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LIVESTOCK RESEARCH
WAGENINGEN UR

Feed4 Foodure

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M.M. van Krimpen, M.M. Hulst, J. van der Meulen, D. Schokker[1]
H.F.J. Savelkoul, E.J. Tijhaar[2]
V.P.M.G. Rutten[3]

1 Wageningen UR Livestock Research

2 Wageningen UR

3 Utrecht University

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Samenvatting NL

Het huidige rapport omvat een literatuurstudie naar diverse aspecten die gerelateerd zijn aan de immuun competentie van landbouwhuisdieren. Deze aspecten, die in hoofdstuk 1 geïntroduceerd worden, zijn:

- Het aantonen van verbanden tussen functionele componenten in diervoedergrondstoffen en de expressie van genen/biologische processen, die van invloed zijn op de darmgezondheid van landbouwhuisdieren;
- Het beschrijven van modellen die gebruikt kunnen worden bij onderzoek naar effecten van voedingsinterventies op immuun gerelateerde kenmerken in landbouwhuisdieren;
- Het samenvatten van effecten van nutritionele interventies in de maternale, neonatale en post-neonatale fase op de ontwikkeling van het aangeboren en verworven immuunsysteem;
- Een literatuurstudie naar de relatie tussen het immuunsysteem in de darm en in de bovenste luchtwegen, waarbij ingegaan wordt op de vraag hoe voedingsinterventies het immuunsysteem in de bovenste luchtwegen kunnen ondersteunen.

Summary UK

The current study covers a review of literature regarding a number of topics related to immune competence in farm animals, which are introduced in chapter 1. These topics are:

- A demonstration of the relationship between functional feed components and the expression of genes/biological processes that are involved in gut health of farm animals;
- A description of available models, that can be used to investigate the effects of nutritional interventions on immune related parameters in animals;
- A review of the effects of nutritional interventions in the maternal, neonatal and post-neonatal phase on the development of the innate and acquired immune system;
- A review of the relationship between the immune system in the gut and in the upper airways, whereas the question will be addressed how the immune system in the upper airways can be affected by nutritional interventions.

Livestock Research Report 800

Table of contents

Foreword

Feed4Foodure is a public-private partnership between the Dutch Ministry of Economic Affairs, a consortium of various organizations within the animal production chain and Wageningen UR Livestock Research. Feed4Foodure aims to contribute to sustainable and healthy livestock farming in the Netherlands, simultaneously strengthening our competitive position on the global market. The Feed4Foodure program line “Nutrition, Intestinal Health, and Immunity”, aims to contribute to a reduction in the use of antibiotics in livestock farming by increasing general health and disease resistance. The main goals are to develop innovative measurement techniques and to test new health-promoting nutritional additives in the field of gut health and immunity.
The current study covers a review of literature regarding a number of topics related to the focus area of the Feed4Foodure program line “Nutrition, Intestinal Health, and Immunity”. These topics are:
The impact of functional feed components on the expression of genes/biological processes that are related to gut health of farm animals;
Available models, that can be used to investigate the effects of nutritional interventions on immune related parameters in animals;
The effects of nutritional interventions in the maternal, neonatal and post-neonatal phase on the development of the innate and acquired immune system;
The relationship between the immune system in the gut and in the upper airways, and the possibilities to affects the immune system in the upper airways by nutritional interventions.
For the current study, scientist of Wageningen UR Livestock Research, Wageningen University and Utrecht University worked together with representatives from the various private partners, including Agrifirm, ForFarmers, Nutreco, De Heus, Denkavit, and Darling Ingredients International. The authors thank the industry partners of the project team for their worthwhile input.

Dr. Mari Smits, leader Feed4Foodure program line “Nutrition, Intestinal Health, and Immunity”.

Samenvatting

Besmettelijke dierziekten hebben een sterk remmend effect op het welzijn van dieren, het efficiënt gebruik van nutriënten en de ecologische footprint van de dierlijke productie. Voeding kan bijdragen aan het vergroten van de immuun competentie en daarmee aan het beperken van de incidentie van ziekten. Immuun competentie wordt gedefinieerd als het vermogen van het immuunsysteem om op adequate wijze te reageren op een antigene stimulus door middel van een evenwichtige immuunrespons met een goede balans tussen tolerantie en ontsteking. Het belang van immuniteit versterkende diervoeding is toegenomen, omdat bacterieremmers, anti-parasitaire toevoegmiddelen en sommige andere additieven die de diergezondheid bevorderen omwille van de volksgezondheid niet meer toegevoegd worden aan het voer.
Het huidige rapport omvat een literatuurstudie naar diverse aspecten die gerelateerd zijn aan de immuun competentie van landbouwhuisdieren. Deze aspecten, die in hoofdstuk 1 geïntroduceerd worden, zijn:

- Het aantonen van verbanden tussen functionele componenten in diervoedergrondstoffen en de expressie van genen/biologische processen, die van invloed zijn op de darmgezondheid van landbouwhuisdieren;
- Het beschrijven van modellen die gebruikt kunnen worden bij onderzoek naar effecten van voedingsinterventies op immuun gerelateerde kenmerken in landbouwhuisdieren;
- Het samenvatten van effecten van nutritionele interventies in de maternale, neonatale en post-neonatale fase op de ontwikkeling van het aangeboren en verworven immuunsysteem;
- Een literatuurstudie naar de relatie tussen het immuunsysteem in de darm en in de bovenste luchtwegen, waarbij ingegaan wordt op de vraag hoe voedingsinterventies het immuunsysteem in de bovenste luchtwegen kunnen ondersteunen.

Het combineren van informatie over enerzijds de chemische kenmerken van natuurlijke bioactieve stoffen en anderzijds hun mogelijke effecten op expressie van genen die betrokken zijn bij darmgezondheid kan bijdragen aan het voorspellen van hun biologische activiteiten in het dier zelf. Dergelijke informatie is van veel natuurlijk voorkomende bioactieve stoffen vastgelegd in online beschikbare databases en op basis hiervan kunnen bioactieve componenten geselecteerd worden die heel gericht onderzocht kunnen worden in dierexperimenten. Hoofdstuk 2 geeft een overzicht van genexpressie datasets, die gekoppeld zijn aan twee datasets waarin informatie over diervoedingsstudies is opgeslagen. Ook bevat dit hoofdstuk een handleiding die beschrijft hoe de informatie in deze databases te gebruiken is om - op basis van de chemische samenstelling van de betreffende bioactieve stof, die de potentie heeft om de expressie en functie van specifieke genen en processen te beïnvloeden – te voorspellen welk mogelijk effecten er kunnen zijn op de darmgezondheid. Hoofdstuk 3 beschrijft de kenmerken, mogelijke toepassingen en voordelen van verschillende typen *in situ, ex vivo, in vitro* en *in vivo* modellen, die gebruikt kunnen worden voor het onderzoeken van immuno-modulerende onderzoeksvragen. In dit hoofdstuk worden de voordelen en beperkingen van de meeste modellen besproken. De nutritionele interventies die rechtstreeks of indirect (via de microbiota) van invloed zijn op de ontwikkeling van het aangeboren en verworven immuunsysteem, zowel in de maternale, neonatale als post-neonatale fase, worden besproken in hoofdstuk 4.
Het is de vraag of voedingsinterventies, naast een effect op het immuunsysteem in de darm, gelijktijdig ook een effect kunnen hebben op het immuunsysteem van de bovenste luchtwegen. Deze vraag komt aan de orde in hoofdstuk 5.

In hoofdstuk 2 wordt geconcludeerd dat de hoeveelheid databases met genexpressie data en andere 'omics' data in openbare bio informatica databases snel toeneemt. Het koppelen van dergelijke data afkomstig van diervoedingsexperimenten aan die van 'omics' onderzoek gericht op darmgezondheid, zoals in deze studie is uitgewerkt voor het probioticum *L. plantarum,* kan de zoektocht naar nieuwe en

alternatieve diervoedingsadditieven in de juiste richting sturen. Daarnaast kan een dergelijke integrale benadering leiden tot de ontdekking of verbetering van bioactieve componenten. Tevens kan hierdoor het gebruik van dure diermodellen bij het verwerven van de eerste inzichten m.b.t. de potentie van specifieke componenten als nieuw diervoederadditief beperkt worden.

In hoofdstuk 3 zijn de diverse soorten *in vitro, ex vivo, in situ* en *in vivo* modellen beschreven. Bij de *in vitro* modellen wordt onderscheid gemaakt tussen primaire cellen, epitheliale "mini-darmen", cel culturen (een, twee- en driedimensionaal) en darmen-op-een chip. De *ex vivo* modellen worden onderverdeeld in perfusiesystemen, de omgekeerde zaktechniek, de Ussing kamer, de *in vitro* orgaan cultuur (IVOC, zowel gepolariseerd als niet-gepolariseerd), de nieuwe gepolariseerde *ex vivo* orgaan cultuur, de IVOC in de Ussing kamer, de precieze weefselsnede plakjes (PCTS) en het varkensdarm model. Voorbeelden van *in situ* modellen zijn het darmlus model en de techniek, waarbij segmenten in de dunne darm doorgespoeld worden (SISP). Tot slot worden sommige *in vivo* modellen kort besproken. Het hangt van de (wetenschappelijk) vraagstelling af welk model het beste gebruikt kan worden. Hoewel tot nu toe nog niet alle modellen gebruikt zijn voor onderzoek naar immuniteit van mucosaweefsel, zijn alle modellen in principe wel in staat om met behulp van mucosaal weefsel en/of darmvloeistof het effect van interventies op immunologische processen te meten.

Hoofdstuk 4 beschrijft de directe en indirecte (via de microbiota) effecten van voedingsinterventies op de ontwikkeling van het aangeboren en verworven immuunsysteem, zowel in de maternale, neonatale en post-neonatale fase. Met betrekking tot voedingsinterventies in de maternale fase kan geconcludeerd worden dat er positieve transgenerationele effecten op de darmgezondheid en immuun competentie van het nageslacht zijn aangetoond bij zowel zoogdieren (muizen, ratten, varkens, mensen) als bij pluimvee. In deze fase zijn interventies uitgevoerd met vetzuren, zeewierextracten, pre- en probiotica, vitaminen en mineralen. De interventies verschillen in werkingsmechanisme. Afhankelijk van het type interventie zijn er effecten gemeten op de samenstelling van de microbiota, aantal en activiteit van specifieke immuun cellen, morfologie van de darmwand en de expressie van genen die betrokken zijn bij een immuunrespons. Sommige toevoegingen aan het voer van de ouderdieren resulteerden in verbeterde technische resultaten of een lagere incidentie van diarree bij de nakomelingen. Dergelijke transgenerationele effecten zijn aangetoond bij toevoeging van zeewierextract, *Saccharomyces cerevisiae*, zink en β-caroteen. Slechts een heel beperkt aantal studies rapporteerden transgenerationele effecten van de interventies op de expressie van genen die betrokken zijn bij immuun competentie. Alleen in studies, waarin omega-3 vetzuren of vitaminen/mineralen werden toegevoegd zijn deze effecten gemeten. Toevoeging van specifieke vetzuren aan maternale voeders, met name vetzuren afkomstig uit visolie, hadden een sterk positief effect op diverse aspecten van de immuun competentie van de nakomelingen. Op basis van deze resultaten kan daarom gesteld worden dat beïnvloeding van het vetzuurprofiel van het voer van ouderdieren veel perspectief biedt voor verbetering van de immuun competentie van de nakomelingen.
Op basis van de studies die gericht waren op het effect van voedingsinterventies op de immuun competentie in de neonatale fase van biggen kan vastgesteld worden dat met name prebiotica en bloedplasma voor deze diercategorie potentiële additieven zijn. Aanbevolen wordt om deze interventies in vervolgonderzoek mee te nemen. Uit onderzoek met jonge kuikens bleek dat het verstrekken van tarwe/sojaschroot voeders (in vergelijking met maïs/sojaschroot of tarwe/rogge/sojaschroot voeders), het toevoegen van de zwavelhoudende aminozuren methionine + cysteine boven het niveau dat nodig is voor het bereiken van maximale dierperformance, en het verstrekken van vitamine E (100 IU/kg) samen met selenium (0,2 ppm) in vergelijking met een voer dat geen vitamine E en selenium bevatte, positieve effecten had om immuun gerelateerde parameters (o.a. cytokines, T cellen en productie van antilichamen). In deze literatuurstudie zijn ook humane studies verwerkt. In deze studies met baby's is met name gekeken naar het effect van prebiotica (o.a. oligosachariden), probiotica (o.a. lactobacilli en bifidobacteriën) en langketenige onverzadigde vetzuren.
Uit onderzoek naar voedingsinterventies in de post-neonatale fase bleek dat het toevoegen van glutamine de integriteit van de darmwand bevorderde en het aantal CD4+ cellen in het bloed verhoogde. Toevoeging van kruiden en bioactieve plantenstoffen, o.a. zwarte komijn, chitosan en quercitine, verbeterden de darmintegriteit, de lokale en systemische immuunrespons, de samenstelling van de microflora in de darm en de dierprestaties. Bestendig zetmeel bleek t.o.v.

verteerbaar zetmeel een aanzienlijk gunstig effect te hebben op immunologische processen in het colon van varkens en op de samenstelling van de microflora. Bepaalde probiotica (o.a. *Bacillus subtilli* en *Lactobacillus Bulgaricus)* verbeterden de immuun competentie, zoals bleek uit een hoger serum IgG en IgA gehalte, een verlaagd gehalte van de pro-inflammatoire cytokine IL-8, verlaagde aantallen clostridia en coliformen in de blinde darm en langere darmvilli. Toevoeging van antimicrobiële eiwitten, o.a. buforin II en lysozym, verbeterden de dierprestaties en de darmintegriteit en verminderden de fecale uitscheiding van clostridia, *E. coli* en coliformen.

De waargenomen positieve effecten van verhoogde aminozuurgehalten in het voer op de immuun competentie kunnen toegeschreven worden aan het effect dat ze hebben op DNA methylatie, activatie en proliferatie van lymfocyten, NK cellen en macrofagen. Kruiden en bioactieve plantenstoffen hebben een effect op de lokale immuunrespons (minder macrofagen en neutrofielen in de darminhoud) en op de systemische immuunrespons (minder TNF-alfa, haptoglobine, witte bloedcellen en lymfocyten in het serum) na een bacteriële challenge. De positieve effecten van bloedplasma op de immuun competentie van jonge biggen hangen in sterke mate samen met het IgG gehalte van bloedplasma. Het gunstige effect van meervoudig onverzadigde vetzuren hangt samen met een vermindering van ontstekingsreacties (minder macrofagen en T cellen) en met een verminderde acute fase respons. Bovendien stimuleren ze de productie van antilichamen. Antimicrobiële eiwitten hebben een direct effect op de samenstelling van de microbiota door het doden van schadelijke bacteriën (o.a. *E. coli*) of door te voorkomen dat ze zich aan mucosale epitheelcellen hechten. Daarnaast zijn antimicrobiële eiwitten in staat om het aantal gobletcellen te verhogen, evenals de expressie van tight junction eiwitten in de darmwand van het jejunum en ileum. Deze tight junction eiwitten beschermen de mucosa tegen beschadigingen, stabiliseren de mucus laag en bevorderen herstel van het epitheel.
Op basis van deze studie kan geconcludeerd worden dat het voorkómen van deficiënties aan aminozuren, mineralen en vitaminen in het voer een belangrijke voorwaarde is voor een goede werking van het immuunsysteem. Uit de literatuur blijkt dat voedingsinterventies met pre- en probiotica, bioactieve plantenstoffen, antimicrobiële eiwitten, bloedplasma en omega-3 vetzuren het meeste perspectief bieden om de immuun competentie van landbouwhuisdieren te verbeteren.
In de huidige literatuur is nog nauwelijks gekeken naar het effect van voedingsinterventies op de expressie van genen die betrokken zijn bij immunologische processen. Ook is de genoom sequentie techniek nog nauwelijks toegepast in dit type onderzoek, zodat er nog zeer weinig bekend over het effect van deze interventies op verschuivingen in de totale microflora. Aanbevolen wordt om deze technieken in toekomstige studies toe te passen.

In hoofdstuk 5 komt de interactie tussen de darm- en luchtwegimmuniteit aan de orde. De immuun competentie van de diepere luchtwegen (trachea, primaire bronchiën en longen) blijkt niet wezenlijk beïnvloed te kunnen worden door voedingsinterventies. Het mucosale immuunsysteem van de bovenste luchtwegen (neusholte, farynx en larynx) zijn echter gedeeltelijk verbonden met het mucosale immuunsysteem van de darm. Een orale vaccinatie (met een cholera toxine) bleek niet alleen in het maagdarmkanaal de IgA productie te vergroten, maar ook o.a. in de bovenste luchtwegen. Immunologische weefsel in het hoofd/nek gebied bevindt zich vooral in tonsillen, lymfknopen en lymffollikels. Het grootste contactoppervlak tussen het voedsel, neusinhoud en het immuunsysteem bevindt zich in het gebied waar neus en mond samenkomen. Inductie van een mucosale IgA reactie kan ook al in het immunologische weefsel van de mond en keel (denk aan de amandelen) plaatsvinden. Het is daarmee waarschijnlijk dat voedingscomponenten/additieven tijdens het passeren van deze weefsels in de mond- en keelholte, invloed op het mucosale immuunsysteem van de bovenste luchtwegen kunnen uitoefenen. Luchtweg pathogenen en luchtallergenen, die in neusafscheiding aanwezig zijn, kunnen terecht komen in de darm en daar een effect hebben op het immuunsysteem. Dit kan resulteren in immunologische reacties in zowel de luchtweg- als darmmucosa als gevolg van circulerende specifieke B-cellen. Een challenge met pathogenen vanuit keel/mond kan resulteren in de productie van IgA door het mucosale weefsel van de bovenste luchtwegen. Secretoir IgA (sIgA) is een niet-inflammatoire immunoglobuline, dat in staat is te voorkomen dat pathogenen mucosale weefsels binnen te dringen (neutralisatie), zonder dat hierbij een ontstekingsreactie op gang komt, met mogelijk nadelige gevolgen voor de gastheer. Voedingsinterventies die de productie van IgA bevorderen, kunnen bijdragen aan het ondersteunen van het immuunsysteem, zowel in de bovenste luchtwegen, als in de darm en illustreren daarbij hun immuun competentie bevorderende activiteit. Uit de experimenten die in deze studie zijn beschreven blijkt dat toevoeging van *Bacillus*

subtillus, Saccharomyces cerevisiae, chitosan, rijstevoermeel, glutamine en bloedplasma aan het voer resulteerde in een verhoogde IgA productie. In ons instituut is een groot aantal voeradditieven getest als potentiele vervanger van antimicrobiële groeibevorderaars (AMGB). Het betrof o.a. levende gisten, mengsels van organische zuren, lecithinen, boterzuur, humuszuur, bioactieve plantenstoffen en prebiotica. In al deze experimenten werd naast dierprestaties ook het aantal veterinaire behandelingen en uitgevallen biggen als gevolg van luchtweginfecties geregistreerd. In de meeste experimenten was het niveau van veterinaire behandelingen en uitval vanwege luchtweginfecties te laag om verschillen tussen behandelingen statistisch te kunnen toetsen, of was er geen aantoonbaar effect van de interventie. De enige uitzondering vormde een interventie met een mengsel van middellange vetzuren (Aromabiotic). Geen enkele big die het voer met Aromabiotic kreeg ontving een veterinaire behandeling tegen luchtwegaandoeningen, terwijl 7 van de 220 biggen in zowel de negatieve als positieve (behandeling met AMGB) controle groep veterinair behandeld werden. Er is behoefte aan meer experimenten, die specifiek het effect onderzoeken van voedingsinterventies op de immuun competentie in de bovenste luchtwegen.

Summary

Infectious diseases greatly impair animal welfare and efficiency of nutrient use and thus the environmental footprint of animal production. Nutrition may aid in minimising the incidence of diseases by enhancing immune competence. Immune competence is defined as the ability of the immune system to respond adequately on an antigenic stimulus by an appropriate immune response with a balance between tolerance and inflammation. The importance of immune competence enhancing nutrition becomes even more critical as anti-bacterials, anti-parasitics and other additives that promote animal health are eliminated due to consumer demands.
The current study covers a review of literature regarding a number of topics related to immune competence in farm animals, which are introduced in chapter 1. These topics are:

- A demonstration of the relationship between functional feed components and the expression of genes/biological processes that are involved in gut health of farm animals;
- A description of available models, that can be used to investigate the effects of nutritional interventions on immune related parameters in animals;
- A review of the effects of nutritional interventions in the maternal, neonatal and post-neonatal phase on the development of the innate and acquired immune system;
- A review of the relationship between the immune system in the gut and in the upper airways, whereas the question will be addressed how the immune system in the upper airways can be affected by nutritional interventions.

Combined information about gene-chemical associations retrieved from on-line available databases can be used to predict the *in-vivo* biological activity of natural occurring chemical compounds. These knowledge can be used to select bioactive components for further assessment in animal experiments. In chapter 2, an overview of gene-expression datasets of feed-related studies in farm animals posted in two databases were discussed. Secondly, a tutorial is provided how to explore all information in these databases in order to (in-silico) predict chemical compounds and additives that have potential to steer the expression and function of specific genes/processes in the GI tract of farm animals.
Chapter 3 describes the characteristics, applications and benefits of different types of in situ, ex vivo, in vitro as well as *in vivo* models that can be used to investigate immuno modulating research questions. Advantages and limitations of most models have been indicated.
The nutritional interventions that directly or indirectly (via the microbiota) engage the optimal development of the innate and acquired immune system, both in the maternal, neonatal and post-neonatal phase are reviewed. The results of this literature search are described in chapter 4.
It can be questioned whether nutritional interventions may not only affect the immune system in the gut, but simultaneously the immune system of the upper airways. This question is discussed in chapter 5.

In chapter 2, it is concluded that the amount of gene expression databases and other "omics" data in public bioinformatics databases still increases rapidly. Integration of such data from feed-related studies, as well as from other "omics" research studying the function of the gut, as was done in this study for the probiotic bacterium *L. plantarum* , can push the search for new and/or alternative feed additives in the right direction. Besides, such an integrative approach may lead to the discovery or improvement of new functional compounds, it may also reduce the use of expensive animal models to gain the primary data needed to prove the potential of specific compounds as functional feed additives in an early stage of their development traject.

In chapter 3, the principles of in vitro, ex vivo, in situ and in vivo models are described. The following in vitro models are distinguished: primary cells, epithelial "mini guts", cell cultures (one-, two- and three-dimensional), and intestines-on-a-chip. The ex vivo models are subdivided in the perfusion system, the

everted sac technique, the Ussing chamber, in vitro organ culture (IVOC, both polarized or non-polarized), the novel polarized ex vivo organ culture, the IVOC in the Ussing chamber, the precision cut tissue slices (PCTS), and the porcine intestinal model. The intestinal loop model and the small intestinal segment perfusion (SISP) are representatives of in situ models. Finally, some in vivo models are briefly described. It depends on the (scientific) question to be answered, which model should be used. Assessing mucosal immunity had not been applied in all models described, but in principal in all models mucosal tissues and/or gut fluid samples can be used for measuring immunological responses.

Chapter 4 presents the direct or indirect (via the microbiota) effects of nutritional interventions on the development of the innate and acquired immune system, both in the maternal, neonatal and post-neonatal phase. The conclusions regarding the maternal phase are summarized as follows. Trans-generational effects of maternal dietary interventions on improvement of gut health and immune competence has been demonstrated in mammals (mice, rats, pigs, humans) as well as in poultry. The reviewed dietary interventions can be categorized to fatty acids, seaweed extract, pre- and probiotics, and vitamins and minerals. The dietary interventions showed a variety in modes of action. They could affect the composition of the microbiota, the numbers or activity of specific immune cells, the gut morphology, and the expression of genes involved in immune response. In some studies, it was shown that the maternal dietary interventions resulted in improved animal performance or reduced incidence of diarrhea of the offspring. These effects were demonstrated for seaweed extract, *Saccharomyces cerevisiae*, zinc and β-carotene. Only a few studies reported trans-generational effects on gene expression. These studies investigated the effects of supplemented omega-3 fatty acids or vitamins and minerals. Supplementation of maternal diets with specific fatty acids, in particular with fish oil, showed to have a wide impact on the immune competence of the progeny. Therefore, modulating the dietary fatty acid profile of maternal diets seems to be a promising intervention for improving immune competence of the progeny.
Based on the studies that were focused on nutritional interventions in the neonatal phase in pigs, it can be concluded that in terms of immune parameters (e.g. cytokines) prebiotics and spray dried plasma could be potential candidates to further investigation. For chickens, evidence of nutritional effects on improved immune competence was found in supplementation of 'wheat/soy' diets (compared to maize/soy or wheat/rye/soy diets), addition of 'Methionine and Cysteine' above the requirements for maximal growth, as well as 'vitamin E (100 IU/kg) together with selenium (0.2 ppm)' vs. an unsupplemented vitamin E/selenium diet (e.g. cytokines, T cells and antibodies). Human infants were also studied extensively within the context of early life variation and (impact on) immunity. From these studies we concluded that one must avoid a depletion of vitamins and/or minerals in the diet. The most abundant investigated nutritional interventions were prebiotics (e.g. oligosaccharides), probiotics (e.g. lactobacilli and bifidobacteria) and long-chain poly unsaturated fatty acids.
In the post-neonatal phase, it was shown that dietary addition of glutamine improved gut integrity and the number of CD4+ cells in blood. Herbs or functional components in plants, e.g. black cumin, chitosan or quercitine, showed to improve gut integrity, local or systemic immune responses, composition of gut microbiota and performance parameters. Compared to digestive starch, resistant starch showed to have significant improving effects on immunological pathways in the colon of pigs as well as on the composition of the microflora. Some probiotics (*Bacillus subtillis* and *Lactobacillus Bulgaricus*) also positively affected the immune competence, as shown by increased serum IgG and IgA concentrations, less pro-inflammatory cytokine IL-8, decreased numbers of clostridia and coliforms in the ceaca, and greater villus height. Antimicrobial proteins, e.g. buforin II and lysozyme, improved performance levels and gut integrity, and reduced faecal excretion of clostridia, E. coli, and coliforms.

The positive observed effects of amino acids on immune competence can be attributed to their role in DNA methylation, activation and proliferation of lymphocytes, NK cells, and macrophages. Herbs and functional plant components have their effects though reduced local (les macrophages and neutrophils in the ileum) and systemic inflammation (less serum TNF-alpha, haptoglobin, white blood cells and lymphocytes) after bacterial challenges. The immuno competence improving effects of spray-dried animal plasma in young pigs is most likely mediated by the IgG component in the plasma. Poly unsaturated fatty acids exert their effects via reducing inflammatory responses in macrophages and T

cells, and by reducing the acute phase response. Moreover, it was shown that they stimulate the antibody response. Antimicrobial proteins are able to affect the microbial population by directly killing harmful bacteria (e.g. *E. coli*) or by preventing binding of bacteria to the mucosal epithelial cells. Moreover, antimicrobial proteins might stimulate the number of goblet cells and the expression of tight junction proteins in the jejunum and ileum. These tight junction proteins may protect the mucosa from insults, stabilize the mucus layer and affect healing of epithelium.
It can be concluded that an important condition for a well-functioning immune system is the prevention of nutritional deficiencies in terms of amino acids, minerals and vitamins. Based on the results of this review, pre- and probiotics, functional plant components, antimicrobial proteins, spray-dried animal plasma, and omega-3 fatty acids seem the most perspective categories of dietary interventions for improving immune competence of farm animals. Transcriptomics data and microbiota data based on genome sequencing techniques, however, are hardly available in the reviewed literature. For a better understanding of the impact of nutritional interventions on immune competence, the use of these techniques are highly recommended.

Chapter 5 shows the interaction between the gastro-intestinal and airway mucosal immune system. It is concluded that the lower respiratory tract (trachea, primary bronchi and lungs) is not significantly modulated by nutritional modulation. The mucosal immune system of the upper respiratory tract (nasal cavity, pharynx and larynx), however, is partly connected to the mucosal immune system of the gut. Oral vaccination (with cholera toxin) did not only increase IgA secretion in the upper airways, but also in the digestive tract and in breast tissue. Immunological tissues in the head/neck area are mainly structured in tonsils, lymph nodes and lymph follicles. The largest site of contact between food and nasal contents and the immune system occurs in the area where nose and mouse come together. The mouth and throat are important inductive sites for initiating specific IgA secretion at the mucosal surfaces of the upper respiratory tract. Airway pathogens and aeroallergens, present in nasal secretions, can become available in to the intestinal immune system, which may result in immune effects in both mucosal compartments due to recirculating specific B-cells. A challenge with such pathogens from mouth and throat is an important trigger for the secretion of IgA at the mucosal surfaces of the upper respiratory tract. IgA, which is a non-inflammatory immunoglobulin, which can exclude pathogens from entering the tissues, without promoting inflammation.
It is expected that nutritional interventions that stimulate IgA production might be helpful in supporting the mucosal immune system, both in the upper airways as well as in the gut, thereby demonstrating their immune competence enhancing activities. In the current study, it was shown from literature that dietary addition of *Bacillus subtillis, Saccharomyces cerevisiae*, chitosan, rice bran, glutamine, and spray-dried animal plasma resulted in increased secretion of IgA.
In our institute, a large number of feed additives, e.g. live yeast, mixtures of organic acids, lecithin's, butyric acid, humic acid, bioactive plant components and prebiotics, were tested as potential replacers of antimicrobial growth promoters. In all these animal performance experiments, veterinary treatments and mortality due to airway disorders were registered. In most of the experiments, however, veterinary treatment and mortality levels were too low for statistical analysis, or were not affected by the dietary interventions. The only exception was an intervention with a mixture of medium chain fatty acids (Aromabiotic), with no veterinary treatments in the piglets that received the Aromabiotic diet, whereas 7 out of 220 piglets were veterinary treated in both the negative and positive (with antimicrobial growth promoter) control groups. More studies, and probably with a more tailored design, should be performed to determine the possible contribution of dietary interventions on the immune competence of the upper airways.

1 Rationale of the report

The fields of immunology, microbiology, nutrition and metabolism are rapidly converging to induce and maintain gastro-intestinal health of agricultural animals. Diet has a considerable effect on the composition of the gut microbiota, and composition and products of the gut microbiota have profound effects on immune and inflammatory responses. The intestinal microbiota is derived at least in part from the mother during the birthing process and is modified thereafter by factors such as diet, antibiotic use, host genetics and other environmental factors.

This suggests that balanced microbial composition results in symbiosis; this provides regulation of immune and inflammatory responses through anti-inflammatory and/or immunomodulatory dietary products, which helps maintain homeostasis. Dysbiosis would lead to dysregulation of the immune system through lack of beneficial microbial products and an increase in virulence factors, which could leave the host susceptible to inflammation. Dysbiosis could occur through the consumption of a poor diet, as well as through changes induced by factors such as host genetics, maternal transfer and antibiotic use. It has been recognized only recently that the gut microbiota can influence immune function beyond the gut, thereby affecting also immune protection in the upper airways.

Postnatal, bacteria colonize the neonatal intestine immediately, initiating multiple events that affect the development or functional maturation of the mucosa and gut-associated lymphoid tissues. Microbe-associated molecular patterns (MAMPs) sensed by pattern-recognition receptors on intestinal epithelial cells and dendritic cells adjacent to cryptopatches stimulate the further recruitment of B cells and T cells, causing the cryptopatches to develop into mature isolated lymphoid follicles. The isolated lymphoid follicles release IgA-producing plasma cells, which are formed through T-cell-dependent and independent interactions, into the lamina propria. Microbes also cross the epithelium and enter Peyer's patches through M cells, from which they are endocytosed by dendritic cells in the sub epithelial dome. Antigen-loaded dendritic cells in the Peyer's patch interact with local lymphocytes to induce T-cell differentiation and T-cell-dependent B-cell maturation in the germinal centre to induce the development of IgA- producing plasma cells that home to the lamina propria, where they release dimeric IgA for transport into the intestinal lumen. Dendritic-cell-mediated luminal sampling of microbial products or transcytosis of bacteria across the epithelium results in antigen loading of lamina propria dendritic cells, which then migrate through the afferent lymphatics vessels to a draining mesenteric lymph node to induce differentiation of effector T cells that traffic to the lamina propria. Sensing of MAMPs stimulates the proliferation of intestinal epithelial cells in crypts, resulting in their increased depth and, in the small intestine, increased density of Paneth cells. This sensing also arms the intestinal epithelial cells for release of antimicrobial peptides.

Appropriate activation of an immunological activity of production animals is thus considered important to become resistant to infectious diseases, in the respiratory and gastro-intestinal tract. Stress induced by husbandry conditions and management activities can lead to changes in the immune response resulting in both increased and decreased resistance to opportunistic pathogens. Growth-promoting antibiotics have been a major tool in modulating host-pathogen interactions and limiting clinical and subclinical bacterial infections. Regulatory pressures to limit antibiotic use in poultry and pig production have limited the disease-fighting tools available to poultry and livestock producers. There is thus a need to evaluate potential antibiotic alternatives to improve both production and disease resistance in high-intensity food animal production.

The nexus between nutrient metabolism and the immune system occurs at many levels, ranging from endocrine signalling to direct sensing of nutrients by immune cells. Diet shapes gut microbial community structure and function, and the microbiota adapts in ways that promote nutrient processing; the ability of the microbiota to process a given diet affects the nutrient and energetic value of that diet. The microbiota and immune systems co-evolve: malnutrition affects the innate and adaptive immune systems as well as the microbiota. The microbiota acts as a barrier to enteropathogen infection; this barrier function may be disrupted by malnutrition, as well as by perturbations in immune system function. The microbiota affects nutrient processing by the host, including the expression of host genes involved in nutrient transport and metabolism. Immune-cell-associated receptors use information about the local nutrient or metabolite milieu to organize local immune responses. The dietary intake of nutrients shapes microbial community structure, which, in turn, changes the nutritional value of the consumed food. Unmodified dietary components are

absorbed in the intestine, where they can interact with immune cells. Microbial signals in the form of microbe-associated molecular patterns (MAMPs) modify local mucosal immune responses through innate signalling pathways such as the inflammasome or TLRs. Microbe-modified dietary components (such as acetate produced by the fermentation of polysaccharides) provide signals by which the immune system can monitor the metabolic activities of the microbiota. In turn, feed factors can directly modifying intestinal microbial ecology.

There is thus a clear need to create more and improved databases for monitoring changing patterns of food consumption and allowing the identification of new host and microbial biomarkers and mediators of nutritional status, determining the nutritional value of various feeds, and characterizing the function of the immune system, including mucosal barrier integrity and mucosal immunity, and the dynamic operations of the microbiota. These biomarkers should be reliable, robust, relevant and reflect compromised health, and need to be determined in several body fluids like peripheral blood (serum or plasma), saliva, urine and stool in sufficient sensitivity and specificity, in small volumes and with the necessary throughput. Such biomarkers are as yet hardly available. Basic research is required in relevant model and target species to define such biomarkers based on -omics studies (using genomics, proteomics and metabolomics tools) combined with bioinformatics (network analyses). For proper utilization, economically relevant diseases (including respiratory infections in pigs and chicken, and feed-induced inflammatory bowel diseases in husbandry species) should be analysed for suitable application of techniques and biomarkers.

Nutritional approaches to counteract the debilitating effects of stress and infection may provide producers with useful alternatives to antibiotics and increased food safety. Since diet is a major contributing factor to the health status of agricultural animals, the feed industry should consciously adapt their available products. This report provides a solid background based on benchmarking the available literature to accommodate a cost-effective effort to new feed approaches for the industry.

2 Introduction

Infectious diseases greatly impair animal welfare and efficiency of nutrient use and thus the environmental footprint of poultry production [1]. Appropriate nutrition becomes even more critical as anti-bacterials, anti-parasitics and other additives that promote animal health are eliminated due to consumer demands. Nutrition may aid in minimising the incidence of diseases by enhancing immune competence. Immune competence is defined as the ability of the immune system to respond adequately on an antigenic stimulus by an appropriate immune response with a balance between tolerance and inflammation.

2.1 Functional feed components

The immuno modulatory effects of specific feed ingredients is assumed to be related to some functional components in these feed matrices. The mode of action of these components, however, is often unknown.
Integration of "omics" techniques like gene-expression profiles (micro-arrays), proteomics, and metabolomics, could fill in this gap of knowledge. The data gained with these techniques would significantly contribute to the elucidation of the mechanisms how specific components and/or ingredients in feed positively influence functional processes in the GI tract, and with this, the overall performance of farm animals.
Combined information about gene-chemical associations retrieved from on-line available databases can be used to predict the *in-vivo* biological activity of natural occurring chemical compounds, i.e. predict their "defined mode of action". Or contrariwise, this information can be used to find compounds that have potential to influence the function of specific genes/proteins or biological processes. Such a data-mining approach has already accelerates the development of alternative or new dedicated supplements/ additives for human foods. In chapter 2, an overview of gene-expression datasets of feed-related studies in farm animals posted in two databases will be discussed. Secondly, a tutorial is provided how to explore all information in these databases in order to (in-silico) predict chemical compounds and additives that have potential to steer the expression and function of specific genes/processes in the GI tract of farm animals.

2.2 Animal models available for studying immune competence

In vivo experiments are widely used to investigate the effects of nutritional interventions on immune related parameters in animals. In these studies animals are used as goal animals, or as a model for humans. *In vivo* experiments, however, might increase the level of discomfort of the animals, are time and labour intensive, and costly. For some research questions, other types of experiments can be used as well, thereby lowering the level of disadvantages compared to *in vivo* experiments. Chapter 3 describes the characteristics, applications and benefits of different types of in situ, ex vivo, in vitro as well as *in vivo* models that can be used to investigate immuno modulating research questions.

2.3 Nutritional immune modulation

Infectious diseases greatly impair animal welfare and efficiency of nutrient use and thus the environmental footprint of animal production [1]. Appropriate nutrition becomes even more critical as anti-bacterials, anti-parasitics and other additives that promote animal health are eliminated due to consumer demands. Nutrition may aid in minimising the incidence of diseases by enhancing immunity. It can be questioned, however, whether the diets in use today for realizing maximal production efficiency are sufficient for optimal immunity. Klasing [1] distinguished six different mechanisms by which diet might affect immunity: 1) feeding the cells of the immune system, 2) feeding pathogens, 3)

modifying the responses of leukocytes, 4) protecting against immunopathology, 5) influencing the microbial ecology of the gut, and 6) stimulating the immune system.
Growing evidence highlights the importance of a mother's nutrition from preconception through lactation in programming the developing immune system of her offspring [2]. Examples of nutrition-mediated immune programming can be found in the literature on intra-uterine growth retardation, maternal micronutrient deficiencies, and infant feeding. One programming mechanism involves activation of the maternal hypothalamic-pituitary-adrenal axis in response to nutritional stress. Maternal malnutrition may also have a direct influence, as evidenced by nutrient-driven epigenetic changes to developing T regulatory cells and subsequent risk of allergy or asthma. A third programming pathway involves placental or breast milk transfer of maternal immune factors with immuno modulatory functions (e.g. cytokines). Early immune system programming can give rise to changes in the foetal immune system that can persist over the life course [3]. An inappropriate maternal immune activation may contribute to an increased risk in the offspring of neurodevelopmental disorders, auto immune diseases and allergies later in life, but well-controlled maternal immune responses might play a positive physiological role in foetal immune and nervous system development [3]. The foetal immune system is particularly vulnerable to environmental insults (e.g., malnutrition, toxins, infections, and stress). Therefore, adequate maternal/foetal nutrition is necessary to stimulate the appropriate development of foetal and neonatal immune responses and immune cell proliferation, placentation, and the development of oral tolerance. According to Korver [4] nutritional immunomodulation holds great promise as a means to increase poultry productivity and health. The induced changes in immune function, however, may predispose the animals to other diseases, or decrease production characteristics.
This study aims, among others, to review the nutritional interventions that directly or indirectly (via the microbiota) engage the optimal development of the innate and acquired immune system of farm animals and humans, both in the maternal, neonatal and post-neonatal phase. This The results of this literature search are described in chapter 4.

2.4 Relation between immune system of the gut and airways

Farm animals may be vulnerable to respiratory diseases. The upper airways are protected by a mucosal immune system that is partly connected to other mucosa of the body. Together this mucosal immune system is referred to as the "common mucosal immune system". The primary specific defence at this mucosa is formed by IgA antibodies. A hypothesis on the common mucosal immune system suggested that antigenic stimulation at any inductive site of the mucosal immune system results in a specific immune response characterized by IgA antibody production in other mucosa included in this system. Therefore, nutritional interventions may not only affect the immune system in the gut, but simultaneously the immune system of the upper airways. This question is discussed in chapter 5.

3 Feed ingredients and functional components

The need of efficacious, safe and low-cost feed additives displaying a defined mode of action urges the animal production industry to invest more money in more specialized research to discover such new additives. Still most in-vivo feed-intervention studies performed in farm animals today measure conventional “performance” parameters" (e.g. feed and water intake, growth, resistance to infection, general health status). Although these parameters are essential data for the feed industry, they do not provide detailed insight in how functional components in feed-formulations interact with the animal. Integration of “omics” data like gene-expression profiles (micro-arrays), proteomics, and metabolomics, could fill in this gap of knowledge. The data gained with these techniques would significantly contribute to the elucidation of the mechanisms how specific components and/or ingredients in feed positively influence functional processes in the GI tract, and with this, the overall performance of farm animals.
The last two decades a large number of gene-expression datasets describing nutritional intervention studies in the intestines of rats and mice, and in cultured intestinal cells (human and animal) were posted in the Gene Expression databases (GEO-NCBI and ArrayExpress-EMBL). Together with data from conventional biological and immunological in-vitro and ex-vivo experiments, these expression data of genes are linked to interacting chemical compounds in databases like the Comparative Toxicogenomics Database (CTD) and PubChem (NCBI), and interactive web-based resources like STITCH. The power of these databases lies in their comprehensive interconnectivity to other biological and chemical data-resources. Public and commercial resources that store information about gene function (e.g. NCBI-Entrez Gene and Genecards: including Gene Ontology terms), transcriptional regulation (e.g. GNCpro), expression in specific cell-types/tissues (BioGPS), (genetic) diseases (e.g. NCBI OMIN), specific biological processes and pathways (e.g. KEGG, DAVID), enzyme activity and metabolism (e.g. Brenda), and relevant literature (PubMed). Combined information about gene-chemical associations retrieved from all these databases can be used to predict the in-vivo biological activity of natural occurring chemical compounds, i.e. predict their “defined mode of action”. Or contrariwise, to find compounds that have potential to influence the function of specific genes/proteins or biological processes. Such a data-mining approach has already accelerates the development of alternative or new dedicated supplements/additives for human foods (http://www.ncbi.nlm.nih.gov/pubmed/20233651/ http://www.ncbi.nlm.nih.gov/pubmed/17506913).

For farm animals the majority of gene expression profiles recorded in the GI track and posted in GEO and ArrayExpress, were obtained after a challenge with enteric pathogens, mainly bacteria. So far only a dozen gene-expression datasets were posted in GEO and ArrayExpress in which nutritional interventions in the GI tract of farm animals was studied without, or in combination with a challenge with enteric pathogens. Unfortunately, not all these datasets are linked to a scientific paper that summarizes and discusses the outcome of these data. This makes these data less accessible for researches not skilled in analysing gene-expression data. This backlog in available “omics” data compared to human, mouse and rat, does not necessary has to be a limitation to apply data-mining strategies also for farm animal feed-research. A clear advantage compared to a decade ago is the completion of the sequence of the cattle, pig, chicken and sheep genomes. Comparison of these genomes to the more in detail characterized human and mouse genomes has accelerated gene-annotation and identification of regulatory elements in farm animal genomes, making extrapolation of human, mouse and rat gene expression data to farm animals more reliable.

In the first part of this paragraph an overview of gene-expression datasets of feed-related studies in farm animals posted in GEO and/or ArrayExpress will be discussed. In the second part of this paragraph a tutorial is provided how to explore all information in the above mentioned databases in order to (in-silico) predict chemical compounds and additives that have potential to steer the expression and function of specific genes/processes in the GI tract of farm animals.

3.1 Gene expression datasets of nutritional interventions in farm animals

A total of 2, 11, and 12 gene expression datasets of feed-intervention studies performed in cattle, pigs, and chicken, respectively, were posted in GEO and ArrayExpress. In all these studies changes in gene-expression were measured in different parts of the GI track as well as in cultured gut-derived ex-vivo cells or cell-lines derived from gut tissues. In Appendix 1, 2, and 3 an overview of these intervention studies is listed (marked with p1, b1, or c1 etc.), including a brief description of the experimental design and the most important genes and/or processes found regulated. Intervention studies with bacterial endotoxins, LPS and probiotic bacteria were also included in this overview. If available a PubMed web link of the peer-reviewed scientific publications describing the results of these intervention studies is provided in Appendix 1,2, and 3..

Compared to the dozens of gene-expression studies conducted with tissues collected from udders and/or mammary glands of dairy cows, so far only two gene expression datasets were posted in which gene expression was measured in tissue collected from the GI tract of cattle (b1, b2). Both datasets describe changes in gene expression recorded after imposing sub-acute ruminal acidosis by feeding dairy cows with dietary grain or high and low concentrate grain diets (HC). In both these studies genes involved in maintenance of the integrity of the ruminal epithelial layer were found to be regulated.

Most intervention studies, in which gene expression profiles were recorded in the gut of pigs and chickens, were aimed to find alternatives for antibiotic treatment against enteric pathogens. Four gene expression datasets were recorded from intestinal lymphocytes (IEL's) isolated from chickens fed with a diet supplemented with chemicals and/or phytogenic compounds/extracts (anethol [c4], secondary metabolites of garlic [c7], and plant extracts of C. annuum fruits [c12] and Curcuma longa [c6]), all four intended to reduce the negative impact of coccidiosis (Eimeria infection) on overall performance of chickens. Gene expression was measured in the jejunum of chicken fed with mannose-rich oligosaccharides (isolated from *Saccharomyces*) in order to compare this additive to treatment with the antibiotic virginiamycin (c8). In addition, gene expression experiments were conducted in which the effect of toxins produced by enteric pathogens on gene expression in intestinal cells was studied. In-vitro cultured chicken intestinal macrophages (HD11 cell-line; c9) and pig jejunal segments in (in-situ SISP model; see above) were challenged with endotoxins from Salmonella and ETEC (p3), respectively. The genes responding in these studies provided valuable information about the innate immune mechanisms activated by bacterial endotoxins. Challenge of cultured "Intestinal Porcine Epithelial Cells" (IPEC-J2) with the fungal toxin deoxynivalenol (DON), a contaminant present in farm animal diets produced of grains, influenced expression of genes involved in maintenance of the integrity of the epithelial layer (p5). With respect to the integrity and the barrier function of the intestinal epithelial layer, several gene expression studies have been conducted in a pig model developed to study induction of necrotizing enterocolitis (NEC) in human preterm neonates (p3, p8, p9). NEC is often induced in preterm neonates when formula milk is provided using enteral tubes. Feeding of formula milk to preterm piglets was compared to colostrum, and gene expression was measured in the distal small intestine of preterm pigs fed by an enteral tube or orally. Gene expression profiles in these studies showed that enteral tube feeding with formula milk induces a pro-inflammatory insult to the immature intestine of the preterm pigs, resulting in development of NEC. Amniotic fluid (AF) added as additive to formula milk suppressed this inflammatory insult, and it was suggested that components in AF reduced the pathogenesis of NEC (p9). The studies reported above were primarily performed in pigs and poultry, and extrapolation to other species therefore should be administered with caution.

In experiments conducted to relate feed efficiency and daily gain of chickens to their diet, 2 studies used gene expression profiles to investigate the metabolic mechanisms of feed conversion and uptake in the gut. Expression profiles recorded in the jejunum after feeding with organic or conventionally produced starter and grower diets were compared in chicken lines with different genetic backgrounds (c11). In a second study, duodenal expression profiles were compared between groups of chickens showing different efficiencies in food-to-energy conversion (Apparent Metabolizable Energy [c1]). Direct-fed microbials (DFM) have been shown to increase overall performance of daily farm animals.

In one pig (p6), and two chicken studies (c1, c10) gene expression profiles were recorded in the intestine after repeated oral administration with cultures of probiotic bacteria. The expression profiles recorded in the pig study (p6) clearly indicated that repeated oral administration with *Lactobacillus plantarum 299v* had a beneficial effect on the mucosal innate and adaptive immune system. Briefly, genes involved in the intestinal immune network for IgA production were regulated by *Lactobacillus plantarum 299v*, especially in the ileum.

3.2 Functional analysis and data-mining using a gene expression dataset

As depicted in Figure 13 (Chapter 5), chemokine's CCL25 and CCL28 initiate the attraction, migration and homing of (pre)-IgA Ab-secreting cells (ASCs) from the GALT to effector sites in the lamina propia of the small intestine and colon, and to other mucosal effector sites in the body [5]. Gene expression of CCL25, and of the pre-B cell surface markers CR2 (complement component receptor 2) and MME (membrane metallo-endopeptidase) was up-regulated more than 10 times in the colon of pigs after repeated oral administration with *Lactobacillus plantarum 299v* preparations (dataset p6 in Appendix 2a). This led to the hypothesis that infiltration of the colon with activated pIgA+ precursor cells or IgA Ab-secreting cells (ASCs), likely, originating from ileum GALT (e.g. Peyers patches) may have occurred. We used this set of 122 regulated genes as input list in web-based bioinformatics programs and retrieved relevant biological and chemical information from databases in order to predict a set of "candidate" feed-additives which could induce similar processes in the colon of pigs as *Lactobacillus plantarum 299v* did. The bioinformatics analysis of the gene expression dataset recorded in the ileum of this pig study will be presented elsewhere (Hulst et. al., submitted 2014).

In Appendix 4 a point to point tutorial is given to perform such a data-mining strategy. Two sub-lists of genes were extracted from bioinformatics programs DAVID and GeneDecks; 1 representing genes involved in "small molecule metabolic processes" (SMMP list; Appendix 5) and 1 list of genes involved in "immune processes" ("immune phenotype"), cell-adhesion, and cytoskeletal rearrangements (IAC list; Appendix 6). These lists were separately analysed in DAVID and GeneDecks to i) retrieve relevant pathways/processes, ii) identify the most frequently present transcription factor binding sequence (TFBS) within the loci of the regulated genes, iii) predict which type of cell or tissue supports expression of the majority of the regulated genes, and iiii) to find significant associations of the regulated with relevant chemicals. In supplementary Appendix 7 and 8 the results of these bioinformatics analysis are presented for the SMMP list and IAC list, respectively. In addition, to identify functional associations and crosstalk between proteins encoded by differential expressed genes and relevant chemicals, a combined list of SMMP and IAC genes was uploaded in the (protein)-protein-chemical interaction web tool STITCH 3.1 beta. An interactive network was built from 18 genes and 4 chemicals (Figure 1) which all scored associations with a high level of confidence (≥0.7; see Appendix 9). By clicking on the lines which connects two nodes (genes or chemicals), relevant literature and information about the type of association (e.g. a physical interaction, or an enzyme-substrate relation, etc.) can be accessed directly in other biological and chemical data-resources (note; only interactive on-line). Four key-genes, displaying a central role in this network (APOC3, NR1H4, CYP3A4, and CYP4F3), were selected together with 3 genes mapped to the "B Cell Receptor Signaling Pathway" (CR2, APOC3, and SPP1; highlighted in red in Appendix 8). The latter 3 genes were selected based on the "pIgA+ precursor cell" hypothesis (see above), the prediction that a majority of genes from the IAC list can be expressed by "lymphomaburkitts" cells (B lymphoma cel line), and their gene loci contains a TFBS of the transcription factor T (BRACH; Brachyury Homolog Mouse), a transcription factor highly active in B-lymphocytes (highlighted in red in Appendix 8). CYP4F3A catalyses the omega-hydroxylation of leukotrine B4, a potent chemoattractant for polymorphonuclear leukocytes. NR1H4 (alias Farnesoid-X-receptor or ileal bile acid transporter) is a bile acid-activated transcription factor which regulates the expression of genes involved in bile acid synthesis and transport. CYP3A4 is a monooxygenase involved in linoleic acid, steroid, and retinol metabolism. With respect to retinol metabolism, 4 remarkable genes were up-regulated. APOA1, APOA4, APOB, and RBP2, all mapped to the KEGG "Vitamin digestion and absorption" pathway (hsa04977).The proteins encoded by these genes are components of the chylomicrons-mediated exocytosis mechanism, a mechanism by which enterocytes secrete luminal absorbed fat-soluble

vitamins, retinol, and cholesterol esters into the lymphatic system (highlighted in blue in Appendix 7 and 8). APOC3 inhibits the uptake of lymph chylomicrons by cells. According to the literature retrieved from the STITCH network NR1H4 influences gene expression and function of APOC3 (http://www.ncbi.nlm.nih.gov/pubmed/22187655).

The Comparative Toxicogenomics Database (CTD) was explored to find chemicals having potential to influence the expression and/or the function of these seven selected key-genes. In Appendix 10, for each key-gene 3 chemicals with potential were listed (web links provide direct access to literature describing the association between a gene and a chemical). The NR1H4 appeared as a central gene/protein in the STITCH network. In addition to the predicted role of NR1H4 in regulation of the transport of fat-soluble vitamins (see above), this nuclear receptor also plays a role in regulating gene expression in intestinal immune cells (see literature links in Appendix 10). Chemicals like alpha-Linolenic Acid and Epigallocatechingallate (EGCG; a natural phenol with antioxidant properties found in high concentrations in green tea), may therefore be promising "candidate additives" to steer gene expression of NR1H4, and with this, immune processes in the intestine. In addition, EGCG also modulates gene expression of NCK1, a key coordinator of cytoskeletal changes in various types of immune cells (including B-cells), enabling polarization and directional migration of these cells. At first, such "candidate" additives have to be tested in an *in-vitro* test. Preferably, a test capable of measuring parameters reflecting the mechanisms predicted by the here performed data-mining strategy. In case of the *Lactobacillus plantarum 299v* dataset analyzed here, for example, mixed cultures of enterocyte cell lines and *ex-vivo* IEL's and/or B cell fractions may be challenged with these chemicals, and tested for expression level of the 3 key-genes CR2, APOC3, and SPP1, or other genes from the IAC list by quantitative real-time PCR. If the outcomes of such experiments are in agreement with the processes predicted by data-mining, an *in-vivo* intervention study, specifically designed for one chemical or a group of related chemicals, may prove whether the "candidate" additives or their analogs can replace DFM like *Lactobacillus plantarum* 299v as an feed-additive that initiates the attraction, migration and homing of (pre)-IgA Ab-secreting cells.

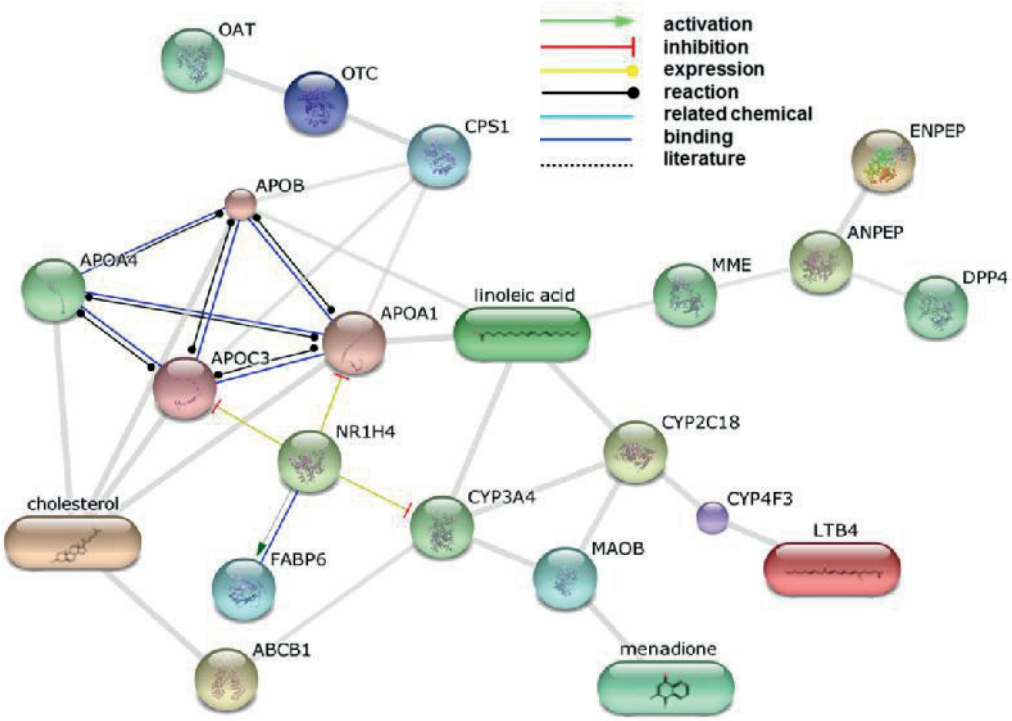

Figure 1 *Interactions between genes/proteins regulated in the colon of pigs in response to repeated L. plantarum administration and relevant chemicals. The displayed network was generated using the (protein)-protein-chemical interaction web tool STITCH 3.1 beta (see also Appendix 9; http://www.ncbi.nlm.nih.gov/pubmed/19897548).*

4 Human and animal models for testing immune competence

The intestine is at the same time the place for nutrient uptake and the barrier against pathogenic antigens. A direct consequence of these physiological functions is a very complex interplay of different cell types leading to a tightly controlled mucosal immune balance in the healthy gut [6].
Using several animal models, intestinal mucosal immunology has been studied *in vivo* (Latin for "within the living")[7] mostly in relationship with pathological conditions, such as inflammatory bowel diseases (Crohn's disease and ulcerative colitis)[8]. As a consequence of, among others, the protection of animals used for scientific purposes and the 3R (reduction, refinement and replacement) approach in animal experimentation, intestinal *ex vivo* models have been developed and employed. *Ex vivo* (Latin for "out of the living") refers to experiments done in or on tissue in an artificial environment outside the organism with the minimum alteration of natural conditions, allowing experimentation under more controlled conditions than possible in *in vivo* experiments, at the expense of altering the "natural" environment [7]. Driven by the ask for faster and cheaper systems, besides pathology related models, intestinal *in situ*, *ex vivo* and *in vitro* models have also been used in research on absorption [9], bacteria-host interactions [10-13] and food science and nutrition [14-17].
In vitro (Latin: in glass) models are those that are conducted using components of an organism that have been isolated from their usual biological surroundings in order to permit a more detailed or more convenient analysis than can be done with whole organisms, and are also known as "test tube experiments" or "petri dish experiments" [7]. *In situ* (Latin: in position) models are intermediate between *in vivo* or *ex vivo* and *in vitro* models. For example, examining cells within a whole organ intact and under perfusion may be an *in situ* investigation. This is not *in vivo* as the donor is sacrificed by the experimentation, but it is not the same as working with the cells alone, a common scenario for *in vitro* experiments [7].
Although not all of the latter models have been used to study intestinal immunology, they may be employed for such an immunological driven approach. In this chapter these models are described, disregarding *in silico* modelling (modelling performed on a computer or via computer simulation)[18].

4.1 *In vitro* models

The intestinal wall is composed of several cell types forming the epithelial barrier (mainly enterocytes) and immune cells on the basolateral side [11, 15]. A perfect *in vitro* cell model has to be a direct substitute of the in vivo environment, reflecting both the natural responses as well as the complex physiology of the intestine. Therefore, cell lines involved in single or co-culture models need to be sufficiently characterized. Such a characterization includes epithelial markers (cytokeratins), brush border (digestive) enzymes, expression of tight junction proteins for the formation of epithelial barrier, integrity and polarity (ZO-1, occludins and claudins, transepithelial resistance (TEER)/transepithelial electrical potential (TEEP) when grown on a microporous membrane, molecular transporters, internal metabolism (cytochromes P450-CYPs) and responsiveness to environmental factors like cytokines and inflammatory molecules (lipopolysaccharides (LPS), cytokines). Depending on the model, cells in different activation/differentiation state can be used. For example, immature non-activated immune cells are useful in host-bacterial intestinal interaction models, while activated cells can be used to study inflammatory intestinal disorders.

4.1.1 Primary cells

Primary cells, isolated from (human or animal) tissue, retain the majority of *in vivo* functionality [11, 15]. They are however rarely used as their survivability is limited and usually need to be derived from different individuals in subsequent tests affecting the reproducibility of the results.
On the other hand, recently commercial sources of primary human intestinal epithelial cells (hInEpCs) have become available [19].

4.1.2 Epithelial mini-guts

However, recently an *in vitro* culture system to grow three-dimensional (3D) intestinal epithelial organoids ('epithelial mini-guts') for periods greater than 1.5 year from $Lgr5^+$-CBC cells has been established [20]. Slender crypt base columnar (CBC) cells are present at the crypt bottom, intercalated between post-mitotic Paneth cells. $Lgr5^+$ marked CBC cells persist for the life-time of a mouse, whereas their progeny include all differential cell lineages of the epithelium. $Lgr5^+$-CBC cells represent cycling, long-lived, multipotent stem cells. Isolated crypts require Matrigel, a 3D laminin and collagen-rich matrix that mimics the basal lamina and a cocktail of R-spondin (a protein interacting with Wnt that constitutes the key pathway to maintain stem cell fate and drive proliferation), epidermal growth factor (EGF) and the polypeptide Noggin is the minimal essential stem cell maintenance cocktail. In vitro-generated organoids occur as cysts with a central lumen flanked by a simple, highly polarized villus epithelium. The basal side of the cells is orientated toward the outside, touching the matrigel, whereas enterocyte brush borders form the luminal surface. Secretion by Paneth and goblet cells occurs toward the lumen. The organoids can be passaged weekly for at least 1.5 years with a phenotype and karyotype that reamin unchanged. To test how normal the epithelial mini-guts are, they were introduced per anum into the colons of mice with chemical-induced mucosal lesions. The engrafted epithelial mini-guts regenerated epithelial patches that were indiscernible from surrounding recipient epithelium and persisted for at least 6 months without changing their histological appearance.

4.1.3 Cell cultures

Most currently used intestinal cell line models are transformed cell lines isolated from cancer affected individuals [21, 22]. Among them, Caco-2 is the most widely used and established cell line. Caco-2 (colon adenocarcinoma) were isolated from a 72-year old Caucasian man and upon reaching confluence, cells were found to express characteristics of enterocyte differentiation and functionality. Another human colon adenocarcinoma cell line, HT-29, was isolated from the colon tumour of a 44-year old Caucasian female, while the Intestine-407 cell line has been established via HeLa cell contamination and CaSki cells were originally isolated from a metastasis in the small intestine. The HIEC-6 cell line was established from normal human crypt small intestinal cells. The H4 cell line was established from human foetal small intestinal cells and subcloned to obtain a small intestinal epithelial cell line, capable of TEER and TEEP formation.
The most widely used rodent lines are IEC-6 and IEC-18 cell lines derived from the rat small intestine [21, 22]. The IPEC-J2 cell line is a non-transformed intestinal cell line derived from jejunal epithelia isolated from a neonatal piglet, maintained as a continuous culture and subcloned to obtain cultures of enterocyte-like and mucin producing cell types.

4.1.4 2D Cell cultures

Cells are mostly cultivated as one-dimensional monolayers on plastic surfaces, where the establishment of a functional, epithelial character is not achieved, possibly resulting in misleading results, especially in adhesion assays [11, 15].
Growing cells on microporous membranes (e.g. insert filters, Transwell models) results in spontaneous cell differentiation and polarization, enabling basolateral feeding of epithelia similar to the *in vivo* setting and facilitating transport studies through the polarised epithelial monolayer [11, 15].
Semi-wet interface culture in combination with mechanical stimulation and DAPT causes HT29, MTX-P8, MTX-E12 and LS513 cells to polarize, form functional tight junctions, form a three-dimensional architecture resembling colonic crypts and produce an adherent mucus layer [23]. Caco-2 and T84 cells also polarize, form functional tight junctions and produce a thin adherent mucus layer after such a treatment, but this is less consistent.
Newly commercially available primary human intestinal epithelial cells (hInEpCs) and induced pluripotent stem cell (iPSC)-derived intestinal cells in Transwell inserts may become an alternative source of human intestinal cells [19].
Epithelial cells growing on microporous membranes can be co-cultured with monocyte/macrophages (derived from the same species) in the lower compartment or on the microporous membrane using an inverted setting [15, 24]. In this way the *in vivo* environment of the intestine is better stimulated and

the chemical crosstalk (chemokine and cytokine release) between intestinal and immune cells (in response to an antigenic stimulus) can be studied. Epithelial cells can also be co-cultured with other more-specialized cell types on microphorous membranes, like mucus-producing Goblet cells.

4.1.5 3D Cell cultures

3D cell models are built from intestinal epithelail cell line growing on a microporous membrane, enabling polarisation of the cells and development of TEER/TEEP [15, 24, 25]. Below the microsporous membrane (basolateral side) epithelial cells are underlaid with immune cells (macrophages, dendritic cells), mimicking mucosal lymphoid tissue (Figure 2).
The pig intestinal cell model is a co-culture composed of the CLAB (adult mucin secreting enterocyte-like) or PSI (partially differentiated cryptic enterocyte-like) intestinal lines and of the PoM monocytes/macrophages that can be differentiated into dendritic cells [15, 24].
Bovine, ovine and goat 3D intestinal cell models are for bovine a co-culture of CIAB (calf intestinal epithelial cells) and BOMA (bovine macrophages), for ovine a co-culture of OSI (ovine small intestinal cells) and MOLT-4 (monocyte/macrophage cell line) and for goat a co-culture of GIE (goat intestinal epithelial cells) and GOMA (goat macrophages) [24].

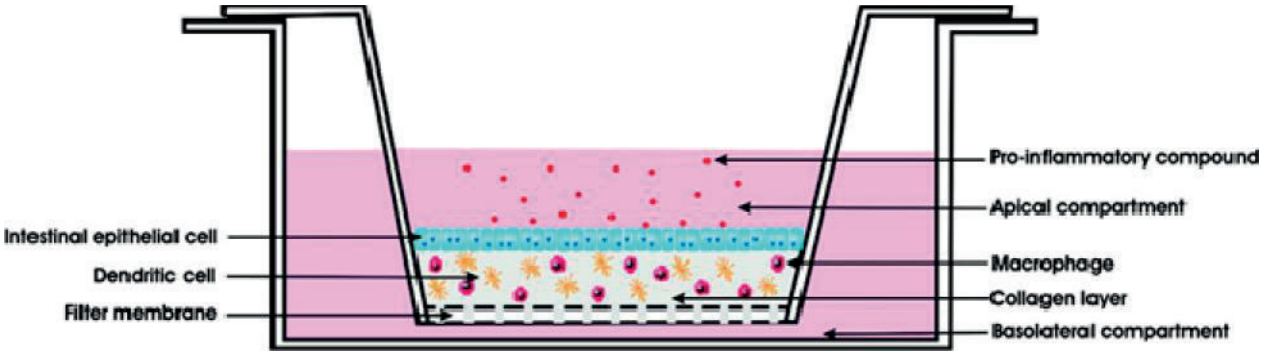

Figure 2 *Setup of the co-culture consisting of intestinal epithelial cell line, macrophages and dendritic cells (from Leonard et al., 2010 without permission)*

4.1.6 Intestine-on-a-chip

3D Cell cultures yield levels of cell differentiation and tissue organization that are not possible in 2D cultures. New advances in 3D culture that leverage microfabrication technologies from the microchip industry and microfluidics approaches to create cell culture micrenvironments that support tissue differentiation are being developed [26-28].
The intestine-on-a-chip recapitulates multiple dynamic physical and functional features of the human intestine that are critical for its function within a controlled microfluidic environment for transport, absorption and toxicity studies, and therefore should have great value for drug testing as well as development of novel intestinal disease models [29]. The biomimetic 'human intestine-on-a-chip' microdevice is composed of two microfluidic channels separated by a porous flexible membrane coated with extracellular matrix (ECM) and lined by human intestinal epithelial (Caco-2) cells (Figure 3). The gut microenvironment is recreated by flowing fluid at a low rate producing low shear stress over the microchannels and by exerting a cyclic strain that mimics physiological peristaltic motions. Under these conditions, a columnar epithelium develops that polarizes rapidly, spontaneously grows into folds that recapitulate the structure of intestinal villi, and forms a high integrity barrier to small molecules that better mimics whole intestine than cells in cultured in static Transwell models. In addition, a microbe (*Lactobacillus rhamnosus* GG) can be successfully co-cultured for extended periods of more than 1 week on the luminal surface of the cultured epithelium without compromising epithelial cell viability which actually improves barrier function.
Ultimately, the development of organ-on-a-chip systems will converge into multi-organ body-on-a-chip systems composed of functional tissues that reproduce the dynamics of the whole-body response [30].

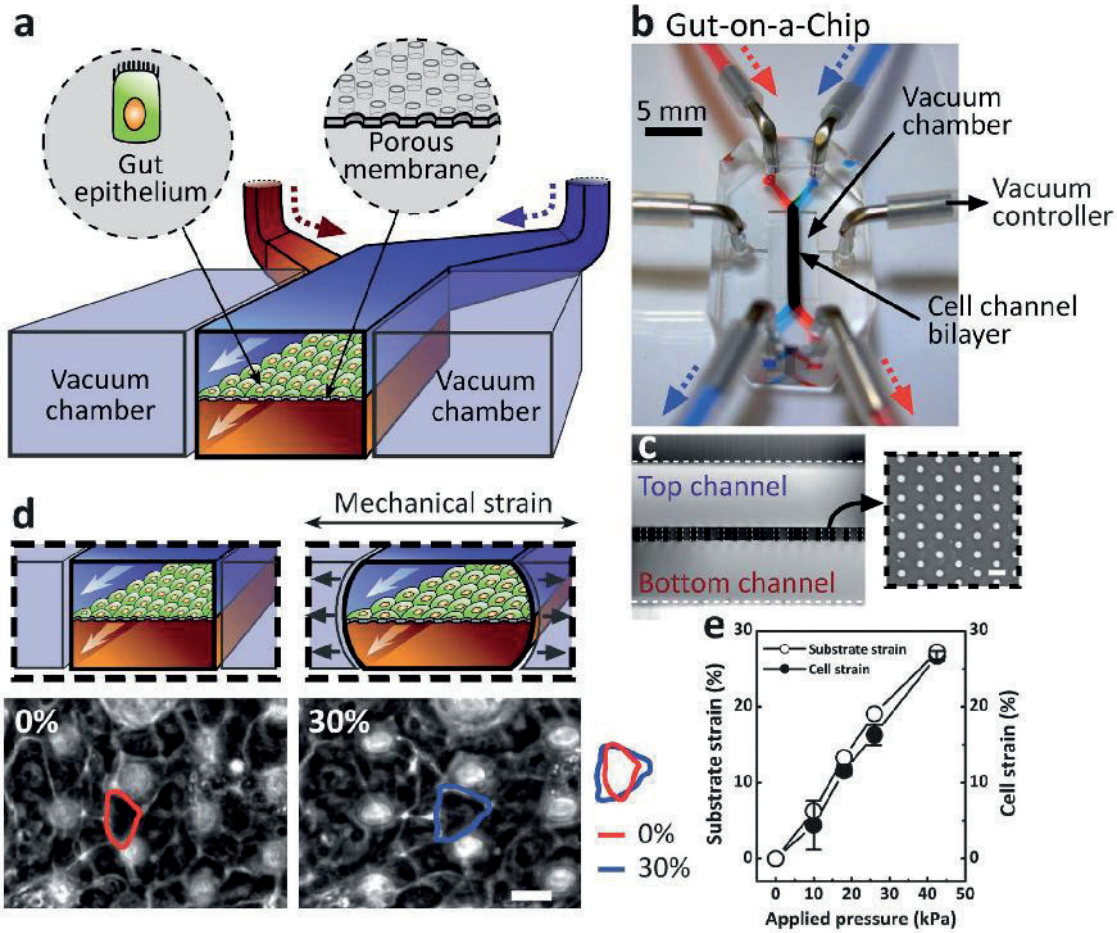

Figure 3 *The human intestine-on-a-chip. (a) A schematic of the intestine-on-a-chip device showing the flexible porous ECM-coated membrane lined by epithelial cells cross horizontally through the middle of the central microchannel and full height vacuum chambers on both sides. (b) A photographic image of the intestine -on-a-chip device composed of clear PDMS elastomer. A syringe pump was used to perfuse (direction indicated by arrows) blue and red dyes through tubing to the upper and lower microchannels, respectively, to visualize these channels. (c) A cross-sectional view of the top and bottom channels (both 150 mm high) of the intestine-on-a-chip; square inset shows a top view of a portion of the porous membrane (10 mm pores; bar, 20 mm). (d) Schematics (top) and phase contrast images (bottom) of intestinal monolayers cultured within the intestine-on-a-chip in the absence (left) or presence (right) of mechanical strain (30%; arrow indicated direction) exerted by applying suction to the vacuum chambers. Red and blue outlines indicate the shape of a single Caco-2 cell before (red) and after (blue) mechanical strain application (bar, 20 mm). Note that the cell distorts in the direction of the applied tension. The regular array of small white circles are pores visible beneath the epithelial monolayer. (e) Quantitation of the mechanical strain produced in the ECM-coated, flexible, porous PDMS membrane (open circles) and in the adherent epithelial cells (closed circles) as a function of pressure applied by the vacuum controller (From Kim et al., 2012 without permission)*

4.2 *Ex vivo* models

4.2.1 Perfusion system

Intestinal fragments or whole organs (rat small bowel, canine colon) are connected with a perfusion circuit: inflow through cannula connected to the main artery inflow and outflow via the main vein [13]. Perfusion via a perfusion circuit under physiological conditions of either whole organs or resected intestinal tissue maintains intestinal explants viability (morphology, histology and physiology) up to several hours of perfusion. The limited duration of an experiment and the need for sufficient quality and quantity of organs are a major disadvantage of such a system.

4.2.2 Everted sac technique

The intestine is divided into segments of 5-6 cm each and each segment is washed with an ice-cold physiological solution [31]. The washed intestinal segment is gently everted over a glass rod. One end of the everted segment is tied off and filled with Krebs solution of 37^{o}C after which the filled intestinal everted segment is sealed with a second tie. The filled everted sac is transferred in an incubation flask with oxygenated medium at 37^{o}C.
The everted sac model is sensitive and specific with several factors having an impact on the results of transport studies, including animal factors (species, age, sex, diet, disease state, chronic treatment, harvesting time, method of killing), intestinal segment used (duodenum, jejunum, ileum, colon) and experimental conditions (e.g. pH, aeration, temperature). The tissue viability and metabolic activity is approximately 2 hours and is one of the limiting disadvantages of this technique.

4.2.3 Ussing chamber

Ussing chambers are mostly used for the characterization of intestinal absorption in different parts of the intestine [32].
Intestinal tissue (from all intestinal segments) is collected in ice-cold Krebs-Ringer bicarbonate (KRB) buffer supplemented with $NaHCO_3$, $CaCl_2$ and HEPES adjusted to pH 7.4 [32]. The adipose tissue and muscle layers are carefully removed and the mucosa is mounted in the Ussing chamber (with an exposed tissue area of 0.46 cm2. Both compartments of the Ussing chamber are filled with pre-warmed KRB-buffer and continuously oxygenated with 95% O_2 and 5% CO_2 gas mixture and maintained at 37^{o}C. The viability of the mounted mucosa is assessed by monitoring the transepithelial potential difference (PD) which remains stable for at least 90 min.

4.2.4 *In vitro* organ culture (IVOC)

Although called *in vitro* organ culture (IVOC), the different forms of IVOC are also described by the term *ex vivo* organ culture [12]. The latter term better approaches reality, as in IVOC survival time of biopsies is limited -notwithstanding the oxygenation of the tissue to achieve adequate exchange of gas and nutrients- and fresh organ tissue is needed for each new experiment [12].
The major advantage of such organ cultures is the anatomical resemblance of the *in vivo* situation [13]. Other advantages of such organ cultures are the use of tissues from different parts of the intestine (from duodenum to colon), from various species representing specific microbiota, genetic and/or disease backgrounds and from animals or humans, which have been subjected *in vivo* to food ingredients [13].
The careful and laborious preparation, relatively low-throughput and short term survival in culture – which may be extended with advanced knowledge in tissue engineering- are the major disadvantages of such organ cultures [13].

4.2.5 Non-polarized (or traditional) IVOC

Intestinal biopsies (obtained from patients receiving upper endoscopy, ileo-colonoscopy or surgical intestinal resection) are placed on a thick supporting sponge or agarose gel base (with the apical side upward and the serosal side downward in direct contact with the underlying sponge) in IVOC medium to create a thin film of medium on the apical sides of biopsies [12](Figure 4A). Bacterial overnight cultures are inoculated on the mucosal side of the biopsies and incubated at 37°C on a rocker in an atmosphere of 95% O_2 and 5% CO_2 with regular replacement of medium to keep the biopsies viable. At harvest, the biopsies were removed and shaken in medium to remove mucus and bacteria. Disadvantages of non/polarized IVOC comprise the inability to apply different conditions to either side of the biopsies [12]. Furthermore, no interactions with deep tissue, different severity of trauma caused by biopsy sampling procedure, diverse underlying diseases of the patients and individual variations in the gut flora associating with the biopsy tissues, are inevitable weaknesses of this model.

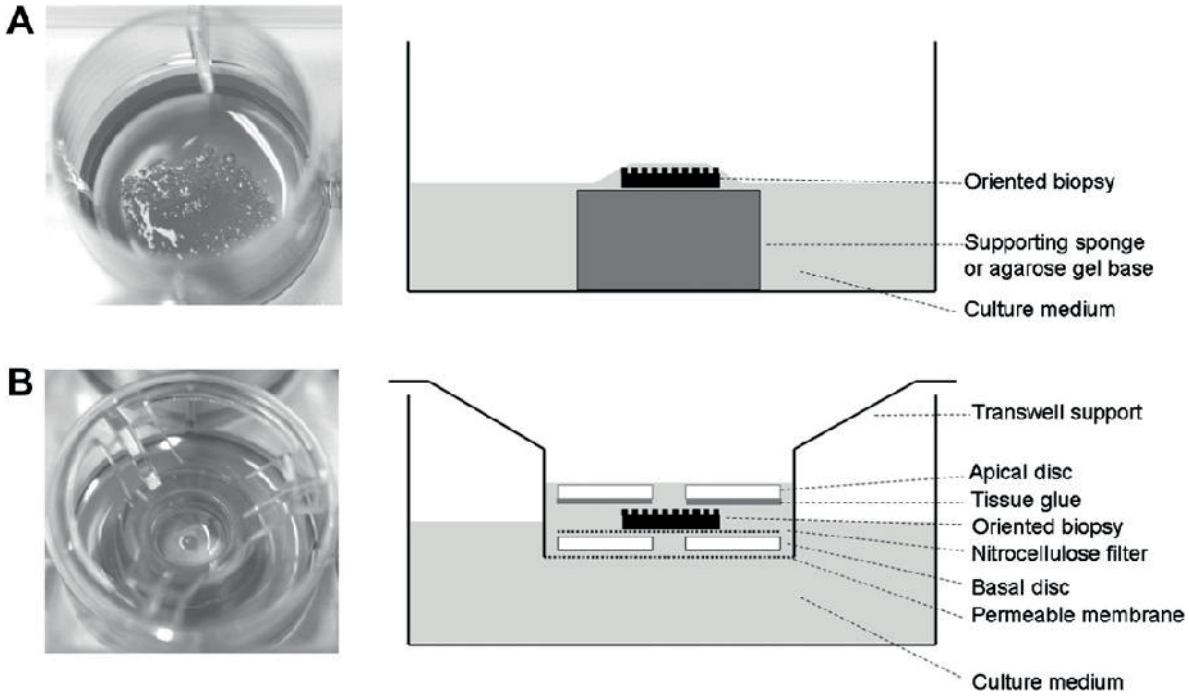

Figure 4 *Mounting of intestinal biopsies for traditional in vitro organ culture (IVOC) and polarized IVOC. (A) A biopsy specimen mounted on a sponge or an agarose plug immersed in the medium with its mucosal surface upward. (B) After orientation of the tissue on a nitrocellulose filter, the apical disc was fixed to the mucosal side of the biopsy via tissue glue. The sandwich was then inserted between a Snapwell support (from Fang et al., 2013 without permission)*

4.2.6 Polarized *in vitro* organ culture (pIVOC)

In the pIVOC each biopsy is positioned as a sandwich between two acrylic glass discs with a central aperture slightly smaller than the size of the biopsy [12](Figure 4B). The upper disc is sealed to the peripheral area of the mucosal side of the biopsy to minimize leakage from the apical to the basal compartment. The sandwich holding the biopsy is then mounted in a Snapwell insert. After transfer into a six/well plate, the explant tissue is submerged in medium and incubated at 37°C on a rocker in an atmosphere of 95% O_2 and 5% CO_2 with regular replacement of medium to keep the biopsies viable. At harvest, the biopsies were removed and shaken in medium to remove mucus and bacteria.

4.2.7 Novel polarized *ex vivo* organ culture

Samples are excised from the intestines of patients at the time of surgery and directly transferred in HBSS buffer supplemented with bacteriostatic antibiotics at 4°C to the laboratory [33, 34]. The clean mucosal layer is washed in Hank's buffer and cut with sterile scalpels into 1 cm^2 pieces. The pieces are placed on sterile metal grids with the basolateral side facing downwards. and the cylinder (cloning

cylinder) is attached with surgical glue under sterile conditions with a pair of forceps (Figure 5). Culture medium (Dulbecco's Modified Eagle medium supplemented with foetal bovine serum, non-essential amino acids, pyruvate, glutamine, epidermal growth factor and insulin) is dispensed in the centre well of the centre-well organ culture dish and incubation is carried out in 100% O_2.

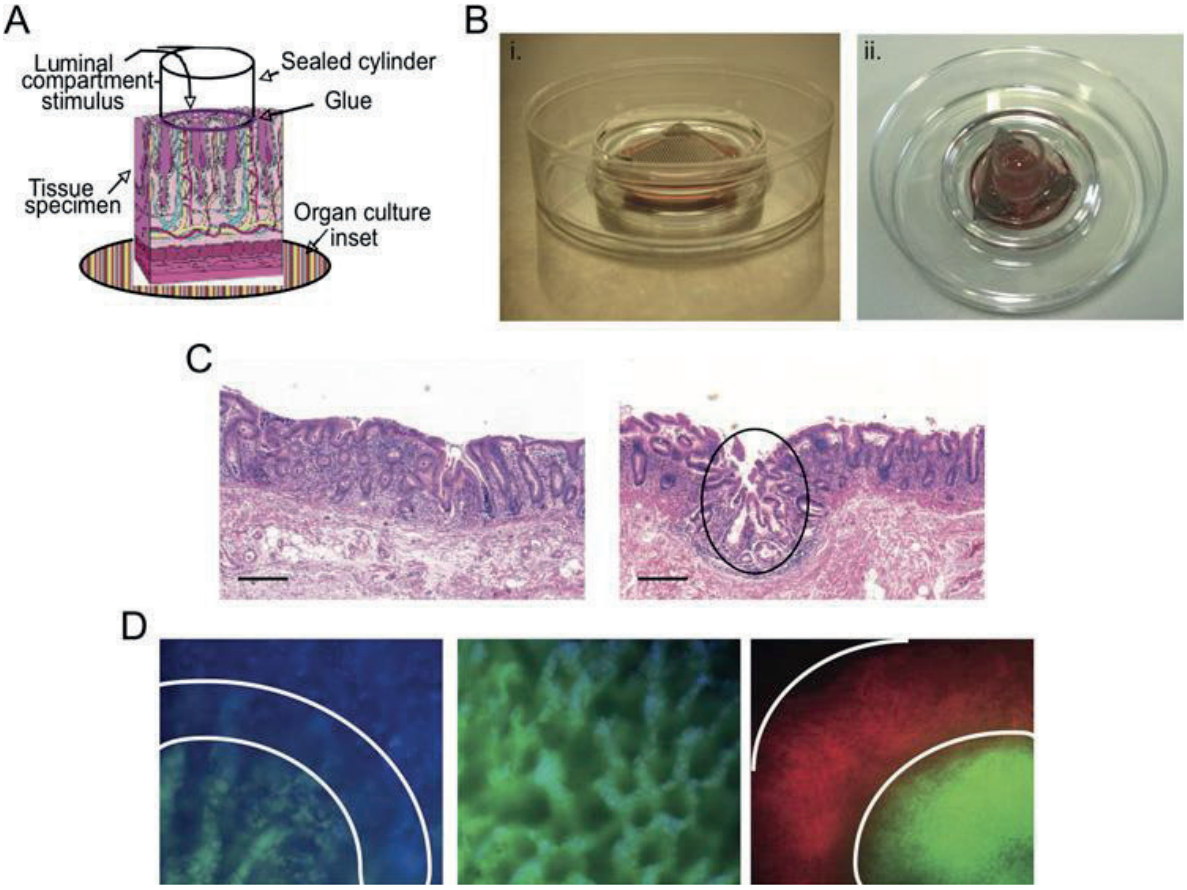

Figure 5 *Application of a cylinder on the luminal face of an intestinal mucosal explant does not impact on tissue wellbeing. (A, B) Schematic representation and photographs of the technical setting for culture and apical stimulation. (C) Tissue cultured in the absence of the cylinder (left panel) and with the cylinder (right panel). Ellipse: cylinder contact point. Scale bars: 500 mm. (D) Left panel: Tissue stained with phalloidin-FITC in the interior of the cylinder (green) and 49,6-diamidino-2-phenylindole (blue, for nuclear staining) once the cylinder was removed. Middle panel: The same piece of tissue inside the cylinder (magnification 43). Right panel: Tissue processed with two cylinders of different size (magnification 1.53). White lines represent cylinder borders. (From Tsilingiri et al., 2012 without permission)*

4.2.8 IVOC in the Ussing chamber

A biopsy or a biopsy sandwiched between two flat discs with a central aperture of 2.5 mm with the serosal side fixed to the support disc, is inserted into a conventional Ussing chamber [12](Figure 6). Apical chambers are perfused with anaerobic or low O_2 levels on the mucosal side of the biopsy to stimulate the microaerobic milieu in the gut, whereas basolateral chambers are maintained in oxygenated conditions to secure good tissue survival. In the IVOC Ussing chamber, temperature and pH of the epithelial are controlled and transmembrane voltage gradients for perfusion and oxygenation can be recorded.

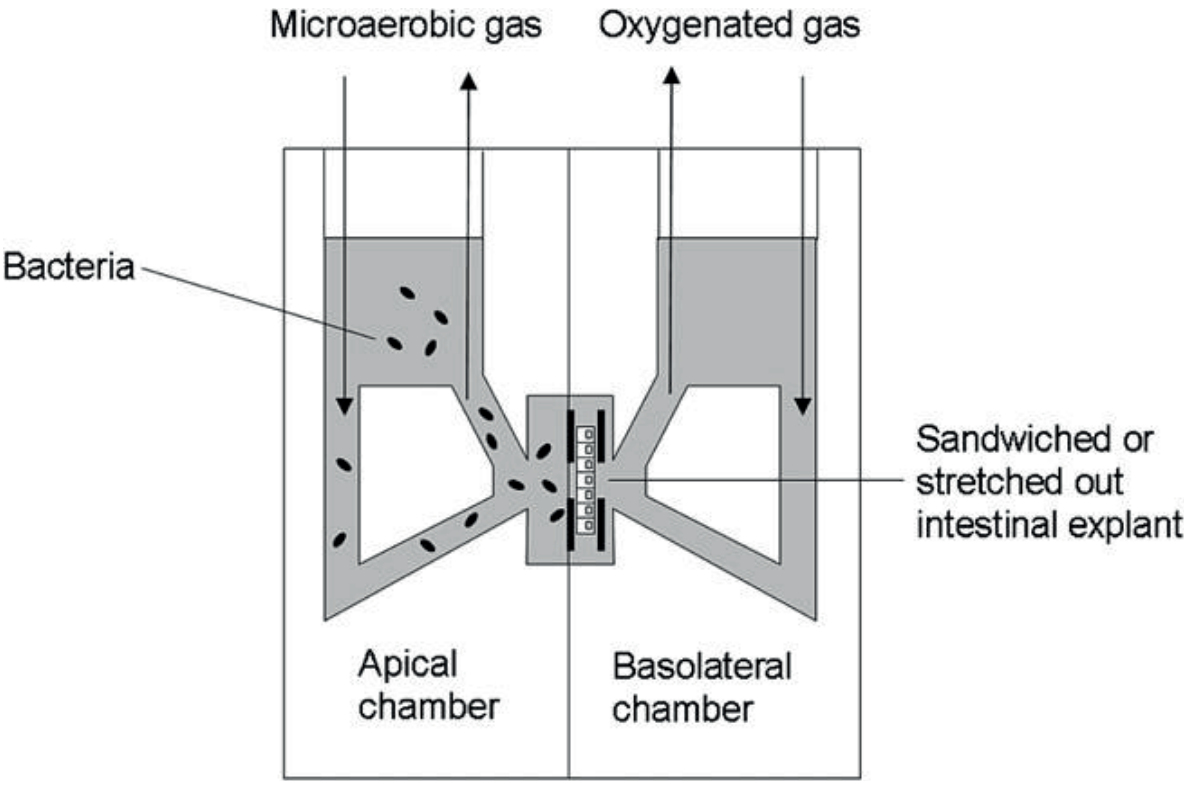

Figure 6 *Bacterial infection within in vitro organ culture (IVOC) in the Ussing chamber. An intestinal explant sandwiched between discs or stretched out by metal pins is mounted between two half chambers and infected apically with bacteria. The apical and basolateral chambers are maintained in microaerobic and oxygenated conditions, respectively (from Fang et al., 2013 without permission)*

4.2.9 Precision-cut tissue slices (PCTS)

The small intestine is transferred to ice-cold oxygenated Krebs buffer [35, 36]. Segments of 3 cm are excised and flushed with ice-cold buffer. One side of the segment is tightly closed, filled with agarose solution at 37°C and then cooled in ice-cold buffer, allowing the agarose solution to gel. Subsequently, the filled segment is embedded in 37°C agarose solution using a pre-cooled tissue embedding unit. After the agarose solution has gelled, precision-cut slices (thickness of 400-450 µm) are cut using a tissue slicer.
Slices are incubated individually in 6- or 12-well culture plates in RPMI medium. The culture plates are placed in a pre-warmed cabinet (37°C) in plastic boxes and incubated in an atmosphere of 95% O_2 and 5% CO_2. Intestinal slices remain viable for 24 h.
PTCS contain all cells of the tissue in their natural environment, leaving intercellular and cell-matrix interactions intact and are therefore appropriate for studying multicellular processes.

4.2.10 Porcine intestinal tissue model

Intestinal segments of pigs are used in a high throughput system in 24 well culture plates [13]. Intestines of pigs killed after approximately 12 h fasting are excised within 15 min after slaughter and stored in ice-cold oxygenated (95% O2, 5% CO2) Krebs Ringer bicarbonate buffer adjusted to pH 7.4 (KRB) and transported to the laboratory (time between excision and studies was approximately 45−60 min)[37]. Upon arrival at the laboratory, the intestine was rinsed with KRB and cut open in a longitudinal direction. With the basolateral side upward, the outer muscle layers are carefully stripped off. The mucosal tissue is placed on gauze with the apical side upward and circles with a diameter of 8 mm (0.5 cm2) are punched out using with a biopsy punch. The intestinal segments are transferred to a 24-well plate kept on ice and filled with KRB. Studies are initiated by replacing the buffer with pre-warmed KRB. The tissues are incubated for 1 h at 37 °C in a humidified incubator at 5% CO2.
This model is further developed to a tractable, medium throughput diffusion chamber based system InTESTine™ [13].

4.3 *In situ* models

4.3.1 Intestinal loop model

In the intestinal loop model, the animal (typically rat, rabbit or mouse) is fasted for approximately 3 days and anaesthetised for the entirety of the surgical procedure [9]. An incision is made in the midline of the abdomen to expose the small intestine. The desired intestinal segments are sliced, held with a clamp, and then washed to remove excess food particles while keeping the intestine attached and in close proximity to the body cavity. The segment is then ligated using suture to form a loop, assuring normal blood flow and muscle movement to the looped segment. An alternative way to perform the initial preparation is to make an incision at the desired location and then ligating that end. To determine particle transport across the intestine, the particle suspension is injected directly into the loop, and the intestinal loop is returned to the body cavity of the animal for up to 2 h while the animals are maintained under anaesthesia. After a chosen time period, the animal is sacrificed and the intestinal loop is removed from the body cavity of the animal. A majority of the time, the closed loop intestine is returned to the body cavity for 2 h and then the entire intestinal segment is removed. Another way to use the closed loop method is to inject a solution into the closed loop, remove the suturing after 2 h, and return the intact intestine to the body, allowing the animal to resume normal digestive processes and movement. After no more than 24 h, the animal is killed and the intestines removed for analysis.

4.3.2 Small intestinal segment perfusion (SISP)

After overnight fasting, a piglet is anaesthetized and in the small intestine maximal 10 segments of approximately 20 cm each are prepared, each with a cranial inflow tube and a caudal outflow tube [38]. The segments are either paired (in one infected and one non-infected) or not, and perfused for up to 8 hours with, in the case of paired segments, up to 5 different and, in the case of non-paired segments, up to 10 different perfusion fluids. For each segment over 8 h net fluid absorption is determined and from each segment mucosal samples (after 8 hours or at different time points after the start of perfusion) can be collected to analyse genome responses of early pathogen-induced inflammation [39].

4.4 *In vivo* models

For pathological human conditions, such as Crohn's disease and ulcerative colitis, different animal models using less complex organisms (roundworm, fruit fly, fish) or rodents (mouse, rat, guinea-pig) have been developed including spontaneous models, chemically-induced models, genetically engineered models (transgenic animals), genetically engineered (knockout mice) and T cell transfer models [6, 8, 40, 41]. In such models blood samples can be taken and by endoscopy or at dissection intestinal tissue samples. For studying the in vivo functions of human cells and tissues and the human immune system, 'humanized' mouse strains have been developed based on immunodeficient mice with mutations in the interleukin-2 receptor common γ-chain locus [42].
Also in the pig a model for chemically-induced colitis has been described [43-46]. Pigs can be fitted with catheters allowing frequent blood sampling and intestinal cannulas for taking intestinal biopsies more frequently than just at dissection [47].

4.5 Correlations between models and real practice in humans and monogastrics

Advantages and limitations of most models have been indicated. Assessing mucosal immunity [48] has not been implied in all models described, but in principal from all models tissue and/or fluid samples can be taken which may be used for immunological analysis.
The correlation between a model and the 'real practice' of humans or monogastrics, depends on the (scientific) question to be answered and the model used. Different *in vitro*, *in situ* or *ex vivo* models, for instance, not always indicate the same:

- *In vitro*, *Lactobacillus gasseri* was found to be effective in reducing *Escherichia coli* adhesion to Caco-2 cells, but in *ex vivo* conditions no exclusion or displacement of *Escherichia coli* by *Lactobacillus gasseri* was observed on jejunal piglet tissue [49].

Furthermore, both studies showing a good correlation and studies showing a bad or no correlation between a model and the 'real practice' of monogastrics have been published. Three examples of both kind of results, in which the *in vitro*, *in situ* or *ex vivo* experiments are reported in the same source as the *in vivo* results, are:

- *In vitro*, in the human small intestinal epithelial Caco-2 cell model, the supernatants of diets containing either pre-fermented cereals or their fermentation end-products (short chain fatty acids) clearly modulated cellular growth, metabolism, differentiation and mucosal integrity [50]. Such effects were not observed in the same study for *in vivo* intestinal characteristics (morphology, mitotic activity) and performance in weanling pigs [50], although in other *in vivo* experiments positive effects of fermented wheat have been reported [51].
- *In vitro*, tempeh, a fermented soya bean, interfered with adhesion of enterotoxigenic *Escherichia coli* (ETEC), *in situ* tempeh reduced ETEC-induced fluid loss in piglet small intestinal segments and *in vivo* severity of diarrhoea was less, feed intake increased and daily weight gain increased in ETEC challenged piglets [52].
- *In vitro*, a porcine *Lactobacillus amylovorus*-like strain (*pLAI*) protected the intestinal permeability of porcine IPEC-1 cells from the perturbation induced by ETEC and *in vivo*, in piglets challenged with ETEC, *pLAI* increased daily weight gain and tended to increase ETEC shedding and total immunoglobulin A (IgA) in saliva and serum [53].

Also advancing insight can affect the way models are looked at. In modern biomedical research, mouse models are often used to explore basic pathophysiological mechanisms, evaluate new therapeutic approaches and make go or no-go decisions to carry new drug candidates forward into clinical trials [54]. But while in humans acute inflammatory stresses from different aetiologies result in highly similar genomic responses, the responses in corresponding mouse models correlate poorly with the human conditions and also, one another. Among genes changed significantly in humans, the murine orthologs are close to random in matching their human counterparts, questioning the use of mouse models to study human inflammatory diseases [54].

A systematic review, focusing on a single question which tries to identify, appraise, select and synthesize all high quality research evidence relevant to that question, as recently introduced for animal models [55], may be employed (and may be technically supported by ZonMw).

5 Nutritional intervention strategies for monogastrics

This chapter aims to review the nutritional interventions that directly or indirectly (via the microbiota) engage the optimal development of the innate and acquired immune system, both in the maternal (par. 4.1), neonatal (par. 4.2) and post-neonatal phase (par. 4.3).

The strategy to search the relevant literature was as follows; the input to the query were 1) general or specific nutritional factors; 2) immune competence related words, i.e. intestinal/gut health, immune system, or immunity; 3) species (e.g. pig, *Sus scrofa* or chicken, broiler, *Gallus gallus*); 4) 'maternal' was used as a key word for searching to studies related to interventions during the maternal stage. It should be noted that the data presented here are incomplete, however it gives an overview of literature describing nutritional interventions in neonatal livestock that may affect performance and/or immune related processes.

5.1 Effects of interventions during the maternal stage on the immune competence of the progeny

5.1.1 Trans-generational effects

Environmental conditions that are experienced in early life can profoundly influence biology and long-term health. Early-life nutrition is a well-documented example of such conditions because they influence the adult risk of developing metabolic diseases. A human Swedish study showed that diabetes mortality increased in men if the paternal grandfather was exposed to abundant nutrition during his pre-pubertal growth period, an effect later extended to paternal grandmother/granddaughter pairs and transmitted in a gender-specific fashion. Poor maternal nutrition has also been associated with increased risk of type 2 diabetes mellitus over several generations in North American Indians, and individuals whose grandparents were in utero during the Dutch Hunger Winter had lower birth weight [56]. The above mentioned examples show that malnutrition during pregnancy exerts negative effects in the offspring. In this study, however, we are interested in dietary interventions that particularly improve the immune competence of the progeny.

5.1.2 Trans-generational transfer of immune competence

Below, the mode of action of dietary trans-generational effects on the immune competence of piglets and broilers is described.

In mammals
According to Salmon et al. [57], immunoglobulins cannot cross the placenta in pregnant sows. Neonatal pigs are therefore agammaglobulinemic at birth. Therefore, they cannot provide rapid immune responses at systemic and mucosal sites. Their survival depends directly on the acquisition of maternal immunity via colostrum and milk. Protection by maternal immunity is mediated by a number of factors, including specific systemic humoral immunity, involving mostly maternal IgG transferred from blood to colostrum and typically absorbed within the first 36 h of life. Passive mucosal immunity involves local humoral immunity, including the production of secretary IgA (sIgA) by the mammary gland (MG), which is transferred principally via milk until weaning. These antibodies are produced in response to intestinal and respiratory antigens, like pathogens and commensal organisms. Protection is also mediated by cellular immunity, which is transferred via maternal cells present in mammary secretions. The hormones addressins and chemokines are involved in the regulation of the cellular immunity. The enhancement of colostrogenic immunity depends on the stimulation of systemic immunity, whereas the enhancement of lactogenic immunity depends on appropriate stimulation at induction sites, an increase in cell trafficking from the gut and upper respiratory tract to the MG and, possibly, enhanced immunoglobulin production at the effector site and secretion in milk. In addition,

mammary secretions provide factors other than immunoglobulins that protect the neonate and regulate the development of mucosal immunity, which is a key element of postnatal adaptation to environmental antigens [57].

In birds

The egg is the main source of maternal antibodies of broilers. Although maternal antibodies are only present in broilers during the first 2 wk of life, they may exert a lifelong determinative influence on the neonatal immune system [58]. Maternal antibodies have strong immuno-regulatory properties which affect the specific neonatal B and T cell binding repertoire. Maternal antibodies, therefore, can be considered as non-genetic, information-carrying molecules that transfer knowledge about the immunologically relevant environment gathered by the mother to her offspring. One of these antibodies, avidin, was expected to guide the gut microbiota composition [1]. It is bacteriostatic and inhibits pathogenic bacterial growth. Moreover, parental natural antibodies might be transferred via the egg shell to the young chicken, thereby preventing the vertical transmission of pathogens. Finally, evidence for heritability of natural and specific antibody responses was found [59].

5.1.3 Impact of nutritional interventions in the maternal diets on trans-generational epigenetic effects

It has been demonstrated that maternal dietary interventions could interfere with different parameters in the offspring, as shown in Table 1. The mode of action, however, differs between intervention strategies. Addition of the probiotic *Saccharomyces cerevisiae* to sow diets was found to increase the IgA concentration in serum of their piglets [60], indicating that this intervention affected the amount of antibodies. Fish oil supplementation in mice reduced the levels of total OVA-specific antibodies and OVA-IgG1 titers in the pup blood [61].

Maternal consumption of seaweed extract, fructo oligosaccharides or zinc resulted in increased cytokine levels in the ileal piglet digesta, in human breast milk, and in 6-month old babies respectively [62-64], whereas maternal consumption of β-carotene reduced cytokine levels in 6-month old babies. Dietary supplementation with probiotic (*Bacillus cereus var. Toyoi*) or with additional vitamins and trace minerals increased the number of T cells and Leukocytes in piglets and broilers, respectively [65, 66]. From a study conducted in rats, it could be concluded that lymphocyte proliferation, which was impaired at birth after prenatal exposure to a low protein diet, could be overcome in postnatal rats if 50% or 90% of corn oil in the prenatal diet was replaced by coconut oil [67]. In another rat study it was shown that a reduced maternal selenium intake of rats reduced the neonatal selenium status which in turn hampers the neonatal immune system development [68]. Replacing sunflower oil by fish oil in broiler breeder diets resulted in the offspring in an increased production of leukotriene B5 by thrombocytes, indicating that modulating maternal dietary n-6 and n-3 fatty acid could lead to less inflammatory-related disorders in poultry [69-71].

Some dietary interventions in the maternal diet (e.g. addition of seaweed extract or vitamins/trace minerals) resulted in an improved gut integrity of the progeny [63, 65]. Addition of probiotics (e.g. *Bacillus cereus var. Toyoi, Saccharomyces cerevisiae*) to sow diets reduced the diarrhea scores of their piglets [60, 66], whereas maternal zinc supplementation would be an effective way to reduce diarrhoeal disease in babies [64].

Supplementation of *Lactobacillus rhamnosus* and *Bifidobacterium lactis* to pregnant women increased the adiponectin content of their colostrum [72].

Until now, transcriptomics data is scarce, but addition of addition vitamins/trace minerals to broiler breeder diets was found to increase the expression of genes that are related to intestinal turnover, proliferation and development, metabolism and feed absorption in the offspring [73].

The improved immune response of the offspring was not always related to improved performance of the progeny. Maternal supplementation with seaweed extract, however, resulted in improved body weight gain of the piglets, and this effect maintained even till the growing finishing phase [63]. Growth depression in broilers that passed through a malabsorption syndrome infection was less if their mothers received additional vitamins/trace minerals [65]. More details regarding the investigated studies are provided in appendix 14.

Table 1 Trans-generational epigenetic effects of dietary interventions in the maternal phase

Nutritional factors	Effect	Marker	Ref.
Fatty acids			
Alkyl-glycerols (AKG) extracted from marine organisms	Giving AKG to sows in late gestation and lactation can improve the passive immunity transfer to piglets at weaning and at 70 d of age.	Performance, specific immune parameters, % of serum bactericidal activity and haemolytic complement at weaning and 70d of age.	[74]
Corn vs. coconut oil x protein content	Reduced lymphocyte proliferation at birth of rats after prenatal exposure to a low protein diet, which could be overcome postnatally if the prenatal diet included a large amount of coconut oil and a small amount of corn oil.	Neonatal lymphocyte proliferation in thyme and spleen; spleen natural killer cell activity.	[67]
Fish oil vs. linseed oil	In mice, maternal intake of fish oil but not of linseed oil changes the offspring's antigen-specific response.	Fish oil reduced the levels of total OVA-specific antibodies and OVA-IgG1 titers in the pup blood, compared to the control and linseed oil groups.	[61]
Fish oil vs. sunflower oil	Early access to n-3 PUFA through in ovo reduces pro-inflammatory cardiac eicosanoid production without affecting hatchability, and may alter leukotriene production in chicks, which could lead to less inflammatory-related disorders in poultry.	Retention of n-3 PUFA in cell membranes, plasma non-esterified fatty acids, production of pro-inflammatory eicosanoids, cell-mediated immunity, and cyclooxygenase-2 protein expression.	[69-71]
Seaweed extract			
Laminarin/Fucoidan	SWE supplementation during lactation improved performance and immune response of weaned piglets.	Performance, pro-inflammatory IL-1a mRNA expression in the ileum of pigs 11 d after weaning, increased villi/crypt ratio.	[63]

Nutritional factors	Effect	Marker	Ref.
Pre- and Probiotics			
Saccharomyces cerevisiae	Dietary supplementation with live yeast S. cerevisiae to sows and piglets in the late gestation, suckling, and postweaning periods can be useful in the reduction of the duration and severity of postweaning diarrhea caused by ETEC.	Performance parameters, duration of diarrhea, shedding of pathogenic ETEC bacteria, IgA levels in serum of piglets.	[60]
FOS	A comprehensive analysis of gene expression in human milk cells to characterise the effect of FOS administration in pregnant and lactating women on the production of immunoactive agents in breast milk.	The consumption of FOS by pregnant and lactating women increases the production of IL-27 in breast milk.	[62]
Bacillus cereus var. Toyoi	Toyoi supplementation of sows and their piglets had a positive impact on the health status of the piglets after a challenge with Salmonella, likely due to an altered immune response.	CD8+ gamma delta T cells in the peripheral blood and the jejunal epithelium; reduced diarrhea scores; less fecal shedding of salmonella.	[66]
Lactobacillus rhamnosus and *Bifidobacterium lactis*	Probiotic supplementation to pregnant women increased the colostrum adiponectin concentration, which has anti-inflammatory properties.	Adiponectin concentration in colostrum	[72]
Vitamins/minerals			
Mixture of vitamins and trace minerals	The immune system of broilers can be stimulated by addition of vitamins and trace minerals to broiler breeder diets, without affecting the growth potential.	Heamatology, spleen weight, humoral response, leukocytes, recovery rate of intestinal lesions and gut histology, gene expression related to intestinal development.	[65, 73]
Selenium	Maternal selenium intake of rats impacts neonatal selenium status which in turn influences the neonatal immune system development.	Selenium content in plasma and milk, neonatal immune cell differentiation and function.	[68]
Zinc + β-Carotene	Addition of zinc to routine iron and folic acid supplements for pregnant women could be an effective way to reduce diarrhoeal	Zinc supplementation increased interleukin-6 production and β-carotene supplementation reduced	[64]

	disease	interferon-g production.	

5.1.4 Conclusions

The most important conclusions were:

- Trans-generational effects of maternal dietary interventions on improvement of gut health and immune competence has been demonstrated in mammals (mice, rats, pigs, humans) as well as in poultry.
- The reviewed dietary interventions can be categorized to fatty acids, seaweed extract, pre- and probiotics, and vitamins and minerals.
- The dietary interventions showed a variety in modes of action. They could affect the composition of the microbiota, the numbers or activity of specific immune cells, the gut morphology, and the expression of genes involved in immune response.
- In some studies, it was shown that the maternal dietary interventions resulted in improved animal performance or reduced incidence of diarrhea of the offspring.
- Only a few studies reported trans-generational effects on gene expression. These studies investigated the effects of supplemented omega-3 fatty acids or vitamins and minerals.
- Supplementation of maternal diets with specific fatty acids, in particular with fish oil, showed to have a wide impact on the immune competence of the progeny. Therefore, modulating the dietary fatty acid profile of maternal diets seems to be the most promising intervention for improving immune competence of the progeny.

5.2 Impact of nutritional interventions in the neonatal phase on immune related parameters

5.2.1 Results from literature

Neonatal development is defined as; day 0 to weaning for pigs and day 0 to 14 for broilers, thus the early development after birth/hatch. Which is hallmarked by the development and differentiation of the intestine. Morphological, functional and immunological development occur simultaneously, and can be influenced by the host (genetics), microbiota, environment (feed, antibiotics, etc.). For both pigs (Table 2) and poultry (Table 3) we have identified a number of macronutrients and micronutrients, as well as microbiota that influence the performance and/or intestinal immune status of the host. Macronutrients can be categorized into carbohydrates (sugars / saccharides), fats (vegetable, animal, etc.), and proteins (polymeric chains of amino acids). Micronutrients are categorized into vitamins (e.g. A, B, C, and E) and minerals (e.g. zinc, copper, selenium, and iodine). The microbiota is more difficult to categorize because of the multitude of processes in which they are involved, however lactobacilli and bifido bacteria are often defined as health promoters. The strategy to search the relevant literature was as follows; the input to the query were 1) general or specific nutritional factor; 2) immune competence related words, i.e. intestinal/gut health, immune system, or immunity; 3) species (e.g. pig, *Sus scrofa* OR chicken, broiler, *Gallus gallus* OR infants, babies). It should be noted that the data presented here are incomplete, however it gives an overview of literature describing nutritional interventions in neonatal livestock that may affect performance and/or immune related processes.
More details regarding the investigated studies are provided in appendix 12 (pigs) and 13 (poultry).

Nutritional factors	Effect	Marker	Ref.
Prebiotics	Composition gut microbiota was influenced by piglet diet and age	Gas production, pH, microbiota changes, SCFA production	[87]
	All prebiotic supplemented groups had numerically higher Lactobacillus populations	Weight, intestinal morphology, inflammation markers, microbiota	[88]
	Enhanced structure and function throughout the residual intestine	Morphology (Crypt-Villus), epithelial cell proliferation (PCNA), nutrient transport (ussing chamber)	[89]
	Increased (luminal) lactobacilli, lactic acid, and SCFA	Morphology, pH, digesta metabolits, microbiota, gene expression (TNFα, IL-8, IL-1β (C), and IL-10)	[90]
Probiotics	Enhanced structure and function throughout the residual intestine	Morphology (Crypt-Villus), epithelial cell proliferation (PCNA), nutrient transport (ussing chamber)	[89]
	Effective against E. coli infections in neonatal piglets	ileal, colon, cecum, rectal swaps, lymph node (specific *E. coli* test)	[91]
	Reduces the portion of piglets suffering from diarrhoea	Diarrhoea patterns, performance (weight, daily gain)	[92]
Other			
Spray-dried animal plasma	upregulated antioxidant system in serum, stimulated intestinal development and improved health, leading to better growth performance, and decreased proinflammatory and anti-inflammatory cytokine responses	Growth performance, morphology, antioxidant capacity, Cytokines (IL-1β, TNF-α, IFN-γ, IL-6, TGF-β, IL-10, IL-4, IL-2, sIL-2R, NF-κB, and SIgA)	[93]

Table 2 Effects of dietary interventions in the neonatal phase on immune related parameters of pigs

Nutritional factors	Effect	Marker	Ref.
Amino acids			
Arginine	Decreased intestinal permeability	CDK1/cyclin B, PKB/Akt, and AMPK	[75]
	Less NEC (preterm piglets)	Intestinal damage	[76]
	Promotes growth performance	Growth performance, ammonia, amino acids (plasma)	[77]
Glutamine / Glutamate	enhance intestinal function and growth performance	O_2 consumption by pig enterocytes , glutamine 'products'	[78]
Threonine	Substrate for mucin production (deficiency leads to less mucin)	Daily-weight gain, diarrhoea incidence, histology, mucin production	[79]
Minerals			
Zn (ZnO)	Both traditionally weaned (> 21 d) and early weaned (< 14 d) pigs grew faster when fed pharmacological (3,000 ppm) concentrations of zinc as zinc oxide during at least the 1st 2 wk after weaning.	Growth performance, blood minerals	[80]
FE	one Fe injection (200 mg) for pigs from sows fed adequate vitamin E will result in adequate growth and hemoglobin concentration	Iron utilization	[81]
Fatty acids			
Medium-chain triglycerides	High dosage decreased milk intake, MCT utilized as fuel	Blood urea nitrogen, milk intake, glycogen levels tissue	[82]
Medium-chain fatty acids	greater villus height and a lesser crypt depth and greater villus/crypt ratio depth The intra-epithelial lymphocyte (IEL) counts per 100 enterocytes were significantly decreased	Performance (weight, FCR), luminal microbial ecology, pH, villi/crypts, IELs	[83]
Poly unsaturated fatty acids	Elevated levels of LC-PUFA enhances acute recovery of ischemia-injured ileum	Transepithelial electrical resistance (TEER), Flux measurements of H3-mannitol and C14-inlulin, and Prostaglandin E2	[84]
Poly unsaturated fatty acids	Improve the repair of small intestine in previously malnourished piglets	DNA/Protein content + lipid composition, fatty acid composition, histology (villi)	[85]
Short chain fatty acids	SCFA butyrate increases epithelial surface area by enhancing proliferation and inhibiting apoptosis	Villus height, crypt cell proliferation, butyrate	[86]

Nutritional factors	Effect	Marker	Ref.
Prebiotics	Composition gut microbiota was influenced by piglet diet and age	Gas production, pH, microbiota changes, SCFA production	[87]
	All prebiotic supplemented groups had numerically higher Lactobacillus populations	Weight, intestinal morphology, inflammation markers, microbiota	[88]
	Enhanced structure and function throughout the residual intestine	Morphology (Crypt-Villus), epithelial cell proliferation (PCNA), nutrient transport (ussing chamber)	[89]
	Increased (luminal) lactobacilli, lactic acid, and SCFA	Morphology, pH, digesta metabolits, microbiota, gene expression (TNFα, IL-8, IL-1β (C), and IL-10)	[90]
Probiotics	Enhanced structure and function throughout the residual intestine	Morphology (Crypt-Villus), epithelial cell proliferation (PCNA), nutrient transport (ussing chamber)	[89]
	Effective against E. coli infections in neonatal piglets	ileal, colon, cecum, rectal swaps, lymph node (specific *E. coli* test)	[91]
	Reduces the portion of piglets suffering from diarrhoea	Diarrhoea patterns, performance (weight, daily gain)	[92]
Other			
Spray-dried animal plasma	upregulated antioxidant system in serum, stimulated intestinal development and improved health, leading to better growth performance, and decreased proinflammatory and anti-inflammatory cytokine responses	Growth performance, morphology, antioxidant capacity, Cytokines (IL-1β, TNF-α, IFN-γ, IL-6, TGF-β, IL-10, IL-4, IL-2, sIL-2R, NF-κB, and SIgA)	[93]

Table 3 Effects of dietary interventions in the neonatal phase on immune related parameters of poultry

Nutritional factors	Effect	Marker	Ref.
Amino acids			
Arginine	Increasing dietary Arg, but not Lys, from 100 to 120% of the NRC recommendation increased day 18 BW gain	Body weight gain, feed intake, feed conversion, and liveability	[94]
	treatment differences in growth responses, lymphoid organ development, and primary antibody titers to SRBC did not occur	Body weight gain, FCR, lymphoid organ weights, and antibody titres	[94]
	-vac birds fed an Arg-deficient diet had lower feed conversion compared to +vac birds Vac+ birds had lower day 15 BW than –vac birds, but higher titers to Newcastle disease virus	Growth, antibody responses, plasma amino acid concentrations	[94]
Glutamine	addition of 4% Gln to the diet or water depressed growth performance	BW gain, feed efficiency, morphology	[95]
	Weight gain improved significantly in 1% Gln for 21 days. Higher concentrations of bile, intestinal, and sera IgA, sera IgG and longer intestinal villi	BW gain, feed efficiency, morphology	[95]
Threonine	Combination of threonine and SC improved growth performance and intestinal morphology traits in broilers.	antibody titre (Newcastle disease) and cell blood count	[96]
Methionine/Cysteine	improvement in the cell mediated PHA-P responses as well as in the IgG (T-cell-dependent) responses	Total Ab, β-met-resistant Abs, IgG	[97]
Feed ingredients			
Wheat/Soy	wheat/rye-based diet compared with a maize-based diet: induces villus fusion, a thinner tunica muscularis, T-lymphocyte infiltration, more and larger goblet cells, more apoptosis of epithelial cells in the mucosa, shift in microbiota	Body weight, feed intake and FCR, morphology, microbiota, T-lymphocyte infiltration	[98]
Corn/Wheat	enzyme supplementation minimized the growth suppression associated with the *C. perfringens* challenge, with the most pronounced effect observed in birds fed the wheat-based diet	BW, FCR, *C. perfringens* counts	[99]

Nutritional factors	Effect	Marker	Ref.
Fatty acids			
Medium-chain triglycerides	organic acids and MCA chicks had a better growth performance, better digestibility, less S. Enteritidis colonization and lower pH in the crop and intestines. FOS chicks tended to have decreased Salmonella colonization in ceca	Performance (weight), microbiota (*Salmonella* counts), pH, cecal SCFA	[100]
Poly unsaturated fatty acids	n-3 PUFA (SALmate) supplementation altered the intestinal Lactobacillus species profiles, but did not alter the overall microbial communities or broiler performance	Performance (weight), microbiota	[101]
Short chain fatty acids	0.4% butyric acid was on par with antibiotic in maintaining body weight gain, and reducing E. coli numbers and found superior for feed conversion ratio	Performance (weight), pH, morphology, microbiota (*E. coli* counts)	[102]
Pre- and probiotics			
Prebiotics	**GOS selectively stimulated the fecal microflora of broiler chickens**	**Performance, microbiota**	[103]
	longer jejunal villi, greater specific activities maltase, leucine aminopeptidase and alkaline phosphatase. Minor improvement in body weight.	Growth, morphology, uptake of L-tryptophan	[104]
	β-glucan feed decreases the incidence of SE organ invasion in neonatal chickens and up-regulating of the functional abilities of heterophils against an SE	Heterophils,% SE killing, oxidative burst	[105]
Probiotics	GOS in combination with a *B. lactis*-based probiotic favoured intestinal growth of bifidobacteria in broiler chickens.	Performance, microbiota	[103]
	1.5g/kg Fermacto increased performance of broilers	Performance (weight), feed intake and feed conversion	[106]
Other			
Vitamin E + Se	Depletion affects both the maturation of specific lymphocyte subpopulations and the functional and proliferative capabilities of the peripheral lymphocytes	CT-1a, CD3, CD4, CD8, sIgs, and Ia	[107]
Essential oils (thyme, cinnamon)	EO could be considered as a potential natural growth promoter for poultry at the level of 200 ppm	Growth performance, blood constituents, carcass characteristics	[108]

5.2.2 Human

Minerals are essential for a proper development of the neonate and the immune system, examples are zinc, selenium, copper, manganese, calcium and iron (Table 4). Suppletion of minerals can have a positive effect on the host, but bear in mind that the (resident) microbiota also make use these minerals or trace elements for their maintenance. In literature many studies conclude that a deficiency of minerals lead to impaired immunity, however an excessive dose can be harmful as well. This means that one must carefully asses what the 'normal' range of these minerals is for the host. Addition of vitamins can have a positive effect on the host's immune system, as with minerals a depletion is detrimental for the immune system. Another category are the fatty acids, in literature mainly the long-chain poly unsaturated fatty acids are described for (young) infants, here two case studies are given. Pre- and probiotics are often used when infants are given formula, instead of mother's milk, both pre- and probiotics try to mimic the mechanism(s) of mother's milk. Prebiotics are often oligosaccharides, whereas probiotics are mainly (specific) Lactobacilli and Bifidobacteria. An overview of case studies describing different nutritional interventions is given in Table 4.

Table 4 Effects of dietary interventions in the neonatal phase on immune related parameters of human

Nutritional factors	Effect	Marker	Ref.
Vitamins			
C + E	Not measured	Time taken to recover from a very ill status, fever, tachypnoea, and feeding difficulty; and improvement in oxidative stress and immune response indicated by thiobarbituric acid reacting substances (TBARS) and response to skin antigens.	[109]
LC-PUFA	Early diet influences both the presence of specific cell types and function of infant blood immune cells	formula fed infants had a higher percentage of CD3+, CD4+CD28+, and lower percentage of CD14+ cells and produced more TNFα and INFγ	[110]
	Plasma Long-chain polyunsaturated fatty acids (LCP) levels similar to those of breast fed infants can be achieved with the LCP supplemented formula used in this trial, without evidence of adverse effects of the LCP enrichment	Urinary malondialdehyde (MDA), marker for oxidative stress, excretion was significantly higher in formula fed infants. α-tocopherol similar between groups, tendency for lower values in formula (group F) fed infants	[111]
Prebiotics			
scGOS/lcFOS	Infants in the intervention group had significantly lower incidence of allergic manifestations	Epidemiological markers / descriptive statistics	[112]
Oligosaccharides	Dosage-dependent effect on the growth of *Bifidobacteria* and *Lactobacilli* as well as softer stool	Bifidobacteria/Lactobacilli	[113]
Nucleotides	supplementation of cow's milk-based formula with NT resulted in a modest improvement in some antibody responses in our population of healthy term infants, with no effect on other markers of immune status and growth	Antibody responses to tetanus toxoid	[114]
Probiotics			
Bifidobacterium* + *Streptococcus	The supplementation of infant formula with *B. bifidum* and *S. thermophilus* can reduce the incidence of acute diarrhoea and rotavirus shedding in infants admitted to hospital	diarrhoeal disease rotavirus shedding	[115]

5.2.3 Conclusions

From these different studies it can be concluded that it is possible to modulate the host by a multitude of nutritional factors. However one must bear in mind that most of the (published) literature is biased towards publishing (significant) measurable effects on the host. Therefor by definition this overview is incomplete, nevertheless these studies do show that different nutritional factors affect the host in different ways. Taken together, no clear biomarker/endomarker can be extracted from this long list of available studies to investigate the effect on the immune competence of the host. Due to the fact that in most studies performance (i.e. body weight (gain) and feed conversion rate) is an important marker to test the impact of the intervention, this is often investigated in conjunction with the morphology of the small intestine. Nevertheless, in a few studies immunological or microbial parameters were used to investigate the impact of the intervention, in this way more information is gained of the possible mode of action associated to immune competence. In other words which genes, proteins and/or microbial groups that contribute to intestinal immune development and health. From Table 2 it can be extracted that in terms of immune parameters (e.g. cytokines) prebiotics (and spray dried plasma) could be potential candidates to further investigate in pigs. For chickens (Table 3), evidence was found in 'wheat/soy' diets, 'Methionine and Cysteine', as well as 'vitamin E together with Selenium', that showed effects on the immune related parameters (e.g. cytokines, T cells and antibodies). Human infants were also studied extensively within the context of early life variation and (impact on) immunity. From these studies we can conclude that one must avoid a depletion of vitamins and/or minerals in the diet. Furthermore the most abundant nutritional factors were; prebiotics (e.g. oligosaccharides), probiotics (e.g. lactobacilli and bifidobacteria) and long-chain poly unsaturated fatty acids.

5.3 Impact of nutritional interventions in the post-neonatal phase on immune related parameters

In this paragraph, the impact of nutritional interventions on the immune competence in the adult phase is discussed. It is based on research in calves, weaned pigs, adult mice, laying hens, ducks, broilers (> 10 d), and humans. The results are shown in Table 5, whereas more details are provided in Appendix 14.

Microbiota parameters
Dietary supplementation of rice bran [116], quercitine [117], alfalfa [118], *bacillus subtillis* [119], antimicrobial peptide A3 and P5 [120], Buforin II [121] and lysozyme [122] reduced the amount of pathogenic bacteria (e.g. *Clostridia, E. Coli)*, whereas in some studies the number of *Lactobacilli* and *Bifidobacteria* was increased

Gut integrity
The gut integrity was improved by dietary addition of glutamine [123], black cumin [124], *bacillus subtillis* [119]*,* antimicrobial peptide A3 and P5 [120], Buforin II [121] and lysozyme [122]. The improved gut integrity was shown by greater villus height and reduced crypt depth, and in some studies an increased production of tight junction proteins, reduced lesion scores and less bacterial translocation from the gut to the spleen.

Immune cells
Several dietary interventions positively affected immune cells. Such effects were found after supplementation of glutamine [123], chitosan [125], garlic botanical [126], rice bran [116], alfalfa [118], black cumin [124], *bacillus subtillis* [119]*,* and *Lactobacillus delbrueckii subsp. Bulgaricus* [127]. These interventions affected a wide range of immune cells:

- Increased IgG, IgA and secretory IgA levels
- Increased numbers of TNF-α, IL-1β cells
- Increased numbers of myeloid dendritic cells ($CD11c^{+}CD11b^{+}$ DCs) in the Lamina propria
- Increased proliferation of T and B lymphocytes
- Increased number of goblet cells
- Increased or decreased number of CD4+ cells and decreased CD8+ cells
- Increased ratio between CD4+/CD8+

- Increased number of monocytes
- Ratio between CD8+/CD3+ in human peripheral blood.
- Ratio between CD16+56 (NK cells)/CD45+ in human peripheral blood.

Gene expression

Gene expression data were only provided in limited number of studies. In these studies, *Lactobacillus delbrueckii subsp. Bulgaricus* [127], lysozyme [122], Buforin II [121], and rice bran [116] were used as dietary interventions. These gene expression data showed:

- a reduced cytokine quantification in human serum
- an increased Lysozyme activity and lysozyme g2 mRNA content
- an increased expression of surface IgA on $B220^{+}$ B cells in Peyers Patches
- an enhanced expression of protective factors (HGF, hepatocyte growth factor, reg-3 gamma, regeneration protein gamma, TFF-3, and trefoil factor-3)
- an enhanced expression of TGF-β1, transforming growth factor β1; VEGF, and vessel endothelium growth factor.

Performance parameters

Dietary addition of quercetin [117], black cumin [124], *bacillus subtillis* [119], antimicrobial peptide A3 and P5 [120], Buforin II [121] and lysozyme [122] resulted in improved animal performance. Improved performance was shown by a decreased FCR and an increased egg mass production, daily gain, nutrient digestibility and retention. In some experiments [121, 122], animals were challenged with pathogenic bacteria, and although animal performance was negatively affected by the challenge, the dietary interventions could partly prevent this reduced performance.

Table 5 Effects of dietary interventions during the post-neonatal phase on immune related parameters

Nutritional factors	Effect	Marker	Ref.
Amino acids			
Glutamine	Improved immune response and increased autophagy of liver cells of weaned calves (35 – 49 d of age).	CD4+, monocytes, the ratio of CD4+/CD8+, villus height and crypt depth of intestine, the autophagy level of liver cells.	[123]
Plants and herbs			
Chitosan	Dietary supplement with chitosan (0.5 – 1.0 g/kg), a natural alkaline polysaccharide, and a key structural component of helminths, arthropods, and fungi, improved humoral and cellular immune responses of weaned piglets.	Serum IgG, secretory IgA in ileum mucosal surfaces, serum specific ovalbumin, CD4, CD8, IL-1 and IL-2, TNF-α.	[125]
Capsicum oleoresin, garlic botanical, and turmeric oleoresin	Supplementation of plant extracts in PRRSV challenged weaned pigs reduces the adverse effects of PRRSV by improving the immune responses.	B cells and CD8+ T cells, TNF-α, and IL-1 β	[126]
Quercetin	Dietary supplementation of quercetin improved performance of laying hens by modulation of the intestinal environment and liver superoxide dismutase content.	Increased rate of lay, decreased FCR, decreased population of total aerobes and coliforms, increased population of *Bifidobacteria*. Increased activity of Cu-Zn-superoxide dismutase (antioxidant).	[117]
Alfalfa	Dietary alfalfa meal supplementation increases intestinal microbial community diversity and improves of the immune response growing egg-type ducks	Linear effect of Alfalfa dosage on T and B cell proliferation. No effects on IgA, IgG and IgM. Molecular analysis of the caecal and faecal DNA extracts showed that the alfalfa meal diet promotes the intestinal microbial diversity.	[118]
Black cumin (*Nigella sativa L.)*	Regardless of supplementation level, dietary inclusion of black cumin decreased E. coli enumeration in ileal digesta and improved serum lipid profile and eggshell quality, whereas the best intestinal health indices and haying hens' performance were obtained by at least 2% black cumin seeds.	Black cumin supplementation resulted in Increased relative pancrease weight, reduced E. coli counts, increased villus height and reduced crypt depth, increased goblet cell numbers and lamina propria lymphatic follicles.	[124]
Prebiotics			
Resistant starch (RS)	Suppression of many immune response pathways, including the adaptive and innate immune system, as well as cell division. RS increased the relative abundance of several butyrate-producing microbial groups and reduced the abundance of potentially pathogenic microbial strains.	Colonic transcriptome profiles, oxidative metabolic pathways, such as the tricarboxylic acid cycle and b-oxidation, the nuclear receptor peroxisome proliferator-activated receptor gamma,	[128]

Probiotics			
Bacillus subtilis fermentation biomass	4.5 g/kg BS 1–2 fermentation biomass had beneficial effects on weanling pigs performance, CTTAD of nutrients, cecal microbiota, serum immunoglobulins and intestinal morphology.	ADG, G:F, digestibility of DM, GE and CP, serum IgA and IgG, cecal *Cl.* spp. and coliforms, villus height and VH:CD (duodenum, jejunum and ileum).	[129]
Nutritional factors	**Effect**	**Marker**	
Lactobacillus delbrueckii subsp. bulgaricus	Immunomodulation induced by *L. delbrueckii subsp. bulgaricus* could favour the maintenance of an adequate immune response.	Slowing the aging of the T cell subpopulations and increasing the number of immature T cells which are potential responders to new antigens.	[127]
Antimicrobial proteins			
Antimicrobial peptide-A3 and P5	AMP-A3 and P5 have potential to improve performance, nutrient digestibility, intestinal morphology and to reduce pathogenic bacteria in weanling pigs	Performance, nutrient digestibility, fecal *Clostridium* spp. and coliforms, anaerobic bacteria, gut morphology	[130]
Buforin II	Buforin II protects small intestinal mucosal membrane integrity of weaned pigs.	Increasing tight junction proteins, enhancing the expression of protective factors, reducing hemolytic E. coli concentrations in the Intestines.	[121]
Lysozyme	Exogenous lysozyme could decrease C. perfringens colonization and improve intestinal barrier function and growth performance of chickens.	A reduction of C. perfringens in the ileum and of the intestinal lesion scores, less overgrowth of E. coli and Lactobacillus in the ileum and less intestinal bacteria translocation to the spleen, and improved intestinal lysozyme activity in the duodenum, and improved feed conversion ratio of chickens.	[122]

5.4 Mode of action of dietary interventions on immunity

In this paragraph, the mode of action of the mentioned dietary interventions is briefly described. This paragraph is partly based on two review reports regarding this topic [131, 132]

Fungal and bacterial cell wall (β-glucans)
β-Glucans bind Dectin-1-like receptor on animal cells (e.g. neutrophils, monocytes). This supports the innate immunity by increased neutrophil functioning (i.e. production of reactive oxygen species, cytokine production), resulting in reduced susceptibility for pathogen invasion (e.g. *Salmonella enteritidis*).

Mannan-oligosaccharide (MOS)
MOS acts as a competition molecule for the adhesion of certain bacterial species in the gut, thereby reducing the risk of diseases. Next to this, MOS also has an anti-inflammatory working, but exact mode of action is not known. However, MOS was also reported to have an increased inflammatory response (i.e. phagocytosis and complement pathways).

Alfalfa
Alfalfa was shown to increase heterophil functioning and was also shown to prevent *Salmonella enteritidis* infection in molting chicken.

Amino acids
Several amino acids were found to alter histone and DNA methylation/acetylation and thereby to regulate gene expressions. A similar working mechanism might be possible in other organs/cells. No direct link of gene expression and immune functioning under influence of amino acids is reported yet. However, amino acids were reported to activate and proliferate lymphocytes, NK cells and macrophages and to alter the acute phase protein response.

Poly unsaturated fatty acids (PUFA)
PUFA react via the PPAR and RXR on cells and thereby affect eicosanoid releases. Additionally, PUFAs are also incorporated in cell membranes and influence cell functioning depending on PUFA type and concentration. Finally, PUFA are known for their regulation of gene expression, possibly via epigenetic mechanisms (in humans). Consequently, PUFAs reduce inflammatory response in macrophages and in T cells and also reduce the acute phase response. They are reported to stimulate antibody response. Chicken become less vulnerable to humoral (B cell) associated diseases, but more vulnerable to cellular (T cell) associated diseases. Also other cells are influenced. PUFA are reported to improve epithelial integrity in the gut. The (n-3) LCPUFA metabolites induce eicosanoid production, alter gene expression, and modify lipid raft composition, altering T-cell signalling; all contribute to immunological functional changes. However, the roles of these mechanisms and the types of T or other immunological cells involved remain unclear at present. Moreover, the effect of (n-3) LCPUFA on the immune system of infants may vary according to dose, time of exposure, and profile of the immune system (T-helper, Th1/Th2). Most of the interventional studies in infancy have been performed for the prevention of allergy. They all confirmed influence on T-cell function and cytokine profiles, but clinically beneficial effects are more conflicting. Supplementation of the maternal diet in pregnancy or early childhood with (n-3) LCPUFA is potentially a non-invasive intervention strategy to prevent the development of allergy, infection, and possibly other immune-mediated diseases. However, any long-term in vivo effects on (n-3) LCPUFA early in life for immuno-modulatory defence in infants and later on immune status and health remain to be assessed [133].

Spray-dried animal plasma
The immune competence improving effects of spray-dried animal plasma in young pigs is most likely mediated by the IgG component in the plasma. The IgG prevents viruses and bacteria from damaging the gut wall, thereby resulting in a more functional intestinal wall, improves intestinal morphology and enzyme activity as evidenced by increased villus surface area, and increased mucosal maltase and lactase activities [134].

Minerals
Mineral deficiencies are well described in literature with often detrimental immuno modulatory influences. Minerals are also reported to affect macrophage functioning and to reduce inflammation (as antioxidants) by protection of neutrophils from oxygen-derived radicals. Also (pro-inflammatory) gene and receptor expression is influenced by minerals. Also lymphocytes require selenium for better functioning. Next to this, the essence of minerals for gut microbiota should not be forgotten.

Vitamins
Vitamin serve as antioxidants in the body and thereby protect cells from oxygen damage. Inflammation and macrophage activity were reported to be reduced, although macrophage activity was also reported to be increased. Vitamins were also reported to induce DNA methylation and alter the immune response, although no direct link between these observations was proven.

Seaweed extract
The reduction in colonic *E. coli* and Enterobacteriaceae populations may be the result of the agglutination properties of seaweed extract, thereby preventing the attachment to epithelial cell surfaces and subsequent colonisation of mucosal surfaces [63]. Seaweed inclusion post-weaning tended to up-regulate colonic MUC2 mRNA expression. Mucin, the primary component of mucus, functions as a biophysical barrier against enzymatic, mechanical insult and pathogenic invasion within the GIT. The fibrous components of Laminarin may stimulate the colonic mucosa or may have a direct effect at the cellular level because of becoming internalized by intestinal epithelial cells and gut-associated lymphoid tissue cells. Alterations in colonic microflora composition may also directly influence mucin synthesis and secretion from goblet cells. The usual up-regulation of intestinal inflammatory cytokine gene expression in ileal and colonic tissues after weaning was not observed or tended to be lower in pigs weaned from seaweed-supplemented sows. Seaweed, therefore, seems to modulate the production of pro- and anti-inflammatory cytokines, which might be beneficial to animal health and performance.

Probiotics
In vitro studies have shown that Sachoromyces *Cerevisiae* secretes anti-inflammatory factors and inhibits the phosphorylation of both p38 and ERK1/2 kinases after co-culture of porcine intestinal epithelial cells with ETEC [135, 136]. Supplementation of *Lactobacillus rhamnosus* and *Bifidobacterium lactis* to pregnant women increased the adiponectin content of their colostrum [72]. The assumed health effects of adiponectin are mediated through its anti-inflammatory properties via regulation of the expression of proinflammatory cytokines and through its insulin-sensitizing properties via inhibition of liver gluconeogenesis and promotion of fatty acid oxidation.

Lysozyme
Toxins produced by C. *perfringens* may destruct the mucosal tissue, causing macroscopic lesions in the duodenum, jejunum and ileum. This results in a disturbance of the interface between enterocytes and the lamina propria, which might induce a strong inflammatory response in birds, thereby negatively affecting digestive functions and impairing nutrient utilization, finally suppressing bird's performance. Lysozyme is suggested to have a direct inhibitive effect on C. *perfringens* proliferation and its immune modulating activity. Based on *in vitro* experiments [137] is was concluded that lysozyme could not only kill C. perfringens, but also reduce the production of α-toxin, the causative agent for intestinal lesions. Other *in vitro* experiments showed that lysozyme stimulates immunoglobulin production of specific blood lymphocytes [138]. After absorption of orally administered lysozyme in the gut, plasma levels of lysozyme will increase, which could produce systemic effects like an increased activity of monocytes and macrophages [139]. Lysozyme has been reported to interact with intestinal bacteria to liberate immune-modulating peptidoglycans and Peyer's patches, and intraepithelial lymphocytes to activate the host's immune system [140].

Rice bran
It is suggested that various bioactive and prebiotic components in rice bran, e.g. γ-oryzanol, polyphenols, fatty acids, essential amino acids and micronutrients, are involved in the enhancement of immunity [141].

Lactobacilli

Lactobacilli are able to stimulate the number and activity of NK cells. NK cells play a critical role in immune surveillance against the development of tumors and viral infections, and intestinal microflora can modulate NK activity [142]. It is suggested that Lactobacilli stimulate the activity of the thymus gland [127]. Lactobacilli could reduce the IL-8 content in the serum, confirming that some probiotics are able to inhibit the production of pro-inflammatory cytokines [143]. Moreover, Lactobacilli supplementation was associated with a very marked increase in hBD-2 antimicrobial molecule in serum, suggesting an enhancement in the innate immunity through defensins induction [144]. Lactobacilli, therefore, do not only have local effects in the intestinal mucosa, but also in the peripheral blood.

Plant extracts

Plant extracts are able to reduce serum TNF-α and haptoglobin, white blood cells and lymphocytes, and to mitigate villus atrophy after an *E. coli* challenge, thereby modulating the systemic inflammation caused by *E. coli* infection [126]. TNF-α is one of the most important cytokines released especially form macrophages and monocytes, in response to bacterial cell wall products and bacterial toxins. The effects of plant extracts on white blood cells and TNF-α showed that they may alleviate the overstimulation of the systemic immunity and early immune response in E. coli infected pigs. Plant extracts could reduce the recruitment of macrophages and neutrophils in the ileum of *E. coli* infected pigs, indicating that these reduce both systemic and local inflammation.

Buforin II

Buforin II kills *E. coli* by penetrating the cell membrane, subsequently inhibiting cellular functions [145]. Buforin supplementation could decrease the jejunal and ileal mucosal lymphocyte amount as well as the calprotectin level. Calprotectin exerts antimicrobial effects through competition for zinc and by preventing bacteria from binding to mucosal epithelial cells. Buforin II significantly increased goblet cell amounts and the expression level of tight junction proteins (VEGF, HGH, Reg-3γ, TGF-β1, and TFF-3) in the jejunum and ileum. HGF acts on a wide variety of epithelial cells, as a mitogen (stimulation of cell growth), a mitogen (stimulation of cell motility), and a morphogen (induction of multicellular architecture), and is considered to be a key molecule for construction of structure during organ regeneration. TFF is a secretory protein expressed in gastrointestinal mucosa and may protect the mucosa from insults, stabilize the mucus layer and affect healing of epithelium [146].

6 Interaction between gastro-intestinal and airway mucosal immune systems

6.1 Immune system of the respiratory tract

The respiratory tract is morphologically and functionally separated in the upper and lower respiratory tract (Figure 7). The lower respiratory tract is formed by the pulmonary parenchyma consisting of the bronchial passages in the lungs, and their associated alveoli (where gas exchange actually takes place). This lower part of the respiratory tract is protected mainly by a locally functioning mucosal immune system. Alveolar macrophages, present in the lumen of the alveoli, participate with other cellular and humoral factors in the non-specific defence of lungs [147, 148]. The humoral component of specific pulmonary defence is represented by the antibodies present in alveolar fluid. Locally produced IgG antibodies prevail in lungs, but lower amounts of IgA antibodies are also present [149]. The locally functioning specific immune system of the lower respiratory tract is not likely to be significantly modulated by nutritional intervention (unless the animal is suffering from an impaired immune system due to overt nutritional deficiencies) and will therefore not be the focus of this literature review.

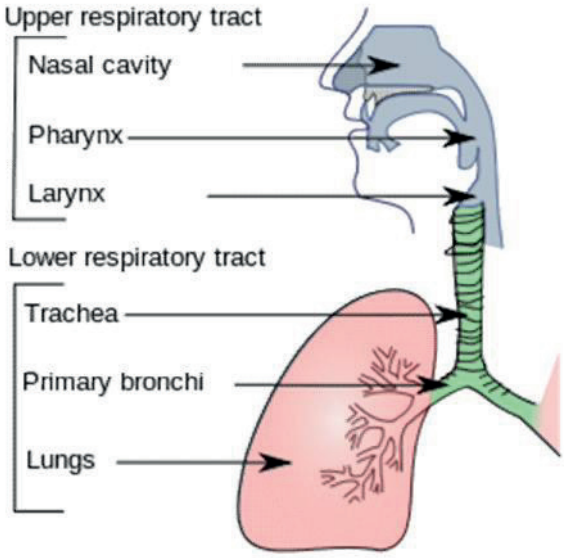

Figure 7 *The upper and lower respiratory tract*

The upper respiratory tract formed by the nasal cavity, the pharynx and larynx. Just like for the lower respiratory tract, a highly complex and effective system of non-specific immune mechanisms operate that can eliminate more than 90% of the microorganisms the penetrate the respiratory tract [150]. The mucociliary apparatus is on one of the most significant systems for the protection of the mucosa. Its primary role is to remove incoming dust particles and microorganisms. The mucociliary apparatus consists of mucus producing cells and ciliary epithelium. Each of these ciliary cells contain 200-3000 ciliae that constantly move the mucus with trapped dust and microbes outwards, till it can be removed by coughing and/or swallowing. In addition the mucus contains a number of substances with antimicrobial activity like lactoferrin, defensins (antimicrobial peptides) and lysozyme (weakens bacteria by breaking down part of its cell wall).

The upper respiratory tract is also protected by a specific mucosal immune system that is partly connected to other mucosa of the body. Together this mucosal immune system is referred to as the "common mucosal immune system". The primary specific defence at this mucosa is formed by IgA antibodies [151].

6.2 The "common" mucosal immune system

The common mucosal immune system referred to the fact that antigenic stimulation at any inductive site of the mucosal immune system results in a specific immune response characterized by IgA antibody production in all of the other mucosa included in this system[152, 153]. Until recently this theory was general accepted [154]. However, it is clear from vaccine studies with cholera toxin that the degree of interconnection varies quite extensively [155, 156]. Whereas vaginal or rectal mucosal vaccination results in IgA production only at the vaccination site, intranasal vaccination induces IgA in the upper and lower airways, as well as in the vagina and head/neck area, and oral immunization results in IgA being produced in the upper airways, head/neck area, digestive system, and in breast tissue (Figure 8).

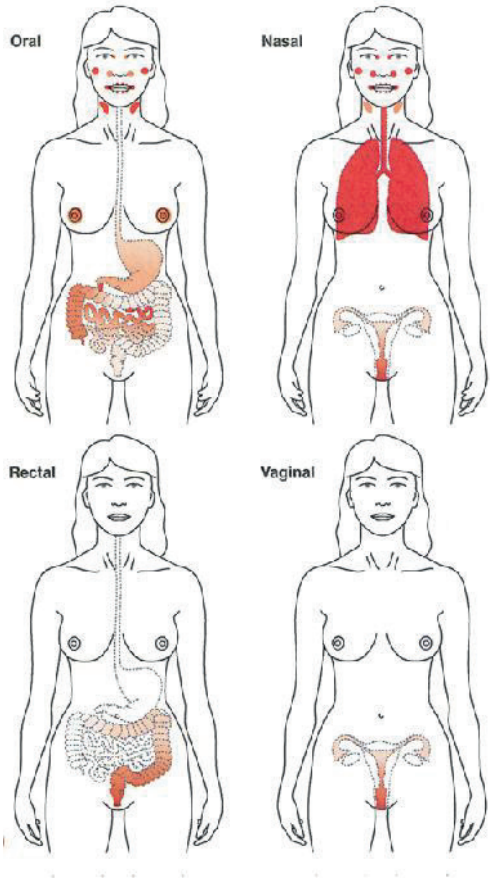

Figure 8 *The "common" mucosal immune system. Depending on the administration route of the mucosal model antigen cholera toxin B (CtxB), specific secretory IgA can be detected at different mucosal surfaces.*

In addition, intragastric feeding results in lower IgA production in the upper airways than normal feeding via the mouth [157].

6.3 Gastrointestinal Immunity.

The intestine is the largest immune organ in the body and actively responds to potentially harmful pathogens and antigens, while creating and maintaining tolerance (unresponsiveness) to other antigens and beneficial commensal and symbiotic micro-organisms. The gastrointestinal mucosal barrier combines a physical barrier with the production of transmembrane TLRs and cytoplasmic nucleotide binding oligomerization domains (NOD) receptors as members of the innate immune system [2]. TLR act as microbial pattern recognition receptors binding ligands from the gut lumen and

determine the interaction with host-immune defence, immune cell recruitment and mucosal inflammation. As shown in Figure 9, the intestinal immune barrier starts from the lumen with an extracellular, luminal mucus layer produced by goblet cells in the epithelium. This barrier blocks access of bacteria and other luminal compounds to the mucosal lymphoid follicles and provides intestinal homeostasis. Immune trafficking mainly occurs in the small intestine, peaks in the ileum, and occurs to a lesser extent in the colon. The gut gatekeepers comprise mucus layer and epithelium consisting of enterocytes responsible for nutrient degradation and uptake, and mucus-producing goblet cells [158]. Enteroendocrine cells and mast cells release hormones, neurotransmitters such as serotonin, and immune-modulatory mediators. Paneth cells in the small intestinal crypts release antimicrobial peptides regulating the microbial ecosystem.

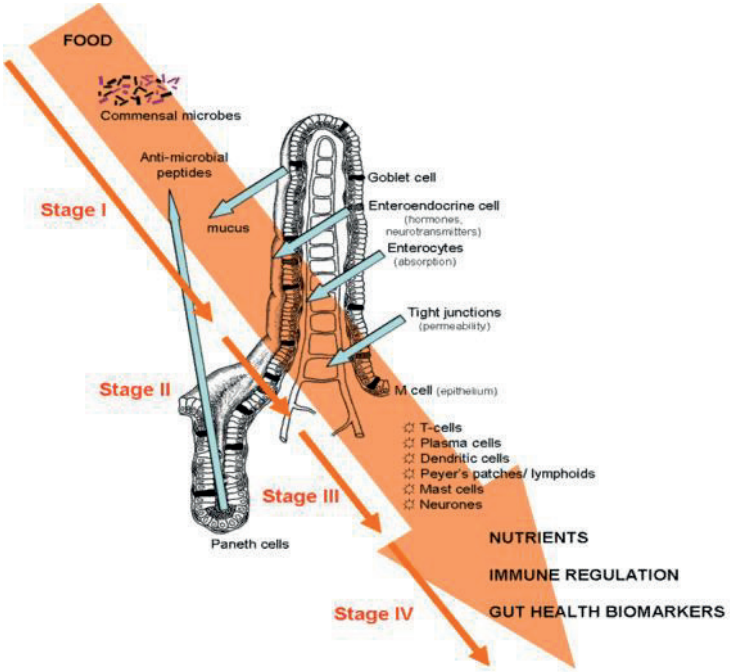

***Figure 9**. Stage I. Luminal processes: Bacterial ecology, metabolites (bacterial, mammalian), and 'defensive' molecules (mucins, antimicrobial proteins). Stage II. Epithelial processes: enterocytes (absorptive functions, permeability), goblet cells (mucin), enteroendocrine cells (signal molecules; hormones, neurotransmitters (5HT)), Paneth cells (antimicrobial proteins, RegIII proteins), and M-cells (transcytosis of bacteria). Stage III. Lamina propria, immunological effects: T-cells, plasma cells (secretory IgA), dendritic cells, Peyer's patches/ lymphoids and mast cells, and neurones (e.g. visceral pain perception). Stage IV. Systemic markers: blood, PBMC, faeces, saliva, immune tissue (e.g., tonsils), and urine.*

The lamina propria, that contains 75% of the body's lymphocytes, harbors intraepithelial T and B lymphocytes and CX3CR1+ dendritic cells (DCs). The T-cells release antimicrobial peptides after epithelial barrier disruption, whereas DCs can actively participate in sampling of bacteria from the gut lumen, activate Th17 cells, and generate regulatory T cells (T-reg). In the lamina propria, NKp46+ROR□t cells are found that release IL-22 in response to microbiota and lead to Th17 expansion. A distinct population of CD103+ DC generates tolerance by inducing T-reg formation after migration to the mesenteric lymph nodes. Finally, secretory IgA plasma cells provide immune deletion of pathogens and toxins within the lumen of the intestine. This intestinal mucosal immune system can be manipulated with functional foods and nutraceuticals impacting on the commensal microbiota in the gut lumen [159, 160]. Any dysfunction sensed by the intestinal immune barrier will immediately result in either local disorders, or infection and can even result in serious systemic disorders all deleterious to health [161].The immune system in the mouth and upper respiratory tract: inductive sites for mucosal immunity.

A proper understanding of the anatomy of mucosal immune tissues is important for designing rational approaches to enhance immunity in the upper airways. This section will focus on the anatomical structure of the upper airways and the presence of immunological tissues in the upper digestive tract, i.e. the mouth and throat.

Immunological tissues in the head/neck area are mainly structured in tonsils, lymph nodes and lymph follicles. The largest area of contact between food and nasal contents and the immune system occurs in the area where nose and mouth come together. As shown in Figure 10, the pharynx is surrounded by a number of structured lymphoid nodules (tonsils), comprising the lingual tonsil, the nasopharyngeal tonsils (adenoids), the palatine tonsils and tubal tonsils. Together these are named Waldeyer's ring.

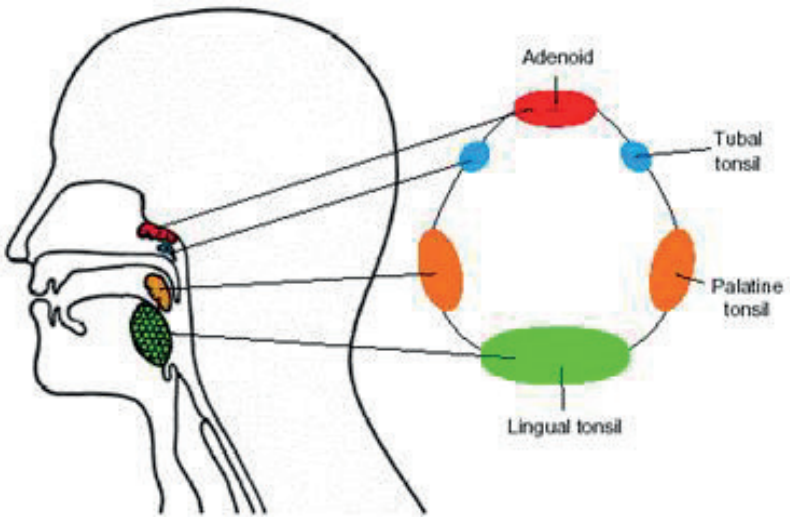

Figure 10 The tonsils are organized in Waldeyer's ring, and line the nasopharynx (from [162]). These immunological structures are the first to come into contact with food components

The term Waldeyer's lymphoid pharyngeal ring is mainly used in literature in human studies. In animals, these tissues are described as nasal associated lymphoid tissue (NALT) [163, 164]. In small laboratory animals the NALT is present in the floor of the nasal cavity as paired lymphoid aggregates, but no comparable aggregates have been found at these sites in farm animals. In contrast, the NALT of farm animals more closely resembles the lymphoid tissues of the Waldeyer's ring, as seen in humans. The tonsils of the pig e.g. are located in the soft palate, between the respiratory and digestive tracts. Like in humans, the tonsils in pigs are part of the immune system of the respiratory as well as the digestive mucosae and they are of great importance in porcine defensive mechanisms against infectious agents. In cattle, sheep, pigs and horses, larynx-associated lymphoid tissue has also been described on the epiglottis [165].

The back of the tongue also contains lymphoid follicles (lingual tonsil). In addition, dispersed in the mucosal tissues of the mouth large numbers of antigen-presenting cells, including Langerhans cell-like dendritic cells (DC), are present. The tonsils and adenoids have antigen-retaining crypts, and the reticulated epithelium lining the tonsillar crypts is probably an antigen entry portal, specialized in transporting antigens to the subepithelial tissue [166, 167]. The adenoids, but to a much lesser extent the palatine tonsils, have epithelia that have well developed tight junctions that are important for their barrier function (Ogasawara et al., 2011). Interspersed are M cells and DC that contribute to antigen sampling. Furthermore, tonsillar epithelium contains the toll-like receptors 2 and 3 (TLR2 and TLR3), and is thus capable of responding to selected bacterial as well as viral ligands [166].
Interestingly, because of the anatomical structure of Waldeyer's ring, the NALT in humans and in pigs is exposed to food as well as to airborne antigens (bacteria, viruses, allergens). Airway pathogens and aeroallergens, present in nasal secretions, can be swallowed and thus become available to the intestinal immune system, which may result in immune effects in both mucosal compartments (i.e. respiratory and gut) due to recirculating specific B-cells. This is in contrast to the situation in mice, in which the oral and nasal cavities are separated. In fact, mice have recently been shown to have respiratory M cells in the nasopharynx that play an important role in sampling airway-related antigens [168], but in humans, and probably also in pigs, sampling does not seem to take place across the nasal epithelium – suggesting that immune sampling of the nose takes place in Waldeyer's ring.
The importance of tonsils as antigen sampling sites is evidenced by the fact that removal of nose and mouth tonsils results in strongly decreased IgA response to live oral polio vaccine, indicating their role in inducing mucosal immunity to this viral pathogen [169].

In addition to the Waldeyer's ring, many lymph nodes are present in the head/neck area (Figure 11). The most important of these are the submandibular lymph nodes located beneath the jaw, and the cervical lymph nodes that are located at the side of the neck. As reviewed by Brandtzaeg [5], after antigens are captured by the lymphoid tissue in the nose and mouth, immune cells traffic to the draining lymph nodes, which are represented by the cervical lymph nodes. Therefore, in addition to the fact that nasal secretions and swallowed foods come into contact with each other in Waldeyer's ring (Figure 12), immune cells in the cervical lymph nodes may simultaneously come into contact with airway pathogens, as well as with food ingredients.

Table 6 MALT structures described in cattle, sheep/goats, pigs and horses

Table I. MALT structures described in cattle, sheep/goats, pigs and horses.

	CALT	NALT	Waldeyer's ring		DALT SALT LDALT	LTALT	BALT	Gastric MALT	GALT
			Nasopharynx	Oropharynx					
Bovine	+	nd	T. pharyngea T. tubaria	T. lingualis T. veli palat. T. palatina	nd	+	(+)	nd	+
Ovine / caprine	+	+	T. pharyngea T. tubaria	T. lingualis T. veli palat. T. palatina	nd	+	(+)	nd	+
Porcine	+	nd	T. pharyngea T. tubaria	T. lingualis T. veli palat.	nd	+	(+)	+	+
Equine	+	+	T. pharyngea T. tubaria	T. lingualis T. veli palat. T. palatina	nd	+	(+)	nd	+

+: constitutively present; nd: not examined / not described; (+): not constitutively present.

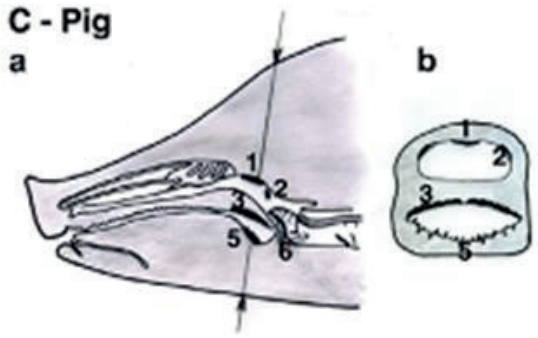

Figure 11 *Location of lymph nodes in the head and neck. Distribution of lymphoid tissues of Waldeyer's ring (1–pharyngeal tonsil, 2–tubal tonsil, 3–tonsil of the soft palate, 4–(entrance to) palatine tonsil, 5–lingual tonsil), LTALT (6) and NALT (7) in pig, median (a) and transversal section (b, plane indicated).*

IgA is the immunoglobulin of choice in mucosal tissues, as it is a non-inflammatory immunoglobulin with limited effector functions that can exclude pathogens from entering the tissues, but does not promote inflammation. More IgA is produced in the mucosal tissues than all other immunoglobulin isotypes in the body, and 3-5 grams of IgA is excreted in to the human GI tract each day [170].

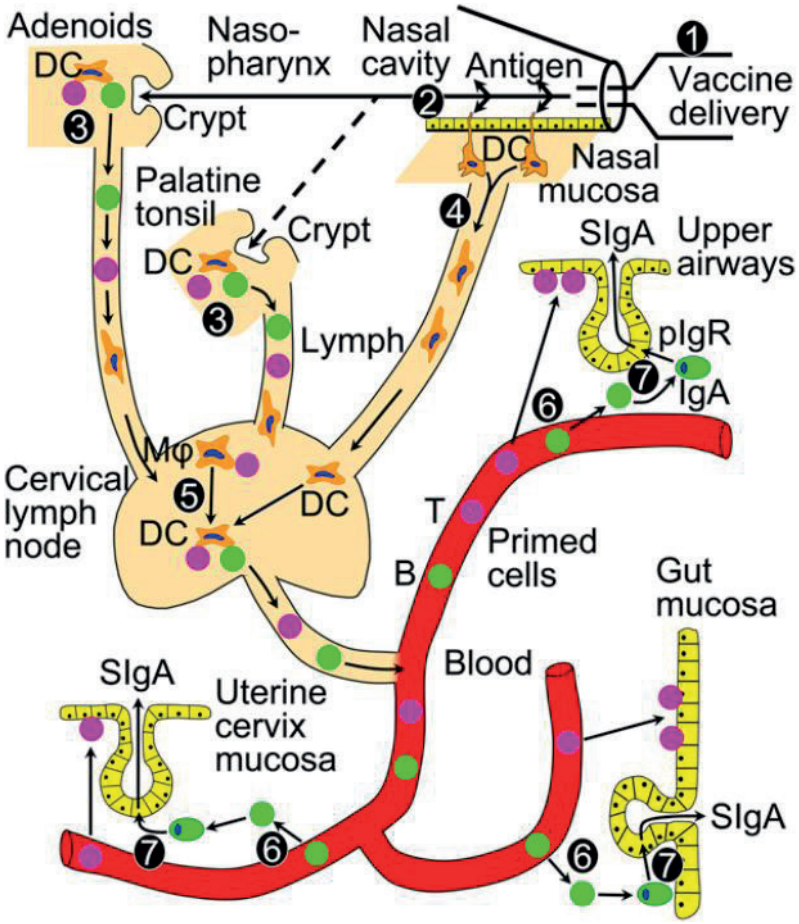

Figure 12 *Antigen sampling and routing of immune cells from Waldeyer's ring to the lymph nodes in the neck (cervical lymph nodes) and mucosal tissues (from Brandtzaeg [5]).* After capturing of antigens in Waldeyer's ring the antigens are transported by immune cells to the cervical lymph nodes, where the immune activation takes place. Upon activation, the immune cells recirculate through the blood and home selectively to the upper airways, where they confer protection against pathogenic substances, e.g. by producing IgA and by memory T cell responses**.**

Furthermore, dispersed Langerhans-like dendritic cells (LC) are present in the mucosal tissues in the mouth [171] suggesting that an effective immune response against pathogens that enter the mouth mucosa already can induce an immune response. These LC-like cells are present in the palatum, tongue (lingua), cheeks (bucca), sublingual, and in the vestibulum. As certain subsets of DC in the GI tract are known to sample their environment across epithelia and mucus layers, the LC in the mouth are expected to sample protein antigens in the oral mucosa in a similar manner. Small molecules can be taken up directly across the epithelium, as it is well established that sublingual administration (under the tongue) of small molecule drugs leads to rapid absorption.

Taken together, these findings demonstrate that the mouth and throat are important inductive sites for initiating specific IgA secretion at the mucosal surfaces of the upper respiratory tract.

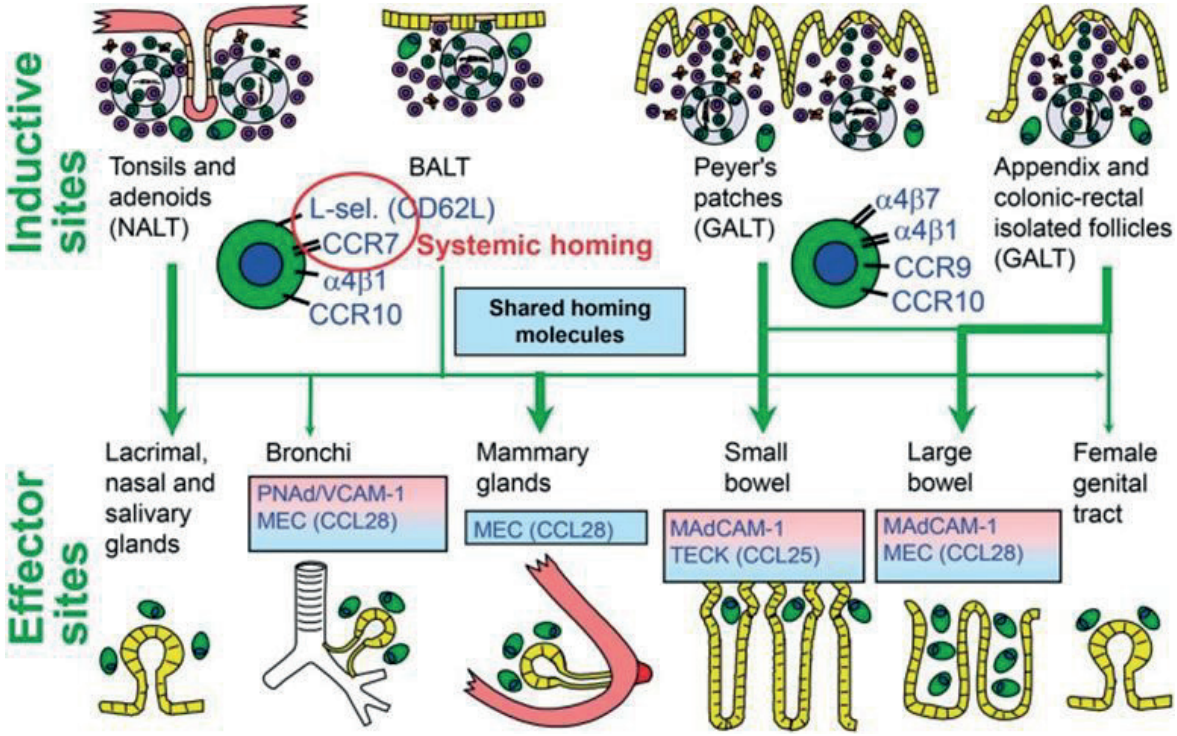

Figure 13 *Putative scheme for compartmentalized migration of B cells from inductive (top) to effector (bottom) sites.*
Depicted are more or less preferred pathways (graded arrows) presumably followed by mucosal B cells activated in nasopharynx-associated lymphoid tissue (NALT) represented by palatine tonsils and adenoids, bronchus-associated lymphoid tissue(BALT), and gut-associated lymphoid tissue(GALT) represented by Peyer's patches, appendix, and colonic-rectal isolated lymphoid follicles. Homing molecules integrating airway immunity with systemic immunity are encircled in red. Adapted from Brandtzaeg [5]. MEC, mucosae-associated epithelial chemokine; TECK, thymus-expressed chemokine.

6.4 The respiratory tract in pigs and its immune system

As shown in Figure 13, the organised mucosa-associated lymphoid tissue (MALT) is strategically located at different antigen entry sites into the body to allow efficient antigen sampling from mucosal surfaces. Based on the anatomical localisation, MALT structures of the respiratory tract can be subdivided into nose-associated lymphoid tissue (NALT), lymphoid tissues of Waldeyer's ring, larynx-associated lymphoid tissue (LTALT) and MALT which is present at the site of bronchial ramification as bronchus-associated lymphoid tissue (BALT) [165]. This organised lymphoid tissue is specialised in the recognition and processing of antigens, and in the primary activation of B lymphocytes. These aggregates of organised lymphoid tissue resemble Peyer's patches of ileum and both are involved in the mucosal immune system, playing a key role in this immune system. They are the sites of mucosal immune response induction [172]. MALT contains lymphatics which transport immune cells and antigens to regional lymph nodes that can therefore be called part of the inductive sites of mucosa and augment the immune responses [165].
BALT does not develop in all pigs and some other animal species such as mice, cats, dogs, humans, primates. It only develops after contact with pathogens, hence usually after an infection of the respiratory tract [173] [174].
Provided BALT develops in pigs, its structure and in particular its topography differ in certain aspects from those in the other animal species studied to date. The T- and B-lymphocyte-containing areas are not as clearly separated from each other as is the case in other animal species [174]; BALT in pigs is usually localised at the site of ramification of the bronchioles and to a lesser extent in small, cartilage non-supported bronchi [175]. Due to the fact that BALT occurs non-constitutively in some animal

species, the essential role of BALT in the induction of mucosal response in respiratory tract is disputable.

6.5 Secretory IgA: Designed for Anti-Microbial Defence and minimal mucosal inflammation

IgA is the most produced (66 mg/kg/day) antibody class in humans and most likely also in farm animals. It is the main antibody in mucosal tissues, including the upper respiratory tract, the gastrointestinal tract and the urogenital tract and is very important to protect against microbial infection of the mucosal tissue. At mucosal sites IgA is present in two distinct forms that are referred to as dimeric IgA (dIgA) and secretory IgA (SigA). Both are produced as dimeric molecules by plasma cells in the mucosal tissue, stimulated by antigens that target the mucose. The IgA dimers are linked via their Fc-tails by a polypeptide called "J-chain". Only this dimeric IgA can bind to the polymeric Ig receptor (pIgR). From the basolateral surface of the epithelial cells the pIg-pIgR complexes are taken up by endocytosis and then extruded into the lumen after apical cleavage of the receptor (Figure 14). Part of the pIgR, the secretory compoment (SC), remains bound to the dimeric IgA and has stabilizing (protease-resistant) and innate functions in the secretory antibodies. Pentameric IgM can be transported by the same mechanism as dIgA and can also be found at many mucosa, but at lower levels than IgA. In the gut, induction of B-cells occurs in gut-associated lymphoid tissue, particularly the Peyer's patches and isolated lymphoid follicles, but also in mesenteric lymph nodes. IgA plasma cell differentiation is accomplished in the lamina propria to which the activated memory/effector B-cells home (□4□7). The airways also receive such cells from nasopharynx-associated lymphoid tissue but by different homing receptors (□4□1). This compartmentalization is a challenge for mucosal vaccination, as are the mechanisms used by the mucosal immune system to discriminate between commensal symbionts (mutualism), pathobionts, and overt pathogens (elimination).

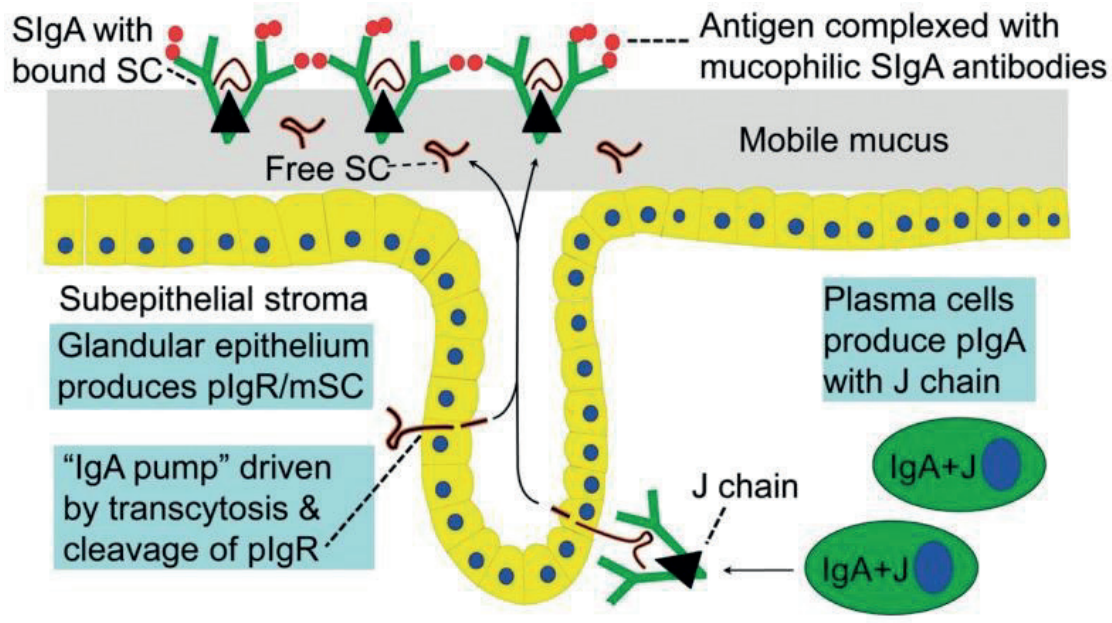

Figure 14 *Receptor-mediated epithelial export of polymeric IgA (pIgA, mainly dimers) to providesecretory IgA (SIgA) antibodies At the mucosal surface, SIgA antibodies together with mucus perform immune exclusion of antigens. The epithelial polymeric Ig receptor (pIgR) is expressed basolaterally, mainly in the intestinal crypts (glands), as membrane secretory component (mSC) and mediates external transcytosis of pIgA (and pentameric IgM, not shown). SIgA is released to the lumen with bound SC by apical cleavage of pIgR, in the same manner as unoccupied pIgR (carrying no ligand) is cleaved to provide free SC. Mucosal plasma cells produce abundantly pIgA with incorporated J chain (IgA + J), which is required for high-affinity epithelial binding of the pIgR ligands. Modified from [176].*

It is expected that nutritional interventions that stimulate IgA production might be helpful in supporting the mucosal immune system, both in the upper airways as well as in the gut. In this study, dietary addition of *Bacillus subtillis, Saccharomyces cerevisiae*, chitosan, rice bran, glutamine, and spray-dried animal plasma resulted in increased secretion of IgA.

In our institute, a large number of feed additives, e.g. live yeast, mixtures of organic acids, lecithins, butyric acid, humic acid, bioactive plant components and prebiotics, were tested as potential replacers of antimicrobial growth promoters. In all these animal performance experiments, veterinary treatments and mortality due to airway disorders were registered. In most of the experiments, however, veterinary treatment and mortality levels were too low for statistical analysis, or were not affected by the dietary interventions. The only exception was an intervention with a mixture of medium chain fatty acids (Aromabiotic), with no veterinary treatments in the Aromabiotic group and 7 out of 220 veterinary treated piglets in both the negative and positive (with antimicrobial growth promoter) control groups [177]. More studies, and probably with a more tailored design, should be performed to determine the possible contribution of dietary interventions on the immune competence of the upper airways.

Modulation of the capacity and activity of the immune system through nutrition and the use of immuno-modulating compounds, results in innovative animal nutrition concepts and feed interventions. The programming, development and maintenance of a properly functioning gastrointestinal tract in pigs, with an improved resistance to pathogens and other stressors will improve the health and welfare of animals and leads to a further reduction in the use of antibiotics in livestock farming and saving on the cost for animal health. The identified bio-indicators of resilience and robustness with predictive value for good animal health will result in improved feed efficiency and ecological footprint. Nowhere did we suggest to use the immuno-modulatory compounds of relevance to this project to treat sick animals, but we focus on strengthening the immune system to better prepare animals for infections and, thereby, improve their resilience.

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Appendix 1 Gene expression datasets of nutritional interventions in cattle

Cattle	Compound (intervention)	Product group	species / tissue or cell-type	administration	process /genes influenced	main conclusion	remarks	GEO or ArrayExpress accession number	Array Platform	PubMed link
b 1	High concentrate (HC) diet (inducing ruminal acidosis) and low (LC) concentrate diet	biological source	dairy cows / ruminal tissue (sub acute ruminal acidosis)	oral	calcium signaling, MAPK pathway and gap junction pathways.	ruminal epithelium function is altered for cows fed with HC diet	ruminal tissue maintenance; association with cAMP dependent protein kinase A catalytic subunit beta (PRKACB) expression.	E-GEOD-19802	A-GEOD-9862 - University of Alberta Bovine 24K long oligo array	PMID: 20732839
b 2	grain-induced sub acute ruminal acidosis (inducing sub acute ruminal acidosis)	biological feed source	dairy cows / ruminal tissue (biopsied papillae)	oral	desmoglein 1 and IGF3, 5, and 6 (genes involved in epithelial growth and structure) and genes involved in ketogenesis and cholesterol homeostasis	structural integrity of the rumen epithelium is compromised and cholesterol homeostasis is altered	luminal increase in sort chain fatty acids affects mucosal cholesterol homeostasis	E-GEOD-17849	A-AFFY-128 - Affymetrix GeneChip Bovine Genome Array Bovine	PMID: 21245418 PMID: 21245418

Appendix 2a Gene expression datasets of nutritional interventions in pigs

pig	Compound (intervention)	Product group	species, tissue or cell-type	administration	process /genes influenced	main conclusion	remarks	GEO or ArrayExpress accession number	Array Platform	PubMed link
p 1	resistant starch (RS)	Carbohydrates □ Polysaccharides □ Glucans	pig 17-wk-old / colon biopsy	oral	stimulation of tricarboxylic acid cycle and β-oxidation and suppression of cell division and adaptive and innate immune system	a diet high in RS provoked major changes in colonic gene expression and microbiome composition	increased abundance of several butyrate-producing microbial groups	E-GEOD-45554	Porcine Gene 1.0 ST Array	PMID: 24132577
p 2	Sorbic Acid	Lipids □ Fatty Acids □ Fatty Acids, Unsaturated	piglets 5 days after weaning / ileum	oral	activation of immune and inflammatory responses are likely are a cause of small intestine atrophy as revealed by a decrease in villus height and villus/crypt ratio	hypothesis; inflammation-induced atrophy in the intestine during weaning	No positive effect of sorbic acid on weaning	E-GEOD-50150	CombiMatrix CustomArrayTM 90K platform	no citation
p 3	broad-spectrum antibiotics (not specified)	Anti-Infective Agents □ Anti-Bacterial Agents	Caesarean-delivered preterm pigs / small intestinal tissue	oral and intramuscularly	modulation of intestinal immunity processes and prevents necrotizing enterocolitis in preterm neonates	prevention of necrotizing enterocolitis in preterm neonates	tissue sampling on day five	E-GEOD-48125	A-AFFY-75 - Affymetrix GeneChip Porcine Genome Array [Porcine]	no citation
p 4	ETEC strain lacking the heat-stable enterotoxin STa and STb	Biological Factors □ Toxins, Biological □ Enterotoxins	pig / jejunum	perfusion of jejunum segments (small intestinal segment perfusion technique)	inflammatory response; regulation of immune genes (IL1B and IL17A)	limited amounts of STb elicit an IL17A response	STa alone is sufficient to induce IL17A and ILB up-regulation	E-GEOD-28003	A-AFFY-75 - Affymetrix GeneChip Porcine Genome Array [Porcine]	PMID: 22815904
p 5	deoxynivalenol (fungal toxin DON)	Organic Chemicals □ Hydrocarbons □ Terpenes □ Sesquiterpenes □ Trichothecenes	pig /cultured Porcine Epithelial Cells IPEC-J2	*in-vitro* challenged cell monolayers (transwell plates)	genes involved in pathways of oxidative phosphorylation, ubiquitin mediated proteolysis, proteasomes, RNA degradation, focal adhesion and tight junctions.	apical and basolateral challenge trigger different gene response profiles	higher susceptibility towards basolateral challenge	E-GEOD-33246	A-AFFY-75 - Affymetrix GeneChip Porcine Genome Array [Porcine]	PMID: 22506013
p 6	Lactobacillus plantarum 299v wild type and a isogenic mutant strain lacking the gene encoding the mannose-specific adhesin (msa-)	probiotic bacteria	pig / jejunum, ileum, colon	oral; repeated administration over a period of 14 days	mainly response in the ileum; stimulation of the "Intestinal immune network for IgA production" and repression of NFKB-mediated transcription and PPARG signaling.	increases the alertness of the intestinal immune network for IgA production and tempering of inflammatory reactions	hypothesis; possible chemical cross-talk between immune cells and sub-mucosal adipocytes	E-MEXP-2198	A-AFFY-75 - Affymetrix GeneChip Porcine Genome Array [Porcine]	Thesis Grabriele Gross; http://www.vlaggraduateschool.nl/theses2008.htm

Appendix 2b Gene expression datasets of nutritional interventions in pigs

pig	Compound (intervention)	Product group	species, tissue or cell-type	administration	process /genes influenced	main conclusion	remarks	GEO or ArrayExpress accession number	Array Platform	PubMed link
p 7	Intrauterine Growth Restricted	NA	piglets (age 0, 2 and 5 days of life) / distal small intestine (ileum) and colon	Intrauterine Growth Restricted (IUGR) vs. normal birth weight piglets	ileum and colon: macromolecule metabolism, transport, biosynthesis, physiological processes, signal transduction, cell development, and cell death processes.	IUGR affected proliferation-apoptosis homeostasis in the intestine	Piglets with IUGR possess a thinner intestine and a reduced surface area of exchange	E-GEOD-15384	A-GEOD-7576 - Porcine oligonucleotide microarray version 4 (POM4) (Condensed version)	PMID: 20335628
p 8	porcine colostrum or formula milk	biological source (formulated)	preterm pigs / distal small intestine	oral or enteral (tube) feeding/preterm pig model for necrotizing enterocolitis (NEC)	altered expression of innate immune defense genes (IL-1α, IL-6, IL-18), nitric oxide synthetase, tight junction proteins (claudins), Toll-like receptors (TLR-4), and TNF-α	Switchs of parenteral to enteral nutrition induces diet-dependent functional, and proinflammatory insults to immature intestine.	Formula feeding induced histopathological lesions, colostrum did not	E-GEOD-13516	A-GEOD-7576 - Porcine oligonucleotide microarray version 4 (POM4) (Condensed version)	PMID: 21700903
p 9	amniotic fluid (AF) and or formula milk	biological source (formulated)	preterm pigs / distal small intestine	oral or enteral (tube) feeding/preterm pig model for necrotizing enterocolitis (NEC)	Formula-enteral feeded pigs down-regulated inflammation-related genes (e.g., TNF-α, IL-1α, IL-6, NOS). No effect of enteral feeded AF	AF reduces NEC development in preterm neonates by suppressing the proinflammatory responses to enteral formula feeding	AF down-regulated IL-6 expression in intestinal cells (IEC-6, IPEC-J2)	E-GEOD-13515	A-GEOD-7576 - Porcine oligonucleotide microarray version 4 (POM4) (Condensed version)	PMID: 23518680
p 10	animal or vegetable protein diet	biological source	pigs / jejunum	oral feeding	not specified	evidence that diet can regulates gene expression in the intestinal epithelium	none	E-GEOD-13457	A-AFFY-75 - Affymetrix GeneChip Porcine Genome Array [Porcine]	no citation
p 11	Cholera toxin (CT)	Biological Factors □ Toxins, Biological □ Enterotoxins	cultured Porcine Epithelial Cells (IPEC-J2)	in-vitro challenged cell monolayers (transwell plates)	not specified	mucosal adjuvanticity and immunogenicity of CT derives from rapid alterations in gene expression at the site of first antigen encounter with the intestinal immune system	none	E-GEOD-10314	A-AFFY-75 - Affymetrix GeneChip Porcine Genome Array [Porcine]	no citation

Appendix 3a Gene expression datasets of nutritional interventions in chickens

chicken	Compound (intervention)	Product group	species / tissue or cell-type	administration	process /genes influenced	main conclusion	remarks	GEO or ArrayExpress accession number	Array Platform	PubMed link
c 1	chicken with different efficiencies in food to energy conversion (Apparent Metabolizable Energy = AME)	biological feed source	chicken / duodenum	oral	mostly genes of unknown function.	Differences in duodenal gene expression between high and low AME birds	poorly described	E-GEOD-47213	A-GEOD-17190 - Nimblegen custom 12x135K chicken high-density multiplex microarray	no citation
c 2	direct fed microbials (DFM)	microbials	chicken / intestine (no further details)	oral	no details described	no details described	Effect of dietary supplementation with direct fed microbials (DFM) in chicken	E-GEOD-41934	A-GEOD-13820 - Agilent-015068 Chicken Gene Expression Microarray 4x44k (Probe Name version)	no citation
c 3	pigeon 'milk''	biological feed source	chicken / ileum	oral	no details described	no details described	title; Functional similarities between pigeon 'milk' and mammalian milk: induction of immune gene expression and modification of the caecal microbiota	E-MTAB-1127	A-MEXP-2133 - Nimblegen Custom Whole Chicken Array	no citation
c 4	Anethol (effect after Eimeria acervulina challenge)	Organic Chemicals □ Ethers □ Methyl Ethers □ Anisoles □ anethole	chicken / isolated intestinal lymphocytes	oral	genes of the "Inflammatory Response" and "Disease and Disorders" category in E. acervulina-infected chickens fed the anethole-containing diet.	anethole dietary supplementation may be relevant to host protective immune response to avian coccidiosis.	immune mediators IL6, IL8, IL10, and TNFSF15 in intestinal lymphocytes were increased	E-GEOD-41250	A-GEOD-13820 - Agilent-015068 Chicken Gene Expression Microarray 4x44k (Probe Name version)	PMID: 24046409
c 5	residual feed intake (RFI=deviation of expected feed intake from actual feed intake)	biological feed source	Chickens / Duodenum (at 35 and 42 days)	oral	no details described	no details described	Chickens consuming less feed than expected are efficient (LRFI) and chickens that consume more feed than expected are inefficient (HRFI).	E-GEOD-39824	A-AFFY-103 - Affymetrix GeneChip Chicken Genome Array [Chicken]	no citation
c 6	Curcuma longa extract - curcumin (effect after Eimeria maxima and Eimeria tenella challenge)	Organic Chemicals □ Hydrocarbons □ Hydrocarbons, Acyclic □ Alkanes □ Heptanes □ Diarylheptanoids	chicken / isolated intestinal lymphocytes	oral (supplemented diet)	genes mediating anti-inflammatory effects	Curcuma longa extract could be used to attenuate Eimeria-induced, inflammation-mediated gut damage in poultry production.	Curcuma longa extract feeding reduced fecal oocyst shedding, and decreased gut lesions	E-GEOD-37813	A-GEOD-13820 - Agilent-015068 Chicken Gene Expression Microarray 4x44k (Probe Name version)	PMID: 24046410

Appendix 3b Gene expression datasets of nutritional interventions in chickens

chicken	Compound (intervention)	Product group	species / tissue or cell-type	administration	process /genes influenced	main conclusion	remarks	GEO or ArrayExpress accession number	Array Platform	PubMed link
c 7	secondary metabolites of garlic, propyl thiosulphinate (PTS) and propyl thiosulphinate oxide (PTSO); effect after Eimeria acervulina challenge	Organic Chemicals □ Sulfur Compounds □ Sulfides □ Disulfides □	broiler chickens /isolated intestinal lymphocytes	oral (supplemented diet)	'Inflammatory Response' and 'Cardiovascular System Development and Function'.	PTSO/PTS dietary supplementation are relevant to protective immunity during avian coccidiosis.	PTSO/PTS compound-supplemented diet increases body weight gain, decreased faecal oocyst excretion	E-GEOD-36302	A-GEOD-13820 - Agilent-015068 Chicken Gene Expression Microarray 4x44k (Probe Name version)	PMID: 22717023
c 8	Mannose-rich oligosaccharides (MOS from Saccharomyces) and virginiamycin (antibiotic; VIRG) / Salmonella LPS-induced systemic inflammation	Polycyclic Compounds □ Macrocyclic Compounds □ Peptides, Cyclic □ Streptogramins (Virginiamycin) / Carbohydrates □ Polysaccharides □ Oligosaccharides (MOS)	broilers chickens / jejunum	oral	MOS induced IL-3 expression in not LPS treated chicken /gene up-regulations for intestinal gluconeogenesis in VIRG chicken	MOS counteracted LPS's detrimental inflammatory effects.	MOS terminated inflammation earlier than VIRG and reduced glucose mobilization	E-GEOD-28959	A-GEOD-13457 - NCSU_chicken_JS3 (359 oligos)	PMID: 22272335
c 9	endotoxin from Salmonella typhimurium-798	Biological Factors □ Toxins, Biological □ Enterotoxins	chicken / macrophage HD11 cells	in-vitro challenged cell monolayers (transwell plates)	Consistent induction of NFKBIA, IL1B, IL8, CCL4 and NLRC5 (CARD domain containing, NOD-like receptor family) genes at 1, 2, 4 and 8 hours after challenge with endotoxin.	induction of a NOD-like receptor family member in response to Salmonella endotoxin in chicken HD11 cells	chicken macrophages quickly return to homeostasis after response to endotoxin-caused shock	E-GEOD-23881	A-AFFY-103 - Affymetrix GeneChip Chicken Genome Array [Chicken]	PMID: 20929591
c 10	poultry-derived, Lactobacillus-based probiotic culture	probiotic bacteria	neonatal chicken day-of-hatch / Cecae	oral gavage using an animal feeding needle.	genes associated with the nuclear factor kappa B complex and involved in apoptosis (growth arrest-specific 2 (GAS2) and cysteine-rich, angiogenic inducer, 61 (CYR61))	increased apoptosis may be a mechanism by which the probiotic culture reduces Salmonella infection.	Lactobacillus-based probiotic culture (FloraMax-B11, Pacific Vet Group USA Inc., Fayetteville, AR)	E-GEOD-19887	A-GEOD-6049 - Arizona Gallus gallus 20.7K Oligo Array v1.0	PMID: 21406379
c 11	organic or conventially produced starter and grower diets	biological feed source	chicken (different lines)/ jejunum	oral	cholesterol biosynthesis (SREBP, sterol regulatory element binding protein) / immunological processes (B-G protein, chemokine ah221, immunoglobulin heavy chain)	significant differences in gene expression were shown between animals on diets with organically or conventionally produced ingredients.	Differences in gene expression between different diets as well as between different chicken lines	E-MEXP-1798	A-MEXP-820 - ARK-Genomics G. gallus 20K v1.0	PMID: 19968894
c 12	carvacrol / cinnamaldehyde / capsicum oleoresin extracted from C. annuum fruits) supplemented starter diets	Organic Chemicals □ Hydrocarbons □ Terpenes □ Monoterpenes / Complex Mixtures □ Biological Products □ Plant Preparations □ Plant Extracts	chicken / intestinal intraepithelial lymphocytes	oral	no details described	no details described	start supplemented diet; for 7 days beginning from hatch	E-MEXP-2204	A-MEXP-1487 - AVIELA	no citation

Appendix 4 Tutorial for data mining

action	databas(s) / module	input	selection based on	# selected (genes/compounds)	remarks
consulting general information of genes in list	Genecards-Entrez	122 (list colon)	general function gene	3	high up regulation of CR2, MME and CCL25, Fold change (treated over non-treated) > 10
create sublist 1; SMMP	GeneDecks	122 (list colon)	GO_BIOL_PROC / small molecule metabolic process (SMMP)	25	information of human genes is used in GeneDecks
create sublist 2; IAC	DAVID - functional annotation cart	122 (list colon)	GO_terms immune phenotype / adhesion / cytoskeletal (IAC)	39	DAVID analysis using the human background
create sublist 3; transcription factors-regulators	DAVID - functional annotation cart	122 (list colon)	GO_terms transcription factors (TF) and regulators	3	the 3 transcription factors-regulators were also present in IAC list
Retrieve significant pathways	DAVID pathway module / GeneDecks	25 (SMMP)	significance (p-value and/or number of genes)	16	in case GeneDecks and David retrieve the same pathway the DAVID pathway is removed
Expression in specific Tissue - cells	DAVID - Tissue expression module	25 (SMMP)	significance (p-value and/or number of genes)	5	GNF_U133A_QUARTILE libraries also include subset of immune cells
Inventory of transcription factor binding sites	DAVID -Protein interaction module	25 (SMMP)	significance (p-value and/or number of genes)	5	UCSC_TFBS; human transcription binding sites
Relations with chemicals/substances	GeneDecks	25 (SMMP)	* compounds related to pathways, and > 20% (#>4)	10	remove equivalent terms (e.g. Retinoic acid and Tretinoin)
Retrieve significant pathways	DAVID pathway module / GeneDecks output list	39 (IAC)	significance (p-value and/or number of genes)	27	in case GeneDecks and David retrieve the same pathway the DAVID pathway is removed
Expression in specific Tissue - cells	DAVID - Tissue expression module (GNF_U133A_QUARTILE)	39 (IAC)	significance (p-value and/or number of genes)	3	GNF_U133A_QUARTILE libraries also include subset of immune cells
Inventory of transcription factor binding sites	DAVID -protein interaction module (UCSC_TFBS)	39 (IAC)	significance (p-value and/or number of genes)	4	UCSC_TFBS; human transcription binding sites
Relations with chemicals/substances	GeneDecks output list	39 (IAC)	* compounds related to pathways and > 12.5% (#>4)	13	remove equivalent terms (e.g. Retinoic acid and Tretinoin)
** Summarize and interpretation	researcher / Genecards-Entrez-PubMed	output from above steps	literature / common genes in SMMP and IAC list)	64 genes / 10 chemicals	identification of genes and related chemicals (e.g. enzyme products and substrates, chemical receptorligands)
					"common" metabolites (e.g. calcium, glucose, etc..) were removed
Build a network of transcription factors-regulators	GNCpro selectable Relationships; Protein Interaction, TFactor Regulation, and Chemical Modification	64 (SMMP plus IAC)	DAVID - functional annotation cart	3	no significant network associations
Build network to identify key-genes	STITCH protein-protein-chemical association module	64 (SMMP plus IAC)	confidence level high; "score > 0.7 / no extra interactor(s) added	18 genes / 4 chemicals	non-interactors are removed from network
Identify key-genes from network and pathways	researcher / Genecards-Entrez-PubMed	18 (STITCH network)	central rol in network and/or specific pathway	7 genes	4 from network (NR1H4, APOC3, CYP3A4, CYP4F3) and 3 from the B Cell Receptor Signaling Pathway
Explore alternative chemicals influencing expression/function of key-genes	Comparative Toxicogenomics Database (CTD)	7	literature search-interpretation	21 (3 per chemical)	see supplementary file MS5.
***) > 12.5% (#>4) means; the percentage of genes from the list is higher than 12.5% or more than 4.**					
**** Summarize and interpretation (results are presented in Table MS2 and MS3.**					

Databases -bioinformatics programs

http://www.ncbi.nlm.nih.gov/gene/
http://www.genecards.org/
http://david.abcc.ncifcrf.gov/
http://www.ncbi.nlm.nih.gov/pubmed/
http://stitch-beta.embl.de/cgi/show_input_page.pl?UserId=_qH_JfHor_A4&sessionId=qLz3ImtldNs7&input_page_type=multiple_identifiers
http://ctdbase.org/
http://gncpro.sabiosciences.com/gncpro/gncpro.php

Appendix 5 David subset > Immune system phenotype / cell adhesion / cytoskelet / transcription (IAC)

OFFICIAL_GENE_SYMBOL	Description
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
ADAM12	ADAM metallopeptidase domain 12
ANPEP	alanyl (membrane) aminopeptidase
APOA1	apolipoprotein A-I
APOA4	apolipoprotein A-IV
APOB	Apolipoprotein B-100 precursor (Apo B-100) [Contains: Apolipoprotein B-48 (Apo B-48)]. [Source:Uniprot/SWISSPROT;Acc:P04114]
APOC3	apolipoprotein C-III
CCL25	chemokine (C-C motif) ligand 25
COL1A2	collagen, type I, alpha 2
CR2	complement component (3d/Epstein Barr virus) receptor 2
DAB1	disabled homolog 1 (Drosophila)
DGKA	diacylglycerol kinase, alpha 80kDa
DIAPH2	diaphanous homolog 2 (Drosophila)
DPP4	dipeptidyl-peptidase 4
EGLN3	egl nine homolog 3 (C. elegans)
ENPEP	glutamyl aminopeptidase (aminopeptidase A)
ETV6	ets variant 6
GBP2	guanylate binding protein 2, interferon-inducible
IFIT1	interferon-induced protein with tetratricopeptide repeats 1
MEF2B	MEF2B
MEP1B	meprin A, beta
MGP	matrix Gla protein
MME	membrane metallo-endopeptidase
NCK1	NCK adaptor protein 1
NR1H4	nuclear receptor subfamily 1, group H, member 4
OTC	ornithine carbamoyltransferase
PABPC1	poly(A) binding protein, cytoplasmic pseudogene 5; poly(A) binding protein, cytoplasmic 1
PSEN2	presenilin 2 (Alzheimer disease 4)
ROCK1	similar to Rho-associated, coiled-coil containing protein kinase 1; Rho-associated, coiled-coil containing protein kinase 1
SCIN	scinderin
SEPP1	selenoprotein P, plasma, 1
SLC26A6	solute carrier family 26, member 6; cadherin, EGF LAG seven-pass G-type receptor 3 (flamingo homolog, Drosophila)
SLC3A1	solute carrier family 3 (cystine, dibasic and neutral amino acid transporters, activator of cystine, dibasic and neutral amino acid transport), member 1
SLC7A9	solute carrier family 7 (cationic amino acid transporter, y+ system), member 9
SPP1	secreted phosphoprotein 1
SSX2IP	synovial sarcoma, X breakpoint 2 interacting protein
TAF3	TAFII140 protein (Fragment). [Source:Uniprot/SPTREMBL;Acc:Q9BQS9]
TFF2	trefoil factor 2
XPA	xeroderma pigmentosum, complementation group A

Appendix 6 GENEDECKS subset > small molecule metabolic process (SMMP)

OFFICIAL_GENE_SYMBOL	Description
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2
ABCG8	ATP-binding cassette, sub-family G (WHITE), member 8
ACADL	acyl-Coenzyme A dehydrogenase, long chain
ALDOB	aldolase B, fructose-bisphosphate
AMN	amnionless homolog (mouse)
APOA1	apolipoprotein A-I
APOA4	apolipoprotein A-IV
APOB	apolipoprotein B (including Ag(x) antigen)
APOC3	apolipoprotein C-III
CA7	carbonic anhydrase VII
CPS1	carbamoyl-phosphate synthetase 1, mitochondrial
CYP2C18	cytochrome P450, family 2, subfamily C, polypeptide 18
CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4
CYP4F3	cytochrome P450, family 4, subfamily F, polypeptide 3
FABP6	fatty acid binding protein 6, ileal
GLS	glutaminase
KHK	ketohexokinase (fructokinase)
MAOB	monoamine oxidase B
OAT	ornithine aminotransferase (gyrate atrophy)
OTC	ornithine carbamoyltransferase
PRODH	proline dehydrogenase (oxidase) 1
RBP2	retinol binding protein 2, cellular
SLC2A2	solute carrier family 2 (facilitated glucose transporter), member 2
SLC5A1	solute carrier family 5 (sodium/glucose cotransporter), member 1

Appendix 7 Pathways / Compounds / Tissue expression / Transcription binding sequences

	Term	# genes	%	*p*-value	genes[a]
		(N=25)			
* Category	Term	Count	%	PValue	Genes
REACTOME_PATHWAY	REACT_602:Metabolism of lipids and lipoproteins	8	32	9.16E-06	APOA4, ABCG8, APOB, APOA1, APOC3, AMN, ACADL, FABP6
SUPER_PATHWAY	Statin Pathway, Pharmacodynamics	6	24	4.41E-13	ABCB1, ABCG8, APOA1, APOA4, APOB, APOC3
SUPER_PATHWAY	Lipoprotein metabolism	6	24	2.86E-12	ABCG8, AMN, APOA1, APOA4, APOB, APOC3
SUPER_PATHWAY	Arginine and proline metabolism	6	24	1.65E-11	CPS1, GLS, MAOB, OAT, OTC, PRODH
SUPER_PATHWAY	alpha-Linolenic acid metabolism	6	24	7.56E-11	ABCG8, APOA1, APOA4, APOB, **CYP2C18**, CYP3A4
SUPER_PATHWAY	SLC-mediated transmembrane transport	6	24	7.09E-06	ABCB1, ABCG2, ABCG8, APOA1, SLC2A2, SLC5A1
REACTOME_PATHWAY	REACT_13:Metabolism of amino acids	5	20	1.12E-02	GLS, OTC, CPS1, OAT, PRODH
SUPER_PATHWAY	Retinoid metabolism and transport	5	20	2.31E-09	APOA1, APOA4, APOB, APOC3, RBP2
SUPER_PATHWAY	Bile secretion	5	20	8.19E-09	ABCB1, ABCG2, ABCG8, CYP3A4, SLC5A1
SUPER_PATHWAY	Metabolism of amino acids and derivatives	5	20	9.14E-07	CPS1, GLS, OAT, OTC, PRODH
REACTOME_PATHWAY	REACT_13433:Biological oxidations	4	16	2.52E-02	CYP3A4, **CYP2C18**, MAOB, CYP4F3
SUPER_PATHWAY	superpathway of citrulline metabolism	4	16	1.46E-09	CPS1, GLS, OAT, OTC
SUPER_PATHWAY	Vitamin digestion and absorption	4	16	9.50E-09	APOA1, APOA4, APOB, RBP2
SUPER_PATHWAY	Selected targets of HNF1	4	16	7.25E-08	APOA1, APOB, SLC2A2, SLC5A1
SUPER_PATHWAY	ABC-family proteins mediated transport	4	16	1.38E-07	ABCB1, ABCG2, ABCG8, APOA1
SUPER_PATHWAY	PPAR signaling pathway	4	16	7.05E-07	ACADL, APOA1, APOC3, FABP6
** Category	Term	Count	%	PValue	Genes
UCSC_TFBS	MEIS1B-HOXA9 complex	18	72	4.40E-03	CYP3A4, **CYP2C18**, SLC5A1, OTC, ALDOB, MAOB, ABCB1, AMN, CPS1, ACADL, ABCG8, KHK, APOB, CA7, GLS, CYP4F3, OAT, FABP6
UCSC_TFBS	NF1=Nuclear Factor I/X (CCAAT-Binding Transcription Factor	13	52	3.97E-02	SLC5A1, RBP2, ABCB1, CPS1, AMN, ABCG2, KHK, APOB, GLS, CA7, CYP4F3, OAT, PRODH
UCSC_TFBS	RORA1	14	56	6.92E-02	OTC, ALDOB, MAOB, ABCB1, CPS1, AMN, APOA4, KHK, APOB, APOA1, CA7, GLS, APOC3, PRODH
UCSC_TFBS	FOXO4	16	64	7.37E-02	CYP3A4, **CYP2C18**, SLC5A1, OTC, ALDOB, MAOB, RBP2, ABCB1, CPS1, ABCG2, KHK, APOB, CA7, GLS, FABP6, PRODH
UCSC_TFBS	BRN2	15	60	8.04E-02	SLC5A1, OTC, MAOB, RBP2, ABCB1, CPS1, ACADL, AMN, ABCG2, ABCG8, KHK, APOB, SLC2A2, GLS, FABP6
*** Category	Term	Count	%	PValue	Genes
GNF_U133A_QUARTILE	Prostate_3rd	20	80	NA	CYP3A4, **CYP2C18**, SLC5A1, OTC, ALDOB, MAOB, ABCB1, AMN, ACADL, ABCG2, KHK, APOA1, CA7, SLC2A2, GLS, APOC3, CYP4F3, OAT, PRODH, FABP6
GNF_U133A_QUARTILE	lymphomaburkittsRaji_3rd	18	72	NA	CYP3A4, **CYP2C18**, SLC5A1, OTC, ALDOB, ABCB1, AMN, CPS1, ACADL, APOA4, KHK, APOB, APOA1, CA7, SLC2A2, GLS, CYP4F3, FABP6
GNF_U133A_QUARTILE	salivarygland_3rd	18	72	NA	CYP3A4, **CYP2C18**, SLC5A1, OTC, ALDOB, ABCB1, CPS1, ACADL, APOA4, KHK, APOB, APOA1, CA7, SLC2A2, GLS, APOC3, CYP4F3, FABP6
GNF_U133A_QUARTILE	Lung_3rd	16	64	NA	CYP3A4, **CYP2C18**, OTC, ALDOB, MAOB, ABCB1, CPS1, APOA4, KHK, APOB, APOA1, CA7, SLC2A2, APOC3, CYP4F3, PRODH
GNF_U133A_QUARTILE	fetallung_3rd	13	52	NA	CYP3A4, OTC, ALDOB, MAOB, CPS1, ABCG2, KHK, APOB, APOA1, SLC2A2, APOC3, CYP4F3, OAT
****Attribute type	Descriptor	Score		P Value	Genes sharing this descriptor
COMPOUND	atp	11	44	6.21E-10	ABCB1, ABCG2, ABCG8, ALDOB, APOC3, CPS1, GLS, KHK, OTC, SLC2A2, SLC5A1
COMPOUND	glucose	10	40	1.65E-08	ABCG8, ALDOB, APOA1, APOA4, APOB, APOC3, GLS, OTC, SLC2A2, SLC5A1
COMPOUND	lipid	10	40	1.32E-07	ABCG2, ABCG8, ACADL, APOA1, APOA4, APOB, APOC3, FABP6, OTC, RBP2
COMPOUND	glutamine	9	36	1.12E-10	ABCG2, ALDOB, APOA4, APOB, CPS1, GLS, OAT, OTC, SLC5A1
COMPOUND	cholesterol	9	36	5.20E-09	ABCB1, ABCG2, ABCG8, APOA1, APOA4, APOB, APOC3, FABP6, SLC5A1
COMPOUND	glutamate	8	32	6.85E-07	APOA1, APOB, GLS, MAOB, OAT, OTC, PRODH, SLC5A1
COMPOUND	fatty acid	7	28	1.20E-07	ACADL, APOA4, APOB, APOC3, FABP6, OTC, RBP2
COMPOUND	taurocholate	5	20	6.05E-10	ABCB1, APOA1, APOB, APOC3, FABP6
COMPOUND	choline	5	20	3.38E-07	APOA1, APOB, GLS, MAOB, SLC5A1
COMPOUND	vitamin a	5	20	1.34E-06	APOA1, APOA4, APOB, APOC3, RBP2

Appendix 8 Pathways / Compounds / Tissue expression / Transcription binding sequences

	Term	# genes	%	*p*-value	genes[a]
		(N=32)			
* Category	Term	Count	%	PValue	Genes
SUPER_PATHWAY	Signaling by GPCR	12	32	4.94E-06	ADAM12, APOA1, APOA4, APOB, APOC3, CCL25, COL1A2, DGKA, NCK1, PSEN2, ROCK1, SPP1
SUPER_PATHWAY	Protein digestion and absorption	6	16	2.94E-09	COL1A2, DPP4, MEP1B, MME, SLC3A1, SLC7A9
SUPER_PATHWAY	Statin Pathway, Pharmacodynamics	5	13	1.21E-09	ABCB1, APOA1, APOA4, APOB, APOC3
REACTOME_PATHWAY	REACT_604:Hemostasis	4	10	8.01E-02	APOB, APOA1, COL1A2, SLC7A9
REACTOME_PATHWAY	REACT_602:Metabolism of lipids and lipoproteins	4	10	2.60E-02	APOA4, APOB, APOA1, APOC3
SUPER_PATHWAY	Retinoid metabolism and transport	4	10	1.63E-06	APOA1, APOA4, APOB, APOC3
SUPER_PATHWAY	Lipoprotein metabolism	4	10	5.11E-07	APOA1, APOA4, APOB, APOC3
SUPER_PATHWAY	Selected targets of HNF1	4	10	4.63E-07	APOA1, APOB, DPP4, NR1H4
SUPER_PATHWAY	B Cell Receptor Signaling Pathway	3	8	2.95E-04	CR2, NCK1, SPP1
KEGG_PATHWAY	hsa04510:Focal adhesion	3	8	1.86E-01	ROCK1, COL1A2, SPP1
REACTOME_PATHWAY	REACT_13:Metabolism of amino acids	3	8	1.60E-01	OTC, SLC7A9, SLC3A1
KEGG_PATHWAY	hsa04640:Hematopoietic cell lineage	3	8	4.41E-02	CR2, MME, ANPEP
REACTOME_PATHWAY	REACT_16888:Signaling by PDGF	3	8	3.13E-02	NCK1, COL1A2, SPP1
REACTOME_PATHWAY	REACT_15518:Transmembrane transport of small molecules	3	8	1.41E-02	APOA1, SLC7A9, SLC3A1
KEGG_PATHWAY	hsa04614:Renin-angiotensin system	3	8	1.93E-03	MME, ANPEP, ENPEP
SUPER_PATHWAY	Amino acid and oligopeptide SLC transporters	3	8	4.19E-04	SLC26A6, SLC3A1, SLC7A9
SUPER_PATHWAY	Cell surface interactions at the vascular wall	3	8	3.81E-04	APOB, COL1A2, SLC7A9
SUPER_PATHWAY	Hematopoietic cell lineage	3	8	3.35E-04	ANPEP, CR2, MME
SUPER_PATHWAY	Selenium Pathway	3	8	3.03E-04	APOA1, APOB, SEPP1
SUPER_PATHWAY	alpha-Linolenic acid metabolism	3	8	1.79E-04	APOA1, APOA4, APOB
SUPER_PATHWAY	Endochondral Ossification	3	8	1.51E-04	MGP, SCIN, SPP1
SUPER_PATHWAY	Insulin Processing	3	8	7.18E-05	ANPEP, DPP4, MME
SUPER_PATHWAY	Vitamin digestion and absorption	3	8	7.27E-06	APOA1, APOA4, APOB
** Category	Term	Count	%	PValue	Genes
UCSC_TFBS	BRACH=T or Brachyuri	26	67	1.33E-02	MEF2B, DIAPH2, EGLN3, MME, SLC7A9, DGKA, APOB, DAB1, TFF2, PABPC1, ETV6, DPP4, NR1H4, CR2, TAF3, ROCK1, OTC, MGP, ABCB1, SLC3A1, SLC26A6, NCK1, SCIN, PSEN2, COL1
UCSC_TFBS	CDP=CUX1 or CUTL1	26	67	4.65E-02	DIAPH2, EGLN3, MME, SLC7A9, ENPEP, DGKA, APOA4, APOB, DAB1, APOC3, PABPC1, SEPP1, SSX2IP, ETV6, DPP4, NR1H4, TAF3, ROCK1, OTC, MGP, ABCB1, SLC3A1, NCK1, COL1A2
UCSC_TFBS	FREAC4 = FOXD1	25	64	1.77E-03	DIAPH2, EGLN3, MME, ANPEP, ENPEP, APOA4, APOB, DAB1, PABPC1, SEPP1, SSX2IP, ETV6, DPP4, NR1H4, CR2, ROCK1, MGP, ABCB1, XPA, SLC26A6, NCK1, SCIN, COL1A2, ADAM12,
***Category	Term	Count	%	PValue	Genes
GNF_U133A_QUARTILE	Cerebellum_3rd	31	79	NA	MEF2B, DIAPH2, EGLN3, MME, SLC7A9, ANPEP, ENPEP, DGKA, APOA4, CCL25, APOB, DAB1, APOA1, APOC3, TFF2, SSX2IP, ETV6, DPP4, NR1H4, CR2, ROCK1, OTC, MGP, ABCB1, SLC3A1, SLC26A6, SCIN, PSEN2, COL1A2, MEP1B, ADAM12
GNF_U133A_QUARTILE	BM-CD34+_3rd	31	79	NA	MEF2B, DIAPH2, EGLN3, SLC7A9, MME, ANPEP, ENPEP, DGKA, CCL25, APOB, DAB1, APOA1, APOC3, TFF2, SSX2IP, PABPC1, ETV6, DPP4, NR1H4, CR2, ROCK1, OTC, SLC3A1, XPA, SLC26A6, NCK1, SCIN, PSEN2, MEP1B, ADAM12, GBP2
GNF_U133A_QUARTILE	salivarygland_3rd	30	77	NA	MEF2B, DIAPH2, EGLN3, MME, SLC7A9, ENPEP, DGKA, APOA4, CCL25, APOB, DAB1, APOA1, APOC3, TFF2, SSX2IP, PABPC1, ETV6, DPP4, NR1H4, CR2, ROCK1, OTC, ABCB1, SLC3A1, SLC26A6, NCK1, SCIN, PSEN2, MEP1B, ADAM12
GNF_U133A_QUARTILE	lymphomaburkittsRaji_3rd	28	72	NA	MEF2B, DIAPH2, EGLN3, MME, ENPEP, APOA4, CCL25, APOB, APOA1, DAB1, TFF2, SSX2IP, PABPC1, ETV6, DPP4, NR1H4, CR2, ROCK1, OTC, ABCB1, SLC3A1, SLC26A6, IFIT1, NCK1, SCIN, PSEN2, MEP1B, ADAM12
****Attribute type	Descriptor	Score	%	P Value	Genes sharing this descriptor
COMPOUND	calcium	15	38	8.E-07	ANPEP, CCL25, COL1A2, CR2, DAB1, DGKA, DPP4, ENPEP, MGP, MME, PSEN2, ROCK1, SCIN, SPP1, TFF2
COMPOUND	glutamate	13	33	1.E-10	ANPEP, APOA1, APOB, DAB1, DPP4, ENPEP, MGP, MME, OTC, PSEN2, SLC3A1, SPP1, XPA
COMPOUND	glucose	12	32	2.E-08	ANPEP, APOA1, APOA4, APOB, APOC3, DPP4, MME, NR1H4, OTC, PSEN2, SEPP1, SPP1
COMPOUND	cholesterol	11	28	2.E-09	ABCB1, ANPEP, APOA1, APOA4, APOB, APOC3, DPP4, MGP, NR1H4, PSEN2, SPP1
COMPOUND	lipid	11	28	2.E-06	ANPEP, APOA1, APOA4, APOB, APOC3, DGKA, MME, NR1H4, OTC, SEPP1, SPP1
COMPOUND	estrogen	11	28	1.E-07	APOA1, APOB, APOC3, COL1A2, CR2, MGP, MME, NR1H4, PSEN2, SPP1, TFF2
COMPOUND	retinoic acid	10	25	1.E-06	ANPEP, APOA1, APOC3, DPP4, ETV6, MGP, MME, NR1H4, PSEN2, SPP1
COMPOUND	lactate	8	20	3.E-07	ANPEP, APOA1, APOB, DPP4, MME, OTC, PSEN2, SPP1
COMPOUND	vegf	8	20	1.E-05	APOB, EGLN3, ENPEP, ETV6, MME, NCK1, PSEN2, SPP1
COMPOUND	fatty acid	7	18	3.E-06	APOA4, APOB, APOC3, MME, NR1H4, OTC, PSEN2
COMPOUND	h2o2	6	16	3.E-04	APOA1, APOB, MME, PSEN2, SEPP1, SPP1
COMPOUND	taurocholate	5	13	6.E-09	ABCB1, APOA1, APOB, APOC3, NR1H4
COMPOUND	vitamin a	5	13	1.E-05	APOA1, APOA4, APOB, APOC3, MME
***** pro-pre B-cell markers	Bio-gps database	high expression in			
MME	http://biogps.org/#goto=genereport&id=4311	lymphomaburkitts			
CR2	http://biogps.org/#goto=genereport&id=1380	lymphomaburkitts / B lymphoblasts			
CCL25	http://biogps.org/#goto=genereport&id=6370	small intestine / thymus			
T or Brachyuri	http://biogps.org/#goto=genereport&id=6862	B lymphocytes			

Appendix 9 STITCH interactors (type of association and confidential scores)

#node1	node2	node1_string_id	node2_string_id	node1_external_id	node2_external_id	neighbor-hood	fusion	cooccurence	homology	coexpres-sion	experimental	knowledge	textmining	combined_score
ABCB1	cholesterol	979655	-100000304	ENSP00000265724	304	0	0	0	0	0	0.272	0.8	0.833	0.974
ALDOB	KHK	991083	978593	ENSP00000363988	ENSP00000260598	0	0	0	0	0.089	0	0.9	0.374	0.935
ANPEP	ENPEP	982231	979556	ENSP00000300060	ENSP00000265162	0	0	0.525	0.886	0	0.16	0.9	0.752	0.922
APOA1	cholesterol	977122	-100000304	ENSP00000236850	304	0	0	0	0	0	0.9	0.9	0.954	0.999
APOA1	linoleic acid	977122	-100003931	ENSP00000236850	3931	0	0	0	0	0	0	0.9	0.86	0.985
APOA1	APOB	977122	977017	ENSP00000236850	ENSP00000233242	0	0	0	0	0.823	0.621	0.9	0.92	0.999
APOA1	APOC3	977122	976781	ENSP00000236850	ENSP00000227667	0	0	0	0	0.897	0.302	0.9	0.79	0.998
APOA4	cholesterol	988386	-100000304	ENSP00000350425	304	0	0	0	0	0	0	0.9	0.487	0.947
APOA4	APOA1	988386	977122	ENSP00000350425	ENSP00000236850	0	0	0	0.811	0.67	0	0.9	0.751	0.969
APOA4	APOC3	988386	976781	ENSP00000350425	ENSP00000227667	0	0	0	0	0.43	0	0.9	0.771	0.985
APOA4	APOB	988386	977017	ENSP00000350425	ENSP00000233242	0	0	0	0	0.668	0	0.9	0.565	0.983
APOB	linoleic acid	977017	-100003931	ENSP00000233242	3931	0	0	0	0	0	0	0	0.828	0.828
APOB	APOC3	977017	976781	ENSP00000233242	ENSP00000227667	0	0	0	0	0.754	0	0.9	0.852	0.995
APOB	cholesterol	977017	-100000304	ENSP00000233242	304	0	0	0	0	0	0	0.9	0.963	0.996
APOC3	cholesterol	976781	-100000304	ENSP00000227667	304	0	0	0	0	0	0	0.9	0.688	0.967
CPS1	APOC3	996337	976781	ENSP00000402608	ENSP00000227667	0	0	0	0	0.817	0	0	0	0.817
CPS1	APOA1	996337	977122	ENSP00000402608	ENSP00000236850	0	0	0	0	0.722	0	0	0	0.722
CPS1	OTC	996337	975784	ENSP00000402608	ENSP00000039007	0.438	0.014	0	0	0.568	0	0.9	0.658	0.989
CPS1	APOB	996337	977017	ENSP00000402608	ENSP00000233242	0	0	0	0	0.742	0	0	0	0.742
CYP2C18	linoleic acid	980959	-100003931	ENSP00000285979	3931	0	0	0	0	0	0	0.9	0	0.9
CYP2C18	CYP4F3	980959	976453	ENSP00000285979	ENSP00000221307	0	0	0.52	0.625	0	0	0.9	0.34	0.927
CYP3A4	CYP2C18	986634	980959	ENSP00000337915	ENSP00000285979	0	0	0.519	0.737	0.068	0	0.9	0.652	0.927
CYP3A4	NR1H4	986634	984066	ENSP00000337915	ENSP00000315442	0	0	0	0	0	0	0	0.916	0.916
CYP3A4	linoleic acid	986634	-100003931	ENSP00000337915	3931	0	0	0	0	0	0	0.9	0.27	0.924
CYP3A4	ABCB1	986634	979655	ENSP00000337915	ENSP00000265724	0	0	0	0	0	0	0	0.851	0.851
CYP4F3	LTB4	976453	-100000169	ENSP00000221307	169	0	0	0	0	0	0	0.9	0.699	0.969
DPP4	ANPEP	988888	982231	ENSP00000353731	ENSP00000300060	0	0	0	0	0	0	0	0.752	0.752
FABP6	NR1H4	993380	984066	ENSP00000377549	ENSP00000315442	0	0	0	0	0	0	0	0.954	0.954
IFIT1	GBP2	990429	990142	ENSP00000360869	ENSP00000359497	0	0	0	0	0.193	0	0.9	0.412	0.945
MAOB	CYP2C18	991745	980959	ENSP00000367309	ENSP00000285979	0	0	0	0	0	0	0.9	0	0.899
MAOB	CYP3A4	991745	986634	ENSP00000367309	ENSP00000337915	0	0	0	0	0	0	0.9	0.366	0.932
MAOB	menadione	991745	-100004055	ENSP00000367309	4055	0	0	0	0	0	0.699	0.7	0.8	0.98
MME	ANPEP	988880	982231	ENSP00000353679	ENSP00000300060	0	0	0	0	0	0	0	0.752	0.752
MME	linoleic acid	988880	-100003931	ENSP00000353679	3931	0	0	0	0	0	0	0	0.8	0.8
NR1H4	APOA1	984066	977122	ENSP00000315442	ENSP00000236850	0	0	0	0	0.085	0	0	0.896	0.898
NR1H4	APOC3	984066	976781	ENSP00000315442	ENSP00000227667	0	0	0	0	0.157	0	0	0.899	0.909
OAT	OTC	989773	975784	ENSP00000357838	ENSP00000039007	0.472	0	0	0	0.311	0	0.9	0.543	0.979
SLC2A2	SLC5A1	984947	979723	ENSP00000323568	ENSP00000266088	0	0	0	0	0	0	0.9	0.379	0.933
SLC3A1	SLC7A9	978602	975765	ENSP00000260649	ENSP00000023064	0	0	0	0	0	0	0.9	0.913	0.99

Appendix 10 Predicted chemical (Comparative Toxicogenomics Database hyperlinks)

Gene	compound 1	literature	compound 2	literature	compound 3	literature
CR2	Tretinoin	16443354	Vitamin E	23208437	Copper Sulfate	19549813
APOC3	Ginsenosides	16297877	Drugs, Chinese Herbal	16297877	Dietary Fats	18457598
SPP1	Calcitriol	17171500	Capsaicin	22150557	Galactosamine	19913045
NCK1	Tretinoin	15498508	epigallocatechin gallate	22079256	beta-methylcholine	21179406
NR1H4	Bile Acids and Salts	15725701	epigallocatechin gallate	22178739	alpha-Linolenic Acid	15307955
CYP3A4	Calcitriol	17270371	Curcumin	17270371	9-cis-retinal	16632523
CYP4F3	Tretinoin	17218384	apple polyphenol extract	16369997	Thapsigargin	19442820
userguide; click on the hyperlink of the gene to access the CTD database, and open the tab " chemical interactions".						
Sort and scroll in the list of compounds to the desired compound to access the related literature links.						
Relevant literature about the role of NR1H4 in the immunity of the intestine						
http://www.ncbi.nlm.nih.gov/pubmed/19864602						
http://www.ncbi.nlm.nih.gov/pubmed/21242261						
http://www.ncbi.nlm.nih.gov/pubmed/22993736						
http://www.ncbi.nlm.nih.gov/pubmed/?term=22285937						
http://www.ncbi.nlm.nih.gov/pubmed/?term=16230354						
http://www.jbc.org/content/280/9/7427.long						

Appendix 11 Detailed study information of the experiments that tested trans-generational epigenetic effects (maternal phase)

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Faty acids								
Alkyl-glycerols (AKG) extracted from marine organisms	240 sows and	Sows: d90 of gestation	Sows: until weaning	D90 to 5d before farrowing: 0.34 g AKG/sow/d D5 before to d5 after farrowing: 1.5 g AKG/sow/d. From d5 until weaning: 0.75 g AKG/sow/d.	Giving AKG to sows in late gestation and lactation can improve the passive immunity transfer to piglets	No effect on performance and specific immune parameters. Increased % of serum bactericidal activity and haemolytic complement (weaning and 70d of age)	No data regarding microbiota, gut morphology, and immuno histochemie.	[74]
Corn vs. coconut oil x low (9%) or standard (18%) crude protein	Femal wistar rats and progeny	During gestation from 4 d after mating onwards	Until birth	90 vs. 180 g/kg crude protein; 100 g corn oil or 50 g corn oil + 50 g coconut oil or 10 g corn oil + 90 g coconut oil/kg	Reduced lymphocyte proliferation at birth after prenatal exposure to a low protein diet, which could be overcome in postnatal rats if the prenatal diet included a large amount of coconut oil and a small amount of corn oil.	Lymphocyte proliferation in thyme and spleen; Spleen natural killer cell activity.	No data regarding microbiota, gut morphology, and immuno histochemie.	[67]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Fish oil vs. linseed oil	9 mice and 41 pups	Mating	Throughout gestation and lactation	Coconut fat (control) vs. fish oil vs. linseed oil (40 g/kg)	Maternal intake of fish oil changes the offspring's antigen-specific response.	Fish oil reduced levels of total OVA-specific antibodies and OVA-IgG1 titers in the pup blood. The decrease in IgG1 was paralleled by an increase in IFN gamma-production and a decrease in IL-6-production.	No data regarding microbiota, gut morphology, and immuno histochemie.	[61]
Fish oil vs. sunflower oil	270 Eggs and hatched chicken	8 wk before egg collection	Until egg collection	Fish oil (3.5%), sunflower oil (3.5) or 50/50 mixture	Modulating egg yolk n-3 FA enhances tissue n-3 FA and reduces pro-inflammatory cardiac eicosanoid production without affecting hatchability, and may alter leukotriene production in chicks, which could lead to less inflammatory-related disorders in poultry.	1) increase in the retention of n-3 PUFA in cell membranes, (2) reduction in plasma nonesterified fatty acids, (3) alteration in the expression of pro-inflammatory cyclooxygenase-2 protein, (4) reduced production of proinflammatory eicosanoids, (5) suppression of cell-mediated immunity. In addition, changes in the expression of several genes associated with lipid metabolism	n-3 Fa resulted in metabolic (23 up, 25 down), developmental (19 genes >2-fold), immunity and defence (3 up, 2 down, 10 genes >2-fold) and signalling processes (29 genes >2-fold).	[69-71]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Seaweed extract								
Laminarin/Fucoidan	20 sows 80 piglets	Sows: d107 of gestation	Sows: until weaning	Sows during gestation/lactation: 10 g/d. Weaned pigs: 2.8 g/kg seaweed extract.	Improved piglet performance and immune response.	Improved daily gain and pro-inflammatory IL-1a mRNA expression in the ileum of pigs 11 d after weaning. increased villus height.	No microbiota data.	[63]
Pre- and probiotics								
Sacchoromyces cerevisiae	Gestating and lactating sows, suckling and weaned piglets (n = 38)	Sows: d94 of gestation; Piglets: from birth onwards	Sows: until weaning; Piglets: until 2 wk after weaning.	Sows: 1 g/kg feed Suckling pigs: 3 x 1 g per wk Weaned pigs: 5 g/kg feed.	Improved performance; reduced diarrhea scores	Less shedding of ETEC bacteria in faeces; increased serum IgA-levels in piglets; Less piglet serum specific antibodies against *E. coli* O149:K88.	No data regarding microbiota, gut morphology, and immuno histochemie.	[60]
FOS	84 Women	26 wk of gestation onwards	Until 1 month after delivery	2 x 4 g/d	Higher expression of a set of genes	Especially up-regulation of *IL-27* in 1- month breast milk samples, which is associated with immuno-regulatory functions.	Future studies to the association with the onset of allergic disorders in children.	[62]
Bacillus cereus var. Toyoi	Gestating and lactating sows (n = 8), suckling and weaned piglets (n = 48)	d87 of pregnancy onwards; piglets from d14 onwards	Until 28d after weaning	Sows: 3.14 x 10^5 cfu/g Suckling pigs: 8.7 x 10^5 cfu/g Weaned pigs: 6.5 x 10^5 cfu/g	No effect on performance parameters; a positive impact on health status of the piglets after a challenge with Salmonella, likely due to an altered immune response	Lower fecal shedding of Salmonella; lower diarrhea scores; reduced frequencies of CD8+ gamma delta T cells in the peripheral blood and the jejunal epithelium.	No data regarding microbiota, gut morphology, and immuno histochemie.	[66]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Lactobacillus rhamnosus and *Bifidobacterium lactis*	256 pregnant women	The first trimester of pregnancy	Until max. 6 month after birth	10^{10} CFU/d	Increased adiponectin content in the colostrum.	Adiponectin has anti-inflammatory properties	No direct measurements of immune response.	[72]
Vitamins and Minerals								
Mix of vitamins and trace minerals	400 Broiler breeders (BB) 1200 broilers	BB: 18 wk of age Broilers: Day-old	BB: until 30 wk of age Broilers: 32 d of age; 50% of the broilers was challenged with a MAS infection	Mixture high versus low in vitamins/ trace minerals.	The immune system can be stimulated by addition of vitamins and trace minerals, without affecting the growth potential of the controls.	In the challenged broilers, increased nr. of leukocytes on d1, a faster recovery rate of intestinal lesions, crypts and villus atrophy were observed.	Increased expression of genes that are related to intestinal turnover, proliferation and development, metabolism and feed absorption.	[65, 73]
Selenium	20 female rats	2 wk prior to pregnancy	Until 10d of lactation	0.15 vs. 0.02 mg/kg selenium	Maternal selenium intake impacts neonatal selenium status and the neonatal immune system development.	Maternal plasma and milk selenium and corresponding neonatal plasma selenium; Thymocyte activation; % of CD8 cytotoxic T cells, CD2 T cells, panB cells, and natural killer cells	No data regarding microbiota, gut morphology, and immuno histochemie.	[68]
Zinc and β-carotene	170 pregnant women; 134 new-borns	Pregnant < 20 wk	Until birth	30 mg/d zinc; +/- 4.5 mg/d β-carotene	Addition of zinc to routine iron and folic acid supplements for pregnant women reduced diarrhoeal disease during the first 6 months of life, albeit at the expense of more episodes of cough.	Effect on cytokine production in the newborns. Zinc increased interleukin-6 and β-carotene reduced interferon-g production.	No data regarding microbiota, gut morphology, and immuno histochemie.	[64]

Appendix 12 Detailed study information regarding the effect of nutritional interventions on immune related parameters in pigs (neonatal phase)

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Amino acids								
Arginine	16 (per group)	Challenge at day 6	14 days	0.4 g/kg or 1.5 g/kg	Decreased intestinal permeability	CDK1/cyclin B, PKB/Akt, and AMPK		[75]
	6 in L-Arginine group	<3 days or 2 weeks old	3 hours	600 mg/kg/h	Less NEC (preterm piglets)	Intestinal damage	SISP model	[76]
	6 per group	Day 7	14 days	0.671, 0.871, and 1.071% L-arginine (on the basis of milk replacer powder)	Promotes growth performance	Growth performance, ammonia, amino acids (plasma)		[77]
Glutamine / Glutamate	6 pigs per group/age	Day 0	Intervention at each time-point specifically 0/2/7/14/21	5mM glucose or [U-^{14}C]glucose + 2mM glutamine	enhance intestinal function and growth performance	O2 consumption by pig enterocytes , glutamine 'products'	*in vitro*	[78]
Threonine	7 per group	Day 2	8 days	0.6 g threonine/kg/day fed intragastrically 0.1 g threonine/kg/day fed intragastrically 0.5 g/kg/day fed intravenously	Subtrate for mucin production (deficiency leads to less mucin)	Daily-weight gain, diarrhoea incidence, histology, mucin production	All subgroups are experimental (no 'normal' feeding	[79]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Vitamins/Minerals								
Zn (ZnO)	23 per group	Day 11	28 days	1) adequate Zn (100 ppm added) during the entire 28-d nursery period; 2) high Zn (3,000 ppm added to the basal diet) only during wk 1; 3) high Zn only during wk 2; 4) high Zn during wk 1 and 2; 5) high Zn during wk 2 and 3; and 6) high Zn for the entire nursery period	Both traditionally weaned (> 21 d) and early weaned (< 14 d) pigs grew faster when fed pharmacological (3,000 ppm) concentrations of zinc as zinc oxide during at least the 1st 2 wk after weaning.	Growth performance, blood minerals		[80]
vitamin E and selenium	6 per group	Day 3	11 days	1) FE, 1 mL containing 200 mg of Fe (iron dextran); 2) FEE, treatment FE plus 1 mL containing 300 IU of vitamin E (d-a tocopherol); 3) FESEE, 1.03 mL containing 200 mg of Fe (iron dextran), .15 mg of Se (sodium selenite), and 15 IU of vitamin E (d-a tocopherol)	one Fe injection (200 mg) for pigs from sows fed adequate vitamin E will result in adequate growth and hemoglobin concentration	Iron utilization	No direct impact on immune status	[81]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Fatty acids								
Medium-chain triglycerides	6 per group	Day 1-2	10 hours	0, 12, 24 ml MCT product (reconstituted coconut oil)	High dosage decreased milk intake, MCT utilized as fuel	Blood urea nitrogen, milk intake, glycogen levels tissue		[82]
Medium-chain fatty acids	15 per group	Day 0 is weaning	16 days	Control diet; soybean oil (15 g/kg), Alphacell (25 g/kg) and soy protein isolate (10 g/kg) *Cuphea* diet; seeds form *lanceolata* and *ignea* (50 g/kg) + 500 mg/kg microbial lipase.	greater villus height and a lesser crypt depth and greater villus/crypt ratio depth The intra-epithelial lymphocyte (IEL) counts per 100 enterocytes were significantly decreased	Performance (weight, FCR), luminal microbial ecology, pH, villi/crypts, IELs	Weaner piglets	[83]
Poly unsaturated fatty acids	6 per group	Day 1	Day 10-12	0, 0.5, or 5% ARA or 5% EPA diets (% is total fatty acids)	Elevated levels of LC-PUFA enhances acute recovery of ischemia-injured ileum	Transepithelial electrical resistance (TEER), Flux measurements of H3-mannitol and C14-inlulin, and Prostaglandin E2		[84]
	6 per group	Day 7	30+10 days (slaughtered at day 47)	'healthy' and malnourished - LC-PUFA (n3/n6)	Improve the repair of small intestine in previously malnourished piglets	DNA/Protein content + lipid composition, fatty acid composition, histology (villi)		[85]
Short chain fatty acids	6 per group		acute (4, 12, 24 h) and chronic (3 or 7 d)	36 mmol/L acetate, 15 mmol/L propionate and 9 mmol/L butyrate (SCFA)	SCFA butyrate increases epithelial surface area by enhancing proliferation and inhibiting apoptosis	Villus height, crypt cell proliferation, butyrate	All pigs received total parenteral nutrition	[86]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Prebiotics	Formula-fed (n=4) and Sow-reared (n=5)	Day 9 and 17	12 hours follow-up	human milk oligosaccharides (HMO), lacto-N-neotetraose, scFOS, PDX+GOS (2:1),	Composition gut microbiota influenced by piglet diet and age	Gas production, pH, microbiota changes, SCFA production		[87]
	8 piglets per group	Day 1	7 days	GOS (4 g/L) PDX (4 g/L), or GOS+PDX (2 g/L each)	All prebiotic supplemented groups had numerically higher Lactobacillus populations	Weight, intestinal morphology, inflammation markers, microbiota	Piglets received total enteral nutrition	[88]
	6 pigs (day 1/3) and 9-10 pigs at day 7	Day 2	Depending on examination	10 g/L short-chain fructooligosaccharides	Enhanced structure and function throughout the residual intestine	Morphology (Crypt-Villus), epithelial cell proliferation (PCNA), nutrient transport (ussing chamber)	Piglets received partial enteral nutrition	[89]
	13 pigs per treatment	Day 1	18 days	Polydextrose; 1.7, 4.3, 8.5, and 17.0 g/L or control	Increased (luminal) lactobacilli, lactic acid, and SCFA	Morphology, pH, digesta metabolits, microbiota, gene expression (TNFα, IL-8, IL-1β (C), and IL-10)		[90]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Probiotics	6 pigs (day 1/3) and 9-10 pigs at day 7	Day 2	Depending on examination	1 × 10^9 colony-forming units [CFU] *Lactobacillus rhamnosus* GG [LGG]	Enhanced structure and function throughout the residual intestine	Morphology (Crypt-Villus), epithelial cell proliferation (PCNA), nutrient transport (ussing chamber)	Piglets received partial enteral nutrition	[89]
	40 control and 68 intervention	Day 0	Oral gavage at 12h and day 1, challenge at day 2	5 mL of the porcine competitive exclusion (CE) culture. Challenge 1 X 10^8 cfu of EC 987	Effective against E. coli infections in neonatal piglets	ileal, colon, cecum, rectal swaps, lymph node (specific *E. coli* test)		[91]
	~606 control piglets and 534 intervention piglets	Day 0	24 days (weaning)	Day 0, 1, 2; 2.8 × 10^9 colony forming units (CFU) of *E. faecium* DSM 10663 NCIMB 10415 per g Day 4-onwards; 1.26 × 10^9 CFU *E. faecium* twice a day, in case of diarrhoea 2.9 (week 1) and 5.8 (≥ week 2) × 10^8 CFU of *E. faecium*	Reduces the portion of piglets suffering from diarrhoea	Diarrhoea patterns, performance (weight, daily gain)		[92]
Other								
Spray-dried animal plasma	36	Day 3	18 days	10% SDAP (AP920, American Protein Corporation, IA) or 10% autoclaved SDAP	upregulated antioxidant system in serum, stimulated intestinal development and improved health, leading to better growth performance, and decreased proinflammatory and anti-inflammatory cytokine responses	Growth performance, morphology, antioxidant capacity, Cytokines (IL-1β, TNF-α, IFN-γ, IL-6, TGF-β, IL-10, IL-4, IL-2, sIL-2R, NF-κB, and SIgA)		[93]

Appendix 13 Detailed study information regarding the effect of nutritional interventions on immune related parameters in poultry (neonatal phase)

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Amino acids								
Arginine	48 per group	Day 1	Day 1-18	control diet (100% of NRC) Arg (120% of NRC) Lys (120% of NRC) Arg+Lys (120% of NRC)	Increasing dietary Arg, but not Lys, from 100 to 120% of the NRC recommendation increased day 18 BW gain	Body weight gain, feed intake, feed conversion, and livability	minimal effects were noted in growth and immune system parameters	[94]
	30 per group	Day 1	Day 1-18	Control diet (CD); CD+plus L-Arg (0.20% Arg of diet); CD plus L-Lys HCl (0.20% Lys of diet); and CD plus L-Arg–L-Glu (0.10% Arg of diet)	treatment differences in growth responses, lymphoid organ development, and primary antibody titers to SRBC did not occur	Body weight gain, FCR, lymphoid organ weights, and antibody titers	minimal effects were noted in growth and immune system parameters	[94]
	~8 per group	Day 1	Day 1-15	Arg; 90, 100, 110, and 120% of NRC. Half of birds vaccinations of Newcastle disease virus and infectious bronchitis at day 1.	-vac birds fed an Arg-deficient diet had lower feed conversion compared to +vac birds Vac+ birds had lower day 15 BW than –vac birds, but higher titers to Newcastle disease virus	Growth, antibody responses, plasma amino acid concentrations	minimal effects were noted in growth and immune system parameters	[94]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Glutamine	60 per group (12 samples for measurements)	Day 1	Day 1-21 (samples on day 4, 7, 14, and 21)	1) control diet 2) control diet + 1% Gln 3) control diet + 1% Gln in drinking water, 4) control diet + 1% Gln and 1% Gln in drinking water 5) control diet + 4% Gln 6) control diet + 4% Gln in drinking water 7) control diet + 4% Gln and 4% Gln in drinking water	addition of 4% Gln to the diet or water depressed growth performance	BW gain, feed efficiency, morphology		[95]
	60 per group (12 samples for measurements)	Day 1	Day 1-21 (samples on day 7, 14, and 21)	1) control diet 2) control diet + 1% Gln for 4 days 3) control diet + 1% Gln for 7 days 4) control diet + 1% Gln for 14 days 5) control diet + 1% Gln for 21 days	Weight gain improved significantly in 1% Gln for 21 days. Higher concentrations of bile, intestinal, and sera IgA, sera IgG and longer intestinal villi	BW gain, feed efficiency, morphology		[95]
Threonine	45 per group	Day 1	0-42 days	dietary levels of L-threonine (0, 2.5, 5 and 7.5 g/kg) with or without *Saccharomyces cerevisiae* (SC)	Combination of threonine and SC improved growth performance and intestinal morphology traits in broilers.	antibody titre (Newcastle disease) and cell blood count	No effect immune markers	[96]
Methionine/Cysteine	?	Day 1	Sacrificed at day 18, 21, 24 (day 14 inoculation)	0.063% Met 0.25% Met 0.85% Met 1.45% Met 0.203% Cys 0.063% Met + 0.153% Cys	improvement in the cell mediated PHA-P responses as well as in the IgG (T-cell-dependent) responses	Total Ab, β-met-resistant Abs, IgG	Met more effective than Cys	[97]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Wheat/Soy	200 per group / 5 per group for histology	Day 1	Day 1-42 (4 time-points for histology)	Maize-soyabean Maize-soyabean+Zn-bacitracin (100g/kg) Wheat/rye–soyabean Wheat/rye–soyabean+ Zn-bacitracin (100g/kg)	wheat/rye-based diet compared with a maize-based diet: induces villus fusion, a thinner tunica muscularis, T-lymphocyte infiltration, more and larger goblet cells, more apoptosis of epithelial cells in the mucosa, shift in microbiota	Body weight, feed intake and FCR, morphology, microbiota, T-lymphocyte infiltration		[98]
Corn/Wheat	152 birds per treatment	Day 0	Day 0-39 (starter, d0-21; grower-finisher, d21-39)	effects of diet type (corn- or wheat-based), enzyme addition (none or multicarbohydrase enzyme supplement), pathogen challenge, and their interactions	enzyme supplementation minimized the growth suppression associated with the *C. perfringens* challenge, with the most pronounced effect observed in birds fed the wheat-based diet	BW, FCR, *C. perfringens* counts	oral challenge with C. perfringens (none or 10^8 cfu/bird on d 13	[99]
Soy								
Fatty acids								
Medium-chain triglycerides	150 per group	Day 1	Day 1-45 (Starter 1-21 and Grower 22-45)	control control + 4 g FOS per kg control + lactic acid, citric acid, ascorbic acid and propionic acid (1:1000L water) control + medium chain fatty acids (1:1000 starter, 1:2000 grower)	organic acids and MCA chicks had a better growth performance, better digestibility, less S. Enteritidis colonization and lower pH in the crop and intestines. FOS chicks tended to have decreased Salmonella colonization in ceca	Performance (weight), microbiota (*Salmonella* counts), pH, cecal SCFA		[100]
Poly unsaturated fatty acids	12 per group	Day 0	Day 0-25	control control + ZnB control + 2% n-3 PUFA (SALmate) control + 5% n-3 PUFA (SALmate)	n-3 PUFA (SALmate) supplementation altered the intestinal Lactobacillus species profiles, but did not alter the overall microbial communities or broiler performance	Performance (weight), microbiota		[101]
Short chain fatty acids	48 per group	Day 1	Day 1-35 Control starter (0-3 wk) and finisher (4-5 wk)	Control Furazolidone 0.2% butyrate 0.4% butyrate 0.6% butyrate	0.4% butyric acid was on par with antibiotic in maintaining body weight gain, and reducing E. coli numbers and found superior for feed conversion ratio	Performance (weight), pH, morphology, microbiota (*E. coli* counts)		[102]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Prebiotics	120 per group	Day 0	2 feeding phases; 1-21 and 21-42	Every 25kg portion, 1) 0.15 kg of corn starch + 1.5 kg H2O(T1, control); 2) diet T1 + 0.75 kg of GOS (T2); 3) 0.15 kg of corn starch + 3 kg of GOS (T3), GOS (β-galactosidase-treated lactose solution)	GOS selectively stimulated the fecal microflora of broiler chickens	Performance, microbiota	No in depth measurements (e.g. transcriptomics/ microbiota)	[103]
	18 per group	Day 7	21 days	Bio-MOS (mannan oloigosaccharide *S. cerevisiae*)	longer jejunal villi, greater specific activities maltase, leucine aminopeptidase and alkaline phosphatase. Minor improvement in body weight.	Growth, morphology, uptake of L-tryptophan	Neonatal to adult-like stage	[104]
	30 per group	Day 1	Challenge day 3	β-glucan feed + no SE, β-glucan feed + SE, control feed + SE	β-glucan feed decreases the incidence of SE organ invasion in neonatal chickens and up-regulating of the functional abilities of heterophils against an SE	Heterophils, % SE killing, oxidative burst		[105]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Probiotics	120 per group	Day 0	2 feeding phases; 1-21 and 21-42	Every 25kg portion, 4) 1.5 kg H_2O + 0.15 kg of bifidobacteria (T4); 5) 0.75 kg H_2O + 0.15 kg of bifidobacteria + 0.75 kg of GOS (T5); and 6) 0.15 kg of bifidobacteria + 3 kg of GOS (T6) GOS (β-galactosidase-treated lactose solution)/enterocoated *B. lactis* D300 pure culture (Abiasa Inc.)	GOS in combination with a *B. lactis*-based probiotic favoured intestinal growth of bifidobacteria in broiler chickens.	Performance, microbiota	No in depth measurements (e.g. transcriptomics/microbiota)	[103]
	48 per group	Day 1	41 days	0, 5, 10 mg (healthy matured) cecal cultures per L drink water and 0, 1.5, 3.0 g Fermacto (Aspergillus) per kg feed	1.5g/kg Fermacto increased performance of broilers	Performance (weight), feed intake and feed conversion	No in depth measurements (e.g. transcriptomics/microbiota)	[106]
				Probiotics healthy animals (*Lactobacilluls reuteri*, *L. salivarius*, *Enterococcus faecium*, *Bifidobacterium animals* and *Pediococcus acidilactici*				
Vitamin E + Se	?	?	?	1) Basal diet, without vitamin E or selenium (Se) 2) basal diet supplemented with vitamin E (100 IU/kg) and Se (0.2 ppm)	Depletion affects both the maturation of specific lymphocyte subpopulations and the functional and proliferative capabilities of the peripheral lymphocytes	CT-1a, CD3, CD4, CD8, sIgs, and Ia	Deficiency/depletion	[107]
Essential oils (thyme, cinnamon)	60 per group	Day 1	2 feeding phases; 1-28 and 29-42	1) control group (no essential oil[EO]) 2) 100 ppm EO - thyme 3) 200 ppm EO - thyme 4) 100 ppm EO - cinnamon 5) 200 ppm EO - cinnamon	EO could be considered as a potential natural growth promoter for poultry at the level of 200 ppm	Growth performance, blood constituents, carcass characteristics		[108]

Appendix 14 Detailed study information of interventions that were tested in the post-neonatal phase

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Amino acids								
Glutamine	24 calves	35 d.	14 d.	0, 8, 16 and 32 g/d.	Improved immune response and increased autophagy of liver cells.	Gln increased the abundance of CD4+, monocyte and the ratio of CD4+/CD8+, villus height and crypt depth of intestine. The autophagy level of liver cells was improved with increasing Gln infusion doses.	No transcriptomics data	[123]
Plants and herbs Chitosan	180 weaned pigs	28 d.	28 d. (0-14 and 14-28 d)	0, 100, 500, 1000, and 2000 mg/kg	Improved humoral and cellular immune response; dose dependent effects.	Positive effects on IgG and ovalbumin IgG, secretory IgA, IL-1 and IL-2, decreased effects of CD4 and CD8.	No transcriptomics data	[125]
Capsicum oleoresin, garlic botanical, and turmeric oleoresin	64 weaned pigs	21 d. Halve of the pigs were challenged with PRRS virus	28 d.	10 mg/kg	These plant extract improved the immune response of PRRSV infected pigs.	Feeding GAR increased B cells and CD8+ T cells of PRRSV-infected pigs The PRRSV challenge increased serum viral load, TNF-α, and IL-1β But feeding plant extracts reversed ($P < 0.05$) this increase.	No transcriptomics data	[126]
Rice bran	SPF-mice	4-6 wk of age	28 d.	10% rice bran	Modulation of mucosal immunity against enteric infections and induction of beneficial gut bacteria	IgA in B lymphocytes in the Peyer's patches, Dendritic cells in lamina propria, serum IgA, Faecal Lactobacilli excretion.	No transcriptomics data	[116]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Quercetin	240 laying hens	28 wk	8 wk	0, 0.2, 0.4 and 0.6 g/kg quercetin	Max. rate of lay and feed efficiency at 0.4 g/kg. Reduced nr. of aerobes and coliforms and increased number of *Bifidobacteria*. Increased antioxidant states measured in the liver.	Performance, microbiota, and antioxidant status.	No transcriptomics data	[117]
Alfalfa	128 Shaoxing growing egg-type ducks	7 wk	8 wk	0, 3, 6 and 9% of alfalfa meal	Increased intestinal microbial community and immune response.	Molecular analysis of cecal and faecal DNA extract. Proliferation of T and B lymphocytes		[118]
Black cumin (*Nigella sativ L.)*	100 Laying hens	49 wk	10 wk	0, 1, 2, and 3% of black cumin	Max. performance with at least 2% inclusion level.	Improved villus height and reduced crypt depth, increased numbers of globet cells and lamina propria lymphatic follicles.		[124]
Prebiotics								
Resistant starch (RS)	10 cannulated male pigs	17 wk of age	14 d.	Digestible vs. resistant starch	A diet high in RS provoked major changes in colonic gene expression, which represent induction of oxidative metabolic pathways and suppression of immune response and cell division pathways. RS also favoured the growth of microbes that produces organic acids and inhibited a range of potentially pathogenic microbial groups.	Colonic transcriptome profiles, oxidative metabolic pathways, such as the tricarboxylic acid cycle and b-oxidation, the nuclear receptor peroxisome proliferator-activated receptor gamma,		[128]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Probiotics								
Bacillus subtilis fermentation biomass	240 weaned pigs	21 d.	28 d. 2-phase feeding (0-14 and 14-28 d)	0, 1.5, 3.0, and 4.5 g/kg BS fermentation biomass	Positive effects on performance and nutrient digestibility. Increased serum IgG and IgA concentrations, decreased cecal Clostridium and Coliforms, greater villus height.	Performance, digestibility, serum IgG and IgA, *Clostridium* spp., coliforms, villus height,	No transcriptomics data	[129]
Lactobacillus delbrueckii subsp. Bulgaricus	Elderly humans (n = 61)	> 65 years of age	6 month	3 capsules/d 3×10^7 *L. delbrueckii subsp. Bulgaricus* per capsule.	Improved immune response, mainly by slowing the aging of the T cell subpopulations and increasing the number of immature T cells.	More NK cells, an improvement in the immune risk profile (IRP), an increase in the T-cell subsets, less pro-inflammatory cytokine IL-8, increased antimicrobial peptide hBD-2.	No data regarding microbiota, gut morphology, and immuno histochemie.	[127]
Anti-microbial proteins								
Antimicrobial peptide-A3 and P5	60 weaned pigs	21 d.	28 d. 2-phase feeding (0-14 and 14-28 d)	1) Neg. control 2) Pos. control (150 mg/kg Avilamycine) 3) 60 mg/kg AMP-A3 4) 60 mg/kg AMP-P5	AMP-A3 and P5 have potential to improve performance, nutrient digestibility, intestinal morphology and to reduce pathogenic bacteria in weanling pigs.	Improved ADG, G:F, Digestibility of GE, CP and DM. Fewer fecal Cl. spp. and coliforms, anaerobic bacteria. Greater villus height and ratio villus height : crypt depth.	No transcriptomics data	[130]
Buforin II	3 * 5 E. coli challenged weaned pigs	21 d.	21 d.	1) Control 2) 2 * 5 ml Buforin/d 3) 2 * 5 ml 5 colistin sulphate.	Buforin II protects intestinal mucosal membrane integrity and can reduce hemolytic E.coli concentrations in the intestines of piglets.	Improved piglet performance, tight junction proteins (e.g. calprotectin) and expression of protective factors, (e.g. HGF, hepatocyte growth factor; Reg-3 gamma, regeneration protein gamma; TFF-3, trefoil factor-3; TGF-beta1, transforming growth factor beta 1; VEGF).	Immuno histochemical data available.	[121]

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Lysozyme	186 male broilers	d14	28 days	40 mg/kg lysozyme; +/- challenged with *C. perfringens*	A decreased C. perfringens colonization and an improved intestinal barrier function and growth performance of chickens	Lesion scores, Lysozyme activity in duodenum, bacterial translocation to the spleen, daily gain and FCR.	No transcriptomics data	[122]
Vitamin C + E	174, 89 treatment, 85 placebo	Age groups; 2–5, 6–11, or 12–35 months old	5 days	Twice a day: 200 mg α-tocopherol and ascorbic acid 100 mg or placebo	None meausered	Time taken to recover from a very ill status, fever, tachypnoea, and feeding difficulty; and improvement in oxidative stress and immune response indicated by thiobarbituric acid reacting substances (TBARS) and response to skin antigens.		[109]
LC-PUFA	46 (Formula; n=14) or treatment (n= 16,) or exclusively human milk; n=16).	Age 7-14 days	28 days	Formula or Formula supplemented with the LCP 20 : 4n-6 and 22 : 6n-3 + exclusively fed human milk	Early diet influences both the presence of specific cell types and function of infant blood immune cells	formula fed infants had a higher percentage of CD3+, CD4+CD28+, and lower percentage of CD14+ cells and produced more TNFα and INFγ		[110]
	46 (Breast fed, n=17; Formula, n=14;Formula+LCP, n=15.	Age 26-39 days	28 days	Formula F contained only traces of LCP. Formula LCP-F contained 0.5 g LA metabolites/100 g fat and 0.8 g ALA metabolites/100 g fat derived from egg lipid extracts, black currant seed oil and low EPA fish oil	Plasma Long-chain polyunsaturated fatty acids (LCP) levels similar to those of breast fed infants can be achieved with the LCP supplemented formula used in this trial, without evidence of adverse effects of the LCP enrichment	Urinary malondialdehyde (MDA), marker for oxidative stress, excretion was significantly higher in formula fed infants. α-tocopherol similar between groups, tendency for lower values in formula (group F) fed infants		[111]

Appendix 15 Detailed study information of interventions that were tested in neonatal humans

Nutritional factors	n	Age (start experiment)	Duration of intervention	Dosage	Effect	Marker	Note	Ref.
Prebiotics								
scGOS/lcFOS	134 term infants (placebo68 and intervention group 66 infants)	formula feeding within the first 2 weeks of life	2 years	8 g/L scGOS/lcFOS or placebo (8 g/L maltodextrin)	Infants in the intervention group had significantly lower incidence of allergic manifestations	Infants in the intervention group had lower cumulative incidences for atopic dermatitis, recurrent wheezing, and allergic urticaria, also fewer episodes of physician-diagnosed overall and upper respiratory tract infections, fever episodes, and fewer antibiotic prescriptions were observed	No molecular immune marker	[112]
Oligosaccharides	90 term infants	Day 6 or 7	27 days	Placebo (maldextrin), 0.4 g/L, 0.8 g/L	Dosage-dependent effect on the growth of *Bifidobacteria* and *Lactobacilli* as well as softer stool	Bifidobacteria/Lactobacilli	Enteral nutrition was started with breast-feeding for all infants	[113]
Nucleotide	325 (formula innate NT,n=102; formula fortified with NT (n=98); Breast fed, n=125			control group, NT at 10 mg/l and treatment group, NT at 33.5 mg/l	Cow's milk-based formula with NT resulted in a modest improvement in some antibody responses in a population of healthy term infants, with no effect on other markers of immune status and growth.	T cells, B cells, NK cells, CD4+, CD8+, CD4:CD8, CD45RA+, CD45RO+, CD25+ Antibody responses to tetanus toxoid		[114]
Nutritional factors	**n**	**Age (start**	**Duration of**	**Dosage**	**Effect**	**Marker**	**Note**	**Ref.**

		experiment)	**intervention**					
Probiotics								
Bifidobacterium and *Streptococcus*	55 (control, n=26; treatment, n=29)	Age 5-24 months	Different, but in total 4447 patient-days recorded	control was formula only, in the treatment group the following was added control was formula only, in the treatment *B. bifidum* (1.9x10^8 colony-forming units [CFU]/g powdered formula: 35.8x10^8 CFU/100 kcal) and *S. thermophilus* (0.14x10^8 CFU/g: 2.69x10^8 CFU/100 kcal).	The supplementation of infant formula with *B. bifidum* and *S. thermophilus* can reduce the incidence of acute diarrhoea and rotavirus shedding in infants admitted to hospital	diarrhoeal disease rotavirus shedding		[115]

Appendix 15 A brief introduction to the immune system

1.The innate immune system

The immune system protects against invading pathogens. It is divided the "innate" and the "adaptive" or "acquired" immune system. The innate (also called "natural" or "native" immune system) forms the first line of defence that is already present before its first encounter with a pathogen. It consists of anatomical and (bio)chemical barriers, humoral (soluble) components and cellular components.

1.1 Anatomical and (bio)chemical barriers

The skin forms a strong physical barrier that is hard to break for microbes. Microbes that do attach are usually removed by "flaking" of old skin epithelium. The internal body epithelia, like that of the respiratory and gastrointestinal tract are covered by a mucus layer that is also difficult to penetrate. Microbes trapped in the mucus will be removed with the mucus from the body by the action of the cilia and due to peristalsis.

The anatomical barriers are covered with antimicrobial (bio)chemical factors, lysozyme on the skin and in tears, gastric acid in the stomach and anti-bacterial proteins (defensins) in the mucosa. Commensal bacteria in the gut and on the skin compete with potential pathogens for space and nutrients.

1.2 Humoral components of the innate immune system

The anatomical en (bio)chemical barriers are a passive part of the innate immune system, but the innate immune system is also able to recognize Microbial Associated Molecular Patterns (PAMPs) and will respond to these. These PAMPs consist of microbial structures like lipopolysaccharide (LPS), peptidoglycan, bacterial DNA (CpG motifs) and double stranded RNA, that are conserved between many different microbes. Humoral (=soluble) components of the innate immune system that can recognize PAMPs are the complement system and acute phase proteins (like C-reactive protein). Recognition of bacteria by these systems results in an amplification reaction that can either kill the microbe directly by forming holes in the bacterial membrane or promotes it destruction by recruitment of immune cells (inflammation) and "tagging" the microbe for killing by the recruited phagocytic immune cells.

1.3 Cells of the innate immune system

The cellular part of the innate immune system consists of the granulocytes (neutrophils, mast cells, basophils and eosinophils), the phagocytic antigen presenting cells (macrophages and dendritic cells) and the natural killer (NK) cells.

Mast cells are a type of innate immune cell that reside in connective tissue and in the mucous membranes. When activated directly by certain microbial products or products of the activated complement system, mast cells rapidly release histamine and tumor necrosis factor alpha (TNFa), that will cause recruitment of immune cells (like granulocytes and macrophages) into the tissue a process known as "inflammation". Basophils have a very similar function as mast cells, but are normally present in the blood. They can be recruited from the blood to sites of infection to contribute to the recruitment of other immune cell, e.g. neutrophils. Neutrophils are the most abundant type of phagocyte, normally representing 50 to 60% of the total circulating leukocytes (= white blood cells) whose primary function is to phagocytose and destroy bacteria and single cell fungi at the site of infection.

Natural killer cells, or NK cells, are a component of the innate immune system that destroys virus-infected cells, thereby limiting the viral replication.

Macrophages are large phagocytic cells present in the tissues that are differentiated from monocytes in the blood. They can phagocytose and kill substantial numbers of bacteria or other cells or microbes. Upon recognition of microbes (PAMPs) they can produce cytokines like TNFa, IL-1, -6, and -8 that promote inflammation, to clear the infection.

Dendritic cells (DC) are phagocytic cells present in tissues that are in contact with the external environment, mainly the skin (where they are often called Langerhans cells), and the inner mucosal lining of the nose, lungs, stomach, and intestines. Dendritic cells are very important in the process of antigen presentation, and serve as a link between the innate and adaptive immune systems. With their many Pattern Recognition receptors (PRR), like Toll like receptors, that can bind to PAMPs they can sense microbial danger. They are the most important cell in the immune system for orchestrating the right kind of immune response for a particular microbe. Depending on the microbial danger that is sensed, they will secrete a cocktail of cytokines that will activate the appropriate immune cells. They can activate antigen specific T cells of the adaptive immune system.

Gamma delta T cells (γδ T cells), represent a small subset of T cells in the blood of humans but are present in relatively larger numbers in the blood of cows and pigs. They consists of several subtypes and can perform many of the functions that conventional αβ (alpha beta) T cells can also perform, including killing of infected or stressed cells and secretion of many immune modulating cytokines. Gamma delta T cells exhibit characteristics that place them at the border between innate and adaptive immunity. On one hand, γδ T cells may be considered a component of adaptive immunity in that they rearrange T cell receptor (TCR) genes to produce diversity and because they can develop to a certain extend a memory phenotype. However, the various subsets may also be considered part of the innate immune system where a restricted set of TCR receptors may be used as a pattern recognition receptor (PRR) able to recognize conserved microbial structures or other signs of danger. For example, according to this paradigm, large numbers of Vγ9/Vδ2 T cells respond within hours to common molecules produced by microbes, and highly restricted intraepithelial Vδ1 T cells will respond to stressed epithelial cells. Gamma delta T cells are more abundantly present in the mucosa where they will be one of the first cells to encounter invading pathogens. Still many questions about these cells remain to be resolved.

2. The adaptive immune system

In contrast to the innate immune system the adaptive immune system is able to very specifically recognize pathogens. A second important difference with the innate immune system is that it has "memory" so that in future encounters with the same pathogen it can mount a much faster and stronger immune response compared with the first encounter. The cells of the adaptive immune system are T cells and B cells. The both contain antigen specific receptors on their surface that are generated by a process called "gene rearrangement". This process ensures that many different B and T cells are generated of which each cell has one kind of B or T cell receptor with its own unique antigen binding site. During an infection, only B or T cells that can bind with their receptor to the invading pathogen will be selected and will start to proliferate. The process of selection and proliferation takes approximately a week till enough antigen specific B and T cells are generated to mount an effective adaptive immune response. The T cells consist of cytotoxic T cells (CTLs) that can kill virus infected cells and T-helper (T_h) cells that are involved in "helping" the appropriate effector cells. T-helper-1 (Th1) cells will e.g. help macrophages to become more effective in killing phagocytosed microbes and will promote the development of CTLs, while T_{h17} cells promote the recruitment of neutrophils to the site of infection. B cells often also require the help of T_{h2} or T_{h1} cells to become fully activated and finally develop into antibody secreting plasma cells. The antibodies form the humoral part of the adaptive immune system.

2.1 Antibodies

In humans 5 antibody isotypes are produced: IgA, IgD, IgE, IgG and IgM. They all consist of 2 light and 2 heavy chains that together form a Y-shaped protein (T-shaped in the case of IgA). The variable part of the heavy and light chains form the antigen binding sites (2 identical ones for each monomeric antibody), while the constant part of the heavy chains form the Fc part of the antibody that determines what will happen with the antigen/microbe bound by the antibody. Depending on the antibody isotype this could e.g. be activation of complement or binding to Fc receptors on phagocytic cells to enhance uptake of the antigen/microbe by these cells. IgG is the principle isotype in serum while IgA is the principle antibody in the gastro intestinal tract and of other mucosal surfaces including the upper respiratory tract.

The mucosal immune system

Of all antibody producing cells in the body 70 to 80% are physically located within the tissues of the mucosal immune system. The majority of these are IgA producing B cells.

Mechanistically, the induction process can be divided into the following steps:

• Antigens entering the digestive tract are taken up by specialized mucosal cells called M cells. M cells internalize the antigen and transport it across the epithelium where antigen can be taken up by APCs such as dendritic cells (DC). "M" cells are formed in mucosal epithelium in response to signals from lymphocytes.

• Antigen can be taken up by DC that have dendrites extending through the epithelial tight junction into the lumen (drawing on right). Antigens are then presented to lymphocytes (in the intestine, these are located in Peyer's patches).

• Lymphocytes (both B and T cells) leave the mucosal site and travel to the mesenteric lymph nodes, then into the lymph.

• Via the thoracic duct, the lymphocytes exit the lymph and enter the circulation.

• Circulating lymphocytes "home" to positions within the mucosal lamina propria throughout the body, including sites distant from the original antigenic encounter. The homing of lymphocytes to mucosal sites involves specific interactions of both adhesion molecules and chemokines.

• B Lymphocytes within the peripheral tissues proliferate and differentiate into IgA secreting plasma cells at effector sites.

C. Features of Mucosal Immunity

1. The administration of antigen at one mucosal site results in specific antibody production at distant mucosal sites (the common mucosal immune system). Some regional preference seems to occur, however. For example, induction via NALT (nasal associated lymphoid tissue) leads to a more robust response in the respiratory sites than in induction via mucosal associated lymphoid tissue (MALT) in the intestine.

2. B cells in the mucosa are selectively induced to produce dimeric IgA rather than other isotypes. The selective switch of B cells to IgA is believed to be mediated by specific cytokines produced by T cells in the inductive sites.

3. A distinct population of lymphocytes, mostly CD8+ T cells are found in the gut epithelium. Many of these intraepithelial lymphocytes are γδ T cells that have an innate character. They can recognize microbial structures with their γδ T cell receptor and respond to invading microbes.

4. Conventional T cells, particularly CTLs, are also an important component of the mucosal immune response. The induction and homing requirements for these cells are not as well described as those for mucosal B cells.

5. Induction of a response via a mucosal site generally elicits a systemic immune response as well, such that serum antibodies can be detected. This indicates that a mucosal encounter with antigen generates subsets of T and B cells that home to mucosal sites and also to spleen and regional nodes.

6. Regulatory T cells are a prominent feature at mucosal sites, and may synergize with suppressive dendritic cells. Regulatory populations have been isolated from draining lymph nodes of mucosal sites.

Wageningen UR Livestock Research
P.O. Box 338
6700 AH Wageningen
The Netherlands
T +31 (0)317 48 39 53
info.livestockresearch@wur.nl
www.wageningenUR.nl/en/livestockresearch

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To explore the potential of nature to improve the quality of life

Wageningen UR Livestock Research
P.O. Box 338
6700 AH Wageningen
The Netherlands
T +31 (0)317 480 10 77
E info.livestockresearch@wur.nl
www.wageningenUR.nl/livestockresearch

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