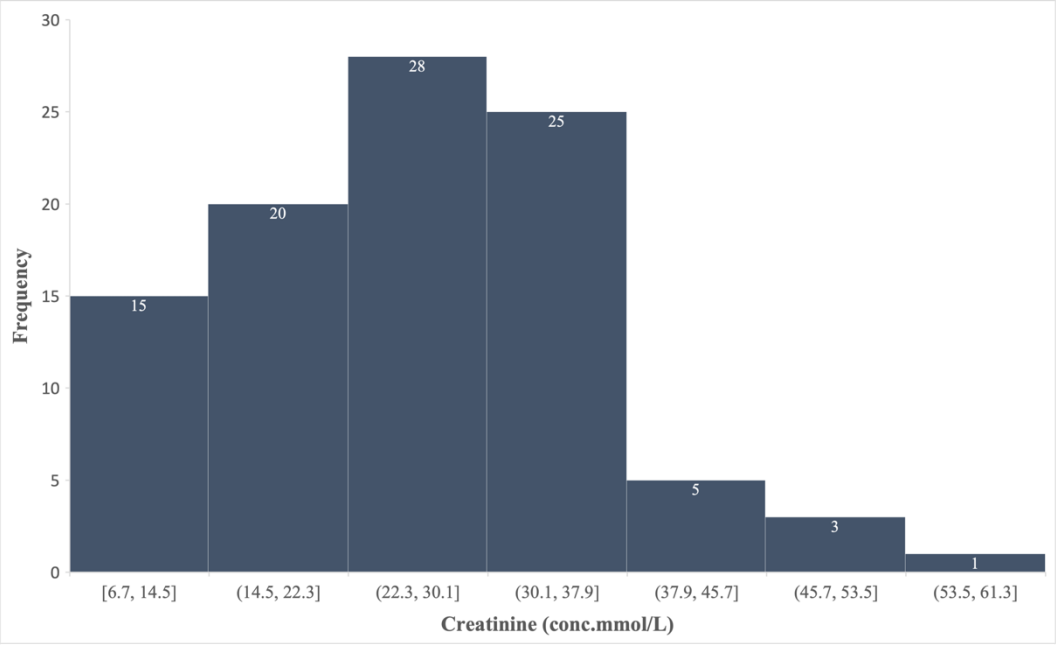

Figure 2. Creatinine concentration (n=97)

4.2 pH measurements

The measured pH from NMR tubes ranged from 7.18 to 7.69 with a mean of 7.40 and therefore, pH 7.40 ± 0.5 was added as a setting to the software library in Chenomx when metabolites were identified.

4.3 Metabolic response

The multivariate PCA model, including fasting - and postprandial urine samples, did not significantly separate the three different experimental feeds; whole-grain wheat, peeled oat, and whole-grain rye. However, a tendency to separation between the different grains could be visualized where whole-grain wheat and whole-grain rye was most separated, and peeled oat was between these groups (Figure 3). The lack of significant difference between the three experimental feeds was also confirmed in the PLS-DA model when all urine samples were included. Neither were there any significant differences between diets when time points fasting - and postprandial urine was separated in two different models, both for PCA and PLS-DA. Therefore, the experimental feeds were tested *groupwise* for both fasting and postprandial respectively; whole-grain wheat with peeled oat, whole-grain wheat with whole-grain rye, and peeled oat with whole-grain rye. The result of the PLS-DA model including whole-grain wheat with whole-grain rye for postprandial urine showed a significant separation regarding the permutation test (1 component: R2Y=0.314, Q2Y=0.173). One more component was added to better visualize the PLS-DA model (Figure 4). The R2Y value represents the goodness of the fit model and the Q2 value represents the predictability of the model. The model was validated by a permutation plot including 100 permutations and 1 component, which fulfills both criteria for validity, 1: All blue Q2-values to the left are lower than the original points to the right; 2: The blue regression line of the Q2-points intersects the vertical axis (on the left) at, or below zero (Figure 5). No significant difference was observed between the experimental feeds in fasting urine.

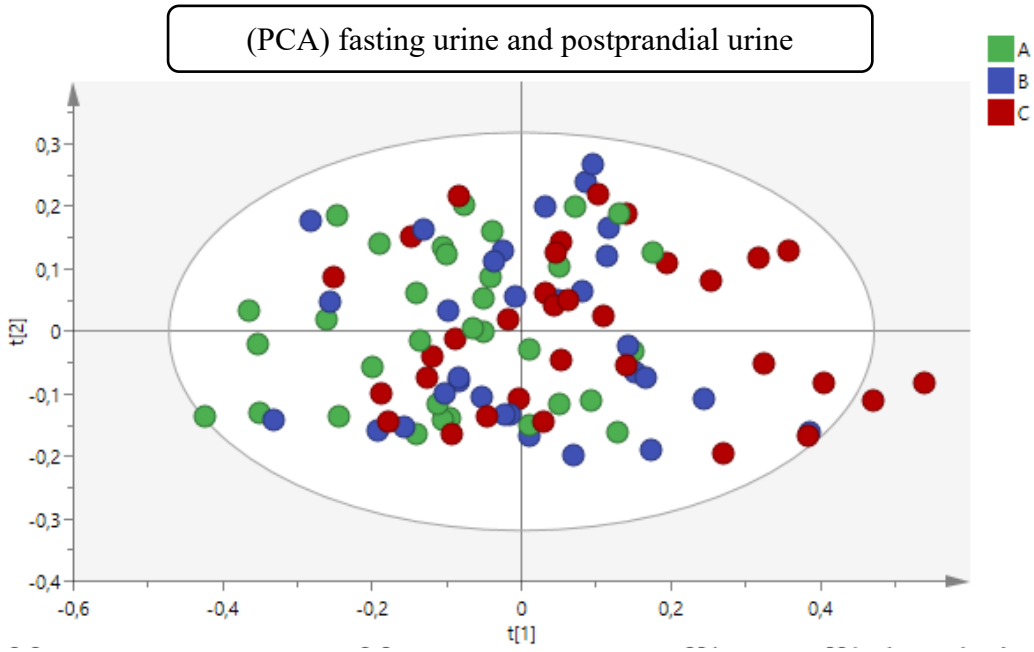

Figure 3. Principal component analysis (PCA) including fasting urine and postprandial urine, A= whole-grain wheat diet, B=peeled oat diet, C=whole-grain rye diet. The horizontal axis shows the score of samples in the first principal component: the interpretable degree of the first principal component R2X[1] = 0.329. The vertical axis indicates the score of samples in the second principal component: the interpretable degree of the second principal component R2[2] = 0.15. T2-Hotelling's distance (95%). (n=97)

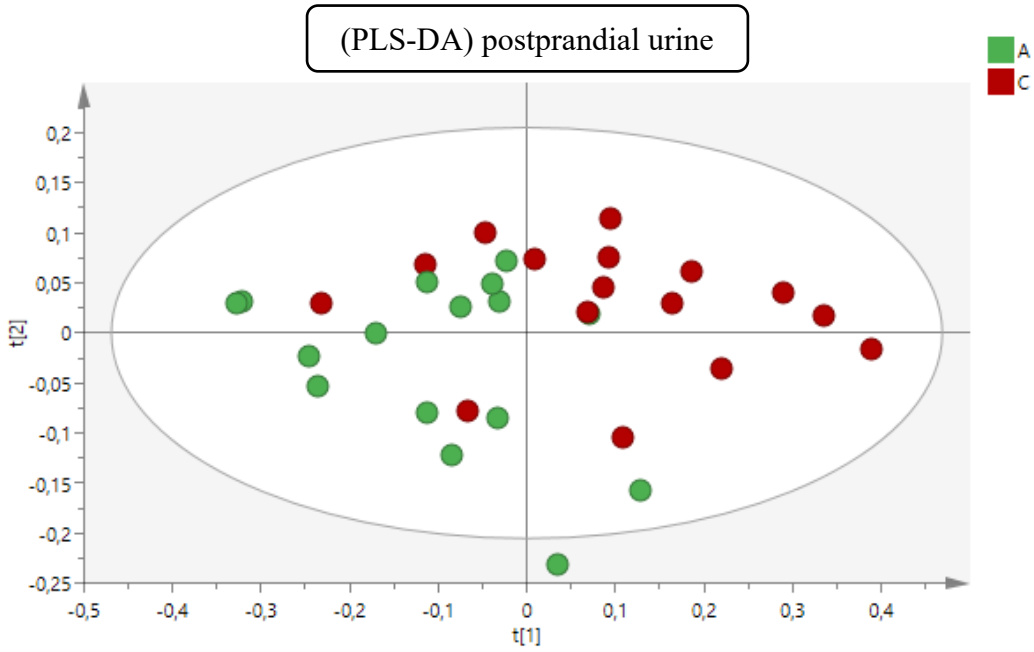

Figure 4. Partial least squares discriminate analysis (PLS-DA) postprandial urine; A= whole-grain wheat diet, C=whole-grain rye diet. The horizontal axis shows the score of samples in the first principal component: the interpretable degree of the first principal component R2X[1] = 0.36. The vertical axis indicates the score of samples in the second principal component: the interpretable degree of the second principal component R2[2] = 0.132. T2-Hotelling's distance (95%). (n=32)

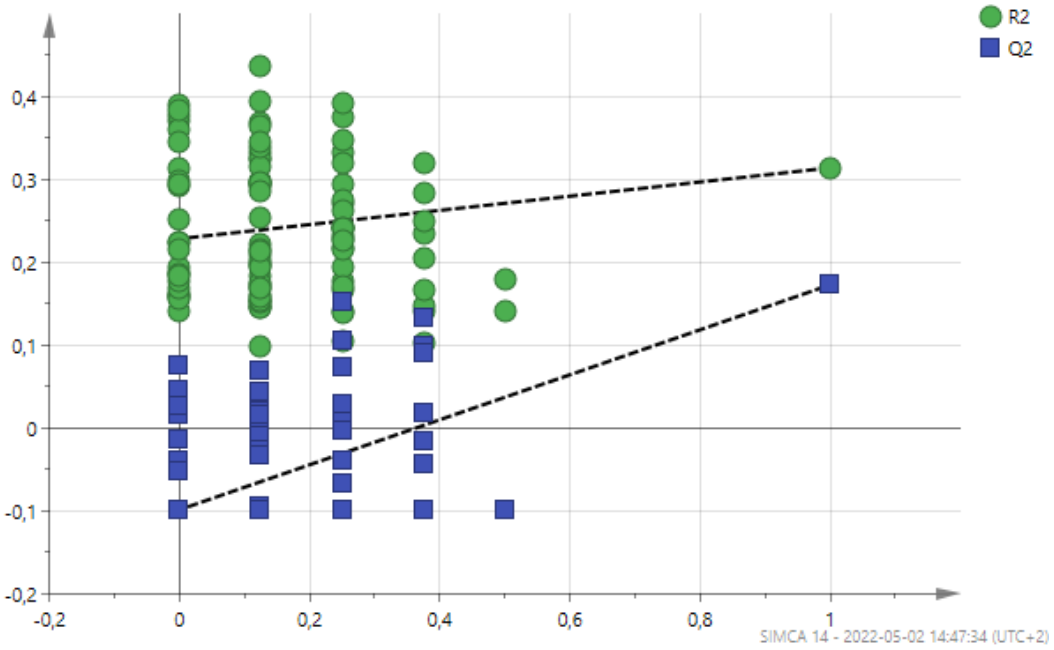

Figure 5. Permutation plot for partial least squares discriminate analysis (PLS-DA) model, 100 permutations and 1 component. Intercepts: R2= (0.0, 0.228), Q2= (0.0, -0.0986). R2Y: goodness of the fit model; Q2: the predictability of the model. Criteria for validity 1: All blue Q2-values to the left are lower than the original points to the right; 2: The blue regression line of the Q2-points intersects the vertical axis (on the left) at, or below zero (n=32)

Variable importance for projection (VIP) was then used to identify the regions in the NMR spectra linked to the differentiation between the experimental feeds; whole-grain wheat and whole-grain rye. A total of 72 VIP:s over the limit of 0.5 were characterized by the PLS-DA model including the feeds with the significant result. Some of the spectrum regions of interest were difficult to identify to a specific metabolite due to overlapping areas and low concentrations. Each VIP region had a "bucket" width of 0.01. Therefore, metabolites with large peaks or peaks spread out in the spectrum contain several VIP values with different importance for differentiation between experimental feeds. Fourteen "buckets" could not be identified as a specific metabolite. However, a total of ten metabolites were identified from the targeted signals from VIP (Table 6). The metabolite urea included most VIP regions (n = 38), and also had the highest VIP value of importance (VIP = 6.01). Mannitol was an identified metabolite containing several VIP regions (n = 9) due to the including signals for mannitol being spread out in the NMR spectrum. Alanine, ascorbate, and taurine had two VIP regions with all VIP values <1. Metabolites with only one VIP region were creatine, methylguanidine, tyramine, n6-acetyllysine, and glutaric acid monomethyl ester (Table 6). Two NMR spectrums from different dogs were randomly selected to illustrate an overview of the metabolomic data generated from the analysis and

visualized in the software Chenomx profiler (Figure 6). The NMR spectrums were generally dominated by the same metabolic signals. However, the signals were clearer with higher peaks in some individuals, while it was more challenging to distinguish the peaks of other individuals because the signals were low and overlapping. The identified ten metabolites were spread along the whole spectrum from a position around 1 part per million (ppm) to 7 ppm.

The creatinine concentrations measured with the urinary creatine ELISA kit from Arbor Assays greatly agreed with the creatinine concentration measured from the NMR spectrum (Pearson correlation coefficient $r = 0.98$, p-value $< 2.2e\text{-}16$). Creatinine concentrations generated from the NMR were used to get a comparable unit of the metabolite concentration between samples (Table 6). Creatine was a metabolite with a standard deviation (SD) $\geq$ the mean, which explains that the concentration has a large variation between dogs included in this study. A large variation among individuals can also be seen in some other metabolites in the time points fasting or postprandial. For example, the metabolite mannitol and taurine had an SD near the mean in fasting urine for the experimental feed of whole-grain rye versus peeled oat.

Table 6. Summary of metabolites identified from NMR spectrum (n=97)

Metabolite	**Concentration (mM)/creatinine[a]**						**VIP[b]**
	Fasting urine Whole-grain wheat	*Postprandial urine* Whole-grain wheat	*Fasting urine* Peeled oat	*Postprandial urine* Peeled oat	*Fasting urine* Whole-grain rye	*Postprandial urine* Whole-grain rye	
Alanine	0.026 ± 0.010	0.030 ± 0.009	0.027 ± 0.008	0.031 ± 0.007	0.030 ± 0.011	0.034 ± 0.014	0.78^{2}
Ascorbate[c]	0.008 ± 0.004	0.006 ± 0.004	0.008 ± 0.003	0.007 ± 0.002	0.008 ± 0.003	0.007 ± 0.003	0.76^{2}
Creatine	0.029 ± 0.030	0.038 ± 0.056	0.038 ± 0.038	0.037 ± 0.047	0.038 ± 0.053	0.049 ± 0.073	0.76^{1}
Glutaric acid monomethyl ester[c]	0.011 ± 0.003	0.011 ± 0.002	0.012 ± 0.002	0.012 ± 0.003	0.011 ± 0.002	0.011 ± 0.003	0.50^{1}
Mannitol[c]	0.048 ± 0.010	0.044 ± 0.009	0.047 ± 0.008	0.044 ± 0.008	0.056 ± 0.040	0.047 ± 0.019	0.95^{9}
Methyl-guanidine[c]	0.012 ± 0.002	0.011 ± 0.002	0.012 ± 0.002	0.013 ± 0.002	0.013 ± 0.003	0.012 ± 0.002	0.65^{1}
N6-Acetyllysine[c]	0.008 ± 0.002	0.007 ± 0.001	0.008 ± 0.002	0.007 ± 0.001	0.008 ± 0.002	0.008 ± 0.002	0.50^{1}
Taurine	0.226 ± 0.088	0.325 ± 0.113	0.252 ± 0.228	0.297 ± 0.192	0.258 ± 0.120	0.305 ± 0.140	0.64^{2}
Tyramine	0.028 ± 0.008	0.029 ± 0.007	0.029 ± 0.008	0.031 ± 0.006	0.029 ± 0.010	0.032 ± 0.009	0.62^{1}
Urea	49.45 ± 7.741	49.47 ± 6.777	48.29 ± 6.426	47.19 ± 6.489	45.40 ± 11.175	45.72 ± 8.386	2.2^{38}

[a]Concentration (mM)/creatinine was calculated by dividing the concentration of the metabolite by the creatinine concentration in each sample (values presented as mean ± SD).

[b]VIP, Variable importance for projection; the mean[n] of the VIP for regions in the spectrum that include the metabolite (n=number of "*bucket regions*" for the metabolite).

[c]Metabolite, low signals in overlapping regions in the spectrum.

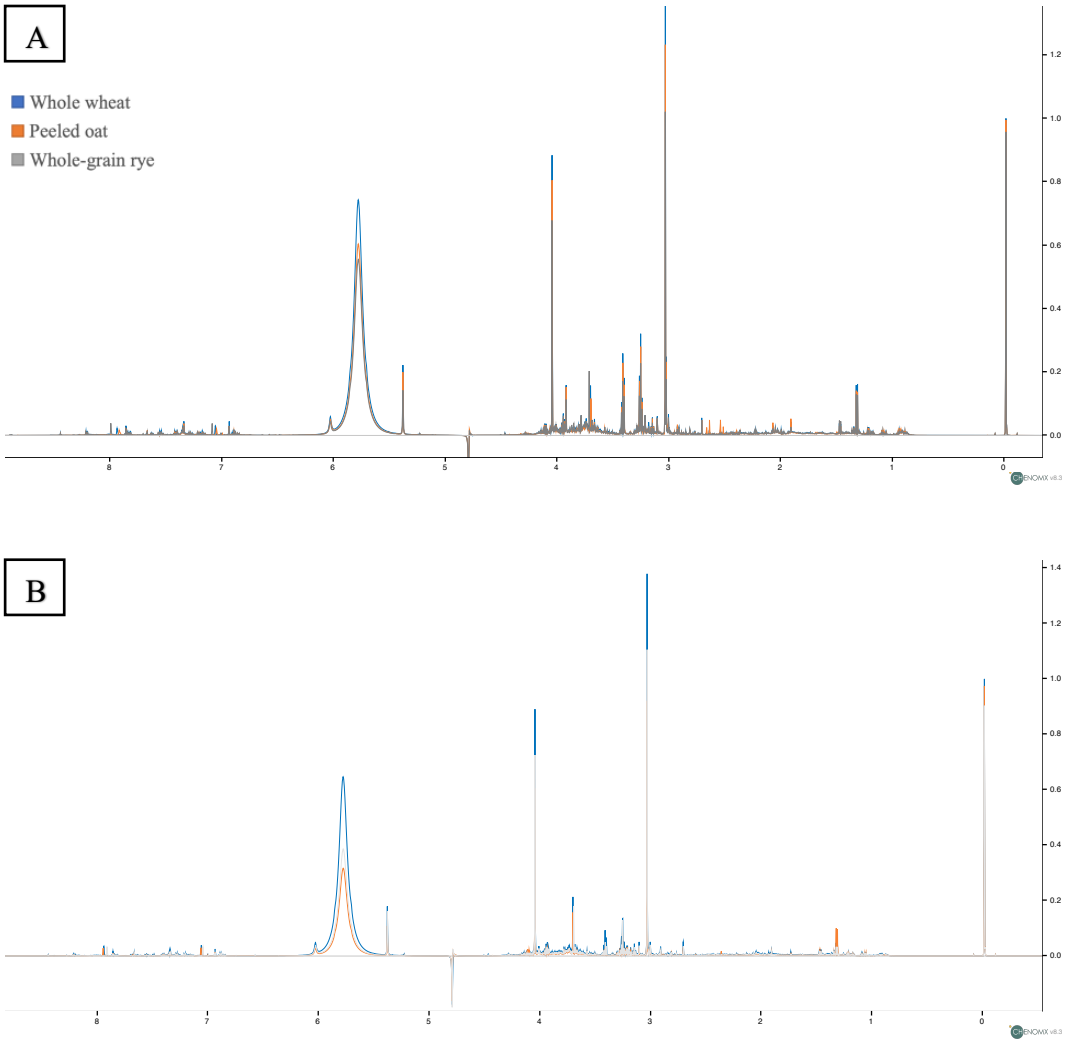

Figure 6. Overview of two randomly selected dogs showing urine spectrums from the three diets (whole wheat, peeled oat, whole grain rye) generated from Nuclear Magnetic Resonance spectroscopy (NMR).

A few dogs had some missing urine samples, and therefore all dogs could not be included in the univariate analysis for the metabolites as the model was based on repeated measurements and did not allow any missing values. Thus, in total 16 dogs were included for the analysis in fasting urine and a total of 13 dogs were included in the analysis for postprandial urine. None of the ten identified metabolites were significantly different between the experimental feeds in fasting urine (n=16 dogs). Three of the metabolites in fasting urine were normally distributed, and the rest of the metabolites (seven) were not normally distributed. Two metabolites differed significantly between experimental feeds in postprandial urine. Seven of the metabolites in postprandial urine were normally distributed, and three were not (n=13 dogs). Methylguanidine differed significantly between the experimental feeds according to ANOVA repeated measure analysis (p=0.045), and the Tukey multiple comparison test showed that the difference was linked to the diet of whole-

grain wheat and peeled oat (p=0.046) (Figure 7). The concentration of methylguanidine was highest in the peeled oat diet and significantly lower in the whole-grain wheat diet. Tyramine tended to differ between treatment in the ANOVA repeated measure analysis (p=0.053). Moreover, the Tukey multiple comparison test showed that the metabolite differed significantly between the experimental feeds of whole-grain wheat and whole-grain rye (p=0.049) (Figure 9). The concentration of tyramine was highest in the whole-grain rye diet and significantly lower in the whole-grain wheat diet. The two significant metabolites from the Tukey multiple comparison test were visualized in the software Chenomx profiler. Spectrum visualization of two randomly selected dogs is presented in Figure 8 and Figure 10. The metabolic response for all three diets is shown in the figures. For a clear visualization, only a part of the spectrum, containing the highest peaks, was chosen for the figures.

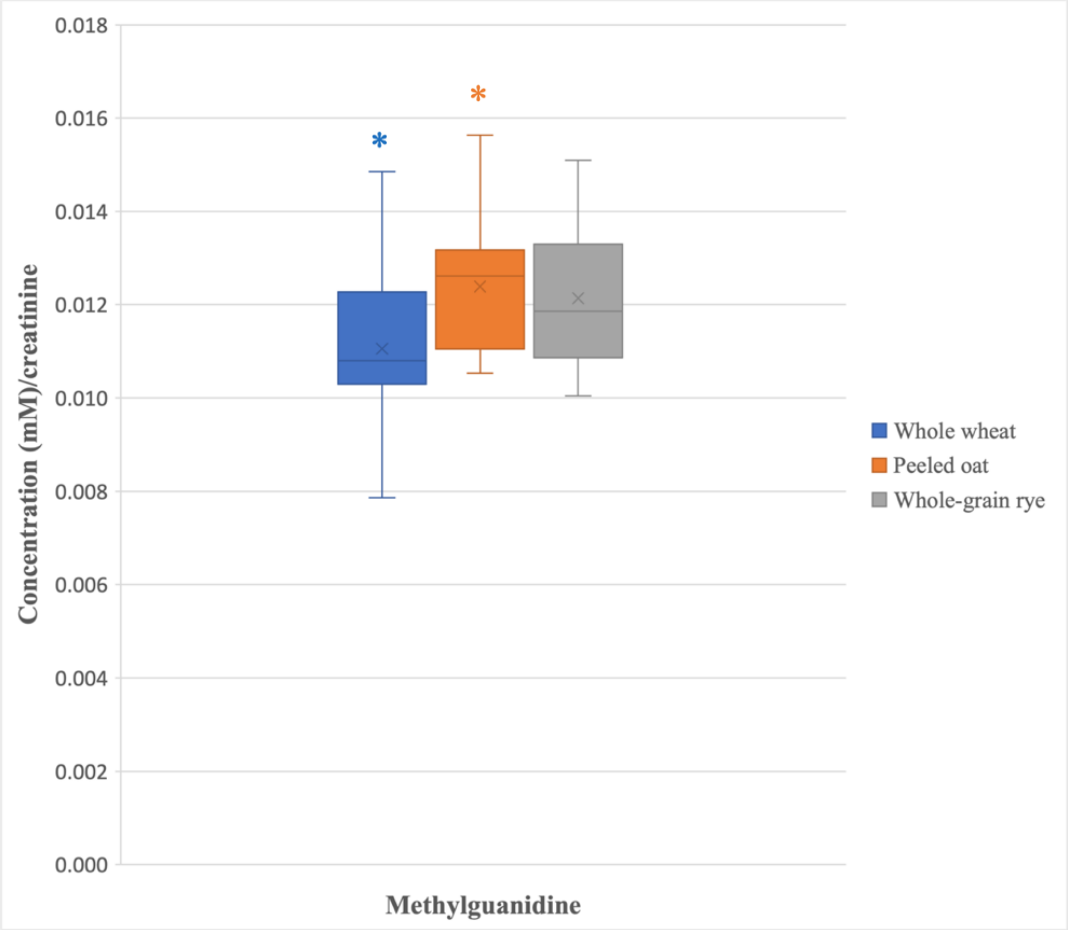

Figure 7. Concentrations of methylguanidine in the three experimental diets; whole-grain wheat, peeled oat, whole-grain rye (= significant differentiation between whole-grain wheat and peeled oat; Tukey multiple comparison test, p = 0.046) p<0.05**

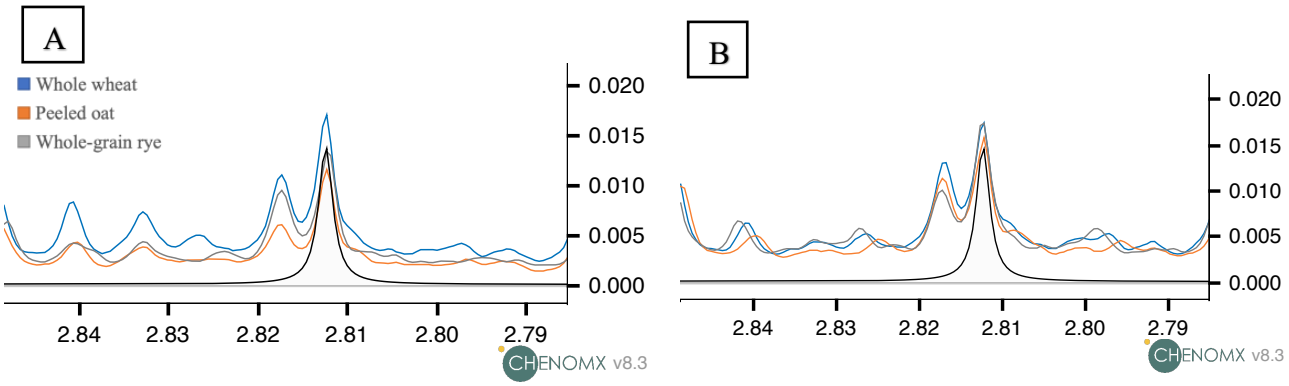

Figure 8. Nuclear Magnetic Resonance spectroscopy (NMR) spectrums from two randomly selected individuals (individual A and B) showing the metabolic response of methylguanidine in the three experimental diets (whole-grain wheat, peeled oat and whole-grain rye). The black peaks in the spectrums shows methylguanidine from the spectral reference library in software Chenomx profiler

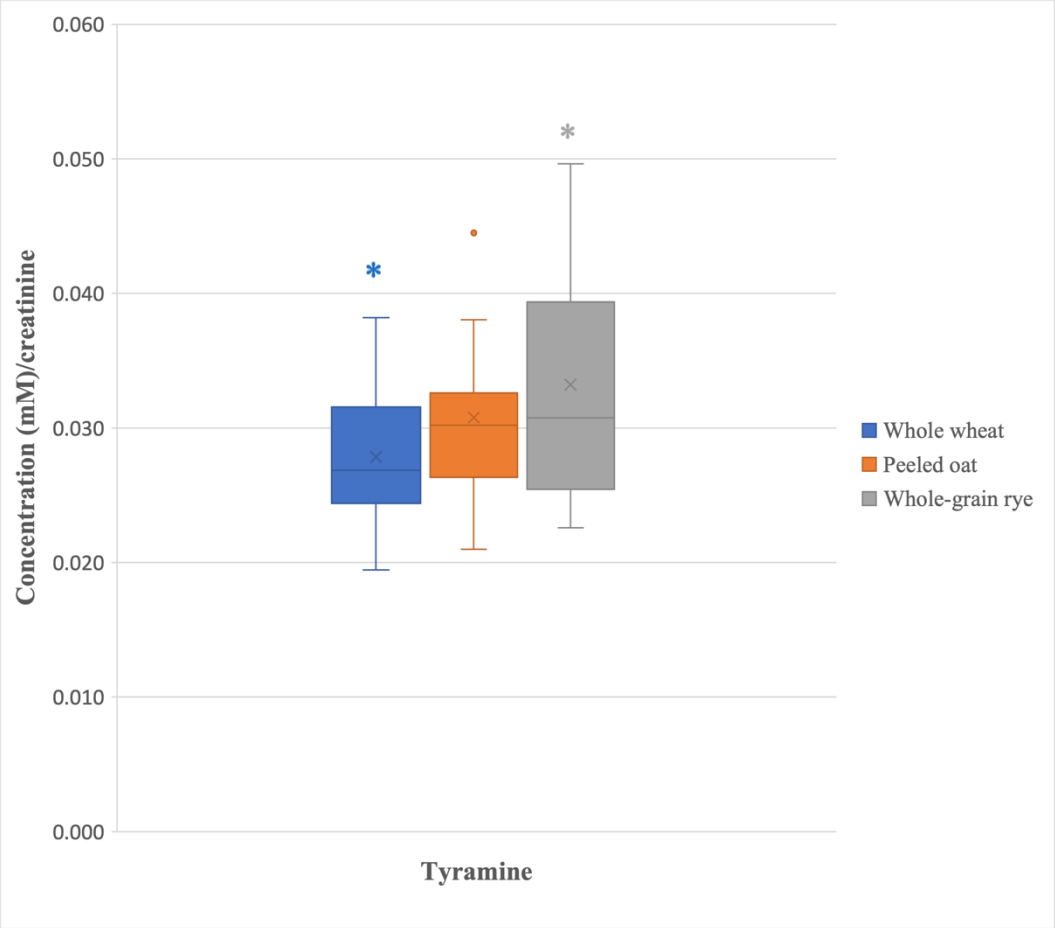

Figure 9. Concentrations of tyramine in the three experimental diets; whole-grain wheat, peeled oat, whole-grain rye (= significant differentiation between whole-grain wheat and whole-grain rye; Tukey multiple comparison test, p = 0.049) p<0.05*, (• = outlier)*

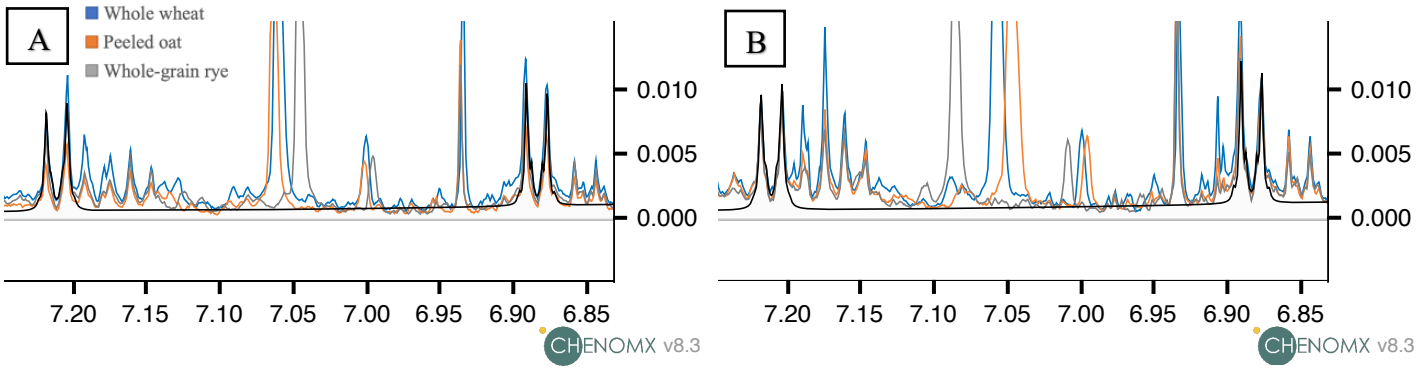

Figure 10. Nuclear Magnetic Resonance spectroscopy (NMR) spectrums from two randomly selected individuals (individual A and B) showing the metabolic response of tyramine in the three experimental diets (whole-grain wheat, peeled oat and whole-grain rye). The black peaks in the spectrums shows tyramine from the spectral reference library in software Chenomx profiler

Six metabolites showed a significant difference in concentration between timepoints (fasting and postprandial) (Table 7). Ascorbate, taurine, alanine, and tyramine were normally distributed; thus, a t-test was performed. Mannitol and n6-acetyllysine did not meet the assumptions for normal distribution, and therefore Wilcoxon's test was performed. The concentration of the metabolite mannitol had a significant differentiation between timepoints both in the whole-grain wheat diet (p=0.016) and the whole-grain rye diet (p=0.043). Ascorbate, mannitol, and n6-acetyllysine had a significantly higher concentration in fasting urine than in postprandial urine (Figure 11 and Figure 13). In addition, the concentration was significantly higher in postprandial urine than in fasting urine for taurine in the whole-grain wheat diet and for both alanine, and tyramine in the peeled oat diet (Figure 11 and Figure 12).

Table 7. Metabolites that differ significantly between time points (fasting and postprandial) in the experimental feeds; whole-grain wheat (n=16), peeled oat (n=15), and whole-grain rye (n=16). The significance levels are 0.05, 0.01, 0.0001

Diet	**Metabolite**	***t* – test**	**Wilcoxon test**
Whole-grain wheat	Ascorbate	p = 0.0063	—
	Mannitol	—	p = 0.0160
	N6-Acetyllysine	—	p = 0.0077
	Taurine	p = 0.0005	—
Peeled oat	Alanine	p = 0.0083	—
	Tyramine	p = 0.0045	—
Whole-grain rye	Mannitol	—	p = 0.0428

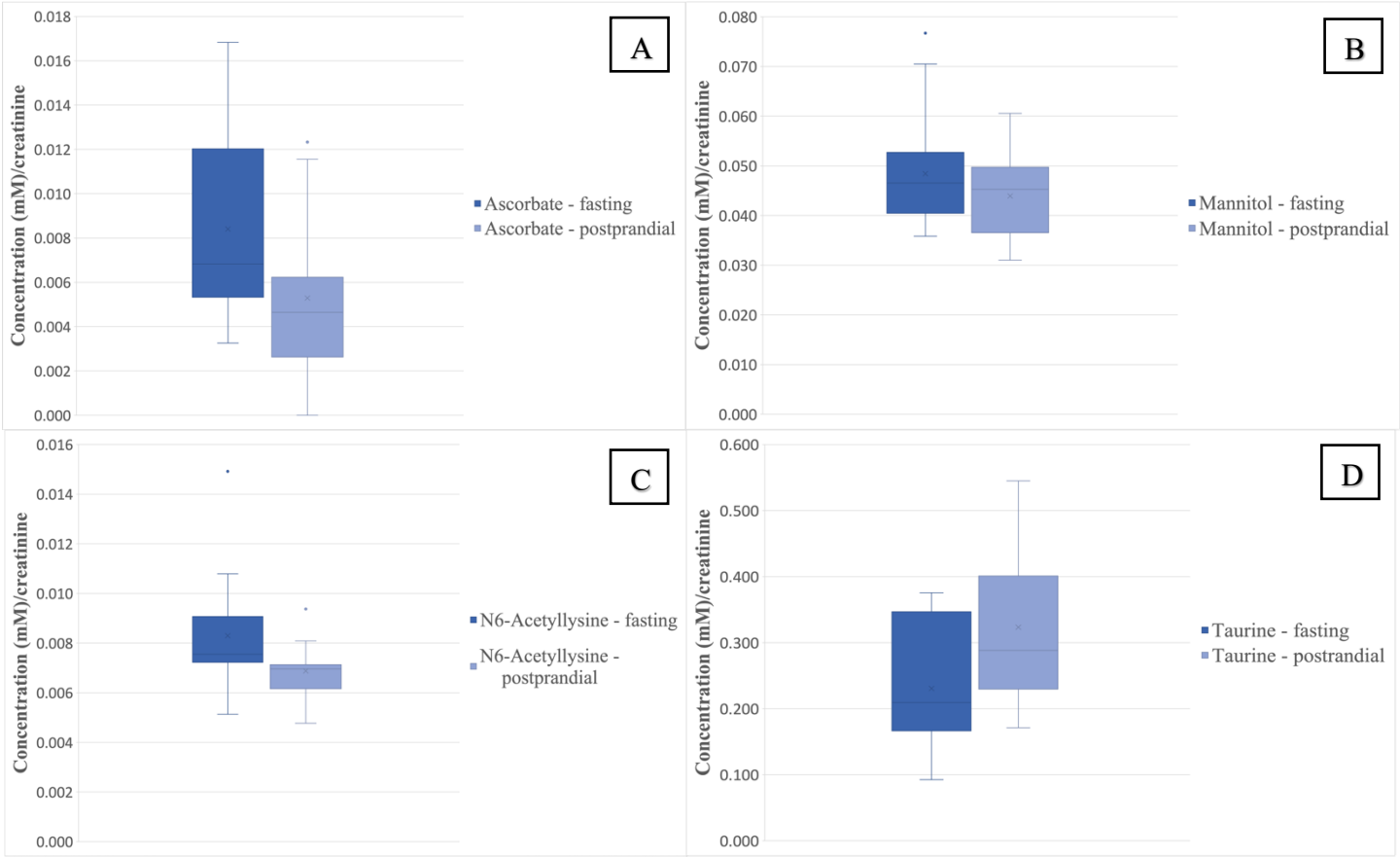

Figure 11. Metabolites that differed significantly between timepoints (fasting and postprandial) of whole-grain wheat diet; A = Ascorbate, B = Mannitol, C = N6-Acetyllysine, D = Taurine (n=16)

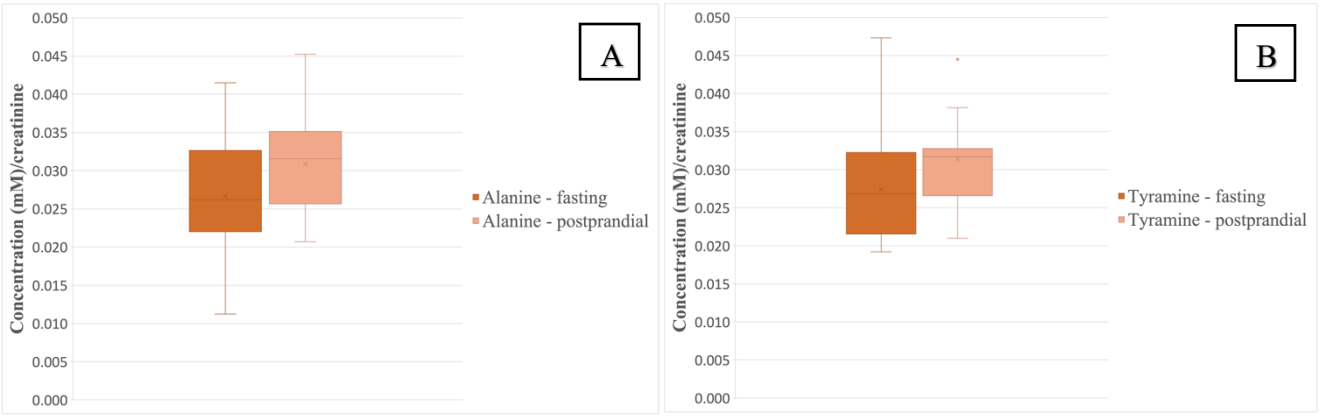

Figure 12. Metabolites that differed significantly between timepoints (fasting and postprandial) of peeled oat diet; A = Alanine, B = Tyramine (n=15)

Figure 13. Metabolite, Mannitol, that differed significantly between timepoints (fasting and postprandial) of whole-grain rye diet (n=16)

5. Discussion

Metabolic effects

NMR-based metabolomics has previously been used in human research studies to differentiate the diet response of different carbohydrates and fibre contents (Bertram et al. 2006). However, the metabolic response and the health effect of different carbohydrates in dog food are still poorly understood. In the present study, metabolic differences were identified by NMR on urine profiles as a response to carbohydrate source in the feed. Firstly, it was a tendency to separation between the experimental feeds, whole-grain wheat, peeled oat, and whole-grain rye, according to the PCA analysis (Figure 3). Secondly, a significant separation was found between the diets of whole-grain wheat and whole-grain rye according to the PLS-DA analysis (Figure 4). A previous study found a clear difference in the metabolic profile in urine and plasma of pigs fed with a refined wheat diet compared with those fed whole-grain rye diet. One of the primary findings in plasma and urine was that the level of betaine was higher in the whole-grain rye diet compared with the refined wheat diet (Bertram et al. 2006). Positive health effects have been reported for betaine. The author Bertram et al. (2006) described a reverse relationship between plasma betaine concentration and excretion of urinary creatinine in the whole-grain rye diet. Additionally, the interaction of these metabolites could be linked to homocysteine metabolism (Bertram et al. 2006).

Creatine is a metabolite that provides the muscles with energy, and creatinine is the by-product created from the body's use of creatine. Therefore, creatine and creatinine are highly linked. The huge variation in concentration between individuals in this study for both of these metabolites can be explained by breed differences with various muscle masses and various water consumption. However, the huge variation presented in creatine SD (Table 6) is contradictory as adjustments have been made in creatinine levels to minimize this variation between individuals which may be due to breed differences and water intake. The purpose behind the adjustment of creatinine was to minimize the error linked to the size of dogs and water consumption to get a representative comparison based on the unit mM/creatinine.

No significant difference was shown in creatinine concentration between the experimental diets in the present study. However, a significantly higher creatinine concentration has previously been found in pigs eating a non-whole grain diet containing wheat compared with a wholegrain diet containing rye (Bertram et al. 2006).

There were regions ("buckets") in the NMR spectrum that according to the PLS-DA differed significantly in abundance between the diets of whole-grain wheat and whole-grain rye. However, due to overlapping regions with low signals, it was not possible to identify a specific metabolite from the spectral reference library in the software Chenomx profiler. Two of the metabolites that were identified from the NMR spectrum showed a significantly different concentrations between the diets. The concentration of methylguanidine was significantly higher in the peeled oat diet compared with the whole-grain wheat diet ($p=0.046$). Tyramine had a higher concentration in the whole-grain rye diet compared with the whole-grain wheat diet ($p=0.049$). It is hard to tell what metabolic functions these findings may have for dogs and more metabolomic studies are needed to provide wider knowledge about how different grains influence metabolic pathways.

Methylguanidine

The concentration of methylguanidine in the urine differed significantly after consumption of the different experimental feeds in this study. The metabolite methylguanidine originates from the guanidine compound, where one of the amino hydrogens is substituted by a methyl group (Wishart et al. 2022). Methylguanidine is involved in protein catabolism and is an inhibitor of nitric oxide synthase, and has been defined as a uremic toxin (Wishart et al. 2022). The Human Metabolome Database explains that the metabolite is involved in physiological states such as renal failure, and it has also been linked to exhibiting anti-inflammatory effects (Wishart et al. 2022). Methylguanidine inhibits iNOS activity and TNF- release, which can contribute to reducing the degree of inflammation and tissue damage linked to endotoxic shock (Wishart et al. 2022). Previous studies have stated that a high concentration of guanidine or guanidine-like compounds can contribute to uremia and hypertension (Chmielewski et al. 2013). It has been shown that uremic concentration of guanidine compounds can act as an inhibitor for neutrophil metabolism (Hirayama et al. 2001). This inhibited effect has further been shown to be linked to adenosine which is a product by inhibition of glycolysis (Hirayama et al. 2001). The connection between renal failure and the metabolite methylguanidine has previously been examined in rats, where the result showed a significantly higher concentration of methylguanidine in the body of rats with renal failure compared with normal rats (Yokozawa & Fujitsuka 1990 see Chmielewski et al. 2013). A decreased concentration of methylguanidine has been detected in serum and urine after a low protein diet with essential amino acid (Ando et al. 1979 see Chmielewski

et al. 2013). Still more studies are needed to map the mechanisms involved in uremic toxicity (Chmielewski et al. 2013). It has not been possible to verify the metabolic significance of the concentrations measured in the present study. The information about metabolic pathways linked to different concentration of methylguanidine is lacking. To our knowledge, there are no previous findings of connection between methylguanidine and dog's metabolism.

Tyramine

Tyramine was the other metabolite with a significant difference in concentration in response to the different experimental feeds. The metabolite tyramine originates from phenethylamines, a class of organic compounds (Wishart et al. 2022). Tyramine is synthesized from the amino acid tyrosine, commonly by the decarboxylation of tyrosine during fermentation or decay (Wishart et al. 2022). According to Human Metabolome Database can large dietary intake of tyramine cause an increase in systolic blood pressure decay (Wishart et al. 2022). Tyramine use G-protein-coupled receptors to act as a neurotransmitter in the brain, and peripheral tissues, including the kidney (Wishart et al. 2022). Information about the effect of tyramine in dogs is lacking as previously described for the metabolite methylguanidine.

Metabolites that differed between fasting and postprandial samples

Six metabolites differed significantly between fasting and postprandial samples in the present study (ascorbate, mannitol, n6-acetyllysine, taurine, alanine and tyramine). A rapid increase in insulin and glucagon in serum as a postprandial response has previously been documented for dogs in the study by Söder et al. (2016). A significant metabolic response for triglyceride, free fatty acid and glucose were also found in serum after dogs were fed a high-fat meal. One of the findings was that it took three hours until the glucose was significantly increased (Söder et al. 2016). One result of this study was that most of the metabolites linked to the differentiation between time points were linked to the intake of the whole-grain wheat diet compared with the peeled oat diet and the whole-grain rye diet. A total of four metabolites (ascorbate, mannitol, n6-acetyllysine, taurine) differed significantly in concentration between fasting and postprandial after intake of the whole-wheat diet. Less metabolic effect was shown in the peeled oat diet, where only the concentration of alanine and tyramine differed, and in the whole-grain rye diet, where the concentration of mannitol differed between fasting and postprandial urine. Comparison of the metabolic effect in the urine of dogs after a high-fat mixed meal has previously shown differences in the metabolites allantoin, taurine, citrate and malonate (Söder et al. 2017). These findings were done 3-hours postprandial which was in agreement with the time of 3 hours between fasting urine sample and postprandial urine sample in the present study. An elevated concentration of taurine

in postprandial urine was a common result in the study by Söder et al. (2017) and in this study.

Study design

The dogs' water intake was not controlled during the study, meaning that urine concentration differed between individuals. To obtain an optimal result from the quantification of metabolites in the NMR analysis, the different urine samples were diluted to reach a common concentration range. The urine concentration of molecules and dissolved salts can be measured in different ways. An indicator of urine dilution can be seen by the color, where light yellow usually is linked to more diluted urine whereas a dark yellow pigment is linked to concentrated urine. In addition to the visual assessment, there are two measurement methods for analyzing urine concentration; USG and determination of creatinine levels. Measurements of the USG were done when the urine samples were collected in 2019 and determination of the creatinine levels in the urine samples were done in this study. A correlation of 0.49 between creatinine concentration and USG has previously been documented in a study by Alessio et al. (1985). This study resulted in a much higher and significant correlation of 0.86 (p-value <2,2e-16) between USG and creatinine, which indicates that the measurements are in good agreement. There was a large variation in creatinine levels in the dogs, ranging from 6.7 to 61.3 mmol/L. This could be explained by individual differences due to different water intake and other physiological traits and states including muscle mass as previously mentioned by Alessio et al. (1985) and Braun et al. (2003). A previous study has found differences in creatinine concentration depending on the amount of protein in the diet (Yamamoto et al. 2019). However, a general statement that creatinine is largely unaffected by the diet, has long been established (Braun et al. 2003). In this study, urine creatinine concentration was not significantly different between fasting and after food intake and no significant difference could neither be linked to the consumption of any of the three experimental feeds. This is in agreement with previous studies stating that urinary excretion is constant and unaffected by the diet (Braun et al. 2003).

Optimization for the NMR protocol was needed due to differences in urine concentration which introduced challenges to generate NMR spectra of good quality. Urine samples are usually very salty, which can influence the sensitivity of the magnetic field in the NMR (Gowda & Raftery 2019). In addition to the influence of salt, it has been shown that protein and lipids in a high concentration in biofluids can contribute to broad and overlapping peaks, thus being problematic for the identification of metabolites in the NMR spectrum (Gowda & Raftery 2019). Moreover, too concentrated urine samples also contributed to the signal of the internal standard, TSP, being too low and close to the limit of detection. The NMR protocol was thus optimized by urine dilution and a higher concentration of TSP

(20mM). The optimization to a higher concentration of TSP and the urine dilution to a creatinine range between 3 – 6.7 mmol/L resulted in a better output signal from the NMR analysis. Additionally, pH for all samples was measured in the NMR tubes to check that the buffering was done properly and to get information on possible pH variation among the samples. All the samples were measured and were in the range of pH 7 (7.18 – 7.69). Therefore, pH 7.40 ± 0.5 were added as a setting to the software library in Chenomx when metabolites were identified. The resonance, represented as the location of the peaks in the NMR spectrum, can be influenced by many parameters such as pH, temperature, the concentration of salts, and the relative concentration of specific ions (Izquierdo-García et al. 2011; Gowda & Raftery 2019). Thus, these parameters were important to take into account when identifying metabolites from NMR. The adjustment of creatinine level and the check-up of pH value minimized identification problems that might otherwise arise. Peak shift across samples can appear and therefore needs to be accounted for (Izquierdo-García et al. 2011). The metabolites were identified in all VIP-specific regions with consciousness of the possible peak shift between the urine samples. The concentrations of the metabolites identified from the NMR spectrums were set to the unit mM/creatinine and were calculated by the area under the peaks estimated from the software, Chenomx profiler. Thereafter, the concentration of each metabolite was divided by the creatinine concentration in each sample. This were done to get comparable values of the metabolites and to minimize the error variance of the water intake of different individuals and the variance in body composition/muscle mass between different breeds.

Further research

It is important to note that our findings regarding the differentiation between the metabolic response in the whole-grain wheat diet and the whole-grain rye diet need follow-up studies to validate the result to a larger extent. Preferably, a further step would be to divide dogs into different population groups where environmental factors can be included in the analysis. With this, it would be of interest to look into what factors in the gut microbiota can affect the metabolic response of dogs and if individual differences linked to environmental factors can be found in the response of different carbohydrate sources. Furthermore, it would be of interest to include lifestyle parameters, such as exercise and how movement in relation to the time of feeding may influence the metabolic response of carbohydrates. An improved understanding of individual nutrient responses of carbohydrate sources will contribute to more knowledge of preventing metabolic diseases and promoting sustainable health for our dogs.

An important factor for the feed companies is to incorporate the effect of food processing on nutrient uptake in the dog. There is little research in this area today, and more is needed.

5.1 Study limitations

The small size of the dataset with variation among breeds, ages, and weights cause a bias to conclude the results for all the dogs as one population. Therefore, statistical tests (repeated measures analysis of variance (ANOVA) or Friedman non-parametric test) were performed to make it possible to study the variation over time, and the effect of the experimental feeds individually. However, there is still a limited ability to draw conclusion when the sample size is small.

In this study, just two of the identified metabolites concentrations significantly differed between the experimental feeds even though the PLS-DA showed significant differentiation among ten identified metabolites. The reason why we could not identify statistical significance for the identified metabolites may be because the response of the feeds had a large individual spread which may be influenced by genetic and lifestyle factors. However, the statistical analysis with repeated measures would handle this variation and conclude if the metabolic response varies individually between the experimental feeds.

Using spot urine samples can cause limitations because the collection does not cover the diurnal variation. The diurnal rhythms of urine excretions can vary between individuals. However, spot samples of fasting urine from the morning have been shown to correspond well to the over-day exertion of urine (Ishibashi et al. 2021).

NMR spectroscopy is a powerful technology that detects macronutrients, including lipids, simple sugars, and amino acids, in biofluid samples (German et al. 2005). This technology provides detailed metabolite structural information (Lehman-Mckeeman & Car 2004). However, the technique is limited to not detecting metabolites less than 100 ng of mass (Lehman-Mckeeman & Car 2004). Complementary technology is needed to identify lower abundant metabolites, such as bioactive products that act at very low concentrations in signalling functions (German et al. 2005). Potentially, biological differentiation between diets can be found in metabolites with a low mass and concentration that the NMR did not detect in this study. There was a limitation with the identification in overlapping regions with low concentration, and no specific metabolite could in this region be identified even though the statistical analysis showed significant differentiation between the diets. Identifying and understanding the mechanisms behind different metabolites

is complex research and time-consuming. The time frame is limited for this master thesis and the results presented here are a start for further research.

6. Conclusions

Metabolic profiles were in this study generated after four weeks of feeding with three experimental feeds containing whole-grain wheat, peeled oat, or whole-grain rye. In conclusion, the result indicated that the metabolic response tended to vary in different types of grains. Statistically, the metabolic profiles differed postprandial for whole-grain wheat and whole-grain rye. The biomarker identification from NMR spectrums resulted in ten metabolites contributing to the differentiating in feed response. However, the variation in concentration of the metabolite was rather low and only two metabolites differed significantly between the experimental feeds. The concentration of some identified metabolites differed between fasting and postprandial urine and most of these metabolites were linked to a significant shift in concentration after feed intake, which were more commonly seen after feeding of whole grain wheat compared with peeled oat and whole grain rye. Methylguanidine was significantly higher in concentration after the feeding period with peeled oat compared with whole-grain wheat. The metabolite tyramine showed a significantly higher concentration after the feeding period with whole-grain rye compared with whole-grain wheat. Today, it is hard to tell what this differentiation can mean for the individual dog and the activation of metabolic pathways due to lacking previous research. According, to the Human Metabolome Database, methylguanidine has been defined as a uremic toxin and is explained as a metabolite involved in physiological states such as renal failure and exhibiting anti-inflammatory effects. Tyramine is involved in the adjustment of systolic blood pressure and acts as a neurotransmitter in the brain, and peripheral tissues, including the kidney. More metabolomic studies are needed to provide wider knowledge about how different grains influence metabolic pathways in dogs and what consequences the grain sources can have for the health and well-being of dogs. According to previous studies, mostly on humans, there is an indication that the grain source rye could contribute to better health effects compared to oats and wheat. However, the findings in this study could not answer the question if the type of grain can be a risk factor for metabolic diseases in dogs.

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Populärvetenskaplig sammanfattning

Hundutfordring är ett omdebatterat ämne. Det finns många frågor som diskuteras inom hundbranschen idag;
Ska jag utfodra min hund spannmålsfritt eller glutenfritt?
Vilken proteinkälla ska jag utfodra till min hund?
Eller vilken kolhydratkälla ska jag välja; potatis, ris, vete, havre eller råg?
Vad är egentligen ett hälsosamt hundfoder?

Frågorna är viktiga att uppmärksamma med tanke på bristen av vetenskapliga studier kopplat till hundens nutrition och det metabola svaret av olika näringsämnen. Mångfalden av ingredienser i kommersiella hundfoder är enorm. Däremot är ett högt spannmålsinnehåll ofta en gemensam nämnare i torrfoder, vanligtvis omkring 30 – 60% av energikällan i torrsubstans. Spannmål i torrfoder har ifrågasatts däremot kan det finnas ett intresse av fullkornsprodukter från spannmål då de innehåller kostfiber. Att studera spannmålens metabola effekter är av betydelse då de innehåller kostfiber och andra bioaktiva ämnen som framförallt inom humanforskningen visat på en rad olika positiva hälsoeffekter. Kostfiber har bland annat visat gynna den goda tarmfloran och därmed bira till god tramhälsa. Andra positiva hälsoeffekter av kostfiber som påvisats hos människa är minskad risk för att utveckla hjärt- och kärlsjukdomar, respiratoriska sjukdomar, cancer och diabetes. En riskfaktor för att utveckla dessa sjukdomar är bland annat övervikt som är ett ökade problem för både människor och hundar. Mellan 30% – 40% av våra hundar världen över bedöms vara överviktiga. Övervikt och fetma klassas som en multifaktoriell sjukdom men foder har en viktig roll för att förebygga utvecklingen av det metabola tillståndet och dess följdsjukdomar.

Syftet med den här studien var att studera hur olika spannmålstyper i hundfoder påverkar hundens metabolism och det metabola svaret. Kostfiber och bioaktiva ämnen skiljer sig åt mellan spannmålstyper men hur de möjligen skulle aktivera olika metabola vägar är okänt. Studien innefattades av tre olika torrfoderdieter där spannmålstyp varierade mellan fullkornsvete, skalad havre och fullkornsråg. Det var 17 privatägda hundar av olika raser som inkluderades i studien och de blev utfodrade med alla tre foder, vardera i en fyraveckorsperiod i slumpmässig ordning. Urinprover samlades in i slutet av foderperioderna, både innan foderintag (fastande)

och cirka 4 timmar efter foderintag (postprandiellt). Metabola profiler kopplat till de olika spannmålstyperna togs fram genom att analysera urinproven med kärnmagnetisk resonansspektroskopi, så kallad NMR-spektroskopi. Resultatet visade på att det metabola svaret av de tre fodertyperna tenderade att skilja sig åt. Genom den statistiska analysmetoden Partial least squares discriminate analysis (PLS-DA) hittades störst variation och en signifikant fodereffekt mellan fullkornsvete och fullkornsråg efter foderintag (postprandiellt). Totalt tio metaboliter kunde kopplas till skillnaden i metabolitprofilerna från dieterna innehållande av fullkornsvete och fullkornsråg. De tio metaboliterna var alanin, askorbat, kreatin, glutarsyramonometyl ester, mannitol, metylguanidin, n6-acetyllysin, taurin, tyramin och urea. Koncentrationen för metaboliterna beräknades och variationen mellan faste och postprandiella urinprover studerades från alla tre spannmålsdieter. Sex av de tio metaboliterna visade sig variera i koncentration mellan fasta och foderintag, där fyra metaboliter varierade efter intag av fullkornsvete. Inga signifikanta skillnader av metaboliternas koncentrationer kunde hittas mellan de olika dieterna för faste urinproverna. Däremot visade resultatet att koncentrationen av två metaboliter skiljde sig åt postprandiellt. En signifikant skillnad för metaboliten metylguanidin mellan fullkornsvete och skalad havre och en signifikant skillnad för metaboliten tyramin mellan fullkornsvete och fullkornsråg. Metylguanidin är en metabolit som är involverad katabolismen av protein och har visat sig vara en inhibitor av kväveoxidsyntas. Denna metabolit har även kunnat kopplas till njursjukdom då den försämrar urinfunktionen. Tyramin är en metabolit som syntetiseras från aminosyran tyrosin och fungerar som en signalsubstans i det centrala nervsystemet. Metaboliten är därmed i huvudsak aktiv i hjärnan men även i delar av kroppen såsom muskler, huden och njurarna. Vidare fördjupning krävs för att ta reda på vad fodereffekten gällande metylguanidin och tyramin skulle kunna innebära för hundens metabolism och om det möjligen skulle kunna kopplas till riskfaktorer för metabola sjukdomar. Sammantaget visar den här studien ansatser till att spannmålen fullkornsvete, skalad havre och fullkornsråg metaboliseras något olika i hunden. Skillnad i hundens metabola svar kunde påvisas mellan fullkornsvete och fullkornsråg efter foderintag men nu kärvs vidare forskning för att kunna bidra med kunskap kring vad skillnaden kan innebära för hundens hälsa och välbefinnande.

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