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VDI-4; In-vitro tests 2013-2014

Marcel Hulst, Arjan Hoekman, Ilonka Wijers, Dirkjan Schokker and Mari Smits

LIVESTOCK RESEARCH
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The objective of this study was to develop an *in-vitro* bioassay using cultured Intestinal Porcine Epithelial Cells (IPEC-J2) and evaluate the capability of this assay to predict enterocyte-specific physiological and immunological processes induced by nutrients/additives in the intestines of farm animals.

Responses to five nutrients/feed-additives, similar to those studied in animal trials, performed in the Feed4Foodure framework, were measured by gene expression analysis of IPEC-J2 cells either under stressed (Salmonella) or non-stressed conditions. Response genes were analysed using bioinformatics web-tools in order to identify dominant biological processes induced by these nutrients/feed-additives and to predict key-genes/proteins important for regulation of these biological processes.

Results obtained with the IPEC-J2 *in-vitro* bio-assay showed similarities and differences to results obtained in other *in-vivo* and *in-vitro* intestinal models. We identified a set of biological processes that could function as potential indicators of "immune competence" of the epithelial layer (enterocytes) of the gut mucosa. Furthermore, we identified a set of key-genes and chemicals/biomolecules which have the potential to represent the capability of the intestinal epithelial layer to properly sense, communicate and respond to hostile pathogens, toxic agents and other environmental changes in the intestinal lumen. Such indicators of "local intestinal immune competence" may predict detriment or beneficial effects of nutritional interventions *in-vivo*.

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Foreword

Feed4Foodure is a public-private partnership between the Dutch Ministry of Economic Affairs, a consortium of various organizations within the animal production chain and Wageningen UR Livestock Research. Feed4Foodure aims to contribute to sustainable and healthy livestock farming in the Netherlands, simultaneously strengthening our competitive position on the global market. The Feed4Foodure program line "Nutrition, Intestinal Health, and Immunity", aims to contribute to a reduction in the use of antibiotics in livestock farming by increasing general health and disease resistance. The main goals are to develop innovative measurement techniques and to test new health-promoting nutritional additives in the field of gut health and immunity.

The current report describes experiments conducted to investigate the effects of five nutrients/feed-additives, similar to those studied in animal trials performed with chickens and pigs in VDI-2,-3, and -5, on gene expression in cultured Intestinal Porcine Epithelial Cells (IPEC-J2) in the presence and absence of a Salmonella challenge. Experiments were performed within the framework of the Feed4Foodure program line "Nutrition, Intestinal Health, and Immunity".

For the current study, scientist of Wageningen UR Livestock Research, and Wageningen UR CVI worked together with representatives from the various private partners, including Agrifirm, ForFarmers, Nutreco, De Heus, Denkavit, and Darling Ingredients International, and from the Universities of Wageningen UR, and Utrecht. The authors thank these partners of the project team for their worthwhile input.

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

Summary

The objective of this study was to develop an *in-vitro* bioassay using cultured Intestinal Porcine Epithelial Cells (IPEC-J2) and evaluate the capability of this assay to predict enterocyte-specific physiological and immunological processes induced by nutrients/additives in the intestines of farm animals. Responses to five nutrients/feed-additives, similar to those studied in animal trials performed with chickens and pigs in VDI-2,-3, and -5 (ZnO, Amoxicillin's [Octacillin and Paracillin], Rye, and Fructo-oligosaccharides [FOS]), were measured by gene expression analysis in IPEC-J2 cells stressed by a Salmonella challenge, and in IPEC-J2 cells under non-stressed conditions (without Salmonella challenge). Response genes were analysed using bioinformatics web-tools in order to identify dominant biological processes induced by these nutrients/feed-additives and to predict key-genes/proteins important for regulation of these biological processes.

Results obtained with the IPEC-J2 *in-vitro* bio-assay showed similarities and differences to results obtained in other *in-vivo* and *in-vitro* intestinal models. Challenging IPEC-J2 cells with Salmonella in the presence of additives revealed some novel mechanisms how porcine intestinal epithelial cells may respond to nutritional interventions when intestinal homeostasis is disturbed by enteric pathogens or other stressors. With respect to intestinal immunity, applying a Salmonella challenge in our IPEC-J2 test proved to be essential for monitoring how additives like ZnO and amoxicillin can modulate cytokine/chemokine responses in a stress situation.
A limitation of the IPEC-J2 bioassay was revealed in tests performed with different concentrations of rye formulated in the control diet. The results of these experiments showed that incubation of a complete diet on IPEC-J2 monolayers induces large changes in gene expression in IPEC-J2 cells that overshadow less dominant effects of the components of interest (rye) within this diet.

Conclusions

- For pigs a medium-throughput bioassay using cultured "Intestinal Porcine Epithelial Cells" was developed which was able to detect enterocyte-specific physiological and immunological processes induced by nutrients/additives.
- ZnO and amoxicillin antibiotics modulated: i) Salmonella-induced cytokine/chemokine response in porcine intestinal epithelial cells; and ii) expression of hypoxia-induced factor 1A (HIF1A)-effector proteins, most likely to rescue these cells from oxidative stress. With respect to reduction and/or banning of antibiotics in the pig and poultry production chains the results of this study are promising. Based on results of the ZnO and antibiotic IPEC-J2 bioassay tests, an array of alternative additives may be selected and pre-screened in the IPEC-J2 bioassay to select for additives with potential to induce similar effects in the gut of pigs and poultry as antibiotics do.
- Rye influences processes related to cell cycle progression in porcine intestinal epithelial cells and showed only limited effect on immune genes. No correlations between viscosity of the tested rye diets and biological processes were found.
- FOS induced no direct biological process in porcine intestinal epithelial cells and showed no effect on immune genes. Changes imposed by "waste metabolites" of fermentation of long chain FOS by Salmonella may have influenced gene expression in porcine intestinal epithelial cells. This suggests that development of an *in-vitro* test in which the interplay between additives, metabolites secreted by specific microbiota in the lumen of farm animals, and enterocytes can be studied, may be feasible using IPEC-J2 cells.
- In this study we identified a set of biological processes that could function as potential indicators of "immune competence" of the epithelial layer (enterocytes) of the gut mucosa. In addition, we identified a set of key-genes and chemicals/biomolecules which have the potential to represent the capability of the intestinal epithelial layer to properly sense, communicate and respond to hostile pathogens, toxic agents and other environmental changes in the intestinal lumen. These indicators of "local intestinal immune competence" may predict detriment or beneficial effects of nutritional interventions *in-vivo*.

1 Background

1.1 Overall aim

The aim of VDI-4 "*In-vitro* models" work-package is the development of a medium-throughput bioassay, based on *in vitro* cultured cells, capable to predict biological and immunological responses to nutritional interventions in the epithelial layer of the gut of pigs, chickens, and cattle. Measuring the functional effects of numerous feed ingredients, i.e. raw materials, feed additives, and diet composition in such a bioassay in the lab, may generate insight in the mechanisms how these interventions influence the immediate early functioning of epithelial cell layers.
Using this knowledge, a panel of alternative ingredients for expensive or controversial feed additives (e.g. antibiotics) may be proposed, and subsequently tested in the bioassay to pre-screen them for their *in vitro* bioactivity before they are tested in expensive animal trials. In addition, the results of this *in-vitro* assay may provide a set of potential read-out parameters which provide information on the competence of the intestinal epithelial layer to properly sense, communicate and respond to hostile pathogens, toxic agents and other environmental changes in the intestinal lumen. Such indicators of "local intestinal immune competence" may predict detriment or beneficial effects of nutritional interventions *in-vivo*.

1.2 Introduction

Lined up enterocytes are the predominant cells of the intestinal epithelial layer. Together with the microbiota in the lumen these enterocytes play a major role in digestion of feeds/foods and absorption and transport of nutrients over the epithelial layer. Cells of the epithelial layer are in direct contact with the content of the lumen, although they are covered by a mucus layer. This mucosal layer functions as a first defence barrier to prevent invasion of enteric pathogens and toxic chemical substances into the body. Together with resident and infiltrating immune cells, enterocytes play a crucial role in the local immune response in the intestinal mucosa. Epithelial cells, specialized cells (e.g. M cells) and enterocyte-conditioned dendritic cells (DC's) embedded in the epithelial layer constantly survey the luminal environment for foreign antigens and toxic residues formed after digestion of feed/foods [Rimoldi et al. 2005]. In case "danger signals" are sensed, enterocytes, M cells and DC's transmit signals (e.g. cytokines/chemokines) to underlying cells/tissues and to the periphery to activate the local innate defence as well as adaptive immune mechanisms. These physiological and immunological properties make enterocytes the cell of choice for development of an *in-vitro* bioassay to study the effect of nutritional inventions.

For farm animals only a well characterized porcine intestinal enterocyte cell line (IPEC-J2) is available for *in-vitro* studies. We [Hulst et. al. 2013] and others [Brosnahan et. al. 2012] showed that IPEC-J2 cells were capable to express an array of cytokines (IL8, IL1A, IL6, IL7, IL18, TNFA and CSF) and several acute phase response proteins when they were challenged with enteric pathogens like Salmonella and ETEC [Hulst et. al. 2013, Geens et. al. 2010, Niewold et. al. 2005 and 2007]. This indicated that these cells mimic the immunological function of enterocytes *in-vivo* [Collado-Romero, et. al. 2010]. Moreover, we also showed that an IL8 response in IPEC-J2 cells induced by Salmonella may be modulated by treatment of these cells with a panel of chemical substances [Hulst et. al. 2013].
We also performed a comprehensive bioinformatics analysis followed by data mining in biological and chemical databases with a set of Salmonella response genes that were generated by gene expression analysis (microarrays) of mucosal scraping dissected from piglets of an *in-situ* Small Intestinal Segment Perfusion experiment. This demonstrated that functional analysis of sets of response genes generated by gene-expression analysis in the intestines of pigs can predict how *in vitro* cultured enterocytes respond to specific chemicals [Hulst et. al. 2013].

An enterocyte cell line derived from the intestines of cattle exists. However, this cell line is derived from "Black Cattle", a native Japanese breed for which the use of biological/genetic material outside of Japan is forbidden by the Japanese authorities [Chiba et. al. 2012]. A few reports describe cell lines derived from the jejunum of chicken and cattle [Cencic et. al. 2013]. However, these cell lines are not

fully cloned yet, grow slowly, and are poorly characterized. Attempts to derive stable lines from these enterocytes are still in progress in the lab in which they were developed.

1.3 Objective and approach

The objective of this study was to develop an *in-vitro* bioassay using cultured Intestinal Porcine Epithelial Cells (IPEC-J2) and evaluate the capability of this assay to predict enterocyte-specific physiological and immunological processes induced by nutrients/additives in the intestines of live farm animals.

In this study we evaluated whether the above described gene-expression analysis/bioinformatics approach also works when applied reversely; i.e. can we predict a response to specific nutrients/additives in the intestine of live farm animals using gene expression data generated in IPEC-J2 cells challenged with these nutrients/additives? We adapted the IPEC-J2 test used in our previous Salmonella study to develop such a predictive bioassay [Hulst et. al. 2013]. Responses to five nutrients/feed-additives, similar to those studied in animal trials performed with chickens and pigs in VDI-2,-3, and -5, were measured by gene expression analysis in IPEC-J2 cells stressed by a Salmonella challenge, and in IPEC-J2 cells under non-stressed conditions (without Salmonella challenge). The sets of response genes were analysed using bioinformatics web-tools in order to identify dominant biological processes induced by these nutrients/feed-additives and predict key-genes/proteins important for regulation of these biological processes. A comprehensive data-mining and literature study was performed to evaluate whether these responses can be translated to an *in-vivo situation*, i.e. to an intact intestinal mucosal layer. Based on these processes, and on the key-genes/proteins linked to these processes, alternative chemicals/additives can be predicted with the potential to induce a similar response in enterocytes as the tested nutrients/feed-additives. For one intervention a set of alternative additives were selected. In addition, with this *in-vitro* assay we identified a set of read-out parameters which potentially provide information on the competence of the intestinal epithelial layer to properly sense, communicate and respond to hostile pathogens, toxic agents and other environmental changes in the intestinal lumen. Such indicators of "local intestinal immune competence" may predict detriment or beneficial effects of nutritional interventions *in-vivo*. Finally, the progress in development of similar bioassays for chicken and cattle, based on enterocyte cell lines or on other epithelial cell lines of these species, are briefly summarized and discussed.

Remarks;
- For relevant literature and background information regarding the *in-vivo* effects of the five nutrients/additives tested in the IPEC-J2 assay, we refer to VDI-2,-3, and -5 reports describing the results of the *in-vivo* intervention studies. In this report some brief information is given at the beginning of each results section and results are discussed briefly in relation to earlier studies with these additives.
- To evaluate the predictive value of the IPEC-J2 bioassay, in VDI-6 an integrated bioinformatics analysis is performed for each of the tested nutrients/additives in order to correlate gene expression data of the IPEC-J2 bioassay to data generated form intact mucosa of the jejunum of chickens and pigs and ileum of pigs. A detailed discussion about overlapping processes and particular genes is given in this VDI-6 report.

2 Material and Methods

2.1 Gene expression analysis in IPEC-J2 cells

2.1.1 Additives tested in the IPEC-J2 assay

The source of the additives used for challenge of IPEC-J2 cells were similar to preparations used in animal trials performed in VDI-2, -3, and -5 in 2013. For detailed specifications and source of these additives we refer to the VDI reports in which the results of these animal trials are described. A brief description of the experimental design of these in-vivo trials and the used additive preparations is given below.

-ZnO; VDI-5, *In-vivo* models pig
-Octacillin® [Amoxicillin antibiotic]; VDI-3, 'Neonatal models'.
-VDI-3, Short and long chain Fructo-oligosaccharides (FOS); 'Neonatal models'.
-Rye; VDI-5, *In-vivo* models chickens).
-Paracillin [Amoxicillin antibiotic]; VDI-2, Maternal effects on intestinal health and immunity of offspring.

VDI-5, ZnO: Pigs were fed a regular (60-100 mg/kg) and higher dose of zinc oxide (2500 mg/kg; [75% ZnO]) over the period from day 14 to 23 post weaning. The pigs were dissected at day 14, 23 and 35. Jejunal and ileal tissue were analysed for gene expression using the porcine Agilent microarrays.

VDI-5, Rye: In the broiler experiment 960 broiler chickens were housed in 24 pens which each contained 40 chickens. The broiler chickens were fed different levels of rye diet containing 0%, 5% and 10% weight/volume. On day 14, 21 and 28, per time-point 6 chickens from each pen were dissected. Jejunal tissue was analysed for gene expression using the chicken Agilent microarrays.
Besides the added rye also the components/nutrients of which the diet is composed contribute to the viscosity of the digesta. Therefore the complete diet formulations, containing different concentration of rye, were tested in IPEC-J2 cells.

VDI-3, Octacillin: Octacillin was administered via the drinking water (67 mg amoxicillin/L) to 1-day-old chickens for a period of 24 hrs. A control group, not exposed to the antibiotic, was included. At three time-points after hatch (day 1=prior to antibiotic administration, day 5, and 14) birds were sacrificed and jejunal mucosa was collected and analysed for gene expression using the chicken Agilent microarrays.

VDI-2, Paracillin-Amoxicillin: Pregnant sows were housed 28 days before expected farrowing date and randomly divided in a control group and a group treated with amoxicillin, started 7 days before expected farrowing date. The control treatment sows were fed regular lactation feed and the amoxicillin treatment sows received a top-coating of 15 mg / kg bodyweight amoxicillin until farrowing.

VDI-3, Short and long chain Fructo-oligosaccharides (FOS): From day 2 or 3 after birth piglets got twice a day an oral administration of 15 ml of 10g/L FOS dissolved in water. A mixture was used containing 10% long chian FOS and 90% short chain FOS (Frutafit® TEX [long chain FOS] and Frutalose® OFP [short chain FOS] from SENSUS). The piglets in the control group got twice a day an oral administration of water. All the piglets of one litter, six FOS piglets and eight control piglets, were reared by their own mother. At day four the FOS mixture was dissolved in water to concentration of 25g/L and a total mix of 6 ml was administrated instead of 15 ml. At three different time-points, day 2, 14, and 25, FOS and control piglets were sacrificed to extract tissue samples of jejunum and colon for gene expression analysis.

All additives, except rye, were dissolved in IPEC-J2 culture medium without Foetal Calf Serum (FCS) and without antibiotics (see below) at a concentration of 10 % w/v and stored in aliquots at -70 °C. Aliquots were diluted to desired concentrations just before they were incubated on IPEC-J2 monolayers. Different concentrations of a Rye suspension were dispersed in medium just before incubation.

2.1.2 IPEC-J2 in-vitro assay, with and without Salmonella challenge

IPEC-J2 cells were seeded in 2 cm^2 tissue culture wells (M24 plate) and grown for 7 days at 37 ºC and 5% CO2 using 1:1 DMEM/Ham's F10 1:1 medium (Gibco-BRL) supplemented with 5% FCS without antibiotics. For all tests, confluent monolayers were washed twice with medium without FCS (hereafter denoted as medium) and incubated for 1 hour with this medium. Hereafter, the medium was discarded and a mixture of Salmonella bacteria and additive dissolved in medium was added.
In a pilot experiment the multiplicity of infection (MOI) that did not induce visible (microscopic) damage to the cells (in the absence of additive) was determined after exposure times of 2 or 6 hrs. Based on this pilot experiment a MOI of 1.0 was used for 2 and 6 hour incubations. After 6 hrs of incubation the pH of the medium was not changed significantly (as indicated by colour of the medium), and there was only a slight increase in turbidity (microscopic visible) due to growth of Salmonella bacteria during the incubation time. In a similar pilot experiment, dilutions of additives in medium with a concentration of 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 % w/v were incubated for 8 h on IPEC-J2 monolayers. At 30', 1h, 2h, 3h, 4h, 6h en 8h monolayers were inspected with a microscope to determine whether the morphology of the cells and the integrity of the monolayers were not disturbed.

The effect of all additives on gene expression in IPEC-J2 was tested in duplicate at one concentration. In each culture plate, duplicate control wells containing no Salmonella (only with chemical) or containing no chemical (only with Salmonella), or without chemical and Salmonella (only medium), were incubated for the same period as was done for wells containing mixtures of chemicals and Salmonella.

2.1.3 RNA extraction from cells

After incubation for 0, 2 or 6 h total RNA was extracted from cells using Trizol (Invitrogen) according to the manufacturer's instructions. RNA was further purified using the QIAamp MinElute Virus Spin Kit (Qiagen Cat no. 57704) [Hulst et. al. 2013]. The quality and integrity of the RNA samples was analysed using an Agilent Lab-on-a-Chip and Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). All samples scored a RNA integrity number (RIN value) of ≥ 9.

2.1.4 Microarray Labelling, Hybridization, Scanning and Feature Extraction

Costum prepared 8x60K Agilent pig micro arrays G2519F Sus scrofa (035953; V2026440) containing 43,803 probes were used for single dye hybridizations with Cy3 labelled cRNA. Labelling, hybridization, scanning and feature extraction were performed in the same manner as described recently (Schokker et.al. 2014) with minor differences. Briefly, 500 ng RNA of each sample was labelled with the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling kit and 600 ng of Cy3 labelled cRNA was used for hybridisation on each patch. Hybridisation and washing of the arrays was performed according to the protocol provided by Agilent Technologies for the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling kit. Arrays were scanned using a DNA microarray scanner with Surescan high resolution Technology (Agilent Technologies). Agilent Scan Control with resolution of 5 µ, 16 bits and PMT of 100%. Feature extraction was performed using protocol 10.7.3.1 (v10.7) for 1 colour gene expression.

2.1.5 Microarray data analysis

The files generated by the feature extraction software were loaded in GeneSpring GX 9.0.5, in which a log2-transformation and a median normalization (75 percentile) was performed on all probes. Probes with a raw intensity of <60 (flooring) and with a corrected *p-value* of >0.05 (OnewayANOVA significance analysis with asymptotic *p-value* computation) were filtered out of the data files. Probes differentially expressed with a Fold-Change (FC) of expression of 2.0 (up-regulation) or 0.5 (down-regulation) in a microarray comparison of two treatments were selected for further analysis. In case probes with a FC>2.0 or 0.5 in specific comparisons were selected with a higher level of significance, (e.g. when they passed a Post-Hoc Tukey's HSD statistical test) this will noted in the results section.

2.2 Functional genomics analysis and Data mining

2.2.1 Preparation of lists of regulated genes.

In addition to the annotation provided by Agilent (pig) oligonucleotide sequences of differentially expressed probes from not annotated yet, or annotated as Unigene, tentative consensus sequences (TC) or mRNA accession number, were compared with the NCBI non-redundant nucleotide databases using blastn to assign a gene-name to these probes. Probes that did not produce a significant match with any other eukaryotic mRNA/gene were excluded from gene lists used for functional analysis. For each comparison of 2 treatments a list of differential probes was prepared containing information about the FC, a brief description of the mRNA/gene for which the probes codes, its regulations (up or down), and the gene-symbol. Throughout this manuscript official human gene-symbols (HUGO Gene Nomenclature Committee: http://www.genenames.org) were used in the text and in all figures, tables and appendixes. In all results paragraphs beneath information about the biological functions of genes was retrieved by consulting the "GeneCards" (Weizmann Institute of Science) and the NCBI Gene reports (Entrez), and from literature linked to these reports (for references about these biological functions of genes we refer to these gene reports).

2.2.2 Bioinformatics analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID version 6.7) website (Huang da et. al., 2009) and the "GeneAnalytics" (LifeMap Sciences, Inc.) were used to assign genes to a specific pathway. List of HUGO gene-symbols differentially expressed were loaded in these programs. Because far more human genes are annotated and more information in databases is available for humans than for pigs, the human background was used for this functional analysis. From DAVID, pathways (KEGG and Reactome) with an EASE score (p-value) of ≤0.1 (default EASE score) were retrieved. From GeneAnalytics output files pathways were retrieved with a high or medium score (p-value <0.05). Pathways retrieved from DAVID were only listed when not called significant by GeneAnalytics or in case more genes were listed by DAVID than by GeneAnalytics. Pathways that contained <3 regulated genes were not retrieved. Associations of genes with compounds (non-synthetic) with a high or medium score were retrieved from GeneAnalytics (corrected *p-value* ≤0.05). Using the protein interaction tool of DAVID, regulated gene-sets were enriched for specific transcription factor binding sequences (UCSC_TFBS module) with EASE score ≤0.1.

Functional associations between chemicals, proteins encoded by differentially expressed genes and enzyme substrates/products linked to these proteins, were established using the (protein)-protein-chemical interaction web tool STITCH 4.0 beta (Kuhn et. al. 2012). Relevant chemicals, related to the additives tested in the IPEC-J2 assay (retrieved from GenAnalytics; see above), or substrates and products of response genes coding for enzymes were uploaded together with lists of response genes/proteins in STITCH. Associations with a confidence score of ≥0.4 (medium level) were selected from output files and displayed in a network. In a supplementary file (appendixes) the type and confidence level of each association is listed. The Comparative Toxicogenomics Database (CTD) was used to explore associations of specific regulated genes with chemical compound and find relevant literature about these associations. Based on consulting this literature, natural (non-synthetic)

chemicals/compounds were selected predicted to induce a similar effect in enterocytes as the additives did in the IPECJ2 assay.

Based on literature study most relevant biological processes/pathways were defined and presented for each tested additive in the results section of this manuscript. Regulated genes (and chemicals related to these genes/proteins) crucial for steering these biological processes are denoted as effector genes/proteins (e.g. transcription factors and other intracellular proteins, and proteins like cytokines and chemokines that induce/steer an extracellular process).

3 Results and Discussion

3.1 Determination of the optimal test concentration of additives

Compared to the *in-vivo* situation, the IPEC-J2 cell surface is not covered by a mucus layer. Diffusion of nutrients/additives from the lumen through this layer is controlled carefully, preventing exposure of epithelial cells to large fluctuations in physiological conditions (like pH, osmolality, etc.) and toxic components, which may disrupt the integrity the cell layer. In general, the concentration and the manner these additives were formulated in the feed matrix and administrated in the intervention trials performed in VDI-2, 3 and 5, were different from the manner additives were applied to the IPEC-J2 monolayers. To prevent additives would impose disturbance of the IPEC-J2 monolayers (detachment) and/or loss of vitality of these cells, a pilot test was performed in which for all tested additives the highest possible concentration of additive was determined at which no microscopic visible damage and morphological changes occurred of the cells during 8 hrs of incubation. In Table 1 this maximum concentration applied to IPEC-J2 cells is presented. For detailed information how these diets containing these additives where formulated for all animal trials, we refer to the specific VDI reports in which the results of these animal trials are described.

3.2 Transcriptome analyses

3.2.1 Microarray comparisons

All RNA samples isolated from IPEC-J2 monolayers showed a RNA integrity number (RIN value) of ≥ 9 when analysed in the Agilent Lab-on-a-Chip and Bioanalyzer, indicating that quality and integrity of the RNA samples was suitable for microarray analysis. All hybridisations were performed in duplicate and repeated in case quality parameters recorded by the scanner software did not met values recommended by Agilent. In Table 1 comparisons performed for all additives at a specific FC cut-off, and the number of up- and down-regulated genes are listed. All lists of regulated genes are presented in separate sheets in the Excel supplementary tables.

Table 1

Microarray comparisons performed for additives with and without Salmonella.

additive	compared to	Concentration (% w/v)	FC (up/down)	# genes * 2h	# genes * 6h
ZnO	Medium	0.0325	2	178	722
ZnO+Salm.	Medium	0.0325	2	35	651
Octacillin	Medium	0.50	2	92	707
Octacillin+Salm.	Medium	0.50	2	169	732
Paracillin	Medium	0.125	2	67	640
Paracillin +Salm.	Medium	0.125	2	23	633
10% rye + Salm.	10% rye	3-fold diluted diet	2	123	894
5% rye + Salm.	5% rye	3-fold diluted diet	2	1049	510
0% rye + Salm.	0% rye	3-fold diluted diet	2	1224	504
10% rye	5% rye	3-fold diluted diet	2	125	47
10% rye	Medium	3-fold diluted diet	3 (2)	282 (571)	4(23)
5% rye	Medium	3-fold diluted diet	3 (2)	102 (208)	8(28)
0% rye	Medium	3-fold diluted diet	2	432	35
Lc FOS	Medium	2.50	2	0	1
Lc FOS + Salm.	Medium	2.50	2	271	309
Sc FOS	Medium	2.50	2	0	2
Sc FOS +Salm.	Medium	2.50	2	522	468

* number (#) of unique genes differentially expressed.

3.2.2 Response to Salmonella

To mimic a stress situation in IPEC-J2 cells, all additives were tested also in the presence of Salmonella. The control experiment gene expression was measured after 2 and 6 hrs of incubation of IPEC-J2 cells with Salmonella alone. Despite IPEC-J2 cells were seeded at a similar density, grown for the same period (7 days) before use, and challenged with the same MOI, gene expression analysis showed that a Salmonella response peaked either at 2h or at 6h, depending on the day the test was performed. Slower growth of the Salmonella culture and/or small differences in the differentiation of the IPEC-J2 cells may account for this difference in response time. Therefore, all incubations with Salmonella alone were performed at the same day as the additives and mixtures of additive plus Salmonella were tested. All differential expressed genes of Salmonella versus medium (=mock) comparisons are presented in the supplementary Table S1, along with lists of differential expressed genes for the additives tested on that particular day. In Tables 3 to 5, provided in the results sections beneath, only Salmonella response genes are discussed in more detail in case IPEC-J2 cells where incubated with a mixture of additive and Salmonella or in case the FC significantly decreased, normalized, or inverted due to the additive. A bioinformatics (pathway) analysis performed on these response genes showed that the response of IPEC-J2 cells reflected processes induced *in-vivo* by Salmonella described in literature by others [Collado-Romero, et. al. 2010] and by our lab [Niewold et. al 2007, Hulst et. al. 2013]. Briefly, the most prominent pathways regulated were the NOD- and Toll-like receptor signalling cascades, Apoptosis and Autophagy (and related to this, Cytoskeleton rearrangements), Glucocorticoid receptor signalling, and the production of cytokines and inflammatory response genes. The cytokines CXCL2, IL1A, CSF2, and IL8 were highly up-regulated (50- to 7.5-fold). In addition, up-regulation of IRF1, FOS, and JUN suggested that higher expression of these cytokines/chemokines was triggered by TLR4 signalling and executed by NF-kB-mediated transcription. Up-regulation of NFKB inhibitors NFKBIA and NFKBIE, and TNFAIP3 indicated that excessive expression of these cytokines/chemokines was prevented. In agreement with this, cytokine mRNA responses that peaked at 2h (e.g. see results of ZnO) were decreased or normalized after 6 h. In all Salmonella versus mock comparisons analysed genes involved in Cell Cycle and "DNA damage/cell cycle checkpoint" were regulated (e.g. DNA-damage-inducible transcript 4 [DDIT4]). No indications were found that oxidative stress (and/or ER stress) was induced in response to Salmonella. Moreover, in some Salmonella/control comparisons genes that normally respond to hypoxia were slightly down-regulated (2- to 3-fold, e.g. HMOX1 and NQO1). A moderate (FC~3) up-regulation at 2 hrs was observed of NRROS (negative regulator of reactive oxygen species), indicating that Salmonella was able to repress ROS production in IPEC-J2 cells, probably to survive in these cells. In summary, the IPEC-J2 cells responded as expected to a stress situation induced by Salmonella.

3.2.3 Response to ZnO

Zinc supplementation would improve health of newly weaned pigs by decreasing the incidence of post weaning diarrhoea induced by enterotoxigenic *E. coli* [Heo et al., 2010] and other enteric viral and bacterial pathogens. The most important pathways retrieved from bioinformatics programs of the lists with genes that responded to ZnO alone (ZnO), and to a mixtures of ZnO and Salmonella (ZnO-Salm) are presented in Table 2. The genes SLC30A1 (transmembrane zinc transporter) and the metallothionein MT1A (intracellular zinc transporter and scavenger of reactive oxygen species) were higher expressed in IPEC-J2 cells incubated with ZnO alone at 2h (note that both these genes were not mapped to a pathway). However at 6 h, their expression reverted to a lower level than was measured in mock treated cells. This suggests that intracellular pools of Zinc were supplemented to a (non-toxic) level tolerated by the IPEC-J2 cells, and that these cells were capable to adjust these levels by a feed-back mechanism. After 2 hrs incubation ZnO alone induced up-regulation of several cytokines/chemokines (IL1A, IL8, IFNL1 [alias IL29] and CXCL2) and a moderate down-regulation of IL6, and several immunological pathways were called significant (see Table 2). Similar as observed for Salmonella-induced expression and for MT1A and SLC30A1, up- or down-regulated cytokine/chemokine expression normalized (IL6, IFNL1) or swapped to down-regulated expression at 6 h (IL8, IL1A, and CXCL2). Moreover, compared to Salmonella alone, a complete different pattern of Salmonella-induced cytokine/cytokine expression was observed after 6 hrs of incubation in the presence of ZnO (summarized in Table 5 paragraph 3.3). Expression of IL1B, one of the most potent inflammatory cytokines, and of IFNA4 was induced specifically in case a mixture of Salmonella and

ZnO was applied. The strong down-regulation of the interleukin 1 receptor antagonist (IL1RN) by ZnO alone and the observation that no IL1B response was induced by Salmonella at 6 hrs suggested that this IL1B response was stimulated by ZnO. However, intensities of IL1B probes on the array were relatively low compared to IL8, IL6 and CXCL20, indicating that IL1B production in IPEC-J2 cells is not as important as IL8 and IL6 production. Induction of IFNA4 expression is probably related to the presence of invaded Salmonella bacteria inside IPEC-J2 cells. Expression of IFNA4s gene may stimulate autophagy.

Table 2

Response to ZnO pathway analysis

hrs	treatment	Score [a]	Pathway term[b]	# total genes	# genes	genes
2	Salm	29.3	TNF Signaling Pathway	110	7	CFLAR, CXCL2, EDN1, FOS, MAP3K8, NFKBIA, TNFAIP3
2	ZnO	25.7	TNF Signaling Pathway	110	10	CFLAR, CSF2, CXCL2, EDN1, FOS, IL6, JUN, MAP3K8, SOCS3, TNFAIP3
6	ZnO-Salm	11.2	TNF Signaling Pathway	110	11	BIRC3, CCL20, CXCL2, EDN1, IL1B, IL6, JUN, , MAP2K1, NFKBIA, TNFAIP3
2	ZnO	27.5	NOD-like Receptor Signaling Pathways	195	13	BCL10, EDN1, FOS, FOSB, HMOX1, IL1A, IL6, IL8, IRF1, JUN, SERPINE1, TICAM1, TLR6
6	ZnO-Salm	10.6	NOD-like Receptor Signaling Pathways	195	15	BIRC3, CASP1, CD209, EDN1, IFNA4, IL1B, IL6, IL8, IRF1, JUN, MAP2K1, NFKBIA, SERPINE1, STAT1, TLR6
2	ZnO	20.1	HIF-1-alpha Transcription Factor Network	65	7	EDN1, FOS, HMOX1, ID2, JUN, MCL1, SERPINE1
6	ZnO	27.6	HIF-1-alpha Transcription Factor Network	65	15	ADM, BHLHE41, CA9, EDN1, EGLN1, EGLN3, FOS, GATA2, HK2, JUN, NCOA1, NDRG1, PFKFB3, PGK1, SERPINE1
6	ZnO-Salm	12.9	HIF-1-alpha Transcription Factor Network	65	9	ADM, BHLHE41, CITED2, EDN1, GATA2, ID2, JUN, NCOA1, SERPINE1
6	ZnO	15.1	Fatty Acid, Triacylglycerol, and Ketone Body Metabolism	217	20	ACADM, AGPAT9, ANGPTL4, APOA2, CEBPD, CPT1A, CYP1A1, ELOVL7, HMGCR, LPIN2, MED1, MED20, NCOA1, NCOA6, NR2F2, PEX11A, RORA, TEAD4, TGS1, TXNRD1
6	ZnO-Salm	13.7	Fatty Acid, Triacylglycerol, and Ketone Body Metabolism	217	18	ABCA1, ACADM, AGPAT9, APOA2, CEBPD, CPT1A, CYP1A1, ELOVL7, GPD1, LPIN1, LPIN2, MED20, MED26, NCOA1, NFYA, NR2F2, RORA, TEAD4
2	ZnO	26.5	Direct P53 Effectors	134	11	BCL6, BTG2, DDIT4, DUSP5, EPHA2, GDF15, JUN, MCL1, PLK3, PRDM1, SERPINE1
6	ZnO	16.8	Direct P53 Effectors	134	16	BNIP3L, BTG2, C12orf5, CASP1, CDKN1A, DDIT4, DGCR8, FOXA1, GDF15, JUN, NDRG1, PMAIP1, PRDM1, RNF144B, S100A2, SERPINE1
6	ZnO-Salm	10.7	Direct P53 Effectors	134	12	BNIP3L, BTG2, CASP1, CD82, DDIT4, GADD45A, JUN, NFYA, PLK3, PRDM1, SERPINE1, TRRAP
2	ZnO	22.3	Senescence and Autophagy	107	9	BMP2, IL1A, IL6, IL8, INHBA, IRF1, JUN, PLAU, SERPINE1
2	ZnO	24.1	IL6-mediated Signaling Events	43	7	FOS, IL6, IRF1, JUN, LMO4, MCL1, SOCS3
2	ZnO	26.0	ATF-2 Transcription Factor Network	55	8	DDIT3, DUSP5, FOS, IL6, IL8, JUN, PLAU, SOCS3
6	ZnO	14.0	Translation Insulin Regulation of Translation	287	23	CBLB, DDIT4, DYRK2, EIF2S2, EIF4A2, EIF4E2, FOS, GSK3B, HK2, JUN, MAP2K1, PCK2, PHKG2, PLAT, PLAU, PPP1R3B, PPP1R3C, PPP3CB, RICTOR, RNF41, SERPINE1, SH2B2, SOCS4
6	ZnO	8.9	TGF Beta Signaling Pathway	55	7	BMP4, CTNNB1, FOS, JUN, NOG, RUNX3, SERPINE1
6	ZnO	9.5	Glucocorticoid Receptor Regulatory Network	82	9	CDKN1A, FOS, GATA3, GSK3B, IL8, JUN, NCOA1, PCK2, SPI1
6	ZnO	13.7	Fatty Acid, Triacylglycerol, and Ketone Body Metabolism	217	18	ABCA1, ACADM, AGPAT9, APOA2, CEBPD, CPT1A, CYP1A1, ELOVL7, GPD1, LPIN1, LPIN2, MED20, MED26, NCOA1, NFYA, NR2F2, RORA, TEAD4
6	ZnO-Salm	10.1	TSLP Signaling Pathway	38	6	IL6, IL8, MAP2K1, MTOR, NFKBIA, STAT1
6	ZnO-Salm	14.0	Transport of Glucose and Other Sugars, Bile Salts and Organic Acids, Metal Ions and Amine Compounds	543	33	AAAS, ABCA4, ABCD3, ADCY4, ADCY8, ANO5, AQP1, ATP2A3, ATP6V0A4, ATP8B1, GABRG3, MYO5B, NUP188, NUP62, PRKAR1A, RAB11FIP2, SLC13A4, SLC16A7, SLC22A7, SLC29A3, SLC2A6, SLC2A8, SLC31A1, SLC35A3, SLC44A5, SLC4A4, SLC7A10, SLC7A2, SLC7A6, SLC7A7, SLC9A9,
6	ZnO-Salm	14.9	Salmonella Infection (KEGG)	113	13	CASP1, CCL4, CXCL2, DOCK1, IFNGR1, IL1B, IL6, IL8, ITGA5, JUN, NFKBIA, PFN2, WASL
6	ZnO-Salm	13.8	Regulation of Cholesterol Biosynthesis By SREBP (SREBF)	89	11	ATF6, FGF21, INSIG1, INSIG2, LPIN1, MBTPS1, MTOR, MVK, NCOA1, NFYA, PMVK
6	ZnO-Salm	13.5	IL-9 Signaling Pathways	161	15	EIF2S2, FYN, GZMA, IL21, IL6, IRF1, JUN, KAT5, LTA, MAP2K1, MTOR, NFKBIA, PRKAA1, RETN, STAT1
6	ZnO-Salm	12.0	Chemokine Signaling	282	20	ADCY4, ADCY8, CCL20, CCL4, CCR3, CXCL11, CXCL2, GRK7, IL8, JUN, MAP2K1, MAPK4, NFKBIA, PLA2G4C, PRKD3, SRF, STAT1, STAT2, VAV3, WASL

[a] GeneAnalytics pathways and enriched GO-BP terms with a high score (>18; corresponding with an FDR corrected p-value ≤ 0.0001) and medium score (>9; corresponding with an FDR corrected p-value ≤ 0.05) were retrieved.

total genes; total number of genes in a pathway. # genes; number of genes differential expressed in IPEC-J2 cells mapped to a pathway. Upper panel; common pathways called significant in all 2 or 3 comparisons. Lower panel; pathways called significant for one of the treatments.

[b] Pathway annotations are from GeneAnalytics (http://geneanalytics.genecards.org).

This time-dependent regulation of cytokines/chemokines may be important for the overall response of IPEC-J2 cells to ZnO. It has been shown that MT1A expression is under control of IL6 and that zinc-finger proteins are necessary for signal transduction from cytokine surface receptors to immunological response genes [Hernández et. al. 1997, Mariani et al. 2008].

In all intracellular activities in which zinc-finger proteins are involved, MT's play a crucial role. At 6 h Salmonella induced down-regulation of RIPK3 expression. Besides that RIPK3 plays a role in sensing of foreign DNA, it also determines the fate of cells in response to DNA-damage and/or oxidative stress by promoting controlled necrosis (necroptosis) via TNF-signalling (the TNF-pathway was called significant in all 3 comparisons) [Newton et. al. 2014]. TNF-signalling in non-immune cells may regulate transcription of many of the cytokine/chemokines for which we observed regulation in this study (see Fig. 1). After 6h of incubation with ZnO we also found up-regulation of BIRC3, an E3 ubiquitin-protein ligase that inactivates the RIPK1-RIPK3 complex in the TNF-cascade. This suggested that TNF-signalling, or parts of this pathway, may account in for the complex regulation of cytokine/chemokine expression in stressed and non-stressed IPEC-J2 cells in response to ZnO.

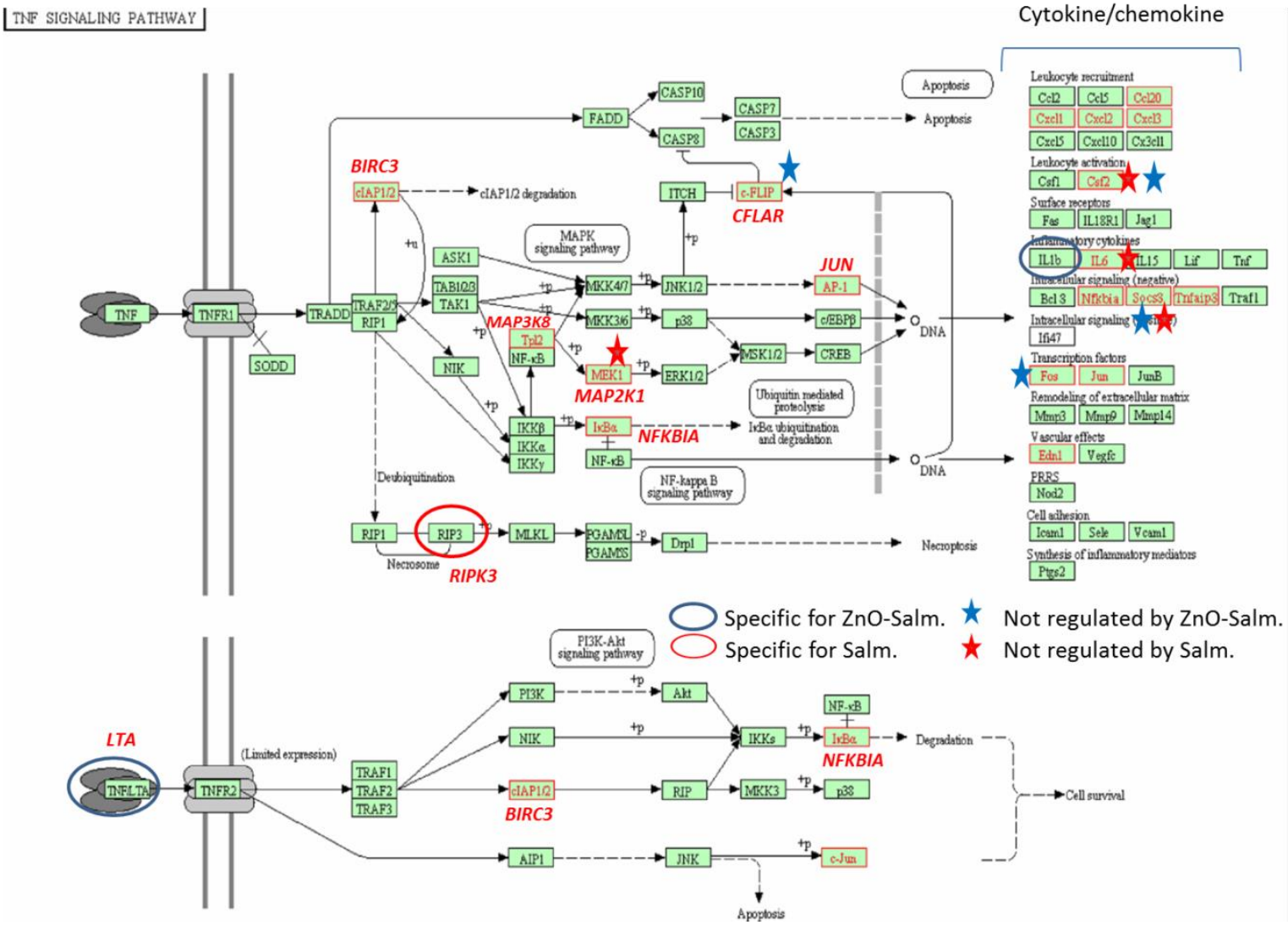

Figure 1 *TNF-signalling pathway (KEGG database). Differentially expressed genes are presented in boxes with red gene-symbols for comparisons performed with ZnO in the presence and absence of Salmonella at 2 and 6 hrs. Genes responding specific to Salmonella (without ZnO) are encircled red and genes responding specific to a mixture of Salmonella and ZnO are encircled blue. Genes not responding to a specific treatment are marked with stars (see panel in the centre of the KEGG-scheme). For non-HUGO gene symbols boxed in the*

original KEGG pathway scheme (green boxes) official HUGO gene-symbols are provided in red near the box.

The most prominent pathway called significant at 6 h was the "Hypoxia-Inducible Factor 1 alpha (HIF1A) signalling pathway". Binding of IL6, LPS, or IFNy to their receptors induces STAT3 or NFKB mediated transcription of HIF1A, and consequently, HIF1A-mediated transcription of several effector genes/proteins for which we observed differential expression after incubation with ZnO for 2 and 6 h. In Fig. 2 all these effector molecules are displayed. In case of a shortage of oxygen exists, these effector molecules can induce extracellular (widening of blood vessels to increase oxygen supply) and intracellular processes (switch to anaerobe sugar metabolism) that allows enterocytes to return to a normal oxygen situation, not harmful for cells. Genes of the insulin pathway called significant at 6h may be involved in regulation of the glucose/sugar metabolism to support this return to normoxia. In addition, incubation with all treatments for 6 hrs also called the "Fatty Acid, Triacylglycerol, and Ketone Body Metabolism" pathway significant. Many genes within this pathway also play a role in regulation of triglyceride fat metabolism and sugar-energy metabolism, two energy-metabolic processes that are tightly linked in mitochondrion of cells. An indication that HIF1A-mediated expression of these effector molecules may be induced by IL6 is the strong up-regulation of the STAT-induced STAT inhibitor SOCS3, differential expressed after 2 hrs incubation with ZnO alone. Together with the NFKB inhibitors NFKBIA and NFKBIE, SOCS3 may prevent overexpression of HIF1A and its effector proteins (see Fig. 2).

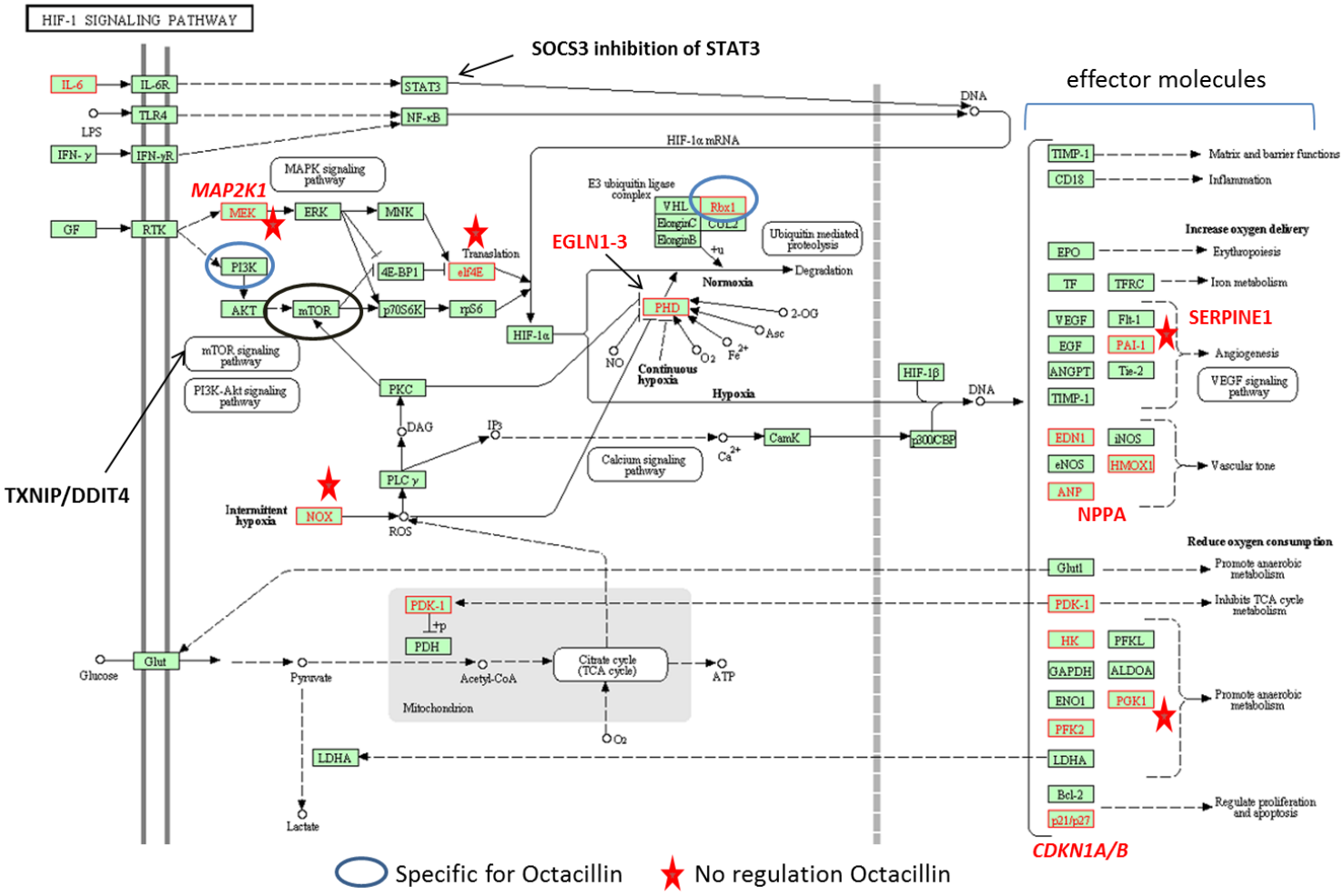

Figure 2 *Effector molecules transcribed by HIF1 signalling. Differentially expressed genes are presented in boxes with red gene-symbols for comparisons performed with ZnO (with and without Salmonella) at 2 and 6 hrs. Genes responding specific to Octacillin (without and without Salmonella) are encircled blue (discussed in paragraph 3.2.5.). Genes not responding to Octacillin are marked with a red star (see panel in the bottom of the KEGG-scheme). HUGO gene-symbols are provided in red text for non-HUGO gene symbols boxed in the original KEGG pathway scheme. The involvement of SOCS3 in inhibition of STAT3 is discussed above, and of TXNIP and DDIT4 in activation of the mTOR complex (encircled black) is discussed in paragraph 3.2.5.1 (response to Paracillin)*

ZnO, Salmonella, and a mixture of both, all regulated the expression of a different panel of effector genes belonging to the ATM/TP53 signalling cascade (ATM cell cycle checkpoint kinase, and TP53 tumour protein p53). Activation of this cascade is induced by DNA-damage and oxidative stress. Compared to a high up-regulation induced by Salmonella alone, the DNA-damaged induced gene DDIT4 was moderately up-regulated by ZnO alone at 2 hrs and slightly down-regulated by a mixture of both. In addition, the "Reprimo, TP53 Dependent G2 Arrest Mediator Candidate1" gene (RPRM) was highly up-regulated by a mixture. This suggested that ZnO prevented DNA-damage induced by Salmonella and cell-cycle arrest. After 6 hrs with ZnO alone, expression of DDIT4 was even further decreased to almost undetectable levels. An interesting observation was that TP53 effector PRDM1 (a PR domain zinc finger protein 1), an repressor of beta-interferon gene expression, showed a similar expression pattern as DDIT4 did.

Along with RPRM, at 2 hrs the Oxytocin Receptor1 (OXTR), mediator complex subunit 23 (MED23; Vitamin D3 Receptor-Interacting Protein) and zinc finger protein 2 (ZFP2) were also highly up-regulated. The two latter genes are transcription enhancers, from which MED23 is the co-activator of transcription factor SP1. In a recent study, HIF2A and SP1 mediated transcription of the copper-transporting ATPase (Atp7a) gene in rat intestinal epithelial cells during hypoxia [Xie et. al. 2013]. This suggests that an SP1 transcription factor might also collaborate with HIF1A in IPEC-J2 cells. Perhaps the above discussed induction of cell cycle arrest byTP53/RPRM allows IPEC-J2 cell to put effort in high-level transcription of a set of genes. Probably, the set of genes for which we found high up-regulation at 6 hrs in case a mixture of ZnO and Salmonella was applied. Interestingly, among these genes were the calcium-dependent NADPH oxidase that generates superoxide radicals (NOX5), a cyclic-AMP-dependent protein kinase inhibitor alpha (PKIA), and HOOK1, a part of the FTS/HOOK/FHIP complex that plays a dominant role in apoptosis/cell survival and in cell cycle progression and arrest regulated by TP53 [Song et al., 2007]. NOX5 and PKIA may be involved in induction of cyclic-AMP/calcium mediated intermittent hypoxia/ROS production, indirectly resulting in the inhibition of radical-sensing and in HIF1A degradation, and consequently, to reduced expression of the vasoactive HIF1 effector proteins like HMOX1, EDN1, SERPINE1 and NNPA (see Fig. 2). This high up-regulation of NOX5 and PKIA may be a mechanism by which Salmonella forces the IPEC-J2 cells to return to a less hypoxic condition, in order to survive in an environment loaded with reactive oxygen species (ROS). The ligand that activates the OXTR, OXT, is a peptide hormone that initiates various endocrine processes. However, in relation to the HIF1 pathway, binding of OXT to OXTR may activate secretion of natriuretic peptide A (NPPA; up-regulated 6-fold by ZnO alone) by granules. NPPA is a hormone implicated in the control of extracellular fluid volume and electrolyte homeostasis, and when cleaved, its N-terminal part is vasoactive.

ZnO alone also induced a high expression of the TP53 effector DUSP6, a protein that negatively regulates mitogen-activated protein (MAP) kinases, and with this, cellular proliferation and differentiation. In the presence of Salmonella a slightly higher expression of DUSP6 was still observed. Because MAP kinases are involved in signalling events part of a wide variety of cellular processes, a specific process inhibited by DUSP6 cannot be predicted in this study. Strong down-regulation by ZnO of NOG at 6hrs, a secreted polypeptide that inactivates members of the transforming growth factor-beta superfamily (TGF-beta; pathway called significant at 6 h for ZnO), and up-regulation of bone morphogenetic protein 4 (BMP4), may point in the direction that cell cycle control, proliferation and cell survival were also steered by a TGF-beta mechanism in IPEC-J2 cells. Moreover, limited up-regulation of SMAD6, an inhibitor of TGF-beta signalling, induced by Salmonella was maintained in case ZnO was present.

Based on the bioinformatics analysis we selected a set of key-genes/effector-molecules that responded in stressed and non-stressed IPEC-J2 cells after treatment with ZnO. Preferably, genes/proteins capable of inducing or influencing processes outside of enterocytes were selected (see section 3.4; Identification of key-genes based on processes/pathways regulated by tested additives).

3.2.4 Response to Rye

From literature it is known that higher concentrations of rye results in a higher viscosity of the digesta with an anti-nutritive activity [Choct and Annison, 1992]. Because components/nutrients in the formulated diets fed to the broilers in the *in-vivo* animal trial also contribute to the viscosity of the digesta, complete formulations containing different concentration rye (0, 5 and 10%) were tested in

IPEC-J2 cells. Furthermore, literature suggests that rye has a positive effect on the mucosal immunity in the intestines. An increase in goblet cells, more infiltration of T lymphocytes, and more immune cell aggregation was observed in the mucosa [Teirlynck et al. 2009]. All gene expression profiles of IPEC-J2 cells were compared to the medium control (mock). Note that 0% Rye is the control diet without Rye, formulated exactly in the same manner as was done in the *in-vivo* study with Rye performed in VDI-5. In addition, in analogy to the *in-vivo* study, gene expression levels between 5% and 10% Rye treatments (without Salmonella) were also compared in the IPEC-J2 test. Gene expression analysis using a cut-of FC >2 and < 0.5 revealed that the control diet (0% rye) induced a strong response in the IPEC-J2 cells at 2 hrs in the absence of Salmonella. After 6 hrs significantly less differential genes were detected in all comparisons with diet alone (0%, 5%, and 10% rye versus mock) and almost no significant biological processes/pathways were retrieved with these differential expressed genes by bioinformatics programs. This suggested that IPEC-J2 cells adapted to the components within the control diet between 2 and 6 hrs of incubation. Because at this day of testing after 2 hrs no clear Salmonella-induced cytokine response was observed yet in the absence of rye diets, the differential expressed genes extracted from data files of the mixtures of Salmonella and 5% or 10% rye diet were not used. Also no explanation was found for the relatively small number of genes differentially expressed at 2 hrs when 10% rye was incubated in the presence of Salmonella (123; see Table 1).

A clear Salmonella-induced cytokine response was observed after 6 hrs incubation in the absence and presence of the rye diets. The Salmonella-induced gene expression of dominant inflammatory cytokines/chemokines IL8, CXCL2, and IL1A at 6 hrs did not change markedly in the presence of rye. Rye slightly reduced gene expression of IL1A and CXCL2 (only at 5% Rye; also observed when comparing 10% to 5% Rye diet) and enhanced expression of CSF2 (also slightly in the absence of Salmonella) at both concentration (5 and 10%). CXCL2 suppresses hematopoietic progenitor cell proliferation, and CSF2 activates macrophages. According to the *in-vivo* intervention study with Rye (VDI-5) there is an effect on cell cycle related processes and the length and depths of the villus and crypts. Although rye does not seem to elicit a strong effect on the Salmonella-induced inflammatory cytokines, the here observed CXCL2 suppression and CSF2 stimulation suggests that it has an slight effect on the immune system in the intestine, perhaps, related to stimulating differentiation of specific subsets of immune cells. Interestingly, IFNA4 expression was reduced moderately by 5% and strongly by 10% rye diet in the presence of Salmonella. This in contrast to results obtained with ZnO, which strongly stimulated IFNA4 expression in case IPEC-J2 cells where challenged with Salmonella.

The strong response of the control diet (0% rye) at 2 hrs in the absence of Salmonella made it impossible to identify Rye-specific responses to 5% and 10% diets with a high degree of confidence. Therefore, the FC cut-off was set to >3 and <0.33 and only genes were extracted in case up- or down-regulation was 2-fold higher than for the 0% control diet. Using this stringent cut-of pathways and processes were retrieved using bioinformatics programs (Table 3). Genes involved in the processes of "Mitochondrial Electron Transport", "gene expression and protein translation", "N-Glycan biosynthesis" and "Cell Cycle/mitosis", were regulated by 5% Rye. The appearance of the Influenza Viral RNA Transcription and Replication pathway is related to activation of the transcription and translation machinery in infected cells. In contrast, at a concentration of 10% Rye "gene expression and protein translation", and "N-Glycan biosynthesis" were not called significant. In addition to "Mitochondrial Electron Transport" and "Cell Cycle/Mitotic", the "DNA Damage/Checkpoint" process was called significant specifically at a concentration of 10% Rye. These results were confirmed by comparing gene expression of the 10% Rye diet directly to that of 5% Rye (results not shown). Among genes up-regulated more strongly by 10% rye compared to 0 and 5 % was ATM, a serine/threonine protein kinase which activates cell cycle checkpoint signalling upon DNA damage, apoptosis and genotoxic stress (see also above). In case Salmonella was present, 10% rye induced transcription of a number of genes part of the TGF-beta pathway, a signalling cascade which regulates cell cycle G1/S arrest and/or cell fate. The relative high up-regulation of an inhibitor of TGF-signalling, ZFYVE16 (alias SARA or Endofin) by 10% Rye compared to a down-regulation observed for all tested mixtures of Salmonella and diets, suggested that both ATM and TGF signalling contribute to steering cell cycle progression in IPEC-J2 cells. ZFYVE16 associates with SMADs and thereby mediates expression of cyclin-dependent kinase inhibitor 2B (cyclin-dependent kinase 4 inhibitor CDKN2B; alias INK4B). In figure 4 a part of the "Cell Cycle control" pathway with down-stream effector proteins INK4B and GADD45 of SMAD's and ATM, respectively, (INK4B and GADD45 were also up-regulated in IPEC-J2 cells by a mixture of rye diet and Salmonella) were highlighted.

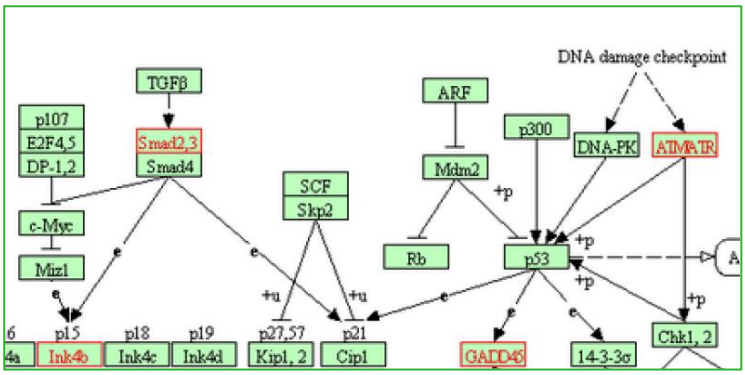

Figure 4 *Part of "Cell Cycle" pathway.*

A total of 9 genes were higher expressed by both concentrations rye (5% and 10%) with a FC>2 and not by the control diet. Three of them, TPM1, TWF1 and UTRN (utrophin) bind to the cytoskeleton protein actin, and all three have a function in calcium-dependent regulation of muscle contraction and motility. Both TPM1 (tropomyosin 1) and TWF1 (twinfilin; a Protein Tyrosine Kinase) can inhibit polymerization of actin stress fibres.

Based on this gene expression analysis the only common process identified for 5% and 10% rye diets was uncoupling of heat production and ATP synthesis. However, we could not find a logical correlation between the changes in this process and a rise in viscosity from 5 to 10% diet.

Based on the bioinformatics analysis we selected a set of key-genes/effector-molecules that responded in stressed and non-stressed IPEC-J2 cells after treatment with Rye. Preferably, genes/proteins capable of inducing or influencing processes outside of enterocytes were selected (see section 3.4; Identification of key-genes based on processes/pathways regulated by tested additives).

Table 3

Response to Rye diets, pathway analysis and enriched GO-BP terms.

hrs	treatment	Score [a]	Pathway or GO-BP term [b]	# total genes	# genes	genes
2	5%	119.0	Influenza Viral RNA Transcription and Replication	293	36	EEF1A1, EEF1G, RAN, 33 RPL and RPS variants
2	5%	87.5	Biosynthesis of The N-glycan Precursor (dolichol Lipid-linked Oligosaccharide, LLO) and Transfer to A Nascent Protein	696	39	ACTB, EEF1A1, EEF1G, MUC13, STS, TUBA1B, 34 RPL and RPS variants
2	5%	59.5	Gene Expression	1210	39	EEF1A1, EEF1G, IGF2BP3, NCOA1, RAN, ZNF750, 33 RPL and RPS variants
2	5%	13.1	Cytoskeletal Signaling	242	8	ACTB, GNB2L1, KRT18, KRT7, RAN, TPM1, TPT1, TUBA1B
2	5%	11.8	Cell Cycle_Spindle Assembly and Chromosome Separation	95	5	ACTB, CSE1L, NEK2, RAN, TUBA1B
2	5%	8.7	Respiratory Electron Transport, ATP Synthesis By Chemiosmotic Coupling, and Heat Production By Uncoupling Proteins.	389	8	ATP5G3, MT-ATP6, MT-CO1, MT-CO2, MT-CO3, SLC18A2, TGM2, UBE2J1
2	5%	119.9	BP-Translation	288	36	EEF1A1, EEF1G, IGF2BP3, 34 RPL and RPS variants
2	5%	100.0	BP-Cellular Protein Metabolic Process	549	39	ACTB, EEF1A1, EEF1G, MUC13, STS, TUBA1B, 33 RPL and RPS variants,
2	5%	77.1	BP-Gene Expression	741	37	EEF1A1, EEF1G, IGF2BP3, RAN, 34 RPL and RPS variants
2	10%	8.3	Cell Cycle, Mitotic	538	4	ATM, CENPF, NIPBL, SYNE2
2	10%	6.7	Respiratory Electron Transport, ATP Synthesis By Chemiosmotic Coupling, and Heat Production By Uncoupling Proteins.	389	3	MT-ND6, NR1H3, SDHA/SDHB
2	10%	9.4	BP-Cellular Response to DNA Damage Stimulus	199	3	ATM, NIPBL, RIF1

[a] GeneAnalytics pathways and enriched GO-BP terms with a high score (>18; corresponding with an FDR corrected p-value ≤ 0.0001) and medium score (>9; corresponding with an FDR corrected p-value ≤ 0.05) were retrieved. For 10% rye pathways or enriched GO-BP terms with a score >6 were only retrieved in case 3 or more differentially expressed genes matched.

total genes; total number of genes in a pathway or GO-term. # genes; number of genes differential expressed in IPEC-J2 cells mapped to a pathways or GO-term.

[b] Pathway annotations are from GeneAnalytics (http://geneanalytics.genecards.org).

3.2.5 Response to Antibiotics

Two different commercial preparations of Amoxicillin were tested in *in-vivo* animal trials performed in VDI-3 (Octacillin; neonatal) and VDI-2 (Paracillin; maternal). The matrix in which the amoxicillin was formulated and the concentrations tested in the IPEC-J2 cells differed between these preparations (see materials and methods). Although many common processes were identified, we also observed responses in IPEC-J2 cells specific for one of the amoxicillin preparations. For Octacillin results will be presented and discussed in detail and for Paracillin only remarkable differences compared to Octacillin are briefly presented and discussed.

3.2.5.1 Response to Octacillin

Octacillin is an antibiotic frequently used in the poultry husbandry to combat bacterial infections. Therefore, it is expected that Octacillin would reduce a Salmonella-induced inflammatory response, as Octacillin kills Salmonella bacteria. This is in accordance with the results we observed when Salmonella gene expression was measured in the presence of Octacillin. Except for CSF2, Octacillin reduced or normalized up-regulation of Salmonella-induced cytokine/chemokine gene expression in IPEC-J2 cells at 2 hrs, and even down-regulated IL1A and CXCL2 expression at 6 hrs. Also Octacillin alone slightly induced expression of these 2 latter inflammatory proteins at 2 hrs. Similar as observed for ZnO alone, IL1RN (IL1 receptor antagonist) expression at 6 hrs was also strongly down-regulated by Octacillin alone at 6 hrs. However in contrast to ZnO, a mixture of Salmonella and Octacillin did not stimulate IL1B and IFNA4 expression.

Table 4a

Response to Octacillin at 2 hrs, pathway analysis

treatment	Score [a]	Pathway term[b]	# total genes	# genes	genes
Oct	9.6	Transport of Glucose and Other Sugars, Bile Salts and Organic Acids, Metal Ions and Amine Compounds	543	8	HMOX1, SGK1, SLC38A1, SLC38A2, SLC3A2, SLC4A7, SLC5A3, SLC7A1
Salm	16.0	Translation Insulin Regulation of Translation	287	6	DDIT4, EGR1, FOS, PLAU, PPP1R3C, SERPINE1
Oct-Salm	11.7	Translation Insulin Regulation of Translation	287	9	DDIT3, DDIT4, EGR1, EIF4A2, FOS, JUN, PLAU, PPP1R3C, SOCS3
Salm	14.3	Toll Comparative Pathway	106	4	FOS, IL1A, IL8, NFKBIA
Oct-Salm	16.0	Toll Comparative Pathway	106	7	FOS, FOSB, IL1A, IL6, IL8, IRF1, JUN
Oct	10.2	Toll Comparative Pathway	106	4	FOSB, IL1A, IL6, IL8
Salm	29.3	TNF Signaling Pathway	110	7	CFLAR, CXCL2, EDN1, FOS, MAP3K8, NFKBIA, TNFAIP3
Oct-Salm	26.3	TNF Signaling Pathway	110	10	CSF2, CXCL2, EDN1, FOS, IL6, JUN, MAP3K8, SOCS3, TNFAIP3, TNFRSF1B
Salm	21.2	TGF-Beta Pathway	686	10	EPHA2, FOS, GDF15, IL1A, IL8, MAP3K8, MYC, NFKBIA, NGF, TCF21
Oct-Salm	18.1	TGF-Beta Pathway	686	18	DUSP6, EIF4A2, EPHA2, FOS, GDF15, IL1A, IL6, IL8, JUN, KLF10, MAP3K8, MYC, NGF, PPBP, RBX1, SOCS3, TNFRSF1B, UBB
Salm	12.6	TGF Beta Signaling Pathway	55	3	FOS, INHBA, SERPINE1
Oct-Salm	14.8	Signaling By Interleukins	120	7	CSF2, IL1A, IL6, MAP3K8, RBX1, SOCS3, UBB
Salm	18.7	Salmonella Infection (KEGG)	113	5	CXCL2, FOS, IL1A, IL8, NFKBIA
Oct-Salm	15.4	Salmonella Infection (KEGG)	113	7	CSF2, CXCL2, FOS, IL1A, IL6, IL8, JUN
Oct	9.9	Salmonella Infection (KEGG)	113	4	CXCL2, IL1A, IL6, IL8
Salm	13.2	P70S6K Signaling	408	6	DDIT4, EPHA2, FOS, GDF15, NGF, PPP1R3C
Oct-Salm	16.4	P70S6K Signaling	408	13	DDIT4, EIF4A2, EPHA2, FOS, GDF15, HBEGF, IGF2R, IL6, JUN, KLF10, NGF, PPBP, PPP1R3C
Salm	19.2	NOD-like Receptor Signaling Pathways	195	6	EDN1, FOS, IL1A, IL8, NFKBIA, SERPINE1
Oct-Salm	21.8	NOD-like Receptor Signaling Pathways	195	11	BCL10, EDN1, FOS, FOSB, HMOX1, IL1A, IL6, IL8, IRF1, JUN, UBB
Oct	9.9	NOD-like Receptor Signaling Pathways	195	5	FOSB, HMOX1, IL1A, IL6, IL8
Salm	13.3	NF-KappaB Family Pathway	249	5	GDF15, IL1A, NFKBIA, NGF, TNFAIP3
Oct-Salm	15.7	NF-KappaB Family Pathway	249	10	BCL10, GDF15, IL1A, IL6, KLF10, NGF, PPBP, TNFAIP3, TNFRSF1B, UBB
Oct-Salm	13.6	Interleukin-1 Signaling	58	5	IL1A, JUN, MAP3K8, RBX1, UBB
Oct-Salm	11.2	Interferon Signaling	367	10	CSF2, EGR1, EIF4A2, IL1A, IL6, IRF1, MAP3K8, RBX1, SOCS3, UBB
Oct-Salm	21.2	IL-9 Signaling Pathways	161	10	FOS, FOSB, IL6, IRF1, JUN, KAT5, MCL1, MYC, SOCS3, TNFRSF1B
Oct-Salm	34.2	IL6-mediated Signaling Events	43	9	CEBPD, FOS, IL6, IRF1, JUN, LMO4, MCL1, MYC, SOCS3
Salm	11.9	HIF-1-alpha Transcription Factor Network	65	3	EDN1, FOS, SERPINE1
Oct-Salm	16.6	HIF-1-alpha Transcription Factor Network	65	6	EDN1, ETS1, FOS, HMOX1, JUN, MCL1
Oct	13.6	Glucose / Energy Metabolism	182	6	ACSL1, ACSS2, IGF2R, NUAK2, SGK1, SLC3A2
Salm	11.0	Glucocorticoid Receptor Regulatory Network	82	3	EGR1, FOS, IL8
Oct-Salm	22.1	Glucocorticoid Receptor Regulatory Network	82	8	CSF2, EGR1, FOS, IL6, IL8, IRF1, JUN, SGK1
Salm	13.0	Direct P53 Effectors	134	4	DDIT4, EPHA2, GDF15, SERPINE1
Oct-Salm	20.2	Direct P53 Effectors	134	9	BCL6, BTG2, DDIT4, EPHA2, GDF15, JUN, MCL1, PLK3, PRDM1
Oct-Salm	11.6	Cell Cycle / Checkpoint Control	229	8	DDIT4, FOS, FOSB, KAT5, NBN, PLK3, TFAP2C, TP53BP1
Salm	16.2	Apoptosis and Autophagy	163	5	CFLAR, GDF15, MYC, TFAP2C, TNFAIP3
Oct-Salm	17.9	Apoptosis and Autophagy	163	9	BCL10, GDF15, MCL1, MXD1, MYC, NUAK2, TFAP2C, TNFAIP3, TNFRSF1B

[a] GeneAnalytics pathways with a high score (>18; corresponding with an FDR corrected p-value ≤ 0.0001) and medium score (>9; corresponding with an FDR corrected p-value ≤ 0.05) were retrieved.

total genes; total number of genes in a pathway. # genes; number of genes differential expressed in IPEC-J2 cells mapped to a pathway.

[b] Pathway annotations are from GeneAnalytics (http://geneanalytics.genecards.org).

Table 4b.

Response to Octacillin at 6 hrs, pathway analysis

hrs	treatment	Score [a]	Pathway term[b]	# total genes	# genes	genes
6	Salm	16.2	Apoptosis and Autophagy	163	5	CFLAR, GDF15, MYC, TFAP2C, TNFAIP3
6	Oct-Salm	13.9	Apoptosis and Autophagy	163	16	BCL2L11, BMF, BNIP3L, CASP10, GZMA, HIPK2, MALT1, MCL1, NDRG1, NUAK2, RIPK1, TFAP2C, TNFAIP3, TRAF6, YWHAG, YWHAH
6	Oct-Salm	15.3	ATM Pathway	48	9	CDC25A, CDK2, CREB1, CREB3, GADD45G, NBN, NFKBIA, RAD50, TP53BP1
6	Oct-Salm	19.5	Cell Cycle / Checkpoint Control	229	23	BRD2, CCND1, CDC25A, CDK2, CDKN1B, DDIT4, DYRK2, EAPP, FEN1, FOSB, HIPK2, KAT5, NBN, NEK7, PLK3, RAD50, RBBP8, SMG1, TFAP2C, TP53BP1, YWHAG, YWHAH, ZBTB17
6	Oct	11.9	Cell Cycle / Checkpoint Control	229	18	BRD2, CCND1, CDC25A, CDKN1B, CSNK1A1, DDIT4, FOSB, KAT5, NBN, NEK7, PLK3, RBBP8, RCC2, SMG1, TFAP2C, TP53BP1, YWHAG, ZBTB17
6	Oct-Salm	10.4	Class I PI3K Signaling Events Mediated By Akt	34	6	CDKN1B, GSK3B, HSP90AA1, SRC, YWHAG, YWHAH
6	Oct	10.6	Class I PI3K Signaling Events Mediated By Akt	34	6	CDKN1B, HSP90AA1, MAP3K5, RICTOR, SRC, YWHAG
6	Salm	13.0	Direct P53 Effectors	134	4	DDIT4, EPHA2, GDF15, SERPINE1
6	Oct-Salm	11.5	Direct P53 Effectors	134	13	BCL6, BNIP3L, BTG2, CASP10, DDIT4, DKK1, DUSP5, MCL1, NDRG1, PLK3, PMAIP1, RNF144B, SPP1
6	Oct	13.7	Direct P53 Effectors	134	14	BCL6, BNIP3L, BTG2, CASP10, DDIT4, DKK1, DUSP5, MCL1, NDRG1, PLK3, PMAIP1, PRDM1, RNF144B, SPP1
6	Salm	11.0	Glucocorticoid Receptor Regulatory Network	82	3	EGR1, FOS, IL8
6	Oct-Salm	11.7	Glucocorticoid Receptor Regulatory Network	82	10	CDK5R1, CREB1, EGR1, GSK3B, HSP90AA1, IL6, IRF1, SGK1, STAT1, YWHAH
6	Oct-Salm	10.5	Glucose / Energy Metabolism	182	15	CEBPD, GSK3B, HK2, IDH2, MAP4K3, MARK1, NUAK2, PASK, PDK1, PGAM1, PTPN1, SDHA, SGK1, SLC3A2, TXNRD1
6	Salm	11.9	HIF-1-alpha Transcription Factor Network	65	3	EDN1, FOS, SERPINE1
6	Oct-Salm	9.8	HIF-1-alpha Transcription Factor Network	65	8	CITED2, CREB1, EGLN1, EGLN3, HK2, HMOX1, MCL1, NDRG1
6	Oct	12.3	HIF-1-alpha Transcription Factor Network	65	9	CA9, CITED2, EDN1, EGLN1, EGLN3, HK2, HMOX1, MCL1, NDRG1
6	Oct-Salm	13.8	IL6-mediated Signaling Events	43	8	CEBPD, HSP90B1, IL6, IRF1, LMO4, MCL1, SOCS3, STAT1
6	Oct	11.4	IL6-mediated Signaling Events	43	7	CEBPD, IL6, IRF1, LMO4, MCL1, SOCS3, STAT1
6	Oct-Salm	24.0	IL-9 Signaling Pathways	161	21	BCL2L11, CCND1, CDK2, CDKN1B, CISH, CREB1, FOSB, GSK3B, GZMA, IL6, IRF1, KAT5, MCL1, NFKBIA, PIK3CG, PTK2B, SERPINA12, SHC1, SOCS3, SOS2, STAT1
6	Oct	16.4	IL-9 Signaling Pathways	161	17	CCND1, CDKN1B, CISH, FOSB, GZMA, IL6, IRF1, KAT5, MCL1, NAMPT, NFKBIA, PIK3CG, PTK2B, SERPINA12, SOCS3, SOS2, STAT1
6	Oct-Salm	13.9	Insulin Signaling	163	16	EGR1, FLOT1, GRB10, GSK3B, MAP4K3, MAPK6, PIK3CG, PTPN1, RRAD, SGK1, SHC1, SOCS3, SOS2, SRF, TSC1, XBP1
6	Oct	10.0	Jak/Stat Pathway	37	6	CCND1, CDKN1B, CISH, IL1RN, SOCS3, STAT1
6	Salm	19.2	NOD-like Receptor Signaling Pathways	195	6	EDN1, FOS, IL1A, IL8, NFKBIA, SERPINE1
6	Oct-Salm	12.6	NOD-like Receptor Signaling Pathways	195	17	FOSB, HMOX1, IL18, IL1A, IL1RN, IL6, IRF1, MALT1, NFKBIA, RIPK1, SHC1, SOS2, SRC, STAT1, TICAM1, TLR6, TRAF6
6	Salm	29.3	TNF Signaling Pathway	110	7	CFLAR, CXCL2, EDN1, FOS, MAP3K8, NFKBIA, TNFAIP3
6	Oct-Salm	12.2	TNF Signaling Pathway	110	12	CASP10, CREB1, CREB3, CXCL2, IL6, JAG1, MMP9, NFKBIA, PIK3CG, RIPK1, SOCS3, TNFAIP3
6	Oct	14.6	TNF Signaling Pathway	110	13	CASP10, CCL20, CREB3, CXCL2, EDN1, IL6, MAP3K5, MAP3K8, MMP9, NFKBIA, PIK3CG, RIPK1, SOCS3
6	Salm	21.6	Toll-like Receptor Signaling Pathway	242	7	CFLAR, FOS, IL8, MAP3K8, MYC, NFKBIA, SERPINE1
6	Oct-Salm	14.9	Toll-like Receptor Signaling Pathway	242	21	CASP10, CCND1, CDK2, CDKN1B, CREB1, CREB3, CTSK, E2F3, IL6, MMP9, NFKBIA, PIK3CG, PPP2R2C, PTK2B, RIPK1, SPP1, SRC, STAT1, TICAM1, TLR6, TRAF6
6	Salm	16.0	Translation Insulin Regulation of Translation	287	6	DDIT4, EGR1, FOS, PLAU, PPP1R3C, SERPINE1
6	Oct-Salm	14.4	Translation Insulin Regulation of Translation	287	23	DDIT3, DDIT4, DYRK2, EGR1, EIF2S1, EIF4A2, ELK4, FLOT1, GSK3B, HK2, PIK3CG, PPP1R3B, PPP1R3C, PTK2B, PTPN1, RNF41, SHC1, SOCS3, SOCS4, SOS2, SRC, STAT1, TSC1
6	Oct-Salm	10.7	Translational Control	161	14	AGO3, DDIT3, DDX6, EDC3, EGLN1, EIF2S1, EIF4A2, ERO1L, HIPK2, HSP90B1, HSPA5, PTBP1, TSC1, XBP1
6	Salm	22.5	Validated targets of C-MYC transcriptional repression	62	8	NDRG2, NDRG1, S100A7, DNMT3A, DKK1, CLU, ZBTB17, DDIT3
6	Oct-Salm	17.6	Validated Targets of C-MYC Transcriptional Repression	62	12	CFLAR, CCND1, CDKN1B, CEBPD, CLU, CREB1, DDIT3, DKK1, NDRG1, RBL1, S100A7, ZBTB17
6	Oct	17.9	Validated Targets of C-MYC Transcriptional Repression	62	12	CFLAR, CCND1, CDKN1B, CEBPD, CLU, DDIT3, DKK1, NDRG1, NDRG2, RBL1, S100A7, ZBTB17

[a] GeneAnalytics pathways with a high score (>18; corresponding with an FDR corrected p-value ≤ 0.0001) and medium score (>9; corresponding with an FDR corrected p-value ≤ 0.05) were retrieved.

total genes; total number of genes in a pathway. # genes; number of genes differential expressed in IPEC-J2 cells mapped to a pathway.

[b] Pathway annotations are from GeneAnalytics (http://geneanalytics.genecards.org).

Compared to Salmonella alone, specific sets of genes were up-regulated in case a mixture of Octacillin and Salmonella were incubated together. For example, the pathways "Direct P53 Effectors" and "Apoptosis and Autophagy" were called significant at 2 and 6 hrs (see Table 4). This indicated that Octacillin not only negatively affected the vitality of Salmonella but also stimulated IPEC-J2 cells to kill and breakdown Salmonella bacteria by phagocytosis and/or to go into apoptosis. In contrast to ZnO, the TP53 effector PRDM1, which acts a repressor of beta-interferon gene expression, was not down-

regulated by Octacillin alone and by a mixture. In agreement with this, the "interferon signalling pathway" was called significant, in which RBX1 was down-regulated. RBX1 is an E3 ubiquitin ligase which specifically targets HIF1A for ubiquitin mediated proteolysis. Together with NBN (a DNA damage sensor), RBX1 are also important for cell cycle arrest (G1/S and S/G2) regulation by the ATM/ATR-TP53 checkpoint mechanism. Likely, IPEC-J2 cells reacted completely different to extracellular and/or intracellular stress signals (e.g. invaded Salmonella bacteria and Salmonella-effector proteins) in case both Salmonella and Octacillin were presence. Salmonella growth and vitality was, most likely, repressed by Octacillin, which may leads to failure of these bacteria to invade cells or inject their effector proteins into cells. Also Octacillin may induce ROS [Rizzo, et al. 2009] in IPEC-J2 cells (see below), which may also affect the vitality of Salmonella. Interestingly, the kinase HIPK2 was specifically up-regulated in case a mixture of Salmonella and Octacillin was incubated for 6 hrs. HIPK2 is a transcriptional co-suppressor of HIF1A and is unstable in unstressed cells and stabilizes in case DNA damage is sensed. By suppressing HIF1A transcription HIPK2 indirectly represses a hypoxia response in cells. Recent research showed that this repression can be reversed by zinc supplementation [Puca et. al. 2008]. Because we observed a moderate up-regulation of genes coding for the transmembrane Zinc transporter SLC39A1 and the intracellular zinc transporter MT1A (see above) when Octacillin alone was applied for 2 hrs, a response of IPEC-J2 cells to hypoxia/ROS, and perhaps also to other stress-signals like DNA damage, might be balanced by raising intracellular Zn levels. This also indicates that MT1A is a functional component of the cellular defence response to oxidative stress in IPEC-J2 cells. Furthermore, our pathway analysis revealed that the IL9-signaling was regulated in the presence of Octacillin at 6 hrs (Table 4). Besides IL9 signalling promotes immunoglobulin production in B-cell cells it also promotes goblet cell hyperplasia and mucus production in the epithelial layer of the lung (IL9 signalling http://www.rndsystems.com/Pathway) However, based on up-and down-regulation of specific genes within this process, no clear conclusion can be drawn about which of these processes is steered by this signalling cascade.

When Octacillin alone was incubated we observed a higher expression in IPEC-J2 cells of genes from which it is known that they respond to oxidative stress and ROS. Genes like "oxidative stress responsive serine-Rich protein 1 (OSER1)", "oxidative stress induced growth inhibitor 1 (OSGIN1)" and HMOX1 were regulated. Together with a transcriptional activator of c-FOS, FOSB itself, MAFB (a transcription factors forming heterodimers with FOSB), adenosine monophosphate deaminase 1 (AMPD1), HSP70, and GADD45G (growth arrest and DNA-damage-inducible, gamma), HMOX1 was extremely high up-regulated at 6 hrs. For most of them no significant regulation was observed when Salmonella was incubated alone. Moreover, HMOX1 was slightly down-regulated and FOSB was moderately up-regulated at 2 hrs. Octacillin alone also slightly stimulated expression of genes involved in transport of metabolites and nutrients at 2 hrs, and at 6 hrs many heat shock proteins were up-regulated. With respect to the HIF1 pathway "egline homolog" 1 and 3 (EGLN; alias egl-9 family hypoxia-inducible factor) were down-regulated. EGLN proteins function as a cellular oxygen sensor and modify under normal oxygen conditions proline residues of HIF1 to 4-hydroxyprolines, resulting in targeting of HIF1 for degradation in proteasomes. This indicates that HIF1 levels were maintained until 6 hrs and with this probably the HIF-mediated transcription of effector genes like HMOX1. Products generated by the enzyme HMOX1 possess antithrombotic properties and it is well accepted that a high HMOX1 level lowers blood pressure by vasodilation [Hosick and Stec, 2012, Davis et. al. 2013]. In addition, endothelin 1 (EDN1), a potent vasoconstrictor, and also an effector gene transcribed by HIF1A machinery, was down-regulated. This indicates that both these "HIF1-transcribed" effector proteins may cooperate to widen blood vessels, probably to supply the intestinal mucosa with sufficient oxygen and glucose to compensate for induced oxidative stress and to return to normoxia. Also, in agreement with this was the down-regulation at 6 hrs of the sugar metabolising enzymes HK2, PDK1, which both promote anaerobic metabolism, and at 2 hrs, the uptake of nutrients by membrane transporters and down-regulation of many enzymes involved in oxidative phosphorylation (energy production). In Fig. 2 (page 17) all genes regulated by Octacillin within the HIF1-signaling pathway are shown along with the genes regulated by ZnO. In Fig. 4a and b interactions between genes/proteins here proposed to be involved in sensing of DNA damage and oxidative stress processes are displayed. Genes responding to oxygen shortage in cells and genes involved in sensing of, and responding to, DNA-damage are highlighted red in Fig. 4a and b. Based on this network we identified key-genes that play a central role in the control of these cellular stress responses (see Table 7 paragraph 3.4). Some of these genes are similar to the key-genes we assigned for ZnO intervention.

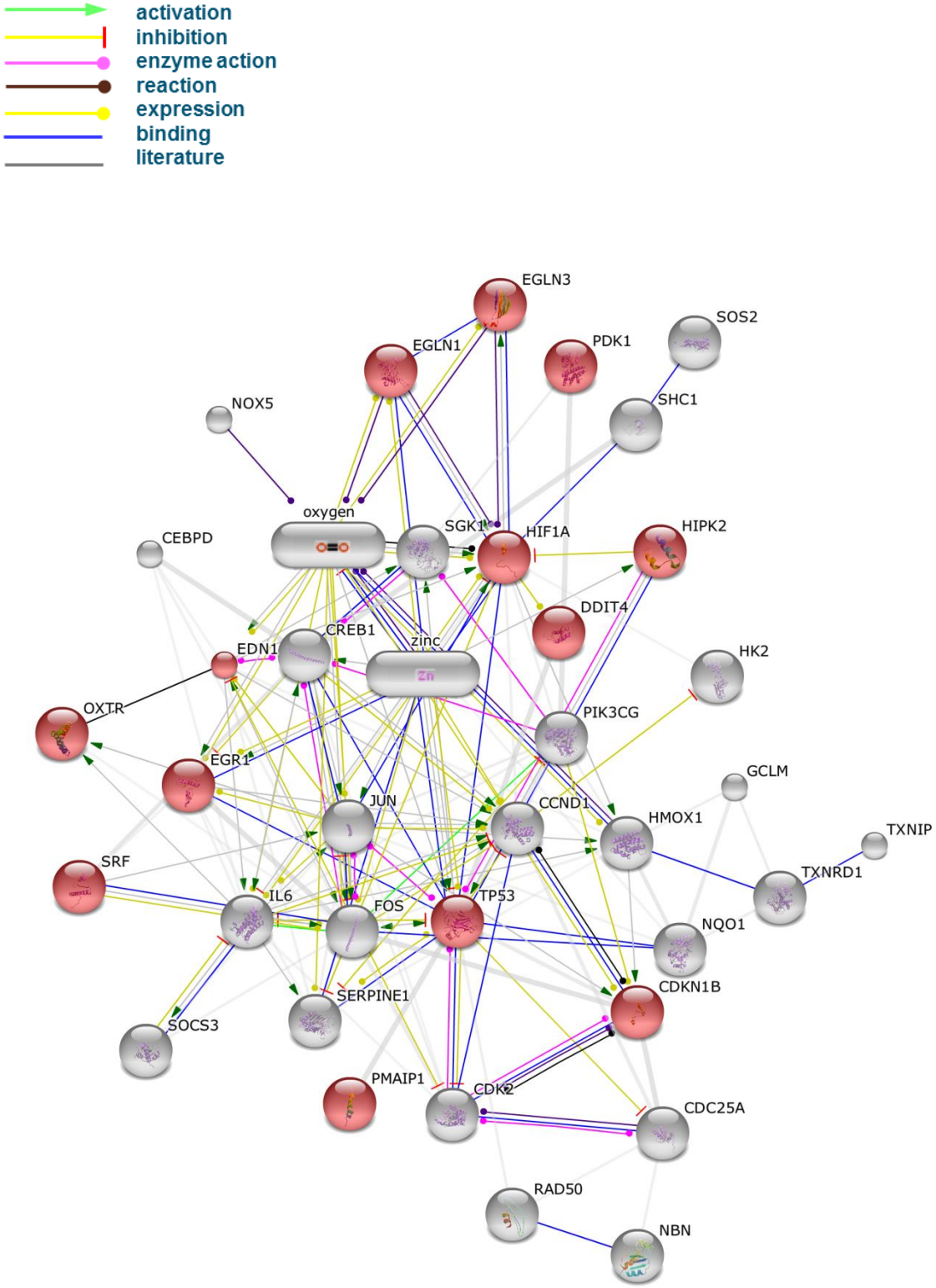

Figure 4a *STITCH network of associations between genes responding to Octacillin in the presence and absence of Salmonella. Chemicals and TP53 (tumour protein p53) were added to the network. Genes responding to oxygen shortage in cells are highlighted in red.*

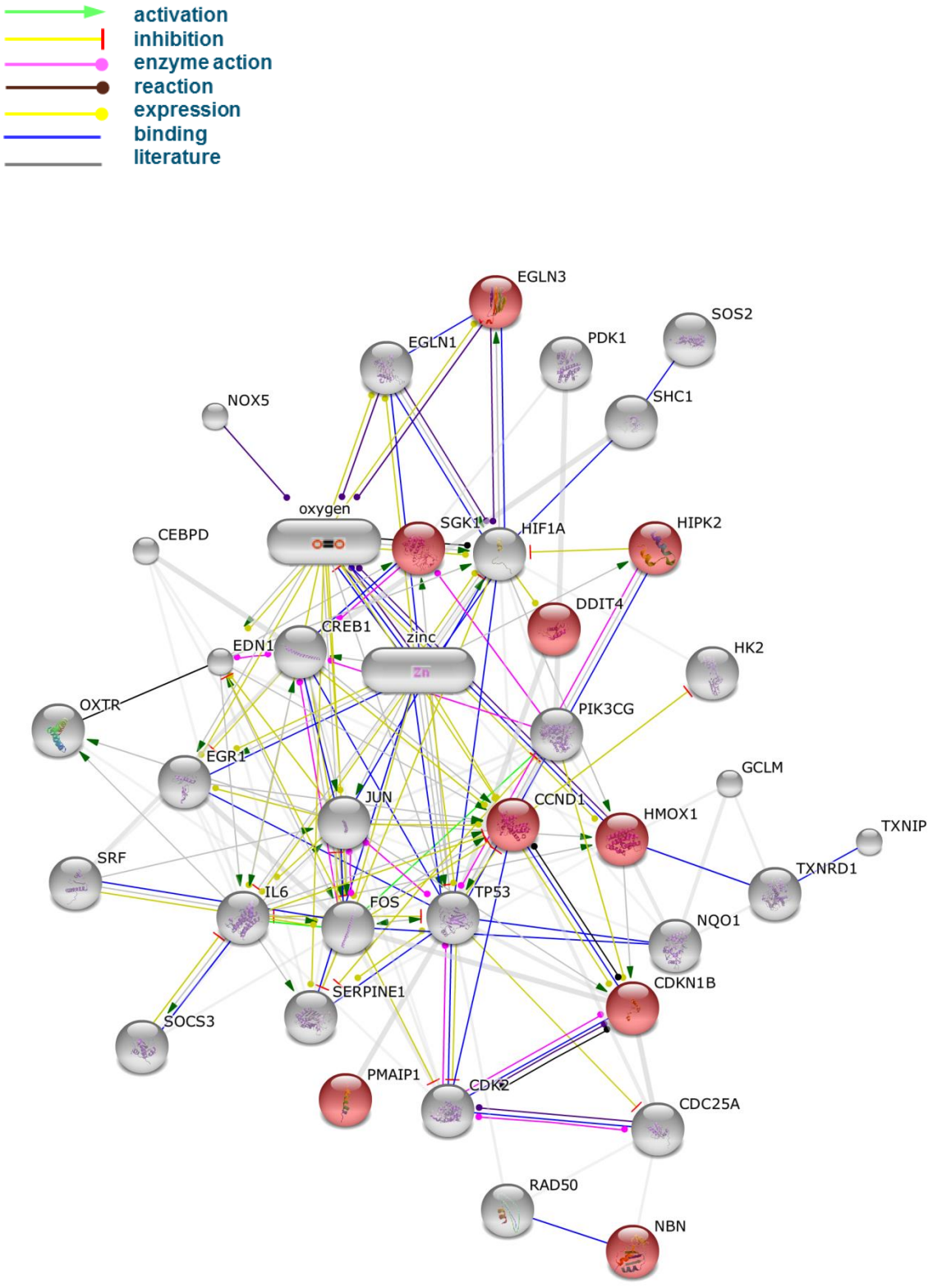

Figure 4b *STITCH network of associations between genes responding to Octacillin in the presence and absence of Salmonella. Chemicals and TP53 (tumour protein p53) were added to the network. Genes sensing and responding to DNA-damage are shown in red.*

Interestingly, the polymeric immunoglobulin receptor (PIGR) and the adhesion molecule PECAM1 were found up-regulated at 6 hrs. PIGR facilitates IgA and IgM transport over the epithelial cell layer,

thereby releasing these antibodies into the lumen where they can bind to "hostile" components. PECAM1 binds to the surface of leukocytes and discloses tight junctions in the cavity between cells, thereby supporting migration of leucocytes over an (epithelial) cell layer. Up-regulation of these latter two genes in the absence of Salmonella is remarkable, because it suggests that Octacillin not only affects pathogenic bacteria but also stimulates the intestinal innate immune response to attack enteric pathogens. However, intensity levels measured for these 2 genes on the array were just above the background suggesting that expression levels of these proteins on the surface of IPEC-J2 is low.

3.2.5.2 Response to Paracillin

In accordance with the results of Octacillin, Paracillin also reduced or normalized up-regulation of Salmonella-induced cytokine/chemokine gene expression of IL8, IL1A, and CXCL2 at 2 hrs. In contrast to Octacillin, Paracillin also reduced a CSF2 response induced by Salmonella to almost a normal level. One of the most striking differences between Paracillin and Octacillin was the much higher down-regulation (almost 100-fold) at 6 hrs of DNA-damage-inducible transcript 4 (DDIT4; alias HIF1 Responsive Protein and component of mTOR complex) by Paracillin alone (4-fold for Octacillin). Such a strong down-regulation of DDIT4 was also observed for ZnO alone at 6 hrs. In relation to this, Octacillin down-regulated expression of the gene TXNIP, an inhibitor of proteasomal degradation of DDIT4, earlier (at 2 hrs) and 2-fold more than Paracillin did. Less TXNIP could result in more DDIT4 protein, and consequently, in more activity of the mTOR complex that activates HIF1 by phosphorylation (see Fig. 2 page 17; encircled in black). DDIT4 gene expression was up-regulated by Salmonella at 2 hrs but decreased to a normal level at 6 hrs. Moreover, in all 6 hrs incubations of a mixture of Salmonella and antibiotics (or ZnO) expression levels of this gene were only slightly changed. DDIT4 regulates TP53-mediated apoptosis in response to DNA damage, also in case damage to cells is induced by oxidative stress.

In agreement with the results of Octacillin several genes belonging to the HIF1 signalling pathway were also regulated by Paracillin alone. Moreover, a to Octacillin related set of HIF1-transcribed effector genes was differentially expressed after 2 and 6 hrs incubation with Paracillin (e.g. HMOX1, EDN1, EGLN1-3). Pathway analysis also confirmed regulation of effector genes of the TP53 signalling pathway and many apoptosis related genes. However, responses of all these effector molecules differed in time and in quantity of regulation between Octacillin and Paracillin, especially when mixtures of these amoxicillin preparations were incubated with Salmonella. This different regulation of the HIF1 signalling may be due to a difference in potency of both preparations to affect vitality of Salmonella bacteria, and to differences in the formulation/composition of Octacillin and Paracillin powders.

Based on the bioinformatics analysis we selected a set of key-genes/effector-molecules that responded in stressed and non-stressed IPEC-J2 cells after treatment with Amoxicillin. Preferably, genes/proteins capable of inducing or influencing processes outside of enterocytes were selected (see section 3.4; Identification of key-genes based on processes/pathways regulated by tested additives).

3.2.6 Response to short and long chain fructo-oligosaccharides (FOS)

In literature it is described that human galacto-oligosaccharide (GOS) and related products such as fructo-oligosaccharide (FOS) will lead to changes in the composition, diversity and activity of the micro-flora in different parts of the gastrointestinal tract [Verdonk et. al. 2005, Biedrzycka et. al. 2004, Meyer and Stasse-Wolthuis 2009]. The difference between GOS and FOS is that GOS is based on milk oligosaccharides and FOS is made from plant products. Studies have shown that inulin-type fructans (FOS) have positive effects on the microbiota, gut integrity, and on animal production performance (weight gain and feed efficiency). In the *in-vivo* experiments (VDI-3; 'Neonatal models') piglets received a mixture of two commercial short (O-ScFos) and long chain FOS (T-LsFos) preparation in a ratio of 9:1, respectively. In IPEC-J2 cells these long and short preparations were tested separately.

Salmonella-induced cytokine/chemokine expression of IL8, IL1A and CXCL2 peaked at 6 hrs in the absence and presence of FOS preparations. No higher expression level of CSF2 at 6 hrs was observed. Short and long chain FOS did not affect Salmonella-induced cytokine/chemokine expression seriously. Only IL8 expression was reduced 2-fold by short chain FOS. Moreover, the number of Salmonella-regulated genes for which expression was affected significantly by FOS was limited to a dozen of genes. Salmonella-induced expression of the gene ryanodine receptor 2 (RYR2; receptor involved in release/transport of Ca2+ from the endoplasmic reticulum [ER] to the cytosol) and ERO1L (see below) were reduced by both short and long chain FOS. Among genes reduced by long chain FOS, there were several genes associated with intracellular membrane-bounded organelles/vesicles and/or are involved in release of sequestered calcium ions into the cytosol (like RYR2). In addition, the genes IRF2BP2 (IRF2 binding protein 2) and ZFP36L1 were reduced almost 2-fold by long chain FOS. This latter gene, also named butyrate Response Factor 1 (BRF1), is an early response gene that binds to AU-rich elements in the 3' region of mRNAs and recruits exosomes and enzymes to degrade mRNA. Interestingly, 3' regions of cytokine mRNA's contain relatively more AU rich elements. These elements make these mRNA's more favourable substrates for RNases, and consequently, shorten their half-life [Maclean et. al. 1998, Savan, 2014]. In addition, expression of the gene P4HA1, a component of the propyl 4-hydroxylase (PH4) enzyme complex was reduced by long chain FOS. P4H41 catalyses protein disulphide formation in the ER, and the ER oxidoreductase ERO1L (see above) re-oxidizes this PH4 complex to sustain disulphide formation activity. Following P4H re-oxidation, ERO1L produces electrons that are passed to oxygen, resulting in the production of reactive oxygen species (ROS) in the cell. An association between the butyrate response factor ZFP36L1 and the P4HA1/ERO1L complex could be exosomes associated with the ribosomes located at the ER-membrane. ZFP36L1 interacts with core components of the exosome (EXOSC's) which is specialized in degradation of RNA (see Figure 5). Cellular compartment GO-term annotation (CC) for TPM4 and RYR2 revealed that these proteins may be part of "extracellular vesicular exosomes". However, there are many kind of different exosome (-like) vesicles produced by cells, all with a specific function.

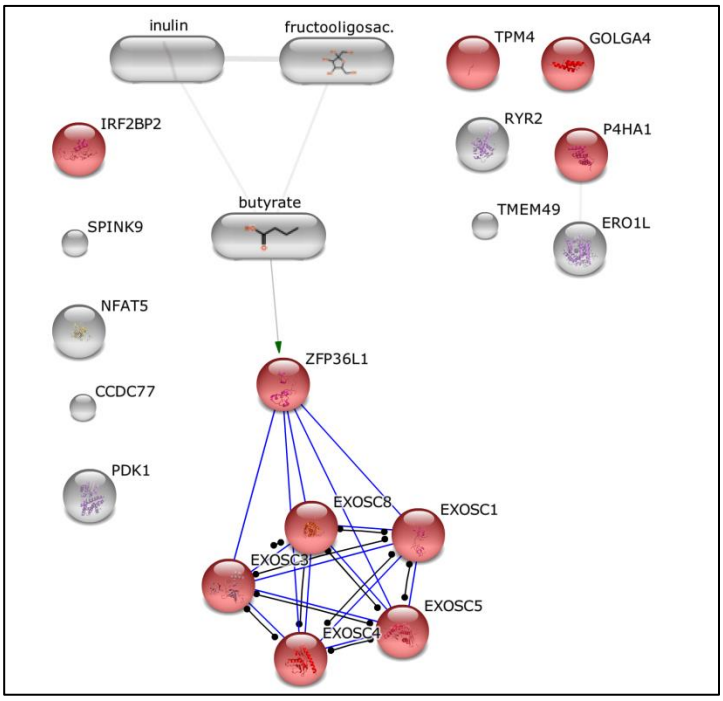

Figure 5 *STITCH chemical-protein interaction network of Salmonella-induced genes reduced in expression by Lc-FOS. Genes/proteins associated with membrane bound organelles are shown in red. All chemicals and EXOSC genes were added to the network*.

Only a few genes were weakly up-regulated by long and short chain FOS preparations alone, and only at 6 hrs. This suggests that the response in IPEC-J2 cells incubated with a mixture of Salmonella and FOS was, most likely, due to fermentation of FOS by Salmonella. These changes in Salmonella energy-metabolism may lead to shedding of a different profile of "waste metabolites" and effector proteins by *Salmonella* into the culture medium, to which IPEC-J2 cells may have responded. It has been described that rapid fermentation of FOS by bacteria increases production and shedding of short chain fatty acids (SCFA) like butyrate [Tsukahara et. al. 2008]. Elevated butyrate levels in the intestines of

pigs reduced colonisation of Salmonella in the intestines of pigs and also translocation of other bacteria over a "stressed" intestinal epithelial layer [Maurer et. al. 2002, Boyen et. al. 2008, Lewis et. al. 2010]. In the IPEC-J2 assay we did not observe a visible decrease in turbidity of the culture medium, indicating that the concentration *Salmonella* was not dramatically changed in case FOS was present. Moreover, elevated expression of most of the Salmonella response genes was not reduced by FOS. This indicated that vitality of the Salmonella population was not changed, and supports our hypothesis of an IPEC-J2 response to "waste products" of FOS fermentation. Because enhanced expression of the butyrate response factor ZFP36L1 was not completely normalised by long-chain FOS, this indirect effect on ZFP36L1 expression has to be investigated further to draw a more solid conclusion about this proposed interplay between IPEC-J2 cells, FOS, and *Salmonella* bacteria. Gene expression of ZFP36L1 induced by Salmonella was not influenced/normalized by rye, amoxicillin's or short chain FOS. However, in the absence of Salmonella ZnO up-regulated ZFP36L1 expression at 2 hrs, and down-regulated expression 6 hrs was observed. For a zinc-finger binding protein this response to ZnO may be logical. However, this gene may also participate in other cellular processes like triglyceride metabolism, a process linked to energy-sugar metabolism in the mitochondrion of cells with oxidative stress.

Based on the bioinformatics analysis we selected a set of key-genes/effector-molecules that responded in stressed and non-stressed IPEC-J2 cells after treatment with FOS. Preferably, genes/proteins capable of inducing or influencing processes outside of enterocytes were selected (see section 3.4; Identification of key-genes based on processes/pathways regulated by tested additives).

3.3 Overlapping processes/pathways regulated by all tested additives

In Table 5 the effect of all tested additives on Salmonella-induced cytokine/chemokine expression in IPEC-J2 cells is summarized. Rye and FOS alone did not affect the expression of cytokines/chemokines significantly. After 6 hrs of incubation the Salmonella-induced cytokine/chemokine response was most affected by ZnO. ZnO and Octacillin modulated expression of IL1A and CXCL2 comparable (boxed in Fig.5).

Table 5

Modulation of Salmonella-induced cytokine/chemokine response by additives.

gene	hrs	Salm./M	ZnO/M	ZnO-Salm./M	Oct/M	Oct-Salm./M	5% rye-Salm./5% rye
IL8	2	↑↑	↓↓	_	↑	↑	_
CXCL2	2	↑↑	↑	↑	↑	↑	↓
CXCL2	*6*	↑	↓↓	↑	↓↓	↓↓	_
CSF2	2	↑↑	↑↑	↑	↑↑	↑↑	_
CSF2	6	↑	_	_	_	_	↑↑
IL1A	2	↑↑	↑	_	↑	↑	_
IL1A	6	↑↑	↓↓	_	↓↓	↓↓	_
IL6	2	_	↓	_	↓	↓	↓
IL6	6	_	_	↑	↓	↓	_
CCL20	6	↑	↓	_	_	↓	_
IFNA4	2	_	_	_	_	_	↓↓ *
IFNA4	6	↓	_	↑↑	_	_	_
IFNL1	2	_	↑↑	_	_	_	_
IFNL1	6	_	_	_	↑↑	↑↑	_
IL18	6	↓	_	↓	_	↑	_
IL21	6	↑	_	↑↑	_	_	_
IL1B	6	_	_	↑↑	_	_	_

LTA	6	_	_	↑↑	_	_	_

-Up-regulated; (↑) FC >2 and <5, (↑↑) FC > 5.

-Down-regulated; (↓) FC<0.5 and >0.2, (↓↓) FC<0.2.

-Significant changes in expression between different treatments are highlighted in red.

*Gene expression of IFNA4 decreased with an elevated rye concentration.

In Table 6 common pathways, scoring the highest confidence level in bioinformatics programs, are listed. Because the response to Paracillin closely resembled that of Octacillin only pathways and biological processes for Octacillin were listed. Based on literature describing earlier *in-vitro* and *in-vivo* experiments with the here tested additives (if available) the most relevant/dominant pathways were boxed in Table 6. For rye and FOS no overlapping pathways with a similar term were detected. For rye pathways related to cell cycle showed some overlapping genes with the Cell Cycle/Checkpoint Control pathway called significant for Octacillin. The "HIF-1-alpha Transcription Factor Network" and "Direct P53 Effectors" were called significant for almost all gene expression comparisons conducted for ZnO and Octacillin, indicating that sensing of DNA damage and HIF1A transcriptional regulation are the major processes by which IPEC-J2 cells respond to oxidative and/or genotoxic stress, irrespective of the nature of how the stress was induced. Interestingly, the pathway "Validated Targets of C-MYC Transcriptional Repression" was called significant only for Octacillin at 6 hrs in the absence and presence of Salmonella. Transcription of target genes by this complex initiates apoptosis and/or cell cycle arrest of the S phase [Rajabi et al., 2004]. MYC also responds to DNA damage [Barzilai and Yamamoto, 2004]. Important inhibitors of apoptosis (Caspase 8 inhibitor CFLAR) and cell cycle progression (RBL1-tumour suppressor and ZBTB17, both also inhibitors of C-MYC transcription) within this pathway were differently regulated by Octacillin at 6 hrs than by ZnO. in the presence and absence of Salmonella. This suggests that these inhibitors/repressors are crucial for determining cell fate in response to cellular stress (CFLAR; marked with a blue star in Fig. 2 page).

Table 6

Common pathways induced by ZnO and Amoxicillin's in the presence and absence of Salmonella.

Pathway term[a]	average % matched genes [b]	Oct (2h)	Oct (6h)	Oct-Salm (2 h)	Oct-Salm (6 h)	Salm (2h)	Salm (6 h)	ZnO (2 h)	ZnO (6 h)	ZnO-Salm (2 h)	ZnO-Salm (6 h)
IL6-mediated Signaling Events	18.0		+	+	+			+			
Validated Targets of C-MYC Transcriptional Repression	14.5		+		+		+				
HIF-1-alpha Transcription Factor Network	11.5		+	+	+	+	+	+	+		+
IL-9 Signaling Pathways	9.8		+	+	+						+
TNF Signaling Pathway	9.1		+	+	+	+	+		+		+
Glucocorticoid Receptor Regulatory Network	8.0			+	+	+	+		+		
Direct P53 Effectors	7.7		+	+	+	+	+	+	+		+
TGF Beta Signaling Pathway	5.6			+		+			+		
NOD-like Receptor Signaling Pathways	5.3	+		+	+	+	+	+			+
Translation Insulin Regulation of Translation	4.7			+	+	+	+		+		
Transport of Glucose and Other Sugars, Bile Salts and Organic Acids, Metal Ions and Amine Compounds	3.8	+									+

[a] Pathway annotations are from GeneAnalytics (*http://geneanalytics.genecards.org*).

[b] average % matched genes; overall average calculated from the percentage of matched genes scored in each pathway called significant for a single treatment.

3.4 Identification of key-genes based on processes/pathways regulated by tested additives.

Literature regarding the effect(s) that in this study tested additives induce in biological systems was consulted to judge whether specific genes from our IPEC-J2 datasets may have the potential to induce a desired effect *in-vivo*. Preferably genes/proteins were selected which were highly up-or down-regulated and capable to transmit, execute, or inhibit an effect induced by a dominant biological

process/pathway identified in this study. Genes which are constitutively active in many cellular processes when gene transcription/translation takes place (like Proto-Oncogene C-Fos [FOS], JUN, and specific MAPK's), or may provoke an overreaction and/or an adverse effect *in-vivo (*e.g. in other cells than intestinal epithelial cells) were not selected. Also, cytokines/chemokines like IL8, IL6, IL1B, and CXCL2 were not selected because manipulation of their expression level could induce an uncontrolled inflammatory reaction *in-vivo* or an unwanted response of the local and/or systemic immune system. In Table 7 for all tested additives these key-genes and the processes in which they may act are presented.

Based on our bioinformatics analysis the most important biological process regulated in IPEC-J2 cells by ZnO and Octacillin was "oxidative stress" in the presence as well as in the absence of Salmonella. IPEC-J2 cells responded to oxidative stress by regulation of expression mainly of genes part of the HIF1-signaling pathway and by regulation of genes that sense free radicals and/or sense DNA-damage (genotoxic stress). The fact that expression of most of these genes were not, or only weakly, regulated by Salmonella alone indicated that HIF1-signaling was a generic pathway by which IPEC-J2 reacted to an "oxidative stress" situation. Down-regulation of genes involved in sugar metabolism (HK) suggests that oxidative stress was also induced by a shortage of glucose as energy source for these cells. Intracellular genes/proteins that sense free oxygen radicals and DNA damage in this pathway and secreted genes/proteins exerting the effect of HIF1-signaling were selected as key-genes. In addition, the Zn-transporter MT1A was selected based on its possible role in steering cytokine production in IPEC-J2 cells, especially, also because of the involvement of MT1A in the production of IL6, a cytokine that regulates HIF1-signalling [Hernández et. al. 1997, Mariani et al. 2008]. Such indirect regulation by MT1A is probably more controlled and prevents overexpression of IL6 (see above). CSF2 was selected as immune modulator. CSF2 production by enterocytes may stimulate secretion of cytokines/chemokines by a small number resident macrophages/dendritic cells in the lamina propia in order to attract and activate other immune cells. The Oxytocin receptor (OXTR) and natriuretic peptide A (NPPA, regulated by ZnO) were selected based on the possible involvement in the control of extracellular fluid volume and electrolyte homeostasis.

Cell cycle progression/mitosis was detected as the main biological process induced by Rye and TGF-beta signalling probably plays a role in steering this process. However, the strong response of the control diet alone and the lack of highly regulated genes made it difficult to accurately predict a dominant pathway specifically induced by rye alone. Only 9 genes were extracted from both the 5% and 10% rye data sets (without Salmonella) that did not respond to the control diet alone (0%). From these 9 genes, TWF1 was the only gene for which a hybridisation intensity more than 2-fold above the background intensity was observed. Therefore, beside ZFYVE16, an inhibitor of TGF-signalling (see above), TWF1 was selected as key-gene for rye. For Rye the cytokine CSF2 was also selected as key-gene.

Based on our hypothesis that the response of IPEC-J2 cells to a mixture of Salmonella and FOS was due to "waste products" of FOS-fermentation, the butyrate Response Factor 1 (ZFP36L1) was selected as key-gene along with the genes P4HA1 and ERO1L genes. However, because of the limited number of genes regulated by FOS alone and in the presence of Salmonella, for these to later genes no plausible relation with a cellular process/pathway could be found that may be modulated by alternative additives linked to these genes. The same applies for the RYR2, which expression was induced by long and short chain FOS preparations in the absence of Salmonella. RYR2 may be an important gene in modulating calcium concentration gradients between the cytosol and the ER.

Table 7

Selected key-genes/effector molecules.

additive	gene	description	function
Amoxicillin	DDIT4	DNA-damage-inducible transcript 4	Regulates cell growth, proliferation and survival
Amoxicillin	HIPK2	homeodomain interacting protein kinase 2	corepressor and a coactivator of transcription factor (TP53)
Amoxicillin	NBN	nibrin	DNA damage sensing
Amoxicillin	NOX5	NADPH oxidase, EF-hand calcium binding domain 5	calcium-dependen NADPH oxidase generating superoxide
Amoxicillin	TXNIP	thioredoxin interacting protein	inhibitor thioredoxin/inhibits proteasomal degradation of DDIT4
ZnO / Amoxicillin	EDN1	endothelin 1	vasoconstrictor
ZnO / Amoxicillin	EGLN1	egl-9 family hypoxia-inducible factor 1	intracellular radical sensor
ZnO / Amoxicillin	HMOX1	heme oxygenase (decycling) 1	enzyme in heme catabolism, cleaves heme to form biliverdin
ZnO / Amoxicillin	SERPINE1	serpin peptidase inhibitor, clade E member 1	cytoskeleton actin-binding protein 1
ZnO	HK2	hexokinase 2	phosphorylate glucose to produce glucose-6-phosphate
ZnO	MT1A	metallothionein 1A	intra and extracellular zinc transporter
ZnO	NPPA	natriuretic peptide A	hormone
ZnO	OXTR	oxytocin receptor	hormone receptor
ZnO / rye	CSF2	colony stimulating factor 2 (granulocyte-macrophage)	cytokine
rye	IL1A	interleukin 1, alpha	cytokine
rye	TWF1	twinfilin actin-binding protein 1	Inhibits actin polymerization by sequestering G-actin
rye	ZFYVE16	zinc finger, FYVE domain containing 16	scaffold proteinand inhibitor in the TGF-beta signaling pathway
FOS	ERO1L	ERO1-like (S. cerevisiae)	Oxidoreductase forming disulfide bonds in ER / ROS production
FOS	P4HA1	prolyl 4-hydroxylase, alpha polypeptide I	component PH4 enzyme complex ER/ ROS production
FOS	RYR2	ryanodine receptor 2 (cardiac)	intracellular calcium channel
FOS	ZFP36L1	ZFP36 ring finger protein-like 1	early response gene / Butyrate Response Factor

3.5 Alternative additives with the potential to mimic a specific response in IPEC-J2 cells

As example for selecting alternative additives, for the key-genes HMOX1 and MT1A, both involved in the most important biological pathway/procces influenced by ZnO and Octacillin, associations with compounds/chemicals in the Comparative Toxigenomics Database (CTD) were explored and judges for relevancy by consulting literature linked to these associations. In Table 8 for both these key-genes, to our opinion, meaningful associations with alternative chemicals/compounds are presented. Toxic and synthetic compounds (drugs) were not selected.

Table 8

Alternative compounds for key-genes/effector molecules HMOX1 and MT1A.

Key-gene	compound (CTD hyperlink)[a]	# references	# different species	description
HMOX1	Ferroprotoporphyrin	57	5	Heme; colour-furnishing portion of hemoglobin.
	sulforafan	46	4	Anticarcinogenic Agents (ISOTHIOCYANATE)
	zinc protoporphyrin	76	3	Zn chelated in the precursor of heme Protoporphyrin IX
	Hemin	88	4	Chloride salt of Ferri-Heme
	Selenium	16	4	Metal
MT1A	Butyrates	6	1	Short chain fatty acid
	manganese sulfate	2	2	Inorganic compound
	sulforafan	2	1	Anticarcinogenic Agents (ISOTHIOCYANATE)
	Copper	1	1	Metal
	sodium propionate	1	1	Short chain fatty acid

[a] Comparative toxigenomics database (CTD) hyperlinks of selected alternative compounds. The number of references describing an effect on key-genes HMOX1 and MT1A, and the number of different species in which these effects were measured, are provided.

3.6 Progress in development of a similar bioassay using chicken and cattle cultured cells

From a research group of the University of Maribor (Slovenia) cell cultures of chicken and cattle intestinal epithelial cells were purchased. These researchers claimed that the cattle cells were cloned and that they grow as a line [Cencic et. al. 2013]. For the chicken cell line they informed us that they were still making efforts to clone them properly to a stable line. However, in our hands both cell cultures were instable and grew very slow and were extremely sensitive to trypsin treatment and low-speed centrifugation (needed to passage and amplify these cells). Because robustness of cells is mandatory in case all different kind of interventions/additives have to be tested, no further efforts were undertaken with these cells. As alternative, more robust "cloned" epithelial cell lines derived for bovine kidney epithelia (MDBK; Madin-Darby Bovine Kidney) and from chicken liver (chicken hepatocellular epithelial cells; LMH) were selected as candidate cell lines for an *in-vitro* assay. Efforts are currently undertaken to analyse the response of the MBDK cells to ZnO, with and without the viral pathogen bovine viral diarrhea virus (BVDV) as inducer of stress, and the response of LMH cells to Octacillin (with and without Salmonella). Salmonella can invade these latter cells [Shah et. al. 2012]. The use of BVDV virus as inducer of cellular stress may generate additional information regarding immunological responses of epithelial cells to viral enteric pathogens.

A porcine macrophage cell line previously developed and tested [Chitko-McKown et. al. 2013] was obtained from the USDA Meat Animal Research Centre. Together with feeder cells, which produce immunological co-factors for these cells, these cells were amplified and stored in ready to use batches in liquid nitrogen. These cells may be used for gene expression analysis in co-culture studies with IPEC-J2 cells grown in a trans-well system. ZnO will be used as additive to develop such a trans-well co-culture system [Trapecar et. al. 2014]. Measurement of gene expression in enterocytes and macrophages may provide valuable information about the cross-talk between these cells. For instance, (mutual) crosstalk mediated by cytokine/chemokines and secreted effector proteins produced by IPEC-J2 cells (e.g. HMOX1, MT1A) in response to ZnO.

4 Summarising discussion and conclusions

4.1 Generic "stress" response of IPEC-J2 cells to Salmonella and additives

IPEC-J2 cells differentiated to jejunal enterocyte-like cells responded to a challenge with Salmonella bacteria as expected. Several cytokines and chemokines were up-regulated by Salmonella. However, within 6 hrs no sign of a generic stress response (ER-stress or oxidative stress) was observed, despite Salmonella is able to invade enterocytes and translocate over the epithelial into the periphery. Likely, Salmonella and/or its effector molecules are able to suppress such a stress response. In line with this, Salmonella induced a high expression of the DNA-Damage-Inducible Transcript 4. For DDIT4, which transcription is induced by HIF1, it was demonstrated that it functions as a negative regulator of HIF1 expression [Regazzetti et. al. 2010]. Both ZnO and Octacillin reduced expression of DDIT4 significantly in the presence and absence of Salmonella. Moreover, in response to the other amoxicillin preparation, Paracillin, DDIT4 expression was down-regulated to almost undetectable levels, indicating that IPEC-J2 cells express a basic level of this gene in case these cells were not stressed. Together with our observation that ZnO and amoxicillin preparations in the absence of Salmonella induced several effector molecules of HIF1-mediated transcription, this strongly suggested that HIF1 transcriptional control is the major and generic process by which IPEC-J2 cells respond to a stressed situation. Hypoxia and ROS production is induced by ZnO and antibiotics, and various HIF1 effector proteins may be expressed to normalize intracellular and activate extracellular processes to overcome this stressed situation (discussed beneath in more detail). Zinc supplementation and antibiotic treatment are controversial feed additives. Therefore, nutritional interventions modulating the expression of the HIF1-related key-genes/effector" has prospect, and may lead to alternative additives. For instance, such additives may also improve health of newly weaned pigs by decreasing the incidence of post weaning diarrhoea induced by enteric bacterial [Heo et. al., 2010] and other enteric (viral and parasite) pathogens. Because ZnO and both amoxicillin preparations strongly influenced Salmonella-induced cytokine/chemokine responses, it is preferable to test these alternatives first in IPEC-J2 cells. In case an extraordinary cytokine/chemokine profile is induced *in-vitro,* which could provoke an overreaction of the immune system *in-vivo*, it will not be wise to apply these additives in a similar manner/dose to live animals.

No ER-stress or oxidative-stress response was induced by rye and FOS. One important similarity found between Rye and ZnO/amoxicillin preparations was the possible involvement of the ATM/ATR-TP53 checkpoint mechanism that controls cell cycle progression/arrest in response to DNA-damage. However, no regulation of genes was observed that sense DNA-damage (like NBN and DDIT4), indicating that no serious genotoxic stress was induced by rye.

4.2 Concordance of additive-responses to responses described in literature

Although the HIF1/hypoxia process was identified as a dominant response of IPEC-J2 cells for ZnO and amoxicillin preparations, there were several remarkable differences between responses measured for these additives. The HIF1-mediated response for ZnO was faster than for amoxicillin's and also a different expression profile of HIF1-mediated effector genes/proteins was observed. Moreover, after 2 hrs in the presence of ZnO IPEC-J2 cells expressed a broad set of p53-induced effectors which were not regulated by Octacillin. Likely, the presence of ZnO was more stressful for IPEC-J2 cells than the presence of amoxicillin. Also, only in case Salmonella and amoxicillin were both present an extremely high expression level for HMOX1 was observed. This not only illustrated that the interplay between IPEC-J2 cells, Salmonella, and amoxicillin influenced the response in IPEC-J2 cells, but it also indicated that the severity of the cellular stress determines how IPEC-J2 cells respond. Surprisingly, no vigorous

stress or cytokine response was induced by a mixture of Salmonella and ZnO suggesting that such interplay also exist for ZnO.
Striking differences were observed between additives with regard to regulation of DDIT4 (discussed above) and MT1A. From MT1A it is known that it also induces oxidative stress in bronchial epithelial cells [Li et. al 2015]. In fact, regulation of a related set of response genes was observed in this study, including MT1A, the HIF1-effector HMOX1, and oxygen-sensor EGLN3. Moreover, in these cells several genes were regulated involved in the control of blood flow and blood vessel homeostasis. Likely, in response to ZnO/amoxicillin induced-oxidative stress enterocytes react by secretion of HIF-effectors like HMOX1, SERPINE1 and EDN1 to increase blood flow, and consequently, the supply and uptake of oxygen and/or glucose from the blood. In addition, to survive from shortage of oxygen enterocytes promote anaerobic energy metabolism in enterocytes by regulating genes involved glucose, pyruvate, and fructose transport (enzymes like HK, SLC2A1, PDK1, PFKFB3 and PGK1). Recently it was shown that Akt-mTOR-HIF1 regulated transcription of sugar metabolizing enzymes triggered a shift towards glycolytic energy metabolism to support epigenetic reprogramming and execution of an innate memory response in macrophages and dendritic cells (so-called trained immunity; Kelly and O'Neil 2015). It would be interesting to research whether a similar mechanism of "immune training" supported by Akt-mTOR-HIF1 transcriptional regulation also exist in, for example M-cells, enterocyte-like cells imbedded in the intestinal epithelial layer that are specialized in sensing antigens in the lumen.
Our results suggest that MT1A is a functional component of a mechanism of IPEC-J2 cells to cope with oxidative stress induced by extracellular signals, and that MT1A together with IL6 may be an important regulator of the HIF1 pathway. A concerted action of IL6 and MT1A was also observed in a study in which sheep airway epithelium was physically damaged [Yahiya et. al. 2013]. In this study several genes for which we proposed a regulatory function in handling oxidative stress in IPEC-J2 cells (Salmonella-induced and/or additive-induced), like SOCS3, CXCL2, IL8, SERPINE1 and FOS, were also regulated in damaged airway epithelium. Zn supplementation also influenced the expression of both IL6 and MT1A in blood of healthy elderly [Mariani et al. 2008]. In this latter study, polymorphic alleles in the genes of IL6 and MT1A were positively correlated to a higher expression of both these proteins. In addition, it was shown in the brain of mice that MT1 production was dependent on IL6 activity [Hernández et. al. 1997]. Therefore, the interplay between IL6 and MT's may be a crucial mechanism for neutralizing ROS, and thereby, in protecting IPEC-J2 cells from a ROS overload. Not only by the function of MT1A as "scavenger of reactive oxygen species", but also as regulator of gene expression. Zinc-finger proteins are necessary for signal transduction from cytokine receptors to response genes and MT's are involved in regulation of this signalling inside the cell. Our observation that MT1A was not up-regulated by ZnO in the presence of Salmonella indicates that also IL6-MT independent mechanisms are involved in case IPEC-J2 cells are stressed by other environmental factor like enteric pathogens. In line with this, in this study ZnO was able to reduce/normalise a cytokine response induced by Salmonella without inducing MT1A expression.

Most studies in which the effects of beta-lactam antibiotics on gene expression in cultured cells were monitored were performed in combination with a challenge with pathogenic bacteria. Mainly to investigate the lethal effects of ROS and other intracellular radicals as part of the mechanisms of killing and clearance of bacterial pathogens by phagocytosis, and mostly, using monocytes. [Bogomolnaya et. al. 2013, Mosel et. al. 2013, Hébrard et. al. 2008]. Only a few reports in literature describe that beta-lactam or macrolide antibiotics itself induce ROS production in bronchial epithelial cells [Brooks et. al. 2005, Clerici et. al. 2009] or to study dose-toxicity relation [Ferrara et. al. 2001]. In our study we clearly showed that IPEC-J2 cells exposed to ZnO and Amoxicillin responded by modulation of the HIF1-process to cope with induced oxidative stress, independently from a challenge with enteric pathogens. In two studies, oxidative stress/ROS was experimentally induced in IPEC-J2 cells by H_2O_2 and/or by xanthine-xanthine oxidase [Cai et. al. 2013 and 2014]. Para-cellular permeability of IPEC-J2 cells was increased by H_2O_2 treatment suggesting that oxidative stress may weaken the integrity of intestinal epithelial cell layer and its barrier function *in-vivo.* The antioxidant Alpha-lipoic acid (LA;Thioctic Acid) was able to reduce ROS levels in IPEC-J2 cells. Interestingly, in response to arsenic-induced oxidative stress, mouse endothelial cells increased expression of HMOX1, VEGF and IL6, and human THP-1 monocytes and macrophages treated with LA increased HMOX1 expression and restored the redox state of these cells [Wang et. al. 2011 and 2012]. Together with the results of our ZnO and antibiotic interventions, these results clearly demonstrate that transcriptional regulation of HIF1 effector molecules by specific additives in enterocyte cells of the

epithelial layer has prospect, especially for protection of the intestinal epithelial layer from pathogen-induced damage [Schumann et. al. 2005]. The effect of food additives on HIF1-mediated processes has already been studied in many types of cells [Losso and Bawadi, 2005]. Importantly, inducing overexpression of HIF1 effector molecules like HMOX1 has to be studied *in-vivo* first to demonstrate that no negative side-effects will be induced. For instance, it was shown that the catabolite bilirubin, produced by breakdown of heme by enzymes (including HMOX1), stimulated ROS-induced membrane disruption of *E. faecalis*, a Gram-positive human commensal bacterium, but protected the Gram-negative *enterohemorrhagic E. coli* (EHEC) from ROS [Nobles et. al. 2013].
Recently it was shown that Akt-mTOR-HIF1 regulated transcription of sugar metabolizing enzymes triggered a shift towards glycolytic energy metabolism to support epigenetic reprogramming and execution of an innate memory response in macrophages and dendritic cells (so-called trained immunity). It would be interesting to research whether a similar mechanism of "immune training" supported by Akt-mTOR-HIF1 transcriptional regulation also exist in enterocyte(-like) cells, for instance in M-cells that are specialized in sensing antigens in the lumen of the intestine.

Particularly soluble non-starch polysaccharides in rye (like arabinoxylans) create a viscous environment in the intestinal lumen, which impairs digestibility and absorption of nutrients [extensively discussed in VDI-5 report]. It was hypothesized that such an increase in viscosity negatively affects gut wall morphology [Haenen et al. 2013]. However, in response to rye no dominant sets of genes involved in processes related to breakdown/damage of the epithelial barrier function and/or mucus layer were observed in IPEC-J2 cells. In contrary, genes related to cell-cycle progression were the only group of genes that were specifically regulated by rye. In cultured human skin cells acidic arabinoxylans increased expression of keratinocyte growth factor [Uchida et. al. 2007]. However, we did not detect up-regulation of an "enterocyte-specific" growth factor to initiate cell-cycle/mitoses progression in response to rye. High grain diets fed to cattle induces expression of genes involved in cholesterol homeostasis in the epithelial layer of the rumen, a process believed to be imposed by changes in short chain fatty acid (SCFA) production by the microbiota in the ruminal biomass [Steele et. al. 2011]. In above described experiments with FOS regulation of the butyrate response factor (ZFP36L1) suggested that IPEC-J2 cells can respond to changes in SCFA level imposed by Salmonella. However, in response to rye no regulation of ZFP36L1 gene expression was observed in Salmonella-stressed and non-stressed IPEC-J2 cells. The effect of fermentation of components within the control diet (without rye) by Salmonella was probably too large and could have overshadowed specific processes induced by rye-specific components.

Gene expression analysis showed that FOS did not directly regulate biological processes in IPEC-J2 cells. However, the observation in this study that the "butyrate response gene" ZFP36L1 responded to changes in concentration of "waste product" secreted by Salmonella corresponded with results of several studies in which the positive effects of inulin-type fructans like FOS on intestinal function were reported (discussed above). Direct positive effects on the host, as well as indirect effects induced by fermentation of these fructans by the microbiome in the intestine (reviewed in Scholz-Ahrens and Schrezenmeir 2007] were reported in these studies. Although further studies in IPEC-J2 cells has to prove that the effects we measured were specifically induced by the fermentation of FOS by Salmonella bacteria, our observation that ZFP36L1 is relatively highly expressed by IPEC-J2 cells and was also regulated in case hypoxia/ROS processes are managed suggested that this gene may be an important connector between intracellular processes in stressed IPEC-J2 cells (e.g. mineral absorption, ROS production/hypoxia and triglyceride fat and sugar-energy metabolism) and the environment. Further understanding of this mechanism may also offer the opportunity to develop a test in which this interplay between nutritional additives, (waste)-metabolites secreted by specific bacteria strains of the microflora, and enterocytes may be studied *in-vitro*.

4.3 Reliability/limitations of the medium high-throughput IPEC-J2 test for prediction of responses in enterocytes of pig, chicken and cattle

Results obtained with the IPEC-J2 *in-vitro* bio-assay showed a relative high degree of similarity compared to the results obtained in several other *in-vivo* and *in-vitro* intestinal models. Measuring the functional effects of feed ingredients in this *in vitro* system may generate insight in the mechanisms how these ingredients influence the immediate early functioning of epithelial cell layers. Challenging

IPEC-J2 cells with Salmonella in the presence of additives revealed some novel mechanisms how porcine intestinal epithelial cells may respond to nutritional interventions when intestinal homeostasis is disturbed by enteric pathogens or other stressors. With respect to intestinal immunity, applying a Salmonella challenge in our IPEC-J2 test proved to be essential for monitoring how additives like ZnO and amoxicillin can modulate cytokine/chemokine responses in a stressed situation. Regulation of cytokine/chemokine signalling is not only important for transmission of "danger signals" from the lumen to the periphery, but also for induction of tolerance or prevention of overreactions of the (local) immune system, which may induce damage and loss of the epithelial barrier function. On the biological process-level as well as on gene-level, results of the IPEC-J2 test showed a strong overlap with results described in studies in which human, rat, and mouse intestinal (and bronchial) epithelium cells were challenged with similar or related additives. Although chickens are no mammals, and the GI tract of herbivores differs considerably from omnivores, we have confidence that at least at process-level results of the porcine bio-assay can be translated to chickens and cattle. In the VDI-6 report (in development) the correlation between responses to rye and Octacillin measured in the intestine of chickens and in porcine IPEC-J2 cells will be evaluated and discussed. For pigs, the concordance of responses in IPEC-J2 cells to measured responses in other mammals make it's, to our opinion, already possible to identify alternative additives for ZnO and antibiotics that have potential to elicit a similar response in the intestines of pigs. Moreover, the set of identified key-genes in this study may also be used as parameters for measuring specific effects of these potential additives in an *in-vivo* trial.
A limitation of the IPEC-J2 bioassay was revealed in tests performed with different concentrations of rye formulated in the control diet. The results of these experiments showed that incubation of a complete diet on IPEC-J2 monolayers induces large changes in gene expression in IPEC-J2 cells that overshadow less dominant effects of the components of interest (rye) within this diet.

4.4 Potential readout parameters for measuring immune-competence in-vivo

The in this *in-vitro* study identified highly up-regulated genes responding to additives alone and to mixtures of additive and Salmonella that have potential to be indicators of the "immune competence" of the epithelial layer. This competence is the capability of the intestinal epithelial layer (enterocytes) of live animals to properly sense an imbalanced (hostile) or balanced luminal environment, and to produce correct signals to activate specific biological processes in other type of intestinal cells and immune cells that restore the intestinal balance or maintain balance. Biological processes representing this capability or "immune competence" are e.g. cell-survival and regeneration of epithelial cells, barrier integrity of the mucosal layer, electrolyte homeostasis, and (controlled) inflammatory reactions. In figure 6 these potential "indicator genes" of local immune competence, with the biological processes in which they function, are depicted schematic. Note that most of these genes were also selected as key-genes (see Table 7) and that also cytokines/chemokine (see Table 5), products and substrates of enzymes (e.g. HMOX1), ligands of receptors (e.g. OXT), and downstream effects of hormones and ligands (e.g. peptide hormone NNPA and SCFA as indicator of dysbiosis related to activation of ZFP36L1=Butyrate response factor 1) may function as indicators of "immune competence". Eventually, a proper response of the epithelial layer, which is the first cell-layer in contact with the content of the lumen, will be important for overall intestinal function, and consequently, for overall performance of animals. It should be noted that the here identified read-out parameters probably represent immediate early indicators of the competence of the intestinal epithelial layer to properly sense, communicate and respond to hostile pathogens, toxic agents and other environmental changes in the intestinal lumen. Such indicators of "early local intestinal immune competence" may predict detriment or beneficial effects of nutritional interventions *in-vivo* and may be used to develop a sort of "measuring stick" for immune competence.

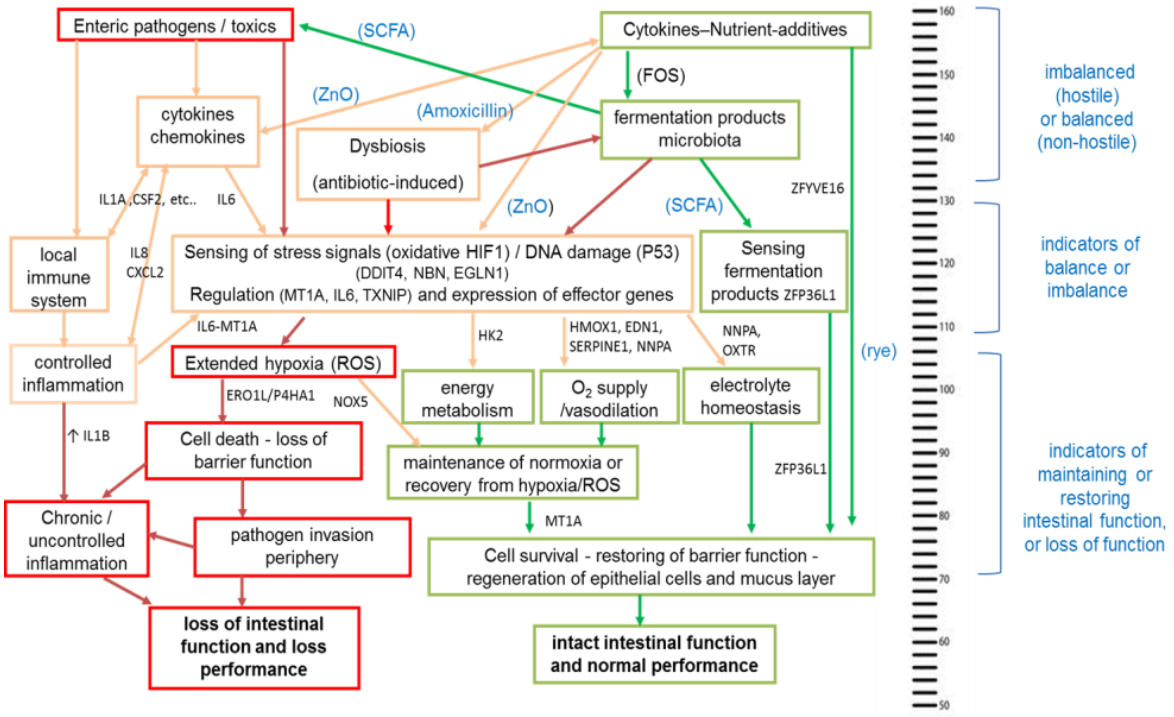

Figure 6 *Potential indicators of "immune competence" of the epithelial layer (enterocytes). Hostile factors and processes negatively affecting intestinal function and overall performance are shown in red (boxes and arrows). Non-hostile and processes positive affecting and restoring intestinal function are shown in green. Neutral processes (participating in negative as well as in positive effects) are shown in orange. Additives and chemical compounds are presented as blue text between brackets and genes are shown in black. (↑); overexpression.*

4.5 Overall conclusions: preface for further research

- For pigs a medium-throughput *in-vitro* bioassay using cultured "Intestinal Porcine Epithelial Cells" was developed which was able to detect enterocyte-specific physiological and immunological processes induced by nutrients/additives.

- ZnO and amoxicillin antibiotics modulated: i) Salmonella-induced cytokine/chemokine response in porcine intestinal epithelial cells; and ii) expression of HIF1A-effector proteins, most likely to rescue these cells from oxidative stress. . In a co-culture experiments the cross-talk between porcine macrophage cells and IPEC-J2 cells can be measured with gene expression analysis after challenge of IPEC-J2 cells with ZnO.

- Rye influences processes related to cell cycle progression in porcine intestinal epithelial cells and showed only limited effect on immune genes. No correlation between viscosity of rye diets and biological processes was found.

- FOS induced no direct biological process in porcine intestinal epithelial cells and showed no effect on immune genes. Changes imposed by "waste metabolites" of fermentation of long chain FOS by Salmonella (e.g. SCFA's) may have influenced gene expression in porcine intestinal epithelial cells. This suggests that development of an *in-vitro* test in which the interplay between additives, metabolites secreted by specific bacteria in the lumen of farm animals, and enterocytes can be studied, may be feasible using IPEC-J2 cells.

- Since no robust intestinal epithelial cell lines are available for chicken and cattle, alternative epithelial cell lines from other tissues (bovine kidney and chicken liver) can be used and validated with ZnO and Octacillin interventions.

- In this study we identified a set of biological processes that could function as potential indicators of "immune competence" of the epithelial layer (enterocytes) of the gut mucosa. In addition, we identified a set of key-genes and chemicals/biomolecules which have the potential to represent the capability of the intestinal epithelial layer to properly sense, communicate and respond to hostile pathogens, toxic agents and other environmental changes in the intestinal lumen. These indicators of "local intestinal immune competence" may predict detriment or beneficial effects of nutritional interventions *in-vivo*.

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