SEVENTH FRAMEWORK PROGRAMME
THEME 2
Food, Agriculture and Fisheries, and Biotechnology

Project acronym: **INTERPLAY**
Project full title: **Interplay of microbiota and gut function in the developing pig – Innovative avenues towards sustainable animal production**
Grant agreement no.: **227549**

Final Report for the period from September 2009 to August 2013

List of Beneficiaries

Beneficiary Number	Beneficiary name	Beneficiary short name	Country	Date enter project	Date exit project
1(coordinator)	Wageningen Universiteit	WU	The Netherlands	Month 1	Month 48
2	University of Bristol	UNIVBRIS	UK	Month 1	Month 48
3	Institut National de la Recherche Agronomique	INRA	France	Month 1	Month 48
4	Otto-van-Guericke Universität Magdeburg	OVGUMagDE	Germany	Month 1	Month 48
5	Istituto Nazionale Di Ricerca Per Gli Alimenti E La Nutrizone	INRAN	Italy	Month 1	Month 48
6	ASG Veehouderij B.V.	ASG Veehouderij B.V.	The Netherlands	Month 1	Month 48
7	Nanjing Agricultural University	NAU	China	Month 1	Month 48
8	Parco Tecnologico Padano s.r.l.	PTP	Italy	Month 1	Month 48
9	Institute of Microbiology of the ASCR, v. v. i.	IMIC	Czech Republic	Month 1	Month 48
10	University of Helsinki	UH	Finland	Month 1	Month 48
11	Universita' Di Bologna	UNIBO	Italy	Month 1	Month 48
12	ID Lelystad B.V.	Biomedical Research	The Netherlands	Month 1	Month 5

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1. EXECUTIVE SUMMARY

Low input farming occurs under non-SPF (specific pathogen free) conditions. The European ban on in-feed antibiotics exposes the piglets to a higher microbial pressure. The postnatal priming of piglets with a diverse microbiota may affect the development of the piglet's host-defense and gut function. By reversal, the piglets developing host-defense may affect the development of the gastro-intestinal (GI) microbiota. Moreover, this intricate interplay between gut microbiota and its host during the early phases of life is expected to also affect animal health and performance later in life.

The gut microbiome is an immensely diverse ecosystem that has co-evolved with its host. Recent research on microbe-host interactions has provided novel insights into the role of commensal microbes in several physiological processes, ranging from epithelial barrier development to immune development as well as neurological aspects. Nevertheless, we only start to understand molecular mechanisms of the host microbe cross-talk. Recent conceptual as well as technological advances have set the stage for the integrated application of a complementary set of high throughput approaches for the comprehensive profiling of GIT microbiota composition and functionality as well as the animal's intestinal function.

To this end, in a multidisciplinary consortium of 12 public and private partners from across and beyond Europe with complementary expertise in gut microbiomics, immunology, physiology, animal genomics and nutrition, INTERPLAY has used an integrated approach towards a sound understanding of the interaction of early colonization of the intestine and gut function development. This knowledge can be exploited for the identification of innovative management strategies that address host genotype as well as nutritional means to provide a framework for sustainable animal production at high food and consumer safety and improved animal health and welfare.

The strategic aim of the research carried out in the framework of the INTERPLAY project was to contribute to maintain European pig production in a worldwide leading position for the implementation of sustainable management strategies that promote optimal animal health and welfare, alongside food and consumer safety. INTERPLAY has generated a knowledge base needed to support the Common Agricultural Policy objectives aiming to provide sustainable development of agriculture.

Dissemination is considered to be an integral task of the project and relevant stakeholders were involved from the very beginning and in public dialogue. The INTERPLAY project provided at the European level a bottom-up approach to help the process of consensus-forming around the development and use of new scientific and technological developments in support of sustainable farming systems. To this end, a public website has been set up allowing easy access to information concerning project objectives and partners from outside of the consortium. Furthermore, a stakeholder forum facilitated and strengthen the possibilities not only for the interactive dissemination of project results into the scientific community, but also to further support one of the key objectives of INTERPLAY, namely to provide the necessary understanding for the rational design of improved management strategies towards more sustainable animal production.

In addition to that, results generated by INTERPLAY partners were disseminated through scientific and popular publications, participation in a large number of conferences, workshops and other events as well as through two international workshops.A first international scientific workshop was held at the premises of partner 7 (Nanjing Agricultural University) in order to foster the scientific dialogue with Asia. A second workshop geared more towards industrial stakeholders was organized at the end of the INTERPLAY project, as a specific session at one of the major conferences in animal production, EAAP, in August 2013.

2. SUMMARY DESCRIPTION OF THE PROJECT CONTEXT AND THE MAIN OBJECTIVES

2.1. Brief introduction to INTERPLAY

The **INTERPLAY** project aims at understanding the interaction between early microbial colonisation of the gastrointestinal tract (GIT) of pigs and the development of gut function, and its implications for health and welfare throughout life. Such knowledge is urgently needed in order to provide a sound basis for the design of innovative management strategies as alternatives to in-feed antibiotics, especially for low-input farming systems. The microbiota colonising the GIT of farm animals is an immensely diverse ecosystem that has co-evolved with its host, playing a pivotal role in animal health through its influence on nutritional, physiological and immunological status of the host. Accumulating evidence indicates the requirement for an intimate interplay between GI microbiota and the host defence mechanisms to develop and maintain intestinal homeostasis, and balanced immune function, avoiding exaggerated responses to luminal antigens while protecting from pathogens. The only recently established ban on in-feed antibiotics has resulted in the increase in microbial disease pressure on the GIT of farm animals, and especially of young piglets. Manipulation of microbial colonisation of the GIT can be a powerful tool to strengthen intestinal barrier function, robustness and immunological competence of young piglets. INTERPLAY examines this interaction under conditions in which the early colonisation can be rigorously controlled with sustainable effects on final colonisation, gut robustness and disease resistance. This is achieved by applying beyond state of the art approaches for the understanding of GIT microbiota and gastro-intestinal function in four experimental models of increasing complexity, i.e. in vitro cell culture systems, and gnotobiotic, isolator-housed and conventionally raised piglets.

To this end, the INTERPLAY project brings together complementary front-line expertise of 12 academic and industrial partners from across Europe and beyond, combining the accumulated wealth of knowledge on gastrointestinal functionality with fresh multidisciplinary approaches from gut microbiomics, immunology and physiology, and animal genomics and nutrition.

The central hypotheses that drive INTERPLAY are

1. The spatio-temporal kinetics of early colonisation of the neonatal piglet GIT by commensal and potentially pathogenic microbiota drives the dynamics of microbiota composition and activity, intestinal function and host-microbe interaction after birth and later in life
2. Sows can affect the co-development of intestinal microbiota and gut function either directly through their genotype, or indirectly through their own microbiota, which they transmit to their offspring at or immediately after birth
3. The rearing environment, including postnatal antibiotic treatment, affects the interplay of microbial colonisation and gut function development
4. Improved management strategies can be developed based on innovative pre- and probiotics towards sustainable pig production, capitalizing on animal health and welfare, as well as food and consumer safety

2.2. Scientific and technological objectives

In the context of the above, the strategic aim of INTERPLAY is to provide the necessary knowledge base to place European livestock production in a worldwide leading position for the implementation of sustainable management strategies that aim at optimal animal health and welfare, and food and consumer safety.
To reach this overall aim, the INTERPLAY consortium has addressed the following specific and measurable scientific and technological objectives.

- **Scientific objectives**: in the field of Biology the project aimed to:
  - S1. Generate a knowledgebase on the kinetics of colonisation by commensal as well as potentially pathogenic microbiota along the GIT of young pigs, and concomitant impact on GIT microbiota composition as well as gastro-intestinal function throughout life
  - S2. Provide understanding at the cellular level of host-microbe interactions that drive gut function development
  - S3. Identify the extent to which the sow influences the microbial colonisation process, and concomitantly gut function in the offspring, either directly through genotype or through the sows own microbiota
  - S4. Expand our knowledge on the short- and long term effects of antibiotic treatment early in life on microbiota composition and gut function throughout life
  - S5. Provide novel insight in the effect of the rearing environment, including the farm hygienic status, on the development of microbiota and gut function
  - S6. Categorise the impact of innovative pre- and probiotic treatments on the co-development of microbiota colonization and gut function
  - S7. Generate novel hypotheses and leads towards the rational design of management strategies for improved farm animal health and robustness, and food safety

- **Technological objectives**: in the fields of Biotechnology and Animal Production the project were to:
  - T1. Establish standardized high-throughput methodology beyond the state of the art for the understanding of microbiota-composition and gut function
  - T2. Establish a correlation database that allows identification of diagnostic microbiota- and gut function biomarkers of animal health & robustness from multidimensional datasets
  - T3. Provide knowledge as to whether breeding and/or choice of host genotype can be used as a tool to select for GIT microbiota structure and functionality associated with increased robustness
  - T4. Identify nutritional interventions, including peri- and postnatal treatment based on novel pre- and probiotics, which can be exploited towards improved farm animal health and welfare, and food safety
  - T5. Generate guidelines for the rational design of sustainable management strategies, capitalizing on animal health and food safety, and based on a sound understanding of the interplay of microbial colonization and gut function development

3. Main Scientific and Technological Results and Foregrounds achieved by the INTERPLAY Project

WP 1: HTP Toolbox & Knowledge Base for profiling microbiota and gut function

WP1 had a pivotal and project-spanning function within INTERPLAY in order to provide the necessary tools and knowledge base to perform the experiments described for the following workpackages with a specific focus on high throughput (HTP) approaches for the profiling of microbiota and gastrointestinal tract function, and providing a data warehouse for the analysis of the multivariate datasets generated by INTERPLAY in order to allow identification of potentially novel biomarkers.

Workpackage 1 Facts

Leading House: UH
Participants: UH, WU, Univbris, INRA, OVGUMagDE, INRAN, ASG, NAU, PTP, IMIC, UNIBO, BMR

Objectives:

1. Optimise and standardise innovative high throughput approaches to explore the diversity and identity of the colonising microbiota
2. Provide a comprehensive and standardised set of methods for the profiling of GIT function, covering aspects of physiology, immunology and morphology of the developing GIT, specifically focussing on innovative in vitro cell culture and ex situ perfusion and tissue explant and models, and functional genomics approaches
3. Establish and exploit a comparative genome sequence database for porcine strains of *Lactobacillus sobrius/amylovorus*
4. Establish a data warehouse for the storage and integrative analysis of multidimensional data obtained by the different approaches throughout the project

This endeavour turned out to be successful, as partners compiled and further optimized a broad range of state of the art methodologies and protocols, starting with how to adequately collect and process biological material obtained from the various in vitro, ex vivo and in vivo animal experiments carried out in subsequent workpackages, as well as a broad range of complementary approaches to measure indicators of intestinal function.

Another major aspect of this workpackage concerned the application of functional genomics approaches towards a better understanding of biology and in situ functionality of key commensals in the porcine GIT belonging to *Lactobacillus amylovorus*. To this end, the genomes of an extended set of 9 isolates have been elicudated by Partner 10, and compared to other members of the lactobacilli. Furthermore, functional characteristics of *L. amylovorus* isolates were tested in a series of experiments using in vitro cell culture based approaches.

Task 1.1. Standard Operating Procedures

During the first six months of the project, a number of essential procedures were defined for the standardized handling, conservation and processing of biological samples for downstream analyses performed by the different partners, focusing specifically on those sample types that have not been subjected to routine analyses in the past, including e.g. rectal swab samples for fecal microbiota analyses from large groups of animals (Partner 8) as well as processing of gastric samples (Partner 11). This ensured optimal comparability of all data generated throughout the project, further improving the odds for identification of novel biomarkers from multivariate analyses.

Task 1.2. Microbiota composition

Activities in the framework of task 1.2 have largely focused on the development and optimization of different high throughput approaches for the measurement of microbiota composition in intestinal samples in order to allow for evaluating microbiota dynamics along spatio-temporal gradients, as well as in response to dietary, environmental and genetic factors. To this end, P1 completed the design of an updated version of the Porcine Intestinal Tract Chip (PITChip 2.0) , a phylogenetic oligonucleotide array designed for the comprehensive profiling of pig intestinal microbiota composition based on publicly available information regarding the occurrence of bacterial phylotypes along the porcine intestinal tract. In addition, several partners have contributed by evaluating applicability of next generation sequencing technology, including pyrosequencing and Ion Torrent sequencing technology for the extreme throughput and cost-effective sequence analysis of 16S rRNA gene amplicons.

Task 1.3. Genomics-based analysis of microbiota functionality

Genomic analyses of *L. amylovorus* strains

The genome sequences of nine *Lactobacillus amylovorous* strains with genome sizes between ~1.8 to ~2 Mb were determined by pyrosequencing. These genomes were found very similar to each other (about 70% sequence identity) and the closest relatives were the genomes of *L. acidophilus* and *L. helveticus*.

The pan-genome of these nine *L. amylovorus* strains consists of 3637 genes, while the core-genome (genes present in all nine *L. amylovorus* genomes) contains 1232 genes. In comparison, the entire *Lactobacillus* pan-genome consists of 15801 genes, while the *Lactobacillus* core genome consists of 160 genes.

Preliminary comparative genomic studies of the nine *L. amylovorus* strains and mining of the genomic data are not in line with functional studies. As an example, we identified several mucus binding protein encoding genes in all of the genomes analyzed here, however, none of the strains functionally studied showed any mucus binding capability (see below). Nevertheless, this is not unexpected, as we currently don't have any data regarding the true functionality or expression of those genes. On the other hand, no genes encoding proteins with similarity to known epithelial cell adhesins were found, even though adhesive properties could be shown in the functional assays. Based on the genome properties presented here, it is very much likely that the mechanisms of putative probiotic properties in these strains are regulated by a combination of several putative probiotic traits and features. Furthermore, genomic data suggest considerable strain-to-strain variation in the probiotic trait complement.

Functional analyses of *L. amylovorus* strains

Functional studies have been performed with seven surface (S) –layer carrying strains of pig intestinal origin (DSM 16698, GRL 1112-GRL 1118) and one S-layer carrying reference strain isolated from silage (DSM 20531^T). The adherence of the strains to porcine gastric mucus (Sigma), to mucus isolated from the small intestine of a piglet and to the porcine intestinal epithelial cell line IPEC-1 have been studied using established methods optimized by P10. Further, the abilities of the strains to inhibit the adherence of pathogens causing post-weaning diarrhea in piglets have been tested using the IPEC-1 cell model.

S-layer protein (Slp*)* genes in lactobacilli are very difficult to knock out and the proteins are poorly water-soluble due to the self-assembly property of S-layers, hindering the study of the role of *Lactobacillus* Slp:s in adherence. To avoid unspecific effects associated with protein aggregation due to poor water-solubility, either beads or purified bacterial cell wall fragments (CWF) were used as carriers for the Slp:s in the adhesion experiments.

In the **bead-based approach**, magnetic beads able to bind the recombinant S-layer proteins through their hexahistidine tags were used. There was no correlation between the binding of the S-layer proteins and the corresponding *L. amylovorus* strains to IPEC-1 cells. For instance, the S-layer protein of the poorly adhering strain GRL 1118 adhered to IPEC-1 cells in a similar way as SlpA of the well-adhering strain DSM 16698. However, a role for the S-layer proteins in adherence could not be excluded, as the regular lattice-like orientation of the S-layer proteins, present on the bacterial cell but apparently not achieved on the beads, may be crucial for the interaction with epithelial cells. This prompted us to stick to the original idea of using bacterial cell walls as carriers.

In the **cell wall-based approach**, the immobilization of the recombinant S-layer proteins is based on their inherent self-assembly on surfaces. The new coating method developed was based on the fact that a small fraction of the Slp molecules remains in solution even in a water-based buffer. Briefly, the recombinant Slp:s were first solubilized (and denatured) by 5 M guanidium hydrochloride at a low protein concentration and then dialyzed against a neutral Tris-based buffer not containing salt, followed by centrifugation at a high speed to get rid of precipitated S-layer proteins. The resulting very low-concentration soluble Slp fraction was then directly incubated with the purified cell wall fragments for coating.

Based on the experiments, we conclude that none of the major S-layer proteins of the *L. amylovorus* strains alone mediated the binding of the strains to IPEC-1 cells, but the Slp-like protein SlpB of DSM 16698 might have a role. In contrast, the major S-layer proteins of some of the poorly adhering strains like DSM 20531^T, GRL 1118 and especially that of GRL 1117 had affinity for IPEC-1 cells in our carrier system. Either some components on the native bacterial cells shield the Slp:s and prevent them from interacting with IPEC-1 cells, and/or the partial coverage of the cell wall by the Slp:s in the carrier system exposes such IPEC-1 cell-interacting regions in the S-layer proteins that are not accessible when the proteins form the fully continuous layer on the bacterial cell.

Receptor studies

Receptor studies with a biotin label transfer reagent, performed with IPEC-1 cells and whole cells *L. amylovorus* DSM 16698, revealed two prominent protein bands of approximately 75 and 130 kDa. In the following tryptic digestion, mass spectrometric analysis and similarity search of the receptor proteins, the best hits for the peptides obtained were swine proteins NP 998931 (Hsp70, 70 kDa heat shock protein) and XP 001929500 (Exportin-5), respectively. Hsp70 has been reported to be a moonlighting protein present on the eukaryotic cell surface in addition to its originally described cytoplasmic location (Multhoff & Hightower 2011, Cell Stress Chaperones 16:251-255), and Exportin-5 is a protein binding RNA and trafficking between nucleus and cytoplasm (Leisegang et al 2012, Biol Chem 393:599-604). However, the interactions observed were found not to be specific for any particular bacterial cell wall

component. As the adherence of plain CWF to IPEC-1 cells was negligible, and SlpA did not confer any significant binding to cell wall fragments, this strongly suggests that the interactions detected are irrelevantly weak in terms of bacterial binding, and merely indicate a very high sensitivity of the method and reflect the high propensity of chaperons and other binding proteins to interact with diverse targets. We conclude that the true IPEC-1 cells receptor(s) of the bacterial interacting component(s) (SlpB and/or other) are most probably non-proteinaceous and/or of very high molecular weight and not detectable by the method used.

Task 1.4. Technology Base for HTP approaches to understand GIT function

A broad range of different approaches are used by a number of partners within the INTERPLAY consortium to address gastrointestinal tract function from a host perspective. These methods have been extensively used in subsequent workpackages during the third reporting period, where specific sets of approaches are used to for example assess development and status of immune function and intestinal physiology. Approaches include, but are not limited to, in vitro cell culture, ex vivo explant models, Ussing Chamber experiments, and the Small Intestinal Segment Perfusion test. This is complemented by a broad range of approaches to measure host response by measuring gene expression (porcine microarrays and quantitative RT-PCR) and protein production in the different host compartments.

Task 1.5. Data Warehouse. Relational database (DB) for integration of results & identification of biomarkers

Partner 1 has developed a relational database system for the handling and analysis of microbial composition data based on MySQL, an OpenSource relational database management system. Furthermore, as an additional component of the INTERPLAY data warehouse, files could be stored on the INTERPLAY intranet.Throughout the runtime of the project and beyond, the database and data repositories, as well as the mentioned multivariate analyses tools, have been used extensively for the analysis of data collected in the framework especially of WP5, WP6, WP7 and WP8 that comprised the main animal experiments.

WP 2: Spatio-temporal kinetics of early microbial colonization and it's impact on the development of immuno-competence

WORKPACKAGE 2 FACTS

LEADING HOUSE: UNIVBRIS
PARTICIPANTS: WU, Univbris, IMIC, UNIBO

OBJECTIVES:

1. To determine the effect of early microbial colonisation upon gut immune development and function.
2. To determine the effect of colonisation with specific bacteria upon the development of innate and acquired immunity.
3. To investigate the cellular and molecular mechanisms by which specific bacteria modulate the development of immunity in young piglets.
4. To determine the effect of age of colonisation upon immune development and function around the time of weaning.
5. To determine the effect of neonatal colonisation upon intestinal microbiota throughout life.

The "ideal" Gut Mucosal Immune System is adapted to respond to complex array of antigens (diet v pathogen v commensal). The piglets gut Immune System is immature during the first few weeks of life, and experiments performed as part of this work package were carried out to determine the effects of rearing environment and gut microbial colonisation upon the development of defined mucosal immune cell populations and there functional activity.

The key findings arising from this work package are:

- Rearing environment can influence gut microbiota and the maturation of the mucosal immune system.
- Gut mucosal immune cells mature in a programmed sequence (antigen presenting cells, CD4 T cells, CD 8 T cells) and the stage of maturation impacts upon the response to mucosally presented antigens.
- Intestinal vascular endothelial cells are a primary target for the effects of early-life rearing environment. For example exposure to a novel microbiota during the first 24 hours of life determines immune phenotype for at least the first 8 weeks of life.
- Rearing environment can alter rate or maturation of different antigen presenting cell and effector cell populations in the gut lamina propria.
- Gut microbiota can be manipulated by early exposure to antibiotics and the response to dietary antigens.

Task 2.1. Effect of early microbial colonisation upon the ability to develop oral tolerance to novel dietary antigens and respond to challenge with pathogens

Two studies were carried out to investigate the effect of early microbial colonistion on the development of oral tolerance. In the first set of studies groups of pregnant sows were dosed daily with oral antibiotics (amoxicillin) from 10 days before the expected date of farrowing through to 21 days post farrowing. Piglets remained with their sow until weaning at 28 days. Control piglets were born to sows not treated with antibiotics. At 28 days, both groups of piglets were weaned onto a commercial weaner diet containing barley (45.3%) and soybean meal (17.5%). Piglets were bled at 14 days, the day of weaning and 14 days post-weaning (14, 28, and 42 days respectively). Amoxicillin treatment of sows altered the gut microbiota of both sows and their piglets. Although amoxicillin treatment had no effect on total serum IgG1, it reduced levels of maternally derived IgG2 in piglet serum for up to 42 days. Following weaning there was a significant reduction in the IgG1 anti-soya response in the antibiotic treated group but not in the non-antibiotic treated controls. These results provide further evidence in support of the hypothesis that gut microflora plays a critical role in the development of mucosal responses to dietary antigens.

In the second set of experiments three sows from an indoor commercial farm were artificially inseminated and at 24h post-partum, two piglets from each litter were removed to the isolator unit and the rest of the litter were left on the sow. The piglets in the SPF unit received bovine-based sow replacer formula milk. At four weeks, the six isolator piglets and two from each experimental sow (six in total) were bled as before and then mixed into two groups, each containing three isolator-reared and three sow-reared animals. Both groups were then weaned onto a soya-based diet. Animals were bled again at five weeks and six weeks.

The results showed that the increases in both serum IgG1 and IgG2 anti-soya antibody levels were significantly greater in the isolator reared pigs when compared with littermates that remained on the farm for the first 4 weeks of life ($p=0.0016$ IgG1: $p=0.01$ IgG2). These results would suggest that the early rearing environment significantly impacts upon the piglets' ability to respond to antigens in the post-weaning diet. To address the effect of microbial colonisation on the response to potential pathogens a number of experimental approaches were adopted. In the first "Conventionally" reared outbred sows on a commercial indoor farm were artificially inseminated using semen from a single boar and at 24h post-farrow, two piglets from each litter were removed to individual isolator units within an SPF facility and split into two groups. Both received bovine-based commercial porcine milk replacer and one group started an antibiotic regime consisting of twice daily pulse doses of Baytril® (Bayer Healthcare, Uxbridge, UK) and Amoxinsol 50 (Vetoquinol UK Ltd, Buckingham, UK). The remaining piglets from the litters were left to suckle the sow. At 5 and 28 days old, one piglet from each group was removed and killed and tissues removed. At 29d, the remaining piglets in the isolator and on the sow were given access to creep feed *ad libitum*. The rest of the piglets in the experiment were killed at 56d. This process was carried out concurrently on an outdoor extensive farm located nearby and three replicates were completed in all, giving a total of 108 piglets (18 in each treatment group, six per time point). In a second study, "Germ-free" piglets were maintained in sterile bubble isolators; to determine the effect of colonisation the defined "microbiological cocktail" (simple flora) was administered twice, at least 5 days after derivation. The number of each strain in doses of the new microbiota administered, as quantified by flow cytometry, were: *L.amylovorus* DSM 16698T: 6.1–10.1 $\times 10^6$ bacteria/ml, *C. glycolicum*: 5.6–8.6$\times 10^6$ bacteria/ml and *Parabacteroides* sp.:2.9–4.5$\times 10^6$ bacteria/ml. Control piglets remained sterile.

In these studies we showed that housing environment impacted upon host gene expression, suggesting significant gut-specific gene responses are also related to early life environment. Significantly, indoor housed piglets displayed increased expression of Type 1 interferon genes, Major Histocompatibility Complex (MHC) class 1 and several chemokines. Products of these genes are directly implicated in the killing of virus infected and we therefore addressed the effect of microbial colonistion (as determined by rearing environment) on the development of gut mucosal cytotoxic T Cells.Critically the differences in gene expression were also translated into protein expression, a significant increase in MHC1 staining being detected in the gut tissue of isolator reared compared to outdoor farm reared piglets. The increased expression of MHC1 bearing cells was also reflected by an increase in the numbers of CD8 T lymphocytes in the epithelium of isolator reared piglets. Taken together these results may indicate a skewing of mucosal immune development toward MHC1 restricted CD8 T cell responses.

In order to further understand the role of differing microbiota on development of the immune system, we assessed the effects of intestinal colonisation with three distinct microbiota on gnotobiotic piglets which were caesarian-derived in a sterile surgical isolator unit and maintained in sterile housing. Each group, consisting of three piglets from two separate litters in order to control for litter effect, was orally administered the 'Bristol Microbiota' (BM), *E. Coli* Nissle (ECN) or a combination of the two (BM+ECN) at birth. We were able to demonstrate that colonisation of the intestinal tract with BM+ECN promoted greater involvement of endothelial cells in immune interactions than colonisation with either BM or ECN alone. This is an important finding as this immune interaction involving endothelial cells has been previously demonstrated to occur in neonates but not in adults.

Task 2.2. Effect of colonisation with specific microorganisms upon the development of innate and acquired immunity

The effects of early colonisation have been studied both in gnotobiotic and conventional pigs housed under different rearing environments.

Effect of colonisation on mucosal immune cell architecture: Using quantitative fluorescence immunohistology we quantitated the expression of CD14, CD16, MHCII and MIL11 in in the intestinal lamina propria. Data was subjected to Principal Component Analysis (PCA) with 16 combinations of proportional, cross-correlated areas of staining for the four markers. The PCA identified five orthogonal variables, explaining 84% of the variance. These represented: 1=CD14 (LPS receptor), 2=MIL11+MHCII (endothelial cell presentation), 3=CD16+MIL11+MHCII, 4=MIL11+CD16 (macrophage presentation). The analysis showed that the derived factors distinguish the effects of very early environment (i.e. born on an indoor or outdoor farm) factor 3 (CD16, MIL11, MHCII) and later rearing (kept on the sow, transferred to an SPF isolator of transferred to an SPF isolator and treated with antibiotics), factors 2 & 4. Together these results highlight the importance of the vascular endothelium as a primary target for the effects of early-life environment.

Effect on rearing environment on "regulatory T cells" (T regs): FoxP3 is a transcription factor which through its expression on regulatory T cells plays an important role in the control of allergic and autoimmune responses. In earlier studies we hypothesised that a failure to regulate allergic responses to novel dietary proteins in the diet may play an important role in the aetiology of post-weaning diarrhoea. We therefore went on to investigate if rearing environment played a role in the expression of the FoxP3 in the lamina propria of young piglets. At 28 days after birth, piglets that were transferred to an isolator from the indoor farm, but not the outdoor farm had significantly fewer CD25(+)Tregs/mm^2 in comparison to their siblings that stayed with their mothers on the farm. Treatment with antibiotics did not reduce this number any further. This was also true when piglets are treated with antibiotics. However, outdoor isolator piglets have a significantly higher ratio of CD25(+/-)Tregs/mm^2 in comparison to isolator piglets of indoor origin, but there is no significant difference between indoor/outdoor piglets that stayed on the farm or were treated with antibiotics. On the other hand, outdoor farm piglets have a significantly lower ratio of CD25(+)Tregs/mm^2 in comparison to indoor farm piglets but there is no significant difference between indoor/outdoor origin piglets that were transferred to an isolator or treated with antibiotics. The ratio of CD25(-)Tregs/mm^2 in outdoor farm and isolator piglets is higher in comparison to their indoor siblings. However there is no significant difference between indoor isolator piglets treated with antibiotics. The biggest effects of farm-of-origin and rearing environment were apparent at 28 days old and were less obvious by 56 days old.

Isolator-reared piglets spent only one day after birth on their indoor or outdoor farm of origin and then were in reared the same environmental conditions. Therefore the significant differences we find between indoor outdoor isolator piglets suggest that this first day after birth is very important and can have a sustained effect even after 28 days. However, sows have spent their whole life, and most importantly, their whole gestation period in that environment. Although there is no maternal antibody transfer through the placenta in pigs, the different environment, and most likely different microbiota, could have affected antibodies in the colostrum or even its nutritional composition. This brings us to an important difference between piglets that stayed on the farm and the ones that were transferred to an isolator. Farm piglets were left to suckle from their mother, whereas isolator piglets were fed on formula milk from 24 hours after birth. We suggest that our observations could be explained by a combination of both microbiota and feeding differences, and an interaction between the two.

Studies in gnotobiotic piglets: Hysterectomy-derived colostrum piglets were i) kept as germ-free (GF) piglets, ii) associated with *Lactobacillus amylovorus*, and iii) associated with *E. coli* strain Nissle 1917. The piglets were i.m. treated by iron-dextran complex and vitamin A on the day of hysterectomy and later also by complex of vitamins B. The bacteria-associated piglets were perorally colonized by 10^8 CFU of corresponding bacterial strain in milk diet four hours after hysterectomy. The same doses of CFU were used for re-association of the piglets on days 2^{nd} and 3^{rd}. The piglets were bred till three weeks or less to complete required groups.

The piglets were euthanized by a cardiac puncture under isoflurane anesthesia. Intestinal contents, scrapings and tissues from different parts of the gastrointestinal tract (stomach, duodenum, proximal jejunum, distal jejunum, caecum and colon) and spleen were prepared from gnotobiotic piglets.

Regarding enzyme activities it was found that alkaline phosphatase (IAP) total activity tended to decrease (duodenum) or decreased (ileum) with increasing age (PND21, vs. PND7). Regarding peptidases, aminopeptidase N (APN) activity decreased in the jejunum and ileum, while dipeptidyl-peptidase IV (DPPIV) activity tended to increase in the ileum of aged GF pigs. Contrasting with this, sucrase activity was strongly increased with age in the jejunum and ileum of GF pigs. Piglets (from a single litter) colonized with *L. amylovorus* and then slaughtered at PND7 displayed lower ileal IAP, jejunal DPPIV, jejunal and ileal APN and duodenal sucrase, but higher ileal sucrase activities. Piglets (from three litters) colonized with the Bristol mix and then slaughtered at PND21 displayed higher jejunal APN and duodenal sucrase activities compared to age-matched GF piglets (from a single litter). No differences between treatments were seen for IAP and DPPIV. As the litter was confounded with the treatment in these trials, additional trials were needed. We received additional gut tissue samples earlier this year.

Task 2.3. Microbial imprinting - Does neonatal colonisation determine carriage of bacteria through life?

The present study was carried out in conventionally reared inbred Babraham piglets born by normal delivery. Four different litters born within 24 hours of each other were kept with and allowed to suckle their own "mothers" for 28 days. Piglets were then weaned and "mixed " by allocating them into 5 different pens where then were housed for three more weeks. Piglets were then killed and gut microbiota analysed by DGGE. This study showed that the microbiota acquired during the first four weeks of life profoundly influences the long term enteric carriage into the post-weaning period and later life. These results are of particular significance since they were obtained in inbred Babrahams that share exactly the same genotype. Whilst these results would not eliminate a contributing role of genotype they highlight the importance of early life environment in determining the longer term carriage of enteric bacteria. Bacteria contributing to this micro-environment will be likely to have originated from the sow and her farrowing area.

WP 3: Development of gastro-intestinal function and host-microbe interaction

As the development of the gastrointestinal function is a complex process, several in vitro and ex vivo approaches are necessary. The focus of work lies on one hand on the understanding the mucosal reaction towards the intestinal microbiota (commensals, pathogens, defined nutritional, using intestinal epithelial cell cultures and piglet mucosa explants. Secondly, WP3 aimed to evaluate mucosal growth and maturation in terms of morphology (villus and crypt measurement), mucosal function (enzyme development, ion transport), pattern recognition receptors, inflammatory pathway) and mucosal immune function (immune cell immigration, interaction of immune cells and epithelium) during the colonisation and stabilisation of the intestinal microbiota.

WORKPACKAGE 3 FACTS

LEADING HOUSE: OVGUMagDE
PARTICIPANTS:INRA, OVGUMagDE, INRAN

OBJECTIVES:
1. To understand the mechanisms of the host-microbe interaction at the cellular level using innovative approaches
2. To evaluate the development of gastrointestinal function during the colonisation and stabilisation of the intestinal microbiota in the period after birth

Task 3.1. Role of the Toll-like-receptors (TLRs) on microbiota-gut interaction

The main objective of this task is to investigate the interaction of pathogenic and non-pathogenic bacteria with intestinal cells. In collaboration with P10, P5 has studied *L. amylovorus* S-layer proteins protective activities against membrane damages; in collaboration with P3b experiments were also performed on pig intestinal explants treated with bacteria.

Microbiota, TLRs, mucosal barrier and inflammatory pathway in intestinal cells.

Intestinal cells (1 x 10^6 cells/filter, 12 mm diameter) were untreated (control), infected with 1 mL of medium containing ETEC K88 (5 x 10^6 CFU/mL), treated with *L. amylovorus* DSM 16698^T (5 x 10^7 CFU/mL), or *L. amylovorus* supernatant (equivalent to 5 x 10^7 CFU/mL), either alone or simultaneously with ETEC, for 2.5 h. The levels of TLR4, MyD88, IRAK4, P-IKKα/β, P-IkBα, P-p65, IRAK-M and Tollip were analyzed by Western blot. ETEC increased the expression of the inflammatory proteins involved in the TLR4 cascade and decreased the expression of the negative regulators IRAK-M and Tollip. Simultaneous treatment with *L. amylovorus* and ETEC, as well as with *L. amylovorus* supernatant, reduced the expression of TLR4, MyD88, P-IKKα/IKKβ and P-p65, upregulated Tollip and inhibited the ETEC induced decrease of IRAK-M. In conclusion, *L. amylovorus* and its secreted factor/factors are able to inhibit the proteins of the TLR4 mediated inflammatory cascade through modulation of the negative regulators.

In order to evaluate whether TLR2 could play a role in *L. amylovorus* anti-inflammatory activity, we performed immunoneutralization experiments. For such purpose, neutralizing anti-TLR2 antibody was apically added to Caco-2/TC7 cells for 1 h before the addition of bacteria as above described. Results indicated that TLR2 is required for the anti-inflammatory activity of *L. amylovorus*.

Furthermore, in collaboration with P8 we aimed to evaluate the transcriptome and the regulation of different genes involved in TLR4 signaling in ETEC and *L. amylovorus* treated Caco-2/TC7 cells. A RNA-Seq approach was used, allowing characterization of new transcripts and identifying transcription start sites, splicing variants and differential promoter usage. IPA (http://www.ingenuity.com) was employed to identify the most significant molecular networks, biological functions and canonical metabolic pathways, that resulted for all treatments: 1) cell movement and immune cell traffic; 2) cell to cell signal interaction and 3) inflammatory response. However the gene expression was differently regulated by the different treatments. In addition, an important role of IL-17 pathway and glucocorticoid receptor

signaling pathway was also found. Furthermore, RNAseq showed that IL-8 was more expressed in the cells treated with *L. amylovorus* than in cells treated with ETEC or ETEC+ *L. amylovorus* for 1 h. However, qPCR analysis showed also that IL-8 expression was up-regulated in ETEC infected cells and down-regulated in ETEC + *L. amylovorus* after 2.5 h treatment.

In collaboration with P10, we wished to verify whether the protective effects by *L. amylovorus* on barrier damage induced by ETEC (Roselli et al., 2007) were exerted also by S-layer proteins (Slp) purified from this strain. After ETEC infection, a decrease in occludin, β-catenin and E-cadherin and an increase in P-β-catenin (β-catenin, when phosphorylated, detaches from AJ) were found in Caco-2/TC7 cells. Both pre- and simultaneous treatments of infected cells with *L. amylovorus* Slp-coated cell walls (CoSLP) inhibited these alterations, while a protection was induced by UnCoSLP only when added before ETEC infection. Treatment of the cells with CoSLP and UnCoSLP alone did not induce any change, indicating that Slp exert protective activities against ETEC induced damages by different mechanisms.

Treatment of Caco-2/TC7 cells with both CoSLP and UnCoSLP did not modify ZO-1 and occludin localization. Infection with ETEC caused a TJ opening, as indicated by the ZO-1 delocalization, showing loss of cell-cell contact, and by occludin dissociation from the membrane with scattered distribution inside the cells. Pre- and simultaneous treatments of infected cells with CoSLP protected the cell membrane by maintaining a correct distribution and organization of TJ proteins. Pretreatment with UnCoSLP only partially protected the cells from ETEC induced damages, while simultaneous treatment with UnCoSLP was not able to counteract the damages in TJ. When treated with CoSLP and UnCoSLP, a correct distribution of β-catenin and E-cadherin was found, while ETEC induced several damages in the distribution of both proteins. All treatments, with the exclusion of UnCoSLP simultaneous treatment, were able to maintain a correct distribution and organization of the AJ proteins. Membrane barrier integrity was determined in Caco-2/TC7 cells by paracellular flux of the phenol red marker, measured by spectrophotometric assay. ETEC induced a significant increase of phenol red passage, that was not observed after pre- and simultaneous treatments of infected cells with CoSLP. Pre-treatment, but not simultaneous treatment, with UnCoSLP completely prevented the ETEC-induced phenol red passage increase.

Intestinal porcine explants

1- Beneficial effect of *L. amylovorus*: analysis of immune reponses and intestinal barrier function

L. amylovorus DSM 16698 has been identified as an abundant member of the intestinal microbiota of pigs (Konstantinov et al., 2005; Jakava-Viljanen et al., 2008), and its probiotic potential was demonstrated previously *in vitro* by the study of intestinal pig epithelial cells (IPEC-1) challenged with ETEC K88 (Roselli et al., 2007) and *in vivo* by the evaluation of piglets fed with DSM 16698 and infected with ETEC F4 (Konstantinov et al., 2008). Using a pig intestinal model (Kolf-Clauw et al., 2009, Cano et al., 2013), this part of the project aimed at evaluating if this L*actobacillus* strain was also able to counteract the deleterious effect of mycotoxins. Deoxynivalenol (DON) and T-2 toxin are the two most common trichothecene mycotoxins. They are found worldwide in cereal products and are considered natural contaminants in food causing problems of public health and animal health. They are also well known to act on the intestine and to disrupt the immune responses that the functions of the intestinal barrier.

Treatment of intestinal explants with mycotoxins (10 µM DON or 3 nM T2-toxin) induces an inflammatory and a Th1 response. *L. amylovorus* alone does not induce these responses. Moreover, when explants were pretreated for 1 hour with the bacteria before to be exposed to the toxins, no modulation of the inflammatory (IL-1a, IL-1b, IL-8, TNF-a) and the Th1 (IFN-g and IL-12) responses were observed. The next step was to investigate the impact of *L. amylovorus* on the Th17 inflammatory response. As previously observed, mycotoxins increased the gene expression of IL-6, IL-22, IL-17A and IL-23A. *L. amylovorus* alone decreases the expression of these genes and inhibits the DON induced Th17 response. By contrast *L. amylovorus* was not able to inhibit the T2-toxin induced Th17 response.

As the mycotoxin DON is known to decrease the intestinal barrier function, we next investigate if *L. amylovorus* could restore the barrier function impaired by DON exposure. Intestinal explant were either exposed to DON either in vitro or were obtained from animals exposed to DON (0.3 mg/Kg b.w./day for 14 days). We observed a decrease in TEER in explant exposed in vitro to DON and in the ones obtained from DON exposed animals. In both type of explants, the addition of *L. amylovorus* partially restore the TEER. The para-cellular and trans-cellular passages were also measure using 4KDa FITC-dextran 4KDa and HRP respectively. Paracellular passage was not modulated by the toxin or the bacteria. By contrast, DON decreases the trans-cellular flux and the addition of *L. sobrius* was able to restore it.

In conclusion our data indicate that *L. amylovorus* is able to limit the intestinal Th17 response and to partially restore an altered intestinal barrier function.

Task 3.2. Understanding cellular physiology in challenged enterocytes in vitro

Establishment of an air-liquid interface culture as model for the intestinal barrier

Air-liquid interface (ALI) cultures are the best *in vitro* representation of airway and gastric epithelium (Fig. 3.6). The surface of the intestinal epithelium represents a comparable interface. Thus, a novel culture system was developed to study the differentiation of intestinal epithelial cells in a microenvironment which is closely related to the physiological conditions.

Effects of Lactobacillus strains on cellular morphology in vitro A set of 4 strains of lactobacilli (P10) was applied on confluent (age 7 d) glass bottom cultured porcine intestinal cells IPECJ2. The attachment and distribution of bacteria and the structure of epithelial cell monolayer was analyzed by conventional and confocal fluorescence microscopy. All lactobacilli strains attach to the epithelial cell surface, however, a qualitative difference in the number of bacteria attached could be seen. Furthermore, the presence of lactobacilli affected the cytosceleton and reduced the actin signal below the detection level. Two principle pathways could be responsible for this effect, a direct physical contact between bacteria and epithelial cell or the influence of soluble factors released by bacteria. Shifting the pH of the medium alone could not mimic the change in actin structure. It is also known that lactic acid is a major metabolite of lactobacilli. Lactic acid can be found in the small intestine of weaning pig in a range between 7 and 37 mmmol/L, depending on the feeding regime and the intestinal segment. Whereas the application of lactate at neutral pH was without effect on the actin structure, the application of lactic acid at pH 4 could mimic the effect detected with bacterial supernatants. In the next step we further analysed possible mechanisms modulating the cytoskeletal structure of the epithelial cells. It has been previous shown that reactive oxygen species (ROS) can regulate actin cytoskeleton in endothelial cells (Moldovan et. al. 2006). ROS are side products of the mitochondrial respiratory chain and are detectable by the oxidation of fluorescence dyes on single cell level. A beneficial effect was found with higher glucose concentrations (4.5 g/L). The cellular AntiA-mediated ROS (superoxide) generation was reduced by treatment of the cells with 25 mM lactate, an effect with could be blocked by the application of an inhibitor of the lactate transporter (UK5099, 10µM). The data suggest a protective role of lactate against ROS and a different modulation of actin skeleton. As *in vivo* lactobacilli generate lactate in proximity to epithelial cell this mechanism may contribute to the positive effect of lactobacilli in the gut.

B) Impact of deoxynivalenol (DON) and E.coli LPS on intestinal architecture and integrity in vivo

Barrows (26.2 ± 4.1 kg) were fed restrictedly either a control diet (CON) or a diet naturally contaminated with 3.1 mg DON/kg feed (DON) for 37 d. At d 37, the control group was infused for 1 h, either with 100 µg/kg BW of DON (CON-DON, *n* = 6), 7.5 µg/kg BW of LPS (CON-LPS, *n* = 6), a combination of both (CON-DON+LPS, *n* = 7), or 0.9% NaCl (CON-CON, *n* = 6) and the DON group with 7.5 µg/kg BW of LPS (DON-LPS, *n* = 8) or 0.9% NaCl (DON-CON, *n* = 6). Pigs were euthanized 3.25 h after start of infusion. Immunohistochemistry (BrdU for proliferation) and immunofluorescence (ZO-1 and β-catenin) from duodenum, proximal jejunum, mid-jejunum, proximal ileum, and terminal ileum were analyzed for crypt depth, cell proliferation, and expression of apical junction proteins. The latter was first scored for presence and absence of an apical signal along the villus axis (ZO-1 degradation score) and secondly a ratio was calculated between the fluorescence intensity of the apical and cytosolic cell compartment (1 = equality between apical and cytosolic signal, >1 apical signal stronger than cytosolic). Furthermore, liver morphology was evaluated with a pathological score system using HE-stained tissue sections. Clinical chemistry was performed in order to assess liver function. Results of this experiment demonstrated that epithelial proliferation has a distinct pattern along the small intestine and is not necessarily positively linked to crypt depth in pigs. Furthermore, results indicated that LPS changed the spatial distribution of ZO-1 in the cytosol of the upper small intestine. A synergistic effect of DON and LPS on intestinal architecture could not be verified in the present study. Relative liver weight (g/kg BW) was in general markedly increased in pigs receiving LPS, independent of DON exposure. Haemorrhage was the main cause for this, which could be verified macroscopically and microscopically. The histopathological score was significantly increased in LPS pigs receiving the control diet, whereas LPS pigs receiving the DON diet showed an alleviated score. The main damage comprised haemorrhage and inflammatory infiltration of neutrophils and eosinophils, the latter contributing to the inflammation to a higher degree. Clinical chemistry revealed some minor effects on aspartate-aminotransferase, γ-glutamyltransferase, glutamate dehydrogenase, albumin and total protein, but a dramatic impact of LPS on total bilirubin. This was increased well above the physiological upper limit, indicating the pathological situation of the liver. Interestingly, this drastic increase could only be observed in control-fed LPS-infused pigs, but was only raised to the upper physiological range when DON was fed to LPS-pigs. In conclusion, Don alone had no effect on liver morphology and function, whereas LPS damaged the liver morphologically and functionally already after 180 min. Again, no synergistic effect of DON and LPS could be observed (like for the intestine), with the exception of bilirubin, which displayed an additive effect of both toxins. Interestingly, LPS-infused pigs showed a profoundly alleviated response when prior fed with DON. Further studies are necessary to elucidate this intriguing impact of chronic oral DON exposure and subsequent immunological challenge with LPS on the liver.

Task 3.3. Understanding the intestinal stress response under the influence of microbial and nutritional luminal stimuli

Microbiota, TLRs and stress. The aim of this task was to investigate whether probiotics may regulate the inflammatory pathway through Hsp and TLR regulation. Since extracellular Hsps can induce inflammation and are critical for the regulation of TLR4 complex formation and function, we further investigated whether these proteins, namely Hsp72 and Hsp90, were regulated by ETEC K88 and *L. amylovorus*. For such purpose, Caco-2/TC7 cells differentiated on Transwell filters (1 x 10^6 cells/filter), were untreated (control) or apically treated with 1 mL of medium containing ETEC (5 x 10^6 CFU/mL), *L. amylovorus* (5 x 10^7 CFU/mL) or *L. amylovorus* supernatant (equivalent to 5 x 10^7 CFU/mL), either

alone or simultaneously with ETEC, for 2.5 h. At the end of treatment, cells were lysed and Hsps analyzed by Western blot. The infection of Caco-2/TC7 cells with ETEC caused an increase in Hsp72 and Hsp90 levels, that was inhibited by co-treatment of the cells with ETEC and *L. amylovorus*. Similar effects were obtained when the cells were treated with ETEC and *L. amylovorus* supernatant. No change in the levels of Hsp72 and Hsp90 was induced by treatment with *L. amylovorus* alone. These results indicate another way by which *L. amylovorus* may counteract the ETEC induced inflammation, namely the inhibition of the extracellular secretion of Hsp72 and Hsp90. Our results reasonably indicate that the downregulation of the extracellular Hsp72 and Hsp90 by *L. amylovorus* and its secreted factor/factors contributed to the inhibition of TLR4 inflammatory signals.

Task 3.4. Quantification and characterization of DC processes in the intestinal epithelium and understanding the transport capacity of DC for bacteria/bacterial antigens via lymphatics to the mesenteric lymph nodes

Evaluation of dendritic cell protrusions into the intestinal epithelium in vivo

Barrows (Deutsches Bundeshybridzuchtprogramm) were fed either a control (CON) or a diet naturally contaminated with deoxynivalenol (DON). Diets were based on wheat, barley and triticale, whereby the triticale batch of the control diet was exchanged for a batch contaminated with DON. Starter and finishing diet contained 2.2 and 2.9 mg DON/kg feed, respectively. Pigs were housed individually in slatted floor pens for 11 weeks and 5 animals of each group were slaughtered at a final live weight of 111 kg. Tissue samples were taken from mid-jejunum and terminal ileum (approx. 5 cm proximal to the ileocaecal junction), snap-frozen and stored for further analysis.

The 3D-reconstruction of intestinal sections revealed that lamina propria CD16+ cells were in general in close contact to the BM and that these cells or their dendrites were able to migrate through the BM pores into the epithelium. Average BM pore size amounted to 3.5 µm (diameter) in jejunum and ileum and diet had no impact on it. Number of pores/1,000µm BM was significantly higher in the ileum compared to jejunum for CON (ileum vs. jejunum: 11.3 vs. 6.4 pores/1,000µm BM, $p<0.001$). In the jejunum, pore count was markedly increased due to diet (CON vs. DON: 6.4 vs. 9.7 pores/1,000µm BM, $p=0.055$), whereas in the ileum there was no dietary impact on this parameter. Furthermore, DON showed a trend of increasing the number of intraepithelial CD16+ cells or their dendrites in the jejunum compared to ileum (jejunum vs. ileum: 4.9 vs. 2.8 CD16+cells/1,000µm BM, $p=0.067$), whereas no such effect on intestinal distribution was found in the CON group.

WP 4: Exploring the gastric ecosystem

WORKPACKAGE 4 FACTS

LEADING HOUSE: UNIBO
PARTICIPANTS: WU, OVGUMagDE, UNIBO

OBJECTIVES:

1. Assess the composition of microbiota in the stomach of the young pig
2. Compare the morpho-function of porcine stomach at different times of the early life
3. Evaluate the effect of different degrees of gut colonization on the gastric morpho-function of the young pig

Data collected from several in vivo experiments evidenced that

1) the relevance of the compartmentalisation in different mucosal areas of the pig stomach; this stresses the importance of a proper sampling depending on the research goals;
2) the stomach should be fully considered a component of the digestive tract that has complex organization and relevance in term of immune competence, also in healthy young pigs
3) the maturation of stomach functions, as indicated by several functional markers, is relevant during the suckling period, but is variable after weaning depending of which aspect is considered
4) the porcine stomach can have a role also in the detection of taste sensed molecules, but it is not organized for the detection of sweet signals
5) the humoral immune response in some areas of the stomach has been underestimated, at least for the young pig
6) some aspects of the stomach maturation and regulation can be delayed by the mother microenvironment, as evidenced by the pre- and post-farrowing treatment of sows with antibiotic in the diet.
7) Data on gastric microbiota in weaned pig indicate that the profile of bacteria adhering to the gastric mucosa does not simply represent the one observed in the gastric content, and thus that the gastric environment has a selective pressure on these bacteria. This is also evidenced by a trend of differentiation of the microbiota detected on oxyntic mucosa, as compared with the pyloric and transition mucosae.

Task 4.1. Development of the gastric microbiota in the early life of pig

In mammals, the gastric mucosa is divided in many compartments, each with specific functional characteristics that could create niches for the settlement of resident microbiota. A database of porcine gastric microbiota was created from 8 weaned pigs from the same environmental, dietary and healthy conditions, sampled in three potentially distinct types of microbial niches: oxyntic area (acid production), pylorus (gastrin secretion) and gastric groove, in the small curvature close to cardiac (immunological function); in addition the gastric content was sampled.
Bacterial genomic DNA was isolated and purified and PCR amplification of 16S rRNA hypervariable region V6 was performed with a pool of 5 forward primers and 4 reverse primers [Huber JA et al., 2007, Science 318: 97–100]. Sequencing of the amplicon libraries was carried out on the Ion Torrent Personal Genome Machine (PGM) system using the Ion Sequencing 200 kit (all Life Technologies).
The microbiome clustering did not show an individual effect; however, in cluster 1, 3 out of 4 samples from oxyntic region form a sub-cluster well supported (BP= 84), indicating more similarity in the relative abundance and taxonomic composition of the microbiotas in oxyntic regions from different pigs.
Principal-component analysis of the relative abundances of individual bacterial OTUs confirmed the presence of two main clusters, one included all the samples of stomach contents and one contained the three mucosal samples (Pyloric, Oxyntic, Groove) from all pigs, with a little sub-cluster composed by oxyntic samples. Assignments at phylum level showed a limited number of Phyla: Proteobacteria, Firmicutes, Actinobacteria, Cyanobacteria/Chloroplast and Bacteroidetes (Acidobacteria, Tenericutes, Fusobacteria and Verrucomicrobia < 1%). Content samples were characterized by a greater presence in Cyanobacteria/Chloroplast (28% in average) and Firmicutes (25% in average) in respect to mucosal samples in which these last decrease in favor of Proteobacteria (65% in average) and Actinobacteria (16% in average). Performing Taxonomic Assignment to the level of Genus and then the PCA + Biplot of the relative abundances of individual bacterial genera, can be seen once again the same clusters. The genera that mainly affected the clustering were: *Moritella*, *Brevundimonas* and *Ochrobactrum* for the Cluster 1 (Mucosal samples), *Streptophyta*, *Lactobacillus*, *Streptococcus* and *Pasteurella* for the Cluster 2 (Content samples).

Task 4.2. Influence of the early microbial colonisation on gastric function development

A sufficient research attention to the interaction of age and variations imposed on gut microbiota, on acid secretion function in the young pigs has not been paid.
Eighty four pigs reared from sows fed a diet with or without Amoxicillin (40 mg/kg BW/d, on –10 d to +21 d from farrowing, ATB) were sacrificed at 14 d, 21 d, 28 d (weaning) or 42 d (inside WP6), and sampled for oxyntic (OXY) and pyloric mucosa (PY), for mRNA quantifications (-80°) and immunohistochemistry. Part of the observations were also extended to littermate offspring that were reared up to the age of 5 months, and then kept on the same diet. On the whole these data evidence that in the oxyntic mucosa the functional maturation progresses during the suckling period and it is extended also later. The treatment of the sows with an antibiotic can potentially reduce the portion of cells dedicated to the acid secretion in the suckled pigs .
Gut maturation during suckling and after weaning is influenced by maternal environment. Scarce is the knowledge on the presence and the regulation of taste receptors in the pig gastro-intestinal tract. The same is for the machinery that processes the taste signals, including α-transducin. Our main goal was to assess if the age of offspring and maternal environment, as influenced by a maternal antibiotic treatment, could affect the expression of the receptors for umami and sweet taste in offspring stomach. Neither perinatal ATB treatment nor later fat diet content affected the studied variables. Data show that sensing for umami taste has relevance for two different gastric mucosae. It can change with age and be potentially affected by maternal environment. For transducin, divergent observations on gene expression and positive cell counts require further evaluation. The same set of samples was used also to assess the involvement of the orexigenic control related to ghrelin secretion. The gastric release of the active ghrelin (octanoyl-ghrelin) is under the complex control of 3 genes: preproghrelin, proprotein convertase (PC1/3), for the posttranslational cleavage, ghrelin O-acyltransferase (GOAT), for acylation of ghrelin. Relative content of preproghrelin mRNA increased during suckling ($p = 0.012$) and also in the post weaning, as a trend ($P = 0.088$). GOAT tended to increase during suckling ($P = 0.062$), while PC1/3 was not affected. ATB never affected the relative expression of these genes.

Additional results for this task were obtained inside two experiments done on pigs reared in Lelystad, aimed at evaluating the priming effect of the complexity of the microbiota that enter the digestive tract on several gut response factors and, for this WP, on gastric transcriptome profile. In the 2nd trial a dietary factor was also considered, that could have an effect on gastric lumen environment: an oil source of medium chain fatty acids.
Twelve (Exp.1) or twenty-four (Exp.2) caesarean derived piglets were assigned to two treatment groups by litter and body weight on day of birth (d 0), housed in two separate clean laboratory rooms with a hygiene barrier, received serum from sow blood from d 0 to d 2, and were orally dosed a “simple” microbiota consisting of 10^6 - 10^7 CFU from each *Lactobacillus amylovorus*, *Clostridium glycolium* and *Parabacteroides* sp from d 1 to d 3. In addition, on d 3 and d 4, the groups received a fecal inoculant obtained from a conventional adult sow (Complex Association, CA), or a placebo inoculant (Simple Association, SA). All piglets were fed a milk replacer diet during day 0 to 5 and a moist diet

from day 6 onwards. In exp.2, within each microbial treatment, half of pigs fed a diet where 7% coconut oil substituted the same quantity of soybean oil (CTRL) and palm oil (medium chain triglycerides diet, MCT). Pigs were euthanized on d 14 (exp.1) or 21 (exp.2) and samples of oxyntic tissue in the stomach were obtained and deep frozen. On quality-tested mRNA, the analysis of whole transcript expression per each pig was done by Affymetrix©Porcine Gene 1.1 ST array strips. In general the results suggest that early association of newborn pigs with a complex microbiota favorably prevents the activation of pathways related to cell division (both at 2 and 3 weeks of age, trial 1 and 2) in the gastric mucosa, compared to the association with a simple microbiota. This may be put in relationship with the observation that a short encounter with a complex microbiota in the early life can be sufficient to influence the intestinal microbiota in the following weeks (Jansman et al., 2012; on the trial 1).

MCT diet enriched 184 gene sets, compared with CTRL, including response to virus, chemochine activity & binding, responses to stimuli, neurite development etc. 1st gene in the ranking was Mucin 13. Conversely CTRL diet enriched only 14 gene sets, compared with MCT (mostly related to energy and lipid metabolism). These data in general suggest that MCT created conditions similar to SA for the gastric environment; microbiology data on pigs of this experiment could better substantiate this observation.

Acid secretion function Inside the stomach, the oxyntic mucosa is characterized by a very large and highly expressed array of genes for ion and water protein channels [Colombo et al., 2013, Ital J Anim Sci .12(suppl.1), 125], compared to pyloric mucosa (and also to intestinal mucosa). Thus it was important the observation that several overlapping nodes of genes related to anion channel activity were up-regulated in pigs fed MCT that were early associated to a complex microbiota, compared to SA-associated pigs, fed the same diet. Particularly, the most representative gene of these nodes for differential expression was chloride channel accessory 1 (CLCA1) that ranked 5th in the list of upregulated genes. CLCA1&2 products in the stomach are characteristics of luminal membranes of murine gastric parietal cells (Roussa et al., 2010, J Histoch Cytochem, 58, 653-668) and also of surface mucous. The presence of the up-regulation of groups of genes related to ion channels only in MCT-supplemented pigs may imply that in the control pigs this up-regulation was not necessary and may fit with a better adaptive conditions of this latter group.

Maturation of gastric signal controls In pigs that had been associated to a complex microbiota, whatever was the diet, the connected nodes G_PROTEIN_COUPLED_RECEPTOR_ACTIVITY and RHODOPSIN_LIKE_RECEPTOR_ACTIVITY were up-graded. Several GPCR are important for the maturation of the gastric mucosa (Nagata et al. 1996, PNAS, 93, 11825-11830), and for example for ghrelin secreting cells (Engelstoft et al., 2013, Molec. Metab. 2, 376-392). In the list of upgraded genes of these nodes the highest ranking was for HTR1B5-Hydroxytryptamine (Serotonin) Receptor 1B, G Protein-Coupled.

Development of enteroendocrine control and neuronal control at the gastric mucosal level

CA treatment enriched the complex of nodes related to neurotransmitter binding, neurotransmitter receptor activity and transmembrane receptor activity. The gastric parietal cells are innervated by secretomotor neurons and stimulated to release acid; equally so are also chief cells that release pepsinogen. Gastric acid secretomotor neurons are cholinergic (Furness, 2000. J Auton Nerv Syst 81: 87–96). Thus CA could have better activated neuronal control of the gastric secretions, as indicated particularly by the first rank in the list of the genes of CHRNA2 cholinergic receptor, nicotinic, alpha 2 (neuronal).

Another high-ranking gene was GABRG2 Gamma-Aminobutyric Acid (GABA) A Receptor, Gamma 2. In the stomach, cholinergic enteric neurons present GABAergic neuron immunoreactivity; GABA may also be sensed as hormonal and paracrine signaling (Tsai, 2005, J Biomed Sci.12:255-266). Interestingly, GABA is produced by several common gut commensal, including some lactic acid bacteria (Barrett, et al., 2012, J Appl Microb 113:411-417). Thus, it would be interesting to study in deep the contribution of gastric microbiota to GABA release and modulation of GABA(A) receptors. Our observation in fact could be associated to the presence of a more developed and complex gastric microbiota.

Task 4.3. The gastric immune system

Local induction of the gastric immune system by the microbiota & Local bacteria control of mucosal inflammation

Mucosa-associated lymphoid tissue (MALT) is present in the different layers of the mucosal wall and consists of organized lymphoid tissue which may occur as isolated or aggregated lymphoid follicles (LFs) and interfollicular areas. Gastric MALT has been intensely studied in experimentally infected pigs but few data are available in healthy, non-gnotobiotic or germ-free animals. Conventional pigs were sampled and the gastric MALT of the gastric diverticulum, in the pyloric mucosa, and in the sites of transition from cardiac to oxyntic and from cardiac to pyloric mucosa were described by means of histological and immunohistochemical stains. The majority of LFs were located in the cardiac mucosa and in the transition from the cardiac to the oxyntic mucosa. The LFs were mainly located in the submucosa and reached the mucosa (called submucosal lymphoid follicles, SLFs). In the pyloric mucosa and in the transition sites from the cardiac to the pyloric mucosa, LFs were located in the mucosa (called mucosal lymphoid follicles, MLFs). In SLFs, a compartmental organization of T and B lymphocytes was present; by contrast, in the MLFs, the T and B cells were intermingled, suggesting the possibility of different roles for the two types of follicles. In the epithelium

overlying the lymphoid tissue, numerous T lymphocytes and some cells immunoreactive to cytokeratin-18 were observed.

The polymeric immunoglobulin receptor (pIgR) is a key element of the mucosal immune system, mediating the active transport of polymeric immunoglobulins (pIgs) from the lamina propria to the lumen and also participating in the innate immune defence. For its role in the maintenance of the mucosal homeostasis, pIgR could play a key role in the development of mucosal immune system in the gastrointestinal tract of young piglets. We aimed at investigating the time course pIgR expression and the protein distribution in three functionally different sites of the gastric mucosa of pre- and post-weaning pigs. Results indicated that pIgR is expressed in the gastric mucosa of growing pigs, and its transcript levels are developmentally regulated with regional differences among the three considered sites of the stomach. The pIgR protein and mRNA were localized in the gastric glands located in the lamina propria, indicating that the pIgR is actively synthesized in gastric mucosa and it could play a crucial role in gastric mucosal immune defence of growing pigs. The expression of TLR2, TLR4, TNF-α and IL-8 genes was also tested in the transition region between OXY and PY, where more lymphatic aggregates are seen than in OXY or PY. TLR2 expression increased during the suckling period ($p = 0.003$), but then tended to decreased ($p = 0.062$). TLR4 was not changed by the age. TNF-α increased constantly ($p < 0.001$), while IL-8 increased in the suckling period only ($p < 0.001$). The ATB treatment was never statistically significant.

In addition, data from the Lelystad experiment showed that with the control diet, pigs that had been associated to a complex microbiota had overlapping enriched nodes related to cellular defense response, response to wounding and inflammatory response, compared to SA pigs. Among the genes of these groups there was chemokine (C-X-C motif) ligand 11 (CXCL11) that ranked 2nd in the list of all genes. In pigs supplemented with MCT, no significant enrichment related to the different early microbiota association was seen for these nodes.

These observations are in some way in contrast with that obtained in the 1st trial with 2 weeks old piglets. There, in addition to the already mentioned general mitotic stimulation, the sets of genes for interplay with xenobiota (Toll like receptors signaling), immune response (chemokine receptors binding chemokines) and host interaction with Human Immunodeficiency Virus factors were enriched with SA treatment. Several chemokines characterized by the CXC motif were represented at the top of the core genes characterizing enriched gene sets, including CXCL11. This made us to conclude that an early association of newborn pigs with a complex microbiota favorably prevents the activation of pathways related to cell division and inflammatory development in the gastric mucosa at an age of two weeks compared to the association with a simple microbiota.

Recently it was shown that transient inflammatory-like state and microbial dysbiosis are pivotal in establishment of mucosal homeostasis during colonisation of germ-free mice (Aidy at al., 2013, Beneficial microbes, 1-11).

Thus the observations from the 2nd trial, where piglets were sacrificed at three weeks of age, may imply that the association to a complex microbiota delays the transient inflammatory-like state related to microbial dysbiosis.

Finally it must be added that a diet containing an oil source of medium chain fatty acids (3 weeks of age, trial 2) in general activated chemokine activities and cytokine binding. This could have masked in these pigs any other differential effect of early microbial association on pathways related to defense or inflammatory response.

WP 5: Effect of host genotype on GIT microbiota and functions

Task 5.1. Comparison of distinct breeds

WORKPACKAGE 5 FACTS

LEADING HOUSE: INRA (3B, INRA-TOULOUSE)
PARTICIPANTS: WU, Univbris, INRA, NAU, PTP

OBJECTIVES:

1. Determine to what extend the host genotype affects the microbiota and the gut function of pigs, especially the immune response
2. Determine if animals differing for their immune response have different microbiota.

The aim of this task was to investigate the effects of pig genotype on gut microbiota, gut function and lipid metabolism. Eight litters of neonatal piglet from two breeds (Meishan and Yorkshire) were used and there are four litters for each breed. Half of each litter of one breed piglets were fostered by the sow of another breed, thus there were four groups: Mm (Meishan mother, Meishan piglets), Ml (Meishan mother, Yorkshire piglets), Ll (Yorkshire mother, Yorkshire piglets) and Lm (Yorkshire mother, Meishan piglets). All piglets were weaned at 28 days of age, and one piglet for each replicates was slaughtered on day 28 and day 49, respectively. Fecal samples of sow and piglets on day 1, 3, 7, 14, 21 and 28 were collected. Samples of blood, liver, thymus, spleen, adipose tissue, content and mucous of each intestinal tract were collected. Total bacterial DNA in feces was extracted for the analysis of microbial diversity.

The results showed that offspring of obese dams had higher pancreatic enzyme activities, liver *PPARα*, *FAS* and adipose *ACC* expression compared to offspring of lean dams, while offspring of lean dams had greater average daily gain, serum HDL levels, and adipose *ACOX* expression than offspring of fatty dams. Offspring suckled by obese dams showed an increased serum leptin concentration, liver MDH activities, lowered liver LPL and TL activities compared to offspring suckled by lean dams.

Pyrosequencing analysis of 16S rRNA gene of total bacteria showed that *Streptococcus* and *Clostridium* were dominant among all samples on day 1 and 3, while *Lactobacillus, Ruminococcaceae* and *Lachnospiraceae* were dominant on day 7 and 14. A low numbers (< 3%), high abundance (>50%) shared OUTs were appeared among different groups in the first week, with a rapid change in their composition among different days. The numbers of common OTUs between piglets and their nursing mothers were increased after the first week. The numbers and abundance of OTUs influenced by breed and nursing mother were increased with the growth of piglets. The result suggested that age was the major factor to affect early colonization of piglets, contributions of piglet breed and nursing-sow were increased after the first week.

Pyrosequencing analysis of 16S rRNA gene of methanogens showed that the age of the piglets significantly affected the diversity and richness of faecal methanogenic Archaeal communities; there was no significant difference between the pig breeds. In both breeds, the piglets harboured a higher diversity of faecal metnanogens at 1 and 3 days of age than at 7 and 14 days ($P < 0.05$). All of the OTUs were closely related to genus *Methanobrevibacter*.

Task 5.2. Lines differing in their immune response (Partner 3B)

The aim of this task was to compare animals that differ in their immune response capacity. We initially planned to study two groups of pigs selected for a divergent level of an index comprising four complementary immune parameters (phagocytosis capacity measured from in vitro tests with total blood, levels of IL-2 and IL-10 after in vitro stimulation of diluted total blood, and seric levels of anti-*mycoplasma hyopmeumoniae* IgGs). This divergent selection was started in 2009 in the frame of a French large project referred to as IMMOPIG funded by the National research Agency. Unfortunately, as mentioned in the previous report, all animals from the first selection generation were killed due to unexplained sanitary problems in the farm at Le Magneraud and the experiment was stopped.

We decided to start a comparative study between Large White (LW) pigs and highly inbred mini-pigs homozygous for the major histocompatibility complex (MHC). On the one hand the LW pigs are not inbred and present diversity for MHC haplotypes, and on the other hand, the mini-pigs all harbor the dd haplotype now referred to as the Hp4.0 haplotype (http://www.ebi.ac.uk/ipd/mhc/sla/haplotypes. html). The mini-pigs were imported about 15 years ago at INRA-Nouzilly by Henry Slamon when he came back to France after a sabbatical time in Prof David Sach's laboratory. Since that time, the pig line has been maintained as a closed inbred line. To carry out this refined program, we have included new collaborators: François Meurens, Mustapha Berri and Claire Chevaleyre from the INRA-IASP laboratory (IASP: Infectiologie Animale et Santé Publique), and Elodie Guettier and colleagues who are managing the experimental farm at Nouzilly.

Twelve animals from each dd and LW group (6 sows and 2 piglets per sow) have been compared. The animals were sampled at five different time points after birth: 8, 20, 40, 60 and 100 days. Piglets (at all time points) and sows (at 8 days post-birth) were sampled for feces (storage at -80°C) and blood (sampling in EDTA tubes for hemograms). At 60 days after birth, blood was sampled in PAXGENE tubes for RNA extraction and further transcriptome analyses. Blood sampled in EDTA tubes at 8 days post birth was frozen at -20°C for DNA extraction, providing opportunities for genotyping.

Transcriptome analyses of total blood were performed using customized Agilent chips that comprised a generic set of pig genes together with an additional set of immunity-related genes as described in Gao et al. (2010, BMC Genomics 11:292). Differential expression analyses between the two conditions (breed dd versus LW at 60 days old) revealed a set of 554 DE genes. 201 probes were found over-expressed in LW compared to dd pigs (fold change from approximately 1.5 to 19.5) and 353 probes were found down-regulated (fold change from -1.4 to -51 and a non annotated transcript repressed down to -1375 times). Our results clearly show that the gene profiling in total blood reveals breed specificities. Interestingly, among the top biological function that are enriched in DE genes, we have identified two functions that are related to the nervous system: neurological disease, and nervous system development and function. Since dd animals are known to have a more aggressive behavior than LW pigs and have a also a very low fertility, it will be interesting to go deeper in this DE gene analysis.

Task 5.3. Exploring large resources

The aim of subtask 5.3 is to explore the MISAGEN resource both at level of animal samples and in the form of a relational database focused on disease related traits. A first study using 454 high-throughput sequencing technology targeting the 16S rRNA gene of intestinal microbiota in pigs at different stages of development showed that a strong influence on the microbiota composition has to be attributed to the diet and partial explanation of the diversity is also given by the presence/absence of antibiotics.

In order to determine the effect of breed on the gut microbiota and the microbiota's variation in breeds differing for *Salmonella* positivity, two different studies were performed using the rectal swabs from the large biological repository available at PTP (more than 4.000 piglets) and the corresponding database that has been previously assembled in the framework of a national programme.
In order to study i) the effect of breed on microbiota composition and ii) the effect of microbiota variation in breeds differing for Salmonella positivity (Elisa test), we decided to select samples coming from the same farm and collected in the same period of time to be sure that the animals had received the same diet. The results of the effect of breed on microbiota composition revealed that, in field condition, using a "standard diet", the *Duroc* and *Landrace* breeds have different microbiota compositions that may be influenced by the breed. Although the animals analysed for each breed were not many, the careful selection of the samples should mimic a study in controlled conditions where also a low number of samples should be enough to study the differences between breeds. However, further studies are necessary to confirm the results. Other studies in cattle (Hernandez- Savabria *et al*. Plos one 2013) showed that the frequency of particular microbial rumen phylotypes in the progeny of cattle may be influenced by the sire breed when different diets are fed and ultimately further impact host metabolic functions, such as feed efficiency. We could speculate that also in pig the fecal microbiota composition could be influenced by breed and the differences could be exacerbated by different diets that could have a different impact on important host metabolic functions such as feed efficiency in different breeds.
Our study did not reveal any differences on the effect of microbiota's variation in breeds differing for Salmonella positivity (Elisa test) neither in *Large White* nor in *Landrace* animals. However in these analyses we classified the animal positive or negative to the Elisa test even if the values of positivity were different among the animals. Perhaps other analyses that consider different classes of positivity or that take into account the different values of positivity could change the results.

WP 6: Effect of early antibiotic therapy and hygienic environment on GIT microbiota colonization and the development of GIT function

WORKPACKAGE 6 FACTS

LEADING HOUSE: INRA (3A, INRA-RENNES)
PARTICIPANTS: WU, INRA, ASG, NAU, UNIBO, BMR

OBJECTIVES:

3. Investigate the effect of early alterations of bacterial colonisation by antibiotics in sow-reared piglets on the subsequent development of the microbiota and of GIT anatomy, physiology and immunity, at the end of the suckling period, after weaning and in growing-finishing pigs
4. Elucidate the effect of neonatal environmental hygiene of formula-fed piglets on subsequent development of their microbiota and of GIT anatomy, physiology and immunity in weaned pigs
5. Set up correlates between biomarkers of microbial colonisation/diversity, intestinal physiology and mucosal immunity in pigs subjected to neonatal 'manipulation' of their microbiota
6. Provide basic knowledge and recommendations on rearing factors (antibiotic usage, hygiene) in the neonatal period that can affect GIT development and function after weaning and later in the life of pigs.

A longitudinal experiment carried out by P3 revealed many effects of perinatal administration of antibiotic in mothers on various aspects of gut physiology, biology and immunity in the short-term and in the log-term (in interaction with diet composition in adulthood). These effects include alterations in: ileal and colonic permeability and electrophysiology, nervous regulation of ileal permeability, gut cytoprotection systems (especially HSP70), key digestive enzymes with roles in gut homeostasis (especially intestinal alkaline phosphatase), villus-crypt architecture (especially crypts), lamina propria mononuclear cell responsiveness to various mitogens, and secretion of cytokines (especially TNFa) by gut tissue explants. Furthermore, transcriptomic data as well as information on gut microbiota composition helped findings and to set up correlates between the microbiome and host gut function.
The experiment conducted by Partner 6 showed that i) the protocol for associating caesarean-derived piglets with either a simple or complex microbiota is technically feasible, ii) bacterial composition in faeces and intestinal digesta of CD-derived piglets is influenced in complexity and composition by the inoculation with donor faeces after birth and differences in faecal microbial composition persist until 28 days, iii) the high mortality rate in the group with simple inoculum seems related to an incidental infection. A revised protocol for limiting infection in CD piglets was set up but still raised problems.
The experiment conducted by Partner 7 on the impact of *Streptococcus suis* exposure during the suckling period on the potential pathogen carriages in the gut and its shedding in the faeces of pigs suggest that the defensive barrier of the stomach can be impaired as *S. suis* became dominant after weaning, which may further result in an increase of *S. suis* abundance in the intestine. Oral administration of probiotic *L. amylovorus* can decrease the risk of *S. suis* transmission in pig herds.

Task 6.1. Impact of early antibiotic therapy on gut colonisation, intestinal development, intestinal barrier,cytoprotection systems and immunity in pigs

The main experiment conducted in 2010 aimed at testing the hypothesis that antibiotic-induced alteration in maternal faecal microbiota around parturition would impact offspring microbiota and different aspects of intestinal and colonic function (permeability, enzymes, heat shock proteins, gene expression). Offspring were studied in a short term period (post-natal days PND14, 21, 28 and 42; weaning at PND28), and in a long-term period (PND170) in two nutritional situations (low vs. high fat diet between PND142 and PND170). We observed a number of gut physiology changes in the short and in the long- term periods in offspring born to antibiotic-treated sows as compared to control offspring.

Short-term experiment

Gut permeability ex vivo (Ussing chambers): Ileal basal para-cellular permeability (FD4) was higher in ATBQ than CTRL piglets at day 14. Cholinergic regulation of ileal paracellular permeability was also altered at that age with higher permeability to FD4 of carbachol-stimulated tissues than unstimulated ones in CTRL piglets but not in ATBQ ones. Acetylcholine concentration in ileal mucosa was 48% lower in ATBQ compared to CTRL piglets. Ileal trans-cellular permeability (HRP) was not affected by maternal antibiotic treatment. Under oxidative stress, no treatment or age effects were noted for ileal para-cellular (FD4) and trans-cellular (HRP) permeabilities. Colonic basal para-cellular permeability (FD4) decreased with age but was not affected by antibiotic treatment. Colonic trans-cellular permeability (HRP) was not different between treatments in basal situation. Under oxidative stress, it tended to be higher at day 14 in offspring born to ATBQ sows (treatment by age interaction).

Gut electrophysiology ex vivo (Ussing chambers):

Ileal basal short-circuit current (Isc) tended to be lower in ATBQ offspring than in controls. Trans-epithelial electrical resistance (TEER) was higher (meaning a lower permeability to ions) at day 42 than at day 28 in controls, with no age difference in ATBQ offspring (treatment by age interaction). Under oxidative stress, TEER was lower in ATBQ offspring than in controls. Na-dpt-Glucose absorption and carbachol-induced chloride secretion of the ileum ex vivo were not influenced by the tested factors in either basal or oxidative condition. Colonic Na-dpt-Glucose absorption tended to be higher in ATBQ offspring than in controls (no differences for other variables). Colonic TEER was transiently higher at day 28 in both groups (age effect).

Heat shock proteins: Ileal HSP70 was two- to three-fold lower at day 28 and day 42 in ATBQ offspring than in controls. Ileal HSP27 was not influenced by perinatal ATBQ treatment or age, but interaction approached significance (HSP27 was numerically lower in ATBQ offspring at day 14 and day 42). Ileal HSC70 (cognate HSP70, supposed to be constant) was higher in ATBQ than control offspring, with a significant difference at day 28. Finally, the major HSP transcription factor HSF1 did not reflect these changes since it did not vary at all. We found that ileal HSP60 tended (P=0.070) to be higher in offspring born to antibiotic-treated sows than in controls in the short-term period. Jejunal HSP70 displayed similar effects as in the ileum (ATBQ < Controls). Colonic HSP27 and HSP70 were (surprisingly) higher in ATBQ offspring than in controls (at day 14 for HSP27, and at day 14 and day 42 for HSP70).

Collectively, our data indicate that early disturbances in gut microbiota colonization in pigs influence HSP profiles differently according to HSP type and gut site. Long-term consequences of early alterations in gut microbial colonization did not appear to apply to inducible HSP in our model.

Gut enzyme activities and mRNA levels: Alkaline phosphatase (IAP): Jejunal mucosa specific activity (/g protein) of (IAP) was lower at day 28 and day 42 than at day 14 in ATBQ offspring, with no significant age effects in controls (treatment by age interaction). Ileal specific (g/protein) and total (/g mucosa) IAP activities were 2.5-fold lower in ATBQ offspring than in controls at day 14, with no differences at day 28 and day 42 (treatment by age interaction). These differences were confirmed at IAP mRNA levels. Colonic IAP values were very low and not influenced by the tested factors. IAP activity in offspring caecal and rectal digesta contents was not influenced by ATBQ treatment of sows. Rectal digesta IAP activity decreased with age. Amino-peptidase N (APN): Jejunal mucosa APN activity was unaffected by the tested factors. By contrast, ileal mucosa APN activity was twice lower at day 14 in ATBQ offspring than in controls (treatment by diet interaction). Ileal APN increased with age. Dipeptidyl-peptidase IV (DPP4): Jejunal mucosa DPP4 specific activity was higher in ATBQ offspring than controls at day 42, with no differences at day 14 and day 28 (treatment by age interaction). Ileal mucosa DPP4 activity was higher in ATBQ offspring at day 28 (treatment by age interaction) and it tended to decrease with age. Sucrase: Jejunal mucosa sucrase activity in offspring was unaffected by ATBQ treatment of sows. That of Ileal mucosa was transiently lower in ATBQ offspring at day 14 with no treatment differences thereafter (treatment by diet interaction). Both jejunal and ileal mucosa sucrase activities drastically decreased with age.

Gut villus-crypt architecture: ATBQ treatment effects on the jejunum were seen for (treatment by age interaction): crypt width that was higher in ATBQ offspring at day 14 and day 42, and for absorption surface magnification factor that was lower in ATBQ offspring at day 14. ATBQ treatment of mothers influenced ileal crypt depth (trend), perimeter and surface area that were lower in ATBQ offspring than in controls at day 42 (treatment by age interaction). Both jejunal and ileal mucosa thickness, villus and crypt morphometry, villus height to crypt depth ratio and absorption surface magnification factor were strongly influenced by age in both offspring groups. Finally, colonic crypts were unaffected by ATBQ treatment but crypt depth, width and perimeter varied significantly with age.

Immunology: Levels of milk IgG and IgA were determined at day 14 and 28 after farrowing in sows (n=6 per treatment, second batch). They did not reveal any ATBQ treatment effect. A decrease with age between day 14 and day 28 in milk was observed for IgA but not for IgG. The number of lymphoid follicles in ileal Peyer's patches was unaffected by ATBQ treatment. Their surface area was numerically lower in ATBQ offspring (not significant), but it increased with age. LPMC tolerance to LPS and flagellin apparently took place before day 21, and to LTA between day 21 and day 42 in both groups. LPMC proliferative index was below 1 at day 42. At day 21 and day 42, LPMCs from controls still responded to CpG whereas those from ATBQ offspring were already tolerant. At day 42, no other differences were observed between groups. IFNγ (Th1 cells) secretion was undetectable in response to TLR ligands at all offspring ages. IL-10 (Th2 and Th3 cells) was secreted by LPMCs from both groups at day 21 only, and slightly more by LPMCs from controls than ATBQ offspring, unless in response to CpG. No secretion was detected in both groups at day 42, but an effect of age was noted for responses to LTA, LPS and flagellin. Secretion of TNFα was higher in ATBQ offspring compared to controls at day 21 (significant with CpG).

Gut tissue transcriptomics: Transcriptomic analysis of ileal epithelial cells was performed on ileal samples collected on both 21d-old suckling (at the end of antibiotic treatment) and 42d-old weaned piglets (after the weaning-induced stress). Extracted RNAs were then used to hybridize a Agilent/INRA_Sus scrofa_60K_enriched array with immune system genes. Agilent has developed a transcriptome-wide porcine (pig, Sus Scrofa) microarray v2 with 43603 unique porcine probes represented. A custom repertoire of 3737 60-mer DNA probes targeting transcripts expressed in immune system (Gao et al. BMC Genomics 2010) have been added. Hybridizations were performed at 'INRA LPGP' transcriptomic facilities using dedicated protocol. At each age, differentially expressed genes were considered when the p-value of the difference in ATB vs. CRTL was < 0.001 and <0.01. At d 21, there were 32 (P< 0.001) and 421 (P<<0.01) differentially expressed genes and at d 42, 36 (P< 0.001) and 386 (P<0.01) differentially expressed genes. DAVID enrichment indicated that main antibiotic-induced changes in gene expression (using p values at P < 0.01) concerned immune response and protein metabolism at both stages. In addition differential expression was observed for genes involved in energy metabolism at the end of the antibiotic treatment and for genes involved in cellular organization after the weaning period. Focusing on genes highly differentially expressed (P < 0.001) and considering differentially expressed genes with a FC variation superior to 2 or inferior to 0.5, no common gene was differentially expressed at both d 21 and 42. Genes were clustered according to the time course profile of variation of their FC over the experimental period: genes overexpressed at either d 21 (n=21) or d 42 (n=9) in the ATB group and genes under-expressed at either d 21 (n=11) or d 42 (n= 27) in the ATB group.

At d 21, the decreased expression of BPI fold containing family B, member 1 (BPIFB1, coding a protein that binds to bacterial endotoxins and neutralizes the ability of LPS to stimulate inflammatory cells in vitro and in vivo; Schumann et al. 1990; Elsbach and Weiss 1998), may be a sign of an inflammatory status in the intestine of piglets born from the sows treated with ATB. Decreased expression of BPI has already reported in germfree vs. conventional piglets (Chowdhury et al 2007), indicating a specific response to changes in gut microbial colonization. In addition heparanase gene (HSE) expression was increased 3.2-fold in ATB group. Heparanase has been shown to generate a self-sustaining inflammatory circuit in a model of chronic colitis. Together our results on BPIFB1 and HSE differentially expression may suggest an activation of macrophages and an enhanced secretion of TNFα with the ATB treatment. That corroborates the results we obtained ex vivo with Ussing chamber, an increased permeability at d 14 in the ATB group, and on LPMC cultures, an increased TNFα secretion by LPMC in response to bacterial ligands (LTA, LPS, CpG and flagelin, only significant (P<0.05) with CpG) in d 21 ATB group. Follistatin-related proteins (FRP) interact with CD14, which is known to mediate toll-like receptor 4 (TLR4) signaling. FRP has the function of evoking innate immune responses as one of the endogenous TLR4 agonists when in chronic inflammation conditions (model of rheumatoid arthritis) it decreases secretion of proinflammatory cytokines. That suggests that in ATB conditions the decrease of follistatin-like 4 (FSTL4 that belongs to Follistatin-related protein family) gene expression and that of RAR (that reduce the expression of SOCS3 and its inhibitory action on proinflammatory cytokines) may also favor the secretion of proinflammatory cytokines. Finally the reduced expression of tenascin C gene may suggest specific defects in Th17 cell polarization. Consistent with our hypothesis the ATB treatment affects the molecular cross-talk between epithelial intestinal cells, immune cells and commensal bacteria in the early period of life. It compromised the intestinal barrier and altered the maintenance of intestinal homeostasis. The secretion of TNFα was specifically enhanced by the amoxicillin treatment. Some of the other differentially expressed genes that are involved in control of growth, metabolism and reproduction (AR, CGA, ATP6V0D1) may have additionally a potential immunoregulatory role or be regulated by immune molecules.

At d 42 genes involved in hematopoiesis were differentially expressed in ATB vs CTRL pigs. Specifically decreased CD37, CD33, CCL21, RUNX1T1, CLCF1 and FLT3LG expressions in ATB group suggest a global repression of the immune response in enterocytes. The 2.3-fold increase of RELB gene indicated an activation of NF-κB pathway. Decreased expression of genes encoding cytoskeleton proteins and CDC25C (proliferative activity) and increased expression of an inhibitor of differentiation may reflect a slow-down in speed renewal of intestinal mucosa postweaning that delayed cell shape and migration and wound healing that should occur after weaning. Anecdotally, the expression of C1QTNF2 that has a similar role as adiponectin was 3-fold decreased. Therefore the immunological component of the intestinal epithelial cell was highly affected by the antibiotic treatment at the end of the treatment period, but also later on

after the weaning challenge. Correlation between changes in gene expression and modifications of microbiota diversity and composition would be interesting to evaluate to specify close relationships between bacteria and enterocyte transcriptome.

Microbiota composition in sows and their offspring: In order to characterize the model described here, and to show that antibiotic treatment of sows had repercussion on gut microbiota in both the sows and their offspring, we analyzed the microbial composition in sows' feces as well as in intestinal contents of offspring for a subset of animals using the PITChip phylogenetic microarray. A more detailed description of microbial data will be published separately. ATB treatment of the sows transiently reduced microbial diversity in the ileum of piglets on day 14 after birth. Diversity in the colon was not affected at any time point. Furthermore, composition of ileal microbiota was affected by the maternal ATB treatment, leading to a reduction in the relative abundance of several presumed beneficial bacterial populations such as lactobacilli and bifidobacteria, whereas increased relative abundance of potential pathogenic bacteria, including *Clostridium difficile*, *C. perfringens* and *E. coli*, was associated with the treatment group before day 21. A similar trend was observed for colonic microbiota. Fecal microbiota composition of sows was affected by ATB treatment, leading to a similar reduction in relative abundance of lactobacilli as was also observed for the offspring.

Long-term experiment

Gut permeability ex vivo (Ussing chambers): Ileal para-cellular (FD4) or trans-cellular (HRP) permeabilities were not different between CTRL and ATBQ pigs fed a LF diet. However, while ileal para-cellular permeability was higher with HF than LF diet in CTRL pigs, no such difference was observed in ATBQ pigs. Cholinergic regulation of ileal para-cellular permeability was also affected by both the maternal antibiotic treatment and the HF diet since neither the ileum of CTRL pigs fed the HF diet nor that of ATBQ pigs fed the LF or the HF diet responded to carbachol stimulation. Acetylcholine concentration in the mucosa was not altered by the maternal antibiotic treatment or the diet. Under oxidative stress, ileal para-cellular permeability was twice higher in HF-ATBQ than in LF-ATBQ, LF-Control or HF-Control (interaction). Trans-cellular permeability was not influenced. Colonic basal para-cellular permeability was lower with HF than LF diet in controls while it was higher in HF-ATBQ offspring (interaction). Under oxidative stress, para-cellular permeability tended to be higher in ATBQ offspring than in controls; Trans-cellular permeability was lower in ATBQ offspring than in controls.

Intestinal permeability and sugar absorption in vivo:

Remaining experimental offspring were fasted overnight and then administered orally a bolus of fluorescein sodium (NaF) and D-xylose. Blood plasma was collected at times 0, 0.5, 2 and 24 h after marker administration and markers assayed. Significant [diet by sampling time] and [treatment by diet by sampling time] interactions revealed differences at time 0.5 hour only. At 0.5 h, plasma NaF concentration was lower with the HF than the LF diet (first interaction) and this was essentially due to a two-fold higher NaF concentration in ATBQ offspring fed the LF diet (second interaction). For xylose, a tendency for a diet by sampling time interaction was noted, with lower xylose concentration in HF than LF offspring at time 0.5 hour only. No interaction with ATBQ treatment was observed for xylose.

Gut electrophysiology ex vivo (Ussing chambers): Ileal basal Isc was higher in ATBQ offspring. Na-dpt-Glucose absorption was lower in HF controls than in the other groups. Basal TEER tended to be lower in HF than LF offspring. Colonic tissues under oxidative stress were not influenced by perinatal ATBQ treatment or diet in adulthood.

Microbiota composition in 6 month-old offspring: In contrast to the observations made during the first 6 weeks of life in piglets, no significant effects of ATB on microbiota was observed at the long term, even though there was a tendency for an interaction between ATB treatment and offspring adult diet (P=0.065).

Heat shock proteins: Long-term effects of perinatal ATBQ treatment and effects of diet in adulthood were very limited for HSPs. The only observation was that jejunal HSP27 tended to be higher in HF than in LF controls, with no difference between LF and HF ATBQ offspring.

Gut enzyme activities and mRNA levels:

Alkaline phosphatase (IAP): Jejunal mucosa IAP was twice lower in adult offspring born to ATBQ sows. Also, a treatment by diet interaction was observed: jejunal mucosa IAP was lower in ATBQ offspring fed the LF diet than in the other groups. Jejunal Iap mRNA levels were not significantly affected by perinatal ATBQ treatment or diet in adulthood. However, jejunal Iap mRNA levels were positively and significantly correlated with jejunal IAP activities. Ileal mucosa IAP activity was not affected by ATBQ treatment but it tended to be lower in offspring fed the HF compared to the LF diet. Ileal digesta IAP was not influenced by ATBQ treatment or diet. By contrast, colonic digesta IAP was higher in adult offspring born to ATBQ sows .It was also higher in HF ATBQ offspring than in the other groups (treatment by diet interaction). Amino-peptidase N (APN): Jejunal mucosa APN activity tended to be lower in HF controls and higher in HF ATBQ offspring (treatment by diet interaction). Ileal APN activity did not vary. Jejunal Apn mRNA levels tended to be lower in HF than in LF controls, with no differences due to perinatal ATBQ treatment. However, jejunal Apn mRNA levels were positively and significantly correlated with jejunal APN activities. Dipeptidyl-peptidase IV (DPP4): DPP4 activity was twice higher in the jejunum, and twice lower in the ileum of ATBQ offspring. Sucrase: jejunal mucosa sucrase activity was lower in HF than in LF offspring (diet effect), and were lower in HF than LF controls, with no diet difference in ATBQ offspring (treatment by diet interaction). This interaction was also evidenced at sucrase mRNA level in the jejunum, and strong positive correlations were established between sucrase mRNA level and sucrase activity at that site.

Gut villus-crypt architecture: Jejunal and ileal villi and crypts were not influenced by ATBQ treatment of sows or offspring diet in adulthood, except for jejunal crypt width that was slightly but significantly higher in ATBQ offspring than controls. By contrast, colonic crypt width tended to be lower in ATBQ offspring compared to controls. Crypt depth and perimeter were unaffected.

TLR-stimulants in gut digesta: TLR2- and TLR4-stimulants were determined in ileal and rectal digesta contents. Their concentrations were low and were unaffected by perinatal ATBQ treatment or offspring diet in adulthood. By contrast, both TLR2- and TLR4-stimulants were influenced by these factors in rectal digesta contents. TLR2- and TLR4-stimulants were both higher in ATBQ offspring than in controls. Furthermore, TLR4-stimulants were higher in ATBQ offspring fed the HF diet than in the other groups (treatment by diet interaction). Ileal and rectal dry matter of digesta contents was unaffected by the studied factors.

Immunology: High-fat diet appeared to cancel the tolerance to LTA in ATBQ offspring adults (diet effect and treatment by diet interaction). LPMCs from ATBQ offspring tended to respond less to LPS (or died) than controls. Cytokine secretion by LPMC was unaffected. Secretion of IL-6 and IL-8 by ileal and colonic tissue explants were not affected by the factors tested. In contrast, pokeweed mitogen (PWM)-stimulated secretion of TNFα by ileal explants was blunted in ATBQ offspring compared to controls, irrespective of the diet. Moreover, LPS-stimulated TNFα secretion by ileal explants was lower in HF ATBQ pigs than in the other groups. PWM-stimulated secretion of TNFα by colonic explants was also lower in ATBQ offspring than in controls, irrespective of the diet. LPS-stimulated secretion of TNFα tended to be higher in ATBQ offspring fed the HF than the LF diet.

Task 6.2. Impact of sanitary status after birth on gut colonisation, intestinal development, immunity, disease resistance and performance in pigs

Thirty caesarean derived (CD)-piglets from two sows were delivered (day 0) and were equally divided over two clean experimental rooms. One group of piglets received a simple microbiota on days 1, 2 and 3 (treatment Simple Association; SA), whereas piglets in treatment Complex Association (CA) received additionally a complex microbiota by providing them with a faecal inoculant of an adult sow on day 3 and 4. During the course of the study, starting at day 10, in total nine piglets from the SA treatment group were euthanized due to severe health problems. All of these piglets showed diarrhoea for several days and most of them did not eat and were skinny and lethargic. Autopsy of these piglets indicated signs of a systemic E. coli infection. The piglets from the CA group did not show major health problems during the course of the study. Microbial analyses of faeces collected during the course of the experiment revealed that microbial composition, as determined by Denaturing Gradient Gel Electrophoresis (DGGE) and by Pig Intestinal Tract Chip (PITChip) analysis of 16S ribosomal RNA genes, was less diverse in the SA group compared to the CA group, and a difference in microbial composition persisted until the end of the study (4 weeks after birth). However, in both experimental groups, microbial composition changed over time indicating that the microbiota was not stable in either group of piglets. In the CA group, cluster analysis of the faecal microbial patterns showed that clustering of microbial communities occurred for faeces samples between piglets within this treatment. In addition, the profiles of these faecal samples also clustered with the profile of the faeces from the donor sow. This indicates that transfer and colonization of microbiota derived from donor faeces was effective in the CA group. Less clustering of the bacterial profiles of faecal samples was observed for faeces in the SA group.

Main conclusions were that:

A) The protocol for associating CD-derived piglets with either a simple or complex microbiota is technically feasible.

B) Microbial composition in faeces and intestinal digesta of CD-derived piglets is influenced in complexity and composition by the inoculation with donor faeces after birth. Differences in faecal microbial composition between simple and complex associated piglets persist throughout the period of the study (28 days).

C) The high mortality rate in the “SA group” seems related to an incidental infection with a pathogenic *E. coli* in one piglet and subsequent spreading of the pathogen throughout the entire experimental group, which was housed in one pen. Future studies should be designed to reduce the risk of infection with a pathogen and to avoid spreading of pathogens within experimental groups.

In a **follow-up experiment** the model was extended with an oral enteropathogenic *E. coli* (ETEC) challenge on d 27 after birth and to evaluate the short term response of the piglets to this ETEC-challenge.

Fifty CD-piglets from four sows were delivered (day 0) and were equally divided over two clean experimental rooms and piglets were subdivided in 4 pens per room. One group of piglets received a simple microflora on days 1, 2 and 3 (treatment Simple Association; SA) and piglets in treatment Complex Association (CA) received additionally a complex microbiota by providing them with a faecal inoculant of an adult sow on day 3 and 4.

A total of 9 piglets (4 and 5 of the SA en CA group, respectively) were euthanized during the course of the experiment due to severe health problems. Some piglets were not viable at the start of the experiment and others did not eat and were skinny and lethargic. However, no diarrhoea was observed. Autopsy of these piglets revealed a systemic infection caused by *Klebsiella pneumonia* or *C. perfringens* or *Enterococcus* or haemolytic *E. coli*. In general, in the SA group, piglets encountered health problems from day 6 onwards and in the CA group, piglets showed signs of reduced health from day 11 onwards. Subsequently, affected piglets were treated with antibiotics. On day 16, it was possible to select 6 piglets from the SA group and 6 piglets from the CA group which had not been treated with antibiotics, for

the collection of blood, tissues and chyme. Thereafter, the remaining piglets had to be treated with antibiotics due to severe signs of infection. One week before the end of the study, antibiotic treatment was ceased. On day 27, 6 piglets from the SA group and 6 piglets from the CA group were selected for the collection of blood, tissues and chyme. Finally, on day 27, 8 piglets from the SA group and 9 piglets from the CA group were orally challenged with pathogenic E. coli (ETEC). To reduce the risk of a high mortality rate of CD piglets after an oral ETEC challenge with the standard amount of 1.5E+9 CFU, i.e. the dose used in conventional piglets, the ETEC dose was reduced 15-fold and this dose was chosen "as best guess". The latter dose induced no visible signs of infection and/or diarrhoea in the CD-piglets, neither from the SA group nor from the CA group of piglets. Also no ETEC excretion in faeces could be detected in the two days after the oral ETEC challenge in both the SA and CA group of piglets.
Main conclusions were that:
A) The protocol for growing CD-derived piglets with either a simple or complex microflora needs to be adjusted in order to increase the disease resistance of CD-piglets to bacterial infections during the course of the study, possibly by providing the piglets with irradiated sow's colostrum after birth.
B) It seems safe to use a "conventional" oral dose of ETEC (1.5E+9 CFU) in future experiments with CD-piglets without running the risk of a high mortality among CD-piglets.

Task 6.3. Impact of *Streptococcus suis* exposure during the suckling period on the potential pathogen carriages in the gut and its shedding in the faeces of pigs

The present study investigated the changes in bacterial community composition, with emphasis on *Streptococcus suis* populations as potentially harmful groups, in stomach, jejunum and ileum of piglets after weaning (21 days postpartum) by 16S ribosomal RNA gene-based molecular methods. In addition, effect of oral administration of *Lactobacillus amylovorus S1* on the number of *S. suis* in the colon of piglets was also evaluated by real-time PCR assay.
Six litters of neonatal piglets (8-11 piglets in each litter) from a commercial maternal-line herd (Landrace-Yorkshire-Duroc) were randomly divided into two groups: control and treatment, each with three litters (replicates). Each piglet in the treatment group received one ml of strain S1 preparation (5×10^9 CFU·ml^{-1}) through oral administration at 7 days of age, 2 ml at 9 days, and 3 ml at 11 days. Each piglet in the control group received the same volumes of skimmed milk at the same age. Creep feed was not provided to piglets during the sucking period. Piglets were weaned at 21 days of age. On the day of weaning, sows were removed from the piglets, while piglets remained in the nursing pens. Piglets were fed ad libitum with free access to water.

Denaturing gradient gel electrophoresis (DGGE) profiles of bacterial communities in the stomach, jejunum and ileum of piglets showed that, after weaning, predominant bands related to *Lactobacillus* spp. disappeared and were replaced by potential pathogenic species, such as *Peptostreptococcus anaerobius*, *Moraxella cuniculi*, *S. suis* and *Porphyromonas catoniae*. A specific and sensitive real-time PCR assay was developed for quantification of the important pathogen *S. suis* within gastrointestinal microbiota. The assay showed that *S. suis* predominated in stomach samples of weaned piglets with population levels up to 10^7 copies g^{-1} digesta, while it was not detected in the stomach before weaning. *S. suis* was not dominant in jejunum and ileum digesta before weaning, but became dominant after weaning, with population levels up to 10^7 copies g^{-1} digesta (Table 6.2). The results for the first time demonstrated the post-weaning dominance of the potentially harmful *S. suis* in piglet intestine.
To gain insight into mucosa-associated microbial communities in the intestine, jejunal samples from piglets in L1 were also used to compare predominant digesta- and mucosa-derived microbiota by DGGE analysis. The *L. amylovorus*-related band was also visible in jejunal mucosa of piglets before weaning and faintly visible after weaning, while the predominant band 7, observed only in weaned piglets, did not appear in profiles generated from mucosal samples. These observations were confirmed by quantitative real-time PCR assays on samples of representatives from litter 1. *L. amylovorus*-related populations were detected in mucosal samples until day 21, while numbers decreased below detectable levels after weaning. In contrast, *S. suis* could not be detected in jejunal mucosa of piglets from days 7 to 35. In the control group, no significant difference in the number of *S. suis* in the colon of piglets was found from D21 to D35, however, after adminstation of *L. amylovorus*, the number of *S. suis* on D35 was significantly lower than that on weaning day. The results suggest that the defensive barrier of the stomach can be impaired as *S. suis* became dominant after weaning, which may further result in an increase of *S. suis* abundance in the intestine. Oral administration of probiotic *L. amylovorus* can decrease the risk of *S. suis* transmission in pig herds.

WP 7: Effect of GIT immune modulation on microbiota development. Response to microbial & dietary antigens and effector molecules

WORKPACKAGE 7 FACTS

LEADING HOUSE: ASG
PARTICIPANTS: ASG, UNIBO, BMR

OBJECTIVES:

1. To study the in vivo effects of luminally administered antigenic compounds from bacterial and dietary origin on GIT immune status and the subsequent effects on the microbiota, GIT anatomy, physiology and performance of weaned piglets.
2. To examine in situ, the mechanistic interaction between host-GIT immune status and the microbiota.
3. To integrate the in situ and in vivo effects for a better understanding of the mechanisms underlying the interplay between host-gut immunology, the microbiota and piglet performance.

The results of the research carried out in WP7 show that the early colonization of the GI tract with microbiota in postnatal piglets can be influenced by the intestinal association with a complex microbiota and by diet composition (presence or absence of medium chain triglycerides) in the postnatal period. The effects of the treatments on intestinal microbiota composition last for at least several weeks. Changes in intestinal microbiota composition at early age were found also to influence intestinal gene expression and gut function. E.g. association with a complex microbiota in the postnatal phase, enhanced the capacity of 4-5 week old piglets to detoxify bacterial lipopolysaccharides in the ileum compared to piglets associated with a simple microbiota (SA) at young age. With respect to gene expression specific effects were found on processes related to the (development of the) local immune system in the gut. These results provide the basis for developing further (dietary) intervention strategies for piglets at young age which are helpful in functional development of the gut of young piglets and contribute to the health and productivity of pigs in later stages of life.
Intestinal ETEC challenge as applied during a small intestinal segment perfusion technique (SISP) study is probably too severe to study the more delicate effects of early microbiota association treatments and dietary composition in the first weeks of life on gut functionality and resistance towards ETEC challenge.
Intestinal ETEC challenge changes the degree and nature of the glycosylation of the intestinal mucosa of piglets. The early association of piglets to a complex microbiota (CA) increased the intestinal presence of n-acetylgalactosamine residues and tended to increase galactose residues in the intestinal mucosa of piglets compared to the association with a simplified microbiota (SA). This increased glycosylation is thought to be crucial for proper gut transport functions, defense against microbial agents and immunological activity. The former might contribute to differences in gut responses and functionality between early microbiota association treatments.

Task 7.1. In vivo experiment

An experiment was performed using experimental procedures developed in WP6. CD (caesarian derived) piglets were fed during the first day of life with heat-treated (20 min at 60 °C) colostrum and housed in a clean laboratory environment. Subsequently, all piglets received a starter microbiota (Bristol mix) during the first three days after birth and either (CA) or not (SA) in addition a complex fecal microbiota originating from an adult sow on d 4 and 5. Two dietary treatments were imposed on the piglets from day 5 up to day 21 of age using an automatic feeding system using irradiated diets. The diets contained either soya oil as energy source or medium chain triglycerides in the form of coconut oil. Medium chain triglycerides are dietary ingredients with potential functional properties as energy source, as stimulator of the development of the immune system and having potentially shaping properties towards the intestinal microbiota. The effects of the association and dietary treatments on body weight development, intestinal microbial development, health, gut development and gut functionality were studied. Throughout the experiment the piglets remained generally healthy. Feed intake and body weight gain of piglets did not differ between association and dietary treatments. Analysis of the microbiota composition in jejunal digesta of the piglets on d 21 showed the early microbial association significantly contributed to microbiota variation between treatments ($P<0.05$). There was also an interaction between the microbiota association treatment and the dietary treatment with regard to the intestinal microbiota composition ($P<0.05$). For all CA piglets, there was a significant increase of the abundance of *Streptococcus* species, as well as uncultured *Prevotella*, *Lactobacillus acidophilus*, *Lactobacillus salivarius*, *Clostridium oroticum, Weissella, Mitsuokella multacida* and *Anaerovorax* in approximate genus level. On the contrary, a decrease of *Lactobacillus paracasei*, *Lactobacillus plantarum, Lactococcus, Clostridium cellulosi, Oceanospirillum, Treponema* and *Brachyspira* was detected at the approximate genus of the CA pigs. For piglets receiving the MCT diet, we found an increase of the abundance of *Treponema* and *Lactobacillus paracasei*, whereas the abundance of *Lactococcus* decreased.
Gene expression analysis was of jejunal mucosal tissue of the piglets was evaluated at d 21 of age. The experimental treatments did not clearly separate in the evaluation of the overall genome-wide expression of genes. A tendency, however, was found for differences related to the effect of diet (MCT vs. control). Further statistical testing was

performed to identify which genes were significantly different between the experimental treatments. No significantly different probes/genes were found (FDR (false discovery rate) <0.05). In addition, gene set analysis (GSEA) was performed. In total 109 gene sets were identified as significant between association and/or dietary treatments in a pairwise comparison (FDR<25%). In these comparisons, 71 gene sets were unique. These gene sets were categorized into six 'processes' being 'Immune', 'Putative immune', 'Immune or metabolic', 'Metabolic', 'Generic process' and 'Protein'. These analyses showed that the diet has an effect in both the CA piglets and in the SA piglets. Approximately 50% of the observed enriched gene sets is involved in 'Immune' processes, which suggests that the microbiota association and/or dietary treatments have a direct or indirect effect on the local immune system in the gut. This could be due to the change in intestinal microbiota composition and subsequent alterations in host-microbe interactions. An alternative option is that the association and/or diet have more direct effects on the host, by altering specific genes or gene sets (e.g. dietary antigens and (anti)microbial compounds/constituents in the diet).

Task 7.2. In situ experiment

The small intestinal segment perfusion technique (SISP) was used to understand the mechanistic interaction between host-GIT immune status, microbiota and luminal content. The aim was to investigate whether a neonatal complex microbiota is more effective than a simple microbiota in reducing the effects of an enterotoxigenic Escherichia coli (ETEC) challenge in later life. In addition, the effect of a diet with or without an enrichment in medium chain triglycerides (MCT) was investigated as a tool to modify intestinal microbial colonization. Animals were used originating from the experiment described under Task 7.1. The piglets were studied over a period of two weeks from about three weeks of age onwards using the "in situ" small intestinal perfusion (SISP) technique. Two jejunal and two ileal segments per piglet were perfused with saline for 8 h with or without ETEC ($5x10^9$ CFU). Fluorescein sodium salt (NaF, at 20 mg/kg body weight) was administered intravenously 4 h after the start of perfusion as marker for intestinal permeability and gut barrier function. Throughout 8 h of perfusion, perfusion fluid leaving the intestinal segments was collected and analyzed for NaF and intestinal alkaline phosphatase as a marker for detoxification capacity of bacterial lipopolysaccharides. Total intestinal net fluid absorption was determined as functional read out for intestinal absorption function.Body weights were similar among treatment groups at 4-5 weeks of age (8.3 ± 1.2 kg). ETEC perfusion caused a 5-fold reduction (P<0.001) in net fluid absorption (µl/cm2), a 50% increase (P<0.001) in gut permeability for NaF (ng/cm^2) and a 33% reduction (P<0.02) in IAP concentration (µg/mg protein). Neither neonatal microbiota association nor dietary MCT significantly affected gut net fluid absorption or gut permeability. Piglets expressing a complex neonatal microbiota (CA), however, showed 2-fold higher IAP concentrations in the ileum (P<0.02) but no change in the jejunum compared to piglets associated with a simple microbiota (SA) at young age.
The results show that intestinal ETEC infection has profound effects on gut fluid homeostasis (induction of diarrhea), on gut barrier function (increase in permeability) and on IAP concentrations (decrease in detoxification capacity of bacterial lipopolysaccharides). The ability of neonatal microbiota association and dietary MCT to modulate severe physiological effects of ETEC piglets of 4 - 5 weeks of age is limited. Postnatal ssociation with a complex microbiota, however, may enhance the capacity of 4-5 week old piglets to detoxify bacterial lipopolysaccharides in the ileum.

Task 7.3. Effect of different bacterial and dietary antigens in early life on intestinal glycobiology

The glycosylation of the intestinal mucosa is thought to control several key functions of the gut such as transport functions, defence against microbial agents
or immunological processes. It has been assumed that the gut microbiota may modulate the glycosylation pattern of the intestinal cell layer. To understand if the intestinal glycobiology is affected by the mechanistic interaction between host-GIT immune status, microbiota and luminal content, jejunal mucosa tissue samples from piglets used in the SISP experiment described under Task 7.2 were further analyzed. Intestinal mucosal samples were obtained from 12 piglets previously differently associated with either a simple (SA) or a complex microbiota (CA). Piglets that were subject to the following perfusion treatments were selected for obtaining samples for Task 7.3: enterotoxigenic *E. coli* F4 (ETEC) as pathogenic strain ($5x10^9$ cfu, per each loop), *E. coli* fimbria F4 as pathogen antigen (extracted and purified by partner 11) (0.5 mg, per each loop), *L. amylovorus* (LAB) a supposed beneficial strain ($5x10^9$ cfu, per each loop) and saline as control (CTRL). Perfusion time was 8 h with 8 ml infused fluid per h. Per each loop (total 48 samples), a sample of jejunum tissue was formalin fixed and paraffin-embedded for immunochemistry or snap frozen for molecular analysis. Furthermore, total mRNA was isolated from the small intestinal mucosa collected from each loop. Results for ETEC, LAB and F4 treatments were compared to CTRL. For intestinal glycosylation, as evidenced by the following lectin binding sugars, PNA for galactose, CONA for mannose, SBA for n-acetylgalactosamine, WGA for n-acetylglucosamine, respectively, no interaction was observed between the effect of intestinal loop perfusion treatment during the SISP study and the early life intestinal microbiota association. ETEC challenge during perfusion increased galactose residues and decreased mannose and n-acetylglucosamine residues in intestinal mucosal tissue. LAB treatment during perfusion decreased mannose residues and LAB and F4 marginally decreased n-acetylgalactosamine in this tissue compared to CTRL. The early association of piglets to a complex microbiota (CA)

increased the intestinal presence of n-acetylgalactosamine residues and tended to increase galactose residues, compared to the association with only a simple microbiota (SA).
From the gene expression analysis in intestinal mucosal samples it was concluded that:

- ETEC treatment of the intestinal loops affected the gene expression within hours after the start of the perfusion treatment inducing typical signs of immune and inflammatory responses. The response to F4 and LAB was mostly characterized by the down-regulation of various pathways, not specifically related to distinct pathways.
- The observed upgrade of gene sets related to regulation and activation of lymphocytes in intestinal tissue in piglets associated with a complex microbiota, compared to the association with a simple microbiota is maintained also under conditions of ETEC challenge, indicating that early life microbiota association induces a stable and persistent microbe-host interaction which is not easily disturbed by an acute, severe pathogenic challenge with ETEC.
- In piglets early associated with a complex microbiota the activation of genes related to chemokine and cytokine activity, and in general for responses to external stimuli, induced after infusion with ETEC, was reduced compared with the ETEC challenged loops from piglets associated with simple microbiota, suggesting that a complex microbiota has the potential to dampen anaphylactic or excessive immune responses upon ETEC challenge.

In conclusion, the results showed the complex nature of the interactions between the gut microbiota and the activation of pathways related to changes in the glycosylation of the intestinal mucosa.

WP 8: Peri- and neonatal manipulation of microbiota and gut function–Innovative pro- and prebiotics

Experiments addressing the objectives of the final workpackage within the INTERPLAY project commenced in the second phase of the project, especially because experiments in other WPs, and mainly of WP6, were pivotal in laying the basis for WP8. To this end, activities in the framework of Tasks 8.2 and 8.4 built on more basic experiments dedicated to the establishment of animal models in WP6.

WORKPACKAGE 8 FACTS

LEADING HOUSE: WU
PARTICIPANTS: WU, Univbris, INRA, OVGUMagDE, ASG, NAU, BMR

OBJECTIVES:

1. This workpackage aims to provide novel leads towards the rational design of management strategies for improved farm animal health and robustness based on a sound understanding on the impact of diet and feeding pattern, with special attention to pre- and probiotic supplementation.
2. To understand the impact of diet as well as feeding pattern on GIT microbiota development
3. To test pre- and probiotic approaches for mitigation of adverse effects of neonatal antibiotic treatment, impaired rearing hygiene, as well as exposure to microbial antigens and effectors
4. To exploit phytoestrogens as unconventional prebiotics

Task 8.1. Assessing the effect of diet and feeding on gut microbiota (Partner 2)

The study was designed to test the following two questions: 1) *Does the prebiotic inulin selectively increase the Bifidobacterium and Lactobacillus population in the gut of the neonatal piglet? 2) Does this outgrowth correlate with changes in metabonomics, lipid biology and immune development?*
Litters of piglets from an indoor unit were allowed to suckle for the first 24 hours and then transferred to the SPF isolator and fed on a liquid sow milk replacer (control) or a diet supplemented with either inulin (prebiotic) or starch.
Faecal samples were collected at one, two and three weeks of age and microbial composition analyzed by denaturing gradient gel electrophoresis (DGGE). Piglets were killed at four weeks age and pieces of intestine collected at multiple levels of the gastrointestinal tract for immunological analysis.
Microbial Profiling: Analysis of DGGE profiles revealed that at each sampling point control samples (G1) assembled within a single cluster (with the exception of W3G1_3), in contrast faecal samples from inulin (G2) and starch (G3) fed piglets were mixed and located in separate clusters. Microbial composition of faecal sample from piglets in the control group differed from both the inulin fed and starch fed groups. The variation of microbiota between treatments was confirmed by principal response curve (PRC) analysis (P=0.005). Taken together these results show that both inulin and starch feeding affected the piglets microbiota during the first two weeks of life but by 3 weeks, there was no significant difference in the gut microbiota between control and starch group and only inulin feeding made a significant contribution to microbial variation.
Feeding both starch and inulin have significant effects on both gut microbiota and gut immune cell populations. It is of significance to note that the time course of the effects of starch and inulin differs possibly suggesting either a different site or mode of action. For the present study the choice of starch and inulin was based on differences in their susceptibility to ileal digestibility, inulin being a poorly ileal digestible carbohydrate whilst starch is highly digestible. Starch sources differ in their content of “resistant starch” and therefore there ability to pass through the ileum and into the colon. Potato starch contains only a very small fraction of resistant starch (~ 1.7%) and since it was the source

for this study and it is unlikely to have played a significant role in this experiment. It is therefore unlikely that in contrast to inulin any undigested starch would have passed into the colon. In the present study replacing the carbohydrate source (lactose) with either inulin or starch had no effect growth rate during the first four weeks of life. In contrast by 4 weeks there were significant effects on both gut immune cell populations and gut epithelial barrier function (as indicated by the expression of tight cell junction proteins). Whilst changes in immune cells in the absence of any effect on growth rate might be considered surprising it should be recalled that the present study was performed in SPF isolator reared piglets with only minimal exposure to potentially pathogenic enteric bacteria. The effects of starch may be particularly informative, since feeding this highly ileal digestible carbohydrate elicited changes in immune cell populations and tight cell junctions in the colonic epithelium and lamina propria. It is also noteworthy that by 4 weeks of age when the immunological analyses were performed the faecal microbiota of the starch fed piglets no longer differed from the control fed animals. In contrast the "immunological effects" of feeding inulin were less pronounced or generalized with no effect in the small intestinal lamina propria. Given that the effects of inulin on the faecal microbiota occurred at a later time than in the starch fed group the possibility of later effect on immune function can not be ruled out.

Task 8.2. Ability of a probiotic (e.g. *Lb. amylovorus*) to prevent/attenuate alterations in gut colonisation and intestinal barrier and immune function following neonatal antibiotic therapy in pigs

The experiment conducted in 2012 was a follow-up to the activities performed in WP6 and aimed at testing the hypothesis that these above mentioned gut physiology alterations seen in offspring born to antibiotic-treated sows could be prevented by neonatal administration of the probiotic *L. amylovorus*. This probiotic isolated from pig intestine was shown to reduce the levels of ETEC in the ileum and to stimulate growth in weaned pigs challenged with ETEC. Further investigations on porcine intestinal epithelial cells revealed that *L. amylovorus* was able to protect these cells from an ETEC challenge by different mechanisms (e.g. prevention of pathogen adhesion and IL-10 dependent barrier integrity maintenance). Twenty four parturient sows were distributed into two groups of 12 sows, on the basis of their body weight, parity and sensitivity of some of their faecal bacteria to the antibiotic amoxicillin. All these sows were given oral amoxicillin (40 mg/kg BW/day) between day -10 and + day 21 around farrowing. All the offspring from one group (PROB group) were given orally a (thawed) suspension of *L. amylovorus* (10^9cfu/ml; 1 ml per intervention per piglet) once a few hours after birth, and then 3 times a week for 3 weeks. Offspring from the control litters (CTL group; n=11; one sow farrowed 1 week before the expected date and was, therefore excluded from the trial) received 1 ml of vehicle orally each time, according to the same protocol. Offspring (1/litter) were slaughtered at PND14, PND21 and PND 42 (short term; weaning at PND28; no slaughter at day 28 because of little treatment differences in the first experiment). The rest of the offspring were reared in conventional facilities until PND142 when each group was split into two subgroups. One subgroup in each group continued to receive the 'normal' low fat (LF) diet and the other subgroup in each group started to receive a high fat (HF, palm oil-enriched) diet. Offspring (n=10, from 5 litters in each group; one pair of males or females of close bodyweight within each litter) were slaughtered at PND170 (long term).

Analysis of zootechnical data and plasma proteins of inflammation showed rather limited effects of early probiotic supplementation of offspring born to antibiotic-treated mothers on growth and systemic inflammation status.

Barrier function of the ileum and the colon: Ileal and colonic paracellular (FD4) and transcellular (HRP) permeabilities were measured in (small) Ussing chambers at the time of slaughter in the short- and long-term experiments. The measurements took place in basal condition, under oxidative stress (monochloramine) and after addition of a mast cell degranulation agent (compound 48/80). The results suggested that neonatal *L. amylovorus* supplementation of offspring born to antibiotic-treated sows has diet-dependent long term influence specifically on the transcellular permeability route when gut tissues are facing oxidative challenge or stress involving mast cell degranulation. These data await additional work on tissue density and degranulation state of ileal and colonic mast cells.

Ileal and colonic electrophysiology: Ileal and colonic electrophysiology parameters (basal short-circuit current, Isc; transepithelial electrical resistance, TEER; (electrical) Potential difference, PD; Na-dependent glucose absorption capacity, ΔIsc-Gluc; and carbachol-induced secretory capacity, ΔIsc Carb) were measured in Ussing chambers under voltage-clamp condition, in the different situations outlined above. Early administration of the probiotic *L. amylovorus* to offspring born to antibiotic-treated mothers had contrasted effects on ileal and colonic electrophysiology, sodium-dependent glucose absorption and carbachol-induced chloride secretion capacities. These effects were revealed as 'pure' effects or effects depending on offspring age (short-term exp.) or late diet (long-term exp.) (= interactions). Such effects were sometimes (but not always) revealed under basal condition, while contrasted stress (e.g. oxidative stress or mast cell degranulation) stimulations ex vivo (in Ussing chambers) allowed us to show additional facets of treatment effects in the short and/or long term. Differential responses under oxidative stess could not be related (=were not correlated) with observed differences in tissue expression of inducible HSPs. Conversely, differential permeability responses under mast cell degranulation could actually be correlated with tissue concentrations of mast cell tryptase, a protease involved in pathophysiological responses of the gut (see below).

Intestinal tissue density (ST) - Intestinal and colonic villus and/or crypt architecture: Short-term effects of early probiotic treatment were rather mild and confined to offspring colon while long-term effects were more marked and localized in the distal ileum. These data suggest an influence of *L. amylovorus* on crypt epithelial cell proliferation.
Intestinal and colonic inducible HSPs: A major effect of L. amylovorus early administration to offspring born to antibiotic-treated sows was observed on protein expression of inducible HSPs, with a short-term 'inhibitory' effect on both HSP27 and HSP70 in the distal ileum, and with a long-term 'inhibitory' effect on protein expression of HSP27 (and HSF1) in the proximal colon. This effect tended to indicate lower gut tissue stimulation by the microbiota and was, in addition, site and HSP protein-specific. Links with observed alterations in ileal and/or colonic permeability under stress or mast cell degranulation do not appear to be straightforward. Finally, how the gut microbiota may be involved in inducible HSP modulation remains to be elucidated.
Intestinal and colonic enzyme activities (aminoeptidase N, dipeptidyl-peptidase IV, sucrase and alkaline phosphatase)
Short-term effects of early probiotic treatment of offspring born to antibiotic-treated mothers were mild and restricted to jejunal sucrase and ileal DPP-IV, with a tendency for higher colonic inflammation (IAP) in probiotic-treated offspring. By contrast, long-term effects of this treatment were more marked and affected sucrase (interaction with the diet) and APN activities. These results collectively suggest subtle bacterial-driven modulations of mucosal enzyme profiles.
Gut tissue and plasma concentrations of Mast Cell Tryptase (MCT) (LT exp.): As an attempt to highlight treatment differences observed in gut tissue permeability under mast cell degranulation *ex vivo* (= in Ussing chambers), the assays of mast cell tryptase (MCT) in plasma (ELISA) and in tissues (Western blot) were carried out in the LT exp (LT only, for reasons of time). MCT data strongly suggest an effect of early probiotic treatment on mast cell proteases and activity on gut physiology, as supported by the observed effects of early probiotic treatment on trans-cellular permeability and MCT concentrations in the ileum. Why such probiotic effects on MCT were not significant in the colon, despite observed differences in trans-cellular permeability, may suggest the implication of mast cell compounds other than MCT on colonic permeability (and electrophysiology).
Gut immune response: ***Short-term experiment:***Lamina propria mononuclear cells (LPMC) were isolated from the ileum of piglets at PND21 and PND170. LPMC were cultivated with either concanavaline A (a mitogen), lipopolysaccharide (LPS) or oligonucleotides (CpG). Cell proliferation was assessed by incorporation of 3H-thymidine and TNF-α, IFN-γ and IL-10 release in the supernatants were measured by ELISA. At PND170, ileal and colonic explants were cultivated with LPS or PWM (pokeweed mitogen) to measure secretion of IL-8 and TNF-α. At PND21, no treatment-induced changes in ileal Peyer's patch microstructure and cellular density was observed. Proliferative responses of LPMC isolated from piglets whose mothers had peripartum antibiotic treatment, are similar to the ones that also received direct probiotic supplementation. Probiotic treatment did not change the unresponsiveness to TLR ligands at 21 days. In the first longitudinal experiment (effect of antibiotic treatment), we have shown that antibiotic peripartum treatment of sows affects several aspects of offspring intestinal immunity. LPMC from piglets of antibiotic group secreted less IL-10 and more TNFα to several TLR ligands at 21 days. Here we observed that after treatment with probiotic of piglets from antibiotic mothers, IL-10 secretion by LPMC was dramatically reduced ($P < 0.05$) in response to ConA and even abolished ($P < 0.005$) in response to LPS but unchanged for CpG response at PND21. This result is in contrast with previous *in vitro* findings on porcine enterocytes (IPEC-1 cell line) reporting that the probiotic *L. amylovorus* prevented the pathogen-induced intestinal damages by increasing IL-10 production (Roselli et al 2007). The analysis of transcriptomic data of laser-capture microdissected enterocytes that is going on with the same age pigs, should provide additional data to better understand mechanisms involved in enterocytes and immune cells.
Long-term experiment The rest of the offspring were reared in conventional facilities until PND142 when each group was split into two subgroups. One subgroup in each group continued to receive the 'normal' low fat (LF) diet and the other subgroup in each group started to receive a high fat (HF, palm oil-enriched) diet. Offspring (n=10, from 5 litters in each group; one pair of males or females of close bodyweight within each litter) were slaughtered at PND170 (long term). Probiotic treatment did not change proliferative responses whatever the diet in responses to ConA or LPS. Adult animals were tolerant to LPS and CpG but still responding to ConA: the response was specific to TLR ligands. Neither probiotic treatment nor HF diet seemed to affect cytokine responses in adult animals. In the first longitudinal experiment (effect of antibiotic treatment), we have shown that secretion of TNFα by ileal explants stimulated by PWM was blunted in ATBQ pigs compared to CTRL ones, irrespective of the diet (-57%, $P < 0.05$). Moreover, TNFα secretion by ileal explants was not increased in response to LPS in HF-fed ATBQ pigs as opposed to LF-fed ATBQ pigs or LF-and HF-fed CTRL pigs (+24%, +50% and +48% respectively, $P<0.05$). In the present second longitudinal experiment, probiotic treatment reversed deleterious effect of antibiotics. Indeed, antibiotic-induced depressed secretion of TNFα by ileal explants in response to PWM was elevated in LF-fed pigs early treated with probiotics ($P < 0.05$). In contrast this result was not observed in HF-fed pigs. Moreover, we confirmed that antibiotic-treated pigs were unresponsive to LPS when fed HF diet, as illustrated by no increased secretion of TNFα by ileal explants in response to LPS. When early treated with probiotics, the responsiveness of ileal explants to LPS was recovered whatever the diet ($P < 0.05$).

Task 8.3. Effect of peri- and neonatal administration of phytoestrogens: daidzein, genistein, hesperidin

The aim of this task was to investigate effects of phytoestrogens on pig health and microbiota. Isoflavonic compounds abundant in soy are a group of phytoestrogens. Among these, daidzein can have beneficial effects. Corn-soybean meal type of diet was widely used in pig produce. In previous studies, more attention was focused on the effects of daidzein and other phytoestrogens on growth performance. However, little information is available on their effects on the gut health and microbial ecology of pigs. In this task, we investigated the effects of daidzein on the development of gastro-intestines (GI), immune organs and microbial community in the GI tract of the newly suckling piglets.
Neonatal piglets from three litters were randomly divided into two groups within each litter. Half piglets from each litter were fed with 1, 2 and 3 mg of daidzein-skimmed milk at 7, 9 and 11 days of age, respectively, and other half were fed with skimmed milk as control. Creep feed was not provided to piglets during the sucking period. All piglets were weaned at 21 d. On the day of weaning, sows were removed from the piglets, while piglets remained in the nursing pens. Piglets were fed ad libitum with free access to water. At 14, 21, 24 and 35 d, within each litter, one piglet from each group was weighed and slaughtered, and spleen and thymus were weighted. Sections of stomach bottom, proximal jejunum, ileum, and proximal colon were removed, opened longitudinally, and fixed by immersion in 10% (v:v) phosphate buffered formalin for histologic study. Serum was collected for cortisol measurement.
Results showed that daidzein increased the number of the oxyntic cells in fundic gland significantly ($P < 0.05$). But no apparent difference was observed in the thickness of the fundic gland. Duodenal and jejunal villus had similar development pattern, becoming shorter 3 d after weaning and recovered at 35 d. There was no apparent difference between daidzein group and the control. Mast cell numbers in fundic gland in the control increased just after weaning and decreased at 35 d. Daidzein could significantly reduce the mast cell number at 21 d and 24 d. A decrease in weight of thymus and spleen was observed in the control group but not in daidzein group, at two weeks post weaning, especially the thymus of daidzein group was heavier than that of control group. Histamine concentration increased after weaning in the control in ileum, but daidzein could significantly reduce the level. In jejunum, daidzein did not have apparent effect.

Task 8.4. Pre- and probiotic mitigation of impaired environmental hygiene and antigen exposure

An experiment was conducted using 108 conventionally born piglets kept under normal farming conditions during the suckling phase of their lives. In the postweaning period, all piglets were exposed to impaired environmental hygiene and antigen exposure by oral administration of pathogenic *E coli* (ETEC). The effects of prebiotics (in-feed medium chain triglycerides and organic acids) and probiotics (in-feed Bacillus subtilis) were compared to the effects of neonatal i.m. antibiotic (tylosine) treatment, neonatal oral administration of a complex microbiota (faeces from a healthy mature sow), in-feed antibiotic treatment (tylosine) and control (no administration). In short: 18 sows delivered 6 piglets each for the study. The piglets were born in week 50, 2012. The day after birth, 6 piglets per litter were selected and balanced on gender and body weight over the 6 experimental groups. In practical terms this means that per litter, the day after birth, one piglet (plus one reserve) was systemically treated with antibiotics, one piglet (plus one reserve) was orally inoculated with faeces originating from an adult sow and four piglets (plus two reserves) received a placebo treatment (oral inoculation with saline) for inclusion in the other treatment groups. Eighteen piglets were used in each of the six experimental groups. Blood and intestinal microbiota were studied at 6 and 15 days postweaning, which corresponds with the pre and post ETEC phase.
Throughout the experiment the piglets remained generally healthy with the exception of transient diarrhea after the ETEC challenge. Feed intake and body weight gain of piglets did not differ between treatments. Focusing on ETEC shedding in faeces after ETEC challenge, the probiotic treatment induced the lowest shedding ($P<0.05$) of ETEC compared to the 5 other treatments. With respect to faecal consistency, the probiotic and the in-feed antibiotic treatments induced the thickest faeces ($P<0.05$), the prebiotic treatment induced intermediate thickness of faeces and the other 3 treatments showed the most fluid faeces after ETEC challenge. On day 15 postweaning, plasma acute phase protein (C-reactive protein) concentrations were higher ($p<0.05$) in the probiotic and both antibiotic treatment groups, intermediate in the prebiotic group and lower in the control and complex microbiota groups. Plasma C-reactive protein is thought to reflect the endogenous capacity of the body to repair and counteract tissue damage suggesting that specific pathways of the immune system were activated by the probiotic and antibiotic treatments. The probiotic treatment stimulated several bacterial phylotypes including healthy related *Lactobacillus plantarum* and butyrate producing *Clostridium* such as *Eubacterium rectal*. The prebiotic treatment had a general inhibitory effect on many bacterial phylotypes not specifically related to health and disease. Also, i.m. antibiotic treatment inhibited the relative contribution of many bacterial phylotypes, not specifically related to health or disease. By contrast, in-feed antibiotic treatment stimulated the potential pathogenic microbiota and inhibited the healthy related lactobacilli.
Overall these results suggest that:

1) in-feed probiotic (*Bacillus subtilis*) treatment is effective in reducing colonization of ETEC and diarrhea in ETEC challenged piglets while stimulating several bacterial phylotypes including healthy related *Lactobacillus.*
2) In-feed prebiotic (medium chain triglycerides and organic acids) treatment is intermediate effective in reducing diarrhea in ETEC challenged piglets without affecting specific phylotypes of the intestinal microbiota.

4. POTENTIAL IMPACT, MAIN DISSEMINATION ACTIVITIES AND THE EXPLOITATION OF RESULTS

The strategic aim of the research carried out in the framework of the INTERPLAY project is to contribute to maintain European pig production in a worldwide leading position for the implementation of sustainable management strategies that promote optimal animal health and welfare, alongside food and consumer safety. INTERPLAY aimed to generate a knowledge base needed to support the Common Agricultural Policy objectives aiming to provide sustainable development of agriculture.
Considerable concern has been expressed about the increase in antibiotic resistance after the ban of in-feed antimicrobial additives. Nevertheless, current efforts to replace these compounds in animal production are often based on trial-and-error and black-box approaches. Hence, the INTERPLAY project directly addresses this important issue by aiming at the generation of sound understanding of the interactions of the early colonization by commensal and potentially pathogenic microbiota with the developing gastro-intestinal tract of pigs directly after birth, but also specifically around the time of weaning. This is the time when the growing farm animal is especially vulnerable, as its defences are not fully developed yet. Furthermore, especially the time around weaning, the animal is subject to multiple stresses, making it susceptible to the increasing disease pressure. This situation is especially relevant in pigs. Furthermore, the pig is recognised more and more as a large model for biomedical research with respect to gastrointrestinal function. Similarities between the swine and human include GIT developmental programme, microbiology, anatomy , physiology, mucosal immunity, genomics, protein digestion and metabolism and nutrition. Investigations carried out in INTERPLAY in the pig species are not only of importance to animal production, but also of major interest to human health.

INTERPLAY succeeded in having ***impacts*** **to achieve EU policy objectives** both in support of the science and dissemination strategy (**European Research Area**) and the implementation of the new strategic directive on Animal Health and disease **"Prevention is Better Than Cure",** especially through

- Providing a **novel and appropriate animal health framework** by choosing **a truly holistic approach**, taking into account important aspects that contribute to more robust and healthy animals in the European livestock production setting
- Understanding the development of pig GIT function, starting from a **new concept**, where **health is not defined as the absence of disease in animals**, but aims to provide knowledge-based measures of **robustness**, taking into account the **critical relationship between the health of animals and their welfare**. This also **links to the EU policy on public health and food safety**. Specifically, INTERPLAY addressed two important zoonotic microbial populations, namely *Salmonella* sp. and *Streptococcus suis*, in addition to ETEC (enterotoxigenic *Escherichia coli*).
- Linking **directly into the goals of the new strategy on Animal Health and disease**, INTERPLAY has provided the necessary knowledge that is required for the rational design of more sustainable management practices at the farm level
- **Improving communication between the researchers working at recognised centres of excellence and at the various levels** within the system (microbiota, immune system & disease resistance, performance) through increased awareness of the researchable issues at a European level. The awareness has been enhanced through discussion of the issues across the system through workshops organized by the consortium as well as through active contributions to symposia and conferences.
- **Dissemination and exploitation of EU research** results through the links (**network**) created between research scientists and EAAP, EADGENE, FABRE, and other partner stakeholder contacts across Europe. In particular through adopting a knowledge interaction approach to help solve stakeholder / end user problems and by providing more effective information interfaces (**INTERPLAY website, Stakeholder Forum**).
- Specification **of the RTD needs** in relation to sustainable animal production end users not only in EU member countries but also associate, candidate and neighbour countries, relevant to the problems of these countries. This is best exemplified by the fact that INTERPLAY has embraced a Chinese institution as a full partner, and organized one of its workshops in China.
- **Improving communication to European scientists, stakeholders/ end users** and the public through round table discussions and a public website of the potential benefits of research and technology

Impacts at the Livestock production, Feed industry sector and Farm levels and Consumers to ensure that the new knowledge can be translated into practice have been facilitated by improved application of the research outputs to generate knowledge-based guidelines towards more sustainable pig production that will lead to safer food

- INTERPLAY has provided **science and technology** that is applicable on farm through a participatory approach to research and dissemination
- INTERPLAY has established **knowledge and tools (biomarkers of microbiota)** that can lead to the development of easy-to-use measures to be used by farmers and their advisors to optimise systems
- INTERPLAY has provided **novel leads towards more robust breeds** that can be used in more sustainable farming systems without the need of extensive use of antimicrobials for therapeutic purposes - based on the understanding of the effect of host genotype (i.e. different breeds) on intestinal microbiota composition, and correlation of these effects with health and performance parameters.
- INTERPLAY has provided new insights on novel dietary approaches to reduce the adverse effects on animal health that are associated with intensive rearing systems. It is to be expected that these findings will be exploited by feed additive manufacturers.

Impacts at the European and International science community through "beyond the state of the art" approaches and technologies are

- The technological possibilities to study complex microbial ecosystems are advancing at an impressive rate, allowing for the first time, to (re-)address several fundamental questions related to, microbiota (functional) composition and dynamics, syntrophic relations and interactions, and ecosystem functionality. The INTERPLAY activities further strengthened our understanding of the highly complex and relevant GI-microbiota in the developing pig. Furthermore, investigations performed in the pig species are not only of importance to animal production, but also of major interest to human health.
- INTERPLAY has provided **innovative and high-throughput methods** accurately to describe the host-microbiota-interactome in the developing animal. The molecular signatures obtained by INTERPLAY will enable validation and/or falsification of proposed molecular mechanisms of host-microbe interactions in the porcine intestinal tract
- Collaboration by the recognized centres of excellence in Europe managed to deliver a major improvement in our understanding of the intricate GIT-microbiota interactions in the pig. In particular **the role of the dynamics at which colonising micro-organisms interfere in gut development** is of interest not only to the pig research community
- The project has derived innovative knowledge from a truly holistic and interactive approach of a **complementary and multidisciplinary team**
- INTERPLAY has identified **new biomarkers** in biological samples to understand microbial activities and interaction
- INTERPLAY realized its mission to form a **virtuous circle of strategic research** linked to model development in an innovative knowledge interaction approach and could provide a model for technological development to both enhance competitiveness and achieve environmental objectives

Main Dissemination Activities & Exploitation of Results

The INTERPLAY project has implemented a dedicated workpackage (WP-9) for the organisation and streamlining of activities within the consortium with respect to the dissemination and exploitation of project results. This WP had as a three-fold general objective; i) to ensure appropriate embedding of the INTERPLAY project in the rapidly progressing scientific environment, ii) to achieve the biggest possible technological, economic and public use of the results obtained within the project, and iii) to actively communicate the project outcome and its impact on the general audience to the general public. Thereby, three main audiences have been targeted that are in addition representing the complete chain of stakeholders for the INTERPLAY consortium, providing a bottom-up approach to help the process of consensus-forming around the development and use of new scientific and technological developments. Dissemination is considered to be an integral task of the project and relevant stakeholders were involved from the very beginning and in public dialogue. A public website has been set up allowing easy access to information concerning project objectives and partners from outside of the consortium. Furthermore, a stakeholder forum facilitated and strengthen the possibilities not only for the interactive dissemination of project results into the scientific community, but also to further support one of the key objectives of INTERPLAY, namely to provide the

necessary understanding for the rational design of improved management strategies towards more sustainable animal production.
A first international scientific workshop was held at the premises of partner 7 (Nanjing Agricultural University) in order to foster the scientific dialogue with Asia. A second workshop geared more towards industrial stakeholders has been organized at the end of the INTERPLAY project, as a specific session at one of the major conferences in animal production, EAAP, in August 2013.

INTERPLAY intranet
To facilitate information flow and storage of documents too large for the website, INTERPLAY has its internal server, which is located on intranet server of WU. All the project documents are stored here – presentations of partners during project meetings are placed here as well as documents for preparation of the reports. Partners each have their own access to the platform, which is managed by WU, and can easily upload or download relevant documents..

INTERPLAY Public website
Visibility to the INTERPLAY project was created in first instance by its website that is hosted on the Wageningen University server www.interplay.wur.nl.
The website has a number of separate pages with information, which direct readers to specific aspects of the project, including project goals, partners, and news items and other activities, and is frequently updated.

INTERPLAY Stakeholder Forum
INTERPLAY has initiated a Stakeholder Forum (SF) to facilitate and strengthen the possibilities not only for the interactive dissemination of project results into the scientific community, but also to further support one of the key objectives of INTERPLAY, namely to provide the necessary understanding for the rational design of improved management strategies towards more sustainable animal production, aiming for highest possible impact of the results produced throughout the runtime of the project.
Partner 8 (PTP) played a coordinating role in the SF. PTP is a young and highly active SME with the mission to combine fundamental and pre-competitive research relevant for crops and domestic animal production with technology transfer activities to promote the translation of gained knowledge and diagnostic products into practical applications for animal science and agrifood end users. Hence, PTP is ideally prepared to coordinate INTERPLAY initiatives directed at the following **stakeholders groups, representing the complete chain** concerned by the topics of the call:

- Research networks, including, but not restricted to, those where members of the INTERPLAY consortium play a leading role, such as
    - FP6-EADGENE (*European Animal Disease Genomics Network of Excellence for Animal Health and Food Safety, represented by Dr. Mari Smits, Partner 6*),
    - FP6-SABRE (Cutting edge genomics for sustainable animal breeding, represented by Dr. Chris Warkup, Coordinator),
    - EAAP (*European Association of Animal Production*) can provide an important forum for dissemination, as successfully achieved in the framework of a previous FP6-project (FeedforPigHealth, 2004-2007). The Secretary General of EAAP, *Dr. Andrea Rosati*, has already expressed his strong support for Interplay, and was looking forward to participation in the SF.
    - FP6-COST action *PigNet*, FP7-METAHIT (*Metagenomics of the human intestinal tract*) and FP7-Marie Curie Initial Training Network CROSS-TALK.

It should be noted that activities in the framework of the Interplay project have been complementary to those in above-mentioned programmes (especially with respect to SABRE), rather than mere duplication.

- Industrial stakeholders, including manufacturers of feed stuff and addititives, and breeders.
    - The majority of partners within INTERPLAY has collaborations with industrial stakeholders. In addition, several members of INTERPLAY are partners in FABRE-TP (***F**arm **A**nimal **B**reeding and **Re**production **T**echnology **P**latform*), a platform to support stakeholder involvement in the set up of partnership by industry.
    - Close ties also exist with the *Genesis Faraday Partnership*, a non-profit business with > 100 member organisations across Europe that aims at improving the interaction between researchers and the animal health and animal breeding industries. The Director of this network, *Dr. Chris Warkup* from the beginning of the project expressed his enthousiasm with respect to INTERPLAY.
- Societal stakeholders such as the EFSA (*European Food Safety Association*) can provide the necessary link to consumer organisations with respect to important issues such as food and consumer safety. *Prof. Dr. Atte van Wright*, member of the FEEDAP (Panel on additives and products or substances used in animal feed), participated in the Stakeholder Forum.

INTERPLAY workshops

The scientific community addressed by the project is extremely wide, ranging from a number of different disciplines within the life sciences to computer scientists: in addition to publications and participation in relevant workshops and conferences, INTERPLAY has organised 2 international workshops.

The **first scientific workshop** has been dedicated to an outreach activity in Nanjing, China, at the location of Partner 7 at the occasion of the 110th Anniversity of Nanjing Agricultural University. From November 6th - 8th, 2012, the first of two international workshops was held at the Hanyuan International Conference Hotel, Nanjing Agricultural University (Nanjing, China).

With over 70 participants from China, Europe and Australia, various aspects of the INTERPLAY of Microbiota and Gut Function in Pigs, the central theme of INTERPLAY, were covered in 3 invited key note presentations delivered by Ole Hojberg (Aarhus University, Aarhus, Denmark), Li-Ping Zhao (Shanghai Jiaotong University, Shanghai, China) and John Pluske (Murdoch University, Perth, Australia), as well as a number of overview presentations from INTERPLAY partners and contributions from researchers from across China.

The **second International workshop** was held as a dedicated session at the EAAP conference in 2013 in Nantes, France, on August 29th, 2013. During this ½-day workshop, major achievements of the INTERPLAY project, with specific attention to their application towards more sustainable pig production, were presented in a number of keynote presentations made by project members, as well as several smaller contributions from within and beyond the consortium.

Publications & Participation in Conferences

Members of the consortium have very actively contributed to the dissemination of the generated foreground through publication as well as participation in a large number of general and specific national and international workshops, symposia and conferences as detailed in the periodic reports as well as in Section 4.2 of the final report.