Organic, More Healthy?

A search for biomarkers of potential health effects induced by organic products, investigated in a chicken model

Machteld Huber M.D.
overall editor

LOUIS BOLK INSTITUUT

For more information

Louis Bolk Instituut
Hoofdstraat 24
NL 3972 LA Driebergen, the Netherlands
Phone: +31 343 523860 Fax +31 343 515611
E-mail: info@louisbolk.nl or www.louisbolk.nl

Colofon

2007 Louis Bolk Instituut, publication number M22
or FQH-publication number FQH05

Organic, More Healthy? A search for biomarkers of potential health effects induced by organic products, investigated in a chicken model.
Machteld Huber M.D. overall editor
Second edition 2007
ISBN 978-90-74021-56-2 Price € 35,- without postage
Order through: publicaties@louisbolk.nl

Photography: Anna de Weerd

Keywords: feeding experiment, organic products, organic feed, organic food, organic diet, conventional diet, wheat, soy, barley, triticale, peas, maize, nutrients, biophotons, biocrystallization, microbiology, chicken model, Wageningen Selection lines, biomarkers, immunological parameters, metabolomics, genomics, pathological anatomy, health, robustness, resilience.

Project Consortium

Louis Bolk Institute – Projectleader

Wageningen UR – Animal Sciences Group

TNO – Quality of life

RIKILT – Institute of Food Safety

In cooperation with:

Muvara BV – Statistics

Institute of Risk Assessment (IRAS) – University Utrecht

Wageningen UR – ASG – Lelystad

Wageningen UR – Biological Farming Systems Group

Centrum voor Smaakonderzoek (CSO)

University of Kassel – Department of Food Quality and Food Culture

KWALIS Qualitätsforschung Fulda GmbH

Biodynamic Research Association Denmark

and others

Consortium contact information

Louis Bolk Institute – Drs. Machteld Huber, M.D. , scientific project leader, m.huber@louisbolk.nl,
Hoofdstraat 24, NL-3972 LA Driebergen.

Wageningen UR – Animal Sciences Group – Prof. dr.ir. Huub Savelkoul, Huub.Savelkoul@wur.nl,
Postbus 338, NL-6700 AH Wageningen.

TNO Quality of life – Dr. Leon Coulier, leon.coulier@tno.nl, Postbus 360, NL-3700 AJ Zeist.

RIKILT – Institute of Food Safety – Dr. Ron Hoogenboom, financial project leader, Ron.Hoogenboom@wur.nl,
Postbus 230, 6700 AE Wageningen.

Acknowledgements

An extensive project like this, can only be realized with the support of many people.
We want to acknowledge following people who contributed in very different ways:
The FQH Concept group who helped to design this project: Dr. Kirsten Brandt (DARCOF & University of Newcastle), Dr. Johannes Kahl (University of Kassel), Dr. Franco Weibel and Dr. Gabriella Wyss (FiBL), Dr. Alberta Velimirov (Ludwig Boltzmann Institut), Dr. Ron Hoogenboom (RIKILT), Dr. Len Roza (TNO), Drs. Ruth Adriaansen-Tennekes (Louis Bolk Institute), as well as Prof. dr. ir. Huub Savelkoul from Wageningen UR, Prof. dr. Jan van der Greef (TNO) and Dr. Dré Nierop (Muvara) who, outside this Concept group, helped to design.
The colleagues from LBI and from colleague institutes abroad, who helped to find the appropriate ingredients, as well as the farmers who were willing to supply us the ingredients.
The people who helped to prepare the ingredients, from Wageningen UR mr. Tamme Zandstra, who toasted portions of soy many times, and from Allkraft Ölmühle GmbH mr. Manfred Klingen who was willing to grind the soy beans in his special small machine, during his spare time.
The people from Kruyt Veevoeders, and especially mr. Laus de Vos, who shared his knowledge and was willing, time after time, to weigh all necessary kilograms and grams of ingredients, mix them, store them, mix them again, and code the many batches of feed we used. This all moistened by lots of good coffee!
Dr. Bart van der Elst, who guarded the codes of the feed.
All people from the many labs who analysed aspects of our feeds and ingredients, the labs from project partners RIKILT and TNO, IRAS, Master Lab, but also from Germany, KWALIS and University of Kassel and Denmark, BRAD.
The staff of mr. Ries Verkerk of Animal Experimental Facility De Haar, Wageningen UR, who took excellent care of our chicken, and especially mrs. Marleen Scheer, who was the special care taker of the chicken and who did build the beautiful “chicken-Hilton” for the second generation of animals.
The people from the laboratory of Wageningen UR who gave so much support during the many blood samplings of the chicken, as well as with the huge amount of analyses that were performed afterwards. Of these to be named especially mrs. Ger de Vries Reilingh, mr. Mike Nieuwland and mr. Adrie Groeneveld.
The veterinarians Franciska Velkers and later Sible Westendorp, who gave advice and evaluated the health of our chicken, and contributed during the section. As well as all other people who helped with the section, among whom butcher Ton Molenaar.
All other labs who analysed samples from our animals, being also the TNO-lab, the PA-lab from RIKILT, ASG-Lelystad, CSO-Centrum voor Smaakonderzoek and Prof. Ariena van Bruggen and her staff, who out of sheer enthousiasm took part in the investigations.
All people who helped us with their specialist knowledge to make choices and to support the interpretations, like from Wageningen UR Dr. René Kwakkel and from Wageningen UR & Leuven University Prof. dr. Eddy Decuypere.
As well as the members of our Advisory Board who were willing to give input of time and critical feed-back:
Prof. dr.ir. I. Rietjens from Wageningen UR, Dr. H. van Loveren from RIVM, Dr. R. Pieters from University of Utrecht, Dr. L. Jansen from the Netherlands Nutrition Centre / Schuttelaar & Partners and Dr. J. Meijs from Biologica (chair) and the representatives of the stakeholders Dr. R. Theelen for ministry of LNV, mrs. Ir. A. Habraken for Rabobank, mr.T. van den Bergh for Triodos Bank.

Photographer mrs. Anna de Weerd who made a photo-diary about the animals and workers in the project.
To all project partners, Ron Hoogenboom, as good sparring project leader-partner, Ruth Adriaansen-Tennekes, Henk Parmentier, Huub Savelkoul, Leon Coulier, Elwin Verheij, Jan van der Greef, and later Lucy van de Vijver, Andreas Freidig, Ben van Ommen and Suzan Wopereis, who were good colleagues to work with during these two and a half years: Thank you! A special thank you to project partner Dré Nierop, who was always a sound and supportive sparring-partner!
Ir. Anneke de Vries, of LBI, who during the whole period excellently supported and assisted the project, who organized, , telephoned, searched the internet , wrote, drove soybeans around, etc, etc, etc.
Dr.ir. Lucy van de Vijver, who contributed very valuable, in editing the part on the feeds in the final document.
In the final stage Ir. Alette Visser who had, in the final stage, the patience to lay-out the document till the very end.
And the Scientific Advisory Committee of FQH who took the effort to review the manuscript in the final stage and gave a positive advice for making it an FQH-publication.
Still many other people supported, who are not mentioned personally here. They are also very much thanked!

Last but not least: I thank our stakeholders, the ministry of LNV and the ministry of EZ, Rabobank and Triodos Bank, without whom this project would have not taken place. Thank you for your vision and trust!

Machteld Huber
Louis Bolk Instituut
December 2007

Abbreviations and explanations

Complement (system)	Enzymatic cascade of first defence system enzymes (innate IS)
Complement – alternative pathway	Complement, no antibodies included
Complement – classical pathway	Complement, antibodies included
Con A	Concanavalin A
DOK	Biodynamic (D), Organic (O) and Conventional (K) – trial FiBL
Elisa	Enzyme-linked immuno sorbent assay
IB MA 5	Infectious Bronchitis – chicken vaccination
IS	Immune System
Gumboro	Gumboro – chicken vaccination
KLH	Keyhole Limpet Hemocyanin – protein from moluscs
LPS	Lipopolysaccharide – part of cell wal of gram negative bacteria
LST	Lymphocyte Stimulation Test
LTA	Lipoteichoic acid – part of cell wall of gram positive bacteria
Marek Rispens HVT	Mareks Disease – chicken vaccination
MRL	Maximum Residue Limit
NAbs	Natural Antibodies
NCD	Newcastle disease – chicken vaccination
NO	Nitric Oxide
Paracox	Coccidiosis - vaccination
PDww	PoxDiphteria wing web – chicken vaccination
SI	Stimulation Index = Stimulated Counts / Control counts
SRBC	Sheep red blood cells
GI	Gastro-Intestinal tract

Contributions by the following Authors:

Overall editing: Drs. Machteld Huber M.D., Louis Bolk Institute (LBI)

Summary/Samenvatting: First draft by Machteld Huber, then adaptations included from Project group

1.Introduction: Machteld Huber

2. and 3. Material & methods and Results - Feed

Edited by Dr. Lucy van de Vijver (LBI)

Feed ingredients, Sources and selection procedures, composition and production: LBI – Machteld Huber, Ir. Anneke de Vries (LBI)

Mycotoxins, Pesticide residues, Dioxins, ANF's and Profiling of ingredients: RIKILT – Dr. Ron Hoogenboom

Macro and micro nutrients, Microbiology: TNO – Dr. Leon Coulier

LPS endotoxin residues: IRAS – Dr. Gert Doekes

Delayed Luminisence, Biophotons and Protein ratio: KWALIS – Dr. Jurgen Strübe and Dr. Peter Stolz

Biocrystallization: Louis Bolk Institute – ing. Paul Doesburg, with contributions from Uni Kassel – Dr. Johannes Kahl and BRAD – Dr. Jens-Otto Andersen

Overview of feed analysis: Louis Bolk Institute – Lucy van de Vijver, based on contributions from the research groups.

4. and 5. Material & methods and Results - Chicken experiment

Edited by Machteld Huber, M.D. (LBI)

General data on the chicken, lines, generations, feeding experiment, housing, blood and tissue sampling, weight etc.: LBI – Machteld Huber, Wageningen UR – Marleen Scheer

Immunological assays: analyses and interpretation – Drs. Ruth Adriaansen – Tennekes (LBI/WU),

Dr. Henk Parmentier, Prof. dr.ir. Huub Savelkoul, statistics: Muvara – Dr. Dré Nierop

Metabolomics: TNO-analysis – Dr. Leon Coulier, Dr. Elwin Verheij; interpretation TNO – Dr. Andreas Freidig; statistics – Muvara

Genomics: analysis, statistics: ASG–Wageningen UR – Dr. Annemarie Rebel, Dr. Astrid de Greeff

Pathological Anatomy: analysis and interpretation RIKILT – Dr. Maria Groot, Prof. dr. Erik Gruys, statistics – Muvara

Microbiology gut: analysis, statistics and interpretation Wageningen UR – Prof. dr. Ariena van Bruggen

Sensory Analyses: analyses, statistics CSO – Dr. ir. Bob Cramwinkel

Statistics: Muvara – Dr. Dré Nierop

Overview of chicken results: Louis Bolk Institute – Machteld Huber, based on contributions from the research groups.

6. Summary of feed and chicken analyses and reflection on research questions

Differences in ingredients used for the chicken feed and in the chicken feeds: LBI – Lucy van de Vijver, based on contributions from the research groups.

Differences in effects in the chicken: LBI – Machteld Huber, LBI, based on contributions of the research groups.

7. Discussion

How representative were these feeds? LBI – Lucy van de Vijver

Reflections on the design of the experiment in relation to the choice of crops: LBI – Drs. Geert-Jan van der Burgt

Comparison with literature concerning the ingredients. LBI – Lucy van de Vijver

Comparison with literature concerning the animal experiments: LBI – Machteld Huber

An attempt to integration of the feed and animal results: Wageningen UR – Huub Savelkoul, TNO – Andreas Freiding,

LBI – Machteld Huber, Lucy van de Vijver

Reflections on the working hypothesis of the project: LBI – Machteld Huber

8. Conclusions first draft by Machteld Huber, then adaptations included from Project group

9. Recommendations first draft Machteld Huber, and like all forgoing texts, with feed-back from Project group and Advisory Board

10. Final reflections after decoding confirmed minutes of the meeting

Content

Samenvatting

Het doel van het project 'Biologisch Gezonder?' was het zoeken naar 'biomarkers' die mogelijke gezondheidseffecten van biologische voeding zichtbaar maken. Een belangrijke reden om biologische producten te kopen is voor veel mensen de veronderstelling dat biologisch geteelde producten gezonder zijn dan gangbaar geteelde producten. Indien gezondheidseffecten kunnen worden bevestigd, zal dit de consument motiveren om biologisch geteelde producten te kopen en dat zou de markt van biologische producten kunnen stimuleren. Tot op heden is er echter weinig onderzoek gedaan naar mogelijke effecten van biologische producten op gezondheid.
In meer dan 100 studies zijn gehaltes aan inhoudsstoffen in biologische en gangbare producten vergeleken. Sommige van deze studies laten zien dat biologisch geteelde producten kunnen verschillen van gangbaar geteelde, bijvoorbeeld in hogere hoeveelheden droge stof, meer anti-oxidanten, hogere of gelijke hoeveelheden vitamine C en mineralen, en een vergelijkbare of betere kwaliteit aan eiwitten in biologisch geteelde producten vergeleken met gangbaar geteelde. Verder is beschreven dat er minder contaminanten zoals pesticide residuen, meestal minder of gelijke hoeveelheid nitraat en lagere of gelijke hoeveelheden mycotoxinen in biologische producten voorkomen. Verschillen in voedingsstoffen kunnen de basis vormen voor verschillen in gezondheidseffecten, zo bleek in recent verrichte dierstudies (Lauridsen 2007, Finamore 2004, Staiger 1988).
Voor het onderzoek naar de gezondheidseffecten van biologische producten ontbreken tot op heden representatieve parameters. Met name voor onderzoek naar gezondheidseffecten bij de mens is het van belang om juiste biomarkers te hebben, om gericht onderzoek te kunnen doen. Biomarkers zijn indicatoren voor biologische processen en dienen aan te sluiten bij de te onderzoeken vraagstelling. Er is veel onderzoek verricht naar het vinden van biomarkers voor specifieke ziekten, maar er is tot op heden weinig onderzoek verricht naar biomarkers voor 'de gezonde toestand' in gezonde individuen. In de huidige studie werd gezocht naar biomarkers ten behoeve van onderzoek naar gezondheidseffecten van voeding van verschillende teeltwijzen, uitgevoerd bij gezonde kippen, maar met het oog op toekomstig onderzoek bij de mens.

De voorliggende studie is ontworpen door een internationale groep van onderzoeksinstituten, als leden van de internationale vereniging voor onderzoek naar biologische voedselkwaliteit en gezondheid, FQH. In deze studie werd ten eerste gezocht naar de verschillen in ingrediënten voor kippenvoer afkomstig van verschillende teeltwijzen, biologisch en gangbaar.
Ten tweede is een voedingsexperiment met kippen uitgevoerd om biomarkers te vinden, die indicatief zouden kunnen zijn voor gezondheid.
De studie is verricht door een Nederlands consortium van instituten, het Louis Bolk Instituut, TNO Kwaliteit van Leven, RIKILT Instituut voor voedselveiligheid en Wageningen Universiteit - Departement Dierwetenschappen, en diverse andere instituten in Nederland en daarbuiten. Diverse instituten hiervan zijn lid van FQH.
Het projectontwerp is beoordeeld en goedgekeurd door de Scientific Advisory Committee van FQH, evenals het rapport.
De studie is gefinancierd door de Nederlandse overheid (het ministerie van LNV en het ministerie van EZ), de Rabobank en de Triodos Bank.

Werkhypothese

Biologisch geteelde producten hebben een gunstiger effect op de gezondheid.

Onderzoeksvragen

1. Kunnen verschillen gevonden worden in de ingrediënten voor kippenvoer, verkregen uit biologische en gangbare teeltsystemen?
2. Kunnen biomarkers voor gezondheidseffecten worden geïdentificeerd, gerelateerd aan de consumptie van biologisch voer vergeleken met gangbaar voer?
    a. Bestaat er een verschil in het zich ontwikkelende immuunsysteem bij kippen, gevoerd met twee verschillende voeders?
    b. Treden er verschillen op in de functies van orgaansystemen, in de zin van positieve of negatieve gezondheidseffecten, die gerelateerd zijn aan de consumptie van de verschillende voeders?

Onderzoeksontwerp

De studie betreft een geblindeerd dierexperiment met voeding, in twee generaties kippen, met identiek samengestelde voeders van óf biologische óf gangbare producten. Het betrof het kippenmodel van de zgn. Wageningse Selectie Lijnen, leghorn hennen die over 25 generaties zijn geselecteerd op óf hoge óf lage antilichaamproductie na een injectie met SRBC (schapen rode bloedcellen), op de leeftijd van 37 dagen. Naast deze lijnen was er een random gefokte controlegroep ter beschikking van kippen van dezelfde afstamming, die het 'originele ouderlijke wild type' van de dieren vertegenwoordigde. De dieren zijn beschreven als 'high line' (H-line of Hoge lijn) voor de groep met een hoog reactieniveau op SRBC injectie en een 'low line' (L-line of Lage lijn) voor de groep met een laag reactieniveau. De controlegroep (C-line) betreft de groep dieren die het normale genetische variatiepatroon op een injectie met SRBC vertegenwoordigde. Parallel aan de hennen van de eerste generatie, werd een groep hanen van dezelfde drie lijnen grootgebracht op het experimentele voer.
Er werd ten behoeve van het onderzoek een kippenvoer samengesteld in overeenstemming met de bestaande normen voor biologisch kippenvoer, bestaande uit 6 ingrediënten die zowel biologisch als gangbaar geteeld worden. Als ingrediënten werden gekozen tarwe, gerst, triticale, maïs, erwten en soja. Deze ingrediënten werden gezocht van biologische en gangbare teelt en bij voorkeur van een 'gecontroleerde teelt'-situatie. Waar dit niet mogelijk was werd gekozen voor producten van boerderijen in de directe omgeving van elkaar - de zo geheten 'farm pairs'- met dezelfde bodemsamenstelling en klimatologische omstandigheden en bij voorkeur bekend staand als 'best practice farms'. Het gebruik van dezelfde rassen van producten werd als ideaal beschouwd, maar alleen dán wanneer dat realistisch was binnen het teeltsysteem. Geschikte tarwe, gerst en triticale werden in Nederland gevonden, erwten in Denemarken en maïs en soja in Oostenrijk. Alleen de biologische en gangbare soja was van hetzelfde ras. Voor gerst en tarwe kon worden gekozen uit twee 'farm pairs'. Bij deze keuze van het meeste geschikte 'farm-pair', zijn referenties van het zgn. DOK-onderzoek in Zwitserland (een over 25 jaar lopende vergelijkend onderzoek tussen biologisch-dynamische, biologische en gangbare teeltsystemen) als 'gouden standaard' genomen.
De ingrediënten werden gescreend op residuen van pesticiden of mycotoxines, om negatieve gezondheidseffecten door deze componenten te vermijden en om te voorkomen dat de interpretatie van de resultaten daar door verstoord zou worden. De producten werden geaccepteerd als zij schoon waren, of wanneer residuen onder een bepaalde maximum residu limiet (MRL) lagen. In één maïsmonster werd een mycotoxine gevonden met een gehalte onder de MRL grens, dat niet als gevaarlijk voor de kippen werd beoordeeld. Later in de studie werden nog meer analyses

uitgevoerd op een beperkt aantal andere anti-nutritionele en remmende factoren. De ingrediënten, zowel als het voer, werden uitgebreid geanalyseerd op macronutriënten, micronutriënten, sporenelementen, bioactieve inhoudsstoffen en bacteriën.

Het kippenvoer werd door een bekende producent van biologische diervoeders bereid uit deze ingrediënten.
Om nutriënten-tekorten in het kippenvoer te voorkomen zijn enkele ingrediënten toegevoegd. Toegevoegd werden een geringe hoeveelheid aardappeleiwit (van gangbare teelt omdat dit gegarandeerd geen, mogelijk schadelijk, solanine bevatte), het aminozuur methionine, een kleine hoeveelheid van een mengsel van vitaminen en mineralen (Fx Layers Premix), kalk, grit, zout en $NaCO_3$, in beide voeders gelijke hoeveelheden. Na de productie werd het voer gecontroleerd op het gehalte aan aminozuren en indien er een tekort bleek te bestaan ten opzichte van de ondergrens van de aanbeveling van de voedernorm, werd een essentieel aminozuur toegevoegd om aan het minimum niveau te voldoen. Het betrof steeds een geringe toevoeging van methionine. Het voer werd vervolgens door de fabrikant geblindeerd met de codes A of B en getransporteerd naar Wageningen Universiteit, waar de kippen werden gehouden.

De eerste generatie kippen werd gehuisvest in individuele kooien. Dit was noodzakelijk om de eieren individueel te kunnen identificeren en zo met zekerheid de identiteit van de dieren van de volgende generatie te kunnen vaststellen.
De tweede generatie werd gehuisvest in inpandige rennen, in groepen van 6 dieren, met steeds 2 hennen van elke lijn. De rennen waren ruim en verrijkt, om een optimaal diereigen gedrag van de hennen mogelijk te maken en daarmee het tot uiting komen van mogelijke gezondheidseffecten te bevorderen.

De dieren van de eerste generatie begonnen hun leven met het gebruikelijke gangbare kippenvoer van de Wageningse stallen. Vanaf de leeftijd van 11 weken kregen de dieren de experimentele voeders. De dieren van de tweede generatie kregen vanaf de eerste dag van hun leven de experimentele voeders. Ze konden eten zoveel ze wilden. De tweede generatie leefde tot een leeftijd van 13 weken.

In deze studie was het doel om biomarkers te verkrijgen met betrekking tot mogelijke gezondheidseffecten. Deze konden zowel positief als negatief zijn. Fysiologische markers werden gezocht op het gebied van algemene gezondheidskenmerken, in immunologische parameters, in metingen van metabolieten in het plasma en de lever met behulp van metabolomics, in de functie van de darm met genomics en in de organen met pathologische anatomie. Daar beide groepen dieren voldoende en volwaardig voer kregen, werden er in het algemeen geen grote verschillen verwacht. Een gezondheidsverstoring werd daarom noodzakelijk geacht, om het vermogen van de dieren om te reageren en te herstellen te evalueren. Een veel gebruikte niet-pathogene immunologische trigger, bestaande uit een injectie met een eiwit (KLH), werd als gezondheidsverstoring (‘challenge’) gekozen en toegediend aan de tweede generatie in de 9^{e} week. Algemene gezondheidskenmerken werden geëvalueerd door wekelijks de voerinname te meten, de dieren te wegen, de eiproductie te documenteren (bij de eerste generatie) en gezondheidsverstoringen, ziektes en sterfgevallen te registreren.
De verwachting was dat het immuunsysteem de meest duidelijke effecten zou laten zien, omdat bekend is dat in jonge, zich ontwikkelende organismen, het contact van de darm met het opgenomen voedsel de ontwikkeling van het immuunsysteem stimuleert. Daarom werd er een groot aantal immunologische metingen in beide generaties verricht.

In de eerste generatie werden de immunologische effecten van de overgang van het oorspronkelijke voer op het experimentele voer, in week 11, onderzocht. In de tweede generatie werd het sterkste effect verwacht van de immunologische challenge met KLH in week 9. De periode vóór en na de challenge werd gemonitored middels immunologische metingen. In de tweede generatie werden ook andere analyses toegevoegd. Bloed, afgenomen voor en na de challenge, werd geanalyseerd met behulp van metabolomics.
In week 13 werd het leven van de dieren beëindigd en werd sectie verricht. Er werden weefselmonsters genomen voor metabolomics onderzoek van de lever, voor genomics onderzoek van de darm, voor pathologische anatomie van de organen, voor smaakproeven van het vlees en voor het aanleggen van een biobank (bewaring) van weefsels. De immunologische metingen werden in de gehele groep dieren uitgevoerd. De metabolomics metingen, de genomics en de pathologische anatomie werden in een subgroep van de dieren verricht. In deze beperkte groep werden alle C-lijn dieren geanalyseerd, maar - uit elke voer groep - werden 6 H-lijn dieren en 6 L-lijn dieren random geselecteerd.

De studie werd geblindeerd uitgevoerd tot de meerderheid van de voer- en dieranalyses waren voltooid, inclusief het grootste deel van de interpretaties, om beïnvloeding te voorkomen. Vervolgens werden de resultaten van het voedselonderzoek gerelateerd aan de analyses van de kippen, echter nog steeds gecodeerd t.a.v. de herkomst van de voeders. Dit maakte een betere interpretatie van de voedselparameters mogelijk, die de basis zouden kunnen zijn voor de verschillende effecten in de kippen. Pas kort voor het ter perse gaan van dit rapport werden de voercodes ontsloten. Voer A bleek het biologische en voer B het gangbare voer.

Resultaten van de voeders en ingrediënten

- Het meest consistent was het verschil in de hoeveelheid eiwit, gemiddeld 10% hoger in het gangbare voer. In tarwe, soja en gerst lag het percentage aminozuren 10-40% hoger in de ingrediënten die gebruikt werden voor de productie van het gangbare voer. De verteerbaarheid van de aminozuren bleek beter in het gangbare voer.
- Het gehalte aan phytosterolen was hoger in soja en gerst, in het gangbare voer (voer B).
- De meeste biologische ingrediënten (voer A) bevatten meer vitamine K en biologische soja bevatte meer isoflavonen en vitamine E, met name alpha-tocopherol. De biologische erwten bevatten meer foliumzuur.
- Vitamine B5 en C waren hoger in de gangbare maïs, erwten en tarwe (voer B).
- Wat betreft microbiologie, werden er geen grote verschillen waargenomen tussen de biologische en gangbare voeders, maar in het voer van biologische herkomst (A) werden meer schimmels gevonden en een hogere hoeveelheid aerobe kolonies en Enterobacteriaceae.
- Er werden echter hogere gehaltes aan LPS endotoxinen gemeten in het gangbare voer (B), met name in het voer dat de eerste generatie dieren kreeg.
- Met complementaire methoden, zoals biofotonen-metingen, proteïne-ratio en kristallisaties, konden de ingrediënten, afkomstig van verschillende teeltsystemen, significant worden onderscheiden. De onderzoekers waren in staat, bij die producten waarmee ze ervaring hadden, de herkomst als biologisch of gangbaar correct te identificeren.

Resultaten van de kippen

Beide generaties dieren en alle drie de kippenlijnen werden onderzocht. Bij de evaluatie van de effecten, werden de resultaten van de dieren uit de controle-lijn (C) van de tweede generatie als het meest informatief beschouwd, omdat deze populatie de natuurlijk voorkomende genetische variatie representeert. De resultaten van deze dieren worden hieronder gepresenteerd.

- Alle dieren uit de tweede generatie werden als volledig gezond beoordeeld. Toch is de eindconclusie dat de twee groepen op de verschillende voeders duidelijke verschillen laten zien in diverse aspecten van hun fysiologie.
- De dieren op het gangbaar geteelde voer (B) waren significant zwaarder gedurende het gehele experiment. Relatieve groei was significant groter bij gangbaar gevoerde dieren in de eerste 5 weken van hun leven, maar daarna begonnen de biologisch gevoerde dieren harder te groeien. Na de KLH challenge werd een afname van de groei met 20-30% waargenomen gedurende twee weken in beide groepen. Na deze groeiafname vertoonden de biologische gevoerde dieren een grotere groei (inhaalgroei), dan de gangbaar gevoerde dieren.
- Diverse immunologische parameters toonden een verschil tussen de beide voergroepen. Dit gold zowel voor de humorale als de cellulaire componenten van het immuunsysteem, zowel aangeboren als verworven. De dieren op biologisch geteeld voer toonden hogere LPS-antilichaamtiters in het bloed (C-line, in de H-line omgekeerd); een hogere KLH-geïnduceerde complement-activering (klassieke route), als afspiegeling van de activering van het aangeboren immuunsysteem; hogere vaccinatie-antilichaamtiters (bijvoorbeeld gericht op Gumboro), als blijk van activering van het adaptieve systeem; en een hogere in vitro respons op voedingsextracten in aanwezigheid van ConA van leukocyten uit het perifere bloed. De immunologische resultaten bleken niet volledig consistent, maar werden als geheel geïnterpreteerd als een aanwijzing voor een hoger immunologische reactievermogen bij dieren die voer van biologische herkomst gehad hadden.
- De resultaten van de metabolomics van het bloed toonden een duidelijk verschil tussen de groepen, vooral na de challenge. Een hoger gehalte aan vrije vetzuren en onverzadigde lipopolysacchariden in het bloed van de dieren op voer A, leidde tot de interpretatie van een sterkere reactie op de challenge met een bijbehorend metabolisme, ten teken van een sterkere acute fase reactie bij de dieren op biologisch voer.
- De resultaten van de metabolomics van de lever toonden een toegenomen activiteit van de pentose-fosfaat-route bij de dieren die biologisch gevoerd waren, alsook meer markers voor het levermetabolisme en voedselopname (vitamine E).
- Genomics onderzoek toonde bij de gangbaar gevoerde dieren een lagere expressie van genen aan, die gerelateerd zijn aan de cholesterol-biosynthese. Deze bevindingen werden bevestigd bij een follow-up analyse. De verwachting van een verhoogd plasma cholesterol-gehalte bij deze groep dieren kon echter niet worden bevestigd m.b.v. metabolomics.
- Onderzoek met pathologische anatomie toonde enkele verschillen aan in orgaangewichten, tussen de voer-groepen en tussen de lijnen.

Conclusies

Met betrekking tot de voeders kan geconcludeerd worden dat de analytische verschillen in de ingrediënten en voeders het duidelijkst waren voor het gehalte aan eiwitten en aminozuren en sommige micronutriënten. Hoewel er verschillen werden vastgesteld, waren de voeders voldoende voedzaam voor de opgroeiende kippen, en behalve ten gevolge van de eiwitten, werden er geen grote verschillen in effect op de gekozen parameters voor gezondheid verwacht.

Ofschoon *alle kippen gezond waren*, werden evidente verschillen in gemeten parameters vastgesteld.

De gangbaar gevoerde dieren van de controle-lijn toonden een sterkere gewichtstoename, terwijl de biologische gevoerde dieren een sterkere immuunreactiviteit, een sterkere reactie op de challenge, en een iets sterker herstel van de challenge in termen van inhaalgroei, toonden.

De resultaten zijn gebaseerd op de bevindingen bij de dieren van de controle lijn, daar deze de natuurlijke genetische variatie vertegenwoordigden. Maar de resultaten uit de speciale hoge- en lage lijn kippen in dit onderzoeksmodel, ondersteunden de conclusie ten aanzien van een versterkt immuun-reactief vermogen bij de dieren die biologisch geteeld voer kregen.

Voor wat betreft de voedingsfactoren die dit verschil zouden kunnen verklaren, moet het hogere eiwitgehalte van het gangbare voer als de factor beschouwd worden, die de sterkere gewichtstoename in deze groep veroorzaakt zou kunnen hebben. De factor(en) in het voer die de fysiologische verschillen in reactie op de challenge zou(den) kunnen veroorzaken, zijn nog niet duidelijk. Er zijn aanwijzingen in de literatuur dat een versterkte immuunreactiviteit gerelateerd zou kunnen worden aan het lagere lichaamsgewicht van de betreffende dieren.

De betekenis van de verschillende fysiologische reacties met betrekking tot gezondheid op korte en op lange termijn van deze dieren, is nog onduidelijk. Dit zou in een follow-up studie verhelderd moeten worden.

Alles overziend kan geconcludeerd worden dat de studie een enorme hoeveelheid informatie heeft voortgebracht en tevens effecten heeft opgeleverd, die niet waren voorzien. Een belangrijke uitkomst van deze studie is dat voedingsingrediënten van verschillende herkomst een klein, maar duidelijk effect kunnen hebben op het immuunsysteem en op de stofwisseling van gezonde dieren. Verder werd duidelijk dat het begrip 'gezondheid' en de fysiologie en immunologie van gezondheid een vooralsnog weinig verkend gebied zijn in onderzoeksland.

Evaluatie van de werkhypothese en onderzoeksvragen

De werkhypothese was 'Biologisch geteelde producten hebben een gunstiger effect op de gezondheid'.

Ten aanzien van een potentieel 'groter' gunstig effect op gezondheid van een van de twee voeders, kunnen geen definitieve conclusies worden getrokken. Beide voeders waren als zodanig gezond. Het concept van 'gezondheid' en de daaraan gerelateerde fysiologie en immunologie dienen verder uitgewerkt te worden, voordat definitieve conclusies getrokken kunnen worden.

Met betrekking tot de eerste onderzoeksvraag: 'Kunnen verschillen gevonden worden tussen de ingrediënten van het kippenvoer, verkregen uit biologische en gangbare teeltsystemen?', kan geconcludeerd worden dat de ingrediënten, afkomstig uit biologische en gangbare teelt, het meest verschilden in eiwit- en aminozuurgehalte. Maar tegelijkertijd is duidelijk dat vele, maar niet álle bestanddelen geanalyseerd zijn.

De tweede onderzoeksvraag: 'Kunnen biomarkers voor gezondheidseffecten worden geïdentificeerd, gerelateerd aan de consumptie van biologisch voer vergeleken met gangbaar voer?' en de twee subvragen, kunnen bevestigend beantwoord worden, ook al zijn de implicaties van de waargenomen verschillen in relatie tot gezondheid nog niet

duidelijk en is verder onderzoek noodzakelijk. Opgemerkt moet worden dat in dit project slechts één selectie van producten afkomstig van één oogst zijn onderzocht.

Geïdentificeerde biomarkers

De biomarkers die in deze studie duidelijk representatief zijn voor de verschillende effecten van de twee voedingsregimes zijn groei en, met name na blootstelling aan een challenge, evaluatie van de responsiviteit van het immuunsysteem, van metabole reacties in bloed en lever en genetische regulatie in de darm.

Aanbevelingen

De resultaten van deze studie zijn veelbelovend. De studie toonde aan dat kleine verschillen in voer, ten gevolge van verschillende teeltsystemen, gevolgen kunnen hebben voor immuunreactiviteit, stofwisseling en genexpressie in gezonde dieren. Voordat deze resultaten bij mensen gebruikt kunnen worden (het uiteindelijke doel), dienen de resultaten bevestigd te worden. Dat zou allereerst opnieuw bij kippen moeten plaatsvinden, die gedurende een langere tijd vervolgd moeten worden (wellicht tot de natuurlijke dood), en die onderzocht moeten worden na een sterkere challenge met een infectiemodel of ander ziektemodel.
Voeringrediënten voor dergelijk follow-up onderzoek zouden verkregen moeten worden van ‘best practice’ boerderijen uit dezelfde regio. De voeders die gegeven worden aan dieren, moeten uitgebreid onderzocht worden, om de gevonden effecten bij de dieren te kunnen relateren aan inhoudsstoffen in het voer. Daarnaast zouden de ingrediënten grondig onderzocht moeten worden, ook in relatie tot de producten die de consument koopt, om meer inzicht te krijgen in de representativiteit van de producten.

In de toekomst wordt een onderzoek met zoogdieren aanbevolen, bij voorkeur met varkens, omdat deze dieren het meest met de mens vergelijkbaar zijn. Onderzoek bij mensen is het ultieme doel.

Summary

The aim of the project 'Organic, More Healthy?' was to search for 'biomarkers' that show potential health effects of organic food. An important reason for many consumers to buy organic is the assumption that organic products are healthier than conventional produced products. If beneficial effects on health are confirmed this motivates the consumer to buy organic products and therefore could stimulate the growth of the organic market. Until now, however, very little research has been performed to study the effect of organic food on health.
More than 100 studies have made the comparison of nutrient content in organically and conventionally grown products. Some of these studies provide findings that organically grown products can be different from conventionally grown products, e.g. higher levels of dry matter, more anti-oxidants, higher or equal amounts of vitamin C and minerals, and a comparable or better protein quality in the organic compared to the conventional products. Furthermore, less contaminants such as pesticide residues, in most cases lower or equal amounts of nitrate and often lower or equal amounts of mycotoxins have been described.
Differences in nutritional content may underlie the differences in health effects, found in the few recent studies performed in animals (Lauridsen 2007, Finamore 2004, Staiger 1988).
To study the effect of organic products on health, thus far representative parameters to study health effects are lacking. Especially when health effects on human subjects are to be studied, it is important to have adequate 'biomarkers'. Biomarkers are indicators of biological processes, and should be adequate for the question investigated. Much research has been performed in finding biomarkers for specific diseases, much less is been developed as yet on biomarkers for health or fitness in healthy individuals. In this study an attempt is made to search biomarkers to study the health effects of different feeding regimes in healthy subjects. For this study a chicken model is used. But the final goal is to find biomarkers suitable for studies in humans, in the future.

The present study was designed by an international group of research institutes, members of the international association of organic Food Quality & Health research, FQH. First of all the study searched for differences in feed ingredients from different production systems, organic or conventional. Second a feeding experiment was performed to study biomarkers which may be indicative from the perspective of health.
The study was performed by a Dutch consortium of institutes, Louis Bolk Institute, TNO Quality of Life, RIKILT Institute of Food Safety and Wageningen UR-Department of Animals Sciences, and several other institutes in the Netherlands and abroad, several of them being members of FQH.
The project design was reviewed and approved by the Scientific Advisory Committee of FQH, as is the report.
The study was financed by the Dutch Government (Ministry of Agriculture, Nature and Food Quality and Ministry of Economic Affairs), Rabobank and Triodos Bank.

Working hypothesis

Organically grown products have a more beneficial effect on health.

Research questions

1. Can differences be found between ingredients for chicken feed, obtained from organic and conventional production systems?

2. Can biomarkers be identified for health effects, related to the consumption of organic compared to conventional feed?
    a. Is there a difference in the developing immune system of chicken fed with the two different feeds?
    b. Do differences occur in the functioning of other organ systems, related to positive or negative health effects, connected to the consumption of the different feeds?

Research design

The study comprised a blinded animal feeding experiment with identically composed feeds from either organic or conventional products. The animals were two generations chicken from the Wageningen Selection Lines, laying hens that during 25 generations were selected for their either high or low antibody reaction to SRBC (sheep red blood cells) 5 days after injection at the age of 37 days. Next to these lines a random bred control group (C-line) of chicken was available, from the same original parental stock, representing the 'original paternal wild type' of the animals. The animals are described as high line (H-line) for the group with a high reaction to SRBC and low line (L-line) for the group with a low reaction to SRBC. The control line (C-line) concerns the group of animals, which represents the whole 'normal' genetic variation in reactions to SRBC. Parallel to the hens of the first generation, a group of roosters from the same three lines was raised on both feeds.
A feed was composed, according to existing norms for organic chicken feed, with six ingredients that were produced organically as well as conventionally. Wheat, barley, triticale, peas, maize and soy were sought, preferably from controlled production. If this was not possible, products were chosen from neighbouring farms - so called 'farm pairs'-, preferably known as 'best practice farms', with the same basic soil and climatic conditions. The use of the same variety of produce is considered ideal, but only if this is realistic within the specific farming system. Wheat, barley and triticale were obtained from the Netherlands, peas from Denmark, maize and soy from Austria. Only soy was of the same variety. Of barley and wheat a choice could be made out of two farm pairs. Here the products from the DOK-trials in Switzerland, a 25 year running comparison trial between (bio)Dynamic, Organic and conventional production systems, served as an indication of a 'golden standard' for the choice of the preferred farm pair.
The ingredients were screened for residues of pesticides or mycotoxins, to prevent adverse health effects due to these compounds, which might disturb the interpretation of the results. Products were accepted if they were clean, or if residues were below the maximum residue limit (MRL). In one maize sample a mycotoxin was found, below MRL, which was considered not dangerous for the young chicken. Later in the study more checks were performed for a selected number of other possible anti-nutritional, inhibiting factors. The ingredients that were accepted for feed production, as well as the feeds, were extensively analysed for macronutrients, micronutrients, trace elements, micro-organisms and bioactive ingredients.

The feeds were produced from these ingredients, by a well known manufacturer of organic animal feeds. To prevent shortages in the nutritional needs of the chicken, some additions were made. Added were potato protein (from conventional origin as this was guaranteed without solanin), the amino acid methionine, a small dosage of a mix of vitamins and minerals (Fx Layers Premix), chalk, grid, salt and $NaCO_3$. After production, the feeds were checked for amino acid content and if a shortage existed, essential amino acids were added up to the minimum level of the Norm (recommended level). Then the feeds were blinded by the manufacturer, named either A or B, and transported to Wageningen UR, where the chicken were housed.

The first generation was housed in individual cages. This was necessary to be able to identify each individual egg, and so to secure the identity of the animals of the next generation. The second generation was housed in indoor runs, in groups of 6 animals, 2 hens from each line. The runs were spacey and enriched, to ensure optimal natural behaviour of the animals, and thus to facilitate the expression of possible health effects.

The animals of the first generation started their life on the usual conventional chicken feed. From week 11 of age the animals were fed the experimental feeds. The animals of the second generation received the experimental feeds from the first day of their life. Both generations could eat ad libitum. The second generation lived till 13 weeks of age and was then sacrificed.

In this study the aim was to obtain biomarkers for possible health effects. The health effects could be either positive or negative. Physiological markers were sought in general health features, immunological parameters, metabolite measurements in plasma and liver through metabolomics, gut functioning through genomics, and in a post mortem evaluation through pathological anatomy. As both groups of animals received balanced and sufficient feed, no large differences were expected. A disturbance was considered necessary, to evaluate the animals potential to react to and recover from that. A non-pathogenic, often used, immunological trigger was chosen as disturbing challenge, being an injection with a protein (KLH) at the age of 9 weeks in the second generation.
General health effects were evaluated by measuring weekly feed intake, weighing the animals and documenting egg production, health disturbances, illnesses and deaths.
The immune system was expected to show most obvious effects of the different feeds, as it is known that in young, developing organisms, the contact of the gut with the consumed food stimulates the development of the immune system. Therefore, a broad range of immunological measurements were performed, in both generations.
In the first generation the effects of the change from original feed towards the experimental feeds, in week 11 on immune parameters, were examined. In the second generation the strongest influence was expected from the immunological challenge by KLH, in week 9. The period before and after the challenge, was monitored by immunological measurements. In the second generation even more analyses were added. Blood, drawn before and after the challenge, was analysed by metabolomics.
In week 13 the animals' life ended and section was performed. Tissue samples were taken for metabolomics of the liver, for genomics of the gut, for pathological anatomy of the organs, for sensory analysis of the meat and for biobanking of material. The immunological measurements were performed on the whole group of animals. The measurements of metabolomics, genomics and pathological anatomy were performed in a subgroup of animals. In this reduced group all C-line animals were analysed, but – from each feed group – only 6 randomly selected H-line animals and 6 L-line animals.

The study was performed blinded, till the majority of the examinations of feeds and animals and their interpretation were finished, to prevent interpretations to be influenced. To allow the interpretation with respect to the feed parameters that could underlie the differential effects in the chicken, the results of the feed analyses, were connected to the results of the chicken analyses, however still coded A or B. Only just before the printing of this report the codes of the origin of the feeds were broken. Feed A turned out to be organic, feed B was conventional.

Results of the feeds

- Most consistent were differences observed in the amount of proteins, which was about 10% higher in the conventional feed (B). In wheat, soy and barley the amino acids were 10-40% higher in the ingredients used for the preparation of the conventional feed. The digestibility of the amino acids appeared better in the conventional feed.
- The level of phytosterols was higher in conventional soy and barley (feed B).
- Most organic ingredients (feed A) were higher in vitamin K, organic soy was higher in isoflavones and vitamin E, especially alpha tocopherol, and organic peas were higher in folate.
- The vitamins B5 and C were higher in respectively the conventional wheat and the conventional maize and peas (feed B).
- In the period of the KLH challenge slight differences in fatty acids in the feed applied, with higher levels of unsaturated C18 in feed A.
- With respect to microbiology, no large differences were observed between the organic and conventional feeds.
- Moulds were more common in the feeds from organic origin, and in general the organic feeds (A) had a higher aerobic colony count, as well as a higher amount of Enterobacteriaceae.
- Higher contents of LPS endotoxins were measured in the conventional feeds (B), especially those provided in the first generation.
- Complementary analyses by biophoton measurements, protein ratio and biocrystallizations showed that ingredients from the two agricultural systems could be differentiated. Where experience with the ingredients was available, researchers were able to identify the organic samples blindly.

Results of the chicken

Both generations and all three chicken lines were evaluated. However, in the evaluation of the effects, the results of the control (C-) line animals of the second generation are considered to be most informative, as these reflect the natural genetic variety of the population. If not indicated otherwise, the results of these chicken are presented.

- All animals of the second generation were diagnosed as being perfectly healthy. However, the groups on the two different feeds showed clear differences in several aspects of their physiology.
- Animals on the conventional feed (B) were significantly heavier throughout the experimental period. Relative growth was significantly higher in the animals on the conventional feed in the first 5 weeks of life, but then the animals on the organic feed (A) started to grow slightly more. After the KLH challenge a 20-30% decline in growth was observed in both groups for about two weeks. After the decline the animals on the organic feed showed a larger growth (catch-up growth) than the animals on conventional feed.
- Several immunological parameters showed differences between the animals on the two different feeds. This was true for both the humoral and cellular components of the immune system, both innate and adaptive, by higher LPS-antibody titers in blood (C-line, H-line reverse), KLH-induced classical complement activation (reflecting an activated innate immune system), higher vaccination antibody titers e.g. directed to Gumboro indicating activation of the adaptive system and the in vitro response to feed extracts in the presence of Con A of peripheral blood leukocytes, which were higher in the organically fed animals. The immunological results were not fully consistent, but were overall interpreted as indicating a higher potential for immunological reactivity of the animals fed the organic feed.

- The metabolomics results of the blood showed a clear distinction between the animals on the two feeds, especially after the challenge. In this period an increase of several so called free fatty acids and unsaturated lipo poly saccharides were observed, more in the A- than in the B-animals. This led to the interpretation that the animals on the organic feed showed, after the challenge, a stronger reaction and connected metabolism, indicating a stronger acute phase reaction, than the animals on the conventional feed.
- The metabolomics results of the liver showed an increased pentose phosphate pathway activity in the animals on the organic feed (feed A), as well as more markers of liver metabolism and food intake (vitamin E).
- The genomics showed, in the animals on the conventional feed, a down-regulation of genes connected with cholesterol biosynthesis. These findings were confirmed by a follow-up analysis. The expectation of higher plasma cholesterol levels could however not be confirmed by metabolomics measurements.
- Evaluation by pathological anatomy showed some differences in the weight of specific organs between the feed groups, in the different lines. This finding is not yet understood.

Conclusions

Concerning the feeds it can be concluded that the analytical differences in ingredients and feeds were most consistent in the amount of proteins and amino acids. Further differences in some micronutrients were found. Although differences were observed, the feeds were sufficiently nutritious for the growing chicken, and with the exception of the proteins, no large effects from these differences on the chosen parameters of health were expected. Though *all the chicken were healthy*, a clear difference in the measured parameters was observed. The animals on the conventional feed gained more weight, whereas the animals on the organic feed showed a stronger immune reactivity, a stronger reaction to the challenge, as well as a slightly stronger recovery from the challenge in terms of regained growth.

Results are based on findings in the control line animals, as these represent the natural genetic variety. However results in the special high and low responding chicken in this animal model supported the conclusions of increased potential for immune reactivity in the organically fed animals.

Concerning the factors in the feed that could explain these differences, the higher protein content in the conventional feed is considered to be the factor, causing the stronger weight gain in the animals on this feed. The factor(s) in the feeds that might cause the physiological differences in relation to the challenge, are not yet clear. There are indications in literature that an enhanced status of immune reactivity in the animals on the organic feed, may be related with lower body weight gain.

The implications of these different physiological reactions in the context of short term and long term ‘health’ of these animals, is still unclear. A follow up study should clarify that.

Overall the study provided an enormous amount of information and caused effects which were not foreseen. An important outcome of this study is that feed ingredients from different origins can have small but clear immunological and metabolic effects in healthy animals. Further it became clear that the concept of ‘health’, and the physiology and immunology of health, are a still quite unexplored field of research.

Evaluation of the working hypothesis and research questions

The working hypothesis was that 'Organically grown products have a more beneficial effect on health'.
Regarding a potential 'larger' beneficial effect on health of one of the two feeds, no clear conclusions can be drawn. Both feeds were 'healthy' as such. The concept of 'health' and the connected physiology and immunology, need to be worked out further, before clear conclusions can be drawn.
With respect to the first research question: 'Can differences be found between ingredients for chicken feed, obtained from organic and conventional production systems?', it can be concluded that the ingredients for the chicken feed, obtained from organic and conventional production systems, differed most clearly with respect to protein and amino acid content. However, at the same time it is clear that many but not all nutritional and anti-nutritional factors in the feeds were analyzed.
With respect to the second research question: 'Can biomarkers be identified, for health effects, related to the consumption of organic compared to conventional feed?', and the two sub questions about effects on the immune system and other organ systems, these can be answered confirmatively, although the implications of the observed differences with respect to health, are not clear yet and need further investigation. It must be noted that only one harvest and selection of products has been investigated in this project and that it cannot be excluded that different selections might have produced different results.

Biomarkers identified

Biomarkers which in this study clearly presented the different effects of the two feeding regimes are growth and, especially after exposure to a challenge, immune responsiveness, metabolic reactions, gene regulation in the gut system and observations by pathological anatomy.

Recommendations

The results of the present study are most promising. This study showed that small differences in feeds, because of differences in agricultural background, have implications for immune reactivity, metabolites and gene activity in healthy animals. Before these results can be used in studies in humans (the final goal), the results need to be confirmed. Confirmation should preferably first be sought, again, in chicken, which need to be followed longer (e.g. till natural death) and should be studied during a stronger challenge with an infection model or other disease model.
Feed ingredients for such follow-up research should be obtained from 'best practice' farms in the same area. The feeds need to be extensively analysed, to give the possibility to relate observed effects in the animals to nutritional factors in the feeds. Next to this the ingredients should be analysed thoroughly, also in relation to products as purchased by the consumer, to get more insight in the representativity of the products.
Towards the future a confirmation in mammals is recommended, preferably in pigs, as these animals are most comparable to human beings. Research in humans is the ultimate goal.

1 Introduction

1.1 General introduction to the project

A contribution to a better health can be an important motivation for the consumption of organic products and therefore can stimulate the growth in the market share of these products. There are hardly any studies in which health effects were studied. There are however more than 100 studies have made the comparison of nutrient content in organically and conventionally grown products. But making a fair and correct comparison of these products is more difficult than it seems. First of all, conventional cultivation practices have changed considerably since the 1940ies. Other aspects that complicate the comparison are e.g.: cultivars (often different cultivars are used in organic and conventional production), product type (e.g. fruit, leafy vegetables, etc.), production method (only relatively recently (1991) organic production methods have been defined (Council Regulation (EEC) No 2092/91). On top of that, comparisons do not always reflect normal practice, e.g. use of pesticides within conventional production has changed considerably with the gain of knowledge etc... Two major reviews were produced by Woese (1997) and Worthington (1998, updated in 2001) making attempts to draw conclusions. Since then Heaton (2001) and others have reviewed the literature as well, all with their own perspectives. Heaton was the first to introduce criteria for the design and execution of studies in this field of research. According to these criteria many studies were not optimal.

In the light of the complexity of this type of research the following preliminary conclusion can be made: some of these studies provide findings that organically grown products compared to conventionally grown products can contain higher levels of 'positive' ingredients e.g. higher levels of dry weight, more anti-oxidants, higher or equal amounts of vitamin C and minerals, and a comparable or better protein quality in the organic compared to the conventional products. Furthermore, less 'negative' ingredients such as pesticide residues, lower or equal amounts of nitrate and lower or equal amounts of mycotoxins have been described (Woese 1997, Worthington 2001, Heaton 2001, Benbrook 2005). Repeated findings with respect to taste preference for organically grown products were found, both in animals as well as in humans (Heaton 2001).
This difference in nutritional content may explain the differences in health effects especially in animals, as has been reported by several research groups (Lauridsen 2007, Finamore 2004, Staiger 1988).
Holistic techniques such as the picture forming biocrystallization method and spectral range luminescence (or biophoton-) research, often show different structures and a stronger capacity to retain light with organic products (Adriaansen-Tennekes 2005, Strube 2004, Jensen 2001). This indicates a difference between the products, but whether these differences contribute to beneficial health effects is yet unclear.
The health effects of organic food that have been described are the following: in animals a slightly improved fertility and a lower amount of foetal deaths in animals fed with organic feed (Heaton 2001). This was also true when foods were supplemented in order to compensate the known differences in nutrient content between organic and conventional food (Staiger 1988). Exposure to mutagens in combination with vegetable extracts showed a lower number of DNA mutations in bacteria, in case of organically grown products, in comparison to extracts from conventionally grown products (Ren 2001). Rats fed on an organic feed showed a higher proliferation of white blood cells compared to the group fed conventional feed, in an underfed (protein deficient) group of animals (Finamore 2004). Recently, in organically compared to conventionally fed rats, higher immunoglobulin-G levels (IgG), less

adipose tissue and a better relaxation were documented (Lauridsen 2007). Even more recent are results from a Dutch prospective birth cohort study, KOALA. Here, organic dairy and meat in the maternal diet, was shown to influence the amount of conjugated linoleic acids in breast milk of lactating women (Rist 2007). Among the children in this study till two years of age, a strict use of organic dairy was associated with a third less eczema (Kummeling 2007).

However, most of these findings are incidental and could not yet reproduced by other research groups. Furthermore this type of research is still relatively new and not well defined, mainly due the complexity of the field.

Scientifically it was concluded that little, and especially few reproduced, studies on beneficial health effects of organic products are available, but indications in this direction exist (Minutes LNV 2004). Representative parameters to measure possible health effects are needed, making the search for adequate 'biomarkers' important. October 2003 a Scientific Workshop of the international Association of Organic Food Quality & Health research (FQH) was held on the topic of most promising experimental designs for studying possible effects of organic foods on health. It was concluded that the developing immune system was a promising field of research for defining 'biomarkers'. Other potential fields were stress sensitivity, intestinal health and taste and food preference. Furthermore it was concluded that it is essential that the raw ingredients used as feed in such studies must be highly standardized (cultivation) and characterised (content) (Minutes FQH 2003). This workshop led to the formation of a international group of scientists who designed the underlying research project: 'Organic, More Healthy? A search for biomarkers of potential health effects induced by organic products, investigated in a chicken model'.

Although the ultimate goal is to investigate possible health effects in humans, this is a bridge too far, as no biomarkers are defined yet. In the choice between *in vitro* research and an animal model, it was decided for the latter. As still many unknown factors may play a role in potential effects of organic food, these might be expressed and monitored in an animal, whereas these could be missed in a focussed laboratory model. The choice for chicken was made as a chicken model was available in Wageningen UR, which seemed very suitable for studying the immune system and health and with which much experience existed (Parmentier 2004). The avian immune system is very comparable to the mammalian immune system and chicken are omnivores, so this model was considered to be a good starting point for biomarker search (Tizard 2004, Turner 1994). The idea for this ideal study was that it would comprise two generations, as it is very likely that the parent(s) environment and genome have an effect on the status of the innate immune system of the chicks. The second generation would be the ultimate experimental group and would receive a non pathogenic challenge, generally used for studying 'general immunocompetence' in animals (Knap 2000, Demas 1997). Besides the immune system other organ functions were to be studied. Because of Dutch financing, the execution of the project is finally carried out by a Dutch consortium of institutes, most of them members of the FQH Association, and sub-contracters, three of whom are foreign FQH-partners .
The project design was reviewed by the Scientific Advisory Committee of FQH and qualified by FQH as a 'Project, approved by FQH'.

Project consortium The study was carried out by a project consortium consisting of the Dutch research institutes Louis Bolk Institute, Wageningen UR, RIKILT-Institute of Food Safety and TNO-Quality of Life. The

scientific coordination was in the hands of the Louis Bolk Institute, the financial coordination was taken care of by RIKILT-Institute of Food Safety. Statistics were performed by Muvara BV.
Further contributions came from Animal Sciences Group – Lelystad, Institute for Risk Assessment, Wageningen UR – Biological Farming Systems Group, University of Kassel – Department of Food Quality and Food Culture, KWALIS Qualitätsforschung GmbH, Biodynamic Research Association Denmark (BRAD) and Centrum voor Smaakonderzoek (CSO).

Stakeholders and Advisory Board The project was financed by the Dutch Government (Ministry of Agriculture, Nature and Food Quality and Ministry of Economic Affairs), Rabobank and Triodos Bank.
During the course of the project an independent Advisory Board monitored the scientific developments in the project and advised the Project consortium. Members of this Board were: Prof. dr.ir. I. Rietjens from Wageningen UR, Dr. H. van Loveren from RIVM, Dr. R. Pieters from University of Utrecht, Dr. L. Jansen from the Netherlands Nutrition Centre / Schuttelaar & Partners and Dr. J. Meijs from Biologica (chair). The stakeholders were represented by Dr. R. Theelen for the ministry of LNV, Mrs. Ir. A. Habraken for Rabobank and Mr. T. van den Bergh for Triodos Bank.

Time frame of project The project ran from August 2005 till December 2007.

	Aug 2005	Sep 2005	Oct. 2005	Nov 2005	Dec 2005	Jan 2006	-	June 2006	July 2006	Aug 2006	Sep 2006	-	Dec 2007
Feed	Search of ingredients	Search of ingredients	Check of ingredients residues	Preparing feed	Checking experim. Feed	Ingredient and feed analyses	Ingredient and feed analyses	Ingredient and feed analyses				Analysing	Reporting
1st gener.			Oct. 18th Hatching			Start exp. Feed	feeding	Brooding				Analysing	Reporting
2nd gener.								June 29th Hatching	feeding	KLH challenge	Sep. 27th Section	Analysing	Reporting
Lab-analyses					Preparing	Ongoing	Ongoing	Ongoing	Ongoing	Ongoing	Ongoing	Ongoing	Reporting

Figure 1.1: Schematic overview of the timeframe and activities in the project.

Structure of this report The experiment will be described in two parts: the Feed and the Chicken experiment.
First the Feed will be described by Material & methods and Results in Chapter 2 and 3.
Then the Chicken experiment will be presented by Material & methods and Results in Chapter 4 and 5.
The results of both Feed and Chicken are summarized and related to the research questions.
Discussion, Conclusions, Recommendations, Final reflections after decoding, Literature and Annexes conclude the report. Throughout the report, results are presented as belonging to either group A (in red) or B (in blue), being the codes of the origin of the feeds during the project. The summary was adapted in the finale stage into a decoded text, as were some sentences adapted about the origin of the feeds in the already existing text in the report.

1.2 Project description

1.2.1 Overall hypothesis

Organically grown products have a more beneficial effect on health.

1.2.2 Research questions

1. Can differences be found between ingredients for chicken feed, obtained from organic and conventional production systems?
2. Can biomarkers be identified for health effects, related to the consumption of organic compared to conventional feed?
   a. Is there a difference in the developing immune systems of chicken fed with the two different feeds?
   b. Do differences occur in the functioning of other organ systems, related to positive or negative health effects, connected to the consumption of the different feeds?

1.2.3 Research design

To study potential health effects of organically compared to conventionally grown ingredients, a feeding experiment will be performed, with two chicken lines, divergently selected for immune antibody responses during 25 generations and a control group.
Within the study two primary lines of research can be identified: the ingredients and the chicken.

Ingredients Six ingredients from both organic and conventional produce were obtained from paired farms, with same climatic and soil conditions. Ingredients were screened on contamination. Of these ingredients chicken feeds were manufactured, with identical amounts of ingredients. The feed and the raw ingredients were analysed extensively. The feeds were substituted to avoid deficiencies, but all other existing differences were left intact. Ingredients and feeds are extensively described in Ch. 2 and Ch. 3.

Chicken The research subjects were chicken, of two selected lines, the Wageningen Selection Lines, and a control line. The two lines concern a population of chicken in which during 25 generations the animals were selected on the primary antibody response against Sheep Red Blood Cells (SRBC). These chicken were fed during two generations the experimental feeds. The second generation lived till 13 weeks of age. The animals were extensively monitored during their life and after death.
The chicken are extensively described in Ch. 4 and Ch. 5.

The policy of (de)coding During the project it was decided to delay the decoding till the very last possible moment, as to prevent any influence or assumed influence on the interpretation. Therefore researchers reported and interpreted the results of feed A and B. Only after the definite reporting of results the feeds were decoded.

In search for biomarkers Biomarkers are indicators of biological processes. In 2001 a standardized definition was launched by a NIH working group, the Biomarkers Definition Working Group (Atkinson 2001), which is cited most in literature: 'A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmaceutical responses to therapeutic intervention.' Most biomarker search is performed in relation to specific diseases, diagnostics and effects of treatments. Even in investigations on effects of exposure to specific foods, most search is targeting on disease development (Davis 2007). The ideal is a set of biomarkers that is predictive, validated and sensitive. This level can only be reached after intensive investigations (Rowan 2007).

The subject of the present study, being possible effects of products from different production systems on normal biological processes, is as yet a very underdeveloped field. So the aim in this study was first of all to explore and identify *differences,* in a broad range of physiological parameters. Then, these optional differences are attempted to be interpreted as physiological functions with respect on health.

1.3 What is health? Concepts of robustness and resilience

In search for biomarkers, it is important to have a perspective on health, to work towards. In the present line of research the ultimate perspective is human health. This is not implicitly the same as the perspective of animal health in production systems, which is mostly represented in the literature that is available as references for the present study.

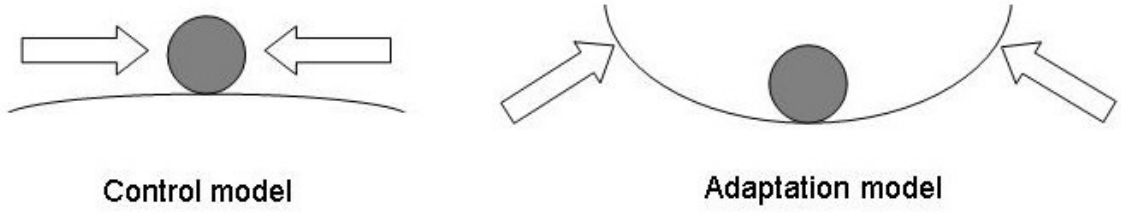
The perspective of human and animal health is partly overlapping and partly different in aspects.

Common ground is the aspect of the ability to survive. Here fitness components are described as the physiological processes to maintain homeostasis, such as in thermoregulation and in the immune system. These are identified as 'maintenance processes'. In animal research also 'production parameters' such as adding weight, production of milk or eggs play an important role, as husbandry has an economic goal (Van Eerden 2005). For human beings production is not so much an important health factor, although fertility is, whereas psychological factors, such as the capacity to experience meaningfulness and a 'sense of coherence', have much influence on health (Antonovsky 1961>1992). Because of this psychological influence on human health parameters, research on effects from nutrition is easier in animals than it is in humans.

Man and animals are comparable where the physical and physiological level are influenced by nutrition, assuming that animals are not bred too dis-harmoniously, e.g. like modern broilers are.

Then, how should 'health' be defined? Health is more and more seen not as static state, but as a dynamical state of wellbeing, influenced by a multitude of factors, with the potential to meet the different challenges, stressors and changes of environment. Disease is seen as a lack of this potential. A healthy organism is able to handle health threats and maintain or restore its homeostasis.

This view is reflected in animal health research, where the concept of 'robustness' meets growing interest. In this context robustness is taken as the capacity to adapt, as a 'resistance' towards disease. In animal husbandry it is important that animals suffer as little as possible from diseases, which means a decrease of productiveness. At the same time there is in society a growing concern about the use of medicines and vaccines, and the wish to reduce the use of these. An increase of robustness in animals would meet interests of farmers as well as of society. So a shift in thinking is occurring from a 'control model', where with medication diseases are overruled, towards an 'adaptation model', where the inborn capacity of being resistant is supported (Ten Napel, 2007).



The aim is to improve this capacity to adapt. Research focuses on factors influencing that.
How should this be evaluated? What are characteristics of a robust animal or system? Kitano (2004) defines robustness 'as a property that allows a system to maintain its functions despite external and internal perturbations. It is one of the fundamental and ubiquitously observed system-level phenomena that cannot be understood by looking at the individual components.' And: 'Robustness is often misunderstood to mean remaining unchanged regardless of stimuli ..... so that the mode of operation is unaffected. In fact, robustness is the maintenance of specific functionalities and this requires often the system to change its mode of operation in a flexible way. Systems that are robust face fragility and performance setback as an inherent trade-off.' So, regarding robustness, functionality is more important than structure.
In a recent Workshop of the Ministry of Agriculture (Workshop LNV, June 2007) on 'natural resistance' in animals, it was stated that until now concepts of 'natural resistance' or 'robustness' are not clearly defined. There was however consensus that the immune system plays a central role.
In this context, the relatively recent notion about the immune system, that the innate immune system (natural immune system) is necessary, and in fact, indispensable in driving subsequent responses from the adaptive (antigen-specific) immune system, is helpful. Effective disease protection and long-term survival, and thus health, are therefore dependent on the interplay between these two arms of the immune system. Besides, the innate immune system is not constant but can be modulated by external (food, infection load, husbandry conditions) and internal factors (stress, hormonal state). The expectation in the present project was that, with respect to robustness, the innate immune system would be most influenced by the different feeding regimes.

How to evaluate functionality? In the context of animal husbandry functionality would comprise continuing production during a disturbance. From the perspective of human health functionality could be considered as good recovery from a disturbance. Here the concept of 'resilience' could be fruitful. Resilience means elasticity. It is currently a prevalent term in ecology and in developmental psychology. In ecology the concept was introduced by Holling in 1973, describing the relationship between resilience and stability (Holling 1973). Currently two definitions of resilience occur: 'engineering resilience' considers how fast a system returns to an equilibrium state after a disturbance (Pimm 1991), while 'ecological resilience' measures how far a system can be perturbed without shifting to a different regime (Holling 1996).
In the field of animal or human physiology, 'resilience' is not yet worked out very much until now. However, to evaluate functionality in relation to health, it might appear logical to study the physiological reaction of an organism, towards a disturbance of the system.
From this perspective, a disturbance is introduced. The choice was made for a non-pathogenic, often used disturbance (further called 'the challenge') and not to take a risk with a pathogen challenge with these valuable

animals at this stage of knowledge. The reaction of the animals to this challenge is evaluated in this study and interpreted from the perspective of resilience.

2 Material & methods – Feed

2.1 Feed ingredients

Primarily the design was simple: chicken were fed with feed from organic or conventional production systems, with an essentially identical feed composition. As primary ingredients for the feed composition were chosen: wheat, barley, triticale, maize, soy and peas. According to organic and conventional norms for chicken feed (Trouw Nutrition-Nutreco Company) a good chicken feed can be composed out of these ingredients, and these products are quite generally produced both organically and conventional. It was considered most ideal if ingredients could be obtained from controlled cultivation systems, like the conventional, organic and biodynamic field trials (DOK-trials) of the Swiss Research Institute of Organic Agriculture, FiBL (www.fibl.org, Mäder 2002). These trials run already since 1978 and are the best documented experiments in this field of research. If for an ingredient no controlled systems would be available, next best was to obtain it from 'best practicing', or at least 'good', neighbouring organic and conventional farms, with the same soil and climate. If possible the same variety (cultivar) of the product is preferred. If for an ingredient this is not realistic, because of cultivation requirements, used varieties should be typical for the production system. Farm management should be documented as complete as possible.
All ingredients were screened for mycotoxins and pesticides, as residues of either of these were not accepted above acceptable standards (Maximum Residu Limits, MRL's) in the feed. The presence of such residues would confuse the interpretation of possible health outcomes.

2.2 Sources and selection procedures

When the project started in summer 2005, it was soon clear that of the amounts of ingredients needed, only barley could be obtained from controlled production, but not from the same variety. It was decided about the other ingredients to search for the best matching, neighbouring, organic and conventional farms. In the Netherlands wheat and triticale and more barley were found, maize and soy were found in Austria and peas in Denmark. The production background of each ingredient was inquired for and documented.

Table 2.1: Overview of the ingredients 2005, background and varieties.

Ingredient	Country	Conventional Location	Organic Location	Conventional Variety	Organic Variety
Wheat	NL	Flevoland province Dronten	Flevoland province Dronten	Bristol	Lavett
Barley	NL	Gelderland province Wageningen	Gelderland province Wageningen	Class	Prestige
Triticale	NL	Gelderland province Veluwe	Gelderland province Veluwe	Talentro	Cairo
Peas	Denmark	Denmark	Denmark	unknown	Unknown
Maize	Austria	Austria	Austria	Mix of 8 varieties	Mix of 5

					varieties
Soy	Austria	Austria	Austria	Essor	Essor

The Swiss DOK-trials of FiBL were taken as 'Golden Standard' for authenticity of the organic and conventional production system. In case a choice was possible between farm pairs, which was so for wheat and barley, protein and amino acid contents were analyzed and compared to analyzed DOK-wheat samples. The samples, which contentwise were closest to the DOK-samples, were chosen to be used in feed manufacturing. For the barley these were the samples from the controlled trial. For wheat it was more complicated. A farm pair which at first appeared appropriate turned to have a mixture of wheat from fields with different soils. Another farm pair was chosen, of which then one wheat turned out to contain some mycotoxin traces. From this same farm, after all, wheat could be obtained which had been ventilated better and which was clean. However, as the chicken of the first generation had already hatched, the first batch of experimental feed was manufactured with another 'safe' wheat, which was not from the farm pair of choice. From the second batch of feed, the used wheats were from the farm pair of choice.
For triticale, peas, soy and maize no choice was available between pairs of farms.
Variety was only the same in the case of the soy. Maize could only be acquired as a mixture of varieties, being however nearly the same for both production systems. The products are more extensively described from an agricultural viewpoint in Annex 2.

2.3 Feed composition and production

The feed was formulated and manufactured in cooperation with the well known Dutch organic animal feed manufacturer, firm Kruyt in Gouda.
All ingredients were transported in July and August 2005 in 50-100 kg bags of either jute or plastic to the Louis Bolk Institute in Driebergen NL, stored in a shed and first checked for residues. Once found clean, all but the soy beans, were transported to the production firm in Gouda. In this factory the ingredients were stored under the same conditions (except for being in small bags) like all other ingredients the firm uses, being dry, quite dark and at a temperature between 10°-17° C.
Freeze drying of the ingredients was considered, but the feed production firm had bad experiences with animals not wanting to eat feed from such storage. Storage in cooling cells was discussed, but would be very impractical and was not considered as necessary by the manufacturer, so was not chosen for. The soy beans were transported to Wageningen UR, where they were stored in a shed, dry, dark and at a temperature between 10°-17° C.

As a template for the feed composition generally accepted guidelines (Trouw Nutrition) for nutritional content in chicken feed were followed, with some adaptations for this experiment. As the aim was to search biomarkers for health effects, the ingredients with their differences were to be processed and supplemented to the least possible extent, so the effect of the differences from the feed origin could be maximal. However, deficiencies in nutritional content had to be avoided as these could produce harmfull effects and thus confuse the results. Because of this the feed was composed in accordance with nutrient guidelines for chicken feed (best quality) and supplemented where necessary to avoid major deficiencies in the menu (ration).

A first adaptation was made in relation to the organic feed. With the available ingredients wheat, barley, triticale, maize, soy and peas, and the common restrictions on maximal percentages of these ingredients in feed, only a feed could be formulated in which a slight protein shortage remained. Best suitable option to solve this shortage was the supplementation with the essential amino acid methionine and potato protein. However, the latter posed a problem. In conventional potato protein the potentially harmful anti nutritional factor solanin is washed out, whereas in organic potato protein this is not the case. As solanin might harm the gut of the young chicken, it was considered unwise to include organic potato protein within this experiment. Omitting this solanin would not exclude something 'typically' organic, so it was decided to supplement both feeds with conventional potato protein, Protastar (Annex 3).
Secondly an adaptation concerned the processing of the soybeans into flakes. In conventional feed production soy pellets are used, produced with the use of chemicals. According to organic regulations, chemical procedures in processing of organic products are restricted, aiming to avoid contamination with chemical residues. For this reason chemically produced soy pellets, that are generally used in conventional chicken feed, were replaced, also in the conventional feed, by mechanically produced flakes.
A third adaptation concerned the standard supplemented ingredients. In organic as well as in conventional feed, standard supplements used are: fat, chalk, salt and a 'premix' of small doses of vitamins and minerals, FX Layers premix (Annex 4). The feed manufacturer considered this a minimum requirement, that could not be excluded. However, an additional stronger supplementation with vitamins, as is usual in conventional chicken feed, was not used.
In general the chickenfeed is adapted to age; young chicks require a different feed composition compared to adult chicken. Therefore three different feeds were composed for the following age groups: Starter feed for 0 – 6 weeks, Grower feed for 7 – 17 weeks and Layer feed for 18 weeks and older. The feed was at all ages supplied to the chicken in form of chicken meal. The composition of the three different feeds used is shown in Table 2.2.
The composition of the feed was checked and agreed on, prior to the start, by the Dutch chicken feed specialist Dr. R. Kwakkel of Wageningen UR.

Table 2.2: The composition of the chickenfeed with the six basic ingredients and additional feed components.

Feed compositions					
Starter, 0-6 weeks		**Grower, 7-17 weeks**		**Layer, from 18 weeks**	
Ingredient	Percentage	Ingredient	Percentage	Ingredient	Percentage
Maize	20,00%	Maize	20,00%	Maize	25,00%
Wheat	30,00%	Wheat	26,42%	Wheat	25,23%
Barley	5,00%	Barley	10,00%	Barley	5,00%
Triticale	12,05%	Triticale	0,00%	Triticale	0,00%
Soybeans heated	0,00%	Soybeans heated	10,17%	Soybeans heated	19,87%
Soy flakes	10,16%	Soy flakes	20,00%	Soy flakes	0,00%
Peas	10,00%	Peas	10,00%	Peas	10,00%
Potato proteins	7,00%	Potato proteins	0,00%	Potato proteins	2,50%

MonoCalcFos	1,13%	MonoCalcFos	0,73%	MonoCalcFos	1,01%
FX Layers premix	1,00%	FX Layers premix	1,00%	FX Layers premix	1,00%
Fat of plant origine	1,50%	Fat of plant origine	0,00%	Fat of plant origine	0,52%
Salt	0,07%	Salt	0,09%	Salt	0,06%
Chalk	1,64%	Chalk	1,16%	Chalk	7,65%
Shells broken	0,00%	Shells broken	0,00%	Shells broken	2,00%
$NaCO_3$	0,09%	$NaCO_3$	0,08%	$NaCO_3$	0,00%
Methionine	0,11%	Methionine	0,04%	Methionine	0,15%
Total (100%)	99,75%	Totaal (100%)	99,69%	Total (100%)	99,99%

The process of feed manufacturing

In the process of feed manufacturing, first an appropriate amount of soy beans was treated at Wageningen UR to inactivate the trypsine inhibitoring factor. This was done by 'toasting' the beans according to the procedure as described by Qin (1996) in his thesis on the theme.

Then these beans were transported to a small company in Germany, 'Alkraft', specialized in organic oil production. Here the soy beans were pressed mechanically, resulting in flakes and oil, both used in the feed; the flakes/beans ratio changing along the developing age of the chicken.

From Germany the soy was transported to Gouda, where a new batch of feed was then made.

For making a batch of feed, individual ingredients were weighed securely and gathered at a wooden pallet, thus resulting in two pallets, one with organic, one with conventional ingredients.

The ingredients were then grinded and mixed. According to the type of feed for different ages of the chicken, different sizes of sieves were used. Before this procedure, the system of the factory was emptied. First the conventional ingredients were poured in, grounded and mixed. The mixed feed was gathered in 50 kg bags and was sampled for amino acid analysis.

When the system was empty, it was 'cleaned' with some organic feed and then the organic ingredients were poured in and the procedure was repeated.

After this production phase the feed was analysed again, because it is known that the level of the amino acid methionine is, to a certain degree, insufficient in chicken feed composed according to organic regulations. So after each round of producing a new batch of feed, an amino acid check was performed on the content of the produced feed. Samples of both the organic and conventional feed were analysed for amino acids by TNO Quality of Life. Awaiting the results, the feed was stored in the factory.

According to the outcomes of the analyses, amino acids, mainly methionine, were added to each feed up to the minimal required level. Consequently the feed was once again mixed in the emptied system.

The feed was packed per 25 kg in paper bags. Then it was blinded and coded by the director of the production firm, as either A or B. Envelops containing the blinding codes were checked for consistency by an independent outsider and further stored in a safe till the end of the project.

The feed was transported the same or next day to Wageningen, where the experimental animals were housed. There the feed was stored in the shed next to the chicken.

Each six weeks a new batch of feed was made.

Table 2.3: The feeds that were fed subsequently in pairs A and B, with their English and Dutch name, code and numbers, are:

Type of Feed	**Dutch name**	**Codes used for the feeds**	
		A	B
Grower	Opfok 2	2	1*
Layer	Legvoer	3 and 7	4 and 8
Starter	Opfok 1	12	11
Grower	Opfok 2	16	15

Nr.1 containing a different wheat than Nr. 15.

Suppletion As deficiencies in the feed should be avoided, samples of the produced feed were analysed by TNO on amino acid composition, after each round of feed manufacturing.

Standards for amino acid content were taken from the guidelines for chickenfeed (Trouw Nutrition). What counts is the (estimated) digestibility of the amino acids, as they come from the different ingredients. Measuring the digestibility was not possible during most of the period of feed manufacturing. Only near the end of the project, TNO did measurements on protein digestibility (Ch.3.2.1)

We acknowledge our colleague institute Schothorst Feed Research, for calculating the expected digestibility of amino acids in the different feed compositions that we used.

Based on these tables, the results of TNO's amino acid analyses were converted into values of digestible amino acids. From these figures, the amounts of amino acids were calculated, that needed to be suppleted, to reach the minimum levels of the guidelines. This meant always a slight suppletion of methioninee and once also lysine and threonine.

Table 2.4:Amounts of suppletion in g/kg of three essential amino acids in the feeds.

	Grower 1st generation		**Layer**		**Starter**		**Grower 2nd generation**	
	Organic	**Conventional**	**Organic**	**Conventional**	**Organic**	**Conventional**	**Organic**	**Conventional**
Methioninee	0,52	0,26	1,02	0,83	0,74	0,66	0,29	0,15
Lysine					1,30	0,24		
Threonine					0,14	0,00		

2.4 Analysis of ingredients and feed

One of the main questions formulated in the project 'Organic, More Healthy?' was whether differences could be found between feeds and their ingredients from organic and conventional production systems. In order to answer this question analyses were performed on contaminations, macro- and micronutrients, microbiology and the complementary measurements biocrystallization and biophotons. Here either *ingredients* or *feed* made out of these ingredients, will be described as such.

Coding of the samples All samples, ingredients, feeds and animal samples, were coded in such a way that no-one, working with certain samples, new the origin. Different, independent people coded. Each lab received different codes. The feeds were finally coded A and B by the manufacturer and these codes were locked away, after being checked for consistency by an independent person.
When it was decided to keep the codes A and B closed till all interpretations were written down, it was agreed that data of the feed and ingredient analyses would be made connectable to the chicken data.
This was realized by the same independent person mentioned earlier, who contacted the feed manufacturer. Just before printing of this report the A and B codes were broken.

2.4.1 Mycotoxins, Pesticide residues, Dioxins, ANF's

Before the processing of the feed, all ingredients were pre-screened for the presence of contamination with mycotoxins or pesticide residues in order to exclude contaminated batches. The analyses were performed by RIKILT Institute of Food Safety in Wageningen, the Netherlands. During the experiment also some feeds were analyzed for undesired substances by RIKILT, Masterlab and IRAS.

Mycotoxins The samples were analyzed, using an LC-MS/MS multimethod, for mycotoxins, including Aflatoxin B1, fumonisins B1 and B2, Ochratoxin, Zearalenone, Deoxynivalenol, T2 and HT2. In short, from each sample two portions of 10 grams are taken. One portion was extracted with water and the other portion with a mixture of acetonitrile/water (84:16 V/V). Both portions were extracted by shaking intensively for at least 3 hours. After filtering from each extract 250 µl was mixed and injected on a LC-MS-MS system in MRM mode.

Pesticides residues Samples were extracted with acetonitrile, in some cases preceded by water. After addition of magnesium sulphate and salt, the mixture was centrifuged and an aliquot of the acetonitrile was purified with primary-secondary amine. The extract was injected on a GC-TOF/MS, equipped with an RTX-50 column (30 m x 0.25 mm). The method quantifies a number of selected pesticides and detects a large number of others qualitatively. If detected, these pesticides would be quantified by injection of standards. LOQs vary between 0.01 and 0.5 mg/kg. The method is able to detect over 300 pesticides.

Dioxins and PCBs Dioxins and PCBs were determined by RIKILT in samples of ten eggs derived from chicken fed feed A or B. Fat was extracted, spiked with labelled standards and purified on a Powerprep system (FMS). The two fractions were analyzed by HRGC/HRMS and the TEQ level determined using the 1998 TEF values. For the DR

CALUX®-assay, fat samples were purified on acid silica and subsequently tested in combination with a set of reference butter fat samples.

Anti Nutritional Factors (ANF's)

Lectins

Samples of untreated soy and peas, as well as of feed were analyzed by Masterlab for the presence of lectins. Samples were extracted with saline and transferred to a N-acetyl-galactosamin-agarose column. After washing of the column with saline, the lectins were eluted with a solution containing galactose. Subsequently the protein was determined with the method of Lowry. The detection limit is 0.05 mg/g.

Anti-trypsine factor

Masterlab also analysed toasted soy for the activity of anti-trypsine factor. Trypsine was extracted from the sample at pH 9.5. The activity was measured with a substrate of benzoyl-l-arginine-p-nitro-anilide (L-BAPA). A spectrophotometer is used to measure the amount of p-nitro-aniline. The detection limit is 0,30 mg/g.

2.4.2 Macro and micronutrients

Macro- and micronutrients were analyzed by TNO Quality of Life, Analytical Research Department, Zeist, both in feeds and in the ingredients from which the feeds were constituted. For feeds, mainly macronutrients, e.g. amino acids, protein, fat and fatty acids, were determined, as well as vitamins E, folate and some trace elements. For all ingredients macronutrients as well as micronutrients, i.e. vitamins and trace elements/heavy metals/minerals and bioactive compounds, i.e. catechines, carotenoids, flavanoids, isoflavones (only for soy samples) and phytosterols, were determined. A short description of the various methods is presented here. All experiments were carried out in duplicate. If the duplicate analysis showed a difference larger than 10%, the analysis was repeated. The mean values of the duplo are reported. In Table 2.5 an overview of the methods for the different analyses and the analyzed compounds is presented.

Table 2.5: Overview and short description of the various nutritional analyses.

Class of nutrients	Nutrients	Method
Amino acids	Cystine, Methioninee, Aspartic acid, Threonine, Serine, Glutamic acid, Proline, Glycine, Alanine, Valine, Isoleucine, Tyrosine, Phenylalanine, Histidine, Lysine, Arginine, Tryptophan	All proteins in the samples are hydrolyzed by boiling in hydrochloric acid. De resulting amino acids are subsequently separated by ion-chromatography, derivatized post-column and quantified using a amino acid analyzer (TNO Standard Operating Procedure AZA/001, AZA/002). For tryptophan the hydrolysis is carried out with bariumhydroxide. Analysis is carried out with HPLC-Flu (TNO Standard Operating Procedure AZA/003)
Macronutrients	Ash content	Samples, after preheating, are heated at high temperature. The residue is weighed (TNO Standard Operating Procedure LNC/167)

	Total carbohydrates	Samples are rendered soluble in boiling water. Amylum is converted to soluble carbohydrates. The carbohydrates are hydrolyzed to mono-saccharides and subsequently analyzed by the Luff-Schoorl method (TNO Standard Operating Procedure LNC/039)
	Raw fiber	Samples are boiled in acid and diluted alkaline solution. Remaining solid substances are ashed (TNO Standard Operating Procedure LNC/011)
	Crude fat	Samples are hydrolysed with hydrochloric acid and subsequently extracted with petroleumether. The extract is evaporated and the residue is weighed (TNO Standard Operating Procedure LNC/139)
	Moisture	Samples are dried and weighed before and after drying (TNO Standard Operating Procedure LNC/069)
	Protein	Samples are destructed in which organic nitrogen is converted to ammonium. Ammonium is converted to ammonia. Protein content is calculated from the nitrogen-amount (TNO Standard Operating Procedure LNC/101)
	Fatty acid composition	Fat is saponificated and subsequently transformed to fatty acid methyl-esters using BF3. The fatty acid methyl esters are analyzed by GC-FID. Quantification is carried using external calibration of reference compounds (TNO Standard Operating Procedure LLC/067)
	Chloride	Samples are extracted with water and diluted nitric acid. The amount of Cl is determined potentiometrically (TNO Standard Operating Procedure LNC/216)
	Choline	All choline containing compounds are isolated by hydrolysis. Choline is subsequently converted to hydrogen peroxide and the latter is determined using extinction at 505 nm (TNO Standard Operating Procedure LNC/130)
Phytosterols	Cholesterol Campersterol Stigmasterol Sitosterol Avenasterol	Sterols are extracted using alkaline water/ethanol. The mixture is subsequently extracted with hexane. The organic phase is evaporated to dryness and subsequently derivatized with MSTFA. The derivatized sterols are analyzed by GC-FID. Quantification is carried out using dihydrocholesterol as internal standard (TNO Standard Operating Procedure LLC/081)
Isoflavones	Daidzin, Genistin, Daidzein, Genistein, Glycetein, Glycetin, Malonyl daidzin, Malonyl	Samples are extracted with ethanol/water. After filtration the extracts are analyzed by HPLC-DAD. Quantification is carried using external calibration of reference compounds (TNO Standard Operation Procedure FYT/009)

	glycetin, Acetyl daidzin, Acetyl glycetin, Malonyl genistin, Acetyl genistin	
Vitamins	Vitamin B1 (total thiamine)	Samples are extracted with acid and subsequently hydrolyzed enzymatically to obtain free thiamine. The total thiamine content is determined by HPLC-Flu (TNO Standard Operating Procedure VIT/010)
	Vitamin B2 (FAD)	Samples are extracted with acid and subsequently hydrolyzed enzymatically to obtain free riboflavine. The total riboflavin content is determined by HPLC-Flu (TNO Standard Operating Procedure VIT/012)
	Vitamin C (ascorbic acid)	Samples are extracted with acid and subsequently enzymatically oxidized. The formed dehydroascorbic acid is derivatized and analyzed by HPLC-Flu (TNO Standard Operating Procedure VIT/014)
	Vitamin E (α,β,γ,δ-tocopherol)	After saponification of the sample, tocopherols are extracted and analyzed by HPLC-Flu (TNO Standard Operating Procedure VIT/113)
	Vitamin K1 (phylloquinone)	After enzymatic digestion of the sample, an organic extract is analyzed by HPLC-Flu after reduction (TNO Standard Operating Procedure VIT/021)
	Vitamin B5 (pantothenic acid)	After acidic extraction, Vit B5 is analyzed by LC-MS (TNO Standard Operating Procedure VIT/035)
	Vitamin B3 (total niacin)	Samples are extracted with acid and subsequently analyzed by HPLC-Flu (TNO Standard Operating Procedure VIT/110)
	Total folate	Sample extracts are added to culture medium. From the growth of bacteria the total folate concentration is determined (TNO Standard Operating Procedure VIT/107)
	Biotin (Vitamin H)	Sample extracts are added to culture medium. From the growth of bacteria the biotin concentration is determined (TNO Standard Operating Procedure VIT/100)
	Vitamin B6 (pyridoxal, pyridoxol, pyridoxamine)	Samples are extracted with acid and subsequently hydrolyzed enzymatically. The subsequent extracts are analyzed by HPLC-Flu (TNO Standard Operating Procedure VIT/015)
Trace elements, heavy metals and minerals	Cd, Ca, Cr, Co, Cu, F, I, Fe, Pb, Mg, Mn, Hg, Mo, Ni, Zn, P, K, Se, Na, As	Samples are destructed by nitric acid digestion or ashing. The resulting solutions are analyzed either by ICP-AES (TNO Standard Operating Procedure LSP/057) or ICP-MS (TNO Standard Operating Procedure LSP/055)

Flavanoids (conjugated and free)	Naringenin Hesperetin Luteolin Apigenin Myricetin Quercetin Kaempferol Isorhamnetin	Homogenized and freeze-dried material is extracted with methanol/water (50:50 v/v). The supernatant is analyzed by HPLC-DAD (290, 345, 370 nm). Quantification is carried using external calibration of reference compounds (TNO Standard Operating Procedure FYT/004) Total flavanoids are determined in a similar way with the addition of a hydrolysis step with HCl (TNO Standard Operating Procedure FYT/005)
Carotenoids	Lutein Zeaxanthin β-cryptoxanthin α-carotene β-carotene lycopene	Samples are extracted with ethanol/tetrahydrofuran/dichloromethane. Carotenoid-esters are hydrolyzed to free carotenoids by saponification. The resulting extract containing all free carotenoids are analyzed by HPLC-UV (450 nm). Quantification is carried using external calibration of reference compounds (TNO Standard Operating Procedure VIT/058)
Catechins	Catechin, Epicatechin, Gallocatechin, Epigallocatechin, Catechin gallate, Epicatechin gallate, Gallocatechin gallate, Epigallocatechin gallate, Theobromin, Theophyllin, Caffein	Samples are extracted with boiling water. The extract is filtrated and acidified. The resulting clear extract is analyzed by HPLC-UV (276 nm). Quantification is carried using external calibration of reference compounds (TNO Standard Operating Procedure FYT/001)

Abbreviations: HPLC: high pressure liquid chromatography; Flu: fluorescence; GC: gas chromatography; FID: flame ionization detector; MSTFA: N-methyl-N-(trimethylsilyl)trifluoroacetamide; UV: ultraviolet; MS: mass spectrometry; LC: liquid chromatography; ICP: induced coupled plasma; AES: atomic emission spectroscopy; DAD: diode array detector.

Protein digestibility/amino acid availability The nutritional quality of proteins is depending on their amino acid profile and their digestibility. Essential amino acids can not be produced by the body and need to be supplied in sufficient quantities in the feed. The amino acid that is in shortest supply in relation to the need is referred to as the limiting amino acid.

A validated in vitro model of the gastrointestinal tract was used to evaluate the amino acid availability. The TNO gastro-Intestinal Model (TIM, Minekus 1995, 1998; Minekus and Havenaar 1996, 1998) simulates very closely the successive dynamic conditions in the gastrointestinal tract, such as the pH curves, (pro-) enzymes in the stomach and small intestine, and bile salts in the different parts of the gut. Gastric emptying, small intestinal passage and secretion of digestive fluids are also controlled, to reproduce realistic species- and meal- depended conditions. Small molecules, such as the products of protein digestion, are removed from the chyme with hollow fibre membranes.

Validation experiments with various types of food products showed the reproducibility and reliability of the results for the digestibility and the absorption of nutrients in comparison to in vivo experiments. For the amino acid availability in the present study a dedicated system was used, specially developed to study protein quality (tiny-TIM) in small animals as chicken and rats.

The amounts of protein and amino acids were determined for all samples prior to the determination of the true digestibility. The amino acid profile was determined according to the AOAC method (1984). Protein nitrogen was analyzed using the Kjeldahl method.
Next, samples were tested by digesting a quantity containing 5 g of protein. After 5 hours of digestion, the dialyzed fraction was sampled to be analyzed for the amino acid profile and/or protein nitrogen. A blank experiment was performed and analyzed to determine the contribution of secreted proteins.
The true amino acid digestibility was determined using the concentration of amino acids in the product and in the digested fraction, using the following formula:

$$\text{True digestibility (\%)} = \frac{\text{digested amount of amino acid from product - digested amino acid amount of from blank}}{\text{amount of amino acid in the product}} \times 100\%$$

Correction with the digestibility and bioaccessibility of the limiting amino acid was carried out.

The limiting amino acid (LAA) was calculated by the following formula:
LAA = (AA/Lys*100)/MAA

Where AA is the amount of dialyzed amino acid; Lys is the amount of dialyzed lysine; MAA is the minimal requirement of the amino acid in growing broilers as compared to lysine.
The determination of the true digestibility and limiting amino acid (LAA) was carried out for most feeds. Due to financial limitations this analysis had to be performed in one run, and not on all feeds available and not all feeds in the same state. Of one feed pair, both the supplemented and non-supplemented samples were analyzed, of some feeds only the supplemented samples were analyzed and of one feed only the unsupplemented version was available. The final feed pair was not analyzed, as it was not available yet.

2.4.3 Microbiology

2.4.3.1 Microbiology by TNO

Microbial diversity of the ingredients and feed was measured to study potential differences in type and number of micro-organisms, as a results of differences in cultivation circumstances. It is known that microbes have an effect on the composition of the intestinal flora in both men and animal (Noverr 2004). Intestine function and with this intestinal health is very closely related to a healthy intestinal flora. From literature it is known that the intestinal flora is also related to the immune system through the mucosal immune system (Guarner 2003).

The experiments were carried out at the department of Microbiology at TNO Quality of Life, Zeist. On arrival the samples were stored at ambient temperature. For Salmonella analysis 25 g of the sample was weighed in 225 ml buffered peptone water. For the other analyses, 5 to 20 g of product was weighed and diluted 10 times with a peptone physiological saline and homogenized in a blender for 1 minute. An overview of the various parameters studied and methods used are shown in Table 2.6.

Table 2.6: Methods used for microbial analysis of feed and ingredients.

International standard	Parameter	Medium	Handling	Incubation		
				T (°C)	t	Conditie
ISO 4833	Aerobic micro-organisms	Plate Count Agar (PCA)	-	30	3 d	Aerobic
ISO 7402	Enterobacteriaceae	Violet Red Bile Glucose (VRBG)-agar	-	37	24 h	Aerobic
ISO 16649-2	Escherichia coli	Violet Red Bile Lactose (VRBL)-agar*	-	44	24 h	Aerobic
ISO 7954	Yeasts and moulds	Oxytetracycline Gist Glucose Agar (OGGA)	-	25	5 d	Aerobic
**	Sulphite reducing Clostridium spp.	Sulfiet polymyxine agar (SuA)	-	30	3 d	Anaerobic
ISO 7937	Clostridium perfringens	Tryptose Sulphite Cycloserine (TSC) agar	-	37	20 h	Anaerobic
ISO 6888-1	Staphylococcus aureus	Baird-Parker (BP)-agar	-	37	48 h	Aerobic
ISO 7932	Bacillus cereus	Mannitol Egg Yolk Polymyxine (MYP)-agar	-	30	24 h, 48 h	Aerobic
ISO 6579	Salmonella spp.	Buffered peptonwater (BPW)	25 g decimal diluted in BPW	37	16-20 h	Aerobic
		Rappaport-Vassiliades Sojapepton (RVS)	0.1 ml of BPW buildup in 10 ml RV-bouillon	42	24 h	Aerobic

	bouillon				
	Muller-Kauffmann tetrathionaat-novobiocine (MKTTn) bouillon	1 ml of BPW buildup in 10 ml MKTTn bouillon	37	24 h	Aerobic
	Phenol red/brilliant green agar (BGA)	Inoculated from RVS and MKTTn after 24 h	37	24 h	Aerobic
	Xylose Lysine Deoxycholaat agar (XLD)	Inoculated from RVS and MKTTn after 24 h	37	24 h	Aerobic

** i.o. TBX agar.*

*** Microbiologie van Voedingsmiddelen, Methoden, principes en criteria, ISBN 90-72072-65-0, 2003.*

2.4.3.2 Microbiology by Wageningen UR – Biological Farming Systems Group

In connection to microbial analyses of the chicken manure of specific animals, after the section, Wageningen UR – Biological Farming Systems Group performed a microbial community analysis on the feed, consumed by the chicken during the period before the section. Starter and Grower feed A and B, used for the second generation of chicken, was stored at -20°C for about one year. After thawing, the feed was sieved (< 2 mm). DNA was isolated from the 4 feed batches (250 mg per batch) in July 2007, using the Bio101® Systems FastDNA® SPIN® Kit for Soil (Qbiogene, Inc., Carlsbad, CA, USA). The 16S rRNA gene of eubacteria was amplified from the extracted DNA with the eubacterial primer pair 968f with an attached GC-clamp and 1401r, using a touchdown scheme for 30 thermal cycles. Denaturing gradient gel electrophoresis (DGGE) was performed using the DCode system (Bio-Rad Laboratories, Hercules, CA, USA) and a 6% acrylamide gel (37.5 acrylamide:1 bisacrylamide) with a 45–60% denaturing gradient to separate the generated DNA amplicons (bands). DGGE electrophoresis was carried out in 0.5x TAE buffer for 16 h at 100 V at a constant temperature of 60°C. Gels were stained with Bio-Rad's Silver Stain (Bio-Rad Laboratories, Hercules, CA, USA). Dendrograms of DNA bands of eubacteria from feed were constructed using the UPGMA clustering method (TotalLab™ TL120 software, version 2006d, Nonlinear Dynamics Ltd.).

2.4.4 LPS endotoxin residues

Samples of the 1st generation Grower feed and first batch of Layer feed, and at a later stage samples of the Starter feed and Grower feed of the 2nd generation, were analysed for LPS endotoxin by Institute of Risk Assessment (IRAS) of University Utrecht. Ground animal feeds (0.7-0.9 gram) were transferred to 50 mL polypropylene tubes (Greiner) and 20 mL extraction fluid (pyrogen-free water with 0.05% Tween-20) was added. After 1 hr incubation in an end-over-end roller at room temperature, the tubes were centrifuged for 15 min at 1,000 g, and four aliquots of 0.1 mL of the supernatant were added to pyrogen-free glass tubes, and stored at -20°C until endotoxin analysis. Endotoxin in

the extracts was measured with the kinetic Limulus Amebocyte Lysate test kit (Bio-Whittaker/Cambrex; Kinetic-QCL; LAL lot no. 3L433E) calibrated with E.coli 055:B5 endotoxin (lot no. 3L2950), essentially as prescribed by the manufacturer. The calibration line was constructed with the standard at 12 two-fold dilutions starting at 25 endotoxin units (EU) per mL. Feed extracts were tested at dilutions 1/1,000, 1/5,000 and 1/25,000, and the mean of the three obtained values (all within the range of the calibration line) was used as the final results. CV values for the three dilutions were for all samples <20%).

2.4.5 Complementary analyses – Delayed Luminisence – FAS – Biophotons

The investigation was performed by KWALIS Qualitätsforschung Fulda GmbH in Germany. The investigation comprised measurement of fluorescence-excitation-spectroscopy (FAS). The method is based on the effect that plant samples after optical excitation emit light (induced luminescence), decreasing during a time ranging from seconds to hours, depending on the kind of sample. The wavelength of the light emitted is longer than the excitation wavelength (fluorescence). The emission is measured by broadband photomultipliers in the spectral range between 260 – 850 nm. In general, the intensity and time course of the emission are sample specific. In order to obtain spectra, multiple excitations are performed using 7 different spectral ranges from dark red (on the border of visibility) to near ultraviolet (780 nm – 360 nm). In addition a broadband (white) excitation is used. The excitation ranges are obtained by filtering white light (tungsten lamp) with standard coloured glass filters (Schott). The excitation ranges have a bandwidth of ≥ 80 nm for uv, blue and green. For excitation at yellow, light red, red and dark red the longpass filters applied have edges at 530, 590, 630 and 695 nm resp.. Spectra are obtained by plotting the emission in dependence of the excitation range (Strube et al. 2004; Strube 2005). A dataset consisting of 120 parameters is calculated out of the emission during one measurement cycle (8 colour-different excitations) of a sample. For the assessment of samples, selected parameters are used. Selection depends on the kind of sample being analysed.

Coded samples of wheat (code E and Z), barley (code K and W), triticale (C and I), maize (G and R), soy (A *(different from the feed-A!)* and S) and peas (M and T), were send to KWALIS (Germany) for analyses. For the investigation the samples were not milled. Equal conditions for drying and equilibration of the samples were provided by bringing them in a common atmosphere dried by silicagel and heated to 40°C. Before measurement the samples were stored in airtight glass flasks. The temperature for intermediate storage and measurement was 15°C. Multiple measurements were performed as sample repetitions. The samples were measured in quartz cells during 10 seconds after 5 seconds of optical excitation. For further details see Strube (Strube 2005).

For assessment of the wheat samples a subset of 10 parameters involving 5 colours was used. A training dataset was generated, using organic and conventional wheat samples, measured in 2004. This dataset was normalized to zero by subtracting the mean value and dividing by the standard deviation. By discriminant analysis a canonical variable was calculated out of the 10 parameters for best separation between organic and conventional samples. For the unknown wheat samples under test (year 2005) the canonical variable was calculated out of the actual measured data and by using the formula, derived from the training dataset of 2004. The canonical data was used to assess the samples of 2005.
For barley, peas, maize, soy and triticale training data are not yet available. So a different approach was used to distinguish between samples. For the assessment of barley, peas and triticale the ratio of the emission (time range 6-

10 seconds), related to excitation by yellow and blue was used. For the assessment of soy and maize the emission (time range 6-10 seconds), related to excitation by white was used.

2.4.6 Complementary analyses – Protein ratio

The investigation was performed by KWALIS Qualitätsforschung Fulda GmbH in Germany. Coded samples of wheat (code E and Z), barley (code K and W) , triticale (C and I), maize (G and R), soy (A and S) and peas (M and T), were analysed for protein ratio by KWALIS.
For determination of raw protein: samples were grinded; digested with sulfuric acid/catalyst, followed by alkalinisation, steamdestillation, titrimetric quantification in 3 replicates. Determination of oven-dry mass, 3 replicates Determination of aminoacids: samples were grinded; hydrolysed with HCl followed by identification and quantification with IC/UV and HPLC/fluorescence; For all samples 2 sample preparations were made with 2 injections per sample preparation. Determination of oven-dry mass, 3 replicates.

The quantification of the proteinogenic amino acids in wheat (with exception of cysteine, methioninee und tryptophane) was carried out according to VDLUFA methods after acid hydrolysis. The homogenized and comminuted sample (ca. 0,8g) was hydrolysed with 6N hydrochloric acid for 24h at 110 °C in a nitrogen atmosphere in a closed bottle. The amino acids were determined by UV detection with amino acid analyser after post-column derivatisation with ninhydrin (570 or 440 nm respectively) and reversed phase hplc with UV detection after precolumn derivatisation with OPA/FMOC (HP-Aminoquant; Hypersil AA ODS; 338 nm).
The quantification of the sulphur containing amino acids cysteine and methioninee in wheat was carried out according to VDLUFA methods with oxidation prior to acid hydrolysis. The homogenized and comminuted sample (ca. 0.8g) was oxidised with formic acid/hydrogen-peroxide for 24h at 0 °C. The oxidation was stopped with sodium disulfite and the further analysis was performed as described above.
The quantification of the tryptophane in wheat was carried out according to VDLUFA methods with alkaline hydrolysis. The homogenized and comminuted sample (ca. 0.8g) was hydrolysed with 4 n LiOH for 24h at 110 °C under nitrogen atmosphere in a closed bottle. The further analysis was performed as described above.

2.4.7 Complementary analyses – Biocrystallizations

The investigation was performed by Louis Bolk Institute. Parallel to the experiments performed at the Louis Bolk Institute, a subset of the above mentioned samples was analysed by the international Triangle network partners: the University of Kassel – Department of Food Quality and Food culture in Germany and the Biodynamic Research Association Denmark (BRAD). The partners received different codes.

Biocrystallizations were performed as a holistic measure for analysing the quality of the ingredients and the feed. This method is developed from the point of view that living organisms contain an 'inner structure', constituting the compounds in the organism.

Analyzed were all ingredients: maize, barley, soy, triticale, pea and wheat and some of the feeds: Layer feed 1st generation, Starter feed 2nd generation and Grower feed 2nd generation. From all samples damaged and broken kernels and remaining foreign particles were removed. 100g of each sample was grinded (Retsch ZM 100; 14.000

rpm; 1.0 mm ring sieve). 50.0g was extracted in 450.0 ml water (25°C) on a horizontal shaker (200 rpm; 30 min). For soy and pea, 25.0g was extracted in 475.0 ml deionised water.
50 ml extract was filtered over respectively Whatmann 41 and 40 paper filters. Due to clogging of the filter, soy and pea extracts were filtered over a nylon sieve (pore size 20 micron). Filtrates were added to $CuCl_2$ solutions and pipetted in 4-6 fold replicate in the crystallisation chamber (Andersen 2001; Kahl 2007). All samples were prepared in replicate, and analyzed over several days (2 or 3 depending on the number of samples).

Table 2.7: Samples with the used concentration ratio (extract/CuCL2) and laboratory performing crystallization.

Ingredient or feed	Sample nr.	Concentration ratio*	Performed by#
Maize	54 and 55	190/120*	LBI/Uni-Kassel
Barley	58 and 59	80/240	LBI/Uni-Kassel
Soy	50 and 53	80/230	LBI
Soy reference (DOK)	60 and 61	80/230	LBI
Triticale	62 and 63	70/130	LBI
Pea	51 and 52	100/250	LBI
Wheat	64, 65	70/90	LBI/Uni-Kassel/BRAD
Wheat reference (DOK)	66 and 68	70/90	LBI
Layer feed	83 and 84	90/120	LBI
Starterfeed	85 and 86	60/90	LBI
Grower feed	87 and 88	60/90	LBI

**190mg maize extract with 120mg $CuCl_2$ in a total volume of 6.0ml.*

 # LBI -Louis Bolk Institute, Uni-Kassel - University Kassel, Dept. of Food Quality & Food Culture; BRAD - Biodynamic Research Association Denmark .

Texture analysis

For the computerized analysis, the crystallizations were scanned and a circular Region Of Interest (ROI 1-100% of the crystallisation surface around the geometric centre) was analysed with the ACTA software (Andersen et al. 1999). Results are presented using three selected ROIs (50, 70, 90%). The software based evaluation system is described in Busscher 2007 (in concept). The computed variables can be grouped in three groups, on the basis of a positive correlation. Variance analysis with a 'linear-mixed-effects' (lme) model Programme R was applied for the statistical analysis of the variables (Meelursarn 2007). Output generates the p- and F-values for the different variables relative to the ROI. Only combinations of ROIs and the 3 group-variables yielding data with a non-significant Shapiro-Wilk and Bartlett-test and a stable progression over ROI were used for the evaluation.

Visual Evaluation

For all analysis the Visual Evaluation criteria for conventional profiling were applied (Huber 2007).
'Two Group Testing' is a visual classification method to assess the statistical significance of the classification of crystallizations originating from 2 samples. For this, individual crystallization pictures were classified as belonging to

group X or Y. This procedure was repeated till 100 pictures are classified. The Chi squared test was used to test the hypothesis that the classification was not due to chance.
The 'Simple descriptive test' was applied to describe the main characteristics and the gesture of the crystallisation pictures. An overall characterisation of these two groups of pictures is given. An assumption was made which groups of pictures belong to the same production system, and which system is organic or conventional.

2.4.8 Profiling of ingredients

A metabolomic study of the polar fractions of the chicken feed used has been carried out by RIKILT-Institute for Food Safety to see if differences in the low molecular weight polar components of the different feeds could be observed by ^{1}H NMR.
The samples analysed in this study were 8 chicken feeds, 4 of which were given to the first generation of chicken and the other 4 that were used to feed the second generation. Analysed were Grower feed, coded as 2 and 1) and Layer feed A and B (resp. code 3 and 4) of the 1st generation and Starter feed A and B (resp. codes 12 and 11) and Grower feed A and B (resp. codes 16 and 15) of the 2nd generation.

For the extraction and analysis of the low molecular weight polar components, the chicken feeds were finely ground, to get a more homogeneous sample and to facilitate the extraction of the low molecular weight polar components. Six replicas of each sample were extracted, in order to check the reproducibility of the method. After weighing the sample, the polar components were extracted with methanol and water in a proportion of 60:40 (v/v), using an adapted and improved version of a method previously developed to extract low molecular weight polar components from tomatoes. The extracts were analysed by ^{1}H NMR with special emphasis on reproducibility, in order to obtain comparable spectra. The scaling of the spectra was made using the international standard trimethyl silyl propionic acid (TMSP).
Once the ^{1}H NMR spectra of the polar fraction of all samples were acquired, they were aligned and compared using a program developed at the RIKILT by Dr. A. Lommen. Data so obtained were then analysed using multivariate statistics in order to know whether significant differences existed between the different samples in relation to their composition in low molecular weight polar components.

3 Results Feed

In this chapter the most important data on analyses of *feeds* and *ingredients* are presented in the tables.
In Annex 6, extensive tables are presented with the analytical results of all analysis performed on the feeds and ingredients of which the feeds are constituted.

All ingredients and feeds were coded A or B. Code A = organic, code B = conventional.

Table 3.1: The feeds that were fed subsequently in pairs A and B, with their English and Dutch name, code and numbers, are:

Type of Feed	**Dutch name**	**Codes used for the feeds**	
		A	B *
Grower	Opfok 2	2	1
Layer	Legvoer	3 and 7	4 and 8
Starter	Opfok 1	12	11
Grower	Opfok 2	16	15

** Nr.1 containing a different wheat than Nr. 15.*

3.1 Mycotoxins, Pesticide residues, Dioxins, ANF's

Mycotoxins The different batches of soy, peas, barley, triticale, maize and wheat were tested for the presence of a number of mycotoxins. In most ingredients no contamination was found. One sample of wheat (Z) contained a zearalenone level of 0.25 mg/kg. Further analysis of wheat Z showed that the upper part (ZB) of the silo was not contaminated in contrast to the lower part (ZO). For safety reasons this upper part was used for feed production (group B). Based on the literature the levels of zearalenone in Z was considered too low to cause any adverse effects in the chicken (EFSA).
One sample of maize (G, from group B) contained fumonisin B1, B2 and B3 at levels of respectively 0.39, 0.31 and 0.15 mg/kg. The levels of fumonisins were evaluated and based on the literature and personal comments by Prof. J. Fink from Utrecht University, considered too low to cause any adverse effects in the chicken (Henry 2000, Li 1999, Espada 1997).

Pesticides None of the samples investigated contained any of the 300 pesticides which can be detected by the method. One sample of barley contained 0.11 mg nicotin/kg. Further investigations and repeated analyses showed that a first sample was contaminated during the sampling and as the contamination was not in the ingredient itself, it was decided that the barley could be used for the preparation of the feed.

Dioxins and PCBs No dioxins could be detected in the two egg samples obtained from hens fed either feed A or B. Upperbound levels were 0.29 pg TEQ/g fat for both samples.

Small amounts of non-dioxin-like PCBs were detected, resulting in upperbound levels of 0.17 and 0.19 pg TEQ/g fat. For comparison, current EU limits for dioxins and the sum of dioxins and dl-PCBs are 3 and 6 pg TEQ/g respectively. This demonstrates that the feed did not contain dioxin and dl-PCB-levels above current background levels and that these compounds cannot be responsible for potentially observed effects. Levels of the 6 indicator PCBs (28, 52, 101, 138, 153 and 180) were also very low, being respectively 1.8 and 1.6 ng/g fat. In the DR CALUX®-assay, the response of the 2 samples was also very low, comparable to a reference sample with 1 pg TEQ/g fat, indicating the absence of stable Ah-receptor agonists like dioxins, dl-PCBs and related compounds.

Lectins The soy samples A and S, used for the feed, contained lectin levels of respectively 4.0 and 2.55 mg/g. In practice, these soy beans were toasted prior to their use for feed production, which should result in the destruction of the lectins. This was confirmed by the fact that none of the feeds measured (1, 2, 3, 4, 11, 12, 15 and 16) contained lectin levels above the detection limit of 0.05 mg/g.
The two batches of peas, M and T, contained lectin levels of respectively 0.06 and 0.12 mg/g. Peas were not pretreated prior to feed production, but the final feed contained only 10% of peas, thus explaining why the differences in the content of the lectin pisum sativum agglutinin (PSA) were not observed in the feeds.

Antitrypsine activity assessment
Antritrypsine activity was assessed in toasted soybeans. Values were respectively 1.34 mg/g and 1.48 mg/g for soy A and B.

3.2 Macro and micronutrients

Macro and micronutrients were analyzed both in the feeds and in the ingredients out of which the feeds were composed. In order to compare the two origins, the ratio of these concentrations was calculated by dividing the concentration of feed or ingredient B by the concentration of feed or ingredient A, resulting in a ratio B/A. In case of a ratio > 1, this means a higher concentration in the corresponding group B feed or ingredient, while a ratio < 1 means higher concentration in the corresponding group A feed or ingredient.

For all samples amino acids and macronutrients were analyzed in duplicate. As feed samples 15 and 17, as well as feed samples 16 and 18 were the same sample, a total of four analyses were carried out for each sample. Therefore it was possible to determine the Residual Standard Deviation (RSD %, n=4) for the various analytical methods. If differences observed between samples A and B are larger than the RSD, it can be concluded that a significant difference between the two origins exist. The RSD for protein was 2%, and for the specific amino acids 1-3% with the exception of tryptophan (9%). For all macronutrients RSD was well below the 10%, so if differences of 10% and higher are reported this means that the difference between samples A and B are significant.

3.2.1 Comparison between the feeds A and B

Macro nutrients The results for the macronutrients in the feeds are shown in Tables 3.2 and 3.3. From the ratio B/A in Table 3.3 it can be seen that the protein content is about 10% higher for all group B feeds in contrast to the group A feeds. No differences were observed in metabolizable energy, carbohydrates and moisture content. For raw

fibre and crude fat small differences occurred, however, not consistently over the different feeds. Chloride showed to be about 10-40% higher in most group B feeds compared to the group A feeds. Only the Starter feed of the second generation had 10% less chloride in the B feed.

Table 3.2: Analysis of macronutrients in feeds.

g/kg	Code	Energy KJ/kg	ash content	Raw fibre	carbohydr. total	crude fat	protein	moisture	chloride
1st generation									
Original feed	Or		59	45	556	44	175	122	?
Grower B	1	14610	48	42	539	59	192	120	2,0
Grower A	2	14480	51	43	546	61	173	126	1,7
Layer B	4	14302	114	40	538	64	164	120	1,4
Layer A	3	14487	113	36	555	69	147	116	1,3
Layer B	8	14061	105	42	559	46	168	122	2,1
Layer A	7	14059	116	37	562	51	154	117	1,7
2nd generation									
Starter B	11	14882	51	34	620	42	164	123	2,1
Starter A	12	14729	64	36	624	42	151	119	2,3
Grower B	15	15102	54	39	574	53	199	120	2,3
Grower A	16	15231	55	39	585	62	176	122	1,9

Note: feed 1, 4, 8, 11 and 15 belong to the same group (group B); the same accounts for feed 2, 3, 7, 12 and 16 (group A).

Table 3.3: Ratio of group B/group A and Original feed for macronutrients.

	Energy	ash content	raw fibre	carbohydr. total	crude fat	protein	Moisture	chloride
ratio Or/1		1,2	1,1	1,0	0,7?	0,9	1,0	1,2
ratio Or/2		1,2	1,0	1,0	0,7?	1,0	1,0	1,4
ratio ½	1,0	0,9	1,0	1,0	1,0	1,1	1,0	1,2
ratio 4/3	1,0	1,0	1,1	1,0	0,9	1,1	1,0	1,1
ratio 8/7	1,0	0,9	1,1	1,0	0,9	1,1	1,0	1,2
ratio 11/12	1,0	0,8	0,9	1,0	1,0	1,1	1,0	0,9
ratio 15/16	1,0	1,0	1,0	1,0	0,9	1,1	1,0	1,2

Note: feed 1, 4, 8, 11 and 15 belong to the same group (group B); the same accounts for feed 2, 3, 7, 12 and 16 (group A). Or+ original feed, being fed before the experimental feed.

Amino Acids Table 3.4 and 3.5 show the results of the amino acid analyses for the different feeds. From Table 3.5 it can be observed that in general the amount of amino acids is higher in group B feeds compared to group A feeds. There seems to be a constant difference of about 10% between the group B feeds and group A feeds administered to the chicken during the whole lifetime of the 1st en 2nd generation. Only for methionine, the concentration was lower in 2 out of 5 feeds (respectively 20% and 10% lower in feed B samples than in feed A samples), being fed to the chicken. The results for the amino acids correspond well with the difference in proteins observed in Table 3.1.

Protein digestibility/amino acid availability The determination of the true digestibility and limiting amino acid (LAA) was carried out for most feeds. The results are shown in Tables 3.6 and 3.7, respectively. Not all feeds were analyzed and not all in the same state. Of Layer feeds 3 and 4 both the supplemented and non-supplemented samples were analyzed. Of Grower feeds 1 and 2 and of Layer feeds 7 and 8 only the supplemented feeds were analyzed. Of Starter feeds 11 and 12 only unsupplemented feeds were analyzed. Grower feeds 15 and 16 were not analyzed. Amounts of supplementation are shown in Table 2.4.

From Table 3.6 it can be seen that the digestibility of all amino acids is generally higher for the feeds belonging to group B compared to group A. However, a more important parameter is the LAA, as shown in Table 3.7. Here in red the limiting amino acid (CVB, 1996) is indicated. The stronger this number deviates from 1.00 (Lys), the stronger the limitation. For the supplemented feeds 1, 2, 3 and 4 the LAA is methioninee (Met). Low values are observed for Met for these samples, i.e. 0.5-0.6, indicating a quite strong limitation. As described elsewhere, a deficiency of methioninee is expected for chicken feed composed following organic regulations. Therefore the feed is usually supplemented with Met, as was carried out for these Grower and Layer feeds 1 to 4. When comparing the unsupplemented and supplemented feed 3 and 4, a result of the supplementation can be seen. The number for Met for supplemented feed 3 and feed 4 are significantly higher, compared to the unsupplemented feeds 3 and 4. Supplemented feed 1 and feed 2 cannot be compared to the unsupplemented feed 1 and 2, but the number for Met is still rather low after supplementing.

For supplemented feeds 8 and 7 (Layer feed), and unsupplemented feeds 11 and 12 (Starter feed) high numbers can be observed for Met. For these samples Met is here not even the LAA, but tryptophan (Trp) is. However the numbers for all amino acids for these samples are rather high and therefore no strong limitation is expected. Comparing the values for the LAA for feeds belonging to group A vs. group B, no significant differences can be observed.

Table 3.4: Analysis of amino acids in feeds.

g/100g	Code	Cys	Met	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	Trp
1st generation																			
Orginal	Or	0,30	0,32	1,72	0,65	0,86	3,25	1,06	0,75	0,87	0,82	0,72	1,44	0,61	0,85	0,47	0,89	1,17	0,20
Grower B	1	0,34	0,29	1,87	0,72	0,95	3,88	1,23	0,81	0,86	0,91	0,83	1,54	0,70	0,95	0,49	1,02	1,39	0,24
Grower A	2	0,33	0,26	1,66	0,65	0,84	3,30	1,07	0,71	0,78	0,80	0,73	1,36	0,62	0,82	0,44	0,94	1,21	0,23
Layer B	4	0,29	0,28	1,63	0,64	0,82	3,27	1,01	0,72	0,77	0,81	0,71	1,37	0,65	0,87	0,45	0,91	1,12	0,22
Layer A	3	0,28	0,26	1,49	0,62	0,75	2,83	0,94	0,66	0,72	0,76	0,67	1,30	0,61	0,77	0,40	0,87	1,04	0,19
Layer B	8	0,29	0,40	1,52	0,62	0,80	3,19	1,02	0,69	0,75	0,78	0,70	1,35	0,61	0,81	0,42	0,88	1,15	0,20
Layer A	7	0,30	0,49	1,52	0,63	0,78	2,96	0,96	0,68	0,76	0,77	0,68	1,32	0,58	0,78	0,39	0,88	1,08	0,19
2nd generation																			
Starter B	11	0,33	0,39	1,79	0,73	0,89	3,30	1,23	0,79	0,83	0,94	0,78	1,52	0,71	0,95	0,42	1,02	1,10	0,23
Starter A	12	0,30	0,45	1,52	0,68	0,79	2,97	1,04	0,72	0,76	0,83	0,72	1,41	0,63	0,84	0,37	0,89	1,01	0,20
Grower B	15	0,33	0,30	1,80	0,72	0,96	3,90	1,20	0,81	0,86	0,91	0,81	1,50	0,68	0,95	0,47	1,00	1,40	0,23
Grower A	16	0,32	0,29	1,80	0,68	0,88	3,40	1,10	0,75	0,82	0,85	0,75	1,40	0,62	0,86	0,44	0,98	1,20	0,20

Note: feed 1, 4, 8, 11 and 15 belong to the same group (group B); the same accounts for feed 2, 3, 7, 12 and 16 (group A).

Table 3.5: Ratio of group B/group A for the various amino acids.

	Cys	Met	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	Trp
ratio Or/1	0,9	1,1	0,9	0,9	0,9	0,8	0,9	0,9	1,0	0,9	0,9	1,1	0,9	0,9	1,0	0,9	0,8	0,8
ratio Or/2	0,9	1,3	1,0	1,0	1,0	1,0	1,0	1,1	1,1	1,0	1,0	0,9	1,0	1,0	1,1	0,9	1,0	0,9
ratio 1/2	1,0	1,1	1,1	1,1	1,1	1,2	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,2	1,1	1,1	1,1	1,0
ratio 4/3	1,0	1,1	1,1	1,0	1,1	1,2	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,0	1,1	1,2
ratio 8/7	1,0	0,8	1,0	1,0	1,0	1,1	1,1	1,0	1,0	1,0	1,0	1,0	1,1	1,0	1,1	1,0	1,1	1,1
ratio 11/12	1,1	0,9	1,2	1,1	1,1	1,1	1,2	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,2
ratio 15/16	1,0	1,0	1,0	1,1	1,1	1,1	1,1	1,1	1,0	1,1	1,1	1,1	1,1	1,1	1,1	1,0	1,2	1,2

Note: feed 1, 4, 8, 11 and 15 belong to the same group (group B); the same accounts for feed 2, 3, 7, 12 and 16 (group A).

Table 3.6: True digestability (%) for relevant amino acids in the various feeds.

	Code	Arg	Ile	Lys	Met	Thr	Trp	Val	Met+Cys
1st generation									
Grower B	1 + Suppl.	80	81	76	63	73	44	77	61
Grower A	2 + Suppl.	58	58	61	49	57	36	57	50
Layer B	4	82	80	83	57	79	53	77	60
Layer A	3	70	66	73	53	66	57	68	55
Layer B	4 + Suppl.	69	69	72	66	69	49	69	63
Layer A	3 + Suppl.	60	60	63	67	59	41	60	60
Layer B	8 + Suppl.	67	67	67	55	64	42	68	55
Layer A	7 + Suppl.	58	57	59	44	58	35	56	45
2nd generation									
Starter B	11	57	55	57	43	56	33	52	43
Starter A	12	53	53	55	39	52	28	53	43

Note: feed 1, 4, 8 and 11 belong to the same group (group B); the same accounts for feed 2, 3, 7 and 12 (group A). Suppl. = supplemented feed (e.g. with methioninee).

Table 3.7: Limiting amino acid in the various feeds.*

	Code	Arg	Ile	Lys	Met	Thr	Trp	Val	Met+Cys
1st generation									
Grower B	1 + Suppl.	1,29	1,26	1,00	0,60	1,03	0,88	1,07	0,69
Grower A	2 + Suppl.	1,24	1,18	1,00	0,60	1,02	0,88	1,05	0,70
Layer B	4	1,16	1,14	1,00	0,55	1,04	0,96	1,04	0,62
Layer A	3	1,09	1,04	1,00	0,56	0,99	1,06	1,02	0,64
Layer B	4 + Suppl.	1,12	1,13	1,00	0,74	1,04	1,02	1,07	0,76
Layer A	3 + Suppl.	1,09	1,12	1,00	0,83	1,03	0,88	1,04	0,81
Layer B	8 + Suppl.	1,24	1,22	1,00	0,97	1,04	0,89	1,13	0,89
Layer A	7 + Suppl.	1,13	1,12	1,00	1,08	1,06	0,80	1,01	0,93
2nd generation									
Starter B	11	1,08	1,16	1,00	0,81	1,09	0,82	1,09	0,75
Starter A	12	1,04	1,16	1,00	0,93	1,09	0,72	1,11	0,88

Fatty acids The fatty acid composition in fat was only determined for the second batch of Layer feed for the 1st generation and the Starter and Grower feed of the 2nd generation (samples 7, 8, 11, 12, 15 and 16). The results for the feeds A and B and the group B/group A ratio are shown in Table 3.8. When focusing on the most abundant fatty acids, i.e. C16:0, C18:0, C18:1, C18:2 and C18:3, there seems to be a slightly higher percentage of saturated fatty acids in group B feeds, although not for all feed sample ratios. Grower feeds15 and 16 were used prior, during and after the challenge with KLH. For these two feeds it can be seen that the percentage of unsaturated fatty acids is slightly lower for sample 15 (group B), compared to sample 16 (group A), while the percentage of saturated fatty acids is slightly higher.

Because small differences in crude fat were found between group A and group B feeds, we also calculated the amount of fatty acids per kg, in the feeds (Table 3.9). From these calculations it is concluded that for the above mentioned fatty acids, no differences were found for the saturated fatty acids C16:0 and C18:0. For the unsaturated C18 fatty acids, no consistent difference was found. However, the ratio for 15/16 was lower than 1, meaning that in the period of the KLH challenge, the percentage of these unsaturated fatty acids was lower in feed B.

Tabel 3.8: Analysis of individual fatty acids in fat in feeds and group B/group A ratios.

in total fat%	Layer B8	Layer A 7	Starter B - 11	Starter A – 12	Grower B - 15	Grower A -16	ratio 8/7	ratio 11/12	ratio 15/16
C8:0	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	-	-	
C10:0	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	-	-	-
C12:0	0,2	0,2	0,5	1	0,7	0,2	1,0	0,5	3,5
C14:0	0,3	0,2	0,3	0,5	0,4	0,2	1,5	0,6	2,0
C14:1 c9	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	-	-	-
C15:0	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	-	-	-
C16:0	13,8	12,6	15,2	15,5	14,7	13,2	1,1	1,0	1,1
C16:1 c9	0,1	0,1	0,1	0,1	0,1	0,1	1,0	1,0	1,0
C17:0	0,2	0,2	0,1	0,1	0,2	0,2	1,0	1,0	1,0
C17:1c	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	-	-	-
C18:0	4	3,6	3,2	3,2	4	3,5	1,1	1,0	1,1
C18:1 t	0,1	<0,1	<0,1	<0,1	0,1	<0,1	-	-	-
C18:1 c	21,9	24,2	21,3	23,4	23,2	23,2	0,9	0,9	1,0
C18:2 c9,12	50,9	50,7	50,9	47,3	48,4	51,3	1,0	1,1	0,9
C18:3 c9,12,15	5,8	5,7	5,1	4,5	5,1	5,9	1,0	1,1	0,9
C20:0	0,4	0,4	0,4	0,4	0,4	0,3	1,0	1,0	1,3
C20:1 c11	<0,1	<0,1	0,2	0,2	<0,1	<0,1	-	1,0	-
C20:2 c11,14	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	-	-	-
C22:0	0,5	0,4	0,3	0,3	0,5	0,4	1,3	1,0	1,3
C22:3 c13,16,19	<0,1	<0,1	0,2	0,4	<0,1	<0,1	-	0,5	-
C22:4 c7,10,13,16	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	-	-	-
C23:0	<0,1	<0,1	0,1	0,1	<0,1	<0,1	-	1,0	-
C24:0	0,4	0,4	0,5	0,7	0,5	0,2	1,0	0,7	2,5
C24:1	<0,1	<0,1	0,3	0,4	<0,1	<0,1	-	0,8	-
unidentified	1	1	0,8	1,2	1,5	1	1,0	0,7	1,5

Note: feed 1, 4, 8 and 11 belong to the same group (group B); the same accounts for feed 2, 3, 7 and 12 (group A).

*Suppl. = supplemented feed (e.g. with methioninee) * Limiting amino acids in the specific feed is indicated in red.*

Tabel 3.9: Amount of fatty acids per kg feed, calculated from crude fat.*

g/kg	Feed 8	Feed 7	Feed 11	Feed 12	Feed 15	Feed 16	ratio 8/7	ratio 11/12	ratio 15/16
C12:0	0,09	0,10	0,21	0,42	0,37	0,12	0,9	0,5	3,0
C14:0	0,14	0,10	0,13	0,21	0,21	0,12	1,4	0,6	1,7
C16:0	6,35	6,43	6,38	6,51	7,79	8,18	1,0	1,0	1,0
C16:1 c9	0,05	0,05	0,04	0,04	0,05	0,06	0,9	1,0	0,9
C17:0	0,09	0,10	0,04	0,04	0,11	0,12	0,9	1,0	0,9
C18:0	1,84	1,84	1,34	1,34	2,12	2,17	1,0	1,0	1,0
C18:1 c	10,07	12,34	8,95	9,83	12,30	14,38	0,8	0,9	0,9
C18:2 c9,12	23,41	25,86	21,38	19,87	25,65	31,81	0,9	1,1	0,8
C18:3 c9,12,15	2,67	2,91	2,14	1,89	2,70	3,66	0,9	1,1	0,7
C20:0	0,18	0,20	0,17	0,17	0,21	0,19	0,9	1,0	1,1
C20:1 c11			0,08	0,08				1,0	
C22:0	0,23	0,20	0,13	0,13	0,27	0,25	1,1	1,0	1,1
C22:3 c13,16,19			0,08	0,17				0,5	
C23:0			0,04	0,04				1,0	
C24:0	0,18	0,20	0,21	0,29	0,27	0,12	0,9	0,7	2,1
C24:1			0,13	0,17				0,8	
unidentified	0,46	0,51	0,34	0,50	0,80	0,62	0,9	0,7	1,3

** calculated as crude fat (g/kg) * (% specific fatty acid/100).*

Micronutrients Total folate, and the 4 tocopherols were analyzed in the feeds.

Tocopherols and Folate The results of the analysis of tocopherols (vitamin E) and folate are presented in Table 3.10. In Table 3.11 the ratio B/A is presented. A difference is found for some feeds in α-tocopherol levels, where feed A is higher. Further, we observed differences in the total folate concentration, with higher levels in some group A feeds.

Table 3.10: Vitamin E and total folate measured in feeds.

mg/kg	Code	Alfa-tocopherol	Beta-tocopherol	Gamma-tocopherol	Delta-tocopherol	Total folate
1st generation						
Grower B	1	14	3,7	37	11	0,5
Grower A	2	16	3,7	43	18	0,7
Layer B	4	13	2	32	11	0,5
Layer A	3	16	2	27	12	0,5
Layer B	8	15	2,7	47	15	0,6
Layer A	7	14	2,6	38	14	0,7
2nd generation						
Starter B	11	15	2,7	10	2,1	0,3
Starter A	12	16	2,7	11	1,9	0,4
Grower B	15	13	2,8	25	9,3	0,6
Grower A	16	13	3,1	27	14	0,6

Table 3.11: Ratio of group B/group A for Vitamin E and total folate.

		Alfa-tocopherol	Beta-tocopherol	Gamma-tocopherol	Delta-tocopherol	Total Folate
ratio	**1/2**	0,9	1	0,9	0,6	0,7
ratio	**4/3**	0,8	1	1,2	0,9	1,0
ratio	**8/7**	1	1	1,2	1	0,9
ratio	**11/12**	0,9	1	0,9	1,1	0,8
ratio	**15/16**	1	0,9	0,9	0,7	1,0

Note: feed 1, 4, 8, 11 and 15 belong to the same group (group B); the same accounts for feed 2, 3, 7, 12 and 16 (group A).

Trace elements/heavy metals Some trace elements/ heavy metals were analyzed in the feed, because of differences between the ingredients A and B, and the potential influence of the Premix added to the feeds. Analyzed were selene, chrome, iron, lead, manganese, arsenic, iodide, and in two of the feeds cadmium. Results of these analyses are found in Table 3.12 and 3.13. From the results it is shown that no consistent differences between the feeds A and B were observed, only the amount of iodide was consequently lower in feed B.

Table 3.12: Trace elements/heavy metals in the total feeds.

		selene ug/kg	iron mg/kg	Chrome mg/kg	manganese mg/kg	lead ug/kg	arsenic ug/kg	iodide ug/kg	cadmium ug/kg
1st generation									
Grower B	1	290	250	0,7	82	170	120	1200	39
Grower A	2	260	240	0,6	80	110	70	1300	45
Layer B	4	290	260	3,1	110	200	120	700	
Layer A	3	270	280	3,9	130	250	130	750	
Layer B	8	290	220	2,9	92	190	110	780	
Layer A	7	330	340	5,2	110	240	150	930	
2nd generation									
Starter B	11	290	220	1,3	130	98	110	870	
Starter A	12	330	260	1,4	150	130	100	940	
Grower B	15	280	330	1,5	110	190	110	940	
Grower A	16	270	240	1,3	100	140	64	1000	

Table 3.13: Ratio of group B/group A for the various trace elements/heavy metals in the total feeds.

	selene	iron	chrome	manganese	lead	arsenic	iodide	cadmium
Ratio 1/2	1,1	1,0	1,2	1,0	1,5	1,7	0,9	0,9
Ratio 4/3	1,1	0,9	0,8	0,8	0,8	0,9	0,9	
Ratio 8/7	0,9	0,6	0,6	0,8	0,8	0,7	0,8	
Ratio 11/12	0,9	0,8	0,9	0,9	0,8	1,1	0,9	
Ratio 15/16	1	1,4	1,2	1,2	1,4	1,7	0,9	

Calculated Micronutrient levels in the feeds

Other nutrients were only analyzed in the ingredients (see paragraph 3.2.2), out of which the feeds were composed. In order to have an idea what the consequences of differences in ingredients are for the final content of a specific nutrient in the feeds, these values were *calculated*. The expected amount of a nutrient in a feed was calculated by summing the contributions for the different ingredients (Table 2.2) as: observed concentrations in ingredient x percentage of this ingredient in the final product. Results can be found at page 2 and 3 of Annex 6.
This has been carried out for nutrients that showed significant differences between group B and A ingredients. These calculated values are just an indication, as they are based on the ingredients only and do not take into account the addition to the feed of potato proteins, MonoCalcFos, FX Layers premix, soy fat, salt, chalk, broken shells, $NaCO_3$ and methioninee (Table 2.2).

Phytosterols Calculating the amount of phytosterols in the feeds from the ingredients (shown in Table 3.14), shows a slightly higher level of sitosterol and avenasterol in both Grower feeds of the B group, compared to group A feeds. The main ingredients contributing to this difference are soy and barley. As expected for plant products, cholesterol levels were beneath the detection limit in all samples and therefore not presented in the table.

Table 3.14: Phytosterols in feeds calculated from ingredients.

mg/100 g	**Code**	**Campersterol**	**Stigmasterol**	**Sitosterol**	**Avenasterol**
1st generation					
Grower B	1	12,3	6,9	44,9	11,4
Grower A	2	11,4	7,1	40,8	9,1
Layer B	4	10,3	5,9	39,5	10,3
Layer A	3	10,3	6,1	38,7	9,3
Layer B	8	10,3	5,9	39,5	10,3
Layer A	7	10,3	6,1	38,7	9,3
2nd generation					
Starter B	11	9,6	4,7	37,9	10,5
Starter A	12	9,6	5,0	38,4	10,5
Grower B	15	11,7	7,1	43,1	10,3
Grower A	16	11,4	7,1	40,8	9,1
		Campersterol	**Stigmasterol**	**Sitosterol**	**Avenasterol**
ratio	**1/2**	1,1	1,0	1,1	1,3
ratio	**4/3**	1,0	1,0	1,0	1,1
ratio	**8/7**	1,0	1,0	1,0	1,1
ratio	**11/12**	1,0	0,9	1,0	1,0
ratio	**15/16**	1,0	1,0	1,1	1,1

Note: feed 1, 4, 8, 11 and 15 belong to the same group (group B); the same accounts for feed 2, 3, 7, 12 and 16 (group A).

Vitamins Calculation of the amount of vitamins in the feeds from the values obtained for the ingredients shows some constant differences between the group A and group B feeds. For vitamin C and vitamin B5 (pathothenic acid) a ratio B/A > 1 is observed for various ingredients. For both vitamins the group B/group A ratio in the feeds is always > 1,2. For Vitamin K1 the B/A ratio is < 1 for various ingredients, resulting in a group B/group A ratio < 1 for the feeds. No differences are calculated for the vitamins B1, B2 and B3, for the feeds A and B (see Annex 6 for all results).

Flavonoids Flavonoids were only present in small amounts in the ingredients, showing no differences between the different origins of the ingredients. Therefore, no differences of these compounds between the feeds A and B are to be expected.

Carotenoids Based on the levels in the ingredients, no large differences in carotenoid levels are expected in the feeds. Depending on the ratios of the feed, higher levels of lutein, zeaxanthin, beta-cryptoxanthin, lycopene and beta-carotene were calculated for most A feeds, whereas alpha-carotene was calculated to be higher in the B feeds. (Table 3.15 and Table 3.16).

Table 3.15: Calculated carotenoid content in the total feeds.

mg/kg		lutein	zeaxanthin	beta-cryptoxanthin	lycopene	alpha-carotene	beta-carotene
1st generation							
Grower B	1	3,1	1,5	0,2	0,1	0,0	0,2
Grower A	2	3,3	1,8	0,2	0,1	0,0	0,4
Layer B	4	3,0	1,7	0,2	0,1	0,0	0,2
Layer A	3	3,2	2,0	0,2	0,1	0,0	0,4
Layer B	8	3,0	1,7	0,2	0,1	0,0	0,2
Layer A	7	3,2	2,0	0,2	0,1	0,0	0,4
2nd generation							
Starter B	11	2,6	1,4	0,2	0,1	0,0	0,2
Starter A	12	2,6	1,6	0,2	0,1	0,0	0,3
Grower B	15	3,1	1,5	0,2	0,1	0,0	0,2
Grower A	16	3,3	1,8	0,2	0,1	0,0	0,4

Table 3.16: Ratio of group B/group A for the various carotenoids.

	lutein	zeaxanthin	beta-cryptoxanthin	lycopene	alpha-carotene	beta-carotene
Ratio ½	0,9	0,9	0,9	0,8	1,1	0,5
Ratio 4/3	1,0	0,9	0,9	0,8	1,1	0,6
Ratio 8/7	1,0	0,9	0,9	0,8	1,1	0,6
Ratio 11/12	1,0	0,9	0,9	0,8	1,1	0,7
Ratio 15/16	0,9	0,8	0,9	0,8	1,1	0,5

Note: feed 1, 4, 8, 11 and 15 belong to the same group (group B); the same accounts for feed 2, 3, 7, 12 and 16 (group A).

Calculated amounts of Trace elements/heavy metals Other trace elements/heavy metal contents in the feeds were calculated, based on the content of these in the ingredients. Some trace elements/heavy metals were hardly detected in any of the ingredients, i.e. Co, I, Hg, and F, while others did not show significant differences between group A and B ingredients, i.e. P, K, Mg. Thus no differences were expected in the feeds.

3.2.2 Comparison of the ingredients used to compose the feeds A and B

The ingredients, i.e. triticale, barley, maize, peas, wheat and soy, from which the feeds were composed, were screened for various nutrients. Extensive tables with the results of these analyses are given in Annex 6.

Macronutrients Results for the macronutrients for the separate ingredients are presented in Tables 3.17 and 3.18. For the macronutrients a similar observation can be made for the ingredients as for the total feeds.
A higher protein content is found for the group B barley, wheat and soy samples, compared to those in group A. This corresponds well with the amino acid results for the ingredients (Table 3.19), as well as the macronutrient results for the feeds (Table 3.2 and 3.4). The higher protein content in group B feeds is likely caused by the higher protein content of the group B soy and wheat samples, and to a lesser extent also the group B barley samples.

Table 3.17: Macronutrients in ingredients used to prepare feed A or feed B.

g/kg	code	ash content	Carbohydr. total	raw fiber	crude fat	moisture	protein
Triticale B	**A2**	18	752	32	18	140	72
Triticale A	**Y2**	17	704	29	17	150	112
barley B	**V2**	17	679	43	25	164	116
barley A	**G2**	27	692	43	24	162	95
maize B	**F2**	12	754	28	37	119	78
maize A	**P2**	14	738	32	40	124	84
peas B	**C2**	30	628	61	10	130	202
peas A	**M2**	32	620	56	10	136	202
wheat-2 B	**T2**	17	716	33	19	146	102
wheat-1 B	**CC2***	16	699	33	22	157	106
wheat A	**X2**	16	741	28	21	142	80
soy B	**J2**	48	287	80	209	102	354
soy A	**R2**	52	329	80	228	89	302

*Note: ingredients A2, V2, F2, C2, T2, CC2 and J2 were used to prepare the feeds belonging to group B and ingredients Y2, G2, P2, M2, X2 en R2 were used to prepare the feeds belonging to group A. * wheat CC2 was only used for feed sample 1 (Grower feed B for the first generation).*

Table 3.18: Ratio group B/group A for macronutrients in ingredients.

	Ash content	Carbohydrates total	raw fiber	crude fat	moisture	protein
ratio A2/Y2	1,1	1,1	1,1	1,1	0,9	0,6
ratio V2/G2	0,6	1,0	1,0	1,0	1,0	1,2
ratio F2/P2	0,9	1,0	0,9	0,9	1,0	0,9
ratio C2/M2	0,9	1,0	1,1	1,0	1,0	1,0
ratio T2/X2	1,1	1,0	1,2	0,9	1,0	1,3
ratio CC2/X2	1,0	0,9	1,2	1,0	1,1	1,3
ratio J2/R2	0,9	0,9	1,0	0,9	1,1	1,2

Note: ingredients A2, V2, F2, C2, T2, CC2 and J2 were used to prepare the feeds belonging to group B and ingredients Y2, G2, P2, M2, X2 en R2 were used to prepare the feeds belonging to group A.

Amino acids Amino acids were analyzed in each of the ingredients (Table 3.19 and 3.20). From the results it shows that the amount of amino acids was significantly higher in group B barley, wheat and soy, compared to those ingredients in group A. Taking into account the composition of the feeds (Table 2.2) and the absolute amount of amino acids in the ingredients, it can be expected that the soy and wheat samples will have the largest contribution to the amount of amino acids in the feeds. Earlier it was observed that the amount of amino acids in group B feeds was higher than in group A feeds (Table 3.5).

Fatty acids The fatty acid composition of the fat in the ingredients was determined, as percentage of total fat (Table 3.21 and 3.22). Soy samples have by far the highest fat content, of 209 and 228 g/kg, in group B and A samples respectively. Thus soy will have the largest effect on the fat content and -composition in the feeds. Analysis of the specific fatty acids did not show any difference in unsaturated or saturated fatty acids, between soy sample J2 (group B) and sample R2 (group A). For the feeds only small, non-consistent differences were found between group B and A, which is confirmed by the results for ingredients.

Table 3.19: Amino acid analysis for ingredients.

g/100g	Code	Cys	Met	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	Trp
triticale B	**A2**	0,18	0,14	0,47	0,24	0,30	1,55	0,56	0,30	0,30	0,33	0,24	0,46	0,21	0,30	0,18	0,28	0,42	0,09
triticale A	**Y2**	0,28	0,20	0,58	0,33	0,48	2,93	1,06	0,42	0,40	0,48	0,39	0,72	0,32	0,50	0,26	0,34	0,57	0,12
barley B	**V2**	0,25	0,21	0,68	0,40	0,50	2,78	1,30	0,46	0,47	0,56	0,42	0,79	0,39	0,61	0,29	0,42	0,64	0,16
barley A	**G2**	0,22	0,18	0,59	0,35	0,41	2,11	0,96	0,41	0,40	0,47	0,34	0,65	0,32	0,47	0,25	0,38	0,54	0,13
Maize B	**F2**	0,19	0,17	0,55	0,30	0,38	1,48	0,69	0,32	0,60	0,39	0,29	0,99	0,34	0,40	0,23	0,26	0,43	0,07
Maize A	**P2**	0,18	0,18	0,62	0,31	0,40	1,61	0,76	0,33	0,62	0,42	0,31	1,04	0,35	0,43	0,23	0,28	0,44	0,07
peas B	**C2**	0,32	0,23	2,44	0,80	0,99	3,50	0,85	0,93	0,92	0,99	0,91	1,53	0,75	1,01	0,51	1,54	1,88	0,20
peas A	**M2**	0,30	0,19	2,44	0,77	0,99	3,44	0,83	0,90	0,90	0,99	0,88	1,50	0,73	1,00	0,49	1,53	1,88	0,19
wheat 2 B	**T2**	0,26	0,21	0,58	0,32	0,51	3,21	1,04	0,46	0,41	0,49	0,39	0,77	0,35	0,51	0,27	0,32	0,62	0,16
wheat 1 B	**CC2***	0,25	0,18	0,51	0,31	0,49	3,07	1,02	0,43	0,36	0,43	0,35	0,69	0,33	0,47	0,26	0,29	0,53	0,16
wheat A	**X2**	0,22	0,17	0,46	0,26	0,40	2,46	0,80	0,37	0,32	0,38	0,30	0,60	0,28	0,38	0,20	0,26	0,46	0,12
soy B	**J2**	0,58	0,53	4,20	1,47	1,86	6,60	1,78	1,54	1,58	1,72	1,68	2,85	1,38	1,88	0,98	2,35	2,83	0,48
soy A	**R2**	0,59	0,51	3,60	1,30	1,61	5,51	1,52	1,34	1,38	1,49	1,47	2,46	1,18	1,58	0,85	2,07	2,41	0,43

*Note: ingredients A2, V2, F2, C2, T2, CC2 and J2 were used to prepare the feeds belonging to group B and ingredients Y2, G2, P2, M2, X2 en R2 were used to prepare the feeds belonging to group A. * wheat CC2 was only used for feed sample 1 (Grower feed B for the first generation).*

Table 3.20: Ratio group B/group A for amino acids in ingredients.

	Cys	Met	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	Trp
ratio A2/Y2	0,6	0,7	0,8	0,7	0,6	0,5	0,5	0,7	0,8	0,7	0,6	0,6	0,7	0,6	0,7	0,8	0,7	0,8
ratio V2/G2	1,1	1,2	1,2	1,1	1,2	1,3	1,4	1,1	1,2	1,2	1,2	1,2	1,2	1,3	1,2	1,1	1,2	1,2
ratio F2/P2	1,1	0,9	0,9	1,0	1,0	0,9	0,9	1,0	1,0	0,9	0,9	1,0	1,0	0,9	1,0	0,9	1,0	1,0
ratio C2/M2	1,1	1,2	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,1
ratio T2/X2	1,2	1,2	1,3	1,2	1,3	1,3	1,3	1,2	1,3	1,3	1,3	1,3	1,3	1,3	1,4	1,2	1,3	1,3
ratio CC2/X2	1,1	1,1	1,1	1,2	1,2	1,2	1,2	1,2	1,1	1,1	1,2	1,2	1,2	1,2	1,3	1,1	1,2	1,3
ratio J2/R2	1,0	1,0	1,2	1,1	1,2	1,2	1,2	1,2	1,1	1,2	1,1	1,2	1,2	1,2	1,2	1,1	1,2	1,1

Note: ingredients A2, V2, F2, C2, T2, CC2 and J2 were used to prepare the feeds belonging to group B and ingredients 12, G2, P2, M2, X2 en R2 were used to prepare the feeds belonging to group A.

Table 3.21:Fatty acid composition in fat of ingredients.

%	Triticale B	Triticale A	Barley B	Barley A	Maize B	Maize A	Peas B	Peas A	Wheat-2 B	Wheat-1 B	Wheat A	Soy B	Soy A
Code	**A2**	**Y2**	**V2**	**G2**	**F2**	**P2**	**C2**	**M2**	**T2**	**CC2**	**X2**	**J2**	**R2**
C8:0	0,1	-	-	-	-	-	-	-	-	-	-	-	-
C10:0	0,2	-	-	-	-	-	-	-	-	-	-	-	-
C12:0	1,3	0,1	0,4	0,6	-	-	-	-	-	-	-	-	-
C14:0	0,8	0,1	0,9	0,9	0,1	-	0,2	0,2	0,1	0,1	0,1	0,1	0,1
C14:1 c9	0,1	0,1	0,7	-	-	-	0,1	0,1	0,1	-	-	-	-
C15:0	0,1	0,2	0,3	0,2	-	-	0,2	0,2	0,1	0,1	0,1	-	-
C16:0	17,7	22,0	26,9	21,7	12,5	11,3	12,9	13,5	17,9	16,9	16,5	10,6	10,6
C16:1 c9	0,1	0,1	0,3	0,2	0,2	0,1	-	0,1	0,1	0,1	0,1	0,1	0,1
C17:0	0,9	0,1	0,5	0,5	0,2	0,1	0,4	0,3	0,2	0,2	0,1	0,1	0,1
C18:0	1,6	0,9	3,5	3,8	1,9	1,9	3,3	3,6	1,0	1,0	0,7	4,0	3,8
C18:1 t	0,2	0,1	0,5	0,7	-	-	-	-	0,1	0,1	0,1	-	-
C18:1 c	13,6	13,9	13,9	12,6	28,4	32,5	18,2	16,4	11,0	16,0	16,5	21,6	21,9
C18:2 c9,12	52,9	55,5	47,1	47,0	53,5	50,8	52,9	54,0	61,0	58,6	59,5	54,9	54,3
C20:0	0,1	-	0,4	-	0,4	0,4	0,3	0,4	0,1	0,1	0,1	0,4	0,3
C18:3 c9,12,15	5,7	6,3	2,7	3,3	1,8	1,6	9,8	9,5	5,0	5,0	4,7	7,2	7,8
C22:0	0,3	0,3	0,6	1,2	0,1	0,2	-	0,1	0,2	0,2	0,1	0,4	0,4
C24:0	0,3	0,4	1,2	1,3	0,3	0,3	0,2	0,2	0,4	0,2	0,2	0,3	0,2
Unidentified	3,8	-	-	6,0	0,6	0,7	1,3	1,3	2,5	1,4	1,1	0,4	0,4

Table 3.22: Ratio group B/group A for fatty acid composition in ingredients.

%	ratio A2/Y2 Triticale	ratio V2/G2 Barley	ratio F2/P2 Maize	ratio C2/M2 Peas	ratio T2/X2 Wheat2	ratio CC2/X2 Wheat1	ratio J2/R2 Soy
C8:0	>1	-	-	-	-	-	-
C10:0	>1	-	-	-	-	-	-
C12:0	13,5	0,8	-	-	-	-	-
C14:0	6,0	1,0	>1	0,9	2,0	1,0	1,2
C14:1 c9	1,0	>1	-	1,6	>1	-	-
C15:0	0,6	1,6	-	0,8	1,4	1,0	-
C16:0	0,8	1,2	1,1	1,0	1,1	1,0	1,0
C16:1 c9	0,9	1,6	1,5	<1	1,0	1,0	0,9
C17:0	6,6	1,0	1,4	1,3	1,5	2,0	1,1
C18:0	1,8	0,9	1,0	0,9	1,4	1,4	1,0
C18:1 t	4,1	0,7	-	-	1,0	1,0	-
C18:1 c	1,0	1,1	0,9	1,1	0,7	1,0	1,0
C18:2 c9,12	1,0	1,0	1,1	1,0	1,0	1,0	1,0
C20:0	>1	>1	1,0	0,8	1,8	1,0	1,1
C18:3 c9,12,15	0,9	0,8	1,1	1,0	1,1	1,1	0,9
C22:0	0,9	0,5	0,9	0,4	1,4	2,0	1,0
C24:0	0,7	0,9	1,0	1,0	1,8	1,0	1,5
Unidentified	>1	<1	0,8	1,0	2,2	1,3	1,1

Note: ingredients A2, V2, F2, C2, CC2 and J2 were used to prepare the feeds belonging to group B and ingredients Y2, G2, P2, M2, X2 en R2 were used to prepare the feeds belonging to group A.

Phytosterols Phytosterols were detected in all ingredients with sitosterol being the major phytosterol (see Table 3.23). However, no constant differences between group B and group A ingredients could be observed. Barley and soy showed higher contents of respectively 50% and 30% for the most abundant sterol, being sitosterol, in the group B samples. Also the less abundant sterols reached several times higher levels in the group B samples.

Table 3.23: Phytosterols in ingredients and group B/group A ratios.

mg/100g	Code	cholesterol	campersterol	Stigmasterol	sitosterol	avenasterol
Triticale B	**A2**	<1,5	10,5	2,8	36,4	12,3
Triticale A	**Y2**	<1,5	8,6	3,7	35,0	17,3
barley B	**V2**	<1,5	7,2	3,0	30,1	9,7
barley A	**G2**	<1,5	3,3	1,7	19,9	7,0
maize B	**F2**	<1,5	15,1	7,6	60,3	23,6
maize A	**P2**	<1,5	15,8	8,3	58,6	20,0
peas B	**C2**	<1,5	8,2	6,2	60,9	4,8
peas A	**M2**	<1,5	6,8	4,8	67,9	4,6
wheat B	**T2**	<1,5	8,0	2,5	30,8	9,1
wheat B	**CC2**	<1,5	10,2	1,7	37,4	13,1
wheat A	**X2**	<1,5	9,9	3,5	37,1	11,0
soy B	**J2**	<1,5	16,6	13,3	45,8	5,8
soy A	**R2**	<1,5	15,3	12,9	34,8	3,4
		cholesterol	**campersterol**	**Stigmasterol**	**sitosterol**	**avenasterol**
Ratio	**A2/Y2**		1,2	0,8	1,0	0,7
Ratio	**V2/G2**		2,2	1,8	1,5	1,4
Ratio	**F2/P2**		1,0	0,9	1,0	1,2
Ratio	**C2/M2**		1,2	1,3	0,9	1,0
Ratio	**T2/X2**		0,8	0,7	0,8	0,8
Ratio	**CC2/X2**		1,0	0,5	1,0	1,2
Ratio	**J2/R2**		1,1	1,0	1,3	1,7

Note: ingredients A2, V2, F2, C2, T2, CC2 and J2 were used to prepare the feeds belonging to group B and ingredients Y2, G2, P2, M2, X2 en R2 were used to prepare the feeds belonging to group A.

Flavonoids No significant amounts of free or conjugated flavonoids were detected in the ingredients with the exception of kaempferol in peas and soy. No difference could be observed between the group B and group A. Values for peas were respectively 34 and 39 ug/g and for soy 12 and 14 ug/g for the group B and group A samples (Annex 6).

Carotenoids The carotenoid values for the ingredients are presented in Table 3.24. The major carotenoid detected in most ingredients was lutein, although for the maize samples a relatively high concentration of

zeaxanthine was found (5,7 mg/kg in the group B and 6,3 mg/kg in the group A sample). Taking into account the concentrations in the different ingredients and the feed composition, no major differences in carotenoids are expected in the feeds.

Table 3.24: Carotenoids in ingredients and group B/group A ratios.

mg/kg	Code	lutein	zea xanthine	β-crypto xanthine	lycopene	α-carotene	β-carotene
triticale B	**A2**	1,2	0,3	<0,05	<0,05	<0,05	0,06
triticale A	**Y2**	0,8	0,2	<0.1	<0.1	<0.1	<0.1
barley B	**V2**	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
barley A	**G2**	0,8	0,3	<0,05	<0,05	<0,05	<0,05
maize B	**F2**	6,3	5,7	0,8	0,4	0,1	0,9
maize A	**P2**	5,9	6,3	0,9	0,5	0,1	1,1
peas B	**C2**	4,6	0,9	<0,03	<0,03	<0,03	0,03
peas A	**M2**	6	1,2	<0,03	<0,03	<0,03	0,04
wheat B	**T2**	1,2	0,2	<0,03	<0,03	<0,03	<0,03
wheat B	**CC2**	1,2	0,3	<0.1	<0.1	<0.1	<0.1
wheat A	**X2**	1,2	0,3	<0,03	<0,03	<0,03	<0,03
soy B	**J2**	3,4	0,7	<0.1	<0.1	<0.1	<0.1
soy A	**R2**	3,7	0,9	<0.1	<0.1	<0.1	0,5
		lutein	**zea xanthine**	**β-crypto xanthine**	**lycopene**	**α-carotene**	**β-carotene**
ratio	**A2/Y2**	1,5	1,6	-	-	-	-
ratio	**V2/G2**	<1	<1	-	-	-	-
ratio	**F2/P2**	1,1	0,9	0,9	0,8	1,1	0,8
ratio	**C2/M2**	0,8	0,8	-	-	-	0,8
ratio	**T2/X2**	1,0	0,7	-	-	-	-
ratio	**CC2/X2**	1,0	1,0	-	-	-	-
ratio	**J2/R2**	0,9	0,8	-	-	-	<1

Note: ingredients A2, V2, F2, C2, T2, CC2 and J2 were used to prepare the feeds belonging to group B and ingredients Y2, G2, P2, M2, X2 en R2 were used to prepare the feeds belonging to group A.

Catechins No catechins were detected, at significant levels, in any of the ingredients.

Isoflavones Isoflavones were only analyzed in soy samples. The results are presented in Table 3.25. The amount of isoflavones, especially malonyl daidzin and malonyl genistin, is somewhat higher in soy sample R2 (group A) compared to sample J2 (group B).

Table 3.25: Isoflavones in soy samples.

mg/g	Soy B	Soy A	Ratio B/A
	J2	R2	J2/R2
Daidzin	0,4	0,4	1,1
Genistin	0,6	0,6	1,0
Daidzein	<0,1	<0,1	-
Genistein	<0,1	<0,1	-
Glycetein	<0,1	<0,1	-
Glycetin	0,1	0,0	2,0
Malonyl daidzin	1,7	2,4	0,7
Malonyl glycetin	0,1	0,2	0,8
Acetyl daidzin	<0,1	<0,1	-
Acetyl glycetin	0,2	0,3	0,7
Malonyl genistin	1,8	2,7	0,7
Acetyl genistin	<0,1	<0,1	-

Vitamins The results for the vitamins in the ingredients are shown in Table 3.26. Many differences can be observed between values found between group A and group B ingredients. The most remarkable difference is the high amount of tocopherols (Vitamin E) in soy group A, sample R2, especially α-tocopherol (α-TP). Further, a relatively high amount of total folate in peas group A, sample M2, compared to peas group B, sample C2, resulting in a ratio B/A (C2/M2) of 0.046.

Table 3.26: Vitamins in ingredients and group B/group A ratios.

	Code	Vit B1	Vit B2	Vit C	α-TP	β-TP	γ-TP	δ-TP	Vit K1	Vit B5	Vit B3	Total folate	Vit H	Vit B6
		mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	µg/100g	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
triticale B	**A2**	2,0	0,8	<1,5	10	3,9	<0,1	<0,1	1,7	4,8	15	0,5	0,08	1,2
triticale A	**Y2**	3,0	0,8	<1,5	6,5	1,8	<0,1	<0,1	2,7	5,2	20	0,5	0,07	1,2
barley B	**V2**	3,3	0,9	1,7	2,3	<0,1	2,3	<0,1	1,6	1,3	33	0,5	0,12	1,4
barley A	**G2**	2,8	0,9	<1,5	7,9	0,4	<0,1	<0,1	3,6	1,8	31	0,6	0,14	1,4
maize B	**F2**	4,9	0,9	4,2	6,6	<1,7	55	2,2	4,2	3,2	<9,5	0,4	0,06	1,8
maize A	**P2**	5,6	0,9	3,3	6,1	<1,7	51	<1,7	4,3	2,7	<9,5	0,4	0,05	2,3
peas B	**C2**	6,3	1,3	14	<1,8	<1,8	67	2,2	9,4	9,8	24	0,2	0,16	0,2
peas A	**M2**	6,4	1,3	11	<2	<2	72	2,9	13	10	23	4,3	0,16	0,3
wheat B	**T2**	3,4	0,9	<1	11	4,0	21	<0,4	5,5	5,4	<9,5	0,4	0,09	1,5
wheat B	**CC2**	3,3	0,6	<1,5	8,7	4,4	<0,1	<0,1	1,2	5,1	15,5	0,3	0,06	1,4
wheat A	**X2**	2,5	0,6	<1	12	5,9	22	<0,3	4,4	3,3	<9,5	0,4	0,07	1,1
soy B	**J2**	3,5	1,5	5,3	22	4,2	156	71	16	14	26	1,6	0,30	4,5
soy A	**R2**	4,8	1,6	5,0	368	6,7	217	107	20	12	25	1,8	0,30	4,5
		Vit B1	**Vit B2**	**Vit C**	**α-TP**	**β-TP**	**γ-TP**	**δ-TP**	**Vit K1**	**Vit B5**	**Vit B3**	**Total folate**	**Vit H**	**Vit B6**
ratio	**A2/Y2**	0,7	1,0	-	1,6	2,2	-	-	0,6	0,9	0,7	1,1	1,1	1,0
ratio	**V2/G2**	1,2	1,0	>1	0,3	<1	>1	-	0,4	0,7	1,0	0,8	0,9	1,0
ratio	**F2/P2**	0,9	0,9	1,3	1,1	-	1,1	>1	1,0	1,2	-	0,9	1,1	0,8
ratio	**C2/M2**	1,0	1,0	1,3	-	-	0,9	0,8	0,7	1,0	1,0	0,05	1,0	0,7
ratio	**T2/X2**	1,4	1,3	-	0,9	0,7	1,0	-	1,3	1,6	-	1,1	1,2	1,4
ratio	**CC2/X2**	1,3	1,0	-	0,7	0,7	<1	-	0,3	1,5	>1	0,8	0,9	1,3
Ratio	**J2/R2**	0,7	0,9	1,1	0,1	0,6	0,7	0,7	0,8	1,2	1,0	0,9	1,0	1,0

Trace elements/heavy metals Results for the trace elements/heavy metals are presented in Table 3.27. Some trace elements/heavy metals were hardly detected in any of the ingredients, i.e. Co, I, Hg and F, while others did not show significant differences between group A and group B ingredients, i.e. P, K and Mg. Striking differences were observed for Pb; a high concentration of Pb and to a lesser extent for As was observed in barley, group A (sample G2). This result, however, was not confirmed with renewed analysis of the barley and of the feeds, therefore contamination of the sample, like was described for the nicotin in section 3.1, seems the most likely explanation for this result. Some more subtle differences could be observed for trace elements, like Mo which shows a high B/A ratio for the feeds, which is caused by relatively high concentrations for group B soy and peas. Further, selenium showed to be higher in triticale, maize, peas and wheat used to prepare feed B. From the analyses of the total feed, however, no large differences in Se were observed between the A and B feeds.

Table 3.27: Trace elements, heavy metals and minerals in ingredients and group B/group A ratios.

ug/kg	Code	Cd	Co	I	Pb	Hg	Mo	Se	As
Triticale B	**A2**	10	<100	< 100	29	< 2	877	44	<10
Triticale A	**Y2**	29	<100	< 100	< 15	< 2	856	28	13
barley B	**V2**	17	<100	< 100	32	< 2	762	11	17
barley A	**G2**	15	<100	< 100	714*	< 2	895	16	73*
maize B	**F2**	< 2	< 100	< 100	< 15	< 2	410	110	< 10
maize A	**P2**	3	< 100	< 100	16	< 2	400	13	< 10
peas B	**C2**	19	< 100	< 100	25	< 2	2600	83	< 10
peas A	**M2**	35	< 100	< 100	< 15	< 2	1700	48	< 10
wheat B	**T2**	29	< 100	< 100	20	< 2	410	48	< 10
wheat B	**CC2**	69	< 100	< 100	< 15	< 2	434	151	40
wheat A	**X2**	20	< 100	< 100	19	< 2	760	34	< 10
soy B	**J2**	<50	200	120	<20	< 1	2800	62	21
soy A	**R2**	<50	200	<100	<20	< 1	1700	200	14
		Cd	**Co**	**I**	**Pb**	**Hg**	**Mo**	**Se**	**As**
Ratio	**A2/Y2**	0,3	-	-	>1	-	1,0	1,6	<1
Ratio	**V2/G2**	1,2	-	-	0,0	-	0,9	0,7	0,2
Ratio	**F2/P2**	<1	-	-	<1	-	1,0	8,5	-
Ratio	**C2/M2**	0,5	-	-	>1	-	1,5	1,7	-
Ratio	**T2/X2**	1,5	-	-	1,1	-	0,5	1,4	-
Ratio	**CC2/X2**	3,5	-	-	<1	-	0,6	4,4	>1
Ratio	**J2/R2**	-	1,0	>1	-	-	1,6	0,3	1,5

** This extreme high amount of Pb and As could not be confirmed in additional analysis in the feed and therefore is likely the result of contamination of the sample.*

3.3 Microbiology

3.3.1 Microbiology by TNO

The results of the microbial analyses are shown in Table 3.28 for the ingredients and in Table 3.29 for the feed. Salmonella was not detected in any of the samples. Sulphite-reducing Clostridium, C. perfringens and S. aureus were not found in relevant concentrations in any of the samples. E. coli was only found in 3 feeds, i.e. feed 1(B), 3 (A) and 7(A), being feeds of the first generation. However, the commonly used upper limit of 1000 CFU/g is not exceeded. Furthermore, these three feeds do not belong to the same group of feeds, i.e. group A or B. E. coli can be used as a marker for faecal contamination.
B. cereus was found in some samples. In the feeds, the amount of B. cereus ranges from 10 to $6*10^3$ CFU/g (see Table 3.28). However, the difference in amount of B. cereus is larger within one group of feeds, than between group A and B. Hence no significant difference in amount of B. cereus was observed between group B and group A. The same is true for B. cereus in ingredients with the exception of barley group A, sample G2. For this sample a concentration B. cereus was observed just above the limit of 10^5 CFU/g that is generally excepted as safe in case of human consumption. Above this value there is a chance of formation of hazardous amounts of toxin. However, the limit used for humans is a worst-case limit and because the observed value is around the limit value no significant consequences are expected. Furthermore, for chicken the risk is expected to be less while bacteria in chicken have less time to come into contact with the gut and thus the immune-system. Above all, in neither of the feeds, a B. cereus concentration above the limit was observed.

Moulds were found in most samples in numbers ranging from 10^3 to 10^6 CFU/g, while yeasts was only found in 4 ingredient samples (3 wheats, 1 maize). For moulds, two feeds belonging to group A, i.e. Grower feed 2 and Starter feed 12, have a significant higher number of moulds, than their counterparts in group B.

The aerobic colony counts varied between 10^3 and 10^6 CFU/g while Enterobacteriaceae were found in numbers ranging from 10^3 to > 10^5 CFU/g. In general the feeds belonging to group A showed a higher aerobic colony count and a higher amount of Enterobacteriaceae than the feeds belonging to group B. The same might be reflected by the ingredients where samples P2 (maize) and M2 (peas), i.e. used for feed of group A, show a significant higher aerobic colony count and higher amounts of Enterobacteriaceae than samples F2 (maize) and C2 (peas), used for feed of group B. Note that the soy samples J2 (B) and R2 (A) show the opposite trend.

Conclusion No large differences were observed between the feeds A and B. Barley which was used for the A feeds contained more B. cereus, but no differences were found for the feeds. Moulds were more common in two feeds for group A and in general the feeds belonging to group A had higher aerobic colony count and a higher amount of Enterobacteriaceae.

3.3.2 Microbiology by Wageningen UR – Biological Farming Systems Group

There were no significant differences in bacterial communities found between feed A and feed B, nor between Starter and Grower feed.

Table 3.28: Results of microbial analyses on ingredients.

Sample	Code	Aerobic colony count CFU/g	Enterobac-teriaceae CFU/g	E .coli CFU/g	Yeasts CFU/g	Moulds CFU/g	Sulphite-reducing Clostridium CFU/g	C. perfingens CFU/g	S. aureus CFU/g	B. cereus CFU/g	Salmonella spp. per 25g
Maize B	**F2**	5,8 x 10^4	< 10	< 10	< 1000*	6,2 x 10^4	< 10	< 10	< 10	ca 10	n.d.**
Maize A	**P2**	5,4 x 10^6	4,7 x 10^4	< 10	3,0 x 10^4	8,0 x 10^3	< 10	< 10	< 10^3*	ca 10	n.d.**
Peas B	**C2**	2,0 x 10^3	ca 15	< 10	<100*	1,4 x 10^3	ca 20	< 10	< 10	ca 75	n.d.**
Peas A	**M2**	2,0 x 10^6	> 1,5 x 10^5	< 10	< 10	2,4 x 10^3	ca 20	< 10	< 10	ca 15	n.d.**
Triticale B	**A2,**	3,4 x 10^4	4,9 x 10^3	< 10	< 10	1,2 x 10^5	< 10	< 10	< 10	< 10	n.d.**
Triticale A	**Y2,**	4,4 x 10^4	5,2 x 10^3	< 10	< 10	5,7 x 10^4	< 10	< 10	< 10	< 10	n.d.**
Barley B	**V2**	1,1 x 10^6	3,2 x 10^3	< 10	< 10	3,6 x 10^6	< 10	< 10	< 10	< 10	n.d.**
barley A	**G2**	5,4 x 10^5	4,0 x 10^3	< 10	< 10	8,0 x 10^5	ca 80	< 10	< 10	3,1 x 10^5	n.d.**
Soy B	**J2**	2,2 x 10^5	< 10	< 10	< 10	1,2 x 10^4	< 10	< 10	< 10	ca 40	n.d.**
soy A	**R2,**	3,1 x 10^4	< 10	< 10	< 10	5,3 x 10^2	< 10	< 10	< 10	< 10	n.d.**
Wheat B	**T2**	4,8 x 10^6	1,4 x 10^5	< 10	<100*	7,2 x 10^3	< 10	< 10	< 10	ca 35	n.d.**
wheat A	**X2**	8,6 x 10^6	>1,5 x 10^5	< 10	1,0 x10^3	5,6 x 10^3	ca 15	< 10	< 10	< 10	n.d.**
wheat B	**CC2**	5,8 x 10^4	7,2 x 10^3	< 10	< 10	ca 45	< 10	< 10	< 10	< 10	n.d.**

** increased detection limit due to 'disturb' flora.*

*** not detected.*

Note: ingredients A2, V2, F2, C2, T2 and J2 are used to prepare the feeds belonging to group B, while ingredients Y2, G2, P2, M2, X2 and R2 are used to prepare the feeds belonging to group A.

Table 3.29: Results of microbial analyses on feed.

Sample	Code	Aerobic colony count CFU/g	Enterobac-teriaceae CFU/g	E .coli CFU/g	Yeasts CFU/g	Moulds CFU/g	Sulphite-reducing Clostridium CFU/g	C. perfingens CFU/g	S. aureus CFU/g	B. cereus CFU/g	Salmonella spp. per 25g
1st generation											
Grower B	**1**	7,6 x 10^5	5,5 x 10^4	110	< 10	6,8 x 10^3	< 10	ca 5	< 10	5,8 x 10^3	n.d**
Grower A	**2**	2,6 x 10^6	6,5 x 10^4	< 10	< 10	6,8 x 10^4	< 10	< 10	< 10	2,8 x 10^3	n.d**
Layer B	**4**	8,4 x 10^4	5,3 x 10^3	< 10	< 10	1,7 x 10^3	< 10	< 10	< 10	ca 44	n.d**
Layer A	**3**	1,2 x 10^5	5,3 x 10^4	220	< 10	2,3 x 10^3	< 10	ca 5	< 10	ca 10	n.d**
Layer B	**8**	6,0 x 10^4	ca.10^4	< 10	< 10	1,2 x 10^4	< 10	< 10	< 10	1,7 x 10^3	n.d**
Layer A	**7**	2,2 x 10^6	1,5 x 10^5	ca 33	< 10	3,4 x 10^4	< 10	< 10	< 10	2,8 x 10^3	n.d**
2nd generation											
Starter B	**11**	1,3 x 10^5	1,4 x 10^4	< 10	< 100*	1,1 x 10^3	< 10	< 10	< 10	< 10	n.d**
Starter A	**12**	1,6 x 10^5	2,7 x 10^4	< 10	< 100*	1,7 x 10^4	< 10	< 10	< 10	< 10	n.d**
Grower B	**15**	3,5 x 10^5	1,8 x 10^3	< 10	< 100*	1,9 x 10^4	< 10	< 10	< 10	< 10	n.d**
Grower A	**16**	2,7 x 10^5	9,5 x 10^3	< 10	< 100*	1,0 x 10^4	< 10	< 10	< 10	< 10	n.d**

** increased detection limit due to 'disturb' flora.*

*** not detected.*

Note: feed 1, 4, 8, 11 and 15 belong to the same group (group B); the same accounts for feed 2, 3, 7, 12 and 16 (group A).

3.4 LPS endotoxin residues

The feeds were analysed for LPS endotoxin in portions per generation, with eleven months in between these analyses. The amounts measured differ between the portions, as well as the ratio's. However in all cases the ratio is > 1, meaning that feed B contains more LPS endotoxin than feed A. Results are displayed in Table 3.30.

Table 3.30: Analyses of LPS in feeds.

EU/mg feed	Feed A	Feed B	ratio B/A
1st Generation			
Grower	26,5	64,14	2,4
Layer	17,72	42,75	2,4
2nd Generation			
Starter	11,5	13,89	1,2
Grower	14,53	19,02	1,3

3.5 Complementary analyses – Delayed Luminisence – FAS – Biophotons

For all ingredients: wheat, barley, peas, soy, maize and triticale, a significant difference was found between the two samples of the ingredient with this method. Data are visualized in the following figures.

Decoding of samples in this paragraph.

	organic	conventional
Wheat:	E	Z
Barley:	W	K
Peas:	T	M
Soy:	S	A
Maize:	R	G
Triticale:	I	C

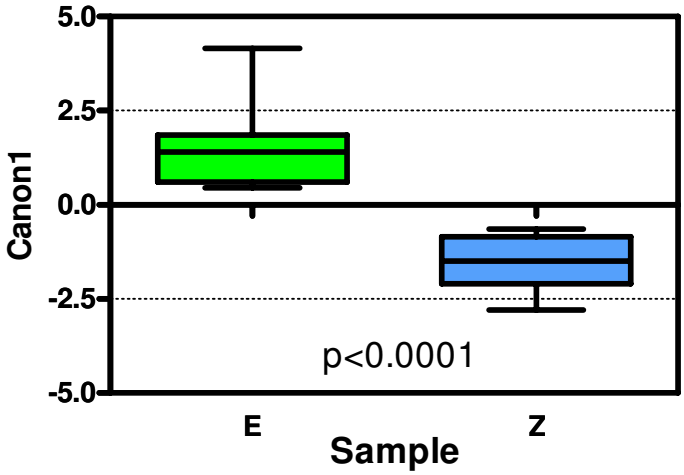


Figure 3.1: Canonical variable canon1 for the evaluation of Wheat samples E and Z. Samples are different with probability p<0,0001 (student's t-test). Boxes contain the 25-75 percentile, whiskers mark the minimum and maximum values.

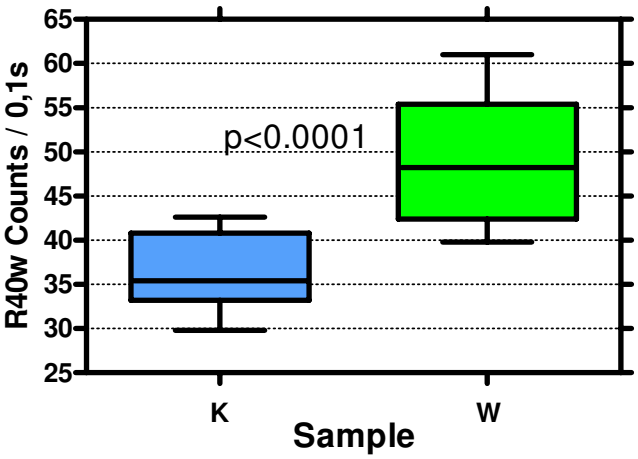


Figure. 3.2: Emission of Barley after white light excitation. Samples are different with probability p<0,0001 (student's t-test). Boxes contain the 25-75 percentile, whiskers mark the minimum and maximum values.

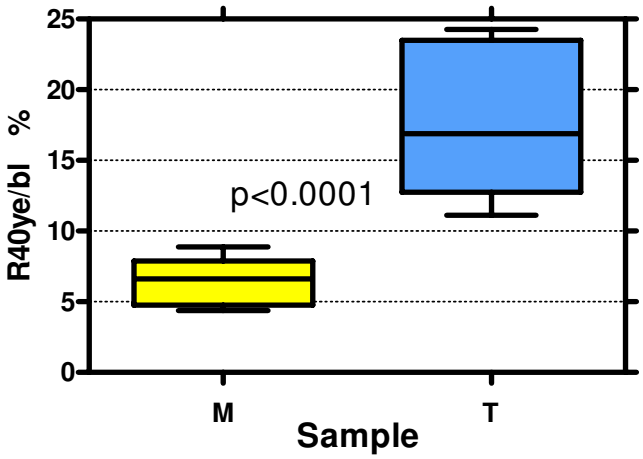


Figure. 3.3: Emission ratio of Peas after yellow and blue light excitation. Samples are different with probability p<0,0001 (student's t-test). Boxes contain the 25-75 percentile, whiskers mark the minimum and maximum values.

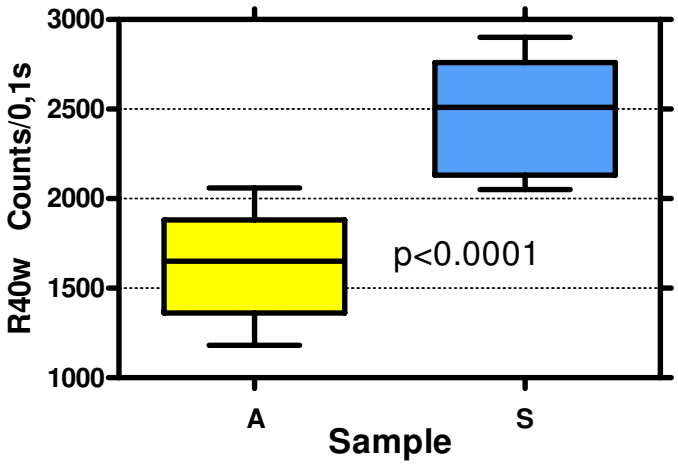


Figure. 3.4: Emission of Soy beans after white light excitation. Samples are different with probability p<0,0001 (student's t-test). Boxes contain the 25-75 percentile, whiskers mark the minimum and maximum values.

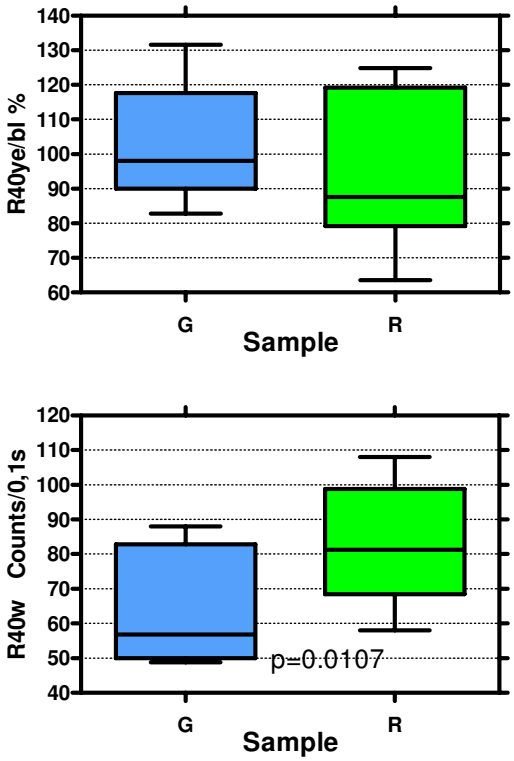


Figure. 3.5: Emission ratio of Maize after yellow and blue light excitation (top graph) and emission after white light excitation (bottom graph). Samples are different with probability p=0,0107 (student's t-test) according to value R40w. Boxes contain the 25-75 percentile, whiskers mark the minimum and maximum values.

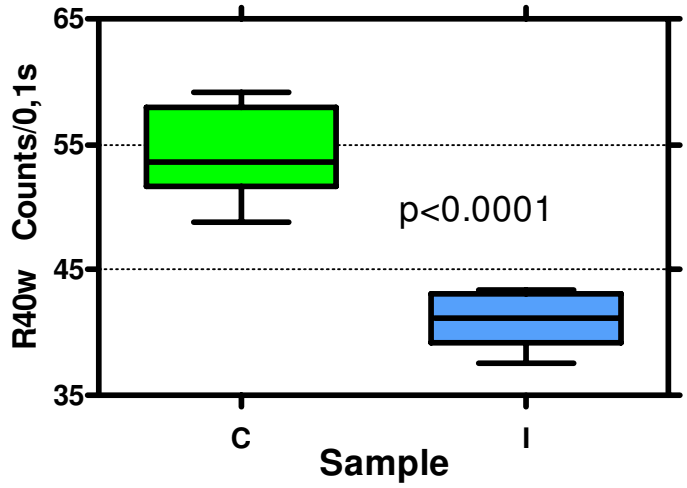


Figure. 3.6: Emission of Triticale after white light excitation. Samples are different with probability p<0,0001 (student's t-test). Boxes contain the 25-75 percentile, whiskers mark the minimum and maximum values.

For wheat and barley significant differences are showing up with several parameters measured. The structure of these data points towards very different growing conditions for these two samples. An approach using discriminant-analysis (see section 2.4.5) turned out to be adequate for wheat, and was used here. For barley and triticale this approach would be adequate, but was not possible yet due to lack of training samples of barley and triticale respectively.

3.6 Complementary analyses – Protein ratio

Decoding of samples in this paragraph.

	organic	conventional
Wheat:	E	Z
Barley:	W	K
Triticale:	I	C
Maize:	R	G
Soy:	S	A
Peas:	T	M

The results of the protein- N and amino acid analyses are presented in Annex 6. The amino acid ratios are presented in table 3.31. The samples of wheat, barley and triticale show differences in protein-N content, the Glu/Lys and Pro/Lys ratio. For maize there is only a little difference and for peas there is practically no difference in the amino acid state of the two pairs. For soy differences in protein-N content and also the ratio of Glu/Lys and Glu/Cys and Glu/Met are observed. The complete dataset can be found in Annex 7.

Table 3.31: Protein content and amino acid ratios in chicken feed ingredients.

	Wheat E	Wheat Z*	Barley K*	Barley W	Triticale C	Triticale I*	Maize G	Maize R	Soy A*	Soy S	Peas M	Peas T
N µmol/100g	11,6	14,7	15,7	12,5	9,3	14,7	10,0	10,6	44,8	38,2	27,0	26,9
Glu/Lys	10,3	11,1	7,4	6,1	6,0	9,6	7,2	7,0	3,5	3,3	2,9	2,9
Pro/Lys	3,9	4,3	3,9	3,3	2,7	4,0	3,6	3,6	1,1	1,0	0,8	0,7
Glu/Cys	12,5	13,5	12,3	10,5	9,5	11,5	7,6	8,6	11,9	9,8	11,2	12,1
Glu/Met	19,8	22,2	17,4	15,0	14,4	18,9	11,1	12,5	16,1	13,9	21,9	22,6
Glu /Trp	37,3	37,9	36,6	32,7	33,7	46,9	40,4	46,0	26,3	24,8	30,6	31,7
Sum AA ‡ µmol/g	770,3	972,1	1047,2	826,5	611,3	985,6	702,4	745,0	3037,7	2575,0	1765,8	1738,9
Sum N ‡ µmol/g	910,4	1148,9	1239,9	986,2	737,7	1161,6	829,7	875,4	3861,6	3257,4	2295,1	2274,1
Nid %	78,2	78,4	79,0	79,0	79,4	78,8	83,3	82,6	86,1	85,4	85,0	84,6

**assumed to be conventional, ‡sum amino acids, sum nitrogen.*

Nutritional Protein Quality In Table 3.32 raw protein as percentage of dry matter is expressed, and the percentage of essential amino acids in the raw proteins. For comparison, one of the samples is normalized to 100%.

Table 3.32: Raw protein content and percentage of the essential amino acids methionine, cystein and lysin in ingredients (normalized to 100%.).

	Wheat E	Wheat Z*	Barley K*	Barley W	Triticale C	Triticale I*	Soy A*	Soy S
Raw Prot. % of DM	100,0	126,0	125,7	100,0	100,0	158,6	117,5	100,0
Met %v of RP	108,7	100,0	100,0	105,7	105,9	100,0	100,0	113,1
Met+Cys % of RP	106,6	100,0	100,0	106,0	101,4	100,0	100,0	115,6
Lys % of RP	104,4	100,0	100,0	110,4	129,6	100,0	100,0	103,4

The raw protein content (RP) of the feed ingredient with the lowest RP is set to 100 %, so the higher raw protein content is shown as 126% for wheat Z. The percentage of methionine based on the raw protein content is set to 100 % for the sample with the highest RP. From this it can be observed that, though less RP is available in wheat E, the relative amount of the essential amino acids in wheat E is higher. Similar observations can be made for the other ingredients.

Based on researcher's experience from other comparison studies (Kahl, 2004), the samples with a higher raw protein content, but a lower protein quality measured in terms of percentage of the essential amino acid of raw protein are classified as being conventional. In this case wheat Z, barley K, triticale I and soy A.

3.7 Complementary analyses – Biocrystallizations

Decoding of samples in this paragraph.

	organic	**conventional**
Wheat	65	64
Wheat DOK trial	66	68
Barley	59	58
Triticale	63	62
Maize	55	54
Soy	50	53
Soy DOK trial	61	60
Peas	51	52
Layer feed	83	84
Starter feed	85	86
Grower feed	87	88

3.7.1 Results Texture analysis

In Table 3.33 the results of the texture analyses of biocrystallizations for the ingredients and feed samples are presented. With the texture analyses all sub-samples of maize, barley, soy, triticale and pea could be grouped, as belonging to one of the two original samples. The statistical difference between the two samples was significant for all these ingredients except for peas. The wheat sub-samples 64 and 65 could be grouped and significantly differentiated, but this was not possible for the DOK samples. For the feeds, only the Grower feeds sub-samples could be grouped correctly. Differentiation was borderline significant (p=0,05).

Table 3.33: Texture analysis results for grouping and differentiation of samples by texture analyses.

Samples	Code	Grouping	Differententiation between samples *	Results confirmed by
Maize	54 and 55	Yes	++	Uni-Kassel
Barley	58 and 59	Yes	++	Uni-Kassel
Soy	50 and 53	Yes	++	
DOK Soy	60 and 61	Yes	++	
Triticale	62 and 63	Yes	++	
Pea	51 and 52	Yes	- (p_{Kappa}=0.08at ROI 70)	
Wheat	64 and 65	Yes	+	Uni-Kassel and BRAD
DOK Wheat	66 and 68	-	-	
Layer feed	83 and 84	-	-	
Starter feed	85 and 86	-	Differentiation borderline p_{Kappa}=0.05 at ROI 70	
Grower feed	87 and 88	Yes	Differentiation borderline $p_{SumVariance}$=0.05 at ROI 90	

** ++ p<0,01, + p<0,05, borderline p=0,05,- not significant.*

3.7.2 Results Visual Evaluation

For the visual evaluation, a 'Two Group Test' was performed. Pictures of the biocrystallizations were coded. Out of the crystallization pictures random sets of photo's were drawn. The researchers divided the pictures in two groups, assuming the pictures to belong to the same sample. In Table 3.34 the results of this grouping are presented. The researchers were capable to correctly group most of the pictures. Only for peas and Layer feed, the grouping was not correct.

Table 3.34: Results visual evaluation 'Two Group Testing'. Percentages per sample.

Samples	Codes	Two group testing*	Percentage correctly classified
Maize	54 and 55	++	94% and 97%
Barley	58 and 59	-	55% and 56% (p=0.26)
Soy	50 and 53	++	100% and 98%
DOK Soy	60 and 61	++	70% and 66%
Triticale	62 and 63	++	77% and 69%
Pea	51 and 52	-	57% and 54% (p=0.30)
Wheat	64 and 65	++	67% and 78%
DOK Wheat	66 and 68	++	72% and 74%
Layer feed	83 and 84	-	57% and 54% (p=0.24)
Starter feed	85 and 86	++	71% and 73%
Grower feed	87 and 88	++	73% and 68%

** ++ p<0,01, + p<0,05, - not significant.*

3.7.3 Simple Descriptive Test

The 'Simple Descriptive Test' was applied to describe the main characteristics and the gestures of the crystallisation pictures. An assumption was made concerning which groups of pictures belonged 'together', to the same production system. An overall characteristic of both groups is given.
In Figure 3.7 till 3.16 representative photos of the ingredients and feeds are displayed.
Pictures are placed either left or right, as assumed to belong to one or the other production system.

The typical features these groups have in common are described.
Group on the left The pictures on the left show broader stems, a less strong perradiation, more curved gestures, a wider angle of ramification, several times more fullness with sideneedles.
Group on the right The pictures on the right show more 'concentrated' stems, a stronger perradiation, more radial gestures, a more narrow angle of ramification, more transverse needles.

Maize

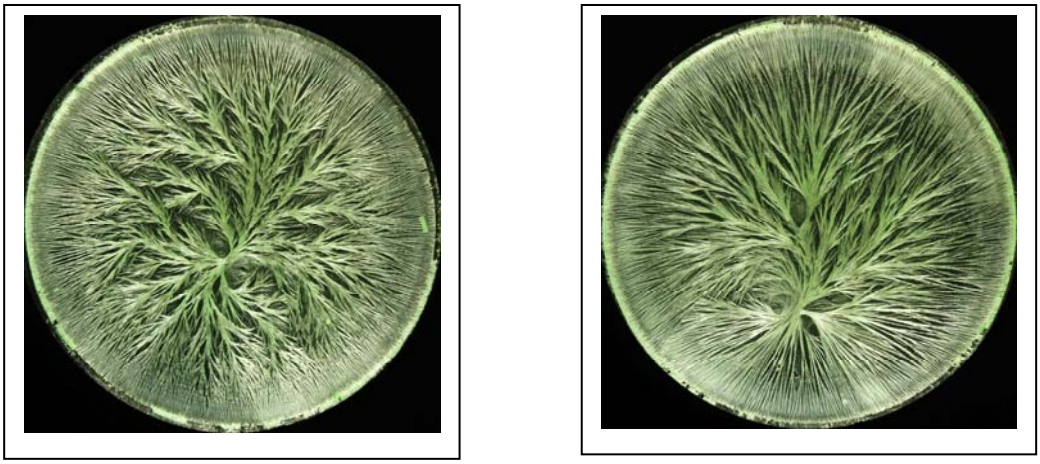


Figure 3.7: Representative pictures of the two Maize samples nr. 54 (left) and nr. 55 (right).

Barley

Figure 3.8: Representative pictures of the two Barley samples nr. 58 (left) and nr. 59 (right).

Soy

Figure 3.9: Representative pictures of the two Soy samples nr. 53 (left) and nr. 50 (right).

Triticale

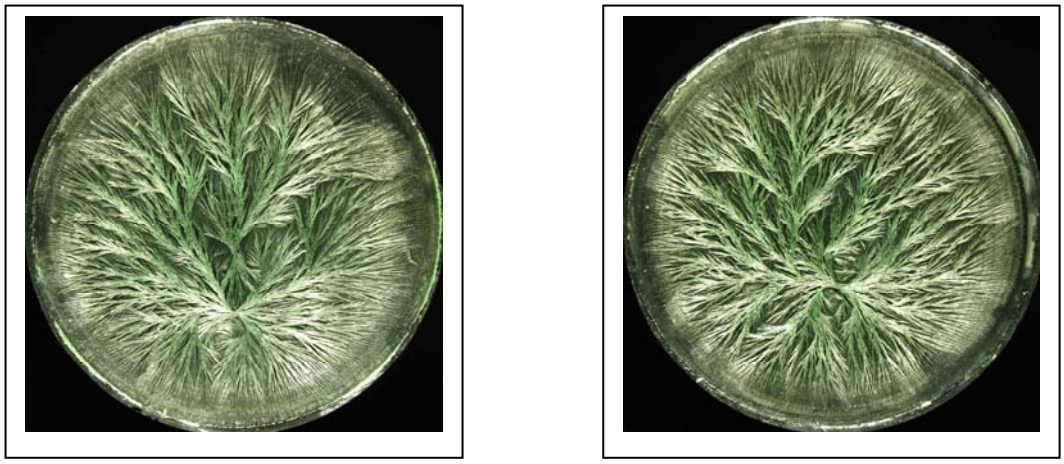


Figure 3.10: Representative pictures of the two Triticale samples nr. 62 (left) and nr. 63 (right).

Pea

Figure 3.11: Representative pictures of the two Pea samples nr. 52 (left) and nr. 51 (right).

Wheat

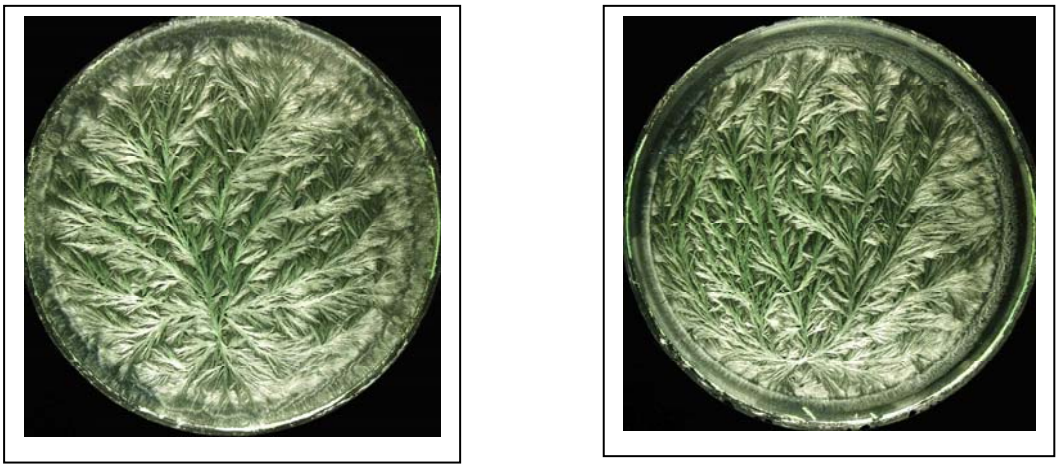


Figure 3.12: Representative pictures of the two Wheat samples nr. 65 (left) and nr. 64 (right).

DOK samples

As reference material 4 DOK-Trial samples, of wheat and soy, originating from conventional and organic production in this trial, were analysed in parallel. These samples were also blinded.

These pictures are placed here, also left and right, according to the assumptions of the researchers.

Figure 3.13: Representative pictures of the two DOK Wheat samples nr. 66 (left) and nr. 68 (right).

Figure 3.14: Representative pictures of the two DOK Soy samples nr. 61 (left) and nr. 60 (right).

Crystallization pictures of the feed

Also for the pictures of the feed an assumption was made concerning the group of pictures belonging together. Also here pictures are placed either left or right, as assumed to belong to one or the other production system.

Layer feed

Figure 3.15: Representative pictures of the two Layer feed samples nr. 84 (left) and nr. 83 (right).

Starter feed

Figure 3.16: Representative pictures of the two Starter feed samples nr. 85 (left) and nr. 86 (right).

Grower feed

Figure 3.17: Representative pictures of the two Grower feed samples nr. 87 (left) and nr. 88 (right).

Conclusions

The 6 ingredients used for making the feeds were analysed with the crystallisation method together with the 3 total feeds used and 4 ingredients from the DOK Trial. The crystallizations of all ingredients and feeds, except the peas and Layer feed, could be differentiated highly significantly ($p<0.0005$) according to the production system by means of computerized image analysis and/or by 'Two Group Testing' in visual evaluation.

By visual evaluation all ingredients were grouped in two groups with comparable gesture. The researchers did not have experience with soy, peas, barley, triticale and maize, which could be used in this study. Where experience was available an assumption was made about the origin of the samples.

3.8 Profiling of feeds

Codes: sample 2, 3, 7,12, 16 - sample A = organic

Sample 1,4,8, 11, 15 - sample B = conventional

Figure 3.17 shows the PCA of the polar fraction of the different chicken feeds analysed. It can be observed that the 6 different replicas of each sample cluster very close together, showing the high reproducibility of extraction and analytical method. At the same time, the different samples are clearly separated from each other, revealing that statistically significant differences between them have been found.

Samples 11 and 12 show differences between them but cluster much closer to each other than to the others, which is logical because both of them have the same composition. The difference between them is due to the origin of the ingredients used to prepare them, that is to say, one of them was prepared with organic ingredients and the other with conventionally produced ones. So, the difference between sample 11 and 12 is due to the different cultivation practices of their ingredients.

On the opposite side of the PCA are placed samples 1, 2, 15 and 16, belonging to Grower feed, which also cluster close to each other. The differences between them are thus again due to the origin of the ingredients used in their preparation.

And finally, the Layer feeds (samples 3 and 4), which are placed close to the Grower feed but slightly more to the left, also show the same behaviour than the former.

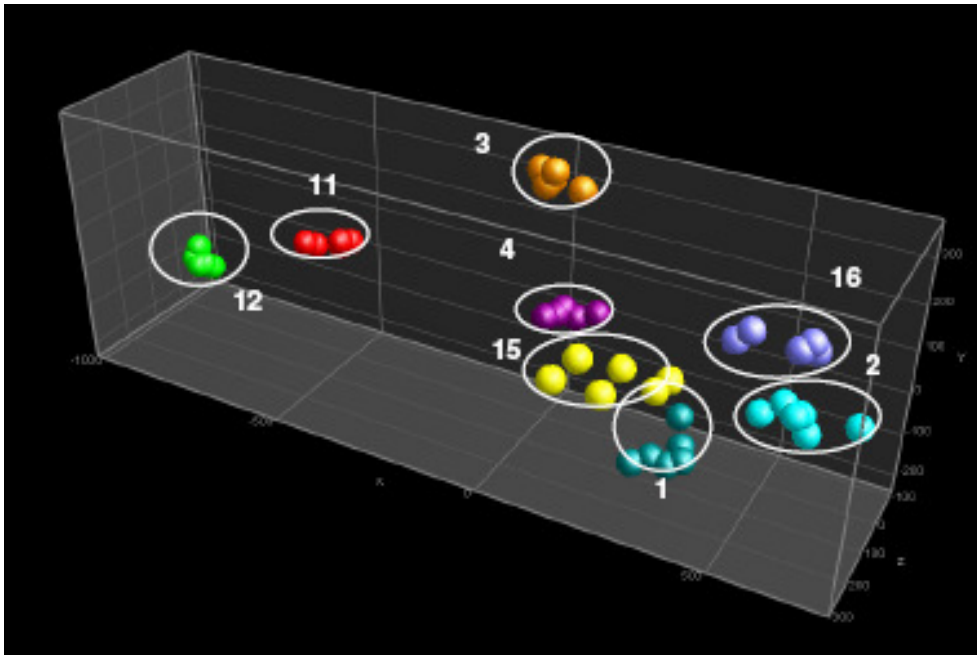


Figure 3.18: PCA-plot of the polar components in the different feeds used in the study.

In order to see which are the signals provoking these differences, the ^{1}H NMR spectra of the samples were overlaid and it was observed that the X axis in the PCA is explained, to a large extent, to the isoflavone derivatives concentration.

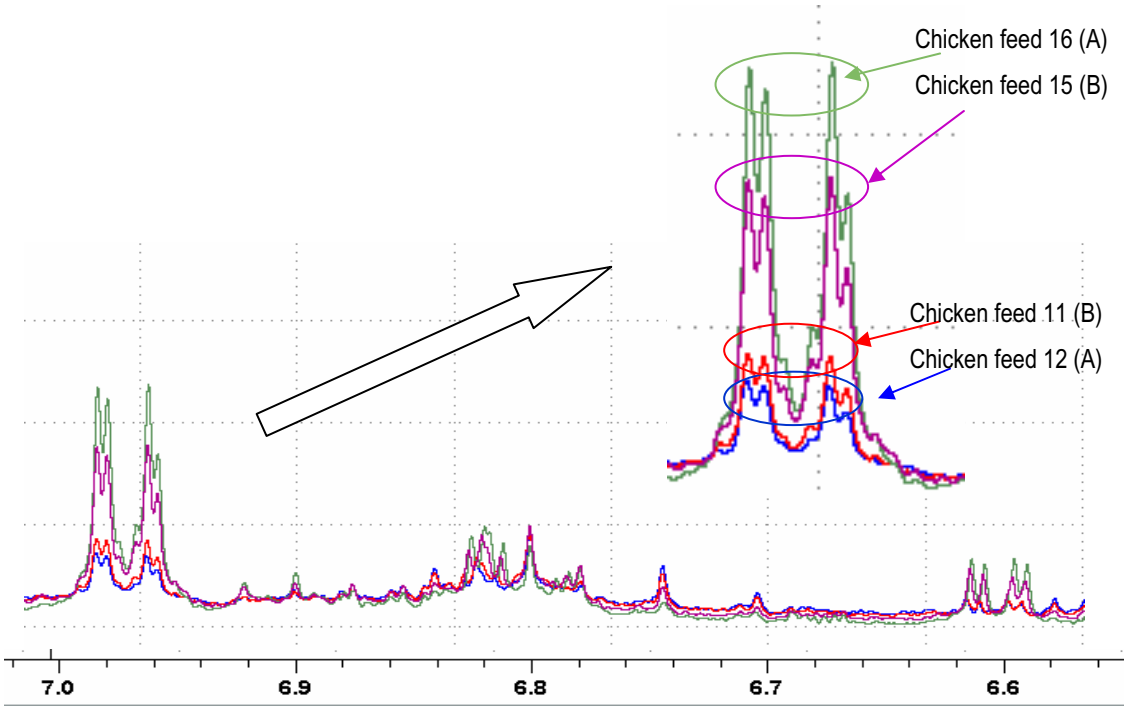


Figure 3.19: NMR-spectrum of the polar fraction of the Grower feeds 15 and 16, and the Starter feeds 11 and 12.

Figure 3.19 shows an expanded ^{1}H NMR spectrum of the polar fraction of the Grower feeds 15 and 16, and of the Starter feeds 11 and 12. Some of the isoflavone derivatives signals appear in this region. In this figure, it can be clearly observed that chicken feeds 15 and 16, that is to say, the Grower feed, have a higher amount of isoflavones than chicken feeds 11 and 12 (the Starter feeds). The estimated ratio of isoflavone content between the 2 types of chickenfeed is approximately of 3.5.

As it was previously shown in the PCA, it is also possible to detect differences in the isoflavone content between the feeds with the same composition, that is to say, between 11 and 12 (Starter feed) and between 15 and 16 (Grower feed). So, sample 11, which was more to the right in the PCA, shows a slightly higher amount of isoflavones than sample 12, and the same occurs with sample 16, which shows a higher amount of isoflavones than sample 15. When the spectra of different samples of the Grower feeds are overlaid, it can be observed that chicken feed 2 has a very similar amount of isoflavones than chicken feed 16 (being both of them prepared with ingredients A), and chicken feeds 1 and 15 show also very similar concentrations of isoflavone derivatives (both of them prepared with ingredients B) and less than the formers.

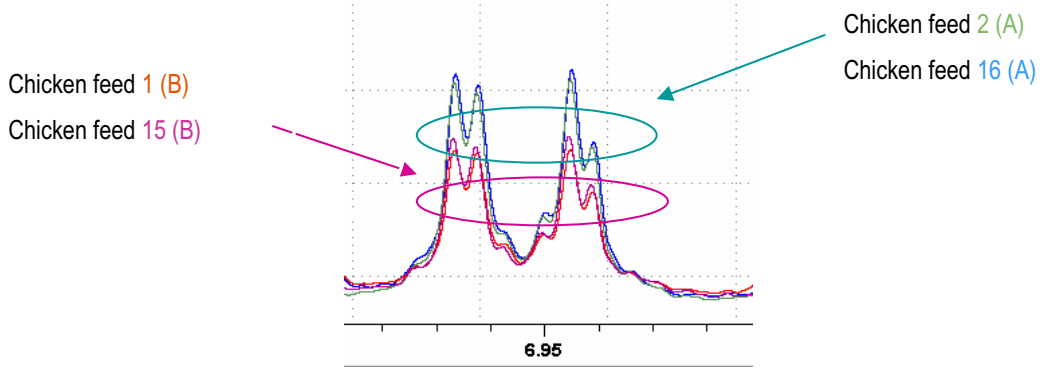


Figure 3.20: NMR-spectrum of the polar fraction of the Grower feeds 1 and 15, and 2 and 16.

Figure 3.21 shows this same expanded spectral region of the chicken feed 2 and 16 (Grower feed) and of 3 and 4 Layer feed. As it could be expected, chickenfeed 3 and 4, which are the Layer feed, have less amount of isoflavone derivatives than the Grower feed, showing feed 3 a slightly higher concentration than the feed 4.

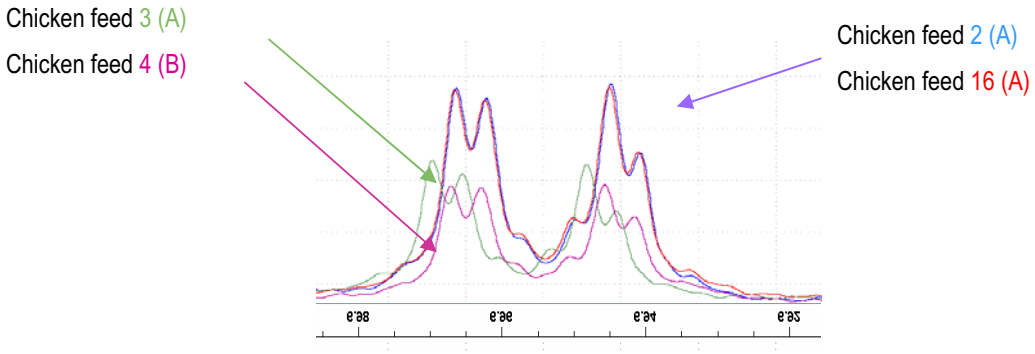


Figure 4

Figure 3.21: NMR-spectrum of the polar fraction of the Layer feeds 3 and 4, and the Grower feeds 2 and 16.

The differences in isoflavone content were also observed in the untreated organic and conventional soy used for the production of the different feeds. Levels in the organic soy were clearly higher.

Conclusions The metabolomic profiling of the polar fractions of the different chicken feeds, based on ^{1}H NMR data, showed clear differences in the composition of the samples, mainly related to their isoflavone content. This study showed not only differences between samples of different composition but also between chicken feeds with the same composition but prepared with organic or conventionally grown ingredients. In fact, all the chicken feeds prepared with ingredients A show higher amounts of isoflavone derivatives than those prepared with ingredients B (except samples 11 and 12, in which the feed prepared with ingredients A show less amount of isoflavones than that prepared with ingredients B).

3.9 Overview of feed analyses

Feeds were intensively analyzed for both nutritive and antinutritive components. In Tables 3.35 and 3.36 the main differences observed by the analyses of TNO Quality of Life are presented. Most consistent differences were observed in the amount of proteins, which was about 10% higher in all the feeds of group B compared to feeds A. Iodide was consistently higher in sample A, alpha-tocopherol and total folate was higher in most of the feeds A compared to the feeds B, whereas chloride was higher in most of the feeds B. Also micronutrient contents calculated from the analyses data from the separate ingredients indicate that differences between the feeds are present. Based on these calculation, feeds B contain more alpha-carotene, vitamin C, vitamin B5 and phytosterols, whereas feeds of group A contain more carotenoids, vitamin K1 and isoflavones. The latter is confirmed by the metabolomic profiling of the polar fractions of the different chicken feeds (RIKILT), which showed clear differences in the composition of the samples, mainly related to their isoflavone content.
With respect to microbiology, no large differences were observed between the feeds A and B. Moulds were more common in two feeds for group A and in general the feeds belonging to group A had higher aerobic colony count and a higher amount of Enterobacteriaceae. Further, it showed that feed B contained consistently more LPS endotoxin than feed A.

Based on the complementary analyses, differences between the feeds from the different origins were found. Where experience was available an assumption on the origin of the samples was made.

Table 3.35: Summary of differences observed between group A and group B feeds.

B>A of A>B	Nutrient	Difference	Remarks
B>A	Amino acids	0-20%	Except for methionine in ratio 8/7 and 11/12
B>A	Protein	10%	
B>A	Chloride	10-40%	Except for ratio 11/12
A>B	Unsaturated C18 fatty acids	20-30%	Not consistent: only for ratio 15/16, during challenge
A>B	Iodide	10%	
A>B	Alfa-tocopherol	0-20%	Except for ratio 8/7
A>B	Total folate	0-30%	Not consistent, no difference in ratio 4/3 and 15/16
B>A	LPS endotoxins	20-240%	
Concentrations based on calculation			
A>B	Carotenoids* (except alpha-carotene)	0-50%	

B>A	Alpha-carotene*	10%	
B>A	Vitamin C*	20%	
B>A	Vitamin B5*	20%	
A>B	Vitamin K1*	10-25%	
B>A	Phytosterols*	0-30%	Except stigmasterol in ratio 11/12
A>B	Isoflavones*	0-30%	

** nutrients not determined directly in feed samples but calculated from feed ingredients.*

Table 3.36: Summary of differences observed between group A and group B ingredients.

B>A of A>B	Nutrient	Difference	Comment
B>A	Amino acids	10-40%	In wheat, soy and barley
B>A	Protein	20-30%	In wheat, soy and barley
B>A	Phytosterols	0-200% depending on specific sterol	In soy, barley
A>B	Isoflavones	20-30%	In soy
A>B	Vitamin E (especially alpha-tocopherol)	30-70%	In soy
B>A	Vitamin C	30%	In maize and peas
B>A	Vitamin B5	50-60%	In wheat
A>B	Total folate	>100%	In peas
A>B	Vitamin K1	20-70%	In various ingredients
A>B	As	80%	In barley

4 Material & methods – Chicken experiment

4.1 General data on the chicken, lines, generations

The research subjects are chicken from the Wageningen Selection Lines, housed in the stables of experimental facility 'De Haar' van Wageningen UR, Animal Sciences Group.

It concerns a population of chicken, originating from cross ISA Brown Warren medium heavy layer hens, of which animals were divergently selected on their primary immune response (Natural Antibody (NAb) titer), being either high or low. During 25 generations these two lines are selected in each generation anew, by intramuscular immunization with Sheep Red Blood Cells (SRBC) at 37 days of age and subsequent measurement of antibody titers to SRBC 5 days after this. Additionally to these lines a random bred control line, originating from the same original parental stock, was maintained (Parmentier 2001, 1998, 1997). After 18 generations of these lines the selection plateau was reached and the chicken were considered homozygous for all loci determining the trait under selection. There are a number of modifications in the immune system caused by the selection. The result of the selection was a 200 fold interline difference between the H and L line for antibody levels and lower threshold dose for T-cell dependent antigens, while there was no difference between H and L line in the response to T cell independent antigens: *Brucella abortus*, *Salmonella* as well as in macrophage activity (Parmentier 1998). T cell activity in vitro was higher in the L line than in H line. H line was more disease resistance to Marek challenge and Coccidiosis (*Eimeria acervulina* challenge) (Parmentier 2001). Only recently there is evidence that neuroendocrine responsiveness has also been affected by the selection procedure. The production, by bidirectional selective breeding, of lines of chicken with extreme opposite phenotypes, was the approach used. The prediction in this work is that major modifications of specific immune responses resulting from this selection also induced profound modifications of resistance/susceptibility to several external factors, including the exposure to different feeds. This would enable us to analyze whether some feeds contribute to a more reactive immune system providing a basis to understanding better health performance.

The three groups used in this study, come from this model:

- H-line, chicken with a high Natural antibody response to SRBC.
- C-line, control animals of randomly bred chicken, representing the whole 'broadness' of the natural population.
- L-line, chicken with a low Natural antibody response to SRBC.

In this study two generations of these chicken lines were included. A generation design was chosen for several reasons. Primarily the expectation is that possible health effects in the second generation of animals, fed with the experimental feed, will be larger. This is based on e.g. Staigers work and work done by the Danish Research Centre for Organic Farming (Lauridsen 2007). Secondly there is an antibody exchange between the hen and her eggs. New feed or ingredients can induce antibody production with various antigen specificities. As antibody production will be one of the outcome parameters, we liked to minimize this effect. Last but not least, the main effect was expected to be found in the innate immune system. It is generally accepted that the parent(s) environment and genome have an effect on the status of the innate immune system of the chicks. Therefore the experiment did cover two generations. As the situation for the two generations was very different, these are described separately.

Animal ethics commission The AEC application for this project has been approved on September 1st 2005 (Annex 5).

4.2 Feeding experiment in chicken, housing, etc.

4.2.1 The first generation

The first generation of animals in the experiment was acquired from the 25th generation of the Wageningen Selection Lines. The experimental animals were randomly chosen from the stock of pullets at 35 days of age, just before the SRBC-immunization would have been administered. So these were not immunized with SRBC, but were considered to represent the lines without testing.
In this way of each line 24 hens were selected. These groups of 24 were randomly divided in two, each group to be fed with one of the two experimental feeds. This resulted in 6 groups of approx. 12 animals each, 71 altogether.

Table 4.1: Number of hens per group in the first generation.

N=	Feed A	Feed B	Sum
H-line	11	13	24
C-line	12	12	24
L-line	12	11	23
Sum	35	36	71

At the same time 22 (8 + 7+ 7) roosters, from the same lines, were selected, to be raised and fed also on one of the two experimental feeds and to take part in creating the second experimental generation. This resulted in 3 to 4 roosters per group.

Housing The animals of the first generation were housed in groups till 8 weeks of age. Then they were housed individually in breeding cages, as is usual in these stables. Individual animals within the lines were randomly divided over the breeding cages. The aim was to be able to identify the eggs of these animals and to ensure identification also of the next generation. The cages were situated in two rows, an upper and a lower one, with a little difference in light intensity between the top and the bottom row. The animals of each feed were housed half at the top, half at the bottom row.

Figure 4.1: Housing of the first generation.

Climate and light exposure The animals were exposed to the recommended temperature, humidity and light scheme, as used in the Wageningen stables.

Feeding regime and registration From the day of hatch, October 18th 2005, the first generation was fed ad libitum, first with the commercial 'Starter feed', normally used for the animals. At 7 weeks of age this feed was changed into the usual commercial 'Grower feed'. This feed is further referred to as 'Original' feed. At 11 weeks of age the animals feed changed from the original Grower feed to the 'experimental' Grower feed, being conventional or organic and blinded as either 'A' or 'B'. This feed was also provided ad libitum. At 18 weeks of age the Grower feed was replaced by Layer feed. This feed was used, till the second generation was present, alive and well. Every six weeks a fresh batch of feed was provided.
Feed consumption was registered weekly till 30 weeks of age. The used time schedule of the 1st generation (in Dutch) can be found in Annex 1a.

Body weight registration The animals were weighed at weekly intervals from the moment they were housed individually, from week 8 onwards till 40 weeks of age.

Vaccination The animals of the first generation received the in this stable usual vaccinations, being on day 1 Marek Rispens, HVT and IB MA 5; at 2 weeks NCD-1; at 3 weeks Gumboro; at 4 weeks NCD-2; at 7 weeks PDww; at 10 weeks IB Primer; at 12 weeks ILT; at 13 weeks AE; at 14 weeks NCD-3; at 16 weeks IB H52.

Table 4.2: Explanation of vaccinations and connected diseases.

Marek Rispens HVT	Mareks Disease
IB MA 5	Infectious Bronchitis
NCD	Newcastles disease
Gumboro	Gumboro
PDww	PoxDiphteria wing web
ILT	Infectious Laryngo Tracheitis
AE	Myoclonia congentia (Trilziekte)

Health checks The animals were seen by the caretaker daily and abnormalities were registered. Routine blood checks were performed randomly every 2 months on Aviary Influenza, Mycoplasm synoviae, Mycoplasma gallisepticum and Salmonella.

Egg registration The first eggs were layed from an age of 19 weeks onwards. The number of eggs, layed within a time period, was registered and the quality, being either first choice (=normal) or second choice (=abnormal), was described. Once a week, for every hen, an egg was weighed.

Breeding From week 31 artificial insemination started and was continued during three weeks.

As not all hens were laying, the 6 line- and feed-groups consisted of 10-12 mother hens per group. Per group 3-4 roosters were available. Hens and roosters were matched in such a way that sibling matches were avoided. From week 31 till week 33 the collecting of (fertilized) eggs for brooding started. Egg weight was on average 51gram.

Brooding The brooding was performed in a climate respiration chamber (80m³), with an automatic tray turning system that turned the eggs every 60 min. at an angle of 90^0. The climate regulation was e.g. controlled by sensors on the eggshells. Brooding started at week 33 of the first generation with 864 eggs, 447 from feed A and 417 from feed B, divided over the 3 lines.
The eggs were checked at day 10 and 18, and 175 eggs were removed because of being infertile, or because the embryo had died. 689 eggs remained, which is 80%.

4.2.2 The second generation

On June 29th 2006 the second generation (or 26th generation of this animal model) hatched.
The chicks were sexed the same day and roosters were removed. Of the remaining 334 hens, 183 were of the A-group and 151 of the B-group, divided over the 3 lines.
The goal was to have 6 experimental groups (3 A- and 3 B-groups) of 25 animals each. As some animals were expected to die in the first week, first 6 groups of 35 animals were formed. The animals were selected in such a way that a maximum variation of mother hens and roosters was reached, with all mothers equally represented.
Altogether 3 animals died, 1 in the A-group, 2 in the B-group. After 10 days the 6 groups were reduced to 28 animals each, in such a way that per mother a chick was removed, randomly if all looked healthy, or if one of the siblings looked weak, that one was removed. Looking weak was considered a risk for dying in the future, while the aim was to have complete groups. After one week another reduction was performed till the ultimate group size, being 26 animals per group. The number of 26, instead of the planned 25 animals per group, had the following reason.
The chicken were housed in groups of 6 animals. To minimize the risk of feather pecking, it was decided to create a natural order among the animals, and to put 2 animals of each line together (2 H-liners, 2 C-liners, 2 L-liners), while avoiding siblings in one group. That resulted in 13 groups of 6 animals per feed, so 2 x 13 x 6 = 156 animals altogether.
It turned out after a few weeks, that there were still some roosters among the animals. As soon as this became clear, the rooster was removed from the group.
Finally the following groups constituted the experimental population:

Table 4.3: Number of animals per group in the second generation.

N=	Feed A	Feed B	Sum
H-line	26	24	50
C-line	24	22	46
L-line	23	26	49
Sum	73	72	145

Housing The animals of the second generation were housed in a different way than the first generation. Once the genetic background of each individual animal was known, it was possible to follow the principle of optimal animal welfare, which meant housing in a group on the floor, with space and an enriched environment. Here an ethical principle counted, as well as a scientific. The presumption was that a maximum effect from the feeding treatment would be best expressed when natural behaviour of the animals would be optimal. Housing outside was no option, as uncontrolled infections needed to be avoided. An indoor housing was build following the organic norms, but even bigger. Two rows of 15 runs (cages) in each row, meeting at the back side (an A- and a B-row). In each run a group of 6 animals was housed. The surface per run was 2.28 m^2, providing 0.38 m^2 space per animal (compare the organic norm: 0.2 m^2 per animal (SKAL). A bigger part was free floor surface, a smaller part covered by a wooden grid. Above the wooden grid a perch was placed, with 0.2 m space per animal (Annex 7). The run was enriched by a sand-bath in a plastic tray and a wooden box, open on one side, to enable shelter. The floor bedding consisted of wood shavings during the first 3 weeks and from the 4th week onwards of sand. The run was cleaned once a week. Feed was provided in a movable hanging trough, water through a movable water pipe with 6 drinking nipples.
The used time schedule of the 2nd generation (in Dutch) can be found in Annex 1b.

Figure 4.2: Housing of the second generation.

Climate and light exposure The animals were exposed to the recommended temperature, humidity and light scheme, as used in the Wageningen stables.

Feeding regime and registration The second generation of animals was fed from the day of hatch, June 29th 2006, ad libitum with experimental 'Starterfeed', organic or conventional, blinded as either 'A' or 'B'.
At 7 weeks of age this feed was changed into the experimental 'Grower feed'. This feed was continued till the animals end of life at 13.3 weeks. Feed consumption was registered weekly per run, for the group as a whole.

Table 4.4: Explanation of vaccinations and connected diseases.

Marek Rispens HVT	Mareks Disease
IB MA 5	Infectious Bronchitis
Paracox	Coccidiosis
NCD	Newcastles disease
Gumboro	Gumboro
PDww	PoxDiphteria wing web

Body weight registration On day 1 the animals were weighed and hereafter weekly on a fixed day.

Figure 4.3: Weighing on day 1.

Vaccination The animals of this generation received the recommended vaccination scheme, and a separate anti-coccodium (Paracox, Schering Plough) as with housing on the floor, an infection with coccidiosis could not be prevented.
On day 1 was given Marek Rispens, HVT and IB MA ; on day 5 Paracox-8 was supplied in separately provided drinking water. At week 2 NCD-1was given; at week 3 Gumboro; at week 4 NCD-2 and at week 6 PDww.
See for the vaccinations also the timeschedule 2nd generation in Annex 1b.

Health checks The animals were seen by the caretaker daily and abnormalities were registered. A routine blood check was performed in week 6 on 10 animals, on Aviary Influenza, Mycoplasm synoviae, Mycoplasma gallisepticum and Salmonella. In week 10 a poultry veterinarian from Veterinary University Utrecht, Dr. S. Westendorp, observed all and handled several pullets to check feather development and general health.

The KLH challenge As in the first generation the change towards the experimental feed at 11weeks was surrounded by blood sampling, in the second generation a challenge with KLH at week 9 was at point 0, before and after which blood was drawn.
KLH is an innocuous respiratory protein derived from the giant keyhole limpet haemocyanin mollusc (*Megathura crenulata*). KLH was used because it generates a robust antigenic response in animals. This non pathogenic model

antigen induces a strong activation of the T-cell system resulting in cytokine release and providing ample B-cell help for antibody formation (Van Eerden 2004, Hangalapura 2003, Demas 1997).

At 13 weeks of age (91days) the animals life was sacrificed.

4.3 Blood and tissue sampling

4.3.1 First generation

Blood sampling The change towards the experimental feed at 11 weeks (point 0) was monitored by the first blood sampling in these animals at 9 weeks (-2), the second at 12 weeks (+1) and two later, at 19 weeks (+8) and 33 weeks (+22).

No tissues were sampled in this generation.

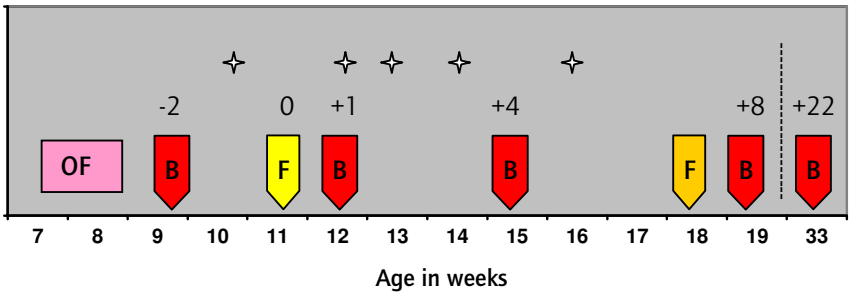


Figure 4.4: Timeframe of the 1st generation with vaccinations, feed changes and blood sampling.
OF= original feed till week 11, B = blood sampling, F = experimental feed.

Vaccinations, marked in this Figure by ✧

10 weeks – IB primer

12 weeks – ILT

13 weeks – AE

14 weeks –NCD -3

16 weeks – IB H52

4.3.2 Second generation

Blood sampling In the second generation a challenge with KLH at week 9 was a point 0, before and after which blood was drawn. Blood was sampled at week 8 (-1), week 10 (+1), week 11 (+2), week 12 (+3) and week 13 (+4). It must be noted that in week 7, so one week before blood sampling -1, the experimental feed had changed from a Starter feed into a Grower feed.

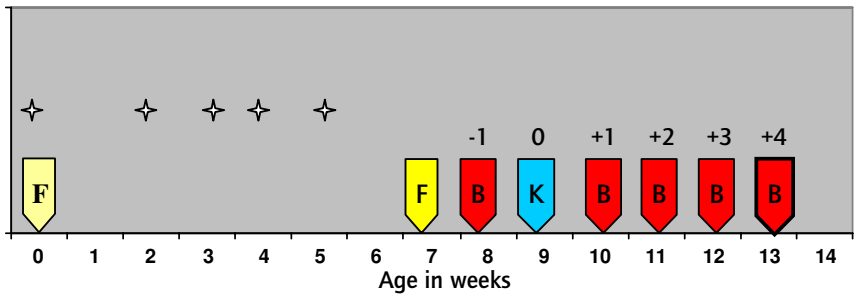


Figure 4.5: Timeframe of the 2nd generation with vaccinations, feed change, KLH challenge and blood sampling. F = experimental feed, B = blood sampling, K = KLH challenge.

Vaccinations, marked in this Figure by ✧

0 weeks –Marek HVT, IB MA5, Paracox-8

2 weeks –NCD -1

3 weeks – Gumboro

4 weeks –NCD -2

5 weeks – PDww

Section and tissue sampling At 13 weeks of age (91 days) the animals of the second generation were killed by cervical dislocation, and were then dissected. This way of ending the animals life was chosen because of the planned analyses on the animals. All 145 animals were handled in one day by a team of eight people. The animals were brought randomly from the stables, when a veterinarian, specialized on poultry, performed the cervical dislocation. The animals were hung upside down during approximately five minutes. Then the animal had quieted and it was weighed. After this the veterinarian opened the body, inspected for abnormalities, took out the complete gastrointestinal (GI-) tract from distal oesophagus till cloaca, including the omentum, liver and gall bladder, spleen and bursa.

These organs were separated in a connecting room. The liver was separated from the gall bladder, weighed, divided and prepared for metabolomics in liquid nitrogen, and for histological analysis in 10% formalin.

The spleen was taken off, divided and prepared for genomics-possibilities and histological analysis. The gastro-intestinal (GI-)tract, without liver, gall bladder and spleen was, weighed, after the stomachs had been emptied. The rest of the gut was left filled as it was, because of practical reasons. Then the two stomachs, ventriculus and proventriculus, and the bursa were taken off and prepared for histological analysis. From the remaining GI-tract of some chicken faecal samples were gathered for microbiological analysis. From the GI-tract samples were prepared of duodenum, jejunum and cecum for genomics analyses, as well as for histological analysis.

In the mean time the vet had taken off the breast skeleton with muscle, for the butcher to prepare in a separate room breast filets for sensory analyses. The veterinarian prepared the thymus, heart, lungs, kidneys, pancreas, ovaries and some bone for histological analysis. From some chicken the brains were taken out and prepared in hapteen for biobanking. The rest of the tissue was discarded.

The section resulted in the following samples for:

Table 4.5: Overview of samples from the chicken of the 2nd generation.

Analysis	**Total**	**H-line**	**C-line**	**L-line**	**Comments**
Pathological Anatomy (PA)/histolo-gical analyses	70 samples	12 animals (6 A and 6 B randomly chosen)	all 46 animals	12 animals (6 A and 6 B randomly chosen)	Thymus, bursa, spleen, heart, liver, ovaries, lungs, kidneys, proventriculus and ventriculus, pancreas, duodenum, jejunum, cecum and some bone from right tibia.
Metabolomics	70 samples	12 animals	all 46 animals	12 animals	Same animals as PA
Genomics	70 samples	12 animals	all 46 animals	12 animals	Same animals as PA
Faecal analyses	60 samples	20 animals	20 animals	20 animals	
Sensory analyses	58 samples	6 animals from feed A	all 46 animals	6 animals from feed A	H- and L-animals; same as PA
Biobanking PA	All minus 70				Thymus, heart, liver, duodenum, jejunum and cecum were stored, fixed in formalin.
Biobanking Genomics	All animals	50 samples	46 samples	49 samples	Frozen gut tissues and spleen
Biobanking Brains	36 animals	12 animals	12 animals	12 animals	Frozen in heptane
Biobanking Plasma	All animals	All	All	All	Frozen plasma

4.4 Immunological assays in 2 generations

Immunological analyses were performed in the laboratories of the Department of Animal Sciences of Wageningen UR, Cell Biology & Immunology group and the Adaptation Physiology group, in a cooperation between Louis Bolk Institute and Wageningen UR.

The Immune System

The starting hypothesis with respect to exploring the immune system in this project is the relatively recent notion that the *innate, natural compartment of the immune system* is necessary, and in fact, indispensable in driving and orchestrating subsequent responses from the *adaptive or specific compartment of the immune system*. Besides, recent studies show that the innate immune compartment is not constant, but can be modulated by external (food (!), infection load, husbandry conditions) and internal (stress, hormonal state) immunomodulatory factors. Effective disease protection and long-term survival, and thus health, are therefore dependent on the interplay between these two compartments of the immune system (see figures).

The *innate compartment* of the immune system consists of two branches: a humoral branch with blood soluble factors such as Natural Antibodies (Nabs) and Complement and a cellular branch with cells like Monocytes, Dendritic cells, Natural Killer cells and Macrophages. Its helps the organism as a first line of defence when challenged by bodily foreign organisms.

The *adaptive antigen-specific compartment* of the immune system also consists of two branches: a humoral, antibody-mediated branch with a prominent role of B-cells, producing e.g. vaccin-antibodies and a cellular branch, with a prominent role of T-cells. Within the T-cell system, $CD4^+$ T-cells are a crucial cell population in coordinating, regulating and orchestrating the immune reaction, whereas $CD8^+$ T-cells perform antigen specific effector function. Specific immunity is the second line of defence.

There are soluble factors called cytokines, that are responsible for the communication between the innate and adaptive immune system, as well as with other systems in the body (e.g. brain).

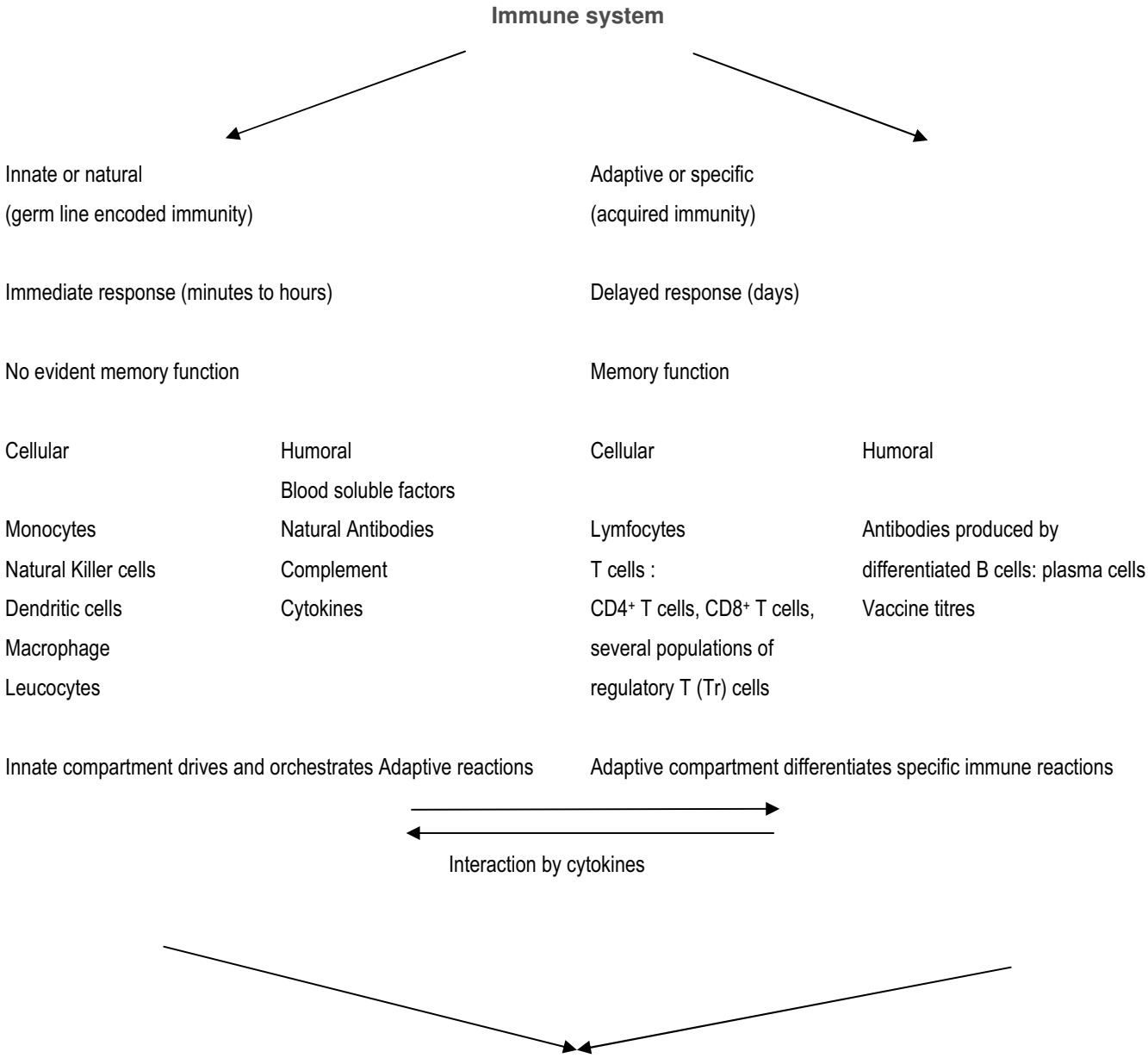


Figure 4.6: Schematic overview of the different compartments and branches of the immune system.

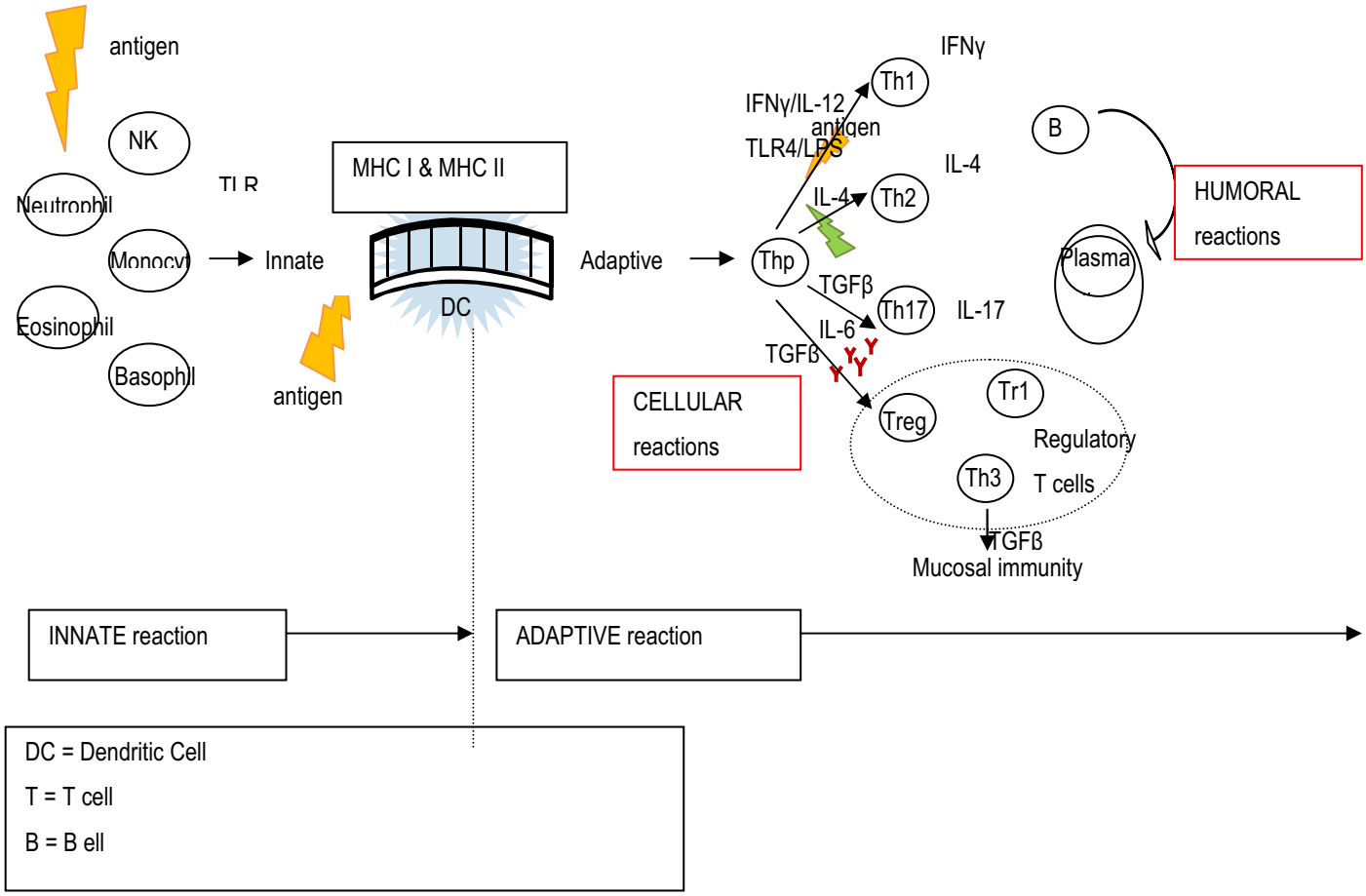


Figure 4.7: Schematic drawing of the immune response.

For various reasons chicken provide a suitable animal model in studying feed-induced immunomodulation of the immune response, as is the hallmark of this project. Precisely the connection between the induction of a rapidly reacting innate immune response and the slower developing, but more specific adaptive immune response can be studied very well in chicken. Since this connection is the target for immunomodulation, chicken are a suitable model to study possible effects of conventionally versus organically produced feed on immune defence.

Both compartments of the immune system will be investigated:

1. the *innate* or *natural* compartment of the immune system
2. the *adaptive* or (antigen-) *specific* compartment of the immune system

The general expectation is that the main effects caused by differences in the feed, will be found in the innate immune system of the second generation. Although the innate immune system was the primary target of research, effects were expected on the specific immune system as well. Therefore, two phases of immune reactions have been chosen within the design, including both generations of chicken.

The first generation The switch to the experimental feed has been monitored in the first generation. Furthermore, studying the first generation also allowed for the optimization of the immune parameters for the second generation. Two weeks before the animals switched to the experimental feed baseline blood samples were taken. After the feed switch blood samples were taken after one, four, eight and twenty two weeks.

The second generation In the second generation blood samples were taken at 8 weeks of age for measuring the innate immune system (i.e. monocyte activity, natural antibodies and concentration of complement), as well as parameters representing the specific immune system (lymphocyte proliferation, immunoglobulins). After a two week recovery period, the specific immune system of the chicken was provoked in a 'challenge' test, using Keyhole Limpet Hemocyanin (KLH) as a model antigen. After this challenge blood samples were taken during four following weeks.

The following parameters were chosen, so all compartments were represented:
Innate immunity:

- Monocyte activity (measured in nitric oxide assay)
- Natural Antibodies (Nab) (measured by titres)
- Complement factors (measured by Von Krogh-entities)

Specific immunity:

- Lymphocyte proliferation in whole blood (measured in proliferation assay)
- Lymphocyte proliferation in isolated white blood cells (measured in proliferation assay)
- Serum antibody concentrations (immunoglobulin vaccin titres) (measured by ELISA)

An originally planned S*tress resistance test* with the animals was called off, as it would interfere with the immunological measurements during the timespan of the project. Prolonging the project was no option, due to shortage of feed.

4.4.1 Innate immune system

4.4.1.1 Cellular parameters

To determine effects of different feeds on cellular parameters of innate responsiveness in blood monocytes, lymphocytes and monocytes were isolated from heparinized blood using a Histopacque gradient. Cells were washed two times in RPMI 1640 and then diluted to a $1*10^7$ cell per ml suspension. Triplicate cultures with 106 cells per well were incubated in flat bottom 96-wells plates for 72 hours at 4°C with 5% CO_2 with or without (= control) LPS in 200 µl culture medium. LPS stimulates selectively monocytes among the peripheral blood leukocytes. After incubation, 50 µl culture medium was extracted from the plates and mixed for 10 minutes at room temperature with 50 µl Griess reagents' in a 96-well flat bottom plate. Extinctions were measured at 540 nm. Monocyte reactivity was calculated using a nitrite calibration and expressed as µM NO production.

4.4.1.2 Humoral parameters

To determine effects of different feeds on humoral parameters of innate responsiveness, levels of natural antibodies and complement factors (indicative of classical and alternative complement activation) were analyzed.

Natural antibodies Plasma from heparinized blood was collected and stored at -20°C until further analysis. All antibodies were determined by a direct enzyme-linked immunosorbent assay (ELISA) as described previously. In short, 96-well high binding ELISA plates were coated overnight with LPS or LTA and plasma was added and diluted four steps and incubated for an hour at 37°C. Detection was performed with horse radish peroxidase labeled

conjugate and substrate tetramethylbenzidine (TMD) and H_2O_2. Extinction was measured at 450 nm. Delta titers were calculated to correct for individual variances.

Complement The determination of functional complement activity is by the total hemolytic (CH50) assay. This assay measures the ability of the test sample to lyse 50% of a standardized suspension of sheep or bovine erythrocytes. Both the classic activation and the terminal complement components are measured in this reaction. Dilution of the serum enables the determination the lysis of approximately 50% of the cells. Cell lysis can be read on a standard spectrophotometer (Baelmans 2005).
Serum from each blood sample was collected and stored at -20°C until analysis. Serum was diluted in 96-well flat bottom plates and either bovine or sheep diluted blood was added. Lysis was measured by measuring extinction at 450 nm.

4.4.2 Adaptive immune system

4.4.2.1 Cellular parameters

To determine effects of different feeds on cellular parameters of adaptive immune responsiveness in lymphocyte proliferation in vitro, the lymphocyte stimulation test (LST) was used to determine specific cellular reactivity. Briefly, heparinized blood was diluted 1:30 in RPMI and 100µl was added per well of a 96-well flat bottom culture plate. Triplicates were incubated with either 100µl Con A (for T-cell stimulation), LPS (for B-cell stimulation) or RPMI culture medium (control) for 48 hours in a humidified incubator at 41°C with 5% CO_2. Then 0.4 µCi 3[H]-thymidine was added per well and incubated overnight. Plates were stored at -20°C before harvesting. After thawing of the plates they were harvested onto fiberglass filters and filters were then counted by liquid scintillation spectroscopy. Data is presented as actual counts as well as stimulation index (SI = stimulated counts/control counts).

4.4.2.2 Humoral parameters

To determine effects of different feeds on specific humoral parameters of vaccine-specific antibody responses, pllasma from heparinized blood was collected and stored at -20°C until analysis. All antibodies were determined by a direct enzyme-linked immunosorbent assay (ELISA) as described previously. In short, 96-well high binding ELISA plates were coated overnight with KLH, NCD or Gumboro and plasma was added, diluted four steps and incubated for an hour at 37°C. Detection was performed with horse radish peroxidase labeled conjugate and substrate tetramethylbenzidine (TMD) and H_2O_2. Extinction was measured at 450 nm. Delta titers were calculated to correct for individual variances.

4.4.3 Feed extraction tests

To determine effects of specific reactivity to feed extracts using lymphocyte proliferation in vitro, the lymphocyte stimulation test (LST) was used to determine specific cellular reactivity. This was performed at week 3 post inoculation for the 2nd generation, and at week 8 after the switch to the experimental feeds for the 1st generation. Briefly, heparinized blood was diluted 1:30 in RPMI and 100µl was added per well of a 96-well flat bottom culture plate. Triplicates were incubated with 50µl Con A (for T-cell stimulation) and 50µl water based extract of either feed A or B for 48 hours in a humidified incubator at 4°C with 5% CO_2. Then 0.4 µCi 3[H]-thymidine was added per well

and incubated overnight. Plates were stored at -20°C before harvesting. After thawing of the plates they were harvested onto fibreglass filters, and filters were then counted by liquid scintillation spectroscopy. Data is presented as stimulation index (SI = Con A + extract counts/Con A counts).

4.5 Metabolomics

Metabolomics was performed on *plasma* and *liver* of the chicken by TNO Quality of Life, Analytical Research Department, Zeist.

Metabolomics

In search for biomarkers, also the modern technique of *Metabolomics* was applied. Metabolomics is the most recent addition to the applied genomics toolbox and involves the non-targeted, comprehensive determination of changes in the complete set of metabolites in the cell, i.e. the metabolome. Metabolomic methods identify and quantify levels of metabolites (small molecules) in the cell and in extracellular fluids (e.g. plasma, urine, cell culture media), or organs (e.g. liver) which may reflect changes in cellular processes due to internal or external stimuli. The analysis of the metabolome is much more complex than that of the genome or proteome. This is due to the fact that the metabolome consists of a large number of different molecules with very diversal physiological properties, and includes organic acids, lipids, amino acids, nucleotides, steroids, eicosanoids, neurotransmitters, peptides, and trace elements. Moreover, the concentration range to be covered in metabolomics can also be extremely large. Due to this heterogeneity and diversity it is clear that a single method or technology cannot provide the performance, necessary for comprehensive metabolic studies. In this study the aim was to find differences in metabolites in plasma and liver that occurred systematically due to the two different feeding regimes and/or the KLH challenges test. It can occur that further interpretation is not really possible, as new metabolites are found on a daily basis and are therefore without clear identification and function. From the planned blood sample collections, plasma samples were stored to be used for metabolomic analyses.

Furthermore, tissue samples of the liver were collected at the time of sacrifice of the chicken to be analysed within this technique as well.

Plasma samples Plasma samples from chicken were provided by Louis Bolk Institute/Wageningen UR. Six 96-well plates containing aliquots of approx. 200 µl of chicken plasma were received on dry ice. Samples were stored in the freezer (-70°C) until further sample work-up.

From the six well plates 210 relevant plasma samples, i.e. 70 chicken x 3 time points, were selected for metabolomics analysis. From these plasma samples aliquots were made in Eppendorf vials for the different analytical platforms:

OS-GCMS 100 µl

Lipid LCMS 10 µl

Global LCMS 2x10 µl

Bile acid/FFA LCMS 50 µl

Each aliquot obtained a unique code and were stored in the freezer (-70°C) until analysis. Deviations in the amount of plasma available were reported. Remaining sample material was kept in the freezer at -70°C until further notice.

Liver samples Pieces of chicken liver were isolated during the section of the chicken and were stored in the freezer (-70°C) until further sample work-up.
Form 70 liver samples, approx. 200-400 mg was cut and freeze-dried. This resulted in a dry weight of liver of approx. 150-200 mg. From the dried liver samples aliquots were made in Eppendorf vials for the different analytical platforms:
OS-GCMS 10 mg
Lipid LCMS 5 mg
Global LCMS 2x 7 mg
Bile acid/FFA LCMS 5 mg
Each aliquot obtained a unique code and were stored in the freezer (-70 °C) until analysis. The exact amount of dry liver sample was reported. Remaining sample material was kept in the freezer at -70 °C until further notice.

Quality control samples

Quality control plasma samples Pooled chicken plasma sample (approx. 10 ml) was provided by Louis Bolk Institute/Wageningen UR. This sample was aliquoted for the different analytical platforms as described above. Each aliquot obtained a unique code and were stored in the freezer (-70°C) until analysis.

Quality control liver samples For liver samples, quality control samples were made for each analytical platform after further sample-work up/extraction. Each aliquot obtained a unique code.

Reference substances The only reference materials used in this study are the various internal standards used for the various analytical methods.

Procedure and methods

OS-GCMS

Principle and performance characteristics Plasma samples were deproteinised by methanol and after centrifugation, decantation and evaporation of the solvent, the metabolites are derivatized to make them amenable to gas chromatography; the samples are then analysed by GCMS. Liver samples were extracted with methanol and after centrifugation, decantation and evaporation of the solvent, the metabolites are derivatized. Before each step internal standards are added in order to monitor the efficiency of each step.
With this method many known and unknown polar metabolites can be analyzed. Approx. 250 standards of metabolites have been analyzed with this method and spectral- and relative retention data for these metabolites are available. However, the performance of the method for the individual metabolites can differ between types of sample, and is monitored in each study through the quality control (QC) samples. More details on and characteristics of the OS-GCMS method are given by Koek (2006) and Smilde (2005).

Randomisation and analysis order In total, approximately 210 plasma samples, corresponding to duplicate analysis of 70 chicken x 3 time points, and 70 liver samples were analyzed. Extraction, derivatization and analysis were carried out in batches of 15 plasma/liver samples, i.e. 5 chicken x 3 time points per batch for plasma samples resulting in 14 batches and 15 chicken per batch for liver samples resulting in 5 batches. It was tried to evenly distribute the different types of chicken, i.e. CL, LL, HL, group A and group B, over the different batches. For

plasma, the three time points (week -1, week 1 and week 3) of each chicken were analyzed in series in the order week 1, week -1, week 3. Per batch 6 injections of QC samples were included.

Quality control The quality control procedure consisted of evaluating parameters per batch. These parameters were obtained by processing the data with Chemstation G1701CA V D.01.02 software (target processing method). Retention time and peak area of all the internal standards in all individual study and QC samples. Peak area ratio of all the internal standards with respect to DCHP in all the study and QC samples (a.k.a. the relative peak area). The performance characteristics of the internal standards in the study and QC samples have a descriptive nature.

Data processing For Quality Control Chemstation G1701CA V D.01.02 software was used for the integration of selected target analytes. After completion of the analysis of all the samples, this software was also used for integration of all peaks of interest present in the chromatograms. The peaks of interest (target metabolites) were either known or tentatively identified, but also unknown metabolites detected in the chromatograms of the study and/or QC samples. Peak integration data for all the metabolites from the target table were obtained. The result was an Excel table containing an integration value for each target compound (columns) and each study or QC sample (rows). The peak areas were subsequently corrected for the internal standard DCHP.

Lipid LC-MS

Principle and performance characteristics Plasma and liver samples were extracted with iso-propanol. After centrifugation, the supernatant was analyzed by reversed-phase LCMS using a water:MeOH gradient and ESI in the positive mode. Several internal standards, one for each class of lipids, is added to the iso-propanol extract. The internal standards are used to check the retention and sensitivity of long sequences and to perform quantitative corrections.

With this method various classes of lipids can be analyzed, e.g. triglycerides (TG), cholesterol esters (ChE) and various phospholipids (e.g. PC and LPC). The performance of the method for the individual metabolites can differ from study to study and is monitored in each study through the QC samples. The performance characteristics, relative standard deviations of selected metabolites in the QC samples have a descriptive nature. More details on the lipid LC-MS method are given by Verhoeckx (2004) and Bijlsma (2006).

Randomisation and analysis order In total, approximately 210 plasma samples, corresponding to duplicate analysis of 70 chicken x 3 time points, and 70 liver samples were analyzed. Plasma samples were extracted and analysed in 4 batches (3 x 16 and 1 x 22 chicken). Liver samples were extracted and analysed in 2 batches (2x 35 chicken). It was tried to evenly distribute the different types of chicken, i.e. CL, LL, HL, group A and group B, over the different batches. For plasma, the three time points (week -1, week 1 and week 3) of each chicken were analyzed in series in the order week 1, week -1, week 3. One injection of a QC sample per 5-6 sample injections was included in every batch.

Quality control The quality control procedure consisted of evaluating parameters per batch. These parameters will be obtained by processing the data with Thermo Xcalibur LCQuan V2.0 (target processing method).
Retention time and peak area of the internal standards in all individual study and QC samples.
The performance characteristics of the internal standards in the study and QC samples have a descriptive nature.

Data processing For Quality Control purposes Thermo XCalibur LCQuan V 2.0 was used for the integration of selected target metabolites. After completion of the analysis of all the samples, this software was also used for integration of all peaks of interest present in the chromatograms. The peaks of interest (target compounds) can be known or tentatively identified, but also unknown metabolites detected in the chromatograms of the study and/or QC samples. Unknown metabolites were characterized by their retention time and at least one qualifying ion. Integration data for all the metabolites from the target table was obtained. The result was an Excel table containing an integration value for each target compound (columns) and each study and QC sample (rows). All peak areas were corrected for the internal standards, either C24:0 PC, C17:0 LPC, C51:0 TG or C17:0 ChE.

FFA/Bile acid LC-MS

Principle and performance characteristics Plasma and liver samples were extracted with methanol containing internal standards. The resulting extract is analyzed by reversed-phase LCMS using a water:MeOH gradient, C18 column and ESI in the negative mode. The internal standards cover both fatty acids and bile acids and are used to check the retention and sensitivity of long sequences and to perform quantitative corrections.
With this method fatty acids and bile acids can be analyzed as well as several unknown metabolites. The performance of the method for the individual metabolites can differ from study to study and is monitored in each study through the QC samples. The performance characteristics, relative standard deviations of selected metabolites in the QC samples have a descriptive nature.

Randomisation and analysis order In total, approximately 210 plasma samples, corresponding to duplicate analysis of 70 chicken x 3 time points, and 70 liver samples were analyzed. Plasma samples were extracted and analysed in 4 batches (3 x 16 and 1 x 22 chicken). Liver samples were extracted and analysed in 2 batches (2x 35 chicken). It was tried to evenly distribute the different types of chicken, i.e. CL, LL, HL, group A and group B, over the different batches. For plasma, the three time points (week -1, week 1 and week 3) of each chicken were analyzed in series in the order week 1, week -1, week 3. One injection of a QC sample per 5-6 sample injections was included in every batch.

Quality control The quality control procedure consisted of evaluating parameters per batch. These parameters will be obtained by processing the data with Thermo Xcalibur LCQuan V2.0 (target processing method). Retention time and peak area of the internal standards cholic acid d4 and C17:0 fatty acid in all individual study and QC samples. The performance characteristics of the internal standards in the study and QC samples have a descriptive nature.

Data processing For Quality Control purposes Thermo XCalibur LCQuan V 2.0 was used for the integration of selected target metabolites. After completion of the analysis of all the samples, this software was also used for integration of all peaks of interest present in the chromatograms. The peaks of interest (target compounds) can be known or tentatively identified, but also unknown metabolites detected in the chromatograms of the study and/or QC samples. Unknown metabolites were characterized by their retention time and at least one qualifying ion. Integration data for all the metabolites from the target table was obtained. The result was an Excel table containing an

integration value for each target compound (columns) and each study and QC sample (rows). All peak areas were corrected for the internal standards, either cholic acid d3 for the bile acids or C17:0 fatty acid for the fatty acids.

Global LC-MS

Principle and performance characteristics Plasma and liver samples were extracted with methanol. The supernatant was subsequently derivatized and analyzed by reversed-phase LCMS using a water:MeCN gradient, C18 column and ESI in the positive mode. Several deuterated amino acids are added as an internal standard. The internal standards are used to check the retention and sensitivity of long sequences and to perform quantitative corrections.
With this method, besides amino acids, many unknown metabolites can be analyzed. The performance of the method for the individual metabolites can differ from study to study and is monitored in each study through the QC samples. The performance characteristics, relative standard deviations of selected metabolites in the QC samples have a descriptive nature.

Randomisation and analysis order In total, approximately 210 plasma samples, corresponding to 70 chicken x 3 time points and duplicate sample work-up, and 70 liver samples were analyzed. Plasma samples were derivatized and analysed in 4 batches (3 x 16 and 1 x 22 chicken). Liver samples were derivatized and analysed in 2 batches (2x 35 chicken). It was tried to evenly distribute the different types of chicken, i.e. CL, LL, HL, group A and group B, over the different batches. For plasma, the three time points (week -1, week 1 and week 3) of each chicken were analyzed in series in the order week 1, week -1, week 3. One injection of a QC sample per 5-6 sample injections was included in every batch.

Quality control The quality control procedure consisted of evaluating parameters per batch. These parameters will be obtained by processing the data with Thermo Xcalibur LCQuan V2.0 (target processing method). Retention time and peak area of the deuterated amino acid internal standards in all individual study and QC samples.
Peak area ratio of all the internal standards with respect to leucine d3 in all the study and QC samples (a.k.a. the relative peak area). The performance characteristics of the internal standards in the study and QC samples have a descriptive nature.

Data processing For Quality Control purposes Thermo XCalibur LCQuan V 2.0 was used for the integration of selected target metabolites. After completion of the analysis of all the samples, this software was also used for integration of all peaks of interest present in the chromatograms. The peaks of interest (target compounds) can be known or tentatively identified, but also unknown metabolites detected in the chromatograms of the study and/or QC samples. Unknown metabolites were characterized by their retention time and at least one qualifying ion. Integration data for all the metabolites from the target table was obtained. The result was an Excel table containing an integration value for each target compound (columns) and each study and QC sample (rows). All peak areas were corrected for the internal standard leucine d3.

4.6 Genomics

4.6.1 Genomics – Microarray

To check effect of the feed on the function of the chicken gut, intestinal gene expression in the jejunum was analyzed by the Animal Sciences Group of Wageningen UR, in Lelystad.

In search for biomarkers, also the function of the intestine, or gut system, was monitored by the use of *Genomics,* to investigate gene expression. Recently, it has been described that different intestinal infections affect gene expression in chicken (Van Hemert 2007; Van Hemert 2004). This was shown by the use of a home-made intestinal cDNA microarray. cDNA microarrays are a recommended technique to study mRNA expression profiles of many different genes simultaneously. It is an unbiased approach where no genes or proteins are chosen beforehand, as is the case with other approaches. With a microarray it is possible to identify new genes involved in the investigated trait. Recently, a 20K chicken oligo array has been developed (ARK Genomics, Roslin, UK). By using whole genome arrays, there is even less bias towards expected results. Pathways involved in cellular processes can be discovered that were not expected beforehand. Recent findings support the idea that diet can influence gene expression (Rebel 2006).

In the present study the gene expression in the jejunum of chicken from both feed groups were compared, using a whole genome array. The use of a whole genome array enables us to find not only immunological differences between feed groups, but also differences in other cellular processes in the intestine. It is important to keep in mind that the chicken in the present study were not challenged with a pathogen, through the gut system. The chicken in this study were animals in good condition, but fed on different feeds. This implies that this study would only identify differences in gene expression in the basal status of the intestine.

Samples Chicken jejunum samples were collected by M. Huber and W. Swinkels according to the ASG protocols.

RNA Isolation RNA was isolated from the jejunum of all individual chicken using the Trizol method as described by Van Hemert et al. (2004). The tissue sample was grinded under liquid nitrogen in a pestle and mortar. A small volume of grinded tissue was dissolved in 1 ml of Trizol (Invitrogen), and homogenized. The RNA was extracted after addition of 1/5 volume of chloroform. Subsequently, the RNA was precipitated with isopropanol, washed and dissolved in DEPC-water. RNA-concentration and -quality was determined using the Nanodrop (Nanodrop technologies), as well as by gel electrophoresis. A sample was used for further processing when the OD260/280 was above 1.8, and no degradation of RNA was observed on the gel image.

RNA pools RNA of 4 – 6 jejunums of individual chicken was pooled according to the scheme that was provided by M. Huber (see Table 4.6). 10 µg of each individual sample was used. After pooling, the RNA-concentration and -quality was monitored again with the Nanodrop.

Table 4.6: Pooling of the RNA samples. 12 H-Liners, 12 L-liners and 46 C-liners. The numbers are chicken numbers.

Pool	Line	Feed	RNA samples from chicken jejunum					
1	H	A	4	5	11	18	20	26#
2	L	A	72	80	89	93	96	101
3	C	A	47	53	62	67	69	
4	C	A	44	48	52	54	60	
5	C	A	40	46	56	59	68	
6	C	A	38	43	45	63	66	
7	C	A	36	39	51	57		
8	H	B	107	113	118	122	136	139
9	L	B	177	182	185	192	203	206
10	C	B	145	148	154#	158	174	
11	C	B	147	152	157	164	169	
12	C	B	142	144	161	173		
13	C	B	143	153	156	162		
14	C	B	141	149	150	165		

 # samples were excluded from pooling due to extensive RNA breakdown.

Microarray A single spotted 20K chicken oligo array from ARK Genomics was used for hybridizations. This array represents the complete chicken genome.

Hybridization of microarrays 5 µg of each pooled RNA-sample was labeled and hybridized using the Micromax TSA Labeling And Detection Kit (Perkin Elmer) according to the instructions of the manufacturer with modifications as described by Van Hemert et al. (2004). Slides were hybridized according to Table 2. Each sample was labeled once with Cy3 and once with Cy5 (dye-swab). A single spot chicken 20K oligo-array (ARK Genomics) was used. Hybridized microarrays were scanned using the Scanarray scanner and software (Perkin Elmer).

Analysis Spot detection was done using GenePix Pro (Molecular Devices). A customized normalization procedure was performed to fit the data as described by Pool (2005). Subsequently, data were analyzed using SAM (Tusher 2001).

Table 4.7: Hybridization of oligo array.

	Cy3			Cy5		
Array No.	Line	Feed	RNA Pool	Line	Feed	RNA Pool
61	H	A	1	H	B	8
62	H	B	8	H	A	1
75	L	A	2	L	B	9
64	L	B	9	L	A	2
76	C	A	3	C	B	10
66	C	B	10	C	A	3
67	C	A	4	C	B	11
68	C	B	11	C	A	4
77	C	A	5	C	B	12
78	C	B	12	C	A	5
79	C	A	6	C	B	13
80	C	B	13	C	A	6
73	C	A	7	C	B	14
74	C	B	14	C	A	7

4.6.2 Genomics – qPCR confirmation

In order to confirm the results of the microarray analysis, Louis Bolk Institute and Animal Sciences Group agreed to perform qPCR on three selected genes. These three genes were selected from the top ten of regulated genes that were based on the analysis of all groups together. These qPCRs were performed on RNA from individual chicken in contrast to the microarray experiments that were performed on pools of five chicken. Thus the expression level of the three selected genes are determined using another technique, independent of the microarray. In this way the microarray results can be confirmed.

Due to the fact that some genes could be hard to amplify using a PCR technique, we followed the approach of selecting 6 genes from the top ten lists of regulated genes. From this 6 genes 3 genes that could be amplified efficiently will be used in the qPCR analysis. The 6 selected genes were ranked in consultation with the Louis Bolk Institute. This means that if all 6 genes are amplified efficiently, the first 3 on the list will be analyzed using qPCR.

RNA Isolation was performed as described in the previous section.

Primer design Primers were designed using Primer Express 3.0 software for Real-Time PCR (Applied Biosystems) based on the gene sequence that is represented by the oligonucleotide found to be regulated on the microarray. Primer sequences are listed in Table 4.8.

Table 4.8: Primer sequences, genes ranked after consultation.

Name	RIGG	Oligo sequentie	GGA no.	Fold induction in line B
Hemoglobin alpha chain	RIGG13934	FW1 AAGAACAACGTCAAGGGCATCT REV1 GCCGGAGGACAGAGAAGGA **FW2 TGCCAACACAGAGGTGCAA** * **REV2 GGGTCTCGGCGCCATAC** *	Gga.2909	+ 2.26
Squalene mono-oxygenase	RIGG19074	FW1 CAGGCACTGTATGAACTTTTTGCT REV1 CTGAGACACATTCTCCACCAAGTC FW2 CTTCAGACTTGGTGGAGAATGTGT REV2 ACGCCACGGCGAAGAA	Gga.22304	- 3.22
Acetoacetyl coA synthase	RIGG11373	**FW1 AGCTGCTGGCACTCCTGAA** * **REV1 TCCTCCACCTTCGGAATCC** * FW2 TATGTGTCCTGTGCACCTACATGT REV2 CAGGAGTGCCAGCAGCTATTT	Gga.22600	- 2.49
Isopenthyl diphosphate deltaisomerase 2	RIGG13515	**FW1 TGTGCAGAAGGATGTAACGCTTA** * **REV1 CGAGGCTTTGTCTAGAAGTTGCT** * FW2 CGAATTCACTACAAGGCCAAGTC REV2 ATTGGGATCAGGATTAAGCGTTAC	?	- 2.42
Methyl sterol oxidase	RIGG20222	FW1 GCATCAGCACAGCCATTTAAATAT REV1 TGAGATTAACAACTCGCATTATCCA FW2 TGATGCAGGTGCAATTGTATATGT REV2 TGATGCCAGTTAGTATGGTCTGCTA	Gga.21297	- 2.37
Chemokine AH221	RIGG20401	FW1 CCCTCCTACTGTCCCACCAA REV1 TTTAGGCATCAGAGCTCCTGACT FW2 TGCGGCACCCACATCTC REV2 CATTTTGTTGTGTTCTGAATCATCTCT	Gga.9133	+ 2.07
28S	NA	**FW CAAGTCCTTCTGATCGAG** * **REV TCAACTTTCCCTTACGGTAC** *	NA	NA

** Selected primer pairs.*

cDNA synthesis cDNA was synthesized using Superscript II transcriptase kit (Invitrogen) according to manufacturer's instructions. Shortly, 200 ng RNA was diluted tenfold and 0,5 ug random hexamers were added. Reaction mixture was incubated at 70°C for 10 minutes. 4 µl buffer, 2 µl 0,1 M DTT, 1 µl transcriptase, 1 µl dNTPS (2mM each), 1 µl RNAsin (Promega) and 8 ul water were added. Reaction mixture was incubated at 37°C for 50 minutes, followed by 70°C incubation for 10 minutes.

Polymerase Chain Reaction (PCR) 2 µl cDNA or colony material was used for PCR. PCR reaction mix contained 5 µl buffer, 1 µl Expand High Fidelity Taq polymerase (Roche), 1 µl dNTPs (10 mM each), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 1 µl MgCl (2 mM), 38 µl water. PCR program was as follows: 96°C for 5 minutes, 40 times (94°C for 1 minute, 58°C for 1 minute, 72°C for 30 seconds), 72°C for 7 minutes. PCR products were analyzed on agarose gels. In case of colony PCR, reaction started with 96∘C for 10 minutes instead of 5 to lyze the bacteria.

Cloning of PCR products PCR products were purified from agarose gel using QIAEX II gel extraction kit (Qiagen) according to manufacturers protocol. Purified PCR products were cloned into TOPO4 using TOPO TA Cloning Kit for Sequencing (Invitrogen). Cloned fragments were transformed to E. coli TOP10 cells (Invitrogen) according to manufacturers protocol.

Plasmid isolation from transformed E. coli E. coli containing TOPO4 – insert was grown in LB medium containing 100 µg/ml ampicillin and 50 µg/ml kanamycin overnight. Isolation of plasmid DNA was performed using the QIAprep Spin miniprep kit (QIAgen) according to manufacturers instruction.

Quantitative Polymerase Chain Reaction (qPCR) qPCR was performed on a ABI 7500 system (Applied Biosystems) using the Powr SYBR ® Green PCR master mix (Applied Biosystems) according to manufacturers instructions. cDNA was diluted ten times before use. Plasmid DNA for each PCR product was added in a concentration range as reference DNA. A negative control containing reagents only and a standard dilution series (28S) were included in each run. For all PCR reactions the same cDNA sample was used in order to standardize and normalize the data. Data were analyzed using the data analysis software from Applied Biosystems. Output data is expressed as relative expression compared to 28S. Statistical analysis on data was performed using independent students T-tests.

4.7 Pathological Anatomy

To investigate if the different feeds and type of immune response of the birds influenced histological characteristics, a pathological anatomy survey was performed by pathologists of RIKILT Institute of Food Safety in Wageningen.

Post mortem

After sacrificing, the animals were examined by the present veterinarian and an assisting butcher. The organs evaluated by *Pathological Anatomy*, through histological staining and macro- and microscopical evaluation, for abnormalities and differences between the groups.

Formalin 10% fixed tissues from 70 animals were received by the RIKILT Institute of food safety. The samples consisted of: thymus, bursa Fabricii, spleen, heart, liver, ovary, lung, kidney, ventriculus and proventriculus, pancreas, duodenum, jejunum, colon and cecum. Tissues originated from 46 Control animals, 12 High responders and 12 Low responders.

After fixation the tissues were routinely processed and embedded in paraffin wax. The thymus and bursa were weighed and all gross pathological alterations were noted during processing of the tissues.
Sections of 4 µm were cut on a microtome (Leica RM 2165) once for each formalin fixed tissue specimen and dried overnight in a stove at 35°C. The sections were stained with Mayer's haematoxylin and eosin (H&E).
For staining T-lymphocytes in the intestines, an immunohistochemical method with anti-CD8 (Rabbit polyclonal antibody, Lab Vision Immunologica Duiven, the Netherlands) was used. Moreover, for apoptosis, sections were stained using Apoptag peroxidase in situ apoptosis detection kit (MP Biomedicals, Amsterdam, the Netherlands). Immunohistochemical staining was performed with an automatic immunostainer (Ventana Benchmark, Ventana Medical Systems Illkirch CEDEX France).

The sections of the duodenum and jejunum were scored for the villus length / crypt lenght ratio by measuring the length of 3 villi and 3 crypts per section from which the mean value was calculated. Measurements were performed using Leica Quips image analysing system (Leica Image Systems, Cambridge England).

4.8 Microbiology gut

It is hypothesized that bacterial communities are different between organic and conventional soils (van Diepeningen 2006), and between feed produced on these soils and consequently between feed residues excreted from intestines (manure). Activation of the immune system is largely affected by carbohydrates and proteins on the surface of, or excreted by microorganisms. Therefore, it is likely that activation of the immune system could be triggered by a change in microbes associated with a change in diet. As microbial diversity is generally greater in organic than in conventional soil, it is also hypothesized that the diversity would be greater on/in organic feed and on/in organic manure. However, the intestinal microbial community is likely not only determined by diet but also by the genetic make-up of an animal.
Based on this hypothesis an explorative community analysis of the chicken manure was included in the study, performed by Wageningen UR – Biological Farming Systems Group.

Chicken manure was collected during the section of the animals of the second generation on 27 September 2006, from the 3 chicken lines in runs 2, 3, 8, 9 and 10, both on the A- and the B-side, and directly frozen at -20°C. So from each line and feed-group 10 samples were available, 60 samples altogether. DNA from manure was isolated starting on 10 October 2006. DNA isolation from 250-300 mg chicken manure per chicken was carried out with the FastDNA® SPIN® Kit as described for DNA isolation from chicken feed. The 16S rRNA gene of eubacteria was amplified from manure DNA in a similar way as from feed DNA. DGGE analysis was also performed as described for feed DNA. Gels were again stained with Bio-Rad's Silver Stain. Dendrograms of bacterial DNA from chicken manure were constructed using UPGMA clustering method (see Microbial community analysis of chicken feed). DNA from selected chicken manure samples were rerun on DGGE gels together with DNA from chicken feed to be able to compare banding patterns of feed and manure DNA. The number of bands per lane was considered the "species" number S, while a combination of band intensity and number constituted the Shannon-Wiener diversity index H. Similarity indices were calculated for all possible pairs of manure samples, and mean similarities calculated for manure from organic versus manure from conventional chicken.

4.9 Sensory analysis of chicken meat

A sensory analyses on the breast muscle was performed by CSO-Centrum voor Smaakonderzoek in Wageningen in two tests. After the sacrificing of the chicken, an assisting butcher prepared chicken breast meat samples from a sub group of chicken. Meat of all C-line animals was compared, whereas from the H- and L-line, 6 animals of each line from just one feed were compared. For this test animals on feed A were chosen by chance.
The 18 selected panelists are regular users of meat, included chicken meat. The QDA method was used. De panelists were asked to score on a scale between 0 and 100. The meat was prepared according standard procedures, being baked in sunflower oil for 12 minutes without salt or pepper. The pieces of meat were served at about 30°C. The products were randomly offered. After finishing the test, the data of all panelists were transformed using the shrink stretch procedure. That means that the scores of the attributes of all panelists become for each attribute the same lowest and highest value. This score is the average of all lowest and highest values of all panelists. All data in between were according both values, proportion recalculated. That means that the correlation between the original data and the recalculated data is +1. Further, the achievements of all respondents were checked by calculating the correlation between the individual scores and the average scores of the whole panel. Only the analytical attributes with significant differences between the samples were used.

4.10 Statistics

4.10.1 Power analysis

Statistical power of the planned group size of 25 animals, in each of the 6 final experimental groups, was calculated by statistician Dr. ir. K. Frankena of Wageningen UR. The power of a test indicates the probability that the null hypothesis (H0: no difference in titre between lines) is being rejected while in reality there is indeed a difference between both lines (it is: H0 is correctly rejected).
A power-analysis showed that at a true difference between lines of one titre unit with a standard deviation of 1.2 units needs at least 25 animals per line to achieve a power of 80%. When the difference between lines is larger or the standard deviation is smaller then the power will increase, given that 25 animals per line are being used. Therefore, it was decided to strive for 25 animals per line.
From logistic reasons 150 animals were the maximum anyway.

4.10.2 Statistics by Muvara

Statistical analyses are predominantly applied on chicken measurements, since most measurements of the ingredients are small in number and can be interpreted directly. All chicken measurements were pre-processed as described in the previous method sections. Data analysis was performed with Matlab software (version 7.3.0 R2006b, The Mathworks).

Investigating feed and line related variance Univariate statistics are computed for measurements on chicken grouped by feed A and B: mean, standard error of the mean (SEM), standard deviation, 95% confidence interval for the mean, median, minimum, maximum. Mean differences between feed A and B are tested with analysis of variance (Dunn & Clark 1974), two-sample T-test with pooled variance (Kreyszig 1970) and Wilcoxon rank sum

test for equal medians (Hollander 1973). Results are given for each of the 3 chicken lines separately and for all lines in total. Differences between the 3 chicken lines are studied in more detail with multiple comparison of means (Hochberg & Tamhane 1987). To facilitate interpretation graphic figures are made of mean measurement values (±SEM) for feed A and B on all measured time points, each figure giving an overview of the high, control and low chicken line with significant differences indicated by stars ($p<0.05$).

Multivariate analysis Data were analysed with principal component analysis (PCA) to explore the structure of the variables (Krzanowski 1988) and their relation with chicken lines and feed. In metabolomics data the number of variables was reduced. The 20 best feed A-B discriminating variables were selected for each platform using cross-validated Linear Discriminant Analysis (Fisher 1936) on all available time points per variable. The 20 selected variables had the highest cross-validated correct rate of classification for predicting feed A or B. The mutual relations between the predictive variables were explored with PCA on standard normalized variables (z-scores). To facilitate interpretation with respect to the influence of feed A and B a slightly modified version of partial least squares discriminant analysis (PLS-DA) (Barker & Rayens 2003; Wold 1982) was applied. In this 'rotated 2LV PLS-DA' a PLS-DA solution is computed with two latent variables and then rotated in such a way that the first rotated component has maximum feed A-B discrimination and the second rotated component has maximum explained variance left. Another helpful visualization tool was to draw SEM ellipses for the PCA and rotated 2LV PLS-DA scores for the 6 different chicken lines and feed combinations. If multivariate analysis was computed on the control line data only, SEM ellipses for high and low chicken lines were projected on the solution to get an impression of the genetic influence. Furthermore the feed A-B discrimination in the discriminant procedures described above was replaced by high-low chicken line discrimination to explore the maximum genetic influence in the metabolomic data. Here fewer variables were selected due to the smaller number of chicken in the high and low line metabolic measurements.

Summarizing Most data will be presented graphically. Significance at a p=0,05 level can be concluded from the * in the graphs.

Note Other statistics have been used by several partners on their own responsibility.

5 Results Chicken experiment

Two generations of animals were included in the experiment. The second generation was the central experimental group, but the first generation was registered also and measured immunologically, which produced valuable information. The data about these generations will be presented chronologically thus, if available, starting with the first generation.

It must be stressed that the situation of the first generation was quite different from that of the second generation. Here once more the time frames are illustrated.

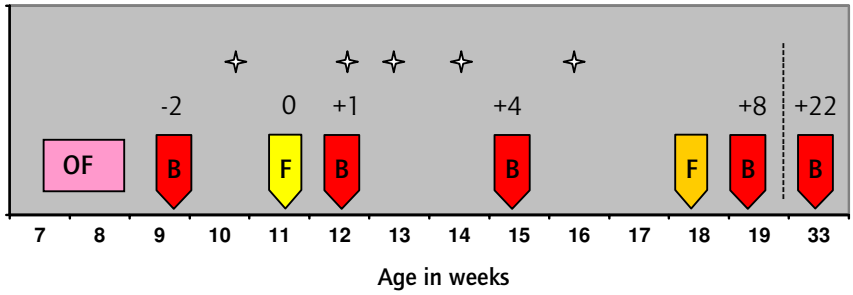


Figure 5.1: Timeframe of the 1st generation with vaccinations, feed changes and blood sampling.
OF= original feed till week 11, B = blood sampling, F = experimental feed.

Vaccinations, marked in this Figure by ✧
10 weeks – IB primer
12 weeks – ILT
13 weeks – AE
14 weeks – NCD -3
16 weeks – IB H52

Table 5.1: Number of hens per group in the first generation.

N=	Feed A	Feed B	Sum
H-line	11	13	24
C-line	12	12	24
L-line	12	11	23
Sum	35	36	71

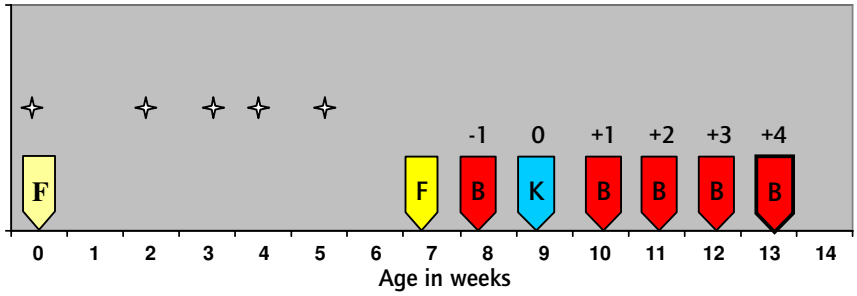


Figure 5.2: Time frame of the 2nd generation with vaccinations, feed change, KLH challenge and blood sampling. F = experimental feed, B = blood sampling, K = KLH challenge.

Vaccinations, marked in this Figure by ✧

0 weeks – Marek HVT, IB MA5, Paracox-8

2 weeks – NCD -1

3 weeks – Gumboro

4 weeks – NCD -2

5 weeks – PDww

Table 5.2: Number of animals per group in the second generation.

N=	Feed A	Feed B	Sum
H-line	26	24	50
C-line	24	22	46
L-line	23	26	49
Sum	73	72	145

*Data will be presented most graphically. Significance in a result can be concluded from the * in the graphs. Numbers at the x-axis represent timepoints in weeks.*

5.1 General health features

As health features in the chicken were evaluated the feed intake, weight, growth, relative growth rate, the condition of feathers, susceptibility to diseases, egg production and observations by the caretaker.

5.1.1 First generation

The 72 hens and 22 roosters of the first generation came on experimental feed from week 11 of age. They developed well. Around 20 weeks of age, the Avian Influenza occurred in the Netherlands, as an unexpected exterior threat. Some animals coughed at the time. Therefore blood and throats were checked by a veterinarian. The animals recovered without medication. No difference was observed between A- and B-animals.
Routine blood checks were performed randomly every 2 months on Aviary Influenza, Mycoplasm synoviae, Mycoplasma gallisepticum and Salmonella. No infections were observed.

Two animals died: one rooster of the H-line on feed B at the age of 25 weeks, from unknown cause, and one hen of the H-line on feed B at the age of 34 weeks, in which an undefined tumor was found. This hen had been weak for months, with often diarrea.
In general the droppings of the experimental animals was greenish, while the manure of the non-experimental flock was brown-red. A veterinarian inspected that, but found it unproblematic.
The animals started laying eggs at age 19 weeks; comparable to their 'sisters'.
Four animals did never lay an egg, one only infrequently.
At 7 months of age the animals were compared by the caretaker with the animals of the same generation, hens and roosters, that were kept on 'original' feed. The hens of the experimental group seemed to have a more pale crest and wattle. However with the roosters this was not the case. They lived in another stable.
Among the hens bloodlice were found, which can cause anaemia and through this paleness. The stable was treated for bloodlice with Solumite Mitex. On this the color of the crest and wattle seemed to improve.
When the second generation of experimental animals was alive and well, the intensity of special observing and weighing of the first generation was reduced. The animals were then 40 weeks of age.

Feed intake From the moment the animals of the first generation were housed individually, at **8 weeks**, till 30 weeks of age, feed intake was registered. The change to experimental feed was at age of **11 weeks.**

Feed intake was once significantly higher, in the C-line animals in week 11 for feed A , just when the experimental feed was introduced. At week 28 there was a dip as, by mistake, no feed was supplied in a weekend. Figure 5.3 displays the feed intake per week, for lines and feed-groups.

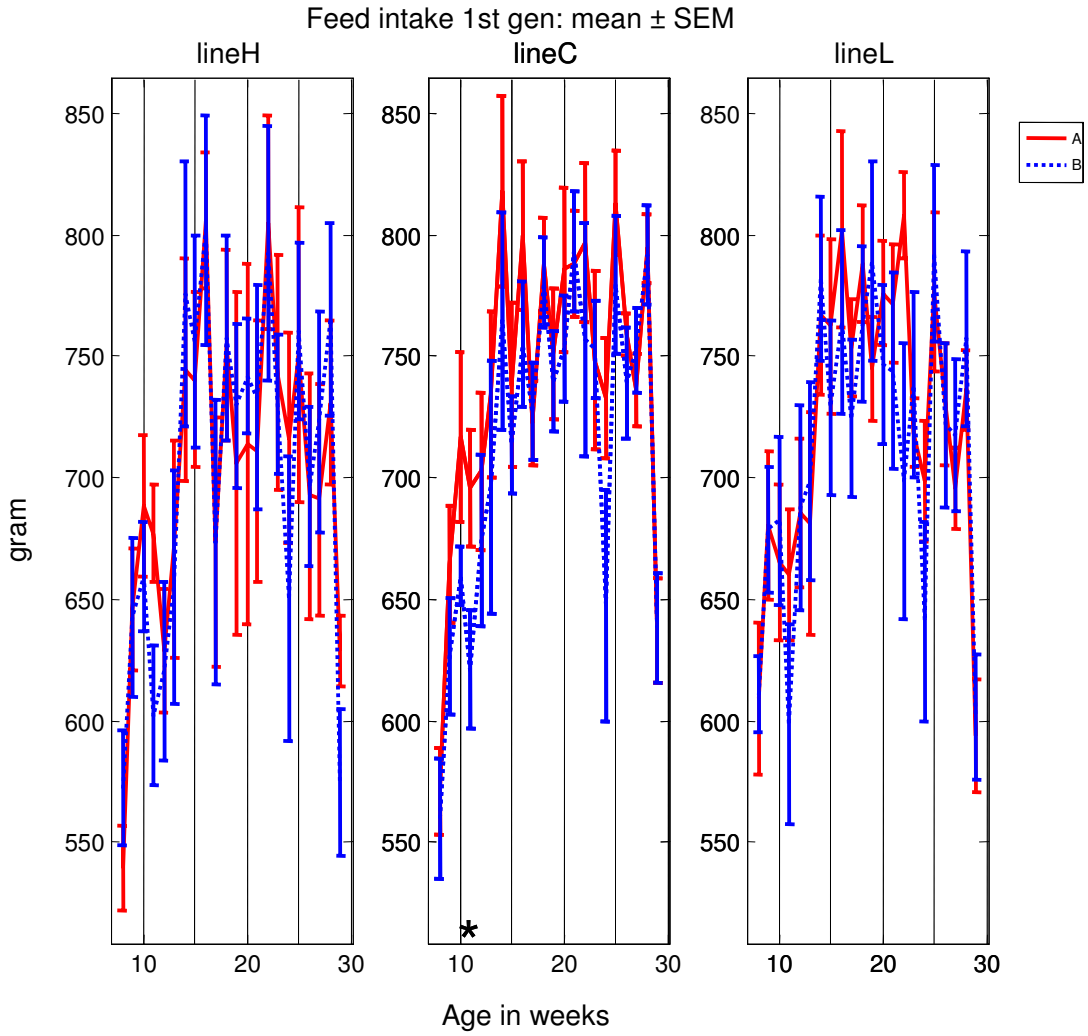


Figure 5.3: 1st Generation. Feed intake per week. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

Cumulative feed intake The cumulative feed intake was slightly higher in the C-line for feed A, but differed not significant. Between the lines, the feed intake was comparable (Figure 5.4).

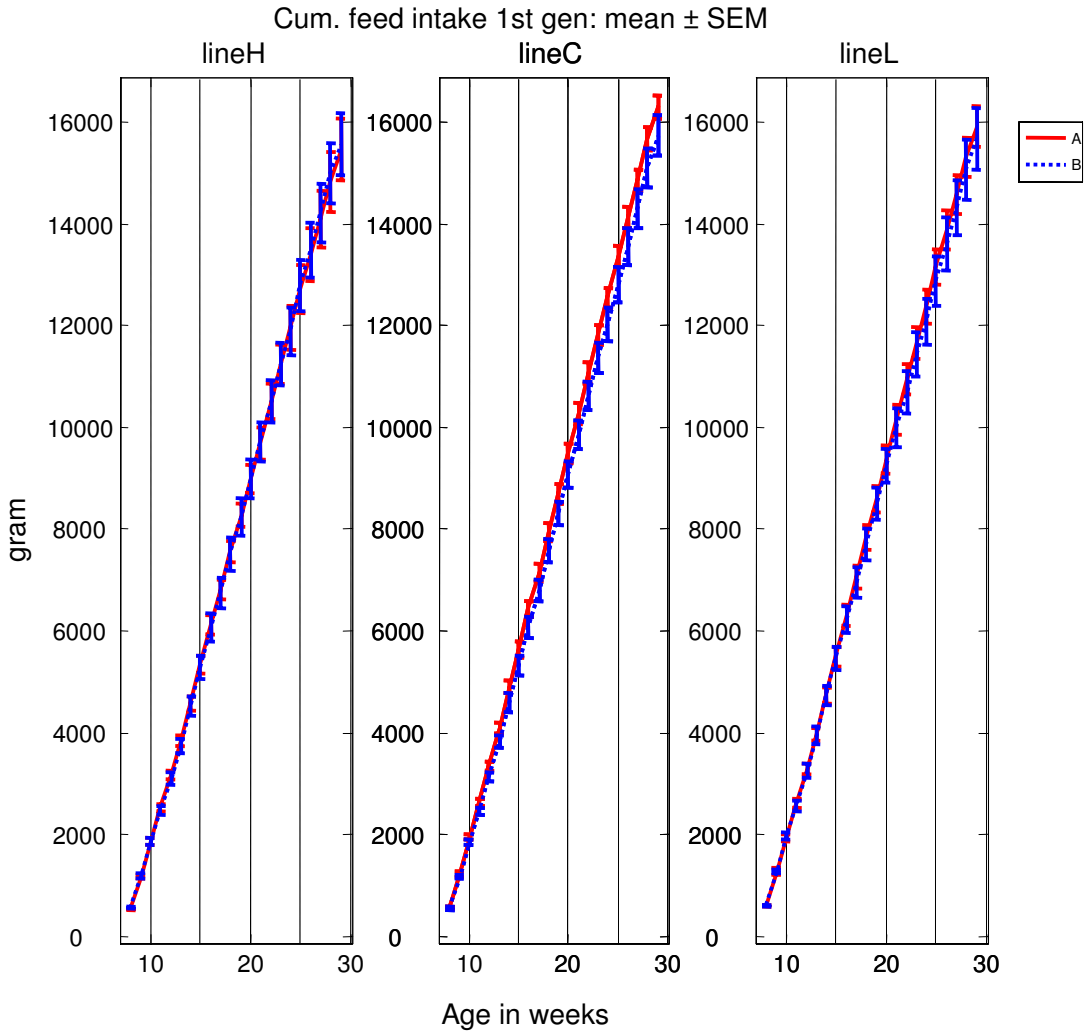


Figure 5.4: 1st Generation. Cumulative feed intake per week. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

Body weight From experience with previous generations with these lines, it is known that the H-line animals are relatively lighter and the L-line animals are relatively heavier, compared to the C-line animals.
Also in this experiment these line-features appeared, the C-group being only slightly heavier than the H-group.
In comparing the effects of the feeds, the animals on feed B were slightly heavier in the C-line from week 20, and in the L-line from week 35, but these differences were not significant (Figure 5.5).

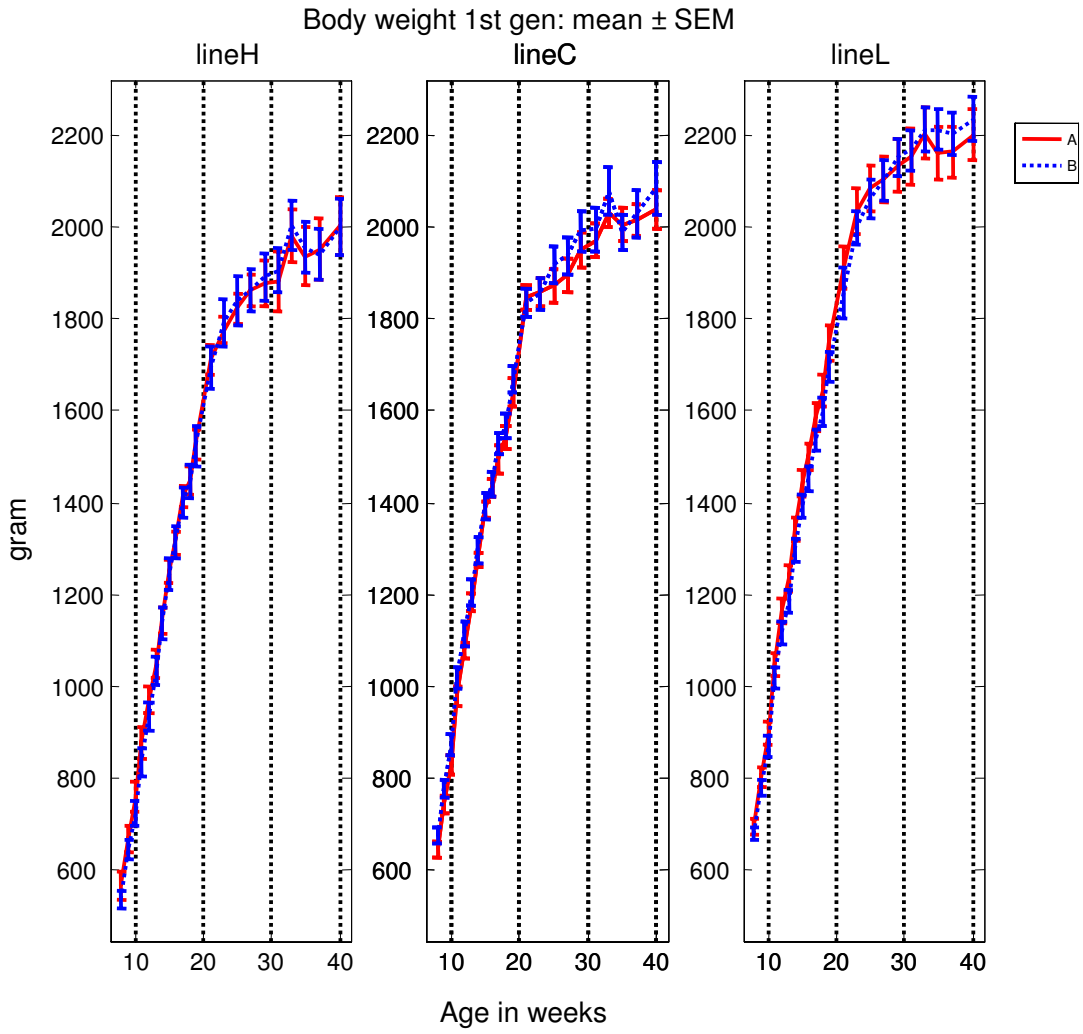


Figure 5.5: 1st Generation. Mean body weight per week. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

Growth and relative growth rate of body weight The growth and relative growth rate of body weight showed a few significant differences. Data on growth are not shown. The relative growth rate, which relates to the body weight of the animals, was for the animals on feed A more in the C-line in week 19 and week 32, and in the H-line in week 33 (Figure 5.6). This seems to coincide with the blood sampling moments.

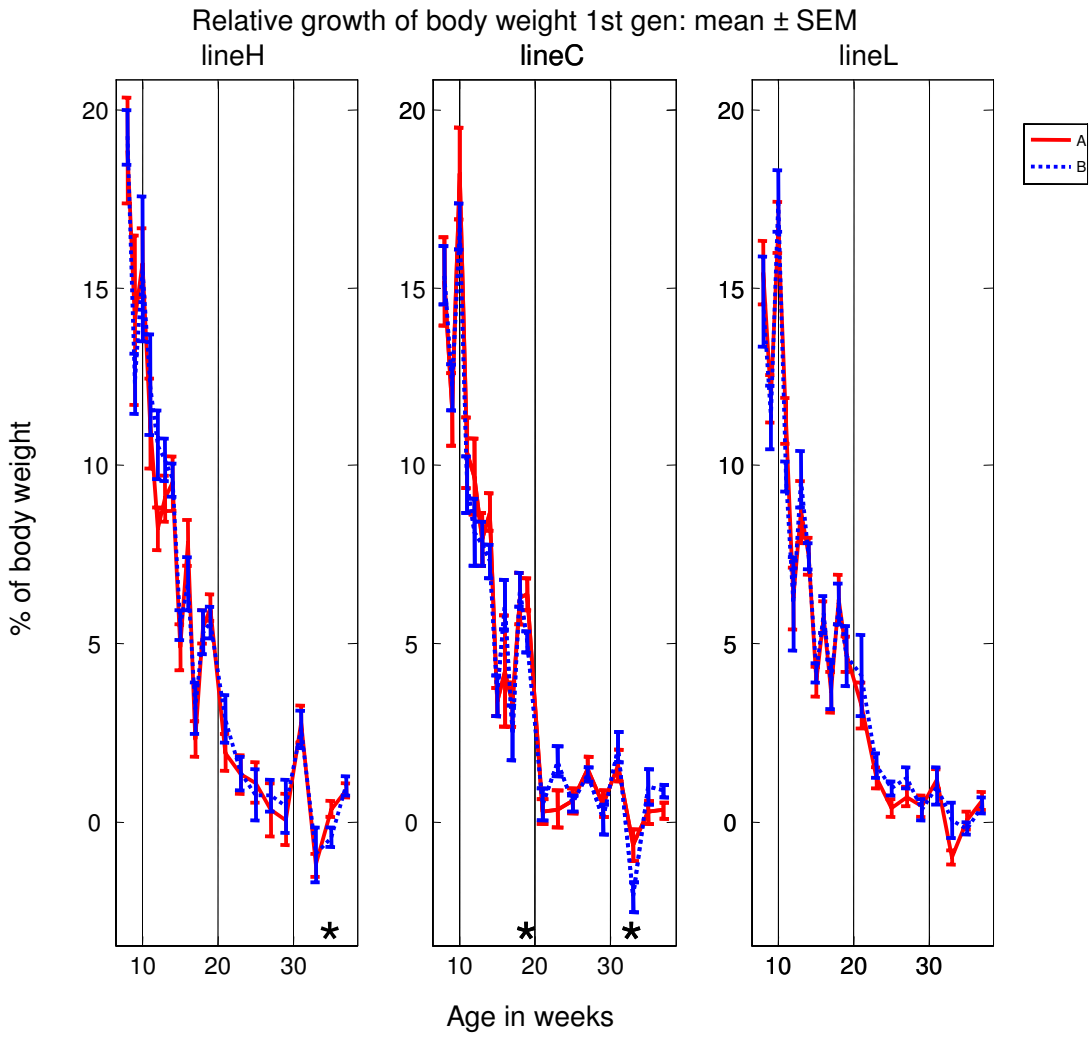


Figure 5.6: 1st Generation. Relative growth rate of body weight. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

Ratio of growth rate and feed intake The ratio growth rate/feed intake was computed. This ratio showed in the first generation no differences (Figure 5.7).

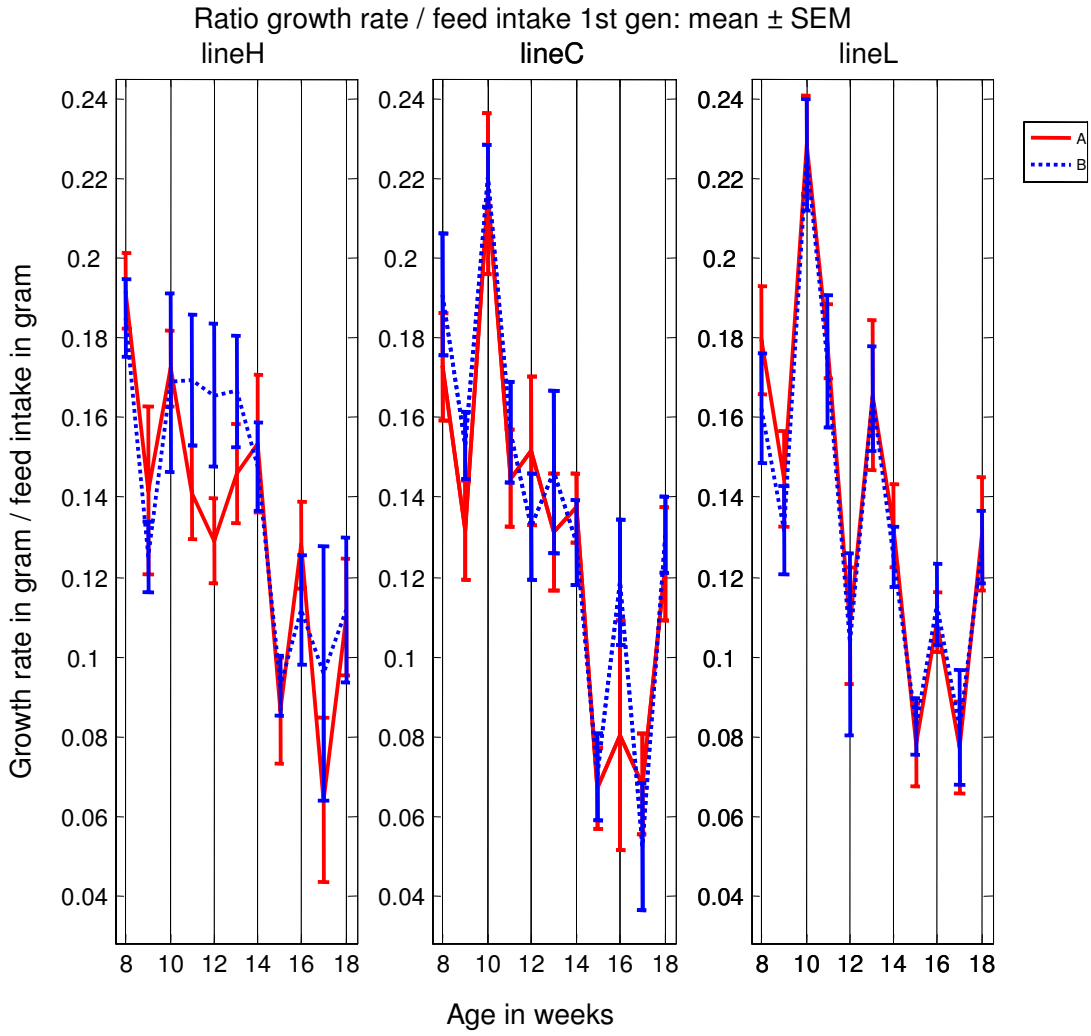


Figure 5.7: 1st Generation. Ratiogrowth rate/ feed intake. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

Egg production first generation The moment the animals started laying eggs, was registered. As can be seen in Figure 5.8 in the top row, the C-line animals started egg laying at a younger age, than the H- and L-line animals. Of the treatment groups, the A-animals started a little earlier, but this was not significant.
Connected to the age of starting, the 'laying period', measured till the end of the registration in week 40, was longer for the animals of the C-line (bottom row in Figure 5.8).
The egg weights had too many missing data to be fruitfully analyzed.

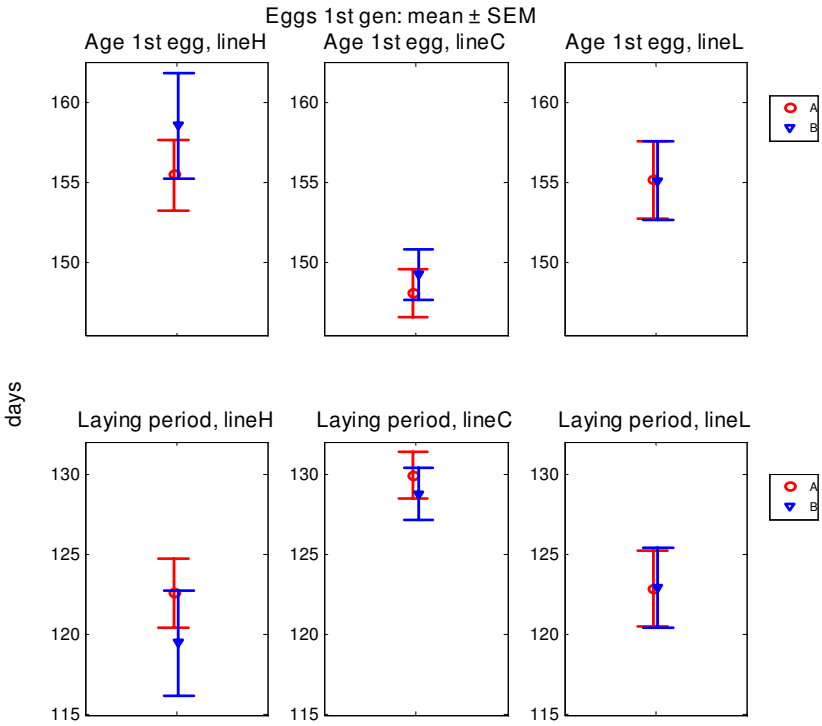


Figure 5.8: 1st Generation. Top row: Age of onset of egg-laying in days. Bottom row: Length of period of egg-laying. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

The total number of eggs layed per period was registered, as well as the type of egg as either first choice (normal) or second choice (several abnormalities possible) (Figure 5.9).
Here slight differences occurred, most obvious in the H-line, where animals on feed A layed a bit more and had relatively more first choice eggs than B-animals. These differences were not significant.

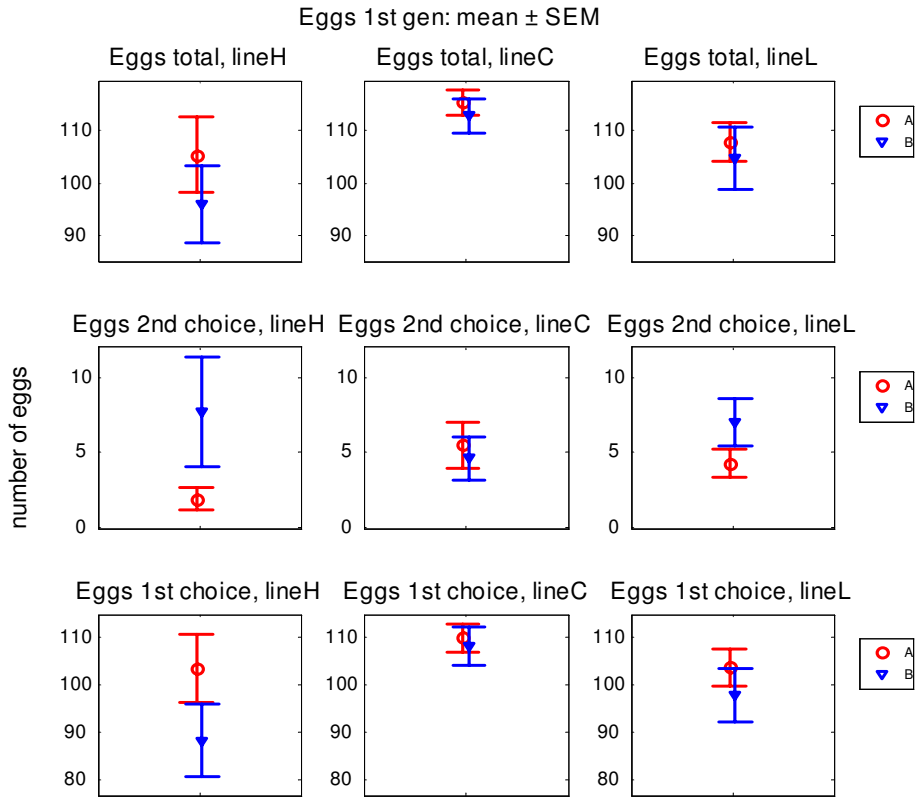


Figure 5.9: 1st Generation. Number and quality of eggs produced. Top row: total number of eggs. Middle row: number of eggs of second choice. Bottom row: number of eggs of first choice. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

Conclusion General health features first generation Concerning general health features a few differences were observed between the A- and B-animals. All animals were considered healthy. Feed intake was at one moment different, being higher in week 10 for feed A in the C-line. Body weight different not. Relative growth was higher for A feed animals, C-line week 19 and 32, H-line in week 33.

5.1.2 Second generation

The second generation of hens lived until 13.3 weeks of age, so did not come into the production of eggs.
The chicks were selected in such a way that obvious weak animals were left out. The distribution of these weak animals was not biased with respect to the feed groups. The rest of the animals was randomly selected. Once the total number of animals was reduced to the required experimental group, resulting in 156 animals. Further reduction occurred if animals turned out to be a rooster instead of a hen.
A routine blood check was performed at 6 weeks on 10 chicken, on Aviary Influenza, Mycoplasm synoviae, Mycoplasma gallisepticum and Salmonella. No infections were observed.
All animals appeared equally healthy. Around 9 weeks there was an impression that the A-group was a bit behind with feather development, compared to the B-group. In week 10 a poultry veterinarian inspected the animals. Although he found the B-group slightly 'creamier' in feather cloak, he could not objectivate any differences. He evaluated all animals in both groups as perfectly healthy.

Feed intake was measured per group of 6 animals, per run. As each group contained 2 animals of each of the 3 lines, no discrimination in feed intake between the lines was possible. In the first generation, where feed intake was measured individually, no differences in feed intake between the lines was registered.
The feed intake differed at several time moments significantly, but these differences switched between A- en B-feed. Life events that occurred, that might have influenced feed intake: In week 2 not enough feed was supplied during a weekend. At **7 weeks** the feed changed from Starter to Grower feed. At **9 weeks** the KLH-challenge was given.

Feed intake Figure 5.10 shows the feed intake. The time points mark the end of the measured week. In week 2 and week 6 Starter feed A was consumed more. Then, after the change to Grower feed, the intake of feed B started to increase, being significantly more in week 9, when the challenge was given.

In week 10 the intake of feed B declined, while the intake of feed A remained nearly the same, B still being taken in significantly more than A.

In week 11 the intake of feed A declined, while the intake of feed B remained the same, B being taken in significantly more than A.

In week 12 the intake of feed A and feed B both increased, B being taken in significantly more than A.

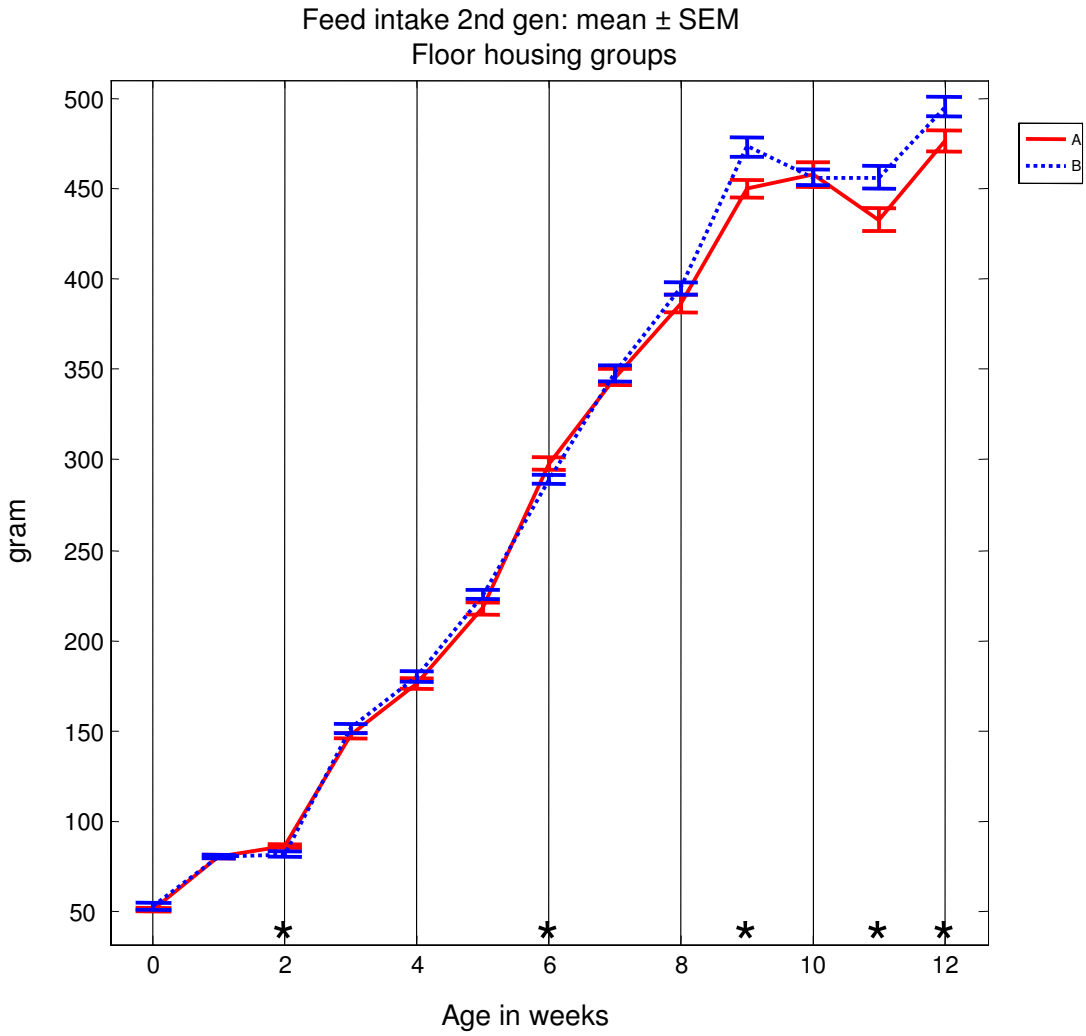


Figure 5.10: 2nd Generation. Feed intake per week, time points mark the end of the week. Results present the mean and SEM for the animals per feed-group, 73 (A) and 72 (B) animals per group.

Cumulative feed intake The cumulative feed intake was significantly higher for feed B in week 11 and 12 (Figure 5.11).

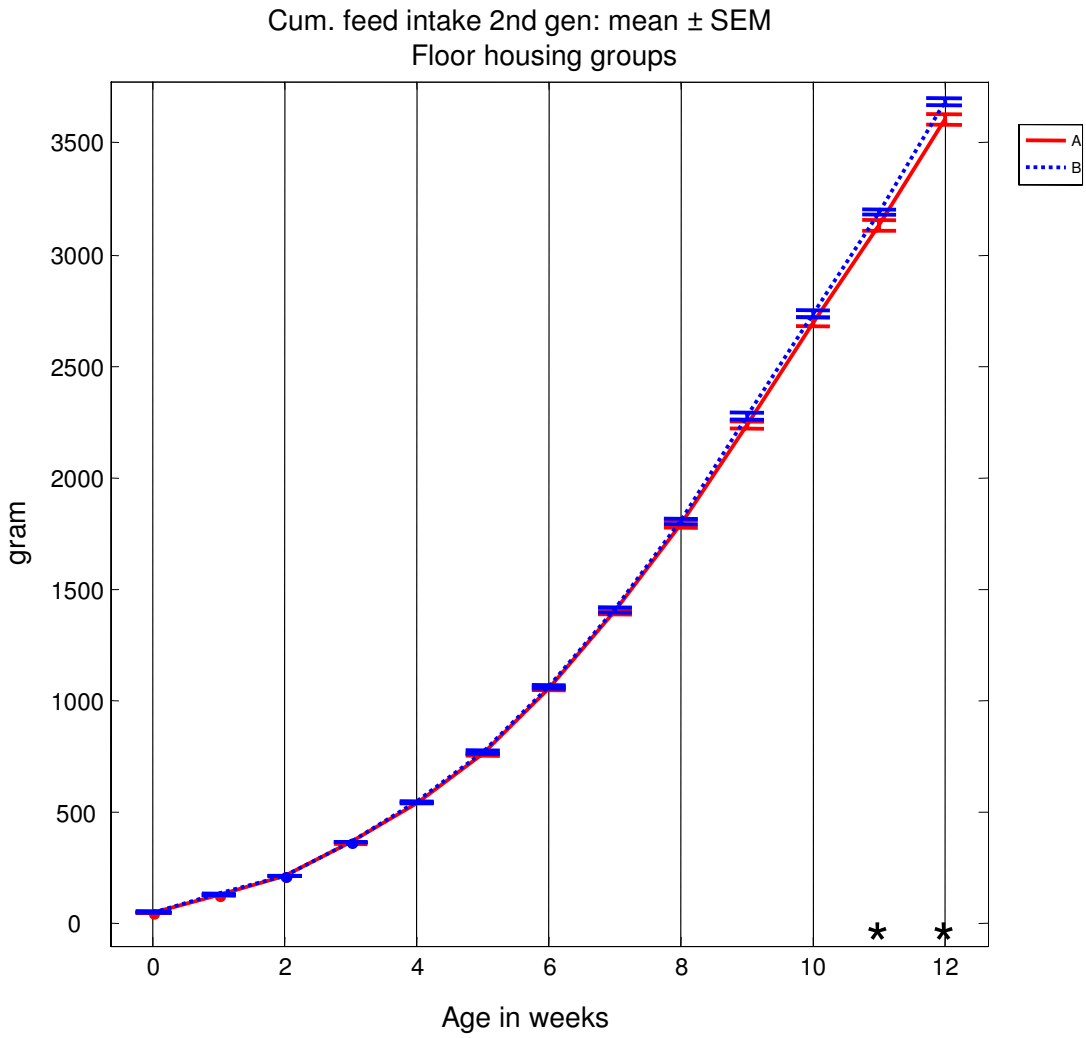


Figure 5.11: 2nd Generation. Cumulative feed intake per week. Results present the mean and SEM for the animals per feed-group, 73 (A) and 72 (B) animals per group.

Body weight Even stronger than in the first generation the line-differences appeared, the H-line animals being relatively lighter and the L-line animals being relatively heavier, compared to the average of the C-line animals (Figure 5.12).

Next to this a difference in body weight related to the treatment appeared, strongest in the C-line animals. The animals on feed B developed a higher body weight than those on feed A. The slope of weight development was steeper. The L-line animals showed a likewise difference during 3 week. In the C-line animals the difference remained and increased throughout their lifespan. Only for the H-line animals the difference was reversed in the first week. Here the bodyweight of the animals on feed A was significantly higher than of those on feed B.

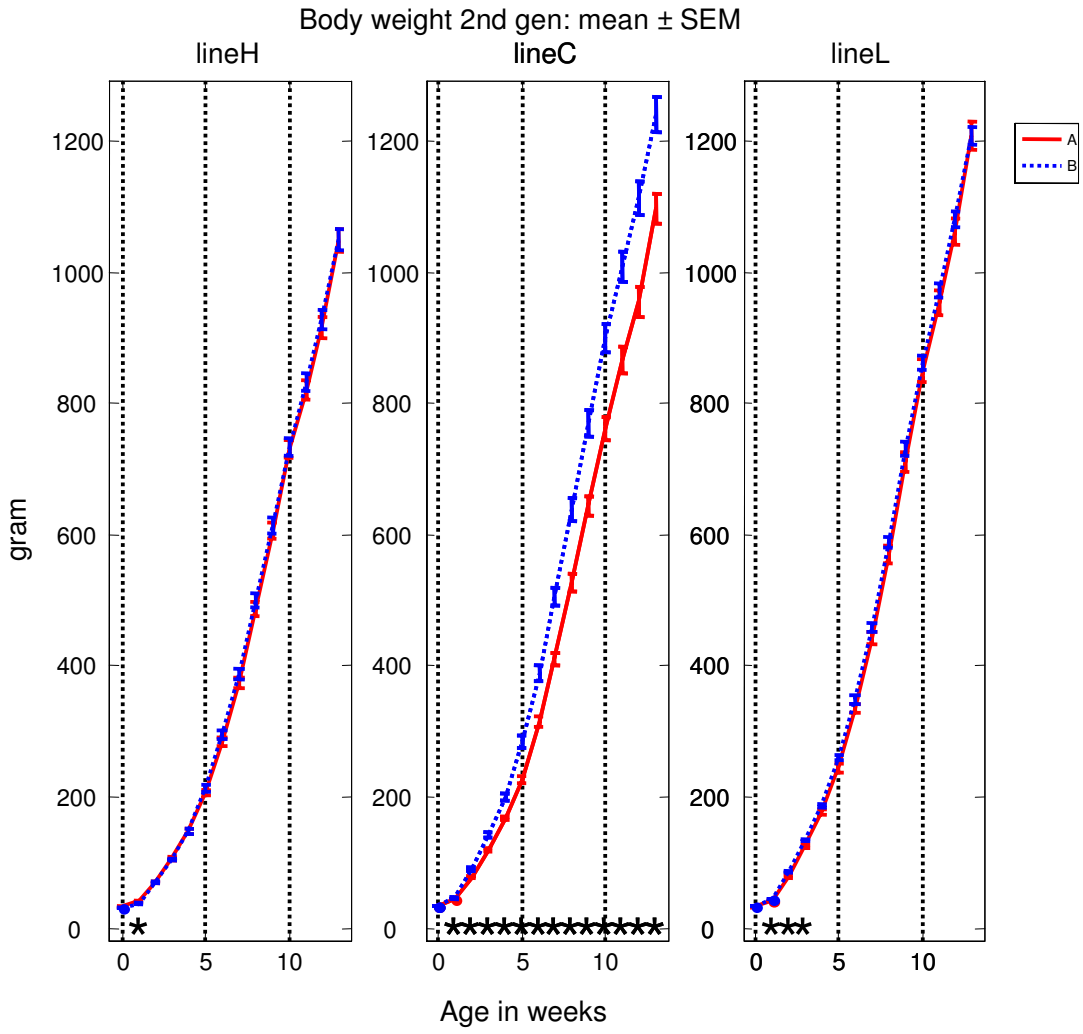


Figure 5.12: 2nd Generation. Mean body weight and SEM per week. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

Growth rate of body weight The growth rate of body weight differs significantly between the feed groups. The animals of the C-line on feed B are significantly ahead till week 8. After week 9, in both A- and B-feed groups, growth declines for a short period. In week 11 for the C-line and in week 12 for all lines the animals on feed A are ahead significantly (Figure 5.13).

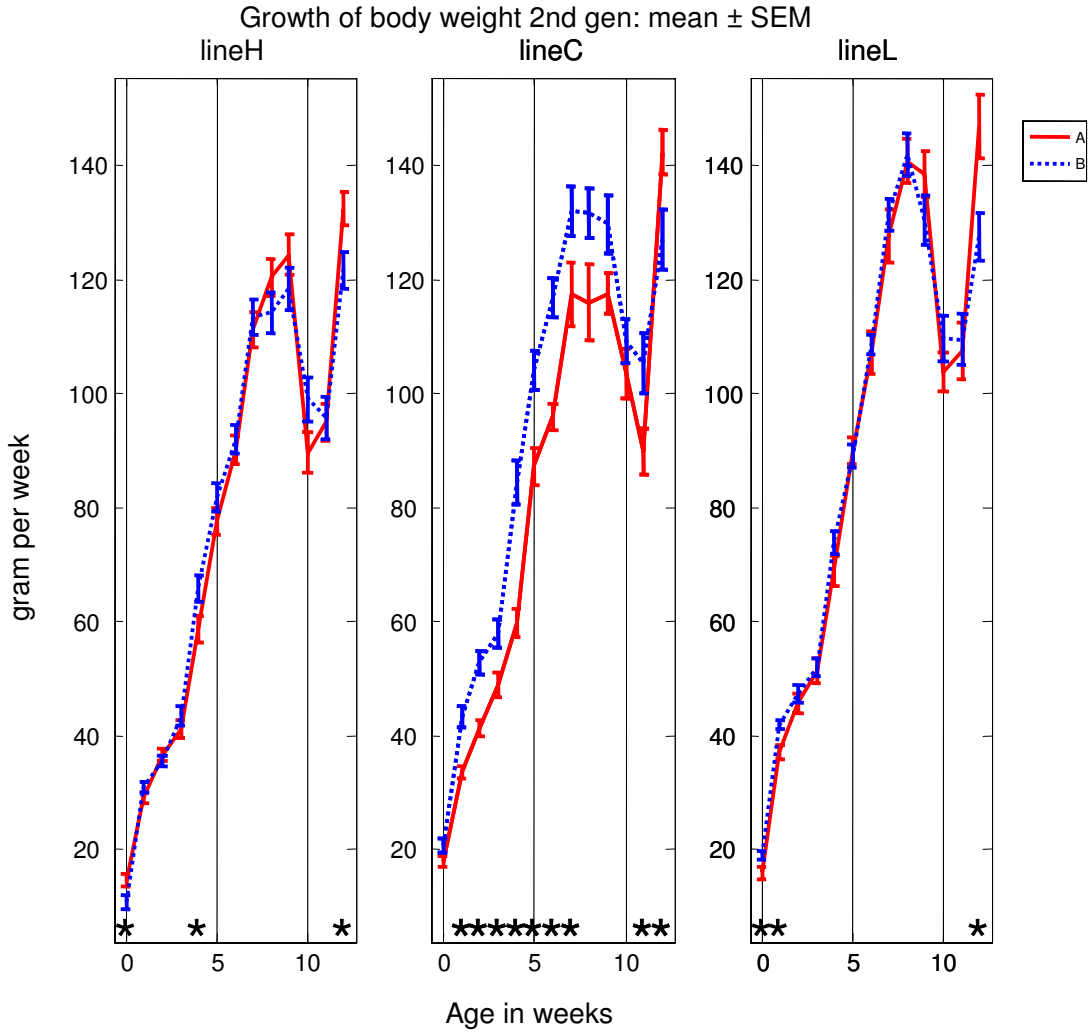


Figure 5.13: 2nd Generation. Growth rate of body weight. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

Relative growth rate From the perspective of relative growth rate of body weight, the animals on feed B are ahead significantly in most of the first 5 weeks. After 5 weeks the A-animals are slightly or significantly ahead. In week 12 the animals on feed A are ahead significantly in all lines (Figure 5.14).

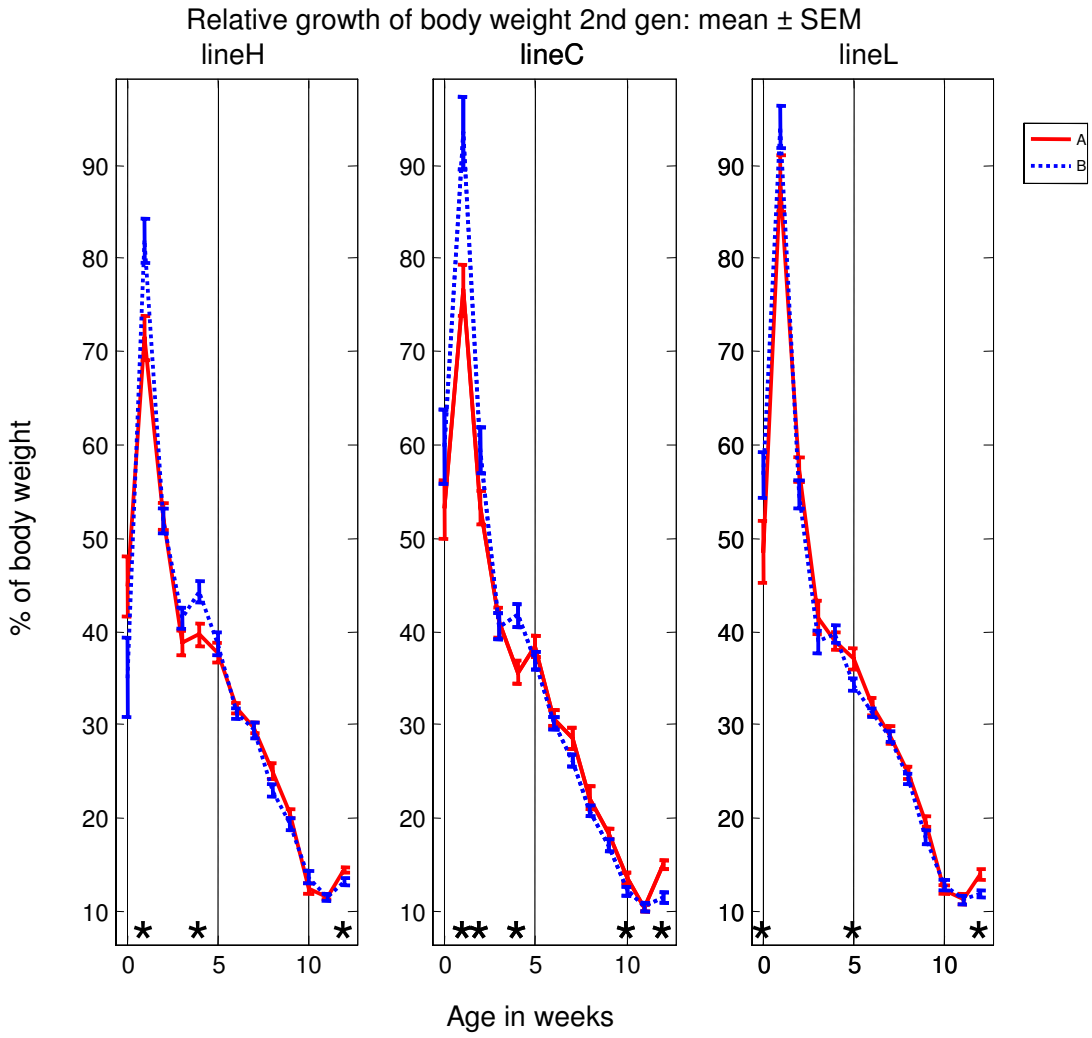


Figure 5.14: 2nd Generation. Relative growth rate of body weight. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

To visualize better the differences between A and B, Z-scores were computed of the relative growth rate of body weight. In the first 5 weeks the animals on feed B are ahead significantly at several time points.
From week 5 the tendency changes, while in week 10 the animals of the C-line, and in week 12 in all lines, the animals on feed A are ahead significantly (Figure 5.15).

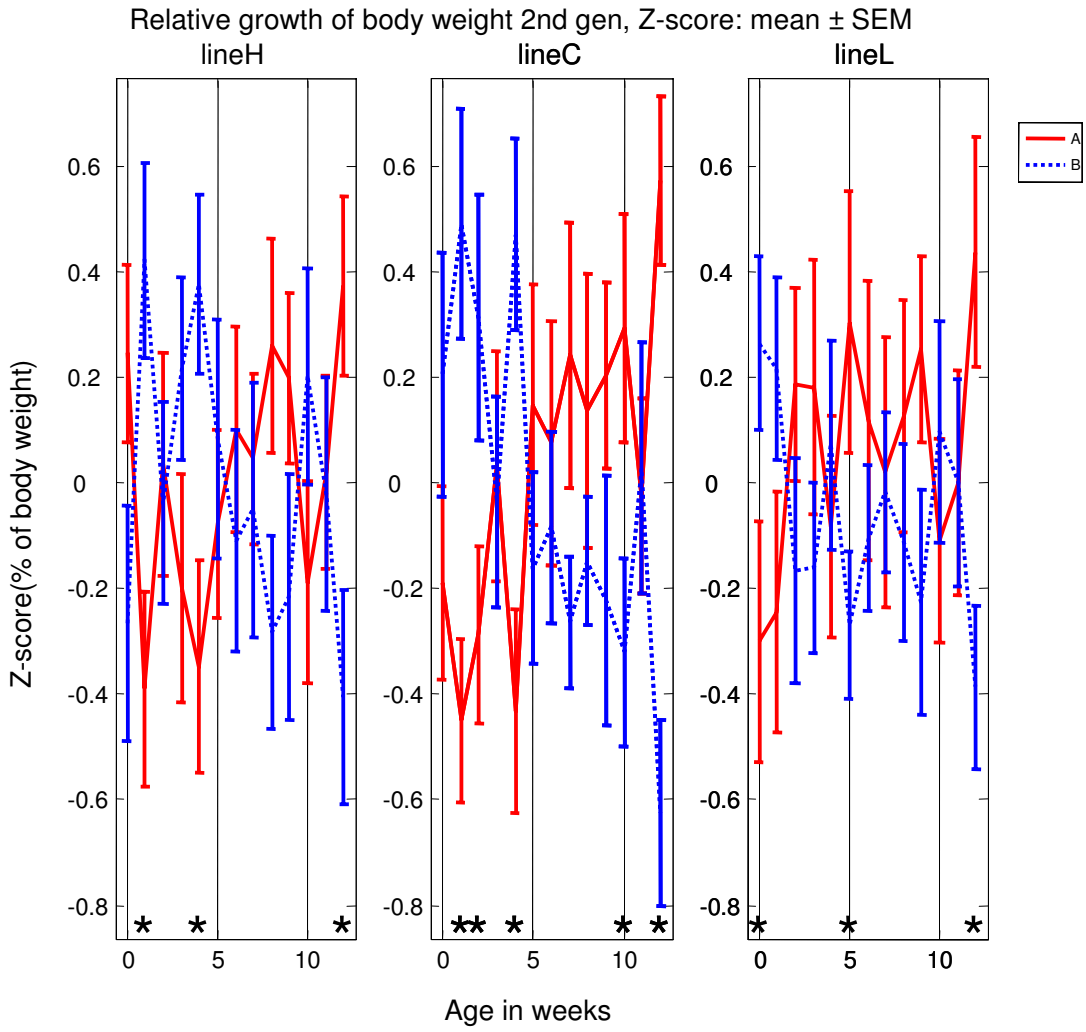


Figure 5.15: 2nd Generation. Relative growth rate of body weight, as Z-scores. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

1st and 2nd generation compared The weight results of the second generation were compared to those of the first generation. To this, Z-scores of the second generation were adjusted for the mean first generation curves. Figure 5.16 plays the comparison for relative growth body weight. The 0 on the y-axis represents the mean of the first generation. At first animals of the second generation grow better than the first generation. Then a steep decline occurs after the KLH-challenge, with a significant faster recovery for animals in all lines on feed A.

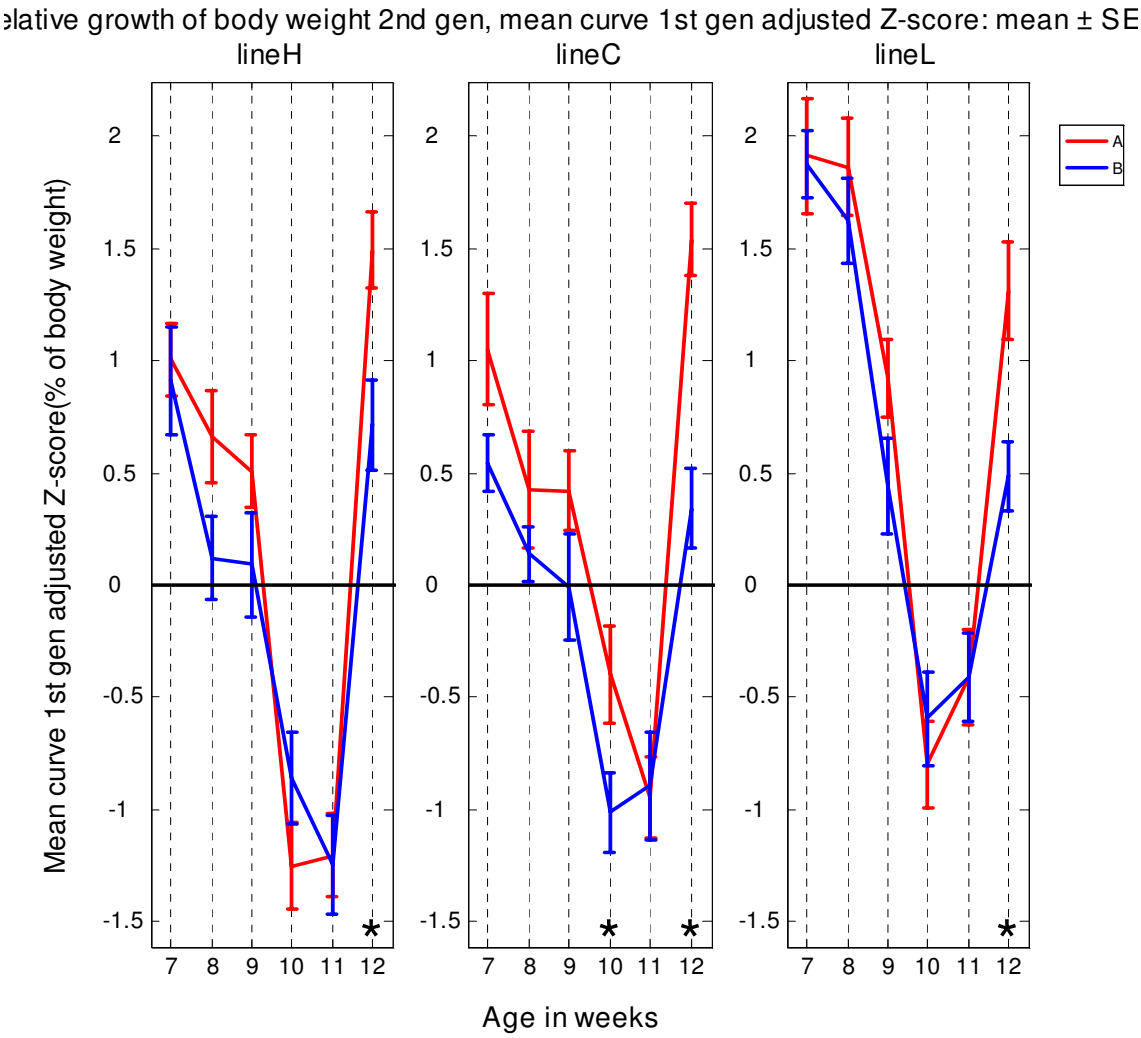


Figure 5.16: 2nd Generation compared to 1st generation. Relative growth rate of bodyweight in Z-scores of 2nd generation, adjusted for the mean (marked 0) of the 1st generation.

Ratio of growth rate and feed intake For the second generation the ratio growth rate/feed intake was computed. This ratio was significantly higher for the animals on feed B in week 1, 2, 4, 6 and 7. In week 12 this changed towards a higher ratio for the animals on feed A (Figure 5.17).

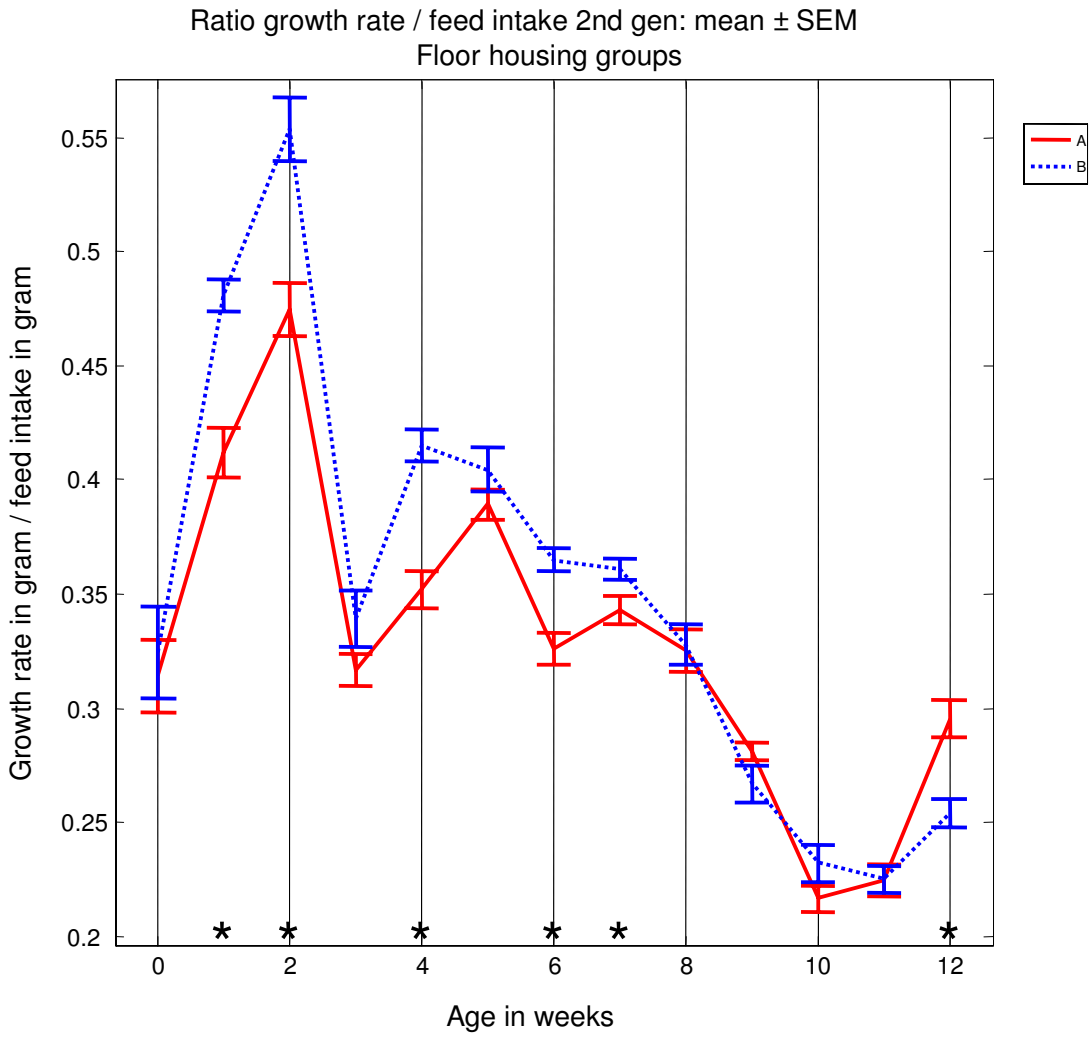


Figure 5.17: 2nd Generation. Ratio growth rate/ feed intake. Results present the mean and SEM for the animals per feed-group, 73 (A) and 72 (B) animals per group.

Organ weight second generation At 13.3 weeks the section took place and the post mortem body weight was weighed (fresh), as well as the gastro-intestinal tract and the liver. Other organs were fixated and weighed afterwards. The relative organ weights showed remarkable differences (Figure 5.18). As a trend, the organ weights in the animals on feed A are higher. It was significantly for the relative weight of the G-I tract in the H-line animals, and for the liver weight in the L-line animals.

Not significant, but different were the relative weights of the formalin fixated thymus and the bursa. The relative weight of the thymus is higher in A, line H. The relative weight of the bursa is higher in A, line L.. See also chapter 5.5.

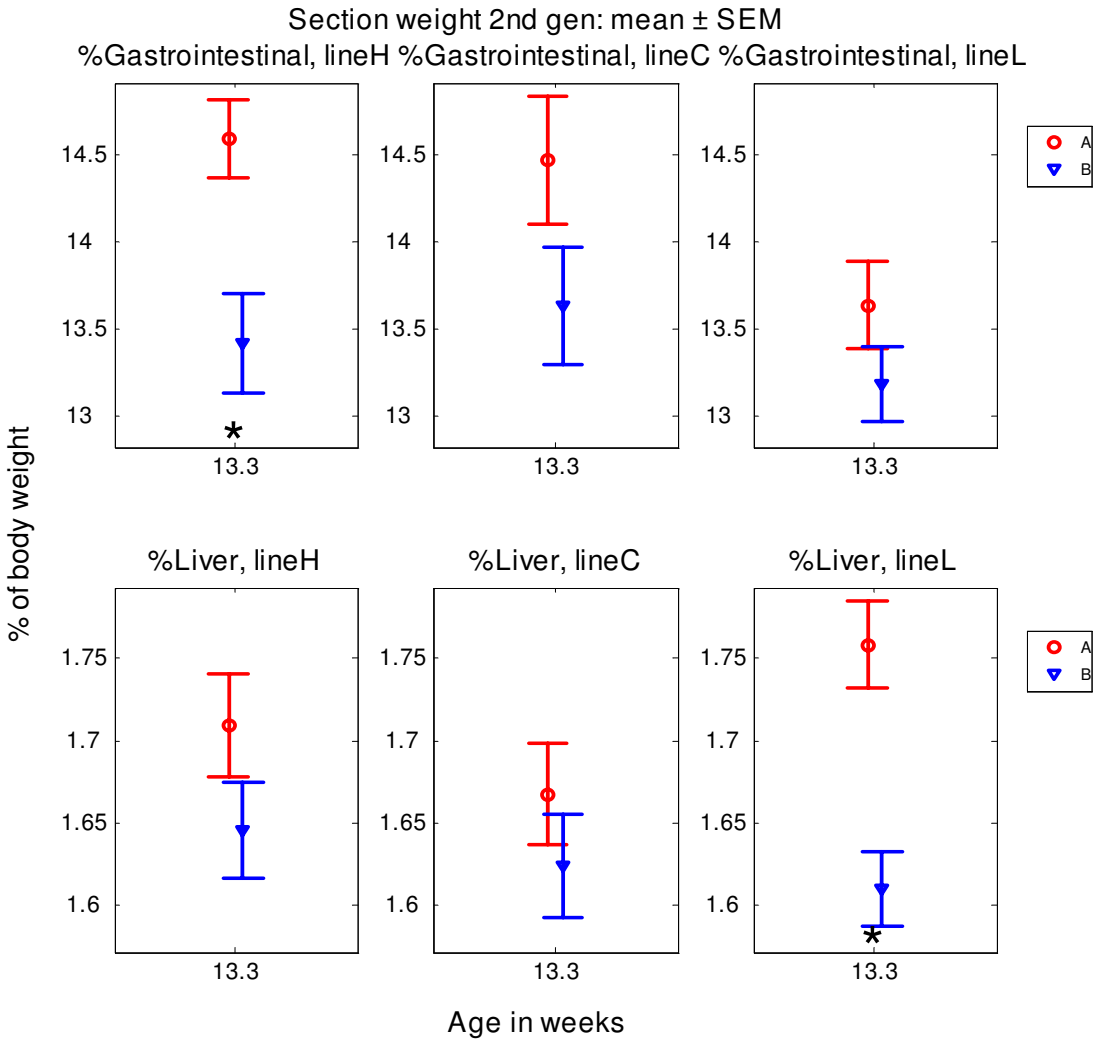


Figure 5.18: 2nd Generation. Mean relative weights of the gastro-intestinal (G-I) tract and the liver, as a percentage of the body weight. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

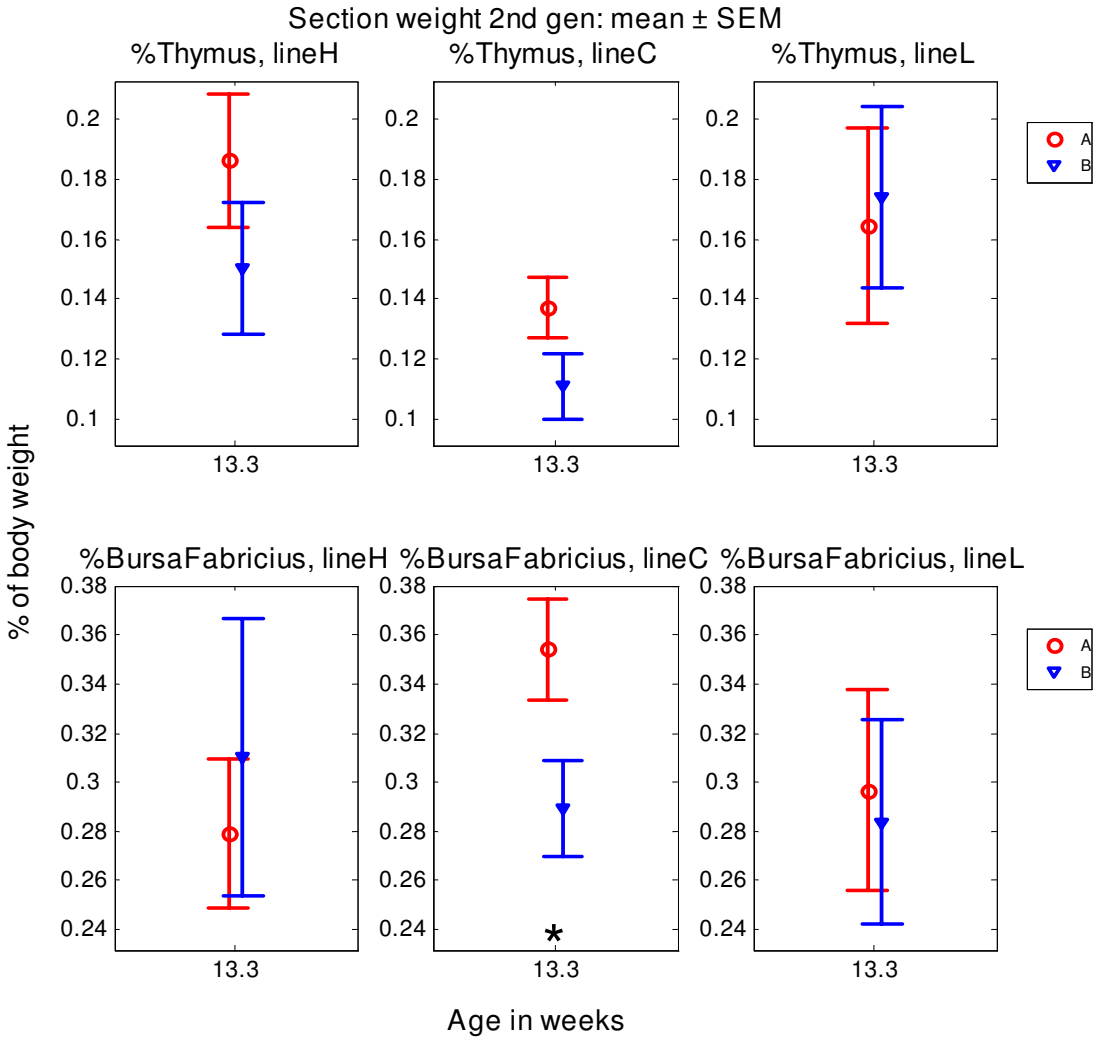


Figure 5.19: Mean relative weights of the formalin fixated thymus and the bursa in the 2nd generation, as a percentage of the body weight. Results present the mean and SEM for the animals per line and feed-group, C-line 24-A and 22-B animals, H and L-line 6 animals each.

Conclusions General health features second generation Differences are apparent, although all animals are healthy. Feed intake differs, A-animals eat more in week 2 and 6, B-animals eat more in week 9, 11 and 12. In week 10, just after the challenge, A- and B-animals eat equal amounts. Growth differs significantly for the C-animals, the B-group being heavier the whole period. H- displayed the same in the first week and L-line in the first three weeks. Growth rate differs, in the C-line B-animals are ahead till week 8, after the challenge there is a decline. From week 11 both groups start to rise again, A growing stronger than B. Line H and line L show the same in week 12. Relative growth rate of bodyweight shows the same pattern after the challenge, in all lines, A-group growing stronger.

5.2 Immunological assays

5.2.1 First generation

5.2.1.1 Innate immune system

5.2.1.1.1 Cellular parameters 1st generation

To analyze the activity of the cellular branch of the innate immune system, monocytes were analyzed for their LPS induced NO production capacity *in vitro*. To this end, heparin blood derived peripheral blood mononuclear cells (containing 5-10% monocytes) were cultured *in vitro* and stimulated with LPS. After 72 hrs NO production was measured. All unstimulated control values are low (max around 1 µM NO), except for the week 1 measurements, the week after the animals were put on the experimental feed. At week 1 only the H line animals on feed A reached significantly increased NO levels, as compared to the other time points and animals of the other lines.

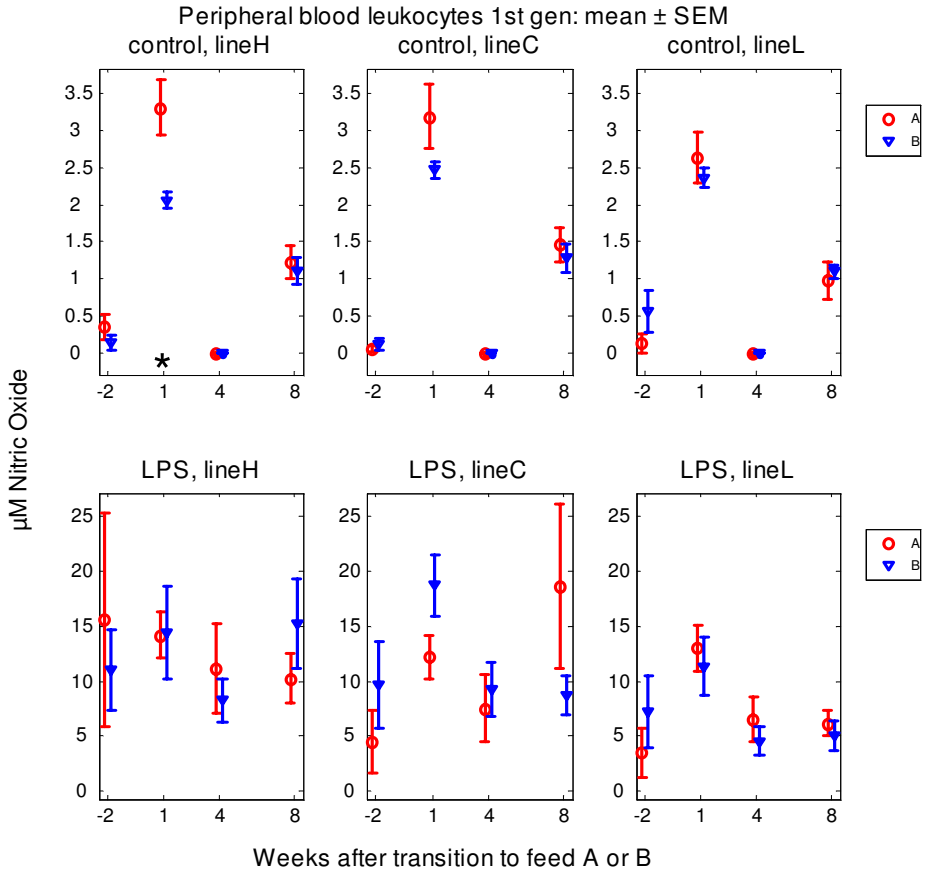


Figure 5.20: 1st Generation. Background and LPS induced NO production in peripheral blood leukocytes. Cells were isolated and incubated in triplicate in the absence or presence of LPS for 72 hrs. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

The LPS stimulated values show less variance due to the feed change, when regarding the absolute values.

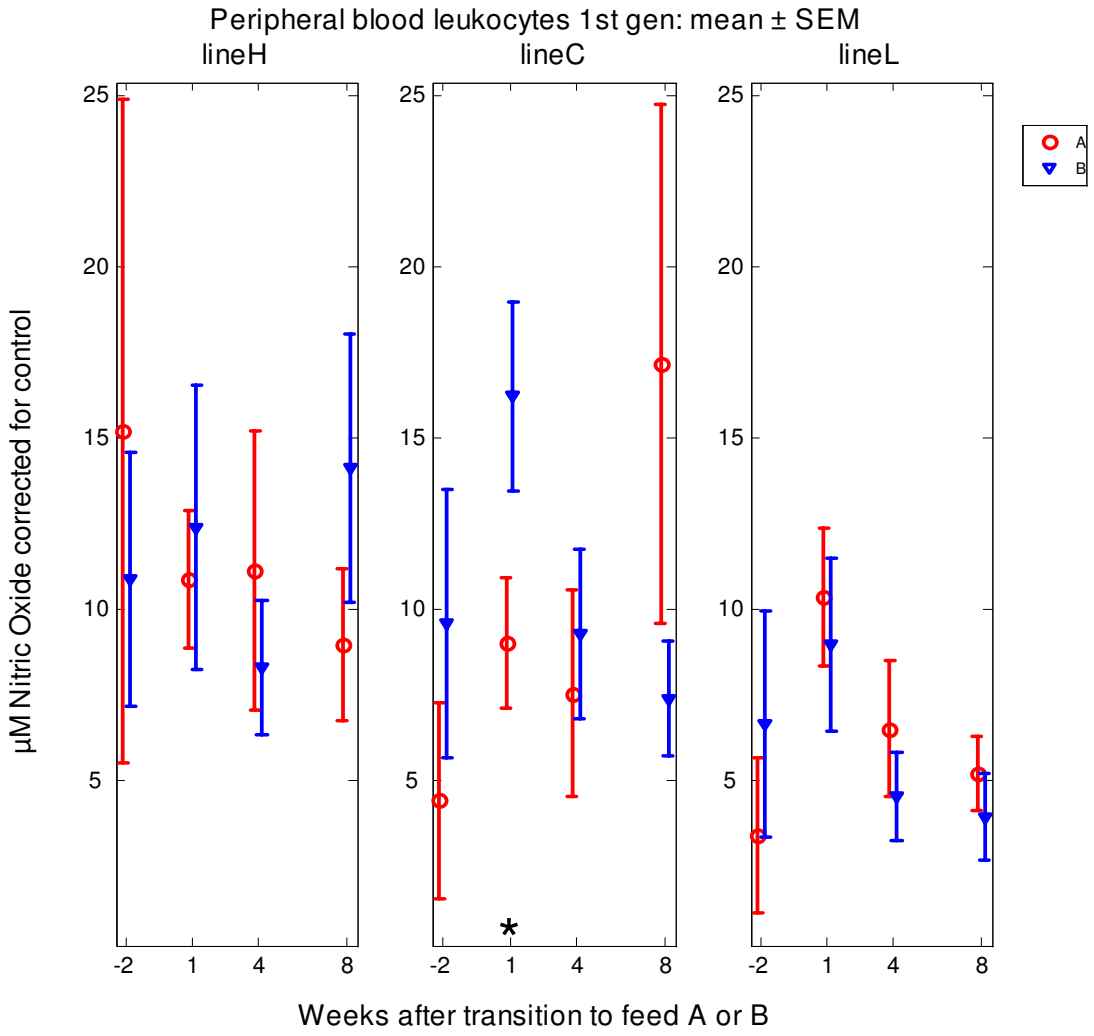


Figure 5.21: 1st Generation. Control corrected LPS induced NO production by peripheral blood leukocytes. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

However, when the stimulated values are corrected for the control values, a significant difference is seen in the C-group at week 1. Here the animals of the control line on feed B show a significantly higher response in the NO production levels in LPS stimulated cultures.

Conclusion The feed change has an effect on the monocyte reactivity. Feed A appears to have an immunomodulatory effect on the control values of all the chicken lines C and H at one week after the feed change, although only significant for the H-line animals. Only the C- animals show a different increase in LPS stimulated cells at one week after the feed change, with the animals on feed B showing the greater immune response.

5.2.1.1.2 Humoral parameters 1st generation

Plasma levels of natural antibodies (binding to LPS, LTA and KLH) and complement factors, indicative of classical and alternative complement activation, were determined by ELISA.

Natural antibodies Large variance of the titers of LPS-binding natural antibodies is seen at week -2 in lines C L , i.e. before the switch to the test feeds. The weeks after the change to experimental feed all titers rise to a plateau value at either week 4 or 8. The L-line animals on feed B show a significant drop in the titers at week 22.

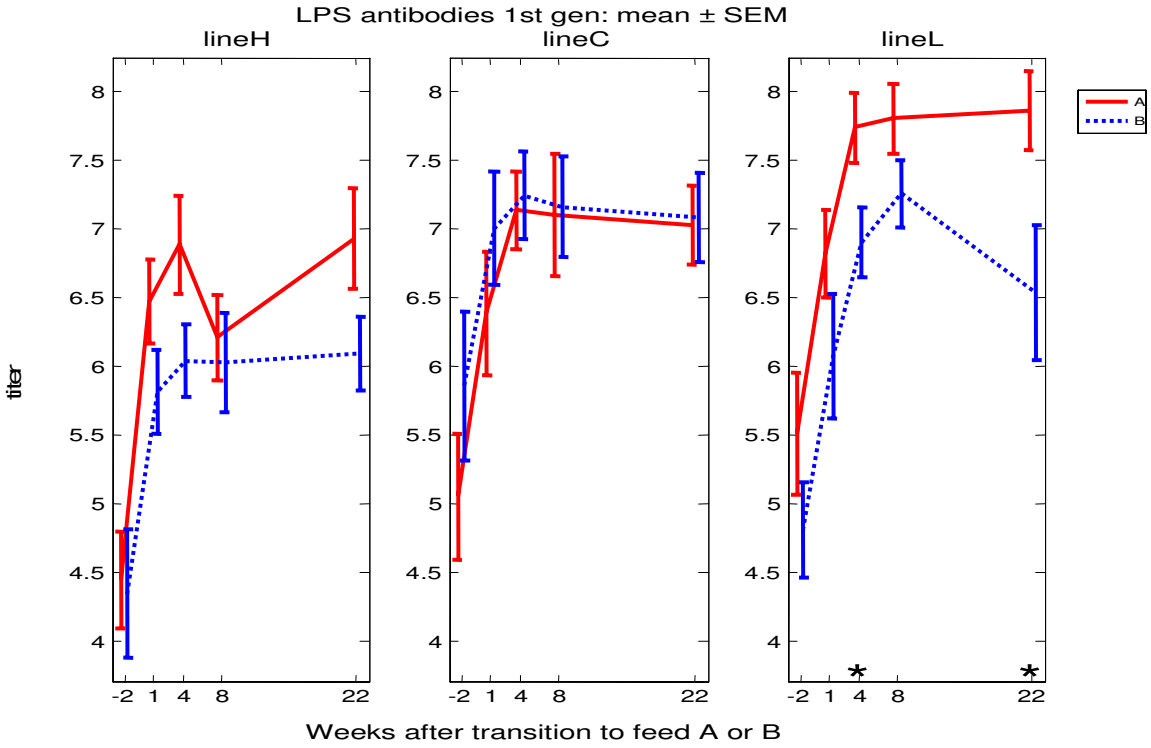


Figure 5.22: 1st Generation. Plasma levels of LPS-binding natural antibodies. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

The LTA (lipoteichoic acid) titers show a slow onset of time-dependent rise early after the change to the experimental feed. All animals show a rise in the titers during the weeks following the feed change. The delta titers (corrected for the titers at week -2), show that the rise in time for all animals is comparable. There were no significant differences between the two feeds.

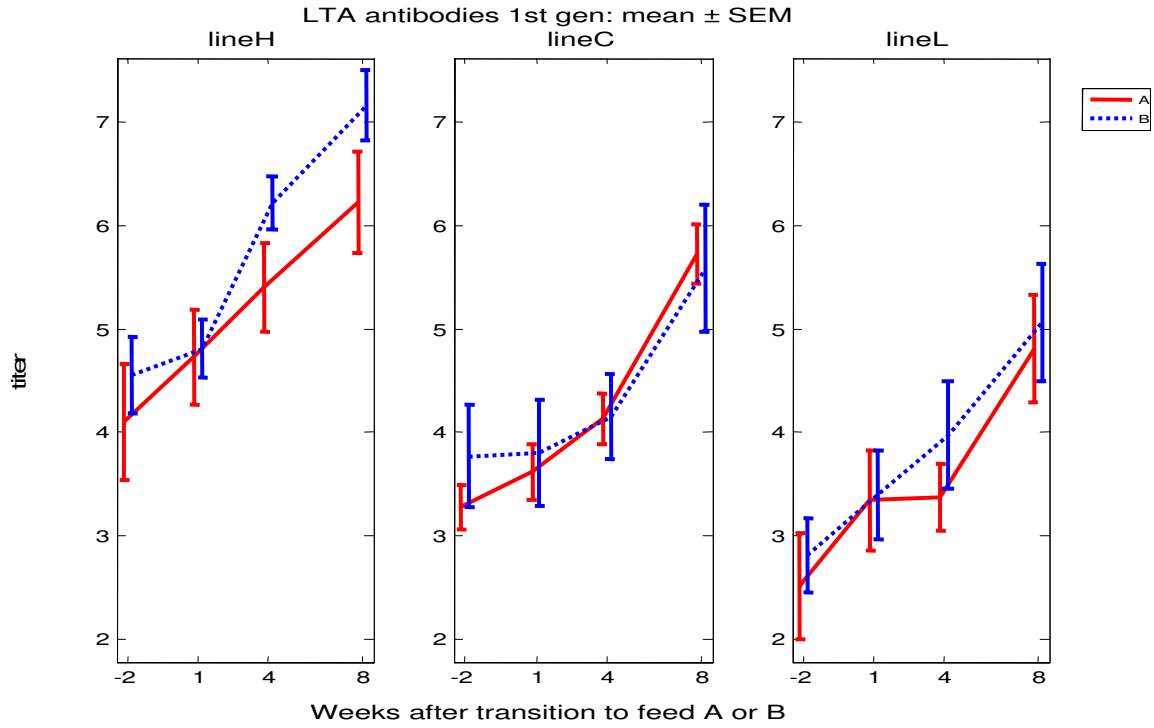


Figure 5.23 1st Generation. Plasma levels of LTA-binding natural antibodies. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

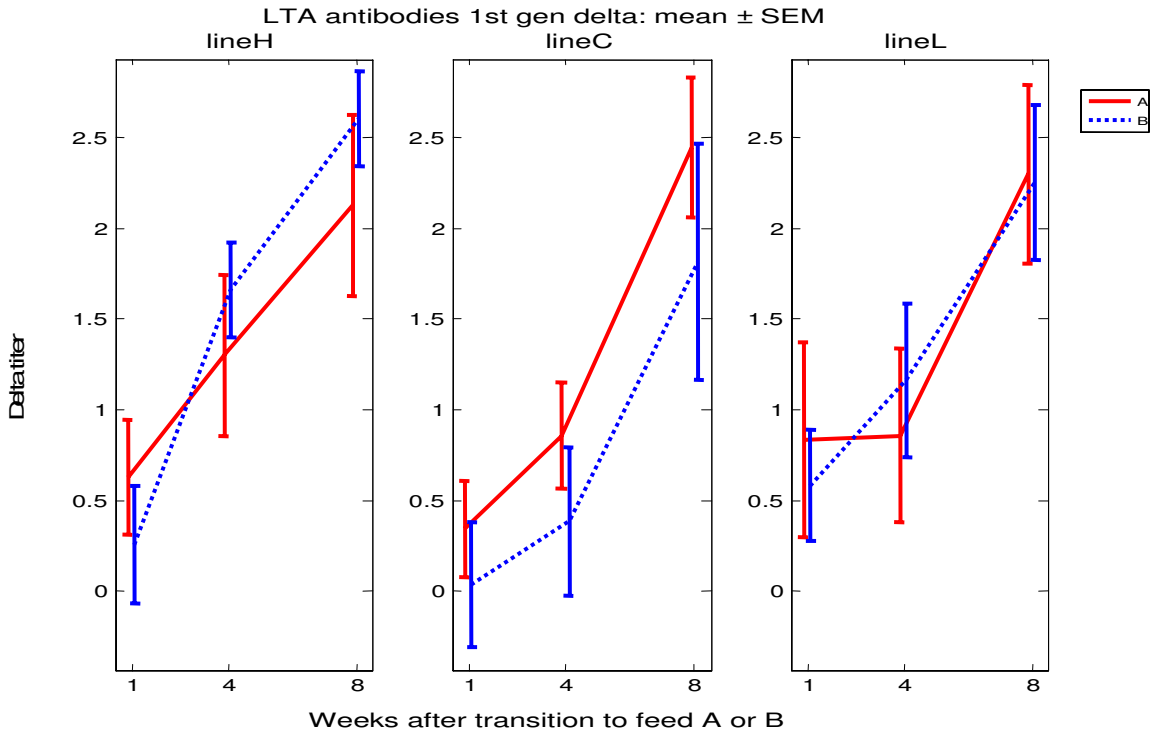


Figure 5.24 1st Generation. Corrected plasma levels of LTA-binding natural antibodies. Levels of individual hens were corrected for the levels measured 2 weeks before the feed change (t =-2). Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

The last natural antibody, represented by the KLH titers, shows the expected line difference between the animals. Chicken of the H- responder line have the highest titers. The C- and L-line respectively have lower titers. There were no significant differences between the two feeds. The KLH and LPS titers seem to mirror each other, when regarding line characteristics, e.g. the L-line shows the highest LPS titers and the lowest KLH titers.

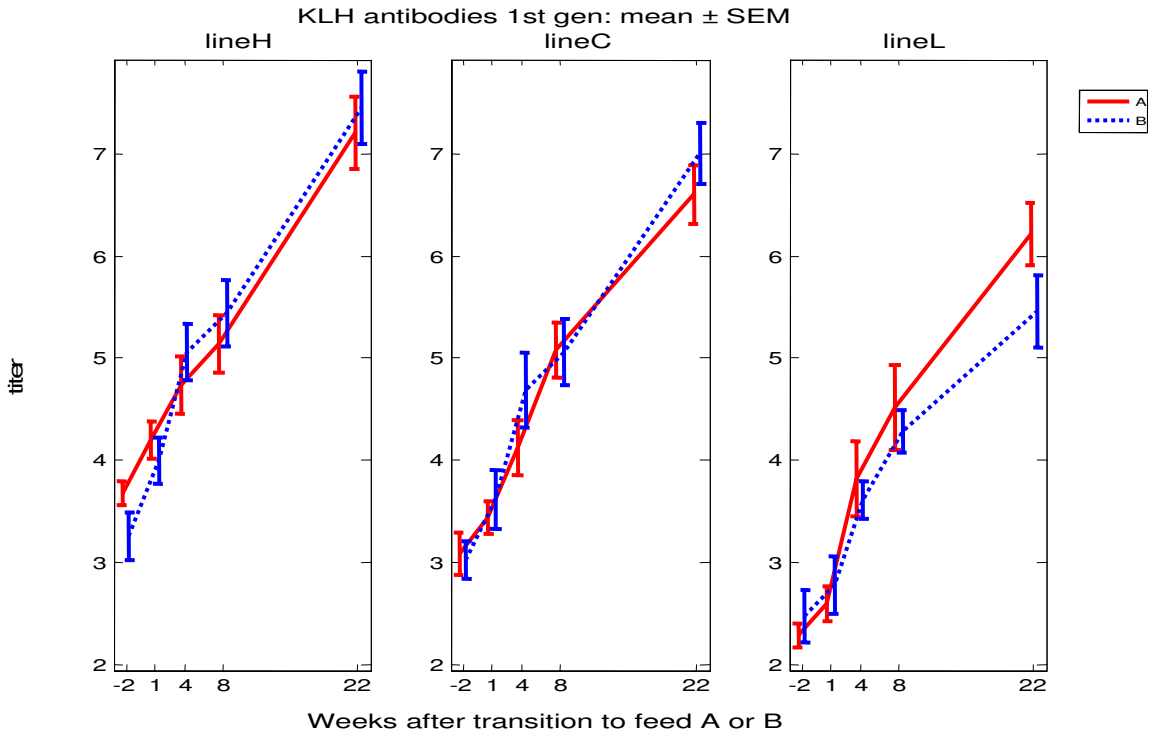


Figure 5.25 1st Generation. Plasma levels of KLH-binding natural antibodies. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

Complement The alternative complement shows a significant difference in the H-line animals 2 weeks before the change to the test feeds. Overall complement activity of the H- and C-line animals is comparable, without divergence due to the treatment. The L-line animals show a different activity pattern throughout time when compared with the other two lines. The activity at baseline is much higher.

The classical pathway activity again shows little differences when comparing lines and treatments. Here too, the L-line animals show statistically different values at t =-2. These differences could be explained by experimental flaws, like double blood drawing.

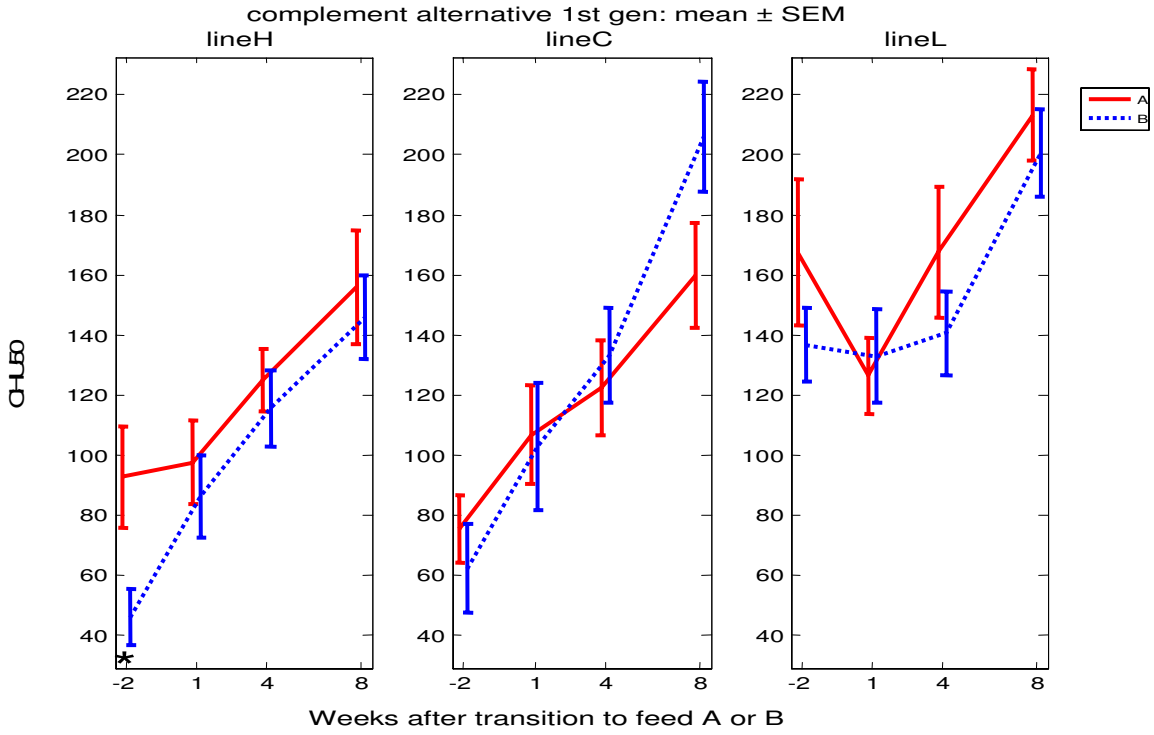


Figure 5.26: 1st Generation. CH50 activity of serum after alternative complement activation. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

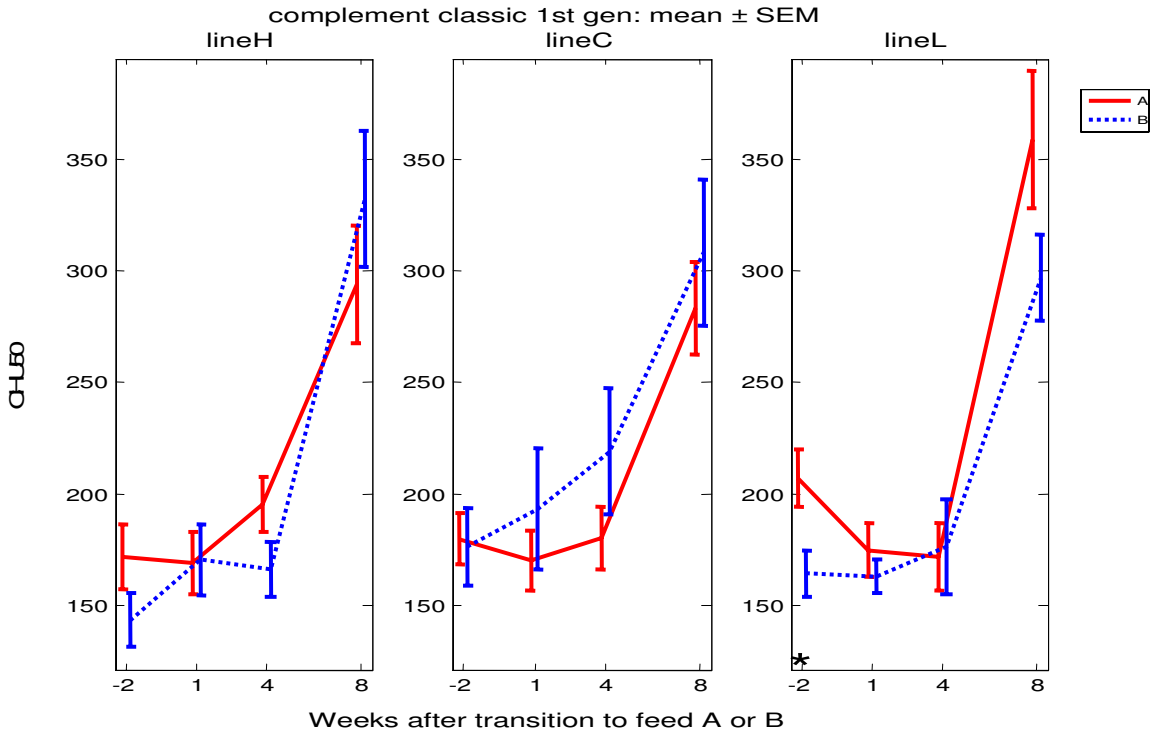


Figure 5.27: 1st Generation. CH50 activity of serum after classical complement activation. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

Conclusion Overall, in the first generation, there is little effect of the treatment with the two experimental feeds on the innate humoral immunity parameters. Complement activity does not appear to be affected by the treatments either.

5.2.1.2 Specific immune system

5.2.1.2.1 Cellular parameters 1st generation

Lymphocyte proliferation in vitro was analyzed to determine effects on cellular parameters of adaptive immune responsiveness. The medium control values of all samples are low as expected, except for the controls of the H- and L-lines of the A group hens at week -2, i.e. before the switch to the test feeds. This rise in medium controls was again caused by double blood drawing from several animals. The high control values also influence the LPS-stimulated values at week -2.

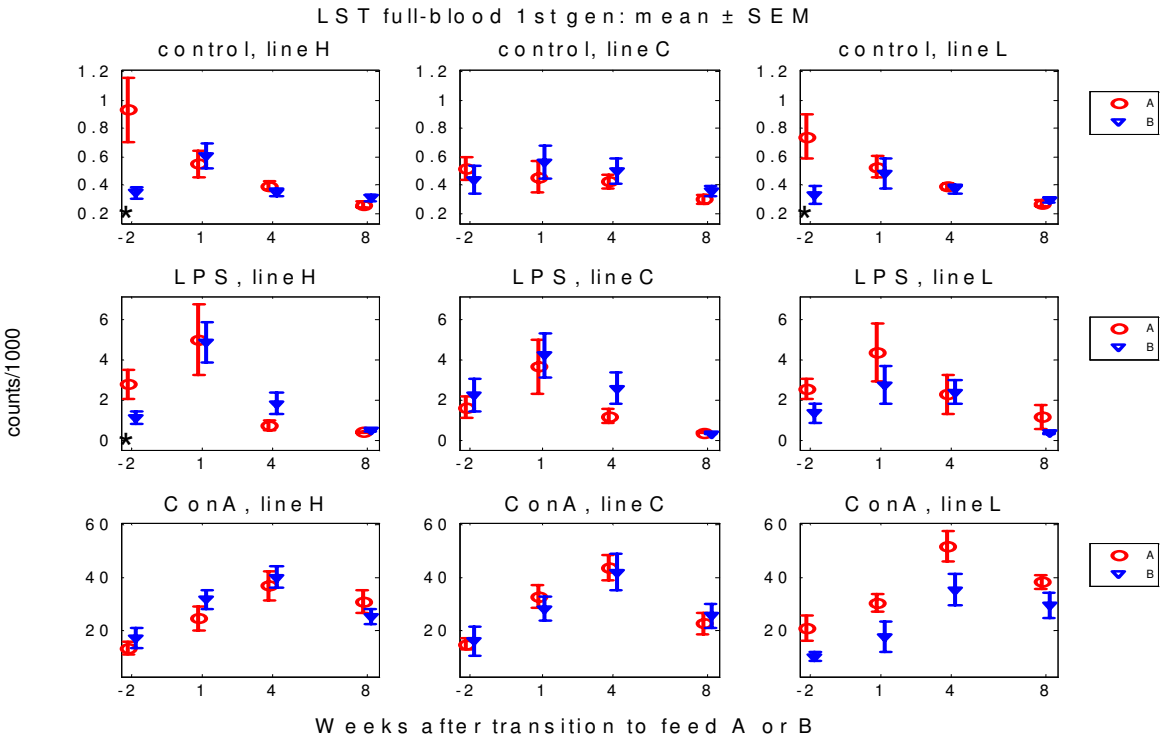


Figure 5.28: 1st Generation. Proliferation of peripheral blood leukocytes in the lymphocyte stimulation test. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

The amount of proliferation is greatly different in the various stimulation conditions and also over time, there are differences with the LPS stimulated cultures peaking at week 1 while the Con A stimulated cultures peak at week 4 with the proliferation. However, the data on proliferation in the controls, as well as the LPS and Con A stimulated cultures show no significant differences with respect to the two feeds at the various time points tested.

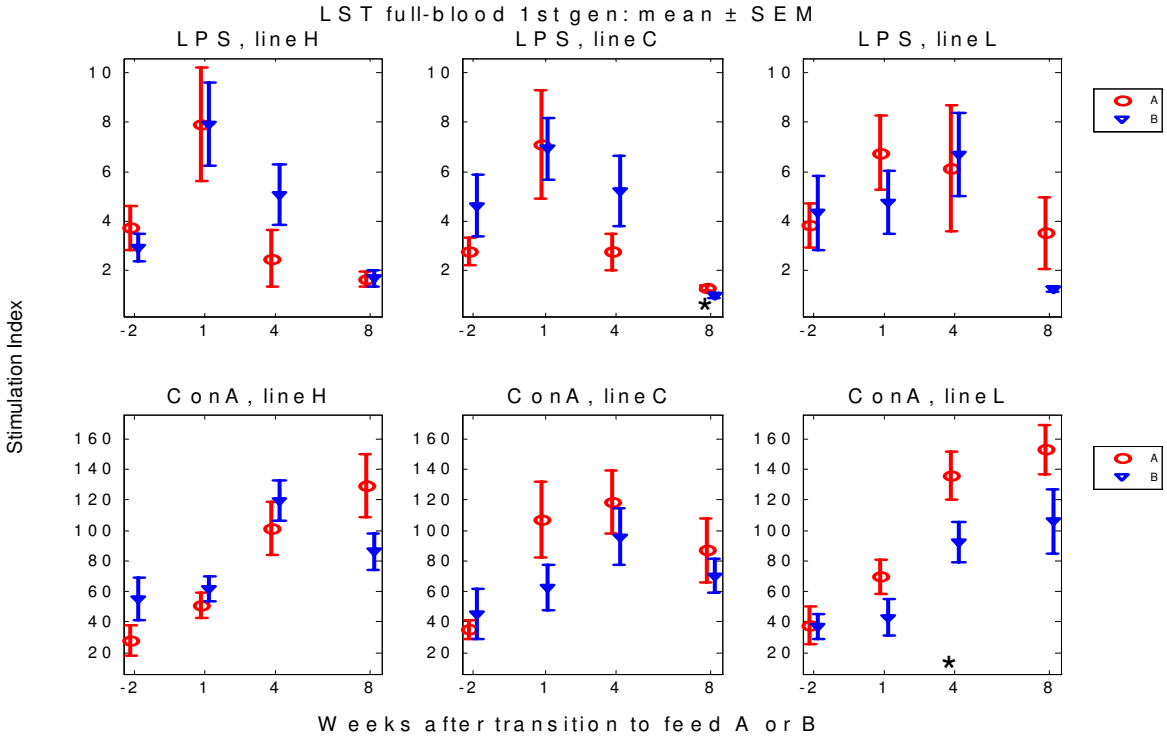


Figure 5.29: 1st Generation. Stimulation Index of proliferation in stimulated peripheral blood leukocytes cultures. The index reflects the cell counts in the stimulated cells divided by those in the non-stimulated cells. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

When expressing the data as SI (Stimulation Index), the results are similar, but more pronounced (Figure 5.29). The LPS responses are highest at week 1 and at week 8 are lower than before the start of the treatment (week -2). The C- and H-line show a possible divergence at week 4, in which the animals on feed B have a higher, but non-significant SI. The T-cell stimulation, as represented by the Con A data, rises in time. Where the animals diverge due to treatment, the animals on feed A show the higher SI but only at week 4 for the L-line animals on feed A, this difference is significant.

Conclusion Few significant effects are seen in the specific cellular parameters as represented by the LPS and Con A stimulated proliferative responses of monocytes of the chicken fed with the two different feeds.

5.2.1.2.2 *Humoral parameters 1st generation*

Vaccine-specific antibody responses were analyzed to determine effects on the antigen-specific humoral immune response. In time, the response of all the animals is similar, the titers rise through the weeks with the greater rise in the H-line animals from week 4 on, most likely in response to the second booster with NCD (week 3). The C- and L-line animals show the greatest rise after week 8. There are little to no differences in the responses of the H-line and C- animals. The L-line animals show the most divergence between to the two treatment groups, with the animals on feed A showing the highest titers. This is primarily caused by a lack of effect in group B until week 4.

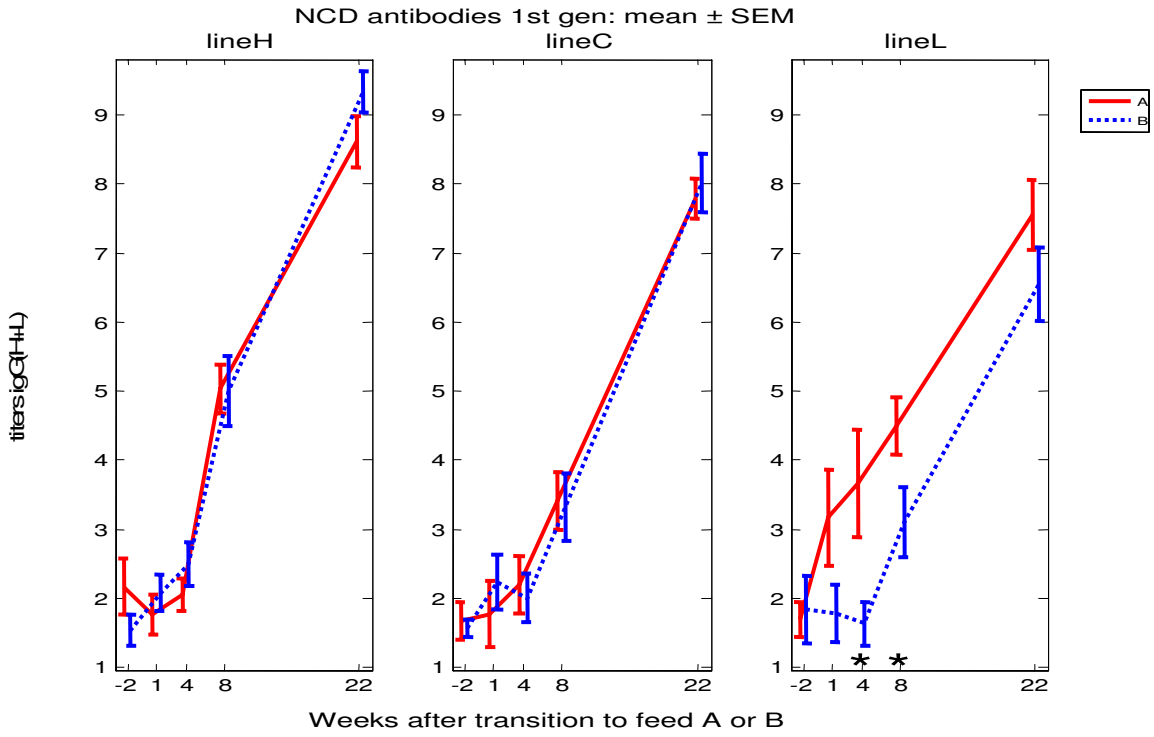


Figure 5.30: 1st Generation. NCD-specific antibody titers in serum. The second booster with NCD was given in week 3. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

When the data are presented as delta titers (corrected for the titers at week -2), comparable results are found. Here it becomes clear that the response in time of the L-line animals on feed A has increased to the level of the C- animals while the L-line animals on feed B are much lower. The H- and L-line groups both show significant differences at week 4. In the H-line animals the animals on feed B tend to have the higher responses, while in the L-line animals the animals on feed A tend to have the higher titers. For the H-line this is primarily caused by the high starting value for group A at t =-2, i.e. before the start of the treatment.

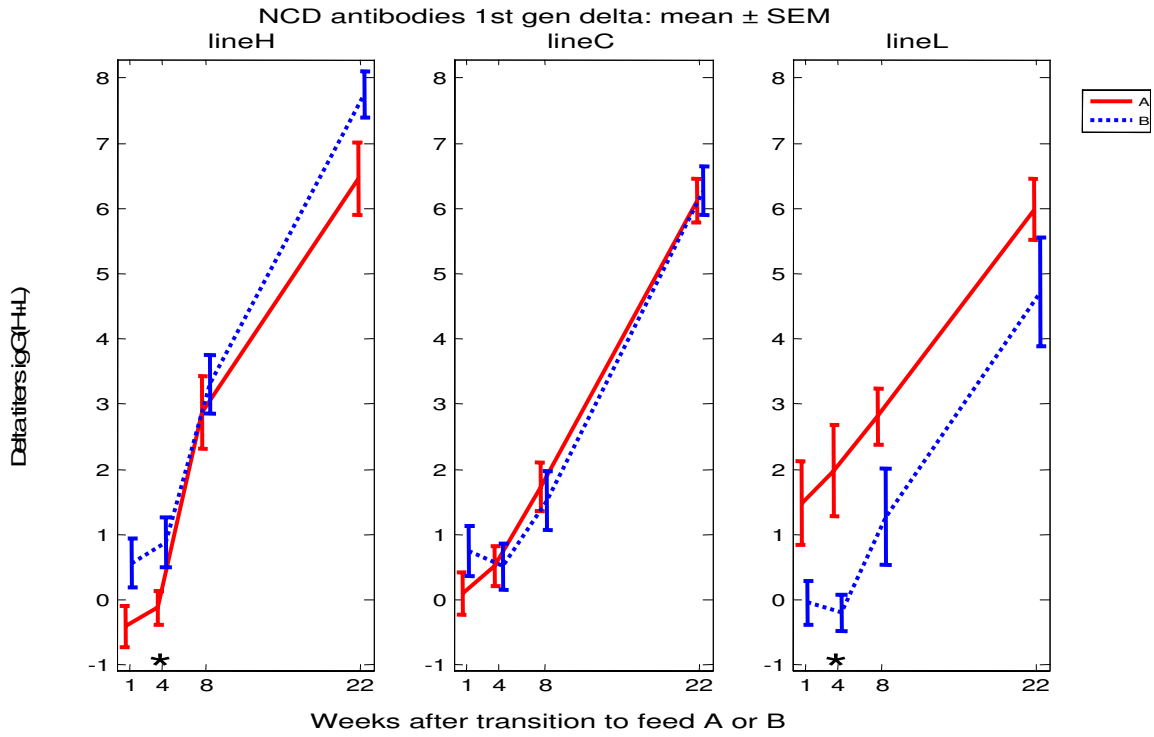


Figure 5.31: 1st Generation. Corrected NCD-specific antibody titers in serum. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

The Gumboro titers of the H- and C-line animals are again quite similar. The titers are high in the first weeks and slowly decline in time. The vaccination was given in week -8. In these groups the responses in time are similar and both show a divergence, albeit not significant, at week 8. At week 8 the animals on feed A show a higher but non-significant response than the feed B fed animals. The L-line animals shows a different response in time when compared to the C- and H-line groups and there is much more variation in the data. There is large variance in the baseline measurement at week -2. The response is almost the same through time.

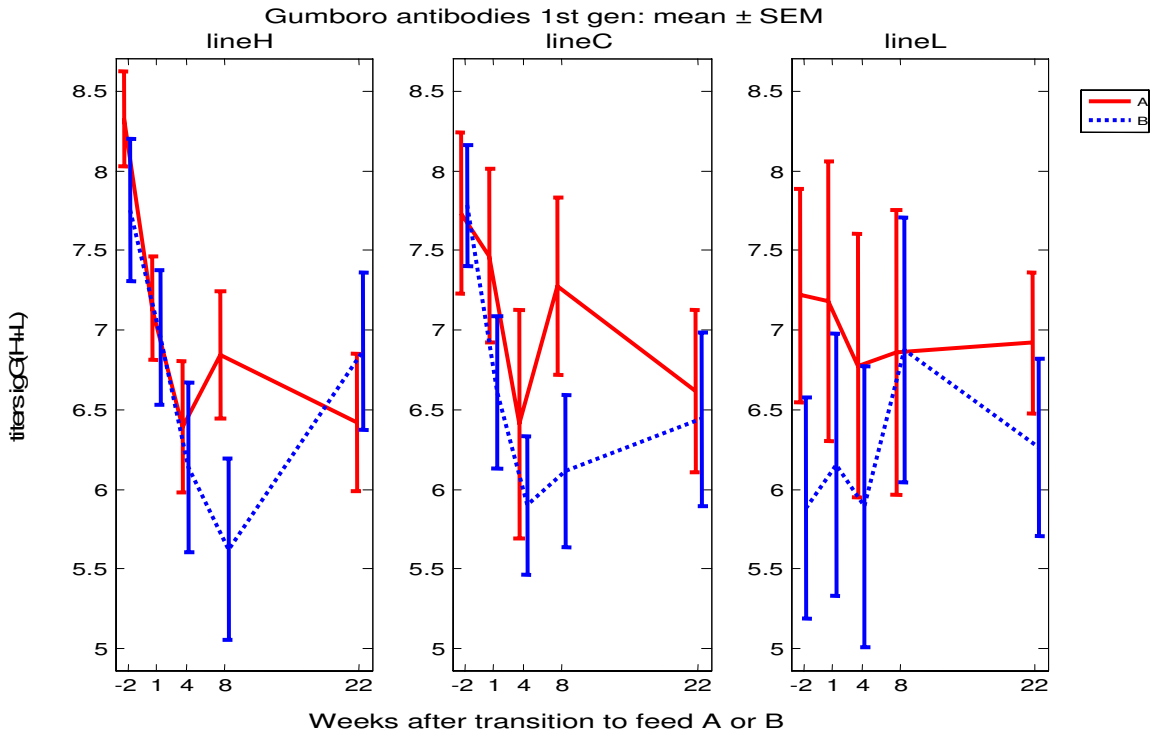


Figure 5.32: 1st Generation. Gumboro-specific antibody titers in serum. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

When the data are expressed as delta (corrected for the titers at week -2), the same effects are seen in the H- and C-line groups. Again, the titers decline in time, with a divergence at week 8 in which the titers of the animals on feed A tend to be higher. The L-line animals on feed B tend to have the higher differential titers.

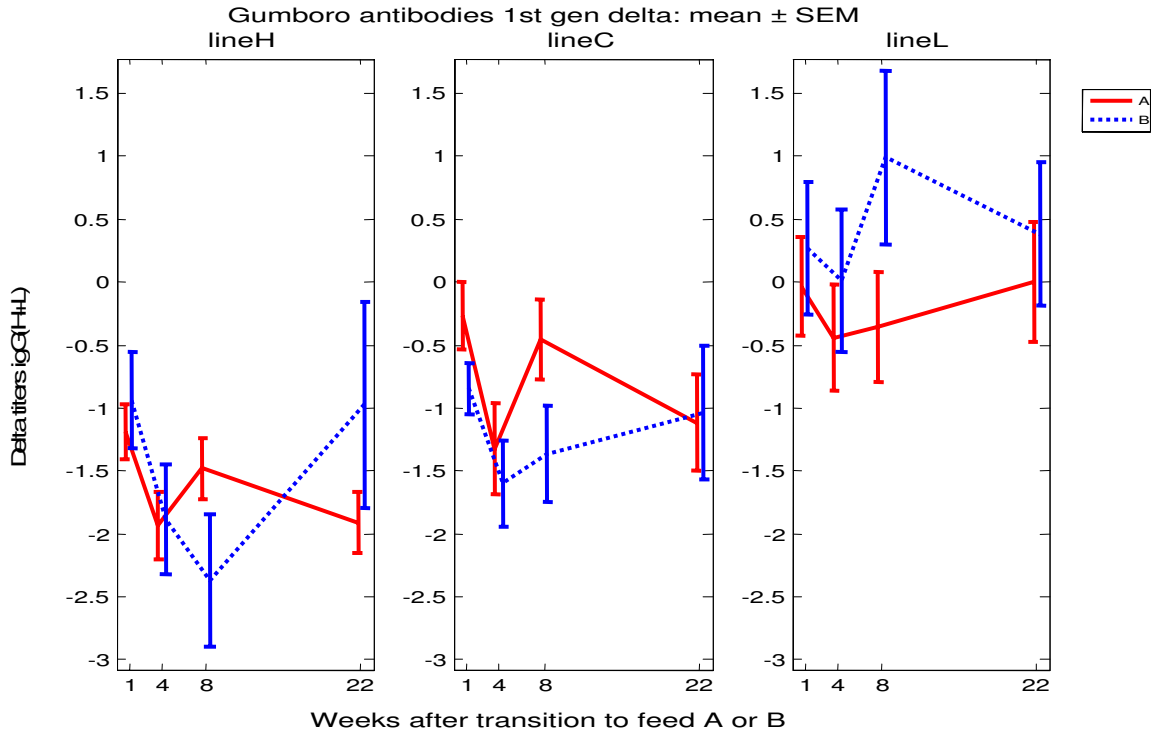


Figure 5.33: 1st Generation. Corrected Gumboro-specific antibody titers in serum. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

Conclusion The H- and C-line animals show little effect of the treatment in their specific humoral responses. The L-line animals show the most divergence due to treatment in these parameters. With the NCD titers the feed A animals in this line show the higher titers and with the Gumboro titers there is a trend that the animals on feed B show higher responses. In the other two lines the opposite was observed for the Gumboro titers. It must be mentioned that it is exceptional to find such differences in the L-line animals. Such effects have not been reported before.

5.2.1.3 *Test with feed extracts 1st generation*

Monocytes were tested in vitro to determine if the extracts of the feeds could enhance the proliferation induced by a suboptimal Con A concentration (2 g/ml). This T-cell specific reactivity of the two different feed extracts was tested in whole blood cells of all three chicken lines.

Different dilutions of the extracts were tested. In this case the index reflects the cell counts in the cells incubated with feed extract and Con A divided by those on Con A only. The final dilution of the feed extract is 2 g/ml ConA.

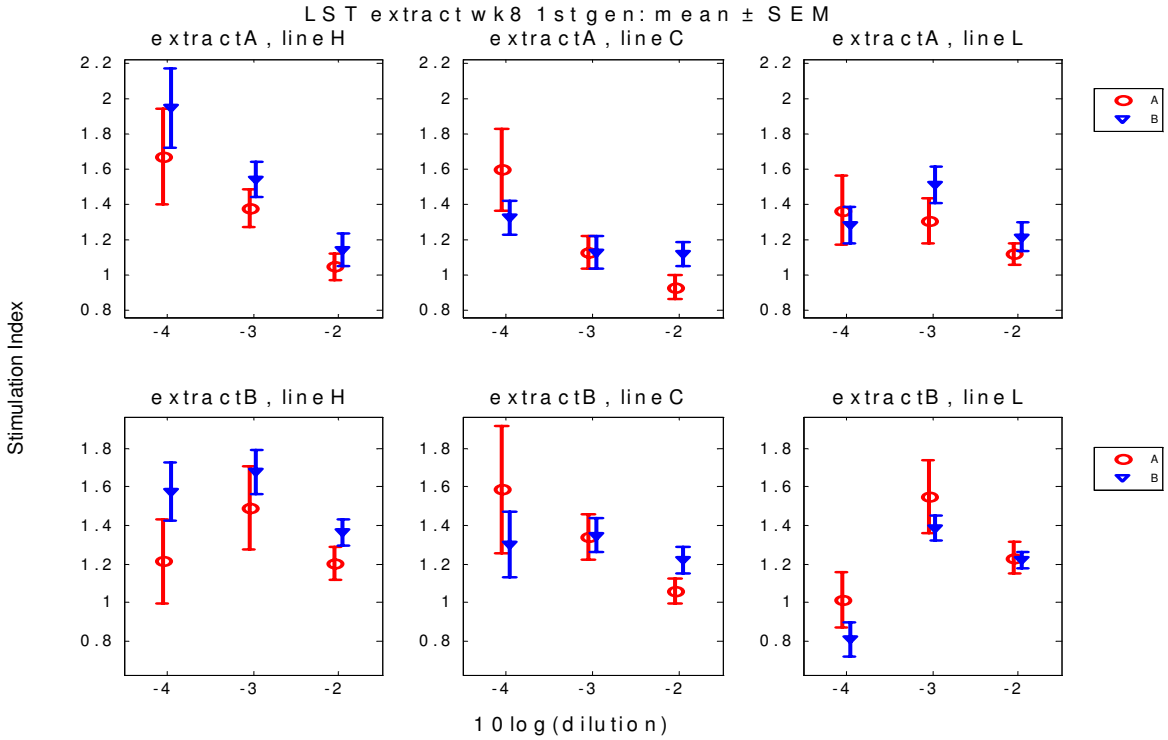


Figure 5.34: 1st Generation. Stimulation index of proliferation in whole blood cell cultures stimulated with Con A in the presence or absence of extracts from Grower feeds A and B. Results present the mean and SEM for the animals per line and feed-group, 11-13 animals per group.

Each chicken line shows a different dose-dependent response to the extracts. In general the response to extract A is similar in the animals fed feed A or B, being the greatest at the highest dilution of the extract. The response decreases in a dose-dependent manner with the lower dilutions and thus higher concentrations of feed extracts. Extract B shows less dose-dependent effects in the groups. The response to extract B observed with the H-line animals is similar to their response to extract A. The C- and L-line groups show stimulation due to extract B, but this is not dose-dependent, the response to all dilutions is comparable. There are no clear differences between the treatments.

5.2.2 Second generation

5.2.2.1 Innate immune system

5.2.2.1.1 Cellular parameters second generation

To analyze the activity of the cellular arm of the innate immune system, monocytes were analyzed for their LPS induced NO production capacity *in vitro*.

When regarding all C- values, regardless of the chicken line and time point, all values are low and close to zero. The variance in the C- animals on feed A at week 1 is large. At week 4 in the L-line animals, a significant difference is seen.

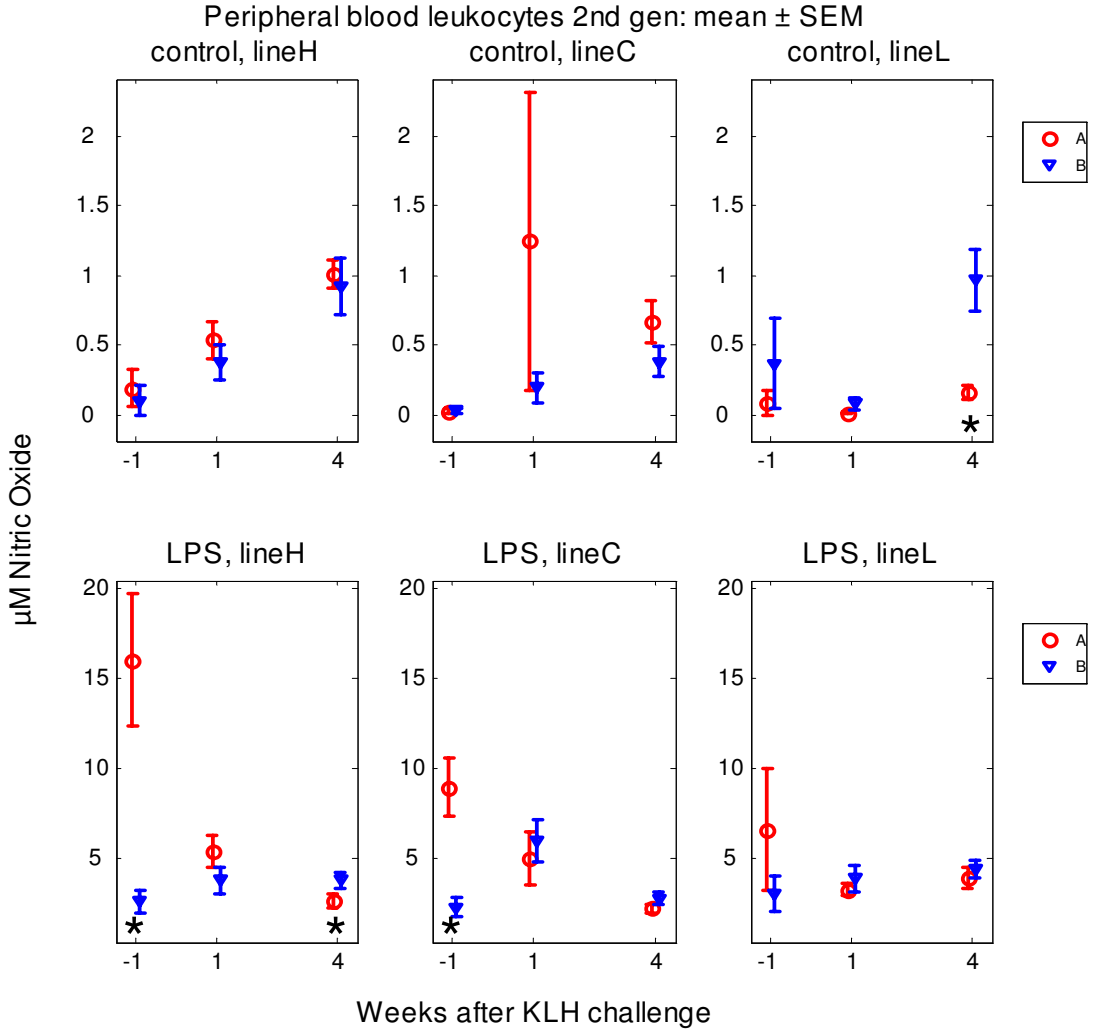


Figure 5.35: 2nd Generation. Control and LPS induced NO production in peripheral blood leukocytes. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

The stimulated values of all lines are relatively low, and most effects are seen at week -1 which was one week after the animals switched from Starter feed to Grower feed.

All animals on feed A show a large rise in the stimulated monocyte values at week -1. The greatest rise is seen in the H-line animals, the least increase in the L-line animals. The differences between A and B are statistically significant for the H- and C- animals. At week 4 a small but statistically significant difference is seen in the H-line animals with the animals on B showing the higher response.

When the stimulated values are corrected for the control values, to correct for individual differences, the observed effects remain the same.

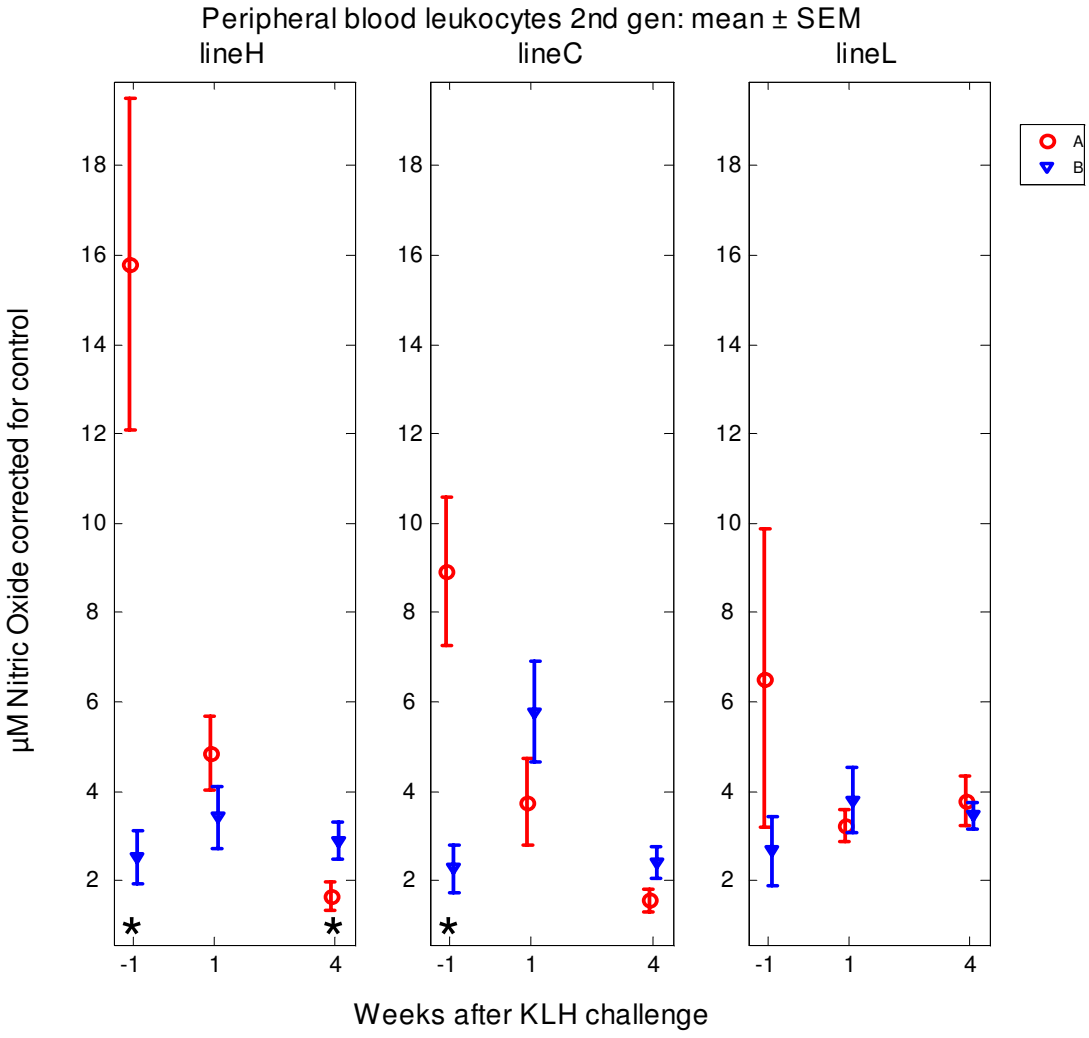


Figure 5.36: 2nd Generation. Control corrected LPS induced NO production in peripheral blood leukocytes. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

Conclusion The feed change has a profound effect on the in vitro monocyte reactivity to LPS in the case of feed A, but only for the H-line and C-line animals this difference is significant.

The KLH challenge has little effect on the monocyte reactivity, let alone when comparing the animals on feed A or feed B.

5.2.2.1.2 Humoral parameters second generation

Plasma levels of natural antibodies (binding to LPS, LTA and KLH) and complement factors, indicative of classical and alternative complement activation, were determined.

Natural antibodies Within the LPS titers, the expected chicken line difference is seen in the week -1 samples, the H-line animals having clearly higher titers than the L-line animals. For all treatments and chicken lines, a strong rise in the titers is seen in the weeks following the KLH challenge, again this increase (6-fold increase in the delta average) is greatest within the H-line animals regardless of treatment and decreases over the C- (delta average of approximately 4.5) and L-line animals (delta average of 4). All H-line animals respond in a similar manner with the highest measured increase in titer at week 1 following with a gradual decrease of the titers in the weeks 2 to 4. When the titers are expressed as delta, weeks 1, 2 and 4 are statistically different, wherein the H-line animals on feed A show the lower response.

The C- group shows a more differential response wherein the C- animals on feed A show the highest response at week 2 and thereafter a gradual decrease. The C- animals on feed B show a response that is still increasing at week 4. The responses are statistically different at week 2 (A>B) and 4 (B>A) when expressed as absolute values.

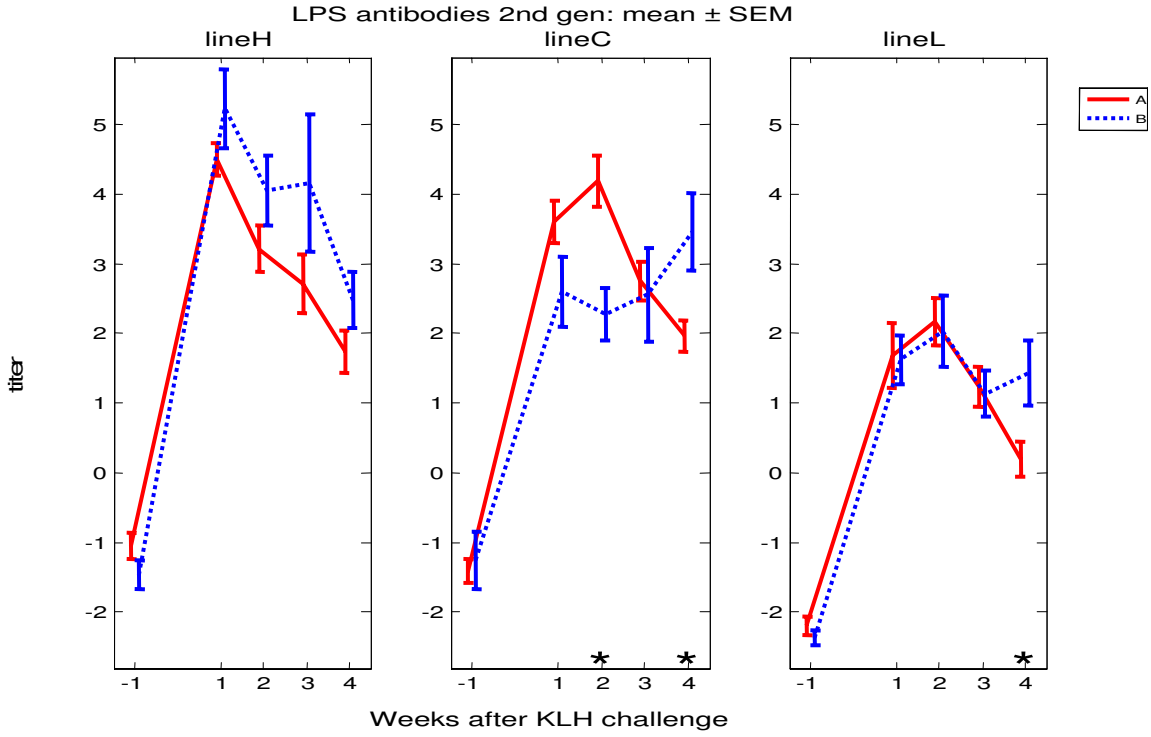


Figure 5.37: 2nd Generation. Levels of LPS binding natural antibodies. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

When expressed as delta, only week 2 is significantly different.

The L-line animals express the least differences in their response, being very similar up to week 4 where the response of the feed A and feed B animals suddenly diverges. The response of the animals on feed B remains at about the same level as before, whereas the response of the animals on feed A further drops, resulting in a significant difference between the groups.

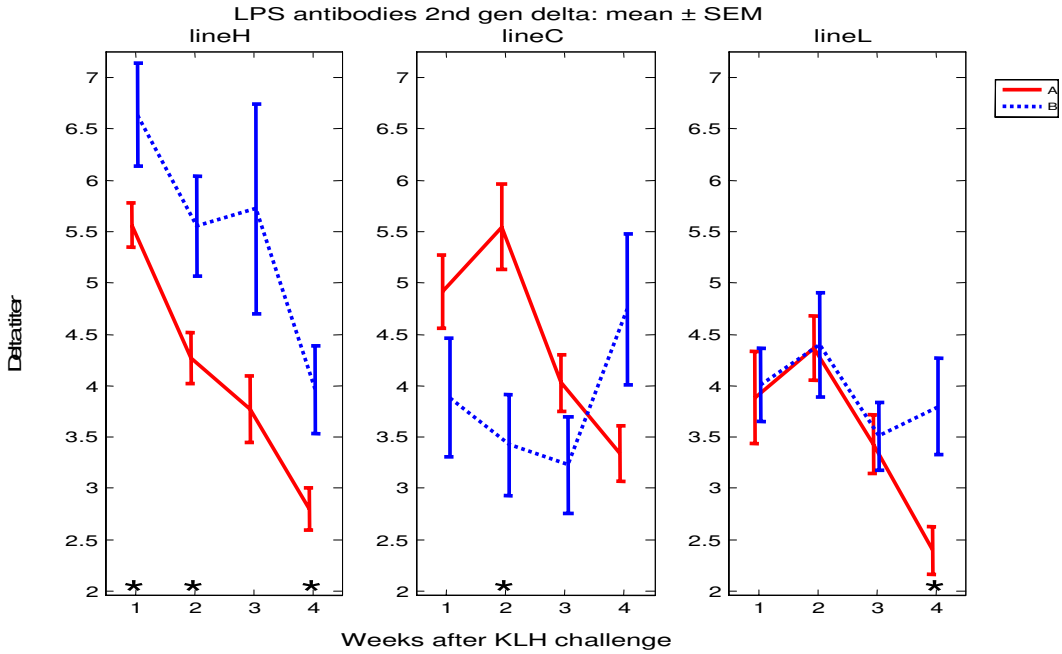


Figure 5.38: Corrected levels of LPS binding natural antibodies. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

When regarding the LTA titers the C- and L-line animals show a significant difference in values at week -1, the week after the feed change. In both cases the animals on feed A show higher titers. There is no discernable difference in the H-line animals at week -1.

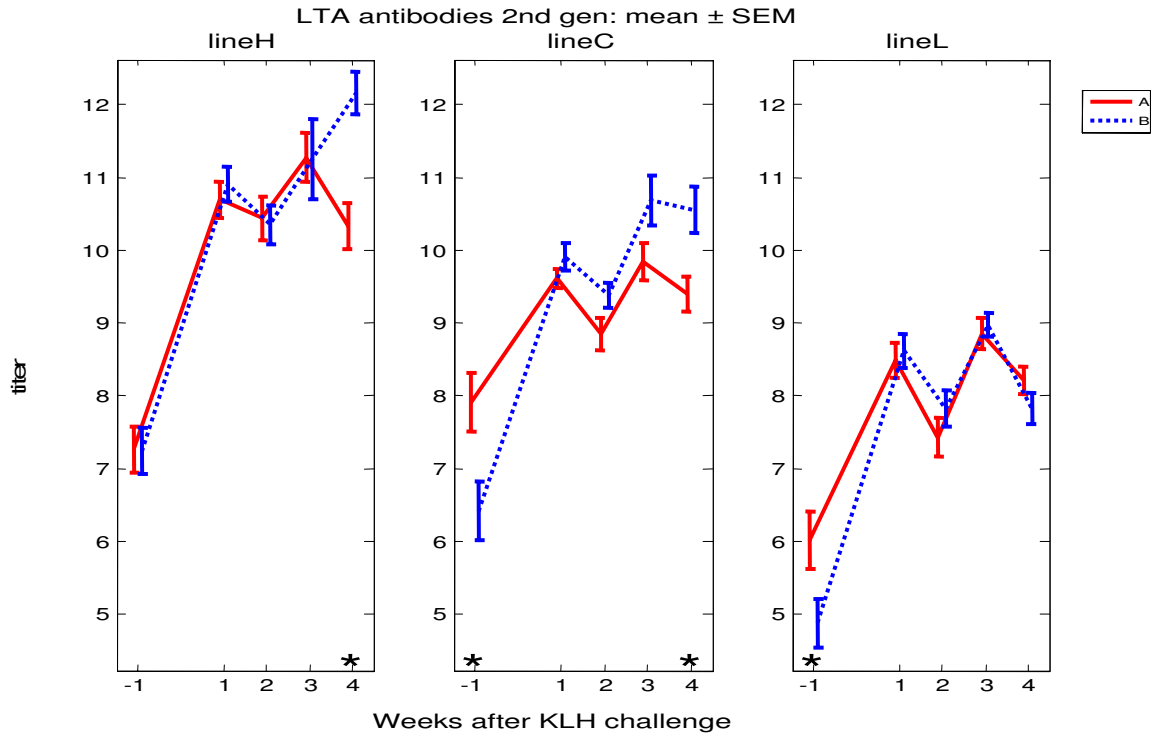


Figure 5.39: 2nd Generation. Levels of LTA binding natural antibodies. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

The H-line animals show a comparable response after the KLH inoculation except for week 4 where the response in the animals on feed A remains at a comparable level, but the response of the animals on feed B rises further. Week 4 is statistically different when the two treatments are compared with each other. Similar is observed in the C-line.

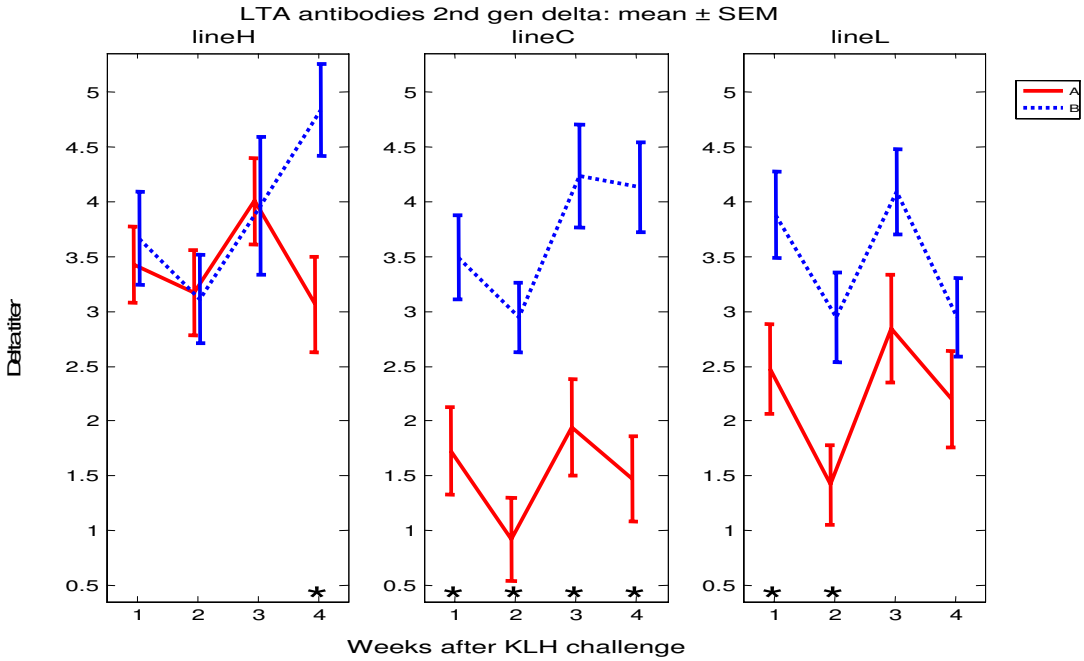


Figure 5.40: 2nd Generation. Corrected levels of LTA binding natural antibodies. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

As the C- and L-line show significant differences at week -1, the delta titers represent the response to the KLH challenge more accurately. The response throughout time is similar in the C- animals as well as the L-line animals and both lines show that the animals on feed B have the relatively higher increase in titers compared to the feed A animals. For the C- animals these differences are significant for all measured time points and for the L-line animals the LTA-antibodies at weeks 1 and 2 are statistically different for feed A and B.

Complement Complement activation through both the alternative pathway and the classical pathway were measured. When regarding the results of the alternative pathway the responses in time are quite similar without distinction between lines or treatments. The H-line animals show the most variance in the weeks after the KLH challenge. At week 4 the response of the H-line animals on feed A seems to rise again and is statistically higher than the response of the H-line animals on feed B.

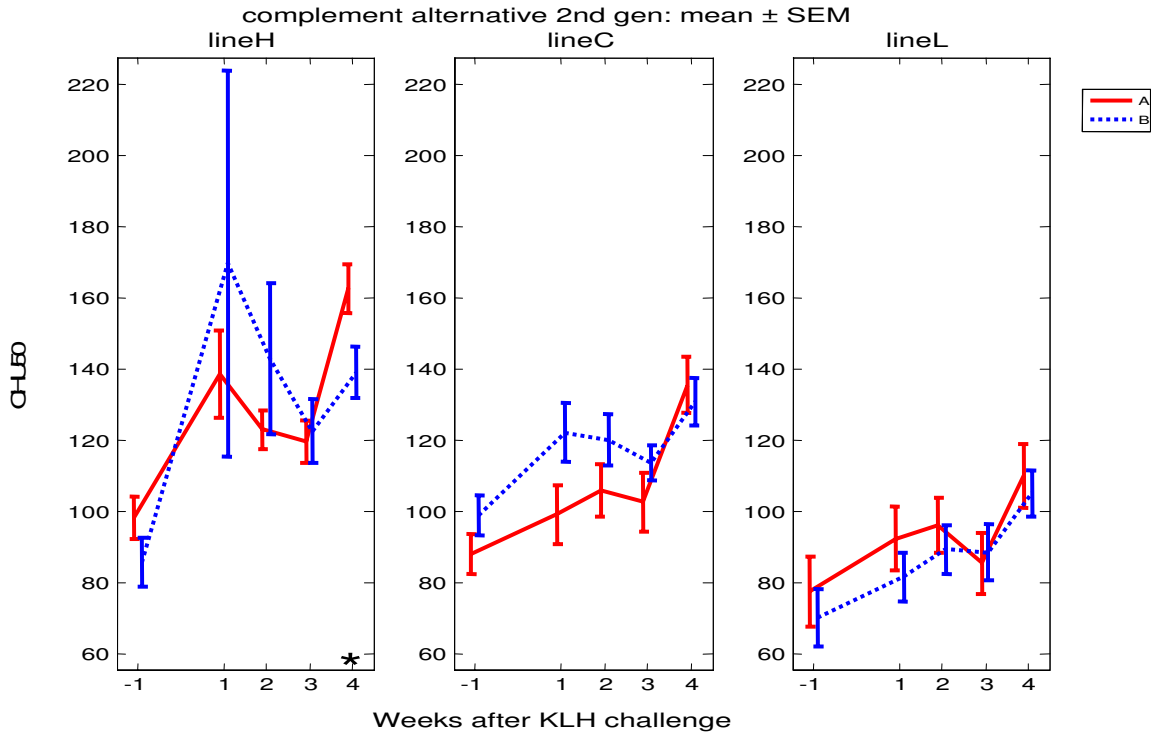


Figure 5.41: 2nd Generation. CH50 activity of serum after alternative complement activation, Y-axis: CHU 50. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

The C- and L-line animals show similar responses in time, albeit the responses of the C-line animals are a bit higher when compared with the L-line animals. The C- animals on feed B show a trend of slightly higher complement activity than the animals on feed A. Within the L-line animals little difference is seen when regarding treatment or response in time.

The results of the classical pathway are very uniform at week -1, the week after the feed change. All complement activity is the same regardless of line or treatment. The response to the KLH challenge is greatest at week 1 after the inoculation for all animals on feed A regardless of the chicken line. The response is highest at week 1 in the H-line animals and is lower for respectively the C- and L-line animals on feed A. The animals on feed B also show an increase at week 1, but this is significantly lower than the response of the animals on feed A. After week 1 the responses of all lines and treatments are again similar, although the L-line animals show slightly lower activity than those of the H- and C-lines.

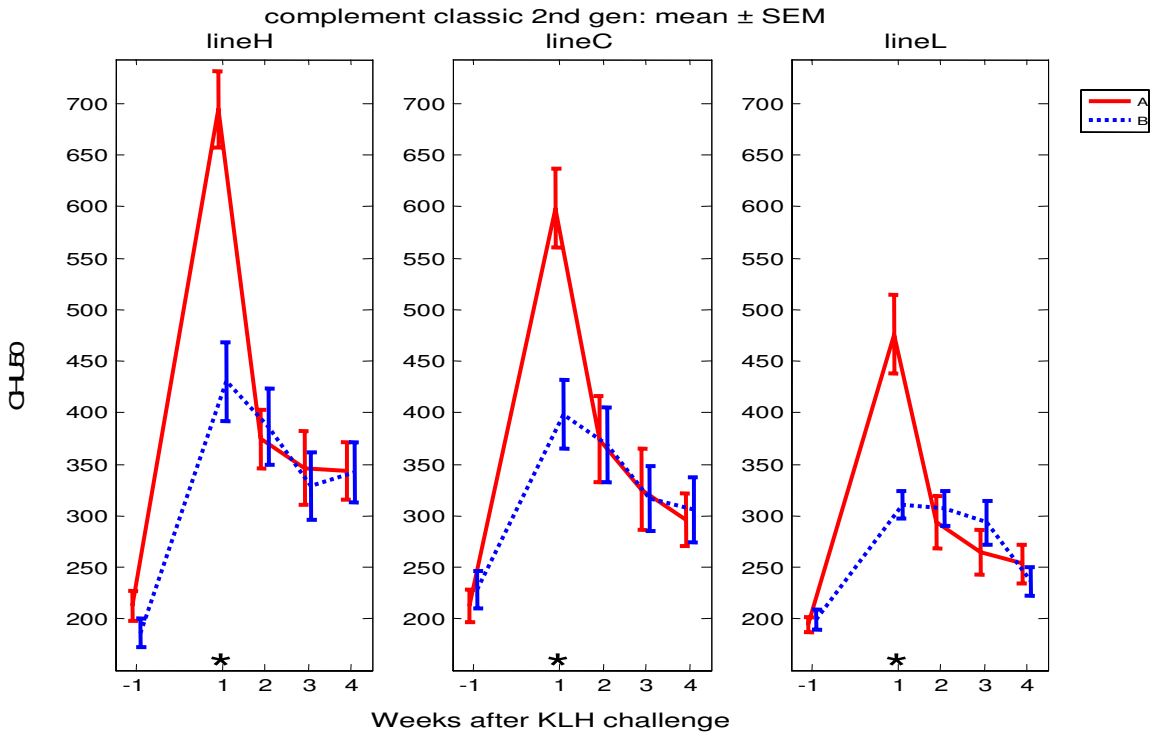


Figure 5.42: 2nd Generation. CH50 activity of serum after classical complement activation. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

Conclusion Overall there are little to no differences in these humoral parameters of innate immunity at one week after the feed change from Starter to Grower feed. Only the LTA titers in the C- and L-line animals on feed A are higher at week -1.

Within lines the measured innate humoral responses to the KLH challenge are similar and the C-line shows the most divergence due to treatment. The LPS responses after the challenge were different in each chicken line as was the effect of the treatment. The greatest differences were seen in the H-line and C-line animals with animals on feed B showing the higher response in line H and those on feed A in line C. At 4 weeks, levels in feed B animals were higher in all lines. The pattern of the LTA responses was similar in time, with the C- and L-line animals on feed B showing a higher increase in antibody titers. Classical complement activity was affected by treatment at week 1 after inoculation. This effect is independent of the genetic line of chickens and was clearly higher on feed A.

5.2.2.2 Adaptive immune system

5.2.2.2.1 Cellular parameters second generation

Lymphocyte proliferation in vitro was analyzed to determine effects on cellular parameters of adaptive immune responsiveness. The control values are all approximately 1000 counts per minute which is relatively high for controls, but is in line with the floor housing (relatively 'dirty' housing conditions).

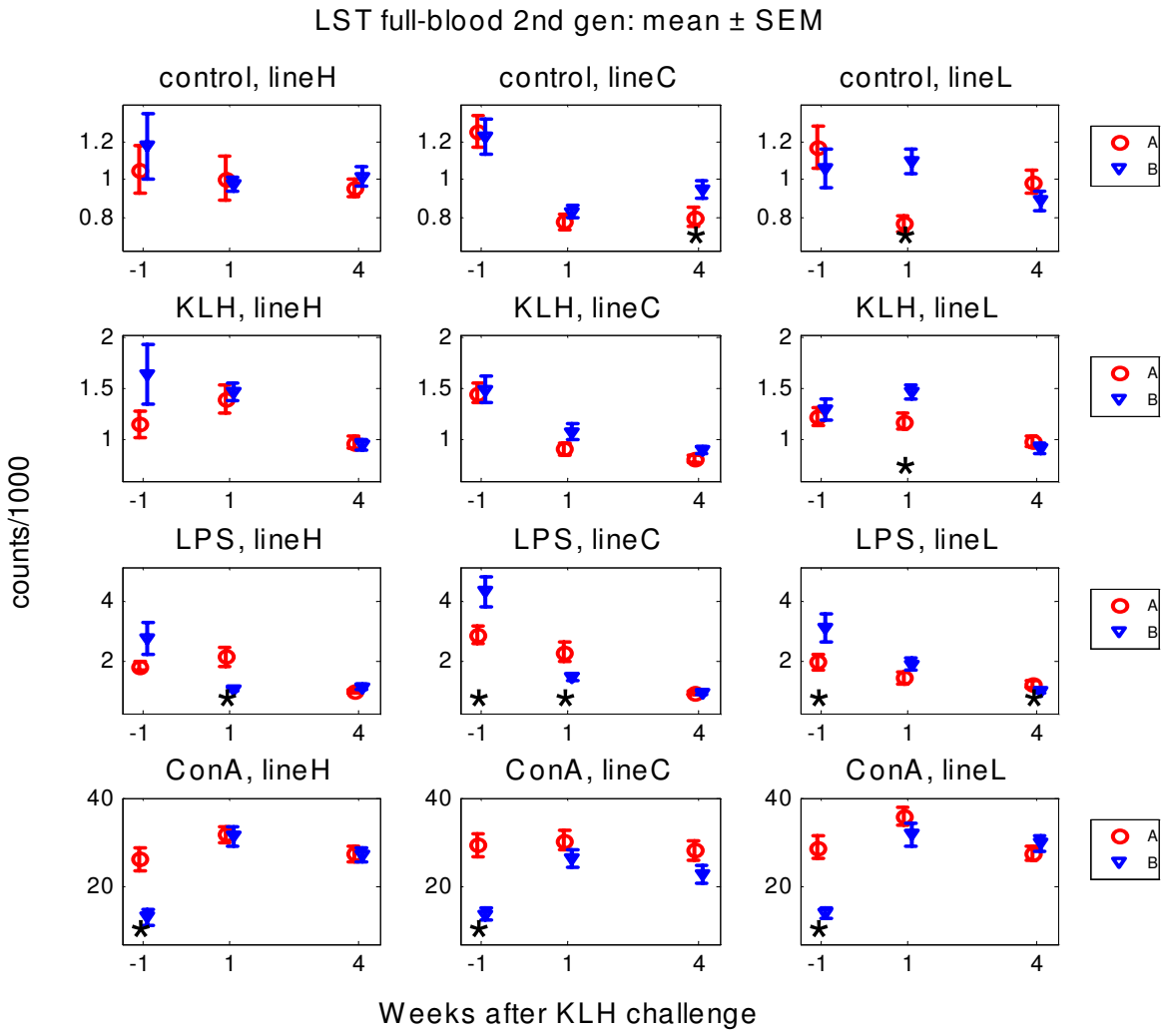


Figure 5.43: 2nd Generation. Proliferation of peripheral blood leukocytes in the lymphocyte stimulation test. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

The control values of the C-line differ significantly at week 4, the feed B animals showing higher control values. The control values of the L-line animals differ statistically at week 1, again with the feed B animals showing the higher values.

When looking at the effects of various stimulants, the results of both data presentations are similar, but somewhat more pronounced when presented as SI (Stimulation Index, being the ratio of stimulated cell counts and control counts). The week after the feed change from Starter to Grower feed, week -1, the H- and L-line animals show a difference in the antigen specific response to KLH, although at this time point the animals were KLH naïve. For both lines the animals on feed B showed a higher proliferative response. After the KLH inoculation there is no more discernable difference in the antigen specific proliferative response to KLH.

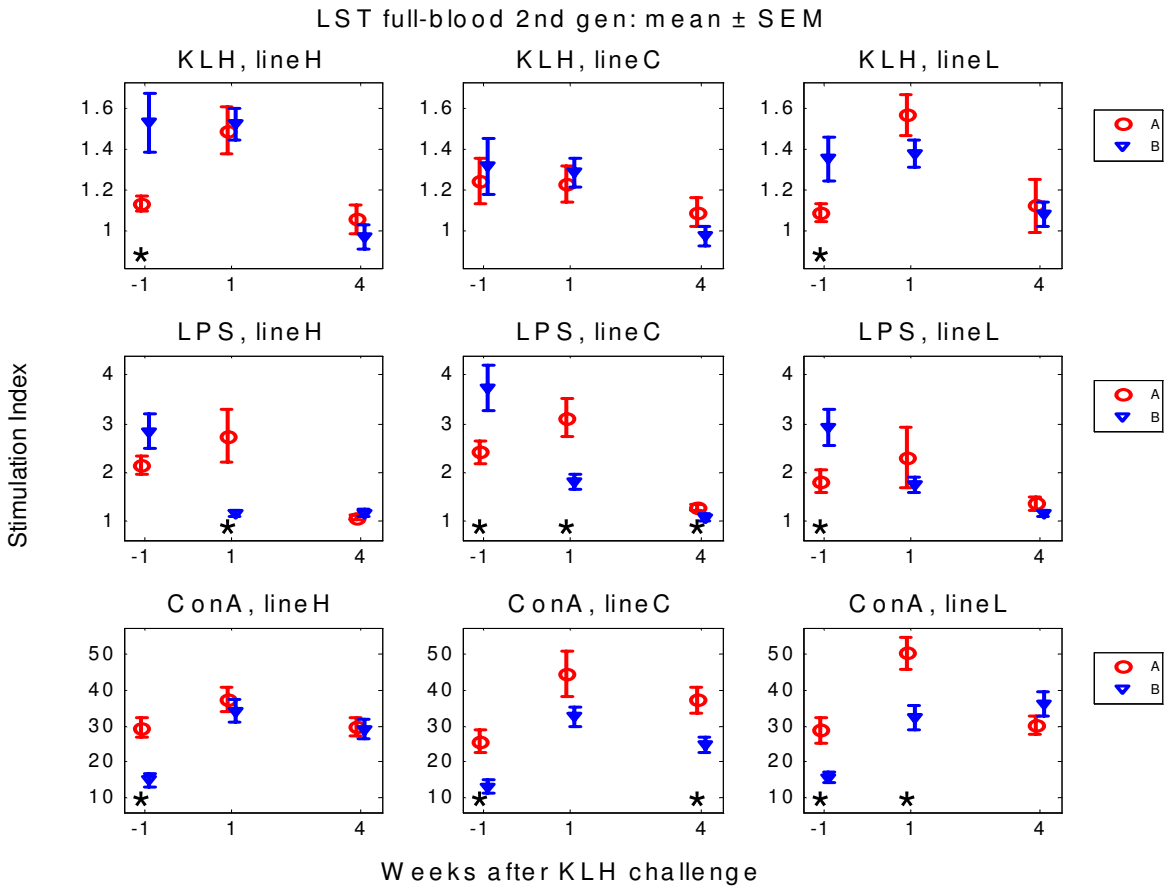


Figure 5.44: 2nd Generation. Stimulation index of proliferation in stimulated peripheral blood leukocytes cultures. The stimulation index is the ratio of the stimulated cell count and the controls. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

When regarding the LPS induced proliferative responses, the measured effects are independent of the presentation chosen. At week -1 all lines show a higher SI of the animals on feed B. This effect is statistically different for the C- and L-line animals. At week 1 the effect is reversed, the feed A animals showing the higher SI the week after the KLH inoculation. Week 4 all responses to the inoculation are the same.

The T-cell response, represented by the Con A stimulation, shows higher responses of the animals on feed A at week -1 and week 1, i.e. after the feed change and after the KLH inoculation. Week -1, after the feed change from Starter to Grower feed, is significant for all animal lines, with the C- and H-line animals on feed A showing a higher response. The measured difference at week 1 is only statistically different in the L-line animals, and week 4 is significantly different in the C- animals (in both cases primarily due to the difference in the control cells).

Conclusion The week after the feed change from Starter to Grower feed, the proliferative responses are diverse and often significantly different between animals on feed A and B. The responses to LPS and KLH at t=-1 are, when different, comparable throughout the genetic lines, with the feed B animals showing the higher responses. The T-cell responses, analyzed by stimulation to the mitogen Con A, show the opposite response where the animals on feed A have a higher response compared to the animals on feed B. Overall, the feed change has a large effect on the measured parameters representing induced proliferation. With feed A, the Con A induced proliferation is largest already before and at early times points after KLH challenge, while LPS induced proliferate responses before KLH challenge show the largest responses in feed B fed chicken. As these effects are apparent in al three chicken lines, the differences are not line-dependent. The KLH challenge, represented by weeks 1 and 4, also influences the specific cellular response. Here the effect for LPS is mainly that the animals fed feed A show a higher proliferation than the animals on feed B. Similar is true for the L-line with Con A stimulation but this is primarily due to the lower response of the controls on feed A.

5.2.2.2.2 Humoral parameters second generation

All chicken received the KLH-challenge and KLH-specific as well as vaccine-specific NCD and Gumboro titers were determined at several time points. The primary antibody level of KLH antibodies is low at week -1 and shows a large increase the week after the KLH inoculation, after which it slowly decreases in the following weeks. There are no significant differences related to the type of feed.

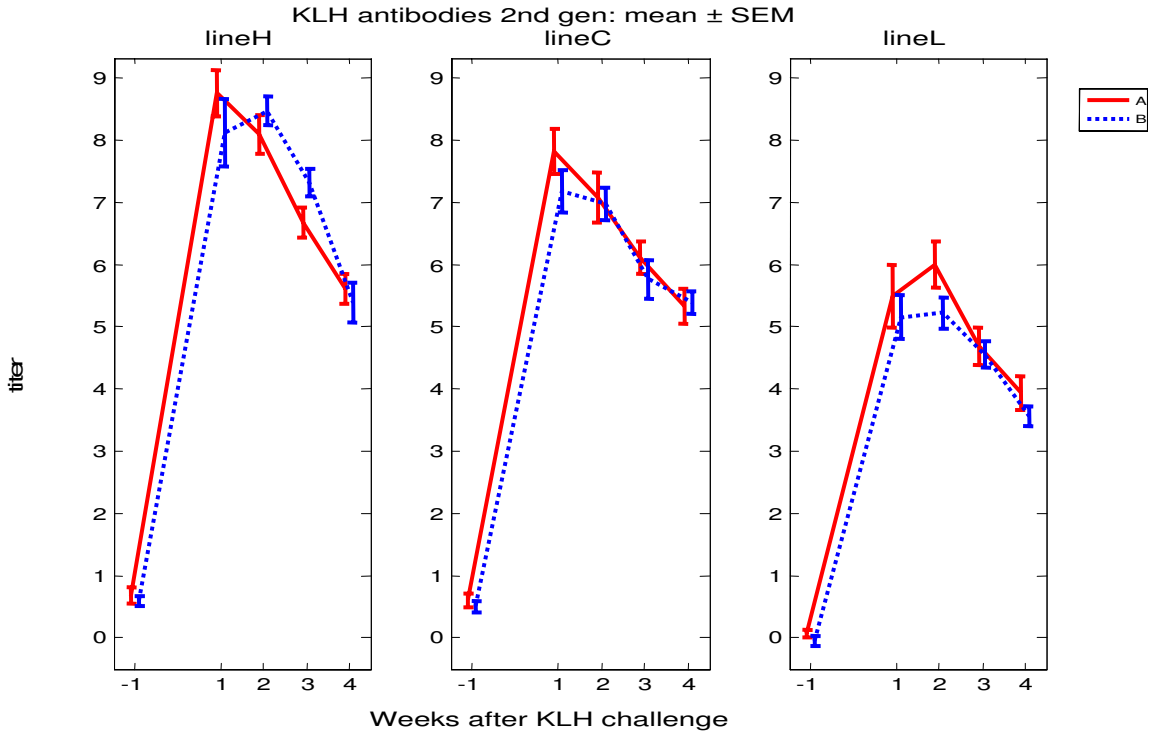


Figure 5.45: 2nd Generation. KLH-specific antibody titers in serum. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

When considering the responses expressed as the net titers corrected for the titers at week –1 before the KLH challenge (delta), again all the animals follow a similar curve. Only the high genetic line animals show a significant difference at week 3, where animals on feed B express higher titers than the animals on feed A.

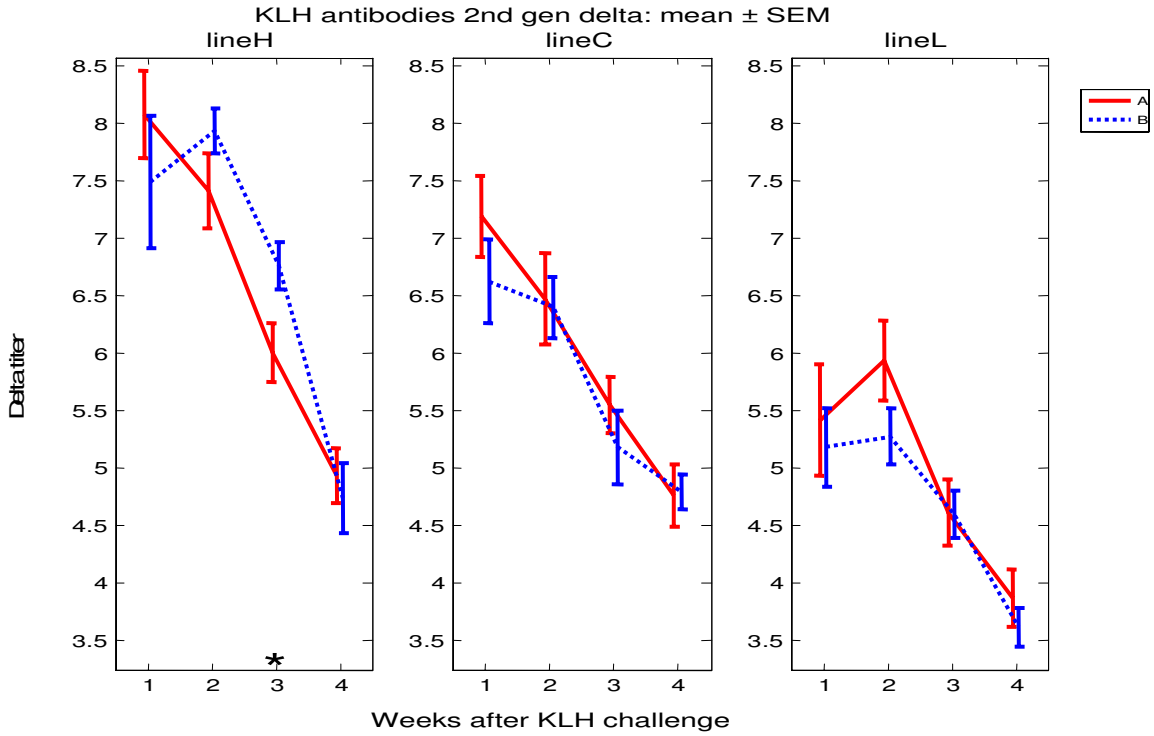


Figure 5.46: 2nd Generation. Corrected KLH-specific antibody titers in serum. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

At week -1 there are no differences in the measured NCD titers. In general, the responses to the KLH inoculation are the same, only the lines differ in the height of the response, in which the H-line animals show the most pronounced increase and this declines respectively with the C-line and the L-line. The response of the animals on feed A shows a peak in the response at week 2 (H- and L-line) or 3 (C-line), suggesting a stronger regulatory response back to normal in the feed A group than in the feed B group.

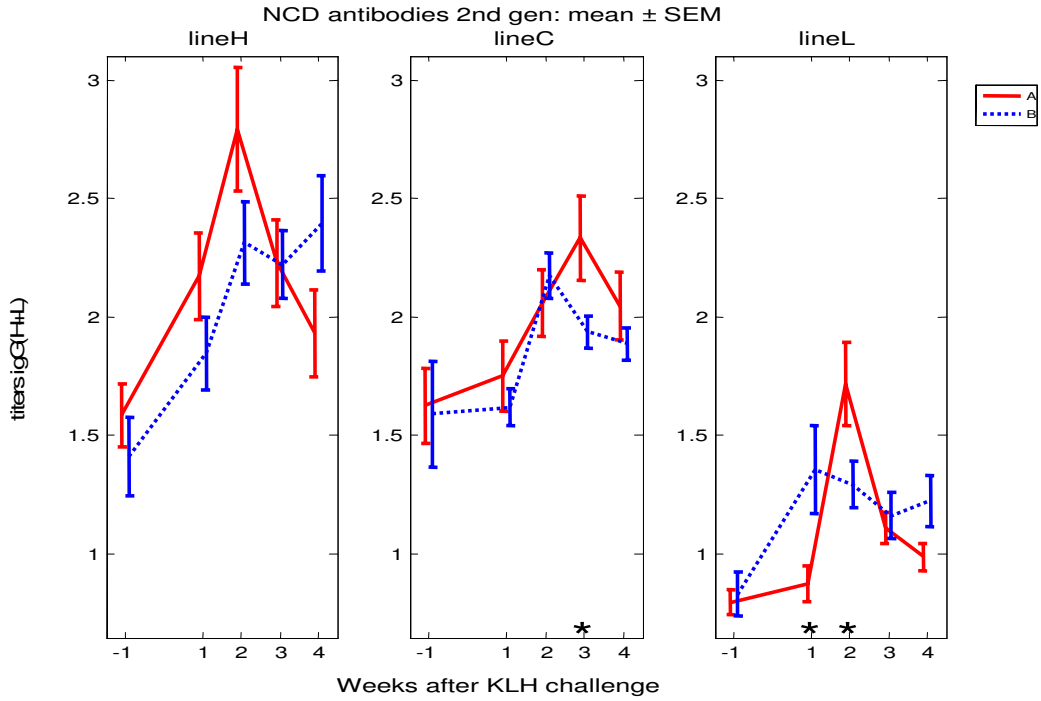


Figure 5.47: 2nd Generation. NCD-specific antibody titers in serum. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

The responses of the animals on feed B show more variation in the pattern of the response, than those of the animals on feed A. Around the peak of the response in the feed A animals, some titers are statistically different when compared to those of the feed B animals. The L-line shows the most divergence in responses due to the treatment. Overall the net response due to the challenge is low (see the net titers corrected for the titers at week -1 before the KLH-challenge (Delta), response is around the 0,5).

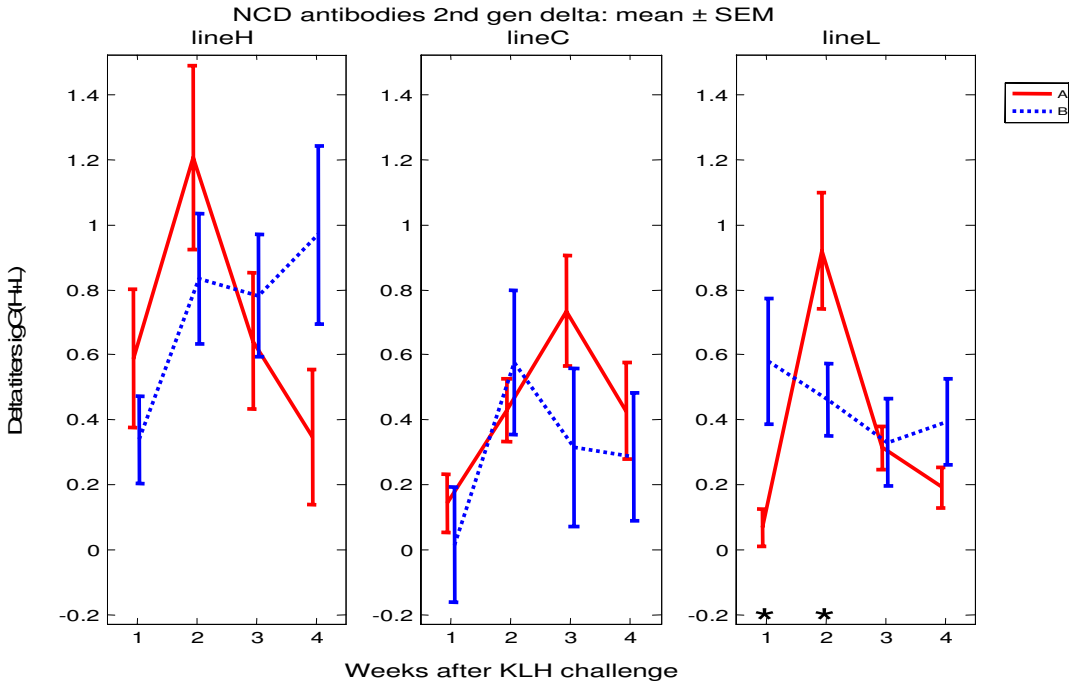


Figure 5.48: 2nd Generation. Corrected NCD-specific antibody titers in serum. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

The Gumboro titers generally show the same response in time, independent of line or treatment. The feed change has no effect on the measurements at week -1. The responses to the KLH challenge are similar in time. The H-line animals show the highest titers and these decrease respectively with the C- and L-line animals.

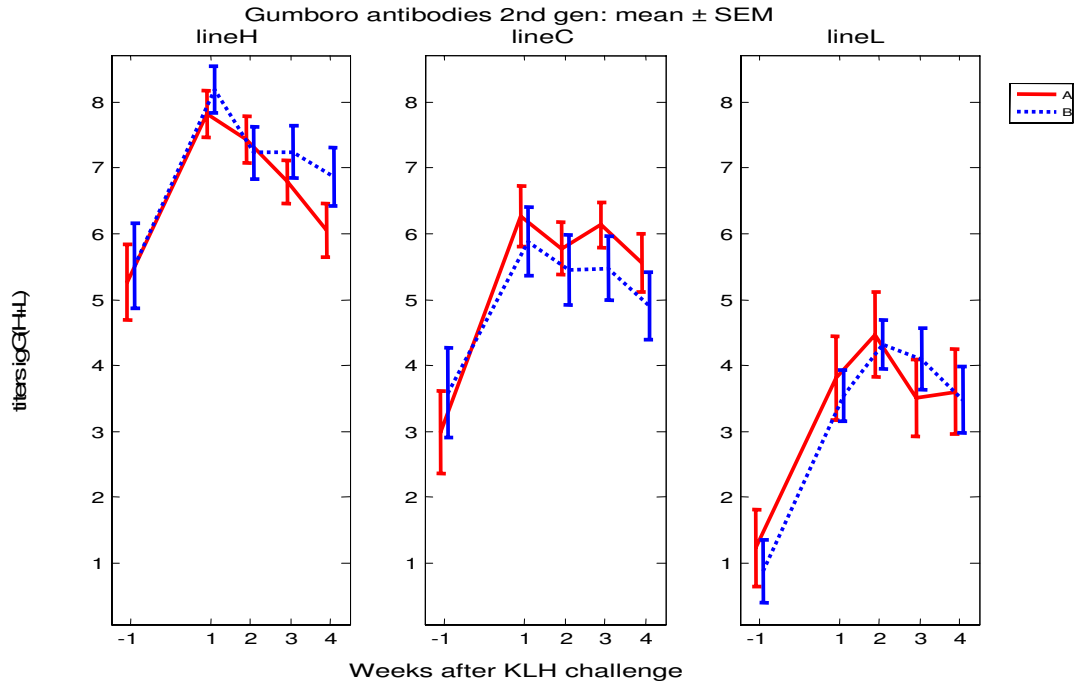


Figure 5.49: 2nd Generation. Gumboro-specific antibody titers in serum. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

When the data are expressed as the net titers corrected for the titers at week –1 before the KLH challenge (delta), therefore showing the average individual response, the C-line shows great divergence due to the treatment. The C-line animals on feed A show a higher increase in the titers after the challenge than the C-line animals on feed B.

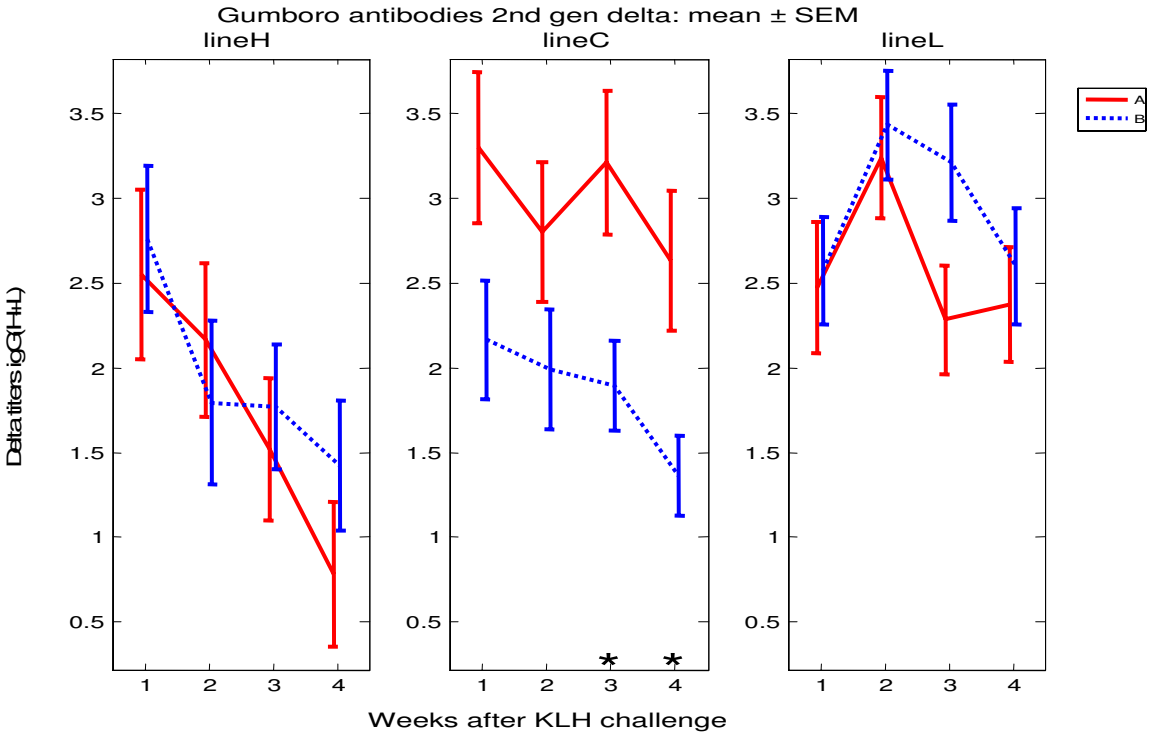


Figure 5.50: 2nd Generation. Corrected Gumboro-specific antibody titers in serum. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

Conclusion Overall the effects seen after the feed change and the KLH challenge on the chosen specific humoral parameters are small. All the presented data confirm the genetic line differences, in which the H-line animals always have the highest titers, and the L-line animals the lowest titers.

No differences were seen in the primary antibody response as measured by the KLH titers. The secondary responses, as measured by the NCD and Gumboro titers, show little divergence as well. Only when the Gumboro titers are expressed as delta titer, the C-line animals on feed A show a higher response.

5.2.2.3 *Tests with feed extracts second generation*

Lymphocyte proliferation was tested in vitro to determine specific reactivity to feed extracts. The proliferative response of T-cells to Con A and the feed extracts was tested and compared to the response to Con A only. When the extract of feed A was added, in general the proliferative response to Con A by T-cells was enhanced in animals that were fed feed A. This effect is only seen in the H- and C-line animals with significant differences between feed A and B animals. With the L-line animals only a significant difference was seen with a less diluted feed extract. Similar was observed for the feed B extract in line H (A>B).

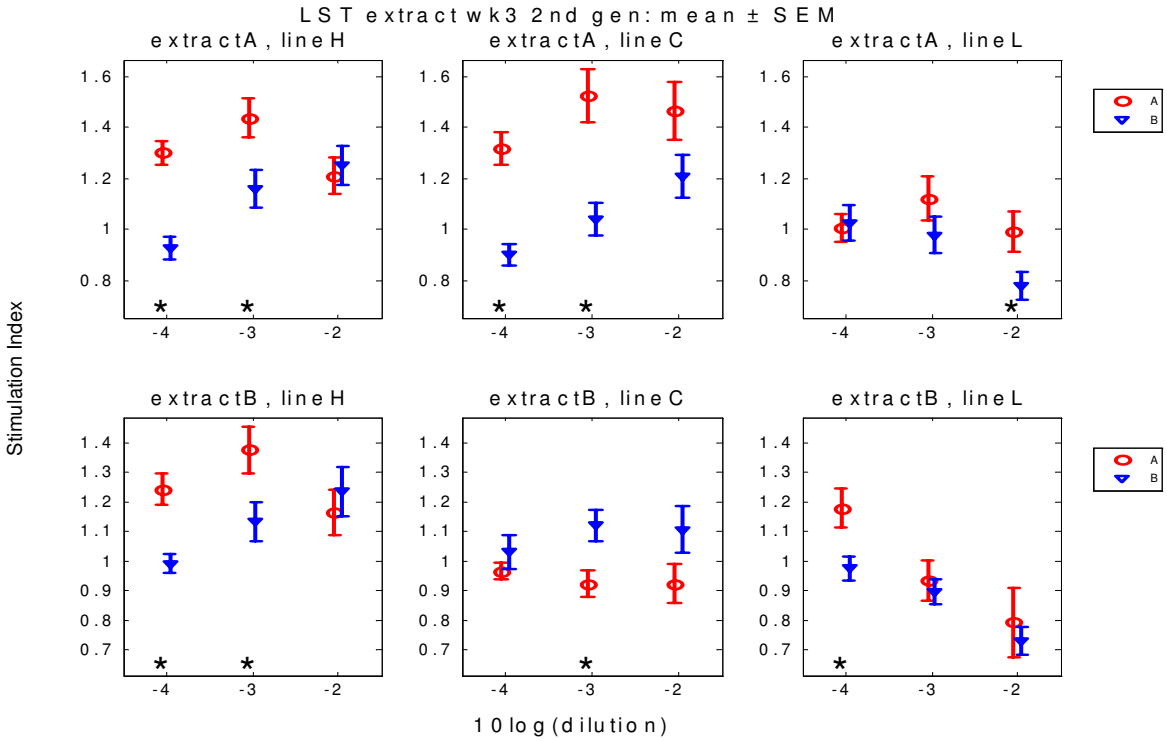


Figure 5.51: 2nd Generation. Stimulation index of proliferation in peripheral blood leukocytes cultures stimulated with extracts from Grower feeds A and B. The final dilution of Con A is 2 µg/m. Results present the mean and SEM for the animals per line and feed-group, 22-26 animals per group.

Conclusion The animals on feed A show higher responsiveness to the feed extracts than the animals on feed B. Overall higher responsiveness of feed A animals refers to the ability to trigger the cellular compartments in vitro.

5.3 Metabolomics

The various plasma and liver samples were analyzed by the different analytical methods:

OS-GC-MS: metabolites detected are amino acids, mono- and di-saccharides, organic acids, amines, alcohols etc.

Lipid LC-MS: metabolites detected are different classes of lipids like triglycerides, cholesterol esters, phosphatidylcholines, lyso-phosphatidylcholines and sphingomyelins.

FFA/bile acid LC-MS: metabolites detected are fatty acids and bile acids.

Global LC-MS: metabolites detected are amino acids, small peptides and organic acids.

Figures 1 to 10 in Annex 9 shows examples of chromatograms obtained of chicken plasma and liver with the different analytical methods. As can be seen from these figures, the four methods together cover a wide range of different classes of metabolites present in chicken plasma and liver.

After analysis the analytical data obtained were further processed as described in the Materials and method section. This resulted in Excel-sheets with relative peak areas of metabolites for all samples analyzed. An example is shown here in Annex 9, Figure 9.11 for lipid LC-MS data. In rows the samples are listed, both study samples and QC samples, while in columns the relative peak areas are present. At the end of the column some statistical values are mentioned (in yellow). Especially the relative standard deviation (RSD) of a metabolite in the QC samples is a good indication of the analytical performance of the method for the specific metabolite. Typically the RSD is below 10%. These Excel-sheets were the input for statistical data-analysis in order to find metabolites that separate between group A and group B chicken.

5.3.1 Plasma metabolomics

Data analysis Two data analysis strategies were applied to the metabolomics data of plasma samples of the chicken. The analyses were always carried out per analytical platform.

First, a univariate discriminant analysis was performed on all data of the control line animals to investigate the differences between the two feed treatments in-depth. This statistical analysis of metabolites in plasma of the control liners resulted in a number of ranked lists of metabolites discriminating between the two feed groups A and B. For plasma samples an analysis based on three sample points was performed for each analytical platform. In total, this discriminant analysis resulted in 4 ranked lists in which each metabolite is ranked according to its discriminating power (fraction of samples correctly classified for treatment). Generally, metabolites with a discriminating power above 0.7 were regarded as discriminative between treatment A and B.

Metabolites with the highest discriminating power in these ranked lists were evaluated for:

a. Known chemical identity
b. Known biochemical or physiological relevance.
c. Association with known specific biochemical pathways or processes.

Second, a multivariate technique was applied on the combined data of all chicken-lines (L, C and H) to investigate whether there were systematic differences between animals from different lines (L, C and H) as well as between animals receiving different feeds. The aim of this analysis was to identify variations in the metabolic phenotype between the different outbread strains of chicken and identify metabolomics platforms that showed a large sensitivity

for the different feed treatments. Using multivariate statistics (PLS-DA) described in section 4.9.2 the data of individual chicken in the whole experiment was combined and analysed. Subsequently, either H and L lines or the treatment groups in the control line (treatment A and B) were used for establishing a regression model and the remaining animals were predicted with the resultant regression. This resulted in two score plots per analytical platform that were used to evaluate the analytical platforms for sensitivity regarding treatment effect and (genetic) animal line effect.

The two data analysis methods were combined as shown in the scheme below. Thereby, the rankings of the individual metabolites were combined with the evaluation of the specific metabolomics platform to obtain the most significant metabolites in analytical platforms that were sensitive for the food treatment. The resulting metabolites were evaluated for their biological activity and for their role as biomarkers.

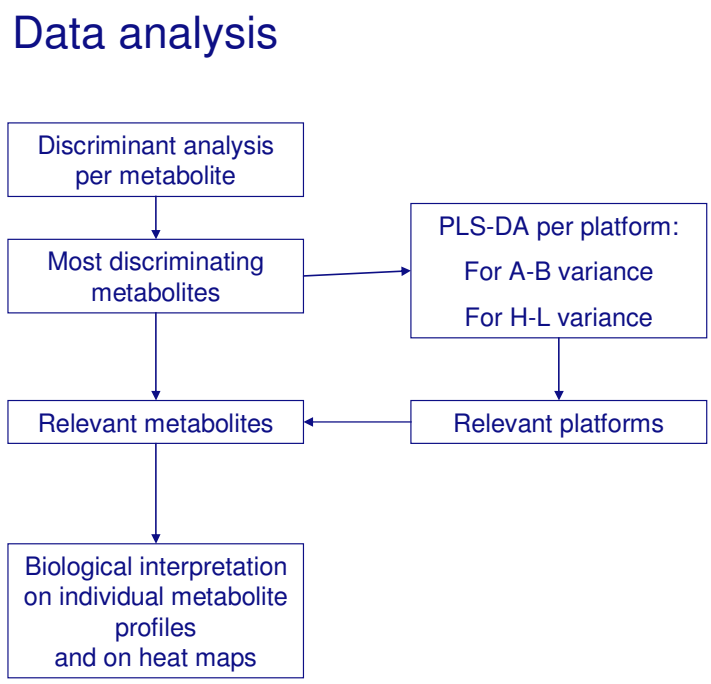


Figure 5.52: Metabolomics data analysis scheme.

Due to the diversity of the metabolomics platforms, this evaluation could not be automated but was carried out by hand.

Lipid platform The multivariate analysis (Figure 5.53) of the metabolite patterns obtained with the lipid platform show that the differences between H-, C- and L- line are modest (left panel). The principal component (RC1) explains 18% of the variance and the error ellipses for each group are large in comparison to the distance between the groups. On the other hand, the two treatments, A and B were well separated throughout the three lines (right panel). Using the discriminant analysis on the data of the C- group many metabolites were found that provided a high degree of correct classification. Notably seven lysophosphatidylcholines (LPC) were identified as highly discriminating metabolites in plasma between treatments A and B (Figure 5.54). The LPC with the largest differences between treatment group A and B were LPC18:0 and LPC16:0. Their response pattern is comparable in the different

treatment groups (Figure 5.55): LPC in group A has a peak after the challenge, LPC levels in group B decreases after the challenge. The two LPC compounds both contain a saturated fatty acid. LPCs are formed by the removal of one fatty acid from phosphatidylcholines (PCs). In phosphatidylcholines the fatty acids on position sn-1 are mostly saturated acids, whereas the fatty acids in position sn-2 are mostly unsaturated. This might indicate that they were formed by a specific class of phospholipases that catalyzes specifically the removal of unsaturated fatty acids from phosphatidylcholines. An example for such an enzyme is soluble phospholipaseA2 (sPLA2), an enzyme know as a marker protein for the acute phase response. This enzyme is present in many species, also in the chicken. Other compounds that strongly discriminate between the two treatments in the C- group are phosphatidylcholines (PC) and cholesterol esters (ChE) (Figure 5.54). The general pattern of response is identical to the pattern observed for the LPC: Group A has increased levels after the challenge, group B starts off higher but decreases in the measurements after the challenge (data not shown). There is not so much known over the expected response pattern of plasma lipids after an immunological challenge in chicken. Recent reviews of immunological responses of plasma lipids in mice and humans have been consulted to help interpreting the observed response in the chicken plasma. It is well known, that plasma lipids react in combination with an acute phase response (APR). Often, injections of e.g. LPS (a powerful bacterial antigen) are used to elicit the acute phase response. Generally, plasma lipids increase due to a higher and altered production of lipoproteins. Furthermore, the composition of the phospholipids has been found to alter during the APR. In the chicken, most conjugated lipids (PCs, LPCs, ChE, SPM) are circulating as HDL lipoproteins. In other species like human or mouse the HDL composition is known to be associated with immunological status (Ansell 2005, Murch 2007, Navab 2006). The turnover of conjugated lipids in plasma is variable, but can range from hours to weeks. So it is probable that changes of certain slow turnover plasma lipids can be traced after one or three weeks after an APR. However, there is little literature on expected lipid turnover in chicken plasma.

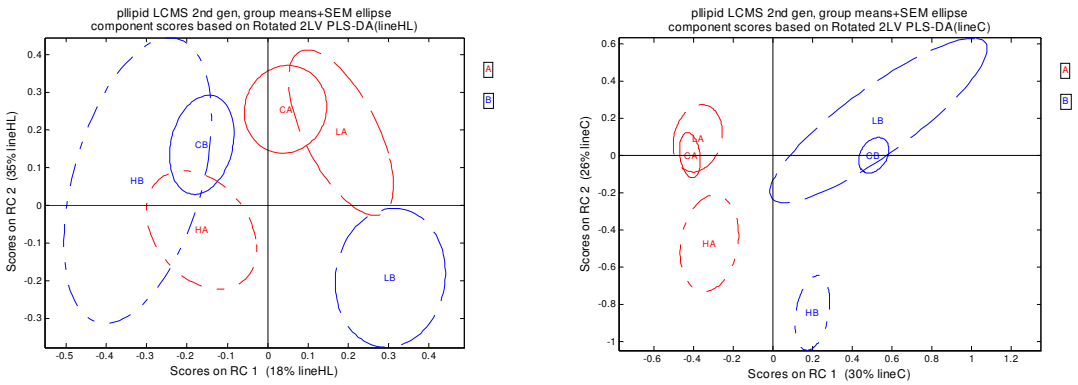


Figure 5.53: PLS-DA of Lipid platform. The left panel shows the rotated PLS-DA that describes the main differences between the high and the low line with RC1 and the main remaining trend with RC2. The C-line is projected in the H-L space. The right panel shows the rotated PLS-DA that describes the main differences between diet A and B with RC1. H and L are projected in the control space.

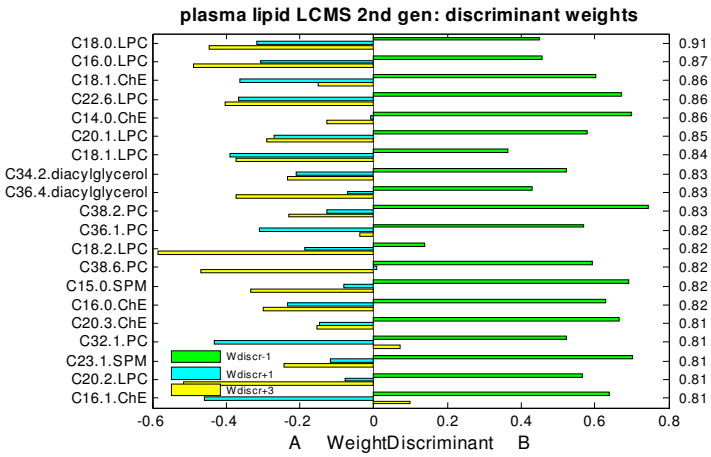


Figure 5.54: Discriminant analysis for treatment of Lipid platform. The green bars show the discriminants on timepoint -1, the blue bars on timepoint +1 and the yellow bars on timepoint +3. On the right hand side, cross-validated rate of correct classification is shown for each metabolite.

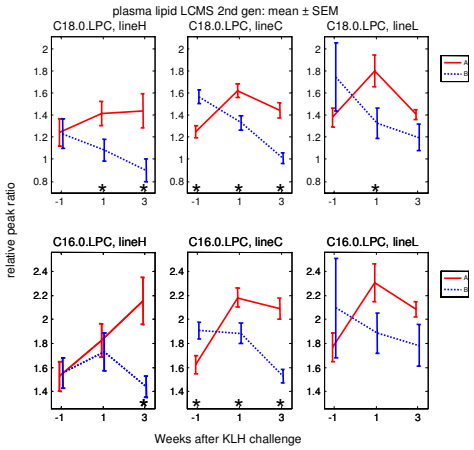


Figure 5.55: Two most discriminating metabolites in the lipid platform.

Bile acids / FFA platform Data from the Bile acid – Free fatty acid platform are shown in Figure 5.56 to 5.58. The PLS-DA analysis of the complete platform data shows, that most variation is associated with the treatment (right panel Figure 5.56) and that the different lines are not separated consistently by this platform. Given the high plasma turnover of the metabolites measured by this platform, it can be considered as a snapshot of fatty acid metabolism and liver function (bile acids). In the weight plot for the discriminant analysis, the fatty acids C22:6, C18:0, C18:1 and C20:3 can be found in the top positions. Only two bile acids, Taucholic acid (TC) and Cholic acid (CA) are found amongst the top 20 discriminating metabolites, having a lower discriminating power than most fatty acids. Using the two plasma bile acids as a marker for liver functionality we can conclude that none of the treatments has dramatically altered the bile acid transport capacity of the liver. The most important fatty acid in this analysis is C22:6, which is the essential omega-3 fatty acid DHA. This compound has been studied in many human and animal experiments and has been shown to have important immuno-modulating functions. This fatty acid is not part of the feed, but has to be synthesized by the body from C18:3 omega-3 fatty acid. In the dietary analysis, the pattern of fatty acids in the two feeds (as percentage of total fat) did not differ statistically, but when the fatty acid composition was expressed as

mg/kg feed, significant differences were detected, notably between the feeds consumed before and during the challenge. When we compare the feed 11/12 (given until 1 week before the first blood sample) with feed 15/16 given during the experiment (week -2 to week 4) we could see that the content of C18:3 increased by 95% for group A and by 26% for group B; content, expressed as mg/kg feed. At timepoint 3, treatments A from all groups (lines L, C and H) have an increased C22:6 concentration, which is higher than in the groups on treatment B. A similar tendency is seen in the FA18:3 metabolite, which includes the precursor of DHA, but can also be observed in the C-line for 18:0, a fatty acid not related to the formation of omega-3 fatty acids. It is likely, that the observed increase in plasma levels of C18:3 and C22:6 in group A during the experiment is primarily caused by an increase in dietary intake of C18:3 after changing to the Grower feed in week -2.

DHA is a bioactive compound which has been implicated in modulating inflammatory responses, among others through interaction with toll like receptors (TLR-4) (Lee 2001). It is not clear, whether the observed extent of the C22:6 fatty acid changes in plasma can have any immunological effects.

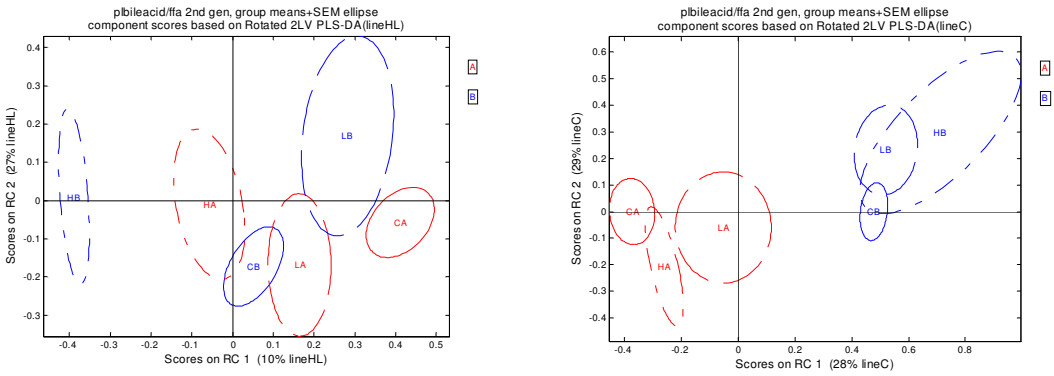


Figure 5.56: PLS-DA of Bile acid / FFA platform. The left panel shows the rotated PLS-DA that describes the main differences between the high and the low line with RC1 and the main remaining trend with RC2. The C-line is projected in the H-L space. The right panel shows the PLS-DA that describes the differences between feed A and B. H and L are projected in the C- space.

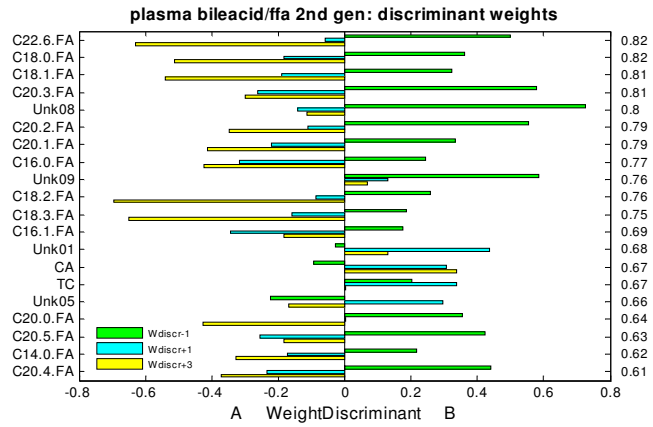


Figure 5.57: Discriminant analysis for treatment of Bile acid / FFA platform. The green bars show the discriminants on timepoint -1, the blue bars on timepoint +1 and the yellow bars on timepoint +3. On the right hand side, cross-validated rate of correct classification is shown for each metabolite.

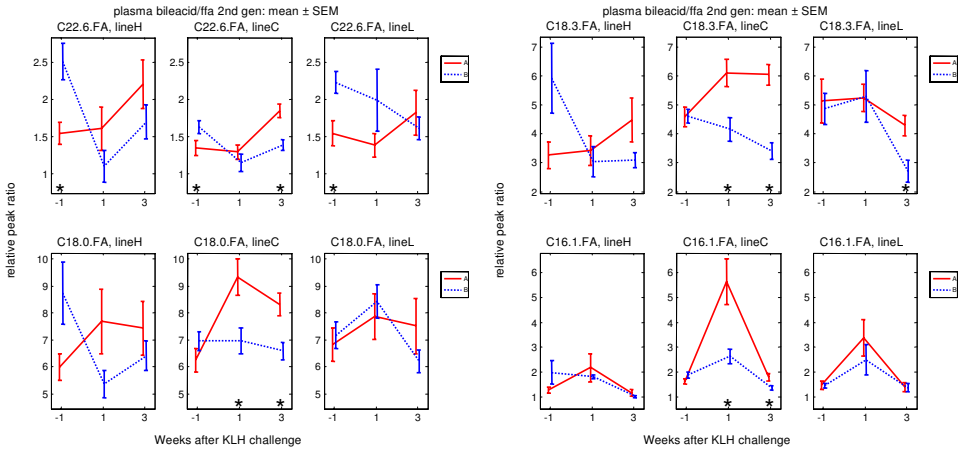


Figure 5.58: Plasma levels of two most discriminating metabolites in the bile acid/FFA platform (left side) and of 18:3, the fatty acid which is a precursor to FA 22:6 (right side).

Global LC-MS platform The LC-MS platform contains many peaks, but hence also several unknown peaks. The PLS-DA analysis reveals again, that there is no clear distinction between the different lines (Figure 5.59 left panel). On the other hand, there is a clear discrimination between treatment group B of the C and L line and treatment group A (Figure 5.59, right panel). Interestingly, the H-line on treatment B clusters together with the chicken on treatment A. The compounds that are most associated with this clustering are shown in the loading plot of the PLS-DA in Figure 5.60. However, the compound most associated with the treatment effect has not been identified yet (Compound identifier: 457.1.240). Besides this unidentified compound, creatine was found to be associated with the difference in treatments across lines.

Only 5 of the top 20 components from the discriminant analysis were known at the moment of biological interpretation. The identified compounds could not be associated with a biological process.

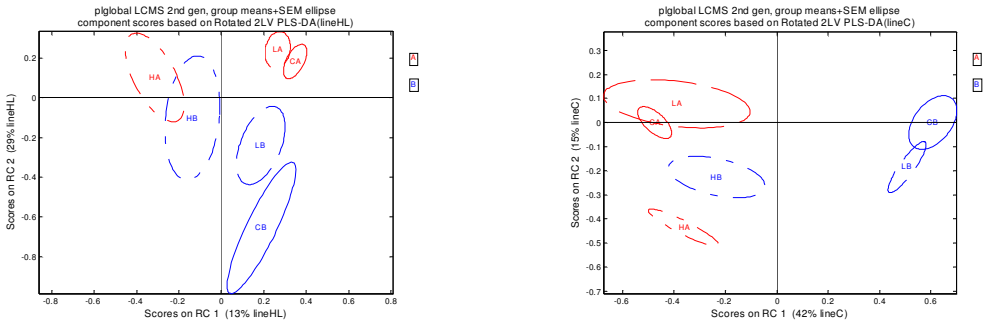


Figure 5.59: PLS-DA of Global LC-MS platform. The left panel shows the rotated PLS-DA that describes the main differences between the high and the low line with RC1 and the main remaining trend with RC2. The C-line is projected in the H-L space. The right panel shows the PLS-DA that describes the differences between feed A and B. H and L are projected in the C- space.

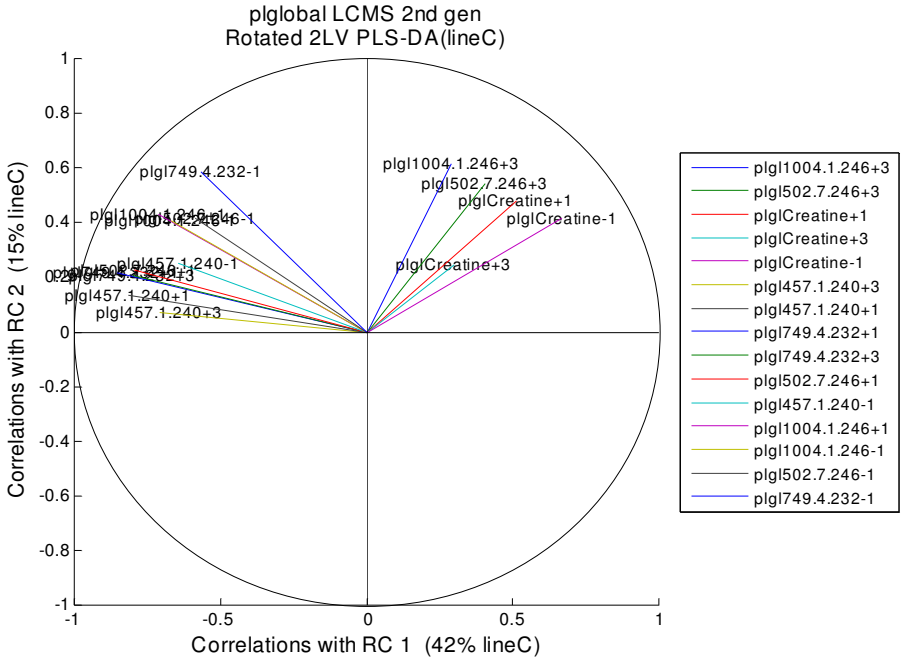


Figure 5.60: Loading of compounds and time points in the plasma global platform, that correlate most with the difference in treatment (PLS-DA score plot on the right hand side of Figure 5.59). The legend is labeled in clockwise order.

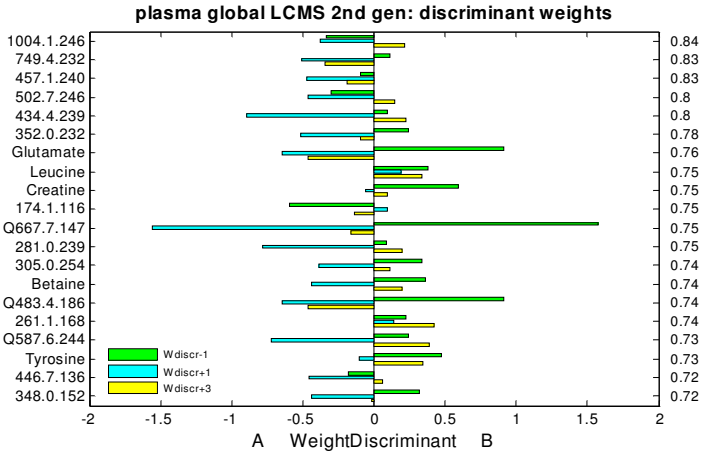


Figure 5.61: Discriminant analysis for treatment of Global LC-MS platform. The green bars show the discriminants on timepoint -1, the blue bars on timepoint +1 and the yellow bars on timepoint +3. On the right hand side, cross-validated rate of correct classification is shown for each metabolite.

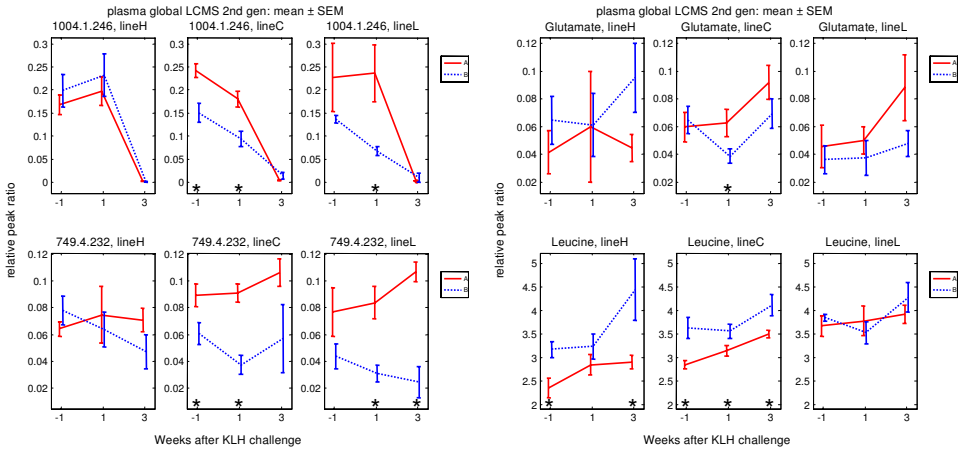


Figure 5.62: Two most discriminating metabolites (left) and two identified amino acids in the LC-MS platform.

GC-MS platform The metabolites from the GC-MS platform allowed a relatively clear separation of the animals according to their genetic background (left panel, Figure 5.63), as well as according to their treatment (right panel, Figure 5.63). Upon closer inspection of the PLS models the metabolites lysine, glycerol and alpha Ketoglutaric acid were most related to line and treatment differences while glycerol, FA 16:1, 18:1 and 18:2 were most related to treatment differences.

The results of the discriminant analysis again reveal glycerol and a number of fatty acids as discriminant compounds. Plots of individual compounds (Figure 5.66 and 5.67) show that glycerol and the monounsaturated fatty acid of group A have a peak at 1 week after the KLH challenge. Glycerol is a metabolite of triglyceride hydrolysis, which along with circulating free fatty acids, is typically increased in the fasting state during increased use of triglycerides. It has been described in the literature that an acute phase response, as eg caused by injection of an immunogenic protein, leads to an increase in triglyceride metabolism (Khovidhunkit 2004), resulting in increased plasma levels of free fatty acids and glycerol.

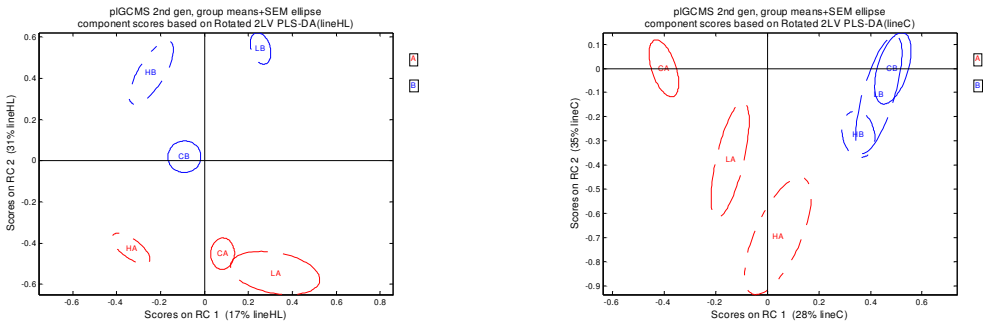


Figure 5.63: PLS-DA of GC-MS platform. The left panel shows the rotated PLS-DA that describes the main differences between the high and the low line with RC1 and the main remaining trend with RC2. The C-line is projected in the H-L space. The right panel shows the PLS-DA that describes the differences between feed A and B. H and L are projected in the C-space.

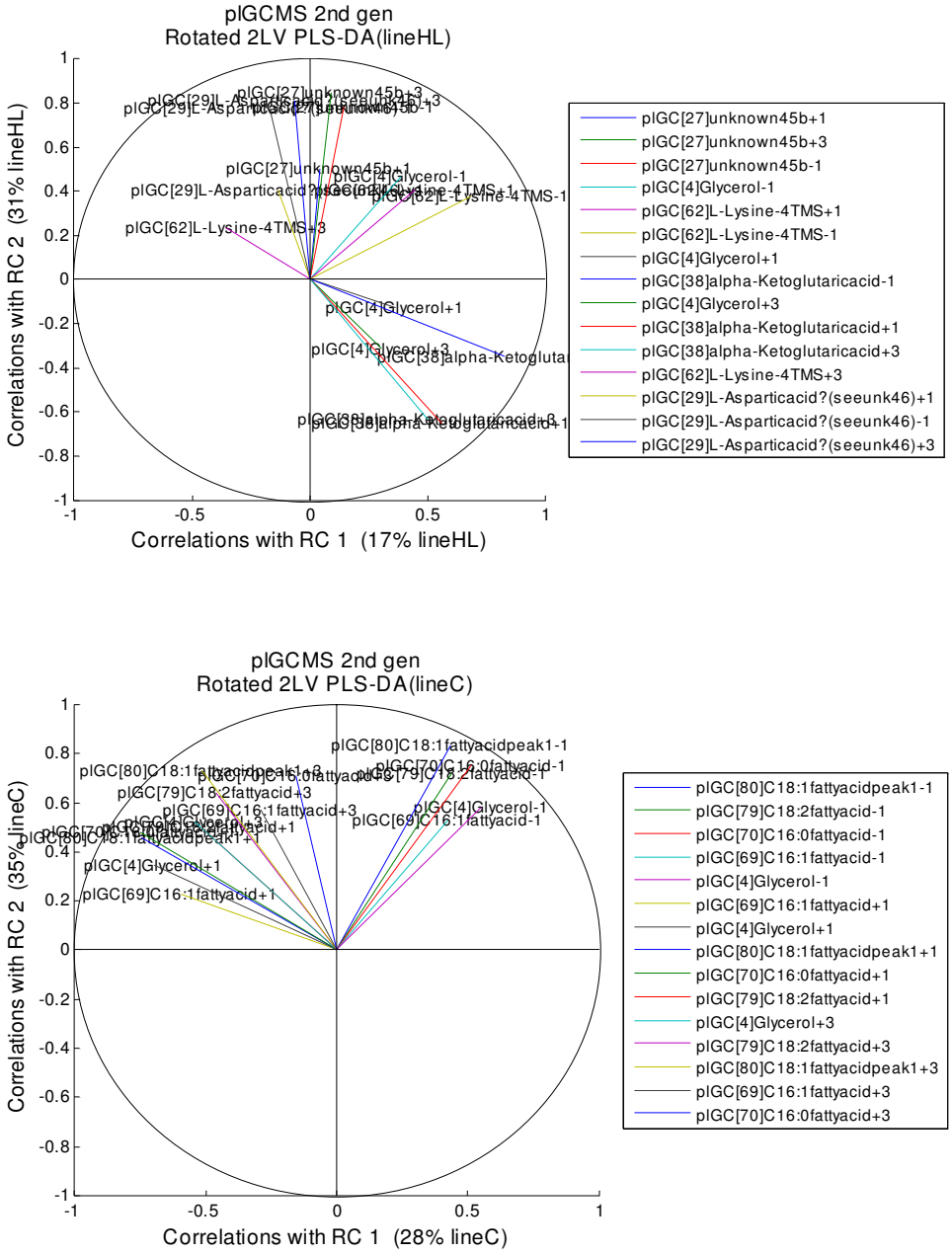


Figure 5.64: Loading plots of PLS-DA regressions for line (upper) and treatment (lower) effect. The legend is labeled in clockwise order.

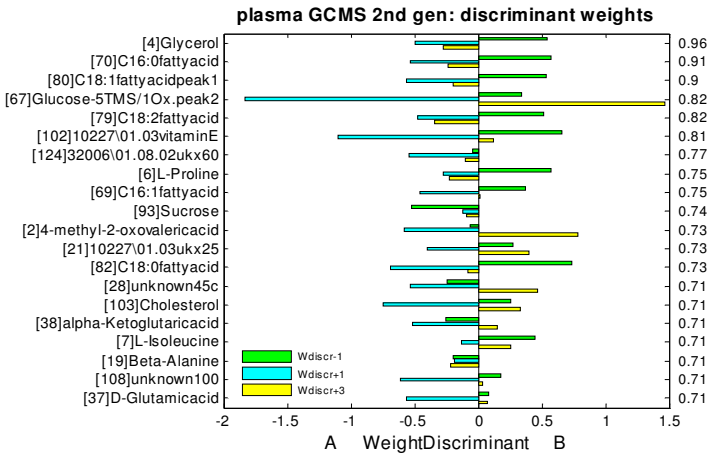


Figure 5.65: Discriminant analysis for treatment of GC-MS platform. The green bars show the discriminants on timepoint -1, the blue bars on timepoint +1 and the yellow bars on timepoint +3. On the right hand side, cross-validated rate of correct classification is shown for each metabolite.

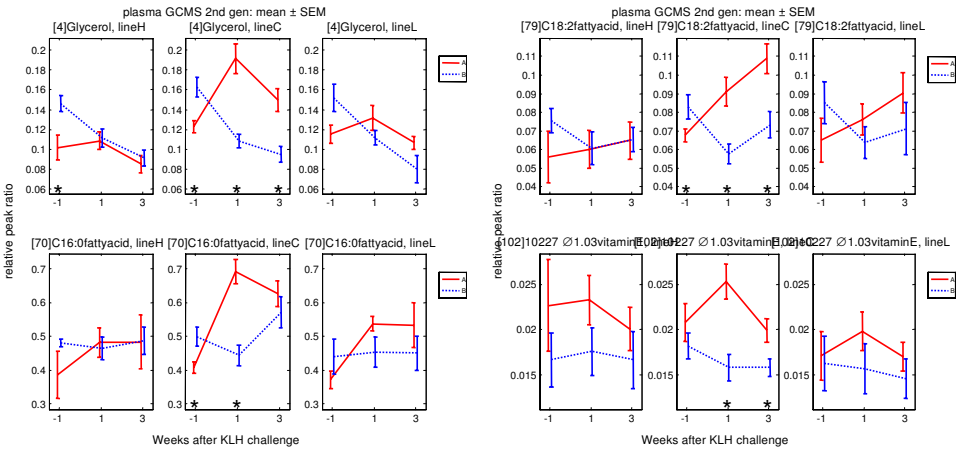


Figure 5.66: Four most discriminating metabolites in the GC-MS platform.

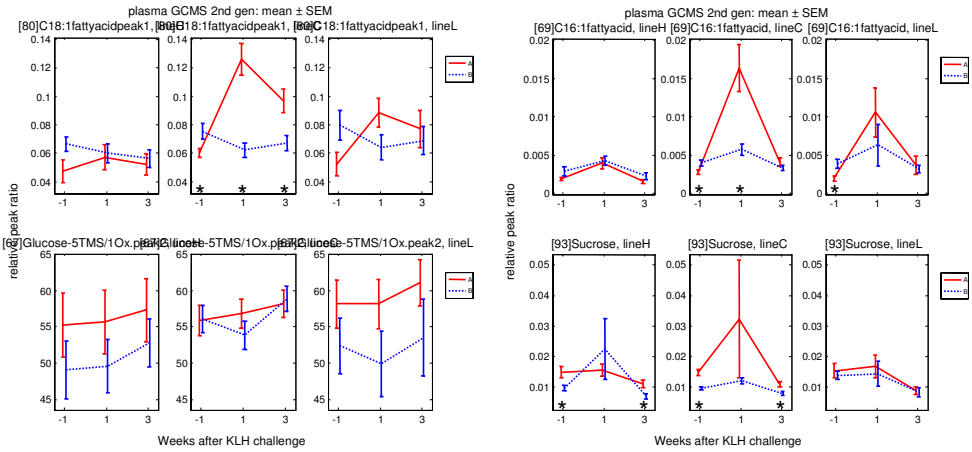


Figure 5.67: Monounsaturated fatty acids, FA 16:1 and FA 18: In the C-line a clear response of these fatty acids can be seen on timepoint 1 in group A.

Summary Plasma Metabolomics

Below, a ranking of the four different analytical platforms regarding plasma metabolomics is given. The indicative ranking (high, medium and low) is based on, interpretation of the PLS-DA discussed above.

Platform:	Sensitivity for A-B difference
Lipid	high
BA+FFA	medium
Global LC-MS	medium
GC-MS	medium

It can be concluded that all four platforms show differences in metabolites between group A and B. The lipid platform shows the most consistent differences. In Figure 5.68, gives an overview of the top 20 plasma lipid monoesters (from the lipid platform) and plasma fatty acids (from the bile acid/ FFA platform). The lipids are sorted according to their chain length and degree of unsaturation. A general pattern can be observed for the plasma lipids, where group A is lower compared to group B at timepoint -1, one week before the challenge, and higher at the measurements after the challenge (timepoints 1 and 3). Specially, LPC's and FFA's show a distinct pattern before and after the KLH challenge in group A compared to group B. In the figures below, taken from the review of Khovidhunkit et al. (2004) an overview of different lipid pathways is shown that react to an acute phase response.

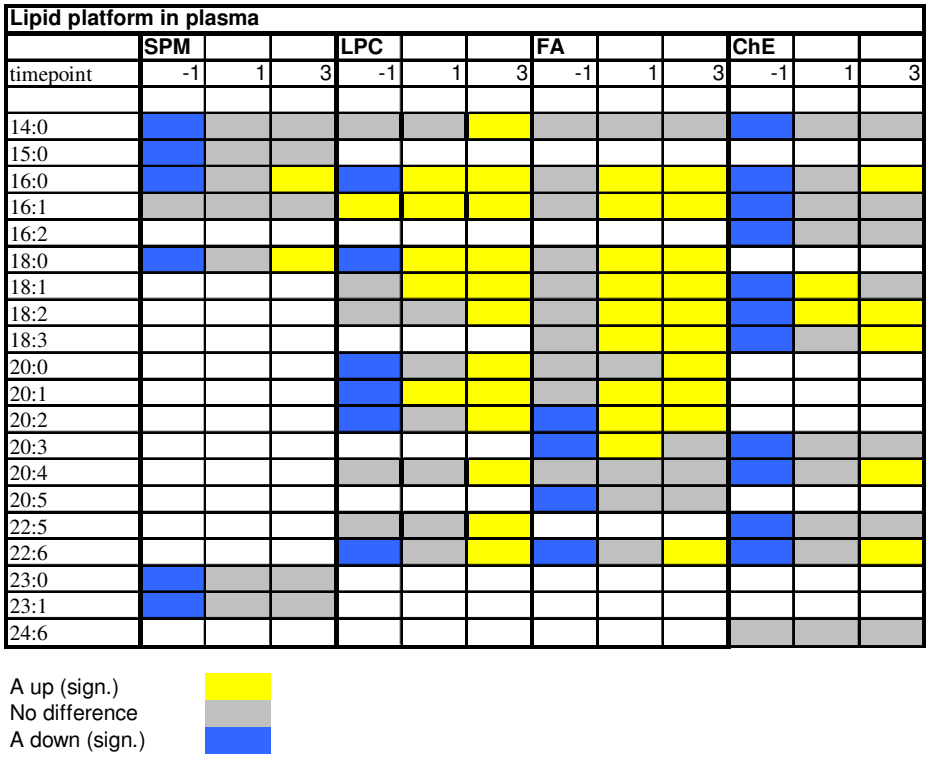


Figure 5.68: General overview of most discriminant lipids and free fatty acids at each timepoint in line C, sorted according to chain length and degree of unsaturation.

Changes in plasma lipids due to acute phase response

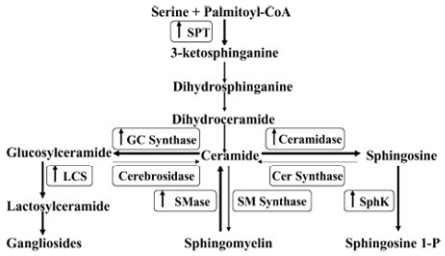


Fig. 5. Changes in sphingolipid metabolism during the APR. LPS

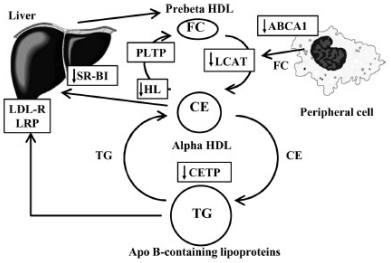


Fig. 4. Changes in reverse cholesterol transport during the APR

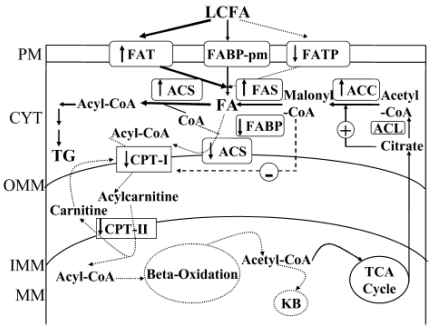


Fig. 1. Changes in hepatic FA metabolism during the acute-phase

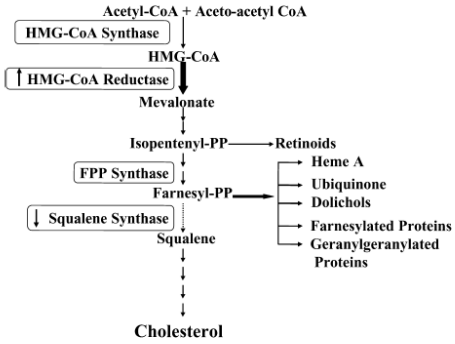


Fig. 2. Changes in cholesterol metabolism during the APR. Infec-

Figure 5.69: Influence of the acute phase response on different lipid classes Khovidhunkit et al. (2004).

Amino acids in plasma were covered by two platforms (GC-MS and Global LC-MS). A summary of the amino acids results obtained with these two platforms is given in Figure 5.70. Although individual amino acids are distinct between A and B, no easy interpretable patterns regarding biochemistry could be identified. Most amino acid levels were equal or higher in group B before the challenge and equal or higher in group A one week after the challenge, whereas there was no predominant pattern across amino acids in plasma three weeks after the challenge.

From the metabolomics analysis in plasma it can be seen that:

- All analytical platforms were able to distinguish between plasma from group A or B.
- Both, treatment and challenge had effect on plasma lipids.
- Increased levels of saturated lysophosphatidyl cholines at t=1 and t=3 were observed for group A in plasma. This specific modification of membrane lipids can be caused by a transient increased activity of secretory phospholipase (sPLA-2) (Six 2000), an enzyme specifically expressed in an Acute Phase Response (APR).
- Increased levels in free fatty acids and glycerol were found in plasma of group A after the (t=1 and t=3). Many fatty acids reached highest levels at t=1, indicating a relation between that peak and the KLH challenge. The acute phase response is known to increase triglyceride metabolism and fatty acid turnover and might be the cause of such a peak.
- Increased plasma levels of C18:3 and C22:6 in group A during the experiment might be caused by an increase in dietary intake of C18:3 after changing to the Grower feed in week -2.

- Before the challenge the levels of the majority of amino acids were equal for group A and B. However, for approximately 7 amino acids the levels in group B were higher. One week after the challenge, still the majority of amino acids showed equal levels for group A and b, but now for 7 amino acids the levels in group A were higher. Three weeks after the challenge no differences in amino acids between group a and B were present.

Metabolomics in C-line animals				
		plasma		liver
Amino acid	**-1**	**1**	**3**	**-4**
[1] L-Valine				
[3] L-Leucine				
[6] L-Proline				
[7] L-Isoleucine				
[8] Glycine-3TMS				
[14] L-Serine				
[15] L-Threonine				
[52] Beta-Alanine				
Alanine (LC-MS)				
[25] L-Methionine				
Aspartate (LC-MS)				
[36] L-Phenylalanine				
[37] D-Glutamic acid				
[39] L-Asparagine				
[52] L-Glutamine				
[61] L-Histidine				
[62] L-Lysine-4TMS				
[63] L-Tyrosine-3TMS				
Tryptophan (LC-MS)				
Cysteine (LC-MS)				
Arginine (LC-MS)				

A is sig. up
No sig.diff.
A is sig. down
not determined

Figure 5.70: General overview of most discriminant amino acids at each timepoint in line C.

5.3.2 *Liver metabolomics*

Liver Metabolomics: Data analysis The same data analysis strategy as in plasma metabolomics was applied (see also Figure 5.52). For the discriminant analysis, only one timepoint (t=4) was available.

Lipid platform Metabolites from the lipid platform were only related to treatment but not to genetic background (Figure 5.71, left panel). Treatment differences were clearly present in the C- and the H- line, but not present in the L-line (Figure 5.71, right panel). The discriminant analysis of the liver lipid data shows, that there are no individual metabolites with discriminating power (maximum of 67 % with a correct classification between treatments). No biological pattern was identified within the top 20 metabolites. It is, however, clear that for the C-line no difference is observed regarding triglycerides, so it is unlikely that differences in liver weight were caused by lipid content of the liver.

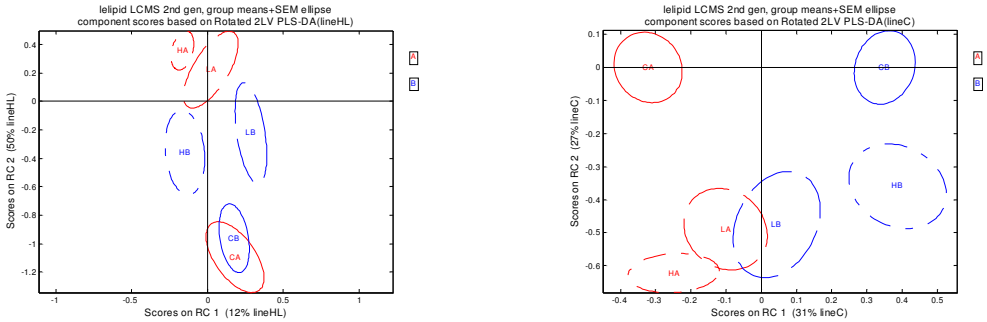


Figure 5.71: PLS-DA of Lipid platform. The left panel shows the rotated PLS-DA that describes the main differences between the high and the low line with RC1 and the main remaining trend with RC2. The C-line is projected in the H-L space. The right panel shows the PLS-DA that describes the differences between feed A and B of the C- group. H and L are projected in the C- group space.

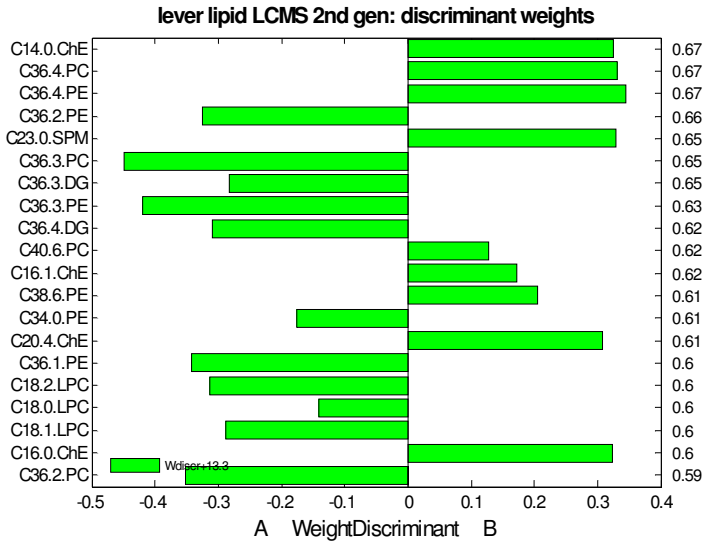


Figure 5.72: Discriminant analysis for treatment of Lipid platform. The green bars show the discriminants on timepoint +4. On the right hand side, cross-validated rate of correct classification is shown for each metabolite.

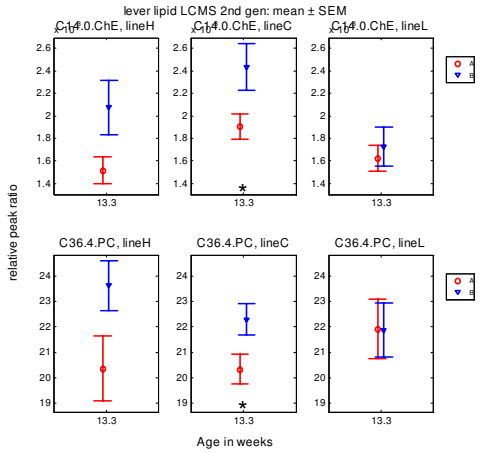


Figure 5.73: Two most discriminating metabolites between the treatments in the lipid platform.

Bile acid / FFA platform The free fatty acid/bile acid platform is the only metabolomics platform that shows a genetic background difference in the liver (Figure 5.74, left panel). The loading plot for this PLS-DA reveals that, although non of the metabolites directly correlates with the discriminant -axis (x-axis, along which the high – low difference has been projected), there are two bile acids tauroursodeoxycholic acid (TUDC) and cholic acid (CA) which have highest loading on the x axis. No clear treatment difference could be observed in the free fatty acid/bile acid platform (Figure 5.74, right panel). It can be concluded that 4 weeks after the challenge there was a difference in liver physiology, which distinguished the H- line from the other two lines. As for the lipid platform, the univariate discriminant analysis (Figure 5.76) did not provide metabolites with high discriminating power between the treatments.

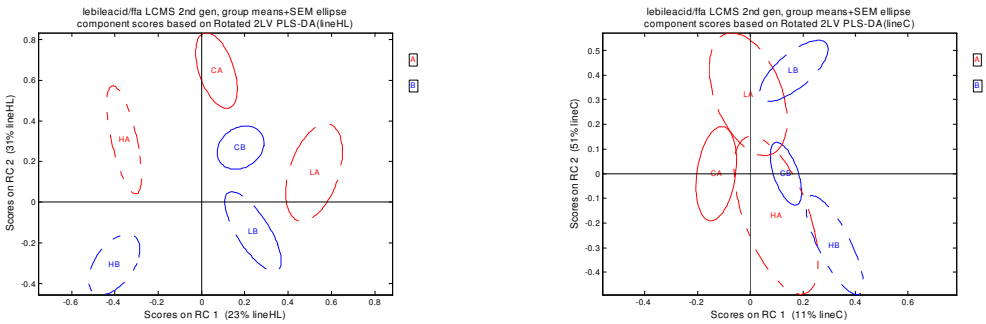


Figure 5.74: PLS-DA of FFA/BA platform. The left panel shows the rotated PLS-DA that describes the main differences between the high and the low line with RC1 and the main remaining trend with RC2. The C-line is projected in the H-L space. The right panel shows the PLS-DA that describes the differences between feed A and B of the C- group. H and L are projected in the C- group space.

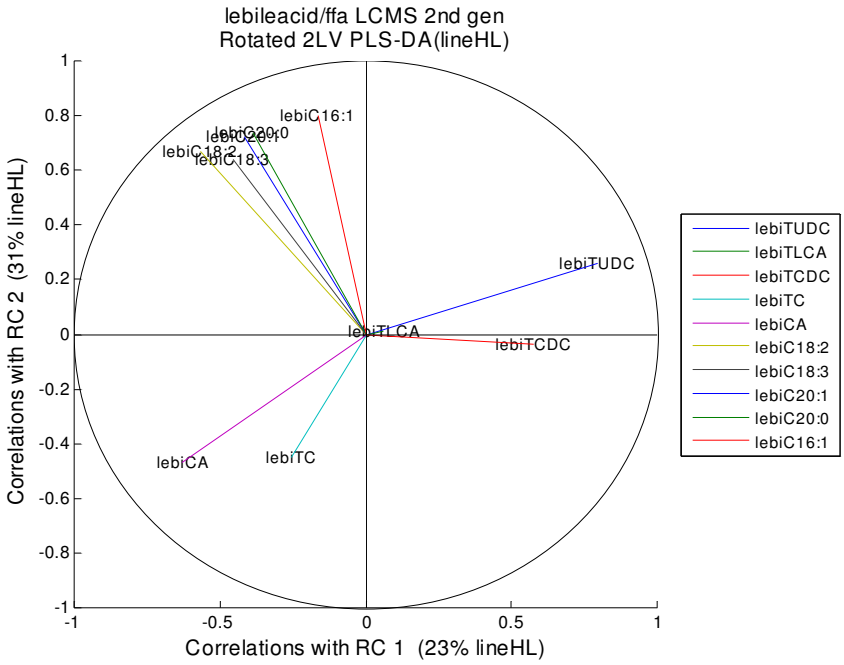


Figure 5.75: Loading plot of PLS-DA regressions for line effects. The legend is labeled in clockwise order.

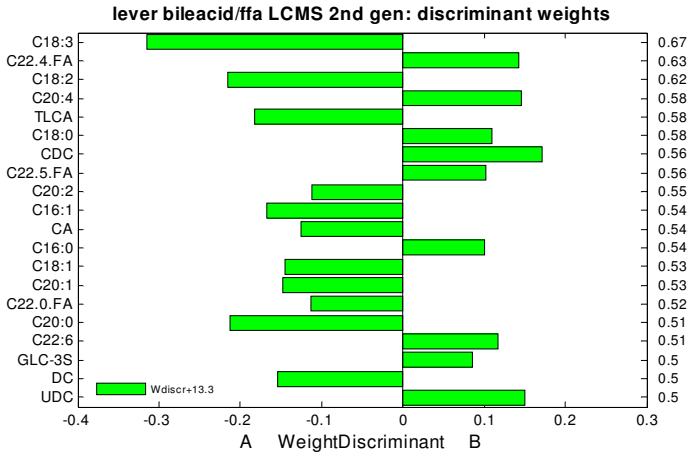


Figure 5.76: Discriminant analysis for treatment of FFA/BA platform. The green bars show the discriminants on timepoint +4. On the right hand side, cross-validated rate of correct classification is shown for each metabolite.

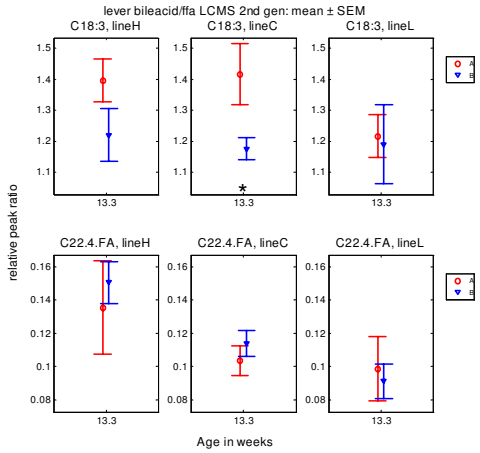


Figure 5.77A: Two most discriminating metabolites between the treatments in the FFA/BA platform.

Global LC-MS platform The multivariate analysis of the global LC-MS platform did neither reveal line nor treatment differences (Figure 5.77B). The C-line showed treatment differences, but these were not consistent for the H- and the L-line. In the univariate analysis of the C-line (Figure 5.78) only histidine was known among the discriminating metabolites. Animals from the C-line (and not the H and L line) on group A had higher levels of histidine in their liver.

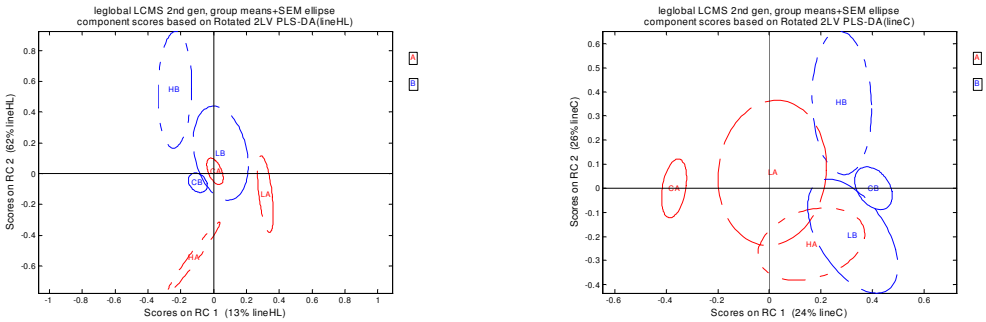


Figure 5.77B: PLS-DA of global LC-MS platform. The left panel shows the rotated PLS-DA that describes the main differences between the high and the low line with RC1 and the main remaining trend with RC2. The C-line is projected in the H-L space. The right panel shows the PLS-DA that describes the differences between feed A and B of the C- group. H and L are projected in the C- group space.

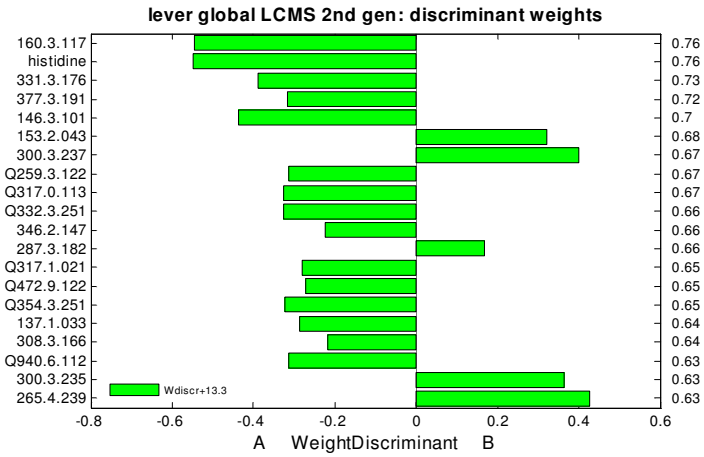


Figure 5.78: Discriminant analysis for treatment of global LC-MS platform. The green bars show the discriminants on timepoint +4. On the right hand side, cross-validated rate of correct classification is shown for each metabolite.

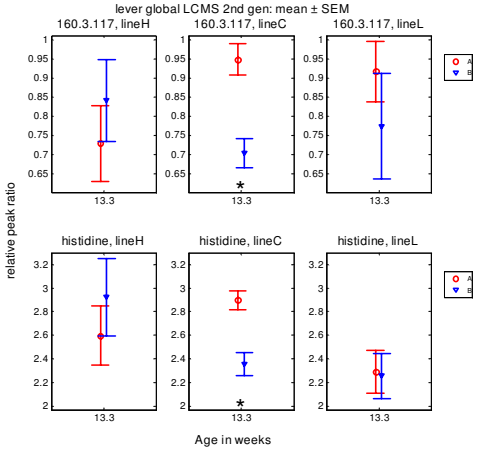


Figure 5.79: Two most discriminating metabolites in the global LC-MS platform.

GC-MS platform Data from the PLS-DA analysis shows, that there is a clear treatment effect for metabolites measured in the GC-MS platform. All lines receiving the same treatment cluster tightly together along the X-axis (Figure 5.80, right panel). The loading plot (Figure 5.81) shows that a number of sugar metabolites, along with glyceric acid, alanine, monomethylphosphate and some unidentified metabolites are responsible for the clear clustering of the liver samples. A similar result emerges from the univarate discriminant analysis, where many metabolites reach the level of 0.7 in discriminating power (Figure 5.82). The biological function of the top metabolite monomethylphosphate is not known. It is possible that it is a breakdown product of less stable intermediates. The suger metabolites D-Ribose, D-Ribulose and Fructose are linked to intermediates of the pentose phosphate pathway. The liver of the animals of group A had higher concentrations of these sugars in the liver. Furthermore, the amino acids Alanine and L-Methionine, Vitamine E and alpha- Ketoglutarate were increased in livers of animals of group A.

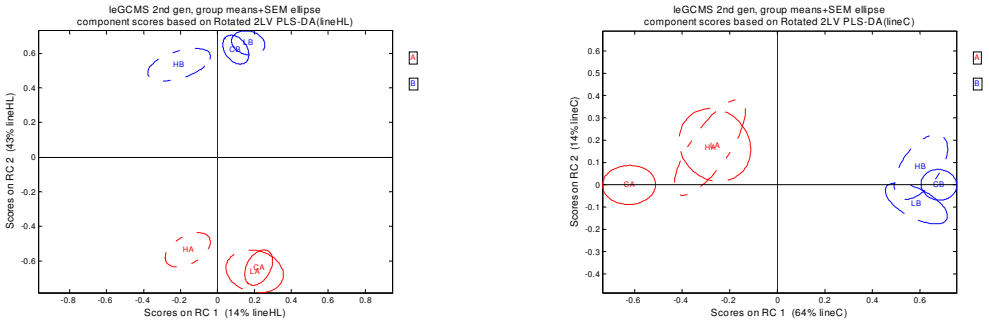


Figure 5.80: PLS-DA of GC-MS platform platform. The left panel shows the rotated PLS-DA that describes the main differences between the high and the low line with RC1 and the main remaining trend with RC2. The C-line is projected in the H-L space. The right panel shows the PLS-DA that describes the differences between feed A and B of the C- group. H and L are projected in the C-group space.

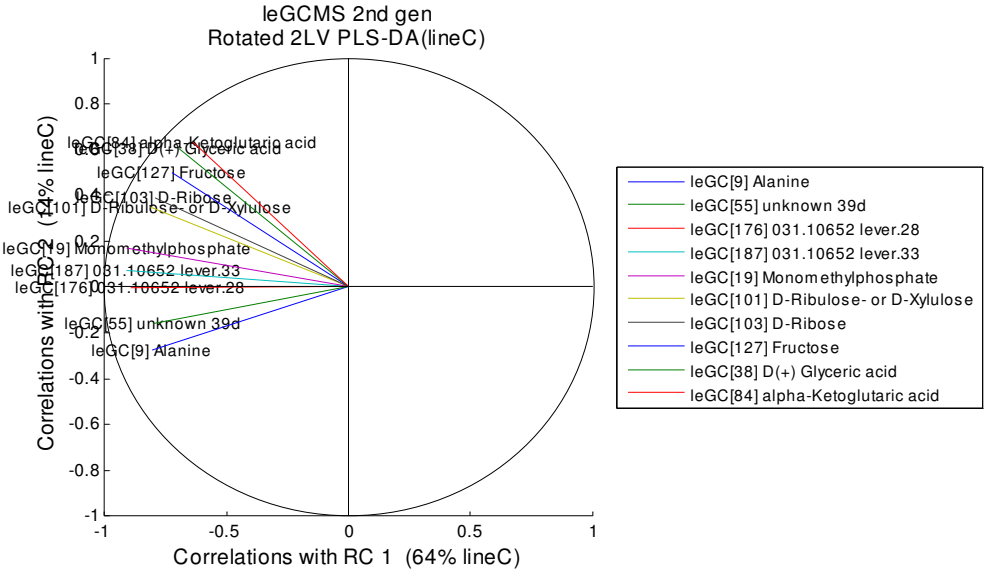


Figure 5.81: Score plot of PLS-DA regressions for treatment effects. The legend is labeled in clockwise order.

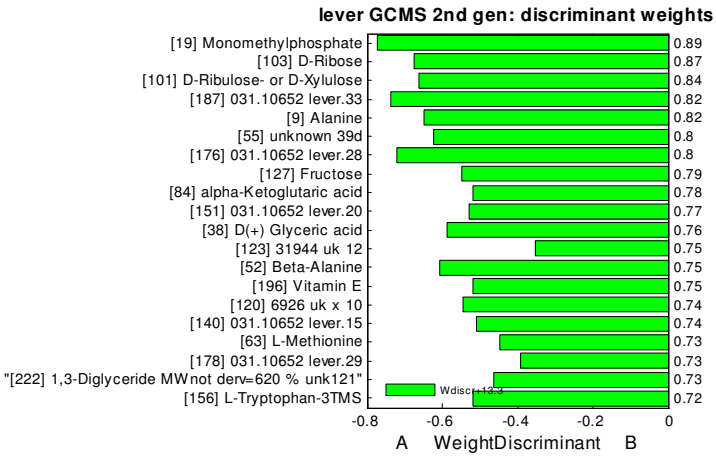


Figure 5.82: Discriminant analysis for treatment of GC-MS platform. The green bars show the discriminants on timepoint +4. On the right hand side, cross-validated rate of correct classification is shown for each metabolite.

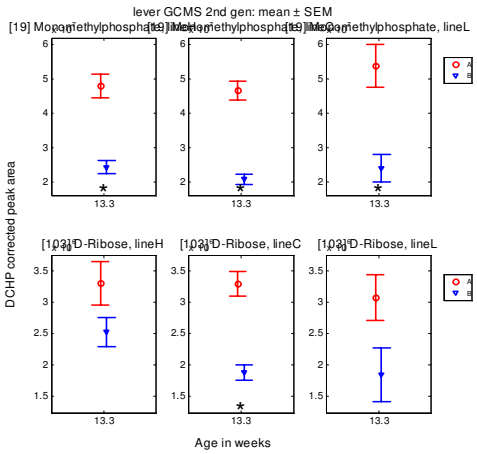


Figure 5.83: Two most discriminating metabolites in the GC-MS platform.

Summary Liver Metabolomics Below, a ranking of the four different analytical platforms regarding liver metabolomics is given. The indicative ranking (high, medium and low) is based on interpretation of the PLS-DA discussed above.

Platform:	Sensitivity for A-B difference
Lipid	high
BA+FFA	medium
Global LC-MS	medium
GC-MS	medium

From the metabolomics analysis in liver at 4 weeks after the challenge, it can be seen, that

- Treatment had no effect on liver lipids or bile acids.
- There is no difference between treatments regarding liver free fatty acids and glycerol.
- Increased liver level of pentose phosphate pathway intermediates were observed in group A animals.
- All liver levels of amino acids were equal or higher in group A, 4 weeks after the challenge. (Figure 5.70).
- None of the liver metabolomics parameters could be linked to the challenge with KLH.

5.4 Genomics

5.4.1 Genomics – Microarray

RNA was isolated from the jejunums of chicken described in Ch 4, Table 4.6 and analyzed. Two samples were excluded from further analysis based on the degradation of the RNA as observed on the gel image (Figure 5.84). All other RNA samples were of good quality and therefore were used for further micro array analysis. Pools of RNA were made by mixing 10 µg of each RNA sample according to the scheme in Chapter 4, Table 4.7.

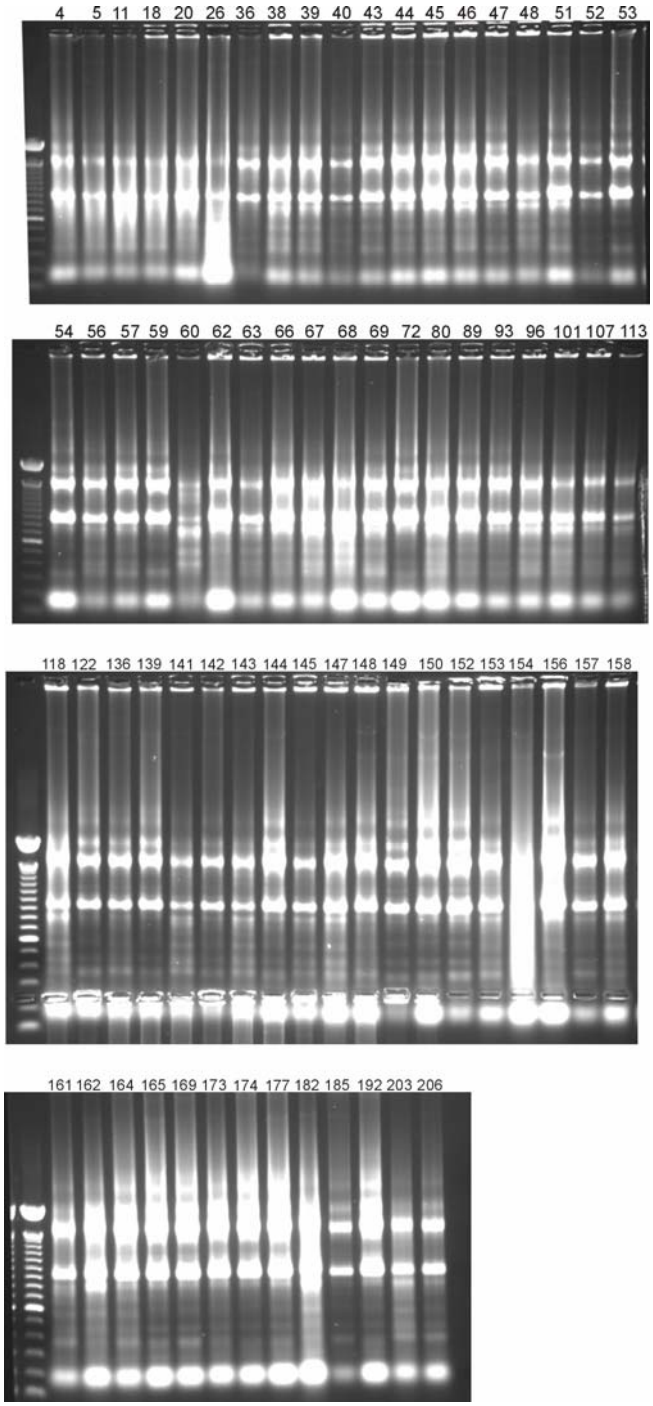


Figure 5.84: RNA analysis on 0,7% TBE agarose gel. No. 26 and 154 are partially degraded.

Hybridization of microarrays 20K chicken oligo arrays were hybridized with the pooled RNA samples according to the scheme in Table 4.7. Each pooled RNA sample was hybridized together with its biological reference (feed group A vs. feed group B). Subsequently, the same hybridization was performed with the dye colors swapped between the samples. Therefore, each pooled RNA sample was hybridized to two microarrays, once with every dye color.

Analysis of data Data were analyzed as described, with in house developed microarray analysis software. First, all data were normalized. Results of the normalization procedure are included for each array. The normalization procedure involves several steps. Steps of the normalization process are depicted in figures that allow to evaluate the normalization process.

Raw intensity plot

The raw values of both channels (Cy3 and Cy5) are plotted against each other. Ideally this results in a sigar shaped cloud of dots, centered along a 45° angle through 0.

Values for background correction

Background correction is based on the value of blank spots on the array. This figure represents the values of those blank spots, plotted against the spatial distribution of those spots on the array.
Non-normalized vs normalized M-values
M-value is the 2log ratio of both channels, and is the usual way to represent fold induction for microarray data. E.g. an M-value of 3, indicates that a gene is 23 = 8 times upregulated. These figures show that the data are more Gaussian distributed after normalization, indicating that the normalization procedure was performed well.

MA plot - first panel

The MA plot is an excellent visual representation of the normalization procedure. The Lowess fit is based on the unnormalized MA plot. Normalization procedure aims to center all values around 0. The reason for this is that overall genes will not be regulated. After normalization, the data points should be cigar shaped around the horizontal 0 line.

MA plot – spline – second panel

After normalization, one last step is performed. This step corrects for heterogeneity of variance over the slides via a monotonic spline function. This figure depicts this function and the outcome of the function.
All figures were interpreted, and all 14 microarrays were considered to be of good quality for further analysis.

Three different analysis were performed. In the first analysis all 14 arrays were included in the analysis. This analysis yielded regulated genes, that were regulated food-dependent but chicken line-independent. The most significant genes are summarized in Tables 5.3A and 5.3B. In this analysis no genes were found with a false discovery rate (q-value) below 5%. Therefore, the top-15 genes with a q-value of 6,6 that were regulated at least 3,2 fold were considered to be the most significant genes.

Subsequently, the selected chicken lines (H and L) were combined and analyzed as one group. This analysis yielded no significant data. Only genes with a false discovery rate higher that 50% were found. Those results were not included in further analyses.

In the last analysis five groups of the chicken C-line were included in the analysis. This analysis yielded genes that were regulated food-dependent and chicken line dependent (Tables 5.4A and 5.4B). The response of those genes in the L-line and H-line is uncertain, because the statistical power of those analyses was too low to interpret the results. With a false discovery rate of almost 10% the statistical power of the analyses of the H-line was lower than that of the first analysis of all 14 slides. The top-list of genes from the analysis on the C-line contained genes that were at least 2,6 fold regulated. Some genes were found to be regulated in the C-line alone, as well as in the analyses on all 14 slides. Other genes were only found to be induced in the C-line.

Table 5.3A:. Statistically significant upregulated genes in line B compared to line A from the analysis of all 14 slides.

No.	Gene name	GGA nr	Fold up (^{2}log)	q-value	Homology
1	RIGG13934	Gga.2909	2.26	6.578	Hemoglobin-α-chain
2	RIGG00458	Gga.9133	2.21	6.578	ch CCLi10
3	RIGG19006	Gga.8473	2.19	6.578	ENSGALT00000026140.1
4	RIGG20401	Gga.9133	2.07	6.578	chemokine ah221
5	RIGG03514	Gga.20490	2.07	6.578	Genome Hit Contig 1336.1
6	RIGG03683	ENSGALP00000026092	1.97	6.578	NDR-2 (human) weakly similar
7	RIGG18723	ENSGALT00000025383	1.96	6.578	Early response to neural induction
8	RIGG10214	Gga.7790	1.92	6.578	Nuclear receptor (NroB2), weakly similar
9	RIGG01347	Gga.12608	1.89	6.578	Insulin-induced gene 1 (Insig-1)
10	RIGG01361	Gga.4330	1.85	6.578	Immunoglobulin Heavy Chain
11	RIGG19421	ENSGALP00000027297	1.84	6.578	F-Box/LRR repeat protein 3A
12	RIGG00545	ENSGALT00000017050	1.83	6.578	Cytochrome P450
13	RIGG13628	Gga.2648	1.81	6.578	Hepatocyte growth factor like protein (HGFL)
14	RIGG19772	Gga.13583	1.80	6.578	Thrombospondin receptor (CD#36)
15	RIGG04380	Gga.13280	1.76	6.578	ENSGALT00000016755.1

Table 5.3B: Statistically significant downregulated genes in line B compared to line A from the analysis of all 14 slides.

No.	Gene name	GGA nr	Fold down (^{2}log)	q-value	Homology
1	RIGG00939	Gga.20815	3.45	6.578	Genome Hit Contig 41.179
2	RIGG19074	Gga.22304	3.22	6.578	Squalene mono-oxygenase
3	RIGG11373	Gga.22600	2.49	6.578	Aceteacetyl-CoA-synthase
4	RIGG13515	ENSGALP00000010861	2.42	6.578	isopentyl-diphosphate-deltaisomerase 2
5	RIGG20222	Gga.21297	2.37	6.578	C4 methyl sterol oxidase
6	RIGG01104	Gga.14299	2.36	6.578	B – G protein precursor/MHC 3-G antigen
7	RIGG00018	ENSGALP00000026298	2.29	6.578	Hypothetical Protein
8	RIGG06705	Gga.7215	2.25	6.578	Hydroxysteroid (17 beta) dehydroxygenase
9	RIGG13598	Gga.9957	1.96	6.578	ENSGALT00000011147.1
10	RIGG09214	ENSGALP00000021691	1.84	6.578	ENSGALT00000013304.1
11	RIGG13837	Gga.6433	1.80	6.578	ENSGALT00000011832.1
12	RIGG17787	Gga.3369	1.73	6.578	ENSGALT00000022866.1
13	RIGG04157	------	1.72	6.578	Genome Hit Contig 190.26
14	RIGG16191	Gga.1714	1.72	6.578	α2-macroglobulin precursor α2
15	RIGG15016	Gga.737	1.69	6.578	soluble carrier family 1

Table 5.4A: Statistically significant upregulated genes in group B compared to group A from the analysis of slides from the C-line. Highlighted genes were also found regulated in the analysis on all 14 slides.

No.	Gene name	GGA nr	Fold up (^{2}log)	q-value	Homology
1	RIGG01361	Gga.4330	2.06	9.336	Immunoglobulin heavy chain
2	RIGG00545	ENSGALT00000017050	1.68	9.336	nuclear factor of activated T-cells cy (NF-Atc3)
3	RIGG01229	Gga.11423	1.66	9.336	Glutathione S-transferase subunit gYc
4	RIGG03514	Gga.20490	1.62	9.336	Genome Hit Contig 1336.1
5	RIGG01351	Gga.17637	1.59	9.336	Partial Contig
6	RIGG00458	Gga.9133	1.54	9.336	ch CCLi10
7	RIGG03683	ENSGALP00000026092	1.51	9.336	N-myc downstream regulated gene 2 (NDR-2)

Table 5.4B: Statistically significant downregulated genes in group B compared to group A from the analysis of slides from the C-line. Highlighted genes were also found regulated in the analysis on all 14 slides.

No.	Gene name	GGA nr	Fold down (^{2}log)	q-waarde	Homology
1	RIGG00939	Gga.20815	2.55	9.336	Genome Hit Contig 41.179
2	RIGG00018	ENSGALP00000026298	2.35	9.336	Hypothetical Protein
3	RIGG01104	Gga.14299	2.16	9.336	B – G protein precursor/MHC 3-G antigen
4	RIGG02841	Gga.2785	1.85	9.336	Contig Hit 355900.3
5	RIGG02736	Gga.10286	1.63	9.336	oxidoreductase/probable isomerase
6	RIGG03868	Gga.6272	1.55	9.336	Genome Hit
7	RIGG04385	Gga.1022	1.43	9.336	Glutathion S-transferase Zeta
8	RIGG01190	Gga.20415	1.41	9.336	Protein tyrosin phosphatase-like protein P

Microarray analysis revealed that transcriptional differences exist in the jejunum between chicken that were fed organically grown feed ingredients, compared to chicken fed conventionally grown feed ingredients. Three different chicken lines were used; two selected lines and an unselected chicken line. No line-dependent differentially regulated genes were identified.

The most striking result is down regulation of several independent genes that code for enzymes involved in cholesterol biosynthesis and the subsequent pathway of steroidogenesis in chicken fed with feed B. Squalene mono-

oxygenase, acetoacetyl-CoA-synthetase and C4 methyl sterol oxidase are genes involved in cholesterol biosynthesis. Isopentenyl-diphosphate-deltaisomerase 2 and hydroxysteroid-17ß-dehydroxygenase (17-HSD) are genes involved in sterioidogenesis. Squalene mono-oxygenase was identified as a positional candidate gene for an obesity QTL (Stylianou 2004), thereby linking squalene mono-oxygenase expression to dietary intake. Kim et al. showed that in mice that were on a long-term high-fat diet (Kim 2004) cholesterol biosynthesis was also downregulated. Those mice also had very high concentrations of circulating cholesterol, that could explain the down-regulation of genes involved in cholesterol synthesis by a feedback mechanism involving the cholesterol molecule itself as an end-product repressor (Goldstein and Brown 1990). Acetoacetyl-CoA synthetase is also regulated by cholesterol. Feeding cholesterol or mevalonate depresses acetoacetyl-CoA syntetase activity (Bergstrom 1984). These findings will have to be combined with physiological parameters like fat content of feed A and B, and circulating cholesterol of both feed groups. However, the microarray data show a very strong tendency towards downregulation of cholesterol biosynthesis (Figure 5.85). Three of the downregulated genes are directly involved in cholesterol biosynthesis, while two other downregulated genes are involved in downstream processing of cholesterol. This could either be due to high concentrations of circulating cholesterol in feed group B, or due to other regulatory mechanisms of cholesterol biosynthesis.

Other genes found to be regulated can also be linked to the mevalonate pathway (Figure 5.85).
Insulin-induced gene 1 (Insig-1) was found to be upregulated in chicken fed on feed B. Insig-1 is also found to be upregulated in fat tissue of mice with diet-induced obesity (Li et 2003). This upregulation was explained by the fact that insig-1 regulates transcription of several metabolic processes, among which cholesterol biosynthesis. This is not a direct effect insig-1 binds to sterol regulatory element binding protein (SREBP), thereby preventing proteolytic processing of this protein. SREBP is a direct positive regulator of cholesterol biosynthesis. So, one trigger for the observed upregulation of insig-1 expression in the chicken intestine could be high circulating cholesterol levels. Another known regulator of Insig-1expression is the insulin concentration, which is also diet dependent.
ERNI, another gene found to be upregulated in chicken fed on feed B, is also an insulin regulated gene (Patwardhan 2004). However, it is predominantly known to be involved in early neural development during embryogenesis. A diet dependent role in the chicken intestine, is not obvious.
CD36 was found to be upregulated in chicken fed on feed B. CD36 is the major scavenger receptor for the uptake of oxidized LDL into macrophages, thereby forming foam cells (Steinbrecher 1999). This is a very important step early in the disease progression of atherosclerosis (Nicholson 2001). CD36 is upregulated by oxidized LDL, which is a form of cholesterol.
Chemokine ah221 (homologous to human MIP-A1), is another upregulated gene in chicken fed on feed B, that is also involved in atherosclerosis development (Zhao 2004). MIP-1A promotes chemotaxis of T lymphocytes.
Other immunological genes were found to be regulated feed dependent. B-G protein precursor was found to be down regulated in the chicken fed on feed B. B-G protein precursor are considered to be part of the major histocompatibility complex of the chicken, and are strongly correlated with disease resistance in chicken (Lamont 1998). There is no previous reporting of diet dependent regulation of B-G protein. This could implicate that chicken fed on feed B have a lower resistance towards infection, although disease resistance is a multifactorial process depending on more than one gene.
Another gene that was also found to be upregulated is the immunoglobulin heavy chain.

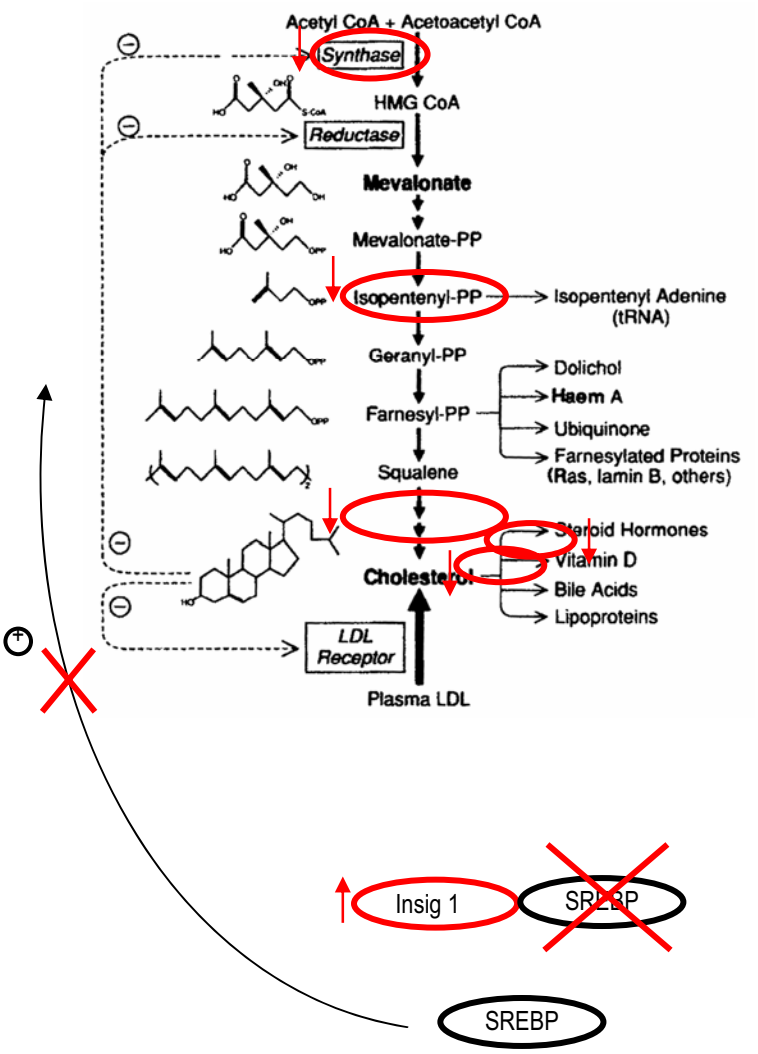


Figure 5.85: Regulation of the cholesterol/mevalonate pathway (adapted from Goldstein et al., 1990). All genes circled in red are found to be differentially expressed in chicken fed on feed B.

The finding of at least 7 regulated genes that are involved in cholesterol biosynthesis or cholesterol processing, greatly improves the statistical power of the analysis. The q-value of individual genes may be low, but the finding of more than 1 gene in a pathway confirms the regulation of that pathway. Therefore, the 7 regulated genes in the cholesterol pathways confirms that this pathway is truly regulated diet dependently. Although, we are unable to calculate a false discovery rate (q-value) for a complete pathway, the data indicate that the statistical power is improved greatly by this finding and therefore that the q-value is decreased to more significant levels.
Two analyses were described in the results; one analysis contained microarray data of all 14 slides from chicken lines H, C, and L. The other analysis only contained data from chicken line C. When those analyses were compared, no discrepancies were found in terms of regulation, so genes were not found differentially regulated between the two analyses. There were homologies between both analysis, that are indicated in Table 4. Three out of 7 upregulated genes in chicken line C and L out of 8 downregulated genes were also found in the overall slide analysis. The remaining regulated genes (n = 9) were not in the toplist of the overall analyses. It cannot be determined whether these genes are specifically regulated in chicken line C, because the results of chicken lines H and L cannot be

analyzed with sufficient statistical power to draw conclusions. Therefore, we can only state that feed dependent regulated genes are identified in chicken line C that are not found in the overall analysis.

In conclusion Feed dependent regulated gene expression in the chicken intestine was found. A high number of differentially expressed genes between the two feed groups are found to be involved in cholesterol biosynthesis or cholesterol processing. This finding suggests that the chicken fed on feed B have a higher circulating cholesterol concentration, or a completely different regulation of the mevalonate pathway. However, this has to be confirmed by physiological parameters of the chicken.

5.4.2 Genomics – qPCR confirmation

RNA material RNA isolation from chicken jejunum was described in the previous study for this project. Two RNA samples were omitted from the microarray study due to extensive breakdown. A second RNA isolation was performed on those samples (#26 and #154). Agarose gel analysis showed no breakdown, so samples were included in the qPCR study.

Selection of genes Six candidate genes were selected for qPCR analysis in consultation with Louis Bolk Institute: hemoglobin alpha chain, chemokine AH221, squalene mono-oxygenase, acetoacetyl coA synthase, isopenthyl diphosphate deltaisomerase 2, and methyl sterol oxidase. Two primer pairs were designed for each gene (Table 1). After constructing a reference plasmid for each PCR product, qPCRS were performed on serial dilutions of these reference plasmids. Based on this screening the top three combinations of primer pair and reference plasmid were chosen for qPCR analysis: primer pair 1 for acetoacetyl coA synthase, primer pair 1 for isopenthyl diphosphate deltaisomerase, and primer pair 2 for hemoglobin alpha chain. Besides target genes, 28S was included as an internal control for cDNA synthesis efficiency and qPCR efficiency.

qPCR analysis

1. Hemoglobin Alpha Chain (HAC)

HAC gene expression is slightly higher in chicken fed on feed B (Figure 5.86-B). This is in agreement with the data found in microarray analysis, in which a 2.3 fold upregulation in chicken fed on diet B was found. This difference was not significant ($p>0,1$). When the different chicken lines are analyzed separately (Figure 5.86-A), HAC expression patterns differ among the chicken lines. Animals of line C and L react similar as found with the microarray. Within line L chicken fed on diet B show a significant higher HAC expression ($P<0,05$). Within line C the difference is nearly significant ($p<0,1$). Animals within line H on the contrary show a significant lower HAC expression in chicken fed on diet B. The difference between line H feed A, and line C feed A is significant, as well as the differences between line H diet B and line C diet B, and line C diet B, line L diet B (Figure 5.86-A).

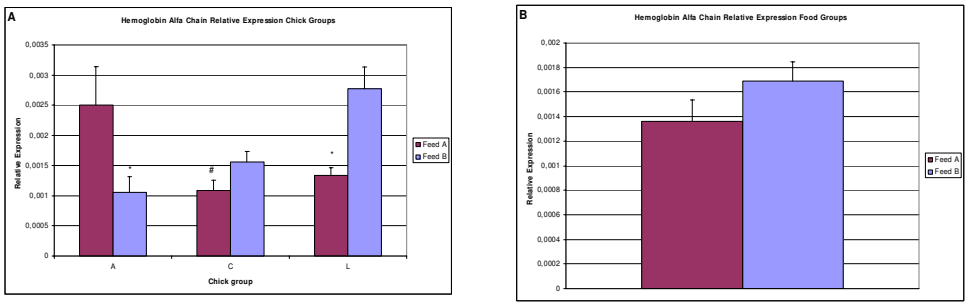


*Figure 5.86: Expression profile of Hemoglobin Alfa Chain. Relative expression per chicken line (A); relative expression per food group (B). * p < 0,05; # p < 0,1, error bars SEM.*

2. Acetoacetyl CoA Synthase (AACS)

Microarray results showed that AACS gene expression is 2,5 times lower in chicken fed on diet B. qPCR data show the same trend, although the difference is not significant (p>0,1) (Figure 5.87-D). When the chicken lines are studied separately, lines H and C show the same non significant trend, chicken fed on diet B show a lower AACS expression than chicken fed on diet A (Figure 5.87-C). Chicken from line L show a higher AACS expression due to diet B. This difference is not statistically significant. The three lines behave differently in their AACS expression, the difference between line C diet B and line L diet B is statistically significant.

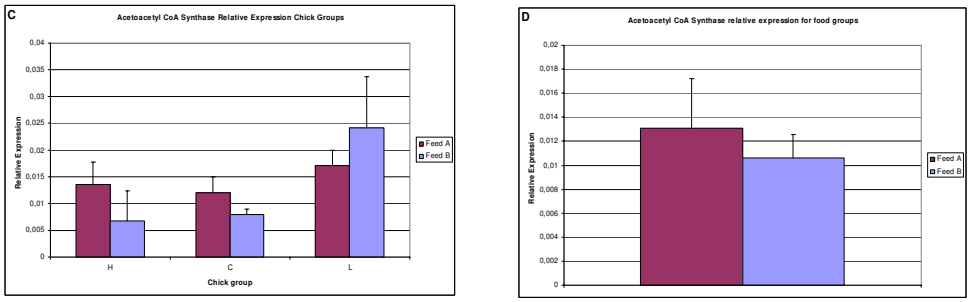


*Figure 5.87: Expression profile of Acetoacetyl CoA Synthase. Relative expression per chicken line (C); relative expression per food group (D). * p < 0,05; error bars SEM.*

3. Isopenthyl Diphosphatase (IPD)

Microarray results showed that AACS gene expression is 2,5 times lower in chicken fed on diet B. qPCR data show the same trend, although the difference is not significant (p>0,1) (Figure 5.88-F). When the chicken lines are studied separately, lines H and C show the same non significant trend, chicken fed on diet B show a lower AACS expression than chicken fed on diet A (Figure 5.88-E). Chicken from line L show a higher AACS expression due to diet B. This difference is not statistically significant. The three lines behave differently in their AACS expression, the difference between line C diet B and line L diet B is statistically significant.

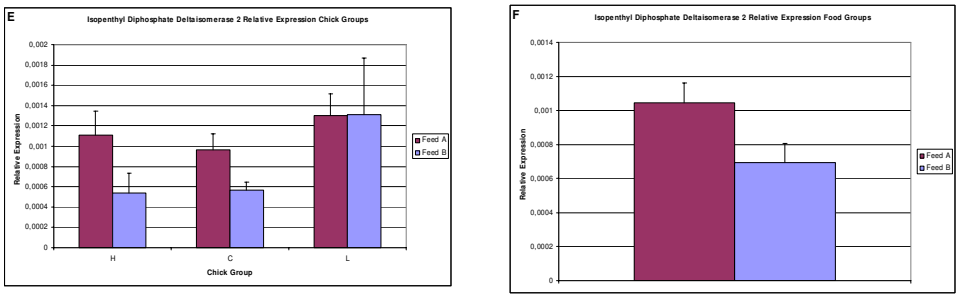


*Figure 5.88: Expression profile of Isopenthyl Diphosphatase. Relative expression per chicken line (E); relative expression per food group (F). * p < 0,05; # p < 0,1. error bars SEM.*

General conclusions qPCR analysis that gene expression profiles that were found using microarrays could be confirmed with an independent technique. The expression of three regulated genes was studied on RNA from individual chicken, whereas on the microarray only RNA pools of five chicken were analyzed. When all chicken lines are studied as one group, expression of all three genes show the same expression pattern as described for the microarray. Thus when the expression level was found to be higher due to feed A on the microarray, the expression level of this gene was also found to be higher due to feed A in the qPCR. The expression difference between chicken fed on the different diets of IPD was found to be statistically significant. The expression differences of HAC and AACS showed the same tendency as found on the microarray, but the differences in expression found with qPCR were not statistically significant.

The qPCR data were also used to study differences between the three chicken lines, whereas the microarray data could not be analyzed in this way. This analysis revealed that the chicken lines do not respond uniformly to the different diets. Several statistically significant differences exist between the three chicken lines. Lines C and L show a higher HAC expression in chicken fed on diet B, this is comparable to the data of the microarray. Line H on the contrary shows a lower HAC expression in chicken fed on diet B. Lines H and C show a lower expression for both AACS expression and IPD expression in chicken fed on diet B, similar to the microarray data. Chicken from line L show a higher AACS expression and no regulation of IPD expression in chicken fed on diet B.

These differences in expression between the different chicken lines were not observed in the microarray analysis. This was mainly caused by the fact that only one RNA pool of five chicken was tested for lines H and L. Therefore the statistical power of this experiment was too low to analyze line H or L independently. From the qPCR data it becomes very clear that differences between the three chicken lines exist at the level of gene expression. For the three genes studied, either line H or line L behaves differently, when compared to the microarray data.

IPD and AACS are both genes from the cholesterol/mevalonate pathway, that were both found to be downregulated in chicken fed on diet B in the microarray data. Not only did we confirm this observation, we also showed in this analysis that the different chicken lines showed different expression patterns for IPD and AACS. Line H and C confirm the microarray data, whereas line L either shows an opposite effect (IPD) or no difference in expression (AACS).

Based on the microarray data and the qPCR data we conclude that differences in gene expression exist between animals that were fed on different diets. Besides, the qPCR data indicate that differences exist in gene expression between the chicken lines that could not be identified in the microarray analysis.

5.5 Pathological Anatomy

The veterinarian, dissecting the organs of the animals did not perceive abnormalities.
The butcher preparing the chicken breast meat, observed that the meat of the A-animals was more red, while the meat of the B-animals was more pale and had more fat tissue among the muscles. This was not objectivated.
In preparing for histological evaluation, no gross alterations were noted during processing of the tissues.
The results of the weights of the thymus and bursa are presented in Annex 10.
The villus / crypt ratio is listed in Annex 11. The CD 8 staining is listed in Annex 12.
Histological results of the tissues are listed in Annex 13, the results of the apoptosis staining are in the same table.

General histology

- The **thymus** showed a normal aspect, large medulla, some small hemorrhages and incidentally small clusters of granulocytes.
- The **bursa** was variable in size, with much lymphoid tissue and sometimes many apoptotic cells. In the epithelium sometimes fluid-filled cysts were observed.
- The **spleen** had a good developed white pulp with PALS (periarteriolar T lymphocyte sheat)
- and PELS (peri-ellipsoid B lymphocyte sheat), and contained a varying amount of blood. Sometimes some follicles were seen.
- In the **heart** the endocardium revealed myxomatous connective tissue and cartilage in the wall of the aorta. Some small lymph follicles were found often at the epicardial aspect.
- The **liver** showed a normal architecture and hepatocytes. Periportally some infiltrates were seen, often as small follicles.
- The **ovaries** showed follicles of varying size and stage, some atretic follicles and some corpus luteum like formations. At the hilus area amounts of nervous tissue were present and the ovaries were located adjacent to the adrenal glands.
- The **lung** was in most animals normal, the amount of blood varied, much BALT was present.
- The **kidney** had smaller and bigger glomeruli as is normal for birds, and some lymphatic infiltrates.
- The **proventriculus** showed no alterations, some lymphocytic infiltrates with follicles in the submucosa. Sometimes the lymphoid tissue was also present in the glandular tissue below the submucosa.
- The **ventriculus**, muscular part: Thick layer of keloid on the glandular layer which showed a lamina propria that contained a varying amount of connective tissue, sometimes very much Sometimes the deeper layers of the glands were dilated.
- The **pancreas** was normal; incidentally some small follicles occurred.
- The **duodenum** showed varying amounts of lymphoid tissue in the mucosa.
- The **jejunum** showed less lymphoid tissue, and mostly nice long villi.
- The **cecum** showed sometimes submucosal fat deposits a varying amount of lymphoid cells in the lamina propria and in some animals apoptosis in the upper regions of the mucosal layer.
- The **tibiae** were investigated in particular for osteoclast activity in the epiphysial area. All animals showed normal histology and no differences between the groups could be observed.

Special Observations

The thymus and bursa weights showed some differences between the groups.

Feed A animals had a heavier thymus in the H-line and slightly in the C-line, but not in the L-line.

The C-line animals showed a heavier bursa.

Feed B birds had a somewhat heavier thymus in the L-line. All weights were within the normal range (Table 5.5, Figure 5.89).

Table 5.5: Mean weights of formalin-fixed thymus and bursa.

Line	Feed A Thymus	Feed B Thymus	Feed A Bursa	Feed B Bursa
H-line	1.92	1.6	2.91	3.25
C-line	1.48	1.37	3.87	3.53
L-line	1.94	2.17	3.47	3.58

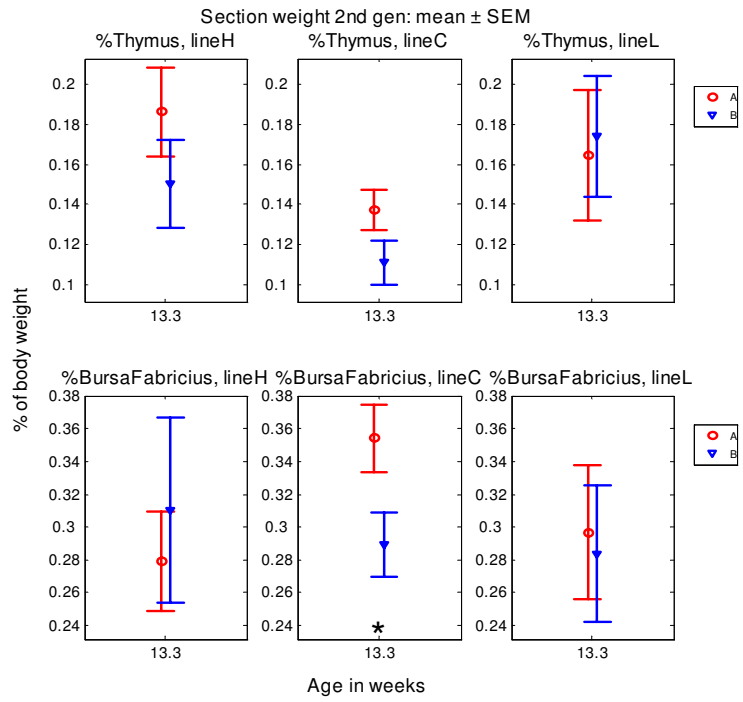


Figure 5.89: Mean relative weights of the formalin fixated thymus and the bursa in the 2nd generation, as a percentage of the body weight. Results present the mean and SEM for the animals per line and feed-group, C-line 24-A and 22-B animals, H and L-line 6 animals each.

Some C-Line animals in the feed B groups showed fat depositions in the cecum. Fat deposition may de due to the general fatness of the animals (Figure 5.90).

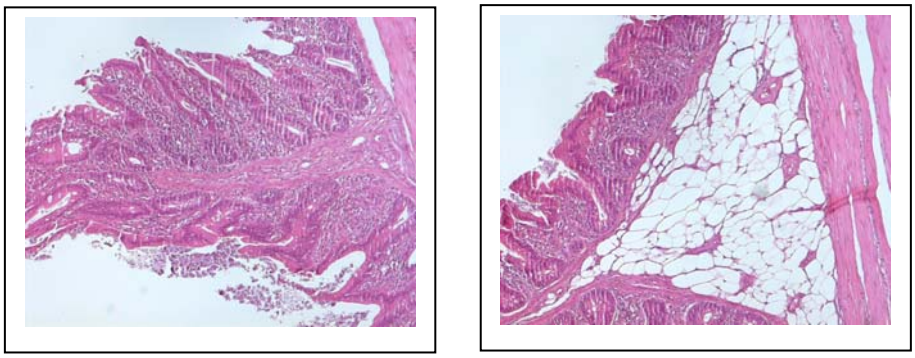


Figure 5.90: Cecum submucosa without (left) and with (right) fat deposits.

Feed A L-line birds showed some dilated tubules of the deep layers of the ventriculus. The meaning of this feature is not clear and may be part of the normal variation in the microscopical morphology.

The villus/crypt ratio showed the following data (Table 5.6).

Table 5.6: Mean villus length / crypt length ratios.

Line	Feed A Duodenum	Feed B Duodenum	Feed A Jejenum	Feed B Jejenum
H-line	8.6	8.4	10.93	7.7
C-line	9.5	8.8	8.6	8.7
L-line	7.8	8.1	8.6	10.5

The C-line showed almost no differences between the feed groups.
The H-line chicken showed longer villi in the jejunum but not in the duodenum in the feed A birds. The L-line birds showed somewhat longer villi in the jejunum on feed B. In both cases the higher mean values were due to one animal with longer villi. When these animals are dismissed no differences are observed.

The CD 8 staining for T-Lymphocytes is listed in Table 5.7.

Table 5.7: Mean semiquantitative number of lymphocytes in the intestines.

Line	Feed A Duodenum	Feed B Duodenum	Feed A Jejenum	Feed B Jejenum	Feed A Cecum	Feed B Cecum
H-line	1	1.7	1.2	1.7	1	1
C-line	1.3	1.3	1.9	1.75	1.3	1.3
L-line	1.7	1.1	2	2	1.7	1.5

In feed A, the H-line animals showed the lowest numbers of lymphocytes stained, whereas L-line showed the highest number of lymphocytes.

In feed B the H-line was higher or comparable to C- and L-line, except for the cecum in which organ also for H-line the lowest amount of lymphocytes was stained.

The L-line varied in the part of the intestine with respect to the amount of lymphocytes stained in feed B, more than feed A animals. Figure 5.91 shows representative duodenum slides stained for T-lymphocytes.

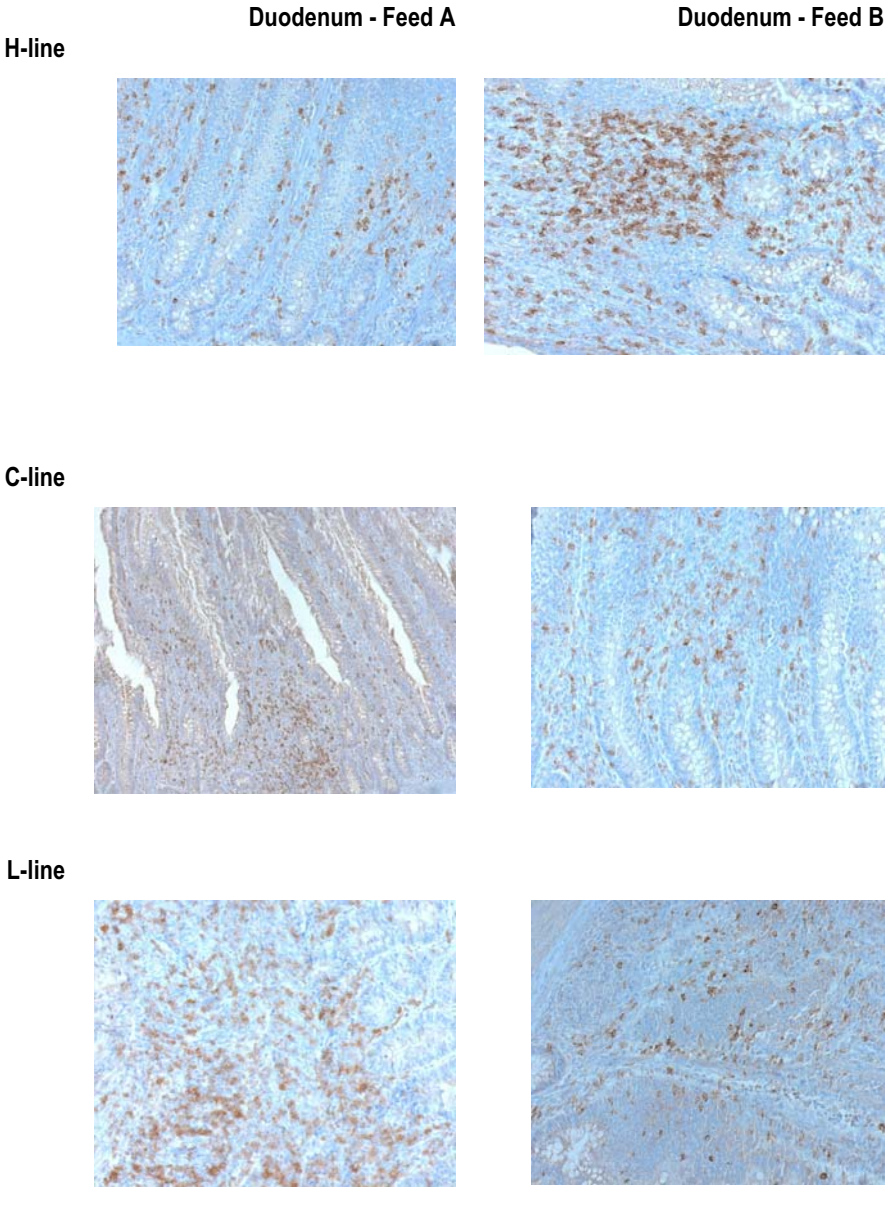


Figure 5.91: Duodenum slides with CD 8 staining for T-Lymphocytes.

5.6 Microbiology gut

On the basis of the dendrograms as well as similarity values, manure samples from organic and conventional chickens grouped more or less together. Similarities were greater for manure from feed A with other manures from A and for manure from feed B with other manures from B, than the similarities between manures from feed A compared to feed B. The diversity indices did not differ significantly among cages or feeds. Animals of the L-line had a slightly higher bacterial diversity than those of lines H or C (data not shown).

Bacterial community composition in the manure was affected significantly by feeding regime, but not by chicken line. Bacterial diversity in manure was not affected by feed source, but differed slightly among chicken lines.

5.7 Sensory analysis of chicken meat

The butcher preparing the chicken breast meat, observed that the meat of the A-animals was more red, while the meat of the B-animals was more pale and had more fat tissue among the muscles. This was not objectivated.

The chicken breasts of the C-line animals on the two feeds differed significantly on the aspect of juiciness. The meat the B-animals was evaluated as juicier.

The chicken breasts of the H and L-line animals on feed A differed significantly on the aspects of typical chicken taste and appreciation. The meat of the H-line animals was evaluated as of more typical chicken taste and was appreciated more.

5.8 Overview of chicken results

In Annex 14 all significant differences between the A and B-fed chicken are schematically represented.

A selection of the results is summarized in Table 5.8.

Table 5.8: Summary of differences observed between animals group A and group B.

B>A or A>B	Measurements	Parameter	Comment
B>A	**General health 2nd generation**	Weight	Consistent with higher protein percentage in feed B
B>A	General health 2nd generation	Relative growth of body weight	Till 5 weeks of age
A>B	General health 2nd generation	Relative growth of body weight	From week 10-12 of age; after KLH-challenge
A>B	**Immune System 1st generation**	Innate Immune System - cellular Intrinsic activ. of monocytes higher	Comparing levels of NO production
A>B	Immune System 1st generation	L-line Innate Immune System – humoral- LPS-antibodies	4 & 22 weeks after change to experimental feed
A>B	Immune System 1st generation	L-line Specific Immune System – humoral- Vaccination titre NCD	4 & 8 weeks after change to experimental feed.
A>B	Immune System **2nd** generation	Innate Immune System- cellular Monocyte activity, LPS-stimulated	1 week before KLH, 1 week after change of feed
A>B	Immune System 2nd generation	Innate Immune System - humoral LPS- antibodies	2 weeks after KLH
B>A	Immune System 2nd generation	Innate Immune System -humoral LPS-antibodies	4 weeks after KLH
B>A	Immune System 2nd generation	Innate Immune System -humoral LTA-antibodies	4 weeks after KLH LTA opposite pattern to LPS
A>B	Immune System 2nd generation	Innate Immune System -humoral Complement – Classical pathway	1 week after KLH
A>B	Immune System 2nd generation	Specific Immune System -humoral Vaccination titre NCD - Delta	3 & 4 weeks after KLH
A>B	Immune System 2nd generation	Stimulation Index feed extraction test Feed A & B lymphocytes to Feed A extract	Dilution -4 & -3 for C- & H-line. Dilution-2 for L-line
B>A	**Metabolomics**	Majority of Amino Acid levels in	Consistent with increased protein

	2nd generation	plasma	percentage in feed of group B
B>A	Plasma metabolites	Plasma Lipids - Lysophosphatidyl choline (LPC), FFA and glycerol.	1 week before KLH challenge
A>B	Plasma metabolites	Plasma Lipids - LPC, FFA and glycerol	1 week after KLH challenge. Consistent with a stronger acute phase response in group A
A>B	Liver metabolites	D-ribose, D-ribulose and Fructose	4 weeks after KLH challenge. Indicative for increased pentose phosphate pathway activity in livers of group A
A>B	Liver metabolites	Histidine, Alanin, L-methionin, alpha Ketoglutarate and Vitamin E	4 weeks after KLH challenge. Markers of liver metabolism and food intake (vitamin E)
B ↓	Genomics –confirmed	Cholesterol biosynthese pathway	Diverse genes downregulated
A>B	Organ weight fresh	Relative Gastro-intestinal weight in H-line	Significant
A>B	Organ weight fresh	Relative Liver weight in L-line	Significant
A>B	Pathol. Anat-fixated	Relative Bursa weight in C-line	Significant

6 Summary of feed and chicken analyses and reflection on research questions

In this chapter results will be summarized and connected to the research questions about feed ingredients and chicken.

6.1 Feed analyses

6.1.1 Differences in ingredients used for the chicken feed

The first research question: 'Can differences be found between ingredients for chicken feed, obtained from organic and conventional production systems?' will be worked out here.
In this study all feed ingredients were extensively analysed to investigate differences between ingredients from the different production systems, organic and conventional. Ingredients under investigation were: triticale, wheat, barley, maize, peas and soy. Wheat, barley, triticale and maize are grain products. Peas and soy are leguminosa. In this study differences between the ingredients were studied in macronutrients (e.g. protein level, amino acids, some fatty acids) and in micronutrients, trace elements/heavy metals, mycotoxins, pesticide residues, dioxines, ANFs.

Macronutrients

Protein and amino acids From the analyses of the ingredients it showed that the level of total protein and (almost) all amino acids were higher in the ingredients used to compose feed B. These differences were observed in wheat (20-30% more of specific amino acids in ingredient B), barley (10-30% higher amino acid levels in ingredient B than ingredient A), soy (10-20% higher amino acid levels in ingredient B than ingredient A). For peas, only the methionin level was about 20% higher in the B sample. Triticale showed a clear different pattern, resulting in amino acid levels to be 20-45% lower in ingredient B. In half of the amino acids in maize no differences were observed, whereas the other half had 10% lower levels in the samples used to prepare feed B.

Fatty acids For fatty acids no consistent differences between the ingredients from different origins were observed. Largest difference was observed in triticale for the saturated fatty acids C14:0 and C17:0, which were 6 times higher in the B ingredient. Both fatty acids comprise less than 1 % of the total fatty acids. For the most abundant fatty acids, C16:0, C18:1c and C18:2 c9,12, comprising resp. >10%, >10% and >50% of the fatty acids in the ingredients, no major differences were observed.

Micronutrients

- Phytosterols: In barley and soy higher levels of the most abundant phytosterol, sitosterol, were observed in the ingredient B. Levels were 50% higher for barley and 30% higher for soy.
- Flavonoids: no differences were observed between ingredients A and B.
- Carotenoids: In triticale levels of lutein and zeaxanthine, the most abundant carotenoids, were higher (resp. 50 and 60%) in the B ingredient. In peas and soy these carotenoids were 20% higher in the A ingredient.
- Catechins: no catechins were detected in significant levels in any of the ingredients.

- Isoflavones were analyzed only in soy. The most abundant isoflavones, malonyl genistin and malonyl daidzin, reached highest levels in the soy used to prepare the feed A (30% higher then the B soy).
- Vitamins: many, but not consistent differences between ingredients A and B in the vitamin contents are observed. Most remarkable is the high amount of tocopherols in soy A and total folate in peas A. For the overview of differences larger than 10% (ratio ≥1,2 or ≤0,8) see Table 6.1.

Trace elements/heavy metals Some trace elements were hardly detected or showed hardly differences between the samples. An observed difference was the higher level of selenium in triticale, maize, peas and wheat, with an about 8.5 times higher level in maize used to prepare the B feed. For selenium, the main factor of influence is the soil. It is not clear if this difference can also have a relation to differences in farming systems.

Mycotoxins, pesticide residues, dioxines, endotoxins, ANFs First all ingredients were prescreened on potentially hazardous compounds. Most samples were free of contamination with mycotoxins or pesticides. Only in two of the analyzed samples (wheat B and maize B), small amounts of mycotoxins were found. Levels were below risk levels for chicken and one (wheat) could be avoided by different sampling. Further, no differences between ingredients from the two production systems were observed with respect to pesticides (all beneath detection limit), dioxins and PCB's (all lower than background value). Both batches of soy and peas contained levels of lectin. Levels in soy were 4.0 and 2.55 mg/g in respectively samples B and A and 0.06 and 0.12 mg/g in batches peas B and A. Antritrypsine activity was assessed in toasted soybeans and was respectively, 1.34 mg/g and 1.48 mg/g for soy A and B. Further, by analysing the feeds it showed that the B feeds had higher levels of LPS endotoxin residues. As the separate ingredients were not analysed for this, it is not known which ingredients have contributed to this.

Complementary analyses

- With bipohoton analyses (Delayed Luminiscence – FAS – Biophotons) all samples could be significantly differentiated. Wheat, barley and maize were classified correctly as being organic or conventional, triticale not. Due to lack of experience with these products no assumption about the origin of peas and soy was made.
- Differences in amino acid ratios are observed for wheat, barley, soy and triticale. The raw protein content between the samples differed and also the percentage of the essential amino acids methionin, cystein and lysine, as percentage of the protein, differed. Based on these parameters, the researchers have classified wheat, barley and soy correctly as being conventional or organic. Triticale not. For peas and maize no clear difference was observed.
- The crystallizations of all ingredients, except peas, could be differentiated highly significantly ($p<0.0005$) according to the production system. By visual evaluation researcher could correctly identify the organic wheat sample. All other ingredients were grouped in two groups with comparable gestures. Due to lack of experience, identification of these groups as organic or conventional was not possible. After decoding the grouping showed to be correct.

6.1.2 Differences in the chicken feeds

Proteins and amino acids The amount of proteins was consistently higher (relatively circa 10%) in the feeds of group B compared to feeds A. The higher protein levels in the wheat, barley and soy samples used to prepare feed B, are consistent with this finding. Also all the specific amino acids were higher in feed B and in wheat, barley and soy used to compose the feeds of group B. To prevent shortages, amino acids that were below the norm for chicken feed, were suppleted before the blinding. This was always the case for methionine, which was suppleted in both feeds up to the lowest level of the norm, more in organic than in the conventional feed, resulting theoretically in equal levels. Once lysine was supplied and once threonine.

Fatty acid composition The fatty acid composition was slightly different between the feeds, though not very consistently so. At the time that the KLH challenge was given, feed B contained 20% (as percentage of total feed) more unsaturated C18, which might be important for the interpretation of the effects found in chicken (Ch 7.4).

Micronutrients Alpha-tocopherol and total folate were equal or higher in most of the feeds A compared to the feeds B. The main ingredient influencing the folate content was peas, though with the exception of triticale also the other ingredients used for feed A had higher levels. The higher alpha-tocopherol levels can be explained by the higher levels in barley, wheat and especially soy. Triticale and to a lesser extent maize showed to have higher levels in the samples used to prepare the B feeds. Iodide was consistently higher in sample A feeds. Chloride was, with the exception of ratio 11/12 higher in the feeds B.

Based on calculation, feeds B contain more alpha-carotene, vitamin C, vitamin B5 and phytosterols, whereas feeds of group A contain more carotenoids, vitamin K1 and isoflavones. The latter is confirmed by the metabolomic profiling of the polar fractions of the different chicken feeds, which showed clear differences in the composition of the samples, mainly related to their isoflavone content. Alpha-carotene was detected only in maize, with higher levels in B. The higher content of vitamin C was observed in all ingredients used to prepare feed B with levels above the detection limit (barley, maize, peas and soy). Vitamin B5 was especially high in wheat used for feed B and higher phytosterol levels in the B feeds are the result of higher levels in soy and barley.

Microbiology With respect to the microbiological analyses of the various feed and ingredient samples, these analyses have not shown any concentration of microbial species in any of the samples that might have a serious direct inverse effect on the health of chicken. Main observed differences between the group A and group B feed and ingredients were the aerobic colony count and the amount of Enterobacteriaceae which were higher in (some of) the feed samples of group A. Analyses of the feed ingredients indicated that this might be due to the barley, maize and pea samples. Moulds were more common in two feeds for group A.

Endotoxins LPS endotoxin was found to be relatively higher in all measured feeds B. This toxin is produced by Gram-negative bacteria like E. Coli, Enterobacteriaceae and Salmonella. However in the microbiological analyses these were not systematically higher in feeds B. On the contrary, Enterobacteriaceae were more present in feeds A. Thus the source of this contamination is not clear.

Complementary analyses – Biocrystallizations Two out of three feeds could be differentiated with this method as belonging to either production origin. The samples could not be identified as belonging to organic or conventional.

6.1.3 The first research question evaluated

The first research question: 'Can differences be found between ingredients for chicken feed, obtained from organic and conventional production systems?' can be answered positively. Between the ingredients from the two agricultural origins differences were observed. Most consistent were differences in protein and amino acid levels, which were > 10% higher in wheat, barley and soy. In Table 6.1 the main differences for the individual ingredients of group A and group B are summarized. In the table all differences of 10% and more in macronutrients and of 20% and more for the micronutrients are presented in the table.

Table 6.1: verview of comparison in nutrient content in the ingredients from organic (A) and conventional (B) origin.

	Wheat*	Barley	Triticale	Maize	Soy	Peas
Dry matter‡	=	=	=	=	=	=
Proteins	**B>A** (30%)	**B>A** (20%)	**A>B** (40%)	**A>B** (10%)	**B>A** (20%)	=
Amino Acids	**B>A** (10-40%)	**B>A** (10-40%)	**A>B** (20-50%)	**A>B** (0-10%)	**B>A** (0-20%)	=
Fatty acids	=	=	=	=	=	=
Fibre	**B<A** (20%)	=	=	=	=	=
Phytosterols		**B>A** (50%)			**B>A** (30%)	
Carotenoids	=	=	**B>A** (50%)		**A>B** (>10%)	**A>B** (20%)
Isoflavones					**A>B** (30%)	
Vit B1	**B>A**	**B>A**	**A>B**	=	**A>B**	=
Vit B2	**B>A**	=	=	=	=	=
Vit B3	=	=	**A>B**			=
Vit B5	**B>A**	**A>B**		**B>A**	**B>A**	
Vit B6	**B>A**	=	=	**A>B**	=	**A>B**
Total folate	**A>B** =	**A>B**	=	=	=	**A>B**
Vit C				**B>A**		**B>A**
Alpha-tocopherol	**A>B**	**A>B**	**B>A**	=	**A>B**	
Beta-tocopherol	**A>B**		**B>A**		**A>B**	
Gamma-tocopherol	=				**A>B**	=
Delta-tocopherol					**A>B**	**A>B**

Vit H	=	B>A	=	=	=	=	=
Vitamin K	A>B	B>A	A>B	=	A>B	A>B	A>B
Cd	B>A		B>A	A>B			A>B
Mo	A>B		=	=	=	B>A	B>A
Se	B>A		A>B	B>A	B>A	A>B	B>A

A>B or B>A – the concentration in the A, resp B ingredients was >10% higher for the macronutrients or >20% higher for the micronutrients, =: concentration differences were <10% for macro- and <20% for micronutrients. Empty boxes, ratio could not be calculated because one, or both concentrations were beneath detection level.

** for wheat two comparisons were made CC2/X2 and T2/X2. CC2 was used in the first chicken feed. When both ratio's were in the same direction, only A>B or B>A is indicated. When the ratio's deviated from 1.0 in different directions, both results are presented in the table, with the results for the CC2/X2 ratio presented first.*
‡ dry matter is defined as: total weight minus moisture.

From these results it can not be concluded that in general ingredients from organic or conventional production systems show the same pattern with respect to protein content or other macro or micronutrients. In Chapter 7 the representativity of these samples for conventional or organic cultivation will be discussed.
Table 6.2 summarizes the differences for the feed groups A and B, measured and calculated.

Table 6.2: Summary of differences observed between group A and group B feeds.

B>A or A>B	Nutrient	Difference	(possible) origin
B>A	Amino acids	0-20%	Wheat, soy and barley
B>A	Protein	10%	Wheat, soy and barley
B>A	Chloride	0-40%	All
A>B	Iodide	10%	All
A>B	Alfa-tocopherol	0-20%	Soy
A>B	Total folate	0-30%	Peas
B>A	LPS endotoxins	20-240%	Source unknown
Concentrations based on *calculation*			
A>B	Carotenoids (except alpha-carotene)	0-50%	Maize, peas and soy
B>A	Alpha-carotene	10%	
B>A	Vitamin C	20%	Various ingredients
B>A	Vitamin B5	20%	Wheat, soy, maize
A>B	Vitamin K1	10-25%	Barely, peas, soy
B>A	Phytosterols	0-30%	Soy, barley
A>B	Isoflavones	0-30%	Soy

6.1.4 Differences in effects in the chicken

The second research question: 'Can biomarkers be identified, for health effects, related to the consumption of organic compared to conventional feed? ' will be worked out here. Biomarkers are taken here as differences, and the research question is thus differentiated:
Is there a difference in the developing immune system of chicken fed with the two different feeds?
Do differences occur in the functioning of other organ systems related to positive or negative health effects connected to the consumption of the different feeds?
Differences will be summarized in general health features, immunological effects and other physiological measurements. An overview with respect to the main significant differences in the animals will be presented.

Differences occurred in most physiological areas being investigated, occasionally differing between the lines. Results of the second generation of the C-line animals are weighed most heavily, as they represent the whole range of

genetic and biological diversity and have been fed the experimental feeds A or B from day one. Results in the H- and L-line animals are especially interesting if they differ from the C-line animals, as they then express an interaction between their genetic makeup and the environment, being the feed. However, in metabolomics, genomics, pathological anatomy and some other small investigations these H-and L-line animals were represented only in smaller numbers (6 per group), while in principle all analyses were performed on all C-line animals (approx. 25 per group).

General health features – General conclusions

There was no difference in health problems: both generations and feeding groups (A and B) were considered healthy. Weight and growth rate were discriminating factors between A- and B- feed for the C-line animals in the second generation. The H- and L-line animals did not show these differences. The C-line animals on feed B gained significantly more weight than the animals on feed A. Growth rate showed the same dynamics for A and B in this line, but this changed after the challenge with KLH. Then, in both feed groups growth declined strongly for a short period. After two weeks the animals of both groups started to grow more again, while the animals on feed A regained growth significantly stronger than the B-feed animals. This was also the case for the H- and L-line animals (Figure 5.13). Feed intake discriminated in the second generation alternating between A- and B-animals. In general group A ate less. Only in week 10, after the challenge, feed intake between group A and B was almost equal.
A statistical extrapolation towards the animals adult age, resulted in an equal adult weight for both groups.

The first generation did not show a difference in weight between the A- and B-animals and slight differences in growth-rate. Two factors may have been of influence here. First the moment in life when the feeds started to be consumed, being at 7 weeks for the first generation and from hatch in the second generation. Secondly the second generation might be influenced, through maternal influence in the egg, by the feed in the first generation.
Egg production in the first generation did not differ significantly between A and b-animals, but A-animals started to produce earlier and produced relatively more 1st choice eggs.

Feather development in the second generation differed, but not significantly, such that B-animals were enhanced in feather development. This might also have had an influence on weight development differences between A and B-animals. This will be discussed in Ch. 7.

Immunological assays – General conclusions

In this study various immunological parameters were studied in the two generations of laying hens, focussing on both the innate and specific immune system. Typical innate parameters are the in vitro production of NO by monocytes and their response to stimulation with LPS, plasma levels of natural occurring antibodies against LPS, LTA and KLH (only first generation), and the presence of complement enzymes, by classical and alternative activation. The specific immune system was characterized by measuring the in vitro proliferation of peripheral blood cells, and their response to LPS and Con A, two well-known stimulants, the antibodies against a number of vaccines applied to the animals by convention (NCD, Gumboro) and the effect of water-soluble feed extracts on the Con A induced proliferation of isolated blood cells. The feed changes, as well as the intervention by KLH in the second generation, were considered as triggers. Clear differences were observed related to both the different chicken lines, the different feeds and the changes in feed. In this regard it is important to realize that the first generation of chicken switched from Original feed

to Grower feed (termed week 0) and later on to Layer feed (week 7), whereas the second generation started on Starter feed and later on switched to Grower feed (termed week -2).
From section 3.4 it appears that feed B contains higher endotoxin contamination than feeds A, particularly for the first generation. This might have induced an LPS-tolerance in the B-animals which could explain several results. This will be discussed in Ch. 7.4.

First generation – conclusions In the first generation the monocytes in the whole blood cells of chicken fed with feed A reacted more with NO production in medium cultures than those of feed B. Therefore, the intrinsic activity of the monocyte compartment in the blood, in the absence of a stimulus, seemed stronger to feed A than to feed B. LPS stimulation in vitro resulted in more NO production from monocytes of chicken of line C, fed with feed B (Figure 5.20). The NO production in LPS stimulated cultures however, was 10-20 times higher than the NO production in non-stimulated cells, but was not significantly affected by feed. This differential feed effect was clearly not observed in the in vivo serum levels of natural antibodies and reactivity of the complement system, although levels of anti-LPS antibodies were higher in L-line chicken on feed A (Figure 5.22-5.27).

Second generation – conclusions In the second generation, the feed change from Starter feed to Grower feed, two weeks before the KLH challenge, had effect on chicken of all three genetic lines. In particular, animals from the feed A group displayed higher in vitro LPS-stimulated NO production by monocytes (Figure 5.35-5.36).
LPS-binding natural antibodies showed, during the KLH challenge, first a significantly increased titer in the chicken fed with feed A, and later in the animals on feed B (Figure 5.37). The latter could be due to KLH induced T-cell reactivity and cytokine production overcoming a neonatally induced LPS tolerance (See also Ch 7.4). This would lead to an overshoot in LPS binding natural antibodies, particularly in the H-line (Figure 5.38). When subsequently the LPS-binding natural antibodies drop, there is again increased room for expansion of LTA-binding natural antibodies in animals on feed B (Figure 5.39-5.40).
The reactivity of the classical complement system was stronger in all feed A animals, shortly after the challenge (Figure 5.42).
Of the vaccination parameters, reflecting the specific compartment of the immune system, the rise of the Gumboro-titers was significant for the A-animals (Figure 5.50).
In the lymphocyte stimulation assay, addition of both feed extracts to Con A stimulated whole blood cells cultures, did result in more proliferation of feed A lymphocytes, as compared to the Con A only control (Figure 5.51).

Overall conclusions immunology The change in feed reflects in the first generation the global reactivity of the chicken's immune system. The observed activation of monocytes supports the potential adjuvant-like activity of endotoxin or mitogen contamination, which can potentially modify the gut bacteria induced by the change in feed. The LPS and LTA natural antibody levels reflect the possible differences in the gut microbiota in the two feed groups which are of crucial importance in shaping the immune repertoire. Since the underlying T-cell driven immune activation in generating LPS vs LTA natural antibodies are mutually different, the level of LPS-binding natural antibodies mirrors the level of LTA-binding natural antibodies in kinetics and concentration. The continuous rise in LTA natural antibodies is accompanied by a rise in KLH-binding natural antibodies. The NCD vaccine is a living vaccine with a strong and continuous increasing specific antibody formation. In the L-line the NCD-specific antibody response appears to be suppressed in the feed B group, like the LPS and the KLH natural antibody responses. From

these data it appears that the feed A group shows a stronger immunomodulatory capacity than the feed B group. The L-line is able to distinguish these activities in a more sensitive way than the two other lines.

In the second generation the KLH challenge showed the most profound effect in the C-line animals. Vaccine-specific antibody responses display their individual kinetics. Gumboro-specific antibody titers are possibly decreasing while NCD-titers continue to rise. As yet not many data are available on possible interference of these responses in vivo. In some parameters of immunity divergence between A and B starts at week 4, which is the last time point analyzed. Therefore, there is no information beyond this point in time. This could reflect that either the analysis was stopped too soon after the induction of measurable differences in immune responses, or that most of the immunomodulatory activities have already occurred at earlier points in time.
As is evident from literature, the primary immune response as such is not easy to alter (Chowdury 2005). All the immune mechanisms, when in place, function directly and in concert to result in activation of the innate immune system, induction of T-cell reactivity, and antibody formation. Our KLH challenge experiments in the second generation, besides being a strong T-cell dependent antibody inducer, did not result in significant changes in KLH-antibodies between the two groups of chicken. A repeated, secondary immune response is much more sensitive to reveal small, but significant, changes in immune reactivity resulting in differential antibody responses. Due to the limitation of the feed compounds, a second KLH challenge of the second generation, which would have provided more information, was not possible.

Based on the qualitative and quantitative aspects of the immune system analyzed here it can be concluded that the feeds A and B have different immunological effects in both generations. This could be explained by different composition of macro- and micronutrients, anti-oxidants or mitogens, or contamination by e.g. endotoxin. Moreover, the second generation analyzed here may have, due to genetic imprinting from the parental strains, altered their epigenetic control and the kinetics of their gene transcription profile. In this experiment these possibilities have not been studied.
In total, it appears that the chicken fed on feed A show the highest potential for immune reactivity as reflected in vitro by enhanced innate responses by monocytes prior to KLH immunization (Figure 5.36), by LPS-titers in blood (Figure 5.37), by KLH-induced classical complement activation (reflecting an activated immune system) (Figure 5.42), by Gumboro titers of the adaptive system (Figure 5.50) and the in vitro response to feed extracts in the presence of Con A (Figure 5.51).

Metabolomics – General conclusions

The metabolomics analysis was set forth as an additional study parameter next to the immunological measurements around the KLH challenge. The scope of the metabolomics analysis was to investigate biomarkers for the influence of organic and conventional food and to investigate whether the consumption of this food by test animals (chicken) would lead to measurable physiological differences. The study was carried out blinded, and the treatments were only known as treatment A and treatment B.

Major results of metabolomics in animals

- Using metabolomics data from plasma and liver a clear distinction was achieved between the treatment A and B was visible. This distinction was achieved in all three chicken lines H, L and C.
- In plasma several metabolites were found to react differently to the KLH challenge in the two treatment groups. In liver significant differences in concentration of several metabolites were found between the two treatment groups.
- 1 week after the KLH challenge, observed plasma metabolome changes include several FFA's and unsaturated LPC's, which can be interpreted as the remains of an acute phase reaction.
- In liver differences in concentration of several metabolites were found between treatment A and B.
- 4 weeks after the challenge liver metabolomics revealed increased levels of pentose phosphate pathway metabolite intermediates and several amino acids in group A.
- Metabolomics revealed only small differences between the three chicken lines (H, C and L).
- Observed differences in the metabolome could not be associated with any disease state.

Genomics – General conclusions

Microarrays The most striking result is down regulation of several independent genes that code for enzymes involved in cholesterol biosynthesis and the subsequent pathway of steroidogenesis in chicken fed with feed B. The statistical power of the results is quite weak; normally results would be considered significantly regulated when the q-value is below 5%. In this study the lowest q-value is 6.6%. This low statistical power could be caused by the fact that there are no large biological differences between the two feed groups. Both groups received the same food ingredients, only the way of growing the ingredients differed. The chicken intestines are not stimulated one way or another before analysis. The intestines are of normal fed animals in good condition. Under these conditions no extreme differences are expected between the two groups. The finding of at least 7 regulated genes that are involved in cholesterol biosynthesis or cholesterol processing, greatly improves the statistical power of the analysis.
Other genes found to be regulated can also be linked to the mevalonate pathway. Insulin-induced gene 1 (Insig-1) and ERNI were found to be upregulated in chicken fed on feed B. CD36 was found to be upregulated in chicken fed on feed B as well as Chemokine ah221, both involved in atherosclerosis development. B-G protein precursor was found to be down regulated.

q PCR confirmation The results of the microarray were could be confirmed by use of q PCR-analysis. When all chicken lines were studied as one group, expression of all three genes showed the same expression pattern as described for the microarray. The expression difference between chicken fed A or B feed of the isopenthyl diphosphatase gene (IPD) was found to be statistically significant. The expression differences of hemoglobin alpha chain gene (HAC) and acetyl coA synthase gene (AACS) showed the same tendency as found on the microarray, but the differences in expression found with qPCR were not statistically significant.

Pathological Anatomy – General conclusions

With the small numbers of birds in the H- and L-line groups and the small variation between the animals, no clear conclusions can be drawn concerning the effects of nutrition type on the histological patterns of the chicken. In general, the histological observations showed a variation that normally can be expected in the chicken. Remarkable

were the differences in relative weight of organs between A- and B-animals in the special lines. In the H-line animals the relative weight of the G-I tract was significantly higher in the animals on feed A than of those on feed B. In the L-line animals the relative weight of the liver was higher in the A-group, compared to the B-group. In the C-line the relative weight of the bursa was higher in the A animals.

Microbiology gut

Bacterial community composition in the manure was affected significantly by feeding regime, but not by chicken line. Bacterial diversity in the manure was not affected by feed source, but differed slightly among chicken lines.

Sensory analysis

The meat of the C-line animals on the two feeds differed significantly on the aspect of juiciness. The meat the B-animals was evaluated as juicier.

The meat of the H and L-line animals on feed A differed significantly on the aspects of typical chicken taste and appreciation. The meat of the H-line animals was evaluated as of more typical chicken taste and was appreciated more.

6.1.5 The second research question evaluated

The second research question: 'Can biomarkers be identified, for health effects, related to the consumption of organic compared to conventional feed? ' and the differentiation of the question:

Is there a difference in the developing immune system of chicken fed with the two different feeds?

Do differences occur in the functioning of other organ systems related to positive or negative health effects connected to the consumption of the different feeds?

can be answered positively. Many significant differences were measured, the full amount of which is displayed in Annex 14. Table 6.3 summarizes the main differences, as observed between the group A and group B in C-line animals, and L-line as exception, selected as most relevant by the different researchers. These differences are taken as biomarkers.

Table 6.3: Summary of biomarkers – considered relevant significant differences – observed between group A and group B in C-line animals. H- and L-animals mentioned as exceptions.

B>A or A>B	Measurements	Parameter	Comment
B>A	General health 2nd generation	Weight	
B>A	General health 2nd generation	Relative growth of body weight	Till 5 weeks of age
A>B	General health 2nd generation	Relative growth of body weight	From week 10-12 of age; after KLH-challenge
A>B	Immune System 1st generation	Innate Immune System - cellular Intrinsic activ. of Monocytes higher	After change to experimental feed
A>B	Immune System 1st generation	L-line Innate Immune System – humoral- LPS-antibodies	4 & 22 weeks after change to experimental feed
A>B	Immune System 1st generation	L-line Specific Immune System – humoral- Vaccination titre NCD	4 & 8 weeks after change to experimental feed.
A>B	Immune System 2nd generation	Innate Immune System- cellular Monocyte activity, LPS-stimulated	1 week before KLH, 1 week after change of feed
A>B	Immune System 2nd generation	Innate Immune System - humoral LPS- antibodies	2 weeks after KLH
B>A	Immune System 2nd generation	Innate Immune System -humoral LPS-antibodies	4 weeks after KLH
B>A	Immune System 2nd generation	Innate Immune System -humoral LTA-antibodies	4 weeks after KLH Pattern of LTA opposite to LPS
A>B	Immune System 2nd generation	Innate Immune System -humoral Complement – Classical pathway	1 week after KLH
A>B	Immune System 2nd generation	Specific Immune System –humoral- Vaccination titre Gumboro -DELTA	3 and 4 weeks after KLH
A>B	Immune System 2nd generation	Stimulation Index feed extraction test Feed A & B lymphocytes to Feed A extract	Dilution -4 & -3
B>A	Metabolomics 2nd generation	Majority of Amino Acid levels in plasma	Consistent with increased protein percentage in feed of group B
B>A	Plasma metabolites	Plasma Lipids - Lysophosphatidyl choline (LPC), FFA and glycerol.	1 week before KLH challenge
A>B	Plasma metabolites	Plasma Lipids - LPC, FFA and	1 week after KLH challenge.

		glycerol	Consistent with a stronger acute phase response in group A
A>B	Liver metabolites	D-ribose, D-ribulose and Fructose	4 weeks after KLH challenge. Indicative for increased pentose phosphate pathway activity in livers of group A
A>B	Liver metabolites	Histidine, Alanin, L-methionin, alpha Ketoglutarate and Vitamin E	4 weeks after KLH challenge. Markers of liver metabolism and food intake (vitamin E)
B ↓	Genomics - q PCR confirmed	Cholesterol biosynthesis pathway	Diverse genes downregulated
A>B	Organ weight fresh	Relative Gastro-intestinal weight in H-line	Significant
A>B	Organ weight fresh	Relative Liver weight in L-line	Significant
A>B	Organ weight fixated	Relative Bursa weight	Significant

7 Discussion

In this chapter several items are discussed; the representativity of our ingredients, the research design concerning the ingredients, literature on ingredients and animal experiments, as well as an attempt to integration of the feed and animal results. The chapter closes with a reflection on the working hypothesis in this project.

7.1 How well do the ingredients used represent the organic or conventional systems?

For the translation of the results from this feeding experiment to more general conclusions, it is important to know how representative our feeds and feed ingredients were for organic and conventional agriculture. We have searched for reference values for organic ingredients, both by contacting relevant feed producers or persons otherwise involved in feed production and by searching the literature. Contacting the feed producers (Schothorst, ForFarmers, Ekorundvee, dhr. Heusinkveld; CVB, Centraal Veevoeder Bureau, onderdeel Productschap Diervoeder) showed that no reference values for organic feed are available. Values for conventional ingredients are available (CVB) and these experts assume that no real differences with organic has to be expected (dhr. Heusinkveld). From the literature however, some differences between organic and conventional produced grains and other products have been reported (Heaton 2001; Woese 1997; Worthington 2001; Winter 2006) and therefore it seems reasonable to assume that differences between the ingredients will be reflected in differences in the feeds. From the results of our study these differences were indeed observed.

For the comparison of our results to the 'general' organic or conventional products, a comparison can be made for wheat and soy with the available information from the Swiss ongoing DOK trial (www.fibl.org). In this study biodynamic, organic and conventional cultivation is practiced for more then 25 years now, thus being the longest running study into this field. The samples were presented blinded to TNO and analyzed generally and microbiologically in the same run as the ingredients used in the feeding experiment.

In this study the wheat and soy samples from the DOK trial were considered as the 'golden standard' and were analyzed for amino acid content. In Table 7.1 and 7.2 the results for the amino acids in wheat and soy are presented. Comparing the results of the DOK trial with the results of our study shows that the highest amino acid levels are found in DOK sample D. This might partly be due to the low water content in this sample. On average the contribution of each of the amino acids to the total amino acid content is very well comparable between our samples and the DOK samples. Based on the amino acids, DOK wheat sample D and our sample B were assumed to have the same cultivation origin, and DOK wheat sample E was considered to be from the same origin as our sample A. After decoding this assumption showed to be correct. As our samples resemble the DOK samples, it can be concluded that for wheat good representative samples were used.

Table 7.1: Comparison of our results (B and A) with results from the golden standard (D and E) for wheat.

		DOK	DOK	T2	CC2 (feed1)	X2
	Sample	D	E	B	B	A
Nutrient	Unit					
Cystine	g/100 g	0,36	0,21	0,26	0,25	0,22
Methionine	g/100 g	0,26	0,15	0,21	0,18	0,17
Aspartic acid	g/100 g	0,68	0,44	0,58	0,51	0,46
Threonine	g/100 g	0,43	0,27	0,32	0,31	0,26
Serine	g/100 g	0,7	0,4	0,51	0,49	0,4
Glutamic acid	g/100 g	4,48	2,35	3,21	3,07	2,46
Proline	g/100 g	1,49	0,78	1,04	1,02	0,8
Glycine	g/100 g	0,58	0,36	0,46	0,43	0,37
Alanine	g/100 g	0,48	0,31	0,41	0,36	0,32
Valine	g/100 g	0,61	0,37	0,49	0,43	0,38
Isoleucine	g/100 g	0,51	0,29	0,39	0,35	0,3
Leucine	g/100 g	0,99	0,57	0,77	0,69	0,6
Tyrosine	g/100 g	0,48	0,28	0,35	0,33	0,28
Phenylalanine	g/100 g	0,66	0,37	0,51	0,47	0,38
Histidine	g/100 g	0,34	0,22	0,27	0,26	0,2
Lysine	g/100 g	0,37	0,27	0,32	0,29	0,26
Arginine	g/100 g	0,73	0,46	0,62	0,53	0,46
Tryptophan	g/100 g	0,17	0,12	0,16	0,16	0,12
Moisture	g/100 g	11,08	16,01	14,6		14,2

For soy, the values found in our study, are within the range of amino acids found in the DOK samples. The DOK sample N2 shows dramatically lower amino acid levels, which only partly can be explained by differences in water content. With respect to the amino acid pattern sample A looks more alike DOK sample B2 than DOK sample N2, which has less than half of the amino acid content of both our samples. After decoding DOK sample B2 and our sample A showed to be both from organic origin. Considering that these DOK samples give a good reflection of amino acid content in the specific agricultural systems (organic or conventional), it must be concluded from our data that our soy samples were no good examples of these agricultural systems. After decoding it showed that the conventional DOK soy sample had the lowest amino acid content. The organic DOK sample and both the organic and conventional soy sample used in this experiment had higher levels of amino acids. In our experimental samples

the amino acid content was even approximately 20% higher in the conventional than in the organic soy. The soy was from the same variety. It was reported from Austria, where our soys came from, that there is little difference between the organic and conventional management of soy production. This may explain the relatively small difference between our soy samples as compared to the DOK samples, in which organic and conventional management largely differs.

Table 7.2: Comparison of our amino acid results (B and A) with results from the golden standard (N2 and B2) for soy.

		6730-4339	6730-4340		
		DOK	DOK	J2	R2
	Sample	N2	B2	B	A
Nutrient	Unit				
Cystine	g/100 g	0,26	0,65	0,58	0,59
Methionine	g/100 g	0,18	0,53	0,53	0,51
Aspartic acid	g/100 g	0,59	4,31	4,2	3,6
Threonine	g/100 g	0,34	1,47	1,47	1,3
Serine	g/100 g	0,54	1,91	1,86	1,61
Glutamic acid	g/100 g	3,46	6,7	6,6	5,51
Proline	g/100 g	1,14	1,78	1,78	1,52
Glycine	g/100 g	0,47	1,58	1,54	1,34
Alanine	g/100 g	0,38	1,61	1,58	1,38
Valine	g/100 g	0,47	1,8	1,72	1,49
Isoleucine	g/100 g	0,38	1,71	1,68	1,47
Leucine	g/100 g	0,76	2,87	2,85	2,46
Tyrosine	g/100 g	0,38	1,37	1,38	1,18
Phenylalanine	g/100 g	0,51	1,87	1,88	1,58
Histidine	g/100 g	0,28	0,95	0,98	0,85
Lysine	g/100 g	0,31	2,39	2,35	2,07
Arginine	g/100 g	0,57	2,88	2,83	2,41
Tryptophan	g/100 g	0,14	0,5	0,48	0,43
Moisture	g/100 g	14,99	7,3	10,2	8,9

In the literature, the differences in proteins between two cultivation systems are reported more often. In the reviews performed in this area (Bourne 2002, Heaton 2001, Worthington 2001, Woese 1997) it was concluded that organic grain contains on average less proteins than the conventional grains, though some studies found that organic grain is

of a higher quality, because it contains more essential amino acids. In most of the studies, wheat has been investigated. To our knowledge no information on triticale has been published. The lower protein content in organically grown products can be connected to the lower nitrogen availability compared to the conventionally produce were nitrogen fertilisers, with easy available nitrogen, are used. When specific amino acids were investigated, organically grown maize was reported to have higher amounts of lysine, methionine, histidine and threonine, but lower isoleucine, leucine and phenylalanine levels (reviewed by Magkos 2003).
In the samples investigated within this study we did see a difference in protein and amino acid content between the wheat samples A and B, with wheat sample B having higher amounts of amino acids. The reported differences in levels of specific amino acids, as has been reviewed by Magkos et al., could not be confirmed in our wheat samples (Magkos 2003), though, researchers from KWALIS did report a relative difference between amino acids between ingredients A and B, with lower amounts of lysine, cysteine and methionine (relative to glutamine) in the A ingredients.
The microbiological researchers concluded that within the group of soy samples a clear difference can be seen between soy samples J2 and R2 on the one hand and samples N2 and B2 on the other hand. Both the aerobic colony count and moulds are lower for the latter samples. The latter samples are the samples from the DOK trial, the J2 and R2 samples are the samples used within the feeding experiment.
A similar difference can be observed for our wheat samples T2 and X2 vs. J33 and D33 from DOK. Samples T2 and X2 have a higher aerobic colony count and higher Enterobacteriaceae concentration but a lower concentration of yeasts compared to samples J33 and D33. Wheat sample CC2, used in the first feed, shows a lower aerobic colony number and lower concentrations of Enterobacteriaceae, yeasts and moulds compared to the other wheat samples. However it should be stressed that although differences were observed, a large variation in microbiological analyses like moulds, aerobic colony count and Enterobacteriaceae is expected and therefore it is difficult to state that the observed differences are significant. In Table 7.3 the results are presented, now with the decoding for sample A, B or DOK sample.

Table 7.3: Comparison of our results (B and A) with results from the DOK trials for wheat and soy.

Sample	Code	Aerobic colony count CFU/g	Enterobac-teriaceae CFU/g	E .coli CFU/g	Yeasts CFU/g	Moulds CFU/g
Soy B	J2	2,2 x 105	< 10	< 10	< 10	1,2 x 104
Soy A	R2	3,1 x 104	< 10	< 10	< 10	5,3 x 102
Soy DOK –conv	N2	1,9 x 103	< 10	< 10	< 10	< 10
Soy DOK – org	B2	5,7 x 103	< 10	< 10	< 10	ca 15
Wheat B	T2	4,8 x 106	1,4 x 105	< 10	<100*	7,2 x 103
Wheat A	X2	8,6 x 106	>1,5 x 105	< 10	1,0 x 103	5,6 x 103
Wheat DOK – org	J33	9,2 x 105	2,0 x 104	< 10	1,1 x 104	2,4 x 103
Wheat DOK – conv	D33	7,9 x 105	2,0 x 104	< 10	1,6 x 104	1,2 x 103
Wheat B (feed 1)	CC2	5,8 x 104	7,2 x 103	< 10	< 10	ca 45

Though the researchers did not know of the origin of the samples, they have observed clear differences between the samples from our study and the DOK trial samples. From this we must conclude that with respect to aerobic colony count and moulds differences exist and that our samples from this point of view are not comparable with the 'golden standard' DOK-trial. This may be due to different countries with different soil and weather conditions from which the products were collected (see Annex 2). The differences between the place of origin of the sample therefore seems larger than the difference in cultivation practice (organic or conventional).

Complementary analyses

In this feeding experiment we used complementary analyses. Based on experience of the researchers from KWALIS (biophoton assessment, protein ratio) and Louis Bolk Institute, University of Kassel and BRAD (biocrystallization) researchers have made assumptions on the organic or conventional background of the analysed samples.

Biophotons From existing experience in comparing wheat from different backgrounds, the researchers assumed that sample E was organic and Z was the conventional sample. Because of the lack of barley and triticale training samples, no discriminant analysis was performed. Based on the wheat experience led to the assumption that barley sample W and triticale sample C were organic. After decoding it showed that the identification was correct for wheat and barley, not for triticale. From this result is shows that our samples do resemble other wheat samples from organic and conventional origin, with respect to biophoton emission.
On the basis of the biophoton measurements, also maize was correctly identified. For peas and soy no interpretation was given because of lack of experience with these legiminosa.

Protein ratio Based on the amino acid ratios (Table 3.31) researchers from KWALIS made an assumption with respect to the origin of the samples. In general it was said that conventional samples have a higher raw protein content, but a lower protein quality measured in terms of percentage of the essential amino acid of raw protein. This assumption was based on earlier experience with wheat samples. The samples of wheat, barley and triticale showed differences in protein-N content, the Glu/Lys and Pro/Lys ratio, similar to those which have been measured between the organic and conventional production systems for wheat in the samples of the DOK-Trial during 4 different harvesting years. For soy there was no such experience, but difference in protein-N content and also the ratio of Glu/Lys and Glu/Cys and Glu/Met seemed to indicate conventional cultivation of soy sample A. In conventional samples often higher protein-N-content is found. The relative content of rare amino acids as the sulphuric amino acids Met and Cys in relation to Glu (Glu/Cys; Glu/Met) are often higher in organic grown plants. From this it was concluded that samples wheat Z, barley K, tricale I and soy A were from conventional production according to their amino acid ratios. For maize there was only a little difference and for peas there was practically no difference in the amino acid state of the two pairs, so no prediction of the cultivation system was made. After decoding it showed that the researchers correctly classified wheat, barley and soy. Triticale not.

Biocrystallizations The crystallizations of all ingredients and feeds, except the peas and Layer feed, could be differentiated highly significantly according to the production system, by means of computerized image analysis and/or by 'Two Group Testing' in visual evaluation. Thus, with this method a difference between the agricultural background of the products can be observed. With the crystallizations, the researchers had experience with some of the ingredients used in this study. With the experience in wheat, researchers from the LBI and University Kassel

were able to identify the DOK samples correctly. Wheat samples were correctly identified as being organic by the researchers of LBI and University Kassel was able to correctly identify barley on the basis of their experience with this ingredient.

Conclusions with respect to representativity

In this experiment the DOK samples were considered the golden standard, because these samples are the result of 25 years controlled production of organic and conventional agriculture. When comparing our data with this golden standard, we may conclude that there is a resemblance for the wheats, however, for soy this can not be confirmed. Especially in the complementary analyses it appeared that experienced researchers saw the same 'pattern' in our samples, as seen in samples in previous comparisons between organic and conventional products. This does imply that, although maybe not totally comparable, a general 'picture' of samples from organic or conventional origin can be formed by these methods.

At the start of the study, also because of the state of knowledge at that time within FQH, it was decided to try to have samples as standardised as possible and to have the samples characterized. Characterization was performed. Within this project all macro and micronutrients of the ingredients have been analysed. Standardization was hardly possible. Though all possible effort was made to find samples as standardised as possible, or at least from neighbouring farms, differences in varieties and other factors will inevitably have influenced the final product. Standardization therefore seems a good idea, though it does not reflect practice. Within the FQH-research association, standardization as such is not the general goal anumore. The search for 'best practice' organic or conventional farmers, from the same neighbourhood, seems a more realistic way to do research in this area. This also implies that differences in varieties, crop rotation etc. will occur as part of the agricultural system of the farm.

7.2 Reflections on the design of the experiment in relation to the choice of crops

Research on organic versus conventional agriculture: farm comparison or market average comparison? A comparison between organic and conventional agriculture seems clear but is not clear at all. 'Conventional agriculture' is not a well-defined object. 'Organic agriculture' has a set of well defined restrictions (the minimum norms, in Europe defined by law) and a set of open-described intentions (published by IFOAM 2004). The agronomic practice can and will differ substantially from farm to farm, both in conventional and organic agriculture. This wide definition of the two systems implicates that many parameters which might be part of a research question can be expected to have only limited power for discrimination between conventional and organic (Figure 7.1). An exception might be the presence of pesticide and herbicide residues on products: organic products are supposed to have none while conventional might have a certain level. And even this parameter is not without exceptions: drift from neighbour farm spraying might infect an organic crop; organic produce might become polluted during storage or transport in uncleaned storage boxes and trucks, and organic farmers might use pesticides although it is not allowed.

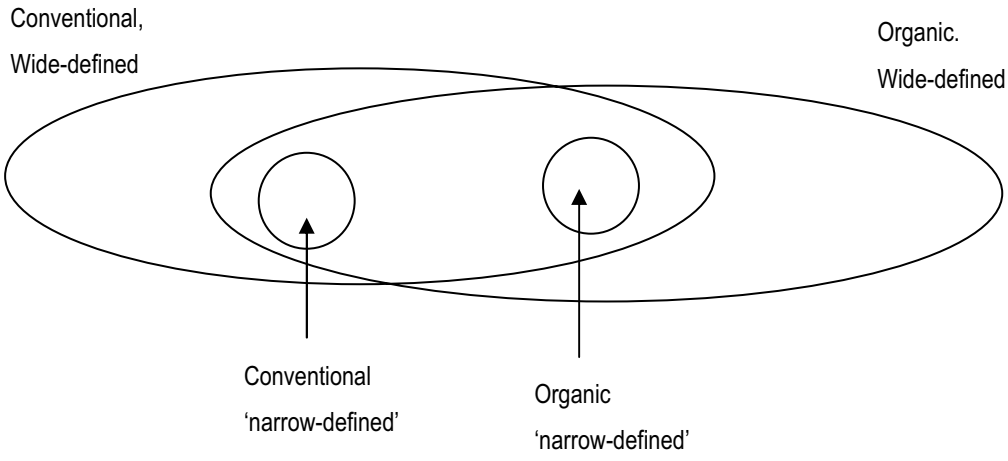


Figure 7.1: The range of differences between organic and conventional production methods.

We are interested in a difference between organic and conventional products in a feeding experiment. At the start of the experiment we did not know which parameters of the chicken feed would be discriminating between organic and conventional agriculture nor did we know which parameters might possibly have an effect on chicken health.

There are four ways to design such an experiment. The first two are based on samples out of the market; the second two are based on products from one farm each.

1. For each feed component, take many samples out of the market, both conventional and organic. In this way you suppose that you reach an acceptable average on all parameters. All product charges are analysed separately. From these products you make chicken feed in all possible combinations of the original components. This experiment can answer the question: has the average organic chicken feed produced this year in this country a different effect on chicken health parameters than conventional chicken feed, conventional being defined as 'not-organic'. This experiment is not possible because of the exploding number of chicken feed charges if you want to investigate all combinations.
2. For each feed component, take many samples out of the market, analyse them separately and make a mixture of samples for each component. Then make chicken feed out of the mixed components. This experiment can answer the question: has this unique mixture of organic chicken feed produced this year in this country a different effect on chicken health parameters than this unique mixture of conventional chicken feed, conventional being defined as 'not-organic'. This experiment is possible, but since we do not know what parameter will be discriminating nor the variability of this component between the samples, we will have to take many samples.
3. Give clear and narrow definitions of what we call 'conventional' and 'organic' with substantial differences in agronomic properties from which we can expect effects on the product properties (Figure 7.1). Then start or use an existing experimental farm with these two systems side by side, and produce the requested feed components. This experiment can answer the question: has this narrow-defined organic chicken feed produced

this year in this country a different effect on chicken health parameters than this narrow-defined conventional chicken feed? This is a perfect test design but not unproblematic in practice since it is nearly impossible to produce all components in one climatic zone.

4. Give relatively narrow definitions of what we call 'typical conventional' and 'typical organic' and try to find nearby 'best practice' farms, which fit in the definition and can produce the requested component. This experiment can answer the question: has this narrow-defined organic chicken feed produced this year on these farms in this country a different effect on chicken health parameters than this narrow-defined conventional chicken feed?

Two main differences might effect product quality: the use or absence of crop protection sprays, and the way the crop is supplied by nitrogen: nitrogen dynamics. Besides this, variety choice is an important factor. For a comparison study the variety used in conventional and organic system would ideally be the same, but on the other hand variety choice is also a system-bound component and then a forced variety 'choice' introduces a non-system property in a system comparison, which is nonsense. Then, a whole set of parameters might have influence such as soil type, local weather conditions and year to year variations of the weather, crop rotation, farm biodiversity etc. These are partly system dependent and partly system independent, and they all complicate the answer to the question: is organic better for whatever purpose, compared to conventional production?

Synthetic crop protection is a well-defined difference between conventional and organic agriculture. The amount used in conventional agriculture will vary from field to field and from year to year, but no sprays at all is very uncommon in Dutch and west-European agriculture. In organic arable farming normally no direct crop protection is used. We don't expect a big 'overleap' as suggested in Figure 7.1, giving 'crop protection' the status of a good discriminatory parameter in the context of a broad definition of conventional and organic agriculture.

The nitrogen dynamics is point of concern. The overall available nitrogen level in organic agriculture is lower than in conventional agriculture (Mäder 2002), but yields are lower too, so relative to the yield the differences are small. The use of slurry and the use of manure pellets or vinasse in organic agriculture also diminishes the difference between conventional and organic. Nitrogen out of these fertilisers is easily available (Janmaat 2005), creating peaks in soil available nitrogen. We indeed expect a big 'overleap' as suggested in Figure 7.1, giving 'nitrogen dynamics' the status of poor discriminatory parameter in the context of a broad definition of conventional and organic agriculture.

Nitrogen dynamics and overall available nitrogen level are known to have a big influence in crop development, yield and product properties, for example protein content, amino acid composition and nitrate content (Bloksma 2001, Bloksma 2004, Northolt 2004). This on its turn might have influence on chicken health properties. This was an important reason to choose for an experimental design resembling option 4, including a relatively narrow definition of both conventional and organic agriculture including aspects of the nitrogen dynamics. The answer of this experiment might thus be that organic products in general potentially have a different effect on chicken health than conventional products, but it will not prove it in general. The last could be realized in experimental designs from option 1 and 2, or could be proven by finding the parameter(s) which cause the health effects and then do a market survey on this (these) parameter(s) to find the variability of it.
In the present study, the mentioned option 4 and secondly option 2 were the only realistic ones. To choose between these two options we had a look at possible differences between conventional and organic agriculture which have an

effect on product quality, and we considered the Dutch organic practice, as well as foreign practice for several ingredients.

7.3 Comparison with literature concerning the feed ingredients

From the literature differences in composition of products from the different cultivation systems have been reported. Not all nutrients and contaminating factors have been reported in literature. Here we report an overview of the existing literature on compounds which have been studied thus far.

Mycotoxins In both conventional and organic grain production, the risk of mycotoxin contamination exists. From studies performed thus far it seems that the risk of mycotoxin contamination is slightly lower or equal in organic than in conventional food production. In a study in the Netherlands (Hoogenboom 2006) contamination occurred at a same level in both production methods. Levels were under detection level till a rainy period, after which mycotoxins were present in both cultivations. In a review of the organic center (Benbrook 2004) it was concluded that in the 24 comparison studies, mycotoxins were detected in conventional food about 50% more often than in organic food and levels reached in conventional food a little over twice as high as in the corresponding organic food (Benbrook 2004). Levels up to 106 ug/kg were found in organic wheat and 69 ug/kg in organic barley. Doll et al. (2002) reported higher levels of DON in conventional wheat (1.540 ug/kg vs. 780 ug/kg organic) and rye (490 ug/kg vs 130 ug/kg) and Birzele et al (2002) in conventional wheat harvested 1997 (250 vs 100 ug/kg) and 1998 (485 vs 300 ug/kg in conv vs. org). That no or small levels of mycotoxins were found in our feed ingredients leads to the conclusion that in the cultivation year in which our products were produced, the weather conditions were preventive for mycotoxin production.

Fatty acid composition From the DOK trials or data presented in literature, no information on fatty acid composition in grains is available, therefore we can not compare our results with other information.

Vitamins and Phenolic compounds From the literature it is argued that organically produced foods may contain a higher amount of vitamins and phenolic compounds (Brandt 2001). Two hypotheses are postulated which might explain this possible increase in vitamins and phenolic substances in organic versus conventional foods. The first hypothesis is based on the growth and differentiation balance (Bloksma 2002). A plant growing on conventional fertilisers with ready available nitrogen may accelerate plant growth resulting in a reduced production of secondary plant metabolites. The second hypothesis considers the response of plants to stressful conditions such as attacks from insects and weeds. Because of limited use of insecticides and herbicides plants may have greater stresses and therefore require a better own chemical defence mechanism (Winter 2006). In several studies higher phenolic compounds were reported in berries, leek, onions, tomatoes from organic compared to conventional production systems (Alfödli 2006, Winter 2006, Benbrook 2005). For grains only a comparison in vitamin B content has been reported (reviewed by Woese, 1997). In the two studies in this area, no difference between organically and conventionally produced grains was observed. For the other vitamins, no comparison studies have been reported in literature. From a study in which 11 conventional wheat varieties were compared (Adom 2003) it is known that large differences in carotenoid levels can occur between different varieties. In this study differences were reported with 5-fold, 3-fold and 12 fold differences in lutein, zeaxanthin and beta-cryptoxanthin between different wheat varieties,

respectively. In our study differences in vitamin Bs were reported, most consistently in wheat were higher levels of several vitamins B were reported in wheat B. Based on calculations out of the ingredients, higher levels of carotenoids were calculated in the feed samples for group A. The most abundant carotenoid in the feed was lutein. When the ingredients were investigated, the lutein and zeaxanthin levels for all ingredients, with the exception of triticale, were higher in the samples A. This observation is in agreement with the previous mentioned hypothesis on increased vitamins and micronutrients in organically produced products, Following the observations in the study of Adom et al (2003), it however should be noted that in the observed differences in carotenoid levels the differences in varieties may have played a role.

Vitamin C is commonly said to be higher in organic products. In fact, on the basis of the literature it can be concluded that on average vitamin C content is higher in organic leafy vegetables. For other products, no information exists or no consistent pattern is observed (Huber 2006). In our study we found higher vitamin C levels in the B sample (which turned out to be the conventional samples) for peas and soy. In how far this coincides with the organic and conventional practice can not be concluded due to lack of information with respect to grains or leguminosa.

Organic products are generally said to have a higher dry weight content. It should however be noted that this statement is based on studies investigating dry weight in vegetables and fruit (Woese, 1997). In our study no differences between feed A and B ingredients in dry weight (calculated as total weight minus water content) were observed. Whether this was to be expected in grains or leguminosa can not be concluded on basis of available literature.

7.4 Comparison with literature concerning the animal experiments

The first feeding trials focusing on production methods have been conducted in the 30ies of the last century. Their purpose was to compare the effects of organic resp. bio-dynamic versus mineral fertilisers on product quality and on possible health effects. Wöse (1992) gives a comprehensive description of these early endeavors: The test animals used were laboratory rats and mice as well as chicken. These very first findings showed a higher weight gain and better rearing success in rats and mice (McCarrison 1926, Scheunert 1935, Pfeiffer 1931) and a higher egg production in chicken (Pfeiffer 1932, 1934) when organically fertilised products were fed. Later feeding experiments still testing fertiliser effects detected no difference between the test rats resp. rabbits (Miller 1958, Alter 1978). So far the parameters tested in the feeding trials were growth rate and offspring development.
A new organ-centred approach was used by Ähnelt (1973). In this case the fertility parameters ovulation points, ovary weight and fertilised ova showed advantages of the feed grown with organic fertilisers. Bram (1974) found smaller nuclei and fewer cells in the adrenal cortex when feeding his rabbits with mineral-fertilised feed. Another feeding trial focusing on organic and mineral fertilisers was conducted by Neudecker (1987) with laboratory rats and mice. He found no differences concerning fertility parameters between the feeding groups.
Gottschewski´s feeding experiment with rabbits was among the first ones investigating food from different farming systems as opposed to different fertilising methods (Gottschewski 1975). His findings were later corroborated by two more rabbit experiments concerning significantly better rearing successes due to fewer perinataly dead and more weaned pubs in combination with a superior weight development in the organically fed groups (Edelmüller 1984, Staiger 1986).

Similar results were obtained when feeding laboratory rats with organic versus conventional feed. Again in the organic group significantly fewer offspring were born dead or died within the first week of their lives, the survival rate until weaning time at the age of 28 days and the weight development were slightly more successful and the weight gain of the female rats in connection with litter size and pup weight during lactation was significantly higher (Velimirov 1992, Velimirov 2005b).
In this last case the diets were of the same nutritional quality according to the main components.
Most of these studies are all more than twenty years old and represent farm practices that will be quite different from present farming. Levels of fertilisers will have increased, whereas the use of pesticides will have decreased in conventional agriculture. It is noteworthy that in most cases weight development is described as a marker for dietary effects, being enhanced in organically fed animals. Noteworthy are also the organ effects that are described.

Two recent studies comparing possible health effects of organic vs conventional produce, and of low vs high fertilized feed on animals, are also only partly comparable to our present study. However, both are investing sensitive markers for influences of different feeding regimes on health aspects and from that perspective relevant for our questions.
Finamore (2004) describes a pilot of a novel approach for evaluating food safety. Organic food might contain higher amounts of natural toxic compounds and in this study effects are evaluated of organic and conventional wheat samples, containing different amounts of mycotoxins, on rats in a vulnerable state.
Eight varieties of wheat were grown on neighbouring fields, either organic or conventional. The different varieties were mixed in the feeds. Young, just weaned male rats were divided in two groups receiving organic or conventional wheat during 30 days. Each group was divided in two subgroups of well nourished or protein-energy-malnourished (PEM, being fed 8% protein) rats. With the PEM rats a more vulnerable condition was created. Evaluated were weight, feed intake, lymphocyte proliferation in several tests and acute phase proteins from the liver. Lymphocyte proliferation is known to be damageable by toxins; the acute phase reaction was expected to be a marker and was taken in exploratively. In the well nourished animals no differences were found in these parameters. However in the PEM group, in one of the tests performed, the proliferative response of the lymphocytes was relatively higher in the organically fed group and inhibited in animals in the conventionally feed group. The authors describe this inhibition as a sign for a risk for lymphocyte function in vulnerable situations. They conclude that their design is valuable for evaluating health risks by food. After deblinding it can be stated that these results of influenced lymphocyte proliferation, reflect results in our study, although it is animals were not in a vulnerable state.

Lauridsen (2007) describes a study in rats in two generations, to identify health related biomarkers affected by different growth conditions in plants. Feed ingredients were grown in a controlled and comparable situation, in three different systems: Low (organic) fertilizer input without pesticides (LIminus P), low (organic) fertilizer input with pesticides (LIplusP) and high mineral fertilizer input with pesticides (HIplusP). The authors state that their design is not covering the question of 'organic' vs 'conventional' food. However, as a biomarker study in a field which approaches our question, we consider it as highly relevant.
The feeds were composed from six ingredients in equal proportions, being potatoes, carrots, apples, peas, green kales and rape seed. The feeds were supplemented to be iso-energetic and iso-nutritious, resulting in similar contents of proteins, minerals and dietary fibre. However with regard to vitamins and fatty acids still differences appeared to occur. No pesticide residues were detectable. Feed ingredients and feed were stored freeze dried.

Experimental animals were from the GKmol rat strain, which can develop type 2 diabetes. The rats of the second generation lived till 44 weeks of age. During their lives several nutritional metabolic measurements were performed like a balance test, heat production, a breath test and activity measurements. Post mortem body and organ weights were recorded, activity of liver and intestines was determined through oxygen consumption and respiration measurements, extensive biochemical measurements were performed in tissues and blood, as well as the humoral immunoglobulins IgG, IgM and IgA.

Significant differences were found in activity of the animals, the LIminusP animals having a lower day activity whereas no difference in activity occurred during the night. The aminopyrine breath test was indicative for a higher hepatic metabolic activity in the LIminusP group. The post mortem body weight of HIplusP animals was higher, which was connected to the 14% higher amount of adipose tissue. Of E-vitamins in plasma α-tocopherol was highest in LIminusP and lowest in HIplusP animals, whereas γ-tocopherol was lowest in the LIminus P-group. However, in liver and adipose tissues no vitamin E differences were observed. Fatty acid profile showed e.g. a lower C18:3 in tissues and plasma of the LIminusP. Liver lipid peroxidation was higher in LIminus P, compared to the HIplusP animals. Of the immunological parameters, IgG was lower in the HIplusP group, compared to both other groups. The authors state that the higher weight of the HIplusP animals cannot be explained by the other measured physiological and biochemical responses or the dietary analyses. The positive correlation with night activity is discussed and not clearly understood. Intensive measurements of activity as well as sleep duration are advised for future studies. The influence on the aminopyrine breath test is not understood, however the authors advise this test for future studies as it appears sensitive for diet induced changes in liver function. The higher IgG levels cannot be explained by the nutritional composition, and no conclusion can be drawn whether this response should be considered as an improvement of the immune status or not. The authors state that it is interesting that in the here described Finamore study, also the immune function seems to be affected by feeds from different production systems.

They conclude that within these dietary treatments with a similar nutritional quality, they found with regard to health markers notable differences in concentrations of IgG, α-tocopherol, day time activity, weight and volume of adipose tissue, liver metabolic function and liver lipid peroxidation. They advise that for future studies on organic vs conventional production systems, these biomarkers deserve further study.

This study is quite different from our study, first of all as fertilisation levels and the use of pesticides are compared and not organic and conventional systems. However, it should be clear that the LIminusP comes closest to the organic system, whereas the HIplusP is quite like conventional agriculture. A further big difference is that the feeds were supplemented towards e.g. equal protein levels. Nevertheless significant differences in weight and fat tissues are found. Further several differences reflect our findings, although measured in a different way. E.g. the immunological parameter, vitamin E and the liver metabolic function reflect our results. After decoding of our feeds it can be stated that, although the study design differed considerably, the results in the described and our study tend toward similarities, concerning the found differences in weight, in immune functions, in vitamin E and in liver metabolism in relation to feeds low fertilizer input or from organic sources.

7.5 An attempt towards integration of feed and animal results

Adverse effects as a results of contamination of feed?

Mycotoxins/pesticides No adverse effects on the chicken are expected from contamination with mycotoxins or pesticides. All feed ingredients were screened for the presence of mycotoxins and if a sample would have contained mycotoxins above the maximum residu level (MRL) it would have been omitted for the feed production. One maize samples (group B) did contain fumonisin B1, B2 and B3 at levels of respectively 0.39, 0.31 and 0.15 mg/kg. This sample was used for feed preparation because from the low levels in the final feed no adverse effects in the chicken were expected (comment Prof. J. Fink).

Raw soy contained small amounts of lectins. In practice, these soy beans were toasted prior to their use for feed production, which should result in the destruction of the lectins. This was confirmed by the fact that none of the feeds (81, 82, 83, 84, 93, 94, 95 and 96) contained lectin levels above the detection limit of 0.05 mg/g.Therefore, influence of lectins on chicken health is not expected.

Heavy metals In one sample of barley (sample G2 of group A) high levels of the heavy metals arsenic (As) and lead (Pb) were found. However, we specifically analyzed the feeds for Pb and As and the results did not show consistent differences between the group A and group B feeds. Therefore, it is unlikely that potentially adverse effects of these elements can have influenced chicken's health in only one of the two groups.

Microbiology and endotoxins The microbiological analyses of the various feed and ingredient samples have not shown any concentration of microbial species or any pathogenic contamination in any of the samples that might have had a direct adverse effect on the health of chicken. Between feed samples, differences in colony forming units (CFU) were evident but neither consistently found in one of the two feed groups, nor were they of pathological relevance. The source of the LPS endotoxin difference of B > A is unclear. Endotoxin levels are known to influence immunological processes. A possible effect will be discussed below. Further, in both samples of soy small amounts of anti trypsine activity was observed. As these were beneath the reference value of 3 mg/kg (Schouten Products), no harmful effect is expected.

Macronutrients and growth rate To prevent any deficiencies because of insufficient essential amino acids or specific trace elements, we have supplemented the feed with the necessary nutrients. As methionin is an essential amino acid, known to be low in organic feed, we specifically tested the feed before use and supplemented till the minimally needed level. Therefore, any deficiencies are not to be expected in the chicken. However the digestability results suggest a low methioninein feeds 1-4. This feed was used only for the first and not for the second generation of chicken. One might consider if this explains the lack of difference in weight development between the animals on feed A and B in the first generation. However, amounts of methionine, supplied in feed 1 and 2 were even higher than the amounts added to the same feeds in the second generation, thus shortages are not very probable. There is no explanation for this discrepancy.

Two important factors influence chicken growth and development during the first period of development, through pullet phase till the prepuberty phase, being energy intake and the amount of amino acids in the feed. Consistent

differences were found regarding the amino acid composition of the feeds. Most amino acids, i.e.12 out of 18, were higher for group B in all analyzed feeds. The total protein content was approximately 10 % higher (w/w) in all feeds for group B compared to group A, which is in accordance with the higher amino acid content. The difference in protein content was compensated by a decreased fat percentage in Layer and Grower 2nd generation and by decreased ash content in Grower 1st generation feeds. Using an equation (shown in Equation 1) by Watt and Merrill (1976) metabolizable energy was estimated for the different feeds and the results are shown in Table 6.4.

Equation 1:
Metabolizable Energy=17 kJ/g protein + 37 kJ/g fat + 17 kJ/g total carbohydrate.

The calculated difference in metabolizable energies between the feeds of group A and B are minimal. The resulting energy to protein (E/P) ratio, however has a 10 % difference between B and A feeds. Changes in energy to protein ratio have been shown to affect the growth rate, plasma composition, energy metabolism, and endocrine functions (Swennen 2005, 2004). However, in the experiments of Swennen et al. two feeds with an E/P ratio of 31.5 and 16.5 respectively, were compared, whereas in the present study the E/P ratio ranged between 20 and 23.5 for feed A and 18.2 and 21.7 for feed B, respectively. Further, these reported effects were all investigated in broilers and not in laying hens. The magnitude of the reported effects by Swennen et al. is therefore not representative for potential effects in this study. Nevertheless, the study by Swennen et al. shows that feed differences in E/P ratio may cause a broad physiological response in chicken. For laying hens the energy/protein ratio seems of influence once laying eggs, and is of less influence in the period till puberty (the moment that our second generation chicken was investigated). For a good development of young layer hens the main factor of interest is a sufficient amount of amino acids, and especially the essential amino acids.

Table 7.4: Calculated metabolizable energy (ME) and energy to protein ratio for feed A and B and ratio between feed A and B for the different batches of feed.

Batch	**calc. ME (kJ/kg):**	**Energy per protein (kCal/g)**
1 (B)	14610	18.18
2 (A)	14480	20.00
Ratio B/A	1.01	0.91
4 (B)	14302	20.83
3 (A)	14487	23.54
Ratio B/A	0.99	0.88
8 (B)	14061	20.00
7 (A)	14059	21.81
ratio B/A	1	0.92
11 (B)	14882	21.68
12 (A)	14729	23.30

Ratio B/A	1.01	0.93
15 (B)	15102	18.13
16 (A)	15231	20.67
Ratio B/A	0.99	0.88

In layer hens deficiencies in amino acids may lead to a restricted growth. Further, it might lengthen the period till puberty, because this is highly dependent on the growth of the chicken (personal remarks by chicken feed and physiology specialists Dr. R. Kwakkel and Prof. E. Decuypere). Amino acids most essential to these chicken are the sulphur containing amino acids cysteïne, methionine and lysine.
In this study the bioavailability of the amino acids was studied. For the second generation, only the Starter feeds were tested. It was shown that in these feeds methionine was not a limiting amino acid. The most limiting amino acid was tryptophan, but even this was in sufficient amounts available that no real limiting effect is expected.
Though not limiting, there was a 10% difference in amino acid content in both feeds. This might have resulted in an intensified growth and development of the group B chicken, which can also have resulted in an increased fat deposit (observation of the butcher). Comparably it might have resulted in the group A chicken, in a relatively delayed growth and a slightly delayed feather development, which was an impression and could not be objectivated. However, if existing in a slight way, this factor of delayed feather development could have had an influence on the growth, because the 'naked' chicken will need more energy for keeping their temperature (personal comment of Prof. Decuypere).

Feed efficiency In the second generation the ratio growth rate / feed intake was higher for the B-fed group during nearly the whole period of observation. As the digestibility of the protein was higher in feed B, this could be expected. This relation showed however after the challenge a change. Two weeks after the challenge the ratio started to turn in a significant advantage of A. This coincided with a sudden stronger growth in the A animals. As the animals were lighter anyway, ratio turned strongly positive for that period.

Protein content and immune functions As feed B was 10% richer in protein, the question arises if this influenced the immune functions. A relationship between protein and immune competence is known, but mainly through the negative impact of protein deficiencies. Lochmiller (1992), Chandra (1992) and Mengeheri (1988) decribed effects from protein malnutrition, resulting in impaired immune competence. Here malnutrition meant approx. 8% protein instead of the 'norm' of approx.18% in chicken feed. However, information about effects of protein enriched feeds on immunological functions was not found in literature and among consulted specialists.

Bodyweight gain and immune functions Literature indicates that an enhanced status of the immune reactivity can relate to a lower bodyweight in chicken (Ploegaert 2007, Parmentier 2002, Klasing 1997, Parmentier 1996). This seems to coincide in our study as well.

Fatty acids and immune functions Small differences in fatty acid composition were found. From literature, some effects of specific fatty acids on immune responses have been reported (Parmentier 1997, Sijben

2001). In the same chicken lines, as used in this study, it was shown that the addition of linoleic acid (C18:2 n-6) enhanced antibody titres to SRBC (sheep Red blood cells) in the H-line as compared to a normal diet and a linolenic enriched diet. In the C-line, the linolenic acid (C18:3 n-3) enriched diet decreased antibody titres. No effects were observed in the L-line, thus resulted in the conclusion that an effect of diet is affected by genotype (Parmentier 1997). From human studies, it is known that especially the ratio n-6 to n-3 fatty acids is of importance for the homeostasis and a normal development (Simopoulos 2000). In all feeds, the n-6 to n-3 ratio was comparable, thus no health impact is to be expected. Only the ratio saturated to unsaturated fatty acids fluctuated over the different feeds. In the period of the challenge the feed sample of group A had about 10% higher values of both linoleic and linolenic acid as compared to the feed sample of group B. This might have had some influence on antibody titres as was shown in the study of Parmentier et al. (1997). However, in this study the level of the unsaturated fatty acids in the experimentally enriched diets was much higher (7% of feed weight), then that in our study. No effect on body weight was reported as a result of addition of fatty acids (Sijben 2001).

Endotoxins and immune functions It appeared that feed B contained a higher endotoxin contamination than feed A, particularly for the first generation. One might expect a stimulation of immune responsiveness through this in feed B animals. However, this could also imply that H-line chicken fed with feed B were confronted with high zone tolerance, resulting in decreased levels of LPS-binding natural antibodies. This would provide more room for expansion of LTA-binding natural antibody producing B-cells. In the L-line this level of LPS contamination could activate LPS-specific tolerance mechanisms that suppress antibody formation again providing expansion of LTA-specific antibodies. However, because of the low antibody producing potential in the L-line animals, this effect is far less pronounced than the effect in the H-line. In the literature there are ample examples of neonatal induced tolerance to LPS (Spencer 2006, Ellis 2006, Walker 2006, Elenkov 1999, Chowdhury 2005).

Micronutrients, trace element and bioactive ingredients and immune functions Numerous studies have studied the impact of bioactive ingredients such as Vitamin E, Lutein, or omega-3 unsaturated fatty acids on immune functions in chicken and have shown that such effects are present. The magnitude of differences in dietary supplementation for the bioactive ingredients is always considerable and typically exceeds a factor of 4 between treatment groups (He 2007). In the present study, the differences found between the group A and group B feeds were, if present, 10-30% maximum. It is therefore not likely, that the observed differences of any of the bioactive ingredients will lead to an effect similar to those reported in previous studies, even if in this study some supplementation took place.

Gut microbiology and immune functions No relationship was found between microbial communities in a particular feed batch and the manure, resulting from that particular feed. This may be due to the long storage of the feed batches before being analysed for microbial community composition. Thus, unfortunately, the hypothesis about the cycling of microbial communities could not be tested, not even for a small part of the cycle (feed -> manure). However, the differences in intestinal microflora between chicken with organic versus conventional feed could have contributed to differences in the development of the immune system.

Metabolomics results in relation to nutrition To relate the dietary composition with potential plasma or liver biomarkers, a distinction between a direct and an indirect link should be made. The composition of the diet can

have a direct effect on the plasma or liver composition or can indirectly have an effect on the physiology and the development of the animals, which in turn affects the plasma or liver composition.
In the present investigation, direct relationships would be expected for slow metabolizing compounds like certain micronutrients (vitamin E) and food contaminants like heavy metals. For many macronutrients, the relation between food intake and plasma levels can be both direct and indirect, so that an increase or difference in intake will not automatically lead to a stable increase in plasma steady state levels of this compound. For amino acids, a more direct relationship between dietary intake and plasma levels is expected, because the turnover of amino acids in the protein pool is relatively stable. For fatty acids and carbohydrates however, the relation between dietary uptake and plasma levels is highly complex, due to extensive post absorption metabolism, extensive physiological feedback controls and high fluxes between different organs. More likely, different dietary macronutrients will shift metabolic pathways and result in changed energy budgets or changed growth.
Significant changes in plasma levels of the free fatty acid C22:6 (DHA) can be caused by food intake differences of essential fatty acids, predominantly the omega-3 linolenic acid (C18:3), in food pair 15 and 16, during the challenge. DHA has been implicated in modulating inflammatory responses, among others through interaction with toll like receptors (TLR-4) (Lee 2001).

Metabolomics in relation to immunology Different plasma compounds in metabolomics were interpreted as reflecting an acute phase response after the KLH challenge, having occurred stronger in the animals on feed A. This could coincide with the stronger immune reactivity of the A-animals.

Metabolomics in relation to genomics The (confirmed) downregulated genes of the cholesterol pathway in the gut of feed B animals suggests a higher circulating cholesterol. This could not be confirmed in the plasma metabolomics.

7.6 Reflections on the working hypothesis of the project

The working hypothesis was ‘Organically grown products have a more beneficial effect on health.’
It can be concluded that ingredients from different farming systems have produced effects in the different feed groups. For several participants in the team these effects were quite unexpected.
Even when the codes are broken it is clear that this hypothesis can be confirmed, nor denied.
First of all because of the problem of the interpretation of the observed effects, from a perspective of health. This is a complex question. From the start of the project it was clear that a definition of health was lacking, so markers to evaluate the health question were also lacking. Very recent this was again confirmed by several specialists on animal health at the Workshop of LNV, June 2007, as mentioned in Ch. 1.3.
An attempt can be made, concerning this question, to use the concepts of ‘robustness’ and ‘resilience’, as introduced in Ch. 1.3, as a context for evaluating the different reactions of the A and B-animals on the KLH-challenge. The different observed physiological parameters showed clearly that the KLH-injection meant a quite strong disturbance to the animals. A baseline reaction on KLH in chicken is not known. In mice, however, Demas (1997) described the effect of KLH, as provoking an increased metabolism, increased body temperature (fever among several animals), accompanied by body catabolism connected to anorexia. Demas connects this process as energy costs of a mounting immune response.

The observed symptoms in our animals reflect the ones Demas describes. The decline in feed intake, especially among the B-animals and the decline in (relative) growth, reflects the described anorexia and catabolism. Body temperature was not measured, but an increased metabolism can be recognized in several parameters of an acute defence reaction, most clearly in the metabolomics results. Overall the impression is that the animals on feed A showed a stronger reaction.
The next observations concern the weeks after the challenge. There the phenomenon occurred that the A-animals, after an as strong decline in growth as the B-animals showed, started to regain weight at the same time as the B-animals, but significantly stronger. They showed, in medical terms, a stronger catch-up growth'. It could be speculated that the increased relative growth in the A animals, 3-4 weeks after the challenge, could be the reason for the differences in liver metabolomics parameters observed at week 4.

Reflecting on the two definitions of resilience, one might speculate that group A animals show a stronger 'engineering resilience', measured by the time a system needs to return to an equilibrium after a disturbance. The group B animals might show a stronger 'ecological resilience', which is measuring by how far a system can be perturbed without shifting to a different regime. As the B-group represented a system that was perturbed less strongly by the disturbance, this might be interpreted as positive.
It must be stated that these observations were only of short duration and incidental and cannot predict any long term health outcomes for the organisms animals.

Immune competence versus immune responsiveness It appears that the animals on feed A have the highest potential or competence for immune reactivity. Immune competence of animals can differ due to several factors, as can be seen in the selection lines used here. The random bred C-line could show the maximum flexibility to adapt to altering antigenic exposure induced by changes in feeds. This is due to the unselected genetic background of these animals. When considering the immune competence of the selection lines used throughout the experiments described here, the high line responders are considered more immune competent than the low line responders, as they always have the higher levels of e.g. natural antibodies, antigen-specific antibodies, etc. Even though these selection lines show different levels of immune competence, their immune responsiveness (IR) can be the same, as is often seen in data expressed as the delta (IR-baseline) immune parameters.
Higher immune competence or greater immune responsiveness can not necessarily be considered more beneficial, as there are always situations wherein the costs of one (or more) highly evolved organ system in some individuals can be in their (dis)advantage under specific circumstances. Again, the effects of an epidemic makes this clear: only a small group will survive and which animals these are, the high immune competent or the low immune competent, is totally dependent on the characteristics of the infectious agent and other environmental circumstances (e.g. famine, cold, etc.). This could be different for animals that are held under relatively controlled conditions, although generalizations in this area remain difficult.

Second the question should be raised about the statement of 'organically grown products' in relation to the feeds used here. As was described in ch 7.2,the usual organic agriculture shows a broad range of (more ór less) differences from conventional agriculture. The present study is just one study with one set of ingredients, from specific locations, in a specific year, etc. etc.
It cannot be excluded that with different varieties, for example, different results would have been obtained.

Thus reflecting on the working hypothesis… regarding a potential 'larger' beneficial effect on health of one of the two feed, no clear conclusions can be drawn. Both feeds were healthy as such. To clarify the mechanisms behind the different patterns of reactions, as well as the long term implications for health, more research will be needed.

A more focussed hypothesis was that organic feed would enhance innate immunological competence.

Indeed the immune system was influenced differently by the feeds.

More insight is needed in the range of product quality, within organic, as well as in conventional products, before it is possible to come further with this working hypothesis about 'the' organically grown products.

8 Conclusions

In this study on two generations of chicken, biomarkers for potential health effects of organic products were investigated. With respect to the first research question: 'Can differences be found between ingredients for chicken feed, obtained from organic and conventional production systems?', it can be concluded that: The ingredients for chicken feed, obtained from organic and conventional production systems, clearly differ with respect to the amount of amino acids and protein. In wheat, soy and barley the amino acids were 10-40% higher in the ingredients used for the preparation of the B feed samples. Further differences were observed in the level of phytosterols in soy and barley, which were higher in the B feed ingredients. Ingredients used for feed A were higher in vitamin K, soy was higher in isoflavones and vitamin E, especially alpha tocopherol, and peas were higher in folate. The vitamins B5 and C were higher in respectively maize and peas and in wheat used for feed B.
In this study only one selection of different ingredients from one harvest and one location has been used, so extrapolation of these results is not possible.

The second research question: 'Can biomarkers be identified, for health effects, related to the consumption of organic compared to conventional feed?', and the two sub questions about effects on the immune system and other organ systems, can be answered confirmatively. A difference in the developing immune system of chicken fed with the two different feeds was observed. From this study it can be concluded that the chicken fed on feed A had a higher potential for immunological reactivity.
Furthermore the two different feeding regimes had a clear impact on the growth of the chicken. The animals fed on feed A had a lower growth rate. However, after the KLH challenge, these animals showed a somewhat stronger recovery. Overall it can be said that the animals in both groups developed well and no clear diseases or unhealthy conditions occurred.
Results are based on findings in the Control line animals, as these represent the natural genetic variety. However results in the special high and low responding chicken in this animal model confirm the conclusions of increased potential for immunological reactivity.
Biomarkers which in this study clearly presented the effect of the two feeding regimes in healthy animals are: growth and, especially after exposure to a challenge, evaluation of immune responsiveness, several metabolites, gene regulation in the gut and pathological anatomy.
Conclusions about health effects cannot be drawn, as the long term implications of the manifestations are unclear.
It must be stated that many observations in this study are still not understood yet.

9 Recommendations

The material and data of the present study are very valuable and still obtain a rich source for further exploration towards more insight in the mechanisms that produced the observed differences in the chicken. Further fundamental research on the available material and data would be very valuable and might result in more insight in the mechanisms that played a role in producing the effects in the animals.

The results of the present study are most promising. This study showed that small differences in feeds, because of differences in agricultural background, have implications for immune reactivity, metabolites and gene activity in healthy animals. Before these results can be used in studies in humans (the final goal), the results need to be confirmed. Confirmation should preferably first be sought, again, in chicken, which need to be followed longer (e.g. till natural death) and should be studied during a stronger challenge with an infection model or other disease model. Feed ingredients for such follow-up research should be obtained from 'best practice' farms in the same area. The feeds need to be extensively analysed, to give the possibility to relate observed effects in the animals to nutritional factors in the feeds. Next to this the ingredients should be analysed thoroughly, also in relation to products as purchased by the consumer, to get more insight in the representativity of the products.

Towards the future a confirmation in mammals is recommended, preferably in pigs, as these animals are most comparable to human beings. Research in humans is the ultimate goal.

10 Reflections after the decoding

Just before the printing of the report, the text being finished except for the summary, the codes A and B of the feeds and the codes of the non-conventional methods, were broken. It turned out that feed A was organic and feed B was conventional.
In the same meeting, the attending researchers of the Project group and the members of the Advisory Committee, were invited to give a reaction or reflection after the decoding.

Their words are summarized here:

Dr. Elwin Verheij, chemist, TNO, projectteam – I am pleasantly surprised by the metabolomics results in this study. Skeptical colleagues did not expect anything from it, as the differences between the feeds were so small, both feeds were of high quality and we worked with healthy animals. However we found very clear differences. So for me these results indicate that we are on the right track!

Dr. ir. Lucy van de Vijver, nutritionist, Louis Bolk Institute, projectteam – I am glad A is organic, as it confirms the expectation I had, based on the literature. And it is the first time that I see such informative metabolomics results.

Dr. Leon Coulier, chemist, TNO, projectteam – I will continue to eat organic anyway! I recognize and confirm the contribution of my colleague Elwin Verheij about the metabolomics results. And it was instructive to work together with statistician Dré Nierop on the results.

Prof. dr.ir. Huub Savelkoul, immunologist, Wageningen UR, projectteam – In this study we found immunological results by analyzing various immune parameters in healthy animals, which is very valuable, as we learn from it which techniques are sensitive enough to detect the expected subtle effects from feeds. Secondly it is informative to find that a feed could indeed trigger the immune system to such an extent that it reveals differential immune responsiveness. Thirdly I conclude that the feeds provoke a difference in immune reactivity, whereby animals on feed A regain their balance quicker. This is usually connected to “health” depending on the genetic background and environmental exposure of the animal. Further research is needed to clarify which components in the feed cause these immunomodulatory effects. We would therefore need additional studies to reveal the underlying mechanisms, to study the interrelatedness of the different immune reactions, and to reproduce the findings.

Dr. ir. Henk Parmentier immunologist, Wageningen UR, projectteam – I would have been disappointed if B had been the organic feed. My expectation was that organic feed is richer in antigens and thus would stimulate the immune system. It is still unclear if feed A is healthier, but if I would be dropped on an isolated island, I would prefer to have food A with me to eat.

Dr. Dré Nierop, statistician, MUVARA, projectteam – For me A gives the most consistent pattern with respect to health. It was interesting to develop during this study new methods to analyse the data in such a way, that patterns could be recognized. My impression is that the metabolomics could be even more informative than immunological results, as with the metabolomics results I could classify the individual chicken of group A and B for a full 100%. Also

the chemical patterns in time seem very informative, but need more time to study, than is available. Anyway, the differences are much bigger than I had expected.
I was surprised to see how little knowledge there is about the physiology of health". This study is a contribution and an impulse to develop more insight in this topic.
Diverse reactions: It is difficult to find money to study the healthy situation. Most fundings go into research about diseases.

Dr. Ron Hoogenboom, toxicologist, RIKILT, projecteam – I had expected A to be organic, based on the amino acids and the protein content of the ingredients. However, dry matter and vitamin c seem to be not consistent with literature.
The results of the biophoton-delayed luminisence analyses on the ingredients fascinate me, as these researchers had identified nearly all ingredients correctly, concerning their origin. What substances in organic products make them retain light longer? I have a question about the representativity of the ingredients used. More research is needed about the spreading among organic products.

Drs. Machteld Huber, MD, Louis Bolk Institute, project leader – I found this project most instructive because of all the different techniques that were included. I had expected A to be organic because of the protein and the chloride content of B.
About the health question it became very clear to me that you need a challenge, to be able to study aspects like flexibility and resistance. It was a pity we could not follow these chicken longer. Many questions are still unanswered.
I find it striking that in literature weight gain has always been described as a marker for effects from different feeds. But in the older studies, animals on organic feed grew stronger, whereas nowadays the conventional feed induces more weight gain, even if protein differences are taken away by supplementation. And it is interesting to see that increased immune responses were found again, connected to organic feeding.

Dr. Raymond Pieters, toxicologist-immunologist, University Utrecht, Advisory Board – I had not expected these differences. Follow up studies are needed to clarify the implications of the differences found. Health is not static, it is a 'flexible system' which is relative and related to a persons situation. If you have an allergic constitution you would not want a more stimulated immune system. And, if I'm on the island, I would also choose A!

Dr. Henk van Loveren, toxicologist-immunologist, RIVM, Advisory Board – I did not expect much from this study at the start, so these results are beyond my expectations. The differences are not big, but I think one can state that the A animals react stronger than the B animals. Follow up studies, with infection models or other disease models, are needed to show if long term resistance of these A animals is indeed different. And, on the island I would not worry about eating B!

Dr. Jac Meijs, animal nutritionist, Biologica & Wageningen UR, Advisory Board – It was not easy to get the project started financially, but it is wonderful that we succeeded and that there is now a sound report available. It is very valuable that so many institutes and disciplines worked together, using techniques that are new for the organic sector. The broad approach has appeared to be fruitful, although I am surprised that it is so difficult to translate

immunological results towards ‘health’. But it is a clear result that we found differences in immune reactivity within healthy animals! And I had expected that A was organic, based on the literature.

Literature

Adom, K. K., Sorells, M. E., Liu, R. H. (2003); **Phytochemical profiles and antioxidant activity of wheat varieties**. J. Agric. Food Chem. 51, 7825-7834.

Adriaansen-Tennekes, R., Bloksma, J., Huber, M. et al. (2005); **Biologische producten en gezondheid. Resultaten melkonderzoek.** Louis Bolk Publicatie, www.louisbolk.nl.

Aehnelt, E., Hahn, J. (1973); **Fruchtbarkeit der Tiere - eine Möglichkeit zur biologischen Qualitätsprüfung von Futter- und Nahrungsmitteln**. Tierärztl. Umschau 4, 155-170.

Albertsson-Wikland, K., Kriström, B., Rosberg, S., Svensson, B., Nierop, A. F. M. (2000); **Validated Multivariate Models Predicting the Growth Response to GH Treatment in Individual Short Children with a Broad Range in GH Secretion Capacities**. Pediatr. Res. 48, 475-484.

Alfödli, T., Granado, J., Kieffer, E., Kretschmar, U., Morgner, M., Niggli, U., Schädeli, A., Speiser, B., Weibel, F., Wyss, G., Schmidt, W., Schmidt., G. (2006) **Qualität und Sicherheit von Bioprodukten - Lebensmittel im Vergleich** [Quality and Safety of Organic Products]. FiBL-Dossier 4, 1. Auflage. Forschungsinstitut für biologischen Landbau (FiBL), CH-Frick.

Alter, G. (1978); **Über den Einfluß von Fütterungsfaktoren auf das Fruchtbarkeitsgeschehen. Den Ascorbinsäuregehalt und den histologischen Aufbau der Ovarien von weiblichen Kaninchen**. Inaugural-Dissertation, Tierärztliche Hochschule Hannover.

Anastassiades, M., Lehotay, S., Stajnbaher, D., Schenck, F. (2003); **Fast and easy multiresidue method employing acetonitrile extraction/ partitioning and „dispersive solid-phase extraction' for the determination of pesticide residues in produce.** J. AOAC Int. 86(2), 412-431.

Andersen, J.-O., (1999); **Computerised image analysis of biocrystallograms originating from agricultural products**. In: Computers and Electronics in Agriculture. 22, 51-69.

Andersen, J.-O. (2001); **Development and application of the biocrystallisation method**. Biodynamic Research Association Denmark, Report 1.

Andersen, J.-O., et al. (2003); **A concentration matrix procedure for determining optimal combinations of concentrations in biocrystallization**. Elememnte der Naturwissenschaft 79, 97-114.

Ansell, B. J., Watson, K. E., Fogelman, A. M., Navab, M., Fonarow, G. C. (2005); **High-density lipoprotein function recent advances.** J. Am. Coll. Cardiol. Nov 15; 46(10), 1792-8. Epub 2005 Oct 19.

Antonovsky (1999); **What keeps people healthy?** Federal Centre for Health Education, Cologne.

AOAC. (1984); **Official Methods of Analysis 14th Ed.**, Arlington, VA, sec. 43260-43264, and (1990) 15th Ed. sec 982.30

Avissar, S. et al. (1999); **The effects of seasons and light therapy on G protein levels in mononuclear leukoytes of patients with seasonal affective disorder**. Arch. Gen. Psychiatry 56 (2), 178-183.

Awad, W. A., Bohm, J., Razzazi-Fazeli, E., Zentek, J. (2006); **Effects of feeding deoxynivalenol contaminated wheat on growth performance, organ weights and histological parameters of the intestine of broiler chickens**. J. Anim. Physiol. Anim. Nutr. (Berl) Feb; 90 (1-2), 32-7.

Baars, E., Baars, T. (2007); **Towards a philosophical underpinning of the holistic concept of integrity of organisms within organic agriculture**. NJAS 54-5, 463-477.

Bacha, W. J., Wood, L. M., (1990); **Color atlas of veterinary histology.** Lea and Febiger, Philadelphia and London.

Baelmans, R., Parmentier, H.K., Nieuwland, M.G., Dorny, P., Demey, F., Berkvens, D., (2005); **Haemolytic complement activity and humoral immune responses to sheep red blood cells in indigenous chickens and in eight German Dahlem Red chicken lines with different combinations of major genes (dwarf, naked neck and frizzled) of tropical interest**. Trop. Anim. Health. Prod. 37,173-86.

Bakalli, R. I., Pesti, G. M., Ragland, W. L. (1995); **The magnitude of lead toxicity in broiler chickens.** Vet. Hum. Toxicol. Feb; 37 (1), 15-9.

Barker, M., Rayens, W. (2003); **Partial Least Squares for Discrimination**. J. Chemometrics 17 (3), 166-173.

Benbrook, C. M. (2005); **Breaking the Mold – Impacts of organic and conventional farming systems on mycotoxins in food and livestock feed**. An Organic Centre State of Science Review. www.organiccenter.org

Benbrook, C. M. (2005); **Elevating antioxidant levels in food through organic farming and food processing**. The Organic Center report www.organiccenter.org

Bergstrom, J. D., Wong, G. A., Edwards, P. A., Edmond, J. (1984); **The regulation of acetoacetyl-CoA synthetase activity by modulators of cholesterol synthesis in vivo and the utilization of acetoacetate for cholesterogenesis**. J. Biol. Chem. 259,14548-14553.

Bijlsma, S., Bobeldijk, I., Verheij, E. R., Ramaker, R., Kochhat, S., MacDonald, I. A., van Ommen, B., Smilde, A. K. (2006); **Large-Scale Human Metabolomics Studies: A Strategy for Data (Pre-) Processing and Validation**. Anal. Chem. 78, 567-574.

Bilbo, S. D. et al. (2002); **Short day lenghts augment stress-induced leukocytre tafficking and stress induced enhancement of skin immune function**. Proceedings of the National Academy of Sciences of the USA 99 (6), 4067-4072.

Birzele, B., Meier, A., Hindorf, H., Kramer, J., Dehne, H. W. (2002**); Epidemiology of Fusarium Infection and Deoxynivalenol Content in Winter Wheat in the Rhineland**, Germany. European Journal of Plant Pathology 108, 667-673.

Bloksma, J., Northolt, M., Huber, M. (2001); **Parameters for Apple Quality. Food Quality and Health FQH report 1**. Louis Bolk Instituut, Driebergen the Netherlands; www.louisbolk.nl.

Bloksma, J., Northolt, M., Huber, M., Jansonius, P., Zanen, M. (2001); **Parameters for Apple Quality-2.** Food Quality and Health FQH report 3. Louis Bolk Instituut, Driebergen, the Netherlands; www.louisbolk.nl.

Bloksma, J., Huber, M. (2002); **Life processes in crops: On growth & Differentiation**. LBI publication GW02, FQH-02; www.louisbolk.nl.

Bloksma J., et al. (2003); **The Inner Quality Concept for food, based on life processes**. In: Proceedings of the Conference 'New approaches in food quality analysis', 13-14 Nov. 2003. Berlin.

Bokhorst, J., Ter Berg, C. (2001); **Mest en Compost, behandelen, beoordelen en toepassen**. Louis Bolk Instituut, Driebergen, the Netherlands; www.louisbolk.nl.

Bourn, D., Prescott, J. (2002); **A comparison of the Nutritional Value, Sensory Qualities, and Food Safety of Organically and Conventionally Produced Foods**. Critical Reviews in Food Science and Nutrition 42 (1), 1-34.

Bram, L. (1974); **Über den Einfluß von Fütterungsfaktoren auf den Ascorbinsäuregehalt und den histologischen Aufbau der Nebennierenrinde von weiblichen Kaninchen unter besonderer Berücksichtigung der Beziehungen zwischen Nebennierenrindenfunktion und Fruchtbarkeit**. Inaugural-Dissertation, Tierärztliche Hochschule Hannover

Brandt, K., Molgaard, J. P. (2001); **Organic agriculture: does it enhance or reduce the nutritional value of plant foods?** J. Sci. Food Agric. 81, 924-931.

Burgt, G. J. van der (2003); **Stikstofdynamiek OBS: indirect maar niet inefficiënt**. In: Wijnands (ed). Duurzame bedrijfssystemen voor de akkerbouw en vollegrondsgroenteteelt deel 1: biologische akkerbouw; Centrale zeeklei. PPO-publicatie 306-1, Praktijkonderzoek Plant en Omgeving, Wageningen UR, Lelystad, the Netherlands.

Burgt, G. J. H. M. van der, Oomen, G. J. M., Habets, A. S. J., Rossing, W. A. H. (2006); **The NDICEA model, a tool to improve nitrogen use efficiency in cropping systems**. Nutrient Cycling in Agroecosystems 74, 275-294.

Busscher, N, Kahl, J., Huber, M., Doesburg, P., Andersen, J.-O. et al. (2004); **Validation and standardisation of the biocrystallisation method: development of a complementary test to assess qualitative features of agricultural and food products**. Int. report of University of Kassel, Louis Bolk Institute and Biodynamic Research Association Denmark.

Carstensen, J.M., (1993); **Co-occurrence feature performance in texture classification**. In: Høgda et al. (Eds.), Proceedings of the Eight Scandinavian Conference on Image Analysis. University of Tromsø, Tromsø, Denmark, pp.831-838.

Chandra, R. K. (2002); **Nutrition and the immune system from birth to old age**. Eur. J. clin. Nutr. 56 (Suppl. 3), S73-76.

Chowdhury, S. R., Smith, T. K. , Boermans, H. J., Woodward, B. (2005); **Effects of Feed-Borne Fusarium Mycotoxins on Hematology and Immunology of Turkeys**. Poultry Science 84, 1698–1706.

Council Regulation (EEC) No 2092/91 of 24 June 1991 on organic production of agricultural products and indications referring thereto on agricultural products and foodstuffs. http://europa.eu.int/eur-lex/lex/LexUriServ/site/en/consleg/1991/R/01991R2092-19990315.

CVB (1996); **Amino acid requirement of laying hens and broiler chickens (translated title)**. CVB Documentatierapport nr 18 (in Dutch).

Davidov, E., Clish, C.B., Oresic, M. et al. (2004); **Methods for the differential integrative omic analysis of plasma from a transgenic disease animal model**. Omics 8 (4), 267-288.

Davis, C. D., Milner, J. A. (2007) **Biomarkers for diet and cancer prevention research: potentials and challenges**. Acta Pharmacol Sin 28 (9), 1262-1273.

Demas, G. E., Chefer, V., Talan, M. I., Nelson, R.J. (1997); **Metabolomic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice**. Am. J. Physiol. 273 (5), 1631-37.

Diepeningen, A.D., van, de Vos, O.J., Korthals, G.W, and van Bruggen, A.H.C. 2006. **Effects of organic versus conventional management on chemical and biological parameters in agricultural soils**. Appl. Soil Ecol. 31: 120-135.

Doll, S., Valenta, H., Danicke, S., Flachowsky, G. (2002); **Fusarium Mycotoxins in Conventionally and organically grown grain from Thuringia/Germany**. Landbauforschung Volkenrode 52, 91-96.

Dunn, O. J., Clark, V. A. (1974); **Applied Statistics: Analysis of Variance and Regression**. Wiley, New York.

Eerden van, E. (2007); **Residual feed intake in young chickens: Effects on energy partitioning and immunity**. Ph. D. Thesis, Wageningen UR, The Netherlands.

Edelmüller, I. (1984); **Untersuchungen zur Qualitätserfassung von Produkten aus unterschiedlichen Anbausystemen (biologisch-dynamisch bzw. konventionell) mittels Fütterungsversuchen an Kaninchen**. Dissertation, Universität Wien.

EFSA (2004); **Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to Zearalenone as undesirable substance in animal feed**. The EFSA Journal 89, 1-35.

EFSA (2005); **Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission related to fumonisins as undesirable substances in animal feed**. The EFSA Journal 235, 1–32.

Elenkov, I. J. (1999); **Stress Hormones, Th1/Th2 patterns, Pro/Anti-inflammatory Cytokines and Susceptibility to Disease**. Trends Endocrino Metab. 10 (9), 359-368.

Ellis, S., Mouihate, A., Pittman, Q. J. (2006); **Neonatal programming of the rat neuroimmune response: stimulus specific changes elicited by bacterial and viral mimetics**. J. Physiol. 571 (3) 695–701.

Emiola, I. A., Ologhobo, A. D., Gous, R. M. (2007); **Performance and histological responses of internal organs of broiler chickens fed raw, dehulled, and aqueous and dry-heated kidney bean meals**. Poultry Science. Jun; 86 (6), 1234-40.

Espada, Y., de Gopegui, R. R., Cuadradas, C., Cabanes, F. J. (1997); **Fumonisin mycotoxicosis in broilers: Plasma proteins and coagulation modifications**. Avian Diseases 41, 73-79.

Finamore et al. (2004); **Novel Approach for Food Safety Evaluation. Results of a Pilot Experiment to Evaluate Organic and Conventional Foods**. Journal of Agricultural and Food Chemistry 52, 7425-7431.

Fisher, R. A. (1936); **The use of multiple measurements on taxonomic problems**. Annals of Eugenics 7, 179-188.

Gifi, A. (1990); **Nonlinear multivariate analysis**. Chichester: Wiley..

Goldstein, J. L., Brown, M. S. (1990); **Regulation of mevalonate pathway**. Nature 343, 425-430.

Gottschewskil, G.H.M. (1975); **Neue Möglichkeiten zur größeren Effizienz der toxikologischen Prüfung von Pestiziden, Rückständen und Herbiziden**. Qualitas Plantarum - Plant Foods for Human Nutrition, 25, 21-42

Greef, J. van der, Davidov, E., Verheij, E.R. et al. (2003); The role of metabolism in systems biology. In **metabolite profiling: its role in biomarker discovery and gene function analysis**. G.G. Harrigan and R. Goodacre, eds. (Kluwer Academic Press, Boston), pp.171-198.

Greef van der, J., Martin, S., Juhasz, P., Adourian, A., Plasterer, T., Verheij, E. R., McBurney, R. N. (2007); **The Art and Practice of Systems Biology in Medicine: Mapping Patterns of Relationships.** Journal of Proteome Research 6 (4), 1540 -1559.

Guarner, F., Malagelada, J. R. (2003); **Gut flora in health and disease**. Lancet 361, 512-519.

He, X., Yang, X., Guo, Y., (2007); **Effects of different dietary oil sources on immune function in cyclophosphamide immunosuppressed chickens**. Animal Feed Science and Technology, In Press, Corrected Proof, Available online 7 March 2007.

Heaton, S.(2001); **Organic Farming, Food Quality and Human Health: A review of the evidence**. Soil Association report. Soil Association of the United Kingdom. www.soilassociation.org

Hemert, S. van, Hoekman, A. J., Smits, M. A., Rebel, J. M. (2004); **Differences in intestinal gene expression profiles in broiler lines varying in susceptibility to malabsorption syndrome**. Poultry Science 83 (10), 1675 – 1682.

Hemert, S. van, Hoekman, A. J., Smits, M. A., Rebel, J. M. (2007); **Immunological and gene expression responses to a Salmonella infection in the chicken intestine**. Vet. Res. 38 (1), 51-63.

Henry, M. H., Wyatt, R. D., Fletcher, O. J. (2000); **The Toxicity of Purified Fumonisin B1 in Broiler Chicks**. Poultry Science 79 (10), 1378–1384.

Hochberg, Y., Tamhane, A.C. (1987); **Multiple Comparison Procedures**. Wiley, New York.

Hollander, M., Wolfe, D. A. (1973); **Nonparametric Statistical Methods.** Wiley, New York.

Hoogenboom, L. A. P., Bokhorst, J. G., Northolt, M. D., Broex, N. J. G., Mevius, D., Meijs, J. A. C, van der Roest, J. (2006); **Contaminanten en micro-organismen in biologische producten; Vergelijking met gangbare producten.** Rapport 2006.002, RIKILT.

Hoogenboom, L. A. P., Northolt, M., Bokhorst, J., Broex, N., Mevius, D., Meijs, J., van der Roest, J. (2006); **Contaminants and micro-organisms in organic versus conventional food.** Biofach, 16-17 febr 2006.

Huber, M. A. S., Adriaansen-Tennekes, R., van de Vijver, L.P.L. (2006); **Verantwoorde en communiceerbare gezondheidsargumenten bij biologische producten.** Louis Bolk Instituut, Driebergen, www.louisbolk.nl.

Huber, M.A.S., et al. (2007); **Development of a methodological instrument for Visual evaluation of biocrystallizations, based on ISO-norms for sensory evaluation by a panel**. Submitted.

Hueza, I. M., Latorre, A. O., Raspantini, P. C., Raspantini, L. E., Mariano-Souza, D .P., Guerra, J. L., Gorniakm, S. L. (2007); **Effect of Senna occidentalis seeds on immunity in broiler chickens**. J. Vet. Med. May; 54 (4), 179-85.

IFOAM (2004); **Principles of organic agriculture**. www.ifoam.org

Janmaat, L., Cuijpers, W. (ed.) (2005); **Bodem en bemesting**. Brochure project Biokas. Louis Bolk Instituut, Driebergen, the Netherlands; www.louisbolk.nl.

Jensen, K. O., Larsen, H. N., Mølgaard, J.P. et al. (2001); **Organic food and human health**, DARCOF report no 14, Danish Research Centre for Organic Farming, Tjele, Denmark.

Jolliffe, I. T. (2002); **Principal Component Analysis**. 2nd ed., Springer.

Kahl, J., Busscher, N., Meier-Ploeger, A. et al. (2004); **Ganzheitliche Untersuchungsmethoden zur Erfassung und Prüfung der Qualität ökologischer Lebensmittel: Stand der Entwicklung und Validierung**. Abschlussbericht 02E170. University Kassel, Germany.

Kahl, J. (2007); **Entwicklung, in-house Validierung und Anwendung des ganzheitlichen Verfahrens Biokristallisation für die Unterscheidung von Weizen-, Möhren- und Apfelproben aus unterschiedlichem Anbau und Verarbeitungsschritten**. Habilitationsschrift, Universität Kassel.

Khovidhunkit, W., Kim, M. S., Memon, R. A., Shigenaga, J. K., Moser, A. H., Feingold, K. R., Grunfeld, C. (2004); **Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host.** J Lipid Res. Jul; 45 (7), 169-96. Epub 2004 Apr 21. Review.

Kim, S., Sohn, I., Ahn, J.-I., Lee, K.-H., Lee, Y. S., and Lee, Y. S. (2004); **Hepatic gene expression profiles in a long-term high-fat diet-induced obesity mouse model**. Gene 340, 99-109.

Kitano, H. (2004); **Biological robustness.** Nat Rev Genet. 5 (11), 826-37. Review.

Klasing, K.C., Korver D.R. (1997); **Leukocytic cytokines regulate growth rate and composition following activation of the immune system**. J. Anim. Sci. 75 (Suppl. 2), 58-67.

Koek, M. M., Muilwijk, B., van der Werf, M. J., Hankemeier, Th. (2006); **Microbial Metabolomics with Gas Chromatography/Mass Spectrometry**. Anal. Chem. 78, 1272-1281.

Koene, P., Zimmerman, P., Parmentier, H. K. (1997); **Behavioural stress response in chicken lines selected for differences in immune response**. In: P. Koene & H.J. Blokhuis (eds), Proceedings of the Fifth European Symposium on poultry Welfare 1997. Wageningen, the Netherlands. pp 99-102.

Köhler, B. (2001); **Der Einfluß von Haltung, Fütterung und Beleuchtung auf die Biophotonenemission (delayed luminescence) sowie herkömmliche Qualitätsparameter von Hühnereiern**. KWALIS Qualitätsforschung Fulda GmbH, Dipperz. 3-935769-00-8.

Kreukniet, M. B., Van der Zijpp, A. J., Nieuwland, M. G. B. (1992); **Effects of immunization, adjuvant and unrelated antigens on the humoral immune respopnse in lines of chickens selected for antibody production against sheep erythrocytes**. Veterinary Immunology and Immunopathology 33, 115-127.

Kreukniet, M. B., Van der Zijpp, A. J., Nieuwland, M. G. B. (1994); **Phagocytic activity of two lines of chickens divergently selected for antibody production**. Veterinary Immunology and Immunopathology 44, 377-387.

Kreukniet, M. B., Gianotten, N., Nieuwland, M. G. B., Parmentier, H. K. (1994); **In vitro T-cell activity in two chicken lines divergently selected for antibody response to sheep erythrocytes**. Poultry Science 73, 336-340.

Kreyszig, E., (1970); **Introductory Mathematical Statistics**. John Wiley, section 13.4.

Krzanowski, W. J. (1988); **Principles of Multivariate Analysis**. Oxford University Press.

Kumar, V., Elangovan, A. V., Mandal, A. B., Tyagi, P. K., Bhanja, S. K., Dash, B. B. (2007); **Effects of feeding raw or reconstituted high tannin red sorghum on nutrient utilisation and certain welfare parameters of broiler chickens**. Br. Poultry. Sci. Apr; 48 (2), 198-204.

Kummeling, I., Thijs, C., Huber, M., van de Vijver, L. P. L., Snijders, B. E. P., Penders, J., Stelma, F., van Ree, R., van den Brandt, P. A., Dagnelie, P. C. (2007); **Consumption of organic foods and risk of atopic disease during the first 2 years of life in the Netherlands.** Br. J. Nutr. 29; 1-8 [Epub ahead of print]

Kwakkel, R. P. (1994); **Multiphasic growth in the layer pullet; Effects of nutrient restrictions during rearing**. Thesis Wageningen Agricultural University, the Netherlands.

Lamers, R. J., De Groot, J., Spies-Faber, E. J. et al (2003); **Identification of disease- and nutrient-related metabolic fingerprints in osteoarthritic Guinea pigs**. J. Nutr. 133, 1776-1780.

Lamont, S. J. (1998); **Impact of genetics on disease resistance**. Poultry Science 77, 1111-1118.

Lauridsen et al. (2005); **Organic Diet Enhanced the Health of Rats**. DARCOF experiment, abstract op IFOAM congres, september 2005. http://www.darcof.dk/research/health.

Lauridsen, C., Yong, C., Helekoh, U. et al. (accepted for publication 2007); **Rats show differences in some biomarkers of health when eating diets based on ingredients produced with three different cultivation strategies**.

Lee, J. Y., Sohn, K. H., Rhee, S. H., Hwang, D. (2001); **Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cylooxygenase-2 mediated through Toll-like receptor 4**. J. Biol. Chem. 276, 16683–16689.

Li, J., Takaishi, K., Cook, W., McCorkle, S. K., Unger, R. H. (2003); **Insig-1 'brakes' lipogenesis in adipocytes and inhibits differentiation of preadipocytes.** Proc. Natl. Acad. Sci. 100, 9476 – 9481.

Li, Y. C., Ledoux, D. R., Bermudez, A. J., Fritsche, K. L., Rottinghaus, G. E. (1999); **Effects of Fumonisin B1 on Selected Immune Responses in Broiler Chicks**. Poultry Science 78, 1275–1282.

Lochmiller, R. L., Vestey, M. R., Boren, J. C. (1993); **Relationship between protein nutritional status and immunocompetence in northern bobwhite chicks**. The Auk 110 (3), 503-510.

Mäder, P., Fliessbach, A., Dubois, D., Gunst, L., Fried, P., Niggli, U., (2002); **Soil Fertility and Biodiversity in Organic Farming**. Science 296, 1694-1697.

Magkos, F., Arvaniti, F., Zampelas, A. (2003); **Organic food: nutritious food or food for thought? A review of the evidence**. Intern. J. Food Sci. Nutr. 54 (5), 357-371.

McCarrison, R. (1926); **The effect of manurial conditions on the nutritive and vitamin values of millet and wheat**. Indian Journal of Medical Research 14, 351-378.

Meelursarn, A. (2007); **Statistical evaluation of texture analysis data from the biocrystallization: Effect of image parameters to differentiate samples from different farming systems**. Diss. Universität Kassel, FB 11, Witzenhausen.

Mengheri, E., Bises, G., Gaetani, S. (1988); **Differentiated cell-mediated immune response in zinc and in protein malnutrition**. Nutr. Res. 8, 801-812.

Miller, E. R., Pastor-Barriuso, R., Dalal, D., Riemersma, R. A., Appel, L. J., Guallar, E. (2005); **Meta-Analysis: High-Dosage Vitamin E Supplementation May Increase All-Cause Mortality.** Annals of Internal Medicine, Vol. 142, Issue 1.

Minekus, M., Marteau, P., Havenaar, R., Huis in 't Veld, J. H. J. (1995); **A multi compartmental dynamic computer-controlled model simulating the stomach and small intestine**. Alternatives to Laboratory Animals (ATLA) 23, 197-209.

Minekus, M., Havenaar, R. (1996); **In vitro model of an in vivo digestive tract**. United States Patent nr. 5,525,305, dated June 11, 1996.

Minekus, M. (1998); **Development and validation of a dynamic model of the gastrointestinal tract**. PhD Thesis, University of Utrecht, Elinkwijk b.v. Utrecht, The Netherlands.

Minekus, M., Havenaar, R. (1998); **Reactor system. European Patent No. 0642382**. European Patent Bulletin 98/07, Art. 97(4) and (5) EPC, dated 11.02.98.

Minutes of 'Organic healthier; fable or fact', workshop in November 2004, organised by order of the ministry of agriculture (LNV).

Minutes workshop Food Quality and Health (FQH), (2003) Louis Bolk Instituut, Driebergen, October 2003.

Minutes Workshop LNV (2007); **Natuurlijke Weerstand - (on)mogelijkheden voor het beleid.** Verslag van een Workshop op 29 juni 2007.

Moore, R.E., Kirwan, J., Doherty, M. K., Whitfield, P. D. (2007); **Biomarker Discovery in Animal Health and Disease: The Application of Post-Genomic Technologies.** Biomarker Insights 2, 185–196.

Murch, O., Collin, M., Hinds, C. J., Thiemermann, C. (2007); **Lipoproteins in inflammation and sepsis**. I. Basic science. Intensive Care Med. Jan; 33(1),13-24. Epub 2006 Nov 9.

Navab M., Anantharamaiah G. M., Reddy, S. T., Van Lenten, B. J., Ansell, B. J., Fogelman, A. M. (2006); **Mechanisms of disease: proatherogenic HDL- an evolving field**. Nat Clin Pract Endocrinol Metab. Sep; 2(9), 504-11.

Neudecker, C. (1987); **Düngung und Qualität von Lebensmitteln – Tierfütterungsversuche mit mineralisch und organisch gedüngten Kartoffeln und Möhren.** In: Akademie für Politische Bildung Tutzing (Hrsg.): Landbaumethoden und Nahrungsqualität. Materialien und Berichte Nr. 60.

Nicholson, A. C., Febbraio, H. J., Silversterin, R. L., Hajjar, D. P. (2001); **Role of CD36, the macrophage class B scavenger receptor, in atherosclerosis**. Ann. N. Y. Acad. Sci. 947, 224-228.

Nierop, A. F. M. (1993); **Multidimensional analysis of grouped variables: An integrated approach**. Leiden: DSWO Press, Leiden University (Dissertation).

Northolt, M., Van der Burgt, G. J., Buisman, T., Vanden Bogaerde, A. (2002); **Parameters for carrot quality**. Food Quality and Health FQH report 4. Louis Bolk Instituut, Driebergen, the Netherlands; www.louisbolk.nl.

Noverr, M. C., Huffnagle, G. B. (2004); **Does the microbiota regulate immune responses outside the gut?** Trends Microbiol. 12, 562-568.

Onderci, M., Sahin, N., Sahin, K., Cikim, G., Aydin, A., Ozercan, I., Aydin, S. (2006); **Efficacy of supplementation of alpha-amylase-producing bacterial culture on the performance, nutrient use, and gut morphology of broiler chickens fed a corn-based diet.** Poultry Science Mar; 85 (3), 505-10.

Parmentier, H.K., Nieuwland, M.G.B., Rijke, E., De Vries Reilingh, G., Schrama, J.W. (1996); **Divergent antibody responses to vaccines and divergent body weights of chicken lines selected for high and low humoral responsiveness to sheep red blood cells**. Avian Diseases 40, 634-644.

Parmentier, H. K., Nieuwland, M. G. B., Barwegen, M. W., Kwakkel, R. P., Schrama, J. W. (1997); **Dietary unsaturated fatty acids affect antibody responses and growth of chickens divergently selecter form Humoral responses to sheep Red Blood Cells**. Poultry Science 76, 1164-1171.

Parmentier, H. K., Abuzeid, S. Y., De Vries, G., Reilingh, G. D., Nieuwland, M. G. B., Graat, E. A. M. (2001); **Immune responses and resistance to Eimeria acervulina of chickens divergently selected for antibody responses to Sheep red blood Cells**, Poultry Science 80, 894-900.

Parmentier, H.K., Bronkhorst, S., Nieuwland, M.G.B., De Vries Reilingh, G., Van der Linden, J.M., Heetkamp, M.J.W., Kemp, B., Schrama, J.W. Verstegen, M.W.A., Van den Brand, H. (2002**); Increased fat deposition after repeated immunization in growing chickens.** Poultry Science 81, 1308-1316.

Parmentier, H. K., Lammers, A., Hoekman, J. J., de Vries Reilingh, G., Zaanen, I. T., Savelkoul, H. F. (2004); **Different levels of natural antibodies in chickens divergently selected for specific antibody responses**. Dev. Comp. Immunol. 28 (1), 39-49.

Patwardhan, V., Gokhale, M., Ghaskadbi, S. (2004); **Acceleration of early chick embryo morphogenesis by insulin is associated with altered expression of embryonic genes**. Int. J. Dev. Biol. 48, 319-326.

Pinard, M. H., Janss, L. L. G., Maatman, R., Noordhuizen, J .P. T. M., Van der Zijpp, A. J. (1993); **Effect of divergent selection for immune responsiveness and of major histocompatibility complex on resistance to Marek's disease in chickens**. Poultry Science 72, 391-402.

Pfeiffer, E. (1931); **Vergleichender Fütterungsversuch mit mineralisch gedüngtem und biologisch-dynamisch gedüngtem Getreide**. Demeter 6 (5), 87-89.

Pfeiffer, E., Sabert, E. (1932); **Vergleichender Fütterungsversuch mit Hühnern.** Demeter 7 (11), 198-200.

Ploegaert, T.C.W. de Vries Reilingh, Nieuwland, G M.G.B., Lammers, A., Savelkoul, H.F.J., Parmentier,. H.K. (2007); **Intratracheally administered pathogen associated molecular patterns affect antibody responses of poultry**. Poultry Science 86, 1667-1676.

Pool, M. H., Hulsegge, B. Janss, L.L.G. (2005); **Background bias on cDNA micro-arrays**. EAPP, Uppsala Sweden, Session G3.3.

Qin, G. (1996); **Processing soy beans of different origins**. WU thesis.

Rebel, J. M. J., Van Hemert, S., Hoekman, A.J.W., Balk, F. R. M., Stockhofe-Zurwieden, N., Bakker, D., Smits, M. A. (2006); **Maternal diet influences gene expression in intestine of offspring in chicken (Gallus gallus)**. Comp. Biochem. Physiol. Part A 145, 502-508.

Ren, H. et al. (2001); **The superiority of organically cultivated vegetables to general ones regarding antimutagenic activities**. Mutation Research 496, 83-88.

Rist, L., Mueller, A., Barthel, C., Snijders, B., Jansen, M., Simões-Wüst, A. P., Huber, M., Kummeling, I., von Mandach, U., Steinhart, H., Thijs, C. (2007); **Influence of Organic Diet on the Amount of Conjugated Linoleic Acids in Breast Milk of Lactating Women in the Netherlands.** Br J Nutr 97, 735-743.

Scheunert, A. (1935); **Vergleichende Untersuchung der physiologischen Wirkung fortgesetzten Genusses von Nahrungsmitteln, die mit und ohne Handelsdünger gezogen sind**. Angewandte Chemie 48, 42-46.

Seber, G. A. F. (1984); **Multivariate Observations**, Wiley.

Sijben, J. W., Nieuwland, M. G., Kemp, B., Parmentier, H. K., Schrama, J. W. (2001); **Interactions and antigen dependence of dietary n-3 and n-6 polyunsaturated fatty acids on antibody responsiveness in growing layer hens**. Poultry Sci. 80 (7), 885-93.

Simopoulos, A. P., (2000); **Human requirement for N-3 polyunsaturated fatty acids.** Poultry Sci.79(7), 961-70. Review.

Siwek, M. et al. (2006); **Detection of QTL for innate: Non-specific antibody levels binding LPS and LTA in two independent populations of laying hens**. Dev. Comp. Immunol. 30 (7):659-66.

Six, D. A., Dennis, E. A., (2000); **The expanding superfamily of phospholipase A(2) enzymes: classification and characterization**. Biochim. Biophys. Acta 1488, 1–19.

Smilde, A. K., van der Werf, M. J., Bijlsma, S., van der Werff-van der Vat, B. J. C., Jellema, R. H. (2005); **Fusion of mass spectrometry-based metabolomics data**. Anal. Chem. 77, 6729-6739.

Spencer, S. J., Boissé, L., Mouihate, A., Pittman, Q. J. (2006); **Long term alterations in neuroimmune responses of female rats after neonatal exposure to lipopolysaccharide**. Brain, Behavior, and Immunity 20, 325–330.

Staiger, D. (1986); **Einfluß konventionell und biologisch-dynamisch angebauten Futters auf Fruchtbarkeit, allgemeinen Gesundheitszustand und Fleischqualität beim Hauskaninchen**. Dissertation, Universität Bonn.

Staiger, D. (1988); **Nutritional value of food from conventional and biodynamic agriculture**. IFOAM bulletin 4, 9-12 & Dissertation Universität Bonn, 1986.

Steinbrecher, U. P. (1999); **Receptors for ocidized low density lipoprotein**. Biochim. Biophys. Acta 1436, 279-298.

Stolz, P., Strube, J. et al. (2000); **Better dietary protein-quality of beans cultivated biodynamically than by hydro-culture.** Proceedings 13th IFOAM Scientific Conference.

Strube, J., Stolz, P. (2001); **Lichtspeicherung und Lebensmittelqualität**. Ökologie & Landbau 117 (1), 15-19.

Strube, J., Stolz P., (2004); **Lebensmittel vermitteln Leben-Lebensmittelqualität in erweiterter Sicht**, herausgeg. KWALIS, ISBN 3-935769-01-6.

Strube, J. (2005); **Fluoreszenz-Anregungsspektroskopie**. in 'Abschlussbericht Projektnummer 02OE170 'Ganzheitliche Untersuchungsmethoden zur Erfassung und Prüfung der Qualität ökologischer Lebensmittel: Stand

der Entwicklung und Validierung''. A. Meier-Ploeger. Bonn, Geschäftsstelle Bundesprogramm Ökologischer Landbau in der Bundesanstalt für Landwirtschaft und Ernährung (BLE) 53168 Bonn S. 61-157.

Stylianou, I. M., Clinton, M., Keightley, P. D., Pritchard, C., Tymowska-Lalanne, Z., Bünger, L., Horvat, S. (2004); **Microarray gene expression analysis of the Fob3b obesity QTL identifies positional candidate gene Sqle and perturbed cholesterol and glycolysis pathways**. Physiol. Genom. 20, 224-232.

Ten Napel, J. (2007); **Robustness**, TLM Presentation 9 january 2007.

Tizard, I.R. (2004); **Veterinary Immunology,** Saunders, Philadelphia, USA.

Trouw Nutrition (Nutreco Company) – **Adviesnormen 2003 voor opfok- en leghennen**.

Tusher, V. G., Tibshirani, R., Chu, G. (2001); **Pathway analysis of microarray data**. PNAS 98 (9), 5116-5121.

Turner, R.J. (1994); **Immunology a comparative approach**.Wiley & Sons, West Sussex, UK.

Velimirov, A., Plochberger, K., Huspeka, U., Schott, W. (1992); **The Influence of Biologically and Conventionally Cultivated Food on the Fertility of Rats.** Biological Agriculture and Horticulture, Vol.8, 325-337 A B Academic Publishers, Great Britain.

Velimirov, A. (2005b); **Reproductive Health of Rats. Proc. of the 1st Scientific FQH Conference 'What we achieved – where we will go'.** Edts.: International Research Association for Organic Food Quality and Health, Nov. 28.-29. 2005, Research Inst. of Organic Agriculture FiBL, Frick, Switzerland.

Verhoeckx, K. C., Bijlsma, S., Jespersen, S., Ramaker, R., Verheij, E. R., Witkamp, R. F., van der Greef, J. (2004); **Characterization of anti-inflammatory compounds using transcriptomics, proteomics, and metabolomics in combination with multivariate data analysis**. Int. Immunopharmacol 4, 1499-1514.

Walker, F. R., Hodyl, N. A., Krivanek, K.M, Hodgson, D. M. (2006); **Early life host–bacteria relations and development: Long-term individual differences in neuroimmune function following neonatal endotoxin challenge**. Physiology & Behavior 87, 126 –134.

Watt, B., Merrill, A. L. (1975); **Composition of foods**. Agricultural Handbook No. 8. Washington, DC: US Department of Agriculture.

Werf, M. J. van der, Jellema, R. H., Hankemeier, Th. (2005); **Microbial metabolomics: replacing trial-and-error by the unbiased selection and ranking of targets**. J. Ind. Microbiol. Biotechnol. 32, 234-252.

Winter, C. K., Davis, S. F. (2006); Organic Foods. J. Food Sci. 71, R117-124.

Woese, K., Lange, D., Boess, C., Bögl, K.W. (1995a+b); **Ökologisch und konventionell erzeugte Lebensmittel im Vergleich.** Eine Literaturstudie, Teil 1+2 (BgVV-Hefte 5/1995). Hrsg.: Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin.

Woese, K., Lange, D., Boess, C., Werner-Bogl, K. (1997); **A Comparison of Organically and Conventionally Grown Foods-Results of a Review of the Relevant Literature**. .J. Sci. Food Agric. 74, 281-293.

Wold, H. (1982); **Soft modelling: the basic design and some extensions**. In K.G. Jöreskog & H. Wold (Ed.), Systems under indirect observation. Part II (pp. 1-54). Amsterdam: North Holland.

Worthington, V., (1998); **Effect of agricultural methods on nutritional quality: a comparison of organic with conventional crops**. Alternative therapies 8 (1), 58-69.

Worthington, V., (2001); **Nutritional Quality of Organic Versus Conventional Fruits**, Vegetables, and Grains. J. Altern. Complement. Med. 7 (2), 161-173.

Zhao, L., Moos, M. P. W., Gräbner, R., Pédrono, F., Fan, J., Kaiser, B., John, N., Schmidt, S., Spanbroek, R., Lötzer, K., Huang, L., Cui, J., Rader, D. J., Evans, J. F., Habenicht, A. J. R., Funk, C. D. (2004); **The 5-lipoxygenase pathway promotes pathogenesis of hyperlipidemia-dependent aortic aneurysm**. Nature Med. 10, 966-973.

Annexes

1a. Timeframe 1st generation
1b. Timeframe 2nd generation
2. Description feed ingredients
3. Feed supplementation Potato protein
4. Feed supplementation Fx Layers premix
5. Animal Ethics Committee approval
6. Tables analyses of ingredients and feeds
7. Complementary analyses – Protein ratio
8. Housing scheme of second generation
9. Metabolomics, Lipid LC-Mc data
10. Weight thymus and bursa
11. Villus/crypt ratio
12. CD 8 staining
13. Histological results of tissues
14. Overview significant chicken measurements
15. Overview all chicken measurements

Annex 1a – Timeframe 1st generation

Tijdspad voor 1ste generatie kippen en voeren								
Jaar	**Maand**	**Week**	**Leeftijd kippen 1ste dagen**	**Leeftijd kippen 1ste weken**	**Leeftijd kippen 2e gen weken**	**Voerschema 1ste generatie**	**Andere handelingen**	**Experimentele handelingen**
2005	oktober	42	0	0		**kuikens uit ei 18 okt 2005**	**Vaccinations**	
	oktober	43	7	1		opfok voer 1 - standaard voer	Marek Rispens HVT, IB MA 5	
	november	44	14	2		opfok voer 1	enting NCD-1	
	november	45	21	3		opfok voer 1	enting Gumboro	
	november	46	28	4		opfok voer 1	enting NCD-2	
	november	47	35	5		opfok voer 1	aselect groepen maken	
	november	48	42	6		opfok voer 1		
	december	49	49	7		start met opfok voer 2	enting PDww	
	december	50	56	8		opfok voer 2		
	december	51	63	9		opfok voer 2		1ste bloedafname oudergeneratie
	december	52	70	10		opfok voer 2	enting IB primer	
2006	januari	1	77	11		opfok voer 2	Start met experimenteel voer	
	januari	2	84	12		opfok voer 2	enting ILT	2de bloedafname (1 week na start voer)
	januari	3	91	13		opfok voer 2	enting AE	
	januari	4	98	14		opfok voer 2	enting NCD-3	
	februari	5	105	15		opfok voer 2	lichtschema van 8 naar 9 uur licht	3de bloedafname (4 weken na nieuwe voer
	februari	6	112	16		opfok voer 2	enting IB H52 + 10 uur licht	
	februari	7	119	17		opfok voer 2	11 uur licht	
	februari	8	126	18		op dag 120 start legvoer	12 uur licht	
	maart	9	133	19		leg voer	13 uur licht	4de bloedafname (8 weken na nieuwe voer
	maart	10	140	20		leg voer	14 uur licht	
	maart	11	147	21		leg voer	15 uur licht	
	maart	12	154	22		leg voer	16 uur licht	
	maart	13	161	23		leg voer		
	april	14	168	24		leg voer		
	april	15	175	25		leg voer		
	april	16	182	26		leg voer		
	april	17	189	27		leg voer	KI	
	mei	18	196	28		leg voer	KI en start met eieren verzamelen	
	mei	19	203	29		leg voer	KI en eieren verzamelen	
	mei	20	210	30		leg voer	uitbroeden	
	mei	21	217	31		leg voer	uitbroeden	
	mei	22	224	32		leg voer	uitbroeden	
	juni	23	231	33	0	leg voer	uitbroeden - uit het ei komen	5e bloedafname (22 weken na nieuw voer)
	juni	24	238	34	1	leg voer	2e generatie opfokvoer 1	
	juni	25	245	35	2	leg voer	opfok voer 1	
	juni	26	252	36	3	leg voer	opfok voer 1	
	juli	27	259	37	4	leg voer	opfok voer 1	
	juli	28	266	38	5	leg voer	opfok voer 1	
	juli	29	273	39	6	leg voer	start met opfok voer 2	
	juli	30	280	40	7	leg voer	opfok voer 2	
	augustus	31	287	41	8	leg voer	opfok voer 2	
	augustus	32	294	42	9	leg voer	opfok voer 2	
	augustus	33	301	43	10	Laatse week voer	opfok voer 2	
	augustus	34	308	44	11		opfok voer 2	
	augustus	35	315	45	12		opfok voer 2	
	september	36	322	46			opfok voer 2	
	september	37	329	47				
	september	38	336	48				
	september		343	49				
	Entingen							
	Marek Rispens HVT		Mareks disease					
	IB MA 5		Infectious bronchitis					
	NCD		New Castle Disease					
	Gumboro		Gumboro					
	PDww		Pox/ Diphteria wing web					
	ILT		Infectious	Laryngotracheitis				
	AE		Myoclonia congentia (trilziekte)					

Annex 1b – Timeframe 2nd generation

Maand	Week	Leeftijd Kippen in dagen	Leeftijd kippen in weken	Tijdpad 2e generatie Voer en experimentele handelingen	ma	di	wo	do	vr	za	zo
					Handelingen	Handelingen		Handelingen			
29-jun-06	26	0	0	opfok voer 1	Leeftijd in dagen		1e kuikens	Marek Rispens HVT + IB MA 5	2	3	4
juli	27	5	1	opfok voer 1	wegen Paracox 5			hok schoonmaken op donderdagen			
juli	28	12	2	opfok voer 1	wegen + NCD-1 selectie 12						
juli	29	19	3	opfok voer 1	wegen 19			Gumboro			
juli	30	26	4	opfok voer 1	wegen 26 zand op bodem			NCD-2			
augustus	31	33	5	opfok voer 1	wegen 33						
augustus	32	40	6	opfok voer 1	wegen 40			PDww			
augustus	33	47	7	start met opfok voer 2	wegen 47						
augustus	34	54	8	opfok voer 2	wegen 54	1e tappen					
augustus	35	61	9	opfok voer 2	wegen 61	challengeKLH					
september	36	68	10	opfok voer 2	wegen 68	2e tappen					
september	37	75	11	opfok voer 2	wegen 75	3e tappen					
september	38	82	12	opfok voer 2	wegen 82	4e tappen					
september	39	89	13	opfok voer 2	wegen 89	5e tap & sectie					

Annex 2 – Description feed ingredients

Crop production The chicken feeds are composed out of fodder components from organic and conventional agriculture. Differences in agronomic practice will cause differences in the product which might influence the nutritional value and health supporting properties of it. Organic agriculture is based on four principles (IFOAM, 2007): health, ecology, fairness and care. This implicates that the targets of organic agriculture are much more differentiated than in conventional agriculture. The agronomic practice *should be based on living ecological systems and cycles, work with them, emulate them and help sustain them* (IFOAM, 2007). This is much broader then the commonly known characterisation 'no use of pesticides and artificial fertilizer'. For arable crops, this implicates that all crops are interrelated in time and space, that variety choice (and breeding) is an essential part of the system, that crop residues and manures are integrated in the existing nutrient and organic matter cycles etc.
Nevertheless, crop protection and fertilization practice are two important differences between these two production systems which can easily be described and quantified, which will be done in the following text. Variety choice is an other, important factor influencing nutritional composition of products.

Crop protection In organic agriculture, no synthetic pesticides are allowed. Some pesticides with natural compounds or bacterial ingredients are allowed. No herbicide is allowed. A healthy crop development is supported by variety choice, crop rotation, a biodiverse environment, moderate fertilizer application and a health supporting soil. In conventional agriculture the crops are kept (if successful) free from plagues and diseases by pesticides, minimising stress, but the application of a pesticide itself is to a certain degree a stress factor. In organic agriculture, after creating optimal circumstances for a healthy crop growth, a certain level of diseases or plagues is accepted, ideally kept at a low level by self-regulation within the production system. Presence of a certain level of stress by diseases or plagues is accepted and considered normal, stimulating to a moderate level the presence of antioxidants and other natural compounds which might play a positive role in product quality. In the organic crops used for this feeding experiment no pesticides were used.

Fertilization In organic farming no synthetic nitrogen fertilizer is allowed. For the other two macro-nutrients, potassium and phosphorus, some natural and some artificial fertilizers are allowed under restrictions. In Dutch organic conditions, the soil fertility status related to phosphorus and potassium is in general sufficient and no shortage is expected under normal conditions. For micro-nutrients this is the same, partly because of a sufficient input of organic matter and a more- than average percentage of soil organic matter under organic conditions. pH of the soils is normally within the optimal range for micro nutrients availability.
The main differences between organic and conventional practice are expected in the type of nitrogen application, the level of nitrogen application and the nitrogen dynamics during the crop growth.

Type of nitrogen.

Artificial fertilizers contain nitrogen which is directly available for plant growth (nitrate) or almost directly available (ammonium, to be transformed into nitrate in the soil). Organic fertilizers contain nitrogen in several forms. Part of it is (almost) directly available (nitrate and ammonium), and part is part of organic compounds. This organically bound nitrogen will become available after decay of the organic matter, which is dependant on soil life, and because of that

dependant on soil moisture, pH, temperature, soil structure, type of soil life and type of organic matter. (van der Burgt et al. 2006)

Nitrogen level

In general, nitrogen application for organic crops is lower than for conventional crops. This will have influence both on yield quantity and quality. Yields are in general lower (Mäder et al. 2002) , and on average protein content and nitrate content are lower, with quite some exceptions.

Nitrogen dynamics

The nitrogen dynamics under organic cultivation can differ substantially from conventional cultivation. Nitrogen becomes available out of two 'sources': direct available nitrogen from artificial fertilizers and (part of the) organic fertilizers, and decomposition of organic materials in the soil. In conventional agriculture, the ratio of 'direct available nitrogen out of artificial fertilizers' versus 'nitrogen out of decomposition' is higher than in organic agriculture (van der Burgt (2003). Conventional agriculture is focussed on direct nitrogen application to the crops; in organic agriculture an important part of the crop nitrogen uptake is not directly applied to the crop but indirectly via the soil and the decomposition of soil organic matter: humus, plant residues, green manure residues, manures, composts (van der Burgt et al, 2006). The pattern of the available nitrogen can differ substantially. When artificial nitrogen fertilizers are applied, a peak of soil available nitrogen appears. The release of nitrogen from decomposition of soil organic matter follows mainly temperature and soil moisture conditions, resulting in an annual increase (spring) and decrease (autumn) in nitrogen release.

Both nitrogen level – in general higher in conventional agriculture) and nitrogen dynamics – peaks when artificial fertilizers are used, increase and decrease during the year in case of soil organic matter decomposition – are influencing both yield and (protein) composition of the product. Still it must be kept in mind that the differences in production level between conventional and organic arable crops can only partly be explained by the level of available nitrogen.

The distinction between conventional and organic agriculture in practice are less pronounced than suggested above. In organic agriculture slurry can be applied in which 50% of the nitrogen is directly available (Bokhorst and ter Berg, 2001), thus causing a peak in nitrogen level. Blood meal pellets and feather meal pellets contain up to 13% N which is easily decomposable (up to 80% of total N within a few weeks) and 'Vinasse' contains up to 2% direct available N and 2% easily decomposable N (Janmaat and Cuijpers, 2005). These fertilizers can all be used in organic agriculture, acting to a certain degree identical to artificial fertilizers.

In conventional agriculture some crops are fertilized moderately, thus relatively increasing the impact of the soil organic matter component in the nitrogen dynamics.

This all means that the above given description of possible differences in the cultivation system must be checked on every crop on its own.

To obtain the ingredients for chicken feed for this project, the pairs of farms – conventional and organic – should show sufficient differences in agronomic practice. As a guideline we used the following descriptions:

Conventional: following the local 'normal agricultural practice' for that crop.

Organic: a 'typical' well-developed organic arable farm: not recently conversed from conventional farming, no crop protection used, and moderate in nitrogen application.
Using these descriptions, both crop protection and nitrogen dynamics might be substantially different between the two systems, contributing to the discriminatory strength.

To characterise the produce which is used in the experiment, information is gathered from the farm and the field where the crop was grown. The information obtained from the farms is limited and in some cases incomplete. In the following tables, a summary of the crop management is given, resulting in a characterisation of the differences between the two cropping systems.

Table A2.1: Agronomic information of wheat.

Wheat	**Conventional**	**Organic**
Location	Flevoland province Dronten	Flevoland province Dronten
Type	Winter wheat	Spring wheat
Variety	Bristol	Lavett
Soil type	Clay	Clay
Fertilizers	Split application of artificial fertilizer 27 kg N/ha (10 April) 40 kg N/ha (20May) Total 67 kg, all available	Composted cattle manure, 25 ton/ha, autumn application, total 130 kg N/ha, estimated available nitrogen 25 kg n/ha
Crop protection	Two spraying with mixtures of five and four components, 5 May and 3 June	None
Yield	Not registered	6800 kg/ha

Characterisation Both conventional and organic are typical for their group. The conventional wheat is cultivated according to the local standard, including nitrogen application dependant on measured available soil-N in spring, and including several crop protection components. The organic wheat has received no crop protection and no fertilizer at all during the growth, being completely dependant on the release of nitrogen out of soil organic matter. The applied manure releases only a minor part of total requested nitrogen. The yield is nevertheless at the top of what is realised in the Netherlands under organic conditions, indicating a stable and optimal organic production system.

Conclusion Strength: both farms typical for their system
Weakness: different varieties and plant types used (winter wheat and spring wheat).

Table A2.2: Agronomic information of triticale.

Triticale	Conventional	Organic
Location	Gelderland province 'Veluwe'	Gelderland province 'Veluwe'
Variety	Talentro	Cairo
Soil type	Sandy soil	Sandy soil
Fertilizers	Artificial fertilizer, Split application, Total 150 kg N/ha	Organic liquid fertilizer ('Vinasse'), Total 160 kg N/ha, estimated available nitrogen 130 kgN/ha
Crop protection	Three sprayings of single pesticides	None
Yield	7200 kg/ha	4500 kg/ha

Characterisation This conventional triticale crop is typical for conventional agriculture, the organic is less typical. In the organic crop, nitrogen has been applied as 'Vinasse', being a type of fertilizer in which 50% of the nitrogen is directly available, comparable with artificial fertilizer (Janmaat and Cuijpers, 2005) and 50% easily decomposable. In this crop, a soil nitrogen peak will have occurred.

Conclusion Strength: conventional farm typical for the system
Weakness: fertilizer type in organic agriculture; use of different varieties.

Table A2.3: Agronomic information of spring barley.

Spring barley	Conventional	Organic
Location	Gelderland province, Wageningen	Gelderland province, Wageningen
Variety	Class	Prestige
Soil type	Sandy soil	Sandy soil
Fertilizers	Artificial fertilizer, 74 kg N/ha, all directly available	Cattle slurry, 15 tons/ha, total 79 kg N/ha, estimated available nitrogen 55 kg N/ha
Crop protection	Four sprayings with single or compound pesticides	None
Yield	6000 kg/ha	3000 kg/ha

Characterisation This conventional spring barley crop is typical for conventional agriculture. The organic crop is for Dutch conditions more or less typical: a moderate amount of slurry, creating a moderate nitrogen peak with a relatively low contribution of direct available nitrogen to total nitrogen turnover.

Conclusion Strength: both farms typical for their system.
Weakness: different varieties used.

Table A2.4: Agronomic information of soy bean.

Soy bean	Conventional	Organic
Location	Austria	Austria
Variety	Essor	Essor
Soil type	Sandy loam	Sandy loam
Fertilizers	No fertilizer	No fertilizer
Crop protection	Unknown	Curry and hoeing
Yield	2000 kg/ha	1400 kg/ha

Characterisation Soy bean, being a leguminous crop, can fix nitrogen out of the air. This means that nitrogen fertilizer application in conventional agriculture normally is limited or none, and in organic cultivation none. In this case, no fertilizer was applied at conventional and organic crop, reducing the difference between the two systems.

Conclusion Strength: both farms typical for their system.
Weakness: no difference in fertilizer application (but this is normal in this crop)

Table A2.5: Agronomic information of maize.

Maize	**Conventional**	**Organic**
Location	Austria	Austria
Variety	Kuxxar, Saxxoo, Disally, Disamanta, Clarica, Monalisa, Soarta, LG 2306.	Kuxxar, Saxxoo, Disally, Disamanta, Clarica.
Soil type	Small gravel, about 20 cm humus-containing topsoil	Small gravel, about 20 cm humus-containing topsoil.
Fertilizers	Ca. 40-60 kg P and 80-100 kg K/ha, 50-60 kg N/h before sowing, 50-60 kg N in June	Crop growers: residues of previous crops (in most cases legumes) Livestock farms: 20m^3 slurry before tillage, 10-20m^3 slurry in June
Crop protection	1X herbicide end of May (Maize-Banvel, Zintan, Pradner,...)	In some cases: Trichogramma against European corn borer
Yield	10.000 kg/ha	6.000-7.000 kg/ha

Characterisation The organic maize is obtained from several farms. Available nitrogen in the organic system was lower than in the conventional system, especially on the arable farms but also on the maize crops on livestock farms. Several varieties were used, both in the conventional and the organic farms.

Conclusion Strength: conventional farm typical for the system. Organic farms probably to, but detailed information of each farm is absent.
Weakness: several farms and several varieties used.

Table A2.6: Agronomic information of peas.

Peas	Conventional	Organic
Location	Denmark	Denmark
Variety	Unknown	Unknown
Soil type	Clay loam	Clay loam
Fertilizers	2000 kg/ha, 4% potassium 21% phosphorus	No fertilizer
Crop protection	2x weed sprayings (0,5 l STOP + 0,5 l Basagra) 2x spraying against aphids (Fasttak, the last time (1st July) including dithane (2 kg/ha) against fungal diseases	None
Yield	5000 kg/ha	1500 kg/ha

Characterisation Comparable to soybean, no nitrogen fertilizer is applied to the conventional and organic pea crops. This is normal for this crop, but reduces the agronomic differences between the conventional and the organic crop.

Conclusion Strength: both farms typical for their system.
Weakness: variety choice unknown.

Annex 3 – Feed supplementation Potato protein

AVEBE FEED

P.O. Box 15
9640 AA Veendam
The Netherlands
FeedInfo@AVEBE.COM
FEED.AVEBE.COM

PROTASTAR®

Product Information

Amino acid composition

(mean values per kg product as such)

Moisture	g	100
Crude protein	g	800
Crude fat	g	30
Crude ash	g	7
Crude fibre	g	7
NFE	g	56
lysine	g	64.0
methionine	g	19.2
cysteine	g	12.8
meth. + cyst.	g	32.0
threonine	g	48.0
trytophan	g	10.4
valine	g	56.8
leucine	g	86.4
isoleucine	g	47.2
phenylalanine	g	55.2
tyrosine	g	46.4
histidine	g	19.2
arginine	g	44.0
glycine	g	42.4
alanine	g	40.8
aspartic acid	g	103.2
glutamic acid	g	97.6
proline	g	41.6
serine	g	45.6
Ca	g	0.5
P	g	2.0
Na	g	0.05
K	g	0.05
Cl	g	3.0
Mg	g	0.25
Total aerobic mesophilic count	CFU/g	< 50 000
Yeasts and moulds	CFU/g	< 1 000
Enterobacteriaceae	CFU/g	< 100
Escherichia coli (1 g)		absent
Salmonella (25 g)		absent

1/1

ref.no. 209006.0, issued August 01, 2005

Annex 4 – Feed supplementation Fx layers premix

27-110.2 Fx(v)LEGHENNEN + ZOUT 1% **Versie: 5 Ingangsdatum: 28-01-04**

Verpakking: 20 kg

Poedervormig voormengsel voor voeders voor: Leghennen

Kleur: Bruin Geur: Neutraal

Inmengingspercentage: 1.000%

Naam	Per kg premix		Per kg eindvoer	
Stortgewicht	1.086	kg	0.011	kg
Liters	1.280	l/kg	0.013	l/kg
Vitamine A	800000.000	IE	8000.000	IE
Vitamine D3	250000.000	IE	2500.000	IE
Vit.E(-acetaat)	1001.000	IE	10.010	IE
Vitamine K3	100.000	mg	1.000	mg
Vitamine B1	50.000	mg	0.500	mg
Vitamine B2	400.000	mg	4.000	mg
d Pantotheenzuur	600.000	mg	6.000	mg
Niacine	2000.000	mg	20.000	mg
Vitamine B12	2000.000	mcg	20.000	mcg
Foliumzuur	30.000	mg	0.300	mg
Vitamine B6	200.000	mg	2.000	mg
Choline Chloride	20001.000	mg	200.010	mg
Cd Max.	0.638	mg	0.006	mg
Pb Max.	21.210	mg	0.212	mg
Fe:Fe(II)Sulf.	7000.000	mg	70.000	mg
Cu:CuSO4.5H2O	1500.000	mg	15.000	mg
Zn:ZnOxyde	6000.000	mg	60.000	mg
Mn:Mn(II)Oxyde	8001.000	mg	80.010	mg
Co:Co(II)Carbon.	25.000	mg	0.250	mg
I:K-Jodide	100.000	mg	1.000	mg
Se:Na-Seleniet	20.000	mg	0.200	mg
Ox(E310/E320/E321	10000.000	mg	100.000	mg
Zout	100.000	g	1.000	g
Na-Bicarbonaat	100.000	g	1.000	g
Calcium totaal	150.000	g	1.500	g
Fosfor totaal	1.880	g	0.019	g
Kalium totaal	3.112	g	0.031	g
Natrium totaal	69.120	g	0.691	g
Chloor totaal	65.209	g	0.652	g
Zwavel totaal	6.200	g	0.062	g
Ruw Eiwit	40.376	g	0.404	g
Ruw Vet	10.423	g	0.104	g
Ruwe Celstof	16.790	g	0.168	g
As	724.000	g	7.240	g
Vocht	39.880	g	0.399	g
Zetmeel Ewers	41.220	g	0.412	g
Suiker	8.876	g	0.089	g
Suiker/Zetmeel	50.805	g	0.508	g
Lysine	1.101	g	0.011	g
Methionine	0.444	g	0.004	g
Meth+Cys	1.012	g	0.010	g
Threonine	0.805	g	0.009	g
Tryptophaan	0.391	g	0.004	g
Isoleucine	0.870	g	0.009	g
OE sk	240.974	kcal	2.410	kcal
OE leg	345.351	kcal	3.454	kcal
V.LysPLV	0.416	g	0.004	g
V.MetPLV	0.166	g	0.002	g
V.M+CPLV	0.304	g	0.003	g
V.ThrPLV	0.239	g	0.002	g
V.TryPLV	0.225	g	0.002	g
oP'97 plv	0.515	g	0.005	g
oP'97 leg	0.515	g	0.005	g

HOUDBAARHEID: zie etiket of geleidedocument

VERWERKING: zie FARMIX-instruktie "Veilig werken met voormengsels."

De authorisatie van deze specificatie is electronisch vastgelegd.

Annex 5 – Animal Ethics Committee approval

Aanmeldingsformulier voor proeven met gewervelde dieren.

Secretariaat DEC
p/a Mw M. Dohmen
Costerweg 50
6701 BH Wageningen
tel. 0317-483906
fax 0317-484292

Aanvrager: Henk Parmentier (henk.parmentier@wur.nl)
Afdeling: Adaptatie Fysiologie (ADP)
bode 128
Zodiac, Gebouwnummer 531
Postbus 338, 6700 AH Wageningen

Titel dierproef: Invloed van 'organic' voer op de immuunstsatus van legpluimvee

Aanmeldcode / Protocol: 2005062.c
Stadia van de proef:

29-08-2005	Aangemeld	Henk Parmentier
01-09-2005	Goedgekeurd	Secretaris van de DEC

Is deze proef wetenschappelijk getoetst en goedgekeurd? Ja
Toelichting:
Dit experiment is onderdeel van een samenwerkingsproject "Biologisch gezonder?" tussen achtereenvolgens het Louis Bolk instituut, RIKILT, TNO Kwaliteit voor Leven, WUR-Dierwetenschappen (LSG's Celbiologie & Immunologie; en Adaptatiefysiologie) in opdracht van het ministerie van LNV (Taskforce Marktontwikkeling Biologische Landbouw). Tevens is dit project onderdeel van een internationaal samenwerkingsverband Food Quality & Health association (FQH). Dit voorstel is een eerste experimentele benadering van de vraag of 'organic' voer een gunstige uitwerking heeft op de gezondheidsstatus (en vleeskwaliteit) van kippen, en is opgesteld door prof. H. Savelkoul en dr. H.K. Parmentier

1.a. Met dit onderzoek te beantwoorden concrete vraag:
. Wetenschappelijke vraag m.b.t.van dieren
In dit experiment zal de invloed van 'organic' voer op de immuunstatus van legpluimvee worden bestudeerd. Dieren van de Wageningse selectielijnen zullen of conventioneel (gangbaar dieet) gevoerd worden, of met een voer samengesteld uit voornamelijk biologisch geteelde producten. De voeders zullen in principe identiek van samenstelling zijn, en voldoen aan de standaard normen voor opfokhennen. Allereerst zullen ouderdieren gevoerd worden met een menu samengesteld uit een mengsel van biologisch geteelde producten of een menu samengesteld uit gangbaar geteelde producten. Deze behandeling is nodig om effecten van mogelijke overdracht van hen op ei te incorporeren. Deze generatie is uitsluitend nodig voor de productie van 25 hennen per behandelings (voer/lijn) groep. Deze laatste dieren vormen het eigenlijke experiment.

De meeste ingrediënten voor het voer zijn afkomstig van de gecontroleerde teelten uit het QLIF programma (tarwe en gerst) in Engeland en DOK programma van FiBL (soja en erwten) in Duitsland en Zwitserland. Beide organisaties hebben jarenlange ervaring in het onderzoeken van verschillende teelt wijzen (dus meer dan alleen biologisch vs. gangbaar). Zij hebben daartoe speciale proefvelden. Het mag duidelijk zijn dat bij de teelt van deze producten alle variabelen die controleerbaar zijn gelijk worden gehouden (ras, bodem, oogstmoment, etc.).
Enkel maïs is niet uit deze programma's te verkrijgen vanwege het klimaat in de deelnemende landen die niet afdoende is om maïs tot volle wasdom te laten groeien. Daartoe wordt maïs verkregen via onze contacten bij het Ludwig Boltzmann Instituut in Oostenrijk. In dit geval is er geen sprake van proefvelden maar is er nauw contact met de boeren uit de omgeving. Zo worden bedrijven paarsgewijs uitgekozen, bij voorkeur twee "buurbedrijven" om bodem verschillen en weersverschillen te minimaliseren. Verder wordt erop gelet dat de ras, bodem, oogstmoment etc. hetzelfde is. Alle teelt informatie wordt verzameld om dit te kunnen onderbouwen.
Het doel is dat minimaal 75% van het voer wordt samengesteld uit producten van ofwel biologische teelt of de gangbare teelt en dat de rest van het voer wordt aangevuld met identieke producten. De andere producten die benodigd zijn voor het voer (vitaminen, kalk, zout etc.) wordt van één bron genomen, zodat dit niet tot verschillen kan leiden.
De samenstelling van het voer wordt gebaseerd op verschillende normen voor leghennen en er is ook contact met een producent van kippenvoer. Tevens wordt uitgezocht wat de samenstelling van het huidige voer is van de huidige generaties.

Aanvullende info van bovenstaande instituten:

QLIF = Quality Low Input Food: www.qlif.org
FiBL = Forschungsinstitut für biologischen Landbau : www.fibl.org
Ludwig Boltzmann Instituut : www.natur-wien.at/partner/boltzmann

1.b. Het uiteindelijk doel (Maatschappelijke en wetenschappelijke relevantie):
Het 'gezondheidsbevorderende' effect van biologische producten is nog steeds onderhevig aan heftige debatten. Een van de redenen waarom er sprake is van een grote mate van scepsis is dat een aantal claims niet direct of eenvoudig terug te voeren zijn op gangbare onderzoeksmethodieken, of algemeen geaccepteerde technologieen, of dat de verschillen tussen groepen dieren marginaal zijn, of omdat de gevonden effecten niet op een mechanisme terug te voeren zijn. In dit experiment wordt het effect van biologische voeding op de immuunstatus bestudeerd door in het zelfde experiment dieren of voer gebaseerd op gangbare productietechnieken of voer samengesteld uit biologisch geteelde producten te meten en de dieren te houden onder dezelfde omstandigheden. Uit dit experiment moet naar voren komen of biologische voeders de immuunstatus van legpluimvee (kunnen) veranderen. De kwaliteit en samenstelling van het te testen biologische voer wordt door andere instituten (RIKILT en TNO) getest, waarnaast het Bolk instituut, respectievelijk het RIKILT en TNO de kwaliteit (nutritionele waardes, bacteriele diversiteit, aanwezigheid van pesticiden en mycotoxines) van het vlees, respectievelijk het meten van biomarkers (biofotonen) in bloed, vlees en lever van de te testen dieren zal uitvoeren.

2. Gepland vanaf: 01-09-2005 **tot** 01-09-2006

3. Specificatie diergroepen:

oudergeneratie	60	kippen
testgeneratie	150	kippen

4.a. Nadere aanduiding gebruikte dieren:
De oudergeneratie (60 dieren) zal geselecteerd worden uit de nieuwe in september 2005 te fokken 25e generatie van de Wageningse SRBC selectielijnen. De te testen dieren (150 hennen) zijn afkomstig van deze oudergeneratie en vormen dus een parallele generatie met een toekomstige 26e generatie van de Wageningse SRBC selectielijnen. De grootouderdieren (hanen en hennen) waaruit de 60 ouderdieren worden gefokt zijn divergent geselecteerd op hun anti-SRBC agglutinatietiter op 5 dagen na immunizatie met SRBC op 7 weken leeftijd, en behoren tot de huidige 24e generatie.

4.b. Motivatie waarom is gekozen voor deze diersoort:
Pluimvee, met name legpluimvee, zijn een belangrijke afnemr van 'organic' voer. Behalve dat het immuunsysteem van vogels in grote mate overeenkomt met dat van zoogdieren of de mens biedt het pluimvee model de kans gen * omgevingsinteracties in de studies mee te nemen, omdat er vele verschillende rassen en houderijsystemen voorhanden zijn. Daarnaast is de kip een eenvoudig hanteerbaar en 'goedkoop' edoch landbouw relevant proefdier.

4.c. Toelichting voor het aantal gebruikte dieren:
Vijfentwintig hennen (testgeneratie) per behandeling/lijn groep zijn gekozen omdat a. er in principe eerst aandacht wordt besteed aan innate immuunparameters (d.w.z. de dieren worden in eerste instantie niet geimmuniseerd) waarvan bekend is dat de spreiding in 'reactiviteit' (bijv. complementniveau's) groot kan zijn, b. ook productieparameters: groei; eileg en voerconversie wordt gemeten, en de te verwachten effecten van 'organic' voer vooralsnog niet groot verondersteld worden, en c. de dieren gedurende ruim 4 maanden worden gevolgd, hetgeen betekent dat er mogelijk sprake van uitval kan zijn. Twintig ouderdieren (6 hanen en 14 hennen) zijn berekend voor het verkrijgen van 50 hennen per (hoge, lage, controle) lijn.

4.d. Herkomst:

oudergeneratie	A. Geregistreerde fok/toelevering in Nederland
testgeneratie	A. Geregistreerde fok/toelevering in Nederland

Toelichting:
De 60 ouderdieren worden geselecteerd uit de nieuw te fokken 25e generatie selectielijnen, en de

testgeneratie: 150 hennen (50 per lijn) zijn 'vergelijkbaar' met een nieuwe toekomstige 26e generatie van de Wageningse selectielijnen, geselecteerd op hoge, dan wel lage antistoftiters tegen SRBC, naast een random bred controle lijn.

5.a. Accommodatie: De Haar varkens / pluimvee

5.b. Huisvesting & Verzorging:
Normaal voor opfokpluimvee.

5.c. Voeding:
Zes hanen en 14 hennen per lijn zullen uit de nieuwe 25e generatie van de SRBC selectielijnen geselecteerd worden. Drie hanen en 7 hennen per lijn worden gevoerd met gangbaar voer vanaf hatch en opfok (oktober 2005 ev). Drie hanen en 7 hennen per lijn worden zo vroeg mogelijk tijdens de opfok en vervolgens verder gevoerd met voer op basis van biologisch geteelde producten die geleverd gaat worden door het Louis Bolk Instituut. Op een leeftijd van niet eerder dan 6 maanden oud (mei 2006) worden de hanen getrained en de hennen van de oudergeneratie geinsemineerd voor het verkrijgen van 300 broedeieren. De te verwachten uitkomst van de 300 broedeieren is niet eerder dan in juni/juli 2006. Vanaf hatch zullen deze te testen hennen of biologisch of gangbaar opfokvoer krijgen.

6.a. Proefschema / proefbehandelingen:
Van de nieuwe 25e generatie SRBC selectielijnkippen zullen zes hanen per lijn, en 24 hennen per lijn als oudergeneratie zullen worden gebruikt voor het verkrijgen van ongeveer 300 broedeieren, waarvan uiteindelijk 150 hennen (50 per lijn (tesgeneratie) gebruikt gaan worden voor het meten van de effecten van organic voer op de immuunstatus. De helft van de ouderdieren zal gevoerd blijven met conventioneel voer, de andere helft zal vanaf een maand voor de aanvang van het trainen en insemineren gevoerd gaan worden met door het Louis Bolk Instituut te leveren 'organic' voeder. De samenstelling en kwaliteit van de voeders wordt bepaald door het RIKILT en TNO.

Proefopzet testgeneratie:
Vanaf de uitkomst zullen de kuikens direct met of het biologisch voer, of het gangbare voer worden gevoerd (ad lib). De dieren (hennen) zullen in 6 groepen (25 dieren per groep) worden verdeeld: 3 lijnen (Hoge lijn, Lage lijn, en Controle lijn) en twee voergroepen, en zullen middels grondvloerhuisvesting gehuisvest worden.
Dieren zullen in 5 replicates van 5 dieren worden gehuisvest. Dit geeft de mogelijkheid voeropname, gewicht en voederconversie te bepalen.

*Op een leeftijd van 8 weken zal per dier 1 ml bloed voor serum en 1 ml bloed voor plasma/cellen worden afgenomen. Deze monsters zullen worden gebruikt voor achtereenvolgens de volgende bepalingen: aantallen en aktiviteit van NK cellen; idem monocyten; idem (T/B) cellen; concentratie natuurlijke antistoffen, cortisol, klassiek en alternatief complementgehalte; niveau's van cytokines (mRNA), en in vitro activatie van T cellen.

*Op een leeftijd van 12 weken zullen de dieren subcutaan worden geinoculeerd met 1 mg van het modelantigeen KLH, waarna op 3, 7, 10, 14, 21 en 28 dagen 1 ml bloed wordt afgenomen voor het bepalen van specifieke antistoftiters tegen KLH, en een aantal van bovenstaande parameters. Deze frequentie is nodig voor het correct meten van effecten van de voeders op de kinetiek van de specifieke immuniteit.
Op dag 28 na immunizatie met KLH zullen de dieren worden geeuthaniseerd. Darmweefsel zal worden gemonsterd voor morfologische bepalingen aan de darm; vlees en lever zal middels een 'metabolomics' techniek gescanned worden voor diverse metabolieten (TNO), en materiaal zal ook worden opgeslagen voor later te meten mRNA expressie of andere vormen van genomics.

6.b. Mate van ongerief:
oudergeneratie B. Gering/Matig
testgeneratie D. Matig/Ernstig

6.c. Waaruit bestaat het ongerief en hoe bent u tot uw inschatting van de mate van ongerief gekomen?
Ouderdieren: Vangen, trainen en insemineren. Dieren zijn individueel gehuisvest.
Testgeneratie: 6 bloedafnames om de ontwikkeling van de immuunrespons in de tijd te volgen. Dieren zijn in 5-tallen gehuisvest.

7. Welke maatregelen heeft u getroffen om het ongerief tot een minimum te beperken?
Anesthesie:
oudergeneratie A. Niet toegepast (geen aanleiding).
testgeneratie A. Niet toegepast (geen aanleiding).
Pijnbestrijding:

oudergeneratie A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.
testgeneratie A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.

Oudergeneratie: De dieren worden voorzichtig gehandeld. Trainen en insemineren geschiedt door ervaren personeel. Het trainen van de hanen door rug en buikmassage en het opvangen van sperma leidt nauwelijks tot ongerief. Het cloaca uitstulpen bij de hennen en het insemineren met sperma is ook gering ongerief.
Testgeneratie: bloedafnames en wegen geschiedt door ervaren personeel

8. Toestand van dieren na einde van de proef:
oudergeneratie Het dier is na de proef in leven gelaten.
testgeneratie Het dier is gestorven of gedood ter beindiging van de proef.

Toelichting:
De ouderdieren worden niet i.v.m. de proef gedood! Voor deze dieren, die in wezen tot de 25e generatie van de selectielijnen behoren zal of in overleg met de proefaccomodatie een bestemming gezocht, of ze zullen weer in het selectieexperiment als ouderdieren voor de regulier toekomstige 26e generatie ingezet worden. De 150 testdieren worden op 16 weken leeftijd d.m.v. T 61 geeuthaniseerd.

9. Welke alternatieven (vervanging, verfijning, vermindering) zijn voor de beschreven experimenten overwogen en waarom zijn deze verworpen?
Het bepalen van gezondheidsbevorderende effecten van voeding op de immuunstatus en vleeseigenschappen vergt het gebruik van levende dieren. Echter binnen het project is ruimte voor het meten van de effecten van organische voedingsbestanddelen op cellen in vitro. Het gebruik van speciaal op grond van hun immuunstatus geselecteerde dieren biedt de mogelijkheid gen * omgevings/behandelingsinteracties te meten.

10. Namen van direct betrokkenen bij de dierproef (artikel 9- en 12-functionarissen):
dr. ir. H.K. Parmentier, art 9
Prof. dr. ir. H.F.J. Savelkoul , art. 9
drs. R. Adriaansen, art. 9, Bolk instituut
M.G.B. Nieuwland, art 12
A. Groeneveld, art 12

Tabel registratiecode opties voor aanvraag 2005062.c (K14):

	1	2	3	4	5	6	7	8	9	10	11	12	13
					36	1	1	01					
oudergeneratie	1	51	1	60					01	1	1	2	3
testgeneratie	1	51	1	150					01	1	1	4	1

Ingredients measured = A =B

Submissie Codering TNO		6130-6265 A2	6130-6275 Y2	ratio	6130-6273 V2	6130-6268 G2	ratio	6730-4335 F2	6730-4336 P2	ratio	6730-4337 C2	6730-4338 M2	ratio	6130-6278 CC2	6730-4342 X2	ratio	6730-4341 T2	6730-4342 X2	ratio	6730-3933 J2	6730-3934 R2	ratio
		Triticale	Triticale		Barley	Barley		Maize	Maize		Peas	Peas		Wheat	Wheat		Wheat	Wheat		Soy	Soy	
Nutrient	Sample unit	1	2	1/2	1	2	1/2	1	2	1/2	1	2	1/2	1	2	1/2	1	2	1/2	1	2	1/2
cystine	g/100 g	0,18	0,28	0,64	0,25	0,22	1,14	0,19	0,18	1,06	0,32	0,3	1,07	0,25	0,22	1,14	0,26	0,22	1,18	0,58	0,59	0,98
methionine	g/100 g	0,14	0,2	0,70	0,21	0,18	1,17	0,17	0,18	0,94	0,23	0,19	1,21	0,18	0,17	1,06	0,21	0,17	1,24	0,53	0,51	1,04
aspartic acid	g/100 g	0,47	0,58	0,81	0,68	0,59	1,15	0,55	0,62	0,89	2,44	2,44	1,00	0,51	0,46	1,11	0,58	0,46	1,26	4,20	3,60	1,16
threonine	g/100 g	0,24	0,33	0,73	0,4	0,35	1,14	0,3	0,31	0,97	0,8	0,77	1,04	0,31	0,26	1,19	0,32	0,26	1,23	1,47	1,30	1,13
serine	g/100 g	0,3	0,48	0,63	0,5	0,41	1,22	0,38	0,4	0,95	0,99	0,99	1,00	0,49	0,40	1,23	0,51	0,40	1,28	1,86	1,61	1,15
glutamic acid	g/100 g	1,55	2,93	0,53	2,78	2,11	1,32	1,48	1,61	0,92	3,5	3,44	1,02	3,07	2,46	1,25	3,21	2,46	1,30	6,60	5,51	1,20
proline	g/100 g	0,56	1,06	0,53	1,3	0,96	1,35	0,69	0,76	0,91	0,85	0,83	1,02	1,02	0,80	1,28	1,04	0,80	1,30	1,78	1,52	1,18
glycine	g/100 g	0,3	0,42	0,71	0,46	0,41	1,12	0,32	0,33	0,97	0,93	0,9	1,03	0,43	0,37	1,16	0,46	0,37	1,24	1,54	1,34	1,15
alanine	g/100 g	0,3	0,4	0,75	0,47	0,4	1,18	0,6	0,62	0,97	0,92	0,9	1,02	0,36	0,32	1,13	0,41	0,32	1,28	1,58	1,38	1,14
valine	g/100 g	0,33	0,48	0,69	0,56	0,47	1,19	0,39	0,42	0,93	0,99	0,99	1,00	0,43	0,38	1,13	0,49	0,38	1,29	1,72	1,49	1,16
isoleucine	g/100 g	0,24	0,39	0,62	0,42	0,34	1,24	0,29	0,31	0,94	0,91	0,88	1,03	0,35	0,30	1,17	0,39	0,30	1,30	1,68	1,47	1,14
leucine	g/100 g	0,46	0,72	0,64	0,79	0,65	1,22	0,99	1,04	0,95	1,53	1,5	1,02	0,69	0,60	1,15	0,77	0,60	1,28	2,85	2,46	1,16
tyrosine	g/100 g	0,21	0,32	0,66	0,39	0,32	1,22	0,34	0,35	0,97	0,75	0,73	1,03	0,33	0,28	1,18	0,35	0,28	1,25	1,38	1,18	1,17
phenylalanine	g/100 g	0,3	0,5	0,60	0,61	0,47	1,30	0,4	0,43	0,93	1,01	1	1,01	0,47	0,38	1,24	0,51	0,38	1,34	1,88	1,58	1,19
histidine	g/100 g	0,18	0,26	0,69	0,29	0,25	1,16	0,23	0,23	1,00	0,51	0,49	1,04	0,26	0,20	1,30	0,27	0,20	1,35	0,98	0,85	1,16
lysine	g/100 g	0,28	0,34	0,82	0,42	0,38	1,11	0,26	0,28	0,93	1,54	1,53	1,01	0,29	0,26	1,12	0,32	0,26	1,23	2,35	2,07	1,13
arginine	g/100 g	0,42	0,57	0,74	0,64	0,54	1,19	0,43	0,44	0,98	1,88	1,88	1,00	0,53	0,46	1,15	0,62	0,46	1,35	2,83	2,41	1,17
tryptophan	g/100 g	0,09	0,12	0,75	0,16	0,13	1,23	0,07	0,07	1,00	0,2	0,19	1,05	0,16	0,12	1,33	0,16	0,12	1,33	0,48	0,43	1,11
As	g/kg	18	17	1,06	17	27	0,63	12	14	0,86	30	32	0,94	16	16	1,02	17	16	1,06	48	52	0,92
carbohydrate total	g/kg	752	704	1,07	679	692	0,98	754	730	1,02	620	620	1,01	699	741	0,94	716	741	0,97	207	329	0,67
chloride	g/kg	<0,4	<0,4		0,85	0,93	0,91	<0,8	<0,8		0,95	<0,8		0,66	<0,8		<0,8	<0,8		<0,8	<0,8	
choline	g/kg	0,8	1,0	0,81	1,1	1,1	1,07	0,52	0,52	1,00	1,17	1,31	0,89	0,9	1	1,00	1	1	1,04	2	2	0,91
raw fiber	g/kg	32	29	1,08	43	43	0,99	28	32	0,88	61	56	1,09	33	28	1,17	33	28	1,18	80	80	1,00
crude fat (zure hydrolyse)	g/kg	18	17	1,05	25	24	1,03	37	40	0,92	10	10	0,97	22	21	1,04	19	21	0,91	209	228	0,92
moisture	g/kg	140	150	0,93	164	162	1,01	119	124	0,96	130	136	0,96	157	142	1,11	146	142	1,03	102	89	1,15
protein	g/kg	72	112	0,64	116	95	1,22	78	84	0,93	202	202	1,00	106	80	1,33	102	80	1,28	354	302	1,17
C8:0		0,15	0,00	#DEEL/0!	0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00	
C10:0		0,17	0,00	#DEEL/0!	0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00	
C12:0		1,34	0,10	13,53	0,43	0,55	0,78	0,01	0,03	0,43	0,04	0,03	1,52	0,03	0,02	1,60	0,04	0,02	2,49	0,00	0,01	0,88
C14:0		0,81	0,14	5,99	0,91	0,92	0,98	0,06	0,04	1,43	0,19	0,22	0,87	0,07	0,06	1,20	0,13	0,06	2,00	0,07	0,06	1,17
C14:1 c9		0,08	0,08	1,03	0,70	0,00	#DEEL/0!	0,01	0,03	0,42	0,11	0,07	1,57	0,04	0,04	1,10	0,08	0,04	2,29	0,00	0,00	1,92
C15:0		0,10	0,17	0,58	0,33	0,21	1,58	0,02	0,03	0,63	0,18	0,23	0,79	0,10	0,10	0,99	0,13	0,10	1,39	0,02	0,02	1,33
C16:0		17,73	21,99	0,81	26,90	21,67	1,24	12,55	11,28	1,11	12,93	13,52	0,96	16,93	16,47	1,03	17,91	16,47	1,09	10,64	10,56	1,01
C16:1 c9		0,09	0,10	0,90	0,31	0,20	1,60	0,18	0,12	1,52	0,05	0,07	0,66	0,10	0,15	0,69	0,14	0,15	0,98	0,09	0,09	0,93
C17:0		0,95	0,14	6,63	0,52	0,49	1,05	0,16	0,11	1,41	0,38	0,28	1,34	0,17	0,13	1,28	0,20	0,13	1,52	0,12	0,11	1,09
C18:0		1,62	0,89	1,82	3,51	3,76	0,93	1,92	1,86	1,03	3,34	3,63	0,92	1,01	0,74	1,35	1,03	0,74	1,39	3,97	3,83	1,04
C18:1 t		0,24	0,06	4,09	0,49	0,70	0,71	0,05	0,03	1,61	0,03	0,01	4,93	0,07	0,06	1,05	0,07	0,06	1,04	0,02	0,03	0,71
C18:1 c		13,61	13,90	0,98	13,87	12,65	1,10	28,40	32,55	0,87	18,23	16,36	1,11	16,04	16,52	0,97	11,03	16,52	0,67	21,56	21,92	0,98
C18:2 c9,12		52,88	55,49	0,95	47,15	46,96	1,00	53,52	50,81	1,05	52,88	54,02	0,98	58,60	59,47	0,99	60,98	59,47	1,03	54,87	54,31	1,01
C20:0		0,12	0,00	#DEEL/0!	0,40	0,00	#DEEL/0!	0,40	0,42	0,95	0,32	0,39	0,83	0,09	0,08	1,13	0,14	0,08	1,83	0,36	0,33	1,09
C18:3 c9,12,15		5,71	6,25	0,91	2,70	3,28	0,82	1,76	1,58	1,11	9,79	9,52	1,03	4,97	4,69	1,06	5,04	4,69	1,08	7,22	7,85	0,92
C22:0		0,27	0,30	0,90	0,56	1,24	0,45	0,14	0,16	0,88	0,05	0,11	0,43	0,16	0,13	1,24	0,18	0,13	1,39	0,40	0,39	1,04
C24:0		0,28	0,38	0,74	1,21	1,34	0,90	0,26	0,25	1,03	0,22	0,22	0,99	0,20	0,23	0,90	0,42	0,23	1,84	0,27	0,17	1,54
unidentified		3,85	0,00	#DEEL/0!	0,00	6,03	0,00	0,56	0,70	0,80	1,26	1,32	0,95	1,42	1,12	1,27	2,47	1,12	2,21	0,40	0,35	1,14
cholesterol	mg/100 g	0,3	0,2	1,50	0,2	0,4	0,50	0,5	0,3	1,67	0,1	0,1	1,00	0,4	0,1	4,00	0,1	0,1	1,00	0,3	0,3	1,00
campersterol	mg/100 g	10,5	8,6	1,22	7,2	3,3	2,18	15,1	15,8	0,96	8,2	6,8	1,21	10,2	9,9	1,03	8	9,9	0,81	16,6	15,3	1,08
stigmasterol	mg/100 g	2,8	3,7	0,76	3	1,7	1,76	7,6	8,3	0,92	6,2	4,8	1,29	1,7	3,5	0,49	2,5	3,5	0,71	13,3	12,9	1,03
sitosterol	mg/100 g	36,4	35	1,04	30,1	19,9	1,51	60,3	58,6	1,03	60,9	67,9	0,90	37,4	37,1	1,01	30,8	37,1	0,83	45,8	34,8	1,32
avenasterol	mg/100 g	12,3	17,3	0,71	9,7	7	1,39	23,6	20	1,18	4,8	4,6	1,04	13,1	11	1,19	9,1	11	0,83	5,8	3,4	1,71
Daidzin	mg/g																			0,4	0,4	1,05
Genistin	mg/g																			0,6	0,6	0,98
Daidzein	mg/g																			<0,1	<0,1	
Genistein	mg/g																			<0,1	<0,1	
Glycetein	mg/g																			<0,1	<0,1	
Glycetin	mg/g																			0,1	0,0	2,00
Malonyl daidzin	mg/g																			1,7	2,4	0,73
Malonyl glycetin	mg/g																			0,1	0,2	0,78
Acetyl daidzin	mg/g																			<0,1	<0,1	
Acetyl glycetin	mg/g																			0,2	0,3	0,69
Malonyl genistin	mg/g																			1,8	2,7	0,67
Acetyl genistin	mg/g																			<0,1	<0,1	
Vitamin B1 (total thiamin)	mg/kg	2	3	0,67	3,3	2,8	1,18	4,9	5,6	0,88	6,3	6,4	0,98	3,3	2,5	1,32	3,4	2,5	1,36	3,5	4,8	0,73

Ingredients measured (continued)

Submissie Codering TNO		6130-6265 A2	6130-6275 Y2	ratio	6130-6273 V2	6130-6268 G2	ratio	6730-4335 F2	6730-4336 P2	ratio	6730-4337 C2	6730-4338 M2	ratio	6130-6278 CC2	6730-4342 X2		6730-4341 T2	6730-4342 X2	ratio	6730-3933 J2	6730-3934 R2	ratio
		Triticale	Triticale		Barley	Barley		Maize	Maize		Peas	Peas		Wheat	Wheat		Wheat	Wheat		Soy	Soy	
Nutrient	Sample unit	1	2	1/2	1	2	1/2	1	2	1/2	1	2	1/2	1	2	1/2	1	2	1/2	1	2	1/2
Vitamin B2	mg/kg	0,8	0,8	1,00	0,9	0,9	1,00	0,86	0,94	0,91	1,3	1,3	1,00	0,6	0,63	0,95	0,85	0,63	1,35	1,5	1,6	0,94
Vitamin C	mg/kg	<1,5	<1,5		1,7	<1,5	#WAARDE!	4,2	3,3	1,27	14	11	1,27	<1,5	<1		<1	<1		5,3	5,0	1,06
alfa-tocopherol	mg/kg	10,1	6,5	1,55	2,3	7,9	0,29	6,6	6,1	1,08	<1,8	<2		8,7	12	0,73	11	12	0,92	22	368	0,06
beta-tocopherol	mg/kg	3,9	1,8	2,17	<0,1	0,4	#WAARDE!	<1,7	<1,7		<1,8	<2		4,4	5,9	0,75	4	5,9	0,68	4,2	6,7	0,63
gamma-tocopherol	mg/kg	<0,1	<0,1		2,3	<0,1	#WAARDE!	55	51	1,08	67	72	0,93	<0,1	22		21	22	0,95	156	217	0,72
delta-tocopherol	mg/kg	<0,1	<0,1		<0,1	<0,1		2,2	<1,7	#WAARDE!	2,2	2,9	0,76	<0,1	<0,3		<0,4	<0,3		71	107	0,66
Vitamin K1 (phylloquinone)	ug/100 g	1,7	2,7	0,63	1,6	3,6	0,44	4,2	4,3	0,98	9,4	13	0,72	1,2	4,4	0,27	5,5	4,4	1,25	16	20	0,80
Pantothenic acid (B5)	mg/kg	4,8	5,2	0,92	1,3	1,8	0,72	3,2	2,7	1,19	9,8	10	0,98	5,1	3,3	1,55	5,4	3,3	1,64	14	12	1,17
Vitamin B3 (total niacin)	mg/kg	14,5	19,7	0,74	32,6	31,3	1,04	<9,5	<9,5		24	23	1,04	15,5	<9,5		<9,5	<9,5		26	25	1,04
Total folate	mg/kg	0,52	0,46	1,13	0,45	0,56	0,80	0,36	0,42	0,86	0,17	4,3	0,04	0,34	0,35	0,97	0,39	0,35	1,11	1,55	1,8	0,86
Biotin	mg/kg	0,08	0,07	1,14	0,12	0,14	0,86	0,058	0,054	1,07	0,16	0,16	1,00	0,06	0,074	0,81	0,086	0,074	1,16	0,3	0,3	1,00
Vitamine B6	mg/kg	1,2	1,2	1,00	1,4	1,4	1,00	1,8	2,3	0,78	0,2	0,3	0,67	1,4	1,1	1,27	1,5	1,1	1,36	4,5	4,5	1,00
Cd	ug/kg	9,8	28,8	0,34	17,17	14,6	1,18	< 2	3	#WAARDE!	19	35	0,53	69,3	20	3,47	29	20	1,45	<50	<50	
Ca	mg/kg	405	221	1,83	373	410	0,91	63	57	1,10	550	658	0,84	338	264	1,28	437	264	1,65	2000	2000	1,00
Cr	mg/kg	1,23	3,65	0,34	8,18	3,38	2,42	9,30	7,20	1,29	3,50	3,60	0,97	2,77	2,20	1,26	1,50	2,20	0,68	6,2	4,3	1,44
Co	ug/kg	<100	<100		<100	<100		< 100	< 100		< 100	< 100		<100	< 100		< 100	< 100		200	200	1,00
Cu	mg/kg	5,4	5,9	0,92	2,9	3,4	0,85	1,8	1,3	1,42	7,4	8,6	0,86	4,9	2,4	2,07	3,0	2,4	1,26	13	17	0,76
F	mg/kg	< 0,2	< 0,2		< 0,2	0,2027	#WAARDE!	< 0,2	< 0,2		< 0,2	< 0,2		< 0,2	< 0,2		< 0,2	< 0,2		< 0,2	< 0,2	
I	ug/kg	< 100	< 100		< 100	< 100		< 100	< 100		< 100	< 100		< 100	< 100		< 100	< 100		120	<100	#WAARDE!
Fe	mg/kg	27	38	0,71	95	161	0,59	20	17	1,19	59	54	1,09	51	29	1,76	44	29	1,53	130	93	1,40
Pb	ug/kg	28,7	< 15		31,6	714	0,04	< 15	16	#WAARDE!	25	< 15	#WAARDE!	< 15	19		20	19	1,09	<20	<20	
Mg	mg/kg	1053	1062	0,99	1197	1143	1,05	1111	1007	1,10	1164	1246	0,93	1024	1072	0,96	1047	1072	0,98	2200	2500	0,88
Mn	mg/kg	30	64	0,47	17	15	1,13	4	5	0,89	8	9	0,96	17	17	1,01	25	17	1,50	30	24	1,25
Hg	ug/kg	< 2	< 2		< 2	< 2		< 2	< 2		< 2	< 2		< 2	< 2		< 2	< 2		< 1	< 1	
Mo	ug/kg	877	856	1,02	762	895	0,85	410	400	1,03	2600	1700	1,53	434	760	0,57	410	760	0,54	2800	1700	1,65
Ni	mg/kg	0,59	1,2	0,49	4,72	1,36	3,47	4,80	3,10	1,55	13,70	14,90	0,92	1,43	2,20	0,65	2,80	2,20	1,27	67	63	1,06
Zn	mg/kg	34	62	0,55	39	37	1,05	17	17	1,00	32	41	0,78	24	18	1,30	25	18	1,37	47	52	0,90
P	mg/kg	3304	3556	0,93	3223	3187	1,01	2485	2738	0,91	3896	4432	0,88	3257	3680	0,89	3740	3680	1,02	5100	7000	0,73
K	mg/kg	4973	4575	1,09	3765	4165	0,90	3174	3450	0,92	10294	10825	0,95	4116	4084	1,01	3779	4084	0,93	17000	18000	0,94
Se	ug/kg	44,4	28,1	1,58	11	16,3	0,67	110	13	8,46	83	48	1,73	150,6	34	4,43	48	34	1,41	62	200	0,31
Na	mg/kg	120	61	1,97	105	189	0,56	78	93	0,84	142	121	1,18	185	83	2,23	98	83	1,18	200	150	1,33
As	ug/kg	<10	13	#WAARDE!	17	73	0,23	< 10	< 10		< 10	< 10		40	< 10		< 10	< 10		21	14	1,50
naringenin	ug/g	<1	<1		<1	<1		<1	<1		<1	<1		<1	<1		<1	<1				
hesperetin	ug/g	<1	<1		<1	<1		<1	<1		<1	<1		<1	<1		<1	<1				
luteolin	ug/g	<1	<1		<1	<1		<1	<1		<1	<1		<1	<1		<1	<1				
apigenin	ug/g	<2	<2		<2	<2		<2	<2		<2	<2		<2	<2		<2	<2				
myricetin	ug/g	<3	<3		<3	<3		<3	<3		<3	<3		<3	<3		<3	<3				
quercetin	ug/g	<2	<2		<2	<2		<2	<2		<2	<2		<2	<2		<2	<2				
kaempferol	ug/g	<2	<2		<2	<2		<2	<2		<2	<2		<2	<2		<2	<2				
isorhamnetin	ug/g	<2	<2		<2	<2		<2	<2		<2	<2		<2	<2		<2	<2				
naringenin	ug/g	<8	<8		<8	<8		<8	<8		<8	<8		<8	<8		<8	<8				
hesperetin	ug/g	<8	<8		<8	<8		<8	<8		<8	<8		<8	<8		<8	<8				
luteolin	ug/g	<5	<5		<5	<5		<5	<5		<5	<5		<5	<5		<5	<5				
apigenin	ug/g	<8	<8		<8	<8		<8	<8		<8	<8		<8	<8		<8	<8				
myricetin	ug/g	<20	<20		<20	<20		<20	<20		<20	<20		<20	<20		<20	<20				
quercetin	ug/g	<15	<15		<15	<15		<15	<15		<15	<15		<15	<15		<15	<15				
kaempferol	ug/g	<10	<10		<10	<10		<10	<10		34	39	0,87	<10	<10		<10	<10				
isorhamnetin	ug/g	<10	<10		<10	<10		<10	<10		<10	<10		<10	<10		<10	<10				
luteine	mg/kg	1,2	0,8	1,50	<0,1	0,75	#WAARDE!	6,3	5,9	1,07	4,6	6	0,77	1,2	1,2	1,00	1,2	1,2	1,00	3,4	3,2	0,92
zeaxanthine	mg/kg	0,31	0,2	1,55	<0,1	0,33	#WAARDE!	5,7	6,3	0,90	0,92	1,2	0,78	0,3	0,33	0,91	0,23	0,33	0,70	0,7	0,9	0,78
beta-cryptoxanthine	mg/kg	<0,05	<0,1		<0,1	<0,05		0,79	0,85	0,93	<0,03	<0,03		<0,1	<0,03		<0,03	<0,03		<0,1	<0,1	
lycopeen	mg/kg	<0,05	<0,1		<0,1	<0,05		0,35	0,45	0,78	<0,03	<0,03		<0,1	<0,03		<0,03	<0,03		<0,1	<0,1	
alpha-caroteen	mg/kg	<0,05	<0,1		<0,1	<0,05		0,11	0,1	1,10	<0,03	<0,03		<0,1	<0,03		<0,03	<0,03		<0,1	<0,1	
beta-caroteen	mg/kg	0,06	<0,1	#WAARDE!	<0,1	<0,05		0,88	1,1	0,80	0,03	0,04	0,75	<0,1	<0,03		<0,03	<0,03		<0,1	0,5	#WAARDE!
catechine	ug/g	<29	<29		<29	<29		<29	<29		<29	<29		<29	<29		<29	<29				
epicatechine	ug/g	<64	<64		<64	<64		<64	<64		<64	<64		<64	<64		<64	<64				
gallocatechine	ug/g	<33	<33		<33	<33		<33	<33		<33	<33		<33	<33		<33	<33				
epigallocatechine	ug/g	<105	<105		<105	<105		<105	<105		<105	<105		<105	<105		<105	<105				
catechine gallaat	ug/g	<26	<26		<26	<26		<26	<26		<26	<26		<26	<26		<26	<26				
epicatechine gallaat	ug/g	<71	<71		<71	<71		<71	<71		<71	<71		<71	<71		<71	<71				
gallocatechine gallaat	ug/g	<28	<28		<28	<28		<28	<28		<28	<28		<28	<28		<28	<28				
epigallocatechine gallaat	ug/g	<180	<180		<180	<180		<180	<180		<180	<180		<180	<180		<180	<180				
theobromine	ug/g	<22	<22		<22	<22		<22	<22		<22	<22		<22	<22		<22	<22				
theophylline	ug/g	<24	<24		<24	<24		<24	<24		<24	<24		<24	<24		<24	<24				
caffeine	ug/g	<153	<153		<153	<153		<153	<153		<153	<153		<153	<153		<153	<153				

Feed measured

	Submissie			110016810				110017952				110022818			110023251			110024617		
	Codering TNO			6130-6894	6130-6895		6730-0684	6730-0683			6730-4173	6730-4172			6730-4485	6730-4486		6730-5535	6730-5536	
				Grower	Grower		Layer	Layer			Layer feed 8 with suppl	Layer feed 7 with suppl			Starter	Starter		Grower	Grower	
	Nutrient	unit	Original feed	mix 1	mix 2	ratio 1/2	mix 4	mix 3	ratio 4/3				ratio 8/7		mix 11	mix 12	ratio11/12	mix 15	mix 16	ratio 15/16
amino acids	cystine	g/100 g	0,298	0,34	0,33	1,03	0,29	0,28	1,04		0,29	0,30	0,97		0,33	0,30	1,08	0,33	0,32	1,03
	methionine	g/100 g	0,324	0,29	0,26	1,12	0,28	0,26	1,08		0,40	0,49	0,81		0,39	0,45	0,88	0,30	0,29	1,03
	aspartic acid	g/100 g	1,719	1,87	1,66	1,13	1,63	1,49	1,09		1,52	1,52	1,01		1,79	1,52	1,18	1,80	1,80	1,00
	threonine	g/100 g	0,649	0,72	0,65	1,11	0,64	0,62	1,03		0,62	0,63	0,99		0,73	0,68	1,07	0,72	0,68	1,06
	serine	g/100 g	0,86	0,95	0,84	1,13	0,82	0,75	1,09		0,80	0,78	1,02		0,89	0,79	1,12	0,96	0,88	1,09
	glutamic acid	g/100 g	3,241	3,88	3,3	1,18	3,27	2,83	1,16		3,19	2,96	1,08		3,30	2,97	1,11	3,90	3,40	1,15
	proline	g/100 g	1,058	1,23	1,07	1,15	1,01	0,94	1,07		1,02	0,96	1,07		1,23	1,04	1,18	1,20	1,10	1,09
	glycine	g/100 g	0,753	0,81	0,71	1,14	0,72	0,66	1,09		0,69	0,68	1,02		0,79	0,72	1,10	0,81	0,75	1,08
	alanine	g/100 g	0,87	0,86	0,78	1,10	0,77	0,72	1,07		0,75	0,76	0,99		0,83	0,76	1,09	0,86	0,82	1,05
	valine	g/100 g	0,829	0,91	0,8	1,14	0,81	0,76	1,07		0,78	0,77	1,02		0,94	0,83	1,13	0,91	0,85	1,07
	isoleucine	g/100 g	0,722	0,83	0,73	1,14	0,71	0,67	1,06		0,70	0,68	1,04		0,78	0,72	1,08	0,81	0,75	1,08
	leucine	g/100 g	1,443	1,54	1,36	1,13	1,37	1,3	1,05		1,35	1,32	1,03		1,52	1,41	1,08	1,50	1,40	1,07
	tyrosine	g/100 g	0,609	0,7	0,62	1,13	0,65	0,61	1,07		0,61	0,58	1,06		0,71	0,63	1,12	0,68	0,62	1,10
	phenylalanine	g/100 g	0,85	0,95	0,82	1,16	0,87	0,77	1,13		0,81	0,78	1,03		0,95	0,84	1,13	0,95	0,86	1,10
	histidine	g/100 g	0,47	0,49	0,44	1,11	0,45	0,4	1,13		0,42	0,39	1,08		0,42	0,37	1,12	0,47	0,44	1,07
	lysine	g/100 g	0,887	1,02	0,94	1,09	0,91	0,87	1,05		0,88	0,88	0,99		1,02	0,89	1,15	1,00	0,98	1,02
	arginine	g/100 g	1,169	1,39	1,21	1,15	1,12	1,04	1,08		1,15	1,08	1,06		1,10	1,01	1,09	1,40	1,20	1,17
	tryptophan	g/100 g	0,2	0,24	0,23	1,04	0,22	0,19	1,16		0,20	0,19	1,05		0,23	0,2	1,15	0,23	0,20	1,15
macronutrients	ash content	g/kg		48	51	0,94	114	113	1,01		105	116	0,91		51	64	0,80	54	55	0,98
	carbohydrates total	g/kg	555,8	539	546	0,99	538	555	0,97		559	562	0,99		620	624	0,99	574	585	0,98
	chloride	g/kg	2,3	2	1,7	1,18	1,4	1,3	1,08		2,1	1,7	1,24		2,1	2,3	0,91	2,3	1,9	1,21
	choline	mg/100 g		123,2	126,7	0,97	96,7	105	0,92		88,9	91,3	0,97		114,9	114,3	1,01	114,9	127,4	0,90
	raw fibre	g/kg	44,7	42	43	0,98	40	36	1,11		42	37	1,14		34	36	0,94	39	39	1,00
	crude fat (acid hydrolysis)	g/kg	43,7	59	61	0,97	64	69	0,93		46	51	0,90		42	42	1,00	53	62	0,85
	moisture content	g/kg	121,8	120	126	0,95	120	116	1,03		122	117	1,04		123	119	1,03	120	122	0,98
	protein	g/kg	174,8	192	173	1,11	164	147	1,12		168	154	1,09		164	151	1,09	199	176	1,13
	ratio protein/crude fat		4,00	3,25	2,84	1,15	2,56	2,13	1,20		3,65	3,02	1,21		3,90	3,60	1,09	3,75	2,84	1,32
	saturated fatty acids										20	18,1	1,10		20,8	22,1	0,94	21,5	18,3	1,17
	mono saturated fatty acids (cis)										22,1	24,3	0,91		21,9	24,2	0,90	23,3	23,3	1,00
	polysaturated fatty acids										56,8	56,6	1,00		56,4	52,4	1,08	53,6	57,2	0,94
	unsaturated fatty acids (trans)										0,1	0,1	1,00		0,1	0,1	1,00	0,1	0,1	1,00
	alfa-tocopherol	mg/kg		14	16	0,88	13	16	0,81		15	14	1,07		15	16	0,94	13	13	1,00
	beta-tocopherol	mg/kg		3,7	3,7	1,00	2	2	1,00		2,7	2,6	1,04		2,7	2,7	1,00	2,8	3,1	0,90
	gamma-tocopherol	mg/kg		37	43	0,86	32	27	1,19		47	38	1,24		10	11	0,91	25	27	0,93
	delta-tocopherol	mg/kg		11	18	0,61	11	12	0,92		15	14	1,07		2,1	1,9	1,11	9,3	14	0,66
	Total folate	mg/kg		0,5	0,7	0,71	0,5	0,5	1,00		0,6	0,7	0,86		0,2	0,4	0,75	0,6	0,6	1,00
	Se	ug/kg		290	260	1,12	290	270	1,07		290	330	0,88		290	330	0,88	280	270	1,04
	Fe	mg/kg		250	240	1,04	260	280	0,93		220	340	0,65		220	260	0,85	330	240	1,38
	Cr	mg/kg		0,7	0,6	1,17	3,1	3,9	0,79		2,9	5,2	0,56		1,3	1,4	0,93	1,5	1,3	1,15
	Mn	mg/kg		82	80	1,03	110	130	0,85		92	110	0,84		130	150	0,87	110	100	1,10
	I	ug/kg		1200	1300	0,92	700	750	0,93		780	930	0,84		870	940	0,93	940	1000	0,94
	Cd	ug/kg		39	45	0,87														
	Pb	ug/kg		170	110	1,55	200	250	0,80		190	240	0,79		98	130	0,75	190	140	1,36
	As	ug/kg		120	70	1,71	120	130	0,92		110	150	0,73		110	100	1,10	110	64	1,72

Feed measured (continued)

Submissie			110016810				110017952			110022818		110023251			110024617		
Codering TNO			6130-6894	6130-6895		6730-0684	6730-0683		6730-4173	6730-4172		6730-4485	6730-4486		6730-5535	6730-5536	
			Grower	Grower		Layer	Layer		Layer feed 8	Layer feed 7		Starter	Starter		Grower	Grower	
Nutrient	unit	Original feed	mix 1	mix 2	ratio 1/2	meng 4	mix 3	ratio 4/3	with suppl	with suppl	ratio 8/7	mix 11	mix 12	ratio11/12	mix 15	mix 16	ratio 15/16
C8:0									<0,1	<0,1		<0,1	<0,1		<0,1	<0,1	
C10:0									<0,1	<0,1		<0,1	<0,1		<0,1	<0,1	
C12:0									0,2	0,2	1,00	0,5	1	0,50	0,7	0,2	3,50
C14:0									0,3	0,2	1,50	0,3	0,5	0,60	0,4	0,2	2,00
C14:1 c9									<0,1	<0,1		<0,1	<0,1		<0,1	<0,1	
C15:0									<0,1	<0,1		<0,1	<0,1		<0,1	<0,1	
C16:0									13,8	12,6	1,10	15,2	15,5	0,98	14,7	13,2	1,11
C16:1 c9									0,1	0,1	1,00	0,1	0,1	1,00	0,1	0,1	1,00
C17:0									0,2	0,2	1,00	0,1	0,1	1,00	0,2	0,2	1,00
C17:0 anteiso (17:1 c)									<0,1	<0,1		<0,1	<0,1		<0,1	<0,1	
C18:0									4	3,6	1,11	3,2	3,2	1,00	4	3,5	1,14
C18:1 t									0,1	<0,1		<0,1	<0,1		0,1	<0,1	
C18:1 c									21,9	24,2	0,90	21,3	23,4	0,91	23,2	23,2	1,00
C18:2 c9,12									50,9	50,7	1,00	50,9	47,3	1,08	48,4	51,3	0,94
C18:3 c9,12,15									5,8	5,7	1,02	5,1	4,5	1,13	5,1	5,9	0,86
C20:0									0,4	0,4	1,00	0,4	0,4	1,00	0,4	0,3	1,33
C20:1 c11									<0,1	<0,1		0,2	0,2	1,00	<0,1	<0,1	
C20:2 c11,14									<0,1	<0,1		<0,1	<0,1		<0,1	<0,1	
C22:0									0,5	0,4	1,25	0,3	0,3	1,00	0,5	0,4	1,25
C22:3 c13,16,19									<0,1	<0,1		0,2	0,4	0,50	<0,1	<0,1	
C22:4 c7,10,13,16									<0,1	<0,1		<0,1	<0,1		<0,1	<0,1	
C23:0									<0,1	<0,1		0,1	0,1	1,00	<0,1	<0,1	
C24:0									0,4	0,4	1,00	0,5	0,7	0,71	0,5	0,2	2,50
C24:1									<0,1	<0,1		0,3	0,4	0,75	<0,1	<0,1	
unidentified									1	1	1,00	0,8	1,2	0,67	1,5	1	1,50

Feed calculated

Nutrient	Sample unit	Orginal feed	Mix 1 Grower	Mix 2 Grower	ratio 1/2	Mix 4 Layer	Mix3 Layer	ratio 4/3	Mix 8 Layer	Mix 7 Layer	ratio 8/7	Mix 11 Starter	Mix12 Starter 1	ratio 11/12	Mix 15 Grower	Mix 16 Grower	ratio 15/16	average ratio
cholesterol	mg/100 g		0,33	0,23	1,44	0,23	0,19	1,21	0,23	0,19	1,21	0,22	0,17	1,24	0,25	0,23	1,09	1,24
campersterol	mg/100 g		12,26	11,40	1,08	10,27	10,33	0,99	10,27	10,33	0,99	9,55	9,57	1,00	11,68	11,40	1,02	1,02
stigmasterol	mg/100 g		6,90	7,13	0,97	5,94	6,09	0,98	5,94	6,09	0,98	4,73	5,03	0,94	7,11	7,13	1,00	0,97
sitosterol	mg/100 g		44,86	40,80	1,10	39,54	38,71	1,02	39,54	38,71	1,02	37,93	38,39	0,99	43,12	40,80	1,06	1,04
avenasterol	mg/100 g		11,38	9,09	1,25	10,31	9,26	1,11	10,31	9,26	1,11	10,49	10,54	0,99	10,32	9,09	1,14	1,12
Daidzin	mg/g		0,12	0,12	1,05	0,08	0,08	1,05	0,08	0,08	1,05	0,04	0,04	1,05	0,12	0,12	1,05	1,05
Genistin	mg/g		0,17	0,17	0,98	0,11	0,11	0,98	0,11	0,11	0,98	0,06	0,06	0,98	0,17	0,17	0,98	0,98
Daidzein	mg/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
Genistein	mg/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
Glycetein	mg/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
Glycetin	mg/g		0,02	0,01	2,00	0,01	0,01	2,00	0,01	0,01	2,00	0,01	0,00	2,00	0,02	0,01	2,00	2,00
Malonyl daidzin	mg/g		0,52	0,72	0,73	0,35	0,48	0,73	0,35	0,48	0,73	0,18	0,24	0,73	0,52	0,72	0,73	0,73
Malonyl glycetin	mg/g		0,04	0,05	0,78	0,03	0,04	0,78	0,03	0,04	0,78	0,01	0,02	0,78	0,04	0,05	0,78	0,78
Acetyl daidzin	mg/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
Acetyl glycetin	mg/g		0,06	0,09	0,69	0,04	0,06	0,69	0,04	0,06	0,69	0,02	0,03	0,69	0,06	0,09	0,69	0,69
Malonyl genistin	mg/g		0,54	0,80	0,67	0,35	0,53	0,67	0,35	0,53	0,67	0,18	0,27	0,67	0,54	0,80	0,67	0,67
Acetyl genistin	mg/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
Vitamin B1 (total thiamin)	mg/kg		3,87	4,15	0,93	3,57	3,76	0,95	3,57	3,76	0,95	3,39	3,50	0,97	3,89	4,15	0,94	0,95
Vitamin B2	mg/kg		1,00	1,06	0,95	0,90	0,89	1,02	0,90	0,89	1,02	0,85	0,81	1,05	1,07	1,06	1,01	1,01
Vitamin C	mg/kg		4,01	3,27	1,23	3,59	2,92	1,23	3,59	2,92	1,23	2,86	2,27	1,26	4,01	3,27	1,23	1,23
Vitamin K1 (phylloquinone)	ug/100 g		7,08	9,72	0,73	6,64	7,64	0,87	6,64	7,64	0,87	5,34	6,02	0,89	8,22	9,72	0,85	0,84
Pantothenic acid (B5)	mg/kg		7,32	6,21	1,18	5,99	4,98	1,20	5,99	4,98	1,20	5,31	4,47	1,19	7,40	6,21	1,19	1,19
Vitamin B3 (total niacin)	mg/kg		17,60	12,97	1,36	9,20	8,83	1,04	9,20	8,83	1,04	8,42	8,78	0,96	13,50	12,97	1,04	1,09
Biotin	mg/kg		0,15	0,15	0,97	0,12	0,11	1,03	0,12	0,11	1,03	0,10	0,09	1,05	0,15	0,15	1,01	1,02
Vitamine B6	mg/kg		2,25	2,28	0,99	1,81	1,85	0,98	1,81	1,85	0,98	1,50	1,49	1,01	2,27	2,28	1,00	0,99
Cd	ug/kg					10,05	10,02	1,00	10,05	10,02	1,00	12,61	14,30	0,88	11,25	10,84	1,04	0,79
Ca	mg/kg	890	797,58	791,49	1,01	597,01	564,71	1,06	597,01	564,71	1,06	469,29	406,88	1,15	823,70	791,49	1,04	1,06
Co	ug/kg		60,34	60,34	1,00	39,74	39,74	1,00	39,74	39,74	1,00	20,32	20,32	1,00	60,34	60,34	1,00	1,00
Cu	mg/kg	6	6,60	7,21	0,92	4,66	5,32	0,88	4,66	5,32	0,88	4,11	4,44	0,93	6,09	7,21	0,85	0,89
F	mg/kg		0,00	0,02	0,00	0,00	0,01	0,00	0,00	0,01	0,00	0,00	0,01	0,00	0,00	0,02	0,00	
Mg	mg/kg	1700	1392,58	1477,67	0,94	1155,29	1200,59	0,96	1155,29	1200,59	0,96	1062,95	1086,62	0,98	1398,65	1477,67	0,95	0,96
Hg	ug/kg		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
Mo	ug/kg		1377,62	1053,18	1,31	1060,40	844,29	1,26	1060,40	844,29	1,26	893,26	798,62	1,12	1371,28	1053,18	1,30	1,25
Ni	mg/kg		23,39	21,83	1,07	16,83	15,41	1,09	16,83	15,41	1,09	10,28	9,38	1,10	23,76	21,83	1,09	1,09
Zn	mg/kg	32	30,91	31,66	0,98	24,95	25,06	1,00	24,95	25,06	1,00	24,86	27,53	0,90	31,22	31,66	0,99	0,97
P	mg/kg	5100	3608,09	4393,69	0,82	3128,98	3606,46	0,87	3128,98	3606,46	0,87	3086,05	3393,88	0,91	3735,69	4393,69	0,85	0,86
K	mg/kg	9100	8257,06	8698,76	0,95	6342,47	6760,43	0,94	6342,47	6760,43	0,94	5312,55	5586,22	0,95	8167,97	8698,76	0,94	0,94
Na	mg/kg	151	149,58	116,82	1,28	103,55	95,58	1,08	103,55	95,58	1,08	99,38	87,68	1,13	126,67	116,82	1,08	1,13
naringenin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
hesperetin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
luteolin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
apigenin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
myricetin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
quercetin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
kaempferol	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
isorhamnetin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
naringenin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
hesperetin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
luteolin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
apigenin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
myricetin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
quercetin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
kaempferol	ug/g		3,40	3,90	0,87	3,40	3,90	0,87	3,40	3,90	0,87	3,40	3,90	0,87	3,40	3,90	0,87	0,87
isorhamnetin	ug/g		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		0,00	0,00		
luteine	mg/kg		3,06	3,29	0,93	3,01	3,15	0,96	3,01	3,15	0,96	2,57	2,65	0,97	3,06	3,29	0,93	0,95
zeaxanthine	mg/kg		1,52	1,77	0,86	1,72	1,97	0,87	1,72	1,97	0,87	1,41	1,61	0,88	1,50	1,77	0,85	0,86
beta-cryptoxanthine	mg/kg		0,16	0,17	0,93	0,20	0,21	0,93	0,20	0,21	0,93	0,16	0,17	0,93	0,16	0,17	0,93	0,93
lycopeen	mg/kg		0,07	0,09	0,78	0,09	0,11	0,78	0,09	0,11	0,78	0,07	0,09	0,78	0,07	0,09	0,78	0,78
alpha-caroteen	mg/kg		0,02	0,02	1,10	0,03	0,03	1,10	0,03	0,03	1,10	0,02	0,02	1,10	0,02	0,02	1,10	1,10
beta-caroteen	mg/kg		0,18	0,37	0,48	0,22	0,38	0,59	0,22	0,38	0,59	0,19	0,27	0,68	0,18	0,37	0,48	0,56

Annex 7 – Results complementary analyses – Protein ratio

Tab.1: Aminoacid and protein–N content of feed ingredients in µmol/g DM with coefficient of correlation (%RSD) of analytical procedure.

	02852		02853		02854		02855		02856		02857	
	Wheat E		Wheat Z		Barley K		Barley W		Triticale C		Triticale I	
	µmol/g	% RSD	µmol/g	% RSD	µmol/g	% RSD	µmol/g	% RSD	µmol/g	% RSD	µmol/g P.	% RSD
Asp	42,38	1,29	48,85	0,93	60,76	1,39	52,74	1,02	44,79	1,40	53,83	0,46
Glu	219,79	0,85	286,29	0,85	260,77	0,45	188,73	0,42	141,29	1,19	277,07	0,45
Ser	44,80	1,82	56,45	0,71	55,75	0,90	44,47	0,84	34,37	1,20	56,29	0,97
His	14,82	0,14	19,39	1,39	18,91	0,99	15,34	0,79	12,59	1,20	18,58	0,69
Gly	55,94	1,10	65,08	0,49	69,20	0,62	59,91	0,84	46,91	1,07	66,19	1,13
Thr	27,42	0,58	31,04	1,47	38,93	1,56	33,34	1,04	24,27	0,60	35,01	0,30
Ala	40,12	1,41	49,35	1,38	59,43	1,18	50,70	0,74	38,78	1,27	51,49	0,75
Arg	27,74	2,49	34,93	1,42	37,49	0,51	30,73	0,63	24,50	0,55	34,69	0,60
Tyr	15,97	3,42	20,53	0,97	22,67	1,34	17,56	1,48	12,75	0,57	19,02	0,60
Val	37,94	0,94	47,47	0,55	57,33	0,61	46,51	0,50	32,96	0,37	49,48	0,45
Met	11,11	1,19	12,88	1,28	14,95	0,74	12,58	0,87	9,81	0,89	14,70	0,24
Cys	17,63	1,06	21,15	1,30	21,29	0,67	17,99	0,22	14,86	2,88	24,04	1,14
Trp	5,89	0,80	7,55	1,33	7,13	0,83	5,77	0,78	4,19	0,33	5,91	0,54
Phe	26,46	0,69	35,02	0,90	43,25	0,58	32,37	0,80	21,37	0,75	35,84	0,43
Ile	26,09	1,00	33,55	1,23	37,30	1,06	29,39	0,57	21,53	0,68	34,32	0,58
Leu	51,10	1,12	65,06	0,44	70,52	0,41	56,01	0,26	40,28	0,70	63,81	0,33
Lys	21,31	1,52	25,73	1,23	35,34	1,43	31,04	0,84	23,55	0,99	28,82	1,39
Pro	83,78	0,96	111,75	0,69	136,12	0,70	101,28	0,54	62,51	0,57	116,49	0,82
N (%)	1,63	0,33	2,05	0,15	2,20	0,35	1,75	0,08	1,30	0,31	2,06	0,07
moisture (%)	8,77	0,79	7,81	0,32	8,65	0,53	7,83	0,29	8,60	0,07	8,03	0,07

Tab.2: Aminoacid and protein–N content of feed ingredients in µmol/g DM with coefficient of correlation (%RSD) of analytical procedure.

	02858		02859		02860		02861		02862		02863	
	Maize G		Maize R		Soy A		Soy S		Peas M		Peas T	
	µmol/g	% RSD	µmol/g	% RSD	µmol/g	% RSD	µmol/g	% RSD	µmol/g	% RSD	µmol/g	% RSD
Asp	47,34	0,87	52,14	1,82	316,65	1,01	265,77	0,85	198,07	0,92	195,31	0,47
Glu	128,05	0,29	136,81	1,05	564,71	1,09	468,60	0,34	321,48	0,38	316,59	0,32
Ser	41,91	1,13	43,59	1,33	197,13	0,81	166,78	0,20	108,13	0,70	106,74	1,28
His	15,97	1,18	16,63	0,66	64,26	1,17	56,84	0,81	37,48	0,87	36,24	0,46
Gly	46,06	0,61	47,24	0,90	216,35	0,85	183,76	0,56	130,35	0,40	126,88	0,97
Thr	29,32	0,49	29,88	0,50	135,16	0,86	114,04	0,98	74,60	1,01	74,15	1,05
Ala	70,14	0,49	75,10	0,88	189,86	2,09	163,67	0,50	115,22	0,60	113,19	0,73
Arg	24,77	0,77	24,89	1,30	171,19	1,33	136,20	0,95	110,84	0,90	114,27	0,69
Tyr	18,16	0,72	18,79	4,47	81,66	0,28	70,51	0,44	44,64	0,98	44,18	0,88
Val	36,66	0,32	38,98	1,08	162,43	0,83	138,75	0,43	97,38	0,37	96,08	0,98
Met	11,49	0,65	10,96	0,84	35,14	1,00	33,82	0,91	14,66	0,73	13,98	1,27
Cys	16,96	1,44	15,91	1,03	47,62	0,20	47,80	1,32	28,64	1,19	26,11	1,37
Trp	3,17	0,79	2,98	1,20	21,48	0,12	18,90	1,90	10,51	1,19	9,98	0,44
Phe	25,98	1,14	27,87	1,25	124,57	0,73	103,71	0,26	70,08	0,37	69,20	0,21
Ile	23,78	0,73	25,31	1,36	140,41	0,38	119,32	0,51	76,97	0,65	76,42	0,83
Leu	80,67	0,39	87,20	1,42	236,09	0,57	200,70	0,16	131,21	0,24	129,62	0,40
Lys	17,90	1,01	19,53	1,40	160,37	1,58	141,18	1,34	111,22	1,00	109,92	0,40
Pro	64,05	0,67	71,16	0,76	172,61	0,58	144,70	0,36	84,33	0,34	79,98	0,18
N (%)	1,39	0,69	1,48	0,45	6,28	0,27	5,35	0,18	3,78	0,24	3,76	0,28
moisture (%)	11,81	0,22	11,58	0,53	11,52	0,13	10,07	0,38	12,79	0,32	13,39	0,47

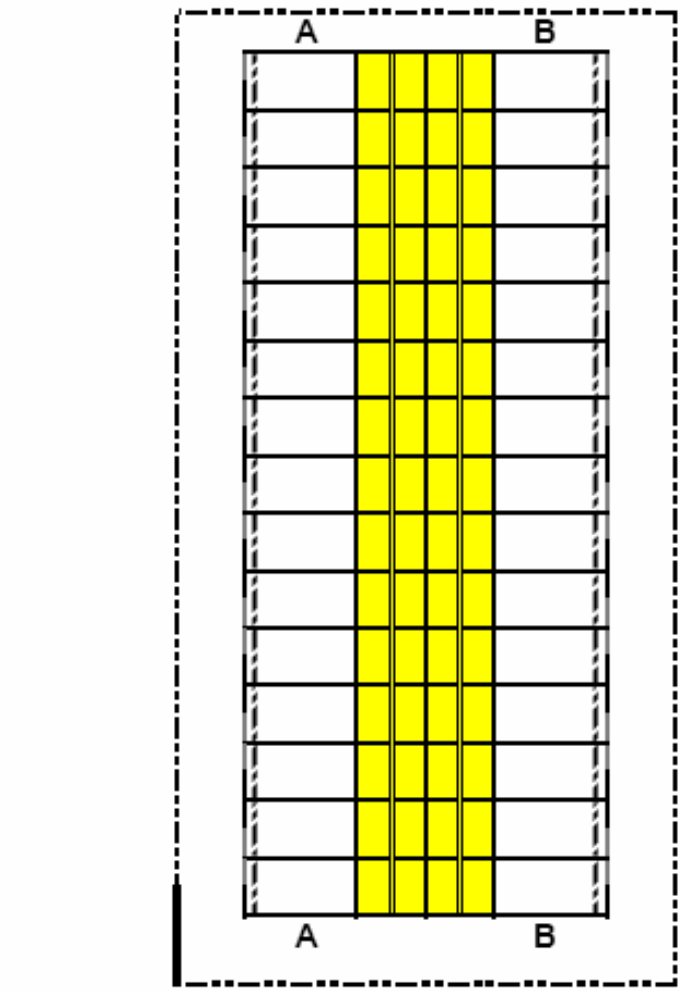
Tab. 3: Raw protein content and percentage of the essential amino acids methionin, cystein and lysin in chicken feed ingredients of Louis Bolk Institute 2006.

	Wheat E	Wheat Z*	Barl K*	Barl W	Triti C	Triti I*	Maize G	Maize R	Soy A*	Soy S	Peas M	Peas T
Raw Prot. % of DM	10,19	12,84	13,74	10,94	8,14	12,91	8,72	9,28	39,25	33,41	23,64	23,53
Met %v of RP	1,63	1,50	1,62	1,72	1,80	1,70	1,97	1,76	1,34	1,51	0,93	0,89
Met+Cys % of RP	3,72	3,49	3,50	3,71	4,01	3,95	4,32	3,84	2,80	3,24	2,39	2,23
Lys % of RP	3,04	2,91	3,73	4,12	4,20	3,24	2,98	3,06	5,93	6,14	6,83	6,78

Tab. 4: Raw protein content and percentage of the essential amino acids methionin, cystein and lysin in chicken feed ingredients of Louis Bolk Institute 2006 (normalized to 100%).

	Wheat E	Wheat Z*	Barl K*	Barl W	Triti C	Triti I*	Soy A*	Soy S
Raw Prot. % of DM	100,0	126,0	125,7	100,0	100,0	158,6	117,5	100,0
Met %v of RP	108,7	100,0	100,0	105,7	105,9	100,0	100,0	113,1
Met+Cys % of RP	106,6	100,0	100,0	106,0	101,4	100,0	100,0	115,6
Lys % of RP	104,4	100,0	100,0	110,4	129,6	100,0	100,0	103,4

Annex 8 – Housing scheme of second generation



Run/cage 1.90 m long surface 2.3 m^2
1.20 m broad

grid 0.80 m long
1.20 m broad

seating tick above grid
per animal 0.24 m

waterpipe with nipple every 0.20m
watersupply 100ml/ min/nipple

deur 0.70 m breed

walls of the stable

cage door

Annex 9 – Metabolomics

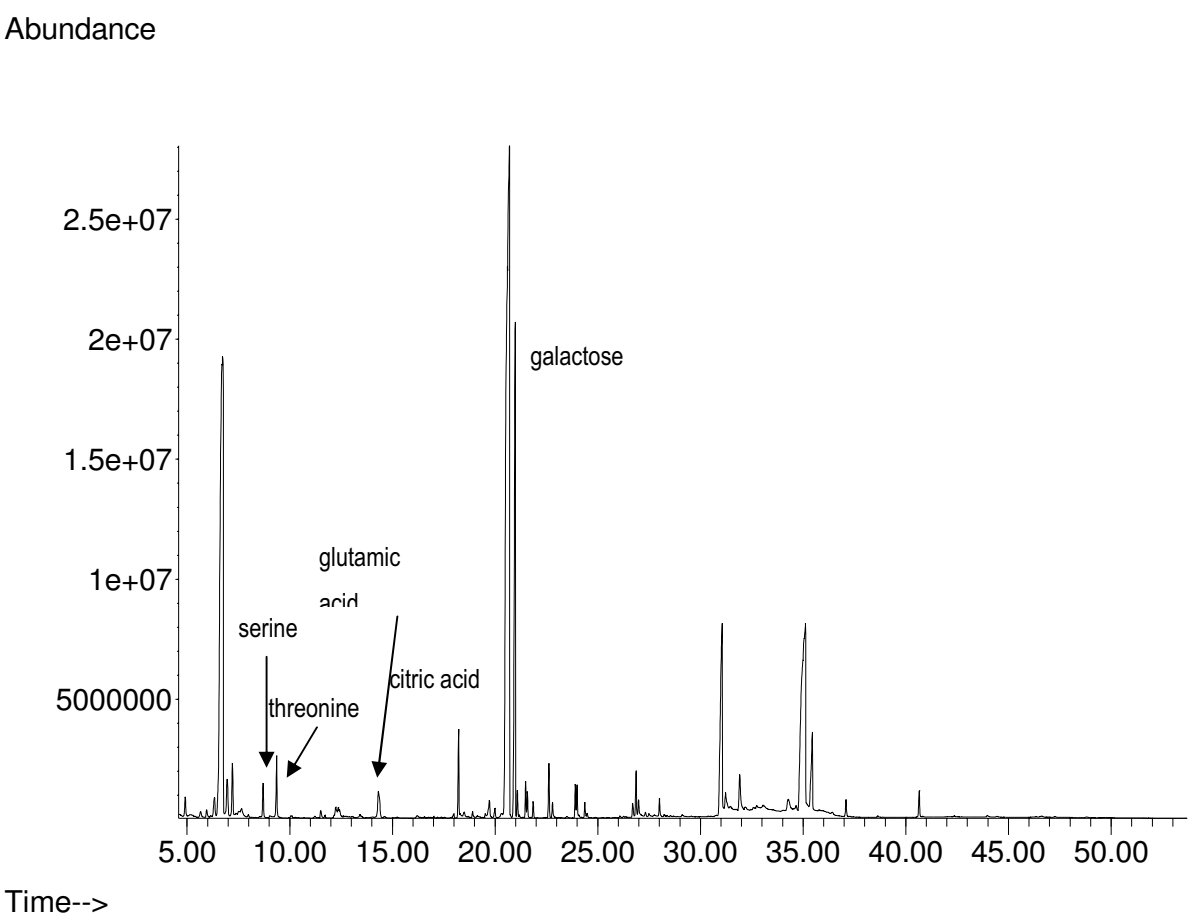


Figure A9.1: Example of a GC-MS chromatogram of chicken plasma obtained with the OS-GC-MS method.

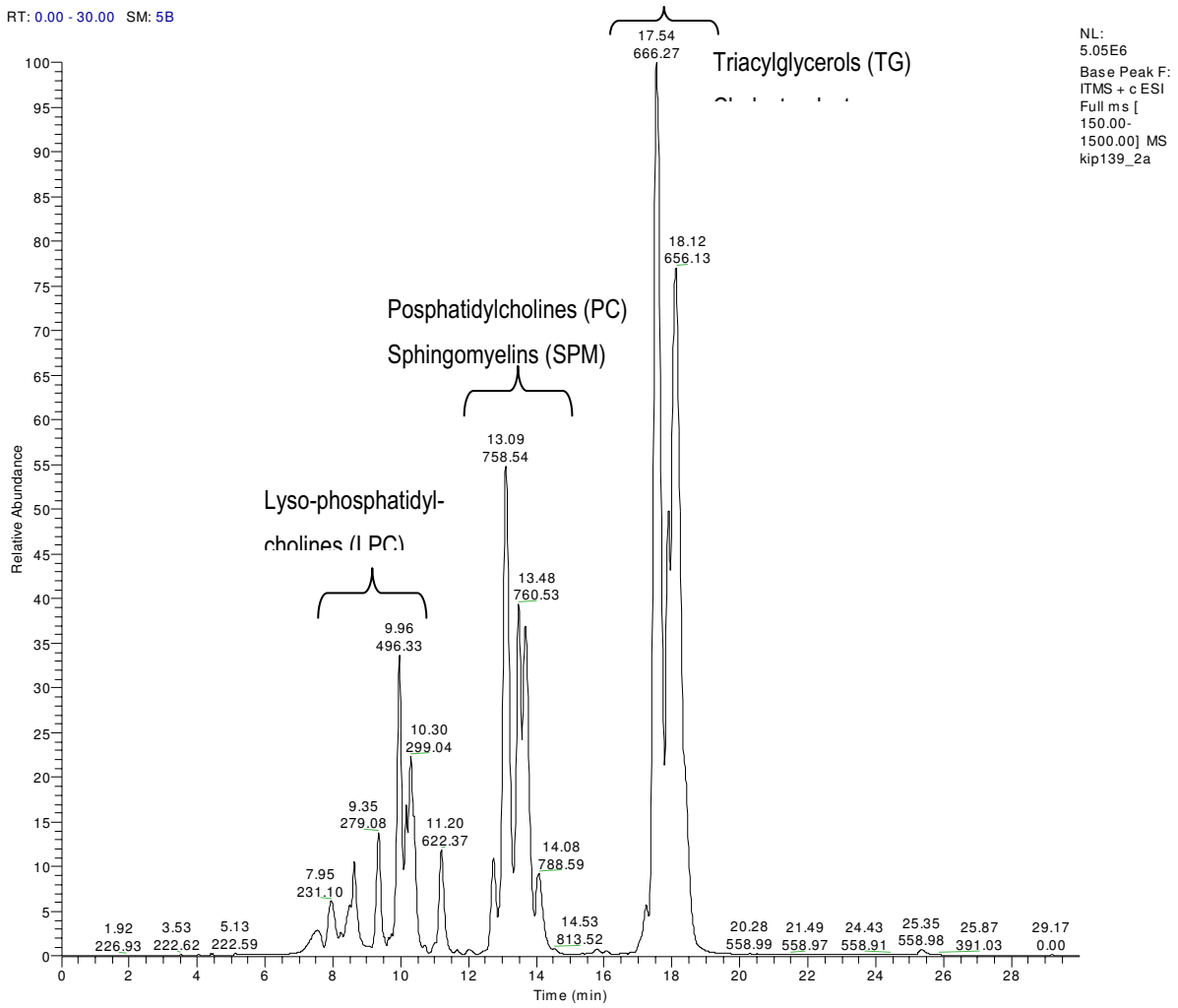


Figure A9.2: Example of a LC-MS chromatogram of chicken plasma obtained with the lipid LC-MS method.

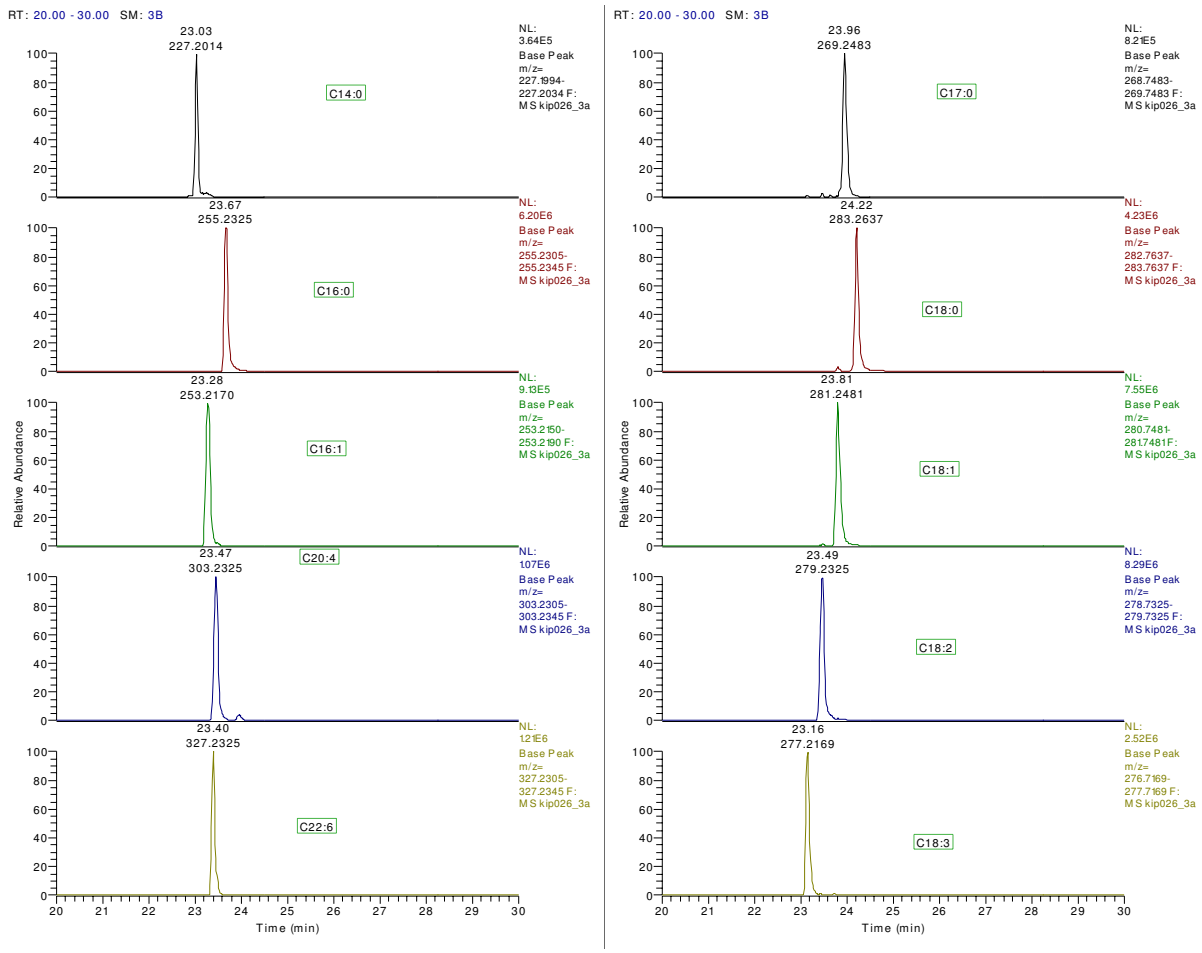


Figure A9.3: Example of fatty acids detected in chicken plasma using the FFA/bile acid LC-MS method.

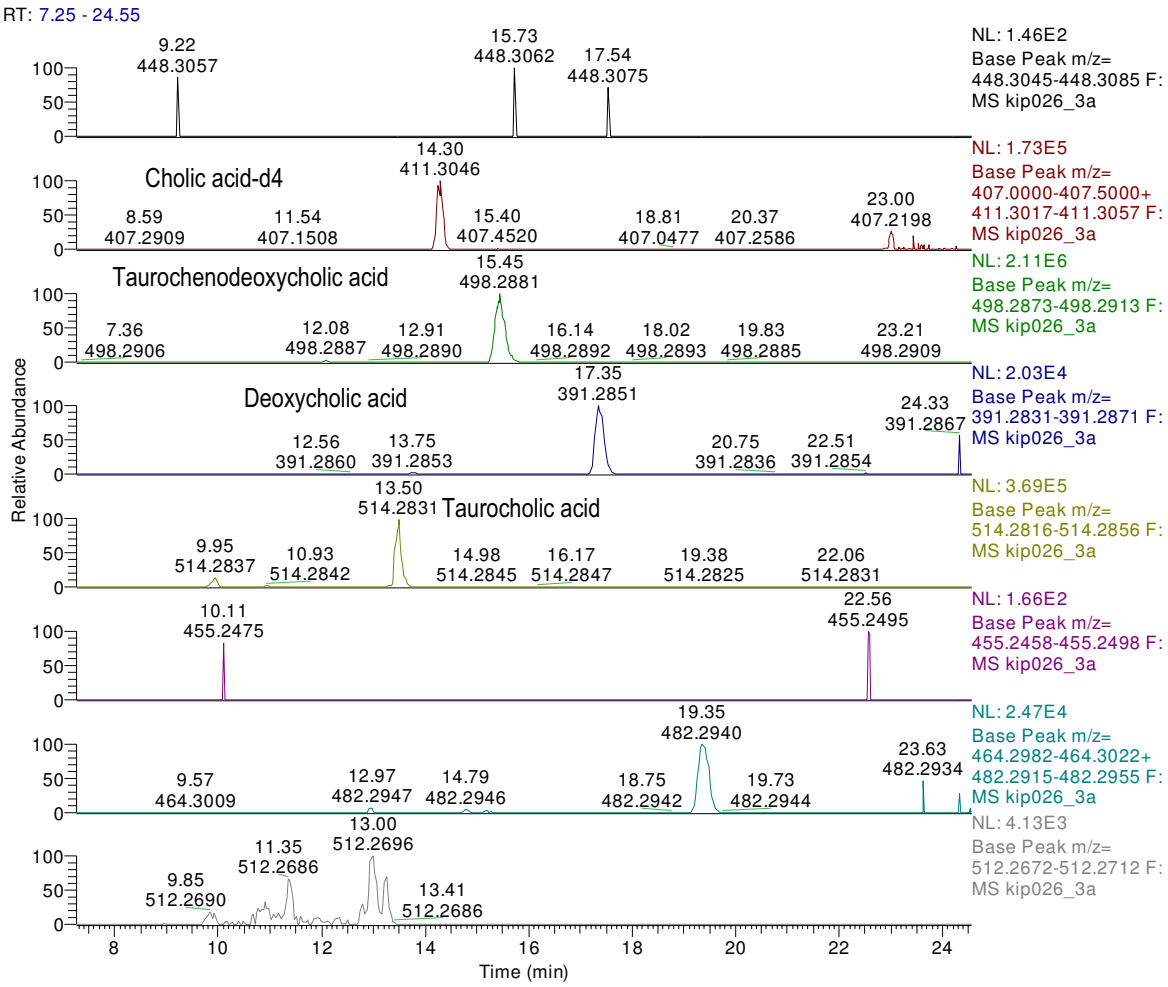


Figure A9.4: Example of bile acids detected in chicken plasma using the FFA/bile acid LC-MS method.

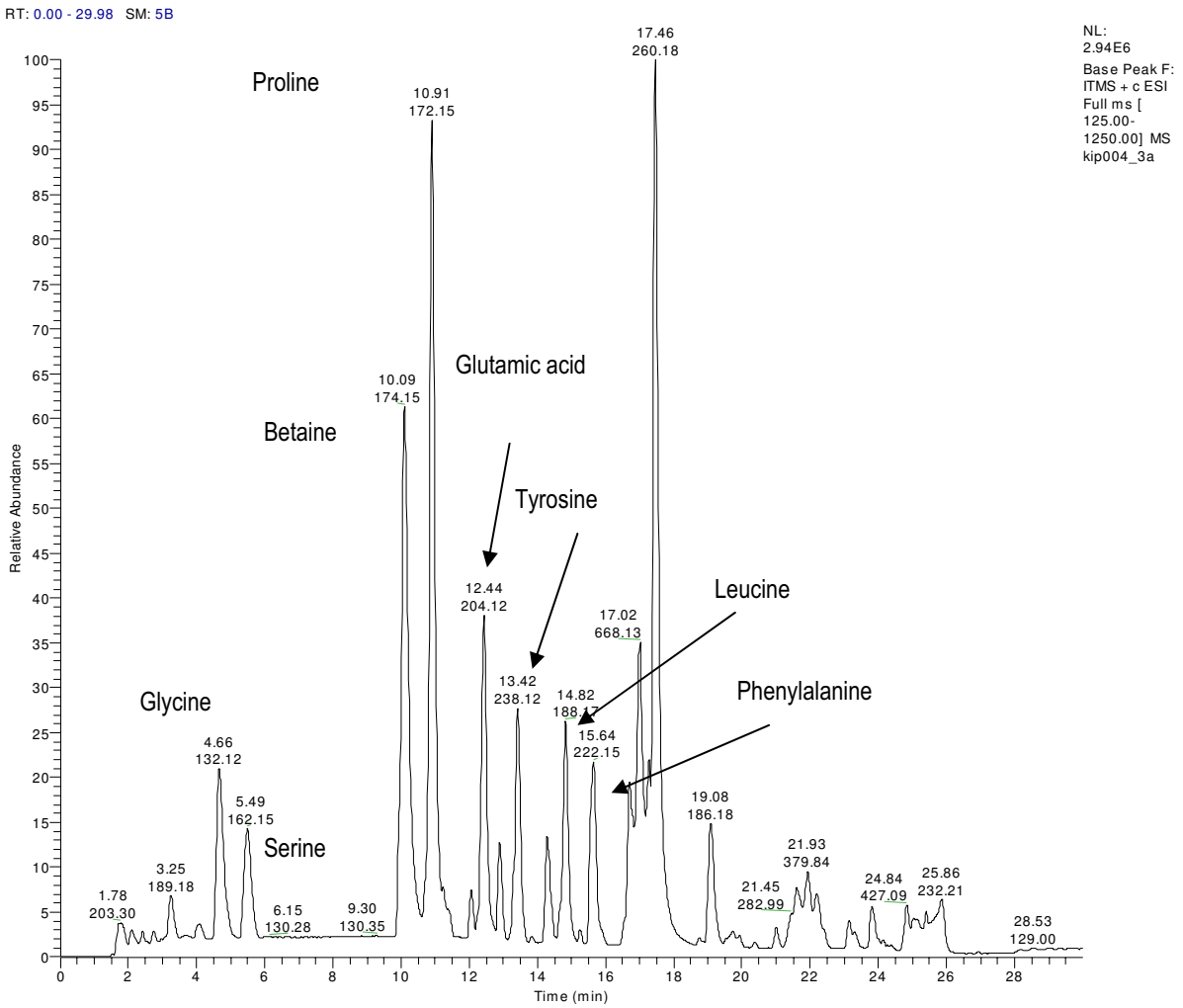


Figure A9.5: Example of a LC-MS chromatogram of chicken plasma obtained with the polar LC-MS method.

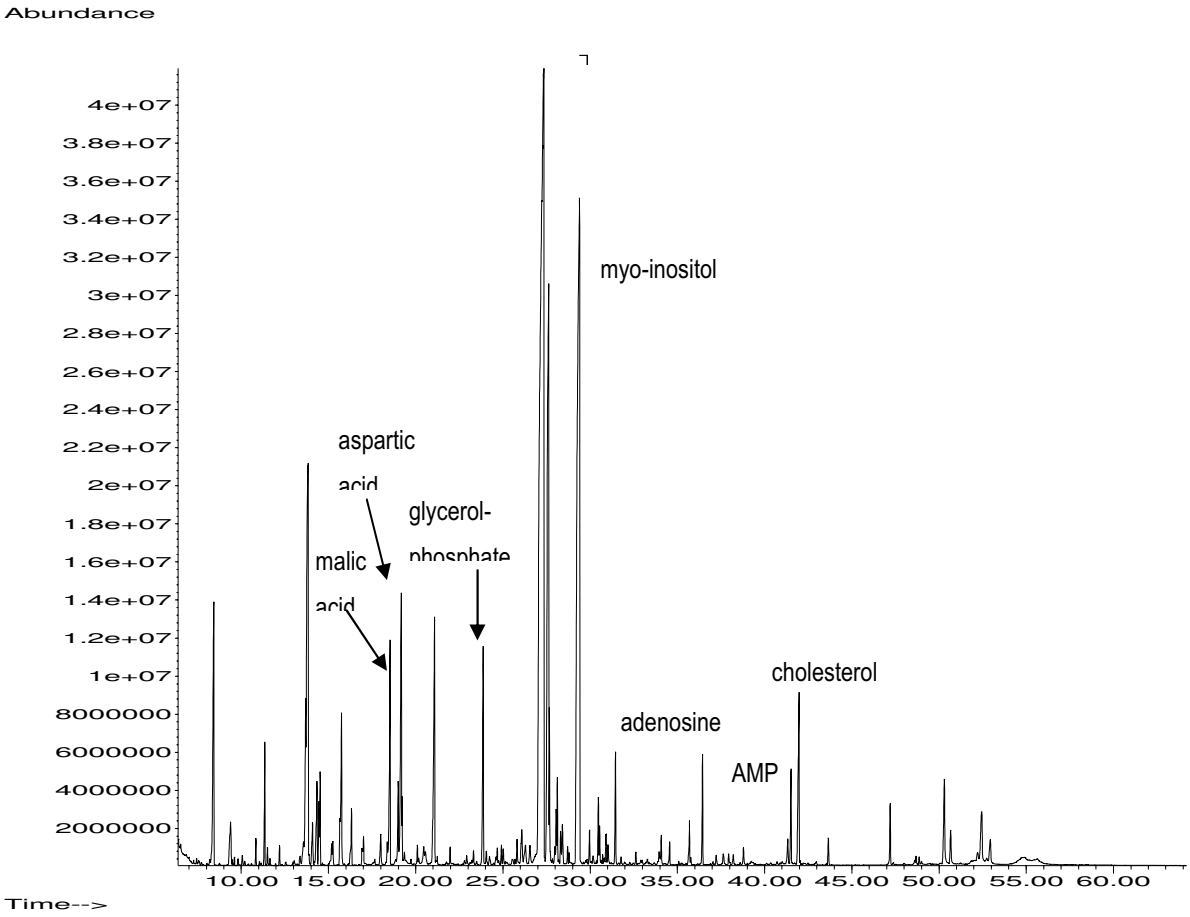


Figure A9.6: Example of a GC-MS chromatogram of chicken liver obtained with the OS-GC-MS method

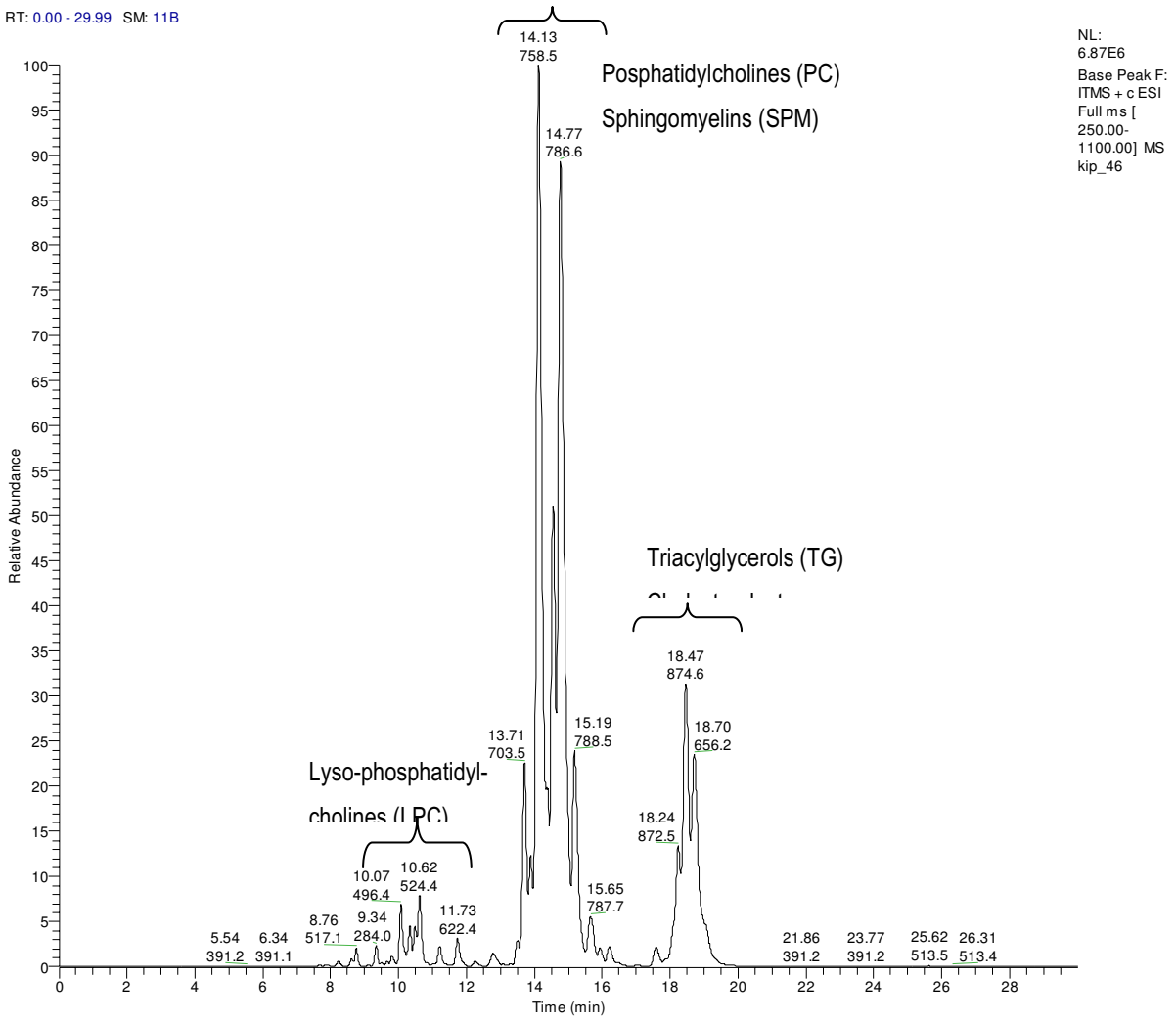


Figure A9.7: Example of a LC-MS chromatogram of chicken liver obtained with the lipid LC-MS method.

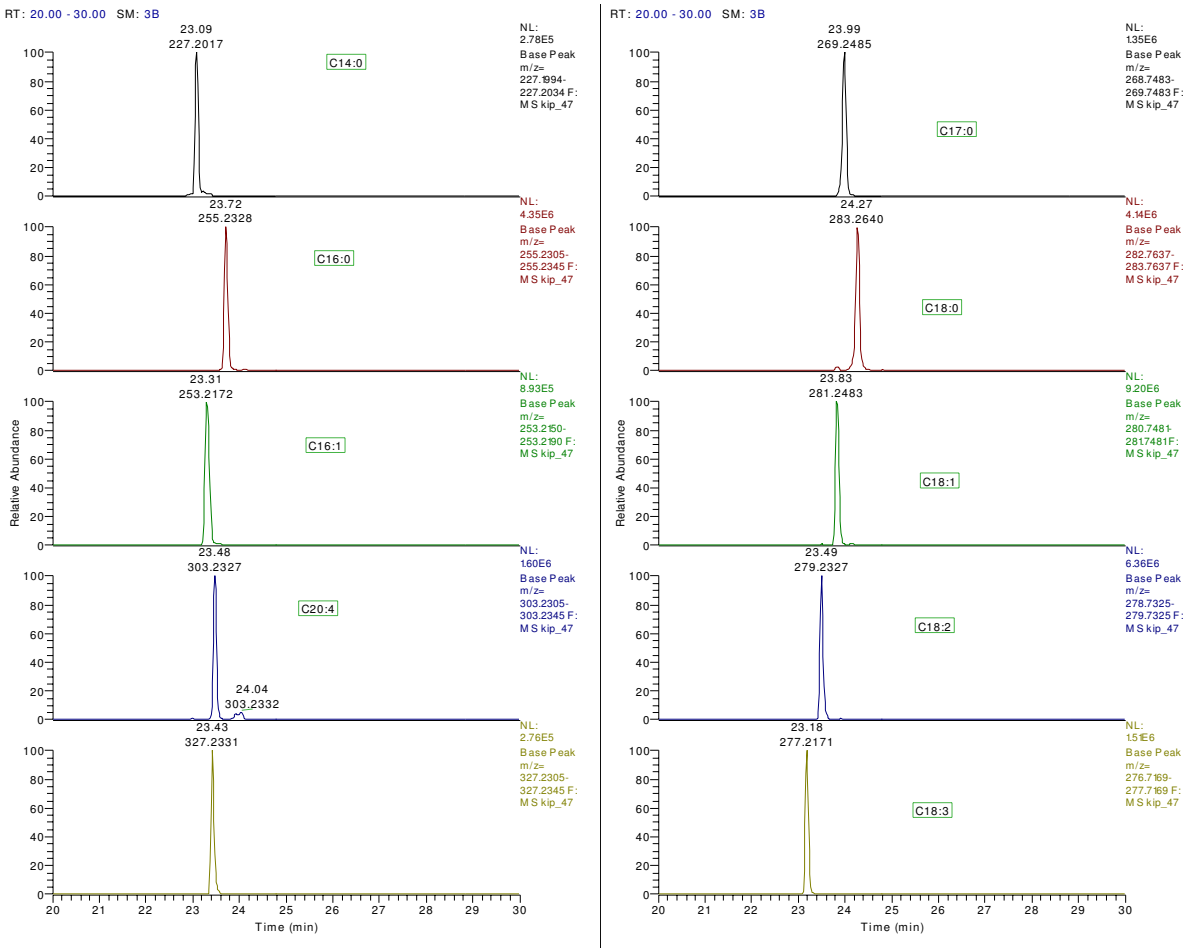


Figure A9.8: Example of fatty acids detected in chicken liver using the FFA/bile acid LC-MS method.

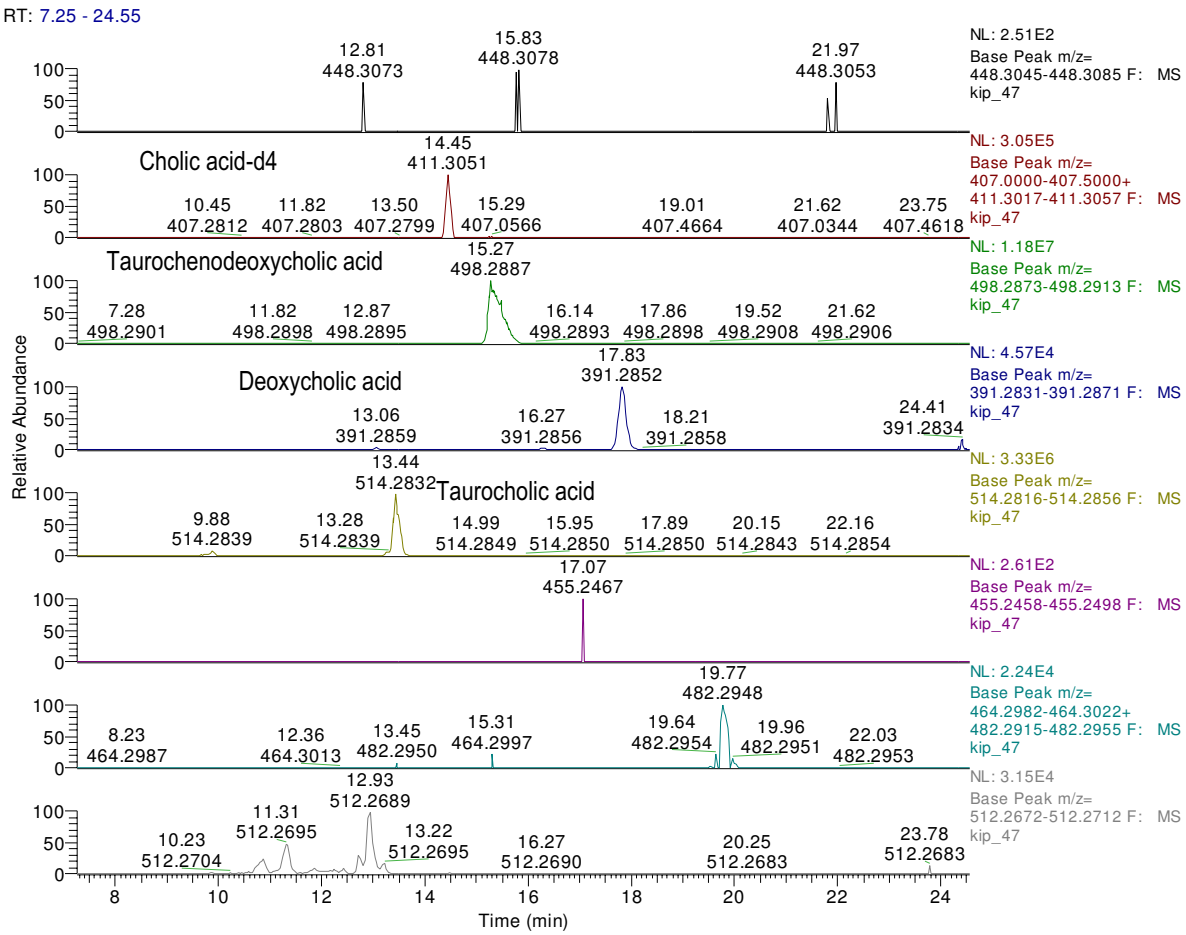


Figure A9.9: Example of bile acids detected in chicken liver using the FFA/bile acid LC-MS method.

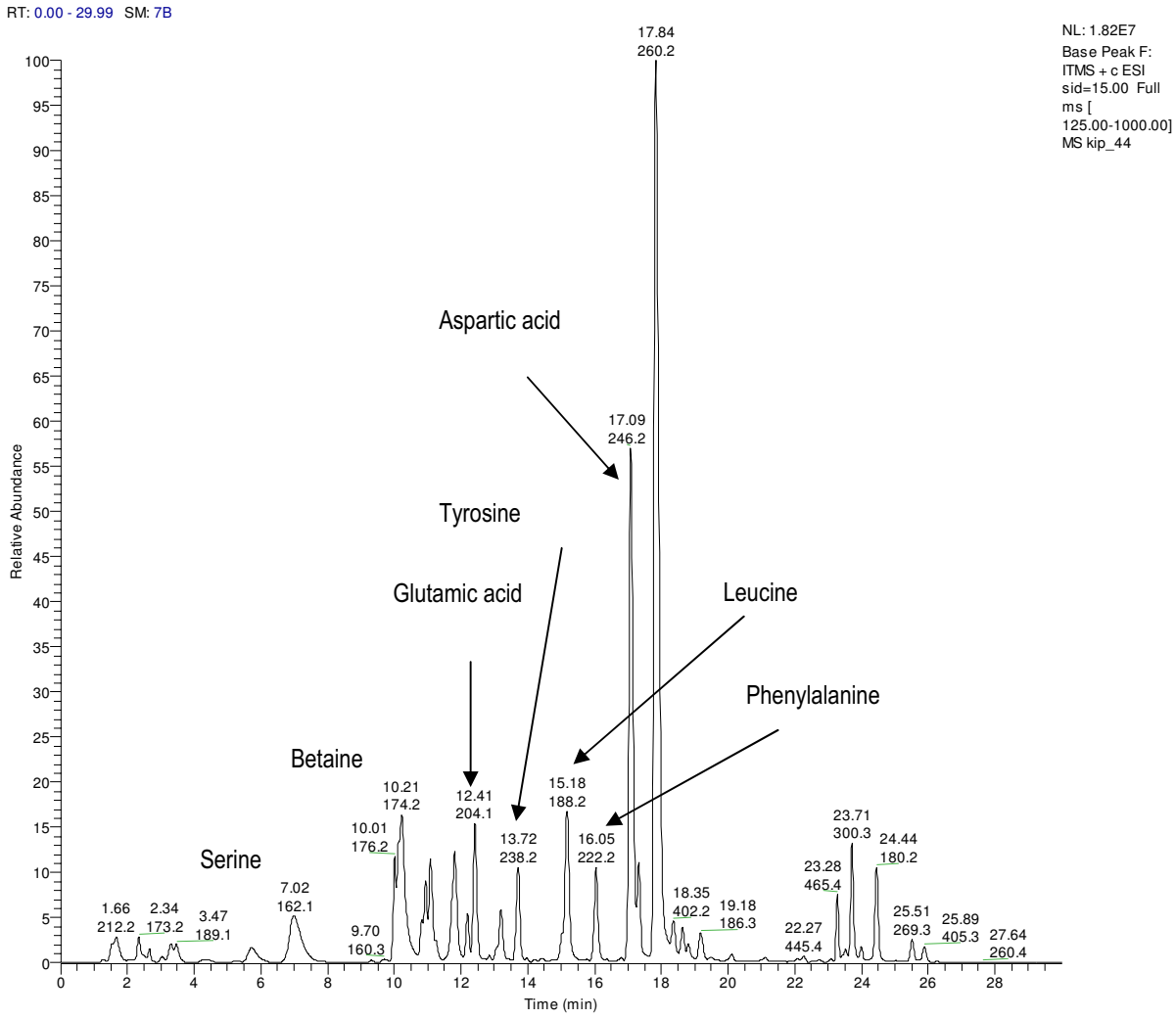
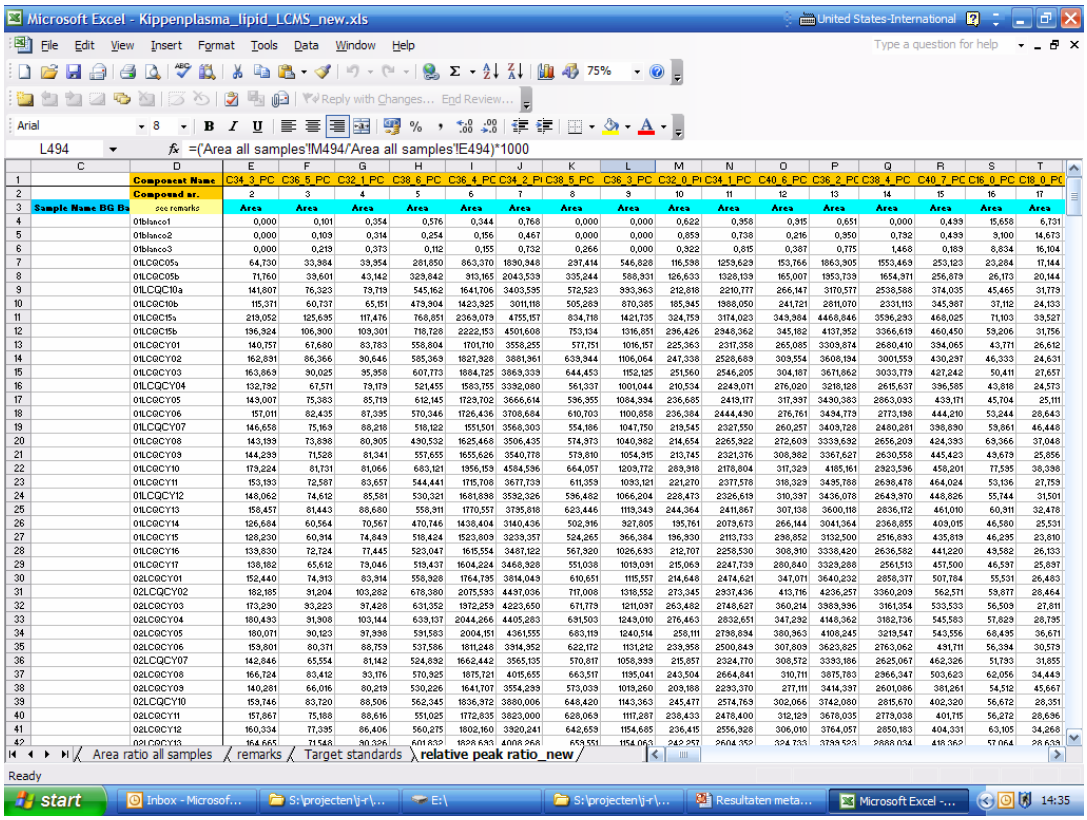


Figure A9.10: Example of a LC-MS chromatogram of chicken liver obtained with the polar LC-MS method.



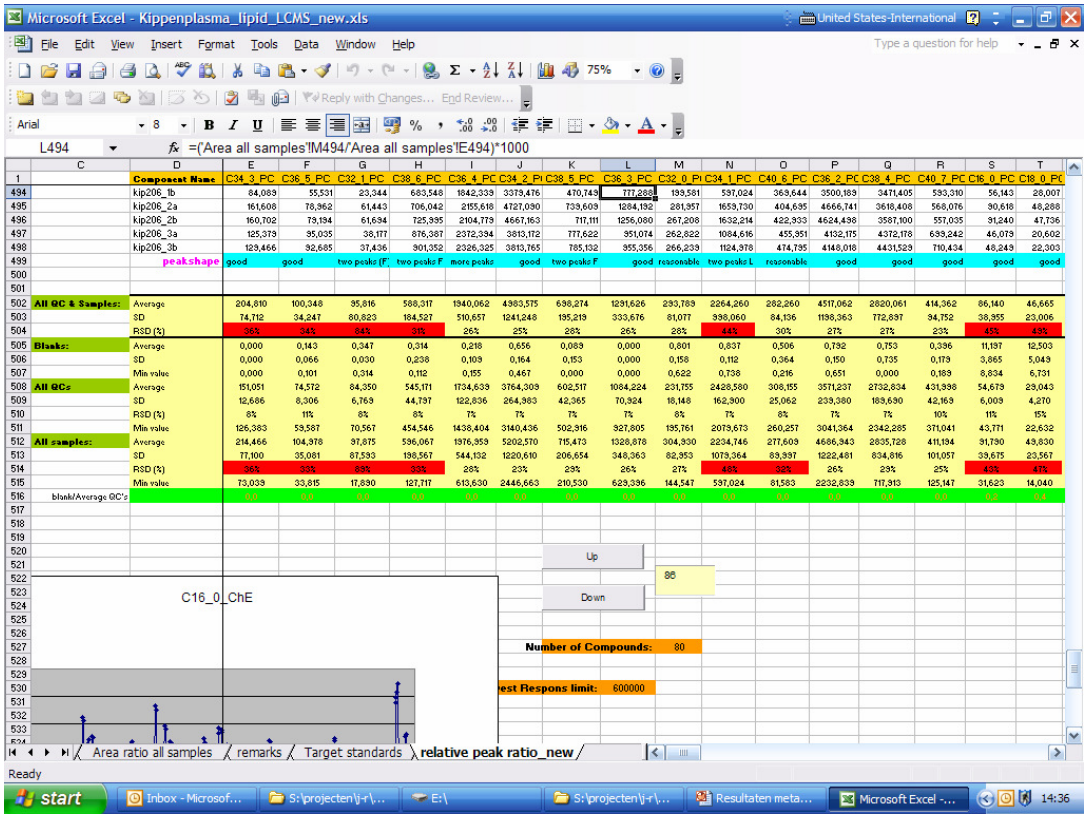


Figure A9.11: Example of an Excel-sheet containing processed metabolomics data.

Annex 10 – Weights of thymus and bursa Fabrici

	Line 1	feed A		Line 1	feed B
	Line 3	Feed A		Line 3	feed B
	Line 2	feed A		Line 2	feed B
Feed A					
Animal number	Histology number	Thymus	Bursa Fabricius		
4	06O0KI174	-	4		
5	06O0KI175	1,28	3,25		
11	06O0KI176	1,84	3,06		
18	06O0KI177	1,99	1,85		
20	06O0KI178	2,35	2,64		
26	06O0KI179	2,16	2,66		
mean		**1,924**	**2,91**		
72	06O0KI204	2,62	5,59		
80	06O0KI205	2,24	3,56		
89	06O0KI206	-	-		
93	06O0KI207	2,17	3,22		
96	06O0KI208	0,71	2,31		
101	06O0KI209	-	2,65		
mean		**1,93**	**3,46**		
36	06O0KI180	1,99	(1) 3,57 - (2) 2,87		
38	06O0KI181	1,26	3,17		
39	06O0KI182	1,88	5,79		
40	06O0KI183	1,17	4,7		
43	06O0KI184	1,26	3,9		
44	06O0KI185	2,09	3,42		
45	06O0KI186	0,69	4,09		
46	06O0KI187	1,1	4,62		
47	06O0KI188	1,35	4,45		
48	06O0KI189	1,53	3,94		
51	06O0KI190	1,51	4,59		
52	06O0KI191	2,34	1,58 *		
53	06O0KI192	1,91	1,44		
54	06O0KI193	0,96	3,39		
56	06O0KI194	1,37	-		
57	06O0KI195	2,43	3,46		
59	06O0KI196	1,18	5,21		
60	06O0KI197	1,64	4,27		
62	06O0KI198	1,14	3,16		
63	06O0KI199	1,8	5,35		
66	06O0KI200	1,37	5,13		
67	06O0KI201	1,59	2,99		
68	06O0KI202	0,29	4,36		
69	06O0KI203	1,55	2,77		
mean		**1,47**	**3,77**		
Feed B		1,924			
107	06O0KI210	2	2,74		
113	06O0KI211	-	2,05		
118	06O0KI212	1,95	3,7		
122	06O0KI213	0,79	4,96		
136	06O0KI214	1,93	4,49		
139	06O0KI215	1,31	1,59		
mean		**1,59**	**3,25**		
177	06O0KI238	afw	4,58		
182	06O0KI239	-	-		
185	06O0KI240	1,73	1,68		
192	06O0KI241	3,18	4,3		
203	06O0KI242	1,48	4,4		
206	06O0KI243	2,27	2,92		
mean		**2, 16**	**3,57**		
141	06O0KI216	1,68	4,25		
142	06O0KI217	1,47	4,33		
143	06O0KI218	1,5	4,41		
144	06O0KI219	1,12	3,35		
145	06O0KI220	1,19	2,67		
147	06O0KI221	-	2,82		
148	06O0KI222	1,84	4,85		
149	06O0KI223	2,17	1,99		
150	06O0KI224	3,09	3,19		
152	06O0KI225	2,21	3,92		
153	06O0KI226	1,27	5,14		
154	06O0KI227	0,47	4,54		
156	06O0KI228	1,2	5,08		
157	06O0KI229	0,36	4,115	6,05 2,18	
158	06O0KI230	1,23	3,03		
161	06O0KI231	0,77	1,67		
162	06O0KI232	0,92	1,81		
164	06O0KI233	2,13	2,31		
165	06O0KI234	1,27	3,29		
169	06O0KI235	0,96	3,2		
173	06O0KI236	0,56	4,99		
174	06O0KI237	1,29	2,68		
mean		**1,37**	**3,52**		

	Feed A	Feed B	Feed A	Feed B
line	thymus	thymus	Bursa	Bursa
line 1	**1,92**	**1,59**	**2,91**	**3,25**
line 2	**1,47**	**1,37**	**3,77**	**3,52**
line 3	**1,93**	**2,16**	**3,46**	**3,57**

Annex 11 – Villus / crypt ratio

Cheicken experiment LBI

Villus crypt ratio from the duodenum and jejunum. In all sections 3 villi and 3 cryptes are measured and the ratio and mean values are calculated

NTB = not able to measure due to autolysis

animal number	Hisnr	Duodenum				Jejunum			
		villus	crypt	ratio	sum and mean	villus	crypt	ratio	sum and mean
4	06O0KI174	1501	171	8,8	29,0	1020	161	6,3	23,6
		1601	138	11,6	**9,7**	973	118	8,2	**7,9**
		1176	136	8,6		1010	112	9,0	
5	06O0KI175	1560	166	9,4	31,3	1003	125	8,0	29,2
		1368	131	10,4	**10,4**	1081	114	9,5	**9,7**
		1288	112	11,5		1065	91	11,7	
11	06O0KI176	1726	199	8,7	24,5	1216	79	15,4	45,5
		1403	197	7,1	**8,2**	1265	84	15,1	**15,2**
		1044	120	8,7		1220	81	15,1	
18	06O0KI177	1051	151	7,0	18,9	NTB			
		1041	163	6,4	**6,3**				
		990	178	5,6					
20	06O0KI178	1164	120	9,7	25,2	NTB			
		1095	165	6,6	**8,4**				
		1084	122	8,9					
26	060OK179	NTB				NTB			
mean					**8,6**				**10, 93**
36	06O0KI180	1566	218	7,2	24,0	1044	137	7,6	23,3
		1451	160	9,1	**8,0**	1040	130	8,0	**7,8**
		1481	192	7,7		1012	131	7,7	
38	06O0KI181	1395	241	5,8	23,7	NTB			
		1393	150	9,3	**7,9**				
		1426	166	8,6					
39	06O0KI182	1359	175	7,8	22,3	NTB			
		1343	173	7,8	**7,4**				
		1110	164	6,8					
40	06O0KI183	1720	207	8,3	26,3	1190	170	7,0	26,6
		1817	170	10,7	**8,8**	1219	118	10,3	**8,9**
		1745	240	7,3		1182	128	9,2	
43	06O0KI184	1803	155	11,6	39,7				
		1809	123	14,7	**13,2**				
		1758	132	13,3					
44	06O0KI185	1720	203	8,5	26,4	1125	118	9,5	26,5
		1684	198	8,5	**8,8**	1006	164	6,1	**8,8**
		1760	187	9,4		842	78	10,8	
45	06O0KI186	1810	244	7,4	29,5	1139	132	8,6	21,7
		1908	178	10,7	**9,8**	1005	188	5,3	**7,2**
		1356	119	11,4		1175	153	7,7	
46	06O0KI187	1590	223	7,1	19,8	1157	136	8,5	
		1690	226	7,5	**6,6**				
		1480	283	5,2					
47	06O0KI188	1957	218	9,0	32,9	1043	155	6,7	28,3
		1935	174	11,1	**11,0**	1069	104	10,3	**9,4**
		2029	159	12,8		1084	96	11,3	
48	06O0KI189	1479	285	5,2	18,4	878	73	12,0	31,7
		1183	159	7,4	**6,1**	811	76	10,7	**10,6**
		1400	242	5,8		788	88	9,0	
51	06O0KI190	1764	162	10,9	23,1	1195	166	7,2	26,9
		1542	188	8,2	**7,7**	1226	170	7,2	**9,0**
		1371	345	4,0		1177	94	12,5	
52	06O0KI191	NTB				962	167	5,8	17,5
						817	146	5,6	**5,8**
						974	158	6,2	
53	06O0KI192	1687	109	15,5	39,7	1127	156	7,2	28,7
		1598	118	13,5	**13,2**	1253	175	7,2	**9,6**
		1484	139	10,7		1102	77	14,3	
54	06O0KI193	NTB				689	90	7,7	19,9
						638	118	5,4	**6,6**
						852	125	6,8	
56	06O0KI194	1366	195	7,0	19,9	797	182	4,4	15,5
		1281	197	6,5	**6,6**	809	147	5,5	**5,2**
		1342	209	6,4		712	126	5,7	
57	06O0KI195	1449	152	9,5	29,4	858	104	8,3	27,5
		1420	114	12,5	**9,8**	753	122	6,2	**9,2**
		1509	205	7,4		970	74	13,1	
59	06O0KI196	1573	138	11,4	34,1	1480	122	12,1	31,4
		1455	145	10,0	**11,4**	1476	199	7,4	**10,5**
		1530	121	12,6		1456	123	11,8	
60	06O0KI197	1714	154	11,1	31,7	NTB			
		1554	163	9,5	**10,6**				
		1281	116	11,0					
62	06O0KI198	1657	205	8,1	22,4	1182	140	8,4	31,6
		1339	167	8,0	**7,5**	1283	103	12,5	**10,5**
		1439	227	6,3		1200	112	10,7	
63	06O0KI199	1645	156	10,5	31,5	1139	108	10,5	31,3
		1237	145	8,5	**10,5**	975	104	9,4	**10,4**
		1812	146	12,4		1137	100	11,4	
66	06O0KI200	1720	216	8,0	22,9	991	106	9,3	32,4
		1450	206	7,0	**7,6**	947	84	11,3	**10,8**
		1483	187	7,9		1040	88	11,8	
67	06O0KI201	NTB				991	106	9,3	32,4
						947	84	11,3	**10,8**
						1040	88	11,8	
68	06O0KI202	1621	147	11,0	28,6	940	140	6,7	22,9
		1540	143	10,8	**9,5**	907	136	6,7	**7,6**
		1465	215	6,8		925	97	9,5	
69	06O0KI203	1475	171	8,6	23,4	993	173	5,7	16,7
		1386	181	7,7	**7,8**	889	146	6,1	**5,6**
		1183	167	7,1		948	194	4,9	
mean					**9,5**				**8,6**
72	06O0KI204	1896	251	7,6	19,3	1287	140	9,2	25,2
		1874	298	6,3	**6,4**	1186	147	8,1	**8,4**
		1774	323	5,5		1265	159	8,0	
80	06O0KI205	1884	220	8,6	23,4	1028	89	11,6	34,7
		1840	260	7,1	**7,8**	1012	80	12,7	**11,6**
		1860	240	7,8		915	87	10,5	
89	06O0KI206	1495	127	11,8	22,6	874	124	7,0	27,0
		1700	283	6,0	**7,5**	1037	115	9,0	**9,0**
		1414	295	4,8		1047	96	10,9	
93	06O0KI207	1756	178	9,9	30,4	1163	160	7,3	19,6
		1704	168	10,1	**10,1**	1129	196	5,8	**6,5**
		1432	138	10,4		1831	280	6,5	
96	06O0KI208	1392	229	6,1	18,2	955	156	6,1	22,8
		1625	274	5,9	**6,1**	948	122	7,8	**7,6**
		1403	225	6,2		1062	119	8,9	
101	06O0KI209	1723	150	11,5	27,3	NTB			
		1358	158	8,6	**9,1**				
		1492	207	7,2					
mean					**7,8**				**8,6**
107	06O0KI210	1151	150	7,7	19,1	NTB			
		916	158	5,8	**6,4**				
		1166	207	5,6					
113	06O0KI211	1695	127	13,3	32,1	1299	126	10,3	29,1
		1299	134	9,7	**10,7**	1226	125	9,8	**9,7**
		1240	137	9,1		1282	143	9,0	
118	06O0KI212	1642	234	7,0	19,9	1201	284	4,2	13,2
		1383	245	5,6	**6,6**	994	207	4,8	**4,4**
		1418	196	7,2		1154	280	4,1	
122	06O0KI213	1582	177	8,9	21,0	1177	131	9,0	25,5
		1565	283	5,5	**7,0**	1174	135	8,7	**8,5**
		1245	191	6,5		1163	149	7,8	
136	06O0KI214	1425	119	12,0	39,7	1322	177	7,5	20,5
		1439	86	16,7	**13,2**	1277	208	6,1	**6,8**
		1297	118	11,0		1255	182	6,9	
139	06O0KI215	1635	232	7,0	21,1	1189	117	10,2	28,1
		1565	221	7,1	**7,0**	1182	108	10,9	**9,4**
		1377	198	7,0		1065	153	7,0	
mean					**8,4**				**7,7**

141	06O0KI216	1610	139	11,6	26,8	1045	92	11,4	31,7	
		1478	230	6,4	**8,9**	1141	111	10,3	**10,6**	
		1590	180	8,8		985	98	10,1		
142	06O0KI217	NTB				937	97	9,7	27,9	
						873	107	8,2	**9,3**	
						927	92	10,1		
143	06O0KI218	1766	185	9,5	29,7	937	97	9,7	27,9	
		1658	161	10,3	**9,9**	873	107	8,2	**9,3**	
		1711	173	9,9		927	92	10,1		
144	06O0KI219	1584	176	9,0	29,2	1430	129	11,1	36,6	
		1637	153	10,7	**9,7**	1428	156	9,2	**12,2**	
		1603	168	9,5		1240	76	16,3		
145	06O0KI220	NTB				1054	153	6,9	22,8	
						985	112	8,8	**7,6**	
						1025	144	7,1		
147	06O0KI221	2130	268	7,9	24,7	1562	135	11,6	37,6	
		1878	320	5,9	**8,2**	1503	89	16,9	**12,5**	
		2070	190	10,9		1640	180	9,1		
148	06O0KI222	1968	173	11,4	32,3	NTB				
		2041	182	11,2	**10,8**					
		1750	181	9,7						
149	06O0KI223	1420	150	9,5	31,1	1369	199	6,9	23,8	
		1548	166	9,3	**10,4**	1402	168	8,3	**7,9**	
		1591	129	12,3		1476	173	8,5		
150	06O0KI224	1686	205	8,2	33,3	NTB				
		2440	187	13,0	**11,1**					
		1943	161	12,1						
152	06O0KI225	2041	150	13,6	28,5	NTB				
		2017	267	7,6	**9,5**					
		2047	278	7,4						
153	06O0KI226	2229	272	8,2	20,7	1285	249	5,2	17,4	
		2138	371	5,8	**6,9**	1330	186	7,2	**5,8**	
		2256	335	6,7		1276	251	5,1		
154	06O0KI227	1604	107	15,0	35,4	NTB				
		1507	161	9,4	**11,8**					
		1490	135	11,0						
156	06O0KI228	1977	183	10,8	28,3	NTB				
		1955	186	10,5	**9,4**					
		1820	261	7,0						
157	06O0KI229	1690	158	10,7	29,5	1105	143	7,7	25,9	
		1721	155	11,1	**9,8**	1121	139	8,1	**8,6**	
		1665	217	7,7		1097	109	10,1		
158	06O0KI230	1287	133	9,7	25,2	1088	96	11,3	31,4	
		1546	198	7,8	**8,4**	1125	115	9,8	**10,5**	
		1311	170	7,7		1083	105	10,3		
161	06O0KI231	1386	217	6,4	18,9	913	136	6,7	18,7	
		1349	224	6,0	**6,3**	810	171	4,7	**6,2**	
		1280	198	6,5		765	106	7,2		
162	06O0KI232	1635	197	8,3	26,4	1020	94	10,9	34,9	
		1680	150	11,2	**8,8**	917	81	11,3	**11,6**	
		1427	207	6,9		1043	82	12,7		
164	06O0KI233	1888	281	6,7	16,8	963	116	8,3	23,6	
		1663	356	4,7	**5,6**	941	103	9,1	**7,9**	
		1742	324	5,4		1006	162	6,2		
165	06O0KI234	1668	167	10,0	31,5	1202	212	5,7	19,6	
		1464	109	13,4	**10,5**	1344	207	6,5	**6,5**	
		1443	179	8,1		1289	173	7,5		
169	06O0KI235	1104	167	6,6	19,0	NTB				
		1159	206	5,6	**6,3**					
		1169	173	6,8						
173	06O0KI236	1369	206	6,6	20,2	1270	257	4,9	19,9	
		1475	201	7,3	**6,7**	1310	212	6,2	**6,6**	
		1484	239	6,2		1261	143	8,8		
174	06O0KI237	1738	210	8,3	22,0	1325	212	6,3	18,6	
		1476	190	7,8	**7,3**	1297	201	6,5	**6,2**	
		1248	210	5,9		1272	215	5,9		
mean					**8,8**				**8.7**	
177	06O0KI238	1645	300	5,5	19,7	1618	93	17,4	51,2	
		1863	218	8,5	**6,6**	1597	91	17,5	**17,1**	
		1829	321	5,7		1528	94	16,3		
182	06O0KI239	1675	323	5,2	22,9	1430	202	7,1	24,7	
		1862	221	8,4	**7,6**	1446	220	6,6	**8,2**	
		1720	186	9,2		1251	113	11,1		
185	06O0KI240	1455	218	6,7	22,5	1428	158	9,0	28,1	
		1360	156	8,7	**7,5**	1477	148	10,0	**9,4**	
		1419	199	7,1		1471	162	9,1		
192	06O0KI241	1800	200	9,0	29,7	1051	109	9,6	25,4	
		1771	151	11,7	**9,9**	1048	142	7,4	**8,5**	
		1573	175	9,0		965	115	8,4		
203	06O0KI242	1603	199	8,1	26,0	886	91	9,7	25,6	
		1552	186	8,3	**8,7**	938	114	8,2	**8,5**	
		1489	155	9,6		928	122	7,6		
206	06O0KI243	1620	212	7,6	24,2	1538	162	9,5	34,3	
		1677	220	7,6	**8,1**	1559	145	10,8	**11,4**	
		1683	188	9,0		1461	104	14,0		
					8,1				**10,5**	

	Feed A	Feed B	Feed A	Feed B
line	duodenum	duodenum	jejunum	jejunum
line 1	**8,6**	**8,4**	**10, 93**	**7,7**
line 2	**9,5**	**8,8**	**8,6**	**8.7**
line 3	**7,8**	**8,1**	**8,6**	**10,5**

Annex 12 – CD 8 staining.

animal number	Hisnumber		duodenum	jejunum	cecum	
4	06O0ki174		1	1	1	
5	06O0ki175		1	2	1	
11	06O0ki176		1	1	1	
18	06O0ki177		1	1	1	
20	06O0ki178		1	1	1	
26	06O0ki179		1	1	1	
mean			1	1,2	1	
36	06O0ki180		1	1	1	
38	06O0ki181		1	2	1	
39	06O0ki182		1	2	2	
40	06O0ki183		1	1	1	
43	06O0ki184		1	2	1	
44	06O0ki185		1	2	2	
45	06O0ki186		1	2	1	
46	06O0ki187		1	2	1	
47	06O0ki188		1	1	1	
48	06O0ki189		1	1	1	
51	06O0ki190		1	2	1	
52	06O0ki191		1	2	1	
53	06O0ki192		2	2	2	
54	06O0ki193		1	2	1	
56	06O0ki194		1	2	1	
57	06O0ki195		1	1	2	
59	06O0ki196		1	1	1	
60	06O0ki197		2	2	1	
62	06O0ki198		2	2	1	
63	06O0ki199		2	2	2	
66	06O0ki200		2	3	2	
67	06O0ki201		2	1	1	
68	06O0ki202		1	2	2	
69	06O0ki203		2	1	1	
mean			1,3	1,9	1,3	
72	06O0ki204		2	2	1	
80	06O0ki205		2	2	2	
89	06O0ki206		2	2	2	
93	06O0ki207		2	2	2	
96	06O0ki208		2	2	2	
101	06O0ki209		1	2	2	
			1,7	2	1,7	
Feed B						
107	06O0ki210		2	1	1	
113	06O0ki211		2	2	1	
118	06O0ki212		2	2	1	
122	06O0ki213		1	2	1	
136	06O0ki214		2	2	1	
139	06O0ki215		2	2	1	
mean			1,7	1,7	1	
141	06O0ki216		2	2	2	
142	06O0ki217		1	1	1	
143	06O0ki218		2	2	1	
144	06O0ki219		1	2	1	
145	06O0ki220		1	2	1	
147	06O0ki221		1	1	1	
148	06O0ki222		1	1	1	
149	06O0ki223		2	2	1	
150	06O0ki224		1	1	1	
152	06O0ki225		1	2	1	
153	06O0ki226		1	2	1	
154	06O0ki227		1	1	1	
156	06O0ki228		1	3	2	
157	06O0ki229		1	2	2	
158	06O0ki230		1	2	2	
161	06O0ki231		2	2	2	
162	06O0ki232		1	1	1	
164	06O0ki233		1	1	afw	
165	06O0ki234		1	2	1	
169	06O0ki235		2	2	1	
173	06O0ki236		1	2	1	
174	06O0ki237		2	3	2	
mean			1,3	1,75	1,3	
177	06O0ki238		2	2	2	
182	06O0ki239		1	2	2	
185	06O0ki240		1	2	1	
192	06O0ki241		1	2	1	
203	06O0ki242		1	2	1	
206	06O0ki243		1	2	1	
mean			1,1	2	1,5	

	Line 1	feed A		Line 1	feed B	
	Line 3	Feed A		Line 3	feed B	
	Line 2	feed A		Line 2	feed B	
	Feed A	Feed B	Feed A	Feed B	Feed A	Feed B
line	duodenum	duodenum	jejunum	jejunum	cecum	cecum
line 1	1	1,7	1,2	1,7	1	1
line 2	1,3	1,3	1,9	1,75	1,3	1,3
line 3	1,7	1,1	2	2	1,7	1,5

Annex 13 – Histological results of tissue

histology chicken

nr = no remarks — absent = not sampled

Feed A

thymus and bursa amount of lymfoid tissues: 1 = little, 2 = more , 3 = normal amount of lymfoid tissue

diemummer	Hisnummer	thymus	bursa	spleen	heart	liver	ovaries	lung	kidney	pancreas	proventriculus	ventriculus	duodenum	jejunum	cecum
4	06O0ki174	absent		3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	apoptosis
5	06O0ki175		3	2 nr	follicle pericard	nr	nr	bronchitis	nr	small infiltrate	nr	nr	nr	nr	nr
11	06O0ki176		3	2 nr	nr	nr	nr	BALT	nr	small infiltrate	surface many follicle	nr	nr	nr	apoptosis in propria
18	06O0ki177		3	3 nr	nr	nr	nr	BALT	nr	small follicle	deeper follicles	nr	many follicles	many follicles	nr
20	06O0ki178		3	3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	nr
26	06O0ki179	3, some granulocyte		3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	nr
36	06O0ki180		3	3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	nr
38	06O0ki181		2	3 nr	follicle epicard	nr	nr	BALT	nr	nr	nr	nr	nr	nr	protozoa
39	06O0ki182		2	3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	nr
40	06O0ki183		2	3 nr	nr	nr	nr	BALT	nr	nr	deeper follicles	nr	nr	nr	apoptosis
43	06O0ki184		3	3 nr	myxomatous	larger follicles	nr	BALT	nr	small follicle	nr	nr	flinke follikels	nr	nr
44	06O0ki185		3	2 nr	nr	nr	nr	BALT	nr	nr	nr	dilated deep tubule	nr	nr	nr
45	06O0ki186		3	3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	nr
46	06O0ki187		3	3 nr	myxomatous ends	nr	nr	BALT	nr	nr	nr	nr	nr	nr	nr
47	06O0ki188		3	3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	fat
48	06O0ki189		3	3 nr	nr	nr	nr	BALT	small infiltrate	nr	deeper follicles	nr	nr	nr	nr
51	06O0ki190		3	3 nr	myxomatous, inf.	nr	nr	BALT	small follicle	nr	nr	nr	nr	nr	nr
52	06O0ki191		3	absent	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	apoptosis
53	06O0ki192		3	1 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	protozoa
54	06O0ki193		3	3 nr	nr	nr	nr	BALT	nr	nr	nr	much propria	nr	nr	nr
56	06O0ki194		3	3 nr	myxomatous	nr	nr	BALT	nr	small infiltrate	nr	nr	nr	nr	apoptosis
57	06O0ki195		3	3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	nr
59	06O0ki196		2	3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	apoptosis
60	06O0ki197		2	3 nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
62	06O0ki198		2	2 nr	insufficient fixation	nr	nr	BALT	nr	nr	nr	nr	nr	nr	nr
63	06O0ki199		3	3 nr	nr	nr	nr	nr	small infiltrate	nr	nr	nr	nr	nr	nr
66	06O0ki200		3	3 nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	much apoptosis
67	06O0ki201	2, strange epith.		3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	nr
68	06O0ki202		3	3 nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	apoptosis
69	06O0ki203		3	3 nr	nr	nr	nr	nr	nr	nr	nr	dilated deep tubule	nr	nr	nr
79	06O0ki204		3 absent	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
80	06O0ki205		3	3 nr	nr	nr	nr	nr	small infiltrate	nr	nr	dilated deep tubule	nr	nr	nr
89	06O0ki206		3	3 nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
93	06O0ki207		3	3 nr	nr	nr	nr	BALT	small infiltrate	nr	nr	dilated deep tubule	nr	nr	nr
96	06O0ki208		3	3 nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
101	06O0ki209	absent	absent	absent	nr	nr	nr	nr	nr	nr	nr	dilated deep tubule	nr	nr	apoptosis
Feed B															
107	06O0ki210		3	3 nr	follicle epicard	nr	nr	BALT	nr	nr	nr	dilated deep tubule	nr	nr	nr
113	06O0ki211	absent		3 nr	nr	nr	nr	BALT	nr	nr	nr	dilated deep tubule	nr	nr	nr
118	06O0ki212		3	3 nr	nr	nr	nr	BALT	nr	nr	nr	dilated deep tubule	nr	nr	nr
122	06O0ki213		3	3 nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
136	06O0ki214		3	3 nr	small infiltrate	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
139	06O0ki215		2	2 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	fat deposition submucosa, apoptosis
141	06O0ki216		3	3, cysten	nr	myxomatous	lymph follicle	nr	nr	nr	nr	nr	dilated deep tubule	nr	nr	nr
142	06O0ki217		2	3 nr	myxomatous	nr	nr	nr	nr	nr	nr	nr	nr	nr	fat deposition submucosa
143	06O0ki218		3	3 follikels	myxomatous	nr	nr	nr	nr	nr	nr	dilated deep tubule	nr	nr	fat deposition submucosa
144	06O0ki219		3	3, cysten	nr	nr	nr	cyste, c.l.	nr	nr	nr	much propria	nr	nr	nr	fat deposition submucosa, apoptosis
145	06O0ki220		3	3 nr	myxomatous	nr	nr	BALT	small infiltrate	nr	nr	nr	nr	nr	nr	fat deposition submucosa, apoptosis
147	06O0ki221	absent		3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	nr	fat deposition submucosa
148	06O0ki222		3	3 nr	small infiltrate	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	fat deposition submucosa, apoptosis
149	06O0ki223		3	2 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	nr	fat deposition submucosa
150	06O0ki224		3	3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	dilated deep tubule	nr	nr	full propria and apoptosis
152	06O0ki225		3	3 nr	small infiltrate epi	nr	nr	BALT	nr	nr	nr	dilated deep tubule	nr	nr	nr	apoptosis
153	06O0ki226		3	3 nr	nr	nr	nr	nr	nr	nr	much propria	nr	nr	nr	apoptosis
154	06O0ki227		3	3 grote foll	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
156	06O0ki228		3	3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	apoptosis
157	06O0ki229		3	3 nr	nr	nr	nr	BALT	nr	nr	nr	nr	nr	nr	nr
158	06O0ki230		2	1, cystes	apoptosis	nr	nr	nr	nr	nr	nr	dilated deep tubule	nr	nr	nr
161	06O0ki231		2	1, verkalking	nr	nr	nr	nr	nr	small infiltrate	nr	nr	nr	nr	nr
162	06O0ki232		3	1, cysten	nr	nr	nr	corpus luteum	BALT	nr	nr	nr	nr	nr	nr
164	06O0ki233		2	3 nr	nr	nr	more follicles	nr	nr	small infiltrate	nr	nr	nr	nr	nr
165	06O0ki234		2	3 nr	nr	nr	nr	BALT	nr	small infiltrate	nr	nr	nr	nr	fat deposition submucosa, apoptosis
169	06O0ki235		3	3 nr	nr	nr	nr	nr	nr	small infiltrate	nr	nr	nr	granulocytes	apoptosis, fat deposition submucosa
173	06O0ki236		2	1 nr	nr	nr	nr	nr	nr	small infiltrate	nr	nr	nr	nr	apoptosis, protozoa
174	06O0ki237		3	3 nr	nr	nr	granulocytes	BALT	nr	nr	nr	nr	nr	nr	apoptosis
177	06O0ki238	absent		3 wat foll	nr	nr	granulocytes	nr	nr	small infiltrate	nr	nr	nr	nr	nr
182	06O0ki239		3	3 nr	nr	nr	nr	BALT	small infiltrate	nr	nr	nr	nr	nr	nr
185	06O0ki240		2	3 nr	nr	nr	nr	nr	small infiltrate	nr	nr	nr	nr	nr	apoptosis
192	06O0ki241		3	3 nr	nr	nr	nr	nr	small infiltrate	nr	nr	nr	nr	nr	apoptosis
203	06O0ki242		3	3 nr	nr	nr	absent	absent	absent	nr	nr	nr	nr	nr	nr
206	06O0ki243		3	3 nr	nr	small heamorraghe	nr	nr	nr	nr	small infiltrate	nr	nr	nr	nr	apoptosis

	Line 1	feed A		Line 1	feed B
	Line 3	Feed A		Line 3	feed B
	Line 2	feed A		Line 2	feed B

Annex 14 – Overview significant chicken measurements

Significant differences, observed between **C-line animals** on feed A or feed B, in 1st and 2nd generation

B>A or A>B	**Measurements**	**Parameter**	**Comment**
A>B	General health 1st generation	Relative growth of body weight	In week 20 and week 35
B>A	General health 2nd generation	Weight	Consistent with increased protein percentage in feed of group B
B>A	General health 2nd generation	Relative growth of body weight	Till 5 weeks of age
A>B	General health 2nd generation	Relative growth of body weight	From week 10-12 of age; is after KLH-challenge
A>B	General health 2nd generation	Relative weight of Bursa	Organ weight after fixation
B>A	Metabolomics 2nd generation	Majority of Amino Acid levels in plasma	Consistent with increased protein percentage in feed of group B
B>A	Plasma metabolites	Plasma Lipids - Lysophosphatidyl choline (LPC), FFA and glycerol.	1 week before KLH challenge
A>B	Plasma metabolites	Plasma Lipids - LPC, FFA and glycerol	1 week after KLH challenge. Consistent with a stronger acute phase response in group A
A>B	Liver metabolites	D-ribose, D-ribulose and Fructose	4 weeks after KLH challenge. Indicative for increased pentose phosphate pathway activity in livers of group A
A>B	Liver metabolites	Histidine, Alanin, L-methionin, alpha Ketoglutarate and Vitamin E	4 weeks after KLH challenge. Markers of liver metabolism and food intake (vitamin E)
B>A	Immune System 1st generation	Innate Immune System - cellular anal. Monocyte activity, corrected for control	1 week after change to experimental feed

A>B	Immune System 1st generation	Specific Immune System -cellular anal. LST – LPS stimulated- Stimulation Index	8 weeks after change to experimental feed
A>B	Immune System **2nd** generation	Innate Immune System- cellular anal. Monocyte activity, response to LPS	1 week before KLH, 1 week after change of phase 1 feed to phase 2 feed
A>B	Immune System 2nd generation	Innate Immune System - cellular anal. Monocyte activity, response to LPS	1 week before KLH, 1 week after change of phase 1to phase 2 feed
A>B	Immune System 2nd generation	Innate Immune System - humoral anal. LPS- antibodies	2 weeks after KLH
B>A	Immune System 2nd generation	Innate Immune System -humoral anal. LPS-antibodies	4 weeks after KLH
A>B	Immune System 2nd generation	Innate Immune System -humoral anal. LPS-antibodies - **DELTA**	2 weeks after KLH
A>B	Immune System 2nd generation	Innate Immune System -humoral anal. LTA-antibodies	1 week before KLH, 1 week after change of phase 1 to phase 2 feed
B>A	Immune System 2nd generation	Innate Immune System -humoral anal. LTA-antibodies	4 weeks after KLH
B>A	Immune System 2nd generation	Innate Immune System -humoral anal. LTA-antibodies - DELTA	Week 1, 2, 3, 4 after KLH
A>B	Immune System 2nd generation	Innate Immune System -humoral anal. Complement – Classical pathway	1 week after KLH
B>A	Immune System 2nd generation	Specific Immune System -cellular anal. LST - control - counts	4 weeks after KLH
B>A	Immune System 2nd generation	Specific Immune System -cellular anal. LST – LPS stimulated-adj. for control - counts	1 week before KLH, 1 week after change of phase 1to phase 2 feed
A>B	Immune System 2nd generation	Specific Immune System -cellular anal. LST – LPS stimulated-adj. for control - counts	1 week after KLH,
A>B	Immune System 2nd generation	Specific Immune System -cellular anal. LST – Con A stimulated	1 week before KLH, 1 week after change of phase

			1to phase 2 feed
B>A	Immune System 2nd generation	Specific Immune System -cellular anal. LST – LPS stimulated - Stimulation Index	1 week before KLH, 1 week after change of phase 1to phase 2 feed
A>B	Immune System 2nd generation	Specific Immune System -cellular anal. LST – LPS stimulated - Stim Index	1 week after KLH
A>B	Immune System 2nd generation	Specific Immune System -cellular anal. LST – LPS stimulated - Stim Index	4 weeks after KLH
A>B	Immune System 2nd generation	Specific Immune System -cellular anal. LST – Con A stimulated	1 week before KLH, 1 week after change of phase 1to phase 2 feed
A>B	Immune System 2nd generation	Specific Immune System -cellular anal. LST – Con A stimulated	4 weeks after KLH,
A>B	Immune System 2nd generation	Specific Immune System -humoral anal. Vaccination titre NCD	3 weeks after KLH
A>B	Immune System 2nd generation	Specific Immune System -humoral anal. Vaccination titre Gumboro -**DELTA**	3 weeks after KLH
A>B	Immune System 2nd generation	Specific Immune System -humoral anal. Vaccination titre Gumboro - **DELTA**	4 weeks after KLH
A>B	Immune System 2nd generation	Stimulation Index feed extraction test **Feed A & B** lymphocytes to Feed A extract	Dilution -4 & -3
B>A	Immune System 2nd generation	Stimulation Index feed extraction test **Feed A & B** lymphocytes to Feed B extract	Dilution -3
B>A	Genomics gut & qPCR	Cholesterol biosynthesis pathway	Diverse genes, IPD signif.
B>A	Sensory analysis	Panel test chicken breast	Meat evaluated as more juicy

*Significant differences observed between **H and L-line animals** on feed A or feed B, 1st and 2nd generation*

B>A or **A>B**	Measurements	Parameter	Comment
A>B	General health 1st generation	**H-line** Relative growth of body weight	In week 35
A>B	General health	H-line Gastro-intestinal weight, relative to body	Organ weight after

	2nd generation	weight	section
A>B	General health 2nd generation	L-line Liver weight, relative to body weight	Organ weight after section
A>B	Immune System **1st** generation	**H-line** Innate Immune System -cellular anal. Monocyte activity, control	1week after change to experimental feed
A>B	Immune System 1st generation	**L-line** Innate Immune System -humoral anal. LPS-antibodies	4weeks after change to experimental feed
A>B	Immune System 1st generation	**L-line** Innate Immune System -humoral anal. LPS-antibodies	22 weeks after change to experimental feed
A>B	Immune System 1st generation	**H-line** Innate Immune System -humoral anal. Complement – Alternative pathway	2 weeks before change to experimental feed (!)
A>B	Immune System 1st generation	**L-line** Innate Immune System -humoral anal. Complement – Classical pathway	2 weeks before change to experimental feed (!)
A>B	Immune System 1st generation	**H-line** Specific Immune System –cellular anal. LST - control	2 weeks before change to experimental feed (!)
A>B	Immune System 1st generation	**L-line** Innate Immune System –cellular anal. LST- control	2 weeks before change to experimental feed (!)
A>B	Immune System 1st generation	**H-line** Specific Immune System -cellular anal. LST – LPS stimulated- counts. Adj. for control	2 weeks before change to experimental feed (!)
A>B	Immune System 1st generation	**L-line** Specific Immune System -cellular anal. LST – Con A stimulated- Stimulation Index	4 weeks after change to experimental feed
A>B	Immune System 1st generation	**L-line** Specific Immune System -humoral anal. Vaccination titre NCD	4 weeks after change to experimental feed
A>B	Immune System 1st generation	**L-line** Specific Immune System -humoral anal. Vaccination titre NCD	8 weeks after change to experimental feed
B>A	Immune System 1st generation	**H-line** Specific Immune System -humoral anal. Vaccination titre NCD - **DELTA**	4 weeks after change to experimental feed
A>B	Immune System 1st generation	**L-line** Specific Immune System -humoral anal. Vaccination titre NCD - **DELTA**	4 weeks after change to experimental feed
B>A	Immune System **2nd** generation	**L-line** Innate Immune System -cellular anal. Monocyte activity, control	4weeks after KLH
A>B	Immune System	**H-line** Innate Immune System -cellular anal.	1 week before KLH,

	2nd generation	Monocyte cellular response to LPS	1 week after change of phase 1to phase 2 feed
B>A	Immune System 2nd generation	**H-line** Innate Immune System -cellular anal. Monocyte cellular response to LPS	4 weeks after KLH
A>B	Immune System 2nd generation	**H-line** Innate Immune System -cellular anal. Monocyte activity, corrected for control	1 week before KLH, 1 week after change of phase 1to phase 2 feed
B>A	Immune System 2nd generation	**H-line** Innate Immune System -cellular anal. Monocyte activity, corrected for control	4 weeks after KLH
B>A	Immune System 2nd generation	**L-line** Innate Immune System -humoral anal. LPS-antibodies	4 weeks after KLH
B>A	Immune System 2nd generation	**H-line** Innate Immune System -humoral anal. LPS-antibodies - **DELTA**	1, 2, 4 weeks after KLH
B>A	Immune System 2nd generation	**L-line** Innate Immune System -humoral anal. LPS-antibodies - **DELTA**	4 weeks after KLH
B>A	Immune System 2nd generation	**H-line** Innate Immune System -humoral anal. LTA-antibodies	4 weeks after KLH
A>B	Immune System 2nd generation	**L-line** Innate Immune System -humoral anal. LTA-antibodies	1 week before KLH, 1 week after change of phase 1to phase 2 feed
B>A	Immune System 2nd generation	**H-line** Innate Immune System -humoral anal. LTA-antibodies - **DELTA**	4 weeks after KLH
B>A	Immune System 2nd generation	**L-line** Innate Immune System -humoral anal. LTA-antibodies - **DELTA**	1 and 2 weeks after KLH
A>B	Immune System 2nd generation	**H-line** Innate Immune System -humoral anal. Complement – Alternative pathway	4 weeks after KLH
A>B	Immune System 2nd generation	**H-line** Innate Immune System -humoral anal. Complement – Classical pathway	1 week after KLH
A>B	Immune System 2nd generation	**L-line** Innate Immune System -humoral anal. Complement – Classical pathway	1 week after KLH
B>A	Immune System 2nd generation	**L-line** Specific Immune System -cellular anal. LST - control - counts	1 week after KLH
B>A	Immune System 2nd generation	**L-line** Specific Immune System -cellular anal. LST – KLH stimulated-adj. for control - counts	1 week after KLH

A>B	Immune System 2nd generation	**H-line** Specific Immune System -cellular anal. LST – LPS stimulated-adj. for control - counts	1 week after KLH
B>A	Immune System 2nd generation	**L-line** Specific Immune System -cellular anal. LST – LPS stimulated-adj. for control - counts	1 week before KLH, 1 week after change of phase 1to phase 2 feed
A>B	Immune System 2nd generation	**H-line** Specific Immune System -cellular anal. LST – Con A stimulated	1 week before KLH, 1 week after change of phase 1to phase 2 feed
A>B	Immune System 2nd generation	**L-line** Specific Immune System -cellular anal. LST – Con A stimulated	1 week before KLH, 1 week after change of phase 1to phase 2 feed
B>A	Immune System 2nd generation	**H-line** Specific Immune System -cellular anal. LST – KLH stimulated - Stimulation Index	1 week before KLH, 1 week after change of phase 1to phase 2 feed
B>A	Immune System 2nd generation	**L-line** Specific Immune System -cellular anal. LST – KLH stimulated - Stimulation Index	1 week before KLH, 1 week after change of phase 1to phase 2 feed
A>B	Immune System 2nd generation	**H-line** Specific Immune System -cellular anal. LST – LPS stimulated - Stimulation Index	1 week after KLH,
B>A	Immune System 2nd generation	**L-line** Specific Immune System -cellular anal. LST – LPS stimulated - Stimulation Index	1 week before KLH, 1 week after change of phase 1to phase 2 feed
A>B	Immune System 2nd generation	**H-line** Specific Immune System -cellular anal. LST – Con A stimulated - Stimulation Index	1 week before KLH, 1 week after change of phase 1to phase 2 feed
A>B	Immune System 2nd generation	**L-line** Specific Immune System -cellular anal. LST – Con A stimulated - Stimulation Index	1 week before KLH, 1 week after change of phase 1to phase 2 feed
A>B	Immune System 2nd generation	**L-line** Specific Immune System -cellular anal. LST – Con A stimulated - Stimulation Index	1 week after KLH
B>A	Immune System 2nd generation	**H-line** Specific Immune System -humoral anal. KLH-titre -**DELTA**	3 weeks after KLH
B>A	Immune System 2nd generation	**L-line** Specific Immune System -humoral anal. Vaccination titre NCD	1 week after KLH
A>B	Immune System	**L-line** Specific Immune System -humoral anal.	2 weeks after KLH

	2nd generation	Vaccination titre NCD	
B>A	Immune System 2nd generation	**L-line** Specific Immune System -humoral anal. Vaccination titre NCD - **DELTA**	1 week after KLH
A>B	Immune System 2nd generation	**L-line** Specific Immune System -humoral anal. Vaccination titre NCD - **DELTA**	2 weeks after KLH
A>B	Immune System 2nd generation	Stimulation Index Feed extraction test **H-line Feed A** to Feed A extract	Dilution -4 & -3
A>B	Immune System 2nd generation	Stimulation Index feed extraction test **L-line Feed A** to Feed A extract	Dilution -2
A>B	Immune System 2nd generation	Stimulation Index Feed extraction test **H-line Feed A** to Feed B extract	Dilution -4 & -3
A>B	Immune System 2nd generation	Stimulation Index feed extraction test **L-line Feed A** to Feed B extract	Dilution -4
A>B B>A	Q PCR on genomics genes	Differences between the lines in regulation of several genes of cholesterol biosynthesis pathway	
H > L in A-animals	Sensory analysis	H line evaluated as having more 'chicken-typical' taste, and getting more appreciation	H and L compared, just tested in A-animals

Annex 15 – Overview all chicken measurements

Overview Results Chicken, 3 lines, 2 generations, 2 feeds =A =B

Δ /▲	=higher resp. significantly higher compared to animal line fed with different experimental feed
○/●	=in combination with ▲/Δ, to indicate which specific groups differ significantly
-	= no difference
	= not measured
↑/↓	= down regulation of genes (only in genomics)

1st generation measured around start of experimental feed in week 11, resulting in week -2, 1, 4, 8, 22
2nd generation measures around KLH-challenge in week 9, resulting in week -1, 1, 2, 3, 4
2nd generation animals sacrificed in week 13

		Generation 1						Generation 2						Remarks
		Feed A			Feed B			Feed A			Feed B			
	Animal line	H	C	L	H	C	L	H	C	L	H	C	L	
Feed intake		-	-	-	-	-	-	-	-	-	-	-	-	
Body weight (BW)		-	-	-	-	-	-	-	●	-	-	▲	-	
Relative growth of Body weight	till week 5*	-	-	-	-	-	-	●	●	●	▲	▲	▲	* for 2nd generation
Relative growth of Body weight	week 5-10	-	-	-	-	-	-	-	Δ	▲	-	○	●	
Relative growth of Body weight	from week 10	▲	▲	-	●	●	-	▲	▲	▲	●	●	●	for 1st generation: H in week 35, C in week 20 &35
Egg production		Δ	-	-	○	-	-							
First choice eggs		Λ	-	-	○	-	-							
Health check by poultry veterinarian								All animals healthy						
Immunology														
Innate cellular responses		*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	
Monocytes-control	week -2/ -1*	-	-	Λ	-	-	○	-	-	-	-	-	-	* -2 for 1st generation, -1 for second generation
Monocytes-control	week 1	▲	Δ	-	●	○	-	-	-	-	-	-	-	
Monocytes-control	week 4	-	-	-	-	-	-	-	-	●	-	-	▲	
Monocytes-control	week 8	-	-	-	-	-	-							
Monocytes - LPS	week -2/ -1	-	-	-	-	-	-	▲	▲	-	●	●	-	
Monocytes - LPS	week 1	-	○	-	-	Δ	-	-	-	-	-	-	-	
Monocytes - LPS	week 4	-	-	-	-	-	-	●	-	-	▲	-	-	
Monocytes -corrected for control	week -2/ -1	-	-	-	-	-	-	▲	▲	-	●	●	-	
Monocytes -corrected for control	week 1	-	●	-	-	▲	-	-	-	-	-	-	-	KLH challenge does not seem to have an effect
Monocytes -corrected for control	week 4	-	-	-	-	-	-	●	○	-	▲	Δ	-	
Innate humoral responses														
LPS antibodies	week -2/ -1	-	-	-	-	-	-	-	-	-	-	-	-	
LPS antibodies	week 1	Δ	-	Δ	○	-	○	-	Δ	-	-	○	-	Contradictory before and after challenge
LPS antibodies	week 2							-	▲	-	-	●	-	
LPS antibodies	week 3							-	-	-	-	-	-	
LPS antibodies	week 4	Δ	-	▲	○	-	●	-	●	●	-	▲	▲	
LPS antibodies	week 8	-	-	Δ	-	-	○							
LPS antibodies	week 22	Δ	-	▲	○	-	●							
LPS Nabs DELTA	week 1,2,4							●	▲	●	▲	●	▲	H in week 1,2,4; C in week 2; L in week 4
LTA antibodies absolute	week -2/ -1	-	-	-	-	-	-	-	▲	▲	-	●	●	
LTA antibodies absolute	week 1, 2, 3	-	-	-	-	-	-	-	○	-	-	Δ	-	Contradictory before and after challenge
LTA antibodies absolute	week 4	○	-	-	Δ	-	-	●	●	-	▲	▲	-	
LTA antibodies - DELTA	week 1-4	-	-	-	-	-	-	●	●	●	▲	▲	▲	H in week 4; C in week 1,2,3,4; L in week 1,2
Nab KLH	week -1	-	-	-	-	-	-							
Nab KLH	week 1-4	-	-	-	-	-	-							
Nab KLH	week 22	-	-	Δ	-	-	○							
Nab KLH - DELTA	week 4	●	-	Δ	▲	-	○							
Complement, alternative pathway	week -2/-1	▲	-	-	●	-	-	-	-	-	-	-	-	
Complement, alternative pathway	week 4	-	-	-	-	-	-	▲	-	-	●	-	-	
Complement, alternative pathway	week 8	-	○	-	-	Δ	-							
Complement, classical pathway	week -2/-1	-	-	▲	-	-	●	-	-	-	-	-	-	Line L for 1st generation, hence feed change causes significant difference.
Complement, classical pathway	week 1	-	-	-	-	-	-	▲	▲	▲	●	●	●	
Complement, classical pathway	week 2,3							-	-	-	-	-	-	
Complement, classical pathway	week 4	-	-	-	-	-	-	-	-	-	-	-	-	
Specific cellular responses		*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	
LST full blood control - counts	week -2/-1	▲	-	▲	●	-	●	-	-	-	-	-	-	
LST full blood control	week 1	-	-	-	-	-	-	-	-	●	-	-	▲	
LST full blood control	week 4	-	-	-	-	-	-	-	●	-	-	▲	-	
LST full blood-KLH-adjusted for control	week 1							-	-	●	-	-	▲	
LST full blood-LPS-adjusted for control	week -2/-1	▲	-	-	●	-	-	○	●	●	Δ	▲	▲	
LST full blood-LPS-adjusted for control	week 1	-	-	-	-	-	-	▲	▲	-	●	●	-	
LST full blood-LPS-adjusted for control	week 4	○	○	-	Δ	Δ	-	-	-	▲	-	-	●	
LST full blood-LPS-adjusted for control	week 8	-	-	-	-	-	-							
LST full blood -ConA (T-cell)	week -2/-1	-	-	Δ	-	-	○	▲	▲	▲	●	●	●	
LST full blood -ConA (T-cell)	week 1	-	-	Δ	-	-	○	-	-	-	-	-	-	
LST full blood -ConA (T-cell)	week 4	-	-	Δ	-	-	○	-	-	-	-	-	-	
LST full blood -ConA (T-cell)	week 8	-	-	Δ	-	-	○							
LST full blood-KLH-Stim Index (SI)	week -1							●	-	●	▲	-	▲	
LST full blood-LPS-SI	week -2/-1	-	○	-	-	Δ	-	○	●	●	Δ	▲	▲	
LST full blood-LPS-SI	week 1	-	-	-	-	-	-	▲	▲	-	●	●	-	
LST full blood-LPS-SI	week 4	○	○	-	Δ	Δ	-	-	▲	-	-	●	-	
LST full blood-LPS-SI	week 8	-	▲	Δ	-	●	○							
LST full blood-ConA-SI	week -2/-1	○	-	-	Λ	-	-	▲	▲	▲	●	●	●	
LST full blood-ConA-SI	week 1	-	Δ	Δ	-	○	○	-	-	▲	-	-	●	
LST full blood-ConA-SI	week 4	-	-	▲	-	-	●	-	▲	-	-	●	-	
LST full blood-ConA-SI	week 8	Δ	-	Δ	○	-	○							
Specific humoral responses														
KLH antibody 2nd generation	week -1							-	-	-	-	-	-	
KLH antibody 2nd generation	week 1-4							○	Δ	Δ	Δ	○	○	A higher than B, except H-line in week 3
NCD titres absolute	week -2/-1	Δ	-	-	○	-	-	-	-	-	-	-	-	
NCD titres absolute	week 1	-	-	Δ	-	-	○	-	-	●	-	-	▲	
NCD titres absolute	week 2							Δ	-	▲	○	-	●	
NCD titres absolute	week 3							-	▲	-	-	●	-	
NCD titres absolute	Week 4	-	-	▲	-	-	●	○	-	○	Δ	-	Δ	
NCD titres absolute	Week 8	-	-	▲	-	-	●							

Overview Results Chicken, 3 lines, 2 generations, 2 feeds (continued) =A =B

Δ / ▲	= higher resp. significantly higher compared to animal line fed with different experimental feed
○ / ●	= in combination with ▲/Δ, to indicate which specific groups differ significantly
-	= no difference
	= not measured
↑/↓	= down regulation of genes (only in genomics)

1st generation measured around start of experimental feed in week 11, resulting in week -2, 1, 4, 8, 22
2nd generation measures around KLH-challenge in week 9, resulting in week -1, 1, 2, 3, 4
2nd generation animals sacrificed in week 13

		Generation 1						Generation 2						Remarks
		Feed A			Feed B			Feed A			Feed B			
	Animal line	H	C	L	H	C	L	H	C	L	H	C	L	
NCD titres absolute	week 22	-	-	Δ	-	-	○							
NCD titres DELTA	week 1	○	○	Δ	Δ	Δ	○	-	-	●	-	-	▲	
NCD titres DELTA	week 2							-	-	▲	○	-	●	
NCD titres DELTA	week 3							-	-	-	-	-	-	
NCD titres DELTA	week 4	●	-	▲	▲	-	●	○	-	-	Δ	-	-	strange contradiction
NCD titres DELTA	week 8	-	-	Δ	-	-	○							
NCD titres DELTA	week 22	○	-	-	Δ	-	-							
Gumboro titres absolute	week -2, 1, 4	-	-	-	-	-	-							
Gumboro titres absolute	week -1 /4							-	-	-	-	-	-	
Gumboro titres absolute	week 8	Δ	Δ	-	○	○	-							
Gumboro titres absolute	week 22	-	-	-	-	-	-							
Gumboro titres DELTA	week -2, 1, 4	-	-	-	-	-	-							
Gumboro titres DELTA	week 1-4							-	▲	○	-	●	Δ	C: in week 3+4 sign.; L: in week 3 trend higher
Gumboro titres DELTA	week 8	Δ	Δ	○	○	○	Δ							
Gumboro titres DELTA	week 22	-	-	-	-	-	-							
Feed extraction tests		*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	
LST extract Feed A - Stim Index	dil. -4,-3,-2	-	-	-	-	-	-	▲	▲	▲	●	●	●	H & C in dilution -4 en -3 sign.; L in dil -2
LST extract Feed B - Stim Index	dil. -4,-3,-3	-	-	-	-	-	-	▲	●	▲	●	▲	●	H sign. in dilution -4,-3; C in dil -3 ; L in dil -4
Metabolomics		*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	
Plasma (most discriminating metabolites)														
LPC 18:0 and 16:0	week -1							-	●	○	-	▲	Δ	LPC clear marker for feed in H and C line
LPC 18:0 and 16:0	week 1							▲	▲	▲	●	●	●	
LPC 18:0 and 16:0	week 3							▲	▲	Δ	●	●	○	
LPC 18:1, 22:6 and 20:1	week -1							-	●	-	-	▲	-	
LPC 18:1, 22:6 and 20:1	week 1							-	▲	-	-	●	-	
LPC 18:1, 22:6 and 20:1	week 3							-	▲	-	-	●	-	
Cholesterol esters 18:1 and 14:0	week -1							-	●	-	-	▲	-	
Cholesterol esters 18:1 and 14:0	week 1							-	▲	-	-	●	-	
Cholesterol esters 18:1 and 14:0	week 3							-	-	-	-	-	-	
Free fatty acid 22:6	week -1							●	●	●	▲	▲	▲	
Free fatty acid 22:6	week 1							Δ	-	○	○	-	Δ	
Free fatty acid 22:6	week 3							Δ	Δ	-	○	○	-	
Global LC-MS peak 1004 and 749	week -1							-	▲	-	-	●	-	Chemical identity of peak 1004 and 749 not
Global LC-MS peak 1004 and 750	week 1							-	▲	▲	-	●	●	resolved
Global LC-MS peak 1004 and 751	week 3							-	-	▲	-	-	●	
GC-MS platform glycerol, FA 16:0 and 18:1	week -1							●	●	○	▲	▲	Δ	glycerol = marker with highest discriminating
GC-MS platform glycerol, FA 16:0 and 18:1	week 1							-	▲	-	-	●	-	power across all metabolomics platforms
GC-MS platform glycerol, FA 16:0 and 18:1	week 3							-	▲	-	-	●	-	
Liver (most discriminating metabolites)														
Cholesterol ester 14:0	week 4							○	●	-	Δ	▲	-	
Free fatty acid 18:3	week 4							Δ	▲	-	○	●	-	
Global LC-MS peak 160 and histidine	week 4							-	▲	-	-	●	-	
GC-MS Monomethylphosphate	week 4							▲	▲	▲	●	●	●	
GC-MS D-ribose, D-ribulose and Fructose	week 4							▲	▲	▲	●	●	●	
GC-MS Alanin and L-methionin	week 4							-	▲	-	-	●	-	
GC-MS Vitamin E	week 4							Δ	▲	▲	○	●	●	
GC-MS alpha Ketoglutarate	week 4							-	▲	-	-	●	-	
Pathological Anatomy														
Gastro Intestinal weight relative to BW								▲	-	-	●	-	-	weighed "fresh" after section
Liver weight relative to BW								-	-	▲	-	-	●	weighed "fresh" after section
Thymus weight relative to BW								-	Δ	-	-	○	-	weighed after preparation
Bursa weight relative to BW								-	▲	-	-	●	-	weighed after preparation
Genomics		*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	
Cholesterol biosyntheses pathway											↓	↓	↓	At least 7 genes, connected to this pathway, are down regulated. Indicating circulating cholesterol?
q PCR														
HAC gene								▲	●	●	●	▲	▲	
AACS gene								Δ	Δ	○	○	○	Δ	
IPD gene								Δ	Δ	-	○	○	-	
Microbial composition manure		*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	
Bacterial composition								-	-	-	-	-	-	Difference between A- en B-manure, non-identified, but different from bacterial comp in feed
Observations of the butcher		*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	
Redness of meat								Δ	Δ	Δ	○	○	○	
Fat connected to meat								○	○	○	Δ	Δ	Δ	
Taste Panel		*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	*H*	*C*	*L*	
Juiciness of the meat									●			▲		
Typical 'chicken' taste									Δ			○		
H-L comparison. Typical 'chicken' taste and appreciation	A-animals only							▲		●				