Chapter 1

General introduction and work hypothesis

1 General introduction and work hypothesis

1.1 General Introduction

In livestock industry, animals are bred to perform and grow as cost efficiently as possible (Ewing and Tucker, 2008). As a consequence, strain is put on their physiology and metabolic processes and in addition, high stocking rates increase the risk of horizontal transmission of diseases (Ewing and Tucker, 2008). Factors with a negative impact on health of the animals are often related to composition of the intestinal microbiota, e.g. diarrhea is frequently associated to enterotoxin producing strains of *Escherichia coli* (Fairbrother et al., 2005). Therefore, dietary modulation to promote growth and activity of beneficial members of the indigenous intestinal microbiota in monogastric animals such as pigs is gaining increasing interest to improve health and performance of livestock. The beneficial members of the intestinal microbiota may then aid the host, for example, by production of vitamins or stimulation of immune functions (Gibson and Roberfroid, 1995) and help to prevent colonization of the intestinal tract by newly ingested bacteria, including potential pathogens (van der Waaij et al., 1971). Some of the commonly used strategies for dietary modulation of the intestinal microbiota include supplementation of pig diets with e.g. probiotics, prebiotics, organic acids or zinc (Pettigrew, 2006; Stein and Kil, 2006). Besides the supplementation with feed additives, the major components of pig diets including cereal grains may be used as dietary modulators. Grains contain varying quantities of starch and nonstarch polysaccharides (NSP) with differences in the composition of the NSP fraction among grains (Stein and Kil, 2006), but also within cultivars of the same grain species (Oscarsson et al., 1996; Rosenfelder et al., 2013). In addition, fermentable carbohydrates are also present in plant protein sources used in livestock nutrition, such as soybean meal, peas, faba beans or lupins (Bach Knudsen, 1997). Diets containing fermentable carbohydrates have proven to modulate the intestinal microbiota through changes in intestinal viscosity, selective stimulation of specific bacteria, and they may also influence the amount and composition of metabolites produced by the microbiota in the gastro-intestinal tract of pigs (e.g. O'Connell et al., 2005; van der Meulen et al., 2010). Besides carbohydrate composition, protein content is another important nutritional factor influencing the intestinal microbiota (Rist et al., 2013). Fermentation of proteins is generally linked to formation of branched-chain fatty acids and detrimental metabolites, such as ammonia, amines, indoles and phenols (Williams et al., 2005). An appropriate selection of feedstuffs containing fermentable carbohydrates may support a stable microbial community with enhanced fermentation of beneficial carbohydrates, and reduced detrimental protein fermentation to support health and well-being of the animals.

1.2 Work hypothesis

Based on a comprehensive literature review (Chapter 2), modulation of the pigs' intestinal microbiota may be achieved by proper selection of feedstuffs as well as dietary addition of feed supplements. Therefore, two experiments were carried out to evaluate the effects of feed supplements and whole grain cereals on microbial activity and composition of the intestinal microbiota. To evaluate the effects of different cultivars of barley and wheat on pigs' ileal and fecal microbiota, growing pigs were fitted with simple T-cannulas at the distal ileum in experiment 1 (Chapter 3). As representatives for important members of the pigs' intestinal microbiota, total eubacteria, *Bacteroides-Prevotella-Porphyromonas*, *Bifidobacterium* spp., *Clostridium* cluster IV, *Enterobacteriaceae*, *Lactobacillus* spp. and *Roseburia* spp. were determined by use of qPCR in ileal and fecal samples. In experiment 2 (Chapter 4), an *in vitro* approach was used to determine the impact of micronized fibers from pea, lupin and wheat added as supplements to assay substrates varying in CP level on microbial activity. Inulin was used for comparison. Furthermore, *in vitro* fermentation was performed under regular and increased salinity. Metabolites of fermentation (total gas, ammonia, SCFA) and bacterial cell numbers (total eubacteria, *Bifidobacterium* spp., *Clostridium* cluster IV, *Clostridium* cluster XIVa, *Enterobacteriaceae*, *Enterococcus asini*, *Lactobacillus* spp.) were determined by use of qPCR. Aim of this thesis was to expand knowledge on the dietary manipulation of the intestinal microbiota in pigs by use of different cereal species and their cultivars as well as feed supplements.

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Chapter 2

Potential of cereal grains and grain legumes in modulating pigs' intestinal microbiota – A review

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Submitted to Livestock Science. Major revision.

2 Potential of cereal grains and grain legumes in modulating pigs' intestinal microbiota – A review

2.1 Abstract

Dietary modulation seems to be a promising tool to promote growth and activity of the beneficial members of the indigenous intestinal microbiota in monogastric animals such as pigs. Several approaches, including the use of various feed additives such as pro- and prebiotics have proven to be efficient in modulating pigs' intestinal microbiota. Furthermore, ingredients of a typical pig diet such as cereal grains contain considerable amounts of fermentable carbohydrates including e.g. resistant starch, mixed-linked β-glucan or arabinoxylan, which can serve as substrates for bacterial fermentation in the gastrointestinal tract. Thus, research has been increasingly focusing on the potential of fermentable carbohydrates present in cereal grains to steer intestinal microbial activity and composition in a beneficial way. In this review, special interest will be directed to wheat and barley due to their frequent use as main ingredients in pig diets, in addition to rye and oat, as these grains are known to contain considerable amounts of fermentable carbohydrates. Furthermore, the potential of grain legumes including peas, faba beans and lupins in beneficially modulating pigs' intestinal microbiota and activity will also be assessed as these feed ingredients contain not only varying amounts of protein but also different contents of various fermentable carbohydrates. This review includes a comparison of studies, in which either whole cereal grains and grain legumes or purified carbohydrate fractions manufactured from these grains were included in pigs' diets to study their effect on intestinal microbiota and metabolic activity. It also takes into account variations between several cereal genotypes, as content and composition of fermentable carbohydrates may not only vary between grains, but also between different genotypes of the same grain species. Finally, results of in vitro studies will be presented to assess the potential of fermentable carbohydrates originating from cereal grains and grain legumes in modulating microbial composition activity in a beneficial and health promoting way.

2.2 Introduction

Livestock raised in intensive production systems is exposed to increasing risks of multifactorial health problems with possible negative impacts on well-being, production performance and product quality (van Nevel et al., 2006). Major challenges are the growing susceptibility of livestock, including pigs, to harmful bacteria, such as enterotoxin producing *Escherichia coli*, *Salmonella* spp., *Clostridium perfringens* and *Campylobacter jejunum* (Ewing and Tucker, 2008). Previous research has shown that the intestinal microbiota can support healthy animals in efficient digestion and absorption of nutrients, as well as in increasing their resistance to infections, thereby protecting the host against enteropathic diseases (Kyriakis et al., 1999; Rolfe, 2000). Especially the weaning period with transition from liquid to solid feed represents a critical point in the life of piglets (Konstantinov et al., 2004a), as animals experience a multitude of changes in intestinal morphology and function, often accompanied by weaning-associated disorders (Lallès et al., 2004). During this transition period, the intestinal microbiota is particularly susceptible to pathogens until the microbiota stabilizes (Williams et al., 2001). Thus, in the attempt to minimize post-weaning associated disorders in pigs, there is growing interest in exploring dietary means aiming to beneficially modulate the animals’ intestinal microbiota. Dietary inclusion of feed additives such as probiotics and prebiotics has been suggested to stimulate proliferation and metabolic activity of beneficial bacteria such as bifidobacteria and lactobacilli (Metzler et al., 2005). Beneficial effects due to the presence of these bacteria include the production of short-chain fatty acids (SCFA) which can be used as energy source by the host (Gaskins, 2001), but which also contribute to a stable microbiota through colonization resistance against non-indigenous microorganisms (van der Waaij et al., 1971). Besides feed additives, the use of fermentable carbohydrates (fCHO) naturally occurring in feed ingredients such as cereal grains and grain legumes, would be another approach to modulate intestinal microbial fermentation pathways, thereby possibly improving gut health conditions (Awati et al., 2006). Several fCHO have been identified as non-glycaemic carbohydrates entering the large intestine as substrate for fermentation, in contrast to glycaemic carbohydrates, which are digested and absorbed in the small intestine to be utilized in the animals’ metabolism (Englyst and Englyst, 2005). From a quantitative perspective, the major fCHO in all cereal grains are arabinoxylan (AX), and also mixed-link β-glucan (BG) in barley and oat (Bach Knudsen, 1997). Grain legumes are mainly used as protein source in pig diets, but they also contain considerable amounts of fCHO (Jezierny et al., 2010) including resistant starch (RS) as the major source of fCHO, but also non-digestible oligosaccharides of the raffinose family (RFO) (Mul and Perry, 1994). Traditionally, NSP such as AX or BG are considered as potentially anti-nutritive factors that negatively affect growth performance of pigs (Garry et al., 2007). However, diets containing these fCHO from cereal grains and grain legumes have proven to modulate the intestinal microbiota through changes in intestinal viscosity, selective stimulation of specific bacteria, and they may also influence the amount and composition of metabolites produced by the microbiota in the gastro-intestinal tract (GIT) of pigs (O'Connell et al., 2005; van der Meulen et al., 2010).

The present review summarizes the effects of cereal grains (wheat, rye, barley and oat) and grain legumes (pea, faba bean and lupin) as the major ingredients of pigs' diets on composition and metabolic activity of their intestinal microbiota. In this context, attention will also be paid to *in vivo* and *in vitro* studies pertaining to the use of different cereal by-products, and purified AX and BG fractions isolated from cereals in pigs' diets. In the following, a brief introduction focusing on the composition of the intestinal microbiota in pigs and its interaction with the host will be given (Chapter 2). As the fraction of fermentable carbohydrates probably is the most important substrate for microbial metabolism, a short description of the different fermentable carbohydrates present in cereal grains and grain legumes (Chapter 3.1 and 4.1, respectively) is provided, followed by the assessment of cereal grains and grain legumes as potential modulators of pigs' intestinal microbiota (Chapter 3.2 and 4.2, respectively).

2.3 The microbial ecosystem of pigs' GIT: composition and interactions with the host

The intestinal microbiota of adult pigs comprises more than 400 different phylotypes, mainly from the Firmicutes and Bacteroides phyla (Hill et al., 2002; Leser et al., 2002). The main bacterial groups in the porcine GIT include the following bacteria: *Bacteroides* spp., *Butyrivibrio* spp., *Clostridium* spp., *Escherichia* spp., *Eubacterium* spp., *Fusobacterium* spp., *Lactobacillus* spp., *Peptostreptococcus* spp., *Prevotella* spp., *Ruminococcus* spp., *Selenomonas* and *Streptococcus* spp. (Gaskins, 2001; Leser et al., 2002). The intestinal microbiota is influenced by environment, diet, genetics and health status of the animal (e.g. Gaskins, 2001; Leser et al., 2002; Ewing and Tucker, 2008). The highest density of bacteria can be found in the large intestine with up to 10^{12} bacteria/g intestinal content (Gibson and Roberfroid, 1995). Due to mechanisms referred to as “colonization resistance”, the indigenous gut microbiota of adult and healthy pigs assists in preventing colonization with newly ingested bacteria including potential pathogens (van der Waaij et al., 1971; Vollaard and Clasener, 1994). This mechanism is mediated *via* a more successful competition for nutrients and adhesion sites of the indigenous gut microbiota (van der Waaij et al., 1971; Ewing, 2008). Further beneficial functions of a healthy indigenous microbiota include e.g. stimulation of immune functions, aid in digestion and/or absorption of nutrients and synthesis of vitamins (Gibson and Roberfroid, 1995). It needs to be considered that most of the information on the composition of the intestinal microbiota is still based on the use of culture techniques, with their well-known short-comings regarding the ability to grow specific microbial groups or species in culture. Therefore, new molecular techniques such as next generation sequencing may give a more detailed insight into this subject, and may also change our perception which bacterial groups are central for animals’ health *in vivo*.

The major metabolic pathways of the intestinal microbiota are saccharolytic and proteolytic fermentation activities. Proteolytic metabolism results in the production of branched-chain SCFA from the degradation of branched-chain amino acids (Macfarlane et al., 1992), and in the accumulation of harmful fermentation end products such as ammonia, indoles, phenolic compounds and amines (Gibson and Roberfroid, 1995; Williams et al., 2005), and is often linked to the growth of potential pathogenic bacteria (Ball and Aherne, 1987). Thus, as recently reviewed by Rist et al. (2013), excessive fermentation of dietary protein should be avoided.

On the other hand, saccharolytic microbial metabolism results in the production of straight-chain SCFA, associated with a pH decrease of the intestinal content (Gibson and Roberfroid, 1995). A lowered pH plays an important role in inhibition of the growth of several bacterial species or groups such as *E. coli* (Fuller, 1977) which may be detrimental to animals’ health (Gibson and Roberfroid, 1995). Acetate, propionate and butyrate account for the largest fraction of SCFA, with acetate being the most prominent SCFA, comprising approximately two thirds of the total SCFA in the GIT (Cummings and Englyst, 1987). Acetate is extensively produced by many bacterial groups, while groups producing propionate and butyrate are more limited (Louis et al., 2007). Propionate is transported to the liver (Morand et al., 1992) and may inhibit *de* *novo* synthesis of fatty acids (Nishina and Freedland, 1990). Butyrate is of specific interest due to various potentially beneficial effects (Louis et al., 2007) such as serving as preferred source of energy for colonocytes (Roediger et al., 1982). The most important saccharolytic bacteria in the gut of single-stomached animals that are considered to be beneficial belong to the genera *Bifidobacterium*, *Lactobacillus*, *Eubacterium* and *Bacteroides* (Cummings and Macfarlane, 1991; Gibson and Roberfroid, 1995; Verstegen and Williams, 2002).

2.4 Cereal grains as potential modulators of pigs' intestinal microbiota

Fermentable carbohydrates naturally occurring in cereal grains have the potential to modulate intestinal microbial fermentation pathways, thereby possibly improving gut health conditions (Awati et al., 2006). For example, diets containing AX and/or BG from cereal grains have proven to modulate the intestinal microbiota through changes in intestinal viscosity, selective stimulation of specific bacteria, and affecting the amount and pattern of microbial metabolites produced (O'Connell et al., 2005; van der Meulen and Jansman, 2010). Thus, in the following, possible effects of cereal grains (wheat, rye, barley and oat) as major ingredients of pigs' diets on composition and metabolic activity of their intestinal microbiota will be assessed.

2.4.1 Carbohydrate composition

Starch and different non-starch polysaccharides (NSP) represent the main carbohydrates in cereal grains, comprising in total between 787 and 850 g/kg dry matter (DM) (Bach Knudsen, 1997). An overview of the carbohydrate composition of different genotypes of wheat (*Triticum spec*.), rye (*Secale cereale*), barley (*Hordeum vulgare*) and oat (*Avena sativa*) is given in Table 1.

The variation in carbohydrate composition between cereal grains reflects not only differences between grain species and genotypes, but is also influenced by harvest year and geographic location. For example, harvest year has a greater influence than genotype on variations in contents of total dietary fiber, water unextractable dietary fiber, total AX, water unextractable AX and BG, both in wheat (Kim et al., 2003) and rye (Hansen et al., 2003), for instance, due to differences in annual rainfall (Kim et al., 2003).