Chicory *(Cichorium intybus* L*)* As Fibre Source in Pig Diets

Effects on Digestibility, Gut Microbiota and Performance

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Abstract

The aim of this thesis was to increase our understanding about chicory (forage and root) as fibre source for pigs, by studying the effects of diets with inclusion of chicory on digestibility, digestion site, performance, gut microbiota and environment.

In total 62 pigs were used, including newly weaned and growing intact pigs and growing post valve t-caecum (PVTC)-cannulated pigs. The weaned pigs were fed cereal-based diets with inclusion of 40, 80 and 160 g/kg of either chicory or ribwort forage. The growing pigs were fed cereal-based diets with inclusion of 80 and 160 g/kg of chicory forage and/or root. The cannulated pigs were fed diets with similar non-starch polysaccharides (NSP) content, comprising of a basal diet and four diets with one of four fibre sources, two pectin-rich (chicory forage and sugar beet pulp), and two arabinoxylan-rich (wheat bran and grass meal).

The results showed that the total tract digestibility of organic matter (OM) and energy of chicory forage was similar to commonly used forage crops. The total tract digestibility of NSP in both chicory forage and root was higher than the NSP digestibility in cereals. Inclusion of 80 g chicory forage/kg did not reduce the digestibility of OM, crude protein and energy. Inclusion of 160 g chicory forage/kg did not reduce growth performance and did not increase gastrointestinal organ weights. Chicory forage and root affected the gut microbiota differently with higher lactobacilli:coliform ratio when combined in the diet which indicates a synergistic effect. Chicory forage increased the abundance of *Bacteroides-Prevotella-Porphyromonas* in faeces. Increased total amount of fermentable dietary fibre in the diet increased the abundance of the same groups in ileal digesta.

The effects of different fibre sources on the microbiota were to a high degree ingredient-specific. The effect on organic acids was NSP-structure specific with increased butyric acid concentration on arabinoxylan-rich diets. The effect of pectin-rich diets was dependent on the intra-molecular structure of pectin, with highest proportion of acetic acid on chicory forage diet.

In conclusion, these studies shows that there is potential for both chicory forage and root to be used as regular feed ingredients in pig diets.

Keywords: Chicory, dietary fibre, growing pigs, digestibility, gut microbiota, pectin arabinoxylan, organic acids, molecular weight distribution, performance

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You only learn to be a better writer by actually writing

Doris Lessing

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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 Ivarsson, E., Frankow-Lindberg, B., Andersson, H.K. and Lindberg, J.E. (2011). Growth performance, digestibility and faecal coliform bacteria in weaned piglets fed a cereal-based diet including either chicory (*Cichorium intubys* L.) or ribwort (*Plantago Lanceolata* L.) forage. *Animal* 5 (4), 558-564.

II Ivarsson, E., Liu, H.Y., Dicksved, J., Roos, S. and Lindberg, J.E. (2012). Impact of chicory inclusion in a cereal-based diet on digestibility, organ size and faecal microbiota in growing pigs. *Animal* doi:10.1017/S1751731111002709.

III Ivarsson, E., Andersson, R. and Lindberg J.E. (2012). Digestibility of fibre sources and molecular weight distribution of fibre fractions in ileal digesta of growing pigs (*submitted*).

IV Ivarsson, E., Roos, S. and Lindberg J.E. (2012). Fermentable dietary fibre increases the abundance of *Bacteroides-Prevotella-Porphyromonas* in ileal digesta of growing pigs (*manuscript*).

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Abbreviations

GI	Gastrointestinal
DM	Dry matter
OM	Organic matter
CP	Crude protein
NSP	Non-starch polysaccharides
NDF	Neutral detergent fibre
ADF	Acid detergent fibre
SCFA	Short chain fatty acids
DGGE	Denaturing Gradient Gel Electrophoresis
TGGE	Temperature Gradient Gel Electrophoreses
T-RFLP	Terminal restriction fragment length polymorphism
TRF	Terminal restriction fragment
PCR	Polymerase chain reaction
qPCR	quantitative PCR
CFU	Colony forming units
PVTC	Post valve t-caecum
s.e.	Standard error

1 Background

In modern pig production, pig feed is mainly based on cereals. Many alternative feedstuffs have a high dietary fibre content which is associated with decreased nutrient utilization and low net energy values (Noblet & Le Goff, 2001). In spite of this, there has been an increasing interest in research related to dietary fibre in pig diets during the last decades. This is due to the demand of cereals for direct human consumption and for bio-fuels has increased as well as the availability of cheap by-products from the food industry with a high dietary fibre content such as oat hulls, soybean hulls and sugar beet pulp (De Leeuw *et al.*, 2008). Today it is well known that dietary fibre has an important role in pig diets. It may stimulate gut health, increase the satiety, affect pig behaviour and overall improve animal well-being (Presto *et al.*, 2009; De Leeuw *et al.*, 2008; Wenk, 2001).

Gut health is a complex concept and much research has been performed within the area. Still, enteric diseases such as weaning diarrhoea and swine dysentery contribute to large production losses in pig production and decrease the welfare of the affected animals. For a long time antibiotics were used as growth promoters on a regular basis to control the problems. Since 2006, antimicrobial growth promoters have been banned within EU and the goal is minimal use of antibiotics in food production. Therefore, it is important to find ways to obtain a good animal health with other means. One possible way is changing diet composition and to use various dietary interventions. A huge amount of research has been performed in the area that aims to change the gut microbial composition, such as supplementation of probiotics, prebiotics, organic acids and dietary fibre (De Lange *et al.*, 2010). It is believed that these types of dietary interventions have the potential to improve gut health.

2 Introduction

2.1 Definition of dietary fibre

The definition of dietary fibre was first introduced by Hipsley (1953) for non-digestible constituents that make up the plant cell wall. These constituents were known to include cellulose, hemicelluloses and lignin. Since, dietary fibre has been redefined several times, and there is at present no universal agreement (DeVries & Faubion, 1999). However there are two definitions that are commonly used, the physiological "all polysaccharides and lignin, which are not digested by the endogenous secretions of the human digestive tract" (Trowell *et al.*, 1976), and the chemical "the sum of non-starch polysaccharides (NSP) and lignin" (Theander *et al.*, 1994). According to Champ *et al*. (2003) most scientists in the food industry agree that non-digestible oligosaccharides and resistant starch should be added to the latter definition. That definition is more complete and contains the constituents, resistant starch and non-digestible oligosaccharides, that do not participate in the cell wall structure but have similar physiological effects as NSP and lignin.

2.2 Dietary fibre in pig diets

Plant carbohydrates including monosaccharides, disaccharides, oligo-saccharides and polysaccharides (Bach Knudsen *et al.*, 2011) are quantitatively the most important energy source for pigs (Bach Knudsen & Canibe, 2000). The polysaccharides can be divided into starch and NSP. The NSP can further be divided into cell wall NSP and non-cell wall NSP. Based on their solubility in water, the NSP fraction can be classified as insoluble and soluble, which is regarded as being a nutritionally important trait (Bach Knudsen *et al.*, 2011; Bach Knudsen, 2001). The dietary fibre in pig diets derives mainly from cell wall NSP and most plants have a mix of soluble and insoluble NSP in the cell

wall, where the ratio differs between plants and with stage of maturity (Montagne *et al.*, 2003).

A minimum level of dietary fibre has to be included in the pig diet to maintain normal physiological function in the digestive tract (Wenk, 2001). However, dietary fibre is not included in the nutrient requirement table of NRC (1998). The amount and composition of dietary fibre vary widely between and within feedstuff (Wenk, 2001). Consequently, the dietary fibre content in a typical pig diet varies between production systems and countries due to availability of feed recourses. Högberg & Lindberg (2006; 2004) used control diets that resembled typical Swedish slaughter pig and piglet diets. The diets contained 951 and 881 g cereals/kg feed (barley, triticale and wheat) and a total NSP content of 139 and 147 g/kg dry matter (DM). Jørgensen *et al*. (1996) considered a content of 52 g NSP/kg DM as a low fibre diet and 256 g NSP/kg DM as a high fibre diet to growing pigs. Moreover, in organic pig production, there is a requirement to offer roughages, as fresh or dried fodder, or silage, in the daily ration (EC, 2008). Thus, in this production system, dietary fibre is even more important and functions as an important energy source.

2.2.1 Determination of dietary fibre

There are several analytical methods available to measure fibre in animal feeds. The methods can be divided into two categories 1) non-enzymatic-gravimetric methods and 2) enzymatic-gravimetric methods.

The first category is commonly used in the feed industry and is mainly adapted to forage. However it does not quantify both soluble and insoluble dietary fibre. This category includes the crude fibre method and the detergent method (Champ *et al.*, 2003). The crude fibre method has been used since the middle of the 18th century (Bach Knudsen, 2001) and measures mainly cellulose and lignin (Horwitz, 1975). The detergent method determines acid detergent fibre (ADF) and neutral detergent fibre (NDF). ADF quantifies mainly cellulose and lignin whereas NDF quantifies hemicellulose, cellulose and lignin (Van Soest & Wine, 1967).

The second category was developed in the early 1980s to be able to quantify both soluble and insoluble polysaccharides. This category has followed the development of the dietary fibre definition in human nutrition, and aims to measure the carbohydrates that are not digested in the human small intestine (Champ *et al.*, 2003). This category includes the total dietary fibre method (Prosky *et al.*, 1985), the Englyst method (Englyst & Hudson, 1987) and the Uppsala method (Theander *et al.*, 1994). The enzymatic-gravimetric methods include removal of starch with enzymes, followed by ethanol treatment to precipitate moderately soluble NSP. The total and insoluble NSP

is separated and quantified gravimetrically. The methods quantify mainly cellulose, hemicelluslose, pectins, lignin and a part of the resistant starch. Some indigestible polysaccharides that are soluble in 78-80% ethanol, such as inulin, β-glucans and poly-dextrose are not included in this method. However there are several AOAC methods available to quantify these components separately (Champ *et al.*, 2003).

2.2.2 Composition and physicochemical properties of dietary fibre

Identical monomeric composition of NSP does not correspond to the same solubility. For example, cellulose and mixed linked β-glucan are both polymers of glucose. Cellulose is insoluble in water because of the presence of only one β-D-1,4 linkage, whereas β-glucan is far more soluble in water because of the presence of interrupting β-D-1,3 linkages (Bach Knudsen, 2001). The composition of the cell wall of plants is a key factor to determine the digestibility and physiological effects in animals. Presence of lignin inhibits the degradation of polysaccharides, whereas non-lignified polysaccharides are more readily utilized. The plant cell wall composition is dependent on the plant species, tissue type and maturity of the plant (Bach Knudsen, 2001; McDougall *et al.*, 1996).

The major plants used in pig diets are monocotyledon plants such as cereals or dicotyledon plants such as legumes and herbs. In the cell walls of monocotyledon plants the major non-cellulosic polysaccharide are arabinoxylans (Andersson *et al.*, 1994). The structure of arabinoxylan in cereals consists mainly of four differently linked xylose residues in the polymer chain (Andersson & Åman, 2001; Andersson *et al.*, 1994). There are also more heterogenous polymers of β-1,4-linked xylose residues substituted with acetyl, arabinosyl and glucuronic acid residues (Louis *et al.*, 2007).The degree of substitution of the xylose residue can be indicated by the arabinose/xylose ratio, where a ratio above 1 indicates a highly substituted polymer (Schoonevald-Bergmans *et al.*, 1999). It has been concluded that arabinoxylans becomes water-soluble because of their substituent's (Andrewartha *et al.*, 1979).

The cell walls of dicotyledon plants contain high levels of pectic polysaccharides, particularly in the mid lamella and primary cell walls (Sun *et al.*, 2006; Voragen *et al.*, 2001). Pectins are complex polymers consisting of four domains; homogalacturonan, rhamanogalacturonan-I, rhamanogalacturonan-II and xylogalacturonan (Sun *et al.*, 2006). Voragen *et al.* (1995) divided pectins into two families, the galacturonans and the rhamnogalacturonans. In the galacturonan family the dominating structure constitutes of long linear chains of α-1,4-linked galactosyluronic acid as the

backbone that can be branched to different degree. This family includes linear homogalacturonan as well as xylogalacturonan and rhamnogalaturonan II where the backbone is branched with side chains containing sugars such as apiose and rahmnose. In the rhamnogalacturonan family the backbone is composed of altering α-(1→2)-linked L-rhamnosyl and α-1,4-linked galactsyluronic acid and are branched with different neutral oligo- and polysaccharides such as arabinans, galactans and arabinogalactans. Pectins also have intra-molecular distribution of regions with mainly galactosyluronic acid that are called "smooth" regions and regions that are branched and forming rhamnogalacturonans "hairy" regions (Voragen *et al.*, 2001). Pectins can differ in galacturonic acid content, neutral sugar content, glycosidic linkage composition, degree of methyl esterification and acetylation, amide content and molecular weight (Voragen *et al.*, 2001). The rate of bacterial fermentation is dependent on the methylation and branching of the polymer (Sun *et al.*, 2006).

Inulin and oligofructose are widely distributed nutritionally important non-cell wall plant storage carbohydrates (Van Loo *et al.*, 1995). Inulin is a fructan where the fructose units is a mixture of linear fructose polymers and oligomers linked by β-(2,1) bonds. A glucose molecule typically is present at the end of each fructose chain and is linked by an α-(1,2) bond. Generally, inulin with a degree of polymerization (DP) <10 is highly soluble in water, rapidly fermented and has a high impact on the microbiota. Inulin with a DP >10 is more slowly fermented and is also fermented more distally than the shorter chains. Oligofructose is entirely composed of chains with DP <10 and contains 2 to 8 monosaccharide residues connected by glycosidic linkages. Oligofructose can be obtained by partial degradation of inulin and contains β-(2-1) fructose chains with or without terminal glucose units (Flickinger *et al.*, 2003). It is the presence of β-(2,1) bonds that gives inulin and oligofructose its special properties, and prevent them from being hydrolytically digested and instead are available as a substrate for the microbiota (Fishbein *et al.*, 1988).

The physicochemical properties of dietary fibre are dependent on the polysaccharides that make up the cell wall and their intermolecular association which determine their solubility (McDougall *et al.*, 1996). The major physicochemical properties that have been considered in pig nutrition are cation exchange capacity, hydration properties, viscosity and organic compound adsorptive properties. Characteristic for insoluble dietary fibres is that they increase rate of passage and faecal bulk whereas soluble dietary fibre increases the viscosity and hydration properties (Bach Knudsen, 2001).

2.3 Utilization of dietary fibre by the pig

The utilization of dietary fibre in pigs shows great variability and is highly dependent on the chemical composition of the polysaccharide. The polysaccharide composition also has a high influence on the digestion site of dietary fibre. The digestibility of NSP in the small intestine of growing pigs has been estimated to be on average 24% with a range between experiments from 10 to 62% (Bach Knudsen & Jørgensen, 2001). The large intestine is the main site for dietary fibre degradation. The average digestibility coefficient for dietary fibre in the total tract is 0.40-0.50, with a range from around zero in highly lignified NSP to 0.90 for some water-soluble NSP (Noblet & Le Goff, 2001). The most important factors for the total tract digestibility of NSP is the NSP source, solubility, degree of lignification, inclusion level in the diet, transit time, age and weight of the animal and gut microbial composition (Bach Knudsen & Jørgensen, 2001).

Fermentation of carbohydrates in the large intestine by the pig results in production of short chain fatty acids (SCFA) and gases such as CO_2, H_2 and CH_4, where CH_4 is of particular interest because of its impact on green house gases (Jørgensen, 2007). Increased amount of NSP in the diet increases the microbial activity (Jensen & Jorgensen, 1994) and the production of SCFA and CH_4 increases. The SCFA are absorbed from the intestine (Giusi-Perier *et al.*, 1989), while CH_4 is lost to the environment (Jørgensen *et al.*, 2007). The production of CH_4 varies considerably and except a high individual variation between pigs, it depends on fibre type and the age of the pig; values equivalent to 0.1-3.3% of digestible energy has been reported (Jørgensen, 2007). The highest CH_4 production has been reported for dry sows fed highly fermentable fibre sources (Jørgensen, 2007). The produced SCFA can be used by the host and provides up to 24% of the maintenance energy for a growing pig (Lindberg *et al.*, 2001; Yen *et al.*, 1991).

2.3.1 Age in relation to dietary fibre utilization

Adult sows has a more developed and larger gastrointestinal (GI) tract, a lower feed intake per kg body weight, a slower digesta transit time and a higher cellulytic activity than young pigs. This results in a superior capacity of sows to digest fibrous components compared to young pigs (Bach Knudsen & Jørgensen, 2001; Shi & Noblet, 1993). Jørgensen *et al*. (2007) found that sows digest a larger part of the NSP in the small intestine than growing pigs. They also showed that sows have a higher capacity to digest insoluble NSP, whereas the difference in digestibility of soluble NSP between growing pigs and sows were only marginal. Noblet & Le Goff (2001) suggested that because of the increased capacity to digest fibrous feedstuff by increased age and body

weight, at least two different energy values, one for growing-finishing pigs and one for sows should be used for most feed ingredients in pig diets. This has been implemented in the INRA net energy system for pigs (EvaPig, 2012).

2.3.2 Determination of digestibility

Digestibility, i.e., the amount of a certain dietary component that is absorbed in the digestive tract of animals, can be assessed by either direct or indirect methods. In the direct method all feed consumed and all faeces excreted are quantitatively collected during a certain number of days. The indirect method is based on qualitative spot sampling of faeces or ileal digesta at several occasions. The indirect method uses an indigestible marker to calculate the apparent digestibility of the diet with the indicator technique (Sauer *et al.*, 2000). The marker can be either internal or external. An internal marker is a natural component in the diet, while the external marker has to be added to the diet. A common internal marker is the acid-insoluble ash, whereas titanium dioxide (TiO_2) and chromium oxide (Cr_2O_3) are common external markers (McDonald *et al.*, 2002).

2.4 Microbiota in the pig GI tract

The complex microbial community in the GI tract consists of different groups of microbes including bacteria, archaea, ciliate and flagellate protozoa, anaerobic phycomycete fungi and bacteriophages. Bacteria are the most abundant and studied microbes in this community. They are provided with substrates from the diet as well as components deriving from the host such as mucopolysaccharides, mucins, epithelial cells, and enzymes (Zoetendal *et al.*, 2004). It is now generally accepted that only a minority of the GI microbes have been isolated by culture based methods (Vaughan *et al.*, 2000) and we have to accept that the knowledge we have today most likely needs to be revised in the future.

2.4.1 Development of the microbiota

The sterile GI tract of the piglet starts to be colonized during birth, and as early as 12 h after birth the bacterial counts in colon reach 10^9 CFU/g colonic content (Swords *et al.*, 1993). Within a few days after birth the dominating bacterial groups in the GI tract are streptococci and coliforms, but lactobacilli and clostrida may also be present (Mackie *et al.*, 1999). The gut microbiota remains relatively stable during suckling although some qualitative changes occur (Inoue *et al.*, 2005). Weaning is associated with many stressors including abrupt feed changes from a diet based on milk to solid feed based on complex

plant materials, which has a huge influence on the gut microbiota (Bach Knudsen *et al.*, 2011). Generally, stress is associated with decrease in the counts of lactobacilli, which allows potential pathogens such as coliforms to increase in abundance (Ewing & Cole, 1994). After weaning it takes about 5-14 days for the gut microbiota to become stable and diverse (Pieper *et al.*, 2008; Bauer *et al.*, 2006; Hillman, 2001). The introduction of solid feed induces a both qualitative and quantitative alteration of the bacterial composition. For example the number of strict anaerobes increases, e.g., *Bacteroides* and the numbers of facultative anaerobes decreases (Konstantinov *et al.*, 2004). However, with exception from one day post weaning lactobacilli remain as the dominating bacterial group in small intestine (Pieper *et al.*, 2008). Swords *et al.* (1993) found that *Bacteroides* started to increase in abundance after weaning and continued to increase until four months of age. The microbiota is considered to remain quite stable in a healthy adult if the conditions are constant (Conway, 1994). However, both environmental and dietary factors influence the microbial composition (Conway, 1994). The diet can change both the composition and activity of the microbiota (Bikker *et al.*, 2006; Awati *et al.*, 2005). Differences in conditions in the GI tract between growing pigs and adult sows also result in differences in the microbial composition. Varel & Yen (1997) reported that adult sows had about seven times higher numbers of cellulolytic bacteria in colon than growing pigs. Different conditions in small and large intestine as well are reflected in differences in both number and composition of the microbiota at the different sites in adult pigs.

Small intestine

The small intestine is characterized by secretions of digestive enzymes and the digesta has a high passage rate especially in the proximal parts. Moreover, the microbiota is also in direct competition with the host for nutrients (Metzler *et al.*, 2009). This limits the proliferation of the microbiota at this site. With reference to cultivable bacteria the small intestine harbour about 10^7-10^9 CFU/g digesta (Jensen & Jorgensen, 1994) and the most common belongs to *Enterobacteriaceae, Streptococcus, Clostridium* and *Lactobacillus* (Jensen, 2001).

Large intestine

In the large intestine the passage rate is slower than in the small intestine which together with a beneficial pH creates a favourable environment for bacterial growth. Consequently the large intestine is the main site for microbial fermentation. With reference to cultivable bacteria, this site harbours 10^{10}-10^{11}

CFU/g digesta belonging to more than 500 different species (Jensen, 2001). Konstantinov *et al.* (2004) reported that the majority of the colonic and faecal groups were Gram-positive obligate anaerobes that belong to *Streptococcus, Lactobacillus, Fusobacterum, Eubacterium* and *Peptostreptococcus*. The Gram-negative groups comprise about 10% of the total cultivable bacteria and belong mainly to *Bacteroides* and *Prevotella* (Konstantinov *et al.*, 2004). This was confirmed by Leser *et al*. (2002), using a molecular approach.

2.4.2 Methods to study the microbiota

Woese (1987) suggested that because ribosomal RNA is present in every cell its nucleic sequence can be used for phylogenetic classification. Since, there has been a dramatic increase in methods and studies based on sequence diversity of the 16S rRNA and its encoding gene. The contribution from these methods and studies give new insights in a variety of ecosystems including the GI tract. Molecular microbial ecology involves studies on the *in vivo* activity of microbes present and their interactions with the host (Hungate, 1960). It is a research area that has been focusing on identification of uncultured bacteria, but has the potential to give a complete description of the GI ecosystem. There are many different methods that can be used to study the microbiota. Below some that are commonly used are described and most of them have also been used in the studies of this thesis.

Cultivation

Cultivation has traditionally been used to study the microbes in the GI-tract. The culturing can be performed in either anaerobic or aerobic atmosphere and the media can be selective or non-selective depending on the bacteria of interest. The major advantage with cultivation is that isolates can be saved and further studied for identification and for physiological and biochemical traits (Amann *et al.*, 1995). The major drawback with the method is that it is labour intense and that only a minority of the microbes can be cultured (Vaughan *et al.*, 2000).

Microbial community fingerprinting

Denaturing gradient gel electrophoresis (DGGE) is a fingerprinting method that is polymerase chain reaction (PCR)-based and first was used on environmental samples, such as wastewater, to study the microbial diversity (Muyzer *et al.*, 1993). Since, it has been used to study many different ecosystems and many similar techniques, such as temperature gradient gel electrophoreses (TGGE), have also been developed. Common for these methods is that they separate PCR products on gels due to sequence specific

melting behaviour. A drawback with the method is that only the most dominant bacteria will be represented in the profiles if a domain (e.g. *Bacteria*) specific primer is used (Zoetendal *et al.*, 1998). This limitation could be overcome by using group specific primers.

Terminal restriction fragment length polymorphism (T-RFLP) is another PCR-based fingerprinting method. The PCR products are fluorescently end-labelled followed by digestion with restriction enzymes with specific target sites. The labelled and digested terminal restriction fragments TRFs are separated by electrophoresis. Sequence polymorphism between species gives TRFs with different lengths, and specific fingerprinting profiles are obtained based on the species composition in the sample. The fluorescence intensity is also registered and can be used as a relative measurement of the relative abundance of each TRF. The method is fast and sensitive, and it gives a high throughput (Marsh, 1999). However, due to amplification biases in the PCR (von Wintzingerode *et al.*, 1997) quantitative conclusions may be less precise. The method gives no identification of the different TRFs and the method is therefore often combined with cloning and sequencing.

Cloning and sequencing

Cloning and sequencing of 16S rRNA genes is an appropriate approach for description of bacterial communities in a culture-independent way (Vaughan *et al.*, 2000). The method includes PCR amplification of 16S rRNA genes, construction of clone libraries, and sequencing of cloned genes (Dicksved *et al.*, 2008). The obtained sequences can then be compared to fast growing databases such as the GenBank database with more than 135,000,000 available sequences (NCBI, 2012) and the Ribosomal Database Project 10 which contains more than 2,100,000 16S rRNA sequences (RDP, 2012).

However, it is a work intensive and expensive method, and to monitor community shifts and compare different communities 16S rRNA fingerprinting is more commonly used (Vaughan *et al.*, 2000).

Quantitative PCR (qPCR)

Real-time quantitative PCR or qPCR is a direct quantification method that allows targets present at very low concentrations to be quantified. The data are collected after each cycle of the reaction (real time) instead of at the end of the procedure as for conventional PCR. The method is based on detection and quantification of a fluorescent reporter that reflect the amount of amplified product in each cycle (Smith & Osborn, 2009). When the accumulation of fluorescent signal is significantly greater than the background level the threshold cycle (C_T) is reached. The C_T-value corresponds to the initial amount

of the targeted sequence in the sample (Heid *et al.*, 1996). In absolute quantification the data from unknown samples are interpolated to a standard curve with dilutions of a known amount of the target gene (Smith & Osborn, 2009).

The 16S rRNA gene is commonly used as a target molecule. However, the bacterial species differ in genome size and numbers of 16S rRNA gene copies per genome, which can be misleading for direct comparison of different bacterial groups (Klappenbach *et al.*, 2000). The approach can also be used to quantify other genes, like functional genes specific for a given group or a known function.

2.5 Dietary fibre and health

The interest in the relationship between diet and health is huge both in human and animal nutrition, and it has a long history. The effects of dietary fibre on human health became highlighted in the 1970´s when the health related "dietary fibre hypothesis" was stated. The hypothesis stated that increasing intake of dietary fibre decreases the incidence of colon cancer and heart disease, and was substantiated by several publications (Trowell, 1972; Burkitt, 1971). These studies opened up the field for the dietary fibre research area.

In 1995, Gibson and Roberfroid introduced the concept of prebiotics. They defined a prebiotic as "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson & Roberfroid, 1995). By this definition, almost every food oligosaccharide and polysaccharide (including dietary fibre) has been claimed to have prebiotic activity; however not all dietary carbohydrates are prebiotic. Later, Gibson (2004) stated that there is a need to establish clear rules of what is needed to classify a food ingredient as a prebiotic. He proposed that to be allowed to classify a compound as a prebiotic it should scientifically be demonstrated that it:

1. Resists host digestion, absorption and adsorption processes.
2. Is fermented by the microbiota colonising the GI system.
3. Selectively stimulates the growth and/or the activity of one or a limited number of bacteria within the GI system.

Two of the major enteric diseases in pig production are post weaning colibacillosis caused by colonization of the small intestine by enterotoxigenic strains of *Escherichia coli* and swine dysentery that is caused by colonisation

of the large intestine by *Brachyspira hyodysenteriae (*Hampson *et al*., 2001). The interest in effects of dietary fibre and prebiotics in pig diets and the impact on enteric diseases is high and there are contradictory results on the effect on pig health. Feeding guar gum, a soluble and viscous NSP, increases the proliferation of enterotoxigenic *E. coli* (McDonald *et al.*, 1999), whereas feeding insoluble NSP reduces the occurrence of haemolytic *E. coli,* and reduces the severity of post weaning colibacillosis (Bertschinger *et al.*, 1979). However, Wellock *et al.* (2008) showed that soluble NSP per se is not detrimental to piglet health. Instead they stated that soluble NSP that does not increase the digesta viscosity may beneficially affect gut health by increasing the lactobacilli:coliform ratio and decrease the occurrence of weaning diarrhoea.

The recommendation today for prevention of weaning diarrhoea and for herds with disease problems related to enteropathogenic bacteria is to feed diets with a mixture of soluble and insoluble fibre supplemented with prebiotic carbohydrates other than fibre, in the first week after weaning (Bach Knudsen *et al.*, 2011).

2.5.1 The concept of gut health

Gut health is not a well defined concept (Montagne *et al.*, 2003). Conway (1994) proposed that the concept of gut health contains three major components, namely the diet, the microbiota and the gut mucosa (Figure 1).

Figure 1. A picture of the three major components in the concept of gut health modified from Conway (1994) and Montange *et al.* (2003).

The three components interact with each other and form a delicate and dynamic equilibrium that ensures a functioning digestive system with a high absorptive capacity (Bach Knudsen *et al.*, 2011). The diet should be selected to create a balance between the gut, the microbiota and the gut environment and prevent disturbances in the gut. A dietary component can be valued based on the capacity to either disturb or support this balance (Bach Knudsen *et al.*, 2011; Montagne *et al.*, 2003). Dietary fibre interacts both with the mucosa and the microbiota and consequently has an important role in the control of gut health (Montagne *et al.*, 2003).

2.6 Fibre sources in pig diets

2.6.1 Cereals

Cereal grains can be divided into different structures, including the endosperm, the subaleurone, the aleurone layer and the husk. The cell wall NSP is present mainly in the aleurone layer and the husk. Arabinoxylans, cellulose and β-glucans are the major cell wall NSP in cereals that nearly are free from pectic substances (Bach Knudsen, 2001). There is variation in the composition of cell wall NSP between different cereals and between different cultivars (Andersson & Åman, 2001; Bach Knudsen, 2001). For example, barley and oats have a high content of β-glucans compared with wheat, rye and triticale that have a high content of arabinoxylans in the cell wall (Bach Knudsen, 1997).

2.6.2 Sugar beet pulp

Sugar beet pulp is a by-product from the sugar refining industry of sugar beets (*Beta vulgaris*). The extraction of sugar removes the water soluble nutrients and the dried remains consist mainly of cell wall NSP (McDonald *et al.*, 2002). Sugar beet belongs to the eudicotyledons plants and has consequently high pectin content in the cell wall (Voragen *et al.*, 2001; Van Laar *et al.*, 2000). Bach Knudsen (1997) reported that the total dietary fibre content in sugar beet pulp was 814 g/kg DM, soluble NSP accounted for 52% of NSP, the major component was uronic acid. Voragen *et al.* (2001) reported a total polysaccharide content of 670 g/kg DM of which 40% was pectic substances. The pectins consisted mainly of arabinan, homogalacturonan and arabinogalactan I.

2.6.3 Forage crops

Forages are commonly divided into grass species, legume species, forbes and browse (Van Soest, 1994). The most common legume species in Europe are clovers; widespread species are red clover (*Trifolum pretense*) and white clover (*Trifolum repens*). Both are often sown with grasses. Lucern (*Medicago Sativa*) is another common legume species which is more commonly grown in monocultures. Among the grasses, perennial rye grass (*Lolium perenne*) and timothy (*Phleum pretense*) are common species (Holmes, 1989). Bach Knudsen (1997) showed that the dietary fibre content in grass meal and lucern range from 457 to 595 g/kg DM. Cellulose was the principal NSP constituent in both, followed by xylose. There is a season variation in the cellulose concentration of the grasses with the lowest concentration from the first cut.

Lucerne contains more soluble NSP and uronic acid than grass. This can be explained by the nature of the plants; that is grasses belong to the monocotyledon family and clovers to the dicotyledon family (Sun *et al.*, 2006). Herbs belong to the group forbes, below I will mention two herbs that have been used in the studies of this thesis: chicory and ribwort.

2.6.4 Chicory (*Cichorium intybus* L)

Chicory is a perennial herb with deep roots, a high mineral content and a high drought resistance. It can be complementary to other forage crops and has traditionally been used as a palatable forage crop for sheep, deer and cattle (Hunt & Hay, 1990; Foster, 1988). It is high yielding, can be grown throughout the temperate world and has a high feeding value for grazing ruminants (Barry, 1998). Sanderson *et al.* (2003) reported a yield of 8100 kg DM/ha in monocultures. In southern Sweden, the yield has been 5000-6000 kg DM/ha (Frankow-Lindberg, 2012). The nutritional quality of chicory is better than traditional perennial grasses during summer and autumn (Moloney & Milne, 1993). The proportion of leaf to stem in chicory is important to determine the nutritional value because the leaves have a higher palatability and digestibility than the stem (Li *et al.*, 1997). The reproductive stage and the maturity of a plant are also important for the nutritional value (Buxton *et al.*, 1985). Labreveux *et al.* (2006) showed a higher crude protein (CP) and lower NDF content in autumn harvest than spring harvest. This can be explained by the fact that chicory flowers later in the spring and summer and has a higher content of pectins than traditional grasses (Barry, 1998).

Most studies concerning chicory forage as a feed ingredient has so far been performed on ruminants and it is known that chicory forage is more rapidly degraded in rumen than ryegrass, which has been explained by the higher pectin content in chicory cell walls (Sun *et al.*, 2006; Barry, 1998). Analysis of the polysaccharide composition of the chicory leaf cell walls showed that the proportion of pectins were particularly high in the laminae. The pectins consist mainly of homogalacturans and rhamnogalacturan-I, the proportion of cellulose, xyloglucans, hetroxylans and glucomannans were low (Sun *et al.*, 2006).

Moreover, a part from the profitable traits of chicory forage, the chicory root, is one of the most concentrated sources of inulin and oligofructose (Castellini *et al.*, 2007) both of which are classified as prebiotics (Gibson *et al.*, 2004). The root naturally contains approximately 150-200 g inulin/kg and 80-120 g oligofructose/kg (Flickinger *et al.*, 2003). Inulin extracted from the chicory roots contains approximately 30–50% chains of DP <10, while the rest are longer chains (Van Loo, 2007). Recent research has used chicory inulin as

a supplement in pig diets with different target areas such as decreased boar taint, post-weaning diarrhoea and intestinal helminthes. Hansen *et al.* (2006) and Øverland *et al.* (2011) showed that chicory inulin efficiently decrease the skatole levels in colon, rectum, plasma and backfat. Wellock *et al.* (2008) and Halas *et al.* (2009) showed that chicory inulin decreased the occurrence of weaning diarrhoea and has the potential to protect against post-weaning diarrhoea. Jensen *et al.* (2011) showed a complex effect on intestinal helminthes with reduction of one species and increase of another.

2.6.5 Ribwort (*Plantago Lanceolata* L)

Ribwort or ribgrass is another perennial herb with a high mineral content and high drought resistance. It has historically been used as a minor forage plant in Europe, and it persists well on poor soils (Foster, 1988). One advantage is that it provides leafage in the autumn and winter and it has a high content of both calcium and phosphorus (Foster, 1988). Forage yields of about 7500 kg DM/ha have been reported from monoculture in New Zealand (Stewart, 1996). It is a highly palatable herb to cattle, sheep, deer and horses. Excellent animal performance has been reported for animals grazing mixed swords (Stewart, 1996). Ribwort contains a range of biologically active compounds, such as antimicrobial compounds that may affect the fermentation in ruminants and alter the volatile fatty acid composition (Tamura & Nishibe, 2002) .

3 Aims of the thesis

This is as far as we know the first study to investigate chicory forage as a fibre source in pig nutrition. The aim was to increase our understanding about chicory (forage and root) as fibre source, by studying the effects of diets with inclusion of chicory on digestibility, digestion site, performance, gut microbiota and gut environment. The specific aims of the studies were to evaluate:

- the effect of inclusion level of chicory forage on growth performance, digestibility and faecal coliform bacteria in weaned piglets

- the effect of inclusion level of chicory forage and/or root on digestive organ size, digestibility and faecal microbiota in growing pigs

- the ileal and total tract apparent digestibility of chicory forage in growing pigs

- the digestion site and molecular weight distribution of fibre sources with different NSP solubility in growing pigs

- the effects of fibre sources differing in NSP solubility on gut environment and ileal and faecal microbiota in growing pigs.

3.1 Hypotheses

The hypotheses were *i)* inclusion of chicory in the diet will have a minor effect on the digestibility of dietary carbohydrates, major nutrient components and energy, *ii)* the digestibility of chicory forage will be higher than commonly used forage crops, *iii)* inclusion of chicory in the diet will increase the microbial diversity and increase lactobacilli:coliform ratio, *iv)* inclusion of chicory in the diet will not reduce growth rate and feed conversion but increase digestive organ size.

4 Materials and Methods

4.1 Experimental design

The first experiment (Paper **I**) was organized as a randomized block design, with three replicates for the low and medium inclusion level of herbs, four replicates for the highest inclusion level of herbs and five replicates for the control diet. The second experiment (Paper **II**) was organized as a split-litter design, with five pigs per litter from six different litters. The pigs from the same litter were randomly allocated to one of the five diets. The third experiment (Paper **III** and **IV**), was performed as a change-over experiment with seven post valve t-caecum (PVTC) cannulated pigs, four diets and four periods. Four of the pigs were randomly allocated to the four diets in a 4 x 4 Latin-square design, and three pigs were considered as replicates. Additionally, a pre- and post-period when all pigs were fed the same feed was performed.

4.1.1 Animals and housing

All experiments have been carried out at the Swedish University of Agricultural Sciences in Uppsala and have been approved by the ethical committee of the Uppsala region.

In the first experiment twenty-five 35-day-old weaned castrated male piglets (Swedish Landrace x Yorkshire), were used in a 35-day experiment. The piglets originated from five different litters and had an initial weight of 11.7 kg (s.e. 0.16 kg). The piglets were housed individually in pens and had *ad libitum* access to feed and water. The pig body weight and feed intake were registered weekly.

The second experiment used in total thirty 7-week-old Yorkshire pigs (16 castrated males and 14 females) in an 18-day experiment. The pigs were weaned when they were 5 weeks old and got two weeks adjustment before the experiment started. The pigs had an initial body weight of 15.5 kg (s.e.1.61

kg), were housed individually and had *ad libitum* access to feed and water. The feed intake was registered daily and the pigs were weighed on days 0, 7, 14 and 18.

In the third experiment seven castrated male Yorkshire pigs with an initial body weight of 24.8 kg (s.e. 1.14 kg) were used. The pigs were fitted with a PVTC cannula as described by Van Leeuwen *et al.* (1991) at an average body weight of 22.8 kg (s.e. 0.30). Each experimental period comprised of 14 days. The pigs were fed twice a day with equal portions at 8:00 and 16:00 h. The feed allowance was 4% of the body weight per day until the pigs reached 60 kg then the feeding level was 2.4 kg per day. The pigs were weighed weekly and the feed allowances were adjusted weekly.

4.1.2 Experimental diets

In the first and second experiment the diets comprised of cereal-based basal control diets supplemented with protein, amino acids, minerals and vitamins to meet the nutritional requirement of weaned and growing pigs (Simonsson, 2006; NRC, 1998). In the first experiment the cereals (wheat, barley and oats) were substituted with 40, 80 and 160 g chicory and ribwort forage meal per kg. A total of seven diets were formulated (B, C40, C80, C160, R40, R80 and R160). In the second experiment the cereals (wheat and barley) were substituted with 80 and 160 g chicory forage and/or chicory root meal per kg. A total of five diets were formulated (C, CF80, CF160, CR80 and CFR). In the third experiment the diets comprised of a basal diet based on maize starch, sugar, casein and cellulose, supplemented with minerals and vitamins to meet the nutritional requirements of growing pigs (NRC, 1998). The basal diet was fed as the only feed in the pre- and post-experimental period. In the experimental diets the basal diet was substituted with one of four dietary fibre sources: chicory forage, sugar beet pulp, wheat bran and grass meal. The experimental diets were formulated to have a similar total NSP content.

The chicory and ribwort forage used in the first experiment were harvested in September 2004. In the second experiment the chicory forage was made from a mix (50:50) of two harvests, June and September 2007. In experiment three, chicory forage was harvested in July 2008 and grass meal was harvested in July 2009 from a mixed ley (timothy and meadow fescue). The analysed chemical composition of forages used in Paper **I-IV** is shown in Table 1. The forages were dried with forced air at 30°C for a week and milled through a 5 mm **(I)** or 3.5 mm (**II, III** and **IV**) screen before mixed with the other feed ingredients. The other dietary fibre sources, chicory root meal (Inu60 Inter-Harz GmbH, Germany), sugar beet pulp (Nordic Sugars A/S, Denmark) and

wheat bran (Nord Mills, Sweden) were commercially available. TiO_2 was included in all diets (2.5 g/kg) as an internal digesta marker.

Table 1. *Analysed chemical composition (g/kg DM) of the forages used in the experimental diets in Paper **I-IV** of this thesis*

	$CF_{Sep}2004$	$CF_{June}2007$	$CF_{Sep}2007$	$CF_{July}2008$	$RF_{Sep}2004$	$GM_{July}2009$
Ash	256	149	198	181	134	163
CP	195	137	144	152	169	80
NDF	268	.	.	257	352	.
Total NSP	311	434	400	349	309	406
Insoluble NSP	195	265	206	190	230	405
Total arabinose	13	19	19	18	18	29
Insoluble arabinose	6	9	7	6	10	28
Total xylose	31	50	24	30	32	136
Insoluble xylose	27	49	23	29	27	136
Total uronic acid	97	165	177	124	88	20
Insoluble Uronic acid	21	26	22	12	20	15
Klason lignin	107	100	92	90	78	80
Dietary Fibre	418	534	492	439	386	486
Gross Energy MJ/kg DM	15.0	15.4	14.7	18.2	17.7	18.6

$CF_{Sep}2004$, chicory forage harvested in September 2004.
CFJun2007, chicory forage harvested in June 2007.
$CF_{Sep}2007$, chicory forage harvested in September 2007.
$CF_{Jul}2008$, chicory forage harvested in July 2008.
$RF_{Sep}2004$, ribwort forage harvested in September 2004.
$GM_{Jul}2009$, grass meal harvested in July 2009.

4.2 Sample collection

Faeces samples for digestibility assessment were collected from the carefully cleaned pen floor and stored -20°C until analysis (**I, II** and **III)**. In Paper **I,** the samples were collected for five subsequent days during week three and five of the experiment. In Paper **II,** the samples were collected for four subsequent days during days 15 to 18. In Paper **III** samples were collected during the four first days during the second week (collection week) of each experimental period. The samples were pooled for each pig and period. Feed samples were collected weekly. Additional fresh faecal samples for microbial enumeration (**I** and **II**) and faecal pH (**II**) were collected at days 1, 16 and 35 (**I**) and at days 6, 13 and 17 (**II**).

Faecal samples for molecular microbial assessment were collected at days 0 and 17 (**II**) and at days 1 and 4 of the collection week (**IV**), the samples were stored at -80°C.

In Paper **II** the pigs were killed at day 18 to get organ measurements. In Papers **III** and **IV** ileal digesta samples were collected from the PVTC cannula during days 5 and 7 of the collection period. Samples were pooled for each pig and period. Digesta samples for gut environment and molecular microbial analysis were collected and stored separately (-80°C).

4.3 Sample analyses

4.3.1 Chemical analysis

Feed, faeces and digesta samples for nutritional analyses were freeze-dried and ground through a 1-mm sieve before analysis. In Paper **I, II** and **III,** DM was determined by drying at 103°C for 16 h and for ash after ignition at 600°C for 3 h (Jennische & Larsson, 1990). CP was determined by the Kjeldahl method (Nordic Committee on feed analysis, 2003). Total soluble and insoluble NSP, its constituent sugars and Klason lignin were determined according to a modified Uppsala method (Bach Knudsen, 1997). TiO_2 was analysed according to Short *et al.* (1996).

In Paper **I,** crude fat was determined according to Official Journal of the European Communities (1984). Starch and sugars were analysed by an enzymatic method (Larsson & Bengtsson, 1983). NDF was determined according to Weizhong and Udén (1998), with a 100% neutral detergent solution and the use of amylase and sulphite. In Paper **II**, fructan content was determined according to Association of Official Analytical Chemists with a Fructan Assay Kit (Megazyme Cat. no. K-FRUC, Bray County, Ireland). In Paper **II** and **III,** gross energy was measured with a bomb calorimeter (Parr 6300 Oxygen Bomb Calorimeter, Illinois, USA).

Organic acids (SCFA and lactic acid) analysis in Paper **IV** was performed on thawed and centrifuged (5 minutes at 13,000 x g) digesta samples. Analysis of the samples was performed according to Andersson & Hedlund (1983) by high-performance liquid chromatography (HPLC). Ileal digesta pH in Paper **IV** was measured on thawed samples with a pH-meter (Metrohm 719 S Titrino). To determine the faecal pH in Paper **II**, a subsample of 2 g of each fresh faecal sample was diluted in 20 ml distilled water and the pH was measured with a pH-meter (PHM 210, Radiometer, Cedex, France).

The molecular weight distribution of the soluble NSP fraction in diets and ileal digesta of treatment chicory forage and sugar beet pulp were analysed with a high-performance size exclusion chromatography system (**IV**).

4.3.2 Microbial analysis

Bacterial enumeration

Faecal coliform counts were determined by plating on blood agar plates (**I**). In Paper **II,** lactobacilli were enumerated by plating on Rogosa agar (Merck, Darmstadt, Germany) and coliforms were enumerated by plating on MacConkey agar (Merck, Darmstadt, Germany). All bacterial counts were log10 transformed before statistical analysis.

Molecular microbial analysis

In Paper **II** and **IV,** molecular microbial assessments were performed. Briefly, DNA was extracted using QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany). To obtain a profile of the faecal microbiota composition **(II** and **IV)**, extracted DNA was analysed with the T-RFLP methodology as previously described (Dicksved *et al.*, 2008).

To identify bacteria that corresponded to TRFs of interest, additional cloning and sequencing were performed (**II)**. Small clone libraries from six pigs were created, followed by PCR amplification and T-RFLP analysis (Dicksved *et al.*, 2008). Clones generating TRF sizes of interest were sent for sequencing at a total of 103 sequenced clones. The sequences were compared with the GenBank database using standard nucleotide BLAST at NCBI, as well as with the Ribosomal Database Project 10 Sequence Match. The obtained sequence data in Paper **II** was also used for identification of TRFs of interest in Paper **IV**.

In Paper **IV,** qPCR assays was used to determine the abundance of bacteria belonging to *Lactobacillus*, *Enterobacteriaceae* and *Bacteroides-Prevotella-Porphyromonas*. *Lactobacillus* was analysed according to Karlsson *et al.* (2011). *Enterobacteriaceae* and *Bacteroides-Prevotella-Porphyromonas* were analysed according to Metzler-Zebeli *et al.* (2010). qPCR was also used to determine the abundance of beta-xylanase (EC 3.2.1.37) genes, *xynB*, among *Bacteroidetes* and *Firmicutes*. For this assessment specific primers were designed and the run conditions optimized. Primers targeting the polygalacturonase (EC 3.2.1.15) gene were designed; however the specificity could not be confirmed and the analysis was excluded.

4.4 Calculations

In all papers dietary fibre was calculated as total NSP+lignin.

In Paper **I, II** and **III** the coefficient of total tract apparent digestibility and coefficient of ileal apparent digestibility were calculated using the indicator technique (Sauer *et al.*, 2000).

The relative organ weight was calculated as g organ/kg live weight (**II**).

Simpson's diversity index was calculated (**II** and **IV)** according to Begon *et al.* (2006).

In Paper **III,** endogenous losses of N in ileal digesta was estimated according to Mariscal-Landín & Reis de Souza (2006). Values for standardised ileal digestibility of CP were calculated according to Stein *et al.* (2007). The coefficients of total tract apparent digestibility of the ingredients were calculated by difference calculation. The contribution of a dietary component from the basal diet were corrected and accounted for to calculate the coefficient of total tract apparent digestibility of each fibre ingredient (Bureau *et al.*, 1999).

For the molecule weight distribution three molecular weight intervals (g/mol) were selected; molecular weight large=1 000 000 -10 000 000; molecular weight medium= 200 000-1 000 000 and molecular weight small= 10 000-200 000. The relative distribution of molecules in each interval was calculated as % of total molecules.

4.5 Statistical analysis

The statistical analyses in all papers were performed with the SAS programme (SAS Institute, Cary, NC, USA, version 9.1). Two-way interactions were tested and excluded from the model if $P>0.05$. The level of significance in all papers were set at $P \leq 0.05$.

Additional cluster analyses using the Spearman rank correlation were performed on T-RFLP data in Paper **II** and **IV** with PAST (Paleontological statistics software package for education and data analysis version 2.13).

Performance, digestibility, organ size, bacterial enumeration, pH, organic acids, relative abundance of TRFs, abundance of bacteria, and molecular weight distribution were analysed with procedure Mixed. The models in Paper **I** and **II** included diets and sex as fixed factors and litter as random factor. The model in Paper **III** and **IV** included diet and period as fixed factors and pig as random factor. Carry-over effects from the previous period were tested and excluded from the model if $P>0.05$. For average daily weight gain **(I** and **II)**, the initial weight was used as a covariate in the model. For the relative abundance of TRFs at day 17 (**II**), the relative abundance at day 0 was used as a covariate. Faecal samples used for digestibility (**I**), bacterial enumeration and faecal pH (**I** and **II**) were collected from all pigs repeatedly. Diet effects were therefore analysed with repeated statement with homogeneous autoregressive of order 1 as covariate structure for digestibility and heterogeneous autoregressive of order 1 for the other parameters.

Pearson correlations were used to evaluate the relationships between gut environment, gut microbiota and amount digested dietary components as well as the molecule weight distribution **(IV)**.

5 Major Results

5.1 Digestibility

The total tract apparent digestibility of DM, organic matter (OM) and CP were lower in all diets with chicory and ribwort forage inclusion (40-160 g/kg) compared with the cereal-based control diet (**I**). However, in Paper **II** there was no difference between the cereal-based control diet and the diet with inclusion of 80 g chicory forage/kg. However, inclusion of 80 g chicory root/kg did decrease the total tract apparent digestibility of CP (**II**). The total tract apparent digestibility of NSP and especially uronic acid was higher for the diets with chicory and ribwort inclusion than for the cereal-based control diets, resulting in higher dietary fibre digestibility with increasing herb inclusion (**I** and **II**). Diets with inclusion of chicory forage had a higher total tract apparent digestibility of NSP than diets with inclusion of chicory root (**II**). In general, the digestibility increased with age and was higher at five than at three weeks post weaning (**I**).

The coefficients of total tract apparent digestibility of OM, CP, NSP and energy for chicory forage were (least square means ± s.e.): 0.43 ± 0.04; 0.31 ± 0.100; 0.66 ± 0.08; 0.43 ± 0.04, respectively (**III**). The ileal apparent digestibility of DM and OM of the diet with chicory forage was lower than for the diet with sugar beet pulp, but similar as for the diets with wheat bran and grass meal. The ileal apparent digestibility of NSP and all constituent sugars did not differ between the diet with chicory forage and the diet with sugar beet pulp; however both were higher than the diet with wheat bran.

5.1.1 Molecular weight distribution

The large molecule weight fraction of ileal digesta did not differ between pigs fed the diets with chicory forage and sugar beet pulp (**III**). However, pigs fed the diet with sugar beet pulp had higher medium molecule weight fraction

whereas pigs fed the diet with chicory forage had higher small molecule weight fraction. Distribution change from diet to digesta showed that the large molecule weight fraction in digesta increased in pigs fed the diet with chicory forage whereas it decreased in pigs fed the diet with sugar beet pulp. The medium molecule weight fraction decreased on both diets; this decline was higher in the pigs fed the diet with chicory forage. The small molecule weight fraction increased relatively equally on both diets.

5.2 Microbiota

5.2.1 Faeces

Bacterial enumeration and pH

The coliform counts decreased with increasing age (**I** and **II**). No diet effect was observed in Paper **I**, whereas pigs fed the diet with inclusion of both chicory forage and root had lower coliform counts than pigs fed the control diet (**II**). Those pigs also had higher lactobacilli:coliform ratio than pigs fed the control diet and diets with inclusion of only chicory forage (**II**). The lactobacilli counts were lower in pigs fed the diet with inclusion of 80 g chicory forage/kg than pigs fed the control and the diets with inclusion of chicory root. The faecal pH was lower in pigs fed the diet with inclusion of only chicory root than in pigs fed the other diets (**II**).

Microbial composition

T-RFLP was used to assess the dietary impacts on the composition of the faecal microbiota (**II** and **IV**). In general, cluster analysis revealed two major clusters (**II**), one of the clusters was dominated by pigs fed the diet with the highest inclusion (160 g/kg) of chicory forage. Five of six pigs fed that diet clustered together. However, no clear clusters related to diet were observed in Paper **IV**. The Simpson's diversity index did not differ significantly because of diet (**II** and **IV**), however a tendency was observed in Paper **II**. A total of 62 (**II**) and 66 (**IV**) TRFs were statistically evaluated, of which 12 (**II**) and 9 (**IV**) differed in relative abundance between diets.

Two TRFs (163 and 264) in Paper **II**, were identified as two different species related to *Prevotella*, had a lower relative abundance in pigs fed chicory forage diets. A third TRF (262), identified as another species related to *Prevotella* had higher relative abundance in pigs fed diets with chicory forage. However, the identification did only show 90% sequence similarity and a newly performed BLAST has identified TRF 262 as a species related to *Clostridium.* Three TRFs (261, 411 and 412) in Paper **IV**, all identified as

species related to *Prevotellacea* had highest relative abundance in pigs fed the diet with chicory forage.

TRF 275, identified as *Megashera elsdenii,* was affected by diet both in Paper **II** and **IV**. A higher relative abundance was observed in pigs fed diets with inclusion of chicory root than in pigs fed diets with inclusion of chicory forage (**II**). Pigs fed the diet with wheat bran had a higher relative abundance of TRF 275 than in pigs fed the diet with chicory forage in Paper **IV**.

TRF 331 identified as *Lactobacillus johnsonii* was affected by diet in Paper **II.** Pigs fed the diet with inclusion of both chicory forage and root had a higher relative abundance than in pigs fed the other diets.

Additional qPCR analysis was used to assess the dietary impact on the microbial composition (**IV**). Pigs fed the diet with chicory forage had higher abundance of *Bacteroides-Prevotella-Porphyromonas* than pigs fed the diet with sugar beet pulp (**IV**). In contrast, pigs fed the diet with sugar beet pulp had a higher *Lactobacillus*:*Enterobacteriaceae* ratio ($P<0.05$) than pigs fed diet with chicory forage. Pigs fed the diet with chicory forage had higher abundance of the beta-xylosidase gene from *Firmicutes* and tended to have higher abundance of the beta-xylosidase gene from *Bacteroidetes* than pigs fed the diets with sugar beet pulp, wheat bran and grass meal.

5.2.2 Ileal digesta

Microbial composition

Sixty-two TRFs were statistically evaluated of which 7 differed in relative abundance between diets. No clear clustering related to diets was observed. TRF 257, identified as *Bifidobacterium boum*, had the highest relative abundance in pigs fed the diet with chicory forage. TRF 262 identified as species related to *Clostridium*, was the TRF with highest relative abundance among all diets and was higher in pigs fed the diet with chicory forage than pigs fed the diets with sugar beet pulp and wheat bran. TRF 306 identified as species related to *Clostridiales bacterium*, had a higher relative abundance in pigs fed the diet with chicory forage than pigs fed the other diets. The qPCR analysis showed that pigs fed the diet with grass meal had higher abundance of *Bacteroides-Prevotella-Porphyromonas* than pigs fed the diet with wheat bran. The abundance of *Lactobacillus* tended to differ between diets with the numerically highest numbers for pigs fed the diet with sugar beet pulp.

Pearson correlations showed that amount of digested (g/day) NSP ($r=0.57$; $P=0.002$), xylose ($r=0.53$; $P=0.004$) and dietary fibre ($r=0.60$; $P=0.001$) were positively correlated to gene copy numbers of *Bacteroides-Prevotella-Porphyromonas*.

Organic acids and pH

There was no dietary effect on ileal digesta pH (**IV)**. Pigs fed diets with grass meal and wheat bran had a higher concentration and a higher proportion of butyric acid in ileal digesta than pigs fed diets with chicory forage and sugar beet pulp (**IV**). However, pigs fed the diet with chicory forage had a higher proportion of acetic acid than pigs fed the other diets.

The abundance of *Lactobacillus* were positively correlated (r=0.52; P=0.008) with the concentration (mmol/L) of lactic acid. There was a negative correlation between amount digested uronic acid and the mol% of butyric acid (r=-0.48; P=0.010). There was a positive correlation between amount digested uronic acid and the mol% of acetic acid (r=0.48; P=0.010). The proportion of the large molecule weight fraction and the medium molecule weight fraction in ileal digesta were negatively correlated to the proportion acetic acid (r=-0.52, P=0.05; and r=-0.62, P=0.02; respectively). The proportion of the medium molecule weight fraction was positively correlated to the proportion propionic acid (r=0.83: P=0.001), whereas there was a negative correlation (r= -0.76: P=0.002) between the small molecule weight fraction and the proportion propionic acid.

5.3 Pig performance and organ size

No significant differences in daily feed intake, daily weight gain and feed conversion ratio were observed between the chicory diets and the cereal-based control diets neither in Paper **I** nor in Paper **II**. The relative weight (g/kg live weight) of the total digestive tract did not differ between the control diet and diets with chicory inclusion (**II**). However, pigs fed the diets with chicory inclusion had a smaller small:large intestine ratio compared with pigs fed the control diet. The relative weight of colon was higher in pigs fed diets with inclusion of 160 g chicory/kg than pigs fed the control diet.

6 General Discussion

This is as far as we know the first study to investigate chicory forage as a fibre source in pig nutrition. The aim was to provide information about chicory (forage and root) and how it can be utilized by the pig. Paper **I-III** of this thesis has given information about that. Moreover, this thesis aimed to evaluate the impact of chicory feeding on the gut microbial composition and the gut environment. To fully understand how different dietary interventions affect gut health more knowledge about the commensal bacteria in the pig is needed. Information about the commensal bacteria in healthy pigs and how it can be affected by different fibre sources is provided in Paper **I**, **II** and **IV** of this thesis.

6.1 Effect of dietary fibre source and inclusion level on digestibility

We hypothesized that inclusion of chicory in the diet would have a minor effect on the digestibility of dietary carbohydrates, major nutrient components and energy, and be a highly digestible forage crop. The coefficient of total tract apparent digestibility of NSP of chicory forage in growing pigs was estimated to 0.66 ± 0.08 (**III**). Chabeauti *et al.* (1991) reported that the NSP digestibility in growing pigs range from 0.16 for wheat straw to 0.79 for soybean hulls. Although the fibre digestibility was high, it is lower than many other dietary components such as starch, sugar, protein and fat which all have coefficients above 0.80 (Noblet & Le Goff, 2001). As a consequence the total tract digestibility of OM, CP and energy decreases with increasing dietary fibre inclusion, resulting in a decreased net energy value of the diet (Anguita *et al.*, 2006; Noblet & Perez, 1993; Just *et al.*, 1983). This was demonstated also in this thesis (**I** and **II**) and confirms our first hypothesis. However, at which inclusion level of chicory forage the digestibilty decreased differed between the two studies. In Paper **I** lower digestibilities were observed already with

inclusion of 40 g chicory forage/kg. In Paper **II** the digestibilties was maintained with inclusion of 80 g chicory forage/kg and declined digestibilities were only observed with inclusion of 160 g chicory forage/kg. The discrepancy in the effect of inclusion level of chicory forage on digestibility may be explained by the age and weight of the pigs. The pigs were younger, had a lower initial weight and grew slower in Paper **I**. However, the digestibility increased from 3 to 5 weeks post weaning in Paper **I**, increased digestibility with age and weight is well in agreement with others (Jørgensen *et al.*, 2007; Longland *et al.*, 1993; Shi & Noblet, 1993). Lindberg & Andersson (1998) reported that growing pigs maintained the digestibility of OM, CP and energy with 100 g inclusion of white clover, lucerne or perennial ryegrass per kg in cereal-based diets. However, with an inclusion of 200 g/kg the digestibility decreased. This is in accordance with the results obtained for chicory forage in Paper **II,** and indicates that inclusion of 80-100 g forage meal of good nutritional quality in a diet to growing pigs can be possible with maintained digestible energy of the diet. This level is higher than the current Swedish recommendation, where maximum inclusion of 50 g grass meal/kg and 30 g lucerne/kg is recommended to growing pigs (Simonsson, 2006). To be able to suggest a changed recommendation, long-term feeding trials with inclusion levels above those currently recommended have to be performed.

In contrast to the OM, CP and energy, the total tract digestibility of NSP and its constituent sugars increased with increasing herb inclusion (**I** and **II**). The NSP source is one of the most important factors determining the dietary fibre digestibility (Bach Knudsen & Jørgensen, 2001). Pectins, arabinoxylans and inulin were the major NSP constituents in the experimental diets of this thesis. In Paper **II** these three constituents were compared and it was demonstrated that pectins from chicory forage was the NSP source with the highest digestibility, followed by inulin from chicory root and last arabinoxylans from wheat and barley. Sugar beet pulp is another pectin-rich fibre source. Estimation of the digestibility of ingredients (**III**) showed a higher NSP digestibility of sugar beet pulp pectins than of cereal arabinoxylans, which is in agreement with others (Noblet & Le Goff, 2001; Graham *et al.*, 1986). Comparing pectins from sugar beet pulp and chicory forage showed a higher total tract NSP digestibility of sugar beet pulp. Pectins are complex polymers and the difference in NSP digestibility between chicory forage and sugar beet pulp indicates that they have a different intra-molecular structure, which was confirmed by the molecular weight distribution in Paper **III**.

We hypothesized that chicory forage would have a higher digestibility than commonly used forage crops. This could not be confirmed for OM, CP and energy (Table 2), instead the digestibility of chicory forage is similar to

commonly used forage crops. However, a high fibre digestibility of chicory forage was observed. It is hard to do direct comparison to previous studies, because of differences in analysis methods (Table 2). Compared to grass meal, chicory forage had numerically higher digestibility of OM, CP, NSP and energy (**III**). However, high individual variation was observed, especially for the grass meal diet. It should be noted that the grass meal values were only based on four pigs, as three of the pigs had a too low feed intake. The inclusion level of the feed ingredients in Paper **III** was low, which made the difference calculation vulnerable to small errors and this may have contributed to the high variation of the digestibility values for the ingredients. To decrease the variation, a higher inclusion level of the ingredient should be used. Another approach to calculate the digestibility of an ingredient is to do a regression analysis, graded inclusion levels of the ingredient is then needed. These options require a quite high availability of the ingredients to be tested, which might not always be the case for new feedstuffs. Another factor that might have affected the digestibility values is the relatively short experimental periods. Longland *et al.* (1993) reported that to get stable values of NSP digestibility 3 to 5 weeks adaptation might be needed. On the other hand, Awati *et al.* (2005) suggested that 72 h is enough to adapt to the fermentation to both sugar beet pulp and wheat starch and shift the dominant microbial species.

It should also be noted that the reported digestibility of chicory forage (**III**) was based on values from only one harvest (July 2008). Variation in the chemical composition of chicory forage from different harvest times is shown in Table 1. The reproductive stage and the maturity of the plant are important for the nutritional value (Buxton *et al.*, 1985). Liu e*t al.* (2011) reported that chicory forage from September harvest had a higher nutritional value in broiler chickens than chicory forage from June harvest. Consequently, the digestibility of chicory forage is expected to be different from different harvest times and needs further investigations. Furthermore, chicory is a forage crop that due to its deep roots and high drought resistance can improve leys when included in mixed swards (Foster, 1988). Therefore, the nutritional quality of mixed sward forages with chicory inclusion also needs to be studied.

Our results also showed that a large part of the NSP fraction of chicory forage was digested before the hindgut. The ileal NSP digestibility was comparable to sugar beet pulp and much higher than wheat bran (**III**). Longland *et al.* (1994) suggested that sugar beet pulp either stimulated the proliferation of gut microbes or rendered the existing population more efficient when compared with cereal fibres. The results from our studies suggest a similar effect for chicory forage; moreover it has been shown that chicory

forage has a higher potential than cereal fibres to manipulate the microbial composition already in the small intestine.

Table 2. *Coefficients of total tract apparent digestibility of chicory forage, lucerne, white clover, red clover and timothy, means ± standard error.*

	Chicory forage	Lucerne	White clover	Red Clover	Timothy
OM	0.43 ± 0.037	0.40 ± 0.025	0.50 ± 0.025	0.42 ± 0.058	0.55 ± 0.03
CP	0.31 ± 0.103	0.49 ± 0.034	0.52 ± 0.026	0.38 ± 0.066	0.47 ± 0.04
NSP	0.66 ± 0.075	-	-	-	-
NDF	-	0.35 ± 0.030	0.42 ± 0.047	0.33 ± 0.083	0.53 ± 0.02
Energy	0.43 ± 0.035	0.35 ± 0.028	0.45 ± 0.027	0.31 ± 0.088	-
Reference	Paper **III**	Andersson & Lindberg, (1997a)	Andersson & Lindberg, (1997a)	Andersson & Lindberg (1997b)	Håkansson & Malmlöf (1984)

6.2 Effect of dietary fibre source on microbiota

The most important control of the microbial fermentation in the GI tract is the amount and type of substrates available (Jensen, 2001). NSP in particular are important as energy substrate for the microbes (Jensen, 2001; Williams *et al.*, 2001). One mechanism behind protection against enteric diseases is thought to be related to reduced availability of substrates for the disease causing bacteria (Hampson *et al.*, 2001). Bach Knudsen *et al.* (2011) reviewed the impact of different carbohydrates on post weaning enteric diseases. Although extensive research has been performed within the area during the last decades, the results are not consistent. No clear conclusions could be drawn of what types of carbohydrates are protective against enteric diseases. It should be noted that there are many other factors than the diet that affect the resilience against diseases. However, Bach Knudsen *et al.* (2011) considered NSP that are both soluble and viscous increases the susceptibility to enteric diseases whereas stimulation of beneficial bacteria like lactobacilli is favourable, which is in agreement with others (Wellock *et al.*, 2008; Hampson *et al.*, 2001; Ewing & Cole, 1994).

We hypothesised that chicory inclusion would increase the lactobacilli:coliform ratio. Ewing & Cole (1994) suggested that the higher the this ratio is, the better is the microbial contribution to growth performance of the host. Interestingly, a synergistic effect was observed by combining chicory forage and chicory root in the diet, with decreased coliform counts and

increased lactobacilli:coliform ratio as a result (**II**). Synergistic effects on lactobacilli and enterobacteria by combining different fibre sources in the diet were also observed by Bikker *et al.* (2006) and Molist *et al.* (2009). The synergistic effects may partly be an effect of metabolic-cross feeding that will be discussed later in this thesis (section 6.2.1). Although most research about prebiotics and the effect on gut microbiota has focussed on the effect on lactobacilli, it is known that also other bacterial groups are stimulated. Mølbak *et al.* (2007) and Halas *et al.* (2010*)* found that supplementation with inulin stimulates *Megasphaera elsdenii*, which we also found (**II**). *M. elsdenii* was also found to be stimulated by wheat bran (**IV)** and is a bacterial species that may play a role in inhibition of the pathogenic bacteria *Brachyspira hyodysenteriae* (Mølbak *et al.,* 2007). Moreover, Hashizume *et al.* (2003) showed that *M. elsdenii* convert lactate to butyrate. Wheat bran also stimulated *Lactobacillus reuteri* in ileal digesta (**IV**). *L. reuteri* is a bacterial species that is able to produce the antimicrobial compound reuterin that have the potential to suppress other bacteria (Cleusix *et al.*, 2008). A numerical but not significant lower diversity was observed on the wheat bran diet (**IV**) and the chicory root diet (**II**). It can be speculated that the increased abundance of *M. elsdenii* and *L. reuteri* suppress the microbial diversity. We hypothesized that inclusion of chicory in the diet would increase the diversity. Although numerical higher values were observed with chicory forage inclusion in Paper **II** and Paper **IV**, this hypothesis could not be confirmed. The link between the microbial diversity and gut health is complex. It is generally considered that a high diversity increase the resilience against pathogens; however, presence of keystone bacterial groups may compensate for a low diversity (Ley *et al.*, 2006).

Prevotella is one of the dominating commensal bacteria in the large intestine of the pig (Leser *et al.*, 2002). Bacteria belonging to the *Prevotellacea* family constituted also the dominating bacterial group in faecal samples in this thesis and were affected by the diet in both Paper **II** and **IV**. The impact of chicory forage on this group was somehow contradictory with generally a lower relative abundance observed in Paper **II**, whereas a higher relative abundance was observed in Paper **IV**. Tajima *et al.* (2001) showed that in rumen *Prevotella ruminicola* and *Prevotella bryantii* respond in opposite directions to hay and grain diets, showing a high variation within the genus. Leser *et al*. (2002) showed that there were few sequence similarities with bacteria identified as *Prevotella* in the pig GI tract, which also was shown in our studies. TRF 262 was one of the dominating TRFs and was stimulated by chicory forage diets (**II** and **IV**). It was first identified as a species related to *Prevotella* but with low sequence similarity and a newly performed BLAST

showed that the TRF may belong to *Clostridium*. However, the similarity was only 96% and to fully understand our dietary effects and how it affects gut health, more knowledge is needed about the commensal bacteria in pigs. It should be pointed out that all the pigs used in our studies stayed healthy without any signs of disturbances in the GI tract.

Bacteroides and *Prevotella* both belong to the phylum *Bacteroidetes* and are among the most frequently isolated xylanolytic bacteria from rumen and human faecal samples (Dodd *et al.*, 2011). Moreover, *Prevotella bryantii* and *P. ruminicola* have been reported to have several genes coding for different xylanases (Flint & Bayer, 2008; Gasparic *et al.*, 1995). A high abundance of the beta-xylosidase gene among the *Bacteroidetes* was found in both digesta and faecal sample in Paper **IV**. Bacteria belonging to *Bacteroides* and *Prevotella* also had the polygalacturonase gene and ability to degrade pectins (**IV**, data not shown). Moreover, a strong postitive correlation between amount digested dietary fibre and the abundance of *Bacteroides-Prevotella-Porphyromonas* was also shown in Paper **IV**. Together the results indicate that the *Bacteroides* and the *Prevotella* genera may be the major dietary fibre degraders in the pig GI tract. However, a high abundance of the beta-xylosidase gene among the *Firmicutes* was also observed in Paper **IV.** This is in agreement with Chassard & Bernalier-Donadille (2006) who showed that bacteria belonging to the *Roseburia* genus have xylanolytic genes. Van Laer *et al*. (2000) reported that the degradation of dietary fibre is complex and assumed to be a result of a combined action of several bacteria like *Bacteroides*, *Bifidobacterium*, *Ruminococcus*, *Eubacterium*, *Lactobacillus* and *Clostridium.* Different bacterial groups are also involved in different steps of the fibre degradation. *Bacteroides* are regarded to utilize mainly NSP although they also can utilize oligosaccharides, whereas other groups such as *Bifidobacteria* utilize smaller fragments, such as oligosaccharides (Van Laere *et al.*, 2000). This complex degradation pattern may contribute to the observed synergistic effects on the microbiota with supplementation of different fibre sources.

The microbial composition in faeces and ileal digesta showed as expected differences in dominating groups, with *Clostridium* as major group in ileum and *Prevotellacea* in faecal samples. Although a difference in dominating groups was observed, the dietary effects in ileal digesta and faeces followed similar patterns (**IV**).

6.2.1 Effect of dietary fibre on gut environment

Awati *et al.* (2005) suggested that it is the structure and availability of substrate that determines the fermentation end-products rather than the microbial community that is present. This was also shown in this thesis: the compositions of organic acids in ileal digesta were highly influenced by NSP structure (**IV**). Feeding pigs diets rich in arabinoxylan either as grass meal or wheat bran, resulted both in the highest concentration and proportion of butyric acid. Results that are in agreement with others (Molist *et al.*, 2009; Bikker *et al.*, 2006; Högberg & Lindberg, 2006). Feeding the pectin-rich chicory forage diet resulted in a higher proportion of acetic acid, which is in agreement with Englyst *et al*. (1987), who showed that acetic acid is the major end-product of pectin degradation. However, feeding the pectin-rich diet sugar beet pulp instead decreased the proportion of acetic acid in accordance with Wang *et al*. (2004). Instead a higher proportion of propionic acid was observed on the sugar beet pulp diet compared to the chicory forage diet. Interestingly, strong correlations were observed between the molecular weight distribution of the pectins and the proportion of acetic and propionic acids. This indicates that the pectin degradation is selective and the difference in SCFA production can be explained by the molecular weight distribution. The results are unique and it would be interesting to investigate if a similar relationship also can be found for other fibre sources. Bach Knudsen *et al.* (2011) expected that the structure of soluble NSP plays a major role for the susceptibility of soluble NSP to predispose to post weaning enteric diseases. Structures that increase the digesta viscosity would likely increase the susceptibility. A rule of thumb is that doubling the molecular weight or the concentration increase the viscosity by factor 10 (Eastwood & Morris, 1992). It is consequently the large molecules that contribute mostly to the increased digesta viscosity. The large molecules were the smallest molecule weight fraction, and did not differ between the sugar beet pulp and the chicory forage diets. It has been shown that both newly weaned piglets (**I**) and broiler chickens (Liu *et al.*, 2011) tolerate high inclusion levels of chicory forage without any signs of digestive disturbances. This indicates that the soluble NSP fraction in chicory forage does not cause a viscosity problem.

Although strong correlations between molecular weight distribution and SCFA were shown, no clear correlation between the molecular weight distribution and specific bacterial groups were found. The relationship between bacterial groups and the production of organic acids is not always straight forward. This can partly be explained by metabolic cross-feeding that commonly occurs within the gut ecosystem. A number of bacterial species can convert lactic acid and acetic acid to butyric acid (Duncan *et al.*, 2004).

Consequently, increased acetic acid and lactic acid production might stimulate the butyrate-producing bacteria. Moreover propionate-producing bacteria can use lactic acid as a substrate (Duncan *et al.*, 2004) and this may explain why lactic acid does not accumulate although there are many lactate-producing bacteria within the gut ecosystem. Acetic acid is the most abundant SCFA in the gut and many bacterial groups can form acetic acid whereas the bacterial groups that form propionic acid and butyric acids are more restricted (Louis *et al.*, 2007). Butyric acid is a major energy source for colonocytes and propionic acid inhibits lipogenesis (Pryde *et al.*, 2002; Williams *et al.*, 2001). Both may be beneficial for health, and especially butyric acid may improve gut health by providing energy for the gut mucosa (Louis *et al.*, 2007; Hashizume *et al.*, 2003; Pryde *et al.*, 2002). The fact that there are strong interactions between dietary fibre sources, microbiota and the production of organic acids is clear. To find how these interactions are arranged and how they affect the gut ecosystem is a key to be able to by dietary intervention manipulate gut health in a controlled way. To map these interactions is a huge challenge for future research, but the availability of many new techniques actually makes it possible.

The pH within the gut is another important factor to determine the major bacterial groups within the ecosystem. An effect on the faecal pH with lower values in pigs fed the diet with inclusion of only chicory root was observed in Paper **II**. An acidic pH may support competitive exclusion by inhibiting *Enterobacteria* and limits the population of potential pathogens (Louis *et al.*, 2007). Walker *et al.* (2005) showed that decreasing the pH in human colon from 6.6 to 5.5 shifted the dominating groups from *Bacteroides* to *Roseburia* and *Eubacterium rectale*. The ileal digesta pH did not differ between diets in Paper **IV** although there were differences in the SCFA content. However, fermentation of a specific substrate is not always accompanied with a decrease in pH, and a pH decrease on its own should not be used as an index for fermentation (Barry *et al.*, 1995).

6.3 Performance and organ development

We hypothesised that inclusion of chicory in the diet will not reduce growth rate and feed conversion but increase digestive organ size. Pigs fed diets with chicory inclusion stayed healthy throughout the experiments; they showed a high daily weigh gain, daily feed intake and feed conversion ratio at all inclusion levels and did not differ significantly from the pigs fed the control diet (**I** and **II**). This confirms our hypothesis. In contrast, decreased performance was observed on the ribwort diet due to feed refusal and feed

spoilage, indicating that ribwort has a low palatability for piglets (**I**). Vestergaard *et al*. (1996) reported decreased performance when grass meal was included in the diet, whereas others have reported maintained performance for weaning pigs fed diets with inclusion of different sources of dietary fibre (Halas *et al.*, 2009; Wellock *et al.*, 2008; Pluske *et al.*, 2003; Longland *et al.*, 1994). However, increased GI organ weight and decreased empty body weight gain were also reported (Wellock *et al.*, 2008; Pluske *et al.*, 2003). There were no differences in the relative weight of the total GI tract in our study, which was in agreement with Halas *et al.* (2009), but in contrast to our hypothesis. However, the gut fill was not measured and increased gut fill with chicory inclusion may have contributed to the maintained body weight (**II**). Although no differences in relative weight of the total digestive tract were detected in our study, inclusion of 160 g/kg chicory resulted in heavier colon compared with the control diet, and the small:large intestine ratio was lower in all diets with chicory inclusion (**II**). This indicates that chicory inclusion stimulates hindgut development. It should be noted that the studies of this thesis have used few animals per treatment in short-term experiments, and to fully explore the potential of chicory feeding on growth performance long-term feeding trials have to be performed.

7 Conclusions

Taken together, the results from studies performed during experimental condition in this thesis show that there is potential for both chicory forage and root to be used as regular feed ingredients in pig diets. However, to fully explore the potential of chicory feeding, long-term feeding trials with on-farm conditions should be performed before a final recommendation can be given. Based on results from the studies performed it can be concluded that:

- The total tract digestibility of OM and energy of chicory forage was similar to commonly used forage crops
- The NSP digestibility in both chicory forage and root was higher than the NSP digestibility in cereals and a high proportion of chicory forage NSP was digested prior to the hindgut
- Inclusion of 80 g chicory forage per kg in diets to growing pigs can be possible without depression in digestibility of OM, CP and energy
- Inclusion of 160 g chicory per kg in diets to pigs can be possible without decreased growth performance and increased GI organ weight
- Chicory forage and root modulated the gut microbiota differently with synergistic effects on lactobacilli:coliform ratio when combined in the diet
- Increasing the total amount of fermentable dietary fibre in the diet increased the abundance of *Bacteroides-Prevotella-Porphyromonas* in growing pigs. The effects of native fibre sources on the microbial composition were to a high degree ingredient-specific rather than NSP-structure specific

- The effect on organic acid was NSP structure-specific and arabinoxylan-rich diets increased the butyric acid production. Pectin-rich diets increased the acetic or propionic acid production depending on the intra-molecular structure of pectin.

8 Future research

The present thesis has evaluated effects of chicory forage in diets to newly weaned pigs and to growing pigs in short-term experiments. To fully explore the potential of chicory forage as a feedstuff in pig nutrition long-term feeding trials with growing pigs as well as with dry and lactating sows should be performed. In addition, the impact of harvest time and different preservation methods (e.g. drying and ensiling) on the nutritional value of chicory forage needs further investigations. Moreover, the economic aspects of chicory feeding, which include feed costs, animal performance and carcass traits, need to be evaluated.

It has been suggested that highly-fermentable dietary fibres have a higher potential to prolong postprandial satiety than low-fermentable (bulky) fibres (De Leeuw *et al.*, 2008). Thus, highly-fermentable chicory forage should have potential to prolong postprandial satiety and could potentially be used to improve the welfare of restrictedly fed dry sows.

Although extensive research related to dietary strategies, aimed at preventing post weaning enteric diseases in pigs, has been performed the problem is still present. Therefore, further studies should be performed with the aim to map the interactions between the intra-molecular structure of NSP, gut microbiota and the production of organic acids as this could be a way to find dietary tools to manipulate the gut ecosystem. Moreover, more knowledge about the commensal bacteria in pigs and how they are affected by diet and other factors, such as the production system, is needed.

Furthermore, the synergistic effects on the gut microbiota observed when combining chicory forage and root in the diet to weaning pigs indicate that this may be a dietary intervention worth exploring further and that could be used to improve gut health and prevent post weaning enteric diseases.

9 Svensk sammanfattning

9.1 Bakgrund

Inblandning av vallfoder i foder till grisar sker idag i mycket begränsad omfattning men kan förväntas öka då vallfoder har positiva effekter på djurens hälsa och välbefinnande. Inom ekologisk produktion är det även ett krav att grisarna ska ha obegränsad tillgång på näringsrikt grovfoder. Cikoria (*Cichorium intybus* L.) är en flerårig ört som växer vild i södra Sverige. Den har egenskaper som gör att den kan komplettera andra vallväxter och ge en mer uthållig och stabil vall. Den vegetativa delen av cikoria innehåller ca 40 % kostfiber varav en stor del består av lättillgängliga pektiner. Även roten har intressanta fiberegenskaper. Den innehåller 15-20 % inulin och 8-12 % oligofruktos, vilka är klassade som prebiotika. Prebiotika stimulerar framförallt tillväxten av de bakterier i tarmen som har hälsofrämjande egenskaper, såsom laktobaciller och bifidobakterier. Studier har visat att pektiner kan ha liknande effekt. Inom smågrisproduktionen är magtarmstörningar ett problem som medför försämrad produktion, lidande för drabbade djur och ekonomiska förluster för producenterna. Det är därför viktigt att skapa en god tarmhälsa eftersom detta kan ge ökad motståndskraft mot magtarmstörningar. Kostfiber kan inte brytas ner av kroppens egna enzymer, utan de bryts ner i olika grad av mikrofloran. Vid denna nedbrytning producerar mikrofloran organiska syror som påverkar tarmmiljön. Ökad inblandning av kostfiber i fodret kan stimulera mikrofloran och därigenom försämra miljön för sjukdomsframkallande bakterier. Målet är att hitta en fiberkälla som ger positiva effekter på tarmhälsan och därigenom en positiv inverkan på produktionen.

Detta är den första studien som utvärderar växtdelen av cikoria som fodermedel till grisar. Syftet var att öka kunskapen om cikoria, såväl växtdel som rotdel, och studera hur denna påverkar:

- Smältbarhet dvs. hur mycket av näringen som grisen tar upp i tarmen och var i tarmen detta sker (tunntarm eller tjocktarm)

- Mikrofloran och tarmmiljön

- Smaklighet av fodret och grisarnas tillväxt.

9.2 Utförda försök

Tre försök har genomförts med växande grisar för att utvärdera möjligheten att använda cikoria som fodermedel. I det första försöket ersatte olika mängder (4, 8 och 16 %) av cikoriablad eller svartkämparblad motsvarande mängd spannmålsbaserat kontrollfodret. I det andra försöket ersatte 8 och 16 % av antingen cikoriablad, cikoriarötter eller en kombination av dessa motsvarande mängd kontrollfoder. Hur detta påverkade fodrets smältbarhet och smaklighet, djurens tillväxt och mikroflora studerades. Mikrofloran i gristarmen studerades genom odling av laktobaciller och koliforma bakterier. Då tidigare studier visat att endast en liten andel tarmbakterierna går att odla så har även molekylärbiologiska verktyg använts.

I det tredje försöket värderades smältbarheten för cikoria och jämfördes med andra vanligt förekommande fiberkällor såsom betfiber, vetekli och gräsmjöl. Samtliga foder hade samma totala fiberinnehåll, men fibrerna hade olika struktur. Detta kan påverka både var i tarmen fibrerna utnyttjas och i vilken omfattning. Betfiber och cikoria har båda hög andel lösliga fibrer i form av pektiner, medan vetekli och gräsmjöl har en hög andel olösliga fibrer i form av arabinoxylaner. För att kunna studera var i tarmen de olika fibrerna utnyttjades användes grisar som hade en fistel inopererad i slutet av tunntarmen. Därmed kunde både tarm och träckprov tas och vi kunde se hur mycket av fibrerna som hade utnyttjats i tunntarm respektive tjocktarm. Vi studerade även mikrofloran i såväl tunntarm som tjocktarm genom att använda molekylärmikrobiologiska metoder. Hur olika fiber påverkar tarmmiljön undersöktes genom att mäta pH och analysera mängden av olika organiska syror i tunntarmen. Slutligen analyserades hur lika strukturen av de lösliga fibrerna i betfiber och cikoria var.

9.3 Resultat och slutsats

Resultaten visade att grisarna växte bra och hade god hälsostatus när de fick foder med cikoria. Smakligheten av cikoriablad var god. Däremot hade svartkämparblad låg smaklighet, vilket begränsar dess användbarhet. Vid låg

cikoriainblandning (8 %) till växande grisar påverkades inte fodrets smältbarhet, medan en viss försämring uppmättes vid hög inblandning (16 %). Smältbarheten av specifika fiberkomponenter, framförallt pektiner ökade vid inblandning av cikoria. Våra resultat visade även att smältbarheten av cikoriablad är helt jämförbar med smältbarheten av vanligt förekommande vallfodergrödor såsom rödklöver och lucern. Cikoria kan alltså användas som en vallfodergröda till grisar. En stor andel av cikoriabladen hade smälts i tunntarmen, vilket skiljer från de olösliga fibrerna i vetekli som var så gott som osmälta i tunntarmen. Detta tyder på att cikoria kan förväntas ha en stor inverkan på mikrofloran i tunntarmen.

Mikrofloran varierade i sammansättning mellan olika grisar. Trots den stora variationen mellan grisarna gick det att urskilja bakteriegrupper som påverkades av inblandningen av cikoria. Kombinationen av cikoriablad och rötter gav en intressant effekt genom att koliforma bakterier missgynnades samtidigt som laktobacillerna gynnades. Cikoriarötter gynnade också tillväxten av en bakterie (*Megasphaera elsdenii*) som man tidigare visat kan hämma tillväxten av en av de bakterier som ger upphov till svindysenteri (*Brachyspira hyodysenteriae*). Såväl cikoriablad som en ökande mängd av smälta fibrer var kopplade till en ökad andel bakterier som tillhör grupperna *Bacteroides-Prevotella-Porphyromonas*. Detta tyder på att det är inom dessa grupper som de flesta fibernedbrytande bakterierna finns.

Tarmmiljön påverkades av fibrernas struktur. Grisar som åt vetekli och gräsmjöl hade högre mängd smörsyra i tunntarmen. Detta anses som positivt för tarmhälsan då smörsyra ger energi till tarmen. Grisar som åt cikoriablad hade en högre mängd ättiksyra i tunntarmen. Betfiber gav högre mängd propionsyra i tunntarmen än cikoriablad. Skillnaden i tarmmiljö mellan cikoriablad och betfiber kunde kopplas till strukturella skillnader i de lösliga fiberdelarna.

Sammanfattningsvis visar resultaten att cikoria kan användas som fiberkälla till växande grisar utan att tillväxt och foderutnyttjande förändras. Inblandning av cikoria i fodret har en stor effekt på såväl mikroflorans sammansättning som tarmmiljön. För att helt kunna förstå hur detta påverkar grisen behövs emellertid mer kunskap om grisens normala mikroflora.

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