Interactions between Dietary Chicory, Gut Microbiota and Immune Responses

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Abstract

This thesis provides a better understanding of interactions between diet, gut microbiota, and immune responses to a specific dietary fiber source, chicory (*Cichorium intybus* L). This was achieved by examining the impact of chicory fiber on animal performance, digestibility, gut development, commensal bacteria community structure in small and large intestine, and follow-up reactions with specific immune components, cytoprotective heat shock protein (HSP) 27 and 72, *in vivo* and *in vitro*.

The impacts of dietary chicory on nutrient utilization, performance, and gut environment and morphology were investigated in chickens and young pigs. One-day-old chicks were fed cereal-based diets with inclusion of 60 or 120 g/kg chicory forage and/or root, with each forage diet derived from two harvests. Growing pigs were fed diets without and with inclusion of 80 or 160 g/kg chicory forage and/or root. The results showed that chicory inclusion maintained good animal performance and was accompanied by changes in gut morphology. Total tract apparent digestibility of non-starch polysaccharides (NSP) and uronic acid in broilers decreased with inclusion of 120 g/kg chicory, but not with inclusion of 60 g/kg. This indicates that chicory can be used as a palatable fiber source for broiler chickens and young pigs.

Gut microbiota complexity and dietary NSP-induced changes in pigs were examined using terminal restriction fragment length polymorphism (T-RFLP). The analysis revealed four primary microbiota clusters: luminal and mucosal ileal microbiota and luminal and mucosal colonic microbiota. In the ileum, lactic acid bacteria (LAB) were dominant and responsive to inulin-type fructan. In the colon, bacteria belonging to clostridial cluster IV and XIVa responded to chicory pectin, whereas *Prevotella* was related to cereal xylan. Mapping of cytoprotective HSP27 and HSP72 occurrence in porcine gut revealed region- and cell type-specific features. Physiological expression of HSP72 was correlated with LAB, representing an important interplay between HSPs and commensal microbes. In-depth studies of interactions between lactobacilli and gut mucosa and their effects on barrier function and HSP expression revealed protective effects from lactobacilli by enhancing HSP and tight junction protein expression under pathogen challenge.

Keywords: dietary fiber, chicory, gut microbiota, heat shock protein, intestinal barrier integrity, probiotic lactobacilli, chickens, young pigs, IPEC-J2

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Dedication

To my grandma, the woman I want to become

The simplest statements evoke the most wisdom.
Robert A.

Contents

List of Publications

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

I Liu, H.Y., Ivarsson, E., Jönsson, L., Holm, L., Lundh, T. and Lindberg, J.E. (2011). Growth performance, digestibility, and gut development of broiler chickens on diets with inclusion of chicory (*Cichorium intybus* L.). *Poult. Sci.* 90 (4), 815-823.

II Liu, H., Ivarsson, E., Dicksved, J., Lundh, T. and Lindberg, J.E. (2012). Inclusion of chicory (*Cichorium intybus* L.) in pigs' diets affects the intestinal microenvironment and the gut microbiota. *Appl. Environ. Microbiol.* 78 (12), 4102-4109.

III Liu, H.Y., Dicksved, J., Lundh, T. and Lindberg, J.E. (2013). Expression of heat shock protein 27 and 72 correlates with specific commensal microbes in different regions of the gastrointestinal tract (*submitted*).

IV Liu, H.Y., Lundh, T., Roos, S., Jonsson, H., Ahl, D., Dicksved, J. and Lindberg, J.E. (2013). Effects of *Lactobacillus johnsonii* and *Lactobacillus reuteri* on gut barrier function and heat shock proteins in porcine IPEC-J2 intestinal epithelial cells (*manuscript*).

Abbreviations

AME	Apparent metabolizable energy
DM	Dry matter
DP	Degree of polymerization
ETEC	Enterotoxigenic *Escherichia coli.*
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
HSP	Heat shock protein
IBD	Inflammatory bowel disease
IECs	Intestinal epithelial cells
LAB	Lactic acid bacteria
MAM	Mucosa-associated microbiota
MOI	Multiplicity of infection
NSP	Non-starch polysaccharides
PPs	Peyer's patches
SCFA	Short chain fatty acids
Sus	Starch utilization system
TEER	Transepithelial electrical resistance
TJ	Tight junction
T_{reg}	Regulatory T cell
T-RFLP	Terminal-restriction fragment length polymorphism
ZO	Zonula occludens

1 Introduction

Trillions of microbial organisms inhabit the gastrointestinal (GI) tract of mammals, resulting in exceedingly complex interactions and networking. Through co-evolution, these interactions provide an impressive example of mutualism between the host and its microbiota (Ivanov & Honda, 2012). Three main interplays are involved in maintaining homeostasis in the gut ecosystem (Figure 1):

1. Dietary substrates resistant to host-mediated digestion, such as fiber fractions, serve as physical stimuli for small intestine development and motility. The fiber is largely fermented in the distal GI tract and provides the host with maintenance energy, fuel for enterocyte growth, vitamins, exogenous amino acids, minerals, *etc.*, and thus influences overall host nutritional status.
2. Gut microbiota composition shifts in response to diet components changes. Commensal bacteria compete for the substrates available, with species-specific preferences, while establishing interspecies metabolism through released fermentation products.
3. Host immunity and gut anatomy primarily define gut microbiota distribution. In turn, changes in bacterial community composition alter the cross-talk with the local mucosal immune system, and further affecting the host systemic immune response. Accordingly, host immunity determines which bacteria are friends or foes and which reaction to promote or inhibit.

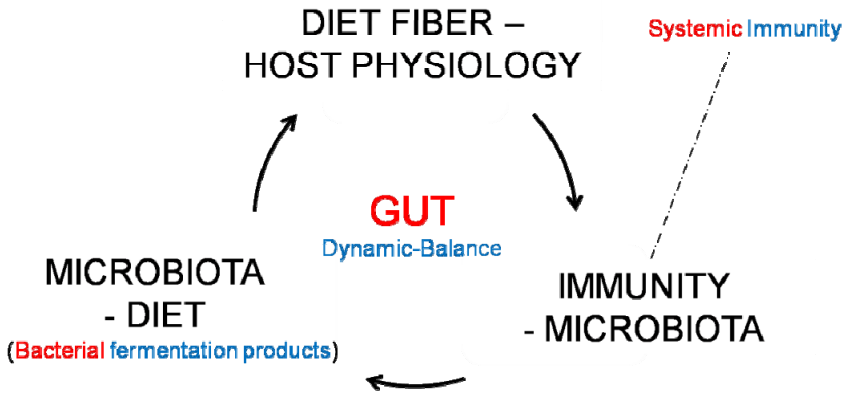

Figure 1. Interactions between diet, host physiology, gut microbiota, and immune system. Intestinal homeostasis is sustained by three mutualistic relationships in the gut ecosystem. Modified from Montagne *et al.*, 2003.

In general, interactions between host physiology and gut microbiota are responsible for the health of individuals from birth, during early life (*e.g.*, children and weaning piglets) and adulthood, and during ageing (Claesson *et al.*, 2012; Robinson *et al.*, 2010; Bailey, 2009). Two contrasting scenarios are possible. One is a dynamic and balanced interaction that enhances the immune system and also improves host health. This can be achieved by a nutritionally smart diet regime. Many studies have associated dietary fiber with gut health-promoting effects in humans and animals (Bach Knudsen *et al.*, 2012; Brownawell *et al.*, 2012). The other scenario is disturbed host-microbiota interaction that leads to dysfunction of the immune system, often seen as autoimmune responses or inflammation. These conditions can be altered by clinical diet interventions, such as addition of prebiotics and probiotics (Ringel *et al.*, 2012; Maslowski & MacKay, 2011). In both scenarios, the response may not be restricted to the local gut microenvironment, but may in certain circumstances have systemic effects. Understanding the network behind these events will allow more precise stimulation of the host immune system by dietary manipulations and associated alterations of the gut microbiota.

The introduction of molecular biology and recent development of sequencing technologies in humans has provided substantial gains in knowledge about the microbial community structure and function (Qin *et al.*, 2010). However, less attention has been paid to farm animals, for instance pigs, on the molecular fingerprinting of gut microbiota and microbiome (the collective genome of the microbiota) (Pang *et al.*, 2007; Leser *et al.*, 2002). Studies of particular diet treatments in livestock, for instance dietary fiber

inclusion, its impact on gut microbiota, and host immune responses, are necessary for several reasons:

1. Livestock production, especially the pig and poultry sector, has been growing rapidly (a 103% increase over the past 30 years), and is very important for global food security. As productivity improved, challenges also arise, such as increasing risk of infectious diseases. Therefore, alternative feeding strategies need to be developed to allow eggs and meat to be produced in a cost-effective and health promoting way (Steinfeld & Gerber, 2010).
2. Fiber inclusion in pig and poultry diets may improve gut health and animal well-being. Moreover, there is increasing concern in society and in the food industry about animal ethics (Høøk Presto *et al.*, 2009; Van Loo, 2007).
3. Pig, in particular, is an excellent model for human studies in terms of gut anatomy and microbiota complexity. There is increasing interest in establishing pig models for studies of infectious intestinal diseases. This will contribute essential knowledge relating to both human and animal health (Bolker, 2012; Meurens *et al.*, 2012).

2 Interactions between dietary fiber and host

2.1 Dietary fiber at a glance

A developing body of evidence suggests that dietary fiber is a major component of a healthy and balanced diet in humans and animals. Chemically, dietary fiber is the sum of non-starch polysaccharides (NSP) and lignin, where a large amount of the NSP is a constituent of plant cell wall components (cellulose, hemicelluloses, pectins, *etc.*) (Bach Knudsen, 1997). Physiologically, dietary fiber contains all the polysaccharides and lignin that are resistant to host-mediated digestion in the upper GI tract due to lack of endogenous enzymes. Nutritionally, inclusion of oligosaccharides, disaccharides, and resistant starch in the definition of dietary fiber has been suggested, as this would better cover health claims relating to the functional and beneficial effects of fiber (Brownawell *et al.*, 2012; Trowell *et al.*, 1976).

Intake of dietary fiber has become an important topic for many reasons. A minimal inclusion of dietary fiber is suggested in human and animal nutrition in order to achieve normal gut function. There is also a need for an adequate amount of dietary fiber to optimize intestinal health and well-being (Bach Knudsen *et al.*, 2012; Brownawell *et al.*, 2012). Moreover, there is huge pressure on livestock production, particularly pig and poultry production, to reduce the use of cereal grain fiber as a feed ingredient due to the increasing cost and the constrained resources for human consumption (Noblet & Le Goff, 2001; Varel & Yen, 1997). In addition, organic pig farming demands appropriate roughage, such as fresh forage, in the daily ration of animals (EC, 2008).

2.1.1 Fiber utilization in chickens and pigs

One fundamental feature for defining the dietary fiber fraction is based on its digestibility, *i.e*., the extent to which the fiber ingredient can be utilized by animals. This varies greatly depending on the chemical composition of the NSP, animal species, age, digestion site, and interactions with non-fiber ingredients. In growing pigs, the average digestibility of NSP is estimated to be 24%, ranging from 10-62% (Bach Knudsen & Jørgensen, 2001), which may lead to a 30% contribution to maintenance energy. Adult sows have an even greater ability to effectively utilize dietary fiber than young pigs (Varel & Yen, 1997). However, increased dietary fiber content has been associated with reduced feed energy content and animal performance (Noblet & Le Goff, 2001). For example in chickens, a negative correlation between the NSP content in wheat and apparent metabolizable energy (AME) has been reported (Annison, 1991). The effect is partly due to the viscous nature of wheat pentosan (arabinoxylans). By adding specific exogenous enzymes or using non-viscous and highly digestible dietary fiber sources, the AME values and bird performance can be improved (Choct *et al.*, 1992).

In Swedish production systems, pig and poultry diets are often formulated on the basis of cereal grains (wheat, barley, oats, rye, and triticale) containing varying levels of dietary fiber (range 115-250 g dietary fiber/kg dry matter, DM) (Högberg & Lindberg, 2006). In addition, cereal by-products are regularly used in feed formulation, as are other fibrous feedstuffs. Moreover, in organic pig and poultry production, forage crops such as white clover, rye grass, timothy, and lucerne are commonly used (Ivarsson, 2012).

2.1.2 Fiber-induced changes in gut development

The small intestine is a quite different organ from the large intestine in terms of anatomy and physiological conditions (Figure 2), which has an impact on gut function, in particular on the microbial community composition and activity (Leser & Mølbak, 2009). The small intestine (duodenum, jejunum, and ileum) has high absorption capacity due to villi and microvilli lining the gut, which results in a large surface area. Depending on physio-chemical properties such as solubility, dietary fiber intake leads to enlarged gut size (length, volume, and weight), increased villus height, greater crypt depth, and increased numbers of goblet cells, indicating an active interaction between fiber and host. Dietary fiber markedly promotes gut development in pigs, resulting in a longer foregut that allows intensive digestion, whereas in chickens a high passage rate and smaller gut size limit both endogenous digestion and exogenous microbial activity (Ito *et al.*, 2009; Wenk, 2001). For example, it is found that a viscous

citrus pectin diet negatively affected chicken's small intestine morphology and microbial activity (Langhout *et al.*, 1999).

Gut-associated lymphoid tissue (GALT) represents the largest collection of lymphoid tissue in the body and is rich in myeloid and lymphoid cells, many of which reside in Peyer's patches (PPs) and lamina propria (Bailey, 2009). Correct feeding is crucial for GALT development. Dietary fiber addition (beet pulp, oligofructose powder, and gum arabic mixture) has been shown to increase $CD8^+$ T-cells among intraepithelial lymphocytes, in PPs, and in lamina propria, and $CD4^+$ T-cells in mesenteric lymph nodes in dogs (Schley & Field, 2002).

The fast passage rate in chickens also applies to the large intestine. Poultry can be assumed to be more sensitive to dietary fiber ingestion than pigs, whereas in pigs adaptations can be developed when the gut is exposed to fiber components over time (Montagne *et al.*, 2003). In pigs and humans, the transit time for dietary fiber fractions to pass through the large intestine (colon, cecum, and rectum) can vary from 10 h to several days to allow intensive microbial activity. Sufficient bacterial fermentation with production of short-chain fatty acids (SCFA), primarily acetate, propionate, and butyrate, plays a fundamental role in the way in which dietary fiber affects large intestinal development (Leser & Mølbak, 2009). In particular, butyrate is the preferred energy source for colonocytes (Fitch & Fleming, 1999). Furthermore, dietary fiber influences the intestinal epithelial ontogeny via stimulating mucus synthesis and mucin excretion. An increased number of mature colonic goblet cells in rats fed a high fiber diet has been reported, indicating an enhanced mucus layer (SchmidtWittig *et al.*, 1996).

2.2 Introducing probiotics and prebiotics

There is widespread interest in developing feeding strategies to improve gut health in farm animals while minimizing in-feed antibiotic usage. One driving factor is the emerging evidence that antibiotic administration has a negative impact on host microbiota, immunity, and health and may spread out the antibiotic resistance gene in pathogens (Ueno *et al.*, 2011). As described above, dietary fiber modulates gut health through an array of interactions with the intestinal epithelium, the mucus layer, the immune system, and the microbiota. It is therefore possible that diet interventions could be a means to alter the microbiota community composition and gut function, and contribute to intestinal homeostasis.

2.2.1 Antibiotic usage

In-feed antibiotic usage has been an efficient and cost-effective approach to manage livestock performance and disease for over 50 years (Looft *et al.*, 2012). However, it has become apparent that antibiotic administration perturbs the interactions between intestinal commensal microbiota and the immune system in human and animals. A severe, broad spectrum antibiotic intervention transforms the gut micro-environment into a barren environment that is difficult to rebuild. One central concern regarding antibiotic use in farm animals is the transfer of the antibiotic resistance gene to human pathogens (Ubeda & Pamer, 2012). A short period of antibiotic administration (14 days) in pigs is suggested to increase the abundance of the resistance gene (Looft *et al.*, 2012), and the bacteria that carry the antibiotic resistance gene may pass on to the offspring from sow (Stanton & Humphrey, 2011).

It is alarming that over 50 years of liberal use of antibiotics in conventional agriculture has led to widespread transfer of the antibiotic resistance gene to human and animal pathogens. Moreover, there appears to be a high background of resistance genes in the non-medicated pig gut microbiota (Looft *et al.*, 2012). In 2006, the European Union banned the general use of in-feed antibiotics as growth promoters, whereas in the United States, over half the antibiotics produced still go to farm animals (Lipsitch *et al.*, 2002).

2.2.2 Probiotics

Probiotics, defined as 'live microorganisms that convey health benefits to the host when administered in adequate amounts' can be strategically used to supplement the deficit caused by antibiotic treatment and boost community diversity (Ringel *et al.*, 2012; Guarner, 2008). For young pigs and poultry, probiotic administration is reported to deliver health benefits, including improved digestive capacity, lower intestinal pH, modulated immunity, and enhanced intestinal barrier function and integrity, at a low cost (De Lange *et al.*, 2010). In practice, lactic acid bacteria (LAB) and bifidobacteria are groups of microbes that most commonly used probiotics. In the gut ecosystem they have known benefits on competitive exclusion (Roselli *et al.*, 2007), antimicrobial substance production (Walter *et al.*, 2011), inhibition of pro-inflammatory cytokines (Shimazu *et al.*, 2012), and fortification of tight junction (TJ) protein expression and thereby strengthened barrier integrity (Karczewski *et al.*, 2010). However, a growing body of data suggests that the efficacy of probiotics is highly plastic, depending on the bacterial species or strains selected, the microbial formulation including dosage, and the product bioavailability (Ringel *et al.*, 2012). Clearly, prudent evaluation and quality control are critical to allow more rational design of novel probiotics.

2.2.3 Prebiotics

Prebiotics are another promising alternative to in-feed antibiotics to manage digestive disorders in pigs and chickens (De Lange *et al.*, 2010; Van Loo, 2007). Although prebiotics developed from the probiotic concept and there is an overlap with dietary fiber classification, by definition prebiotics can be distinguished as a separate group. The following three essential criteria must be fulfilled, underpinning the substrate specificity and selectivity (Roberfroid, 2007), whereas 'adequate amount' or nutritional contribution to host maintenance is not a must:

1. Resistant to host mediated-digestion and absorption in the small intestine.
2. Specifically fermented by beneficial bacteria in distal GI tract.
3. Selectively promote bacteria growth and/or activity and have a confirmed beneficial effect.

In light of these criteria, chicory inulin, composed of fructan and oligofructose, falls into the prebiotic category. Indeed, inulin-type fructan is one of the few thoroughly validated prebiotics in human and animal nutrition, with probiotic lactobacilli and bifidobacteria serving as clear health-promoting bacteria targets (Kolida & Gibson, 2007; Van Loo, 2007). Inulin can be extracted from the root of the chicory plant (*Cichorium intybus* L.) and normally contains a mixture of short and long chains of fructan with a degree of polymerization (DP) from 11 to 65. The shorter chain fructan with lower DP is fermented rapidly and significantly promotes LAB growth, while longer chain fructan with higher DP is fermented more slowly and can reach more distal parts of the GI tract (Macfarlane *et al.*, 2006). Inulin feeding has been directly related to improved growth performance, intestinal health, and well-being in livestock. In broilers, inulin feeding is reported to efficiently inhibit *Salmonella* infections. In pigs, dietary supplementation of inulin is widely used to modify the gut microbiota composition at weaning in order to achieve a better natural resistance to diseases (De Lange *et al.*, 2010; Van Loo, 2007).

2.3 Chicory (*Cichorium intybus* L.) as a novel dietary fiber source

Chicory is a deep-rooted perennial herb that is rich in minerals and resistant to drought. Chicory produces a large quantity of nutritious, high quality forage that is preferred by many types of grazing ruminants such as finishing lambs, deer, and cattle (Li & Kemp, 2005). In southern Sweden, the yield of chicory is around 5000-6000 kg DM/ha (Ivarsson, 2012). In contrast to chicory root (inulin), chicory forage has been little studied in monogastric nutrition. The forage contains 80-90 g/kg DM of uronic acid (building blocks of pectin) that

is highly soluble compared with other pectic substrates (Ivarsson *et al.*, 2011). Pectin of plant origin, such as sugar beet pulp, has been used as a fibrous feedstuff in pig diets and results in an increased LAB population in the small intestine and increased microbiota diversity in the large intestine (Konstantinov *et al.*, 2004). In addition, a high digestibility of uronic acid in growing pigs fed on chicory has been reported, with supplementation of both root and forage modulating gut microbiota community composition and affecting SCFA production (Ivarsson, 2012). Taken together, this warrants further studies to evaluate the potential of chicory as a novel and unique dietary fiber source in monogastric animal nutrition.

3 Interactions between gut microbiota and dietary fiber

3.1 Exploring the porcine gut microbiota

One step in understanding the mutualistic relationships between gut microbiota and the host is to determine the 'normal' microbial community profile and to learn how changes in the composition is linked with health and diseases. Sterile at birth, the mammalian gut is immediately colonized by maternal and environmental microorganisms (Dominguez-Bello *et al.*, 2010). Once developed into an adult pattern, temporal variations in bacterial composition are minimal in the absence of external stress (Costello *et al.*, 2009). For pigs, weaning is an extremely drastic period for gut development, where a re-establishment of microbiota is necessary to adapt to the new feeding conditions, with plant NSP materials introduced (Bach Knudsen *et al.*, 2012).

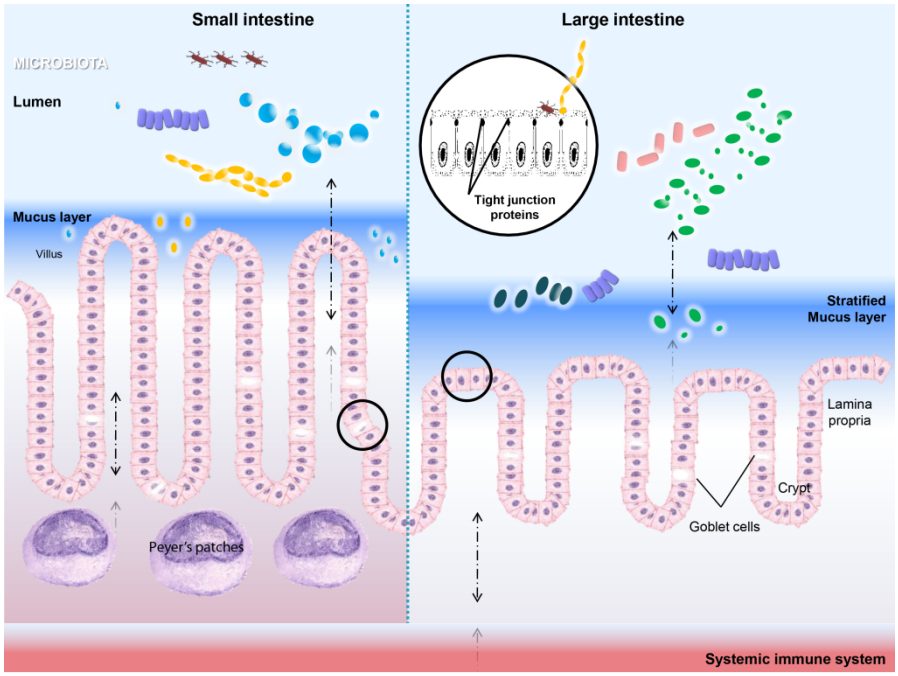

Figure 2. Intestinal microenvironment: small intestine vs. large intestine. Intestinal epithelial cells (IECs) constitute a single cell layer barrier that is sealed by tight junction proteins therefore separate the internal tissue from the external environment. Goblet cells in the intestine produce mucin that is organized into stratified mucus layer. The stratification is more apparent in the large intestine, where a firm inner mucus layer is largely impervious to bacterial penetration. Bacteria (commensal and transient) are present in the intestinal lumen, most of which reside outside the mucus layer, whereas some can achieve mucus colonization. At the mucosal interface, there is a constant signaling between bacteria and the host gut, including the lamina propria and the underlying Peyer's patches containing abundant myeloid and lymphoid cells. The interaction between microbe and IECs and between IECs and immune cells extending beyond the gut (*i.e.* in circulation) is pivotal regarding adaptive immune response activation and the maintenance of host homeostasis.

The trillions of microorganisms that inhabit the human and animal intestine form a diverse and intricate ecosystem (Lozupone *et al.*, 2012) (Figure 2). The large variation of the microbial community between individual subjects obscures the vision to define a 'normal' gut microbiota. However, due to the revolution in using culture-independent methods and an astounding improvement of sequencing technology, our understanding of microbiota diversity has grown tremendously (Leser & Mølbak, 2009). Roughly 400 bacterial phylotypes have been estimated to occur in the porcine gut falling into a few phyla (mainly *Firmicutes* and *Bacteroidetes*), whereas

approximately 80% representing unknown species. The bacteria screening study in porcine gut identified five major clusters of bacterial phylotype, of which four could be correlated with sequences from human GI tract and rumen databases, as few studies have focused on the microbial ecology in the porcine gut so far (Leser *et al.*, 2002). However, more and more attentions are paid to pigs, as the porcine gut resemble human's in microbiota complexity, anatomy, and physiology, and can be used as a model in studies on infectious diseases, contributing knowledge to both human and animal health (Meurens *et al.*, 2012). In general, low-G+C gram-positive bacteria (*Firmicutes*) constitute the largest fraction in the bacterial community of the porcine GI tract (Leser *et al.*, 2002). In the small intestine however, there is a clear dominance of gram-positive LAB, especially *Lactobacillus* spp. (Konstantinov *et al.*, 2004), whereas in cecum and colon, gram-negative *Prevotella* phylotypes and bacteria belonging to clostridial cluster XIVa and cluster IV are the most abundant.

Diversity must be addressed in relation to a healthy gut microbiota and is crucial for conferring the natural resistance of an individual ecosystem. Diversity index entails species richness and eveness (Lozupone *et al.*, 2012). Bacterial diversity is partitioned across gut sites. A longitudinal gradient is reported in pigs, with increasing diversity from ileum to cecum and colon (Leser *et al.*, 2002). Notably, the pig gut has a great number of colonizers in the distal ileum, which can amount to 10^9 bacteria/g content, which is markedly higher than in humans (10^6-10^7 bacteria/g content) (Van Loo, 2007). In the large intestine, bacteria colonize at a density of 10^{11}-10^{12} bacteria/g content. Given that biopsies from healthy human subjects are more difficult to obtain, the axial distribution of gut microbiota (luminal to mucosa), especially in the small intestine, is less clearly described (Macfarlane *et al.*, 2006). Eckburg *et al*. (2005) demonstrated that bacteria clone libraries from stool samples and colonic mucosal samples have distinct lineages in a human study (Eckburg *et al.*, 2005). Due to its proximity to intestinal epithelial cells (IECs), the mucosa-associated microbiota may play a pivotal role in shaping the host mucosal immune system (Macfarlane *et al.*, 2006). Especially in disease states, for example, early study has shown that the mucosa-associated microbial community is markedly altered in inflammatory bowel diseases (IBD) patients compared with healthy individuals (Swidsinski *et al.*, 2002). In contrast, some assume that the luminal microbiota contains its indigenous members and also bacteria shed from the mucosa, due to intestinal peristalsis, cell turnover, and mucus excretion (Leser *et al.*, 2002).

It is commonly accepted that communities with high diversity are less susceptible to pathogen intrusion (Keesing *et al.*, 2010; Stecher *et al.*, 2010). However, diversity alone cannot determine whether the state of an ecosystem

is more or less resistant. In addition, community stability should be considered. A higher diversity may indicate a more chaotic community, especially during the early life of animals. For example, pigs raised in an outdoor environment have been found to have more reduced microbial diversity than their indoor-raised litter mates, yet have more abundant *Lactobacillus* species that are proven to be health promoting (Mulder *et al.*, 2009; Schmidt *et al.*, 2008).

3.2 Core groups of bacteria and individuality

Bacterial enterotype is one reflection of gut microbiota stability, as it is highly conservative across gender, body weight, and landscape. A recent study proposed three enterotypes, namely *Bacteroides*, *Prevotella*, and *Ruminococcus*, in the human GI tract, based on dominant bacterial genera and species composition (Arumugam *et al.*, 2011). Compositionally similar enterotypes have also been identified in animals (Moeller *et al.*, 2012). The enterotype concept demonstrates a stable symbiotic relationship between host and microbiota, and can be of great help in differentiating one microbial community signature from another (Lozupone *et al.*, 2012). For example, obese patients are characterized by a particular enterotype and reduced gut microbiota diversity in comparison with their lean twin siblings (Turnbaugh *et al.*, 2009b). This indicates a possibility to link enterotype with specific physiological conditions and disease states.

Although it remains unclear exactly how the clustering originated, it can be partly explained by the energy harvesting habit of each enterotype member and the abundant co-occurring genera (Arumugam *et al.*, 2011). A core gut microbiome has been found to coincide with a core group of bacteria that share some important metabolic functional genes, including carbohydrate degradation. This has been attributed to the diverse co-occurring genera at lower taxonomic levels, which also enables great inter-individual variability (Turnbaugh *et al.*, 2009b). Under host selection pressure, some microbes that occur in low abundance can influence intestinal homeostasis markedly through specialized functions and features. For instance, the commensal *Escherichia coli*, although low in abundance, is responsible for producing a major proportion of bacterial pilli (key structure for bacterial colonization) assembly proteins and thereby achieving optimal survival in the GI tract (Arumugam *et al.*, 2011).

3.3 Diet-driven changes in bacterial community composition

External factors, especially diet, are crucial in shaping the gut microbiota community structure. It seems that enterotypes are primarily driven by long-term dietary regimes. Individuals on an animal protein- and fat-rich diet have a *Bacteroides* enterotype, which can be distinguished from a carbohydrate diet-associated *Prevotella* enterotype (Wu *et al.*, 2011). Similarly, rural African children living on a fiber-rich diet harbor a gut microbiota in which *Prevotella* dominates, while this fraction of community is completely lacking in European children living on a 'Western' diet (typically high in animal protein, sugar, starch, and fat and low in fiber). Moreover, this difference is also associated with various lifestyles (De Filippo *et al.*, 2010). It has been shown that gut bacterial composition correlates with certain lifestyle factors, including diet, antibiotic use, and environment (Dicksved *et al.*, 2007). Maslowski and MacKay (2011) postulated a diet-microbiota hypothesis that the modern 'Western' diet type and the subsequent alteration of gut microbiota are associated with the increasing incidence of inflammatory disease, such as type 1 diabetes, in European countries (Maslowski & MacKay, 2011; Patterson *et al.*, 2009). These studies together indicate the fundamental role of diet in the host and gut microbiota co-evolution process.

In the studies described above, the timescale of diet effect is life-long. However, gut microbial composition can be rapidly changed by specific diets. Studies in human subjects show that shifting diet from high-fat/low-fiber to low-fat/high-fiber results in marked changes in the gut microbiota within 24 hrs (Wu *et al.*, 2011). Similarly, in gnotobiotic mice a low-fat/fiber-rich diet shifts gut bacterial composition originating from a high-fat/high-sugar diet within one day (Turnbaugh *et al.*, 2009a). Moreover, a number of studies have manifested effects of prebiotic dietary fiber on porcine gut microbiota modulation in experimental periods of 4 days to several weeks (Patterson *et al.*, 2010; Mølbak *et al.*, 2007; Bikker *et al.*, 2006; Konstantinov *et al.*, 2004). Together, these studies indicate that diet manipulation may be a simple and efficient way to affect gut health through changing the composition of gut microbiota. However, very few studies have addressed the long-term effects of prebiotic administration. Santos *et al*. (2006) demonstrated that 6 months of prebiotic addition induced gut microbiota alterations were diminished in the first week upon prebiotic removal (Santos *et al.*, 2006).

3.3.1 Specific NSP fractions utilized by bacteria

A wide range of dietary substrates arrive in the distal GI tract incompletely digested, and the majority of these comprise plant cell wall NSP. Entering the large intestine, NSP fractions are released from plant structures, become

solubilized in various states and sizes, and provide ecological niches for gut bacteria (Flint *et al.*, 2008). A series of metabolic events then occurs, accompanied by changes in the microbiome, including NSP degradation functions. However, limited information is available on interactions between a given NSP fraction and specific commensal bacteria in the gut (Walker *et al.*, 2011). One reason for this is the difficulty in culturing anaerobes *ex vivo*.

Only a few bacteria have the ability to directly break down the intact plant NSP structure. Thus most bacterial species need to produce multiple enzymes in order to degrade isolated plant polysaccharides. *Prevotella* spp. is the main driver for one of the enterotypes and dominates the anaerobic communities of pig large intestine and rumen (Dehority, 1966). This group of gram-negative bacteria is able to produce several xylanases, mannanases, and β-glucanases, and contributes to soluble xylan utilization (Flint & Bayer, 2008). Xylan is one of the structural polysaccharides that makes up the majority of cereal fiber (Andersson & Åman, 2008). One gene cluster has been identified from the genome of *Prevotella bryantii* strain $B_1$4 containing genes encoding proteins such as β-xylosidase, endoxylanase, and other xylan-degrading enzymes (Flint *et al.*, 1997). Due to the complexity of the xylan structure, it is difficult for bacteria to utilize this NSP fraction efficiently without extensive extracellular xylanase activity. Dodd and co-workers (2010) described a cluster of genes that are specifically induced by xylan, termed Xus, which is highly conserved among xylanolytic *Bacteroidetes* members (Dodd *et al.*, 2011; Dodd *et al.*, 2010). It is an example of energy acquisition from xylan NSP based on the Sus-like (starch utilization system) paradigm discovered in human colonic *Bacteroides thetaiotaomicron*. In simplified terms, the Sus system comprises 8 adjacent Sus genes. In coordination, they encode several proteins that are constituents of a cell envelope-associated apparatus, that further facilitating *B. thetaiotaomicron* to bind and degrade starch polysaccharides (Martens *et al.*, 2009). Nevertheless, the xylan-degrading system of gut bacteria is extremely sophisticated, and the molecular machinery remains to be fully investigated (Dodd *et al.*, 2011). In contrast, the utilization of pectin, another major NSP fraction, by specific commensal bacteria is poorly described. A recent study showed that *Faecalibacterium prausnitzii*, a major butyrate producer in the human large intestine, is able to outcompete *B. thetaiotaomicron* and other bacteria on apple pectin, although the metabolic pathways utilized are unclear (Lopez-Siles *et al.*, 2012).

On the other hand, bacteria that do engage in directly breaking down intact plant NSP structures, mainly cellulolytic bacteria, may not use the solubilized product, and will therefore enable bacterial cross-feeding, as well as competition for substrates in the host large intestine (Flint *et al.*, 2008).

3.3.2 Bacterial fermentation

Dietary fiber generally increases SCFA production by the commensal microbiota, which reduces the pH and creates a microenvironment that favors the growth of some members of the microbiota. In the proximal colon, a pH of 5.5 results in a dominance of gram-positive butyrate-producing bacteria rather than gram-negative bacteria, including the opportunistic pathogen *E coli.* (Duncan *et al.*, 2009). Moreover, SCFA serve as energy substrates for colonocytes (butyrate) and peripheral tissues (acetate and propionate). For example, propionate is designated for hepatocyte gluconeogenesis (Samuel *et al.*, 2008).

Whether brought about by diet or other environmental factors, alterations of bacterial community structure is accompanied by changes in the bacterial metabolites profiles. The relative abundance of each bacteria species in gut microbiota influences the final mixture of fermentation end-products by direct production and interactions with other bacteria (Fischbach & Sonnenburg, 2011). There is great interest in bacteria belonging to clostridial cluster XIVa and cluster IV, as they are major butyrate producers that utilize lactate and acetate in the large intestine. This group of bacteria plays a central role in colonic bacterial cross-feeding (De Vuyst & Leroy, 2011). Moreover, dietary fructan promotes *B. thetaiotaomicron* growth in mouse colon, concomitant with production of acetate and formate, which are subsequently utilized by *Methanobrevibacter smithii* for methanogenesis, indicating an efficient energy harvesting pattern (Samuel & Gordon, 2006). Several lines of evidence suggest that SCFA can act as molecular signals and exert immune modulatory properties beyond the mucosal surface. Butyrate has been studied as a de-acetylase inhibitor (a new class of anticancer agents) involved in NF-κB-dependent transactivation regulation (Quivy & Van Lint, 2004). In addition, acetate is suggested to contribute to the protective effect provided by bifidobacteria against *E. coli* infection by inhibiting toxin translocation into the blood (Fukuda *et al.*, 2011).

4 Interactions between the immune system and gut microbiota

4.1 Gut microbiota influences the immune system

As detailed information on gut microbiota complexity and functional diversity accumulates, we are learning more about how diet-microbiota interactions can influence the immune system within and outside the gut, and host homeostasis in general (Maslowski & MacKay, 2011). Since trillions of bacteria have lived inside the human body for millions of years, an interdependent symbiotic relationship must be deeply embedded. One important example is that the gut commensal microbiota dictates the host immune system, especially the maturation of intestinal mucosa and its abundant immune cells. Without appropriate signals from the microbiota, abnormal immune responses such as autoimmune reactions and non-reaction to pathogens can take place (Maynard *et al.*, 2012). In germ-free mice, the absence of commensal bacteria results in an undeveloped intestinal mucosal immune system that contains hypoplastic PPs, fewer germinal centers, largely reduced numbers of various lymphoid cells (*e.g.* IgA-producing plasma cells and lamina propria $CD4^+$ T cells) (Macpherson & Harris, 2004), lack of regulatory T (T_{reg}) cells (Ivanov *et al.*, 2009), and minimal expression of cytoprotective heat shock protein (HSP) 25 and HSP70 (Hu *et al.*, 2010). A disrupted balance between host immunity and microbiota can easily lead to dysbiosis (microbial imbalance) (Tamboli *et al.*, 2004).

4.1.1 Dysbiosis and enteric diseases

It is becoming clearer that microbe-associated disease is not confined to the action of 'one microbe'. Major perturbation of gut microbiota can be caused by single strains of bacteria and/or imbalance of the community structure (dysbiosis) (Clemente *et al.*, 2012). Dysbiosis is best exemplified in IBD,

where the bacterial composition is shifted into a community that contains fewer *Bacteroidetes,* fewer *Firmicutes*, and more bacteria from the phyla *Actinobacteria* and *Proteobacteria* than in healthy subjects (Frank *et al.*, 2007). This is in line with studies on ileal Crohn's disease, in which patients are characterized by lower microbial diversity and a community structure that deviates from a healthy gut (Dicksved *et al.*, 2008; Sokol *et al.*, 2008). A reduction of *F. prausnitzii* is concomitant with loss of anti-inflammatory effects and may increase the risk of recurrence of ileal Crohn's disease (Sokol *et al.*, 2008). Bacteria are highly interdependent in the intestinal microenvironment, therefore depletion of one bacterial species can provoke a chain of reactions that leads to a perturbed community. Whether dysbiosis is a cause or effect of gut disorders is still unknown. Nevertheless, evidence from dietary interventions including antibiotics, prebiotics, and probiotics indicates that the state of microbial imbalance can be modified and reversed (Bäckhed *et al.*, 2012).

Pigs suffer from a broad spectrum of enteric diseases, of which diarrhea is the most common and the major cause of morbidity and mortality in young animals. For instance, post-weaning diarrhea, swine dysentery, and necrotic enteritis (which also occurs in chickens) constitute great challenges for the pig and poultry industry, and cause significant economic losses in many parts of the world every year (Bailey, 2009; Mølbak *et al.*, 2007; Montagne *et al.*, 2003). Studies on gnotobiotic pigs have shown that swine dysentery may be associated with dysbiosis and that colonization by spirochete alone does not cause severe colonic lesions unless it co-occurs with colonization by other anaerobic species (Whipp *et al.*, 1979).

4.2 Intestinal epithelium barrier as frontline defense

Normal functioning of the gut relies on the maintenance of a mucosal barrier that is lined with a single layer of columnar epithelial cells. This monolayer covered with mucus, represents a frontline defense barrier that separates the internal tissue from the external environment, while maintaining nutrient uptake. The epithelial lining is a crucial innate immunity component and has the ability to modulate the adaptive immune response. The intestinal barrier defense strategy includes the commensal microbiota, a stratified mucus layer, epithelial integrity, cell turnover, and finally the underlying lamina propria enriched with immune cells (Maynard *et al.*, 2012). Given that heavy loads of bacteria reside in the gut lumen and in the vicinity of the epithelium, it is not surprising that the IECs actively sense and interact with microbes to achieve homeostatic immune responses (Westendorf *et al.*, 2010).

In order to serve as an efficient barrier, the intercellular space of IECs must be sealed by TJ proteins, which regulate the intestinal permeability. The TJ complex consists of the transmembrane proteins occludins and claudins, tricellulin, scaffolding protein zonula occludens -1 (ZO-1), -2 and -3, and junctional adhesion molecules, containing in total over 50 proteins. The TJ structure is constantly being remodeled in response to external stimuli, including microbes and food antigens. The detailed TJ structure and its multifunction has been reviewed previously (Schneeberger & Lynch, 2004). One crucial step of enterotoxigenic *E coli.* (ETEC) pathogenesis is TJ disruption, achieved by toxin production after bacterial attachment to IECs. The resultant leaky barrier may allow indiscriminate antigen passage, thereby leading to signal dysregulation and dysfunction of the immune system, followed by inflammatory reactions and cytokine production (Croxen & Finlay, 2010). Pro-inflammatory cytokine-induced TJ perturbation and a leaky gut is also characteristic of IBD patients (Edelblum & Turner, 2009). In contrast, increasing evidence suggests that commensal bacteria and probiotics can enhance the intestinal barrier function by altering TJ protein expression and distribution (Ulluwishewa *et al.*, 2011). As discussed earlier, the value of probiotics and prebiotics is the 'gut health-promoting' effect. It is thus important to identify one or more interactive immune components in IECs in order to achieve the most effective host mucosal defense.

4.3 Role of heat shock proteins in gut health

Heat shock proteins are a set of highly conserved proteins that are present and can be induced in all types of cells, from microbes to mammals. They were discovered in 1962 following a laboratory mistake in which the temperature of an incubator where *Drosophila melanogaster* larvae were being kept was accidentally increased, which induced new puffing patterns of the polytene chromosomes in salivary glands (Ritossa, 1962). The term heat shock protein derives from the initial heat shock observations, but these proteins can be induced by a wide range of stimuli other than thermal stress, such as cytokines, oxidative stress, toxins, microbes, dietary components, and SCFA (Henderson & Pockley, 2010; Tissiéres *et al.*, 1974). HSPs are a large and diverse group of proteins categorized into several families on the basis of their approximate molecular weight (*e.g.* HSP27, HSP60, HSP72 and HSP100) and are present in inducible or constitutive forms. They display molecular chaperone functions, for instance assembly, disassembly, folding, refolding, stabilization and translocation of proteins under stress and non-stressful conditions, thereby acting as cytoprotective constituents (Hightower *et al.*, 2000; Hightower,

1991). The following text mainly focuses on HSP27 and HSP72 as the two major cytoprotective HSPs in the GI tract.

4.3.1 Intestinal epithelial HSP27 and HSP72 expression

The physiological role of HSP27 and HSP72 is to carry out crucial housekeeping functions in order to maintain the mucosal barrier integrity against various stimuli in the gut microenvironment. HSP27 is associated with cytoskeleton stabilization through interactions with F-actin (Mounier & Arrigo, 2002). HSP72 (HSP70 is a homologue) displays many specific features, including a chaperone property to confer cell protection (Kampinga & Craig, 2010). It appears that under normal conditions, HSP27 (HSP25 is a homologue) and HSP72 expression is cell type- and region-specific. They are localized in stomach, distal ileum and colon IECs (Hu *et al.*, 2010; David *et al.*, 2002; Jin *et al.*, 1999). It has been suggested that the regional distribution of HSPs is due to the extreme chemical conditions to which the gastric mucosa is exposed and to the abundant bacteria colonizers and their metabolites in the colon, indicating the fundamental role of HSPs in fulfilling certain physiological niches (Arvans *et al.*, 2005).

It is becoming evident that the enteric flora is one of the major determinants of HSP physiological expression in IECs. This has been clearly demonstrated in antibiotic-treated mice, in which a significant reduction of HSP25 and HSP72 occurs in colonic mucosa (Kojima *et al.*, 2003). Moreover, in germ-free mice, the longitudinal expression of HSPs along the GI tract is abolished, which is not the case in conventionally colonized mice (Hu *et al.*, 2010). Recent studies have shown that nutritional components can also affect HSP expression in the GI tract, *e.g.*, addition of butyrate, glutamine, dietary pectin, and arginine conferred beneficial effects through HSP induction in the gut (Wu *et al.*, 2010; Ren *et al.*, 2001; Wischmeyer *et al.*, 1997). However, results from studies concerning disease-associated HSP expression in the gut are conflicting. For example, stronger HSP72 expression has been observed in IBD patient biopsies compared with control subjects (Ludwig *et al.*, 1999). In contrast, Hu *et al*. (2009) demonstrated that HSP70 expression is down regulated in inflamed mucosa of mouse, which results in more severe tissue injury in the host. Surprisingly, this effect can be attenuated by forcing HSP70 expression in IECs (Hu *et al.*, 2009).

4.3.2 Heat shock protein as a novel immune modulator

One paradox regarding HSPs is that most early studies reported that these chaperones induced pro-inflammatory signals, whereas more recent research reports anti-inflammatory effect of HSPs. This has led to the assumption that

the biological action of HSPs may be multi-factorial. It is therefore critical to understand the means by which a highly conserved protein such as HSP performs multifunctions (Henderson & Pockley, 2010). The 'moonlighting' paradigm states that individual proteins can have multiple functions. In fact, the molecular chaperones are one of the major groups of moonlighting proteins that are increasingly being shown to exert unexpected actions (Henderson & Pockley, 2010; Jeffery, 2003). A review by Kampinga and Craig thoroughly demonstrated that HSP70 achieves multiple functions through a diverse class of cofactors (Kampinga & Craig, 2010). Intriguingly, studies have detected HSPs (*e.g.*, HSP10, HSP27, and HSP72) in extracellular fluids from the bodies of healthy individuals (Shamaei-Tousi *et al.*, 2007; Fanelli *et al.*, 1998; Pockley *et al.*, 1998). It is likely that secreted HSPs can act as molecular signals in various immune responses. Evidence shows that HSP70 is involved in bacterial lipopolysaccharide signal transduction and acts as a mediator of cell activation (Triantafilou *et al.*, 2001). In addition, exogenous HSP27 has been observed to stimulate IL-10 overproduction in human monocytes, indicating that HSPs may be a novel class of immune modulators (De *et al.*, 2000).

4.4 Interplays between specific microbes and immune components

Individual commensal bacteria have been described as influencing specific host immune components in distinct ways. Understanding these interplays may explain how shifts in gut microbial community composition correspond to immune reactions in health and disease, and may identify clear targets for therapeutic manipulation (Ivanov & Honda, 2012). As discussed above, it is believed that HSPs are mediators of bacteria-host interactions. To date, studies of HSP induction and microbes have focused on the use of probiotics. A substantial body of evidence suggests that probiotic *Lactobacillus* spp. confer beneficial effects in the intestinal mucosal barrier, at least partly through interactions with HSP27 and HSP72, although the network behind this has not been fully elucidated (Segawa *et al.*, 2011; Ueno *et al.*, 2011; Tao *et al.*, 2006; Petrof *et al.*, 2004).

Another classic example of interaction between microbe and host immune component is the induction of colonic T_{reg} cells by commensal clostridia species (Atarashi *et al.*, 2011). Within the intestine, the balance between effector immune cells, such as T helper 17 (which produce IL-17 and IL-22) and T_{reg} (which produce IL-10), is essential in fine-tuning the adaptive immune system (Hooper *et al.*, 2012). Administration of 46 strains of bacteria

belonging to clostridial cluster XIVa and cluster IV to germ-free mice not only showed T_{reg} accumulation in colonic mucosa, but also induced anti-inflammatory cytokine IL-10 production, indicating that commensal bacteria may strongly affect the host immune system (Atarashi *et al.*, 2011).

5 Aims of the thesis

The overall aim of the work presented in this thesis was to gain a better understanding of interactions between diet, gut microbiota, and immunity from a specific dietary fiber source, namely chicory (*Cichorium intybus* L). This involved studies concerning the impact of chicory fiber on animal performance, digestibility, gut development, microbiota community structure in small and large intestine, and follow-up reactions with specific immune components- cytoprotective HSP27 and HSP72, *in vivo* and *in vitro*. Specific objectives of the studies described in **Papers I-IV** were to investigate:

- The effects of dietary inclusion of chicory forage or root on growth performance, gut development, and nutrient utilization in young monogastrics.

- The effects of changes in composition of the fiber fraction in chicory diets relative to a cereal-based diet on gut microenvironment and intestinal microbiota in pigs.

- The distribution of cytoprotective HSP27 and HSP72 along the GI tract, interactions between HSP expression and specific microbes, and whether these could be related to the dietary fiber components.

- Interplays between specific strains of lactobacilli, their metabolites and HSP expression in the mucosal barrier, and whether the interactions could elicit cytoprotection against ETEC challenge.

6 Materials and methods

6.1 Experimental design and animals

All experiments were conducted at the Swedish University of Agricultural Sciences (SLU) in Uppsala and animal experiments were approved by the ethical committee for the Uppsala region. The chicken trial (**Paper I**) was structured as a randomized complete block design with 8 treatments in 4 blocks. Each pen that served as a block contained 8 birds. In the pig trial (**Paper II** and **Paper III**), a split-litter design was employed with 30 pigs from 6 different litters (5 pigs per litter) randomly allocated to one of 5 experimental diets, resulting in 6 replicates for each. The *in vitro* cell culture experiment (**Paper IV**) was carried out as a completely randomized design, with 3-7 treatments depending on the assay performed. Each assay was conducted with at least 3 independent experiments.

In the chicken trial, 256 one-day-old broiler chicks (Ross 308, female and male) with an initial BW of 44.0 ± 15.1 g were studied over a 30-day period. During the first week, weak and dead birds (four in total) were replaced. Chickens were kept on wood shaving floors from day 1 to day 27. Thereafter, a net floor was used for three days to facilitate excreta sampling. The birds had free access to feed and water throughout the experiment. The temperature and light in the environment were strictly controlled. Body weight and feed intake were recorded for each pen at arrival and every week thereafter and the data were aggregated for two periods (0-13 days and 14-27 days).

In the pig trial, 30 Yorkshire pigs (castrated male and female) aged 7-weeks, with an initial BW of 11.7 ± 0.16 kg, were studied over an 18-day period. The pigs were housed in individual pens without straw bedding and supplied with feed and water *ad libitum*. Feed intake was recorded daily and pigs were weighed weekly and on the last day of the experiment.

6.2 Diets

Chicory forage or root (Inulin-60, Inter-Harz Gmb, Germany) was used to compose experimental diets and compared with a cereal (wheat and barley) control diet in animal studies. All diets were supplemented with protein, amino acids, minerals, and vitamins to meet the nutritional requirements of the broilers (NRC, 1994) and the growing pigs (NRC, 1998). Prior to mixing with other feed ingredients, chicory forage was dried with forced air at 30 °C for one week and milled through a 3-mm screen for chicken feed and a 3.5-mm screen for pig feed. The diet contained titanium dioxide as an indigestible digesta marker at 5 g/kg (chicken experiment) or 2.5 g/kg (pig experiment), and no antibiotics. The feeds were fed as pellets to the animals in all cases except for the first week for chicks, when pellets were ground.

In brief, in the chicken experiment the cereal basal diet (C) was partly replaced with 60 and 120 g/kg chicory forage harvested in June (CF_1) or September (CF_2) or 60 g/kg chicory root meal (CR) containing 60% inulin, alone or mixed. In total, eight diets were used in the chicken experiment (**Paper I**). In the pig experiment (**Papers II** and **III**), chicory forage from two harvests was mixed and inclusion level was adjusted to 80 or 160 g/kg forage or 80 g/kg chicory root meal, alone or mixed. The resulting diets for pigs were denoted C, CF80, CF160, CR80 and CFR. Detailed data on the chemical composition of the diets and their nutritional content are presented in Table 1. It can be seen from the table that the major fiber fraction constituent was pectin (uronic acid as building block) in the chicory forage diet, inulin-type fructan in the chicory root meal diet and arabinoxylan in the cereal basal diet.

Table 1. *Chemical composition (g/kg DM) and gross energy content (MJ/kg DM) of the experimental diets used in* ***Papers I, II*** *and* ***III***

Item	Diets in chicken experiment (**Paper I**)								Diets in pig experiment **(Papers II** and **III)**				
	C[a]	$CF_1$60	$CF_2$60	CR60	$CF_1$120	$CF_2$120	CF_1R	CF_2R	C[b]	CF80	CF160	CR80	CFR
Gross energy	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.7	18.4	18.2	18.5	18.4
Ash	74	80	82	74	83	94	81	84	45	59	69	48	61
Crude protein	197	201	204	192	191	201	194	190	236	234	232	233	236
Dietary fiber	171	174	191	159	198	193	177	170	137	175	196	135	158
Klason lignin	19	20	25	12	26	16	24	23	27	38	43	24	31
NSP*	152 (38)	174 (53)	166 (44)	147 (37)	172 (47)	177 (59)	153 (40)	147 (44)	110 (22)	137 (39)	137 (39)	137 (39)	137 (39)
Arabinose*	24 (6)	28 (9)	23 (6)	23 (6)	21 (4)	22 (6)	21 (5)	21 (6)	18 (2)	19 (4)	19 (5)	18 (3)	17 (3)
Xylose*	40 (5)	39 (7)	36 (1)	36 (5)	35 (1)	33 (3)	33 (3)	30 (4)	37 (4)	36 (4)	36 (4)	33 (3)	31 (2)
Uronic acid*	18 (10)	25 (16)	29 (20)	17 (8)	35 (25)	39 (29)	29 (19)	30 (20)	4 (2)	17 (15)	24 (19)	8 (5)	17 (12)
Fructan	20	21	21	54	19	17	48	45	8	10	6	49	49

[a] C: control diet in chicken experiment; $CF_1$60: 60 g/kg chicory forage from June harvest; $CF_2$60: 60 g/kg chicory forage from September harvest; CR60: 60 g/kg chicory root; $CF_1$120: 120 g/kg chicory forage from June harvest; $CF_2$120: 120 g/kg chicory forage from September harvest; CF_1R: mix of chicory forage from June harvest and chicory root; CF_2R: mix of chicory forage from September harvest and chicory root.

[b] C: control diet in pig experiment; CF80: 80 g/kg chicory forage; CF160: 160 g/kg chicory forage; CR80: 80 g/kg chicory root meal (Inu60, Inter-Harz Gmb, Germany); CFR: mix of 80 g/kg chicory forage and root meal.

* Values in brackets indicate soluble fiber fraction (g/kg DM).

6.3 Sampling and analysis

In animal experiments (**Papers I** and **II**), feed and feces samples were collected for chemical analysis. In addition, tissue segments were taken from the jejunum (20 cm from duodenal loop) and cecum (one side randomly) in chickens and from the jejunum, distal ileum (50 cm cranial to the ileocecal valve) and proximal colon (20 cm from the cecum) in pigs and stored appropriately for different purposes. In the pig experiment, ileal and colonic digesta were also collected.

In addition, digestive organ size (**Paper I**) was recorded and pH in intestinal contents (**Papers I** and **II**) was measured (PHM 210 meter, Radiometer, Cedex, France) upon animal dissection. Blood samples were collected from the wing vein in chickens (1 mL) and from the jugular vein in pigs (5 mL) using size-appropriate syringes. The blood was allowed to clot at room temperature before being centrifuged at 3,200 x *g* for 10 min. Serum was isolated and stored at -80 ºC until analysis.

6.3.1 Chemical analysis

In **Papers I** and **II**, feeds, feces and digesta were freeze dried for nutritional analysis as previously described. In brief, DM was determined by drying samples at 103 °C for 16 h and ash after ignition at 600 °C for 3 h (Jennische & Larsson, 1990). Crude protein (CP) was analyzed 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 the modified Uppsala method (Bach Knudsen, 1997). Fructan content was analyzed using a Fructan Assay Kit (Megazyme Cat. no. K-FRUC, Bray, Ireland). Gross energy was measured with a bomb calorimeter (Parr 6300 Oxygen Bomb Calorimeter, Illinois, USA). Titanium dioxide was analyzed as previously described (Short *et al.*, 1996). Organic acids (SCFA and lactic acid) was analyzed by HPLC in **Paper II** as previously described (Andersson & Hedlund, 1983).

6.3.2 Gut morphometry

Intestinal histological parameters, including mucosa thickness, villus height, crypt depth, thickness of the muscularis externa (**Papers I** and **II**), and total number of goblet cells, were determined (**Paper III**) following standard procedures. In brief, in the small intestine, villus height was measured from its apex to the transition into the crypt zone, while crypt depth was taken as the difference between mucosa thickness and villus height. In the large intestine,

mucosa thickness (distance from villus top to crypt end) was measured and the muscularis externa was taken to include the inner circular layer and longitudinal outer muscular layer.

6.3.3 Microbial analysis in digesta and gut mucosa

In order to explore porcine gut microbial community composition, an internal terminal restriction fragment length polymorphism (T-RFLP) database was constructed by pooling information on samples from the intestinal digesta (**Paper II**), the gut mucosa (**Paper III**), and feces in pigs. DNA was extracted using QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany). T-RFLP analysis was performed as previously described (Dicksved *et al.*, 2008).

A total of 10 clone libraries were constructed to identify TRFs of interest. In each library, 48 inserts were PCR amplified. The resulting PCR products were analyzed by T-RFLP, and subsequently 202 clones were chosen for sequencing and were aligned to the GenBank database using standard nucleotide BLAST at NCBI. Pintail v1.0 was used to identify chimeric sequences, which were removed from further analyses.

6.4 IPEC-J2 cell line in microbiological investigations

In the cell culture experiment (**Paper IV**), porcine IPEC-J2 intestinal epithelial cell line was employed as an *in vitro* model to study interactions between microbes and mucosa surface. The cell line was derived from the jejunum of a neonatal, unsuckled piglet (less than 1 day old) (Berschneider, 1989). IPEC-J2 is unique in that it is a non-transformed, non-tumorigenic small intestinal cell line that provides high specificity to porcine studies and is analogous to human gut physiology.

6.4.1 Epithelial cell culture

The IPEC-J2 cells were propagated in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma Aldrich, Sweden), supplemented with an antibiotic mixture (penicillin, 100 U/mL; streptomycin, 100 µg/mL), 0.5 mM sodium pyruvate, and 5-10% fetal bovine serum (FBS; Sigma Aldrich, Sweden). The cells were cultured at 37 °C in a 5% CO_2 and 95% air-humidified incubator. Cells were passaged every 3-4 days (by seeding at 1:3 ratio). Serum-free medium supplemented with insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 ng/mL), and epidermal growth factor (EGF, 5 ng/mL; Sigma Aldrich, Sweden) was tested in searching for a replacement for FBS of undefined and variable composition (van der Valk *et al.*, 2010). However, it was found that the serum-free medium with supplement suppressed IPEC-J2

cell attachment and polarization. In order to keep the cell phenotype stable and consistent, cells within 10 passages should be used, especially with high passage numbers (95-105) as in **Paper IV**.

When grown on a permeable filter (collagen-coated membrane with 0.4 μm pore size and 1.12 cm^2 growth area) in optimized conditions, IPEC-J2 cells can become polarized, express tight junction proteins, and develop microvilli. The differentiated monolayer exhibits strong similarities to native intestinal mucosa (Geens & Niewold, 2011). The polarized IPEC-J2 cell monolayer was characterized by scanning electron microscopy. The cells were fixed with 2.5% glutaraldehyde for 2 h at room temperature (RT). Thereafter 2% osmium tetroxide was applied for 2 h (RT) followed by dehydration in graded ethanol. The images were obtained by Hitachi TM-1000-μDeX environmental tabletop electron microscope (Uppsala, Sweden). Another method used for characterization of the polarized cells was immunofluorescent staining of tight junction protein ZO-1. Starting with cell monolayer fixation in 4% paraformaldehyde and permeabilization, localization of ZO-1 was carried out. Samples were subsequently stained with Rabbit polyclonal anti-ZO-1 (diluted at 1:100; N-term, Invitrogen, Sweden) as primary antibody, Alexa fluor 488 goat anti-rabbit (green) as secondary antibody (diluted at 1:200; Invitrogen), and counterstained with DAPI (Invitrogen). Images were acquired using laser scanning confocal microscopy (Nikon C-1 with Plan ApoVC 60 x/1.40 oil objective; Nikon EZ-C1 software). In addition, in order to assess cell monolayer integrity, transepithelial electrical resistance (TEER) was measured every day after cell seeding on transwell filters using the Millicell electrical resistance system (Millipore, Sweden).

6.4.2 Microbial preparation for IPEC-J2 study

In **Paper IV**, *Lactobacillus rhamnosus* strain GG ATCC 53103 (probiotic product Culturelle), *Lactobacillus johnsonii* strain P47-HY, and *Lactobacillus reuteri* strain P43-HUV (intestinal content isolates from pig experiment) and their equivalent supernatants (*sLGG*, s*L. johnsonii*, and s*L. reuteri*) were evaluated in the IPEC-J2 cell line. Bacteria at early stationary phase at a concentration representing multiplicity of infection (MOI) 100 were freshly prepared by cultivation in de Man-Rogosa-Sharp (MRS) broth (Oxoid, U.K.) at 37 °C overnight. Thereafter, bacteria were harvested by centrifugation at 5,000 x *g* at 4°C for 10 min, washed with phosphate-buffered saline (PBS, pH 7.4) and resuspended in cell culture media to the desired concentration, while supernatant was sterilized and diluted (1:10 directly in cell culture medium). In addition, the gene encoding propanediol dehydratase large subunit (*pduC*) was detected in *L. reuteri* strain P43-HUV by PCR analyses, indicating production

of antimicrobial reuterin (Walter *et al.*, 2011). A clinical isolate ETEC strain 853/67 (Handl *et al.*, 1988) at MOI 10 was used as a challenge for IPEC-J2 cells and was grown in Luria-Bertani broth (tryptone 10 g; yeast extract 5 g; NaCl 5 g with distilled water up to 1 L) overnight at 37 °C with vigorous shaking at 120 rpm.

In addition, bacteria adhesion was assessed by agar plating as previously described (Roselli *et al.*, 2007). The capacity of lactobacilli for adhesion and their ability to inhibit ETEC attachment to the IPEC-J2 monolayer were studied during 2-h incubations. As live bacteria were studied, cell viability was tested by trypan blue exclusion and by using a lactate dehydrogenase activity kit (Biovision, Sweden). In different experiments, IPEC-J2 cells were seeded on Transwell®-COL collagen-coated membrane filters (1.12 cm^2; 0.4 μm; Corning, Sweden) or tissue culture plates (VWR international, Sweden) and incubated with bacteria and/or supernatant for different periods. In **Paper IV**, whole cells, cell monolayer, cell culture medium, and cell lysate were sampled.

6.5 Detection of heat shock proteins *in vivo* and *in vitro*

Efforts were made in this thesis to detect inducible HSP27 and HSP72 in normal pig gut mucosa and in blood (**Paper III**). HSP27, HSP72, and heat shock cognate protein (HSC) 73 were analyzed in IPEC-J2 cells (**Paper IV**).

First, immunohistochemical analysis was performed following descriptions in rat studies (Arvans *et al.*, 2005). Paraffin-embedded tissue sections from pig jejunum, ileum, and colon (**Paper III**) were stained with anti-HSP27 rabbit polyclonal antibody (1:200) or anti-HSP72 mouse monoclonal antibody (1:100; Stressgen, CA), developed by Vectastain Elite ABC kit using a Avidin/Biotinylated enzyme Complex (Vector Labs, Burlingame, CA) and finally detected by diaminobenzidine (DAB) solution. A brownish precipitate was indicative of immunoreactivity. Images of the slides were examined using a light microscope equipped with Canon A640 digital camera. The intensity of HSP27 and HSP72 was quantified using a scoring system (0, negative; 1, mild; 2, moderate; 3, strong) described previously (Ludwig *et al.*, 1999). The appearance of ileal PPs was recorded. In **Paper III**, circulating HSP27 and HSP72 concentrations in serum were analyzed using Porcine Heat Shock Protein 27 and Porcine Heat Shock Protein 70 kits (USCNlife, China).

Expression of HSP27, HSP72, and HSC73 (HSC73 served as a protein loading control) was investigated in IPEC-J2 cells by Western Blot analysis (**Paper IV**). IPEC-J2 cells were left untreated or treated with *L. rhamnosus GG* (LGG)*, L. johnsonii*, or *L. reuteri* (MOI of 100) for 6 h at 37 °C. The cells were washed with saline and culture media, and thereafter incubated further for 6 h.

In view of the fact that LGG inducing HSP expression in mouse colonic cells began at 4-6 h of incubation to 20 h (Tao *et al.*, 2006), the time period needed was identified in preliminary experiments which tested 6 h incubation plus 6 h recovery, 6 h incubation plus 12 h recovery, and 12 h incubation and no recovery time. Furthermore, the heat shock control used was IPEC-J2 cells heat-treated at 42 °C for 60 min and left at 37 °C for 2 h based on serial tests (42 °C for 30, 60 min or 24 h, 45 °C for 30 or 60 min, and 50 °C for 30 min or 60 min). Proteins were extracted from samples using a mammalian cell extraction kit (BioVision, Sweden). One aliquot of samples was used for protein determination by the bicinchoninic acid assay (Pierce, Sweden). Proteins were separated by SDS-PAGE with PhastSystem (GE Healthcare, Sweden) and thereafter transferred to a nitrocellulose membrane for blotting. The blotted membrane was subsequently stained with anti-HSP27, anti-HSP72, or anti-HSC73 antibody (Stressgen, CA) and species-appropriate horseradish peroxidase-conjugated secondary antibody (Enzo Life Sciences, Sweden), and finally developed using DAB solution. Densitometry was performed on all membranes under the same conditions and evaluated using a computer-assisted image analysis system (Quantity One, version 4.6.7, Bio-Rad, USA).

6.6 Data analysis

In **Paper I**, the total tract apparent digestibility and ileal apparent digestibility were calculated using the indicator technique (Sauer, 2000). In **Papers II** and **III**, Simpson's index of diversity was calculated (Dicksved *et al.*, 2008). Statistical analyses were performed with various procedures in SAS (SAS Institute, Cary, NC, USA, version 9.2). Two-way interactions were tested (**Papers II** and **III**) and excluded from models if $P>0.05$. Data were presented as least square means (**Papers I-III**) or means ± SEM (**Paper IV**). Significance was set at $P<0.05$.

Treatment effects in terms of diet, gut location, and bacteria treatment were analyzed using PROC GLM with CONTRAST statement (**Paper I**), PROC MIXED (**Papers II** and **III**), and PROC ANOVA with Tukey's post hoc test (**Paper IV**). Furthermore, PROC CORR was carried out to identify relationships between variables with false discovery control over multiple comparisons (Benjamini & Hochberg, 1995) in **Papers II** and **III**. Multivariate analysis was used for analyzing bacterial community structure based on T-RFLP data with Spearman rank correlation cluster analysis (MultiExperiment Viewer, MeV 4.5.1) in **Paper II** and with Principal Coordinates Analysis (PAST 2.13) in **Paper III**.

7 Main results

7.1 Animal performance and nutrient utilization

Feed intake and growth performance were recorded as a measure of feed effectiveness. Improved FCR was seen at 13 days of age in chickens fed diet CF60, whereas birds fed diet CF120 reached the same BW but consumed more feed, which resulted in lower feed efficiency compared with birds fed the control diet throughout the experiment. This may be linked to the fact that a higher inclusion level of dietary chicory fiber resulted in lower total tract apparent digestibility of DM, organic matter (OM), ileal apparent digestibility of CP, and energy content. Fiber content, type, and harvest time affected the total tract apparent digestibility of all fiber fractions (**Paper I**). Pigs utilized chicory fiber well, as the growth performance remained unchanged even with 160 g/kg dietary chicory inclusion (**Paper II**).

7.2 Gut histology

In general, small intestine morphology was not altered by dietary chicory addition except that ileal villus height was shorter on diet CF160 (Figure 3). Mucosa thickness in chicken cecum decreased with increasing fiber inclusion, whereas in pigs the colonic muscularis externa was thicker with diets CF80 and CFR (**Papers I** and **II**).

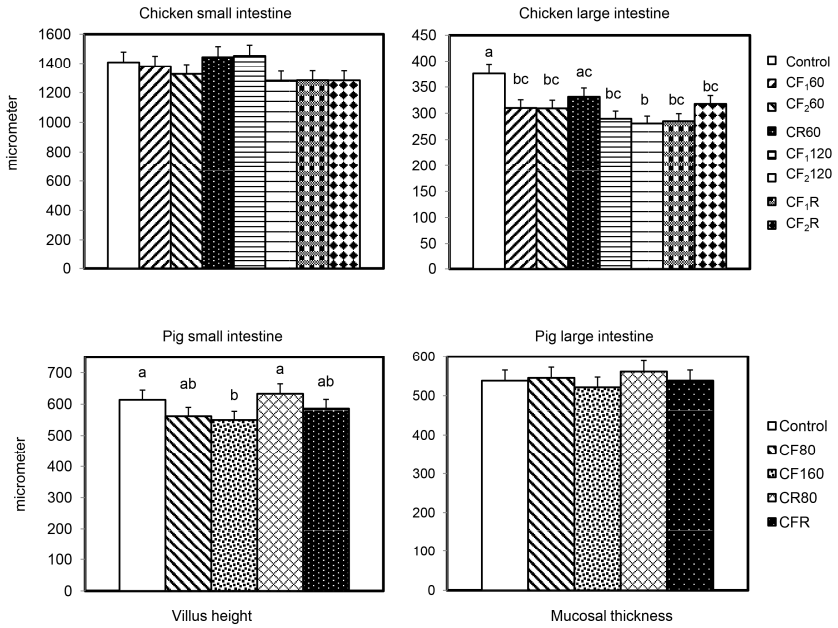

Figure 3. Effect of diet on villus height and mucosal thickness in the small and large intestine of chickens and pigs. In chicken experiment: $CF_1$60: 60 g/kg chicory forage from June harvest; $CF_2$60: 60 g/kg chicory forage from September harvest; CR60: 60 g/kg chicory root; $CF_1$120: 120 g/kg chicory forage from June harvest; $CF_2$120: 120 g/kg chicory forage from September harvest; CF_1R: mix of chicory forage from June harvest and chicory root; CF_2R: mix of chicory forage from September harvest and chicory root. In pig experiment: CF80: 80 g/kg chicory forage; CF160: 160 g/kg chicory forage; CR80: 80 g/kg chicory root meal (Inu60, Inter-Harz Gmb, Germany); CFR: mix of 80 g/kg chicory forage and root meal. Values with different letters differ significantly (P<0.05).

7.3 Dietary fiber-induced changes in gut microbiota

7.3.1 Gut bacterial community structure

The T-RFLP data collected from ileal mucosa-associated microbiota (MAM), ileal luminal microbiota, colonic MAM, and luminal microbiota provided an overview of the microbiota composition in the gut of healthy, young pigs (**Papers II** and **III**). Inter-individual differences were evident within gut sites (Figure 4A). None of the TRFs were distributed among all gut locations. Principal coordinate analysis revealed strong primary clustering by gut segmentation and stratification (Figure 4B), with the luminal microbiota showing higher dissimilarity between ileum and colon than the MAM. The

bacterial diversity was also partitioned across gut sites with the highest diversity in the luminal colonic microbiota (**Papers II** and **III**).

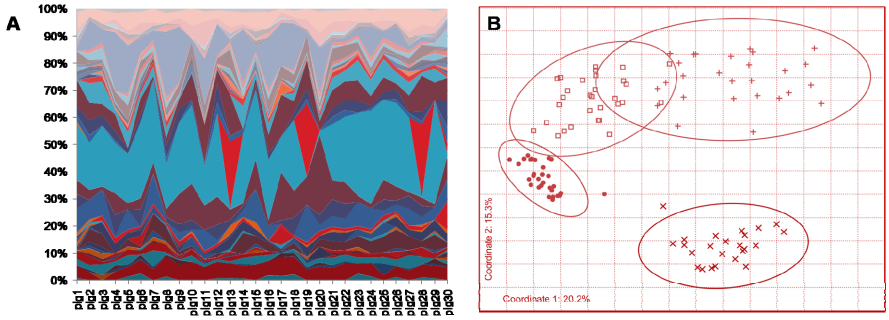

Figure 4. Microbial communities across gut sites (**Paper III**) and individual bacterial profiles. (A) The colonic luminal microbiota in pigs reveals large inter-individual variation in gut microbiota composition residing within the same location. (B) Microbial community clustering using principal coordinate analysis of Spearman rank correlations shows that samples clustered into four primary groups: +, ileal mucosa-associated microbiota; x, ileal luminal microbiota; □, colonic mucosa-associated microbiota; •, colonic luminal microbiota.

7.3.2 Dietary fiber-responsive bacteria

Dominant bacteria groups were identified and responded to dietary components differently (**Paper II**). Terminal restriction fragments (TRFs) identified as LAB were prominent in the ileal luminal microbiota. The relative amount of this group was increased by the CF160 and CFR diets. The colonic luminal bacterial community was dominated by TRFs identified as butyrate-producing bacteria (bacteria belonging to clostridial cluster XIVa and cluster IV) and *Prevotella* phylotypes. With increasing chicory forage inclusion, butyrate-producing bacteria were promoted, whereas *Prevotella* spp. responded to dietary arabinoxylan content changes. Furthermore, one individual species of bacteria, *Megasphaera elsdenii* (TRF275), was linked with diets CR80 and CFR.

7.3.3 Bacterial fermentation products and pH changes

Major bacterial fermentation products, including acetate, lactic acid, butyrate, and valeric acid, changed following microbiota alteration (**Paper II**). The molar proportion of acetic acid was positively correlated with the relative abundance of butyrate-producing bacteria and was correlated with butyrate concentration. The relative abundance of *M. elsdenii* coincided with valeric acid concentration. In addition, the diet containing a mixture of chicory forage and inulin resulted in the lowest colonic pH.

7.4 Host immune responses

7.4.1 Intestinal mucosal immunity

In **Papers III** and **IV**, HSP27 and HSP72 were studied as a cytoprotective measure and were detected in normal porcine jejunum, distal ileum, proximal colon mucosa (Figure 5), ileal PPs, and IPEC-J2 cells. The expression was highest in the surface epithelium, lower in crypt cells, and limited in lamina propria (Figure 5B). Overall, HSP expression in the distal ileum was stronger than that in the proximal colon mucosa. Examining the gut microbiota complexity in order to identify relationships between specific microbes and HSP expression, we found correlations between HSPs and TRFs identified as LAB, butyrate-producing bacteria, and individual bacterial species. Furthermore, chicory uronic acid intake was positively correlated with ileal HSP27 expression and further related to the abundance of mucosa-associated *M. elsdenii* (**Paper III**).

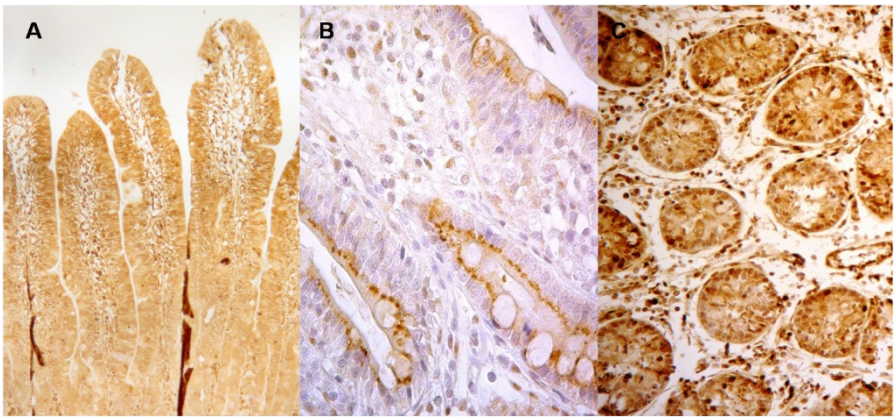

Figure 5. Localization of heat shock proteins (HSP) in normal pig gut. (A) HSP27 expression in the jejunum (strong staining). (B) HSP72 expression in the distal ileum showing a positive staining on the surface of the epithelial cells (C) HSP72 expression in the proximal colon showing strong staining (the tissue section was cut horizontally).

To determine whether the correlation between HSPs and LAB could be confirmed, IPEC-J2 cells treated with one of three lactobacilli strains (LGG, *L. johnsonii*, or *L. reuteri*) were quantified for HSP expression (**Paper IV**). Expression of HSP27 was induced by all lactobacilli and their supernatants after 6 h incubation, whereas HSP72 only was induced by the supernatant of *L. reuteri*, but not by any live bacteria treatment. Further, the protective effect of *Lactobacillus* spp. in the IPEC-J2 monolayer was investigated using ETEC challenge. The results showed that, consistent with TEER values, different

strains of *Lactobacillus* spp. affected tight junction protein ZO-1 expression in IPEC-J2 cells differently and maintained gut barrier integrity to different extents (**Paper IV**) compared with a complete perturbation of the cell monolayer with ETEC treatment alone. In addition, lactobacilli exerted different adhesion capacity and inhibition of ETEC attachment in the IPEC-J2 cell monolayer.

7.4.2 Immune components in circulation

Serum IgG was measured in normal chickens and was not affected by dietary components (data not shown). In normal pigs, circulating HSPs were detected, but in low concentrations and in only 8 out of 30 pigs (Figure 6).

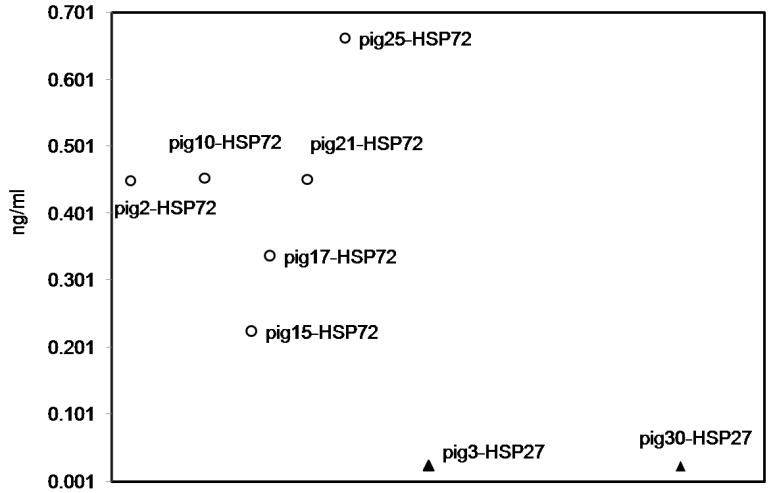

Figure 6. Detection of heat shock proteins (HSP) in normal pig blood. Each spot represents an individual pig. °: individuals detected for HSP72; ▲: individuals detected for HSP27.

8 General discussion

This thesis describes the use of chicory forage and chicory root meal as a dietary fiber source in the nutrition of broiler chickens and young pigs, and how it is utilized in animals, particularly in the GI tract (**Papers I** and **II**). It provides insights into the complexity of gut microbiota and changes in bacterial community composition in response to various fiber fractions in pigs (**Papers II** and **III**). Furthermore, the expression and distribution of cytoprotective proteins HSP27 and HSP72 were mapped and found to be located in different regions of porcine GI tract. The physiological expression of HSP could be correlated with specific commensal microbes, where the interaction between LAB and HSP72 represents one of the important examples identified. The results from **Papers III** and **IV** indicate that chicory diet and microbiota interactions can elicit induction of HSPs in the gut that mediates IEC protection against pathogen invasion.

8.1 Dietary chicory utilization in broilers and young pigs

Our initial work aimed at describing interactions between dietary chicory and young animals. Fiber-rich diets were composed with two levels of chicory addition, two types of fiber source (chicory forage and root), and various mixtures. In addition, the effect of harvest time was evaluated in the chicken experiment. Taken together, our studies have focused on different chemical composition of the experimental diets, in which pectin was the major NSP fraction for chicory forage diets, inulin-type fructan for chicory root diets, and xylan for the cereal (wheat and barley) control diet. It has been reported that the digestibility of dietary fiber is highly variable depending on its origin (Noblet & Le Goff, 2001). In **Paper I**, effects of fiber source and harvest time were observed in chickens. The total tract apparent digestibility of NSP in the chicory forage diet was higher than that in the chicory root diet. Moreover, the digestibility of uronic acid (building blocks for pectin) was higher for

September harvest of chicory than for June harvest, most probably due to a higher soluble uronic acid content. It was in line with the higher CP content at the September harvest, which together resulted in better performance of chickens fed on these diets. A previous study found that increasing dietary pea fiber (from 187 to 375 g/kg) was linked with decreased uronic acid digestibility in chickens (Jorgensen *et al.*, 1996). Similarly, we found that inclusion of 120 g/kg chicory forage decreased the digestibility of uronic acid, but not that of the diet containing 60 g/kg chicory forage, indicating that the capacity of broilers to digest uronic acids is highly dependent on the inclusion level. This effect was also seen on total tract apparent digestibility of NSP, where a reduction was observed in the diet with the highest chicory forge inclusion compared with the control. In the chickens studied in **Paper I**, mean total tract apparent digestibility of NSP was 25.1%, ranging from 17.5% in the diet containing chicory forage and root mixture to 31.2% in control. This is in the same range as reported previously (Jorgensen *et al.*, 1996). In contrast to chickens, growing pigs are able to digest chicory forage to a greater extent, with a total NSP digestibility of 67% (Ivarsson *et al.*, 2011). Consequently, up to 30% of maintenance energy can be obtained from NSP utilization in young pigs (Varel & Yen, 1997), whereas a significantly lower apparent metabolizable energy is observed in chickens fed a fiber-rich diet compared to the control (Choct *et al.*, 1992). Dietary fiber inclusion has been associated with reduced feed energy content and digestibility of other dietary components, such as OM (Noblet & Le Goff, 2001). Similarly, we found decreased digestibility of DM, OM, and AME in chickens fed diet included 120 g/kg chicory forage. In addition, the ileal apparent digestibility of CP was lower in the chicory forage diet (60 g/kg), which may be linked to higher microbial activity in the gut, as reflected by the higher NSP digestibility of this diet. Together, the data in **Paper I** indicate that chicory forage and root can be utilized to some extent in broiler chickens and that the threshold for dietary fiber inclusion differs between NSP sources.

The utilization of dietary fiber was reflected in animal growth performance and gut development. Both broilers and pigs maintained body weight gain and feed intake, irrespective of diet treatments, throughout the experiments (**Paper I** and **Paper II**). One explanation is that the cereal-based control diet was already high in fiber content. Previous studies have shown that high dietary fiber inclusion results in increased gut fill and/or organ weight, which may contribute to the final body weight in chickens and pigs (Wellock *et al.*, 2008; Pluske *et al.*, 2003; Hetland & Svihus, 2001; Jorgensen *et al.*, 1996). Interestingly, improved feed utilization coincided with faster growth at 13 d of age in chickens fed chicory forage compared with those fed the chicory root

diet, and the better feed conversion ratio was maintained to the end of the experimental period. These results indicate that different types of NSP affect animal performance differently (Bedford & Classen, 1992; Choct *et al.*, 1992). We assumed that these dietary effects would also be manifested at gut level, and indeed animal growth performance is interrelated with gut development (Drochner *et al.*, 2004). Several studies have described dietary inulin as a prebiotic that elicits significant enlargement of villus height and crypt depth in the small intestine in monogastric animals (Rehman *et al.*, 2007; Kleessen *et al.*, 2003; Kim, 2002). In our studies, no changes were found in the small intestine morphology of broilers, whereas increased villus height in the ileum was observed in pigs on the chicory root diet (80 g/kg; Figure 3). Chickens have a much shorter foregut and a higher passage rate than pigs, limiting their NSP digestion (Ito *et al.*, 2009; Wenk, 2001). On the other hand, dietary fiber has a more profound influence on the distal GI tract due to active microbial fermentation (Montagne *et al.*, 2003). A thinner cecal mucosa in chickens and a thinner muscle layer in the colon of pigs with increasing dietary fiber level were observed. It is suggested that the water-holding and gel-forming properties of NSP may increase gut distension (Drochner *et al.*, 2004).

8.2 Dietary fiber correlates with dominant bacteria phylotype

A diverse group of dietary NSP fractions arrives at the distal GI tract undigested, thus allowing intensive microbial fermentation. As a consequence, a tremendously complex microbial community is shaped, depending on the type and amount of NSP available (Walker *et al.*, 2011). In **Paper III**, bacterial T-RFLP profile analysis of samples from pig ileal digesta and mucosa and colonic digesta and mucosa revealed four primary clusters dependent on different sample types. Moreover, large inter-individual variation within a particular gut location was observed (Figure 4), and confirmed the findings from earlier studies (Ley *et al.*, 2006; Leser *et al.*, 2002). In humans, the fecal microbiota is commonly used as a reflection of the intestinal bacterial composition due to difficulties in obtaining mucosal samples, especially from the small intestine (Macfarlane *et al.*, 2006). Several studies have however reported that the composition of the fecal microbiota differs from the composition found in colonic biopsies (Eckburg *et al.*, 2005; Lepage *et al.*, 2005; Zoetendal *et al.*, 2002). Eckburg *et al*. (2005) suggested that different microbial populations may fulfill different roles in the gut ecosystem. For example, the microbiota in proximity with the gut epithelium is likely more important for shaping the host innate immunity compared with the luminal microbiota. Interestingly, we found a significant deviation of ileal MAM from

ileal luminal microbiota (Figure 4B). This result could be of high importance for clinical applications. For example, the ileal MAM is markedly influenced by the inflammatory condition in Crohn's disease patients and differs from the fecal microbiota (Willing *et al.*, 2010). On the other hand, we observed that in the proximal colon of pigs, the MAM profiles were distinctly different from the luminal microbiota profiles, yet, not as divergent as for the small intestine. Taken together, we postulate that the luminal microbiota represents a combination of shed mucosal bacteria and separate non-adherent members.

It is becoming apparent now that despite large individual variations, certain bacterial phylotypes occur more commonly than others and determine the gut microbiota community structure (Arumugam *et al.*, 2011). Diet is one of the driving forces in shaping these bacterial communities, and perhaps one of the easy approaches in effecting a beneficial change (Wu *et al.*, 2011). There is evidence that dietary supplementation with prebiotics such as inulin can promote specific groups of bacteria, including LAB in the human and animal gut (Kolida & Gibson, 2007; Van Loo, 2007). By combining the data on controlled dietary NSP intake and utilization, we further identified dominant bacterial species that inhabit the small and large intestine in pigs and investigated to what extent changes in bacteria abundance and their activity are influenced by diet. In **Paper II**, a clear dominance of the LAB group, especially the TRF corresponding to *L. johnsonii* in the ileal luminal microbiota was found, which is in agreement with other studies (Konstantinov *et al.*, 2004; Leser *et al.*, 2002). Interestingly, the LAB group was associated with the diet containing 160 g/kg chicory forage and the chicory forage and root mixture, which was further correlated with a significantly higher daily uronic acid and inulin-type fructan intake, respectively, indicating a substantial effect of different dietary NSP fractions (**Papers II** and **III**). Similarly, a diet containing sugar beet pulp (pectin-rich) and inulin promoted ileal luminal *Lactobacillus* spp. in young pigs compared with a maize starch basal diet (Konstantinov *et al.*, 2004). The LAB group is well recognized as containing beneficial bacteria for the host (Ringel *et al.*, 2012). For instance, *L. johnsonii* has been suggested to regulate IL-12 production in co-ordination with *Lactobacillus casei* in mouse macrophages, which may help to maintain host homeostasis (Shida *et al.*, 2009), whereas *L. reuteri* has been shown to ameliorate dextran sodium sulfate-induced colitis in rats (Dicksved *et al.*, 2012).

Prevotella is a major bacterial phylotype in the porcine colon (Leser *et al.*, 2002) (**Paper II**). It is also described as a dominant genus in one of three enterotypes identified in the human gut microbiota. The *Prevotella* enterotype is responsive to dietary fiber components (Arumugam *et al.*, 2011; Wu *et al.*,

2011; De Filippo *et al.*, 2010). In this thesis, dietary NSP-induced changes in *Prevotella* were observed. A gradual reduction in *Prevotella* spp. in colonic luminal microbiota coincided with increasing chicory pectin inclusion. This may be explained by dietary NSP content variations. The cereal-based control diet contained the highest arabinoxylan content, with arabinoxylans from wheat and barley, whereas in the chicory forage diets cereal arabinoxylans were replaced by chicory pectin at two levels. Xylan from cereal grain has been suggested to be the preferred substrate for *Prevotella* growth, as shown in human colonic microbiota and rumen (Dodd *et al.*, 2011; Flint & Bayer, 2008). *Prevotella* species are able to degrade xylan by producing xylanases, mannanases, β-glucanase, *etc.* in the large intestine. By contrast, in the chicory root diets arabinoxylan was replaced with inulin-type fructan alone or together with pectin. These diets, although they contained less arabinoxylan than the control, resulted in an increased *Prevotella* abundance compared with chicory forage diets. In a study of IBD in a rat model, dietary inulin increased the number of *Bacteroides-Prevotella-Porphyromonas* group in cecal microbiota and reduced chronic inflammation, indicating that bacteria belonging to this group can utilize fructan *in vivo* (Koleva *et al.*, 2012). It was confirmed by an *in vitro* study, which showed that a specific strain of *Prevotella* was promoted by inulin addition, and possibly by encoding enzymes such as fructanase and fructokinase (Fuse *et al.*, 2013). Interestingly, a similar NSP response was observed for the relative abundance of ileal luminal *Prevotella*, confirming that dietary fiber degradation had already taken place in the distal small intestine of pigs. Moreover, this bacteria group was abundant (relative abundance up to 20%) in the ileum in our pig study (**Paper II**). In contrast, ileal *Prevotella* is reported to be almost absent in pigs fed different types of commercial diets (Leser *et al.*, 2002). It appears that a given dietary NSP fraction can significantly interact with specific commensal bacteria in the gut.

Given its important role in the maintenance of intestinal homeostasis and butyrate production (Pryde *et al.*, 2002; Barcenilla *et al.*, 2000), we grouped TRFs correlated with *Firmicutes* that belong to clostridial cluster IV and XIVa, and anticipated diet responses (**Paper II**). There was a marked increase in the relative abundance of these butyrate-producing bacteria, including mucosa-associated members (data not shown), with increasing inclusion of chicory forage pectin in the diet. The shifts in this functional bacterial group were correlated with acetic acid production and further related to butyric acid concentration in the colon. The stimulation of mucosa-associated butyrate producers by chicory pectin may play an essential role in sustaining colonocyte integrity. In order to survive, butyrate-producing bacteria are capable of utilizing acetate, the major SCFA end products of pectin fermentation

(Drochner *et al.*, 2004). Some of the members can also utilize lactate, while some are net acetate consumers, therefore establishing their central role in bacteria cross-feeding in the colon (De Vuyst & Leroy, 2011; Barcenilla *et al.*, 2000). In this thesis, the colonic butyrate-producing bacteria appeared to prefer chicory forage pectin to inulin-type fructan. However, previous *in vitro* cross-feeding studies have shown that inulin-type fructan is the favored substrate for butyrate-producing bacteria growth (Scott *et al.*, 2011; Falony *et al.*, 2009). One reason for this discrepancy could be that *in vitro*, it was a direct inulin addition to the bacteria medium. In pigs, a large proportion of dietary inulin is degraded in the distal ileum (up to 80%) (Patterson *et al.*, 2010), which leaves limited substrate for colonic bacteria stimulation. Nevertheless, our results suggest that butyrate-producing bacteria were responsive to chicory forage pectin through bacteria cross-feeding.

The relative production rates of SCFA provide a potentially important link between diet and colonic microbiota. This thesis found that another butyrate producer, luminal *M. elsdenii*, was associated with dietary inulin. Similarly, a fructan-rich diet increased the relative abundance of colonic *M. elsdenii* in parallel with increased numbers of bifidobacteria in pigs. *M. elsdenii* is not a primary fructan degrader, but this bacterial species is able to utilize lactate produced by other bacteria and convert it to butyrate, propionate, and/or valeric acid (Mølbak *et al.*, 2007; Hashizume *et al.*, 2003). Accordingly, a strong positive correlation was identified between the relative abundance of *M. elsdenii* and the n-valeric acid concentration in the colon, whereas the n-valeric acid concentration was highest in diets containing inulin (**Paper II**).

8.3 Localization of HSP expression in young pigs

The physiological expression of heat shock proteins plays a pivotal role in the maintenance of host homeostasis and cytoprotection. HSP27 is an important member of the small heat shock protein family, whereas the HSP70 family comprises the inducible HSP72 and the constitutive HSC73. They can be induced by a wide range of stimuli (Henderson & Pockley, 2010). It has been suggested that in IECs, microbiota is one of the major determinants of HSP expression (Kojima *et al.*, 2003). Indeed, the IECs that are covered by mucus, constitute a barrier that is constantly exposed to commensal and transient bacteria and their metabolites in the gut. Their normal functioning is essential in preventing antigen passage to the portal circulation while maintaining the uptake of nutrients (Maynard *et al.*, 2012). Given that limited information is available on the role of HSPs in the normal porcine gut (David *et al.*, 2002),

this thesis first aimed to locate HSP27 and HSP72 along the GI tract of young pigs.

Under normal conditions, HSP27 and HSP72 expression was observed along the GI tract, where the ileal mucosa exerted stronger expression of these two proteins than the proximal colonic mucosa (**Paper III**). In addition, HSP27 was found to be expressed in one randomly selected jejunum tissue sample (Figure 5A) and porcine jejunum epithelial cells at a high basal level (**Paper IV)**. This is in agreement with findings in one study in young pigs that HSP27 and HSP70 are expressed in the proximal small intestine, including duodenum and jejunum (David *et al.*, 2002). In contrast, several studies in humans and rodents have suggested that HSPs are almost undetectable in the normal proximal small intestine. Moreover, the strong expression of these chaperones in the proximal colonic mucosa was suggested to be dependent on the high bacterial richness and diversity (Hu *et al.*, 2010; Arvans *et al.*, 2005; Ren *et al.*, 2001). Regarding relationships between bacteria and IECs, the colon shows more constringency by developing a firm inner mucus layer to keep bacteria at a distance, whereas the mucus stratification is less organized in the small intestine to allow nutrient absorption (Dicksved *et al.*, 2012; Johansson *et al.*, 2011). We found a significantly higher bacterial diversity in the luminal colonic microbiota compared with the ileal community (**Paper II**). However, the diversity was not different between ileal and colonic MAM in closer contact with IECs (**Paper III**). The discrepancy in HSP expression could also be due to animal species differences. As discussed previously in this thesis, dietary fiber fermentation takes place earlier along the GI tract in pigs than in humans and rodents (Drochner *et al.*, 2004). It coincides with abundant bacteria inhabitation and enriched immune cells in the underlying PPs (Bailey, 2009; Leser *et al.*, 2002), which may thus have contributed to stronger ileal HSP expression in our young pigs. Nevertheless, the results presented in this thesis indicate that the physiological expression of HSP27 and HSP72 is region-specific.

Furthermore, we found a gradient in expression of both HSP27 and HSP72 along the villus/crypt axis, with the highest expression in the surface epithelium, lower in crypt cells, and limited in the lamina propria (**Paper III**). This is in line with earlier studies showing a similar pattern in the colon (Hu *et al.*, 2010; Arvans *et al.*, 2005; Kojima *et al.*, 2003). In addition, those authors studied antibiotic-treated or germ free mice and found HSPs abolished in the gut. Overall, it is suggested that the axial gradient HSP expression is very likely dependent on dietary components, microbes, and their metabolites to which the mucosa surface is exposed. In **Paper III**, a strong HSP27 expression in ileal PPs *in vivo* was observed for the first time, whereas HSP72 was also

detected, but with a considerably weaker staining. Moreover, the expression of HSP27 was positively correlated with ileal IEC HSP27 expression. It is unclear how the HSP response on the mucosal surface reaches the underlying PPs. One possible mechanism is that microbes are sensed by toll-like receptors (TLR, essential bacteria-recognizing receptors in the innate immunity system) which activate interactions between IECs and immune cells in the lamina propria, further directing immunological responses such as luminal bacteria uptake. This reaction could extend to lymphoid follicles in PPs (Wells *et al.*, 2011). Another example of the interaction of PPs with intestinal luminal compounds is that segmented filamentous bacteria have been shown to be capably of directly adhering to ileal PPs and stimulating T cell response (Gaboriau-Routhiau *et al.*, 2009). The differential results in expression pattern between HSP27 and HSP72 suggest that the expression of these proteins is cell type-specific.

The revised recognition of HSPs is that they are not strictly intracellular proteins but can be excreted extracellular (Henderson & Pockley, 2010). Studies have discovered HSP10, HSP27, and HSP70 in the circulation of healthy individuals (Shamaei-Tousi *et al.*, 2007; Fanelli *et al.*, 1998; Pockley *et al.*, 1998), indicating the possibility that HSP act as intercellular signals. In this thesis, blood samples from healthy pigs were also analyzed and HSP27 and HSP72 were detected in 8 out of a total of 30 pigs, although at very low concentrations (Figure 6). As systemic immune responses in healthy subjects are expected to be subtle, the possibility cannot be ruled out that HSP27 and HSP72 play an immunomodulatory role in the circulation of pigs. Confirmation and elucidation are necessary in future studies.

8.3.1 HSP expression correlates with specific bacteria

Interactions between gut microbes and the mucosal immune components greatly affect host health and disease (Clemente *et al.*, 2012). In **Papers III** and **IV**, several interplays were identified, indicating that specific bacteria interact with HSP27 and HSP72 differently.

The correlation between ileal HSP72 expression and the relative abundance of luminal LAB is one important example presented in the thesis. Moreover, the relationship was positive between HSP72 expression and the population of luminal *L. johnsonii*. In both cases, the correlation was limited to luminal lactobacilli rather than MAM. An increasing body of evidence demonstrates that probiotic lactobacilli, such as *Lactobacillus brevis*, LGG, and VSL3# (probiotic product comprising several strains of bacteria, including four lactobacilli) enhance intestinal barrier function and IEC protection by inducing HSP27 and HSP72 expression (Segawa *et al.*, 2011; Tao *et al.*, 2006; Petrof *et al.*, 2004). The positive correlation in **Paper III** was confirmed in **Paper IV**,

with significantly higher HSP72 expression in *L. reuteri* supernatant-treated IPEC-J2 cells compared with the untreated group. However, HSP72 was only detectable in the other two lactobacilli treatments, which included LGG and a porcine isolate *L. johnsonii* from our pigs. *In vivo*, *L. johnsonii* was dominant in the ileum of pigs, consequently influencing the mucosa to a greater extent (**Paper II)**, whereas *in vitro*, the lactobacilli treatments were at the same MOI. There is evidence that HSP72 induction is more stimuli-specific and the consequent expression plays a major role in cytoprotection against intestinal epithelium injury compared with HSP25/27, whereas this small HSP is associated with cytoskeleton stabilization (Liedel *et al.*, 2011; Hu *et al.*, 2009; Musch *et al.*, 1999). It was confirmed in this thesis that expression of HSP27 was not correlated with HSP72 in any gut segment (**Paper III**). In **Paper IV**, strong induction of HSP27 was observed with *L. johnsonii*, *L. reuteri*, and *L. reuteri* supernatant treatments of IPEC-J2 cells. The protective effect of lactobacilli was further investigated using ETEC challenge. The results showed that ETEC is able to adhere to the epithelium and cause significant loss of cell-cell contact, reduce IPEC-J2 cell viability and integrity, and disrupt TJ protein expression. In contrast, *L. reuteri* treatment substantially counteracted these detrimental effects and preserved the IEC barrier function, while *L. johnsonii* and LGG also achieved barrier function protection, partly by directly interacting with ETEC. *L. reuteri* is a well-studied probiotic and its supernatant carries the bioactive compounds produced from an overnight culture, possibly containing antimicrobial reuterin (Walter *et al.*, 2011). Interestingly, it has been shown that mother's milk-induced HSP expression is co-localized with ZO-1 expression in the small intestine of rat pups, confirming cytoprotection of the immature gut (Liedel *et al.*, 2011). We speculate that there is an association between TJ protein ZO-1 enhancement and HSP27 induction. The possible mechanism is discussed in section 9.1.

Furthermore, we found a positive correlation between ileal HSP27 expression and mucosa-associated *M. elsdenii* (**Paper III)**. This bacterial species is suggested to effectively convert lactate from fructooligosaccharide or inulin fermentation, to butyrate, that will contribute to a more beneficial SCFA profile (Mølbak *et al.*, 2007; Hashizume *et al.*, 2003). *M. elsdenii* was low in abundance in ileal MAM. However, the relative abundance of ileal mucosa-associated *M. elsdenii* was increased by the diet containing 160 g/kg chicory forage (P=0.03, data not shown), in contrast with the inulin-promoted growth of luminal *M. elsdenii* (**Paper II**). This indicates that chicory pectin may play a specific role in intestinal mucosa homeostasis. This was reinforced by the finding of a positive correlation between mucosa-associated *M. elsdenii* and daily uronic acid intake, further related to ileal HSP27 expression (**Paper III)**.

Similarly, ileal HSP25 was specifically induced in rats fed a pectin diet in a previous study, an effect that was attributed to the fermentation end-product, butyrate (Ren *et al.*, 2001).

In the colon, HSP72 expression was positively related to the abundance of both luminal and mucosa-associated *Roseburia inulinvorans*. Moreover, the mucosa-associated *F. prausnitzii* was also positively correlated with colonic HSP72 expression (**Paper III**). Indigenous clostridia species have a confirmed immunomodulatory effect in the colon of mice (Atarashi *et al.*, 2011), whereas *F. prausnitzii* has been demonstrated to exhibit an anti-inflammatory effect in IBD patients (Sokol *et al.*, 2008). Therefore, it is plausible that induction of HSP72 may be one immunomodulatory function of bacteria belonging to clostridial cluster IV and XIVa, such as *R. inulinvorans* and *F. prausnitzii*.

Our results so far demonstrated that specific commensal microbes can have differing influences on the intestinal immune components, *i.e.* cytoprotective HSP27 and HSP72. Furthermore, specific lactobacilli may achieve effective resistance to pathogens depending on the unique host immune response.

9 Conclusions

The overall aim of this thesis was to provide an integrated understanding of interactions between diet, gut microbiota, and host immune components using a specific dietary fiber source, chicory (*Cichorium intybus* L).

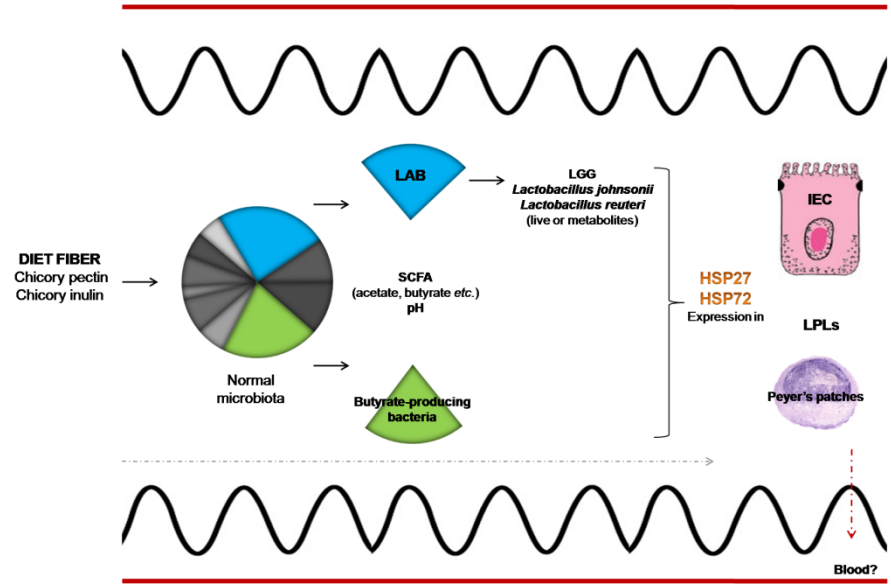

Figure 7. A possible story of this thesis: in brief, we studied effects of dietary chicory inclusion on its utilization, particularly in the intestine, *i.e.* changes in gut microbiota and intestinal microenvironment. We identified diet-responsive, dominant bacteria groups such as lactic acid bacteria (LAB) and butyrate-producing bacteria, concomitant with short chain fatty acid (SCFA) production changes. We demonstrated that heat shock protein (HSP) 27 and 72, which exhibited region-specific and cell type specific expression pattern in healthy pigs (they were located in intestinal epithelial cells, IECs; lamina propria lymphocytes, LPLs; Peyer's patches and may exist in blood), may interact with microbes. Specific lactobacilli were further isolated from LAB group in our pigs, and manifested their interaction with HSPs *in vitro*.

As illustrated in Figure 7, animals were fed various inclusion levels of chicory forage and/or root meal (inulin) in comparison with a cereal-based control diet and the intestinal microbiota composition and microenvironment were found to be altered. Furthermore, major bacteria phylotypes were identified and correlated with HSP expression in different regions of the GI tract, with distinctive patterns. These correlations may mediate IEC protection against pathogen challenge. In summary, this thesis demonstrated that:

- In chickens, dietary fiber source, inclusion level, and chicory harvest time influenced nutrient utilization. The total tract apparent digestibility of OM, DM, and NSP decreased with increasing fiber inclusion level. The diet with chicory forage inclusion had higher uronic acid digestibility than that with chicory root inclusion. Chicory forage from September harvest contained higher CP and uronic acid content than June harvest, which coincided with better uronic acid utilization and growth performance.

- Diets with inclusion of 60 g/kg chicory forage for broilers and 80 g and 160 g/kg for young pigs retained good performance with intestinal morphology alterations. Diets with inclusion of 120 g/kg chicory resulted in higher feed intake and feed conversion ratio in broilers compared with the breed standard, especially during the early rearing period, but did not affect final body weight. These findings suggest that chicory has potential to be as a palatable fiber source for broiler chickens and young pigs.

- Various NSP fractions in diets induced different changes in gut microbial composition in young pigs. LAB was dominant in the small intestine and was associated with diets containing 160 g/kg chicory forage and chicory forage and root mixture. In the large intestine, dominant bacteria groups identified were bacteria belonging to clostridial cluster IV and XIVa, and *Prevotella*, which was linked with chicory pectin and cereal arabinoxylan, respectively. Within diet type, these changes followed a similar pattern in the small and large intestine.

- The physiological expression of intestinal HSP27 and HSP72 showed regional and cell type-specific features. Correlations between luminal LAB and HSP72, HSP27 and daily uronic acid intake in the small intestine and between HSP72 and clostridia species in the large intestine represent important host-microbe interplays. The interaction between lactobacilli and HSPs was found to be associated with tight junction protein distribution and mediation of cytoprotection by IECs under ETEC challenge.

9.1 Perspectives

One of the main objectives in this thesis was to locate HSP27 and HSP72 in the porcine GI tract and to identify their roles in the maintenance of intestinal homeostasis in association with microbiota changes. Several studies have discussed different modes of HSP action, revealing their cytoprotective and anti-inflammatory properties (Borges *et al.*, 2012; Henderson & Pockley, 2010; Malago *et al.*, 2002). Based on the results and speculations in this thesis and those in previous studies, two possible scenarios can be formulated:

In scenario one, intracellular HSP27 functions as a TJ stabilizer (Figure 8A). It has been well demonstrated that HSP27 modulates cytoskeleton dynamics by directly binding to F-actin filament (the two proteins share a common structural motif). The phosphorylated HSP27 oligomers would interact with F-actin and prevent the filaments from breakage, while the non-phosphorylated monomers would coat the actin filament and then become involved in microfilament assembly, therefore achieving cytoskeleton stabilization (Mounier & Arrigo, 2002). Likewise, F-actin is suggested to bind directly to the C-terminus of the TJ protein ZO-1. In a study using a Madin-Darby canine kidney cell model, depletion of ZO-1 resulted in actin disruption that coincided with increased paracellular permeability, indicating an impaired barrier (Van Itallie *et al.*, 2009). ZO-1 is a universal component of the TJ complex and acts as an adaptor with three domains that interact with the claudins, other ZO proteins, and junctional adhesion molecules, respectively. Therefore, they regulate the whole TJ complex dynamics (Ulluwishewa *et al.*, 2011). Probiotic lactobacilli have been shown to preserve intestinal barrier integrity, as discussed earlier in the thesis. The bacteria or molecular signals they produce can be captured by TLR2, which is constitutively expressed in IECs. These further induce protein kinase C activation and result in apical ZO-1 tightening, concomitant with increased TEER values, and indicating barrier function augmentation (Cario *et al.*, 2004). Increasing expression of both ZO-1 and HSP27 in IECs enhances the intestinal integrity. However, a link is missing between ZO-1 and HSP27, *i.e.* which protein is the main regulator in this pipeline.

In scenario two, extracellular HSP72 functions as an anti-inflammatory signal, based on the hypothesis of Borges *et al*. (2012). The HSPs may selectively influence extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and other mitogen-activated protein kinase pathways under various conditions in order to modulate intestinal immune responses (Malago *et al.*, 2002). In simplified terms (Figure 8B), the secreted HSP72 may bind to a lectin-like receptor in different types of cells, such as dendritic cells, signaling through TLR2. This will trigger Myeloid differentiation primary

response gene 88 (MyD88), induce ERK phosphorylation, and elicit IL-10 production. In addition, HSP27 and HSP72 have been shown to prevent IL-10 down-regulation by modulating the JNK pathway (Malago *et al.*, 2002). Thus, we are on the verge of understanding the molecular mechanisms of HSP regulation in host homeostasis. However, it should be noted that each line of HSP networking is multifactorial at multiple levels, thus needing detailed in-depth investigations.

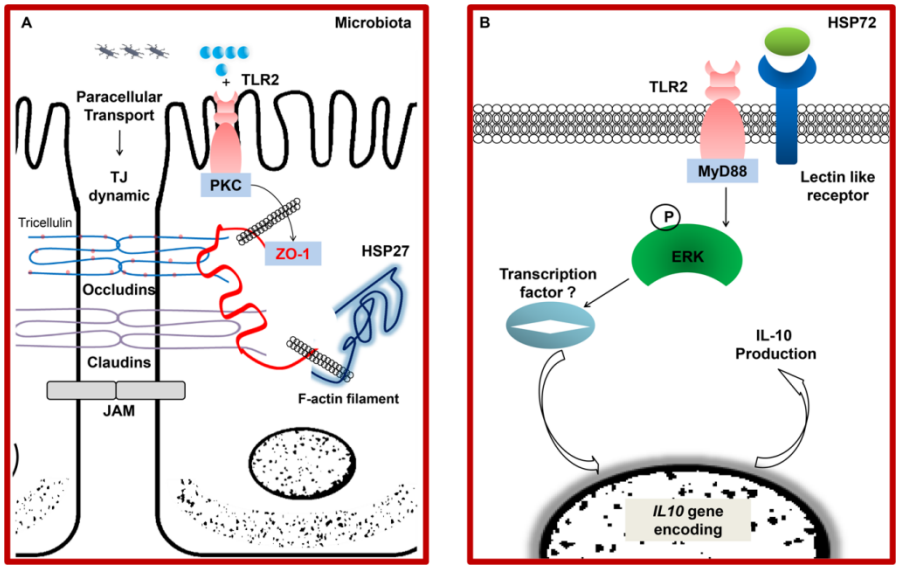

Figure 8. Schematic diagram showing proposed mechanisms of heat shock protein (HSP) actions, emphasizing the intra- and extracellular roles they may play. (A) The tight junction (TJ) is composed of multiple interacting proteins including occludins, claudins, and junctional adhesion molecules (JAM), whereas ZO-1 can bind to F-actin to stabilize cytoskeleton. Meanwhile, intracellular HSP27 can also modulate F-actin, which may further regulate TJ through this pipeline, achieving TJ stabilization. This reaction can be initiated by toll-like receptor 2 (TLR2) in IECs sensing microbial signals, followed by protein kinase C (PKC) induction. (B) Extracellular HSP72 may be sensed by an endocytic receptor *i.e.* lectin-like receptor and initiates a cascade reaction involving TLR2 and myeloid differentiation primary response gene 88 (MyD88) activation and extracellular signal-regulated kinases (ERK) phosphorylation *etc.*, eventually lead to IL-10 production, achieving anti-inflammatory effect (adopted from Borges *et al.*, 2012).

It is important that future research takes a broader consideration of health-promoting diet manipulation in human and animals. Integrating the understanding of interactions between diet, gut microbiota, and immune system will help us to gain a holistic view of health and to transform different data into applicable knowledge.

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