

# Effect of feeding soybean meal and differently processed peas on intestinal morphology and functional glucose transport in the small intestine of broilers

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**ABSTRACT** Peas are locally grown legumes being rich in protein and starch. However, the broad usage of peas as a feed component in poultry nutrition is limited to anti-nutritional factors, which might impair gut morphology and function. This study investigated the effect of feeding raw or differently processed peas compared with feeding a soybean meal-based control diet (C) on intestinal morphology and nutrient transport in broilers. A total of 360 day-old broiler chicks were fed with one of the following diets: The C diet, and 3 diets containing raw peas (RP), fermented peas (FP) and enzymatically pre-digested peas (EP), each supplying 30% of dietary crude protein. After 35 d, jejunal samples of broilers were taken for analyzing histomorphological parameters, active glucose transport in Ussing chambers and the expression of genes related to glucose absorption, intestinal permeability and cell maturation. Villus length ( $P = 0.017$ ) and crypt depth ( $P = 0.009$ ) of EP-fed broilers were shorter compared to birds received C. The villus surface area was larger in broilers fed C compared to those fed with the

pea-containing feed ( $P = 0.005$ ). Glucose transport was higher for broilers fed C in comparison to birds fed with the EP diet ( $P = 0.044$ ). The sodium-dependent glucose co-transporter 1 (SGLT-1) expression was down-regulated in RP ( $P = 0.028$ ) and FP ( $P = 0.015$ ) fed broilers. Correlation analyses show that jejunal villus length negatively correlates with the previously published number of jejunal intraepithelial T cells ( $P = 0.014$ ) and that jejunal glucose transport was negatively correlated with the occurrence of jejunal intraepithelial leukocytes ( $P = 0.041$ ). To conclude, the feeding of raw and processed pea containing diets compared to a soybean based diet reduced the jejunal mucosal surface area of broilers, which on average was accompanied by lower glucose transport capacities. These morphological and functional alterations were associated with observed mucosal immune reactions. Further studies are required elucidating the specific components in peas provoking such effects and whether these effects have a beneficial or detrimental impact on gut function and animal health.

**Key words:** broiler, pea, feed processing, intestinal morphology, intestinal glucose transport

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## INTRODUCTION

In Europe, considerable efforts are being made to promote the usage of locally grown legumes as a protein source for animal feed. The reasons for these attempts are diverse, ranging from the economic interest of being independent of soybean imports to social demands and consumer expectations regarding the absence of genetically modified feed. Legumes can be produced in an ecologically and environmentally friendly manner by local farmers. Peas are a traditional grown protein source rich in essential amino acids and starch, although the nutritional composition and quality may vary based on variety, location, and climate conditions (Nikolopoulou et al., 2007; Barac et al., 2010). Thus,

the protein content can range from 208 to 264 g/kg (Igbasan et al., 1997). In comparison with soybeans, peas contain higher amounts of lysine, similar concentrations of threonine, but lower proportions of sulfur amino acids and tryptophan (Lallès, 1993; Gatel, 1994). The nutritional value of peas is limited due to several anti-nutritional factors (ANF) that could impair the nutrient digestibility, and thus animal performance (Cowieson et al., 2003; Meng and Slominski, 2005; Moschini et al., 2005). In this regard, studies showed that feeding raw peas influenced the development of the intestinal microstructure in pigs leading to villus atrophy and alterations of the villus morphology (Mekbungwan et al., 2003; Mekbungwan and Yamauchi, 2004). Moreover, the feeding of raw and differently processed peas resulted in a quantitative increase of intraepithelial T cells in the jejunum of broilers, suggesting an immune-modulating effect of peas (Röhe et al., 2017). Mucosal damage and the occurrence of mucosal immune

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reactions might be accompanied by an increase of the intestinal permeability and a reduction in intestinal nutrient absorption (Ford et al., 1985; Sun et al., 1998; Musch et al., 2002). Peas contain a wide range of ANF including antigenic proteins, lectins, and non-starch polysaccharides (NSP), which might interact with the gut wall. It is assumed that antigenic proteins and lectins present in peas and soybeans interact with the intestinal mucosa, initiating mucosal immune reactions accompanied by changes in the intestinal morphology (Lorenz-Meyer et al., 1985; Kik et al., 1990; Bush et al., 1992; Dreau et al., 1995). Soluble NSP could increase the viscosity of the intestinal contents, which is associated with a decreased feed passage rate and an inhibition of intestinal enzymatic activity (Ikeda and Kusano, 1983; Van der Klis et al., 1993a). Thus, nutrient absorption and digestion might be impaired (Fengler and Marquardt, 1988).

Different feed processing methods decrease the amount of ANF and thus increase the nutritional value of legumes. It was shown that heat treatment reduced the lectin content of legume seeds (Alonso et al., 1998) and that antigenic proteins can be degraded by fermentation processes using bacteria such as *Bacillus subtilis* (Feng et al., 2007; Wang et al., 2011). Furthermore, carbohydrases can be used as feed additives in order to hydrolyze NSP compounds, reducing the intestinal viscosity when fed to the animal and improving the nutrient digestibility and animal performance (Bedford and Morgan, 1996; Kiers et al., 2000; Cowieson et al., 2003). To our knowledge, studies in chickens regarding the effect of feeding raw peas or differently processed peas on the intestinal morphology and permeability as well as on the nutrient transport are not available. Thus, the aim of this study is to ascertain whether the feeding of different protein sources (soybeans vs. pea) and differently processed peas influence both the jejunal microstructure and the intestinal permeability in broilers determining the jejunal histomorphometry, the expression of tight junction proteins (ZO-1, CLDN-5), and genes related to apoptosis and crypt-villus differentiation (CASP-3, ALP). Furthermore, investigations are focused on the intestinal active glucose transport and the expression of intestinal glucose transporters (SGLT-1, GLUT-2) in order to determine whether nutrient absorption is affected by feeding the different diets. Additionally, results from this study are correlated with recently published data concerning the distribution of intraepithelial immune cells (Röhe et al. 2017) examining potential correlations between the observed immune cell accumulation and morphological and functional parameters in the jejunum of broilers. It was hypothesized that the feeding of peas instead of soybeans has an impact on the development of the jejunal morphology, permeability, and glucose absorption and that the feeding of processed peas might change the nutritive value of peas affecting those parameters.

## MATERIAL AND METHODS

All procedures involving handling and treatments of animals were approved by the local state office of occupational health and technical safety (Landesamt für Gesundheit und Soziales, Berlin, Germany, LaGeSo G. Nr. 0203/14).

### Animals and Rearing Conditions

A total of 360 day-old male broiler chicks (Cobb 500) were randomly allocated to 24 pens (15 birds per pen). Four different experimental diets were randomly assigned to birds within the pens, resulting in 6 replicates per feeding group. For a period of 35 d, broilers were reared on litter-floor pens (softwood shavings) and had ad libitum access to mash feed and water. The pen was regarded as the experimental unit. In the first experimental week, barn temperature was adjusted to 33°C and then gradually reduced by 3°C per week until reaching 24°C. The following lighting schedule was used: From d 0 to d 3 24 h of light, from d 4 until d 7 20 h of light, and from d 8 to the end of the trial 16 h of light. During the experimental trial, the bodyweight (BW) and feed intake (FI) of broilers were recorded weekly and hence the feed conversion ratio (FCR) calculated. At the end of trial (d 35), broilers were slaughtered by stunning and cervical decapitation, followed by the collection of jejunal tissue used for histomorphological analyses, Ussing chambers experiments and gene expression analyses.

### Experimental Diets

Broilers received 4 different diets implementing a 2-phase feeding program based on a starter (1 to 21 d) and a grower (21 to 35 d) diet. The following experimental diets were produced differing in terms of protein source and processing conditions: A control diet (C), based on corn, wheat and toasted (at 110°C for 3 min) soybean meal (SBM) and 3 diets containing raw peas (RP), fermented peas (FP) and enzymatically pre-digested peas (EP), each supplying 30% of required crude protein. Ground peas (*Pisum sativum* L. Madonna), intended for the FP diet, were mixed with water containing  $2.57 \times 10^8$  *Bacillus subtilis* spores/kg pea (GalliPro<sup>®</sup>, Chr. Hansen, Denmark) and fermented for a period of 48 h at 30°C. Ground peas intended for the EP diet were soaked in water, which was added with 3 commercial enzyme products: AlphaGal<sup>TM</sup> (0.1 g/kg pea; Kerry, Beloit, WI) containing an  $\alpha$ -galactosidase, RONOZYME<sup>®</sup> ProAct (0.2 g/kg pea; DSM, Kaiseraugst, Switzerland) containing a protease, and RONOZYME<sup>®</sup> VP (0.2 g/kg pea; DSM, Kaiseraugst, Switzerland) containing a mixture of pectinase and  $\beta$ -glucanase. The process of enzymatic pre-digestion was done for 24 h at 30°C. Finally, the FP and EP peas were dried (at 75°C for less than 3 s)

**Table 1.** Feed composition (% unless noted) and analyzed nutrient content of experimental starter (St: 1- to 21-d-old) and grower (Gr: 22- to 35-day-old) diets.<sup>1</sup>

| Diets  | C <sup>2</sup> |       | RP    |       | FP    |       | EP    |       |
|--|----------------|-------|-------|-------|-------|-------|-------|-------|
|  | St             | Gr    | St    | Gr    | St    | Gr    | St    | Gr    |
| Ingredient (%)                                   |                |       |       |       |       |       |       |       |
| Pea product                                      | 0              | 0     | 31.8  | 26.7  | 30.2  | 25.4  | 31.3  | 26.3  |
| Maize  | 31.5           | 34.8  | 7.6   | 14.7  | 9.8   | 16.7  | 8.3   | 15.3  |
| Wheat  | 20             | 30.0  | 20.0  | 30.0  | 20.0  | 30.0  | 20.0  | 30.0  |
| Soybean meal (CP 44%)                            | 36.7           | 24.9  | 25.0  | 15.0  | 24.5  | 14.6  | 24.8  | 14.9  |
| Soybean oil                                      | 7.40           | 5.70  | 10.0  | 7.9   | 9.8   | 7.7   | 9.9   | 7.8   |
| Premix <sup>3</sup>                              | 1.2            | 1.20  | 1.2   | 1.2   | 1.2   | 1.2   | 1.2   | 1.2   |
| MCP  | 1.42           | 1.13  | 1.6   | 1.3   | 1.6   | 1.3   | 1.6   | 1.3   |
| Limestone  | 1.46           | 1.12  | 1.4   | 1.1   | 1.4   | 1.1   | 1.4   | 1.1   |
| L-Lysine HCL                                     | 0.09           | 0.28  | 0.59  | 0.70  | 0.60  | 0.71  | 0.59  | 0.71  |
| DL-Methionine                                    | 0.26           | 0.25  | 0.51  | 0.46  | 0.51  | 0.45  | 0.51  | 0.46  |
| L-Threonine                                      | 0.02           | 0.12  | 0.32  | 0.37  | 0.32  | 0.37  | 0.32  | 0.37  |
| L-Tryptophan                                     |                | 0.05  | 0.08  | 0.12  | 0.08  | 0.12  | 0.08  | 0.12  |
| TiO <sub>2</sub> <sup>4</sup>                    |                | 0.50  |       | 0.50  |       | 0.50  |       | 0.50  |
| Analyzed Nutrients (g/kg)                        |                |       |       |       |       |       |       |       |
| Crude Protein                                    | 233            | 189   | 229   | 185   | 223   | 187   | 222   | 190   |
| Crude Fat  | 91.9           | 81.2  | 120   | 95.8  | 111   | 88.5  | 117   | 91.5  |
| Crude Fiber                                      | 28.7           | 25.1  | 37.6  | 34.3  | 51.4  | 35.4  | 61.5  | 37.0  |
| Starch   | 261            | 359   | 259   | 340   | 248   | 346   | 267   | 323   |
| Phosphorus                                       | 7.21           | 6.07  | 7.28  | 5.74  | 7.60  | 6.10  | 7.20  | 6.02  |
| Calcium  | 9.16           | 7.38  | 9.26  | 7.13  | 9.37  | 7.44  | 9.17  | 7.66  |
| Sodium   | 1.81           | 1.78  | 2.00  | 1.86  | 1.84  | 1.80  | 1.96  | 1.98  |
| Potassium  | 8.31           | 6.57  | 7.60  | 6.67  | 7.61  | 6.11  | 7.35  | 6.17  |
| Calculated AME <sub>N</sub> (MJ/kg) <sup>5</sup> | 12.57          | 12.65 | 12.57 | 12.65 | 12.57 | 12.65 | 12.57 | 12.65 |

<sup>1</sup>As-fed basis.<sup>2</sup>C = control diet; RP = raw pea diet; FP = fermented pea diet; EP = enzymatically pre-digested pea diet.<sup>3</sup>Contents per kg premix: 400,000 IU vit. A; 40,000 IU vit. D3; 8000 mg vit. E ( $\alpha$ -tocopherol acetate); 300 mg vit. K3; 250 mg vit. B1; 250 mg vit. B2; 2,500 mg nicotinic acid; 400 mg vit. B6; 2000  $\mu$ g vit. B12; 25,000  $\mu$ g biotin; 1,000 mg calcium pantothenic acid; 100 mg folic acid; 80,000 mg choline chloride; 5,000 mg Zn (zinc oxide); 2,000 mg Fe (iron carbonate); 6,000 mg Mn (manganese oxide); 1,200 mg Cu (copper sulfate-pentahydrate); 45 mg I (calcium iodate); 30 mg Co (cobalt- (II)-sulfate-heptahydrate); 35 mg Se (sodium selenite); 130 g Na (sodium chloride); 55 g Mg (magnesium oxide).<sup>4</sup>Indigestible marker (Sigma Aldrich, St. Louis, MO).<sup>5</sup>Estimated according to equation of the World's Poultry Science Association (1984).

and ground by a dryer mill (Ultra-Rotor Type U III a, Jäckering Mühlen und Nahrungsmittelwerke GmbH, Hamm, Germany) and used for the production of the different experimental diets. The used doses of the probiotic strain and enzymes as well as the applied feed processing conditions were derived from previous experiences as well as from pilot tests at the laboratory scale (data have not been published). All diets were formulated to meet the respective nutrient recommendations for broilers (GfE, 1999). The nutrient content of the diets was determined by classical Weende procedures (Naumann and Bassler, 2004). The feed composition and nutrient content of the experimental diets are displayed in Table 1.

## Sampling and Analyses

**Histomorphological Analyses.** Histological examinations were focused on the characterization of the jejunal microstructure of broilers fed the different experimental diets. Jejunal tissue of 12 randomly selected birds (2 animals per pen of 6 replicate pens) per feeding group was used for histological and morphometric analyses. Subsequently after slaughtering, 8 to 10 cm

segments were taken from mid-jejunum, defined as tissue located in the midway between the point of entry of the bile ducts and Meckel's diverticulum. Tissue sections were cut open longitudinally and placed on cork boards by using hedgehog spines and fixed in a 4% phosphate-buffered formaldehyde solution for 48 h. After dehydration and infiltration with solidified paraffin wax, the samples were embedded. The paraffin blocks were cut at 5  $\mu$ m with a sledge microtome (Typ 1400, Leitz, Wetzlar, Germany), and the obtained sections were mounted on glass slides. Tissue slides were stained with hematoxylin-eosin (Merck KGaA, Darmstadt, Germany) and analyzed with a light microscope (Photomicroscope III, Zeiss, Germany), which was equipped with a digital camera (DP72, Olympus, Germany). By using an image analysis software (CellSense software, Olympus, Germany), the following histological parameters were examined: the villus length (measured from the tip of the villi to the villus crypt junction), the crypt depth (defined as the depth of the invagination between adjacent villi), the villus length-to-crypt depth ratio, the villus area (calculated by multiplying the individual villus area by the number of villi per 1,000  $\mu$ m intestinal cross-section) and the villus surface area (calculated by multiplying the

**Table 2.** List of primers used in this study.

| Targets                 | Sequences of primers (5' to 3')                      | A <sub>T</sub> (°C) <sup>1</sup> | Reference               |
|-------------------------|--|----------------------------------|-------------------------|
| $\beta$ -Actin          | GAGAAATTGTGCGTGACATCA<br>CCTGAACCTCTCATTGCCA         | 60                               | Li et al. (2005)        |
| GAPDH                   | GGTGGTGCTAAGCGTGTTA<br>CCCTCCACAATGCCAA              | 60                               | Li et al. (2005)        |
| $\beta$ 2-Microglobulin | AAGGAGCCGCAGGTCTAC<br>CTTGCTCTTTGCCGTCATAC           | 60                               | Li et al. (2005)        |
| SGLT-1                  | GCCATGGCCAGGGCTTA<br>CAATAACCTGATCTGTGCACCAGTA       | 60                               | Gilbert et al. (2007)   |
| GLUT-2                  | TGTTTCAGCTCCTCCAAGTACC<br>ACAACGAACACATACGGTCC       | 60                               | Humphrey et al. (2004)  |
| ZO-1                    | CTTCAGGTGTTTCTCTCTCCCTC<br>CTGTGGTTTCATGGCTGGATC     | 60                               | Osselaere et al. (2013) |
| CLDN-5                  | GCACAAAGCTCTCCCTC<br>CATCACTTCTCCTTCGTCAGC           | 60                               | Osselaere et al. (2013) |
| ALP                     | CTGCTGCCTGTAGTCCTT<br>CTGGCCCCTAAGAAAGCGAA           | 60                               | This study              |
| CASP-3                  | TGCAGAAGTCTAGCAGGGAAACCC<br>AAGTTTCCTGGCGTGTTCCCTCAG | 60                               | Brisbin et al. (2008b)  |

<sup>1</sup>A<sub>T</sub> = annealing temperature.

individual villus surface area by the number of villi per 1,000  $\mu\text{m}$  intestinal cross-section).

**Intestinal Electrogenic Glucose Transport.** Jejunal tissue of 8 randomly selected birds (2 animals per pen of 4 replicate pens) per feeding group was used investigating the active glucose transport in Ussing chambers. Ussing chambers were equipped with microcomputer-controlled voltage/current clamps (K. Mussler Scientific Instruments, Aachen, Germany) detecting transepithelial potential and conductance (**Gt**) during the experiment. As previously described (Ruhnke et al., 2013), the *Tunica mucosa* was stripped from jejunal samples and placed vertically in net-supported Ussing chamber with an exposed area of 0.79  $\text{cm}^2$ . Tissue was bathed in modified Krebs-Henseleit buffer containing (mmol/L) NaCl, 115; NaHCO<sub>3</sub>, 25; mannitol, 20; KCl, 5; Na<sub>2</sub>HPO<sub>4</sub>, 2.4; CaCl<sub>2</sub>, 1.5; MgCl<sub>2</sub>, 1.2; and NaH<sub>2</sub>PO<sub>4</sub>, 0.6 (pH adjusted to 7.4). The buffer solution was continually stirred, heated to 38°C, and oxygenated with carbogen. After equilibration for approximately 8 to 10 min under open-circuit conditions, the tissue was short-circuited by clamping the voltage at 0 mV. After a tissue stabilization period of about 10 min, 10 mmol/L D-glucose (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was added to the buffer solution of the mucosal side and 10 mmol/L mannitol (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to the buffer solution of the serosal side of the chamber maintaining osmotic balance across the mucosa. Since a baseline of the short-circuit current (Isc) was reached 100  $\mu\text{mol/L}$  phloridzin (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany) was applied to the mucosal side. Phloridzin presents a specific inhibitor of the SGLT-1 and binds to the transporter but is not transferred, preventing glucose absorption into the epithelial cell (Ferraris and Diamond, 1986; Awad et al., 2007). Finally, after a stable Isc baseline was reached, 100  $\mu\text{mol/L}$  carbamylcholine chloride (carbachol; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was applied to the serosal side of chambers. The

electrical response was observed as the peak response, obtained approximately 3 min after addition of the different substrates. The electrogenic ion movements by active transport were displayed by the difference between the peak Isc/Gt and the basal Isc/Gt expressed by  $\Delta\text{Isc}$  values.

**Gene Expression Analyses.** Gene expression analyses were performed from jejunal tissue of 12 randomly selected birds (2 animals per pen of 6 replicate pens) per feeding group. From 30 mg of jejunal tissue total RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and the mRNA quality and quantity were analyzed by a Bioanalyzer (Agilent 2100, Agilent, Waldbronn, Germany). Subsequently, reverse transcription of 100 ng of total RNA into cDNA in a final volume of 20  $\mu\text{L}$  was executed using the Super Script III Reverse Transcriptase First-Strand cDNA Synthesis System (Invitrogen, Carlsbad, CA). Primers for the sodium-dependent glucose co-transporter 1 (**SGLT-1**), glucose transporter 2 (**GLUT-2**), zona occludens 1 (**ZO-1**), claudin 5 (**CLDN-5**), alkaline phosphatase (**ALP**) and caspase 3 (**CASP-3**) were used (Table 2). Primers for ALP were developed at the Institute of Animal Nutrition. The real-time quantitative PCR was conducted with a Stratagene MX3000p (Stratagene, Amsterdam, The Netherlands). The reference genes  $\beta$ -actin, glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) and  $\beta$ 2-microglobulin were used for normalization and times-fold expression was determined based on mean cycle threshold values of the housekeeping genes using the relative expression software tool REST<sup>®</sup> (Pfaffl et al., 2002).

## Statistical Analyses

Statistical analyses were performed using SPSS (version 22.0, Chicago, IL). Means and standard deviation of the means are reported for the 4 experimental groups

**Table 3.** Histomorphological analyses of the villus length (VL), crypt depth (CD), villus length-to-crypt depth ratio (VL/CD), villus area (VA) and the villus surface area (VSA) in the jejunum of broilers fed with the different diets.<sup>1,2,3</sup>

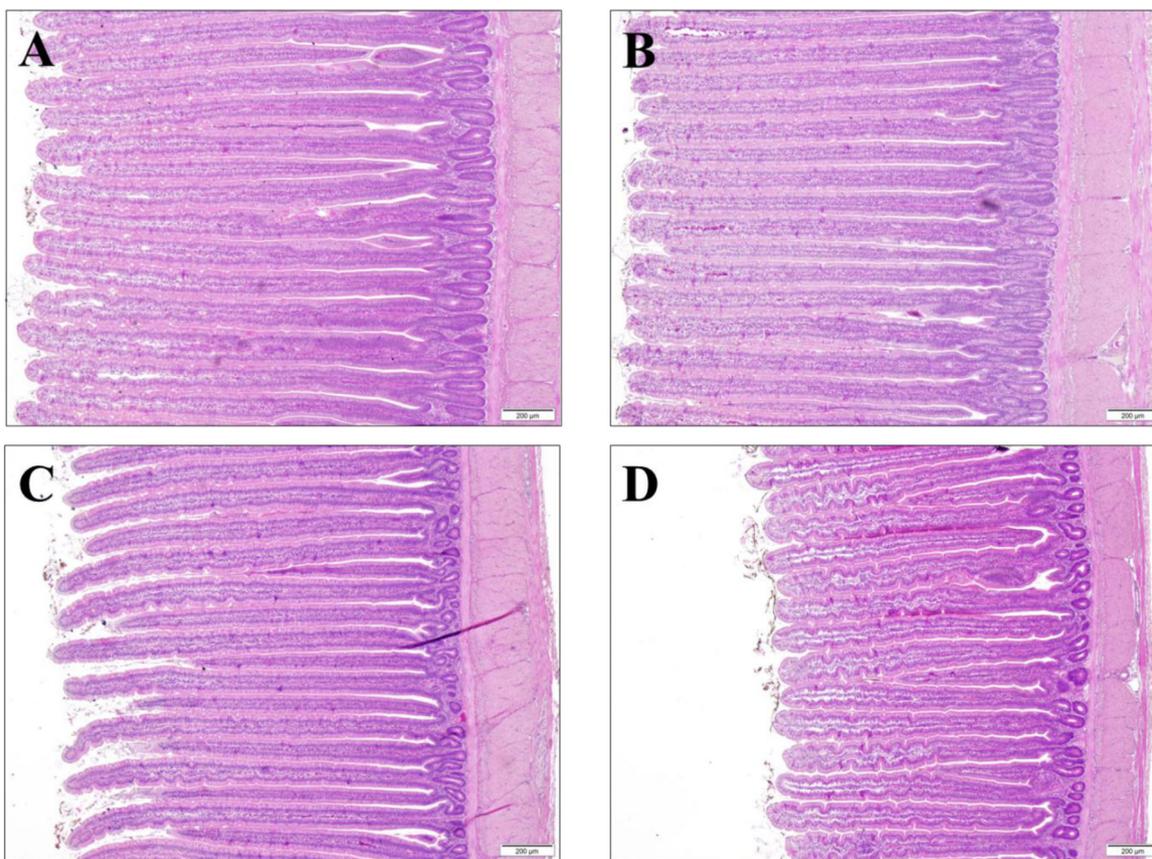
| Item                 | C <sup>4</sup>               | RP                             | FP                             | EP                           | P-value |
|----------------------|------------------------------|--------------------------------|--------------------------------|------------------------------|---------|
| VL ( $\mu\text{m}$ ) | 1556 <sup>a</sup> $\pm$ 101  | 1401 <sup>a,b</sup> $\pm$ 182  | 1394 <sup>a,b</sup> $\pm$ 82.8 | 1305 <sup>b</sup> $\pm$ 101  | 0.017   |
| CD ( $\mu\text{m}$ ) | 214 <sup>a</sup> $\pm$ 40.2  | 176 <sup>a,b</sup> $\pm$ 15.3  | 173 <sup>a,b</sup> $\pm$ 22.1  | 158 <sup>b</sup> $\pm$ 12.3  | 0.009   |
| VL/CD                | 7.44 $\pm$ 1.16              | 7.98 $\pm$ 0.87                | 8.17 $\pm$ 1.34                | 8.30 $\pm$ 0.81              | 0.541   |
| VA ( $\text{mm}^2$ ) | 1.74 <sup>a</sup> $\pm$ 0.17 | 1.49 <sup>a,b</sup> $\pm$ 0.18 | 1.41 <sup>b</sup> $\pm$ 0.13   | 1.37 <sup>b</sup> $\pm$ 0.10 | 0.002   |
| VSA ( $\text{mm}$ )  | 35.8 <sup>a</sup> $\pm$ 3.54 | 29.2 <sup>b</sup> $\pm$ 4.23   | 29.0 <sup>b</sup> $\pm$ 2.96   | 28.7 <sup>b</sup> $\pm$ 2.68 | 0.005   |

<sup>1</sup>Results are reported as means of 6 replicate pens  $\pm$  SD.

<sup>2</sup>Statistical analyses were conducted by ANOVA and post hoc Tukey's test.

<sup>3</sup>a,b Means with different superscripts are significantly different ( $P < 0.05$ ).

<sup>4</sup>Animals fed with: C = control diet; RP = raw pea diet; FP = fermented pea diet; EP = enzymatically pre-digested pea diet.

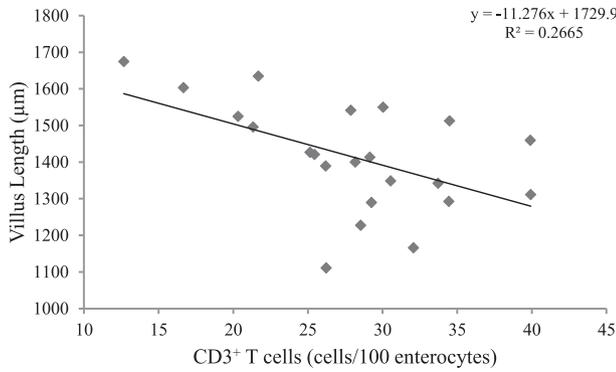


**Figure 1.** Typical light microscopic images of jejunal tissue of broilers fed with the control diet (A), raw peas (B), fermented peas (C) and enzymatically pre-digested peas (D). Tissue sections were stained with hematoxylin and eosin.

(mean  $\pm$  SD). As data were normally distributed, statistical analyses were conducted by analysis of variance (ANOVA) and posthoc Tukey's test. The software tool REST<sup>®</sup> was used for group-wise comparison and statistical analysis of relative gene expression results. Additionally, Pearson correlation analyses were performed illustrating correlations between intestinal morphology, glucose transport and mucosal immune response. For this purpose, recently published data regarding the distribution of intraepithelial immune cells, which were obtained from the same experiment and are based on means of 2 animals per pen of 6 replicate pens (Röhe et al. 2017) were taken into account. Differences were considered significant at  $P < 0.05$ .

## RESULTS

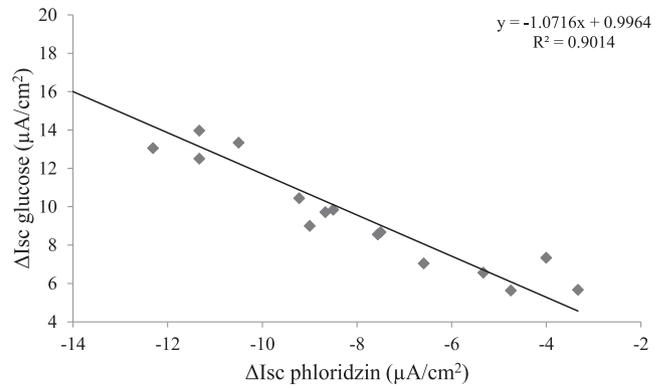
During the feeding trials, broilers were healthy and showed no clinical evidence of disease. Regarding bird performance, the final BW as well as the FCR of broilers fed diets C (BW: 1,884 g; FCR: 1.478), RP (BW: 1,737 g; FCR: 1.469), FP (BW: 1,722 g; FCR: 1.463) and EP (BW: 1,767 g; FCR: 1.447) were comparable ( $P > 0.05$ ). The results of the histomorphological analyses revealed that the jejunal microstructure was influenced by the different protein sources (Table 3). Broilers receiving the EP diet had shorter villi ( $P = 0.017$ ) and crypts ( $P = 0.009$ ) compared to birds fed C (Figure 1) while the villus length-to-crypt depth ratio was not



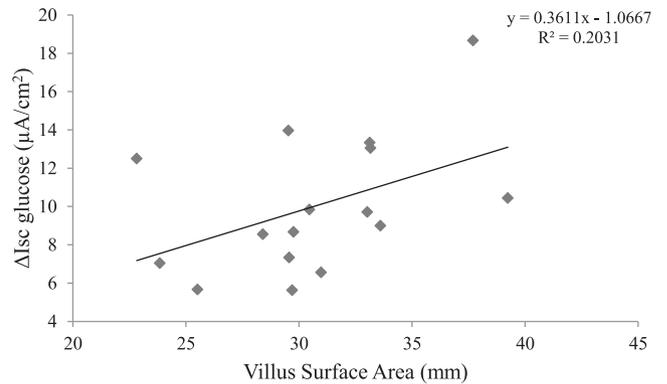
**Figure 2.** Relationship between jejunal villus length ( $n = 6$ ) and number of jejunal intraepithelial  $CD3^+$  T cells ( $n = 6$ ), determined in the villus mid (Röhe et al. 2017) (Pearson coefficient:  $-0.516$ ,  $P < 0.05$ ).

influenced. The villus area was reduced in birds fed the FP and EP diet compared to those receiving C ( $P = 0.002$ ). Furthermore, the villus surface area was higher in broilers of the C group compared to birds fed with the pea containing diets ( $P = 0.005$ ). Pearson correlation analysis illustrates correlations between intestinal morphology and observed mucosal immune response (Röhe et al. 2017). Figure 2 shows that the jejunal villus length negatively correlates with the number of jejunal intraepithelial  $CD3^+$  T cells detected in the villus mid (Pearson coefficient:  $-0.516$ ,  $P = 0.014$ ).

The results of the Ussing chamber experiments are displayed in Table 4. Basal values for Gt were uniform among the feeding groups. Pearson correlation analyses show that there is a strong relationship between  $\Delta I_{sc}$  glucose and  $\Delta I_{sc}$  phloridzin (Figure 3) revealing that SGLT-1 transporters were inhibited by the addition of phloridzin and that measured values of  $\Delta I_{sc}$  glucose representing the active glucose transport across the intestinal epithelia (Pearson coefficient:  $-0.949$ ,  $P < 0.01$ ). Broilers receiving the EP diet showed lower values for  $\Delta I_{sc}$  glucose ( $P = 0.044$ ) and  $\Delta I_{sc}$  phloridzin ( $P = 0.002$ ) than birds fed with C. The addition of carbachol led to comparable  $\Delta I_{sc}$  responses among the feeding groups. Figure 4 displays that there is a relationship between  $\Delta I_{sc}$  glucose and the intestinal surface area, although correlation analysis showed a trend towards significance (Pearson coefficient:  $0.451$ ;  $P = 0.08$ ). Tak-



**Figure 3.** Relationship between the electrical responses after glucose and phloridzin addition during the Ussing chamber experiments ( $n = 4$ ). Pearson coefficient:  $-0.949$ ,  $P < 0.01$ .



**Figure 4.** Relationship between the jejunal, SGLT-1 mediated glucose transport ( $n = 4$ ) and the jejunal villus surface area ( $n = 6$ ). Pearson coefficient:  $0.451$ ;  $P = 0.08$ .

ing into account the recently published data regarding the distribution of intraepithelial immune cells (Röhe et al. 2017), correlation analyses (Figure 5) show that the glucose uptake is negatively correlated with the occurrence of jejunal intraepithelial  $CD45^+$  leucocytes in the villus mid (Pearson coefficient:  $-0.516$ ;  $P = 0.041$ ).

The relative mRNA expression of SGLT-1 was influenced by the diets. In comparison with birds fed C, the SGLT-1 expression was higher in broilers fed the RP ( $P = 0.028$ ) and FP ( $P = 0.015$ ) diet. Broilers receiving the EP diet and C showed comparable SGLT-1 expression patterns ( $P = 0.54$ ). The expression of genes related to intestinal permeability (ZO-1, CLDN-5) and

**Table 4.** Effect of D-Glucose, phloridzin and carbachol on short-circuit current ( $\Delta I_{sc}$ )<sup>1</sup> in isolated jejunal mucosa of broilers fed with the different diets.<sup>2,3,4</sup>

| Item  | C <sup>5</sup>            | RP                          | FP                          | EP                        | P-value |
|---|---------------------------|-----------------------------|-----------------------------|---------------------------|---------|
| Gt (mS/cm <sup>2</sup> )                    | 7.48 ± 2.33               | 7.88 ± 1.73                 | 6.75 ± 1.23                 | 6.01 ± 1.63               | 0.485   |
| $\Delta I_{sc}$ glucose ( $\mu A/cm^2$ )    | 12.8 <sup>a</sup> ± 4.26  | 11.0 <sup>a,b</sup> ± 2.26  | 9.88 <sup>a,b</sup> ± 2.96  | 6.30 <sup>b</sup> ± 0.82  | 0.044   |
| $\Delta I_{sc}$ phloridzin ( $\mu A/cm^2$ ) | -11.3 <sup>a</sup> ± 2.64 | -9.51 <sup>a,b</sup> ± 1.71 | -8.48 <sup>a,b</sup> ± 2.05 | -4.35 <sup>b</sup> ± 0.87 | 0.002   |
| $\Delta I_{sc}$ carbachol ( $\mu A/cm^2$ )  | 3.69 ± 1.42               | 5.51 ± 2.98                 | 4.70 ± 1.25                 | 5.89 ± 1.30               | 0.399   |

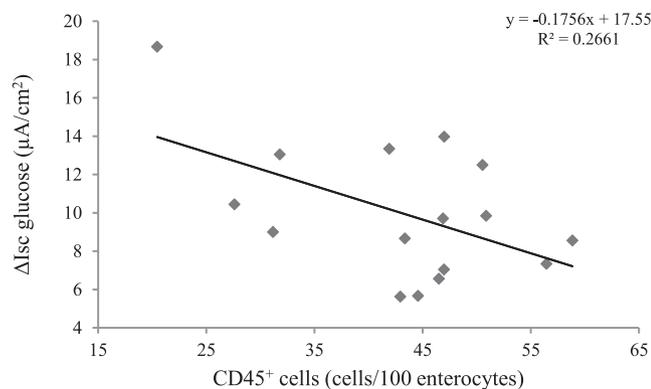
<sup>1</sup> $\Delta I_{sc}$  is the difference between the basal and the maximal value obtained after adding substances.

<sup>2</sup>Results are reported as means of 4 replicate pens ± SD.

<sup>3</sup>Statistical analyses were conducted by ANOVA and post hoc Tukey's test.

<sup>4,a,b</sup>Means with different superscripts are significantly different ( $P < 0.05$ ).

<sup>5</sup>Animals fed with: C = control diet; RP = raw pea diet; FP = fermented pea diet; EP = enzymatically pre-digested pea diet.



**Figure 5.** Relationship between the active glucose transport ( $n = 4$ ) and the number of jejunal intraepithelial  $CD45^+$  leucocytes ( $n = 6$ ), detected in the villus mid (Röhe et al. 2017). Pearson coefficient:  $-0.516$ ,  $P = 0.041$ .

**Table 5.** Relative mRNA expression of genes related to glucose transport (SGLT-1, GLUT-2), intestinal permeability (ZO-1, CLDN-5) and cell maturation (ALP, CASP-3) in jejunal tissue of broilers fed with the different diets.<sup>1,2,3</sup>

| Gene   | Group <sup>4</sup> | Relative expression <sup>5</sup> | P-value |
|--------|--------------------|----------------------------------|---------|
| SGLT-1 | RP                 | 0.796                            | 0.028   |
|        | FP                 | 0.725                            | 0.015   |
|        | EP                 | 0.927                            | 0.54    |
| GLUT-2 | RP                 | 0.789                            | 0.051   |
|        | FP                 | 0.888                            | 0.296   |
|        | EP                 | 0.96                             | 0.759   |
| ZO-1   | RP                 | 1.071                            | 0.409   |
|        | FP                 | 0.893                            | 0.231   |
|        | EP                 | 1.059                            | 0.613   |
| CLDN-5 | RP                 | 1.08                             | 0.379   |
|        | FP                 | 1.057                            | 0.515   |
|        | EP                 | 1.171                            | 0.12    |
| ALP    | RP                 | 0.77                             | 0.207   |
|        | FP                 | 1.055                            | 0.786   |
|        | EP                 | 0.963                            | 0.828   |
| CASP-3 | RP                 | 1.061                            | 0.645   |
|        | FP                 | 1.007                            | 0.947   |
|        | EP                 | 1.039                            | 0.804   |

<sup>1</sup>Results are reported as means of 6 replicate pens  $\pm$  SD.

<sup>2</sup>Statistical analyses performed by a pairwise fixed reallocation randomization test using the software tool REST<sup>®</sup>.

<sup>3</sup>Reference genes were used for normalization of the real-time PCR data.

<sup>4</sup>Animals fed with: C = control diet; RP = raw pea diet; FP = fermented pea diet; EP = enzymatically pre-digested pea diet.

<sup>5</sup>Gene expression of the control group was set to 1.

cell maturation (ALP, CASP-3) were not affected by feeding the different diets (Table 5).

## DISCUSSION

The development of the intestinal microstructure and nutrient transport is influenced by intrinsic and extrinsic factors including dietary composition, level of nutrients and anti-nutrients passing the intestinal tract. The current study investigated the effect of feeding soybeans or peas as protein sources and differently processed peas on the development of the histomorphometry, the active glucose transport as well as on the expression of tight junction proteins and cell maturation markers in the jejunum of broilers.

To the best of our knowledge, data in chickens regarding the effect of feeding raw peas or differently processed peas on the intestinal microstructure and intestinal nutrient absorption are not available. The results of this study showed that the feeding of raw and processed peas resulted in a decreased mucosal surface area in the jejunum of broilers and that gut function, as example jejunal glucose uptake, was related to the villus surface area (Figure 4). Moreover, the expression of the  $Na^+$ -dependent SGLT-1, present in the small intestinal brush border, was down-regulated in broilers fed with the RP and FP diet. Although SGLT-1 expression in the EP fed birds was similar to that of the C group, intestinal glucose transport was reduced emphasizing that the size of the absorptive mucosal surface area is playing an important role in the process of nutrient absorption. Only few studies have been published investigating the effect of feeding peas on the intestinal development of pigs. The feeding of raw peas compared to SBM led to a reduction of duodenal villus length, cell area, and cell mitosis in growing pigs (Mekbungwan et al., 2003). Moreover, a reduced villus length, cell area and cell mitosis in the duodenum, jejunum, and ileum accompanied with a decrease in body weight gain and feed efficiency were observed in piglets fed with raw instead of heated pigeon pea seed meal, suggesting a reduction of ANF by heat treatment (Mekbungwan and Yamauchi, 2004). In the present study, broilers fed the SBM based diet showed on average higher BW compared to those fed the pea containing diets, although differences were not statistically significant. Furthermore, no significant correlations regarding performance data and the examined gut wall characteristics were observed (data not shown). As shown by Mekbungwan and Yamauchi (2004), piglets' performance tended to decrease with increasing dietary levels of raw peas and was significantly decreased by inclusion levels of 40% of raw peas. Thus, with regard to the present study, it can be assumed that differences in the BW development of broilers might become more pronounced by elevating the dietary inclusion level of peas. The current data are in good agreement with results from the same feeding experiment, published previously (Röhe et al. 2017). This data show that the feeding of diets formulated with raw and processed peas in comparison with feeding the SBM initiated a strong mucosal immune response in the jejunum of broilers, indicated by a quantitative increase of intraepithelial T cells (Röhe et al. 2017). By combining data from both studies, a negative correlation between the number of jejunal T cells and the villus length is obvious (Figure 2). Moreover, data clearly show that the measured glucose uptake correlates inversely with the epithelial density of leukocytes (Figure 5). Thus, the observed jejunal immune cell accumulation in pea-fed broilers was accompanied by morphological and functional alterations of the tissue. In this regard, mucosal alterations in connection with mucosal immune reactions are observed in humans suffering from acute or chronic gastroenteritis (Isolauri et al., 1989; Ramachandran et al., 2000).

Particularly, mucosal T cell activation, up-regulation of pro-inflammatory cytokines, mucosal atrophy, and increase of intestinal permeability accompanied by a reduction in the intestinal nutrient absorption are related to inflammatory bowel diseases and diarrhea observed in mice and humans (Musch et al., 2002; Croitoru and Zhou, 2004; Zeissig et al., 2007). In this study, however, birds were healthy and showed no clinical evidence of diarrhea. Furthermore, neither an up-regulation of inflammatory cytokines (Röhe et al. 2017) nor evidence of increased intestinal permeability could be observed. The jejunal expression of tight junction proteins (ZO-1, CLDN-5) and genes related to apoptosis and cell maturation (CASP-3, ALP) were comparable among the different feeding groups. The down-regulation of tight junction proteins is associated with epithelial dysfunction and an increase of the intestinal permeability (Zeissig et al., 2007; Zhang and Guo, 2009) while an enhanced expression of CASP-3 could be observed in apoptotic tissue (Nicholson et al., 1995; Chin et al., 2006). Moreover, results of the Ussing chamber experiments showed that Gt values, which correspond to the reciprocal values of the transepithelial resistance, did also not differ between birds fed the different diets indicating that tissue integrity was comparable.

Viewing the results, the question arises, which components in the used processed and unprocessed pea diets might lead to observed effects on gut function, intestinal morphology and immune system. Peas contain heat-labile lectins which could interact with the intestinal mucosa leading to villus atrophy and a destruction of intestinal microvilli and enterocytes may followed by mucosal immune responses (Pusztai et al., 1979; Rossi et al., 1984; Kik et al., 1990; Buttle et al., 2001). In the present study, peas were not heat treated. Although the lectin content of the different experimental diets were not determined, it seems conceivable that lectins were not eliminated by both processing methods. As highlighted by Lallès (1993), information regarding the lectin content of peas as well as soybeans is scarce due to the lack of analytical methods providing a reliable determination of functional lectins. Lectins can be directly or indirectly measured by methods such as the hemagglutination test or enzyme-linked immunosorbent assay (ELISA) techniques (Kik et al., 1990; Alonso et al., 1998; Wang et al., 2011). However, those tests do not allow a differentiation between non-toxic lectins, which have no negative impact on the gut wall, and toxic lectins (Van Der Poel et al., 1990; Huisman and Tolman, 1992), which are associated with mucosal destruction and intestinal immune responses. Apart from lectins, antigenic proteins, present in peas but also in soybeans, are considered to be responsible for intestinal villus atrophy and intestinal immune reactions in calves and piglets (Seegraber and Morrill, 1986; Bush et al., 1992; Dreau et al., 1995; Lallès et al., 1996). Antigenic proteins could be effectively degraded by fermentation using bacteria such as *Bacillus subtilis* (Kiers et al., 2000; Visessanguan et al., 2005; Song et al., 2010). Feeding of *Bacillus subtilis* fermented SBM compared

to feeding conventional heat treated SBM resulted in increased villus heights in the small intestine of weaned piglets (Feng et al., 2007). In this study, the histomorphological analyses of intestinal villi and crypts showed comparable results between birds fed with diets containing fermented or unprocessed peas. This might be explained by species differences regarding pigs and birds or by the level of antigenic epitopes in the fed pea products. Apart from lectins and antigenic proteins, legumes contain also different non-digestible carbohydrates, including  $\alpha$ -galactosides and dietary fiber, which could have an impact on the digestion of nutrients in general and by that on the intestinal microbiota and gut function (Choct et al., 1996; Teirlynck et al., 2009). In the present study, the crude fiber content of grower diets was on average higher in the pea-containing diets (ranging from 3.4% to 3.7%) compared to the SBM based diet (2.8%). Dietary fiber underwent different definitions, one is that it includes any polysaccharide reaching the large intestine as resistant starch, lignin, soluble and insoluble NSP (Montagne et al., 2003). The NSP content of peas usually range between 14% and 20% (Englyst and Hudson, 1996; Bach Knudsen, 1997) depending on variety, location, and growing conditions while about 25% of NSP represent soluble and 75% insoluble NSP (Englyst and Hudson, 1996; Perriago et al., 1996; Nikolopoulou et al., 2007; Adamidou et al., 2011). Soluble NSP are known to increase gut viscosity resulting in a reduced feed passage rate (Van der Klis and Van Voorst, 1993b; Almirall and Esteve-Garcia, 1994; Choct et al., 1996), which could affect the diffusion rate of substrates, intestinal enzymatic activity, and consequently the nutrient absorption (Ikeda and Kusano, 1983; Fengler and Marquardt, 1988; Anison, 1993; Smits et al., 1997). Moreover, soluble NSP might also affect the development of intestinal mucosa as well as mucosal immune responses (Teirlynck et al., 2009). It is hypothesized that viscous digesta may enhances the rate of mucosal cell losses due to increased shear forces of the digesta (Montagne et al., 2003; Teirlynck et al., 2009). Apart from physiochemical effects it could be demonstrated that the feeding of diets supplemented with isolated soluble NSP increased both the digesta viscosity and the concentration of volatile fatty acids in the ileum of broilers suggesting a proliferation of the fermentative microbiota in the small intestine (Choct et al., 1996). Intestinal bacteria and its fermentation products may influence the development of the intestinal morphology and the mucosal immune system (Sakata, 1987; Chichlowski et al., 2007; Brisbin et al., 2008a; Awad et al., 2009). Alterations of the intestinal mucosa in connection with a mucosal T cell accumulation were also seen in this study. However, it might be expected that applied feed processing methods led to potential changes in the diet's NSP content accompanied by changes in the intestinal viscosity and intestinal microbial composition. By contrast, the observed effects on intestinal villus surface area and mucosal immune system were seen both in birds receiving the raw and processed peas. Thus, further studies are required in

order to investigate which components in peas evoke such effects and to highlight the complex interactions between ingested feed and intestinal microstructure, gut-associated immune system and intestinal microbiota.

## CONCLUSIONS

In conclusion, the results of the study illustrated that the feeding of raw as well as processed peas resulted in a decreased mucosal surface area in the jejunum of broilers, which on average was accompanied by lower jejunal glucose transport capacities. In this regard, it can be assumed that changes in jejunal microstructure and nutrient transport are associated with observed mucosal immune reactions which may be induced by pea associated ANF. Further research is needed in order to clarify which specific factors might be responsible for observed effects, if those effects should be considered beneficial or harmful and to what extent gut function, animal health and performance might be affected.

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