

# The Influence of Apple- or Red-Grape Pomace Enriched Piglet Diet on Blood Parameters, Bacterial Colonisation, and Marker Gene Expression in Piglet White Blood Cells

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## ABSTRACT

*Proanthocyanidins and flavanoids, both subfamilies of the polyphenols, are highly concentrated in different fruits and berries as well as in fruit pomace. They have shown to exhibit anti-cancer, anti-microbial, anti-oxidative, and immune-modulatory effects in vertebrates. Herein the effect of additional apple pomace or red-grape pomace in conventional piglet starter feeds were investigated in 36 young growing piglets. Immunological marker gene expression was quantified by quantitative real-time RT-PCR in white blood cells, and intestinal bacterial flora was investigated from weaning to three weeks post weaning. Polyphenol content in red-grape pomace, gut content and tissues were analyzed with HPLC. Flavan-3-ols (epicatechin and catechin) and proanthocyanidins (B1, B2 and C1) were identified in the gastro-intestinal tract content, whereas only traces could be detected in various piglet organs. The blood parameters, hemoglobin and hematocrit, were affected and down-regulated in all groups over testing period. In both pomace treated groups more thrombocytes were present compared to the standard feeding group. It turns out, that the pomace diets had greatest impact on the bacterial content in the colon. Results demonstrate that feeding apple pomace and red-grape pomace tended to increase the number of total colonic bacteria. *Streptococci/Enterococci* increased in the red-grape pomace. *C. perfringens* was not detectable at the second time point. The number of lactobacilli increased in both applied diets. The number of *Clostridium perfringens* decreased with the age of the piglets. Trends of mRNA expression changes were found in white blood cell (WBC) between different feeding regimens, since the expression variability in the groups was very high. Between the different time points there were significant differences within the apple pomace group, where *TNF $\alpha$*  ( $p = 0.033$ ), *NF $\kappa$ B* ( $p = 0.024$ ) and *Caspase 3* ( $p = 0.019$ ) mRNA expression increased significantly during treatment. We conclude that both polyphenol rich feedings have the potential to positively influence the intestinal flora, blood parameters, and WBC mRNA gene expression pattern of immunological marker genes.*

**Keywords:** Apple Pomace, Red-Grape Pomace, White Blood Cells, mRNA Gene Expression, Blood Parameters, Bacterial Colonization

## 1. Introduction

Grapes and apples contain a large amount of different phenolic ingredients in skins, pulp and seeds, that are only partially extracted, e.g. during wine making process [1]. Therefore high polyphenol content (up to 80%) remains in the pomaces [2-4] and these feeds may therefore be of value in human and animal nutrition. The main phenolic compounds in grape seed [5] are catechin and

epicatechin. The monomeric flavan-3-ols epicatechin and catechin and their oligomeric proanthocyanidins B1, B2 and C1 are concentrated in several fruits and berries. They as well present the major flavonoids in grape skins in addition to various anthocyanins [6]. Polyphenols have been shown to reduce certain types of cancer and the incidence of cardiovascular diseases. They increase the plasma antioxidant capacity and are thought to inhibit

oxidation of LDL [7]. Polyphenols can reduce the systolic pressure and the level of plasma cholesterol in humans and animals. Further they inhibit platelet aggregation both in-vitro and in animal experiments and may thereby prevent thrombosis [8].

Since January 2006 the European Union laws against prophylactic use of antibiotics in animal feed have come into effect (Regulation 1831/2003/EC). It is generally accepted that the use of antibiotics may potentially result in antibiotic residues in the tissue of treated animals, but in recent years, the greatest concern has been the use of antibiotics to support growth. Long-term use of antibiotics in animal diets may select for resistant bacteria which in turn may transfer resistance to other bacterial species [9,10]. Four mechanisms have been suggested to underlie the effects of antibiotics on animal growth: [1] inhibition of subclinical infections, [2] reduction of growth-depressing microbial metabolites, [3] reduction of microbial use of nutrients, and [4] enhanced uptake of nutrients through a thinner intestinal wall [11-13].

The goal of this approach was to test the influence of two different polyphenol rich feeding regimens (freeze dried apple- and red-grape pomace) in young growing piglets. Effects on fecal flora, various blood parameters and mRNA marker gene expression in white blood cells (WBC) were determined after weaning.

## 2. Material and Methods

### 2.1. Experiment Setup and Tissue Sampling

Three groups of 12 cross breed piglets [Pietráin x (Deutsche Landrasse x Deutsches Edelschwein)] were fed three different diets. The animals were housed at the experimental station Osterseeon (Bayerische Landesanstalt für Landwirtschaft, Institut für Tierernährung und Futterwirtschaft). Twelve animals were fed a standard weaning diet (CD) (50% wheat, 23% barley, 22% soy, 1% soy oil, 4% vitamins and minerals), whereas the experimental diets contained in addition 3.5% apple pomace (APD) and red-grape pomace (RGPD) respectively on a dry matter basis (**Table 1**). Energy, protein, fat and starch content were balanced in all three feeding groups. At weaning, piglets were assigned to the three treatments on the basis of age ( $31 \pm 1.8$  days), weight ( $7.5 \pm 1.1$  kg) and sex (50% male, 50% female). Piglets were weighed at the age of 31 days and at day 50. On the day of weaning (at the age of 31 days) and 19 days later (at the age of 50 days) six piglets per treatment were slaughtered.

Piglets were killed by an electric gripper and exsanguinated. Blood was sampled in tubes containing EDTA during exsanguination. A horizontal incision along the midline was made to open the abdominal cavity, and all the internal organs were excised. Samples of three piglets

having received the RGPD diet for 19 days were sampled stomach content, colon content, liver, kidney and ileum for later polyphenol analysis. Samples were frozen at  $-20^{\circ}\text{C}$  until polyphenol extraction. Additionally, pure red-grape pomace and piglet starter with red-grape pomace were sampled.

### 2.2. Sample Preparation

Polyphenol containing samples were lyophilized for four days. One gram dry sample was mixed with 10 ml 100% MeOH and crushed with an Ultra Turrax (Jahnke and Kunkel, IKA, Staufen, Germany). Additionally the piglet starter with red-grape pomace was extracted with water and with HCl (pH 2). The mixture was treated for 30 minutes in an ultrasonic waterbath at  $4^{\circ}\text{C}$  and centrifuged for 10 minutes. The supernatant was poured into another cup and frozen at  $-20^{\circ}\text{C}$  until HPLC analyses.

### 2.3. HPLC Analyses

The HPLC equipment used consists of an autosampler (Gilson-Abimed Modell 231, Langenfeld, Germany), of two pumps (Kontron Modell 422, Eching, Germany), and a diode array detector (Bio Tek Kontron 540 m Eching, Germany). For post column derivatisation a further analytical HPLC pump (Gynkotek Modell 300 C, Germering, Germany) and a VIS-detector (640 nm, Kontron Detektor 432, Eching, Germany) were used. The column ( $250 \times 4$  mm I.D., Macherey-Nagel, Düren, Germany) was pre-packed with Shandon Hypersil ODS 3  $\mu\text{m}$ . The solvents were 5% acetic acid (A) and methanol (B). Gradient range: 0 - 5 min, isocratic, 5% B in A; 5 - 10 min, 5 - 10% B in A; 10 - 15 min, isocratic, 10% B in A; 15 - 35 min, 10% - 15% B in A; 35 - 55 min, isocratic, 15% B in A; 55 - 70 min, 15% - 20% B in A; 70 - 80 min, isocratic, 20% B in A; 80 - 95 min, 20% - 25% B in A; 95 - 125 min, 25% - 30% B in A; 125 - 145 min, 30% - 40% B in A; 145 - 160 min, 40% - 50% B in A; 160 - 175 min, 50% - 90% B in A; 175 - 195, isocratic, 90% B in A; 195 - 210, 90% - 5% B in A; 210 - 235 min, isocratic, 5% B in A [14]. Phenolic acids and flavonols were detected at 280 nm whereas the flavan 3-ols were measured at 640 nm after post column derivatisation with p-dimethyl-amino-cinnamic aldehyde (DMACA) [15]. 6-methoxyflavone was used as internal standard for quantitative analyses.

The single compounds were identified in triplicates by retention times and their UV-absorbance spectra via diode array detection and by comparison with standards. These standards were either commercially available from Roth and Sigma (catechin, epicatechin, chlorogenic acid, phloridzin, rutin) or previously isolated from apple and service tree: procyanidins B1, B2, B5, C1, phloretin derivatives, hydroxycinnamic acids, quercetin glycosides

**Table 1. Dry matter, protein, fat, fiber, Nfe (N-free extract), ME (metabolisable energy) of three diets in g per kg dry matter. The feeding was according to GFE and “Gruber Futterwerttabelle” (www.lfl.bayern.de/Tierernahrung/schwein).**

	dry matter	crude protein	crude lipids	crude fiber	Nfe	ME	Ca	P	Na	K	Mg	Cu	Zn	Lys	Met	Cys	Thr	Trp
feed	[g]	[g]	[g]	[g]	[g]	MJ	[g]	[g]	[g]	[g]	[g]	[mg]	[mg]	[g]	[g]	[g]	[g]	[g]
control diet (CD)	878.0	188.8	25.2	38.3	564.6	12.93	8.4	5.1	2.0	7.6	2.5	179.4	182.4	10.5	3.5	4.6	6.9	2.2
apple-pomace diet (APD)	880.0	182.7	25.2	42.6	571.6	12.95	8.5	5.0	2.0	7.4	2.5	157.2	184.8	10.9	3.2	4.3	6.8	2.1
red-grape pomace diet (RGPD)	880.0	188.8	30.5	45.2	556.8	12.95	7.4	4.8	1.7	7.9	2.3	134.0	167.4	10.4	3.2	4.1	6.4	2.2

[14,16,17].

### 2.4. mRNA Extraction

Blood probes were collected with EDTA (0.2 ml EDTA per 10 ml total blood) for blood cell extraction. For white blood cell separation, 10 ml blood was mixed with 10 ml lysis buffer (0.83 g NH<sub>4</sub>Cl, 3.7 mg Na-EDTA, 1 g KCl in 100 ml, pH 7.4) and centrifuged for 10 min at 220 g. The cell pellet was suspended in 10 ml lyses puffer and centrifuged again. The white blood cell pellet of the 10 ml blood was then suspended in 100 µl TriFast. Total RNA of these white blood cells was isolated using TriFast (Pq-lab, Erlangen, Germany) according to the manufacturer’s instructions. To quantify the extracted RNA concentration, the optical density was determined in triplicates at three different dilutions of the final total RNA preparations at 260 nm. RNA integrity was verified by optical density OD<sub>260 nm</sub>/OD<sub>280 nm</sub> absorption ratio > 1.80.

### 2.5. Real-Time One-Step Quantitative RT-PCR

Relative quantification of mRNA concentration was carried out using a one-step quantitative RT-PCR (qRT-PCR) in the ep realplex Cyler (Eppendorf, Hamburg, Germany). To minimize pipetting errors an eight-channel pipetting robot epMotion 5075 (Eppendorf) was used. 25 ng mRNA in 1 µl volume was inserted as RT-PCR template. Further the master-mix components for the qPCR reactions [0.3 µl iScript (Bio-Rad, Munich, Germany), 0.225 µL (20 pmol) of forward and reverse primer (Table 2) synthesized by MWG Biotech (Ebersberg, Germany), 7.5 µl 2x SYBR Green (Bio-Rad) and up to 14 µl water] was assembled by the robot. One-step qRT-PCR was performed with 40 cycles and product-specific annealing temperature, according to the kit manufactures cycle settings. The crossing points (Ct) were acquired with the automatic Ct-method present in the ep realplex analysis software (Eppendorf). Amplification PCR products underwent a melting curve analysis after the last cycle to specify the integrity of amplification. Finally a cooling step was performed.

### 2.6. Relative mRNA Quantification

A relative quantification was applied, using a panel of physiological and immunological marker genes and Histone H3 as reference gene (Table 2). Single marker gene mRNA expression were normalized by the constant Histone H3 mRNA gene expression over the investigated feeding groups and time points, according to the ΔCP method [18,19], described by the following equations:

$$\Delta CP_{(day\ x)} = CP_{(Histone\ H3)} - CP_{(marker\ gene)}$$

$$\Delta\Delta CP = \Delta CP_{(day\ 50)} - \Delta CP_{(CD\ day\ 31)}$$

First the target gene expression Ct was normalized by the reference gene expression Ct (=>ΔCP). In the second step, the ΔCP value at day 50 was compared with the ΔCP value of the control diet treatment at day 31 (CD day 31), which results in the ΔΔCP value. Positive ΔΔCP values represent an mRNA up-regulation and negative ΔΔCP values represent a mRNA down-regulation of the described expressed marker gene at day 50, compared to day 31.

### 2.7. Hemogramm

A blood cell count including leukocyte differentiation was made at a veterinary laboratory (Vetmed Labor, Ludwigsburg, Germany). Erythrocyte, hematocrit, hemoglobin concentration, hemoglobin concentration per erythrocyte (MCHC), thrombocyte and differential white cell count were determined using the CELL-DYN 3700SL System (Abbott Diagnostika GmbH, Wiesbaden, Germany). Leukocytes were differentiated on blood smears stained with May Grünwald-Giemsa. The percentage of basophilic granulocytes, eosinophilic granulocytes, segmented and non-segmented neutrophilic granulocytes, lymphocytes and monocytes were calculated.

### 2.8. Bacterial Examination of GIT Flora

Chymus samples were taken from the colon immediately after slaughtering and stored on ice from the first and the last slaughtering group. Total aerobic and anaerobic bac-

teria were determined and *Streptococcus*, *Lactobacillus*, *E. coli* and *C. perfringens* were cultured using selective culture media performed at the Tiergesundheitsdienst Bayern e.V. (Grub, Germany). One gram chymus was mixed with 9 ml PBS (Table 3). This mixture was diluted by the factor  $10^1$  to  $10^{10}$ . Of each dilution 0.1 ml was pipetted on an agar plate. Agar plates with 3 to 300 bacteria were counted and the mean was calculated, according to Bollmann [20].

### 2.9. Statistical Evaluations

For the statistical evaluation of the data Sigma Stat 2.03 [21] was used. Data were compared with two way ANOVA (“diet group” and “time point”). *P* values less than 0.05 were considered significant ( $P < 0.05$ ).

## 3. Results

### 3.1. Daily Gain and Body Weight

All animals remained healthy during the feeding experiment and no animal losses were registered. Some of the piglets excreted pasty faeces, but none of the pigs got ill or were treated with veterinary drugs. Energy and feed intake and average daily gain did not differ between dietary treatments. At the different slaughter dates the piglets weighed  $7.5 \pm 1.1$  kg (day 31) and  $13.2 \pm 1.6$  kg (day 50). The empty body weight was not affected by the applied diets [22,23].

### 3.2. Polyphenol Analysis

Figure 1 shows the results from the polyphenol quanti-

fication, in the pure red-grape pomace, the RGPD feed, stomach and gut contents, as well as in three selected tissues (ileum, liver, and kidney). The content of the five major and dominant flavanoids are shown in the Figure 1, the monomers catechin and epicatechin, and the procyanidins B1, B2 and C1. All five compounds were recovered and could be quantified in the feed, in stomach and colon content. In ileum, liver and kidney only traces of catechin, B1 and B2 could be detected. The observed polyphenols are more highly concentrated in stomach and gut content, compared to the 3.5% RGPD mixed diet. The extraction with water and with HCl (pH 2), respectively, yielded comparable proportions of the flavonoids, but the total concentrations differ with the applied extraction methods.

### 3.3. Total RNA and mRNA Expression

Total leukocyte RNA contents showed no significant variations in RNA integrity and quantity between analysed feeding groups. All tested genes were abundant in leukocytes, showed single peaks in melting curve analysis (ep realplex software) and a single band in high-resolution 4% agarose gel electrophoresis (gels not shown). The reference gene Histon H3 mRNA expression remained constant during the entire study and was affected neither by the time ( $p = 0.770$ ) nor by the diet treatments ( $p = 0.615$ ). Beside the reference gene, five different marker genes were determined in one-step qRT-PCR: a pro-inflammatory marker (TNF $\alpha$ ), a transcription factor (NF $\kappa$ B), an anti-inflammatory marker (IL10), an apop-

Table 2. Sequence of the forward and reverse primers used for one-step qRT-PCR.

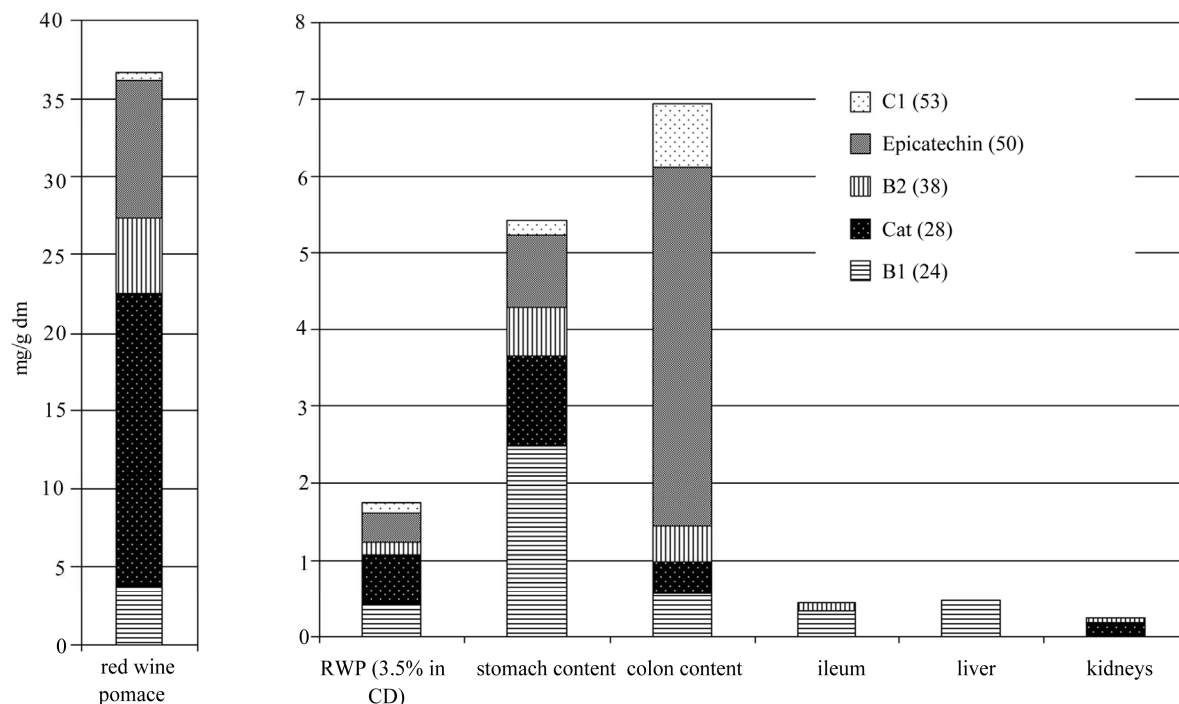
gene	forward primer	reverse primer
Histone H3	act ggc tac aaa agc cgc tc	act tgc ctc ctg caa agc ac
TNF $\alpha$	ccc cca gaa gga aga gtt tc	tgg gcc cct gaa gag gac
IL 10	act tta agg gtt acc tgg gtt g	gta gag acc cct ctc ttg ga
NFKB	ggt gga gaa ctt tga gcc tc	cca gag acc tca tag ttg tcc
Caspase3	tgt gtg ctt cta agc cat gg	agt tct gtg cct cgg cag
Cyclin D1	tcc tgt gct gcg aag tgg a	ggt cca ggt agt tca tgg c

Table 3. The media and culture methods for comprehensive investigation of intestinal flora.

bacteria	medium	incubation method
Aerobes	Columbia-Agar with 5% sheep blood	48 h, 37°C, aerobe
Anaerobes	Zeißler-Agar	48 h, 37°C, anaerobe
<i>E. coli</i>	Gassner-Agar	48 h, 37°C, aerobe
<i>Streptococci/Enterococci</i>	Edwards-Agar	48 h, 37°C, aerobe
<i>Lactobacilli</i>	Method of de Man, Rogosa, Sharpe	48 h, 37°C, anaerobe
<i>Clostridium perfringens</i>	Zeißler-Agar	48 h, 37°C, anaerobe

totic marker (caspase 3), and a proliferation and cell-cycle marker (cyclin D1). Trends of mRNA expression changes ( $0.05 < p < 0.10$ ) were found in white blood cell (WBC) between different feeding regimens, since the expression variability in the groups was very high (Table

4). Between the different time points there were significant differences within the APD, where  $TNF\alpha$  ( $p = 0.033$ ),  $NF\kappa B$  ( $p = 0.024$ ), and Caspase 3 ( $p = 0.019$ ) mRNA expression increased significantly over treatment time (Figure 2).

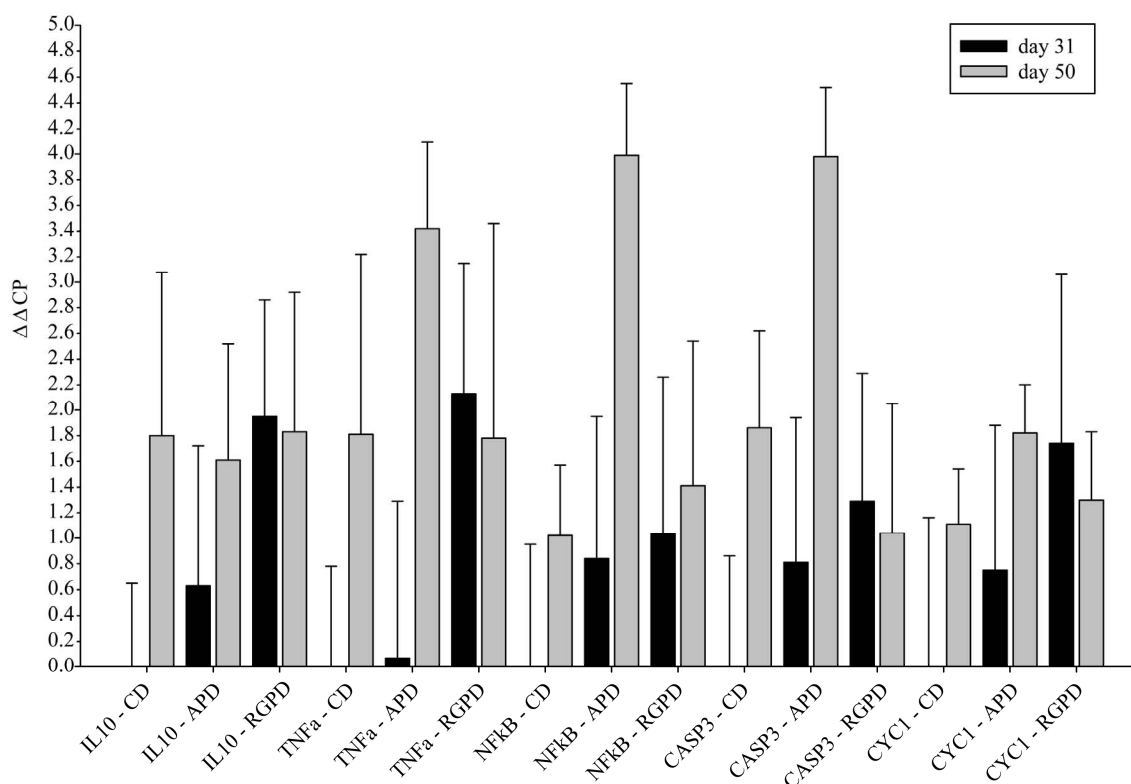


**Figure 1.** Content of the main Flavan-3-ols (epicatechin and catechin) and proanthocyanidins (B1, B2 and C1) in red-grape pomace, the RGPD feed, stomach and colon content and in ileum, liver and kidneys in mg/g dry matter (dm). The procyanidins C1, B2 and B1 and the monomer epicatechin were calculated as epicatechin, catechin as catechin.

**Table 4.** Marker gene mRNA expression in white blood cells (WBC) was quantified by quantitative RT-PCR. Data are shown as  $\Delta\Delta CP$  values from day 31 and 50 ( $n = 6$ ; mean  $\pm$  SD). All gene expression data were normalized to the mean control diet (CD at day 31) gene expression of the respective gene.

marker genes	feeding group	day 31	day 50
<b>IL10</b>	CD	0.00 $\pm$ 0.65	1.80 $\pm$ 1.28
	APD	0.63 $\pm$ 1.09	1.61 $\pm$ 0.91
	RGPD	1.95 $\pm$ 0.91	1.83 $\pm$ 1.09
<b>TNF<math>\alpha</math></b>	CD	0.00 $\pm$ 0.78	1.81 $\pm$ 1.41
	APD	0.07 $\pm$ 1.22 <sup>#</sup>	3.42 $\pm$ 0.68 <sup>#</sup>
	RGPD	2.13 $\pm$ 1.02	1.78 $\pm$ 1.68
<b>NFKB</b>	CD	0.00 $\pm$ 0.95	1.02 $\pm$ 0.55
	APD	0.84 $\pm$ 1.11 <sup>#</sup>	3.99 $\pm$ 0.56 <sup>#</sup>
	RGPD	1.03 $\pm$ 1.23	1.41 $\pm$ 1.13
<b>Caspase 3</b>	CD	0.00 $\pm$ 0.86	1.86 $\pm$ 0.76
	APD	0.81 $\pm$ 1.13 <sup>#</sup>	3.98 $\pm$ 0.54 <sup>#</sup>
	RGPD	1.29 $\pm$ 1.00	1.04 $\pm$ 1.01
<b>Cyclin D1</b>	CD	0.00 $\pm$ 1.16	1.11 $\pm$ 0.43
	APD	0.75 $\pm$ 1.13	1.82 $\pm$ 0.38
	RGPD	1.74 $\pm$ 1.32	1.30 $\pm$ 0.53

Superscript (#) indicates significant differences between treatment day 31 and day 50 ( $P < 0.05$ ).



**Figure 2.** Marker gene mRNA expression in white blood cells (WBC) in apple pomace diet (APD) and red-wine pomace diet (RGPD) treatment was quantified by quantitative RT-PCR. Data are shown as  $\Delta\Delta\text{CP}$  values from day 31 and 50 ( $n = 6$ ; mean  $\pm$  SD). All gene expression data were normalized to the mean control diet (CD at day 31) gene expression of the respective gene.

### 3.4. Blood Parameters

Results from the haematological examination are summarized in **Table 5**. The sex of the piglets had no significant influence on the different blood parameters. Only very few non-segmented neutrophilic granulocytes ( $0.00 \pm 0.00$ ), basophilic ( $0.32 \pm 0.78$ ) and eosinophilic granulocytes ( $0.48 \pm 1.05$ ) were counted, therefore no statistical calculation was made with these parameters (n. d.). The number of erythrocytes was higher in the CD compared to APD at the end of the feeding trial ( $p = 0.049$ ). The hemoglobin concentration decreased significantly over time in CD ( $p = 0.015$ ), APD ( $p < 0.001$ ) and RGPD group ( $p = 0.005$ ), as did the haematocrit (CD  $p = 0.001$ , APD  $p < 0.001$ , RGPD  $p < 0.001$ ). At the first time point we found more thrombocytes in RGPD than in CD ( $p = 0.019$ ), at the second time point more thrombocytes in APD ( $p = 0.042$ ) and RGPD ( $p = 0.029$ ) than in CD. The number of thrombocytes decreased over time in CD ( $p = 0.031$ ) and RGPD ( $p < 0.001$ ), in APD it was only a trend of decrease.

### 3.5. Bacterial Flora

**Table 6** summarizes the results of the bacterial examina-

tion in faeces samples. *Streptococci/Enterococci* increased in the CD ( $p = 0.002$ ) and in the RGPD ( $p = 0.024$ ), whereas the APD ( $p = 0.292$ ) showed no significant change. *C. perfringens* was not detectable at the second time point. The number of *lactobacilli* increased in APD ( $p < 0.001$ ) and RGPD ( $p = 0.033$ ). On day 50, the numbers of *lactobacillus* in the APD and RGPD groups were higher ( $p < 0.001$ ) than in CD.

## 4. Discussion

Weaning involves multiple changes in the physiology of growing piglets. The adverse stressful effects of weaning may be due to nutritional, immunological, environmental or psychological factors [24]. The goal of the experiment was to test if the negative effects of weaning, due to severe nutritional and immunological changes, could be reduced by different polyphenol rich diets. The effect of two flavanoid rich feeds on piglets blood parameters, selected marker gene expression in WBC, and number of bacteria in colon content were investigated. Five flavanoid marker compounds which typically occur in grape pomace were measured in the feeds, in the gut content and in three piglet tissues. The total content of flavanoids in red-grape pomace was comparable with published

**Table 5. Blood cell counts ( $n = 6$ ; mean  $\pm$  SD).**

	feeding group	day 31	day 50
<b>Erythrocytes</b> [ $10^6/\mu\text{l}$ ]	CD	6.55 $\pm$ 0.45	6.58 $\pm$ 0.71 <sup>a</sup>
	APD	6.15 $\pm$ 0.24	5.83 $\pm$ 0.46 <sup>b</sup>
	RGPD	6.42 $\pm$ 0.50	6.05 $\pm$ 0.65
<b>Hemoglobin</b> [g/dl]	CD	12.43 $\pm$ 0.90 <sup>#</sup>	11.30 $\pm$ 0.72 <sup>#</sup>
	APD	12.58 $\pm$ 0.45 <sup>#</sup>	10.87 $\pm$ 0.66 <sup>#</sup>
	RGPD	12.23 $\pm$ 0.77 <sup>#</sup>	10.92 $\pm$ 0.94 <sup>#</sup>
<b>Hematokrit</b> [%]	CD	40.50 $\pm$ 2.81 <sup>#</sup>	36.00 $\pm$ 2.61 <sup>#</sup>
	APD	40.83 $\pm$ 0.41 <sup>#</sup>	33.50 $\pm$ 1.64 <sup>#</sup>
	RGPD	39.17 $\pm$ 1.72 <sup>#</sup>	33.83 $\pm$ 2.93 <sup>#</sup>
<b>Thrombocyte</b> [ $10^3/\mu\text{l}$ ]	CD	299.10 $\pm$ 146.48 <sup>#a</sup>	159.33 $\pm$ 31.59 <sup>#ab</sup>
	APD	413.33 $\pm$ 110.58	316.50 $\pm$ 115.27 <sup>a</sup>
	RGPD	544.83 $\pm$ 103.85 <sup>#b</sup>	300.83 $\pm$ 98.68 <sup>#a</sup>
<b>Leukocytes</b> [ $10^3/\mu\text{l}$ ]	CD	16.05 $\pm$ 3.01	19.92 $\pm$ 3.17
	APD	14.35 $\pm$ 4.24	17.65 $\pm$ 3.12
	RGPD	12.95 $\pm$ 2.03 <sup>#</sup>	17.73 $\pm$ 5.19 <sup>#</sup>
<b>segmented Granulocytes</b> [% of leukocytes]	CD	60.17 $\pm$ 16.93	59.67 $\pm$ 9.44
	APD	65.17 $\pm$ 7.57	62.83 $\pm$ 17.58
	RGPD	54.67 $\pm$ 12.26	60.50 $\pm$ 15.29
<b>non-segmented Granulocytes</b> [% of leukocytes]	CD	n. d.	n. d.
	APD	n. d.	n. d.
	RGPD	n. d.	n. d.
<b>Lymphocyte</b> [% of leukocytes]	CD	34.00 $\pm$ 20.21	34.33 $\pm$ 12.40
	APD	26.33 $\pm$ 12.01	31.17 $\pm$ 20.92
	RGPD	37.00 $\pm$ 12.9	30.33 $\pm$ 17.07
<b>Monocyte</b> [% of leukocytes]	CD	2.50 $\pm$ 3.51	3.33 $\pm$ 5.05
	APD	7.00 $\pm$ 4.38	3.83 $\pm$ 4.75
	RGPD	2.00 $\pm$ 4.43	7.00 $\pm$ 4.65

Superscript (#) indicates significant differences between treatment days ( $P < 0.05$ ). Superscript (ab) indicates significant differences between feeding groups ( $P < 0.05$ ), (n. d. = no detectable).

**Table 6. Number of bacteria on day 31 (weaning) and day 50 of piglets fed 19 days with control diet (CD), 3.5% apple pomace diet (APD) and 3.5% red-grape pomace diet (RGPD). Values are means calculated ( $n = 6$ ) of bacterial counts per gram of faeces.**

	feeding group	day 31	day 50
<b>total aerobes</b> [cfu/g]	CD	3.39E + 08 $\pm$ 2.48E + 08	1.27E + 09 $\pm$ 8.94E + 08
	APD	6.22E + 08 $\pm$ 6.58E + 08	1.97E + 09 $\pm$ 6.88E + 08
	RGPD	9.72E + 08 $\pm$ 9.75E + 08	2.49E + 09 $\pm$ 3.67E + 09
<b>Streptococci/Enterococci</b> [cfu/g]	CD	5.94E + 07 $\pm$ 8.21E + 07 <sup>#</sup>	9.09E + 08 $\pm$ 9.18E + 08 <sup>#</sup>
	APD	5.06E + 07 $\pm$ 5.11E + 07	3.24E + 08 $\pm$ 3.37E + 08
	RGPD	4.07E + 07 $\pm$ 3.28E + 07 <sup>#</sup>	6.48E + 08 $\pm$ 4.51E + 08 <sup>#</sup>
<b>E. coli</b> [cfu/g]	CD	1.21E + 08 $\pm$ 2.69E + 08	1.14E + 08 $\pm$ 2.29E + 08
	APD	2.14E + 08 $\pm$ 4.36E + 08	9.07E + 07 $\pm$ 1.67E + 08
	RGPD	4.99E + 08 $\pm$ 7.43E + 08	2.21E + 07 $\pm$ 2.34E + 07
<b>total anaerobes</b> [cfu/g]	CD	1.51E + 09 $\pm$ 1.96E + 09	1.65E + 09 $\pm$ 1.08E + 09
	APD	1.29E + 09 $\pm$ 1.03E + 09	2.80E + 09 $\pm$ 6.69E + 08
	RGPD	9.33E + 08 $\pm$ 1.01E + 09	1.45E + 09 $\pm$ 1.20E + 09
<b>Lactobacillus</b> [cfu/g]	CD	3.73E + 08 $\pm$ 2.04E + 08	1.58E + 09 $\pm$ 8.45E + 08 <sup>a</sup>
	APD	9.63E + 08 $\pm$ 3.87E + 08 <sup>#</sup>	4.77E + 09 $\pm$ 1.57E + 09 <sup>#b</sup>
	RGPD	6.25E + 08 $\pm$ 4.80E + 08 <sup>#</sup>	4.93E + 09 $\pm$ 8.92E + 08 <sup>#b</sup>
<b>C. perfringens</b> [cfu/g]	CD	2.13E + 06 $\pm$ 2.94E + 06 <sup>[#]</sup>	n. d.
	APD	2.53E + 06 $\pm$ 3.36E + 06 <sup>[#]</sup>	n. d.
	RGPD	7.40E + 06 $\pm$ 1.04E + 07 <sup>[#]</sup>	n. d.

Superscript (#) indicates significant differences between treatment days ( $P < 0.05$ ); Superscript (ab) indicates significant differences between feeding groups ( $P < 0.05$ ); (n. d. = not detectable).

values [8]. In stomach- and colon-content higher levels of total polyphenols were detected. Flavanoids that are not absorbed in the stomach or small bowel will be carried forward to the colon. In addition, flavanoids that are absorbed will be excreted back from the enterocytes to the small intestine and will reach the colon as a metabolite, such as a glucuronide [25]. Quantification resulted in 3-4 folds more flavanoids in the stomach and colon content on dry matter basis. This might be due to the better digestion through gastrointestinal enzymes and metabolism, resulting in better availability [25,26]. Physiologically the colon contains about  $10^{12}$  microorganisms/cm<sup>3</sup> and has enormous catalytic and hydrolytic potential. Deconjugation reactions readily occur and many gastrointestinal bacteria have the potential to metabolize flavanoids. For example the number of bacteria able to use quercetin-glucoside was estimated to be  $10^7$  to  $10^9$ /g dry mass [27]. Absorbed flavanoids are also metabolized in the liver and excreted in the bile. From liver they can also reach the blood stream and thus tissues and organs [25,26]. In the investigated tissues herein only traces of polyphenols could be found in ileum, liver and kidneys. The highest local concentration of polyphenols was found in the gut lumen [8], so the greatest impulse of the polyphenol rich feedings should be found in the gastrointestinal tract.

It turns out, that the pomace diets had greatest impact on the bacterial content in the colon. The intestinal flora has been shown to have beneficial effects on the host health, because these can inhibit the proliferation of harmful bacteria and stimulate the host's immune system [28,29]. *Lactobacilli* and *bacteroidaceae* are composed of a member of the predominant flora of pigs. Results from the bacterial analysis demonstrate that feeding apple pomace and red-grape pomace tended to increase the number of total colonic bacteria with specific alteration observed in the number of *lactobacillus*, *streptococcus* and *enterococcus*. Hara and coworkers [30] observed an increase of the level of *lactobacilli* during tea polyphenol administration. The number of *Clostridium perfringens* decreased with the age of the piglets [30], as investigated in our study. Noxious bacteria including *Clostridia* and *Enterobacteria* may produce certain putrefactive substances [31,32] such as ammonia, amines, phenols and indole, resulting in suppression of growth. The changes in the composition of intestinal flora including the increase of *lactobacilli* during APD and RGPD administration, and the decrease of *clostridia* over time may therefore lead to a decrease in faecal putrefactive products.

The changed flavanoid content had as well an effect on the gastrointestinal morphology [23] and the gene expression pattern in the gastrointestinal tract [22]. The red-

grape pomace inhibits the jejunum villi growth, whereas apple and red-grape pomace showed stimulating effect on crypt size in piglet colon. Apple and red-grape pomace can reduce the gastro intestinal immune activation via the Peyer's patches in the ileum. In conclusion, the flavanoids rich feeding regimen showed positive effects on villi morphology, gastro intestinal immune activation, and result in an improved health situation [23].

Further the feeding regimens effected the tissue individual regulation of mRNA gene expression in various organs. Significant differences were found between the diets and significant changes during feeding time course. Both applied pomace caused a significant up-regulation of NFκB, cyclin D1, IL10, and IGF-1 and Caspase 3 in liver. In the gastrointestinal tract the treatment groups showed an inhibitory effect on gene expression mainly in stomach and jejunum for NFκB, Cyclin D1 and Caspase 3 [22]. In jejunum and stomach the cell cycle turn over was reduced, whereas in liver the cell turn over was highly accelerate. The influence on inflammatory marker gene expression was mainly relevant in stomach. Sehm and coworkers [22] presumed that both flavanoid rich feeding regimens had beneficial effects on piglet health, and the potential to modulate the mRNA expressions of inflammatory, proliferation and apoptotic marker genes in gastro intestinal tract and piglet organs [22].

In this study the effects on the blood composition cells as well the mRNA gene expression changes in WBCs was investigated. Only apple pomace group showed significant changes in expression analysis over treatment time. Only trends of gene regulation could be shown between treatment groups, due to the high variation in the experimental groups. TNFα is a potent cytokine produced by many cell types, including macrophages, monocytes, lymphocyte, keratinocytes and fibroblasts, in response to inflammation, infection, injury and other environmental challenges [33]. Exposure of cells to TNFα can result in activation of a caspase cascade leading to apoptosis [34]. One of the main markers is caspase 3, which plays a key role in the regulation of apoptosis. In our study TNFα and caspase 3 mRNA expressions were induced similarly by the apple pomace treatment. TNFα causes activation of two major transcription factors, AP-1 and NFκB, which induce genes involved in chronic and acute inflammatory responses [35]. In this study NFκB mRNA expression was as well up-regulated in the APD. But the process of inflammation is self-limiting because the production of pro-inflammatory cytokines is followed almost immediately by production of anti-inflammatory cytokines like IL10 and IL13 [36]. Herein we could not measure any anti-inflammatory response through IL10. Summarizing all the mRNA expression



result in WBCs we can conclude an activation of the immune system through TNF $\alpha$  or NF $\kappa$ B and an increased apoptotic activity through apple active ingredients [3].

Piglet's blood parameters of the piglets were in the normal physiological range [37]. The feeding regimes had minor influence on the different parameters. New-born pigs normally experience a decrease in haemoglobin concentration and haematocrit [38]. This decrease is attributed to the expansion of blood volume from absorbed colostrums [39], an increase in body size, and an increase in plasma volume [40]. The results in this study are comparable. There are only few studies available, regarding the effect of grapery and apple juice by-products on haematology. Bentivegna and Whitney [41] investigated the effects of grape seed extract and grape skin extract in rats and found no clinical relevant changes in haematology, as we did. The slight increase of leukocytes over all feeding groups during this trial, was in accordance with some other studies [42,43]. This might be the reason of immuno-stimulation through TNF $\alpha$  or NF $\kappa$ B. The number of thrombocytes decreased in both pomace groups and might be the result of increased apoptotic activity described above. Plant flavanoids are found to inhibit platelet adhesion, aggregation, and secretion and protect against cardiovascular diseases [44,45]. The pomace administration contributed to an improvement of the intestinal flora, by increasing the amount of *lactobacilli* and in the reduction of *C. perfringens*. Further positive effects were the activation of the immune system in WBC by apple pomace.

We conclude that both polyphenol rich feedings have the potential to positively influence the intestinal flora, blood parameters, and WBC mRNA gene expression pattern of immunological marker genes.

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