

Super-dosing of vitamin B2 induces beneficial hindgut transcriptome responses in weaned piglets

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Abstract

High dietary levels of vitamin B2 on the caecal transcriptome of weaned piglets was explored, to evaluate if super-dosing of riboflavin could reach the hind gut, and consequently trigger a beneficial host response. Caecal gene expression was evaluated using Agilent transcriptome microarray, 14 and 28 days after supplementation in three experimental groups; a control group (recommended dose of riboflavin: 10 mg/kg diet), and two groups additionally supplemented to reach 50 or 100 mg/kg of riboflavin, respectively. Both supplementation levels significantly changed the caecal transcriptome pattern compared to the control. The 100 mg/kg dose resulted in the highest number of differentially expressed genes. At day 14, the top modulated genes were related to oxidative stress and immune response, confirming the antioxidant effect of riboflavin. Furthermore, pathways involved in the response of the host to changes in the microbiota were modulated, indicating modulatory effects of riboflavin on the microbiome, and indirectly the host response. Finally, the highest dose of riboflavin triggered molecular mechanisms involved in aldosterone and bile acid metabolism, demonstrating the potential of vitamin B2 in improving hind gut health status. A lower impact of the 100 mg/kg supplementation of riboflavin on caecal gene expression was found at day 28. Nevertheless, some positive gene responses were shown against DNA damage for both supplementation levels, and some pathways related to cell signaling were modulated. The data presented in this study confirm that super-dosing vitamin B2 affects caecal physiology, and further investigation is needed to support the findings of the possible modulation on the microbiota and bile acid metabolism. In conclusion, super-dosing of vitamin B2 positively impacts the transcriptome response of the weaned piglets' caecum. This creates the opportunity for considering riboflavin as part of a novel solution to reduce the incidence and severity of digestive problems associated with the period immediately post-weaning.

Keywords: transcriptomics, microarray, piglets, riboflavin, vitamin

DEG: Differentially Expressed Gene

PCA: Principal Component Analysis

PCR: Polymerase Chain reaction

UPLC: Ultra Performance Liquid Chromatography

RNA: Ribonucleotide Acid

LC/MS: Liquid Chromatography / Mass spectrometry

Ct: Cycle threshold

RIN: RNA Integrity Number

FDR: False Discovery Rate

KEGG: Kyoto Encyclopedia of Genes and Genomes

HSP: Heat Shock Protein

ROS: Reactive Oxygen Species

BA: Bile Acid

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INTRODUCTION

Weaning imposes tremendous stress (environmental, nutritional- and psychological) on piglets and is accompanied by marked changes in gastrointestinal physiology, microbiology and immunology (1). Consequently, the period following weaning is generally characterized by diarrheic symptoms and other intestinal disturbances, which can result in decreased growth performance and mortality. Traditionally, antibiotic growth promoters and heavy minerals, especially zinc (ZnO) and copper (CuSO₄), were used to reduce the complications associated with weaning. However, currently the use of in-feed antibiotics is either being minimized or completely eliminated (European Union) and there are concerns about environmental accumulation of minerals resulting from high dietary levels of inorganic zinc and copper. It is therefore imperative to find alternative strategies to modulate gastrointestinal functionality, the gut microbiome, the innate immune system, the intestinal barrier integrity and the intestinal enzyme activity. In this framework, B vitamins play a key role in the regulation of the immune response in the gut (2,3).

Among B-vitamins, riboflavin (vitamin B₂) in humans and in *in vitro* studies demonstrates a positive impact on oxidative stress, inflammation and nutrient absorption (4–7). Gut morphology is also sensitive to riboflavin depletion, leading to impairment of villi and crypts, emphasizing the importance of this vitamin in the gastrointestinal functionality (8,9). Furthermore, riboflavin is known to modulate the gut microbiota of patients with Crohn's disease (7) and recently, a pilot trial proves it with broiler chickens supplemented with different levels of vitamin B₂ (10). The gut microbial ecosystem plays a major role in B vitamin metabolism, as both a consumer and also a producer. The caecal microbiota is the main endogenous producer of riboflavin and the hind gut is a major absorption site for vitamin B₂ (11–14). Among the hind gut ecosystem, one of the most abundant players is *Faecalibacterium prausnitzii*. This anaerobe is known to be depleted in colonic diseases, is a producer of the short-chain fatty acid butyrate (15) and is suggested as a potential biomarker of a healthy colon (16,17). The metabolism of *F. prausnitzii* allows the utilization of the riboflavin for its growth (18) and therefore it is suggested that the beneficial anti-oxidant and anti-inflammatory effect of the riboflavin may potentially be indirectly due to the modulation of this commensal microbe (7). In conclusion, the stimulation of the host immune function and gut health status is mediated by both diet and microbiota-derived riboflavin.

In order to reap the benefits of riboflavin on the host gastrointestinal tract, feeding animals with the correct dose of vitamin B₂, depending on their life stage, is crucial. According to the National Research Council (NRC) (19) riboflavin requirements for piglets between 5–30 kg body weight is between 2–4 mg/kg feed, and the Optimum Vitamin Nutrition (OVN) guidelines from DSM (20), recommend between 10–15 mg/kg feed in order to maximize the properties of vitamin B₂ modulating the gastrointestinal functionality. Nevertheless, independently of the dosage used, NRC or OVN, dietary supplementation of riboflavin is rapidly absorbed in the small intestine, and very little amounts of exogenous riboflavin may reach the large intestine, where only the endoge-

nous microbial riboflavin is present. Thus, one option proposed is increasing the amount of vitamin B₂ in the diet in order to reach the hind gut and reap the benefits on host immune status and modulation of the microbiome as Biagi et al. demonstrate in broiler chickens (10). The research testing super-dosing of vitamin B₂ on zootechnical performance and immune status in swine is limited. Only a few studies report effects of high doses of B-vitamins on performance and potential response to stress, suggesting a higher requirement of B-vitamins during stress conditions. No clear consistent effects of super-dosing on performance or carcass characteristics is reported (21,22). Lutz and Stahly estimated that the need of riboflavin for protein accretion is higher than the dose recommended by the NRC, supporting the idea that a higher level of riboflavin would be beneficial for pigs (22). However, Böhmer and Roth-Maier showed effects on performance, but not on protein accretion when animals were supplemented up to 800% of the required vitamin B₂ level, corresponding to 18.4 mg of riboflavin per kg of diet. It is also described that by-products of vitamin B₂ show a positive effect against stress by reducing norepinephrine level and on improving the meat quality (color and lean meat percentage) (23).

The objective of the present study was to evaluate the impact of different dietary levels of vitamin B₂ on the piglet transcriptome response at caecum level in order to consider riboflavin as part of a novel solution with other feed additives in an additive/synergistic way, reducing the incidence and severity of digestive problems associated with the period immediately after weaning.

MATERIAL AND METHODS

Animals, housing and diets

The 28-day trial was performed at the Research Center for Animal Nutrition and Health (DSM Nutritional Products France, F-68305 Village-Neuf) according to the official French guidelines for experiments with live animals.

A total of 54 Redon x Large-White castrated pigs weaned at 28 days of age with initial body weight of 7.1 ± 1.0 kg were used. Pigs were obtained from a French commercial supplier at weaning and transported to DSM facilities. Pigs were allocated into 3 equal groups of 18 animals each, in 9 pens of 6 animals. They were housed in flat-deck pens in an environmentally controlled room. Each pen had a plastic-coated welded wire floor and was equipped with two water nipples and two stainless-steel individualized feeders. Water and feed were provided *ad libitum*. The room temperature was maintained at 27 °C on the day of weaning and reduced at regular intervals until 21–22 °C. Humidity percentage was 50 %. The lighting program was set to 16 h light and 8 h dark. The diets pre-starter from d1–14 of the study and starter from d14–28 of the study were based on wheat-barley-corn-soybean meal and were formulated to meet or exceed the requirements of weaning piglets (Table 1).

Supplementary table 1: Primers used for qPCR analysis

Gene	Forward primer	Reverse primer	Amplicon length	Reference
GAPDH	AAGGAGTAAGAGCCCTGGGA	TCTGGGATGGAAACTGGAA	140	Li et al.
β -act	CTCGATCATGAAGTCCGACGT	GTGATCTCCTCTGCATCCTGTC	114	Woyengo et al.
HSPH1	ACTCAGACACCGAGGTAATG	GGTAGCTCAACATTCACCACT	88	This study
HSP70.2	GCCGAGAAGGACGAGTTTGA	TGGTACAGTCCGCTGATGATG	77	This study
HSP90AA1	ATCGCCAGTTGATGCTGTT	AGCATCGGACGAGTTGGAAA	90	This study
MX1	CAGAGGACGCGGAATTGTG	TGACTTTGGCCCTCCATTCTGT	89	This study
TLR7	AGCACTTGACAGCGATTCTCT	GCTGGAGTGATGCTCGCTAT	88	This study
IL12B	CCTGCCATTGAGGCTGCTG	GAGGCTGTGGTTTGATGATGTC	98	This study
NFE2L2	GCCGACTATCCCAGGTTTCTT	GAGCCTGTTAGGAGCAATGA	100	This study
RAD17	GCTGAGTGCTTTGCTTACA	TTAAAGTACAATTGCGCTTCTGGT	99	This study
SLC51B	GGCTGTGGTGGCTGTGATAA	GGCAGGCTTTTGATTTCTGT	75	This study
NR1H4	AGAAACTGTGAACAAACCGGAGA	TGATCCCATCCAAACTTTCTGT	90	This study

The treatments included A) a control diet including in the pre-mix 10 ppm of Riboflavin (DSM Nutritional Products), B) the control diet supplemented with 40 ppm of Riboflavin in order to get 50ppm of Riboflavin in total, and C) the control diet with 90 ppm of Riboflavin to get 100ppm of Riboflavin in total.

Sampling collection

On days 14 and 28 of study; 9 pigs per treatment were selected (3 pigs/pen) according to the ranking of body weight by pen: 1st, 3rd and 5th on d14. These pigs were euthanized by stunning with a captive bolt, followed immediately after by exsanguination in the jugular area. Following euthanasia, the ileal (upper 0–80 cm of the ileum-cecum junction) and caecal digesta were collected and stored at -20°C until further analyses. Caecal tissue samples were collected and stored in RNA Later for transcriptomic analysis.

Riboflavin determination in digesta

Vitamin B2 concentration in digesta was quantified at days 14 and 28 of the study using UPLC 1290 Infinity II LC System (Agilent Technologies, Basel, Switzerland) coupled with MS detection API4000 (SCIEX, Baden, Switzerland). Briefly, protein precipitation followed by extraction was performed, and the vitamin B2 was analyzed with the LC-MS/MS system. Data acquisition, integration and quantification were performed by Analyst® software (SCIEX, Baden, Switzerland). The riboflavin concentration in digesta was reported and averaged for the 9 piglets in the same treatment. The Intestinal flow of riboflavin in ileum and caecum was expressed in mg/kg digesta, corrected by the dry matter (DM).

RNA extraction

Approx. 100 mg of caecal tissue was collected for transcriptome analysis. Total RNA was extracted from the tissue (stored at -20°C in RNA later) by lysing tissue with FastPrep® 24 (MP Biomedicals, Illkirch, France), using the phenol-chloroform method (TRIzol reagent; Invitrogen, Invitrogen, Cergy Pontoise, France) followed by purification using RNeasy columns by automated method with the Qiacube HT (Qiagen, Courtaboeuf, France).

The concentration of RNA was measured by a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Illkirch, France) and the purity was estimated by A260/A280 ratio. RNA integrity was assessed by using the Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). The threshold of the RNA Integrity Number (RIN) was set at 7.5 to validate sufficient quality of the RNA.

Microarray analysis

Gene expression analysis was performed by One-color microarray-based analysis using the porcine (V2) Gene Expression Microarray, 4×44K (Agilent: G2519F-026440). 100 ng of total RNA was labelled with Low Input Quick Amp Labelling Kit following the manufacturer's instructions. The quality of the Cy-3 labelled cRNA was checked using the NanoDrop and the yield and specific activity were calculated. Hybridization and scanning were done following the protocol described by Agilent. Signal intensities obtained were extracted using Feature Extraction software version 12.1.

Differential expression analysis and pathway analysis

The extracted data were analyzed using Partek® Genomics Suite® version 7. Examination of differentially expressed genes (DEG) was evaluated, as well as outliers and batch effect assessment, using PCA plots and hierarchical clustering.

The analysis was based on 3 experimental group comparisons for both periods:

- Supplementation of vitamin B2 at 50mg/kg (group B) versus control (group A)
- Supplementation of vitamin B2 at 100mg/kg (group C) versus control (group A)
- Supplementation of vitamin B2 at 100mg/kg (group C) versus supplementation of vitamin B2 at 50mg/kg (group B)

The pathway analysis for the three comparisons at each time point was performed using the IPA (Ingenuity Pathway Analysis) software from Qiagen, against the human pathway database based on orthologous genes alignment from the list of significant DEG.

qPCR confirmation of selected genes

Analyses of selected target genes were performed by quantitative PCR to validate microarray results. The reverse transcription was performed using iScript™ Reverse Transcription Supermix for RT-qPCR (BioRad, Marnes-la-Coquette, France) with 100 ng of total RNA, following the manufacturer's instructions. The resultant cDNAs were diluted 5 times to be amplified by real-time PCR. qPCR reactions were performed for all individual samples (n=8 per treatment) in triplicate, with 20 µL reaction volume, using SsoAdvanced Universal Sybr Green Master Mix (BioRad, Marnes-la-Coquette, France), with a final concentration of 500 nM of each primer. Sequences of the primers used are given in supplementary Table 1. The thermal cycling was run on a Light Cycler 96 (Roche Diagnostics, Meylan, France) with the following program: 95°C 30 sec, followed by 40 cycles of denaturation at 95°C 15 seconds and hybridization/elongation at 60°C 60 seconds. A melting curve was performed to check the amplicon specificity. Analysis of qPCR data was carried out by averaging the three technical replicates of the Ct values for each of the 8 samples per treatment. Each mean Ct value of the target genes was normalized to the geometric mean Ct value of the housekeeping genes for the same sample. The Delta Ct method was used to determine expression of target genes (24).

Results presentation and statistical analyses

The significance of gene expression from the microarray analysis was assessed by a one way ANOVA and the P-values were tested for

false discovery rate (FDR) using the Benjamini-Hochberg method with Partek® Genomics Suite® version 7 (25). All 8 samples per group were used. A cut-off of fold changes was set at >1.5 or <-1.5 and unadjusted P-value at 0.05, comparing all pairs of conditions; vitamin B2 50mg/kg vs Control, vitamin B2 100mg/kg vs Control and vitamin B2 100mg/kg vs vitamin B2 50mg/kg. Differentially expressed genes (DEG) were presented in Venn diagram format and hierarchical clustering is illustrated by a heatmap. Selected individual gene expression results were presented in tables with P-values based on student-test.

For the pathway analysis, as with the differential expression analysis, significance values were FDR tested using Benjamini-Hochberg. The pathways were significantly enriched if they had FDR adjusted P-values of less than 0.05.

The significance of gene expression from the qPCR results was evaluated by student test by comparing the groups supplemented with riboflavin compared to control group (Supplementary table 2).

RESULTS AND DISCUSSION

Riboflavin determination in digesta

Riboflavin was detectable in the ileum and caecum of weaned pigs fed diets supplemented with different levels of Vitamin B2 after 14 and 28 days. Riboflavin flow linearly increased at the ileal and caecal level ($P < 0.001$ and $P = 0.002$, respectively) after 14 days of dietary vitamin B2 at both 50 and 100 ppm supplementation compared to the control group. This indicates that vitamin B2 reached the end of the small intestine and the beginning of the hind gut (Table 2). However, after 28 days of supplementation, a larger variation was noted between animals, and riboflavin flow values were not significantly affected by vitamin B2 supplementation. In addition, the control group animals showed higher caecum flows of Riboflavin compared to ileum flows, independently of the time point, suggesting that bacterial populations present in the caecum of pigs were producing vitamin B2. Nevertheless, in the groups supplemented with 50 and 100 ppm of riboflavin this effect was not so evident. Since we could not discriminate between the dietary and the bacterial riboflavin, we could speculate that microbial population in the caecum has been modulated by piglet diets supplemented with super-dosing of vitamin B2 as Biagi et al. (10) demonstrate it in a broilers chicken trial.

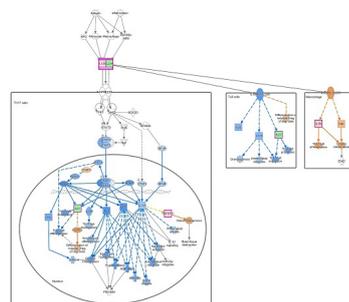
Supplementary table 2: Confirmation of microarray gene expression by qPCR

Gene	Vit B2 50mg/kg vs Control				Vit B2 100mg/kg vs Control			
	Microarray		qPCR		Microarray		qPCR	
	Fold change	p-value	Fold change	p-value	Fold change	p-value	Fold change	p-value
HSPH1	-1.79	8.90E-05	-1.64	0.003	-1.75	2.20E-05	-1.55	0.002
HSP70.2	-1.49	0.034	-1.38	0.008	-1.64	0.014	-1.58	4.26E-04
HSP90A1	-1.72	4.80E-06	-1.35	0.008	-1.67	8.00E-06	-1.46	1.93E-04
MX1	-1.57	9.30E-04	-1.66	0.004	-1.15	0.156	-1.17	0.186
TLR7	-1.56	0.069	1.00	0.97	-1.72	0.117	-1.02	0.888
IL12B	1.72	0.052	2.96	0.034	1.01	0.984	1.84	0.039
NFE2L2	-1.22	0.026	-1.07	0.484	-1.61	4.80E-05	-1.23	0.019
SLC51B	-1.89	0.027	-1.76	0.023	-1.56	0.062	-1.49	0.029

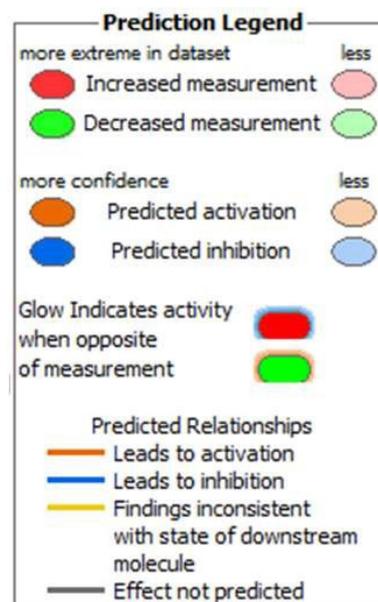
Determination of differentially expressed genes (DEG) and sample clustering

When looking at the overall number of DEG, at day 14 it was evident that the comparison between the group receiving 100mg/kg of riboflavin compared to the control group had the highest

number of DEG before FDR correction (1828, Figure 1A). Furthermore, the comparison between the group with 50mg/kg of riboflavin and the control group revealed 604 genes differentially expressed. These results showed a dose response of dietary riboflavin on caecal gene expression, with more differentially expressed genes at the highest dose. A similar comparison was performed at day 28 (Figure 1B), where the number of DEG was again highest between the group fed the highest dose of riboflavin compared to the control group. A total of 958 genes were differentially expressed between these groups, confirming the dose response effect of dietary riboflavin on caecal gene modulation. However, the effect on the number of DEG before FDR correction was lower at day 28 than at day 14, indicating an increased impact of riboflavin during the earlier period of supplementation, in line with the results shown on riboflavin flow. After FDR correction, the number of DEG was too low to interpret any data when the group with 50mg/kg of riboflavin was compared to either the control group or to the group with 100mg/kg of riboflavin. However the comparison of the group receiving 100mg/kg of riboflavin to the control group resulted 521 DEG after FDR correction, showing that the highest dose of supplementation still impacted the hind gut. In order to compare the data between experimental groups at the same statistical level, further investigation presented in this paper were based on non-adjusted P-value data.

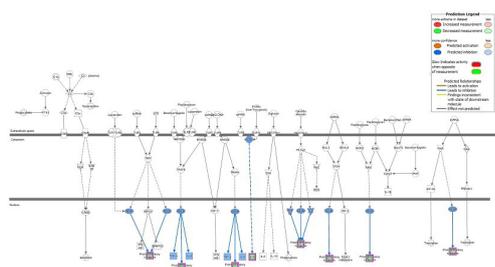


Supplementary Figure 1: IL-23 signaling pathway.



To investigate if different doses of riboflavin supplementation changed gene expression in the hind gut in a consistent way,

whole gene expression pattern clustering was performed in the caecal tissue by creating heatmaps. At day 14, clustering of samples based on the dosage of riboflavin could be observed (Figure 2A). The clearest differentiation was observed between the control group and samples from the groups receiving additional riboflavin. Interestingly, the gene expression of the highest supplemented group was mostly downregulated (indicated in green in the figure). At day 28, a similar clustering was observed, with the clearest separation between the control group and the groups receiving additional riboflavin (Figure 2B).



Both the DEG determination and the clustering analysis confirmed that the super-dosing of riboflavin had an impact on gene expression patterns in the caecal tissue of pigs. Additionally, a dose-response could be observed with the highest supplementation (100 mg/kg) showing a larger differentiation to the control, compared to the group supplemented at 50 mg/kg.

In the following sections, the focus will be on the comparison between 100mg/kg and the control group.

Comparing gene expression pathways of super-dosing Vitamin B2 (100mg/kg) versus Control at day 14

Genes most modulated

At day 14, the genes most significantly modulated between the control and vitamin B2 super-dosing showed a strong antioxidant response with 5 out of the top 10 genes that are known to be related to oxidative stress response (HSPH1, TIMM21, NFE2L2, LYAR and RAD17, (26–30)) (Table 3). Interestingly, all these genes were downregulated in supplemented versus control animals. Heat shock proteins (HSPs) contribute to cellular protection and cell survival against environmental stress, and HSPH1 avoids accumulation of denaturated proteins in cells under stress (31,32). Different studies in chickens and pigs show that HSPs are sensitive to different stress conditions (high stocking density, temperature, weaning, transport) and showed that feed supplementation with vitamins or essential oils can reduce HSP gene expression (33–35).

Supplementary table 3: Top 10 DEG between group with 100mg/kg and group with 50mg/kg of vitamin B2 at day 14

Gene	p-value (Vit B2 100mg/kg D14 VS Vit B2 50mg/kg D14)	Fold-Change (Vit B2 100mg/kg D14 VS Vit B2 50mg/kg D14)
SCIN	0.0003	-1.81
MASP2	0.0003	-1.69
TEX47	0.0004	-2.86
PDE3B	0.0007	-1.94
RGN	0.001	-1.72
IFNA4	0.0011	-1.60
DGKK	0.0012	-2.15
CD55	0.0015	-1.57
ADGRB3	0.0019	-1.80
TNFSF8	0.002	-1.82

This is of importance especially in weaning piglets, as they have an imbalanced and immature antioxidant system in the intestine and are therefore easily attacked by oxidative stress which impairs intestinal function, intestinal turnover, and cell survival (36).

Pathway analysis

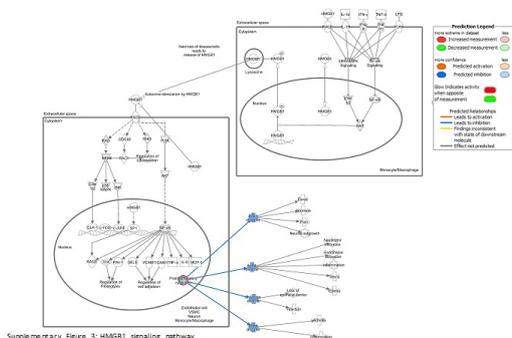
Using KEGG pathway analysis, the most impacted pathways were determined and showed that most were related to HSPs response (Table 4).

Supplementary table 4: Top modulated pathways between group with 50mg/kg of vitamin B2 and control group at day 14

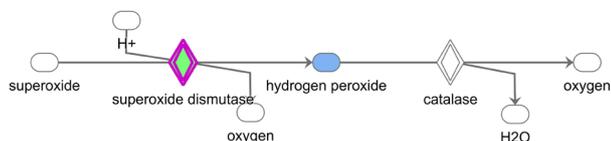
Canonical Pathways	-log(p-value)	Ratio	Involved modulated genes
IL-23 Signaling Pathway	5.06	0.114	IL12B,IL11B,IL21,IL23A,TNFSF11
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	4.45	0.0493	DDX58,IL12B,IL18,IL21,OAS1,TLR7,TNFSF11
Interferon Signaling	4.2	0.118	ISG15,MX1,OAS1,PSMB8
Th17 Activation Pathway	3.72	0.0602	HSP90AA1,IL12B,IL18,IL21,IL23A
Role of Cytokines in Mediating Communication between Immune Cells	3.57	0.0816	IL12B,IL18,IL21,IL23A
LXR/RXR Activation	3	0.042	IL1B,LPL,NR1H4,S100A8,TTR
FXR/RXR Activation	2.9	0.04	IL1B,LPL,NR1H4,SLCS1B,TTR
BAG2 Signaling Pathway	2.64	0.0732	HSP90AA1,HSPA5,HSPA6
Aldosterone Signaling in Epithelial Cells	2.52	0.0327	DNAJC6,HSP90AA1,HSPA5,HSPA6,HSPH1
Granulocyte Adhesion and Diapedesis	2.4	0.0307	CLDN16,CLDN22,HRH4,IL1B,MMP12
Unfolded protein response	2.28	0.0545	HSPA5,HSPA6,HSPH1

The “Aldosterone Signaling in Epithelial Cells” pathway (KEGG reference) showed the most significant modulation between the control and super-dosing riboflavin group. All genes modulated in this pathway were significantly down-regulated compared to the control group (Figure 3), showing a clear antioxidant effect of riboflavin in the caecum through the down-regulation of the expression of genes encoding members of the HSPs, such as DNAJC5B, HSP90AA1, HSP90B1, HSPA12A, HSPA5, HSPA6, HSPD1 and HSPH1. The HSP70 (which contains the HSPA12A, HSPA5 and HSPA6 genes) is one of the key proteins for protein folding and against heat stress, but also against oxidative stress. The HSP90 also responds to stress conditions by maintaining cell homeostasis (31,37–41). In addition, down-regulation of the SCN-N1G gene was observed in this pathway. The SCNN1G encodes an epithelial sodium channel (ENaC), and is responsible for the flow of Na⁺ ions from the lumen to the intestinal cell, a process which is known to be regulated by aldosterone (42). Aldosterone consequently regulates water absorption in the gut (43,44) and low levels of aldosterone may reduce activation of the HSP, reduc-

ing the reactive oxygen species (ROS) (45). Taken together, the hypothesis arises that a high level of riboflavin in the diet would impact the level of aldosterone, which would be beneficial to the animals by regulating the water in the intestinal content and reducing oxidative stress.



Another significantly altered pathway worth highlighting was the FXR/RXR pathway (Figure 4). All the related genes modulated in this pathway were downregulated and involved in the response (46), transport (47–49) or metabolism (50) of bile acids (BAs). This pathway plays a crucial role in the signalling and regulation of bile acids, which governs bile, glucose and lipid metabolism (46,48). Excess BAs entering the colon can cause watery stool (51). The downregulation of this pathways might indicate a reduction in BAs escaping enterohepatic circulation into the caecum.



This pathway analysis suggested that super-dosing riboflavin could potentially regulate water content in the lumen by both aldosterone and bile acid metabolism, which would have beneficial effects on the gastrointestinal functionality of animals. These findings are in accordance with the beneficial effect of riboflavin on gut health (52), however the link to bile acid metabolism is new and requires further investigation. A reduction of watery faeces would be very relevant for the swine industry especially to combat the current issues of diarrhoea at weaning and during stress conditions (53–55).

Other comparisons at day 14

Genes most modulated

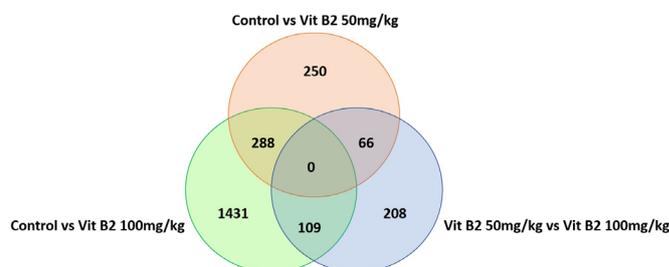
The most significantly altered genes in animals supplemented with 50 mg/kg of vitamin B2 compared to the control are presented in supplementary Table 2, and showed a similar response in the down-regulation of heat shock proteins (HSPH1, HSP90AA1 and HSP70.2). These observations confirm the positive effect of riboflavin supplementation on oxidative stress in the caecum.

Comparing the two super doses of riboflavin (100mg/kg versus 50mg/kg), the highest dose showed a downregulation of genes involved in modulation of the immune response (supplementary Table 3). The most significantly regulated genes were involved in innate immunity through the regulation of complement factors (MASP2, CD55) (56,57) and the antiviral activity (IFNA4) (58), *Transcriptomics 2020, Vol.7 Iss.4 DOI:10.4172/2329-8936.1000e152*

or adaptive immunity (TNFSF8) (59). In previous studies, riboflavin enrichment is known to have immunomodulatory effects (60,61).

Pathway analysis

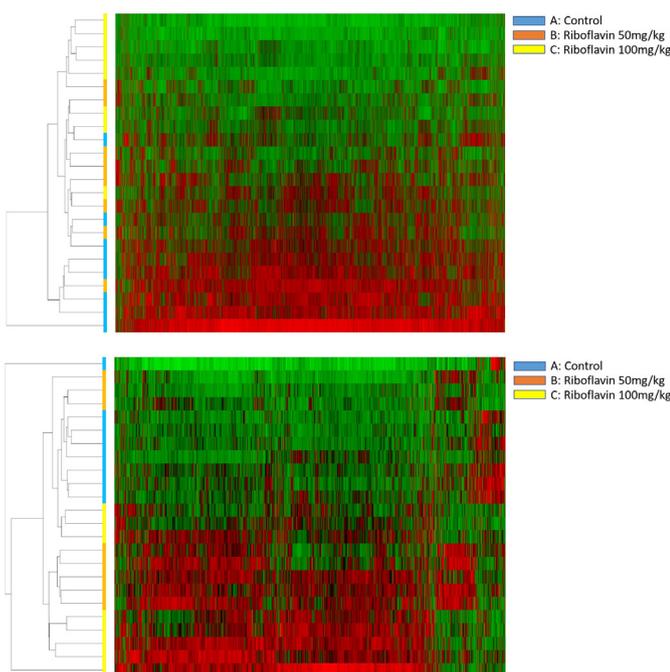
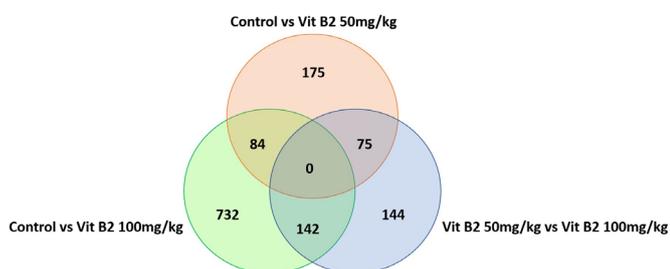
Pathway analysis comparing animals receiving 50mg/kg to the control, showed that the most impact was related to the immune response (supplementary Table 4). The most significantly impacted pathway was the IL-23 signalling pathway, showing an up-regulation of IL-1B, IL12B and TNFSF11 compared to the control group. However, IL23A, IL21 and TLR7 were down-regulated. The overall modulation of this pathway would reflect a down-regulation of the TH17 response and antimicrobial response, depicted by the blue colour in supplementary Figure 1. We hypothesize that this might reflect the host's response to modulation of the caecal microbiota induced by riboflavin. More research is needed to confirm this hypothesis.



The pathways modulated between the two super doses of riboflavin are presented in the supplementary Table 5. The pathways “Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses” and “HMGB1 signaling pathway” were characterized by the same list of modulated genes and were downregulated in animals receiving the highest dose of riboflavin (supplementary Figures 2 & 3). These two pathways are closely related, as the mechanism of binding to TLR2 and TLR4 mediates HMGB1-dependent activation of macrophage cytokine release (62). Additionally, HMGB1 is able to interact with TLR4 on neutrophils to stimulate the production of reactive oxygen species (ROS) by NADPH oxidase (63). In relation to ROS, the gene encoding the NQO1 was also down-regulated with the highest dose of vitamin (supplementary Table 5 and supplementary Figure 4). Therefore, the higher dose of 100 mg/kg riboflavin seems to have a strong impact on the genes interacting with the microbiota, and ROS. These findings might confirm that riboflavin alters the microbiome and thereby influences gene expression. We hypothesize that riboflavin modulates the most abundant colonic bacteria, *F. prausnitzii* (16,18), a butyrate producer, which can stimulate the intestinal epithelium. This would also be in line with the observations from Biagi et al, who show that after 14 days of supplementation, a super-dose of vitamin B2, at 100mg/kg diet, in broilers increases the abundance of bacteria belonging to the genus *Faecalibacterium* (10). In this same study at days 28 and 42, more butyrate is still produced in supplemented animals, but a switch of the microbial populations is observed; an increase of the lactate producer *Bifidobacterium* but there is no longer a difference in *Faecalibacterium* populations, compared to control group. Thus, the lactate produced could be utilized by butyrate producers other than *Faecalibacterium*. In our study, this hypothesis would require further investigation, by investigating the modulation of the

caecal microbiome in response to riboflavin super-dosing.

Comparing gene expression pathways of super-dosing Vitamin B2 (100mg/kg) versus Control at day 28



Genes most modulated

At day 28, the genes most significantly modulated between control and vitamin B2 super-dosing are presented in Table 5. In contrast to the pattern at day 14, all the genes were up-regulated compared to the control. No clear common determinant could be identified between the most significantly regulated genes, several genes were involved in non-specific and various processes such as protein modification (ART3), or transcription and translation process (RBM24, DIP2C).

Supplementary table 5: Top modulated pathways between group with 100mg/kg of vitamin B2 and group with 50 mg/kg at day 14.

Canonical Pathways	-log(p-value)	Ratio	Involved modulated genes
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	3	0.0282	FASLG, IL12B, TNFSF11, TNFSF8
HMGB1 Signaling	2.84	0.0255	FASLG, IL12B, TNFSF11, TNFSF8
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	2.64	0.087	DEFB1, IL12B
Complement System	2.25	0.0556	CD55, MASP2
IL-23 Signaling Pathway	2.08	0.0455	IL12B, TNFSF11
Xenobiotic Metabolism Signaling	2.05	0.0153	GAL3ST2, GCLC, NQO1, PPP2R2C
Superoxide Radicals Degradation	1.61	0.125	NQO1

Pathway analysis

In animals supplemented with 100mg/kg of Riboflavin, the pathway analysis showed several modulations of different physiological processes such as calcium signalling, ILK signalling, and inflammation related pathways such as IL-6 signaling (Table 6).

Supplementary table 6: Top 10 DEG between group with 50mg/kg of vitamin B2 and control group at day 28

Gene	p-value (Vit B2 50mg/kg VS Control D28)	Fold-Change (Vit B2 50mg/kg vs Control D28)
TEX47	1.52E-04	3.83
DGKK	6.96E-04	1.95
TRPM6	1.84E-03	2.14
MAT1A	2.28E-03	-1.89
MAFA	4.04E-03	1.56
AOX2	4.87E-03	1.71
TFR2	5.12E-03	-1.50
MTTP	5.46E-03	2.07
ULK4	5.82E-03	1.53
FBXO32	5.86E-03	1.51

The calcium signalling pathway links back to the finding at day 14, as many Ca²⁺ channels at the cell surface and intracellular organelles, including the endoplasmic reticulum and mitochondria, are regulated by redox modifications. In turn, Ca²⁺ signaling can influence the cellular generation of ROS (64).

Other comparisons at day 28

Genes most modulated

The most significantly altered genes between animals supplemented with 50mg/kg of vitamin B2 compared to the control are presented in supplementary Table 6, and included genes related to lipid metabolism (DGKK, MTTP)(65,66), regulation of ROS (AOX2)(67) and iron metabolism (TFR2)(68). Interestingly, the latter is known to be modulated by the riboflavin at the gut level (69).

Comparing the two different super doses of riboflavin showed no clear pattern in the most significantly altered genes (supplementary Table 7).

Supplementary table 7: Top 10 DEG between group with 100mg/kg and group with 50mg/kg of vitamin B2 at day 28

Gene	p-value (Vit B2 100mg/kg VS Vit B2 50mg/kg D28)	Fold-Change (Vit B2 100mg/kg VS Vit B2 50mg/kg D28)
HAND1	4.49E-05	2.11
DGKK	1.44E-04	-2.32
RBPM5	1.70E-04	1.66
TEX47	3.28E-04	-3.19
RPRM	6.55E-04	2.15
ITGBL1	6.84E-04	2.19
MAPK10	7.34E-04	2.18
ART3	8.94E-04	1.86
KCNG1	1.17E-03	1.61
TPM1	1.46E-03	1.80

Pathway analysis

Pathway analysis comparing animals receiving 50mg/kg to the control animals, showed a lower number of genes than at day 14 involved in the modulation of pathways (supplementary Table 8). However, the comparison between the two super doses of riboflavin showed modulation of more pathways (supplementary Ta-

ble 9), suggesting that after 28 days of supplementation only the higher dose of riboflavin was capable of altering transcriptomic pathways in the caecum.

Supplementary table 8: Top modulated pathways between group with 50mg/kg of Vitamin B2 and control group at day 28

Canonical Pathways	-log(p-value)	Ratio	Involved modulated genes
DNA damage-induced 14-3-3 sigma Signaling	2.91	1.05E-01	AKT3,SFN
Glucose and Glucose-1-phosphate Degradation	2.87	1.00E-01	PGM1,RGN
LPS/IL-1 Mediated Inhibition of RXR Function	2.44	1.75E-02	ABCA1,ABCB1,FABP7,UST
Inhibition of ARE-Mediated mRNA Degradation Pathway	2.34	2.46E-02	AKT3,PPP2R2C,SFN
p70S6K Signaling	2.21	2.22E-02	AKT3,PPP2R2C,SFN
P2Y Purigenic Receptor Signaling Pathway	2.20	2.19E-02	ADCY10,AKT3,P2RY2
Xenobiotic Metabolism Signaling	2.08	1.38E-02	ABCB1,ESD,PPP2R2C,UST
Wnt/beta-catenin Signaling	1.91	1.72E-02	AKT3,CDH12,PPP2R2C
CXCR4 Signaling	1.91	1.71E-02	ADCY10,AKT3,CXCR4
PI3K/AKT Signaling	1.88	1.68E-02	AKT3,PPP2R2C,SFN

Supplementary table 9: Top modulated pathways between group with 100mg/kg and group with 50mg/kg of Vitamin B2 at day 28

Canonical Pathways	-log(p-value)	Ratio	Involved modulated genes
Calcium Signaling	8.25	5.58E-02	ACTA1,ACTC1,CASQ2,GRIA1,GRIA4,GRIK MYH2,MYL2,TNNC1,TPM1,TPM2
GP6 Signaling Pathway	3.48	4.31E-02	COL1A1,COL1A2,COL6A3,COL8A1,LAMA
ILK Signaling	3.41	3.24E-02	ACTA1,ACTC1,MAPK10,MYH2,MYL2,PTG
Prostanoid Biosynthesis	2.94	2.00E-01	PTGIS,PTGS2
Intrinsic Prothrombin Activation Pathway	2.91	7.32E-02	COL1A1,COL1A2,CLK15
Glutamate Receptor Signaling	2.52	5.36E-02	GRIA1,GRIA4,GRIK1
Gap Junction Signaling	2.49	2.59E-02	ACTA1,ACTC1,GRIA1,GRIA4,GRIK1
MSP-RON Signaling Pathway	2.48	5.17E-02	ACTA1,ACTC1,CLK15
Citrulline Degradation	2.29	1.00E+00	OTC
Epithelial Adherens Junction Signaling	2.13	2.68E-02	ACTA1,ACTC1,MYH2,MYL2

CONCLUSION

In this study, super-dosing vitamin B2 showed a clear impact on the caecal transcriptome, at either 50 or 100 mg/kg of vitamin B2. The combination of the transcriptomic data and the riboflavin level measured in ileal and caecal content proved that the riboflavin fed at a high dosage reached the hind gut of piglets, and triggered a dose-response effect on host gene expression.

The strongest modulation was observed at the highest dose of vitamin B2, after only 14 days of supplementation. As several genes encoding heat shock proteins were downregulated by riboflavin supplementation, the main effect of riboflavin on gene expression in the caecum appears to be related with antioxidant capabilities. Additionally, pathways of inflammation emerged in the data analysis, suggesting an immune modulation by vitamin B2. This transcriptomic data also provided new insights on the possible mechanisms behind the positive gastrointestinal health benefits of riboflavin, linked to aldosterone or bile acid metabolism.

In conclusion, super dosing riboflavin in piglet diets could be considered as a promising solution to optimize gastrointestinal functionality in weaned piglets.

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