



DOCTORAL THESIS NO. 2023:65  
FACULTY OF VETERINARY MEDICINE AND ANIMAL SCIENCE

# Whole grain cereals in dog food

## Effects on metabolism and gut microbiota

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SWEDISH UNIVERSITY  
OF AGRICULTURAL  
SCIENCES

DOCTORAL THESIS

Uppsala 2023

Acta Universitatis Agriculturae Sueciae  
2023:65

Cover: På äventyr i spannmålsfältet  
(Illustration: Nadia Nörbom)

ISSN 1652-6880

ISBN (print version) 978-91-8046-182-5

ISBN (electronic version) 978-91-8046-183-2

<https://doi.org/10.54612/a.1cn95jv40c>

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Print: SLU Grafisk service, Uppsala 2023

# Whole grain cereals in dog food. Effects on metabolism and gut microbiota

## Abstract

During domestication from the wolf, the gastrointestinal tract in dogs adapted to a diet containing starch, which is often present in substantial amounts in commercial dog food. In humans, diets with whole grain, compared with refined, are reported to have beneficial metabolic effects, possibly through the microbiota and short-chain fatty acids (SCFA) produced during fermentation of dietary fiber by certain bacteria, *e.g. Prevotella*. The objective of this thesis was thus to explore the effects of whole-grain cereals; wheat, oats and rye, in dog diets on metabolism, gut microbiota and SCFA. Rye is an uncommon ingredient in dog food, thus an initial study with six Beagle dogs evaluated suitable inclusion rate and effects on microbiota compared with refined wheat. In a subsequent study, 18 privately-owned dogs were fed three diets with whole grains of wheat, oats or rye in a cross-over experiment, and effects on fecal microbiota, SCFA and postprandial metabolic response were studied.

Whole grain rye included at 25% of dry matter was acceptably digested and tolerated by the dogs. A 50% inclusion rate induced a significant shift in fecal microbial composition with an increase in relative abundance of *Prevotella*. Whole grains from wheat, oat or rye did not have differing effects on general fecal microbial composition, but microbial diversity was higher following wheat compared with rye. *Bacteroides* abundance was lower after rye than after wheat or oats, and inversely related to *Prevotella* abundance. Fecal acetate and propionate concentrations were higher after rye than after oats. The oat diet resulted in higher postprandial blood concentration of glucose, glucagon-like peptide-1 (GLP-1) and triacylglycerol compared with wheat. Fasting insulin was higher after rye than after wheat or oats. Wheat tended to result in more insulin per glucose and GLP-1 than the other two diets. Overall, the results in this thesis indicate that whole grain rye can affect dog gut microbiota composition and function in ways that could be beneficial to health. Further studies on the metabolic effects of whole grains in dog food are warranted.

Keywords: *Prevotella*, *Bacteroides*, rye, oat, wheat, SCFA, GLP-1, insulin, glucose

# Fullkorn i hundfoder. Effekter på metabolism och mikrobiota

## Sammanfattning

När hunden domesticerades från vargen anpassades dess digestionssystem till en diet med stärkelse, som i dagens hundfoder kan utgöra en stor del av innehållet. För människor kan en kost som innehåller fullkorn, istället för raffinerat mjöl, ha metabola hälsofördelar, möjligen delvis kopplat till mikrobiotan och de kortkedjiga fettsyror (SCFA) som vissa bakterier, t.ex. *Prevotella*, bildar vid fermentation av kostfiber. Syftet med avhandlingen var att utforska vilka effekter fullkorn av vete, havre och råg, har på hundens metabolism, tarmmikrobiota och SCFA. Råg är en ovanlig ingrediens i hundfoder. I en första studie, med sex beaglar, utvärderades därför lämplig inblandningsnivå av fullkornsråg och effekter på mikrobiota jämfördes mot raffinerat vete. I nästa delstudie utfodrades 18 privatägda hundar i en cross-over design med tre olika foder, innehållandes fullkorn av vete, havre eller råg. Effekter på mikrobiota, SCFA och den postprandiella metabolismen studerades.

Foder med 25 % inblandning av fullkornsråg accepterades av hundarna och hade godtagbar smältbarhet. När inblandningsnivån av råg var 50 % sågs förändringar i den generella mikrobiotasammansättningen med ökad relativ förekomst av *Prevotella*. Inga skillnader i generell mikrobiotasammansättning sågs mellan foder med vete, havre eller råg i delstudie två, men diversiteten var högre efter vete än efter råg. Förekomsten av *Bacteroides* var lägst efter råg och negativt korrelerad till förekomsten av *Prevotella*. Acetat- och propionat-koncentrationen i feces var högre efter råg än efter havre. Havrefodret gav högre postprandiell blodkoncentration av glukos, glucagon-like peptide 1 (GLP-1) och triacylglycerol jämfört med vete. Fastenivån av insulin var högst efter råg. Insulinfrisättning per glukos och GLP-1 tenderade att vara högst efter vete. Sammantaget indikerar resultaten att fullkornsråg kan påverka hundens mikrobiota och dess funktion i hälsofrämjande riktning. Vidare studier angående de metabola effekterna av fullkorn i hundfoder behövs.

Keywords: *Prevotella*, *Bacteroides*, råg, havre, vete, kortkedjiga fettsyror, GLP-1, insulin, glukos

# Dedication

Till pappa





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## List of publications

This thesis is based on the work contained in the following Papers, referred to by Roman numerals in the text:

- I. Palmqvist, H., Ringmark, S., Höglund, K., Pelve, E., Lundh, T., & Dicksved, J. (2023). Effects of rye inclusion in dog food on fecal microbiota and short-chain fatty acids. *BMC Veterinary Research*, 19(1), 70
- II. Palmqvist, H., Höglund, K., Ringmark, S., Lundh, T., & Dicksved, J. (2023). Effects of whole-grain cereals on fecal microbiota and short-chain fatty acids in dogs: a comparison of rye, oats and wheat. *Scientific Reports*, 13(1), 10920
- III. Palmqvist, H., Ringmark, S., Dicksved, J., Lundh, T., & Höglund, K. Metabolic and hormonal effects of rye, oats or wheat included as whole grains in dog food. (manuscript)

Papers I-II are reproduced with the permission of the publishers.

The contribution of Hanna Palmqvist to the Papers included in this thesis was as follows:

- I. Planned the study together with the supervisors. Organized and carried out the study, with practical help from the supervisors. Performed parts of the laboratory analyses. Performed the univariate statistical analyses. Interpreted the results with help from the supervisors. Wrote the first draft of the manuscript and adjusted the manuscript in collaboration with the supervisors.
- II. Planned the study, with help from the supervisors. Organized and carried out the study. Oversaw the laboratory analyses. Performed the statistical analyses and interpreted the results with help from the supervisors. Wrote the first draft of the manuscript and adjusted the manuscript in collaboration with the supervisors and corresponded with the journal.
- III. Planned the study, with help from the supervisors. Organized and carried out the study, with practical help from the supervisors. Oversaw the laboratory analyses. Performed the statistical analyses and interpreted the results with help from the supervisors. Wrote the first draft of the manuscript and adjusted the manuscript in collaboration with the supervisors

## Related publication not included in the thesis

- Höglund, K., Palmqvist, H., Ringmark, S., Svensson, A. (2022). Quantification of normetanephrine in canine urine using ELISA: evaluation of factors affecting results. *Journal of Veterinary Diagnostic Investigation*, 34(1), pp. 28-35





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## Abbreviations

ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
ATTD	Apparent total tract digestibility
AUC	Area under curve
BCS	Body condition score
BW	Body weight
DM	Dry matter
ELISA	Enzyme-linked immunosorbent assay
FDR	False discovery rate
FFAR	Free fatty acid receptor
GI	Gastrointestinal
GLP-1	Glucagon like peptide-1
ME	Metabolizable energy
NEFA	Non-esterified fatty acid
OTU	Operational taxonomic unit
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PYY	Peptide tyrosine tyrosine
SCFA	Short-chain fatty acids

TAG	Triacylglycerol
TDF	Total dietary fiber

# 1. Introduction

## 1.1 The dog - a facultative carnivore or an omnivore?

The domestic dog (*Canis lupus familiaris*) is considered a facultative carnivore, but there is no clear distinction between facultative carnivores and omnivores since both groups can and does eat vegetable material as well as meat (Sjaastad *et al.* 2016). However, in contrast to obligate carnivores such as the domestic cat, dogs have a lower protein requirement and possess the ability to synthesize many of the essential nutrients, such as taurine and vitamin A, that are found in meat but not in vegetable material (NRC 2006). The wolf (*Canis lupus*), from which the dog evolved at least 10,000 years ago (Skoglund *et al.* 2011) is also defined as a facultative carnivore. However, genetic studies comparing the dog and wolf genome have revealed that three genes crucial to digestion of starch and uptake of the resulting glucose molecules have in dogs evolved, increasing their ability to digest starch (Axelsson *et al.* 2013). This adaptation to a more starch-rich diet is suggested to have been a driving force behind domestication of the dog at the time when humans started to establish settlements and cultivate the land. Hence, the domestic dog could be considered an omnivore rather than a carnivore. Given the strain on the planet that production of meat entails (Swanson *et al.* 2013) and considering that the physiological features of the dog's digestive system are suited to a diet of both plant and animal origin, it is reasonable to suggest that dogs in general should be fed a mixed diet that is more environmentally sustainable than a diet composed for more pronounced carnivores.

## 1.2 Carbohydrates, grains and health in humans and dogs

Carbohydrates are a diverse group of organic compounds differing in composition, structure, size and physicochemical properties. In nature they serve as structural components and an energy store, and are important elements in many other structures vital for living organisms. The function as an energy store is an important aspect of their role in nutrition. The sub-group of carbohydrates categorised as dietary fiber also have important, but more intricate, functions in the field of nutrition.

Despite the many important functions of carbohydrates, they have had a rather negative image among the public in recent decades as components of the diet of humans themselves and their pets (Gunnarsson & Elam 2012; Laflamme *et al.* 2014; Knight 2015; Rankovic *et al.* 2019). In humans, refined carbohydrates have been associated with increasing incidence of lifestyle diseases such as obesity and diabetes mellitus, together with allergies and cancers, but with varying strength of evidence (Clemente-Suarez *et al.* 2022). Domestic pets share the living conditions and way of life of humans and are now commonly treated as family members (Arañuri *et al.* 2017). As a result, obesity and several other lifestyle diseases that affect humans also affect pets (German 2006; Courcier *et al.* 2010; German *et al.* 2018). Dedicated pet owners want what is best for their pet, and food is one way in which they can show their love and by which they can manage their pet's health (White *et al.* 2016; Morelli *et al.* 2019). One of the ingredients that animal owners tend to avoid for their dog is cereal grains, often based on allergic symptoms in their dog (Banton *et al.* 2021). As a result, grain-free dog food has become popular during the past decade. Cereal grains and carbohydrates are, however, not synonymous. Cereals contain a great deal of starch, but also considerable amounts of fiber, vitamins and minerals (Frølich *et al.* 2013). Thus when cereals are included in the diet as whole grains, they are considered to have beneficial effects on human health (Aune *et al.* 2016; Reynolds *et al.* 2019). Despite the fact that this has been known for many years, studies on the health effects of whole grains in the diet of dogs are scarce.



## 2. Background

### 2.1 The digestive tract of dogs

When the dog consumes a mixed meal containing all three main nutrient classes (carbohydrate, protein and fat), digestion starts in the oral cavity, with mastication reducing the size of food particles in preparation for enzymatic digestion in the stomach. From the stomach, partly digested food passes on to the small intestine, where enzymatic processing continues. The resulting monosaccharides, amino acids and lipids are then absorbed over the epithelium of the small intestine. By the time food reaches the large intestine, most readily available nutrients have been digested and absorbed. However, the dietary fiber fraction of the food still remains relatively intact, as it contains chemical bonds that mammalian endogenous enzymes cannot dissolve (Sjaastad *et al.* 2016). The microbiota in the large intestine has the capacity to break these chemical bonds and degrade fiber for energy extraction. At the same time, the environment becomes more hospitable to microorganisms, and hence the concentrations of bacteria in the luminal contents increase (Suchodolski *et al.* 2005). The caecum and colon of the dog are relatively short compared with those of the omnivorous pig, yet somewhat longer than those of the domestic cat. The main function of the colon in the dog is absorption of water and electrolytes, but an important influence of the colon microbiome on metabolism, the immune system and intestinal health in dogs is increasingly being recognised (Tizard & Jones 2018; Wernimont *et al.* 2020).

## 2.2 Metabolism and satiety

### 2.2.1 Metabolites and hormonal response after a meal

The monosaccharides and amino acids absorbed through the epithelium of the small intestine are released into the portal vein, which carries them to the liver. In the liver, hepatocytes take up some of these nutrients, while the remainder are released into the general circulation (Frayn 2010). The timing and magnitude of the elevation in peripheral blood glucose depends on the composition of the meal (Nguyen *et al.* 1994). The increases in blood glucose and amino acids stimulate secretion of insulin from beta-cells in the pancreas (Frayn 2010). Amino acids from a mixed meal also stimulates the pancreas to release glucagon from the alpha-cells (Schmid *et al.* 1992). Thus the concentrations of both insulin and glucagon in the blood increase following a mixed meal. However, as the increase in insulin concentration is greater than that in glucagon concentration, the insulin to glucagon ratio increases, which results in the liver switching from glycogenolysis to glycogenesis (Frayn 2010). Insulin and the increasing glucose concentration in blood eventually have an inhibitory effect on glucagon secretion (Frayn 2010). Glucose that is absorbed via the intestine elicits a higher insulin response than glucose administered intravenously, because glucose present in the intestinal lumen causes specific endocrine cells in the small intestine to release incretins, such as glucagon-like peptide 1 (GLP-1), from L-cells, which potentiates insulin secretion from the pancreas. Furthermore, GLP-1 has an inhibitory effect on glucagon when the glucose concentration increases from fasting levels (Holst 2007).

Lipids in food mainly constitutes triacylglycerols (TAG) (Sjaastad *et al.* 2016). These are taken up by the epithelial cells in the small intestine and combine with lipoproteins to form chylomicrons, which are transported out to the lymphatic system, and thus do not pass through the liver, before reaching the systemic circulation via the thoracic duct (Frayn 2010). The chylomicrons transport TAG to the adipocytes, where insulin inhibits lipolysis within the adipocyte while activating the lipoprotein lipase needed for disassembly of TAG to free fatty acids and monoacylglycerol, which then diffuse into the adipocytes for storage (Frayn 2010).

Uptake from the large intestine largely consists of water, but the fermentation products of the microbiota, *i.e.* short-chain fatty acids (SCFA),

are absorbed by the epithelial cells of the large intestine and used locally as an energy source or enter the blood stream via the portal vein (Frayn 2010).

### 2.2.2 Satiety signals

Appetite and satiety are controlled by centres in the arcuate nucleus of the hypothalamus (Sjaastad *et al.* 2016). Numerous factors intricately affect the control mechanisms, by direct or indirect routes. Early satiety signals arise from the walls of the stomach being stretched due to filling, irrespective of the specific nutrients in the food (Pappas *et al.* 1989). When nutrients enter the small intestine and come into contact with specific cells, hormones such as cholecystokinin (CCK), gastric inhibitory polypeptide (GIP), peptide tyrosine tyrosine (PYY) and GLP-1 are released, all inducing satiation by central signalling and/or local actions such as induction of the “ileal break” (Karhunen *et al.* 2008). The ileal break is a negative feed-back mechanism which slows down gastric emptying and transit time through the small intestine. It is induced by undigested nutrients present in the ileum (Read *et al.* 1984). Insulin from the pancreas acts centrally as a signal to terminate a meal, when enough energy (mainly glucose) has been consumed, and as a sensor of energy homeostasis in a longer-term perspective (Woods *et al.* 2006). Aside from hormones acting as satiety signals, some nutrients (*e.g.* glucose) can have such effects (Sjaastad *et al.* 2016). Acetate, one of the SCFA produced during bacterial fermentation of fiber in the colon, can also cross the blood-brain barrier and has been suggested to reduce appetite directly in the hypothalamus (Frost *et al.* 2014).

## 2.3 The gut microbiota in dogs and its function in health

### 2.3.1 Healthy microbiota

In the gut of healthy dogs, a multitude of bacteria, archaea, viruses, protozoa and fungi reside, together forming what is called the microbiota. The bacterial component is the predominant and most studied part, and the only part considered in this thesis. The dog gut microbiota, primarily evaluated based on rectal and fecal samples, is mainly composed of members of the phyla Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria and Proteobacteria (Handl *et al.* 2013; Honneffer *et al.* 2017; Söder *et al.* 2022). On genus level, the reported composition differs between studies, which can

be attributed at least in part to differences in methodology (Suchodolski 2022). However, in studies comparing the microbiota in the dog gut on lower taxonomic levels the inter-individual variation is generally reported to be large (Handl *et al.* 2013; Söder *et al.* 2022). This variation makes it difficult to establish what constitutes a healthy microbiota. The gut microbiota in humans has been shown to be an ecosystem, where some bacteria rely on other for cross-feeding of substances or for provision of a hospitable environment and several different species occupy the same niche (Lozupone *et al.* 2012). Thus diversity and species richness are important for the resilience of the system. Likewise, in dogs with gastrointestinal (GI) diseases, such as acute diarrhoea and chronic idiopathic inflammatory bowel disease (IBD), disturbances in microbial diversity have been observed and are often referred to as “dysbiosis” (Suchodolski *et al.* 2012b; Guard *et al.* 2015; Vazquez-Baeza *et al.* 2016). These observations indicate similar importance of microbiota diversity and richness for gut health in dogs as in humans. Alterations in specific bacterial groups are also reported to be associated with GI diseases in dogs, and thus an index for assessing the degree of dysbiosis based on fecal samples from dogs with GI disease has been developed (AlShawaqfeh *et al.* 2017). It should be noted, however, that it is difficult to assess whether a microbial disturbance is the cause of GI disease or a symptom. The dysbiosis index is based on quantitative polymerase chain reaction (qPCR), which quantifies specific bacterial groups. In the study used for the development of the index total number of bacteria was higher in healthy dogs compared with dogs with GI disease (AlShawaqfeh *et al.* 2017). Some of the taxa that were associated with healthy dogs were *Faecalibacterium*, *Fusobacterium* and *Clostridium hiranonis*, while increased numbers of *Escherichia coli* and *Streptococcus* were observed in dogs with GI diseases (AlShawaqfeh *et al.* 2017). The genus *Faecalibacterium* has likewise been identified as a healthy component of the human gut microbiota (Sokol *et al.* 2008), while *Fusobacterium* harbours species that are considered not to be beneficial for humans (Pilla & Suchodolski 2019). Thus, what is considered to be part of a healthy microbiota differs between dogs and humans.

Even though the bacterial composition of the gut microbiota differs widely between individuals, diet or, more precisely, the macronutrient composition of the diet can have a large impact on the microbiota (Li *et al.* 2017; Mori *et al.* 2019). Gut microbiota composition in dogs fed a diet high

in complex carbohydrates is often dominated by the phyla Firmicutes and Bacteroidetes, which contain fiber-fermenting genera that are considered beneficial in humans and dogs, such as *Faecalibacterium* and *Prevotella* (Pilla & Suchodolski 2019). A diet high in protein, on the other hand, generally favours the phylum Fusobacteria and to some extent also Proteobacteria (Pilla & Suchodolski 2019). The latter includes the family Enterobacteriaceae, which has been found to occur in dysbiosis in dogs (Vazquez-Baeza *et al.* 2016).

In humans, the existence of enterotypes has been suggested where, depending on their gut microbiota, individuals can be divided into three clusters, dominated by *Bacteroides*, *Prevotella* and *Ruminococcus* (Arumugam *et al.* 2011). Two of these clusterings (*Prevotella*- and *Bacteroides*-dominated) have been further corroborated (Wu *et al.* 2011). Studies on these two enterotypes have shown that they differ in their fiber-utilizing capacity and SCFA production (Chen *et al.* 2017; Pirkola *et al.* 2023). They have also been observed to result in different metabolic outcomes (Kovatcheva-Datchary *et al.* 2015; Sandberg *et al.* 2019; Eriksen *et al.* 2020). The basis for the enterotypes is suggested to depend on long-term dietary patterns, with diets high in fiber and carbohydrates correlated with *Prevotella* and diets high in protein and fat with *Bacteroides* (Wu *et al.* 2011). The corresponding concept has not been established in dogs, but a similar pattern of inverse relationships between these two genera has been reported (Li *et al.* 2017; Schauf *et al.* 2018; Martinez-Lopez *et al.* 2021). However, it is probably not possible to transfer the human enterotype clustering approach directly to dogs, since the dog gut microbiota is often dominated by the phylum Firmicutes, with genera such as *Clostridium*, *Turicibacter* and *Blautia*, and also often includes the genus *Fusobacterium* to a considerable extent (Mori *et al.* 2019; Pilla & Suchodolski 2019; Söder *et al.* 2022).

### 2.3.2 Local functions of the microbiota and SCFA

The gut microbiota provides several functions that are beneficial to the host. Some produce substances that are antimicrobial or block pathogens from interacting with the hosts cells (Moal & Servin 2006), while others interact with the immune system in the gut, thereby influencing its development and function (Tizard & Jones 2018). Other important functions include production of vitamin K (Conly & Stein 1992) and modification of bile acids

(Collins *et al.* 2023). A critical function of the gut bacteria is production of SCFA as a result of microbial metabolism. The major SCFA produced during microbial fermentation of (mainly) dietary fiber are acetate, propionate and butyrate. As SCFA are acids, their production keeps the pH in the colon on a level that favours beneficial commensals (Pinna & Biagi 2014). Butyrate is the preferred energy source for colonocytes and is thus mainly utilised in the gut epithelium, where it is reported to play an important role in intestinal barrier function and to have local anti-inflammatory effects (Knudsen *et al.* 2018). Furthermore, an anaerobic environment is created during  $\beta$ -oxidation of butyrate, ensuring a beneficial environment for commensals, which in the colon are mostly anaerobic (Byndloss *et al.* 2017).

### 2.3.3 Short chain fatty acids systemically and as signal molecules

Acetate absorbed into the blood can be converted to acetyl-CoA and be oxidised for energy or stored by lipogenesis (Frayn 2010). Propionate can function as a substrate for gluconeogenesis in the liver (Koh *et al.* 2016), but has also been observed to reduce hepatic lipogenesis and cholesterol synthesis in mice (Berggren *et al.* 1996). Much of the interest in SCFA is because of observed metabolic aspects, *e.g.* SCFA are reported to have beneficial effects on glucose homeostasis and body weight in humans and rats (Koh *et al.* 2016). These effects are thought to be mediated by free fatty acid receptors (FFAR) 2 and 3, both on L-cells and on adipocytes peripherally (Robertson 2007; Koh *et al.* 2016). Upon SCFA binding to the receptor on L-cells, GLP-1 and PYY are proposed to be released to the blood and act to decrease energy intake and potentiate insulin release (Koh *et al.* 2016). However, contradictory results have been reported in a study on pigs, where the SCFA concentration in portal blood was high and the GLP-1 concentration was low following a diet with oat  $\beta$ -glucan (Hooda *et al.* 2010). In dogs fed diets containing a mix of highly fermentable fiber, GLP-1 concentrations have been reported to increase in plasma (Massimino *et al.* 1998) or to remain unchanged (Bosch *et al.* 2009b), even though, in that study, a higher SCFA concentration was observed following a high fermentable diet compared with a low fermentable diet. Bosch *et al.* (2009b) further did not observe any changes in plasma PYY concentration. Ferreira *et al.* (2018) studied metabolic response in dogs fed a diet supplemented with oat  $\beta$ -glucan and did also not find any differences in plasma PYY, however, they did not measure SCFA. Thus, it is not clear whether SCFA binding to

FFAR on L-cells leads to secretion of GLP-1 and PYY, or if SCFA have more of a modulatory effect. Interaction of SCFA with peripheral FFAR2 and 3 on adipocytes is suggested to increase insulin sensitivity by decreasing non-esterified fatty acid (NEFA) release and increasing adipogenesis (Robertson 2007). However, results from studies examining the metabolic effects of different SCFA administered as a supplement are not conclusive (Cherta-Murillo *et al.* 2022).

## 2.4 Dietary fiber

The definition of dietary fiber has been a topic of discussion among scientists and interested parties for many years. In 2009, the Codex Alimentarius Commission published its definition, which has since been adopted by the Food and Agriculture Organisation of the United Nations and the World Health Organisation (Jones 2014). The definition essentially states that dietary fiber means carbohydrate polymers with 10 or more monomeric units that are not hydrolysed by endogenous enzymes in the small intestine of humans. Two footnotes followed the initial definition, one leaving room for national authorities to include compounds with 3-9 monomeric units and one including lignin and other compounds when associated with polysaccharides in plant cell walls (Jones 2014). On including both these footnotes, the Codex Alimentarius definition aligns closely with that of the European Food Safety Authority (Jones 2014).

Dietary fibers are often classified into different groups based on their physicochemical properties. One way to group fiber types is by their degree of solubility in water. Another is by their ease of fermentation by bacteria in the colon. These classifications are not always straightforward since there are no clear borders between them, and since the source of the fiber and possible processing can influence both solubility and fermentability (Lovegrove *et al.* 2017). Overall, however, these properties determine the role that fiber will play in the intestine and in the health of the consumer. Soluble fiber can have more or less viscous properties, meaning that it forms a gel when mixed with water. In humans this property, rather than solubility, is believed to lower cholesterol and decrease glucose response after a meal due to the trapping of nutrients, which delays enzymatic digestion and subsequent absorption (McRorie & McKeown 2017). Fermentable fiber acts as an energy substrate for gut bacteria, which during the fermentation process

produce health-promoting SCFA. Since this fiber type can affect the health of the host through the microbiota, it can be used as a supplement or can be added to food and is then termed “prebiotic”. Non-fermentable fiber passes relatively unchanged through the GI tract and thus increases bulk and can have beneficial laxative effects (McRorie & McKeown 2017).

#### 2.4.1 Dietary fiber in dog food

The energy contribution of dietary fiber to dog food is rather small and varies with the fermentability of the fiber, non-fermentable fiber contributing very little energy (NRC 2006). Thus insoluble, non-fermentable fiber, often cellulose, is commonly added in commercial dog food in order to dilute the energy content in weight management diets. Fermentable fiber, on the other hand, is used for its prebiotic potential. A commonly used fiber source is beet pulp, which is moderately fermentable because it contains a mix of viscous, soluble and insoluble, non-fermentable fiber (de Godoy *et al.* 2013). However, in recent years it has become more common to add purified fiber as a prebiotic. Inulin is a popular prebiotic, as are oligosaccharides with a low degree of polymerization, such as fructooligosaccharides and galactooligosaccharides (Pinna & Biagi 2014).

## 2.5 Whole grain cereals

The concept of whole grain is generally considered to entail the naked seed of the grain with all anatomical components (endosperm, germ and bran) included in the same relative proportions as they exist in the intact grain (Frølich *et al.* 2013). The most commonly grown cereal crops in the Nordic countries are wheat, rye, oats and barley (barley is not considered further in this thesis). Oats differ from rye and wheat in that the grain has an outer shell or hull which does not come off during threshing in the field and has to be removed at milling. Following dehulling, the oat groat is naked and considered a whole grain.

#### 2.5.1 Fiber characteristics of wheat, oat and rye

Rye generally has a higher total dietary fiber (TDF) content than oats and wheat (Frølich *et al.* 2013; Bach Knudsen *et al.* 2017). Arabinoxylan is the dominant fiber type in both rye and wheat, constituting around 9%, respectively 6-7%, of the grain DM. The ratio of soluble to insoluble



arabinoxylans is generally higher in rye compared with wheat (Knudsen 2014). Oats differ from rye and wheat by having a rather low arabinoxylan content of about 2%, but relatively high content of  $\beta$ -glucan, constituting around 4-5% of grain DM (Frølich *et al.* 2013; Bach Knudsen *et al.* 2017). Rye contains moderate amounts of  $\beta$ -glucan (1.5-2% of DM) and, unlike both oats and wheat, moderate amounts of fructan (3-4% of DM) (Frølich *et al.* 2013; Bach Knudsen *et al.* 2017).

Arabinoxylan consists of a xylose backbone with arabinose side chains to varying degrees (Siurek *et al.* 2012). Some of the arabinose molecules carry a ferulic acid, which has antioxidant properties. Fructan is made up of fructose molecules with a fairly low degree of polymerisation, which makes it highly soluble and fermentable (Frølich *et al.* 2013). The fiber  $\beta$ -glucan is composed of chains of glucose molecules linked together with  $\beta$ -bonds. The fiber is soluble, generally has high molecular weight and is therefore highly viscous (Knudsen 2014).

Whole grain cereals further contain varying amounts of other bioactive compounds, which are believed to contribute to the observed health effects (Siurek *et al.* 2012; Bach Knudsen *et al.* 2017). Rye has the highest content of phytosterols and alkylresorcinols of the three grains, and together with wheat the highest content of tocopherols (E-vitamin) and phenolic acids (Siurek *et al.* 2012; Frølich *et al.* 2013). Oats, on the other hand, contain the antioxidant avenanthramide, which the other grains do not (Frølich *et al.* 2013).

### 2.5.2 Metabolic health effects

Studies of health effects of rye inclusion in dog food are lacking, but multiple studies on humans show that rye can have beneficial effects on glucose metabolism compared with refined control ingredients (Leinonen *et al.* 1999; Juntunen *et al.* 2003; Nilsson *et al.* 2008a; Rosen *et al.* 2009; Sandberg *et al.* 2016). Amount of fiber alone does not seem to be the only explanation for the beneficial effects, since rye products differing in fiber content have been found to have similar lowering effects on insulin (Juntunen *et al.* 2003) and since including rye in pig diets has been shown to have greater insulin-lowering effects than whole grain wheat (Theil *et al.* 2011). Presence of bioactive compounds in rye might be one factor contributing to the beneficial effects on glucose metabolism, since higher amounts of phenolic acids in rye have been observed to be related to lower glucose and insulin response (Rosen *et al.* 2011). Rye has further been observed to induce increased

feelings of satiety in humans compared to refined products (Rosen *et al.* 2011; Isaksson *et al.* 2012).

In dogs, one study compared postprandial glucose and insulin response following diets with whole wheat or oat groats and found a lower insulin response following the wheat diet (Kempe *et al.* 2004). Results from human and pig studies on whole grain wheat are conflicting when it is compared with whole grain rye, with some studies reporting equally beneficial effects of wheat and rye (McIntosh *et al.* 2003; Rosen *et al.* 2011), while others slightly less or no beneficial effects of whole wheat (Nilsson *et al.* 2008a; Theil *et al.* 2011).

Oat and purified  $\beta$ -glucan have been studied in dogs, with some results suggesting beneficial effects on plasma cholesterol, but no significant glucose- or insulin-lowering effects (Kempe *et al.* 2004; Ferreira *et al.* 2018; Traugher *et al.* 2021). In humans and pigs, on the other hand, postprandial studies of oat  $\beta$ -glucan, in various forms, have reported an attenuated blood response of glucose and insulin and lowered cholesterol compared with control (Björklund *et al.* 2005; Hooda *et al.* 2010; Hartvigsen *et al.* 2014). Oat has further been reported to increase satiety in humans (Rebello *et al.* 2016).

### 2.5.3 Changes in microbiota and short-chain fatty acids

Studies investigating the effects of rye on microbiota in humans have shown varying results, some with minor changes and some with no changes (Vuholm *et al.* 2017; Eriksen *et al.* 2020; Iversen *et al.* 2022). Prykhodko *et al.* (2018) reported a higher abundance of genus *Prevotella* and reduction in *Bacteroides* following short-term rye consumption. Iversen *et al.* (2022) likewise observed an increase in *Prevotella* following rye for a longer period of time, but it did not reach statistical significance. Butyrate in fecal or plasma samples is frequently reported to increase following rye consumption in both humans and pigs (McIntosh *et al.* 2003; Knudsen *et al.* 2005; Sandberg *et al.* 2016; Eriksen *et al.* 2020; Iversen *et al.* 2022).

Studies in humans using diets with whole grain wheat have not shown significant effects on general microbiota composition compared with control or baseline (Vitaglione *et al.* 2015; Vuholm *et al.* 2017; Eriksen *et al.* 2020). One study reported some changes from baseline in specific taxa, among them an increase in *Prevotella* (Vitaglione *et al.* 2015). When compared with

whole grain rye, wheat has been reported to result in lower fecal butyrate concentration (McIntosh *et al.* 2003; Eriksen *et al.* 2020).

In dogs, a diet with oat groats, compared with a conventional diet, did not result in significant changes in general microbial composition, but some smaller changes in specific taxa was observed, for example an increase of *Prevotella*, and of the family *Lachnospiraceae* (Traughber *et al.* 2021). These changes were accompanied by an increase in butyrate, propionate and total SCFA. Hooda *et al.* (2010) in a study on pigs observed an increase in propionate and butyrate in portal blood following dietary supplementation of oat  $\beta$ -glucan. In humans, consumption of  $\beta$ -glucan oat bran bread increased acetate, propionate and butyrate compared with baseline (Nilsson *et al.* 2008b).



### 3. Aim and objectives

Since the dog is more of an omnivore than carnivore, often sharing the owner's lifestyle and fed a diet with refined grains, the effects of including different whole grains in the diet of dogs need to be investigated, given the beneficial effects of whole grains in human studies. The overall aim of this thesis was thus to explore and compare effects of including whole-grain cereals in dog food on canine metabolism, gut microbial composition and fermentative end-products. Wheat, oats and rye were chosen as the dietary cereals, as they are three of the most commonly grown crops in the Nordic countries.

Specific objectives of the work reported in Papers I-III were to:

- Evaluate a suitable level of inclusion of whole grain rye in dog food in terms of digestibility and acceptance by the dog (Paper I)
- Investigate effects on fecal microbiota and SCFA production of whole grain rye, compared with refined wheat, in dog food (Paper I)
- Compare effects of extruded diets with different whole grains (rye, oat and wheat) on gut microbiota composition and diversity and production of SCFA (Paper II)
- Investigate and compare metabolic and hormonal responses in dogs fed extruded diets with whole grains from rye, oat or wheat (Paper III)



## 4. Comments on material and methods

Detailed information on the materials and methods used in Papers I-III can be found in the individual papers. This chapter briefly describes and comments on the two cohorts of participating dogs, experiment designs and methods used for sampling and statistical analysis.

Examinations of the dogs and analyses were performed at the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. The studies were approved by the Ethics Committee for Animal Experiments, Uppsala, Sweden (Approval no. 5.8.18-18808/2017-7). Written informed consent was obtained from all dog owners before the start of the study (Paper II and III). Information on the owners was handled in accordance with general European Union (EU) data protection regulations (Regulation (EU) 2016/679).

### 4.1 Study populations

In Paper I, purpose-bred Beagle dogs owned by SLU were used. These dogs are primarily kept for educational purposes in the veterinary and veterinary nursing programmes, and thus were available for research to some extent. Six animals were available for the study in Paper I. The participating dogs were assessed as healthy based on physical examination and routine haematological and biochemistry blood analyses. None had been treated with antimicrobial drugs within the preceding six months and all had been dewormed in accordance with their regular routine. The dogs were all males aged 1 to 7 years (mean 4.6 years, standard deviation (SD)  $\pm 2.3$  year). Mean body weight (BW) at the start of the study was 14.6 kg (SD  $\pm 0.78$  kg) and mean body condition score (BCS) on a 9-point scale (Laflamme 1997) was 5.3 (SD  $\pm 0.52$ ). The dogs were housed according to their regular living conditions in two separate groups, of two and four individuals, respectively.

At night they were kept in indoor pens and in daytime they were housed in outdoor pens on gravel.

For the second study (Papers II and III), a larger and more diverse group of dogs was needed, so that the results were more applicable to the general dog population. Thus a decision was made to recruit privately-owned dogs. To increase the probability of a high level of compliance, these dogs were recruited among staff and students at SLU and the University Animal Hospital in Uppsala, Sweden. Inclusion criteria were minimum age 12 months, minimum body weight (BW) 7 kg. Furthermore, they had to be assessed as healthy based on physical examination, routine haematology, serum biochemical analyses and urine analysis. Criteria for exclusion were treatment with antibiotics within three months before the start of the study, intolerance or allergy to any of the ingredients used in the experimental diets or sensitivity to diet change. Clear indications of systemic or organ-related disease also led to exclusion, as did any GI reaction to the diets that affected the dog's general condition. Initially, 22 dogs were recruited and all were deemed healthy, but four of these dogs were excluded during the first acclimatization period or early in the first experimental diet period, due to problems with loose stools, signs of possible cutaneous adverse food reaction or issues with palatability. Thus 18 dogs were included. These comprised 13 purebred dogs (two of the same breed) and 5 dogs of mixed breeds. Mean age was 5.7 (SD  $\pm$  2.6) years, mean BCS on the 9-point scale (Laflamme 1997) was 5.2 (SD  $\pm$  0.6) and mean BW was 18.4 (SD  $\pm$  9.5) kg.

## 4.2 Experimental diets and feeding

The diets used in Paper I were formulated and produced at SLU. The aim in formulation was to create diets that were as similar as possible in terms of energy and protein content (Table 1). Metabolizable energy (ME) content of ingredients was estimated using modified Atwater factors (NRC 2006) prior to formulation, and portion size was then calculated based on the resulting estimated ME of the diet. Three diets were compared in Paper I: a wheat diet (W) containing (DM basis) 50% refined wheat flour; a rye and wheat diet (RW) comprising a mixture of 25% coarsely ground whole grain rye meal and 25% refined wheat flour; and a rye diet (R) containing 50% coarsely ground rye meal. Titanium dioxide (TiO<sub>2</sub>) marker was added in an amount of 2 g/kg food for measurement of apparent total tract digestibility (ATTD).



Flours and marker were mixed with meat, water and rapeseed oil and baked to a core temperature of 90-100 °C in a steam oven, in order to enable gelatinization of starch granules. Fish oil, vitamin premix and minerals were added to the baked food after cooling, thus ensuring preservation of heat-labile nutrients, together with water to compensate for losses during baking. The final dog food was stored at -20 °C.

The ideal body weight of each dog (n=6) was estimated before the first diet period (Paper I), based on BCS (Laflamme 1997). Daily energy requirement was then calculated for the ideal BW, using the recommendation from the National Research Council (NRC 2006) for the average laboratory kennel dog, *i.e.* 543 kJ ME/kg metabolic BW ( $BW^{0.75}$  kg). The daily food allowance was divided into two meals per day. The dogs were moved to individual indoor pens at feeding and allowed to eat for 10-15 minutes. Water was available *ad libitum* during the entire study period. Since the digestibility of rye in dogs was not known, an ethical exclusion limit of maximum 5% weight loss per week was set, or alternatively 10% weight loss during the complete diet period or a two-point decrease in BCS over two weeks. The palatability of rye was also unknown, so a limit of three days of complete food refusal was set.

Experimental diets used in Papers II and III were formulated and balanced with help from, and produced by, Doggy AB, Vårgårda, Sweden (Table 1). As in Paper I, the diets were formulated to be similar in protein and ME content. Three diets were compared, with 25% inclusion of whole-grain flour of rye (RYE), wheat (WHE) or steam-rolled oats (OAT). All ingredients were ground in a hammer mill before mixing and extrusion.

The initial daily feed allowance in Papers II and III was calculated based on the amount of calories in each individual's normal feed intake, as reported by the owners. Daily caloric intake was then adjusted throughout the experiment to maintain original BW. The owners were instructed to keep to the dog's normal feeding routines and to feed the experimental diet as the main source of energy. Treats were allowed to increase compliance, but the instruction was to keep to approximately the same amount during each diet period and to give nothing but the experimental diet during the last three days before sampling in each diet period.

Table 1. Chemical composition (% of dry matter (DM)) of the experimental diets compared in Papers I-III

Item	Paper I				Paper II & III		
	W diet	RW diet	R diet	WHE diet	OAT diet	RYE diet	
Dry matter	49.9	51.4	49.6	93.9	93.5	93.9	
Gross energy (MJ/kg DM)	24.7	24.6	24.5	20.9	21.7	21.5	
Metabolizable energy (MJ/kg DM)	21.3	21.2	21.0	17.2	18.0	17.8	
Organic matter	95.9	95.4	95.0	92.9	93.1	92.7	
Crude protein	22.5	20.8	20.5	30.5	30.4	29.5	
Nitrogen-free extract	43.3	41.4	42.2	46.8	43.6	44.7	
Crude fat	29.5	32.3	31.1	13.7	17.6	16.7	
Crude fiber	0.58	0.89	1.24	1.9	1.5	1.8	
Neutral detergent fiber	2.9	6.3	6.6				
Total dietary fiber				8.6	7.6	9.1	
Soluble dietary fiber				1.4	2.0	1.7	
Insoluble dietary fiber				7.2	5.6	7.4	
Total starch				38.3	34.2	34.8	
Resistant starch				0.2	0.2	0.3	
Non-resistant starch				38.1	34.0	34.5	

Nitrogen-free extract (calculated). Metabolizable energy calculated in accordance with NRC (2006).

W = 50% refined wheat; RW = 25% refined wheat, 25% whole rye; R = 50% whole rye.

WHE = 25% whole wheat; OAT = 25% rolled oats; RYE = 25% whole rye.

### 4.3 Study design

In all papers, the intention was to use a cross-over design in which all dogs received all diets. This would allow each dog to act as its own control, eliminating the need for a control group and reducing the impact of inter-individual variation, meaning that the number of dogs used in the experiment could be reduced. This would partly compensate for the limited number of dogs in Paper I and the less controlled environment for the dogs in Papers II and III. However, the dogs in Paper I lived together in two groups and, to avoid the risk of coprophagia causing interference between diets, it was decided to let all dogs eat the three test diets in the same order. This meant that a cross-over design could not be used and there might be a risk of carry-over effects. Thus the diets were fed with a washout period of 2-3 months, during which the dogs were fed their standard maintenance diet (Figure 1). To minimize the possible influence of seasonal effects which this could entail, the gut microbiota was evaluated by comparing samples collected before and after each diet period. A six-day acclimatization period in which the dogs' standard food was mixed with increasing amounts of the experimental diet was applied before each diet period. The dogs were then fed the experimental diet at 100% for 15 days.

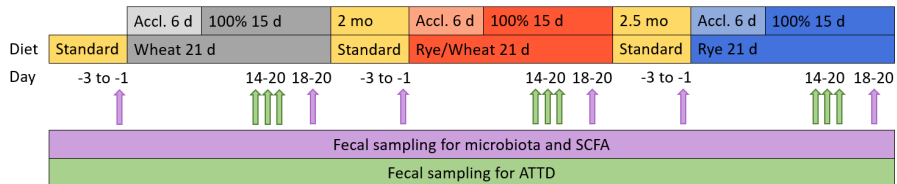


Figure 1. Flowchart of the study design in Paper I. SCFA = short-chain fatty acids. ATTD = apparent total tract digestibility. Accl = acclimatization period. (From Paper I).

In Papers II and III, dogs were categorised by gender and size, anonymised and then randomly divided into three groups (1-3) that were as diverse as possible, which were allocated to different diet orders. A cross-over design in the form of a reduced 3x3 Latin square was used, with the diet order WHE-OAT-RYE, OAT-RYE-WHE and RYE-WHE-OAT for dog group 1, 2 and 3, respectively. To ensure a high level of compliance for pairs of dogs living in the same household, they were kept together and followed the same diet order. At the end of each diet period, dog owners were asked

to complete a form with closed and open questions (Appendix) regarding compliance and the dog's digestive function and appetite behaviour. Each experimental diet in Papers II and III was fed for a minimum of four weeks before sampling. Before starting the first experimental diet period, all dogs were first fed WHE for three weeks, including a 4-7 day transition period, in order to make the starting conditions as similar as possible.

## 4.4 Sampling

### 4.4.1 Fecal sampling (Papers I and II)

In Paper I, fecal samples for analysis of microbiota composition and SCFA concentration were collected before the start of each diet period to obtain a baseline value, with which a sample collected at the end of the study was compared (Figure 1). For ATTD determination, samples were collected once a day for three days in the middle of each diet period for all dogs except two, from which (in one period each) only two samples could be collected. A fecal scoring system for dogs (Royal Canin SAS 2013, [www.royalcanin.ca](http://www.royalcanin.ca)) with a scale from 1 (very loose) to 5 (dry and hard) was used to assess fecal consistency.

In Paper II, fecal samples for microbiota and SCFA analyses were collected by the owner once, during one of the two last days of each experimental period. These samples were placed in a freezer as soon as possible and kept at -20 °C until analysis.

Studies investigating effects on microbiota often use fecal samples, which are easy to collect and cause no discomfort to the study object, but this means that events taking place more proximally in the intestine might be missed (Suchodolski *et al.* 2008). Furthermore, it is possible that the bacteria of greatest importance to the animal are located closer to the intestinal wall, hidden in the mucus, and that bacteria present in the digesta do not necessarily provide a representative picture of the microbiota (Ringel *et al.* 2015). However, the primary interest in this thesis was the microbiota involved in the fermentation process and its end-products. Given that most fermentation takes place in the luminal contents of the colon, which is short in dogs compared with in pigs and humans, fecal samples from dogs should give a fairly representative picture of the microbiota (Honneffer *et al.* 2017). However, due to uptake of SCFA in the caecum and proximal colon, some

information regarding production of SCFA might be lost. This needs to be considered at the interpretation of the results.

#### 4.4.2 Blood sampling (Paper III)

Following an overnight fast of minimum 12 hours, dogs were brought to the clinic, where a peripheral vein catheter was placed in the cephalic vein and a fasting blood sample was collected. Each dog was placed in a separate area together with its owner, to reduce stress which could affect blood results. The dog was then offered half of its daily caloric allowance of the current experimental diet. The postprandial period started when the dog started to eat and it was allowed 10 minutes to consume the food. Blood samples were collected at 20, 40, 60, 120, 180 and 240 min postprandially, in serum tubes and K2 EDTA plasma tubes.

### 4.5 Analyses

#### 4.5.1 Microbiota (Papers I and II)

Microbial DNA was extracted from samples and PCR amplicons were generated from the hypervariable V3-V4 region of the 16S rRNA gene. In a second PCR run, each sample was barcoded individually. In Paper I, amplicons were sequenced on the MiSeq Illumina platform at the National Genomics Infrastructure (NGI), SciLifeLab (Solna, Sweden). In Paper II, amplicons were generated and sequenced on the MiSeq Illumina platform at Novogene (Beijing, China). The sequences obtained were quality-filtered, clustered into operational taxonomic units (OTU) and compared against the SILVA database for species annotation (Quast *et al.* 2013).

#### 4.5.2 Short-chain fatty acids (Papers I and II)

Acetate, propionate and butyrate were analyzed by high-performance liquid chromatography (HPLC). Unfortunately, the mode of housing in Paper I led to some dogs consuming gravel and sand from the outdoor pen, as later discovered in the fecal samples. Thus, in Paper I concentrations were reported as a proportion of the sum of all three SCFA, since the varying proportion of gravel and sand prevented absolute determination.

#### 4.5.3 Metabolic and hormonal parameters (Paper III)

A canine enzyme-linked immunosorbent assay (ELISA) kit (Canine Insulin ELISA, Mercodia, Uppsala, Sweden) was used for analysis of plasma insulin concentration and a human ELISA kit (Glucagon ELISA (25µL), Mercodia, Uppsala, Sweden), previously validated for dogs (Söder *et al.* 2016), was used for analysis of serum glucagon concentration. Plasma glucose concentration was analysed with a kit based on an enzymatic UV-method (D-glucose, Boehringer Mannheim, Germany) previously used in dogs (Hesta *et al.* 2001). Serum concentrations of total cholesterol and TAG were analysed by routine automatic methods using an enzymatic colorimetric assay, at the Department of Clinical Chemistry, Uppsala University Hospital.

For analysis of plasma GLP-1, it was decided to use a human ELISA kit (Total GLP-1 NL-ELISA, Mercodia, Uppsala, Sweden) that had not previously been validated for use in dogs. The active form of GLP-1 has a very short half-life in plasma, of about 1-2 minutes (Holst 2007) and this kit was chosen as it could detect total GLP-1, *i.e.* both the active form (6-36 amide) and the metabolite (9-36 amide), ensuring that possible differences in GLP-1 secretion due to diet effect could be detected. As the GLP-1 gene is highly conserved among all mammals, a human ELISA kit should be able to detect canine GLP-1 (Holst 2007). Furthermore, previous studies in dogs have successfully used kits to detect the amidated form of GLP-1, although only the active form (Massimino *et al.* 1998; Bosch *et al.* 2009b). Verification of assay performance for dog plasma was performed in Paper III. To detect the active form, use of a dipeptidyl peptidase (DPP)-IV inhibitor is recommended, but when measuring total GLP-1 this should not be necessary. However, to confirm this, blood was collected in EDTA tubes with or without the DPP-IV inhibitor and a pilot study analysing total GLP-1 in the same sample with and without the DPP-IV inhibitor was performed on three samples (with 7 replicates each). On average, the samples without inhibitor had 0.18 pmol/L (SD ± 0.13) higher GLP-1 concentration than the samples with inhibitor. The concentration range in these samples was 1.49-6.54 pmol/L, which was representative of the range observed for most of the samples in Paper III.

As the samples were stored in a freezer for a longer period than originally planned, a pilot study was also performed on additional samples from two dogs participating in an unrelated study, which were analysed within a few days of sampling. The GLP-1 concentration in these fresh samples did not

deviate from that in the study samples, making it reasonable to assume that the concentration in the study samples did not decrease notably during storage. Storage time should therefore not have had any substantial impact on the results in Paper III.

#### 4.5.4 Statistical analysis

In Papers I and II, effects of the diets on overall microbial composition was investigated by principal coordinate analysis (PCoA) based on Bray Curtis dissimilarity index on relative abundance data from all OTUs, using the software PAST (Hammer *et al.* 2001).

To compare the effects of the different diets on specific microbiota, SCFA and blood variables in Papers I, II and III, analysis of variance (ANOVA) was employed as the statistical test, using R software (R Core Team 2022). Linear mixed effects models were used for all univariate analyses using *lme4* package (Bates *et al.* 2015) in Paper I and *nlme* package (Pinheiro *et al.* 2022) in Papers II and III.

In Paper I, a limit for OTUs tested through ANOVA was set arbitrarily at minimum mean general abundance of 1%, in order to limit the number of tests performed but still include the most abundant OTUs in all dogs. The study design in Paper I entailed that the interaction effect of diet and time was the most interesting outcome of the statistical analysis. However, as it was not possible to control for seasonal effects through the study design, comparison of baseline abundances was performed to minimize possible confounding effects of environmental conditions.

In Paper II, in which a more explorative approach was used, the limit was set at 0.1% relative abundance for OTUs and genera analysed with ANOVA test, yet with an inclusion criteria of a maximum 25% of samples with zero counts. Due to the resulting increased number of ANOVA tests run, correction was made for this by using false discovery rate (FDR) adjustment (Benjamini & Hochberg 1995) and reporting both *p*- and *q*-values. Values were considered significant at  $p \leq 0.05$  and  $q \leq 0.1$ .

#### *Additional statistical tests performed specifically for this thesis*

An analysis of similarities (ANOSIM) was performed on the OTU data from Paper II with regard to the three dog groups. The analysis was performed in the statistical software PAST (Hammer *et al.* 2001), using Bray-Curtis dissimilarity index on all fecal samples from all three diet periods.

Bonferroni correction was used for *post hoc* comparisons. This analysis was performed in order to assess whether there was in general any statistically significant difference in microbiota composition between the dog groups with different diet orders. This analysis had not been performed in paper II, because results did not suggest dependency of diet. Furthermore, this was not part of the study aim for paper II. As complementary information for this thesis essay, this difference and its possible effects on blood parameters were investigated.

Spearman rank correlation tests were performed in PAST to explore correlations between abundance of the genera *Prevotella\_9* and *Bacteroides*, concentrations of the three SCFAs, and fasting value and total area under the curve (AUC) for the different blood parameters. The comparisons were Bonferroni-corrected, but for explorative purposes uncorrected comparisons were also made. To investigate the correlation between *Prevotella\_9* and *Bacteroides*, least square regression analysis was performed in PAST.



## 5. Main results

### 5.1 General outcomes of feeding diets with rye to dogs

The dogs in Paper I generally showed equally good acceptance of the RW and W diets. The ATTD of protein, fat and gross energy (GE) was also high for both diets, although for GE slightly lower with the RW diet (emmeans  $\pm$  SE: diet RW:  $90.5 \pm 0.66\%$ , diet W:  $93.6 \pm 0.66\%$ ;  $p < 0.05$ ). Some refusals were left by some dogs, primarily during the R diet period, and one dog lost more weight than the set limit of 5% in one week during the R diet period and was hence excluded from that period. Based on the results obtained in Paper I, it was decided to use the lower level of rye inclusion (25%) in the diets in Paper II. The owners of the dogs in Paper II did not report any significant problems with refusals, or any significant problems with loose stools or other GI issues in their dogs, for any of the diets.

### 5.2 Gut microbiota (Papers I and II)

In Paper I, consumption of the diet with 50% inclusion of rye resulted in a significant difference in overall microbial composition in fecal samples, compared with samples collected before the diet period (ANOSIM;  $p = 0.014$ ,  $R = 0.67$ ) (Figure 2). According to a similarity percentage (SIMPER) test, the bacterial taxa contributing most to the observed difference were *Prevotella\_9*, *Catenibacterium*, *Bacteroides*, *Romboutsia* and *Megamonas*. There were no differences when comparing baseline values with values in samples collected after the RW diet, or after the W diet. *Prevotella\_9* was the most abundant genus following both the R and RW diets (mean 54% and 36% of sequences, respectively). *Prevotella\_9* abundance increased

numerically after all diets, but the difference was only significant ( $p < 0.001$ ) following the R diet. *Prevotella\_9* abundance was also higher after the R diet compared with after the W diet ( $p < 0.007$ ). Genus *Romboutsia* and an unclassified member of the family Peptostreptococcaceae both decreased significantly in abundance after the R diet period ( $p < 0.02$  for both). Even though the change in *Bacteroides* had large impact on the ANOSIM test, no differences were detected in the ANOVA analysis. Figure 3A shows the abundances of *Prevotella\_9* and *Bacteroides* before and after each diet. The ratios of relative abundance of *Prevotella\_9*/*Bacteroides* and Firmicutes/Bacteroidetes were examined, but no interaction effects were found.

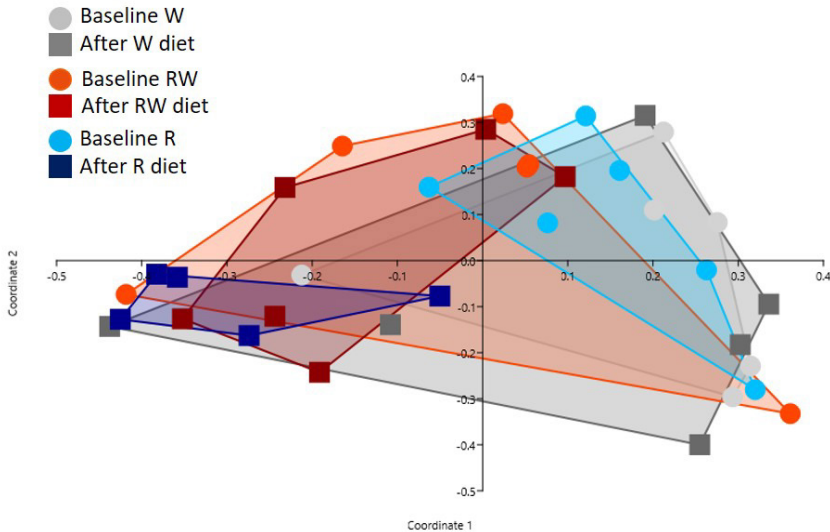


Figure 2. Principal coordinate analysis (PCoA) plot of fecal microbial composition before (baseline, dots) and after each experimental diet (filled squares) in Paper I. Diet periods are represented by different colors: grey = wheat diet (W); red = rye+wheat diet (RW); blue = rye diet (R). (From Paper I).

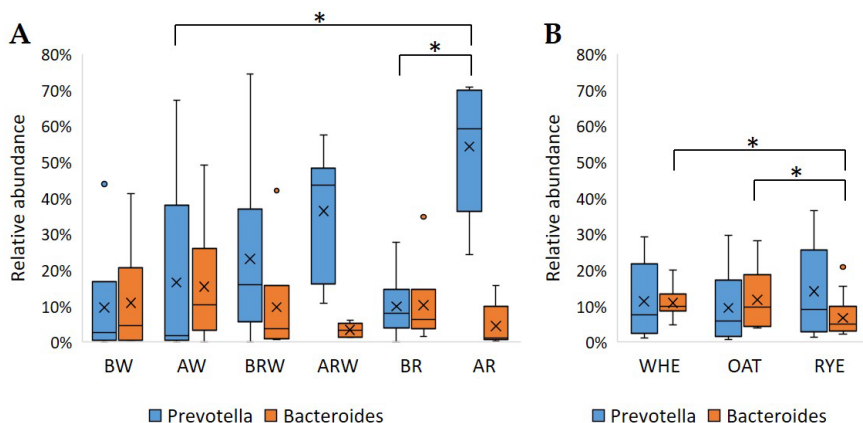


Figure 3. Relative abundance of the genera *Prevotella\_9* and *Bacteroides* in dog fecal samples collected in (A) Paper I (BW = baseline wheat, AW = after wheat, BRW = baseline rye+wheat, ARW = after rye+wheat, BR = baseline rye, AR = after rye) and (B) Paper II (WHE = wheat, OAT = oat, RYE = rye). Each box indicates the 1<sup>st</sup> and 3<sup>rd</sup> quartile, line within box marks the median and X marks the mean. Whiskers show samples within the 1.5 interquartile range and dots outside whiskers are outliers. \*indicates significant difference ( $p < 0.05$ ).

In general, fecal microbial composition did not differ following consumption of diets containing the three different whole grains in Paper II (Figure 4a). However, there was a pattern in the PCoA analysis indicating a difference between the three groups of dogs (Figure 4b). ANOSIM tests performed for this thesis confirmed a difference between the groups ( $p=0.0001$ ,  $R=0.17$ ), with group 1 differing from groups 2 and 3 ( $p=0.0036$  and  $p=0.0003$ , respectively). Evenness and richness of microbial composition was higher in fecal samples from dogs fed diet WHE compared with RYE ( $p=0.011$  and  $p=0.012$  for Shannon diversity and PD whole tree, respectively). One OTU belonging to the family Lachnospiraceae, with lowest abundance following rye, was significant after FDR correction of ANOVA results. None of the genera was still significant after FDR correction. However, some interesting results from the uncorrected p-values are worth highlighting. Relative abundance of genus *Bacteroides* was lower in samples following the RYE diet compared with samples collected after WHE ( $p=0.004$ ) or OAT ( $p=0.014$ ) (Figure 3B). Genus *Prevotella\_9* was numerically most abundant in samples collected after RYE ( $p=0.098$ ). The initial difference between the three groups of dogs seen in multivariate analysis was also evident in univariate analysis ( $p=0.0033$ ), as genus *Prevotella\_9* was generally more abundant in group 1 compared with groups

2 and 3 ( $p=0.012$  and  $p=0.0048$ , respectively). Genus *Bacteroides* also tended to differ between the groups ( $p=0.066$ ). Moreover, there was a negative correlation between *Prevotella\_9* and *Bacteroides* ( $p=0.0006$ ) (Figure 5).

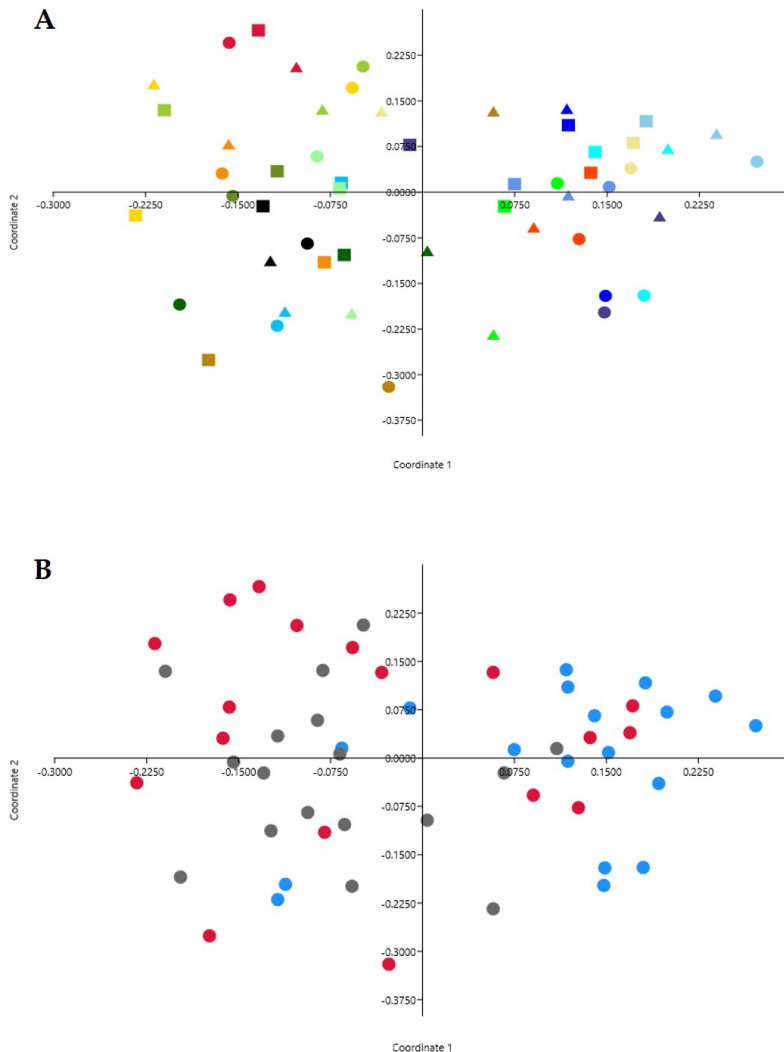


Figure 4. Principal coordinate analysis plots based on Bray Curtis distance in operational taxonomic unit data from Paper II. Each point represents one fecal sample from one dog following a diet period. Both plots represent the same data, but colored differently. In (A), different colors represent individual dogs. Square = wheat diet (WHE), triangle = oat diet (OAT), dot = rye diet (RYE). In (B), different colors represent groups 1-3 with different diet orders. Blue = 1, Red = 2, Grey = 3. (From Paper II).

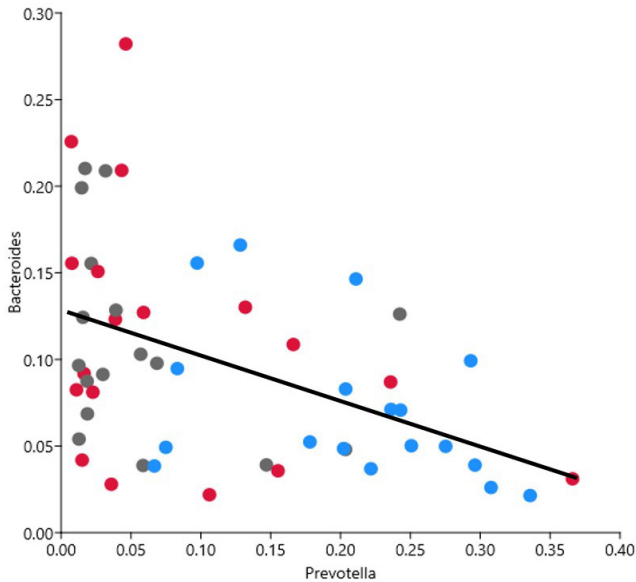


Figure 5. Correlation between genera *Prevotella* 9 and *Bacteroides* ( $R=-0.45$ ) in samples from dogs in groups 1-3 with different diet orders. Blue = 1, Red = 2, Grey = 3.

### 5.3 Short chain fatty acids (Papers I and II)

Comparisons of SFCA proportions in fecal samples following each diet in Paper I revealed a trend ( $p=0.057$ ) for an interaction effect of diet and time on the proportion of acetate, which was numerically around 8% higher in samples collected after R diet compared with before that diet (baseline) (Figure 6). No corresponding increase in SCFA proportions following the other two diets was observed.

An effect of rye on acetate proportion was also observed in Paper II. The acetate and propionate concentrations in fecal samples were higher after consumption of RYE compared with after OAT ( $p=0.044$  and  $p=0.018$ , respectively) (Figure 7). The relative proportions of the three SCFA did not differ between the diets.

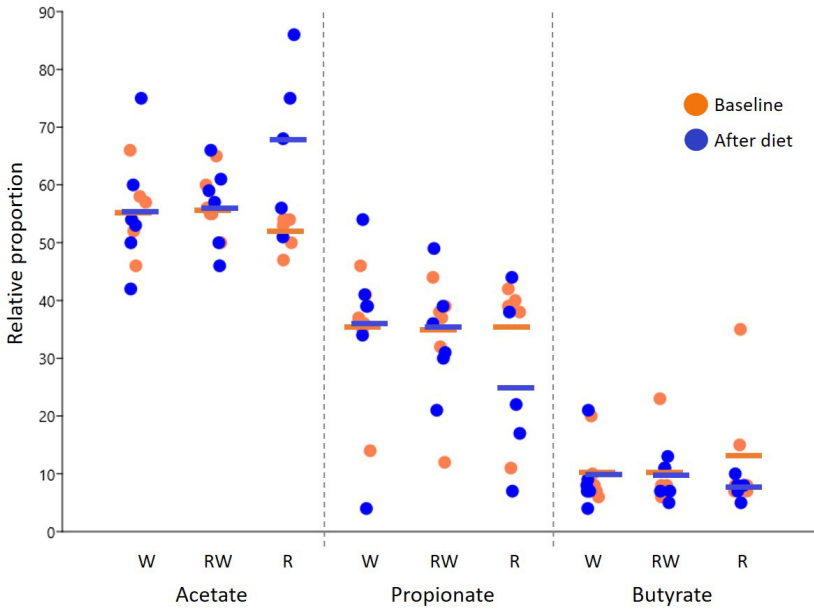


Figure 6. Jitter plot showing acetate, propionate and butyrate as proportions of total short-chain fatty acids (SCFA) in dog feces following consumption of different diets in Paper I (W = wheat, RW = rye+wheat, R = rye). Each dot represents a sample from an individual dog, lines mark the mean. There were no differences between the diets ( $p > 0.05$ ). (Modified from Paper I).

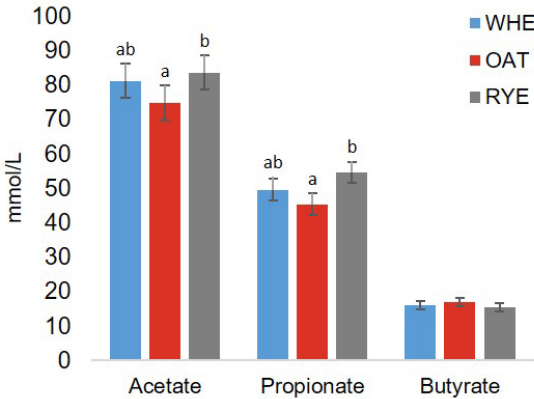


Figure 7. Concentrations (estimated marginal mean  $\pm$  SEM) of acetate, propionate and butyrate in dog feces following consumption of the three experimental diets (WHE = wheat, OAT = oats, RYE = rye) in Paper II. Different letters indicate significant differences in concentration between diets. (From Paper II).

## 5.4 Blood metabolites and hormones (Paper III)

There were no main effects of diet on the postprandial blood response curve for any of the blood variables evaluated. There was also no interaction of diet and time, *i.e.* the average concentration throughout the postprandial sampling time and the shape of the curves were both similar for all diets (Figure 8). However, on analysing the responses of variables in more detail, some interesting observations were made.

The postprandial plasma glucose concentration did not rise significantly from the fasting concentration when the dogs were fed the WHE diet, while for both the OAT and RYE diets the glucose concentration was elevated from 120 minutes after feed intake and did not return to the baseline value within the sampling time (Figure 8A). Total glucose AUC concentration was higher following the OAT diet than following the WHE diet ( $p=0.035$ ) (Table 2).

The fasting plasma insulin concentration was higher in samples collected when dogs were fed the RYE diet compared with the two other diets ( $p=0.0053$ ) (Table 2). However, no differences in insulin AUC concentration were found. The OAT diet resulted in greater GLP-1 AUC concentration than the WHE diet ( $p=0.0064$ ) (Figure 8C). In the early postprandial phase, diet affected the ratio of insulin to GLP-1 ( $p=0.042$ ) (Table 2), with the WHE diet resulting in numerically most insulin per GLP-1 of all three diets.

Diet did not have any significant effect on glucagon concentration (Figure 8D), but dog group and period both had some effect, where dog group 2 had higher glucagon AUC concentration in the early postprandial phase than group 3 ( $p=0.038$ ) and dogs had higher AUC concentration of glucagon during period 2 compared with period 3 ( $p=0.02$ ).

Total TAG AUC concentration (Table 2) was higher following the OAT diet compared with the WHE diet ( $p=0.025$ ). The fasting TAG concentration was affected by period ( $p=0.0007$ ), and was lowest in period 3 for all diets. There were no differences between the diets in terms of cholesterol concentrations (Figure 8F).

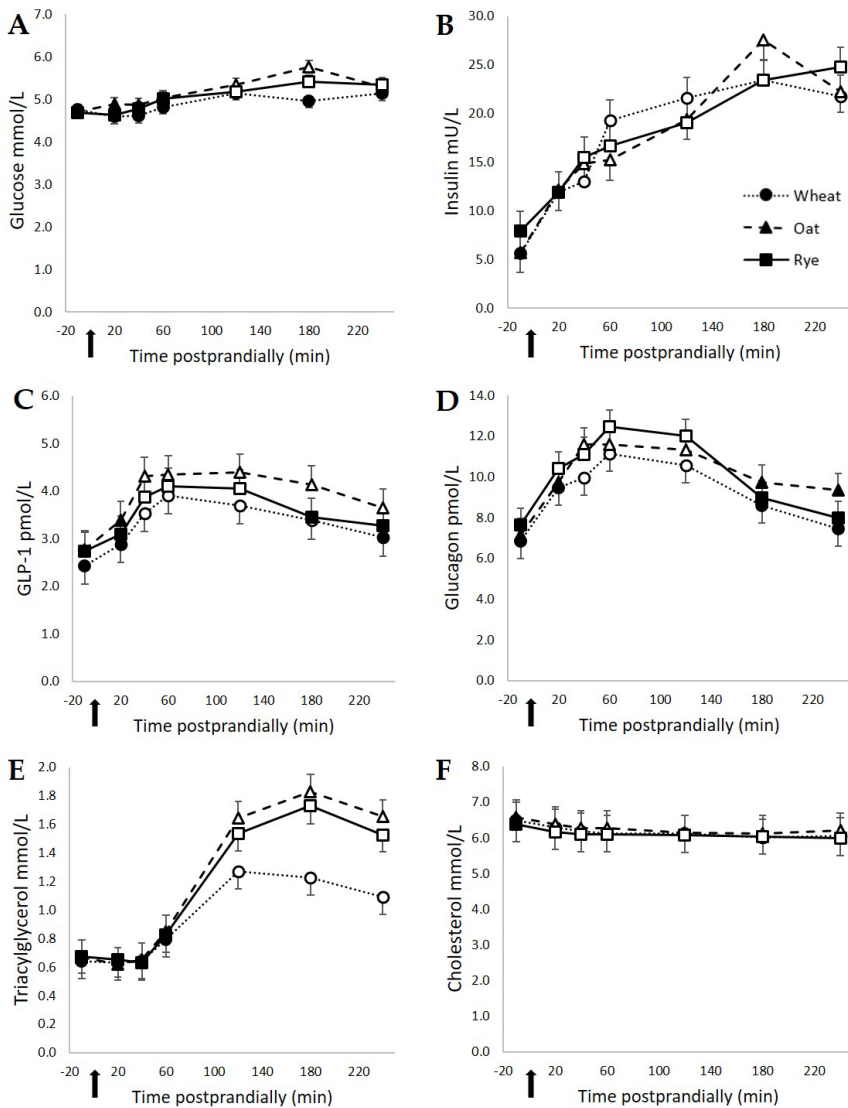


Figure 8. Fasting concentrations and postprandial blood response curves of (A) glucose, (B) insulin, (C) glucagon like peptide-1 (GLP-1), (D) glucagon, (E) triacylglycerol and (F) total cholesterol to experimental diets. Shapes and bar indicate estimated marginal mean, with standard error only shown in one direction. The arrow indicates time point 0 when dogs were fed each test diet. Non-filled shapes are significantly different from the fasting value. Note that for insulin time point 20, non-filled, significant shapes for the OAT and WHEAT diets are masked by the non-significant square of rye. (From Paper III.)



Table 2. Blood hormone and metabolite concentrations in dogs fed extruded diets containing whole grains of wheat, oats and rye after a 12 hour fast and for 4 hours postprandially as area under curve (AUC). Estimated marginal means  $\pm$  standard error (SE). *p*-values from natural logarithmical transformed values if needed. (Modified from Paper III).

	<b>Wheat <math>\pm</math> SE</b>	<b>Oat <math>\pm</math> SE</b>	<b>Rye <math>\pm</math> SE</b>	<b><i>p</i>-value</b>
<b>Fasting concentration</b>				
Glucose mmol/L	4.76 $\pm$ 0.13	4.71 $\pm$ 0.13	4.69 $\pm$ 0.13	0.86
Insulin mU/L	5.45 $\pm$ 0.59 <sup>a</sup>	5.88 $\pm$ 0.57 <sup>a</sup>	7.78 $\pm$ 0.57 <sup>b</sup>	0.005
GLP-1 pmol/L	2.45 $\pm$ 0.15	2.77 $\pm$ 0.15	2.71 $\pm$ 0.15	0.13
Triacylglycerol mmol/L	0.66 $\pm$ 0.038	0.68 $\pm$ 0.038	0.67 $\pm$ 0.038	0.97
<b>AUC total</b>				
Glucose mmol/L	1197 $\pm$ 33.1 <sup>a</sup>	1260 $\pm$ 32.4 <sup>b</sup>	1236 $\pm$ 31.9 <sup>ab</sup>	0.045
Insulin mU/L	4614 $\pm$ 380	4758 $\pm$ 369	4581 $\pm$ 361	0.85
GLP-1 pmol/L	821 $\pm$ 93.1 <sup>a</sup>	987 $\pm$ 92.4 <sup>b</sup>	919 $\pm$ 92.4 <sup>ab</sup>	0.009
Triacylglycerol mmol/L	260 $\pm$ 24.9 <sup>a</sup>	324 $\pm$ 24 <sup>b</sup>	305 $\pm$ 24 <sup>ab</sup>	0.025
Ratio insulin/glucose	3.86 $\pm$ 0.28	3.77 $\pm$ 0.28	3.66 $\pm$ 0.27	0.71
Ratio insulin/GLP-1	6.01 $\pm$ 0.56	5.24 $\pm$ 0.56	5.47 $\pm$ 0.55	0.22
<b>AUC 0-120</b>				
Glucose mmol/L	583 $\pm$ 14.1	601 $\pm$ 13.9	593 $\pm$ 14	0.21
Insulin mU/L	1816 $\pm$ 175	1748 $\pm$ 172	2145 $\pm$ 182	0.27
GLP-1 pmol/L	429 $\pm$ 46.6 <sup>a</sup>	491 $\pm$ 46.6 <sup>b</sup>	474 $\pm$ 46.6 <sup>ab</sup>	0.040
Triacylglycerol mmol/L	104 $\pm$ 6.25	116 $\pm$ 6.09	111 $\pm$ 6.09	0.28
Ratio insulin/glucose	3.51 $\pm$ 0.27	3.07 $\pm$ 0.27	3.02 $\pm$ 0.27	0.082
Ratio insulin/GLP-1	5.09 $\pm$ 0.50	4.12 $\pm$ 0.50	4.16 $\pm$ 0.50	0.042
<b>AUC 120-240</b>				
Glucose mmol/L	620 $\pm$ 20.8 <sup>a</sup>	663 $\pm$ 20.1 <sup>b</sup>	638 $\pm$ 19.8 <sup>ab</sup>	0.049
Insulin mU/L	2674 $\pm$ 255	2980 $\pm$ 241	2737 $\pm$ 235	0.51
GLP-1 pmol/L	398 $\pm$ 47.3 <sup>a</sup>	495 $\pm$ 46.8 <sup>b</sup>	447 $\pm$ 46.9 <sup>ab</sup>	0.010
Triacylglycerol mmol/L	156 $\pm$ 20.4 <sup>a</sup>	209 $\pm$ 19.8 <sup>b</sup>	193 $\pm$ 19.8 <sup>ab</sup>	0.008
Ratio insulin/glucose	4.32 $\pm$ 0.37	4.48 $\pm$ 0.35	4.24 $\pm$ 0.34	0.78
Ratio insulin/GLP-1	7.40 $\pm$ 0.77	6.57 $\pm$ 0.76	6.91 $\pm$ 0.75	0.52

<sup>a-b</sup>Values within rows with different superscripts differ significantly (*p*<0.05)

## 5.5 Effect of microbiota on blood variables and SCFA

No correlations between abundance of *Prevotella\_9* or *Bacteroides* and blood variables were detected when Bonferroni correction was applied. Without correction, a weak positive correlation was observed between *Prevotella\_9* and total TAG AUC and a weak negative correlation between *Bacteroides* and total TAG AUC ( $p=0.045$ ,  $R=0.29$  and  $p=0.025$ ,  $R=-0.32$ , respectively) (Figure 9).

On examining correlations between SCFA and *Prevotella\_9* and *Bacteroides* without correction, it was found that *Prevotella\_9* was positively correlated to propionate ( $p=0.0005$ ,  $R=0.46$ ) and negatively correlated to butyrate ( $p=0.0021$ ,  $R=-0.41$ ).

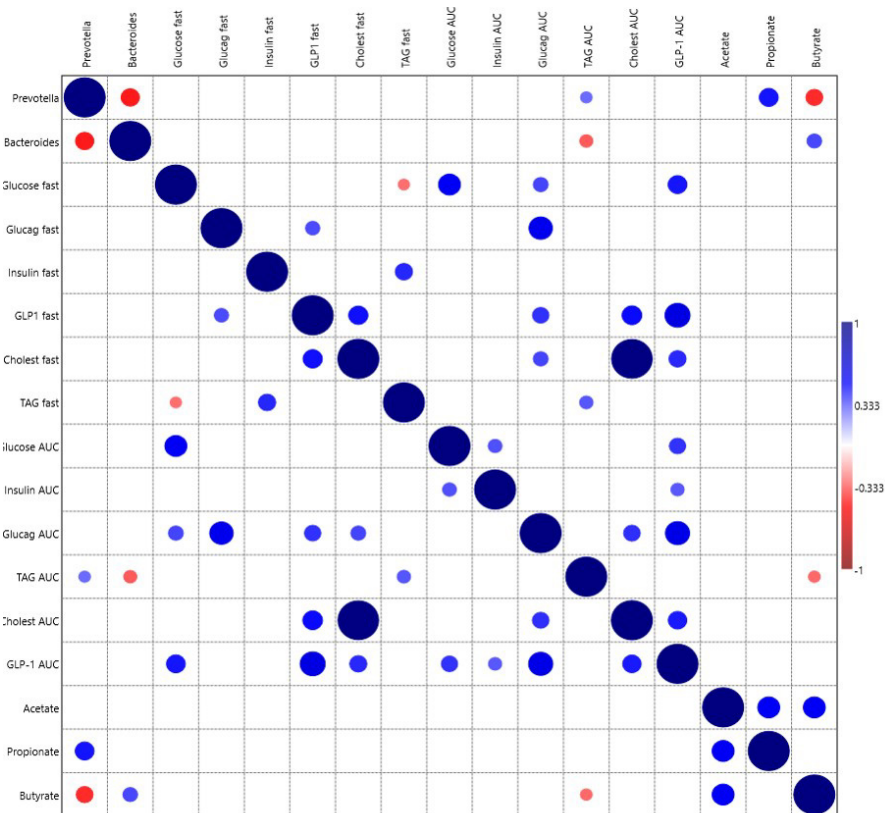


Figure 9. Correlation plot of genera *Prevotella\_9* and *Bacteroides* against blood variables and short-chain fatty acids. Blue dots represent positive correlations and red dots negative correlations (the size of the dot represent the correlation coefficient (R-value)). Cholest = cholesterol, Glucag = glucagon, fast = fasting concentration.

## 6. General discussion

The overall aim of this thesis was to explore and compare effects of including three whole-grain cereals in dog food on dog metabolism and on gut microbial composition and fermentative end-products.

On comparing the results in this thesis to previous studies some general differences in design and methodology should be highlighted as they may explain some of the differences in outcome. First: Studies of whole grain effects in dogs are scarce, thus human and pig studies have also been used in comparisons. Needless to say, there are differences in physiology between dogs and these species. Second: Many studies conducted in dogs and humans compare experimental diets with a control diet that does not contain the studied fiber or is based on refined ingredients and thus contains very little fiber in general. The experiment in Paper I followed this type of design. The experiment in Papers II and III involved comparisons between three diets which all contained dietary fiber, but with different properties, an approach that best met the aim of those studies. Studies comparing the effects of the same whole grains as in this thesis are scarce in both humans and dogs. Thus the results obtained were also compared with findings from comparisons of whole grain with refined wheat in some cases, or with findings for isolated arabinoxylan and  $\beta$ -glucan, which are not fully comparable to the whole grain with bioactive components included. Furthermore, in many human studies, effects of fiber on glucose and insulin have been investigated using fiber in the form of bread and other isolated carbohydrate-rich food products with little protein, which makes comparison of postprandial results of the mixed meals in Paper III complicated. Third: Studies comparing effects of different fiber types in the diet of dogs often use laboratory dogs. Use of privately-owned dogs (Papers II and III) in a study examining the effects of

fiber sources could be viewed as somewhat controversial in the animal science field since environmental factors are hard to control in a home setting. However, most human studies are carried out in a home setting, and therefore this approach should give reliable results. The use of a cross-over design, with the dog acting as its own control, should have minimized possible environmental effects on the results. There is a possibility that the animal owners gave their dog treats and leftovers from the table, but humans generally eat a much more varied diet than dogs. Dogs are generally given the same complete kibble diet every day, which may entail more consistency in fiber intake. Results from a study with privately-owned dogs should further better represent possible effects in the general population than a study with laboratory dogs.

## 6.1 Gut microbiota

### 6.1.1 General microbial composition and diversity

In Paper I the general microbial composition differed in fecal samples collected following diet R compared with baseline samples (Figure 2). No such changes were detected following the RW or W diets in Paper I. The baseline samples from the different periods varied widely in composition and did not differ significantly between the three diet periods, supporting that the difference observed following R diet was in fact due to diet and not period. In Paper II there were no changes in general microbial composition for any of the diets (WHE, OAT, RYE). The fecal microbiota results from both papers revealed large variation between individual dogs in both baseline values and in responses to diet. The individually colored PCoA plot obtained in Paper II (Figure 4a) further revealed that in about 30% of the individuals the microbiota was rather stable over the sampling periods, with only minor differences between diets. For another 30% of the dogs, samples from two of the three different diet periods clustered together, but not consistently for any specific diet. These results suggest that individual or other environmental factors were more influential than diet. A strong influence of individual has been reported previously in dogs (Suchodolski *et al.* 2005; Handl *et al.* 2013; Söder *et al.* 2022). The large individual variation, together with the limited number of dogs, especially in Paper I, made potential differences between diets difficult to detect. Another factor that may explain why there were no

major changes in microbial composition in Paper II could be that the dietary fiber composition of the three diets were similar. The RYE diet had the highest content of dietary fiber, which is in accordance with reported fiber content of the three grain types (Frølich *et al.* 2013), but the small differences in specific fiber composition might not have been enough to have significantly different effects on general microbial composition. However, the diversity in Paper II was higher in fecal samples when dogs were fed WHE diet compared with RYE, indicating that the microbial composition was affected differently by the two diets. For both diets the diversity was within what has previously been reported for healthy dogs (Suchodolski *et al.* 2012b; Guard *et al.* 2015).

### 6.1.2 Changes in specific microbial taxa

On investigating the response in more detail on genus and OTU level, the abundance results from many taxa in Papers I and II varied. However, they indicated a consistent response to rye diets in genera *Prevotella\_9* (henceforth called *Prevotella*) and *Bacteroides*, which, even though the changes were not significant in both papers, were numerically affected in opposite directions (Figure 3); *Prevotella* increased and *Bacteroides* decreased following rye compared with baseline in Paper I and/or with the other diets in Papers I and II. When adjusting for multiple statistical comparisons using FDR, *Bacteroides* abundance was not significantly different between the diets in Paper II. These results are however in line with findings in a study on human fecal microbiota following consumption of rye kernel bread or refined wheat bread as evening meals, for one or three days, where an increase in *Prevotella*, with a simultaneous decrease in *Bacteroides*, was observed following rye intake (Prykhodko *et al.* 2018). However, other studies in humans on consumption of whole rye compared with refined wheat and whole wheat have observed no differences in *Prevotella* or *Bacteroides* (Vuholm *et al.* 2017; Iversen *et al.* 2022). A sole previous study on rye consumption in dogs investigated microbial effects of a vegetarian diet supplemented with feather meal, alone and together with either corn meal, rye or fermented rye but reported microbial abundance only at phylum level (Hankel *et al.* 2020), preventing identification of differences between *Prevotella* and *Bacteroides*. No significant differences were found in that study, but phylum Bacteroidetes, which includes both *Prevotella* and *Bacteroides*, increased markedly in some dogs following rye

supplementation, fermented or not (Hankel *et al.* 2020). Interestingly, *Prevotella* abundance has been found to be decreased in dogs with inflammatory bowel disease (Suchodolski *et al.* 2012a), indicating a possible beneficial effect of *Prevotella* in the dog gut.

In Paper II several OTU belonging to the family Lachnospiraceae were significantly less abundant when dogs were fed RYE diet compared with WHE. One unclassified member was significant also following FDR correction. This is in line with a human study where consumption of whole grain rye induced a decrease in OTUs belonging to Lachnospiraceae, while whole grain wheat consumption did not (Eriksen *et al.* 2020).

### 6.1.3 Factors possibly affecting abundance of *Prevotella* and *Bacteroides*

The studies in this thesis indicated that inclusion of rye in dog food promotes *Prevotella*, while *Bacteroides* decreases. The increase in *Prevotella* abundance seen in this thesis was, however, significant only for the R diet with 50% inclusion of rye flour in Paper I, while the decrease in *Bacteroides* was significant for the RYE diet in Paper II. There was however also a trend ( $p < 0.1$ ) for *Prevotella* abundance to increase following RYE diet in Paper II. The apparent pattern of the response to rye in the two genera is interesting and the factors behind deserves some investigation. There were also some differences between the diets in the two studies which may provide some information as to why the genera were not significantly affected by rye in both studies.

The relative abundance of *Prevotella* to *Bacteroides* is reported to be affected by the carbohydrate to protein ratio in the diet of humans (Wu *et al.* 2011) and dogs (Li *et al.* 2017; Martinez-Lopez *et al.* 2021), with *Prevotella* being positively correlated with carbohydrates and fiber, and *Bacteroides* with protein. The diets in Papers I and II differed in terms of macronutrient content. On a DM basis, the fat content in the diets in Paper I was almost twice that of the diets in Paper II (Table 1), while the protein content was about 30% lower. The carbohydrate content (nitrogen-free extract) was approximately the same in both diets. The low protein content in the diets in Paper I might have posed a disadvantage to *Bacteroides*, leaving more room for *Prevotella* to increase. The diets in Paper I were not analysed for TDF or for soluble and insoluble fiber, but the crude fiber content and neutral detergent fiber content (Table 1) provided an indication that the dietary fiber

content was slightly lower in the R and RW diets, and considerably lower in the W diet, compared with the diets in Paper II. This indicates that the fiber content alone does not explain the increase in *Prevotella* following consumption of the R diet in Paper I.

Other studies in dogs investigating the effects of different fiber sources have found highly variable abundances of *Prevotella* and *Bacteroides*, ranging from around 0.25% to 30% for *Prevotella* and 0.5% to 20% for *Bacteroides* (Panasevich *et al.* 2015; Nogueira *et al.* 2019; Beloshapka *et al.* 2021; Traughber *et al.* 2021). Some have found small but significant differences in response to fiber sources for one or both of these genera (Panasevich *et al.* 2015; Traughber *et al.* 2021), while others have not found any significant differences (Nogueira *et al.* 2019; Beloshapka *et al.* 2021). Traughber *et al.* (2021) found that *Prevotella* abundance in fecal samples increased following a diet with oat groats compared with a control diet with rice and that *Bacteroides* abundance decreased simultaneously, although not significantly. No differences in these two genera were found following OAT diet in Paper II.

Some characteristic compound in rye may promote *Prevotella* or be a disadvantage to *Bacteroides*. Rye is characterized by a high content of arabinoxylan, fructan and other bioactive compounds. Whole wheat also contain reasonable amounts of arabinoxylan, yet less soluble than arabinoxylan in rye (Knudsen 2014). Species of *Bacteroides* and *Prevotella* both have the ability to utilize arabinoxylan (Bai *et al.* 2021) and fructan (Sonnenburg *et al.* 2010; Fuse *et al.* 2013). One *in vitro* study (Chen *et al.* 2017) using inocula from two human donors with *Prevotella*- or *Bacteroides*-dominated microbial community compared degradation and subsequent SCFA production from two arabinoxylan sources (corn and sorghum). The results showed that the *Prevotella*-dominated inocula fermented the substrates faster, and with higher SCFA production, than the *Bacteroides*-dominated inocula (Chen *et al.* 2017). In the same study, diversity in the *Bacteroides* cultures was higher after 24 h than in the *Prevotella* cultures, where one *Prevotella* OTU outcompeted other major taxa when fermenting the substrates. Another similar *in vitro* study examining fermentation and microbial shifts in inocula of human fecal origin dominated by either *Prevotella* or *Bacteroides* using rye, oat or wheat breads as substrates did likewise observe faster fermentation rate in the *Prevotella*-dominated inoculum for all substrates (Pirkola *et al.* 2023). However, after

24 h the initially *Prevotella*-dominated inocula was still dominated by *Prevotella* in oat and wheat substrates, whereas in rye substrate the genus *Subdoligranulum* dominated. Similarly to the study by Chen *et al.* (2017), they also found that the *Bacteroides*-dominated inocula had a more diverse composition after 24 h compared with the *Prevotella*-dominated inocula (Pirkola *et al.* 2023). Thus it is not clear why consumption of rye would promote *Prevotella* and in fact it does not consistently do so. However, it appears that when *Prevotella* is enriched it may outcompete other taxa, as was also indicated by the results in Paper I following the diet with 50% rye.

There were some differences in structural properties of the diets in Papers I and II. In Paper II the diets were extruded, with flours that were milled similarly, while in Paper I the diets were baked and the wheat flour was finely milled, while the rye flour was coarsely milled. These structural differences might have affected the accessibility of fermentable material, which in turn might have affected the microbiota composition, with the more coarse flour possibly favouring *Prevotella*. In the study by Prykhodko *et al.* (2018), where a similar pattern in the two genera was observed, the breads used likewise differed in structure, with the rye bread being baked from whole kernels and the wheat bread from white flour. An increase in *Prevotella* and decrease in *Bacteroides* have also been reported in a study using barley kernels (Kovatcheva-Datchary *et al.* 2015). Participants in the studies by Vuholm *et al.* (2017) and Iversen *et al.* (2022), in which no differences in the two genera following rye consumption were observed, could choose from a variety of rye products of varying structure. All in all, these findings indicate that the structure of the fiber source might be of importance.

#### 6.1.4 The existence of enterotypes in dogs

Enterotype is a concept that has been investigated in humans in recent years (Arumugam *et al.* 2011; Wu *et al.* 2011; Sandberg *et al.* 2019; Eriksen *et al.* 2020). The two types often highlighted are *Prevotella*-dominated and *Bacteroides*-dominated. These two genera are also often reported in dogs, but to the best of my knowledge the concept of enterotypes in dogs has not been investigated to any considerable extent. The microbiota of dogs is often dominated by the phylum Firmicutes and to some extent Fusobacteria (Mori *et al.* 2019; Pilla & Suchodolski 2019; Söder *et al.* 2022), as generally also observed in Papers I and II in this thesis. However, some dogs in Paper II had consistently higher relative abundance of *Prevotella*, ranging from



around 15% to 30%, while *Bacteroides* abundance was about 5-10%. In other dogs the abundance of *Prevotella* was consistently lower, around 1-5%, and *Bacteroides* showed correspondingly higher relative abundance, of around 10-20%. This was also reflected in the correlation analysis (Figure 5). By coincidence, most of the dogs with high *Prevotella* abundance proved to have been randomly assigned to group 1 (starting on diet WHE), and consequently clustered separately from the other two groups in the PCoA analysis (Figure 4b). This difference was likely not dependent on diet, because the same pattern was seen in samples collected after the initial adaptation period when all dog groups had been fed the wheat diet (unpublished data). The observations of high abundance of either *Prevotella* or *Bacteroides* in Paper II are based on a limited number of dogs and did not apply to all dogs, but it would be interesting to investigate the concept of canine microbiota enterotypes further in future studies.

## 6.2 Short-chain fatty acids

The results of the short-chain fatty acid analysis are in Paper II presented as concentrations of acetate, propionate and butyrate in absolute values, while, in Paper I, they are presented as proportions relative to the total concentration of all three. In Paper II, concentration of acetate was significantly higher in fecal samples following RYE diet compared with OAT diet (Figure 7). In Paper I there was a trend for an increase in the proportion of acetate following the diet with 50% rye compared with baseline (Figure 6). These findings are in accordance with a study by Bai *et al.* (2021) comparing arabinoxylan (although from corn) with oat  $\beta$ -glucan. Propionate concentration was also higher after RYE than OAT in Paper II, but the proportion was not affected. The proportion of propionate was also not affected following any of the diets in Paper I. *Prevotella*-dominant microbiota has previously been reported to correlate with increased propionate production (Chen *et al.* 2017; Poeker *et al.* 2018; Eriksen *et al.* 2020), but also with acetate (Bai *et al.* 2021), depending on substrate. There was a trend in Paper II for total SCFA concentration to be higher after RYE compared with OAT. The OAT diet had the highest inclusion of soluble dietary fiber, therefore it was expected to be easily fermentable. However, this also means that digestion of fiber in the OAT diet could have occurred more proximally in the intestine, and thus SCFA could also have been absorbed more proximally. However, the results

from an *in vitro* study comparing fermentation on breads made from rye or oats indicated that rye resulted in more SCFA, especially when the inoculum was *Prevotella*-dominated (Pirkola *et al.* 2023).

Butyrate did not differ between the diets in either Paper I or II. In contrast, butyrate in fecal samples has been reported to be higher following rye compared with whole grain wheat consumption in human studies (McIntosh *et al.* 2003; Eriksen *et al.* 2020). Butyrate has also been observed to increase in plasma following rye or arabinoxylan consumption in both humans and pigs (Ingerslev *et al.* 2014; Iversen *et al.* 2022). The SCFA concentration in plasma was not measured in Paper II, because only a limited volume of blood was available for analysis. It is possible that higher butyrate formation could have been detected in plasma, since most of butyrate produced is absorbed before the intestinal content reach the rectum.

### 6.3 Blood metabolites and hormones

The postprandial response curves were similar between diets (WHE, OAT and RYE) (Figure 8) for all investigated blood variables in Paper III, and concentrations were within the range observed previously in dogs following mixed meals (Kempe *et al.* 2004; Carciofi *et al.* 2008; Söder *et al.* 2016). However, there were some differences in the response to the three diets. The AUC concentrations of plasma glucose, GLP-1 and serum TAG were greater following consumption of the OAT diet compared with WHE, while fasting plasma insulin concentration was higher during the RYE diet period compared with the WHE and OAT periods (Table 2). Moreover, the WHE diet tended to result in higher postprandial plasma concentration of insulin per glucose and GLP-1 than the other two diets.

The finding of higher blood glucose concentration following OAT than WHE was unexpected, since  $\beta$ -glucan is generally reported to give an attenuated glucose response compared with control diets in humans and pigs (Biörklund *et al.* 2005; Hooda *et al.* 2010; Hartvigsen *et al.* 2014). However, two previous studies in dogs, one with oat groats and one with purified  $\beta$ -glucan supplement, also found no glucose-lowering effects (Kempe *et al.* 2004; Ferreira *et al.* 2018). The mechanism by which  $\beta$ -glucan is hypothesized to have such effects in humans and pigs is to form a viscous gel which delays starch breakdown, and glucose uptake from the small intestine, thereby flattening the postprandial curve (McRorie & McKeown

2017). However, a canine *in vitro* study by Bednar *et al.* (2001) found that rolled oats were more easily digestible than wheat grain. Those authors suggested that processing of the oats made the starch more available to enzymatic digestion, which could also be a plausible explanation for the glucose results in Paper III.

Similar to glucose, plasma GLP-1 concentration was higher after the OAT diet. This could be because  $\beta$ -glucan did increase the viscosity and thus more glucose reached distal parts of the small intestine and was available to stimulate GLP-1 secretion from L-cells (Holst 2007). Another possible explanation for the high GLP-1 concentration following the OAT diet, is that SCFA produced during fermentation of fiber in OAT could have activated receptors FFAR2 and FFAR3 present on L-cells, which is suggested to result in GLP-1 release (Chambers *et al.* 2015; Koh *et al.* 2016). For dogs, deviating results regarding the effect of fermentable fiber on plasma GLP-1 concentration have been reported (Massimino *et al.* 1998; Bosch *et al.* 2009b). Massimino *et al.* (1998) observed an increase in plasma GLP-1 in dogs subjected to an oral glucose tolerance test after 14 days of consuming a diet supplemented with high fermentable fiber compared with a diet with low fermentable fiber, but they did not measure SCFA. On the other hand, Bosch *et al.* (2009b) found no differences in postprandial plasma GLP-1 in dogs following a diet with high fermentable fiber compared with low fermentable fiber even though fecal SCFA concentration was higher following the high fermentable fiber diet. There were no signs of increased SCFA production in feces following consumption of the OAT diet in Papers II and III but, as pointed out earlier, fecal SCFA may not give a fully representative picture of fermentation taking place more proximally in the large intestine. Plasma SCFA, rather than fecal SCFA, is reported to be positively correlated with fasting plasma GLP-1 (Muller *et al.* 2019). The finding of higher GLP-1 following the OAT diet is interesting as GLP-1 is known to contribute to the ileal break in humans (Holst 2007), and thus might have increased satiety following OAT diet.

The higher fasting plasma insulin concentration for the RYE diet, compared with the other two diets, could not be explained by differences in fasting glucose or GLP-1 concentrations. However, while the postprandial insulin curve for OAT and WHE seemed to peak within the 240-min sampling time, no peak was detected for the RYE diet. This may indicate that insulin in fact had a prolonged elevation period when dogs were fed the RYE

diet. But the difference at 240 min was small and a longer sampling period might have changed the picture. The trend for lower ratio of insulin to glucose and GLP-1 following the RYE and OAT diets compared with WHE is otherwise interesting, as it may imply that less insulin was needed following the RYE and OAT diets to metabolize the glucose produced. However, this theory is based on observations made during the first four hours following feeding and it would have been interesting to follow the postprandial response for a longer period. Some previous studies in pigs and humans on the effects on insulin of rye consumption have reported increased insulin economy, but displayed as lower insulin and similar glucose, compared with whole and refined wheat consumption (Leinonen *et al.* 1999; Juntunen *et al.* 2002; Rosen *et al.* 2009; Theil *et al.* 2011). This phenomenon, for which the mechanism is not clear, has been called the “rye factor” (Jonsson *et al.* 2018).

Glucagon concentration curve was similar following all diets. The main purpose of glucagon is to increase blood glucose concentration in the event or risk of hypoglycaemia (Frayn 2010). Thus, the strongest signal to increase glucagon is low blood glucose, while high blood glucose and insulin suppress glucagon release. However, glucagon is also stimulated by ingestion of amino acids, as in a mixed meal with carbohydrates and protein, which was likely the reason for the increased glucagon concentration following the experimental diets in our study.

The postprandial AUC concentration of TAG was higher following OAT compared with WHE. The postprandial TAG concentration has been observed to vary with the fat content of the diet (Downs *et al.* 1997). Thus the difference might be explained, at least in part, by the fat content in diet OAT (17.6%) being somewhat higher than that in WHE (13.7%). The uptake of TAG in adipocytes is stimulated by insulin (Frayn 2010), but there were no differences in postprandial plasma insulin concentration between the diets that could explain the differences in TAG concentration.

No effect of diet was detected on cholesterol concentration pre- or post-prandially. One previous study has reported that  $\beta$ -glucan can lower serum cholesterol in dogs (Ferreira *et al.* 2018). However, in that study the  $\beta$ -glucan was provided purified as a supplement and the supplemented diet was compared with the same diet without supplementation.

## 6.4 Associations between microbiota and blood variables and the links to satiety

Metabolic effects on the host from fermentation by colonic bacteria have been a subject of great interest to researchers for many years. More recently, it has been reported that the metabolic response to dietary fiber may differ depending on specific bacterial taxa present in the gut. Kovatcheva-Datchary *et al.* (2015) investigated the glucose response in humans to a standardised breakfast, following evening meals with a barley kernel-based bread or white wheat bread, and found a low glucose and insulin response following barley bread only in a subset of the participants. Individuals in that group had an increased ratio of genus *Prevotella* to *Bacteroides* following consumption of the bread, while the group that did not have a differing response between the two breads had significantly lower *Prevotella/Bacteroides* ratio (Kovatcheva-Datchary *et al.* 2015). This was further investigated by Sandberg *et al.* (2019) who found that individuals with a high ratio of *Prevotella/Bacteroides* at baseline had a lower glucose and insulin response to a standardized breakfast following an evening meal with barley kernel bread or white wheat bread, and in general lower subjective hunger sensations after the breakfast. Individuals with low *Prevotella/Bacteroides* ratio had a low glucose and insulin response after the breakfast only following the barley evening meal and higher hunger sensations than the individuals with high *Prevotella/Bacteroides* ratio.

Given previous results indicating that *Prevotella* may affect how the diet affects metabolism and the finding in Paper II that some dogs had high abundance of *Prevotella*, the correlation between *Prevotella* or *Bacteroides* and blood variables was analysed for this thesis. The only correlation detected was for TAG, which was positively correlated to the abundance of *Prevotella* and negative to *Bacteroides*, but this difference did not persist following correction for multiple comparisons. It is not clear why this correlation would occur. In summary, these two enterotypes did not seem to have an effect on blood variables in this thesis, although the number of dogs was limited and only a few had markedly higher abundance of *Prevotella*. The correlation to SCFA was also investigated in this thesis and showed a positive correlation between *Prevotella* and propionate, corroborated by previous reports (Chen *et al.* 2017; Poeker *et al.* 2018; Eriksen *et al.* 2020), and a negative correlation between *Prevotella* and butyrate. It is not clear to what extent genus *Prevotella* has the ability to produce butyrate. In contrast

to previous findings by Bai *et al.* (2021), no correlation between *Prevotella* and acetate was found even though acetate was highest and *Prevotella* tended to be higher following RYE compared with the other diets.

A possible effect of different *Prevotella/Bacteroides* ratio at baseline on the outcome of the studies could not be investigated because, in Paper I, the baseline samples varied widely between the dogs and no clear consistent microbiota could be detected and in Paper II it was decided to not collect baseline samples, since this was not needed in order to compare the effects of diets and since the dogs had such varying backgrounds. This information would have been interesting, however, in order to investigate whether the basal microbiota influenced the outcome.

#### 6.4.1 Satiety study connected to findings in Paper II and III

In humans, whole grain rye and oats have both been observed to increase satiety, while whole wheat does not seem to have equally satiating effects (Cooper *et al.* 2015). One of the factors that is believed to contribute to the increased satiety is that SCFA binding to FFAR 2 and 3 on L-cells in the intestine stimulates release of GLP-1 (Koh *et al.* 2016), which contributes to the ileal break (Holst 2007). In dogs, studies have indicated that high-fermentable fiber is more satiating than low-fermentable fiber (Jackson *et al.* 1997; Bosch *et al.* 2009b). The connection to GLP-1 is however not clear (Bosch *et al.* 2009b). The satiety in dogs in those previous studies was evaluated by measuring voluntary food intake (Jackson *et al.* 1997; Bosch *et al.* 2009b) and assessing interprandial behaviour (Bosch *et al.* 2009a). In Paper III, the postprandial concentration of GLP-1 was higher following the diet OAT compared with WHE, while for diet RYE it was numerically higher compared with WHE. Thus oats, and perhaps rye, could have had an increased satiating effect compared with wheat on the dogs. The question is, was this reflected in the appetite behaviour of the dogs?

Parallel to the study presented in Papers II and III, a separate study was performed investigating feed motivation in 11 dogs fed diets WHE, OAT and RYE (Hellström 2021). Six of the dogs also participated in the study for Papers II and III. Feed motivation was studied in a runway test, where the latency to reach a bowl with a food reward at the end was measured at 15 minutes and at 3 and 6 hours post morning feeding (Hellström 2021). The results showed that the dogs generally had higher feed motivation during the WHE diet period compared with the other two diet periods, indicating that

whole wheat was less satiating than oats and whole rye. An attention bias test was also performed, where the dogs at 15 minutes and 6 hours post morning feeding were presented with a toy and their own food bowl, to investigate the time to approach the bowl. No difference between diets were observed during this test (Hellström 2021). These findings together with the results from paper III of higher plasma AUC concentration of GLP-1 following the OAT diet and numerically higher concentration following RYE compared with WHE, suggests that oats and whole rye in dog diets are more satiating than whole wheat and that GLP-1 might have had a role in the satiating effect of the two diets.





## 7. Conclusions

The general conclusions of this thesis were that whole grain rye in dog food may affect the fecal relative abundance of specific bacterial taxa and increase fecal concentration of acetate and propionate, which could benefit dog health. The overall metabolic responses were similar following whole grain wheat, oat and rye, while detailed investigation of responses showed differences which warrant further studies.

Specific conclusions:

- Whole grain rye at an inclusion rate of 25% of DM in dog food had similar apparent total tract digestibility as refined wheat, and was well accepted and tolerated by the dogs
- Whole grain wheat, oats and rye in dog food did not have clear differential effects on general microbial composition
- Inclusion of whole grain rye in dog food may increase the relative abundance of genus *Prevotella* and decrease abundance of genus *Bacteroides* in the gut, but may also decrease microbiota diversity
- Inclusion of whole grain rye may lead to an increase in concentrations of acetate and propionate in the gut
- Inclusion of whole grain oat in dog food may induce a greater blood response in glucose, GLP-1 and TAG than a diet including whole grain wheat
- Inclusion of whole grain rye may give higher fasting concentration of insulin compared with inclusion of whole grain wheat and oats



## 8. Future perspectives

Whole grain wheat and oats are currently used to some extent in commercial dog food and the results in this thesis indicate that whole grain rye could also be a suitable ingredient in commercial diets, to replace part of the refined grain component and potentially improve dog gut health by promoting fiber-fermenting bacteria.

The genera *Prevotella* and *Bacteroides* were detected in relatively high abundance in the gut of dogs consuming whole grain diets in this thesis. In humans, these two genera have been found to each dominate one of the three enterotypes that have been suggested (Arumugam *et al.* 2011). The enterotypes are believed to have developed as a result of long-term consumption of diets rich in fiber and carbohydrates (*Prevotella*) or protein (*Bacteroides*) (Wu *et al.* 2011). Different enterotypes may respond differently to dietary interventions (Prykhodko *et al.* 2018; Eriksen *et al.* 2020). It would be interesting to study the concept of enterotypes in dogs further. As *Fusobacterium* and *Clostridium* generally increases in dogs fed a diet higher in protein (Pilla & Suchodolski 2019) *Bacteroides* might not cover the same niche in the gut of dogs as in humans. In this thesis, changes in the gut microbiota of dogs fed different whole grain diets were examined with a bacterial community analysis based on sequencing of PCR amplicons, which means that abundances of specific bacteria were determined in relation to that of all other bacteria in the sample. This use of relative abundance data might have obscured changes in specific taxa following a general increase or decrease in all bacteria. Moreover, a decrease (or increase) in relative abundance does not necessarily mean a decrease (or increase) in absolute abundance. In order to study changes in specific taxa, quantitative PCR (qPCR), which measures abundances in absolute numbers,

should be used in future work, as it could provide information that increases understanding of the effects of diet.

In this thesis, it was observed that acetate and propionate concentrations in feces increased in dogs consuming a diet with rye compared with a diet with oats. However, the majority of SCFA produced in the intestine are absorbed into the bloodstream and it is this absorbed fraction, rather than that in the feces, which could affect the health of the animal. Therefore future studies should analyze SCFA concentrations in the blood of dogs consuming whole grain diets, to obtain additional information that could assist in interpretation of the metabolic response. The work in this thesis also revealed slightly higher fasting insulin following the rye diet than the other diets, but also an indication of lower postprandial insulin:glucose ratio compared with the wheat diet. In human and pig studies, inclusion of rye in the diet can have been observed to result in better insulin economy compared with refined and whole wheat (Jonsson *et al.* 2018). It has been suggested that circulating NEFA can decrease insulin sensitivity but that the release of NEFA from adipocytes is decreased by circulating SCFA (Robertson 2007). Future studies should therefore also analyze NEFA concentrations at fasting, which may help make the picture clearer.

In this thesis, the postprandial metabolic response was followed for four hours and within that time only glucagon had returned to the fasting concentration by the end of sampling. Thus a longer sampling period should be applied in future studies to capture the decline to baseline in other blood parameters following the postprandial metabolic response. The sampling period should preferably be 12 hours, which was the minimum time the dogs in this thesis had fasted before coming to the clinic on the morning of sampling. Such a long sampling time was difficult to achieve when working with privately-owned dogs. However, postprandial sampling for up to eight hours might be possible, and might better reflect the fasting period of dogs during the day until the next meal. A longer sampling time could give a better picture of the insulin curve following consumption of different diets and make it possible to detect dips in glucose concentration that could influence satiety. The postprandial metabolic response should in further studies also be investigated by untargeted metabolomics. This would make it possible to detect smaller but possibly important differences in the metabolic response to the different whole grains.

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## Populärvetenskaplig sammanfattning

Domesticeringen av hunden från vargen tros ha börjat för minst 10 000 år sedan i samband med att människor började bruka marken och odla, bland annat, spannmål. En teori om varför domesticeringen skedde är att de vargar som kunde tillgodogöra sig energin från den föda som fanns nära människornas boningar vande sig vid människor. Med tiden kom dessa vargar att bli till sällskap och nytta för människorna i utbyte mot att de fick tillgång till föda. Denna teori stöds av att man i hundens gener, i jämförelse med vargens, kunnat hitta förändringar som leder till en förbättrad nedbrytning av stärkelse, vilket utgör en stor del av innehållet i spannmål. I dagens hundfoder är spannmål en vanlig ingrediens och stärkelseinnehållet i torrfoder kan vara upp emot 60 %. En viss mängd stärkelse behövs för processen i vilken foderkulan produceras och i stärkelsen finns energi som hunden kan använda. Ofta används spannmål i hundfoder i raffinerad form, vilket innebär att stärkelse är det huvudsakliga näringsämnet som spannmålet tillför. Spannmål i form av fullkorn innehåller dock flera andra näringsämnen som protein, mineraler, vitaminer och, inte minst, kostfibrer.

För människor har studier visat att en kost med fullkornsprodukter, i stället för raffinerade produkter, ger bättre mättnad och en minskad risk för bland annat diabetes typ 2, hjärt-kärlsjukdomar och olika cancerformer. För hund finns det ännu väldigt lite forskning om hälsoeffekter av fullkorn. Vad det är som gör att fullkorn har dessa effekter på människor är inte klarlagt, men forskningen pekar på att det har att göra med kostfibrerna i fullkorn. Människan, liksom hunden, har i sin tarmkanal inga enzymer som kan bryta ner kostfibrerna, så de passerar relativt oförändrade till tjocktarmen. I tjocktarmen finns en stor mängd olika bakterier. Många av dessa har förmågan att bryta ner kostfibrer genom jäsning och därmed tillgodogöra sig energi ifrån dem. En kost som innehåller kostfibrer främjar dessa bakterier,

som därmed kan skydda tarmen från potentiellt skadliga, sjukdomsframkallande bakterier, vilka oftast inte använder kostfibrer som energikälla. I jäsningprocessen bildas korta fettsyror, bland annat acetat, propionat och butyrat. Fettsyrorna tas upp av tarmcellerna och kan användas som energi, men de har också visats kunna ha en mängd andra effekter, bland annat öka frisättning av hormoner som ökar mättnad och påverka hur insulin frisätts och används i kroppen.

I och med de hälsofrämjande effekter som rapporterats att fullkornsprodukter har på människa var syftet med denna avhandling att utforska vilka effekter olika fullkorn i hundfoder har på hundens tarmbakterier och de korta fettsyror som dessa producerar samt vilka effekter som kan ses i ämnesomsättningen efter måltid. Vete, havre och råg är tre av de vanligast odlade spannmålen i Norden. Råg och havre har i flera studier på människa visats ha hälsofrämjande effekter, medan vete inte lika ofta har visats ha det. Råg är en ovanlig ingrediens i hundfoder, medan vete, och till viss del havre, är vanligare. Vete används dock oftast i raffinerad form.

Två studier utfördes för denna avhandling. Den första studien utfördes på sex beaglar som fick äta tre olika foder under vardera tre veckor. De tre fodren innehöll antingen 50 % raffinerat vete, 25 % vardera av raffinerat vete och fullkornsråg, eller 50 % fullkornsråg. Syftet var att undersöka lämplig inblandningsnivå av fullkornsråg i hundfoder eftersom det inte var känt hur bra hundar är på att smälta det och huruvida det skulle vara smakligt i hundfoder. Dessutom undersöktes om några skillnader i förekomst av olika bakterier kunde ses i träckprover.

Studien visade att hundarna accepterade och kunde smälta fodret med 25 % inblandning av fullkornsråg i liknande hög utsträckning som fodret med raffinerat vete, medan 50 % inblandning av råg hade något sämre smältbarhet och några av hundarna lämnade en del foder med denna inblandningsnivå. Därför bedömdes att 25 % inblandning var lämpligt. Fodret med 50 % råg gav dock upphov till en förändring i bakteriesammansättningen i träckprover och speciellt en bakterieart med namnet *Prevotella* ökade. *Prevotella* har förmågan att jäsa fibrer och producera några av de korta fettsyror som rapporterats vara hälsofrämjande på människa. Vid 25 % inblandning av råg kunde ett liknande mönster i *Prevotella* skönjas, men inte säkerställas statistiskt.

I den andra studien jämfördes foder med inblandning av 25 % fullkorn av vete, havre eller råg. Fodren gavs till 18 privatägda hundar som fick äta de

tre fodren under fyra veckors tid vardera. I slutet av varje period samlades träckprover som undersöktes för bakteriesammansättning och korta fettsyror. Sista dagen i varje period kom djurägaren in med hunden till kliniken. På kliniken togs ett fastebloodprov och sedan utfodrades hunden. Bloodprover togs därefter under fyra timmar efter måltid för att följa hur innehållet av blodsocker, fetter, kolesterol och de tre hormonerna insulin, glukagon och GLP-1 förändrades efter måltiden.

Resultatet från träckproverna visade att en bakterieart med namnet *Bacteroides* var lägst efter att hundarna ätit råg, samtidigt kunde en tendens till ökning av *Prevotella* ses, men det kunde inte säkerställas statistiskt. *Prevotella* och *Bacteroides* har tidigare visats ha detta omvända samband både hos hund och människa. I träckproverna från rågperioden var också de korta fettsyrorerna acetat och propionat högre än proverna tagna under havreperioden, vilket kan betyda att mer av dessa fettsyror producerades i tarmen när hundarna hade ätit råg.

Bloodanalyser visade att ämnesomsättningen efter måltid var relativ lik efter de tre olika fodren, men några mindre skillnader fanns. Hormonet GLP-1, som tros öka mättnad, var högre när hundarna åt havre jämfört med när de åt vete, men även blodsocker och fett var högre efter havre. Vidare var fastenivån av hormonet insulin högst hos hundarna under rågperioden. Skillnaderna var små och betydelsen av dem är svårt att avgöra med den information som framkom i studien.

Sammanfattningsvis visade studierna i denna avhandling att råg kan ha främjande effekter på vissa bakterier som kan ha goda hälsoeffekter på hunden i och leda till en ökning av korta fettsyror. Effekterna av fullkorn på hundens ämnesomsättning behöver undersökas vidare i framtida studier.



## Acknowledgements

I would first like to acknowledge the **Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences** and **Agria/Svenska Kennelklubben Forskningsfond** with my warmest thanks for providing funding for the studies of this thesis.

To my main supervisor, **Johan Dicksved**, thank you for always believing in me and guiding me through this journey with patience and calm advice. For supporting and reassuring me when I was in doubt and providing the opportunity to develop my skills and independence as a researcher. Thank you for having my back and being there to help with all things, big and small.

My sincerest thanks to assistant supervisor **Torbjörn Lundh** for letting me take part of all your knowledge in nutrition and biochemistry, making it logical. Thank you for always encouraging me to follow my mind and for supporting me through the digestibility dilemmas, without you I would probably have given up on it.

Assistant supervisor **Katja Höglund**, my warmest thanks for always checking in on me and making sure I take care of myself. Thank you for interesting and important discussions and invaluable input in the veterinary medicine aspects. I am so happy that you joined the group, you have enriched it both with joy, warmth and a lot of wisdom.

My warmest thanks also to assistant supervisor **Sara Ringmark** for all your encouragement and support. You always spurred me on, believing that I could do it, which truly helped me keep going! Thank you for asking critical questions and always having an eye for the important things.

My sincere thanks to all **dog owners** and **dogs** for daring to participate in my study and taking the time to come here for all the sampling, for keeping track of all the instructions, collecting samples and all the while with so much kindness and patience.

A sincere thank you also to the company **Doggy AB** for generously producing the diets used in Papers II and III. Thank you especially to **Cecilia Hjelm** for tireless help with all my requests for, and questions about, the diet formulation.

Thank you also to the **Beagle boys** and to **Emma Hörnebro** for all your help in my first study, feeding my strange diets and checking the gray poop 😊

**Claudia von Brömssen**, thank you so much for your swift and kind help with my endless questions about statistics, I am amazed at your efficiency and patience. Thank you co-author **Erik Pelve** for your help with the bioinformatics in the first study, I am so glad you wanted to join.

To **Anna-Greta, Astrid, Jorge, Camilla, Thomas** and **Delal**, my warmest thank you for help with analyses, sample handling, marking test tubes, keeping track on all my samples and never getting tired of my questions and teaching me so many things and also for lovely chitchat in between about all things in life. Thank you also to **Anna Svensson** and **Inger Lilliehöök** for invaluable advice and help with interpretation of lab results.

Thank you **Malin Hellström** and **Else Verbeek** for taking on my vague idea and then developed and performed the behavior study. I am so happy you were willing to do it and think you did a fantastic job.

A very warm thank you to all **present and former PhD colleagues** for all the fun times that has enriched my days as a PhD student. Thank you to **Lidija**, I am so glad that you were the one who I was going to share room with. We've had so many good times and so many long talks about all the good and the annoying things, you've helped me and encouraged me and you will always be in my heart. **Anna** thank you so much for all your help and for cheering me on these last months. **Katrin** and **Malin**, thank you for being

there to listen and for all the laughs. Thank you to **Emy, Sofia, Heidi, Johanna F, Lisa, Johanna K, Eliška, Josef, Aprajita**, who have been along for a large part of my journey here at HUV, I am so glad that I could share it with you. I am also thankful for getting to know **Annica, Claire, Melania, Markos, Léonie, Ashwath, Pontus, Anne, Shouwen**, who have lightened up my days more recently. I feel blessed to have met so many wonderful people who always have made me feel lighter when my work felt heavy.

Thank you to all colleagues at **HUV department** for laughs and interesting discussions at fika and lunch. Everyone at **HUV Administration**, thank you for kind and efficient help with every practicality, **Johan**, thank you for always having a solution to all problems, **Majsan**, thank you for all the joy you bring, I know Vilmer agrees ☺

Thank you to my former and future **colleagues at KV-DOV** for asking when I will come back, I feel so welcome and am looking forward to joining you. A special thanks to **Josefin** for checking in on how I'm doing and cheering me on. I'm so happy we'll work together soon.

Tack till min kära vän **Nadia**, för den underbara illustrationen, men mest för att du är du, vem vore jag annars? ☺

Mina underbara vänner **Linda, Jenny, Rana** och **Cecilia** är så glad och tacksam att jag har er, som alltid finns där, hur sällan vi än ses.

Mina veterinärpolare **Malin, Veronica** och **Carro**, tack för att ni hjälpte mig ta mig igenom veterinärutbildningen med en massa skratt och härliga minnen.

Min älskade **Pappa** och **Mamma** för att ni alltid varit stolta över mig, funnits där och inspirerat mig, och **Hille** och **Sara**, utan er hade livet varit väldigt tomt och tråkigt, är så glad för allt roligt vi har tillsammans och alla underbara barn ni har ♥

Sist, men egentligen mest, tack min älskade familj, **Vilmer** och **Teodor**, ni är min lycka och glädje, älskar er till stjärnstopp. **Linus**, tack för allt stöd, det har varit ovärderligt under den här sista intensiva tiden, tack för att du är

min klippa och trygghet, för att du alltid får mig att skratta och för att du verkligen lyssnar. Jag älskar dig ♥

Finally - If I forgot to mention you, please know that you are in my heart, though, unfortunately, at the moment, not in my tired head.



# Appendix

These two questionnaires were used in the study for Papers II and III in order to retrieve some information from the owners regarding the dogs' wellbeing and behavior while being fed the three diets. They also provided information about compliance and other factors which might have affected the results, such as medications.

**Questions for animal owners post-trial period nr: \_\_\_ Diet: \_\_\_ Date: \_\_\_\_\_**

Dog's name: \_\_\_\_\_

Your answers should be based on your experience during the last two weeks of the trial, compared to pre-trial, unless otherwise stated.

My dog's appetite has been:

                                                                                         
Very decreased      Decreased      Unchanged      Increased      Very increased

Optional comment: \_\_\_\_\_

My dog's behaviour regarding begging for food has been:

                                                                                         
Very decreased      Decreased      Unchanged      Increased      Very increased

Optional comment: \_\_\_\_\_

My dog's urge to look for extra food has been:

                                                                                         
Very decreased      Decreased      Unchanged      Increased      Very increased

Optional comment: \_\_\_\_\_

My dog's average fecal score, in my opinion. (See attached score sheet)

\_\_\_\_\_

Variation of fecal score, lowest to highest. (If applicable)

\_\_\_\_\_

My dog's defecation frequency has been:

Very decreased     Decreased     Unchanged     Increased     Very increased

Optional comment: \_\_\_\_\_

My dog's fecal volume has been:

Very decreased     Decreased     Unchanged     Increased     Very increased

Optional comment: \_\_\_\_\_

My dog's physical activity has been:

Very decreased     Decreased     Unchanged     Increased     Very increased

Optional comment: \_\_\_\_\_

My dog's energy level has been:

Very decreased     Decreased     Unchanged     Increased     Very increased

Optional comment: \_\_\_\_\_

During the final three days of the trial, has your dog eaten anything other than the trial food? Please list any other intake.

\_\_\_\_\_  
Compared to before the study, how much extra energy in the form of treats, chew bones, food scraps etc. has the dog eaten in the last 2 weeks (excluding the last 3 days)?

Very decreased     Decreased     Unchanged     Increased     Very increased

Optional comment: \_\_\_\_\_

Has your dog been given any medication or supplement during this trial period? Please list any medication or supplement.

\_\_\_\_\_

Please share any observations you deem significant during this trial period.

\_\_\_\_\_

**Post-trial questions**

**Date:** \_\_\_\_\_

Dog's name: \_\_\_\_\_

Did you find that any of the trial foods made your dog more satisfied/more full than any of the other trial foods? If so, which food and in what way did it manifest itself?

\_\_\_\_\_

Did you find that any of the trial foods seemed more/less palatable than another did? If so, which one?

\_\_\_\_\_

Did you find that your dog's stomach was better or worse from any of the trial foods? If so, which one and how?

\_\_\_\_\_

Other reflections on differences between the trial foods?

\_\_\_\_\_

Would you consider continuing giving your dog any of the foods used during the trial periods? If yes – which one? If no – why not?

\_\_\_\_\_







RESEARCH

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# Effects of rye inclusion in dog food on fecal microbiota and short-chain fatty acids

Hanna Palmqvist<sup>1</sup>, Sara Ringmark<sup>2</sup>, Katja Höglund<sup>2</sup>, Erik Pelve<sup>2</sup>, Torbjörn Lundh<sup>1</sup> and Johan Dicksved<sup>1\*</sup>

## Abstract

**Background** Rye intake has been associated with beneficial effects on health in human interventions, possibly due to dietary fiber in rye. In dogs, few studies have explored the effects on health of dietary fiber in general, and rye fiber in particular. The aim of this study was to investigate how inclusion of rye, compared with wheat, influenced fecal microbiota composition, short chain fatty acids (SCFA) and apparent total tract digestibility (ATTD) in dogs. Six male Beagle dogs (mean age 4.6 years, SEM 0.95 years; mean body weight 14.6 kg, SEM 0.32 kg) were fed three experimental diets, each for 21 days, including an adaptation period of six days and with 2–2.5 months between diet periods. The diets were similar regarding energy and protein, but had different carbohydrate sources (refined wheat (W), whole grain rye (R), or an equal mixture of both (RW)) comprising 50% of total weight on a dry matter (DM) basis. The diets were baked and titanium dioxide was added for ATTD determination. Fecal samples were collected before and in the end of each experimental period. Fecal microbiota was analyzed by sequencing 16S rRNA gene amplicons and fecal SCFA by high-performance liquid chromatography. Crude protein, crude fat, neutral detergent fiber, and gross energy (GE) in food and feces were analyzed and ATTD of each was determined. Univariate and multivariate statistical methods were applied in data evaluation.

**Results** Faecal microbiota composition, differed depending on diet ( $P=0.002$ ), with samples collected after consumption of the R diet differing from baseline. This was primarily because of a shift in proportion of *Prevotella*, which increased significantly after consumption of the R diet ( $P<0.001$ ). No significant differences were found for SCFA, but there was a tendency ( $P<0.06$ ) for higher molar proportions of acetic acid following consumption of the R diet. The ATTD of crude protein, crude fat, neutral detergent fiber, and GE was lower after consumption of the R diet compared with the other diets ( $P<0.05$ ).

**Conclusions** Consumption of the R diet, but not RW or W diets, was associated with specific shifts in microbial community composition and function, but also with lower ATTD.

**Keywords** Arabinoxylan, Canine, Diet, Fiber, *Prevotella*

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## Background

The composition of gut microbiota and its metabolic activities have significant impacts on the health of the host [1]. The gut microbiota is essential for several metabolic functions, such as degradation of dietary fiber, production of vitamins, and biotransformation of bile acids. It is also important for development of the immune system and in preventing colonization by pathogenic bacteria, among many other vital functions [2]. Specific members of the microbiota are known to be key species in this host-microbe interface, e.g., fiber-fermenting bacterial species such as members of the *Ruminococcaceae* family and *Prevotella* have been associated with healthy intestinal and metabolic status in both dogs and humans [3–6].

Dietary fiber escapes enzymatic digestion and absorption in the small intestine, and thus reaches the large intestine relatively unchanged. Dietary fiber includes both soluble and insoluble fiber [7]. Soluble fiber, e.g., arabinoxylan,  $\beta$ -glucan, and fructans, is readily fermented by the gut microbiota and is currently attracting attention due to its ability to create a healthy intestinal environment and metabolic homeostasis, and to its satiating effects [8]. The mechanisms behind these effects are not fully understood, but several factors seem to be involved. One factor is production of short-chain fatty acids (SCFA), primarily acetate, propionate and butyrate, as specific metabolites during bacterial fermentation [9]. Butyrate is the preferred energy source of colonocytes and is vital for their barrier function [10]. Moreover, SCFA lower the pH in the colon, favoring potentially beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* [11, 12], while also restricting proliferation of bacteria associated with gastrointestinal disease in dogs, such as *Clostridium perfringens* and *Escherichia coli* [3, 4]. SCFA have been shown to increase satiety, both directly through central appetite regulation [13] and indirectly by stimulating release of satiety hormones [14, 15]. The species composition of microbiota is a key determinant for the type and levels of SCFA produced during fermentation of dietary fiber, but SCFA production is also dependent on the type and solubility of dietary fiber [16].

Whole grain cereals are rich in dietary fiber. In particular, rye, a cereal crop widely grown in Scandinavia, has a high content of dietary fiber, with arabinoxylan constituting the major part [17]. Studies on humans and pigs have shown effects of whole grain dietary fiber on microbiota, SCFA, and host metabolism, and associated health benefits [18, 19]. Whole grain rye products in particular are reported to be associated with these effects [19–22].

Some studies in dogs have investigated effects on the gut microbiota of consumption of high-carbohydrate or high-protein diets [23, 24], but these studies have mainly

focused on the dietary starch content. Other studies have investigated the effects on microbiota and fermentation profile of specific fiber types, often included as dietary supplements [25, 26]. However, few studies have investigated the effects of whole grain in general on canine gut microbiota and their fermentation characteristics and, to our knowledge, only one published study has used rye as a carbohydrate source [27]. Given the interesting effects on gut microbiota and metabolism reported after rye inclusion in human interventions, it is relevant to study the effects of rye inclusion in dog food.

The aim of the present study was to investigate the effects of inclusion of rye, compared with wheat, in dog food on fecal microbiota, production of SCFA, and apparent total tract digestibility (ATTD).

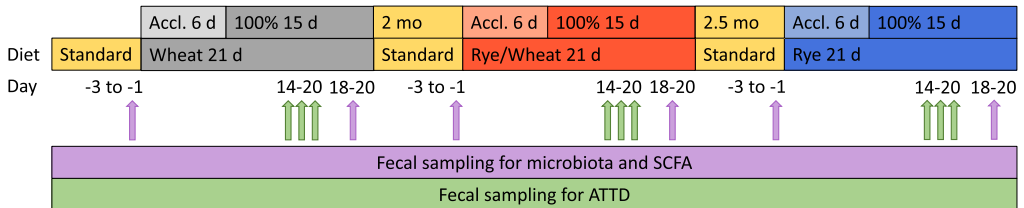
## Methods

### Animals and housing

Six male purpose-bred Beagle dogs aged 1 to 7 years (mean 4.6 years, SEM 0.95 year) were subjected to three experimental diet periods with three different carbohydrate sources: wheat (W), mixed rye/wheat (RW), and rye (R). Mean body weight at the start of the study was 14.6 kg (SEM 0.32 kg, total range 13.6–15.7 kg) and mean body condition score (BCS) on a 9-point scale [28] was 5.3 (SEM 0.21, total range 5–6). Body weight and BCS were recorded once per week during the diet periods. The dogs were housed according to their regular routines, in indoor pens during evening and night and in outdoor pens, on gravel, in daytime. They were divided into one group of four and one group of two dogs, in accordance with their normal living conditions. Mean daytime outdoor temperature in the three periods was: Diet W: +4.4 (range -4.4 to +9.5) °C; diet RW: -4.0 (range -14.3 to +2.1) °C; and diet R: +18.5 (range +5.8 to +27.3) °C. Before the experiment started and between diet periods, the dogs were fed a standard commercial dog food with the following nutrient content on a dry matter (DM) basis: protein 23.1%, fat 16.2%, crude fiber 1.7%, and ash 5.1% (Science Plan, Medium, Adult, Advanced Fitness; Hills Pet Nutrition Inc., Topeka, KS, USA). Metabolizable energy (ME) content was 15.6 MJ/kg of food.

No signs of disease were detected in routine hematological and biochemical blood analyses (alanine aminotransferase, albumin, protein, creatinine, C-reactive protein) performed before the first diet period. Before each diet period, all dogs underwent physical examination [29] by the same veterinarian and were assessed as healthy. None of the dogs had been treated with any antimicrobial drugs during the six months preceding the study. All dogs were dewormed with Milbemax vet. (milbemycinoxim/prazikvantel) (Elanco, Stockholm, Sweden) prior to the experiment.





**Fig. 1** Flowchart of the study design. All dogs ate the three diets in the same order, with a washout period of their standard food in between. Three to one days before each diet period, one fecal sample per dog was taken to establish a baseline value of microbiota and short-chain fatty acids (SCFA) for that specific period. In the last week of each diet period fecal samples were collected for analysis of microbiota, SCFA and apparent total tract digestibility (ATTD). Accl = acclimatization period

**Table 1** List of ingredients used in the experimental diets (g/kg)

Ingredient	Wheat (Diet W)	Rye/Wheat (Diet RW)	Rye (Diet R)
Rye meal, whole grain	0	132	264
Wheat flour, white	269	135	0
Beef meat	213	196	182
Chicken meat	328	348	366
Fish oil	13	12	12
Rapeseed oil	0	4	6
Water	133	131	128
Titanium dioxide	2	2	2
Vitamin	21	20	20
Mineral	21	20	20

**Study design**

Because the dogs were housed in groups and were known to consume feces occasionally, a cross-over study design was not possible. Thus it was decided to subject all dogs to the three different diets in the same order. An outline of the study design is shown in Fig. 1. The W diet was fed in the first period and the order of the two other diets was randomized to RW followed by R. Each diet period

started with a six-day acclimatization period, in which the experimental diet was mixed with the dogs’ standard food in increasing amounts. The experimental diet was then fed to the dogs at 100% for 15 days. The diet periods were separated by 2–2.5 months, during which the dogs were fed their standard commercial food as described above.

**Diets and feeding**

The experimental diets (ingredient list in Table 1, composition in Table 2) were designed to be as similar as possible in terms of energy and protein, but with differences in type of carbohydrates. Modified Atwater factors (protein 14.6 kJ/g, fat 35.6 kJ/g, nitrogen free extract 14.6 kJ/g) [7] were used to estimate the ME of the diet ingredients before formulation. Portion size was calculated based on this estimation. In all diets, the carbohydrate source consisted of food-grade flour (Kungsörnen, Sweden). The W diet contained refined wheat flour, the RW diet a mixture of coarsely ground whole grain rye meal and refined wheat flour, and the R diet rye meal alone. The flours were added in the respective diets to comprise 50% of weight (DM basis). In the mixed (RW) diet, the two flours were added to comprise 25% each of

**Table 2** Chemical composition (% of dry matter) and energy content (MJ/kg dry matter) of the experimental diets

Diet	Wheat (Diet W)	Rye/Wheat (Diet RW)	Rye (Diet R)
Dry matter, %	49.9	51.4	49.6
Crude protein, %	22.5	20.8	20.5
Crude fat, %	29.5	32.3	31.1
N-free <sup>1</sup> extract, %	43.3	41.4	42.2
Crude fiber, %	0.58	0.89	1.24
Neutral detergent fiber, %	2.9	6.3	6.6
Ash, %	4.1	4.6	5
Gross energy, MJ/kg	24.7	24.6	24.5
Metabolizable energy <sup>2</sup> , MJ/kg	21.3	21.2	21.0
Metabolizable energy <sup>2</sup> , MJ/kg food	10.6	10.9	10.4

<sup>1</sup> Nitrogen-free extract (calculated)

<sup>2</sup> Metabolizable energy calculated in accordance with NRC[7]

DM. In all diets, the flour was mixed with minced beef (Bravo Hundfoder AB, Klippan, Sweden) and chicken (OY MUSH Ltd, Jakobstad, Finland), water, and rapeseed oil (in the RW and R diets to balance the energy) in an industrial mixer (Elektro Helios, Stockholm, Sweden). For measurement of ATTD, the inert marker titanium dioxide ( $\text{TiO}_2$ ) was added in an amount of 2 g/kg food. The mixture was spread on an oven tray and baked for 25–30 min at 180 °C in a steam oven to a core temperature of 90–100 °C. The baked food was allowed to cool at room temperature for an hour. To compensate for water loss during cooking and cooling, the baked food was then weighed and cold water was added to make up for the difference in weight compared with before cooking. Finally, the food was mixed with fish oil, vitamin premix (WorkingDog Multivitamin, Trikem AB, Malmö, Sweden; for composition see Supplementary Table 1, Additional File 1), and minerals (Calphosum D, Aptus, Orion Pharma Animal Health, Danderyd, Sweden; for composition see Supplementary Table 2, Additional File 1), portion-packaged, and immediately frozen to -20 °C. Before feeding, the food was thawed in a refrigerator.

Daily energy requirement for each dog was calculated according to National Research Council [7] recommendations for the average laboratory kennel dog, i.e., 543 kJ ME/kg metabolic body weight ( $\text{BW}^{0.75}$  kg), based on estimated ideal body weight of the dogs at the beginning of the first diet period. The food was divided into two meals per day, offered to the dogs at approximately 08.00 h and 16.00 h. Before feeding, the dogs were moved to individual indoor pens, where they were allowed to eat for 10–15 min and then returned to the kennel. Water was made available ad libitum. Since the capability of the dogs for digestion of rye was not known, an ethical exclusion limit was set at maximum 5% weight loss per week, or 10% during the complete diet period, or a two-point decrease in BCS over two weeks, along with a limit of three days of total food refusal. In order to avoid interference with dietary effects on gut microbiota or ATTD, the intention was to keep the portion size unchanged during the diet periods. However, for four dogs during the RW period and one dog during the R period, the portion size had to be increased in the last three days of the period to maintain body weight above exclusion level (by 25% for RW and by 10% for R).

#### Fecal samples

Fecal samples for analysis of microbiota composition and SCFA were collected immediately after voiding, once at 1–3 days before the start of each period to get baseline values, and once during the last three days of each experimental period. At sampling, care was taken not to collect feces that had been in contact with the ground. Samples

were stored at -80 °C until analysis. Fecal samples for determination of ATTD were collected once a day for three days in the middle of each diet period, except from one dog in the W period and one dog in the R period, where only two samples could be collected. These samples were kept at -20 °C until analysis. Fecal consistency was assessed on a scale from 1 to 5, where 1 was described as very loose (diarrhea), 5 was dry and hard, and 4 was assessed as optimal (fecal scoring system for dogs; Royal Canin SAS 2013, [www.royalcanin.ca](http://www.royalcanin.ca)). Scoring was performed immediately after voiding, by either the first author or the animal keeper, during walks or in the morning when the dogs were released into their outdoor pen.

#### Microbiota analysis

DNA was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol, but with the modification of using bead-beating to break down bacterial cell walls. The bead-beating step was carried out by adding 0.3 g sterilized 0.1 mm zirconia/silica beads to the samples and running them in a Precellys24 sample homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France), 6500 rpm, for 2×1 min. The extracted DNA was kept at -20 °C until further analysis. PCR amplicons were generated from the V3-V4 region of the 16S rRNA gene using the primers F341 and R805 and with Phusion<sup>®</sup> High-Fidelity PCR chemistry (Thermo Fisher Scientific Inc., Waltham, MA, USA). The first PCR run was initiated with denaturation at 98 °C for 30 s, followed by 30 cycles with denaturation at 98 °C for 10 s, hybridization at 60 °C for 30 s, and elongation at 72 °C for 4 s, and the run was ended with a final elongation at 72 °C for 2 min. Amplicons were cleaned using Agencourt AMPure XP magnetic beads according to the manufacturer's instructions (Beckman Coulter Inc., Bromma, Sweden). In the second PCR run, forward and reverse barcode primers were added to barcode each sample individually. The primers contained both barcode and Illumina adaptor sequences and the PCR amplicons were generated with Phusion<sup>®</sup> High-Fidelity Master Mix (Thermo Fisher Scientific Inc.). The conditions for the second PCR run were the same as for the first except for a 5 s elongation in each cycle and 10 cycles in total. Amplicons were then cleaned as after the first PCR. The samples were quantified using a Qubit<sup>®</sup> 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific Inc.), and samples were then pooled in equimolar amounts. Amplicons were sequenced on the MiSeq Illumina platform, using the v3 kit (2×300 bp) at the National Genomics infrastructure (NGI) hosted by SciLifeLab, Solna, Sweden.

The raw sequence dataset contained in average 84,397 (inter quartile range: 63,114 to 102,119) paired sequences/sample. The amplicon sequences were analyzed with the software Mothur v.1.41.0 [30]. Paired-end reads joined by the 'make.contigs' command were filtered by the 'screen.seqs' command to remove sequences deviating from the 90% majority of sequences with regard to overlap length and number of mismatches, as well as minimum and maximum length of the joined reads. In addition, sequences with homopolymers larger than eight nucleotides and sequences with ambiguous bases were removed. The sequences were aligned to the Silva database version 132 [31], and the aligned sequences were filtered to remove sequences deviating from the 90% majority with regard to start and end position of the aligned sequences compared with the reference. Classified sequences (cutoff value 80) were filtered to remove hits to chloroplasts, mitochondria, and eukaryotes, and unknown hits. The filtered dataset contained in average 54,676 (inter quartile range: 40,243 to 65,363) merged sequences/sample. Phylotypes were produced with the phylotype, make.shared, and classify.otu commands.

#### Short-chain fatty acid analysis

Three SCFA (acetate, propionate, and butyrate) were analyzed in 0.5 g fecal matter dissolved in 1 mL 5 mM H<sub>2</sub>SO<sub>4</sub>, as previously described [32], using a high-performance liquid chromatography system consisting of an Alliance 2795 separation module and a 2414 RI Detector (Waters Corp. Milford, MA, USA). Column packet ReproGel H 9μ 300\*8 mm was used as the separation column and a ReproGel H, 9μ 30\*8 mm (Dr. A. Maisch, Ammerbuch, Germany) was used as a pre-column. Due to interference from ingested gravel in some fecal samples, the concentrations of the individual SCFA were converted to proportions before statistical analysis.

#### Analysis of food and feces

The experimental food and fecal samples were dried in a freeze dryer for 72 h before further analysis. For DM determination, the samples were dried at 103 °C for 16 h, followed by cooling in a desiccator before weighing [33]. Ash was determined by incinerating the dried samples in an oven at 550 °C for three hours and then cooling in a desiccator before weighing. Total nitrogen was determined according to the Kjeldahl method [34], using a 2020 digester and a 2400 Kjelltec analyzer (FOSS Analytical A/S, Hilleröd, Denmark), and crude protein was calculated as N×6.25. Crude fat was analyzed according to Commission Directive EC/152/2009 [35] using a Soxtec extraction unit (FOSS Analytical A/S, Hilleröd, Denmark). Crude fiber was analyzed by boiling a sample first in H<sub>2</sub>SO<sub>4</sub> solution and then in KOH

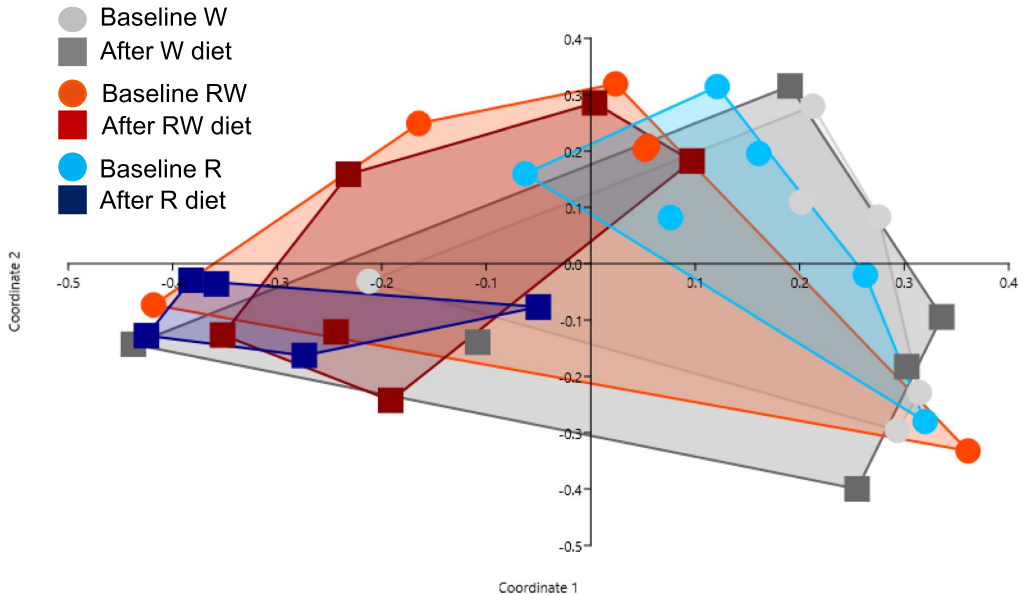
solution [36]. Neutral detergent fiber was analyzed as previously described [37]. Gross energy (GE) was measured on a Parr isoperbol Bomb Calorimeter 6300 (Parr Instrument Company, Moline, Illinois, USA). Titanium dioxide was analyzed according to Short et al. [38] and ATTD was calculated as: ATTD (%) = 100 - [(% TiO<sub>2</sub> in food / % TiO<sub>2</sub> in feces) × (% Nutrient in feces / % Nutrient in food) × 100] [26].

#### Statistical analyses

Principal coordinate analysis (PCoA) based on Bray Curtis distances and analysis of similarity (ANOSIM) was used to analyze microbial community structure and evaluate the effects of the dietary interventions on the microbiota. A similarity percentage test was used to identify taxa primarily responsible for differences between groups identified in ANOSIM analysis. To assess changes in relative abundances of microbial taxa due to the intervention, analysis of variance (ANOVA) was performed with a linear mixed effects model fitted with diet, time, and the interaction diet:time as fixed effects and dog as random effect. The time factor had two levels denoting if samples were collected before or after the diet period. The model for each taxon was checked with diagnostic plots for homoscedasticity and normality. If the model did not meet the criteria, but a model with natural logarithm-transformed data did, the latter was used instead. *Post-hoc* comparisons of estimated marginal means were only made between baseline and post-diet samples within each diet, or between different diets but at the same time point. These comparisons were made with Tukey's adjustment. Only microbial taxa with a total mean relative abundance of minimum 1% were analyzed. A linear mixed effects model with a similar structure was used to test for differences between molar proportions of SCFA. For ATTD, verification of equal body weight and BCS at the start of each period, and fecal score and DM, a linear mixed effects model was fitted with diet as fixed effect and dog as random effect. The PCoA and ANOSIM analyses were carried out using the software Past [39] and the linear mixed effects models using the lmer-function in R [40]. Differences were regarded as significant at P ≤ 0.05.

#### Results

All dogs but one completed all three diet periods. One dog lost more weight than the set limit of 5% in one week when fed the R diet, and was excluded from that period. All dogs but one ate all food provided during all periods. The dog that refused some food primarily did so in the morning and mostly during the R diet period. To compensate, that dog was offered up to 50% extra at the afternoon meal, but still ate 23% less during the period than the calculated requirement. Mean food consumption per



**Fig. 2** Principal coordinate analysis (PCoA) plot of fecal microbial composition before (baseline) and after each experimental diet. Baseline samples are represented by point symbols and samples collected after the diets are represented by filled squares. Diet periods are represented by different colors: grey = wheat diet (W); red = rye-wheat diet (RW); blue = rye diet (R)

kg body weight and day for the periods was: W 24.6 g (range 23.4–25.7 g), RW 26.3 g (range 24.6–27.7 g), and R 24.4 g (range 21.1–26.1 g). Mean weight loss per diet period ranged from 1.8% to 4.0% (values from excluded dog in R diet not included). Body weight and BCS at the start and end of each diet period are presented in Supplementary Table 3 and Supplementary Table 4, respectively, in Additional File 2. Body weight and BCS at the start of each diet period did not differ between periods. We discovered small amounts of gravel in some of the fecal samples, likely after ingestion in the outdoor pen.

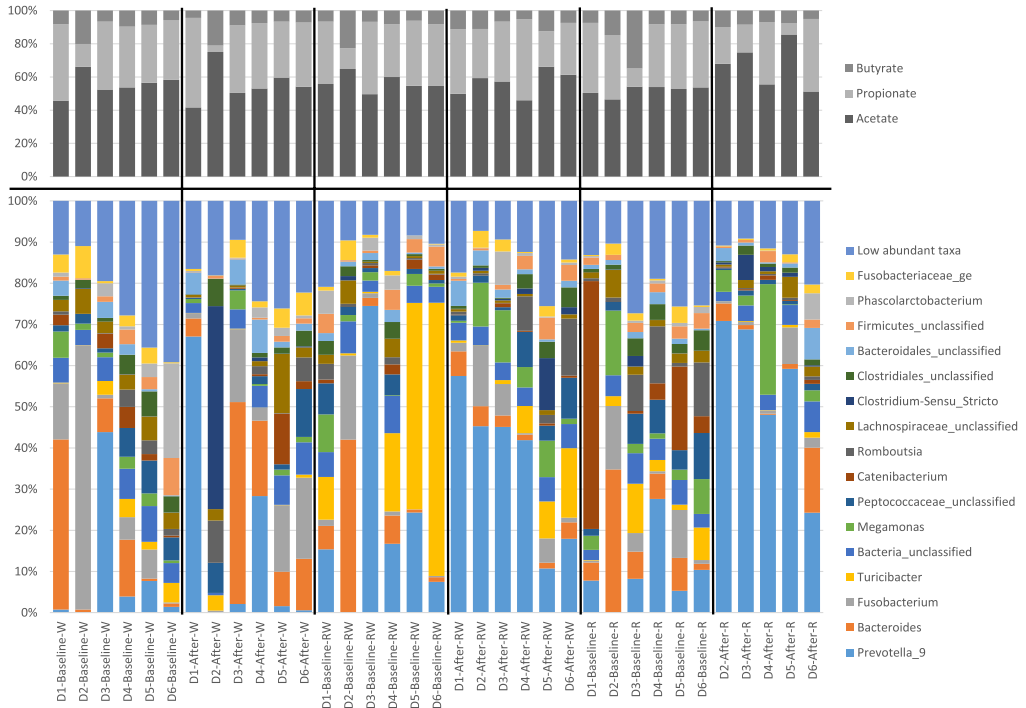
**Microbiota**

Microbial composition in the baseline fecal samples did not differ significantly between the three periods according to PCoA and ANOSIM (Fig. 2). However, there was variation between individual dogs as well as temporal variation between individual samples within dogs (Fig. 3). Following both the R and RW diet periods, *Prevotella* was the overall most abundant genus in feces and clearly dominant (mean 54% and 36% of sequences, respectively). PCoA and ANOSIM revealed differences in microbial composition linked to diet (ANOSIM;  $P=0.002$ ,  $R=0.19$ ). There were no differences when comparing baselines and samples collected after the W

and RW diet periods, but in dogs fed the R diet microbiota composition in feces differed from baseline (ANOSIM;  $P=0.014$ ,  $R=0.67$ ) (Fig. 2). Similarity percentage test revealed that the taxa of highest importance for the observed difference following the R diet were: *Prevotella*, *Catenibacterium*, *Bacteroides*, *Romboutsia*, and *Megasphaera* (Fig. 4).

ANOVA was performed on 22 taxa detected in mean relative abundance >1%. This represented in total 86% of the generated sequences. Fourteen of these taxa displayed differences in at least one effect in ANOVA (Table 3). Natural logarithm-transformed values were used for 16 taxa.

Relative abundance of *Prevotella* in feces increased numerically after all diet periods, but the difference was only significant after the R diet period ( $P<0.001$ ). Relative abundance was also higher following the R diet compared with the W diet ( $P<0.007$ ). Furthermore, diet had an effect from baseline to after diet, i.e., the interaction was significant, for *Romboutsia* and for an unclassified member of the family *Peptostreptococcaceae*, with abundance of both decreasing after the R diet period ( $P<0.02$  for both). For the other taxa with significant changes detected in ANOVA, differences were found for time and in some instances also for diet, but not for



**Fig. 3** Barcharts showing relative proportions of short chain fatty acids (upper barchart) and microbiota (lower barchart) for individual samples. D1-D6 in sample legends represent the individual dogs whereas “baseline” and “after” indicate if it was before or after the diet period. For easier interpretation, only taxa with an average relative abundance > 2% are shown in the figure. All taxa with lower average relative abundance are shown as low abundant taxa. W = wheat diet, RW = diet with equal mixture of whole grain rye and refined wheat, R = whole grain rye diet

their interaction. *Prevotella/Bacteroides* ratio showed a tendency for differentiation depending on diet and time ( $P < 0.08$  and  $P < 0.06$ , respectively), but not for their interaction. Firmicutes/Bacteroidetes ratio was different for time ( $P < 0.05$ ), but the interaction effect was not significant.

**Short-chain fatty acids**

The relative proportions of acetate, propionate, and butyrate in feces showed no overall differences for any of the diets ( $P > 0.05$ ), but for acetic acid there was a tendency for a difference in time ( $P < 0.08$ ) and for the interaction between time and diet ( $P < 0.06$ ) (Fig. 3 and Fig. 5). *Post-hoc* comparisons of the marginal means with Tukey’s adjustment revealed higher molar proportions of acetic acid after the R diet (mean before 52.2% and SEM 1.4%, mean after 67.0% and SEM 6.3%;  $P = 0.005$ ).

**Apparent total tract digestibility**

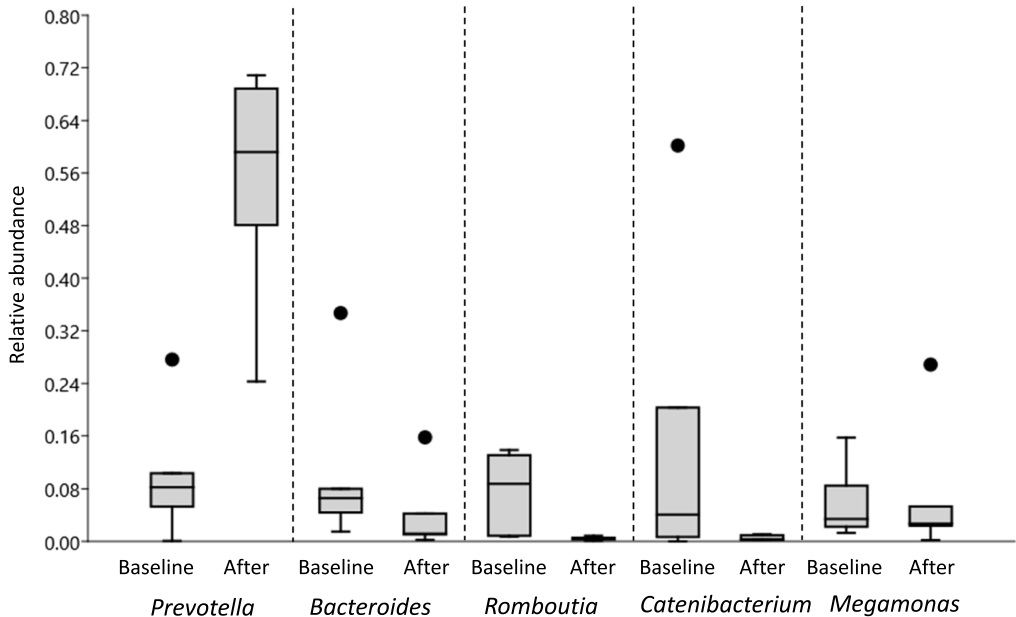
Mean ATTD of crude protein, crude fat, neutral detergent fiber, and GE was lower in all cases for the R diet compared with the other two diets, while the RW diet only differed from the W diet in terms of lower ATTD for GE (Table 4).

**Fecal score and dry matter**

Mean fecal score for the last seven days of each diet period did not differ between diets (W: mean 3.5, SEM 0.2, range 1–5; RW: mean 3.6, SEM 0.2, range 2.5–5; R: mean 3.1, SEM 0.2, range 2–4). Mean fecal DM also did not differ between diet periods (W: mean 32.1, SEM 5.6, range 20.4–41.8%, RW: mean 28.0, SEM 0.6, range 26.2–29.6%, R: mean 32.3, SEM 1.9, range 27.5–38.1%).

**Discussion**

This study investigated how diets with different inclusion levels of whole grain rye influenced fecal microbiota composition, SCFA profile, and ATTD in dogs. The main



**Fig. 4** Box plots showing relative abundance, at baseline and after the R diet, of the top five taxa of importance for the difference seen in analysis of similarity (ANOSIM), according to similarity percentage tests. The box represents the 25–75 percentage quartiles and the horizontal line in the box represents the median value. Whiskers represent minimum and maximum values and dots represent outliers

differences were observed with the highest inclusion level of rye (50% of DM), which caused a change in microbial composition, mainly due to an increase in the relative abundance of *Prevotella*.

Multivariate analysis showed a difference in microbial composition after the R diet compared with the other diets. This difference was primarily driven by an increase in *Prevotella* and a decrease in *Catenibacterium*, *Bacteroides*, *Romboutsia*, and *Megamonas* (Fig. 4). Univariate analysis showed that the relative abundance of several taxa changed significantly between the baseline and after the diet periods in general. For *Prevotella*, *Romboutsia*, and an unclassified member of *Peptostreptococcaceae*, the model revealed that the changes were significant only for the R diet. The detection of higher relative abundance of *Prevotella* is in line with findings in a previous study on humans examining the effect of rye-kernel bread versus white wheat bread on gut microbiota [41]. That study found that *Prevotella* increased after rye bread compared with white wheat bread consumption, but that *Bacteroides* showed a tendency to decrease with rye bread consumption compared with wheat bread. In a study on pigs, increased abundance of *Prevotella* following consumption of an arabinoxylan-rich diet, although derived from

wheat, has been reported [42]. A recently published study on Beagle dogs fed a vegetarian diet supplemented with feather meal and either corn meal, rye, or fermented rye did not find any significant influence on microbiota composition, but addition of rye, fermented or not, increased the proportion of the phylum Bacteroidetes, in particular in some dogs [27]. *Prevotella* is a dominant member of the Bacteroidetes phylum, and thus our results point in the same direction as those in that study. Our finding of increased relative abundance of *Prevotella* after the R diet is interesting, since a previous study in humans and mice found that high abundance of *Prevotella* had a favorable impact on glucose metabolism when the test subjects consumed a fiber-rich diet compared with a refined carbohydrate product [6]. It would be interesting to investigate, using a larger sample, whether this connection also exists in dogs.

Since the abundance of the different taxa was measured as relative proportions, it is plausible that the difference seen in PCoA and ANOSIM was driven by the large increase in *Prevotella* after the R diet and that the reductions in the other taxa were mainly a consequence of that. However, there was large variation in relative abundance of taxa, both between and within the dogs,

**Table 3** Mean microbial relative abundance at baseline when the dogs were fed a commercial diet and after each three week experimental diet period

	Wheat (Diet W)		Rye/Wheat (Diet RW)		Rye (Diet R)		D <sup>1</sup>	T <sup>2</sup>	D:T
	Baseline	After	Baseline	After	Baseline	After			
<i>Prevotella</i> <sup>‡</sup>	9.6±6.9	16.6±11.0	23.1±10.8	36.4±7.4	9.9±3.8	54.3±8.5	*	**	*
<i>Bacteroides</i>	10.9±6.5	15.4±7.2	9.7±6.5	3.4±0.8	10.2±5.0	4.5±2.9			
<i>Fusobacterium</i> <sup>‡</sup>	15.3±10.0	9.7±3.7	4.1±3.3	5.3±2.2	5.6±2.6	2.7±1.6			
<i>Turicibacter</i>	2.4±0.8	0.8±0.6	24.5±11.3	5.7±2.7	4.4±1.9	0.5±0.3	**	**	
<i>Megamonas</i>	2.6±0.8	1.5±0.7	2.8±1.3	6.5±2.1	5.6±2.3	7.5±4.9			
Peptostreptococcaceae uncl	4.2±1.2	3.8±1.9	2.9±1.1	4.3±1.6	5.9±1.5	1.0±0.2		*	*
<i>Catenibacterium</i>	2.2±0.8	2.5±2.0	1.3±0.4	0.4±0.1	14.9±9.6	0.6±0.2		*	
<i>Romboutsia</i> <sup>‡</sup>	1.7±0.7	3.0±1.7	1.2±0.6	4.3±2.3	6.4±2.6	0.4±0.1			*
Lachnospiraceae uncl	4.2±0.6	3.7±2.2	2.3±0.9	0.8±0.1	2.8±0.8	2.1±0.8	*	*	
<i>Clostridium sensu stricto 1</i>	0.1±0.0	8.4±8.2	0.2±0.2	2.8±2.0	0.5±0.4	1.8±1.1		***	
Clostridiales uncl	3.1±0.9	2.2±1.0	1.8±0.7	2.3±0.8	2.9±0.7	1.2±0.3			
Bacteroidales uncl <sup>‡</sup>	1.8±0.7	3.8±1.3	1.4±0.4	2.5±0.8	1.4±0.3	2.6±1.4			
Firmicutes uncl	3.0±1.3	0.7±0.2	3.1±0.9	2.4±0.8	2.4±0.4	1.2±0.5		**	
<i>Phascolarctobacterium</i>	5.2±3.6	0.9±0.4	2.3±0.9	1.7±1.3	0.7±0.2	1.3±1.3	*	*	
<i>Fusobacteriaceae_ge</i> <sup>‡</sup>	3.3±1.1	2.8±1.0	1.3±0.7	1.9±0.6	1.6±0.6	1.0±0.4			
<i>Peptoclostridium</i> <sup>‡</sup>	2.7±0.6	0.7±0.3	1.4±0.6	0.9±0.3	2.8±0.7	0.5±0.1		***	
<i>Alloprevotella</i>	1.4±0.7	2.9±1.0	0.6±0.2	1.6±0.8	0.6±0.3	1.4±0.7			
Prevotellaceae uncl	0.7±0.4	1.8±0.9	1.0±0.3	1.8±0.6	0.5±0.2	1.7±0.6		*	
<i>Faecalibacterium</i>	3.5±1.6	0.04±0.0	0.6±0.2	0.1±0.0	2.4±0.9	0.4±0.2		***	
Clostridiaceae 1 uncl	0.1±0.0	2.6±2.4	0.3±0.2	2.3±1.3	0.6±0.5	1.2±0.6		**	
<i>Sutturella</i>	1.5±0.3	0.8±0.3	0.8±0.2	0.8±0.2	1.3±0.2	1.0±0.2			
<i>Anaerobiospirillum</i>	1.2±0.6	1.3±0.7	0.5±0.2	0.3±0.2	2.8±2.1	0.03±0.0		*	
<i>Prevotella/Bacteroides</i> ratio	3.4±2.1	2.9±2.5	13.9±7.0	13.0±3.9	2.5±1.1	67.4±35.8			
Firmicutes/Bacteroidetes ratio	9.5±5.0	80.4±73.3	2.6±1.2	1.5±0.7	3.6±0.9	0.4±0.1		*	

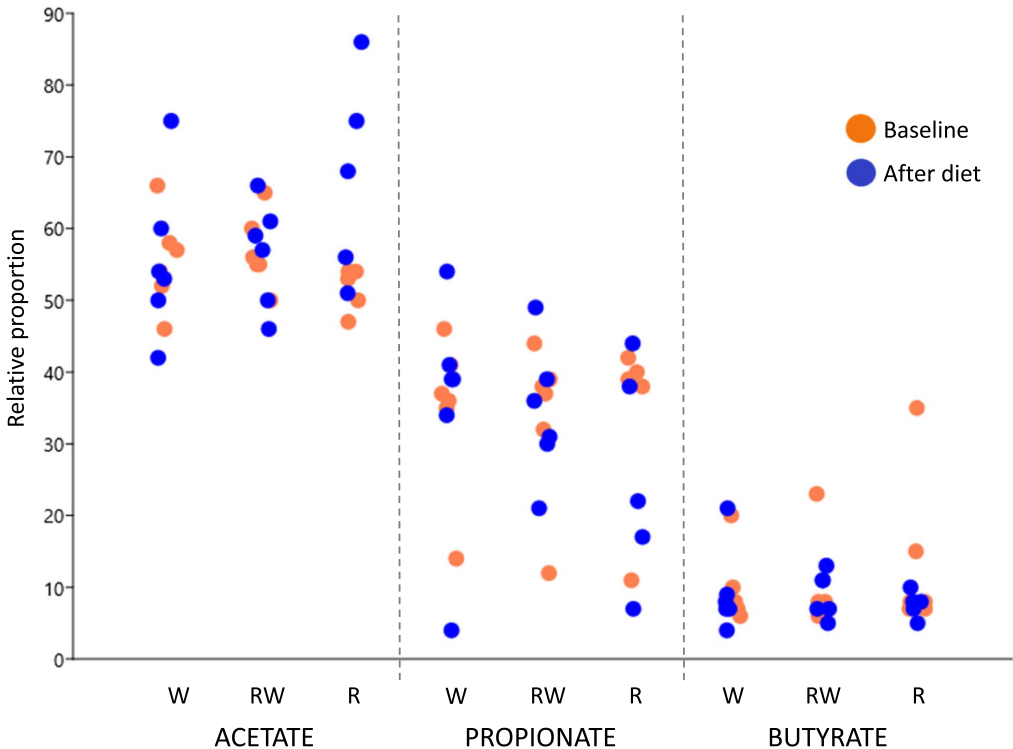
Data shown for taxa with total mean relative abundance > 1%. Values in % ± SEM. <sup>1,2</sup>Significant difference in diet (D) and/or time (T) and/or the interaction (D:T) in analysis of variance (ANOVA) at: \*P ≤ 0.05, \*\*P ≤ 0.01, \*\*\*P ≤ 0.001. The time factor had two levels denoting if samples were collected before or after the diet period. #Non-logarithmic values were used in the model, (uncl = unclassified)

which in combination with a small sample size probably masked other changes in microbial composition linked to the experimental diets.

Firmicutes/Bacteroidetes ratio and *Prevotella/Bacteroides* ratio have previously been shown to be affected by carbohydrate to protein ratio in the diet of humans [43, 44] and dogs [45]. In humans, the amount of complex carbohydrates is suggested to be the main influence [44]. Our study, although based on a limited number of animals, showed similar indications, with a lower Firmicutes/Bacteroidetes ratio after the R diet period and a tendency for higher *Prevotella/Bacteroides* ratio depending on diet and time. The change in *Prevotella* abundance was likely the main contributor to the changes in both ratios. The shift in ratio after the R diet period aligns well with the findings from the previous mentioned study [27], which also observed that inclusion of rye, but not corn, shifted the Firmicutes/Bacteroidetes ratio in favor of Bacteroidetes.

For the other taxa identified in this study, a few published papers have reported associations to dietary fiber. A decrease in *Catenibacterium* abundance was indicated as important for the difference in ANOSIM after the R diet, whereas a previous study in pigs observed an increase in *Catenibacterium* abundance after inclusion of oat bran in the diet [46]. *Megamonas* abundance has been found to increase in dogs fed a diet based on raw meat supplemented with inulin, compared with a control [47]. *Faecalibacterium*, which has previously been associated with fiber-rich diets [12, 48] and colonic health in dogs [3], decreased in relative abundance after all diets in our study.

The three experimental diets were designed to be as similar as possible in terms of fat content, but all experimental diets had a higher fat content than the dogs' standard diet. This change from the normal diet might have contributed to some of the changes detected in microbiota composition, as a previous study on dogs



**Fig. 5** Jitter plot showing molar proportions of acetate, propionate, and butyrate as proportion of total short-chain fatty acids (SCFA). There were no differences between diets ( $P > 0.05$ )

**Table 4** Emmeans of apparent total tract digestibility (ATTD), %  $\pm$  standard error

	Wheat (Diet W)	Rye/Wheat (Diet RW)	Rye (Diet R)	P-value
Crude protein	89.3 $\pm$ 0.79 <sup>a</sup>	87.0 $\pm$ 0.79 <sup>a</sup>	79.6 $\pm$ 0.87 <sup>b</sup>	< 0.001
Crude fat	96.8 $\pm$ 0.68 <sup>a</sup>	97.0 $\pm$ 0.68 <sup>a</sup>	95.7 $\pm$ 0.70 <sup>b</sup>	0.01
Neutral detergent fiber	69.9 $\pm$ 3.62 <sup>a</sup>	64.0 $\pm$ 3.62 <sup>a</sup>	34.0 $\pm$ 3.92 <sup>b</sup>	< 0.001
Gross energy	93.6 $\pm$ 0.66 <sup>a</sup>	90.5 $\pm$ 0.66 <sup>b</sup>	83.1 $\pm$ 0.74 <sup>c</sup>	< 0.001

<sup>abc</sup> ATTD values within rows with different superscript letters differ significantly between the diets ( $P < 0.05$ )

found that consumption of a high-fat, low-starch diet led to a decrease in *Prevotella* compared with a high-starch, low-fat diet [49]. However, the high fat content in the diets in our study did not seem to have a negative effect on *Prevotella*.

Statistical analysis of fecal SCFA indicated a tendency for an increased proportion of acetic acid depending on diet, which was due to a difference between baseline R diet and post R diet, but no other differences were found.

Acetate has been reported to suppress appetite in mice [13], indicating one possible way in which rye can promote satiety. *Prevotella*-dominant microbiota has been shown to correlate with increased relative production of propionate rather than acetate, although with some differences depending on substrate [16, 50]. However, high proportional acetate production has been seen in combination with *Prevotella*-dominated microbiota in finisher pigs fed pea fiber [51]. The total amount of SCFA



produced is also reported to depend on the dominant genera. *Prevotella*-dominated fecal inoculum has been found to increase the amount of SCFA in vitro compared with *Bacteroides* fermenting the same type of fiber, indicating higher fiber-utilizing capacity in *Prevotella* [16]. We expected total fecal SCFA to increase with increased inclusion of whole grain rye, due to the larger quantity of fermentable fiber. However, the absolute levels of SCFA could not be determined, because some of the dogs apparently ingested small amounts of gravel from their outdoor pen, which contaminated the fecal samples.

In the R diet period, ATTD was lower for all nutrients, including GE, compared with the other diet periods. This is in line with findings in a study on pigs comparing whole grain cereals from rye and wheat [52], where lower digestibility (both ileal and total tract) was seen for rye and attributed, in part, to higher viscosity. A study on dogs in which rye was added at 20%, as-is, to a basic diet found no differences in ATTD of crude protein or crude fat [53]. Dog foods containing other cereals have been observed to vary in digestibility of nutrients depending on carbohydrate source [54], with digestibility decreasing with increasing fiber inclusion [55]. The difference in ATTD between diets in our study may also have been due to the rye meal being more coarsely ground than the wheat meal. Our fecal collection period of three days, with only one sample per day, might also have added some uncertainty to the results. The European Pet Food Industry Federation [56] recommends total fecal collection over a period of four days, which was not practically possible in our case. The difference in ATTD could have been expected to affect fecal DM, but no such difference was detected. However, we observed more sand and gravel in the feces following the R diet, and it is possible that the fecal DM content in R period samples would have been lower without this contamination.

Most dogs accepted all diets but the subjects were laboratory Beagle dogs, which are accustomed to eat what they are offered, and dogs of other breeds or privately owned dogs might be more selective. However, all dogs lost weight during all periods, which can be attributed to a number of factors. Apart from differences in ATTD, we calculated the daily energy requirement of the dogs based on the National Research Council recommendation for the average laboratory kennel dog, without considering individual variation. In addition, the outdoor temperature differed between periods and might have affected energy expenditure to a considerable extent. That was the reason for the increment in the daily ration in the RW diet period, the coldest period. Since some adjustments were made to the daily portion size and the weather conditions were different, no statistical analysis was performed on the weight loss.

Fecal samples are often used in studies examining gut microbiota composition and function, due to the non-invasiveness and ease with which they can be collected. We were interested in the effects of different cereal carbohydrate sources on microbial composition and production of SCFA and ATTD, and we assumed that fecal samples would give a fair approximation of these effects. One limitation is that samples taken from different parts of the intestine often differ in microbial composition [57]. However, the differences between colon and rectum have been shown to be non-significant [58], which indicates that fecal samples give a good reflection of the colonic microbiota. The composition and amount of SCFA have also been shown to shift in samples collected along the large intestine of pigs [42], presumably due to colonic uptake of SCFA and depletion of fermentable substrate. To our knowledge, no study has compared SCFA content in cecum/proximal colon fecal samples from dogs. However, given the considerably smaller cecum and shorter colon of dogs compared with pigs, these considerations might not be completely applicable to dogs. Therefore, fecal samples may give a reasonable approximation of SCFA produced along the colon.

Our study was limited by the small sample size. Large inter-individual variation was likely one reason why some of the differences between diets did not prove statistically significant. Further, although we did not observe any coprophagia, it might have occurred. This could have affected the microbiota, SCFA, and ATTD. To mitigate these effects, we did not apply a cross-over design in the experiments, even though some of the effects seen could then have been due to seasonal fluctuations in the microbiota. However, we did not see any differences in the baseline samples that could explain the differences after the diet periods, indicating that the changes were indeed due to diet.

## Conclusions

A diet with 50% rye inclusion (by DM) led to an increase in fecal relative abundance of the genus *Prevotella*, accompanied by a decrease in *Catenibacterium*, *Bacteroides*, *Romboutsia*, and *Megamonas*. This microbial shift seemed to affect the fiber fermentation pattern, as the relative proportion of acetic acid increased compared with other measured SCFA. ATTD was lower for the diet with 50% rye inclusion, compared with the other diets, indicating that a somewhat lower inclusion rate might be advisable. As *Prevotella* has been linked to stability in glucose metabolism and acetic acid has been observed to suppress appetite in humans, further studies on the effects of rye on metabolism and appetite in dogs are warranted.

**Abbreviations**

ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
ATTD	Apparent total tract digestibility
BCS	Body condition score
PCoA	Principal coordinate analysis
SCFA	Short-chain fatty acid

**Supplementary Information**

The online version contains supplementary material available at <https://doi.org/10.1186/s12917-023-03623-2>.

**Additional file 1: SupplementaryTable 1.** Composition of WorkingDog Multivitamin (Trikem AB, Malmö, Sweden) in µg/mL, as stated by the manufacturer. **SupplementaryTable 2.** Composition of Calphosum D (Aptus, Orion Pharma Animal Health, Danderyd, Sweden) per gram, as stated by the manufacturer.

**Additional file 2: SupplementaryTable 3.** Dogbody weight in kg at end of the acclimatization period and at the end of each diet period.

**SupplementaryTable 4.** Dog body condition score (BCS) at the end of the acclimatization period and at the end of each diet period.

**Acknowledgements**

The computations/data handling were enabled by resources provided by the Swedish National Infrastructure for Computing (SNIC) at UPPMAX, partly funded by the Swedish Research Council through grant agreement no. 2018-05973. The authors would like to thank Emma Hörnebro for support in handling the dogs, Claudia von Brömsen for statistical support, and laboratory staff at the Department of Animal Nutrition and Management for help with laboratory analyses.

**Authors' contributions**

HP planned, organized, and carried out the study, performed parts of the laboratory analyses, and interpreted the results with guidance and help from JD, TL, KH, and SR. EP performed the bioinformatics analysis, JD performed the multivariate statistical analyses, and HP performed the univariate statistics. HP wrote the manuscript, with help from JD, and with guidance also from TL, KH, and SR. All authors have read and approved the final version of the manuscript.

**Funding**

Open access funding provided by Swedish University of Agricultural Sciences. This study was funded by the Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences.

**Availability of data and materials**

The datasets used and/or analyzed during the study are available from the corresponding author on reasonable request. The sequence data generated in this project has been deposited in the Sequence Read Archive (SRA): PRJNA816660.

**Declarations****Ethics approval and consent to participate**

The authors confirm that all methods were performed in accordance with relevant guidelines and regulations. The study was carried out in compliance with the ARRIVE guidelines and it was approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden, DNR 5.8.18–18808/2017.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

Received: 2 March 2022 Accepted: 3 May 2023

Published online: 10 May 2023

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# OPEN Effects of whole-grain cereals on fecal microbiota and short-chain fatty acids in dogs: a comparison of rye, oats and wheat

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Dietary fiber in dog food is reported to promote healthy gut microbiota, but few studies have investigated the effects of whole-grain cereals, which contain a variety of fiber types and other bioactive compounds. The aim of the present study was to compare the effects of diets containing whole-grain rye (RYE), oats (OAT) and wheat (WHE) on fecal microbiota and short-chain fatty acid production. Eighteen dogs were fed three experimental diets, each for four weeks, in a cross-over design. Fecal samples were collected at the end of each diet period. Analysis of 16S rRNA gene amplicons showed that family *Lachnospiraceae* and genus *Bacteroides* were the gut microbial groups most affected by diet, with lowest relative abundance following consumption of RYE and a trend for a corresponding increase in genus *Prevotella*\_9. Fecal acetate and propionate concentrations were higher after consumption of RYE compared with OAT. In conclusion, rye had the strongest effect on gut microbiota and short-chain fatty acids, although the implications for dog gut health are not yet elucidated.

Several studies in dogs have investigated potential promotion of a healthy gut microbiota by specific dietary fiber types<sup>1–3</sup>, fiber-rich food by-products, or fiber blends added to the food<sup>4–6</sup>. However, few studies have investigated how gut microbiota composition and function are affected by addition of fiber as part of the whole-grain component in dog food<sup>7,8</sup>. Whole grains contain several bioactive compounds, such as tocots, phytosterols, alkylresorcinols and other phenolic compounds, which can have additive or even synergistic effects with the gut microbiota on host health according to studies in humans and pigs<sup>9,10</sup>. Rye, wheat, oats and barley are the most commonly grown grain crops in the Nordic countries and all contain different combinations of dietary fiber and bioactive compounds. Rye and wheat grains have a high content of arabinoxylan, while oat and barley grains are high in  $\beta$ -glucans<sup>11</sup>. Rye has the highest content of fructan, phytosterols and alkylresorcinols and, together with wheat, the highest content of tocots<sup>10</sup>. Hence, it is likely that these grain types have different effects on the gut microbiota.

Arabinoxylan,  $\beta$ -glucan and fructans are soluble dietary fibers readily fermented by the gut microbiota, which in the process produce short-chain fatty acids (SCFA), primarily acetate, propionate and butyrate<sup>12</sup>. These SCFA serve as substrates in the metabolism and interact with receptors on the intestinal cells, which then release metabolic hormones<sup>13–15</sup>. Furthermore, SCFA are acidic, hence lowering the pH in colon. The low pH favors potentially beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus*<sup>16,17</sup>, and restricts proliferation of bacteria associated with gastrointestinal disease in dogs, such as *Clostridium perfringens* and *Escherichia coli*<sup>18,19</sup>. The absolute and relative amount of the different SCFA produced during fermentation of dietary fiber depends on the species composition of microbiota as well as the type and solubility of the dietary fiber<sup>20</sup>.

We have previously compared the effects of whole-grain rye and refined wheat on the fecal microbiota in dogs<sup>21</sup>. In that study, we observed an increase in genus *Prevotella* relative to baseline values when rye was included as 50% of dry matter in the diet. No such effect was observed when the dogs were fed a refined wheat diet. Some studies in humans have reported beneficial effects of whole grain in the diet on host metabolism, with or without major changes in gut microbiota<sup>22–24</sup>. However, as those studies compared whole-grain diets with refined cereal products, the results could not clarify whether differences observed were due to effects of the grain-specific fibers and bioactive compounds, or effects of a high-fiber versus a low-fiber diet. A few studies on humans

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have compared the effects of whole grains from different sources (mostly rye and wheat) on gut health-related parameters such as SCFA concentration and microbial community composition<sup>25,26</sup>. In dogs, such effects have been studied when feeding dietary oat groats or a number of other ancient grains<sup>8</sup>, but to our knowledge there is no previous comparative study on dogs fed diets including whole-grain wheat or rye. Therefore, the aim of this study was, to explore the effects of dietary inclusion of three different whole grain types (rye, oats and wheat) on fecal microbiota composition and SCFA production in dogs. Our hypothesis was that given the differences in fiber and bioactive component composition of the whole grains, they would promote different bacterial taxa and thus the microbial composition and their fermentative products would differ.

## Material and methods

This explorative, experimental, cross-over diet study was performed in August–December 2019. The dogs were privately owned and lived in their home environment throughout the study period. Examinations of the dogs and analyses were performed at the Swedish University of Agricultural Sciences in Uppsala, Sweden. The study was approved by the Ethics Committee for Animal Experiments, Uppsala, Sweden (Approval no. 5.8.18-18808/2017-7) and complied with ARRIVE guidelines<sup>27</sup>. Written informed consent was obtained from all dog owners before the start of the study. Information on the owners was handled in accordance with general European Union data protection regulations (Regulation (EU) 2016/679).

**Diets.** Three extruded experimental diets, containing whole-grain flour of rye (RYE), wheat (WHE) or ground rolled oats (OAT), were produced by the commercial dog food producer Doggy AB, Vårgårda, Sweden (Table 1).

The diets were nutritionally complete and balanced according to FEDIAF guidelines<sup>28</sup>. The level of grain inclusion was set at 25%, as fed, based on previous results<sup>21</sup>. The diets were also composed to be similar in terms of protein and metabolizable energy content (Table 2).

**Animals and study design.** Healthy dogs were recruited among staff and students at the Swedish University of Agricultural Sciences and the University Animal Hospital in Uppsala, Sweden. This population was chosen as these dog owners could be expected to have high treatment compliance and competence to follow study instructions due to their competence within veterinary medicine and animal science. The criteria for inclusion were a minimum age of 12 months and a minimum body weight (BW) of 7 kg. Exclusion criteria were: antibiotic treatment within three months prior to the study, known intolerance or allergy to any of the experimental food ingredients, or history of sensitivity to diet change. Before the first experimental diet period, all dogs underwent a health assessment, which included a physical examination (all performed by the same veterinarian), routine hematology, serum biochemical analysis and urine analysis (standard dipstick chemistry test, urine specific gravity and protein/creatinine ratio). Dogs were excluded if there were any clear findings indicating systemic or organ-related disease or if they had a gastrointestinal reaction to the diets that affected their general condition (mild, transient alterations of fecal consistency and frequency were allowed).

The study was performed as a reduced 3 × 3 Latin square, in which each dog acted as their own control and with the diet orders WHE-OAT-RYE, OAT-RYE-WHE and RYE-WHE-OAT. The dogs were categorized by gender and size, anonymized and randomly divided into three groups. The groups were then randomly assigned a diet order. Pairs of dogs living in the same household were kept together and followed the same diet order. Each experimental diet was fed for a minimum of four weeks before sampling. A transition period of 4–7 days preceded each diet period. During the transition periods, the owners were instructed to mix the dog's present

Ingredient	Wheat diet	Oat diet	Rye diet
Wheat flour	25	–	–
Oat flour	–	25	–
Rye flour	–	–	25
Maize	15	15	15
Rice	11.7	12.5	10.8
Lignocellulose <sup>a</sup>	1.5	1.5	1.5
Dried chicken meal	29.7	29.9	30.4
Fresh chicken meat	5	5	5
Chicken stock <sup>b</sup>	3	3	3
Pork fat	7.5	6.4	7.6
Premix of minerals and vitamins <sup>c</sup>	1.7	1.7	1.7

**Table 1.** Ingredients in the three experimental diets, expressed as % included. <sup>a</sup>Source of insoluble fiber consisting of lignin, cellulose and hemicellulose. <sup>b</sup>Source of flavor. <sup>c</sup>Nutrients added per kg: Vitamin A (IE) 11,100, Vitamin D3 (IE) 1160, Vitamin E (mg) 299, Vitamin C (mg) 403, Thiamine B1 (mg) 2.9, Riboflavin B2 (mg) 4, Pyridoxine B6 (mg) 2, Niacin B3 (mg) 19.9, Pantothenic acid B5 (mg) 17.6, Biotin (mg) 0.2, Vitamin B12 (mg) 0.06, Folic acid (mg) 0.4, Copper(II) sulphate pentahydrate (mg) 23, Copper (mg) 6, Manganese(II) oxide/ manganese(III) oxide (mg) 9.6, Manganese (mg) 5.8, Zinc sulphate monohydrate (mg) 101, Zinc (mg) 36, Calcium iodate anhydrate (mg) 17.8, Iodine (mg) 1.8.



Item	Wheat diet	Oat diet	Rye diet
Dry matter	93.9	93.5	93.9
Gross energy (MJ/kg DM)	20.9	21.7	21.5
Metabolizable energy (MJ/kg DM) <sup>a</sup>	17.2	18.0	17.8
Organic matter	92.9	93.1	92.7
Crude protein	30.5	30.4	29.5
Crude fat	13.7	17.6	16.7
Crude fiber	1.9	1.5	1.8
Total dietary fiber	8.6	7.6	9.1
Soluble dietary fiber	1.4	2.0	1.7
Insoluble dietary fiber	7.2	5.6	7.4
Total starch	38.3	34.2	34.8
Resistant starch	0.2	0.2	0.3
Non-resistant starch	38.1	34.0	34.5

**Table 2.** Chemical composition of the experimental diets, expressed as % of dry matter (DM) unless otherwise stated. <sup>a</sup>Calculated according to NRC 2006<sup>61</sup>.

food with the new diet in increasing amounts to allow the dog to adapt to the new diet. In order to make the starting conditions as similar as possible for all dogs they were all first fed WHE for three weeks, including a 4–7 day transition period, before starting the first experimental diet period.

All owners were blinded to the content of the diets. The owners were instructed to keep to the dog's normal feeding routines and to weigh their dog on the same scale once a week. The start daily feed allowance was based on the amount of calories in each individual's normal feed intake, calculated before the study. Daily caloric intake was adjusted to maintain original BW by increasing or decreasing the allowance by 5–25%, based on the level of BW change and then evaluating BW the following week. The owners were instructed to feed the experimental diet as the main source of energy, but treats were allowed as long as they were given in approximately the same amount during each diet period. However, owners were instructed to give nothing but the experimental diet during the last three days before sampling in each diet period.

At the end of each diet period, owners were asked to fill in a form with both closed and open questions concerning compliance to the instructions, as well as the wellbeing of the dog, during the diet period.

**Fecal sampling and handling.** Fecal samples for microbial and SCFA analyses were collected once during one of the two last days of each experimental period. All samples were collected immediately after voiding. Samples were either placed in  $-20^{\circ}\text{C}$  within 2 h from sampling, or kept chilled and placed in  $-20^{\circ}$  within 4 h from sampling. Samples were then stored for a maximum of seven months before analysis.

Analyses of the samples were performed by laboratory staff who were blinded to the diets.

**Microbiota analysis.** For DNA extraction, 180–220 g of fecal matter were transferred to a sterile tube containing 0.3 g sterilized 0.1 mm zirconia/silica beads (Biospec products, Bartlesville, Oklahoma, USA), followed by addition of 1 ml InhibitEX buffer (Qiagen GmbH, Hilden, Germany). The samples were vortexed and homogenized in a Precellys24 sample homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 6500 rpm for  $2 \times 60$  s, to disrupt bacterial cell walls. A QIAamp Fast DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) was used according to the manufacturer's protocol to isolate DNA. For generation of 16S rRNA gene amplicon libraries and sequencing, extracted samples were sent to Novogene (Tianjin, China).

**Library preparation and sequencing.** The V3-V4 region of the 16S rRNA gene was amplified using the primers 341F (5-GTGCCAGCMGCCGCGTAA-3) and 805R (5-ACTACHVGGGTATCTAATCC-3). Polymerase chain reactions (PCRs) were performed using Phusion<sup>®</sup> High-Fidelity PCR Master Mix (New England Biolabs) and the amplicons generated were confirmed by gel electrophoresis, purified with Qiagen Gel Extraction Kit (Qiagen, Germany) and quantified using a Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific). The final library including barcodes and adaptors was generated with the NEBNext<sup>™</sup> UltraTM DNA Library Prep Kit. The amplicons were then sequenced using Illumina sequencing (NovaSeq 6000) at Novogene (Beijing, China). The sequence data obtained were deposited in the Sequence Read Archive (SRA), under accession number PRJNA933568.

**Bioinformatics analysis.** Paired-end reads were assigned to each sample based on their unique barcode. After truncating the barcode and primer sequence using FLASH (v1.2.71) paired-end sequences were merged<sup>29</sup> and the raw data sequences were quality-filtered using QIIME (v1.7.02)<sup>30,31</sup>. Sequences were clustered into operational taxonomic units (OTUs) using Uparse software (Uparse v7.0.10013)<sup>32</sup>, where sequences with  $\geq 97\%$  homology were assigned to the same OTU. Representative sequences for each OTU were screened for further annotation. For each representative sequence, the Mothur software was applied to the SSU rRNA data in the SILVA Database for species annotation at each taxonomic rank<sup>33,34</sup>.

**Short-chain fatty acid analysis.** Acetate, propionate, and butyrate were analyzed in 0.5 g fecal matter dissolved in 1 mL 5 mM H<sub>2</sub>SO<sub>4</sub>, as previously described<sup>35</sup>. The high-performance liquid chromatography system consisted of an Alliance 2795 separation module and a 2414 RI Detector (Waters Corp. Milford, MA, USA). ReproGel H 9 $\mu$  300  $\times$  8 mm (Dr. A. Maisch, Ammerbuch, Germany) functioned as the separation column and a ReproGel H, 9 $\mu$  30  $\times$  8 mm, was used as the pre-column.

**Chemical analysis of food.** The dog food samples were analyzed without pre-drying. To determine dry matter (DM) content, the samples were dried at 103 °C for 16 h, and then placed in a desiccator to cool before weighing<sup>36</sup>. For ash determination, samples were incinerated at 550 °C for three hours and then cooled in a desiccator before weighing. The Kjeldahl method<sup>37</sup> was applied to determine nitrogen content, using a 2020 digester and a 2400 Kjeltec analyzer (FOSS Analytical A/S, Hillerød, Denmark). Crude protein was then calculated as N  $\times$  6.25. Crude fat was analyzed in accordance with Commission Directive EC/152/2009<sup>38</sup>, on a Soxtec extraction unit (FOSS Analytical A/S, Hillerød, Denmark). Crude fiber was analyzed as previously described<sup>39</sup>. Gross energy (GE) was measured on a Parr isoperobol Bomb Calorimeter 6300 (Parr Instrument Company, Moline, Illinois, USA). Total dietary fiber (TDF) as well as soluble and insoluble dietary fiber were analyzed in accordance with AOAC 991.43 using a Total Dietary Fiber Assay Kit (Megazyme, Bray, Ireland), while resistant and non-resistant starch were analyzed according to AOAC 2002.02 using a Resistant Starch Assay Kit (Megazyme, Bray, Ireland).

**Statistical analysis.** Effects of diet on general microbial community composition were analyzed by principal coordinate analysis (PCoA), based on Bray Curtis distance and including relative abundance data from all OTUs, using the software PAST<sup>40</sup>. A linear mixed effects model with treatment and treatment order as fixed effects and dog as random effect, together with continuous autoregression correlation of time per dog, was set up using the *nlme* package v3.1.157<sup>41</sup> in R version 4.2.1<sup>42</sup>. The model was used for univariate analyses of genera and OTUs with mean relative abundance > 0.1% and with maximum 25% zero counts, and for data on diversity and SCEA. Models were checked for normality and homoscedasticity with residual plotting. Diversity data from Chao1 index and PD whole tree, as well as relative abundance data, were transformed by the natural logarithm before statistical analysis. A constant of  $1 \times 10^{-5}$  was added to genera and OTUs with zero counts in order to do the transformation. The *p*-values obtained for relative abundance were corrected for multiple testing using false discovery rates (FDR) according to Benjamini-Hochberg<sup>43</sup>. Comparisons of estimated marginal means were corrected using Tukey's adjustment. Differences were considered significant if  $p \leq 0.05$ .

## Results

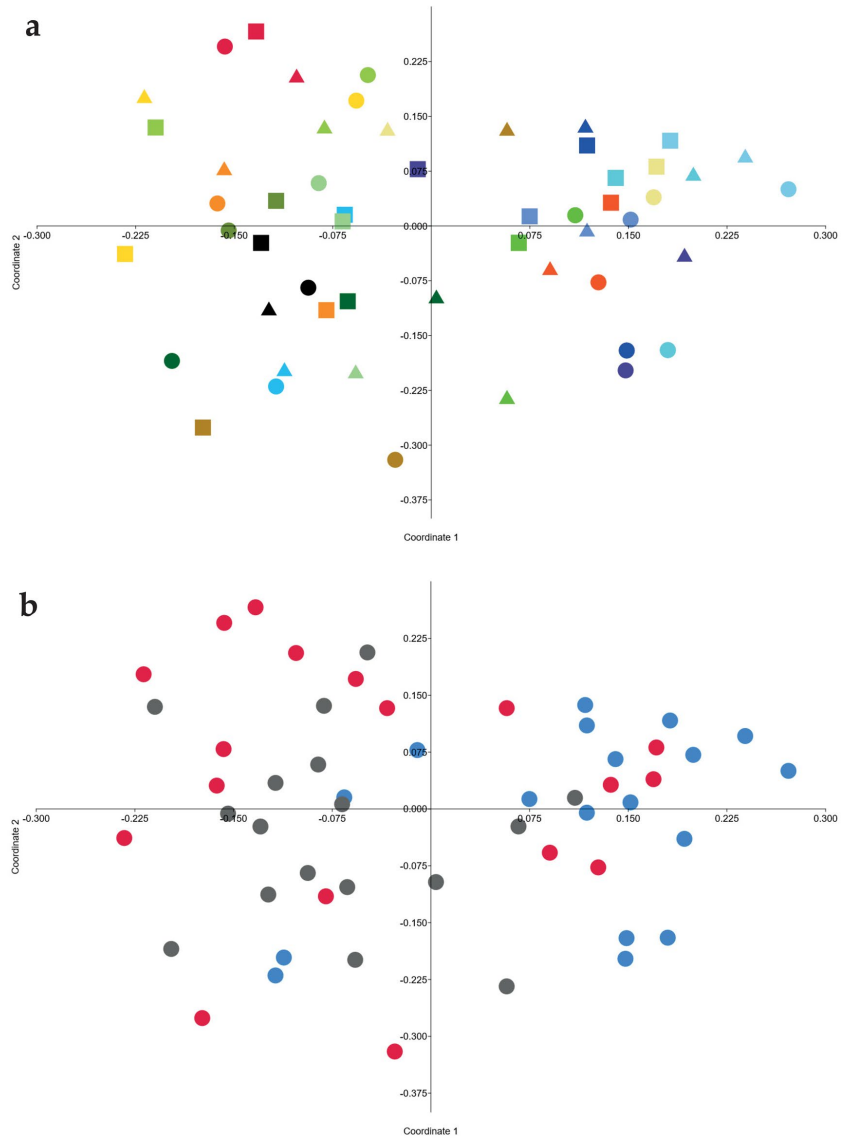
**Participating dogs.** Initially, 22 dogs were recruited. However, four dogs were excluded in agreement with their owners during acclimatization to the diets or early in the first experimental diet period due to problems with loose stools, signs of possible cutaneous adverse food reaction or palatability issues. All remaining 18 dogs were assessed as healthy before the first experimental diet period. The dogs were of 12 different dog breeds (2 dogs were of the same breed) and 5 mixed dog breeds. The mean  $\pm$  SD age was  $5.7 \pm 2.6$  years, mean  $\pm$  SD body condition score (BCS) on a 9-point scale<sup>44</sup> was  $5.2 \pm 0.6$  and mean  $\pm$  SD body weight (BW) was  $18.4 \pm 9.5$  kg. There were three pairs of dogs living in the same household. The pairs were randomized to different groups. A detailed demographic description of all included dogs can be found in Supplementary Table S1.

**Outcome of the intervention.** All dogs remained on the experimental diets throughout the whole study period. Body weight change (mean  $\pm$  SD) from the start of the experimental part to last sampling was  $-0.03 \pm 0.50$  kg for all dogs, while for the different diets it was: WHE:  $-0.1 \pm 0.41$ , OAT:  $0.2 \pm 0.38$ , RYE:  $-0.1 \pm 0.33$  kg. Intake of TDF (mean  $\pm$  SD), in gram per kg BW and day, in dogs on the different diets was: WHE:  $1.0 \pm 0.3$ , OAT:  $0.9 \pm 0.2$ , and RYE:  $1.1 \pm 0.3$  g. There were no indications in the owners' reports of any significant deviations regarding compliance with the diet instructions. All dogs completed the whole study except one dog, which died during the last diet period. Necropsy showed that the dog died for reasons not related to the study. Five dogs were treated with non-steroidal anti-inflammatory medication for at least one short period during the study, for reasons not related to the study. One dog was treated with a combined betametason and fucidic acid ointment for a localized skin infection during the last diet period. The infection had unknown etiology, but healed and did not recur after completion of the medical treatment, which ended three weeks prior to the last fecal sampling. The results from the analysis of that dog's final fecal sample revealed that it did not deviate from the samples collected previously from that dog, and hence it was included in the statistical analysis.

**Microbiota analyses.** The sequence analysis generated on average 112,113 (range: 46,162–126,364) sequences per sample. The most abundant genera in the sample set were: *Fusobacterium* (Fusobacteria), *Prevotella\_9* (Bacteroidetes), *Bacteroides* (Bacteroidetes), *Catenibacterium* (Firmicutes) and *Peptoclostridium* (Firmicutes).

No clear effects of diet on general microbial composition in fecal samples were detected in PCoA analysis (Fig. 1a). Whether clustering could be explained by other traits was explored by coloring the samples in the PCoA plot by the dogs' age, size, body condition score or gender. No such clustering was seen, but the samples from the group that started with WHE seemed to cluster separately from the groups starting with OAT or RYE (Fig. 1b).

There was an overall effect of diet on alpha diversity as measured by Shannon diversity index and PD whole tree (Table 3). Post hoc comparisons based on Shannon diversity and PD whole tree values revealed that microbial diversity was higher in samples collected after WHE compared with RYE ( $p = 0.011$  and  $p = 0.012$ , respectively).



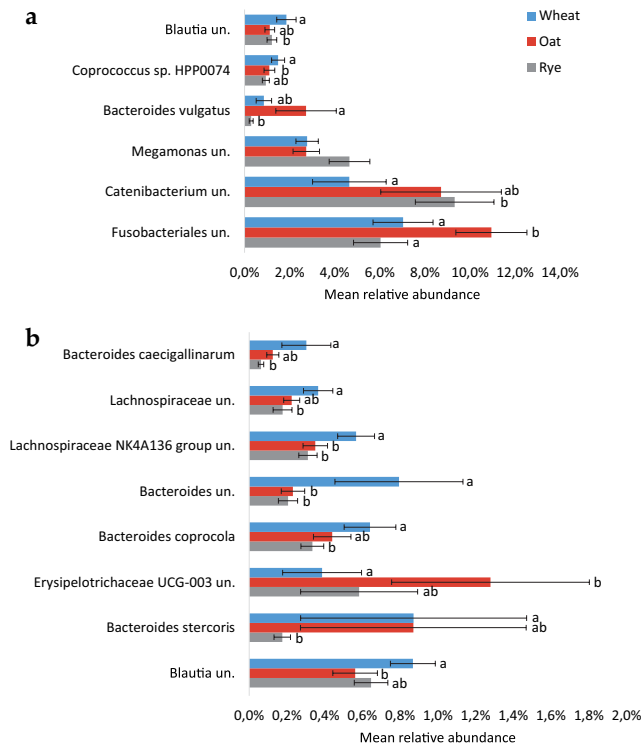
**Figure 1.** Principal coordinate analysis plots based on Bray Curtis distance metrics on operational taxonomic unit data. Each point represents one fecal sample from one dog following an experimental diet period. Both plots represent the same data, but colored differently. (A) Different colors represent individual dogs. Square = wheat diet (WHE), triangle = oat diet (OAT), dot = rye diet (RYE). (B) Different colors represent different diet orders. Blue = WHE-OAT-RYE, Red = OAT-RYE-WHE. Grey = RYE-WHE-OAT.

**Specific changes in fecal microbiota composition linked to the diet intervention.** Explorative univariate analysis of the dominating bacterial genera and OTUs indicated that diet had an effect on genus *Bacteroides*. In the analysis at OTU level, several OTUs classified to *Bacteroides* were least abundant in sam-

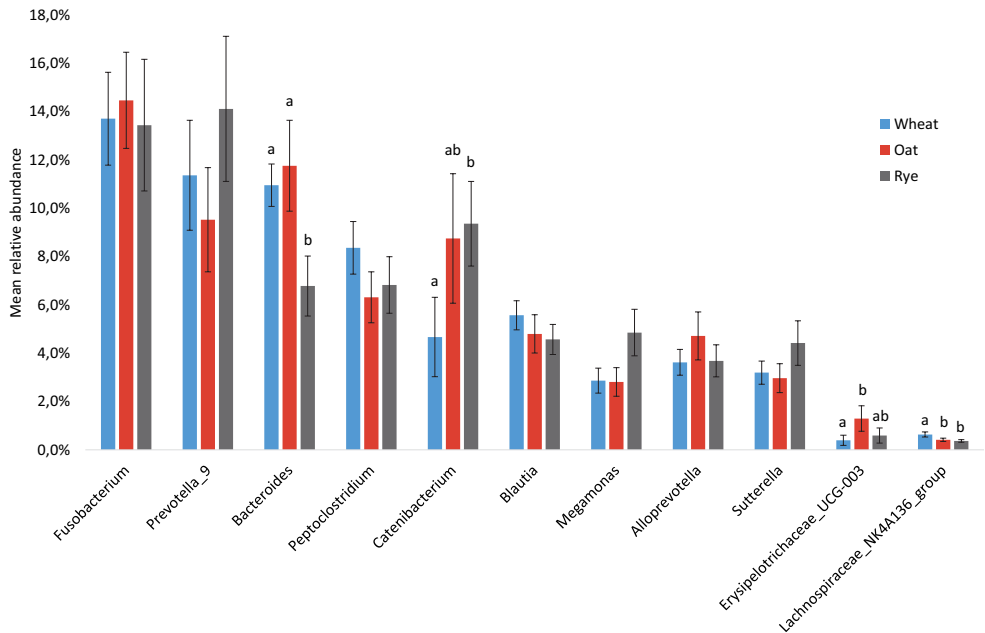
ples collected after RYE and most abundant in samples collected after WHE (Fig. 2). This was confirmed by the data at genus level, where *Bacteroides* was less abundant in samples collected after RYE than in samples collected after WHE ( $p=0.004$ ) or OAT ( $p=0.014$ ) (Fig. 3). There was also a trend for diet order to have an effect on genus *Bacteroides* ( $p=0.066$ ). Several OTUs belonging to family *Lachnospiraceae* were also least abundant in RYE samples and/or most abundant in WHE samples. Of the highly abundant genera, *Catenibacterium* was more abundant in RYE samples than in WHE samples ( $p=0.026$ ). Genus *Megamonas* was affected by diet ( $p=0.046$ ), but no effects could be confirmed in post hoc comparisons, although there was a trend for higher abundance of *Megamonas* in RYE samples compared with OAT samples ( $p=0.057$ ). Among the less abundant genera, *Lachnospiraceae*\_NK4A136\_group and *Erysipelotrichaceae*\_UCG-003 were identified as being affected by diet ( $p=0.008$  and  $p=0.014$ , respectively). There was a trend ( $p=0.098$ ) for a difference between diets in genus *Prevotella*\_9, which was numerically most abundant in samples collected after RYE.

Diversity index	Wheat diet	Oat diet	Rye diet	p-value
Shannon	4.80 ± 0.45 <sup>a</sup>	4.51 ± 0.43 <sup>ab</sup>	4.45 ± 0.45 <sup>b</sup>	0.011
Chao1	373 ± 93	412 ± 180	319 ± 69	0.058
PD whole tree	51.0 ± 16.6 <sup>a</sup>	44.4 ± 12.8 <sup>ab</sup>	38.8 ± 12.5 <sup>b</sup>	0.004

**Table 3.** Microbial diversity index (mean ± SD) for fecal samples collected from dogs following three experimental diets. <sup>abc</sup>Values within rows with different superscript letters are significantly different.



**Figure 2.** Relative abundance (mean ± SEM) of operational taxonomic units (OTU) with mean relative abundance > 0.1% and showing a significant difference in main treatment effect. Different letters within the same OTU indicate significant difference in log-transformed relative abundance between the different treatments. (A) OTUs with relative abundance > 1%; (B) OTUs with relative abundance between 1 and 0.1%.



**Figure 3.** Relative abundance (mean  $\pm$  SEM) of genera with mean relative abundance  $>3\%$  and/or differences due to a treatment effect. Different letters within the same genus indicate a significant difference between treatments.

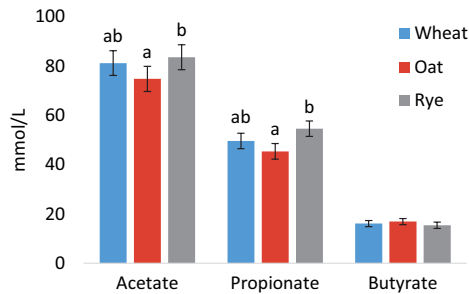
Diet order had a significant effect on genus *Prevotella\_9* ( $p=0.0033$ ), with the group of dogs that started with WHE having higher mean relative abundance of *Prevotella\_9* than the other two groups. Figures 2 and 3 show mean relative abundance of the significant OTUs and dominating genera, respectively. After FDR correction, one OTU belonging to family *Lachnospiraceae* was still significant, while the others were not (Supplementary Table S2).

**Short-chain fatty acids.** There was a trend for effect of diet on the concentration of total SCFA in fecal samples ( $p=0.051$ ) with estimated marginal mean  $\pm$  SE for each diet: WHE:  $147 \pm 8$ , OAT:  $138 \pm 8$  and RYE:  $153 \pm 8$  mmol/L. When comparing the effect of diet on the three different SCFA there were significant differences in acetate and propionate with higher concentrations after RYE than OAT ( $p=0.044$  and  $p=0.018$ , respectively) (Fig. 4). The relative proportions of the three SCFA in fecal samples did not differ between the diets.

## Discussion

This study explored the effects of dietary inclusion of three different types of whole grains (rye, oats or wheat) on fecal microbial composition and SCFA concentration in a population of privately owned dogs, acting as their own controls.

The PCoA analysis showed no clear effects of diet on general microbial community composition. For some dogs, samples from two of the different diet periods clustered together, but this pattern was not consistent for any specific diet. For a few dogs, all three samples were similar in composition, indicating an individual or environmental effect that could not be overruled by the diet. However, microbial alpha diversity (based on Shannon and PD whole tree values) was higher in samples collected after WHE than in samples collected after RYE. For all diets, Shannon diversity values were within the range previously reported for healthy dogs<sup>19,45</sup>. Previous studies comparing inclusion of whole grains in dog food are scarce. However, in studies on dogs, alpha and beta diversity have been reported to be unaffected both when comparing different ancient grains, one of which was oat groats<sup>8</sup>, and when comparing rye flour to fermented rye or cornmeal added to a vegetarian diet supplemented with feather meal<sup>7</sup>. Effects on fecal microbiota of consumption of whole-grain rye and wheat have been compared in a human study<sup>26</sup>, which found no significant effects on microbial alpha or beta diversity. In another human study comparing the same grains, some differences in general fecal microbial composition were observed<sup>25</sup>. Hence the effects of the grains are not clear. However, duration of the intervention and TDF intake varied between the different studies and these factors, together with large individual variation, make it difficult to draw any general conclusions about the effects on the overall microbial community in dogs and humans.



**Figure 4.** Fecal concentration (estimated marginal mean  $\pm$  SEM) of acetate, propionate, and butyrate in dogs following consumption of the three experimental diets. Different letters indicate significant differences in concentration between treatments.

On investigating the effects on individual microbial genera and OTUs, we observed that genus *Bacteroides* and several OTUs belonging to that genus were least abundant in samples collected from dogs after RYE and highest after WHE. In a previous study by our research group, a decrease in *Bacteroides* after a diet with 50% rye inclusion, compared with samples collected before the diet period, was a factor of major importance for the difference in general microbial composition<sup>21</sup>. Decreased abundance of *Bacteroides* has been reported in studies on humans and pigs investigating the effects of rye kernel bread compared with refined wheat<sup>22</sup> and an arabinoxylan-supplemented diet compared with a control diet<sup>46</sup>. A decrease in *Bacteroides* abundance in those studies was coupled with increased abundance of genus *Prevotella*. In the present study, *Prevotella* abundance was numerically highest after RYE and lowest after WHE but there was only a trend in the main diet effect ( $p=0.098$ ). Statistical significance was likely not reached due to large inter-individual variation, and larger sample size would perhaps confirm a significant difference between diets. In agreement with findings in the present study, our previous study showed significantly higher *Prevotella* abundance following the diet with highest inclusion of rye than following the wheat diet<sup>21</sup>. However, in that study whole-grain rye was compared to refined wheat and the dietary inclusion level of rye was higher (50% of DM).

The statistical model in the present study showed that diet order had an effect on relative abundance of genus *Prevotella\_9* ( $p=0.033$ ) and tended to have an impact on abundance of *Bacteroides* ( $p=0.066$ ). On reviewing the data, it emerged that there was a difference between groups in relative abundance already at the start of the study. This was also observed in the PCoA analysis, where the group that started with WHE clustered separately from the other groups. The group that started with WHE generally had lower *Bacteroides* abundance and higher abundance of *Prevotella\_9*. In human studies, the concept of enterotypes has been used to divide people into groups with a gut microbiota dominated by either *Prevotella* or *Bacteroides*<sup>47,48</sup>. High *Prevotella/Bacteroides* ratio has been observed to have positive effects on glucose metabolism in human studies<sup>49,50</sup>. Whether the same is true for dogs has not yet been determined. Human studies have reported that these enterotypes have different fiber-fermenting characteristics and that the same fiber can benefit different bacteria depending on the enterotype<sup>20</sup>. It is not unlikely that these bacteria could have the same characteristics in dogs and that the individual responses observed in the present study were a result of this. There was over-representation of individuals with high *Prevotella/Bacteroides* ratio in the group that started with WHE. This could not have been predicted based on the data available prior to the study. In the ideal situation, we would have analyzed the fecal microbiota before grouping the dogs and taken microbial composition into consideration as a blocking factor. However, in the design used in the present study, the dogs were their own controls, so the initial clustering of dogs should be of minor importance.

*Catenibacterium* is a common bacterial member of the gut microbiota in dogs, yet often of relatively low abundance<sup>45,51,52</sup>. In the present study it was one of the most abundant genera, with highest abundance in samples collected after RYE. In contrast, in our previous study this taxon decreased in abundance from a baseline level on adding rye to the diet<sup>21</sup>. The reason for the discrepancy is unknown.

Of the less abundant OTUs, several belonging to family *Lachnospiraceae* showed significantly lower abundance after RYE compared with WHE. Similar results have been reported in a human study comparing microbial relative abundances after a rye diet with baseline values<sup>23</sup>. In that study, no such difference was observed after a whole-grain wheat diet.

It should be noted that this was an explorative study on the effects on fecal microbiota in a broad perspective, rather than an analysis of effects on specific bacteria. We therefore report both the uncorrected  $p$ -values and the  $q$ -values where a FDR correction was made to account for multiple statistical tests (Table S2). After FDR correction, the only remaining significant difference was for one OTU belonging to family *Lachnospiraceae*. This means that the uncorrected results should be interpreted with caution and that effects on specific taxa should be confirmed in further studies.

The lack of similar previous studies and the explorative nature of the study made it unfeasible to perform power calculations to determine sample size when planning the study. However, one study comparing other

grains used 10 beagle dogs<sup>8</sup> and other studies examining the effect of fiber on microbiota used 8–10 dogs<sup>5,53</sup>. Since we used privately owned dogs of different breeds living in a less controlled environment and with expected larger variation, we aimed to have around twice as many dogs as in those studies.

One possible limitation in the microbiota sampling was the time from defecation to placement in freezer and storage temperature. However, although there does not seem to be a consensus regarding best practice, several previous studies on the stability of microbiota samples have found no major differences when comparing different storage temperatures and duration from sampling to freezing<sup>54–58</sup>. In our study we sought to keep the time in room temperature to a minimum and the owners handled all samples from their dog equally, thus the effect of temperature should be the same for all samples from the corresponding dog and equal for all diets.

The concentrations of acetate and propionate were higher in samples collected after RYE compared with OAT. Similarly, in a previous study on pig fecal inoculum, production of acetate, but not production of propionate, was found to be higher from arabinoxylan substrate than from  $\beta$ -glucan<sup>59</sup>. In the present study, there was a trend for total SCFA concentration to be higher after RYE compared with OAT. Diet OAT had the highest inclusion of soluble dietary fiber, which was expected to make that diet more easily fermentable. However, it is plausible that digestion of oat fiber occurred more proximally in the intestine and thus the SCFA could also have been absorbed more proximally. A previous in vitro study with human inoculum showed faster fermentation rate of rye than oat samples<sup>60</sup>, although in that study only the bran of the grains was used, which means that the fiber content was likely higher than in our diets. Butyrate concentration did not differ between the three diets in the present study. In contrast, in a study in humans assessing the effects on fecal microbiota composition and function of whole-grain rye and wheat, higher production of butyrate after rye consumption was observed<sup>25</sup>. On comparing enterotypes, the same study found that propionate tended to be higher after rye consumption in a test group with *Prevotella* enterotype than in a test group with *Bacteroides* enterotype. In the present study, although there were differences in absolute levels of SCFA, the relative proportions of the three main SCFA were in line with previous reports in dogs<sup>8</sup> and did not differ between the diets.

Using privately owned dogs provides an opportunity to investigate diet effects that are strong enough to have an impact on the nutrition of a broader dog population, but also poses challenges in terms of the less controlled environment than when using laboratory dogs. However, the dog owners participating in this study were likely more knowledgeable about the importance of following the instructions in an experimental research study than the average population, since they were staff and students at a university of agricultural sciences. Hence, the compliance was expected to be high, which was also indicated by the follow-up form in the end of each diet period. Moreover, the cross-over experimental design, in which the dogs serve as their own control, was another way of ensuring that potential differences in the studied effects would indeed be due to the differences in the diets and not in the environment or management. A few of the dogs received short term treatment with NSAID or topical ointment during the study. When interpreting the results, those dogs did not show a deviating pattern during the medical treatment period compared to the other diet periods within the same dog.

## Conclusions

Inclusion of whole-grain rye, oats or wheat in the diet did not have clear differentiating effects on total fecal microbiota composition in dogs in this study. However, family *Lachnospiraceae* and genus *Bacteroides* were the gut microbial groups most affected by diet, with lowest relative abundance following consumption of the rye diet and a trend for a corresponding increase in genus *Prevotella*<sub>9</sub>. This coincided with higher concentrations of acetate and propionate in fecal samples after consumption of the rye diet. Although rye had the largest impact on gut microbiota and SCFA, the relevance of the observed changes for the dog gut health need to be elucidated in future studies.

## Data availability

Sequence data have been deposited in the Sequence Read Archive (SRA) under accession number PRJNA933568. Data from digestibility and SCFA analyses and feed analysis can be obtained upon request from the corresponding author.

Received: 3 April 2023; Accepted: 30 June 2023

Published online: 05 July 2023

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## Acknowledgements

The authors would like to thank all dogs and dog owners who participated in the study; Claudia von Brömssen, Department of Energy and Technology, Swedish University of Agricultural Sciences, for invaluable statistical support and advice; laboratory staff at the Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences; and the company Doggy AB and Cecilia Hjelm for help in producing the experimental diets.

## Author contributions

H.P., K.H., S.R., T.L. and J.D. designed the study. H.P. and K.H. performed the initial health examinations on the dogs. H.P. carried out the experiments and oversaw the laboratory analyses. H.P. and J.D. conducted the initial data analyses and all co-authors were involved in interpretation of the data. H.P. wrote the first draft of the manuscript and all authors provided editorial input. All authors have read and agreed to the published version of the manuscript.

## Funding

Open access funding provided by Swedish University of Agricultural Sciences. This research was funded by Agria/SKK Research Fund, grant number P2020-0008.

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-023-37975-4>.

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ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

DOCTORAL THESIS NO. 2023:65

This thesis investigated effects of diets with wheat, oat and rye on dog metabolism, gut microbiota and short-chain fatty acids. The results indicate that whole grain rye may affect dog gut microbiota composition and function in ways that could be beneficial to health. The overall metabolic response was similar for the three whole grains, but responses in specific blood variables indicate the need for further studies.

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ISSN 1652-6880

ISBN (print version) 978-91-8046-182-5

ISBN (electronic version) 978-91-8046-183-2