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# Effects of feed structure on animal performance, gastrointestinal morphology, gut-associated lymphoid tissue and jejunal glucose transport in laying hens

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## List of abbreviations

| | |
|---|---|
| ANOVA | analysis of variance |
| APC | antigen-presenting cell |
| Approx. | approximate |
| ATP | adenosine triphosphate |
| BCR | B cell receptor |
| BSA | bovine serum albumin |
| BW | body weight |
| BWG | body weight gain |
| CCK | cholecystokinin |
| CD (+; ⁻) | cluster of differentiation (positive; negative) |
| Conc. | concentrated |
| CT | caecal tonsils |
| DNA | Deoxyribonucleic Acid |
| DTT | Dithiothreitol |
| EDTA | Ethylenediaminetetraacetic Acid |
| e.g. | exempli gratia |
| EP | egg production |
| EW | egg weight |
| F | physical form |
| FACS | fluorescence-activated cell sorting |
| FCR | feed conversion ratio |
| FE | feed efficiency |
| FI | feed intake |
| FITC | fluorescein isothiocyanate |
| FS | Forward Scatter |
| GALT | gut-associated lymphoid tissue |
| GLUT-2 | glucose transporter 2 |
| Gt | transepithelial tissue conductance |
| HBSS | Hanks' Balanced Salt Solution |
| HE | Haematoxilin and Eosin |
| i.a. | inter alia |
| i.e. | id est |
| IEL | intraepithelial lymphocytes |
| IFN | interferon |

| | |
|---|---|
| Ig | immunoglobulin |
| IL | Interleukin |
| Isc | short-circuit current |
| LM | light microscopy |
| LP | lamina propria |
| LPL | lamina propria lymphocytes |
| M | mill |
| mAb | monoclonal antibody |
| MALT | mucosa-associated lymphoid tissue |
| MHC | Major Histocompatibility Complex |
| mRNA | messenger ribonucleic acid |
| NK | natural killer (cells) |
| PAMP | pathogen-associated molecular pattern |
| PBS | phosphate buffered saline |
| PE | phycoerythrin |
| PP | Peyer's patches |
| PRR | pattern recognition receptor |
| PS | particle size |
| rpm | revolutions per min |
| RPMI | Roswell Park Memorial Institute Medium |
| Rt | total transepithelial resistance |
| SCFA | short-chain fatty acids |
| SD | standard deviation |
| SGLT-1 | sodium-dependent glucose cotransporter 1 |
| SS | Side Scatter |
| TCR | T cell receptor |
| TLR | Toll-like receptor |
| Th | T helper (cells) |
| Vt | transepithelial voltage potential |
| x *g* | gravity (measurement of centrifugal force) |

# 1 Introduction

The world's population will rise considerably over the coming years and is forecasted to exceed 9 billion people by 2050 (FAO, 2013). This development combined with rising incomes and urbanization will result in a growing demand for livestock products (WHO, 2003). Particularly, the poultry sector is affected by this development, with poultry stocks significantly growing at an average rate of three percent per year (FAO, 2013). Consequently, there is also a growing interest in the opportunities to increase animal health and thus the animal performance. Concerning this matter, the optimization of animal feed and the development of new feeding concepts provide a central starting point. Within the framework of such approaches, the energy consumption used for the feed production is also a factor of interest having an economic and environmental impact.

Therefore, new developments in the production and processing of feed for the use in the poultry sector are promoted in order to increase the quality and safety of feed and to achieve a more energy-efficient feed production. Additionally, innovative resource-conversing feeding concepts are implemented. Hence, different grinding methods, grinding intensities as well as thermal treatment processes are used influencing the structure of feed, which is characterized by the particle size and the particle size distribution as well as by the physical form of the diet. The production of coarsely ground instead of finely ground diets using energy-saving milling methods could reduce the feed production costs and improve the stability, the mixing characteristics and the storability of feed. The further thermal treatment of feed (conditioning) is an energy-intensive process but aims to increase i.a. the nutritive value and thus the digestibility of feed.

As the feed structure is changing by production and processing the question arises whether the animal health and thus the animal performance is influenced by this factor. In contrast to what has been premised so far, recent studies showed that the use of coarsely ground feed and the inclusion of whole grains had no negative impact on the performance of broilers and turkeys, and that in contrast, positive effects regarding the gastrointestinal function and health have been observed (Gabriel et al., 2007; Bank, 2010; Witte, 2012). There is clear evidence that the particle size and the physical form of the diet have an impact on the development of the avian digestive tract, which is also accompanied by changes in physiological processes. Several studies showed that the feeding of coarsely ground as well as mash diets led to an increase of the relative gizzard weights in broilers compared to the feeding of finer particles and thermal processed diets (Nir et al., 1994a; Nir et al., 1994b; Engberg et al., 2002; Peron et al., 2005; Amerah et al., 2007a).

Furthermore, decreased colonization and growth of *Salmonella* in the caecum was observed when broilers were fed with coarsely ground as well as mash diets (Huang et al., 2006; Santos et al., 2008). Thus, feed structure seems to affect the intestinal microbiota, which could help to prevent bacterial infection and reduce the use of antibiotics in the poultry sector.

Moreover, providing laying hens with structural material, such as coarsely ground feed and foraging material, led to a lower incidence of feather pecking and thereby improved animal health and welfare (Blokhuis, 1986; Aerni et al., 2000; El-Lethey et al., 2000; Hetland et al., 2003b) However, data on the effect of different feed structures on laying hens are limited, whereas these studies generally report performance data (Wahlström et al., 1999; Hetland et al., 2003a; MacIsaac and Anderson, 2007; Safaa et al., 2009).

Therefore, the aim of this study was to investigate whether gastrointestinal function of laying hens and consequently their performance and health were affected by the feeding of differently structured diets. For this purpose, investigations were focused on the influence of feed structure on the development of digestive organs including morphometric examinations, on the gut-associated lymphoid tissue as well as on the jejunal glucose transport in laying hens.

# 2 Literature review

## 2.1 Feed processing

Due to the high energy consumption, the reduction of feed particles represents the main cost factor of feed production in the layer industry (Amerah et al., 2007b). While the particle size of protein meals such as soybean meal is usually defined by the pre-ground of the processor, manufacturers of compound feeding stuffs are responsible for the particle size reduction of cereal grains (Amerah et al., 2007b). The hammer mill and the roller mill represent the most commonly used mills in the feedstuff production (Koch, 1996; Waldroup, 1997), whereas the latter plays only a subordinate role (Svihus et al., 2004b). Usually laying hens are fed mash diets, while the feed of broilers is mainly offered in pelleted form (Svihus, 2006). However, there are attempts to use also thermally processed feed such as extruded and expanded diets for laying hens. In the following the hammermill, the roller mill as well as the process of expansion are described and both the advantages and disadvantages of these technologies presented.

The **hammermill** contains a set of hammers, which are attached to a rotor assembly. The rotor is enclosed by a screened grinding chamber and regulates the speed of the hammers and thus the impact between hammers and the grinding material. The grist can leave the grinding chamber when the particles have the appropriate size to pass through the screen hole sizes. Hence, the size and spectrum of particles is determined by the used screen size as well as by the hammer speed (Koch, 1996). After grinding the shape of particles appears spherical with a smooth surface (Reece et al., 1985).
The advantages and disadvantages of the use of hammermills are shown in **Table 1** according to Koch (1996).

**Table 1:** Advantages and disadvantages of the hammermill

| Hammermill | |
|---|---|
| **Advantages** | **Disadvantages** |
| • produce a wide range of particle sizes<br>• work with any friable material and fiber<br>• less initial purchase compared to roller mills<br>• offer minimal expense for maintenance<br>• generally feature uncomplicated operation | • produce greater particle size variability (less uniform)<br>• provide less efficient use of energy compared to roller mills<br>• may generate heat (source of energy loss)<br>• may create noise and dust pollution |

**Roller mills** consist of pairs of rolls, which are horizontally aligned and encompassed by a steel frame. The gap between one pair of rolls as well as the speed of the rolls can be adjusted, so that different particle sizes of feed can be produced. During the grinding process, shearing and compression are the primary forces, which are applied to the feed. The shape of produced particles appears more irregular, being cubic or rectangular (Koch, 1996).

The advantages and disadvantages of the use of roller mills are presented in **Table 2** according to Koch (1996).

**Table 2:** Advantages and disadvantages of the roller mill

| **Roller mill** | |
|---|---|
| **Advantages** | **Disadvantages** |
| • energy efficient<br>• uniform particle size distribution<br>• little noise and dust generation | • little or no effect on fiber<br>• particles tend to be irregular in shape and dimension<br>• may have high initial cost (depends on system design)<br>• when required, maintenance can be expensive |

There is a growing interest in using new technologies for the feed production in the poultry sector. Thermal processed feed such as pellets or expandate is commonly used for broilers, while laying hens are usually fed with mash diets (Svihus, 2006).

The process of **expansion** is referred to as a high temperature, short time (HTST) process. Before the cereals can be fed into this process they have to be ground to mash previously. The expander consists of a screw conveyor, residing in a barrel section, provided with an adjustable annular discharge gap, which regulates the degree of pressure on the feed due to shear and friction (Fancher et al., 1996). Together with the supply of steam, heat is generated reaching temperatures of up to 130°C (Peisker, 2006). In a passage time of 2-5 sec., the screw conveyor forces the feed towards a resistor in the outlet gate of the expander, where it is confronted with outside atmospheric conditions (Williams et al., 1997). Thus, the moister of the expandate evaporates, the material expands in volume and the temperature decreases rapidly (Fancher et al., 1996). Several studies demonstrated that the feeding of expandate enhanced the digestibility of feed in broilers connected with improved animal performance (Plavnik and Sklan, 1995; Williams et al., 1997). The process of expansion can result in a rupture of structural carbohydrates and may lead to an increased gelatinization of starch (Edwards, 1999).

Additionally, productivity benefits were observed regarding improved pellet quality, the ability to add high levels of liquids (particularly fat) to the mash prior to expansion as well as improved hygienic status due to lower microbiological activity in the feed (Fancher et al., 1996; Vest and Calhoun, 1997; Williams et al., 1997; Edwards, 1999; McCracken, 2002).
However, negative effects were observed when diets were thermally processed. During the process of expansion the solubility of non starch polysaccharides could increase resulting in a higher viscosity of the gut content, which may reduce the nutrient digestibility (Edwards, 1999; Svihus, 2006). Furthermore, studies showed that the enzyme activity as well as the vitamin content is reduced when mash feed was further processed to expandate (Inborr and Bedford, 1994; Anderson and Sunderland, 2002). Moreover, the process of expansion is connected with a high energy consumption and accordingly increasing energy costs.
However, it should be emphasized that advantages and disadvantages related to the expansion of feed are always dependent on the used substrate as well as on the processing parameters (Edwards, 1999).

## 2.2 Anatomy, histology and physiology of the avian digestive system

### 2.2.1 Overview of major parts and functions of the digestive tract

As an adaption to the feed intake behavior and special diet of birds, the avian digestive tract shares only few similarities with that of the mammals. The gastrointestinal tract of birds is relatively short and suitable for transforming concentrated diets into nutrients (Larbier and Leclercq, 1992). It begins with a beak, which consists of keratin and allows breaking of e.g. hard grains while teeth and lips are absent. The mouth and pharynx form one unit, namely the bucco-pharyngeal cavity, in which several salivary glands are distributed. The salivary fluid contains mucus, which ensures the lubrication and humidification of ingested feed. However, no amylase activity could be detected in the salivary fluid of chickens, so that the starch digestibility is mainly dependent on the pancreatic amylase (Jerrett and Goodge, 1973). The bucco-pharyngeal cavity is connected to the esophagus, which is relatively long in comparison to mammals. Just before entering the thoracic cavity, the esophagus forms a diverticulum, designated as crop. The crop is used for temporary feed storage and enables the bird to consume significant amounts of feed in a short period of time (Larbier and Leclercq, 1992). Additionally, the crop regulates the transit time of the digesta, which is dependent on several factors including the particle size and humidification of the feed, as well as the filling state of the proventriculus and gizzard. The actual enzymatic digestion of nutrients begins in the proventriculus or glandular stomach, which is cranially connected to the esophagus and represents the first part of the bird’s stomach. Similar to mammals,

secretory cells of tubular glands release hydrochloric acid and the proteolytic enzyme pepsinogen, which compose the gastric juice. By maintaining a pH level of 2 to 4.5 (Svihus, 2011), pathogenic microorganisms can be eliminated and the inactive enzyme precursor pepsinogen is converted to the active enzyme pepsin. Depending on the filling state of the gizzard, the chyme remains in the proventriculus for between a few minutes and an hour (Larbier and Leclercq, 1992). The gizzard, also called the ventriculus, is characterized by its well-developed tunica muscularis with its large mass of strongly myolinated smooth muscles, which can generate remarkable muscular strength during contraction (Svihus, 2011). This allows an effective grinding and a reduction in feed particle size within the digesta. This effect is facilitated by previously ingested stones (grit).

Beside the mechanical function of the gizzard, digestive processes initiated by the secretory activity of the proventriculus also take place, e.g. protein digestion. Furthermore, the gizzard regulates feed intake by influencing the flow of feed from the crop (Chaplin et al., 1992) and influences satiety by mediating vagal and humoral signals. Svihus et al. (2004a) and Hetland et al. (2003a) speculated that enhanced gizzard activity stimulates the release of cholecystokinin, which, in connection with vagal signals, may lead to an increase of pancreatic enzyme secretion.

The small intestine is divided into three sections: The region from the gizzard junction to the pancreatic and bile ducts (duodenum), the area between the end of duodenum and Meckel's diverticulum (jejunum), and the segment between Meckel's diverticulum and the ileo-ceco-colic junction (ileum). The duodenal loop is surrounded by the pancreas, and the bile and pancreatic ducts enter the duodenum near the beginning of the jejunum. Bile aids in the digestion of lipids in the chyme as well as the absorption of fat-soluble vitamins, whereas the pancreatic fluid comprises the enzymes amylase, chymotrypsinogen, trypsinogen and lipase among others, which help to further break down carbohydrates, proteins and lipids (Pubols, 1991). Furthermore, the pancreas secrets bicarbonate, which ensures that the pH of the small intestine is adjusted to a level of 6 to 7. At these pH levels, the majority of digestive enzymes can function efficiently. In addition to the pancreatic and bile secretions, intestinal juice is composed of enzymes released by the brush border of the small intestine. These include saccharase and isomaltase, which are specialized in the hydrolysis of short chain carbohydrates. After passing the ileum, the digesta reaches the large intestine, which can be differentiated into the caeca and the rectum. Within the blind sacks, microbial degradation of particular cell wall components takes place and short chain fatty acids (SCFA) are produced, which serve as energy source for the organism. Moreover, water and electrolytes, in particular sodium, are absorbed. The large intestine terminates with the cloaca, which represents a common passage for digestive, urinary, and reproductive systems.

### 2.2.2 Histological structure of the small intestine

The small intestine is separated into three different sections, namely the duodenum, jejunum and ileum. The intestinal part from the ventriculus to the bile duct junction is regarded as the duodenum; the section from the duodenum end to the Meckel's diverticulum is defined as the jejunum and the ileum comprises the Meckel's diverticulum to the ileo-ceco-colic junction (Yamauchi, 2001; Rougiere et al., 2009).

General structure of the intestinal wall of the small intestine according to König et al. (2008):

- **Tunica mucosa**
  - Lamina epithelialis mucosae
  - Lamina propria mucosae
  - Lamina muscularis mucosae
- **Lamina (Tela) submucosa**
- **Tunica muscularis**
  - Lamina (Stratum) circularis
  - Lamina (Stratum) longitudinalis
- **Lamina (Tela) subserosa**
- **Tunica serosa**
  - Lamina propria serosae
  - Lamina epithelialis serosae

The tunica mucosa of the small intestine is arranged into two fundamental structures, namely villi and crypts. Villi are projections into the intestinal lumen, whereby the core of a villus is considered as an extension of the lamina propria mucosae.
Crypts of Lieberkühn are finger-like invaginations of the epithelium around the villi that are largely lined with younger epithelial cells, while stem cells are situated at the basis. These dividing cells migrate upwards from the depths of the crypts onto the surfaces of the villi, where they are extruded into the lumen. During migration, the cells are subject to differentiation, and develop to mature enterocytes with specialized features in regard to digestion, absorption and mucin secretion. The total transit time in the jejunum of chickens is reported to be approximately 48 hours (Imondi and Bird, 1966).

The single-layered prismatic epithelium of the small intestine mainly consists of enterocytes. Each enterocyte has a basal nucleus and is covered with numerous microvilli located at the luminal surface, which form the brush border. The enterocytes are involved in several transport processes e.g. in the absorption of amino acids, monosaccharides and fatty acids. The structure and function of the epithelium is described in detail in chapter **2.2.3**.

Beside the enterocytes, mucus-secreting goblet cells, intraepithelial lymphocytes (IEL) and enteroendocrine cells are also present. Goblet cells are scattered among the epithelial lining and their luminal surface is covered with microvilli similar to those found on enterocytes. These cells are responsible for the production of the mucins, high-molecular-weight glycoproteins (Forstner, 2009). The secretion of mucins leads to a formation of a protective mucus layer, covering the epithelial surface. This mucus layer provides a physical and chemical barrier that protects the epithelium against harmful external influences, e.g. enteric bacteria, as well as bacterial and environmental toxins (Specian and Oliver, 1991).

The IELs consist of a heterogeneous population of cells, mainly T cells and natural killer cells, as well as to a lesser extent B cells, monocytes, macrophages, dendritic cells and heterophils that reside among the basal and apical part of the epithelium in the gut (Vervelde and Jeurissen, 1993; Bar-Shira and Friedman, 2005; Davison et al., 2011).

Enteroendocrine cells produce peptide hormones, which influence various digestive processes.

The lamina propria mucosae is composed of glandular complexes, smooth muscle cells as well as lymphatic structures. The lamina muscularis mucosae is poorly developed and separates the tunica mucosa from the tunica submucosa, which is rich in vessels and nerves. The tunica muscularis is thin-walled, and the combination of circular and longitudinal smooth muscle gives the intestine an ability to perform complex movements. The lamina subserosa lies immediately beneath the tunica muscularis, followed by the tunica serosa, which encloses the intestinal tract.

### 2.2.3 Epithelial function of the small intestine

The intestinal epithelium has two major functions: Firstly, it constitutes a barrier against the external environment and prevents the invasion of harmful antigens such as microorganisms and their toxins (Madara et al., 1990; Podolsky, 1999).

Secondly, the epithelium acts as a selectively permeable barrier and regulates the transport of nutrients, electrolytes and water from the intestinal lumen into the organism (Powell, 1981; Ferraris and Diamond, 1997).

This transport is mediated by two different pathways: The transcellular and paracellular pathway.

The transcellular route is associated with selective transmembrane energy-dependent channels and carriers, which enable the transport of e.g. amino acids, electrolytes and glucose through the epithelia cells by passing the apical and basolateral membrane (Ferraris and Diamond, 1997; Broer, 2008).

The paracellular pathway or shunt path is found between epithelial cells and consists of multicomponent, multifunctional complexes composing tight junctions, adherens junctions and desmosomes (Schneeberger and Lynch, 2004).

Tight junctions are located as a continuous intercellular barrier at apical-lateral membrane borders and consist of transmembrane proteins such as occludin and claudin (Furuse et al., 1993; Furuse et al., 1998).

In contrast to the transcellular pathway, the paracellular transport is dependent on an electrochemical gradient and regulates the passive diffusion of ions and small noncharged solutes.

The ability of the epithelium to exchange substances depends on the permeability and permselectivity of its epithelial barriers. While the permselectivity is characterized by the specific qualitative tissue property including transporters and carriers, the permeability is determined by the electrical resistance of the epithelia (Powell, 1981).

The total transepithelial resistance (Rt) is defined by Kirchhoff's law; formula adapted from Li et al. (2004):

$$Rt = \frac{(Ra + Rb) \times Rshunt}{Ra + Rb + Rshunt}$$

Rt is calculated by the transcellular resistance, which is composed of the resistance of the apical ($R_a$) and basolateral membrane ($R_b$) and shunted by a parallel resistor ($R_{shunt}$), consisting of tight junctions and intercellular space.

Epithelia can be classified into leaky (gall bladder and small intestine), moderately tight (distal colon) and tight epithelia (urinary bladder) as described by Frömter and Diamond (1972). Powell (1981) describes leaky epithelial as having a total transepithelial resistance of less than 1000 $\Omega \cdot cm^2$ or a paracellular conductance that is greater than 50 % of the total tissue conductance. In leaky epithelia, the paracellular pathway determines transport and charge selectivity, i.e. the preference of tight junctions passing a specific ion more than others (Van Itallie and Anderson, 2004).

On the contrary, in tight epithelia the paracellular permeability and the charge selectivity of tight junctions is negligible, and the high electrochemical gradient is maintained by active transcellular transport (Anderson and Van Itallie, 2009).

### 2.2.4 Absorption of glucose in the small intestine

In all segments of the chicken intestine, except in the coprodeum, glucose absorption occurs and is mediated by the transcellular or paracellular pathway. However, sugar absorption is more efficient in the duodenum and jejunum compared with the ileum and the large intestine (Levin et al., 1983; Savory and Mitchell, 1991; Ferrer, 1993; Amat et al., 1996).

In contrast to other vertebrates, the large intestine of the chicken has the capacity to transport glucose, which has been observed in the proximal region of the caeca (Ferrer et al., 1986) as well as in the rectum (Lind et al., 1980).

Glucose is absorbed across the brush border membrane by the secondary active sodium-dependent glucose cotransporter 1 (SGLT-1). Glucose enters the cell along with two sodium ions, accumulates within the enterocyte and is then transported by the glucose transporter 2 (GLUT-2), situated in the basolateral membrane, into the blood (Ferrer, 1993; Garriga et al., 1999). The latter represents a downhill, passive transport while the electrochemcial gradient across the brush border membrane is maintained by the Na/K-ATPase, which actively transports sodium out of the cell by consuming ATP. This generated sodium electrochemical gradient is the mainspring of glucose uptake in the cell against the concentration gradient, and in consequence the transport rate is dependent on the magnitude of the sodium electrochemical potential gradient (Wright et al., 2007).

Transport rates of glucose are closely related to the degree of villi (Calhaun, 1933) and microvilli development (Planas et al., 1987), as well as to the availability of nutrients i.e. to the luminal carbohydrate concentration (Ferraris and Diamond, 1986).

The SGLT-1 expression is directly regulated by the level of monosaccharides in the lumen of the intestine in many species (Solberg and Diamond, 1987; Shirazi-Beechey et al., 1991; Ferraris, 2001; Suvarna et al., 2005). Specific glucose sensors are located in the apical membrane of the intestine, which are similar to the sweet taste receptors situated in taste cells of the lingual epithelium (Nelson et al., 2001; Shirazi-Beechey et al., 2011). These sensors, namely T1R2 and T1R3, are responsible for detecting luminal sugars and mediate the up and down regulation of SGLT-1 expression (Shirazi-Beechey et al., 2011).

The glucose sensor is able to initiate a G-protein-coupled second-messenger pathway associated with neuroendocrine mechanism, which finally leads to increased SGLT-1 expression (Dyer et al., 2003; Shirazi-Beechey et al., 2011).

However, Shi and Zhang (2006) demonstrated that the T1R2 gene in the chicken genome is absent, which may explain the relatively low level of taste reactivity in chicks during the posthatch period (Ganchrow et al., 1990). There is evidence that in some species of birds, the regulation of the SGLT-1 expression is independent of the luminal glucose concentration (Levey and Karasov, 1992; Afik et al., 1997). Barfull et al. (2001) demonstrated that the SGLT-1 expression and the uptake of a D-glucose analogue across the jejunal brush border membrane vesicles isolated from enterocytes of two day-old chicks were higher compared with those of five week-old chicks, although both feeding groups received equivalent amounts of carbohydrates. It was therefore proposed that there may be other signals besides the luminal glucose level, which control the SGLT-1 expression during ontogenetic development.

The active glucose absorption across the apical brush border can be inhibited by phloridzin, a specific inhibitor of the SGLT-1. Phloridzin, which occurs naturally in plants, binds to the transporter but is not transferred, whereby glucose absorption into the cell is prevented. It is a useful tool to study the function of the SGLT-1 and has therefore been used in several recent experiments (Shapiro, 1947; Ferraris and Diamond, 1986; Ferrer, 1993; Awad et al., 2007).

In addition to the transcellular pathway, glucose is also absorbed passively through intercellular spaces in birds, particularly in wild birds (Karasov, 1994; Caviedes-Vidal and Karasov, 1996; Afik et al., 1997). However, the precise mechanism and its contribution to the total glucose absorption is still unknown. It has been suggested that a cotransport of water with nonaqueous substrates through the SGLT-1 or a water transport driven by osmosis may occur, where the SGLT-1 acts as a water channel (Charron et al., 2006; Zeuthen and Zeuthen, 2007).

## 2.3 Effect of feed structure on animal performance of birds

The assumption that positive performance effects can be achieved in poultry through intensive grinding of feed no longer corresponds to current findings in research (Kluth, 2011). Recent studies showed that using coarsely ground feed and the inclusion of whole grains had no negative impact on the performance of broilers and turkeys, and that in contrast, positive effects regarding the gastrointestinal function and health have been observed (Gabriel et al., 2007; Bank, 2010; Witte, 2012). In addition to the particle size, the structure of the feed is also characterized by the physical form of the diet. Only few studies have been performed regarding the effect of different physical forms of diets in laying hens, presumably as mash is commonly fed in practice (Svihus, 2006). In the following, an overview will be

provided regarding the effect of feed structure in laying hens and broilers, particularly focusing on the factors particle size and physical form of the diet.

Earlier work has shown that the factor particle size in mash diets did not influence the egg production and body weight gains of laying hens (Berg and Bearse, 1951; Deaton et al., 1989). These studies generally report performance data, including egg production, feed intake and feed conversion ratios, in addition to nutrient digestibility. Recent studies also suggest that coarsely ground feed did not impair these performance parameters. Safaa et al. (2009) investigated the effect of feed particles that varied in their geometric mean diameter (fine, medium or coarse mash), on productive performance and egg quality of brown egg-laying hens. The results illustrated that the factor particle size did not affect the productive performance or egg quality of laying hens, except for the parameter feed intake, that was higher for hens fed with the coarsely ground mash. Blair et al. (1973) demonstrated that the egg production of hens, fed with either mash, whole grains or pellets, was similar, but the feed intake increased when feeding whole grains. MacIsaac and Anderson (2007) also examined the effect of feed structure on the productive performance of laying hens. White Leghorn hens were fed with diets that differed in particle size as well as feed, which contained 20 % of whole wheat. The results showed that performance parameters such as feed intake, body weight, weight gain and feed consumption were not impaired by the use of coarsely ground grains or the inclusion of whole wheat, except in regard to egg production, which decreased during the late production phase.

An unique feature of the bird is the existence of the gizzard, which enables a high digestibility when taking up coarse feed particles as well as whole grains. Therefore, an intensive pre-grinding of grains by mills seems not to be beneficial for avian digestion. Furthermore, investigations showed that birds preferred larger feed particles instead of finer ones (Portella et al., 1988). However, the prerequisite appears to be that the digestive system is almost completely developed as animal performance decreased when feeding coarse particles to chicks (Davis et al., 1951; Douglas et al., 1990).

Hamilton and Proudfoot (1995) reported that, with regard to performance parameters of laying hens, the particle size of the feed was of less importance than the physical form of the diet. Leghorn hens were fed with diets differing in their physical form (mash, pellets or crumbles) and particle size (fine or coarse). The results showed that hens given the mash diet had an increased egg production and higher feed intakes than those that received pellets or crumbles, irrespective of whether fine mash or coarse mash was fed.

Pepper et al. (1968) demonstrated that pullets fed with steamed crumbles had higher feed intakes and increased weight gains, but decreased egg production rates in comparison with those that received feed in mash form. Wahlström et al. (1999) observed increased body

weights for laying hens that received crumbled diets in relation to hens fed with mash. Moreover, misplaced eggs and decreased egg weights as well as a decreased feed conversion ratio and an increased energy consumption were detected for laying hens that were fed with the mash diets. However, early studies showed that by feeding laying hens with mash instead of pelleted diets, higher body weights and increased feed consumption could be achieved, although the egg production, egg weights and feed efficiency were comparable within the feeding groups (Heywang and Morgan, 1944; Jensen and McGinnis, 1952; Black et al., 1958). Furthermore, it could be demonstrated that the inclusion of wood shavings led to a higher apparent ileal starch digestibility in laying hens (Hetland et al., 2003a). In a large scale experiment, Hetland et al. (2003b) examined whether the inclusion of 40 % whole wheat, incorporated into 3 mm pellets, had an effect on animal performance. The control group was fed with 3 mm pellets, which did not contain whole wheat. The results showed that laying hens (Hybrids LSL and Shaver 2000), given the experimental diet, had a tendency towards a lower feed conversion ratio ($P$ = 0.07). However, no differences were found with regard to egg production and egg weight between the groups. It was speculated that a better plumage condition of hens, fed with the experimental diet, resulted in a lower feed conversion ratio.

In this context, it is important to stress that animal performance always depends on animal health and welfare. Therefore, several experiments were conducted in order to examine the effect of feed structure on animal health. The results underline that the feeding of pelleted diets instead of mash diets had a negative impact on the welfare of laying hens due to an increased risk of feather picking (Aerni et al., 2000; El-Lethey et al., 2000). Therefore, it has been recommended to provide laying hens with structural material, such as coarsely ground feed and foraging material, which may result in a lower incidence of feather pecking and thereby improve animal health and welfare (Blokhuis, 1986; Aerni et al., 2000; El-Lethey et al., 2000; Hetland et al., 2003b).

Many scientific studies address the examination of effects of different feed structures on performance and nutrient digestibility in broilers. By feeding coarsely ground mash instead of finely ground mash, a higher feed intake and increased animal performance (favorable feed conversion ratio and higher weight gain) could be obtained (Nir et al., 1994a; Amerah et al., 2007c). Peron et al. (2005) investigated the effect of different particle sizes of wheat (coarsely ground vs. finely ground), fed in pelleted form, on the digestibility and performance of broilers. The study showed that the use of coarsely ground pelleted feed compared with finely ground pelleted feed led to decreased digestibility with regard to starch and dry matter. However, the digestibility of proteins and lipids as well as the growth performance was not influenced by the wheat grinding. Jacobs et al. (2010) also demonstrated that the growth performance of broilers were not affected by the diet's particle size, when young chicks were

fed a corn-soybean meal-based diet. The inclusion of whole grains in diets of broilers improved the starch digestibility as well as the feed efficiency in comparison to the use of finely ground commercial compound feeds (Svihus and Hetland, 2001; Hetland et al., 2002; Plavnik, 2002; Hetland et al., 2003a). However, Svihus et al. (2004b) found that broiler performance was not affected by the extent of grinding, when mash or pelleted feed was implemented.

Regarding the influence of thermal feed processing on broiler performance, several studies have shown that higher animal performances, better feed conversion ratios and higher weight gains could be achieved with pelleted feed in comparison with mash feed (Calet, 1965; Douglas et al., 1990; Hamilton and Proudfoot, 1994; Amerah et al., 2007c). In addition, it was demonstrated that broilers fed with pellets containing higher amounts of coarse particles had a similar productive performance to those fed with pellets consisting of finely ground feed particles (Engberg et al., 2002; Svihus et al., 2004b; Witte, 2012).

However, laying hens and broilers, which were provided with mash diets or structural materials such as wood shavings or oat hulls, showed an improvement in starch digestibility (Rogel et al., 1987; Svihus and Hetland, 2001; Hetland et al., 2003a). In this context, it was speculated that birds fed with pelleted diets are becoming overeaters. Thus, the passage rate of ingested feed increases, which may lead to a decreased starch digestion and reduced activity of pancreatic digestive enzymes (Engberg et al., 2002; Svihus, 2006).

Svihus (2006) noted that, with regard to thermal feed processing, negative effects e.g. in terms of the nutrient digestibility (decreased starch digestibility, increased viscosity, reduced nutrient absorption) could occur and concluded that the temperature during processing should be maintained as low as possible.

There is a lack of publications focusing on the effects of feed, produced by different milling methods, on the animal performance. Svihus et al. (2004b) investigated the impact of diets, which had the same geometric mean diameter but were produced either by a hammermill or by a roller mill, on broiler performance and digestibility of feed. While no significant differences were observed regarding the animal performance, a higher digestibility was found for feed ground through a roller mill than for diets produced by a hammermill. It was suggested that conceivably roller mills are rather able to break off the entire cereals by shearing and compression than hammermills, where fragmentation by collision occurs.

## 2.4 Effect of feed structure on the morphology of the avian digestive tract

Several studies have been carried out in order to examine the effect of different particle sizes and the physical forms of diets on the development of the avian digestive tract, although particularly broilers were used as target animals. Particle sizes range from fine to coarse particles and whole grain as well as mash, which may be processed further to pellets, crumbles or expandate. There is clear evidence that the structure of feed has an impact on size and weight of different segments of the digestive tract, particularly regarding the gizzard. Higher relative gizzard weights were observed when feeding coarse particles compared to feeding fine particles in broilers (Nir et al., 1994a; Engberg et al., 2002; Peron et al., 2005; Amerah et al., 2007d).

Several studies illustrated that by feeding whole grain diets in comparison to pelleted diets, the relative gizzard weights of broilers were increased (Preston et al., 2000; Plavnik, 2002; Gabriel et al., 2003; Svihus et al., 2004a; Williams et al., 2008). Furthermore, Hetland et al. (2003a) demonstrated that the implementation of wood shavings to the diet led to increased gizzard weights of layers accompanied with an increase of the total amount of bile acids, suggesting an enhanced gastroduodenal reflux. The gastroduodenal reflux together with the intestine and the cloaca-ceca reflux form the gut reflux, where reverse peristalsis occurs. This reflux ensures that chyme is re-exposed to gastric as well as pancreatic secretions so that digestion and nutrient absorption is promoted (Ferket, 2000)

It was also reported that the physical form of the diet affected the gizzard weight. Pellet-fed birds had significantly lower gizzard weights than mash-fed birds (Nir et al., 1994b; Nir et al., 1995; Engberg et al., 2002). In this context, Hetland et al. (2003b) demonstrated that higher gizzard weights of laying hens could be achieved when feeding 40 % of whole wheat, incorporated into 3 mm pellets, in comparison with feeding 3 mm pellets, which did not contain whole wheat. These results suggest that the microstructure of thermally processed feed, i.e. the particle size distribution within the pellet, is essential for the development of the gizzard. However, it should be noted that thermal processing of feed is always connected with a further size reduction of particles and therefore the factors physical form and particle size of the diet can be interlinked.

The higher gizzard weights in birds fed with diets containing coarse particles are a consequence of an increased frequency of gizzard contractions (Roche, 1981). The intensive grinding reduces feed particle sizes and leads to a greater gizzard development as well as to an increase of the gastrointestinal reflux (Engberg et al., 2002; Gabriel et al., 2003; Hetland et al., 2003a; Amerah et al., 2007b). Conversely, fine particles are not ground extensively for further digestion and a reduction of gizzard weights can subsequently be observed.

Wu et al. (2004) and Witte (2012) stated that the higher gizzard weights of broilers related to coarser particles in feed were not based on an increased tunica muscularis layer thickness but rather on an enlargement of the entire organ.

In this regard, increased relative pancreas weights in combination with increased relative gizzard weights were observed in several studies (Engberg et al., 2004; Gabriel et al., 2007; Williams et al., 2008; Rougiere et al., 2009). Engberg et al. (2002) reported that the relative weight of the pancreas as well as the activity of pancreatic digestive enzymes were affected by feed form, and were significantly higher in mash-fed birds than in pellet-fed birds. In addition, Svihus et al. (2004a) stated that the inclusion of whole wheat leads to an increased secretory activity of the pancreas, potentially as a result of an increased gizzard activity. The enhanced gizzard activity may stimulate the vagus nerve and the release of cholecystokinin (CCK), a duodenal peptide hormone, which influences pancreatic enzyme secretion (Li and Owyang, 1993; Svihus et al., 2004a; Svihus, 2006).

The relative proventriculus weight was not increased by feeding coarse particles in most of the studies, however, a dilatation of this organ could be detected when fine pelleted diets were fed (Jones and Taylor, 2001; Gabriel et al., 2007; Witte, 2012).

Several studies suggest that the dilatation of submucosal glands of the proventriculus may be responsible for this enlargement (O'Dell et al., 1959; Gabriel et al., 2003). In addition, O'Dell et al. (1959) observed degenerated glands, increased cell numbers and mucus secretion, as well as distended musculature in these dilated organs. Witte (2012) reported that broilers fed with fine pelleted diets showed enlarged proventriculi combined with decreased relative gizzard weights and thinner tunica muscularis layers.

The reason for the occurrence of the dilatation of the proventriculus and the underdeveloped gizzards in relation to finely pelleted feed in birds remains unclear. However, it is possible that an overload of the proventriculus may play a role, which might be a consequence of a reduced gizzard motility as a result of fine feed structure (Witte, 2012).

The development of the small intestine tends not to be affected as strongly by the feed structure as compared to the gizzard. Several studies illustrated that coarser particles did not influence the relative organ weight or size of the different parts of the small intestine (Wu et al., 2004; Peron et al., 2005; Gabriel et al., 2007). This may be due to the fact that the gizzard is able to grind feed to a consistently fine particle size irrespective of whether the ingested diet contains large or small particles (Hetland et al., 2004). Thus, by evening out particles size differences, segments of the small intestine might be not affected by different particle sizes in the feed.

In contrast, Amerah et al. (2007a) could demonstrate that duodenal digesta of broilers fed with coarser particles in mash diets contained greater amounts of large particles compared with those fed fine particles in pelleted diets. These results are consistent with findings of

Lentle et al. (2006) who compared the particle sizes of feed with excreta samples from broilers fed with finer or coarser particles. On the basis of these results, it appears that the gizzard does not grind feed to uniformly-sized particles. Therefore, coarser particles from original coarsely ground feed can pass the gizzard and are able to enter the small intestine.
Nir et al. (1994b) reported that pelleting reduced the length of the jejunum and ileum. In addition, Gabriel et al. (2003) observed lower duodenum weights in pellet-fed birds compared with those receiving whole grains. Dahlke et al. (2002) showed that the relative organ weights of the duodenum and jejunum plus ileum were positively related to increased particle size in mash diets.

## 2.5 Effect of feed structure on the microscopic structure of the small intestine of birds

Published data on the effect of the physical form and particle size of feed on the microscopic structure of the intestine are scarce.
Dahlke et al. (2002) investigated the effect of particle size and physical form of diet (mash vs. pellet) on the villus number, villus height and crypt depth in the duodenum of broilers. Pellet-fed birds showed a higher number of villi per transversal duodenum cut and greater duodenal crypt depths when compared to mash-fed birds. An increase in particle size of the diets resulted in deeper crypts and higher duodenal villi, irrespective of the physical form of the diet. Gabriel et al. (2007) observed increased villus to crypt length and surface rations in the duodenum of broilers fed with whole wheat in comparison with mash-fed broilers. This was contrary to the results obtained by Williams et al. (2008) showing no effect of whole wheat on morphological parameters in the duodenum.
A general assumption exists that coarse feed particles and their interactions with the intestinal wall have beneficial effects on the development of the intestinal villi. A resulting enlargement of the absorptive surface can increase the absorption of nutrients and may lead to a better feed conversion ratio (Dahlke et al., 2002; Gabriel et al., 2007).

## 2.6 Gut-associated lymphoid tissue (GALT)

The gut-associated lymphoid tissue (GALT) is a component of the mucosa-associated lymphoid tissue (MALT), which also includes the bronchial, salivary, nasopharyngeal, and genitourinary lymphoid tissue. The MALT has developed specialized features that underline its role as the first line of defense on mucosal surfaces (Lillehoj and Trout, 1996; Bar-Shira et al., 2003). This defense comprises physical, chemical and cellular barriers supported by the

appearance of antigen-presenting cells, immunoregulatory cells, and effector cell types that are distinct from their counterparts in the systemic immune system (Lillehoj and Trout, 1996; Beal et al., 2006).

The GALT contains more lymphocytes than any of the other lymphoid tissues (Kasahara 1993) and more than half of the total lymphocyte pool of the MALT (Yun et al., 2000; Bar-Shira et al., 2003). It represents a key immunological system by initiating and mediating immune function. Thereby the GALT has to on the one hand distinguish between innocuous antigens such as nutrients, and as such should not provoke an immune response, and on the other hand harmful pathogens, which should trigger immune processes in order to defend the body against infections. Handling this balance between tolerance and response is mainly dependent on the interaction of the immune cells of the GALT and precondition for gut health (Bar-Shira and Friedman, 2005; Beal et al., 2006).

The GALT in chicken has organized lymphoid structures, which include all lymphatic cells in the epithelial lining and underlying lamina propria, the so-called intraepithelial cells (IEL) and lamina propria lymphocytes (LPL), as well as specialized lymphoid structures residing in strategic sites along the intestine (Davison et al., 2011). Such definable structures are the bursa of Fabricius, the caecal tonsils (CT), the Meckel's diverticulum, the Peyer's patches (PP) and lymphoid aggregates located within the lamina propria (Lillehoj and Trout, 1996; Davison et al., 2011). In comparison to mammals, the avian immune system lacks structured lymph nodes (Bar-Shira and Friedman, 2005; Casteleyn et al., 2010; Davison et al., 2011). This situation stresses the role of the avian GALT as the major secondary lymphoid organ for the defense against avian intestinal infections (Oláh and Glick, 1984; Muir et al., 2000).

### 2.6.1 Intraepithelial leucocytes in the small intestine

Morphologically, the gastrointestinal mucosa is separated by a basement membrane into two different compartments: The epithelium and the lamina propria (Lillehoj and Trout, 1996; Davison et al., 2011). Within these two layers intraepithelial leucocytes (IEL) and lamina propria leucocytes (LPL) can be found.

The IELs consist of a heterogeneous population of cells, mainly T cells and natural killer cells, as well as to a lesser extent B cells, monocytes, macrophages, dendritic cells and heterophils that reside among the basal and apical part of the epithelium in the gut (Vervelde and Jeurissen, 1993; Bar-Shira and Friedman, 2005; Davison et al., 2011). According to locality, strain, age, and antigen stimulation, the total number and distribution of the IELs differ during the postnatal development (Bäck, 1972; Lillehoj and Chung, 1992; Vervelde and Jeurissen, 1993; Göbel et al., 2001).

### 2.6.2 Innate immune system

The innate immune system is considered as the first line of defense and is able to react quickly so that the spread of pathogens can be limited until adaptive response become mobilized (Davison et al., 2011). It comprises physical and chemical barriers including mucus and antimicrobial chemical components on epithelial surfaces that prevent the entry of pathogens. In case pathogens are able to overcome these barriers, cellular and soluble components are present, which can immediately build on an innate immune response (Murphy et al., 2009; Davison et al., 2011). In addition to phagocytosis and lysis of pathogens, the innate immune system also instigates a cascade of events such as recruitment of diverse immune components and induction and modulation of the adaptive immune system.

The cellular mediators of the innate immune system include leukocytes such as natural killer cells, monocytes and macrophages, dendritic cells and heterophils. These cells are able to identify pathogen-associated molecular patterns (PAMP) by pattern recognition receptors (PRR) such as Toll-like receptors or C-type lectins which are encoded in their germline DNA (Davison et al., 2011). The stimulation of PRRs leads to maturation and activation of e.g. dendritic cells, the most important representative of the antigen presenting cells (APC). By presenting captured antigens and expressing co-stimulatory molecules, activated dendritic cells are able prime naive T cells.

The humoral mediator of the innate immune system is the complement system with its different cytokines and chemokines. These substances are released by innate immune cells in order to stimulate diverse processes of the immune system. Those and the antigen presenting cells represent the connection to the adaptive immune response (Murphy et al., 2009).

### 2.6.3 Cells of innate immune system

*Natural killer cells*

Natural killer cells are considered to be part of the innate immune system (Lanier, 2005; Murphy et al., 2009). Similar to T cells and B-cells, NK cells originate from lymphoid stem cells and are described as large lymphocytes with electron dense granula (Göbel et al., 1994), containing perforin and granzymes. Similar to CD8+ cytotoxic T lymphocytes, they are able to recognize and kill intracellular pathogens, virus-infected cells and neoplastic cells. By expressing, activating and inhibiting receptors, NK cells interact with surface antigens of cells, namely the major histocompatibility complex class 1 (MHC) molecules. They can

distinguish between healthy conventional MHC 1 expressing cells and infected non-conventional MHC 1 expressing cells (Lanier and Phillips, 1995; Lanier, 2005; Murphy et al., 2009). In this process, inteferons such as IFN α and IFN β as well as cytokines e.g. IL-12 secreted by macrophages play an important role as an activator for the NK cells, which on their part release IFN-γ, a immunostimulatory and immunomodulatory cytokine. In opposition to cells of the adaptive immune system, NK cells are unable to generate antigen-specific receptors by somatic cell genetic alterations, and their development is thymus-independent (Pat Bucy et al., 1990; Davison et al., 2011).

Due to the lack of suitable antibodies for detecting NK cells in chicken, early studies were concerned with assaying NK cell-like activity (Sharma and Okazaki, 1981; Chai and Lillehoj, 1988). In 2001 however, Göbel et al. (2001) developed a specific antibody, the 28-4 mAb, in order to identify and characterize NK cells within the intraepithelial leukocytes in the chicken gut. These cells were CD8+ but lacked specific markers for the T cell or B cell lineage, although a small portion of cells were 28-4+ and CD3+, which are probably comparable to mammalian NKT cells (Godfrey et al., 2000; Göbel et al., 2001; Godfrey et al., 2004).

Furthermore, NK cells expressed the common leukocyte antigen CD45, cytoplasmic CD3 and Fc receptor-like Ig-binding activity but did not express MHC class II antigens, CD4 and immunglobulin (Göbel et al., 1994).

Phenotypic studies demonstrated that NK cells in adult chickens are located primarily in the intestinal epithelium and that they may reside in the spleen and blood in low numbers, in contrast to humans, mice and rats (Göbel et al., 2001; Jansen et al., 2010).

*Macrophages*

Macrophages derive from bone marrow stem cells, which develop into monocytes, and after their arrival into the bloodstream are seeded in diverse tissues and organs. Macrophages therefore represent tissue forms of blood monocytes (Dil and Qureshi, 2002).

The principal role of macrophages is in chemotaxis, phagocytosis, killing bacteria and tumor cells, cytokine production and antigen presentation (Qureshi, 2003). Therefore, macrophages are involved in both, the innate and adaptive immune response.

By expressing different pattern recognition receptors (PRR), e.g. scavenger receptors or Toll-like receptors (TLR) are able to detect present pathogens (Dil and Qureshi, 2002). They also secrete cytokines, cytokine inhibitors, endocrine hormones, eicosanoids, neurotransmitters and reactive oxygen intermediates, and thus are essential for regulating immune and inflammatory responses (Klasing, 1998).

*Heterophils*

The complements of mammalian neutrophils are avian heterophils. They represent polymorphonuclear leukocytes which (like neutrophils) form the first line of defense by phagocytosis and elimination of invading pathogens (Beal et al., 2006; Davison et al., 2011). In opposition to mammalian neutrophils, heterophils lack myeloperoxidase and do not produce oxygen radicals.

### 2.6.4 Adaptive immune system

When the non-specific immunity is not effective and overwhelmed by invading pathogens, the adaptive immunity also known as specific immunity or acquired immunity is of fundamental importance. The adaptive immunity involves on the one hand the humoral immunity, which is based on the activity of antibodies produced by B cells, and on the other hand on the cell-mediated immunity, which refers to the activity of lymphocytes, primarily to T cell activity (Erf, 2004; Murphy et al., 2009).

In contrast to the innate immune system, the cells of the adaptive immune system show a high specificity due to the immune response. This specificity enables the immune cells to differentiate between particular pathogens that are mediated by specific pathogen receptors. These receptors, namely the B cell receptor (BCR) and the T cell receptor (TCR), are not encoded in the germ line but instead are acquired through somatic expression processes during maturation. Additionally, the adaptive immune system is characterized by an immunological memory which ensures rapid responses and protection in case of a reinfection with the same pathogen (Davison et al., 2011).

### 2.6.5 Cells of adaptive immune system

*T cells*

T cells represent the antigen-specific component of the cell-mediated immunity (Chen et al., 1991). They originate from haematopoietic stem cells in the bone marrow, colonialize the cortex of the thymus, undergo the process of differentiation and maturation, and finally are released from the thymus to peripheral tissues. T cells express the CD3 polypeptide complex and are characterized by the presence of a T cell receptor (TCR) on their cell surface. Due to the TCR, T cells can be classified into either TCRαβ+ or TCRγδ+ cells, depending on the different protein chains which are connected to each other by disulfide bridges (Davison et al., 2011). In contrast to TCRγδ+ cells, two different subfamilies of TCRαβ+ cells exist, the TCRαβ1 subfamily (expressing Vβ1) and the TCRαβ2 subfamily (expressing Vβ2).

In addition to the TCR, other TCR co-receptors such as CD4 exist, which are expressed by T helper (Th) cells (CD4+) and CD8, located on the cell surface of cytotoxic T cells (CD8+).
Th cells can be assigned into Th1 and Th2 cells. Both cell types recognize antigen peptides, which are presented by APC in association with self-MHC class II molecules. The antigen peptides are derived from endogenous synthesized proteins, e.g. peptides of viral or intracellular bacterial origin, and are displayed by APCs including dendritic cells or macrophages. The interaction with antigen-peptides and the simultaneous binding of the MHC molecules leads to an activation of Th cells. Both subtypes of cells are able to enhance the humoral immune system by activating naive B cells to produce antibodies. Additionally, Th1 cells are able to activate the cell-mediated immunity by reinforcing the killing efficacy of macrophages and the proliferation of cytotoxic CD8+ T cells. All these processes are mediated by cytokines e.g. interferon-γ and interleukin-10 associated with Th 1 cells and interleukin 4 and 13 regarding to Th 2 cells (Murphy et al., 2009; Davison et al., 2011).
Cytotoxic T cells bear the co-receptor CD8 and are specific for MHC class I molecules. Chicken CD8 expresses 2 different isoforms, which are composed of either a CD8αα homodimer or a CD8αβ heterodimer (Tregaskes et al., 1995). The CD8 αα homodimer is expressed on splenic and gut NK cells, intraepithelial γδ T cells and peripheral CD4+ cells while thymocytes as well as cytotoxic T-cells bear CDαβ heterodimer (Göbel et al., 1994; Tregaskes et al., 1995; Luhtala et al., 1997; Luhtala, 1998).
Cytotoxic T cells are able to destroy dysfunctional cells such as cancer cells or virus-infected cells. They recognize antigen peptides, which are presented by any nucleated cells and displayed on MHC class I molecules on the cell surface. If cytotoxic cells recognize infected cells, they can destroy these by inducing apoptosis.
Information regarding to the occurrence and distribution of avian T cells within the population of intraepithelial lymphocytes are scarce. Few studies show that the IEL of the chicken small intestine contains TCRαβ+ and TCRγδ+ CD3+ T cells with minor populations of TCRαβ+ CD4+ (Lillehoj, 1994). Mainly CD8+ T cells could be detected, which primarily express the CD8αα homodimer rather than the CD8αβ heterodimer (Vervelde and Jeurissen, 1993; Göbel et al., 2001; Lillehoj et al., 2004).
In contrast to the IEL population, the lamina propria lymphocytes consist of a larger proportion of TCRαβ+ T cells, and only few TCRγδ+ T cells. The TCRαβ+ T cells predominantly express CD4+, while only a small proportion of CD8+ cells are detectable (Davison et al., 2011).

*Natural Killer T cells*

Although to date only little data exists regarding natural killer T cells (NKT cells) in mammals, it is likely that these cells also exist in chickens (Göbel et al., 2001). NKT cells are a

population of T cells that share some characteristics with natural killer cells, most mentionable the expression of the NK1.1 receptor, the invariant α T cell receptor linked with 3 different β chains, the CD1d (nonclassical MHC class Ib molecules) restriction and considerable cytokine production, in particular interleukin 4, interleukin 10 and interferon γ (Godfrey et al., 2000; Murphy et al., 2009). Instead of NK cells, the NKT cells are thymus – dependent, similarly to T cells, but are not MHC I or II restricted.
NKT cells can be activated in case of infection or innate stimuli, present microbial lipids on their surface and release high levels of IL-4 and IFN-γ. Therefore, these cells have a capable immunoregulatory potential (Godfrey and Rossjohn, 2011).

*B cells*
The bursa of Fabricius, first described by the scientists Glick et al. (1956) and Cooper et al. (1966), is located in the proctodeal region of the cloaca and is unique to birds and essential for the proliferation and differentiation of B cells. B cells represent the humoral immunity arm of the adaptive immune system and are characterized by their ability to produce antibodies against antigens. B cells are distinguishable from other cells by their B cell receptor (BCR), which recognizes specific antigens directly. For an effective activation they need the support of CD4+ T helper cells, which deliver signals via specific cytokines in order to enhance the differentiation of B cells into antibody-producing plasma cells or memory B cells (Murphy et al., 2009). The immunoglobulins (Ig) secreted by plasma cells bind to pathogenic antigens that trigger the immune response. By activating the complement system and stimulating effector cells such as macrophages, IGs ensure that pathogens are removed from the organism.
B cells are almost entirely absent from the IEL, but in contrast, a high amount are detectable in the lamina propria (Davison et al., 2011).

## 2.7 Effect of feed structure on avian intestinal immune system

Few studies are published considering the effect of feed structure on the intestinal immune system. These studies are mainly focused on the effects of feeding different feed structures on the intestinal microbiota. In this context, the use of coarsely ground feed resulted in decreased colonization and growth of *Salmonella* in the intestine, and thereby to a reduced excretion of these zoonotic pathogens by the animal. Thus, the caecal *Salmonella* concentration was reduced by feeding coarsely ground feed or whole cereal grains in broiler chickens (Santos et al., 2008). The physical form of the feed offered also seems to have an influence on the intestinal content of *Salmonella*. The concentration of *Salmonella* in the

caecum was increased by feeding a fine pelleted diet compared to a coarse mash diet (Huang et al., 2006). In addition to the observed positive effects of pH reduction in the gizzard including an increase of microbially produced volatile fatty acids in the caecum and the formation of a thicker intestinal mucus layer, the question arises to what extent the gut-associated immune system is influenced by the feed structure.

Scientific studies investigating the influence of particle size and physical form of the diet on the immune system are scarce. Liu et al. (2006) examined the effect of particle size (coarse vs. fine) and physical form of the diet (mash vs. pellet) on the number and location of mast cells as well as on the histamine content in the small intestine of broilers. By feeding coarsely ground diets, more mast cells were detected in histological sections, which were concentrated in the upper part of the villi of the intestinal epithelium. In the animals fed with finely ground diets, less mast cells were present in the intestinal tissue and these were evenly distributed in the tissue sections. In accordance with the results of the mast cell numbers, the histamine content was higher in the jejunum when feeding coarsely ground diets. With regard to the physical form of feed, no significant differences in the number and localization of mast cells and the histamine content in the intestinal tissue were observed.

## 2.8 Aims of the study

In the light of global population growth, rising incomes and urbanization the demand for livestock products specifically for poultry products is growing continuously (WHO, 2003; FAO, 2013). As a consequence, there is a growing interest in the opportunities to increase animal health and thus the animal performance. In this regard, the optimization of animal feed and the development of new feeding concepts provide a central starting point. The use of different grinding methods, grinding intensities as well as thermal treatment processes influence the structure of feed i.e. the particle size, particle size distribution and the physical form of the diets. The aim of this study was to investigate whether gastrointestinal function and consequently animal performance and health of laying hens were affected by the feeding of differently structured diets.The following hypotheses were derived:

The implementation of different grinding methods (hammermill vs. roller mill), grinding intensities (coarsely ground vs. finely ground) as well as thermal treatment processes (mash vs. expandate) changes the feed structure and has an impact on:

1. Animal performance
2. Development of digestive organs and on the intestinal microscopic structure
3. Intestinal glucose transport
4. Intestinal immune system

# 3 Material and methods

## 3.1 Experimental design

### 3.1.1 Animals and rearing conditions

In eight consecutive trial runs, a total of 384 laying hens (Lohmann Brown) aged 19 weeks were randomly allocated to eight different experimental groups (in total 48 hens per feeding group) with six animals per floor pen each representing a single experimental unit. The lighting program was continuous and consisted of 16 h light and 8 h dark. The ambient temperature was maintained at a constant 21 ± 2°C during the entire experiment. The birds had *ad libitum* access to feed and water, and were reared on litter floor pens (barn system) for a period of 28 days. In the first seven days, all hens were fed with a commercial mash diet in order to adapt them to the experimental conditions. They then received the different experimental diets over a period of 21 days. The laying performance (egg weight and egg production) of each feeding group was recorded daily, and the feed intake and body weight were determined weekly. At the end of each trial run, the hens, aged 23 weeks, were slaughtered by stunning and cervical decapitation, followed by exsanguination.
The study was performed in accordance with the Animal Welfare Act of Germany and all procedures involving handling of animals were approved by the local state office of occupational health and technical safety (Landesamt für Gesundheit und Soziales Berlin, Germany, LaGeSo Reg. Nr. 0117/11).

### 3.1.2 Experimental diets

All diets were produced by the International Research Association of Feed Technology e.V. (Braunschweig-Thune) and based on corn (30 %), wheat (29 %), and extracted soybean meal (22 %). The feed composition and the nutrient content of the experimental diet are shown in **Table 4**. In total, eight different diets were produced: Coarsely ground mash, finely ground mash, coarsely ground and expanded feed, and finely ground and expanded feed, whereby each diet was produced using two different mills: A hammermill (Horizontalrotor-Hammermühle, Tietjen Verfahrenstechnik GmbH, Hemdingen, Germany) and a roller mill (Walzenstuhl, MIAG, Braunschweig, Germany) (**Figure 1**). The roller gap adjustments and the hammermill screen sizes used for the production of the different diets are depicted in **Table 3**.The cereals corn and wheat were separately ground by the mills and afterwards mixed (Zweiwellenpaddelmischer, Dinnissen BV, Sevenum, Netherlands) with the other

components or further processed to expandate The expander (Einwellen-Extruder/Expander, Amandus Kahl GmbH & Co KG, Reinbeck, Germany) was operated at 85 - 90 bar and a processing temperature of 116 °C; mass throughput was in the range of 130 - 150 kg/h. The supply rates of the electrical energy used for the production of the different diets were measured by wattmeters and are shown in **Figure 1**. High readings of expander energy consumption refer to the applied small sized experimental plant (max. 150 - 200 kg/h). Industrial scale plants operate at lower specific energy consumption.

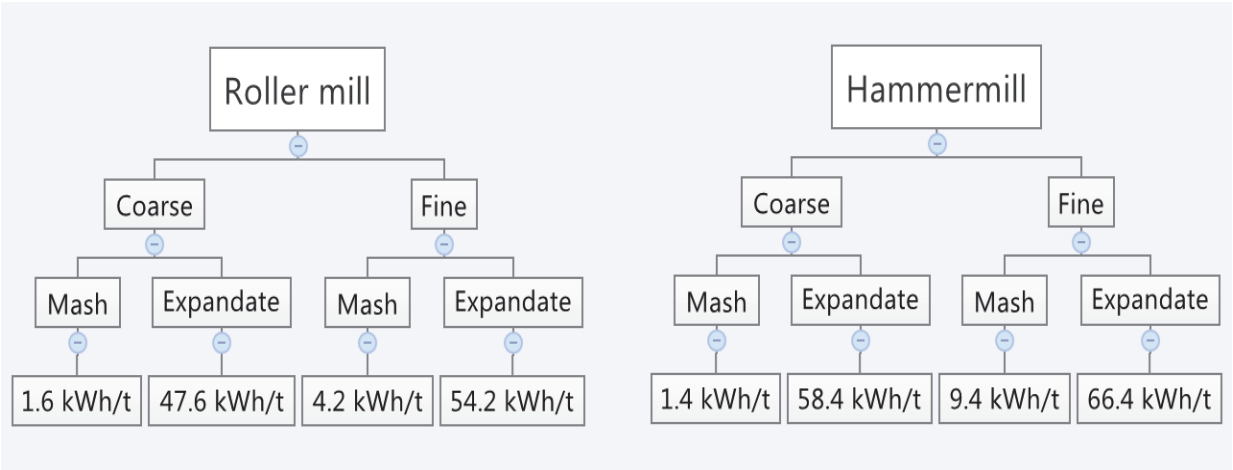


**Figure 1:** Schematic drawing of the produced diets including the electrical energy consumption (kWh/t) required for feed production (data from the production report of the International Research Association of Feed Technology e.V., 2013)

**Table 3:** Roller gap adjustments (mm) and screen sizes (mm) used for the production of the different experimental diets

| | **Roller mill** | | | | **Hammermill** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Mash** | | **Expandate** | | **Mash** | | **Expandate** | |
| **Roller Gap Adjustment/ Screen Size (mm)** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** |
| Wheat | 2 | 0.75 | 2 | 0.75 | – [1] | 5 | – [1] | 5 |
| Corn | 3.25 | 3.25 | 3.25 | 3.25 | – [1] | 6 | – [1] | 6 |

[1] no screen was present

**Table 4:** Feed composition and nutrient content of the experimental diet

| **Ingredients** | **g/kg as fed** |
|---|---|
| Corn | 300.8 |
| Wheat | 290.8 |
| Soybean meal (42 %CP[1]) | 224.8 |
| Sodium carbonate | 86.0 |
| Soya oil | 44.6 |
| Molasses | 30.0 |
| Mineral/Vitamin premix[2] | 12.0 |
| Monocalcium phosphate | 7.8 |
| Salt | 2.0 |
| DL-Methionine | 1.0 |
| L-Tryptophan | 0.2 |
| **Nutrient content** | **g/kg** |
| Dry matter | 881.9 |
| Crude protein | 185.8 |
| Ether extract | 59.7 |
| Crude fiber | 32.7 |
| Starch | 418.5 |
| Ash | 110.6 |
| Calculated ME (MJ/kg) | 11.4 |

[1]CP crude protein

[2] Mineral and Vitamin Premix (Spezialfutter Neuruppin, Germany) containing per kg premix: 400000 IU vitamin A; 40000 IU vitamin D3; 8000 mg vitamin E (alpha-Tocopherole acetate); 300 mg vitamin K3; 250 mg vitamin B1; 250 mg vitamin B2; 2500 mg nicotinic acid; 400 mg vitamin B6; 2000 µg vitamin B12; 25000 µg biotin; 1000 mg calcium pantothenate; 100 mg folic acid; 80000 mg choline chloride; 5000 mg zinc (zinc oxide); 2000 mg iron (iron carbonate); 6000 mg manganese (manganese oxide); 1200 mg copper (copper sulfate-pentahydrate); 45 mg iodine (calcium jodate); 30 mg cobalt (cobalt-(II)-sulfate-heptahydrate); 35 mg selenium (sodium selenite); 35 g sodium (sodium chloride); 55 g magnesium (magnesium oxide).

## 3.2 Slaughtering and sampling

Birds were slaughtered by stunning and cervical decapitation, followed by exsanguination. **Table 5** provides an overview of the samples taken as well as the sampling points used for the different analyses.

In order to determine the organ weights, the pancreas, proventriculus, gizzard and the three segments of the small intestine were extracted, emptied, the adhering fat and mesenteries were removed, and the organs were weighed (**Figure 2**).

The segments removed from the small intestine were:

1. Region from the gizzard junction to the pancreatic and bile ducts (duodenum)
2. Area between the end of duodenum and Meckel's diverticulum (jejunum)
3. Segment between Meckel's diverticulum and ileo-ceco-colic junction (ileum).

For the morphometric analysis, 8 to 10 cm long segments were removed from the duodenum, jejunum and ileum, as described by Uni (Uni et al., 1999):

1. Apex of the duodenum
2. Midway between the point of entry of the bile ducts and Meckel's diverticulum
3. 10 cm proximal to the ileocecal junction

The samples were flushed with cold PBS buffer, the existing fat and mesenteries were removed, and the samples were cut open longitudinally. The open segments were placed on cork boards by using hedgehog spines and subsequently fixed in Bouin's solution.

For the Ussing chamber studies, the entire jejunum was resected and placed in ice-cold-carbogen-gassed Krebs-Henseleit buffer.

The entire duodenum and jejunum used for the flow cytometric analysis were resected, the adhering fat and mesenteries were removed, cut open longitudinally on their mesenteric side, and immediately transferred to the PBS buffer.

**Table 5:** Overview of sampling

| Samples used for | Excised tissue/organs per animal | Number of animals sampled |
|---|---|---|
| Determination of organ weights | Proventriculus<br>Gizzard<br>Duodenum<br>Jejunum<br>Ileum<br>Pancreas | N = 256<br>(4 animals per group x 8 groups x 8 repetitions) |
| Ussing chamber experiments | Jejunum | N = 64<br>(1 animal per group x 8 groups x 8 repetitions) |
| Histology | Duodenum<br>Jejunum<br>Ileum | N = 64<br>(1 animal per group x 8 groups x 8 repetitions) |
| Fluorescence cytometry | Duodenum<br>Jejunum | N = 64<br>(1 animal per group x 8 groups x 8 repetitions) |

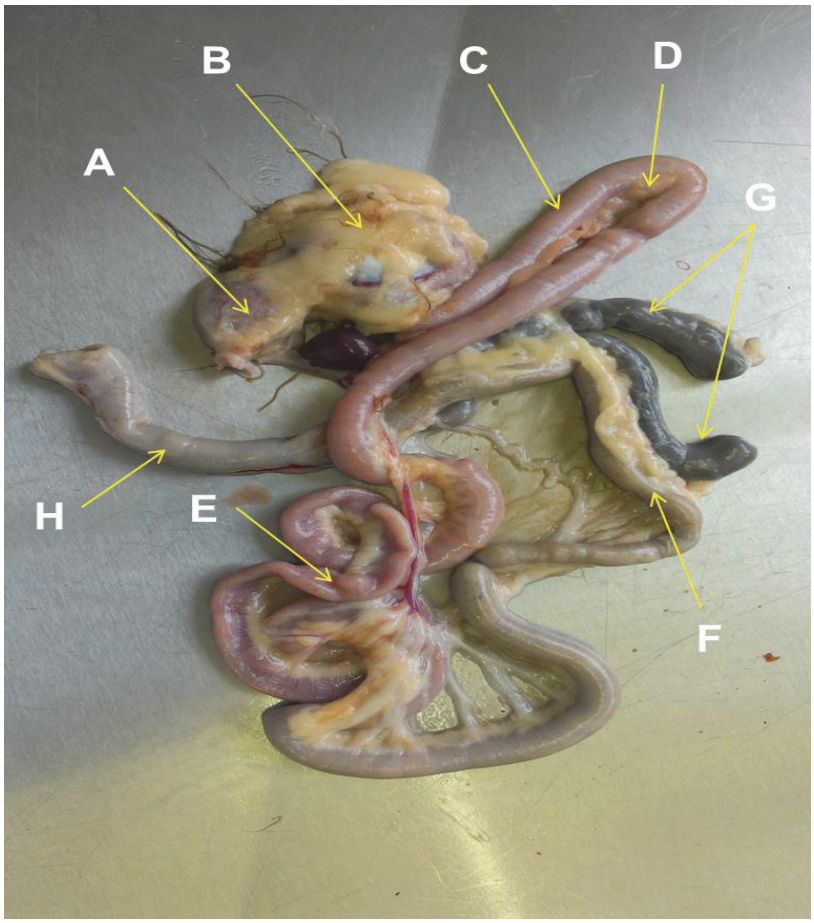


**Figure 2**: The gastrointestinal tract of a laying hen is shown and the different segments are labeled. A = proventriculus, B = gizzard,C = duodenum, D = pancreas, E = jejunum, F = ileum , G = caeca, H = rectum

## 3.3 Measurement of particle size of feed by dry and wet sieving analysis

Feed particle sizes of the eight diets were determined by dry and wet sieving. To date there is no official method or protocol, which describes the procedure of determining the particle size distribution of feed by using dry or wet sieving analysis (Wolf et al., 2012). Therefore, sieve analyses were conducted based on the protocols of previous studies (Laurinen et al., 2000, Wolf et al. 2010, Witte 2012, Wolf et al. 2012).

Representative 100 gram samples of each diet were passed through a sieve stack on a sieve shaker (Analysette 3®, Fritsch, Idar Oberstein, Germany) for 10 min at an amplitude of 7.

The sieve stack consisted of nine sieves with screens of different mesh sizes (4 mm; 2,5 mm; 2 mm; 1,6 mm; 1,25 mm; 1 mm; 0,63 mm; 0,4 mm; 0,15 mm).

The sieves were dried prior to analysis at 103°C in a drying oven, cooled in a desiccator, and individually weighed. After the shaking process, the amount of particles retained on each screen was determined by subtracting the weight of the sieve and the retained feed from the blank weight of the sieve.

In the case of wet sieving, representative 100 gram samples of each diet were previously suspended in 1000 ml water with occasional stirring. After one hour, each sample was washed by flushing with 10 liters of water with corresponding pressures through the sieve stack.

By weighting the sieves again after drying, fractions of feed particles on each screen were detected, and the proportion of particle sizes (% of mass) was calculated in relation to the dry matter content of the original feed sample.

## 3.4 Histological examinations

Tissue samples from the small intestine were used for the histological examinations. 8 to 10 cm long segments were removed from the duodenum, jejunum and ileum as described by Uni (1999):

1. Apex of the duodenum
2. Midway between the point of entry of the bile ducts and Meckel’s diverticulum
3. 10 cm proximal to the ileocaecal junction

After flushing with cold PBS buffer, the excised samples were cut open longitudinally, placed on cork boards, and subsequently fixed in Bouin's solution. In total, samples from the duodenum, jejunum and ileum of eight animals per feeding group were evaluated.
The histological preparation of the intestinal segments was conducted within one week after sampling. After dehydration and infiltration with solidified paraffin wax, the samples were embedded and stored in a refrigerator to cure until sectioning. The paraffin blocks were cut with a sledge microtome, and the obtained sections were mounted on slides. Finally, the sections were stained with Haematoxylin-Eosin (HE) and evaluated using a light microscope for morphometric analyses. The equipment and reagents used for the histological work are listed in **Table 6**. The individual steps of the procedure are described in detail in the following chapter.

### 3.4.1 Histological preparation of the intestinal segments

Tissue samples from the small intestine were fixed in Bouin's solution (according to Böck 1989) for 48 hours. The fixative comprised the following chemicals:

- 1500 ml of saturated picric acid
- 500 ml of 37 % formaldehyde
- 100 ml glacial acetic acid 99 to 100 %

After fixation, the samples were cut into small pieces sized with an area of ca. 5 x 10 mm and a thickness of 5 mm, in order to ensure the rapid penetration of the fixation medium and subsequently an optimal fixation quality (Meyer and Hornickel, 2011). Specimens were passed through a series of liquids during the course of embedding, staining, and mounting (Kiernan, 2008). At first, tissue samples were placed in plastic capsules and passed through an ascending concentration of ethanol for dehydration, in order to make them suitable for the following embedding process:

- 48 hours in 70 % ethanol + few drops of ammonia (conc.)
- 48 hours in 80 % ethanol
- 2 hours in 96 % ethanol
- 2 hours in isopropanol

The drops of ammonia (conc.) were added to the 70 % ethanol. After passing the ascending concentrations of ethanol, samples were transferred into the clearing agent xylene for two hours to remove the ethanol from the tissue.
After this procedure, the samples were stored in paraffin I overnight, placed in paraffin II for 4 hours on the next day, and finally transferred into paraffin III for another 4 hours. The process of tissue infiltration with paraffin wax was carried out in an oven maintained at a temperature of 60°C. In the last step, the tissue samples were embedded with fresh 60°C paraffin in metal molds and afterwards hardened using a cooling plate at 4°C. The paraffin blocks were stored in the refrigerator until sectioning.
The hardened blocks were cut with a sledge microtome. The 5 µm thick sections obtained were placed in a warm water bath (40°C) in order to allow stretching. Then the sections were carefully mounted on adhesive silanized glass slides and dried in an oven overnight at a temperature of 37°C. For further storage, the sections were kept at room temperature.

### 3.4.2 Histological staining of the intestinal sections with Haematoxylin-Eosin

HE staining is a standard staining method implemented in histology that comprises dichromatic staining, which is composed of the nucleus dye haematoxylin and the acidic cytoplasm dye eosin (Böck, 1989). After staining, the nuclear chromatin appears blue to purple while the cytoplasm, collagen and keratin are pink, which allows the differentiation of the individual components of the section (Kiernan, 2008).
The staining method began with the process of deparaffinisation, in which adhering paraffin wax was removed from the tissue by using xylene. Afterwards, the process of rehydration was achieved by using descending concentrations of ethanol. This step was essential because the staining process took place in an aqueous milieu and the agents xylene and alcohol are immiscible with water. After staining with haemalum according to Mayer and eosin, the slides were again dehydrated in ascending concentrations of ethanol, and finally covered with the synthetic resin Histokitt®. The following protocol was applied:

1. Process of deparaffinisation and rehydration
    - Xylene I 10 min
    - Xylene II 10 min
    - Isopropanol 6 min
    - 96 % ethanol 3 min
    - 80 % ethanol 3 min
    - 70 % ethanol 3 min

- 50 % ethanol 3 min
- Aqua bidest I 3 min
- Aqua bidest II 3 min

2. Staining with haemalum and eosin
   - Haemalum after Mayer 5 min
   - Washing in running tap water 10 min
   - 1 % eosin in Aqua dest. 3 min
   - Washing in running tap water 1 min

3. Process of dehydratation
   - 70 % ethanol 2 min
   - 80 % ethanol 2 min
   - 96 % ethanol 2 min
   - Isopropanol 3 min
   - Xylene I 10 min
   - Xylene II 10 min

4. Covering slides with the synthetic resign Histokitt®

### 3.4.3 Microscopic evaluation

After staining with HE, the Bouin-fixed tissue slides of the different intestinal sections were analyzed with a light microscope (Photomicroscope III, Zeiss, Germany), which was equipped with a digital camera (DP72, Olympus, Germany) and a corresponding analysis program (Cell Sense software, Olympus, Germany). The morphometric analyses were focused on the intestinal villi with their crypts of Lieberkühn. The villus height (measured from the tip of the villi to the villus crypt junction) and crypt depth (defined as the depth of the invagination between adjacent villi) were measured for each intestinal segment (**Figure 3**). 20 villi and 20 crypts per section were examined while only vertically oriented villi and crypts were taken into account. Per animal and intestinal segment (duodenum, jejunum, ileum), four sections were analyzed. For the evaluation of the villus height and crypt depth, a 2x respectively 4x magnification was used.

In total, 64 animals (1 animal per group x 8 groups x 8 repetitions) were examined taking the single-blind method into account.

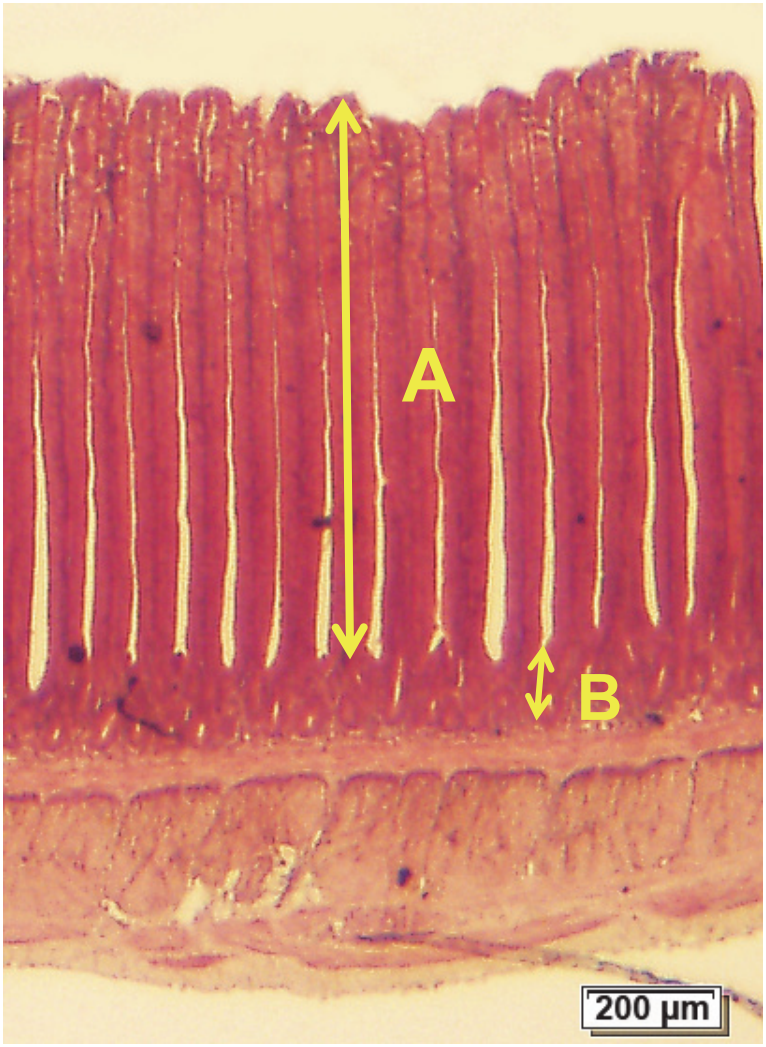


**Figure 3**: Microscopic picture of the intestinal mucosa of the ileum. Morphometric analysis was focused on the villus length (A) and the crypt depth (B), HE

**Table 6:** Overview of equipment and reagents

| Product | Supplier | Based in |
|---|---|---|
| EC-350 paraffin-embedding station | Microm | Walldorf, Germany |
| Sledge microtome, Typ 1400 | Leitz | Wetzlar, Germany |
| Heraeus® drying oven, T5042 | Heraeus electronic | Hanau, Germany |
| Rotilabo®-embedding cassettes Macro | Carl Roth GmbH&Co.KG | Karlsruhe, Germany |
| Magnetic and heating stirrer, RH-KT/C | IKA Labortechnik GmbH&Co.KG | Staufen, Germany |
| Coverslip, 24 x 50 mm | VWR International | Darmstadt, Germany |
| Histobond® microscope slides | Marienfeld GmbH | Lauda-Königshofen, Germany |
| Paraplast® | Carl Roth GmbH&Co. KG | Karlsruhe, Germany |
| Haemalum after Mayer | Carl Roth GmbH&Co.KG | Karlsruhe, Germany |
| Eosin | Merck | Darmstadt, Germany |
| Xylene | Medite | Burgdorf, Germany |
| Ammonia (conc.) | Merck | Darmstadt, Germany |
| 96 % ethanol denatured with Methylethylketon (MEK) | Carl Roth GmbH&Co.KG | Karlsruhe, Germany |
| Isopropanol | Carl Roth GmbH&Co.KG | Karlsruhe, Germany |
| 70 % ethanol | Carl Roth GmbH&Co.KG | Karlsruhe, Germany |
| Roti®-Histokitt | Carl Roth GmbH&Co.KG | Karlsruhe, Germany |
| Glacial acetic acid | Carl Roth GmbH&Co.KG | Karlsruhe, Germany |
| 37 % Formaldehyde | Carl Roth GmbH&Co.KG | Karlsruhe, Germany |
| Picric acid solution (saturated) | Sigma-Aldrich Biochemie GmbH | Darmstadt, Germany |
| PBS (Phosphate Buffered Saline Substance Dubecco's) | Biochrom AG | Berlin, Germany |

## 3.5 Ussing chamber technique

Over 60 years ago, the Danish scientists Ussing and Zerahn (1951) developed an apparatus that was able to simultaneously determine the $Na^+$ transport and electric current through frog skin, today known as the Ussing chamber.

By eliminating transepithelial diffusion forces (osmotic and electrochemical gradients), Ussing detected ion movements that are transported actively by epithelial cells and thus provided the basis for subsequent research regarding transepithelial transport processes (Clarke, 2009).

### 3.5.1 Ussing chamber design

Transepithelial voltage potential (Vt), short-circuit current (Isc) and electrical resistance (Rt) were measured with a microcomputer-controlled voltage/current clamp (K. Mussler Scientific Instruments, Aachen, Germany). The voltage clamp was connected with a modified net-supported Ussing chamber (constructed by Freie Universität Berlin). The net was used in order to protect the fragile tunica mucosa from hydrostatic and hydrodynamic loads. The voltage/current clamp was attached to electrodes as follows:

- Agar salt bridges (mm) located close to the serosal and mucosal surface of the mounted tissue provided contact to Ag-AgCl electrodes, that were used to measure the transepithelial voltage potential Vt.
- Salt bridges (mm), situated in distance to the mounted tissue, connected the Ussing chamber with Ag-AgCl electrodes, responsible for short-circuit current passing across intestinal preparation.

### 3.5.2 Sampling and mounting of tissue in the Ussing chamber

In total, jejunal segments of 64 laying hens (1 animal per group x 8 groups x 8 repetitions) were analyzed in the Ussing chamber.
The jejunum, maintained in oxygenated (95 % $O_2$, 5 % $CO_2$) and ice-cooled Krebs-Henseleit buffer, was cut into sections of 5-7 cm, and existing fat and mesenteries were removed.
A longitudinal incision was placed along the mesenteric border of the jejunum to obtain the tunica mucosa. It was subsequently placed vertically between the two halves of the Ussing chamber (**Figure 4**), as described in detail by Ruhnke et al. (2013). The time span from slaughtering of the animals to the mounting of the tissue in the chamber took place within 10 min.
The serosal and mucosal surface of the tissue, with an exposed area of 0.79 $cm^2$, was bathed in a modified Krebs-Henseleit buffer in glass reservoirs, each filled with 15 ml buffer solution and connected to one of the chamber halves. The buffer was continually stirred, heated to 38°C, and oxygenated with carbogen, a 95 % /5 % mixture of $O_2$ and $CO_2$, in order to simulate the conditions in the chicken's intestine. During the experiment, leads from above permitted the addition of substances into the buffer of the respective half-cell.

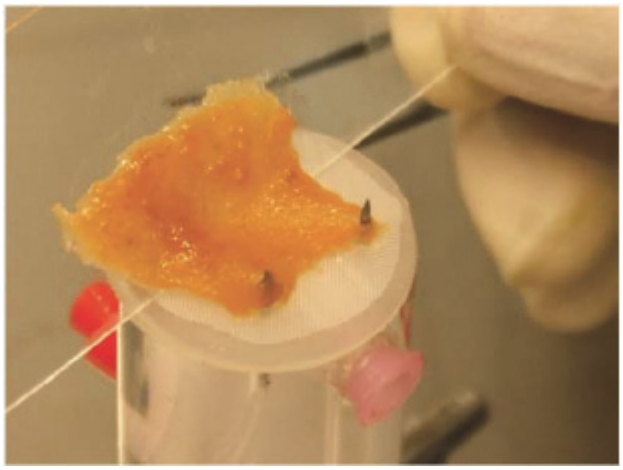

**Figure 4:** Transfer of the jejunal mucosa onto the net supported Ussing chamber cylinder

### 3.5.3 Electrical measurements

Before inserting the tissue in the chamber, the resistance of the system, consisting of fluid resistance, electrode resistance, and the resistance of the agarose bridges was determined in order to obtain a blank value. This parameter was required for adequate calculation of resistance and current.

After tissue insertion, the transepithelial resistance Rt was detected under open-circuit conditions. The transepithelial voltage potential was measured in response to bipolar 50 µA current pulses that were generated for 200 ms, and Rt, was calculated every 6 sec. according to Ohm's law. The transepithelial conductance (Gt) corresponds to the reciprocal value of the transepithelial resistance, and both parameters represent an useful indicator for the integrity of the sample.

After equilibration for approximately 8 to 10 minutes, the tissue was short-circuited by clamping the voltage to 0 mV by passing the appropriate Isc through the Ag-AgCl electrodes.

After a tissue stabilization period of about 10 minutes, D - glucose (10 mmol/l) was added to the buffer solution of the mucosal side of the chamber. Simultaneously, mannitol (10 mmol/l) was added to the serosal side in order to maintain an osmotic balance across the mucosa.

After reaching a baseline of Isc, phloridzin (100 µmol/l) was applied to the mucosal side and finally carbachol (100 µmol/l) was added to the serosal side of the tissue.

The electrical response was observed as the peak response, obtained approximately 1 minute after addition of the different substrates. The difference between the peak Isc/Gt and the basal Isc/Gt expressed by ΔIsc represented the electrogenic ion movements by active transport.

## 3.6 Fluorescence cytometry of lymphocyte subpopulations in gut-associated lymphoid tissue

### 3.6.1 Principle of fluorescence cytometry

The method of Fluorescence Activated Cell Scanning (FACS) allows the measurement and analyses of physical and molecular characteristics of cells or other particles. A fluorescence cytometer is able to detect the size and granularity of single cells. If these cells were previously labeled with a fluorescent dye, fluorescence signals can also be detected. The particles are strung by a hydrodynamically focused stream of liquid, such that they are consecutively hit by a monochromatic laser beam. The light scattered from each particle is collected by band-pass filters. The forward light scatter (FS) is proportional to cell-surface area or size. Side-scattered light (SS) is proportional to cell granularity or internal complexity. Fluorescence signals emitted by the cells while passing the laser beam are detected by photodetectors and amplified by photomultiplier tubes. The cell cytometer used in this study has an argon laser beam that emits light at a wavelength of 488 nm as well as a red diode laser that emits light at a wavelength of 635 nm.
Based on the light scattering and the fluorescence characteristics of individual cells, the flow cytometer allows the differentiation, characterization and quantification of different cell populations. The equipment and chemicals used for flow cytometric analyses are listed in **Table 9.**

### 3.6.2 Preparation of the intraepithelial lymphocytes

At first, a protocol was established, which ensures the reliable determination of different lymphocyte subsets from the gut-associated lymphoid tissue of laying hens using fluorescence cytometry. For the analyses, the entire duodenum and jejunum were resected, and adhering fat and mesenteries were removed. Afterwards, the segments were opened longitudinally at the mesenteric side, flushed with PBS buffer to remove fecal contents, and immediately transferred to the PBS buffer. In order to isolate and purify the intraepithelial lymphocytes from the intestinal epithelium, incubation and centrifugation steps were performed as follows (composition of media used for flow cytometric analyses are listed in **Table 10.**

Isolation and purification of the lymphocyte subsets:

- Transfer of each tissue sample into 100 ml of media containing dithiothreitol (DDT) and ethylenediamine tetraacetic acid (EDTA)
- Incubation of the sample in an orbital shaker at 110 rpm (revolutions per min) for 3 x 30 min at 4°C; filtering of the sample through a synthetic gauze after every 30 min to obtain the cell suspension; transfer of the tissue sample to a fresh 100 ml medium; incubation in order to obtain additional IELs
- Centrifugation of the obtained cell suspension at 600 x *g* for 10 min at 4°C
- Discarding of the supernatant without disturbing the pellet and resuspension of the pellet in Hanks-balanced salt solution (HBSS)
- Addition of 60 µl DNAse (1 : 500 dilution); incubation of the sample after gentle shaking at room temperature for 5 min
- Centrifugation at 600 x *g* for 10 min at 4°C
- Removal of the supernatant and resuspension of the pellet in 25 % Percoll solution so as to eliminate remaining cellular debris and mucus; subsequent centrifugation at 600 x *g* for 20 min at 4°C
- Rejection of the supernatant and resuspension of the pellet in Roswell Park Memorial Institute Medium No. 1640 (RPMI 1640) followed by centrifugation at 300 x *g* for 10 min at 4°C
- Discarding of the supernatant and resuspension of the pellet in RPMI 1640
- Assessment of the cells in regard to their vitality via trypan blue exclusion staining; counting and adjustment in RPMI 1640 at 1 x $10^6$ cells/100µl

In order to ensure that only IELs were isolated from the intestinal tissue, several tissue samples were fixed in Bouin's solution, stained with HE and finally examined histologically. The histological preparations were performed as described in chapter **3.4**.

### 3.6.3 Immunostaining and flow cytometric analysis

In order to characterize the lymphocyte subpopulations of the epithelium, specific fluorescence-labeled antibodies were used, which were directed specifically against certain cell surface proteins. Thus, the different IELs were marked based on their cell surface structure and analyzed in the FACS. The focus lay on the characterization and differentiation of T cells and their subpopulations, NK cells, NKT cells and B cells. The fluorochromes that

were applied for the FACS analysis were fluorescein isothiocyanate (FITC), R-phycoerythrin (R-PE) and Alexa Fluor 647. The primary and secondary antibodies used are listed in **Table 7** and **Table 8**. Additionally, isotype control antibodies were used in order to confirm the specificity of primary antibody binding and to assess the level of background staining.

**Table 7:** Primary antibodies used for phenotyping of the IELs

| Primary antibody | Isotype | Dilution | Antigen specificity | Target cells |
|---|---|---|---|---|
| Mouse Anti Chicken CD45[1] Clone: UM 16-6 | IgG2a | 1:400 | CD45 | All leukocytes |
| Mouse Anti chicken CD3[1] Clone: CT-3 | IgG1 | 1:100 | CD3 | T cells |
| Rat Anti Human CD3:FITC[1] Clone: CD3-12 | IgG1 | 1:5 | CD3 (surface and intracellular CD3 protein) | T cells NK cells (intracellular CD3 protein) |
| Mouse Anti Chicken CD8α:RPE[1] Clone: 11-39 | IgG1 | 1:10 | CD8 alpha | Cytotoxic T cells NK cells |
| Mouse Anti Chicken TCR γ/δ[1] Clone: TCR-1 | IgG1 | 1:50 | Gamma/delta T cell receptor | γδ T cells |
| Mouse Anti Chicken TCRab/Vb1[4] Clone: TCR 2 | IgG1 | 1:100 | Alpha/beta T cell receptor | Subpopulation of αβ T cells |
| Mouse Anti Chicken TCRab/Vb2[4] Clone: TCR3 | IgG1 | 1:100 | Alpha/beta T cell receptor | Subpopulation of αβ T cells |
| Mouse Anti Chicken 28-4[4] | IgG3 | 1:100 | NK cell receptor | NK cells |
| Mouse Anti Chicken AV20[1] | IgG1 | 1:1000 | B cell receptor | B cells |
| Mouse Anti Chicken CD4-FITC[1] | IgG1 | 1:500 | CD 4 | T helper cells |

[1] AbD Serotec, Düsseldorf, Germany
[2] Life Technologies, Darmstadt, Germany
[3] Southern Biotech, Birmingham, USA
[4] Prof. Thomas Göbel, Ludwig-Maximilians-Universität München (LMU), Germany

**Table 8:** Secondary antibodies used for phenotyping of the IELs

| Secondary antibody | Primary antibody | Dilution |
|---|---|---|
| Goat Anti Mouse IgG2a[2] Alexa Fluor 647 | Mouse Anti Chicken CD45[1] | 1:400 |
| Goat $F(ab)_2$ Anti Mouse IgG1[3] R-PE | Mouse Anti chicken CD3[1]<br>Mouse Anti Chicken TCR γ/δ[1]<br>Mouse Anti Chicken TCRab/Vb1[4]<br>Mouse Anti Chicken TCRab/Vb2[4]<br>Mouse Anti Chicken AV20[4] | 1:200 |
| Goat Anti Mouse IgG3[3] FITC | Mouse Anti Chicken 28-4[4] | 1:100 |

[1] AbD Serotec, Düsseldorf, Germany
[2] Life Technologies, Darmstadt, Germany
[3] Southern Biotech, Birmingham, USA
[4] Prof. Thomas Göbel, Ludwig-Maximilians-Universität München (LMU), Germany

For the FACS analysis, 1 x $10^6$ cells/100 µl were transferred into FACS tubes, and the respective primary antibodies were added and incubated on ice for 25 min. Cells were then washed with a solution, consisting of PBS, 0.5 % bovine serum albumin (BSA) as well as 2 mM EDTA, and centrifuged at 300 x *g* for 5 min. After centrifugation, the supernatant was removed and the obtained pellet resuspended. In the case of unlabeled primary antibodies, a secondary antibody was applied, depending on the isotype of the primary antibody. After incubation on ice for 25 min, the centrifugation and washing steps were repeated as described above. Finally, the FACS tubes were analyzed by the FACS Calibur® flow cytometer and the data were evaluated with the help of the software CellQuest pro®.

The results of the measurements were illustrated in density plots and the certain cell populations were differentiated by size, granularity and fluorescence characteristics. According to the FS and SS characteristics, the leucocyte population within the cell suspension was selected. Within the selected leucocyte population, the distribution and relative frequency of the different lymphocyte subpopulations were evaluated and expressed as percentages.

**Table 9:** Overview of equipment and reagents

| Product | Supplier | Based in |
|---|---|---|
| Heraeus®Megafuge®1.0R | Thermo Scientific | Karlsruhe, Germany |
| MaxQShakers | Fisher Scientific GmbH | Schwerte, Germany |
| FACS Calibur® | Becton Dickinson Bioscience | San José, USA |
| CellQuest pro® | Becton Dickinson Bioscience | San José, USA |
| FACS-tubes | Falcon (Becton Dickinson) | Franklin Lake, USA |
| Sefar Nitex (120µm Nilon net) | Sefar AG | Heiden, Switzerland |
| HBSS (Hanks balanced salt solution) | Biochrom AG | Berlin, Germany |
| EDTA > 99 % (ethylenediaminetetraacetic acid) | Carl Roth GmbH&Co.KG | Karlsruhe, Germany |
| 1,4-Dithiothreitol >99 % | Sigma-Aldrich Biochemie GmbH | Hamburg, Germany |
| Instamed 9.55 g/L PBS Dulbecco w/0 $Ca^{2+,}$ $Mg^{2+}$ | Biochrom AG | Berlin, Germany |
| RPMI,1640 (Roswell Park Memorial Institute Medium) | Biochrom AG | Berlin, Germany |
| Percoll™ | GE Healthcare Bio Science | Uppsala, Sweden |
| DNAse | Sigma-Aldrich Biochemie GmbH | Hamburg, Germany |
| Hepes Pufferan® >99 % | Carl Roth GmbH&Co.KG | Karlsruhe, Germany |

**Table 10:** Media and buffer

| | |
|---|---|
| **DTT-EDTA Medium** | |
| 1,4-Dithiothreitol (DTT) | 2 mM |
| Ethylenediaminetetraacetic acid (EDTA) | 1 mM |
| **Percoll (90 %)** | |
| 10 x HBSS | 10 ml |
| $NaHCO_3$ | 0.175 g |
| Percoll (density: 1.131 g/ml) | 90 ml |
| **Percoll (25 %)** | |
| HBSS without $Ca^{2+}$ and $Mg^{2+}$ | 43.34 ml |
| Percoll (90 %) | 16.66 ml |

## 3.7 Statistical analysis

Statistical analyses were conducted using SPSS (version 21.0, Chicago, IL, USA).
The floor pen represented the experimental unit. Means and standard deviations for each of the eight experimental groups (mean ± SD) are reported.
The Shapiro-Wilk Test was used to test whether the data were normally distributed.
Since the sample under investigation was found to be normally distributed, all data were analyzed by using a three-factor analysis of variance to control for interaction and single factor effects with respect to the factors milling method, physical form and particle size of the diet (2 x 2 x 2 factorial arrangement).
Additionally, a one-way ANOVA and the post hoc Tukey's test were performed in order to identify significant differences between the eight feeding groups. Results tables show superscript notation. Group means with different superscripts are significantly different in Tukey's post-hoc test. Differences were considered significant at $P < 0.05$.
Statistical analyses were adjusted with respect to the initial body weight, in order to take into account that minor changes in the initial body weights within the groups could have effects on the results of the overall performance data. Therefore, the variable "baseline body weight" was defined.
With regard to the flow cytometric analyses the fractions of CD4+ and AV20+ cells were not included in the statistical analysis due small sample sizes. Data were expressed in means and standard deviations.
The graphical presentation (box plots) of the data was done with SPSS 21.0 while the scatter plots showing the results of the flow cytometric analyses were performed using the software CellQuest pro®.
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# 4 Results

## 4.1 Particle size distribution of the feed

In order to assess the particle sizes and the particle size distribution of the different diets, dry sieving as well as wet sieving analyses were conducted.
The results of the dry sieving and the wet sieving analysis for each diet are presented in **Table 11** and in **Table 12**.

**Table 11:** Proportion of particle sizes (% of mass) in the diets based on dry sieve analysis

| | Roller mill | | | | Hammermill | | | |
|---|---|---|---|---|---|---|---|---|
| | Mash | | Expandate | | Mash | | Expandate | |
| **Particle size (mm)** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** |
| > 4.0 | 3.05 | 0.37 | 7.63 | 0.38 | 7.63 | 0.08 | 2.55 | 0.00 |
| > 2.5 ≤ 4.0 | 22.0 | 2.11 | 27.0 | 8.54 | 31.0 | 11.1 | 24.6 | 1.96 |
| > 2.0 ≤ 2.5 | 14.6 | 8.82 | 18.91 | 7.86 | 10.4 | 19.3 | 25.1 | 10.6 |
| > 1.6 ≤ 2.0 | 11.0 | 19.8 | 15.36 | 12.1 | 7.42 | 18.3 | 13.2 | 20.9 |
| > 1.25 ≤ 1.6 | 12.1 | 21.4 | 8.66 | 11.0 | 10.1 | 14.4 | 7.41 | 21.0 |
| > 1.0 ≤ 1.25 | 8.21 | 11.8 | 5.73 | 10.8 | 8.97 | 10.7 | 5.51 | 11.8 |
| > 0.63 ≤ 1.0 | 14.2 | 15.5 | 8.47 | 15.4 | 12.2 | 13.3 | 9.64 | 15.0 |
| > 0.4 ≤ 0.63 | 7.93 | 8.85 | 4.12 | 18.7 | 5.62 | 6.50 | 5.64 | 9.22 |
| > 0.15 ≤ 0.4 | 6.02 | 9.36 | 3.36 | 14.0 | 6.64 | 6.10 | 5.42 | 8.10 |
| < 0.15 | 0.93 | 1.97 | 0.78 | 1.12 | 0.11 | 0.32 | 0.96 | 1.37 |
| Σ | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| > 2.5 | 25.1 | 2.49 | 34.6 | 8.92 | 38.6 | 11.2 | 27.1 | 1.96 |
| > 1 to ≤ 2.5 | 45.8 | 61.9 | 48.7 | 41.8 | 36.8 | 62.6 | 51.2 | 64.4 |
| > 0.15 to ≤ 1 | 28.1 | 33.7 | 15.9 | 48.2 | 24.5 | 25.9 | 20.7 | 32.3 |
| < 0.15 | 0.93 | 1.97 | 0.78 | 1.12 | 0.11 | 0.32 | 0.96 | 1.37 |

The results of the dry sieving analysis show that the proportion of coarse particles (> 2.5 mm) exceeded 25 % in coarse feed while the proportion of coarse particles in the finely ground diets ranged from 2 % to 11 %. The proportion of particles with a size of > 1 mm to ≤ 2.5 mm ranged from 37 % to 46 % in coarsely ground mash and was about 50 % in coarsely ground expandate. With regard to the finely ground mash diets, approx. 62 % of the particles were sized between > 1 mm and ≤ 2.5 mm, while in the finely ground expandate, 42 % to 64 % of the particles were of this size. In fine feed, the largest portion of particles was between > 0.15

mm to ≤ 2.5 mm. In all diets, less than 2 % of particles passed through the 0.15 mm sieve. The results of the dry sieving analysis underline the differences in particle size distribution between coarse and fine grinding, independently of the milling method and thermal processing techniques implemented. This is not in accordance with the results of the wet sieving analysis, as shown in **Table 12**.

**Table 12:** Proportion of particle sizes (% of mass) in the diets based on wet sieve analysis

| | **Roller mill** | | | | **Hammermill** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Mash** | | **Expandate** | | **Mash** | | **Expandate** | |
| **Particle size (mm)** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** |
| > 4.0 | 9.39 | 0.23 | 0.00 | 0.00 | 18.2 | 0.11 | 0.11 | 0.00 |
| > 2.5 ≤ 4.0 | 23.3 | 5.32 | 6.47 | 0.00 | 37.4 | 11.8 | 11.3 | 0.34 |
| > 2.0 ≤ 2.5 | 9.28 | 13.7 | 10.9 | 0.23 | 7.96 | 14.9 | 9.74 | 4.18 |
| > 1.6 ≤ 2.0 | 7.81 | 17.3 | 7.61 | 2.04 | 6.82 | 12.4 | 4.87 | 6.22 |
| > 1.25 ≤ 1.6 | 7.58 | 11.7 | 5.34 | 4.08 | 6.48 | 10.3 | 4.53 | 5.65 |
| > 1.0 ≤ 1.25 | 5.32 | 6.23 | 4.20 | 3.40 | 2.73 | 4.92 | 3.17 | 3.28 |
| > 0.63 ≤ 1.0 | 6.79 | 6.23 | 6.47 | 7.37 | 3.18 | 7.66 | 7.14 | 7.57 |
| > 0.4 ≤ 0.63 | 4.41 | 3.51 | 4.20 | 6.35 | 1.25 | 4.46 | 6.34 | 6.67 |
| > 0.15 ≤ 0.4 | 3.85 | 4.30 | 7.72 | 9.86 | 1.48 | 5.03 | 7.70 | 8.70 |
| < 0.15 | 22.3 | 31.5 | 47.1 | 66.7 | 14.5 | 28.5 | 45.1 | 57.4 |
| Σ | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| > 2.5 | 32.7 | 5.55 | 6.47 | 0.00 | 55.6 | 11.9 | 11.4 | 0.34 |
| > 1 to ≤ 2.5 | 30.0 | 48.9 | 28.0 | 9.74 | 24.0 | 42.4 | 22.3 | 19.3 |
| > 0.15 ≤ 1 | 15.0 | 14.0 | 18.4 | 23.6 | 5.91 | 17.2 | 21.2 | 22.9 |
| < 0.15 | 22.3 | 31.5 | 47.1 | 66.7 | 14.5 | 28.5 | 45.1 | 57.4 |

In general, there was an increase of particles sized < 0.15 mm in all diets in comparison with the results of the dry sieving analysis. Concerning the coarsely ground expandate, on average, 34 % of the particles were sized > 1 mm, while about 46 % were sized < 0.15 mm. In the finely ground expandate, the proportion of particles sized > 1 mm were on average 15 % and particles sized < 0.15 mm were on average 62 %. With regard to particles sized > 2.5 mm, there was an increase in the proportion of about 10 % in the coarsely ground mash diets. However, the particle size distribution of the coarsely ground mash diets was comparable with that of the dry sieving analysis. This was also the case for the finely ground mash diets, although a shift occurred from medium-sized particles in favor of particles sized < 0.15 mm.

## 4.2 Animal performance

The laying performance (egg weight and egg production) of each feeding group was recorded daily, and the feed intake (FI) and body weight (BW) determined weekly. From these data, the feed conversion ratio (kg of feed/kg of weight gain; FCR) and the feed efficiency (kg of feed/kg of eggs; FE) were calculated.

**Table 13** provides the collected data for feed intake, body weight, body weight gain (BWG) and feed conversion ratio determined for the different feeding groups during the experimental periods and for the total trial period. The average daily FI (g/hen) ranged from 105 g to 113 g with regard to the total experimental period. On average, the FI increased over the course of the trial, so that in the first week the FI was 99 g to 106 g, whereas it increased to 112 g to 119 g during the last week of the trial.

Similar to the FI, the BW increased during the experimental period. At day one, the animals were randomly allocated to the eight different feeding groups and the initial body weights (baseline body weights) showed no significant differences between the groups, thus, the comparability of the groups and their performances were ensured. However, statistical analyses were adjusted with respect to the baseline body weight, in order to take into account that minor changes in the initial body weights within the groups could have effects on the results of the performance data.

The baseline body weights were about 1500 g per hen on average, and values of about 1700 g were recorded for the final body weights. Average weekly weight gains of the laying hens were highest in the first experimental week and decreased over the course of the experiment. Regarding to the total experimental period, the weekly BWGs were between 54 g and 66 g per hen. The FCR was calculated on the basis of the FI (kg of feed) and BWG (kg of gain). In the first week, FCRs were uniform among the different feeding groups and ranged between 1.0 and 1.5. With regard to the second and third week, FCRs for the different feeding groups were higher and subject to major fluctuations. These variations can be explained by the increased laying performance during this period. Thus, the values for the BWGs decreased during the course of the experiment, while EWs and EP increased. Concerning the total experimental period, FCR values of 1.8 to 2.5 were achieved among the feeding groups.

**Table 13:** Average daily feed intake (g/hen), average weekly body weight (g/hen) and average weekly body weight gain (g) of the different feeding groups, recorded for each experimental week and for total trial period; feed conversion ratio was calculated on basis of feed intake and body weight gain[12]

| | Roller mill | | | | Hammermill | | | |
|---|---|---|---|---|---|---|---|---|
| | Mash | | Expandate | | Mash | | Expandate | |
| **Experimental period (day)** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** |
| | | | | **Feed Intake** | | | | |
| **1 - 7 d** | **106** | **110** | **96.4** | **99.3** | **103** | **106** | **100** | **103** |
| SD | 7.65 | 5.49 | 5.51 | 4.70 | 10.1 | 6.70 | 9.24 | 5.09 |
| **7 - 14 d** | **107** | **110** | **107** | **102** | **105** | **107** | **109** | **105** |
| SD | 6.78 | 9.00 | 4.81 | 10.6 | 7.75 | 10.6 | 7.24 | 8.01 |
| **14 - 21 d** | **115** | **119** | **117** | **115** | **112** | **115** | **119** | **115** |
| SD | 6.72 | 6.43 | 10.4 | 10.3 | 10.6 | 10.4 | 7.90 | 8.01 |
| **1 – 21 d** | **110** | **113** | **107** | **105** | **107** | **109** | **109** | **108** |
| SD | 2.96 | 5.91 | 5.34 | 7.06 | 7.79 | 6.80 | 5.84 | 6.01 |
| | | | | **Body weight** | | | | |
| **1 d** | **1526** | **1539** | **1533** | **1516** | **1507** | **1521** | **1560** | **1536** |
| SD | 40.4 | 88.2 | 62.6 | 84.9 | 88.1 | 56.8 | 62.6 | 98.7 |
| **7 d** | **1647** | **1658** | **1622** | **1617** | **1615** | **1631** | **1646** | **1644** |
| SD | 50.5 | 47.4 | 54.8 | 61.6 | 82.3 | 53.4 | 67.2 | 82.8 |
| **14 d** | **1677** | **1691** | **1660** | **1662** | **1647** | **1662** | **1693** | **1681** |
| SD | 70.1 | 55.1 | 51.0 | 73.7 | 65.6 | 57.1 | 85.8 | 88.6 |
| **21 d** | **1722** | **1738** | **1696** | **1706** | **1676** | **1707** | **1729** | **1708** |
| SD | 60.1 | 56.0 | 40.2 | 81.1 | 70.4 | 47.7 | 69.8 | 69.8 |
| | | | | **Weight gain** | | | | |
| **1 - 7 d** | **121** | **119** | **89.0** | **102** | **108** | **110** | **85.7** | **108** |
| SD | 47.7 | 64.5 | 44.1 | 32.3 | 40.3 | 45.2 | 37.9 | 38.3 |
| **7 - 14 d** | **30.1** | **32.5** | **38.1** | **44.7** | **32.0** | **31.5** | **46.9** | **37.4** |
| SD | 32.0 | 28.5 | 36.9 | 41.8 | 32.3 | 15.3 | 28.2 | 13.1 |
| **14 - 21 d** | **44.5** | **47.2** | **35.7** | **44.9** | **29.3** | **38.8** | **36.2** | **26.7** |
| SD | 26.5 | 12.7 | 45.7 | 41.9 | 19.2 | 34.1 | 25.8 | 28.6 |
| **1 – 21 d** | **65.1** | **66.2** | **54.3** | **63.3** | **56.4** | **62.2** | **56.3** | **57.4** |
| SD | 17.8 | 26.1 | 21.2 | 22.9 | 24.5 | 15.4 | 14.7 | 14.9 |
| | | | | **Feed conversion ratio** | | | | |
| **1 - 7 d** | **1.01** | **1.21** | **1.48** | **1.07** | **1.22** | **1.31** | **1.39** | **1.10** |
| SD | 0.40 | 0.66 | 1.00 | 0.35 | 0.80 | 1.10 | 0.61 | 0.53 |
| **7 - 14 d** | **0.08** | **2.55** | **5.35** | **7.43** | **5.36** | **3.10** | **2.74** | **3.13** |
| SD | 7.47 | 6.49 | 16.1 | 8.54 | 3.52 | 1.19 | 0.89 | 1.08 |
| **14 - 21 d** | **6.03** | **2.71** | **4.24** | **3.15** | **9.18** | **1.43** | **1.39** | **0.36** |
| SD | 9.01 | 0.90 | 4.95 | 1.34 | 10.6 | 14.2 | 5.80 | 5.31 |
| **1 – 21 d** | **1.81** | **1.96** | **2.16** | **1.81** | **2.52** | **1.87** | **2.08** | **1.98** |
| SD | 0.54 | 0.78 | 0.60 | 0.49 | 1.93 | 0.54 | 0.61 | 0.46 |

[1] Results are reported as means ± SD
[2] Data are means of eight replicate pens, 6 animals per floor pen

The laying performance, expressed by the parameters egg weight (EW), egg production (EP) and feed efficiency (FE), also showed no significant differences between the feeding groups as analyzed by ANOVA and posthoc Tukey's test (**Table 14)**.
An increase of EWs and EP was detected during the trial period. At the beginning, average EWs of 47 g to 49 g were found, and the average EP was between 37 % and 46 %.
In the last week of the experiment, EWs were between 53 g and 55 g, and the EP ranged from 89 % to 95 %. The FE was calculated in accordance with FI (kg of feed) and EW (kg of egg weight). The detected weekly FEs were comparable among the feeding groups and barely changed during the course of the experiment. Concerning the total experimental period, FE values of 2.0 to 2.2 were calculated for the feeding groups on average.

In order to control for interaction and single factor effects, a three-factor analysis of variance was conducted on collected performance data concerning the different experimental periods (**Table 15**) and the total experimental period (**Table 16**). Statistical analyses were adjusted regarding the baseline body weight. Statistical significances were detected for the covariate "baseline body weight" in each experimental week as well as in the total experimental period, indicating that there were differences in the initial body weights of laying hens within the feeding groups. The physical form of the diet affected the FI and FE of hens in the first experimental week. The FI and FE of laying hens, fed with mash diets, were significantly higher compared with those that received the expandate (FI: $P < 0.001$; FE: $P = 0.005$). Furthermore, in the last experimental week, the FCR was influenced by the factor particle size and was significantly higher for birds that were fed with coarsely ground feed in comparisons with those fed finely ground feed ($P = 0.042$). With regard to the total experimental period, the three-factor analysis of variance on selected performance data showed no interaction and single factor effects, except for the parameter FI. The FI was significantly influenced by the physical form of the feed ($P = 0.045$). The FI of laying hens that consumed the mash diets were higher than the FI of hens fed with the expandate (**Figure 5**).

**Table 14:** Average weekly egg weight (g) and average weekly egg production (%) of the different feeding groups, recorded for each experimental week and for the total trial period. Average weekly feed efficiency was calculated from the average daily feed intake and the average weekly egg weight [12]

| | Roller mill | | | | Hammermill | | | |
|---|---|---|---|---|---|---|---|---|
| | Mash | | Expandate | | Mash | | Expandate | |
| **Experimental period (day)** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** |
| | | | | **Egg weight** | | | | |
| **1 - 7 d** | **47.8** | **49.0** | **47.1** | **48.2** | **48.4** | **47.9** | **48.0** | **47.6** |
| SD | 3.93 | 3.22 | 3.28 | 4.88 | 3.91 | 2.72 | 3.30 | 1.54 |
| **7 - 14 d** | **50.5** | **51.6** | **51.1** | **51.9** | **51.5** | **51.6** | **52.9** | **50.2** |
| SD | 1.69 | 2.66 | 2.86 | 3.54 | 2.80 | 1.61 | 2.22 | 1.71 |
| **14 - 21 d** | **53.6** | **55.1** | **54.5** | **54.9** | **54.5** | **54.7** | **55.4** | **53.6** |
| SD | 1.68 | 2.45 | 2.53 | 2.80 | 2.14 | 2.24 | 2.28 | 1.67 |
| **1 – 21 d** | **50.6** | **51.9** | **50.9** | **51.6** | **51.5** | **51.4** | **52.1** | **50.5** |
| SD | 1.97 | 2.59 | 2.68 | 3.67 | 2.75 | 2.05 | 2.30 | 0.88 |
| | | | | **Egg production** | | | | |
| **1 - 7 d** | **37.4** | **46.4** | **40.6** | **30.7** | **35.7** | **45.3** | **42.9** | **43.2** |
| SD | 18.7 | 21.8 | 14.8 | 12.3 | 20.3 | 22.8 | 13.9 | 21.0 |
| **7 - 14 d** | **77.3** | **82.7** | **81.3** | **75.6** | **77.6** | **82.0** | **76.5** | **82.5** |
| SD | 13.6 | 9.10 | 11.6 | 14.9 | 10.7 | 12.7 | 13.5 | 10.4 |
| **14 - 21 d** | **89.0** | **92.6** | **89.2** | **89.3** | **92.2** | **95.2** | **91.3** | **90.8** |
| SD | 6.15 | 3.52 | 10.2 | 5.47 | 5.97 | 6.78 | 6.57 | 11.3 |
| **1 – 21 d** | **67.9** | **73.9** | **70.4** | **65.2** | **68.5** | **74.2** | **70.2** | **72.1** |
| SD | 9.45 | 9.42 | 9.01 | 8.55 | 10.7 | 12.3 | 6.48 | 9.54 |
| | | | | **Feed efficiency** | | | | |
| **1 - 7 d** | **2.23** | **2.26** | **2.05** | **2.08** | **2.14** | **2.21** | **2.09** | **2.17** |
| SD | 0.19 | 0.13 | 0.13 | 0.17 | 0.19 | 0.11 | 0.12 | 0.14 |
| **7 - 14 d** | **2.12** | **2.14** | **2.09** | **1.97** | **2.04** | **2.07** | **2.06** | **2.10** |
| SD | 0.12 | 0.12 | 0.07 | 0.19 | 0.09 | 0.18 | 0.18 | 0.15 |
| **14 - 21 d** | **2.15** | **2.16** | **2.14** | **2.09** | **2.06** | **2.10** | **2.14** | **2.15** |
| SD | 0.07 | 0.10 | 0.15 | 0.15 | 0.17 | 0.15 | 0.12 | 0.15 |
| **1 – 21 d** | **2.17** | **2.18** | **2.10** | **2.04** | **2.08** | **2.13** | **2.10** | **2.14** |
| SD | 0.07 | 0.08 | 0.06 | 0.12 | 0.12 | 0.10 | 0.11 | 0.10 |

[1] Results are reported as means ± SD
[2] Data are means of eight replicate pens, 6 animals per floor pen

**Table 15:** Interaction and single factor effects of a three-factor analysis of variance on performance data recorded in the feeding groups for the different experimental periods. Statistical analysis was adjusted regarding to the baseline body weight

| | ***P*-value** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Experimental period: 1 – 7 d** | **Feed intake** | **Body weight** | **Weight gain** | **FCR** | **Egg weight** | **Egg production** | **Feed efficiency** |
| Baseline body weight | 0.208 | **<0.001** | **<0.001** | **0.003** | **0.041** | 0.605 | 0.373 |
| Mill (M) | 0.967 | 0.685 | 0.645 | 0.756 | 0.924 | 0.533 | 0.994 |
| Physical form (F) | **<0.001** | 0.144 | 0.158 | 0.893 | 0.391 | 0.661 | **0.005** |
| Particle size (PS) | 0.094 | 0.426 | 0.394 | 0.605 | 0.690 | 0.622 | 0.198 |
| M * F | 0.061 | 0.188 | 0.163 | 0.329 | 0.963 | 0.402 | 0.069 |
| M * PS | 0.779 | 0.768 | 0.724 | 0.954 | 0.348 | 0.564 | 0.526 |
| F * PS | 0.997 | 0.734 | 0.783 | 0.284 | 0.806 | 0.158 | 0.914 |
| M * F * PS | 0.867 | 0.933 | 0.975 | 0.702 | 0.945 | 0.608 | 0.984 |
| **Experimental period: 7 – 14 d** | ***P*-value** | | | | | | |
| Baseline body weight | **<0.001** | **<0.001** | 0.639 | 0.686 | **0.002** | 0.973 | **0.036** |
| Mill (M) | 0.946 | 0.789 | 0.938 | 0.880 | 0.651 | 0.889 | 0.711 |
| Physical form (F) | 0.212 | 0.768 | 0.168 | 0.340 | 0.937 | 0.768 | 0.212 |
| Particle size (PS) | 0.743 | 0.553 | 0.967 | 0.715 | 0.815 | 0.413 | 0.833 |
| M * F | 0.411 | 0.282 | 0.935 | 0.088 | 0.370 | 0.849 | 0.159 |
| M * PS | 0.974 | 0.874 | 0.527 | 0.398 | 0.054 | 0.393 | 0.196 |
| F * PS | 0.151 | 0.893 | 0.832 | 0.730 | 0.294 | 0.448 | 0.447 |
| M * F * PS | 0.689 | 0.835 | 0.655 | 0.683 | 0.306 | 0.317 | 0.303 |
| **Experimental period: 14 – 21 d** | ***P*-value** | | | | | | |
| Baseline body weight | **0.017** | **<0.001** | **0.009** | 0.357 | **0.001** | 0.267 | 0.383 |
| Mill (M) | 0.532 | 0.360 | 0.233 | 0.407 | 0.973 | 0.216 | 0.467 |
| Physical form (F) | 0.770 | 0.523 | 0.523 | 0.396 | 0.987 | 0.227 | 0.748 |
| Particle size (PS) | 0.856 | 0.378 | 0.519 | **0.042** | 0.791 | 0.383 | 0.936 |
| M * F | 0.504 | 0.191 | 0.658 | 0.656 | 0.359 | 0.648 | 0.186 |
| M * PS | 0.885 | 0.796 | 0.854 | 0.353 | 0.110 | 0.877 | 0.443 |
| F * PS | 0.287 | 0.657 | 0.289 | 0.170 | 0.252 | 0.418 | 0.588 |
| M * F * PS | 0.937 | 0.406 | 0.252 | 0.355 | 0.711 | 0.980 | 0.886 |

**Table 16:** Interaction and single factor effects of a three-factor analysis of variance on selected performance data detected in the different feeding groups for the total experimental period. Statistical analysis was adjusted regarding to the baseline body weight

| | ***P*-value** | | | | |
|---|---|---|---|---|---|
| **Total experimental period** | **Feed intake** | **Body weight** | **Weight gain** | **Egg weight** | **Egg production** |
| Baseline body weight | **0.001** | **<0.001** | **<0.001** | **0.003** | 0.526 |
| Mill (M) | 0.737 | 0.577 | 0.360 | 0.909 | 0.435 |
| Physical form (F) | **0.045** | 0.417 | 0.523 | 0.698 | 0.472 |
| Particle size (PS) | 0.506 | 0.412 | 0.378 | 0.844 | 0.373 |
| M * F | 0.134 | 0.183 | 0.191 | 0.552 | 0.610 |
| M * PS | 0.858 | 0.941 | 0.796 | 0.116 | 0.484 |
| F * PS | 0.236 | 0.985 | 0.657 | 0.567 | 0.149 |
| M * F * PS | 0.836 | 0.702 | 0.406 | 0.677 | 0.443 |

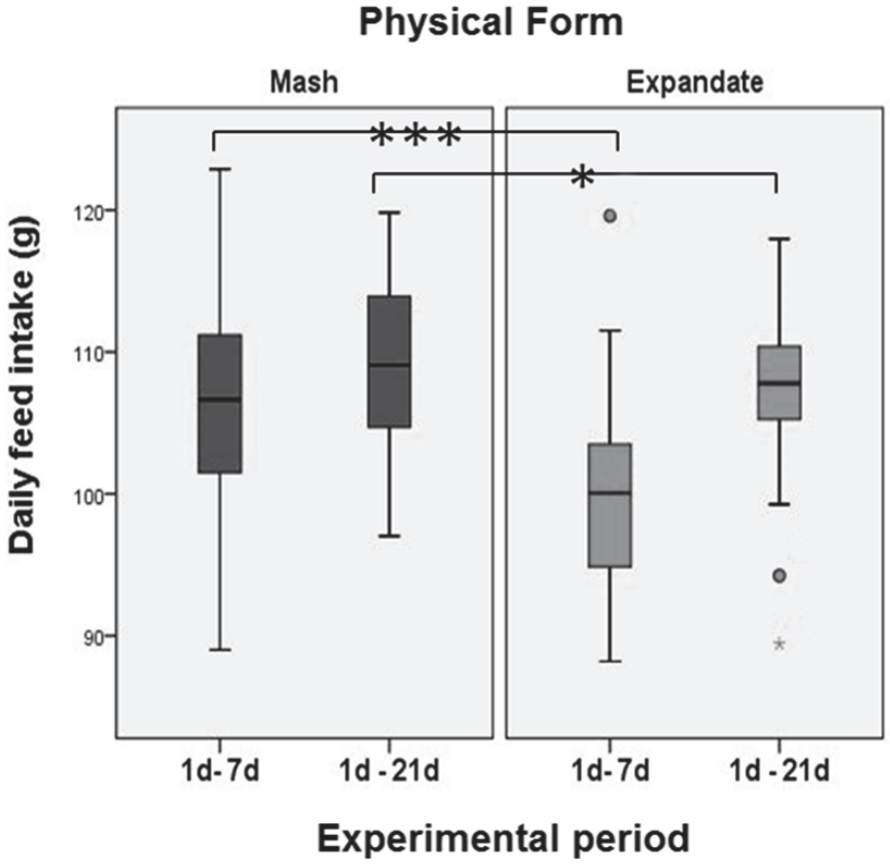


**Figure 5:** Influence of feed form (mash vs. expandate) on the daily FI (g/hen), recorded for the first experimental week ( 1d – 7d) and the total experimental period (1d – 21d).* $P < 0.05$; *** $P < 0.01$

## 4.3 Organ weights of the proventriculus, gizzard, segments of the small intestine and the pancreas

The weights of digestive tract organs were calculated as relative organ weights based on organ-to-body weight ratio (**Table 17**).

**Table 17:** Empty relative weight (g/kg body weight) of the gastrointestinal organs of laying hens, fed with the different diets (N = number of analyzed samples). Statistical analyses were conducted by ANOVA and posthoc Tukey's test

| | Roller mill | | | | Hammermill | | | |
|---|---|---|---|---|---|---|---|---|
| | Mash | | Expandate | | Mash | | Expandate | |
| **Organ weight (g/kg)** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** |
| **Proventriculus** | **3.38** | **3.37** | **3.17** | **3.31** | **3.50** | **3.30** | **3.22** | **3.11** |
| SD | 0.30 | 0.34 | 0.28 | 0.28 | 0.35 | 0.26 | 0.27 | 0.12 |
| N[3] | 32 | 32 | 31 | 31 | 30 | 30 | 32 | 31 |
| **Gizzard[12]** | **16.6$^{a}$** | **15.4$^{ab}$** | **14.2$^{bcd}$** | **13.6$^{cd}$** | **16.5$^{a}$** | **14.9$^{abc}$** | **13.7$^{cd}$** | **12.8$^{d}$** |
| SD | 1.01 | 1.31 | 1.53 | 1.18 | 1.30 | 0.80 | 0.77 | 0.81 |
| N[3] | 32 | 32 | 31 | 31 | 30 | 29 | 31 | 31 |
| **Duodenum[1]** | **7.28** | **7.15** | **7.19** | **6.92** | **7.01** | **6.79** | **6.96** | **6.92** |
| SD | 0.89 | 0.56 | 0.56 | 0.69 | 0.92 | 0.36 | 0.72 | 0.40 |
| N[3] | 32 | 32 | 31 | 31 | 30 | 30 | 32 | 31 |
| **Jejunum[1]** | **11.3** | **11.1** | **11.2** | **11.5** | **11.6** | **10.8** | **10.9** | **11.0** |
| SD | 1.47 | 1.26 | 0.93 | 1.43 | 1.74 | 1.43 | 1.05 | 1.01 |
| N[3] | 32 | 32 | 31 | 30 | 30 | 30 | 32 | 31 |
| **Ileum[1]** | **6.62** | **6.21** | **6.55** | **6.43** | **6.13** | **6.20** | **6.24** | **6.70** |
| SD | 0.66 | 0.84 | 0.55 | 0.71 | 0.52 | 0.33 | 0.89 | 0.69 |
| N[3] | 32 | 32 | 30 | 31 | 30 | 30 | 32 | 31 |
| **In total[1]** | **45.1** | **43.2** | **42.4** | **41.7** | **44.7** | **42.1** | **40.8** | **40.5** |
| SD | 3,43 | 3.11 | 2.88 | 3.21 | 3.33 | 2.54 | 3.14 | 1.57 |
| N[3] | 32 | 32 | 30 | 30 | 30 | 29 | 31 | 31 |
| **Pancreas[1]** | **1.89** | **1.84** | **1.71** | **1.70** | **1.89** | **1.71** | **1.70** | **1.72** |
| SD | 0.12 | 0.16 | 0.25 | 0.16 | 0.33 | 0.16 | 0.14 | 0.14 |
| N[3] | 27 | 27 | 26 | 26 | 26 | 26 | 24 | 24 |

[1] Results are reported as means ± SD.
[2] [a-d] Means with different superscripts are significantly different ($P < 0.05$)
[3] Sample sizes vary due to missing samples

Among the different feeding groups, significant differences were detected concerning the relative organ weight of the gizzard. Relative gizzard weights were on average higher in laying hens fed mash diets compared to those fed expanded diets. The highest gizzard weights were observed in birds fed with the coarsely ground mash diets, followed by birds fed with finely ground mash diets, irrespective of whether either a hammermill or roller mill was used for feed production.

In order to control for interaction and single factor effects, a three-factor analysis of variance was conducted, illustrating significant differences regarding the relative organ weights (**Table 18**).

**Table 18:** Interaction and single factor effects of a three-factor analysis of variance on empty relative organ weight (g/kg body weight) observed in the eight different feeding groups

| | ***P*-value** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Proven-triculus** | **Gizzard** | **Duodenum** | **Jejunum** | **Ileum** | **In total** | **Pancreas** |
| Mill (M) | 0.702 | 0.101 | 0.207 | 0.554 | 0.430 | 0.150 | 0.565 |
| Physical form (F) | **0.011** | **<0.001** | 0.713 | 0.810 | 0.269 | **0.002** | **0.019** |
| Particle size (PS) | 0.517 | **<0.001** | 0.338 | 0.688 | 0.992 | 0.073 | 0.260 |
| M * F | 0.496 | 0.481 | 0.551 | 0.485 | 0.458 | 0.676 | 0.468 |
| M * PS | 0.125 | 0.576 | 0.831 | 0.576 | 0.119 | 0.907 | 0.680 |
| F * PS | 0.416 | 0.298 | 0.944 | 0.312 | 0.311 | 0.236 | 0.303 |
| M * F * PS | 0.838 | 0.922 | 0.627 | 0.775 | 0.890 | 0.754 | 0.428 |

Concerning the proventriculus, gizzard and pancreas, the results show that the physical form of the diet was the main factor which influenced organ weight development ($P < 0.01$), while no combined effects of factors were observed. In general, birds fed with mash diets had significantly higher organ weights with regard to the proventriculus, gizzard, pancreas and total organ weight compared to birds fed the expandate. Additionally, the factor particle size affected the relative organ weights of the gizzard ($P < 0.001$) i. e. hens fed with coarsely ground feed had significantly higher gizzard weights than hens given the finely ground diets. Owing to the increased gizzard weights, the total weight of the investigated organs were also significantly affected by the factor physical form of the diet ($P = 0.002$).

## 4.4 Histological evaluation of villus length and crypt depth in the segments of the small intestine

The intestinal villi length and the crypt depth were determined in the duodenum, jejunum and ileum of birds fed with the different diets. The means of villi lengths are listed in **Table 19** and the means of the crypt depths are illustrated in **Table 20**. The sample sizes (N) vary between the experimental groups due to diverging sample quality, so that only well oriented villi and crypts were examined.

**Table 19:** Histological measurements of the villi length in the duodenum, jejunum and ileum concerning the different feeding groups (N = number of analyzed samples); statistical analyses were conducted by ANOVA and posthoc Tukey's test

| | Roller mill | | | | Hammer mill | | | |
|---|---|---|---|---|---|---|---|---|
| | Mash | | Expandate | | Mash | | Expandate | |
| **Villus length (µm)** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** |
| **Duodenum**[1][2] | **1759**[ab] | **1716**[abc] | **1551**[bc] | **1451**[c] | **1892**[a] | **1707**[abc] | **1545**[bc] | **1526**[bc] |
| SD | 168 | 223 | 94.2 | 129 | 206 | 166 | 153 | 187 |
| N[3] | 8 | 8 | 6 | 7 | 7 | 8 | 8 | 8 |
| **Jejunum**[1] | **1285** | **1364** | **1167** | **1256** | **1325** | **1213** | **1211** | **1269** |
| SD | 114 | 177 | 60.3 | 123 | 197 | 125 | 196 | 124 |
| N[3] | 8 | 6 | 6 | 5 | 8 | 7 | 5 | 8 |
| **Ileum**[1] | **776** | **811** | **875** | **879** | **783** | **756** | **861** | **779** |
| SD | 92.8 | 117 | 146 | 190 | 98.2 | 91.5 | 56.6 | 113 |
| N[3] | 6 | 8 | 6 | 7 | 7 | 7 | 6 | 7 |

[1] Results are reported as means ± SD
[2] [a-c] Means with different superscripts are significantly different ($P < 0.05$)
[3] Sample sizes vary due to sample quality

**Table 20:** Histological measurements of the crypt depth in the duodenum, jejunum and ileum with regard to the eight different feeding groups (N = number of analyzed samples)

| | Roller mill | | | | Hammer mill | | | |
|---|---|---|---|---|---|---|---|---|
| | Mash | | Expandate | | Mash | | Expandate | |
| **Crypt depth (µm)** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** |
| **Duodenum**[1] | **244** | **223** | **237** | **250** | **250** | **218** | **265** | **246** |
| SD | 32.9 | 19.2 | 26.0 | 31.7 | 41.8 | 39.0 | 28.6 | 26.5 |
| N[2] | 8 | 8 | 6 | 7 | 7 | 8 | 8 | 8 |
| **Jejunum**[1] | **209** | **200** | **198** | **196** | **187** | **182** | **216** | **194** |
| SD | 56.3 | 32.6 | 36.4 | 64.0 | 46.7 | 43.5 | 40.5 | 19.8 |
| N[2] | 8 | 6 | 6 | 5 | 8 | 7 | 5 | 8 |
| **Ileum**[1] | **139** | **118** | **126** | **128** | **125** | **111** | **137** | **122** |
| SD | 47.7 | 10.8 | 10.0 | 19.7 | 15.9 | 18.8 | 19.0 | 20.2 |
| N[2] | 6 | 8 | 6 | 7 | 7 | 7 | 6 | 7 |

[1] Results are reported as means ± SD
[2] Sample sizes vary due to sample quality

Among the feeding groups, significant differences were detected regarding the villi length of the duodenum. In general, birds fed with mash diets had significantly longer duodenal villi compared with chicks that consumed expandate. The longest villi were detected for laying hens fed with the coarsely ground mash diets, followed by those fed with finely ground mash diets. No significant differences were found between the feeding groups with regard to the villus length of the jejunum and ileum, as well concerning the crypt depth of the duodenum, jejunum and ileum.

In order to examine interaction and single factor effects, a three-factor analysis of variance was conducted (**Table 21**). Whereas no interaction and single factor effects were assessed concerning the factors mill and particle size, significant differences were observed regarding the factor physical form of the diet. Hens fed with mash diets had longer duodenal ($P < 0.001$) and shorter ileal ($P = 0.47$) villi than those given the expandate. Additionally, the feeding of mash tended to increase jejunal villus height ($P = 0.087$), while expanded fed birds showed tendentially deeper duodenal crypts ($P = 0.053$). Moreover coarsely instead of finely ground feed tended to promote duodenal villus height ($P = 0.057$), duodenal crypt depth ($P = 0.072$) as well as ileal crypt depth ($P = 0.061$).

**Table 21:** Interaction and single factor effects of a three-factor analysis of variance on villus length and crypt depth determined for the eight different feeding groups

| | ***P*-value** | | | | | |
|---|---|---|---|---|---|---|
| | **Villus length** | | | **Crypt depth** | | |
| | **Duodenum** | **Jejunum** | **Ileum** | **Duodenum** | **Jejunum** | **Ileum** |
| Mill (M) | 0.285 | 0.739 | 0.221 | 0.436 | 0.637 | 0.547 |
| Physical form (F) | **<0.001** | 0.087 | **0.047** | 0.053 | 0.597 | 0.407 |
| Particle size (PS) | 0.057 | 0.486 | 0.597 | 0.072 | 0.432 | 0.061 |
| M * F | 0.763 | 0.308 | 0.618 | 0.494 | 0.264 | 0.299 |
| M * PS | 0.733 | 0.178 | 0.267 | 0.201 | 0.756 | 0.689 |
| F * PS | 0.546 | 0.272 | 0.508 | 0.149 | 0.834 | 0.351 |
| M * F * PS | 0.213 | 0.330 | 0.855 | 0.501 | 0.616 | 0.313 |

Additionally, the villus height to crypt depth ratio was calculated for the different feeding groups (**Table 22**). Similar to the results illustrated before, an increased villus height to crypt depth ratio was found for birds fed with the mash diets. A three-factor analysis of variance showed that the feeding of mash led to increased duodenal villus height to crypt depth ratios ($P < 0.001$) and tended to increase jejunal villus height to crypt depth ratios ($P = 0.082$) (**Table 23**).

**Table 22**: Calculation of the villus height to crypt depth ratio in the duodenum, jejunum and ileum regarding to the different feeding groups (N = number of analyzed samples); statistical analyses were conducted by ANOVA and posthoc Tukey's test

| | **Roller mill** | | | | **Hammer mill** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Mash** | | **Expandate** | | **Mash** | | **Expandate** | |
| **Villus/crypt ratio** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** |
| **Duodenum**[12] | **7.33**[ac] | **7.76**[ab] | **6.50**[ac] | **5.89**[c] | **7.76**[ab] | **8.00**[a] | **5.86**[c] | **6.22**[bc] |
| SD | 1.35 | 1.13 | 0.81 | 1.02 | 1.50 | 1.36 | 0.68 | 0.80 |
| N[3] | 8 | 8 | 6 | 7 | 7 | 8 | 8 | 8 |
| **Jejunum**[1] | **6.51** | **6.94** | **6.05** | **6.82** | **7.32** | **6.95** | **5.67** | **6.65** |
| SD | 1.56 | 1.24 | 1.07 | 1.62 | 1.22 | 1.57 | 0.73 | 0.95 |
| N[3] | 8 | 6 | 6 | 5 | 8 | 7 | 5 | 8 |
| **Ileum**[1] | **6.09** | **6.93** | **6.99** | **6.92** | **6.29** | **6.89** | **6.42** | **6.45** |
| SD | 1.87 | 1.09 | 1.24 | 1.58 | 0.66 | 0.95 | 1.26 | 0.98 |
| N[3] | 6 | 8 | 6 | 7 | 7 | 7 | 6 | 7 |

[1] Results are reported as means ± SD
[2 a-c] Means with different superscripts are significantly different ($P < 0.05$)
[3] Sample sizes vary due to sample quality

**Table 23:** Interaction and single factor effects of a three-factor analysis of variance on the villus height to crypt depth ratio detected for the eight different feeding groups

| | *P*-value | | |
|---|---|---|---|
| | **Duodenum villus/crypt** | **Jejunum villus crypt** | **Ileum villus crypt** |
| Mill (M) | 0.812 | 0.878 | 0.514 |
| Physical form (F) | **<0.001** | 0.082 | 0.670 |
| Particle size (PS) | 0.776 | 0.218 | 0.308 |
| M * F | 0.360 | 0.336 | 0.378 |
| M * PS | 0.443 | 0.657 | 0.916 |
| F * PS | 0.384 | 0.258 | 0.281 |
| M * F * PS | 0.277 | 0.495 | 0.797 |

## 4.5 Intestinal glucose absorption

In order to investigate the effect of the different diets on the active glucose transport in the jejunum of laying hens, changes in the short-circuit current (Isc) were detected after applying glucose and phloridzin to the mucosal surface of the tissue.

The basal values for the transepithelial conductance (Gt) were uniform during the experiment and among the different feeding groups, indicating the integrity of the intestinal preparation and ensuring the comparability of the results (**Table 24**). The mean of Gt was 9.07 mS/cm$^2$ ± 3,51 corresponding to the transepithelial resistance Rt of 122 Ω * cm$^2$ ± 36,6.

**Table 24:** Transepithelial conductance (Gt) in the different feeding groups (N = 8 for each group)

| | **Roller mill** | | | | **Hammermill** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Mash** | | **Expandate** | | **Mash** | | **Expandate** | |
| **(mS/cm$^2$)** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** |
| **Conductance (Gt)[1]** | **11.3** | **8.05** | **8.88** | **8.33** | **7.95** | **10.5** | **8.67** | **8.92** |
| SD | 7.21 | 1.98 | 1.96 | 2.70 | 3.40 | 3.02 | 2.70 | 2.08 |

[1] Results are reported as means ± SD

Concerning the different feeding groups, means of Δ Isc for glucose and phloridzin are illustrated in **Table 25**, showing significant differences between the feeding groups. In the mash groups, the Δ Isc values for glucose ranged from 9.13 to 12.3 µA/cm$^2$ and were higher compared to the Δ Isc values detected for the expandate groups, which ranged from 6.08 to 8.30 µA/cm$^2$. The birds fed with the coarsely ground mash showed the highest values for Δ Isc, while the lowest were recorded for birds fed the finely ground expandate. These results were comparable with those determined for Δ Isc phloridzin.

**Table 25:** Effect of D-glucose and phloridzin on short-circuit current in isolated mucosa of chicken's jejunum (N = 8 for each group); statistical analyses were conducted by ANOVA and posthoc Tukey's test

| | Roller mill | | | | Hammermill | | | |
|---|---|---|---|---|---|---|---|---|
| | Mash | | Expandate | | Mash | | Expandate | |
| **Δ Isc ($\mu A/cm^2$)** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** |
| **Glucose**[12] | **9.13**$^{abc}$ | **12.3**$^{a}$ | **7.08**$^{bc}$ | **6.08**$^{c}$ | **11.95**$^{ab}$ | **10.79**$^{abc}$ | **8.30**$^{abc}$ | **8.12**$^{abc}$ |
| SD | 3.69 | 2.89 | 3.26 | 2.68 | 3.01 | 2.84 | 3.00 | 2.91 |
| **Phloridzin**[12] | **-8.15**$^{abc}$ | **-10.5**$^{a}$ | **-6.03**$^{bc}$ | **-5.67**$^{c}$ | **-9.80**$^{ab}$ | **-8.73**$^{ab}$ | **-7.09**$^{abc}$ | **-7.48**$^{abc}$ |
| SD | 2.71 | 2.70 | 2.71 | 2.81 | 2.26 | 1.92 | 2.69 | 2.93 |

[1] Results are reported as means ± SD
[2] $^{a-c}$ Means with different superscripts are significantly different ($P < 0.05$)

In order to control for interaction and single factor effects, a three-factor analysis of variance was performed pointing out significant differences regarding the factor physical form of the diet (**Table 26**). Laying hens fed with mash diets had significantly higher values for Δ Isc glucose and Δ Isc phloridzin compared with those fed expandate. The factor mill and particle size as well as their interactions did not influence the values of Δ Isc glucose and Δ Isc phloridzin.

**Table 26:** Interaction and single factor effects of a three-factor analysis of variance according to Δ Isc glucose and Δ Isc phloridzin detected in the eight different feeding groups

| | ***P*-value** | |
|---|---|---|
| | **Δ Isc glucose** | **Δ Isc phloridzin** |
| Mill (M) | 0.241 | 0.305 |
| Physical form (F) | **<0.001** | **<0.001** |
| Particle size (PS) | 0.556 | 0.611 |
| M * F | 0.343 | 0.253 |
| M * PS | 0.419 | 0.307 |
| F * PS | 0.178 | 0.628 |
| M * F * PS | 0.181 | 0.114 |

## 4.6 Fluorescence cytometry of lymphocyte subpopulations in the gut-associated lymphoid tissue

On the basis of the flow cytometric measurements, the distribution and relative frequency of different lymphocyte subpopulations were determined in the duodenum and jejunum for the different feeding groups. Cell viability was greater than 90 % as determined by trypan blue exclusion. Cells were sorted on the basis of their size and granularity in order to select those representing the pool of leucocytes (**Figure 6**).

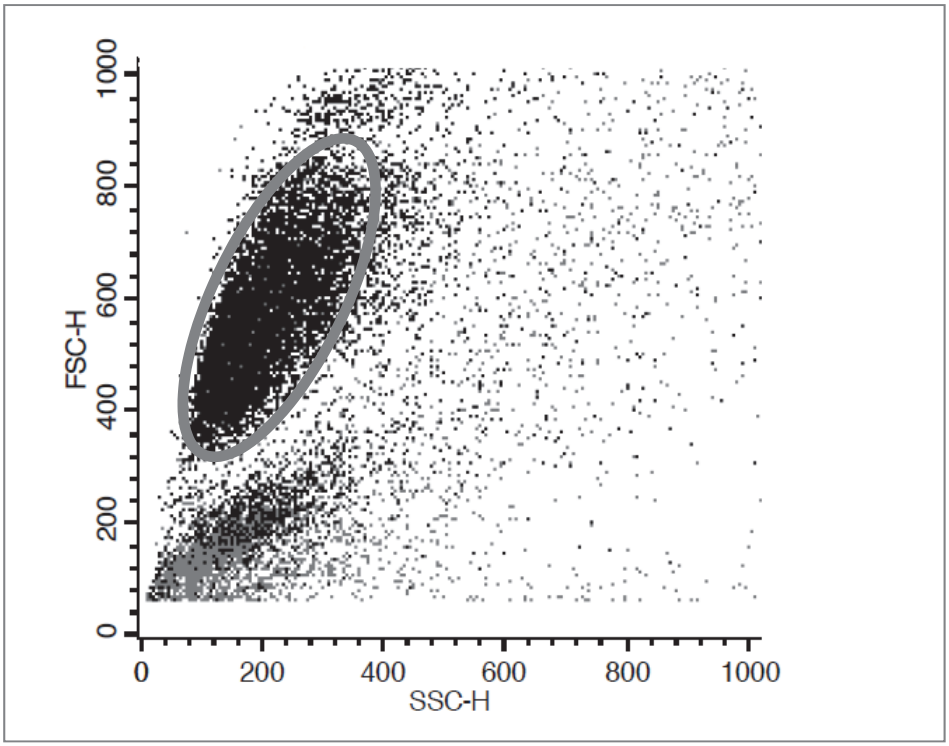


**Figure 6:** Cell population obtained from jejunal tissue was characterized by forward light scatter (FS) and side scatter light (SS). CD45 + cells are marked black and CD45 – cells appear grey. The lymphocyte population is encircled. One representative experiment is shown

The cell population, highlighted in **Figure 6**, was identified as a leucocyte population with cells that expressed mainly the leucocyte common antigen CD45.

Within the selected leucocyte population, the distribution and relative frequency of the different lymphocyte subpopulations were evaluated on the basis of their surface antigens and expressed as percentages (**Table 27** and **Table 28**). The sample sizes (N) vary between the experimental groups because partly not enough cells could be isolated from the tissue in order to evaluate the different lymphocyte subpopulations. Furthermore, the primary antibodies phenotyping 28-4, TCR2, TCR3, CD4 and AV20 positive cells were established during the course of the experiment, which explains the varying sample sizes.

**Table 27:** Proportions (%) of intraepithelial lymphocytes of the duodenum for the different feeding groups, obtained by flow cytometric phenotyping (N = number of analyzed samples); proportion of lymphocyte subpopulations calculated in relation to detected CD45+ cells. IELs were characterized by their specific surface antigens

| | Roller mill | | | | Hammermill | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mash | | Expandate | | Mash | | Expandate | | |
| **Phenotype** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **In total** |
| **CD3+**[1] | **53.9** | **54.4** | **63.6** | **52.4** | **58.9** | **49.8** | **63.3** | **64.1** | **57.6** |
| SD | 23.2 | 27.0 | 23.5 | 23.5 | 12.2 | 24.3 | 12.9 | 21.0 | 21.0 |
| N | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 64 |
| **TCR1+**[1] | **25.9** | **29.1** | **30.9** | **31.6** | **31.6** | **26.5** | **31.6** | **30.2** | **29.6** |
| SD | 9.32 | 8.66 | 7.68 | 6.28 | 4.16 | 12.2 | 12.2 | 10.8 | 9.06 |
| N[2] | 8 | 7 | 8 | 7 | 8 | 8 | 8 | 8 | 62 |
| **TCR2+**[1] | **22.2** | **19.5** | **22.6** | **20.5** | **18.7** | **16.9** | **21.4** | **27.2** | **21.2** |
| SD | 6.54 | 5.88 | 5.63 | 3.77 | 6.55 | 5.25 | 14.2 | 2.61 | 7.00 |
| N[3] | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 32 |
| **TCR3+**[1] | **14.6** | **12.4** | **16.3** | **14.4** | **14.3** | **11.9** | **16.4** | **14.5** | **14.4** |
| SD | 4.43 | 0.73 | 2.36 | 5.13 | 3.34 | 3.61 | 3.15 | 4.83 | 3.57 |
| N[3] | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 32 |
| **CD8α+**[1] | **46.8** | **54.7** | **58.3** | **50.1** | **55.1** | **44.5** | **54.9** | **54.6** | **52.4** |
| SD | 18.9 | 7.04 | 8.05 | 6.12 | 5.31 | 17.8 | 7.15 | 8.39 | 11.5 |
| N[2] | 8 | 7 | 8 | 7 | 8 | 8 | 8 | 8 | 62 |
| **28-4+**[1] | **5.94** | **6.69** | **7.44** | **7.38** | **7.48** | **6.01** | **5.47** | **6.40** | **6.58** |
| SD | 3.21 | 1.75 | 3.66 | 2.90 | 1.21 | 3.61 | 1.25 | 2.16 | 2.58 |
| N[23] | 7 | 6 | 7 | 6 | 7 | 7 | 7 | 7 | 54 |
| **28-4+ CD3+**[1] | **3.75** | **4.49** | **5.05** | **5.84** | **4.80** | **3.56** | **4.28** | **4.03** | **4.45** |
| SD | 2.04 | 1.98 | 2.94 | 2.80 | 1.64 | 2.47 | 1.42 | 1.92 | 2.16 |
| N[23] | 7 | 6 | 7 | 6 | 7 | 7 | 7 | 7 | 54 |
| **CD4+**[1] | **7.32** | **9.74** | **6.03** | **8.56** | **9.85** | **5.32** | **6.62** | **8.37** | **7.58** |
| SD | 0.52 | 4.93 | 2.38 | 4.05 | 1.30 | 1.95 | 3.38 | 3.55 | 3.03 |
| N[23] | 3 | 3 | 3 | 2 | 3 | 4 | 4 | 4 | 26 |
| **AV20+**[1] | **8.85** | **9.25** | **6.26** | **6.68** | **8.76** | **11.5** | **5.67** | **9.99** | **8.47** |
| SD | 6.65 | 0.83 | 0.35 | 4.50 | 3.37 | 4.38 | 4.87 | 2.21 | 3.70 |
| N[23] | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 19 |

[1] Results are reported as means ± SD
[2] Sample sizes vary due to sample quality
[3] Sample sizes vary because antibody was established during the course of the experiment

**Table 28:** Proportions (%) of intraepithelial lymphocytes of the jejunum for the different feeding groups, obtained by flow-cytometric phenotyping (N = number of analyzed samples); proportion of lymphocyte subpopulations calculated in relation to detected CD45+ cells. IELs were characterized by their specific surface antigens

| | Roller mill | | | | Hammermill | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mash | | Expandate | | Mash | | Expandate | | |
| **Phenotype** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **Coarse** | **Fine** | **In total** |
| **CD3+**[1] | **63.1** | **67.0** | **67.7** | **69.0** | **69.5** | **65.4** | **67.7** | **67.3** | **67.1** |
| SD | 22.5 | 24.0 | 18.2 | 16.9 | 16.7 | 11.5 | 15.1 | 18.0 | 17.4 |
| N[2] | 8 | 8 | 8 | 8 | 8 | 7 | 8 | 8 | 63 |
| **TCR1+**[1] | **27.9** | **31.3** | **32.3** | **32.7** | **39.9** | **32.9** | **32.2** | **32.3** | **32.7** |
| SD | 11.2 | 11.0 | 5.74 | 10.8 | 9.08 | 11.5 | 7.02 | 8.55 | 9.51 |
| N[2] | 8 | 8 | 8 | 8 | 8 | 7 | 8 | 8 | 63 |
| **TCR2+**[1] | **23.8** | **23.0** | **23.5** | **22.8** | **23.5** | **22.4** | **20.8** | **27.8** | **23.5** |
| SD | 8.25 | 5.20 | 3.73 | 7.61 | 7.59 | 2.9 | 15.7 | 4.90 | 7.33 |
| N[3] | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 32 |
| **TCR3+**[1] | **15.9** | **16.6** | **15.6** | **15.3** | **15.0** | **15.9** | **15.6** | **17.7** | **15.95** |
| SD | 5.13 | 5.20 | 2.09 | 6.26 | 3.57 | 3.95 | 7.43 | 7.11 | 4.82 |
| N[3] | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 32 |
| **CD8α+**[1] | **51.9** | **53.6** | **61.1** | **55.8** | **57.4** | **56.3** | **57.0** | **57.3** | **56.3** |
| SD | 16.6 | 13.8 | 6.89 | 14.9 | 9.51 | 9.39 | 14.2 | 9.92 | 11.9 |
| N[2] | 8 | 8 | 8 | 8 | 8 | 7 | 8 | 8 | 63 |
| **28-4+**[1] | **6.02** | **6.56** | **5.85** | **7.21** | **7.12** | **8.54** | **6.51** | **7.04** | **6.82** |
| SD | 2.92 | 3.26 | 2.45 | 3.12 | 2.46 | 3.62 | 3.20 | 2.96 | 2.91 |
| N[23] | 7 | 7 | 7 | 7 | 7 | 6 | 7 | 7 | 55 |
| **28-4+ CD3+**[1] | **4.89** | **4.70** | **3.30** | **5.06** | **5.75** | **5.28** | **4.66** | **4.27** | **4.73** |
| SD | 2.74 | 2.88 | 1.17 | 2.29 | 1.40 | 2.62 | 2.39 | 1.82 | 2.20 |
| N[23] | 7 | 7 | 7 | 7 | 7 | 6 | 7 | 7 | 55 |
| **CD4+**[1] | **7.44** | **7.73** | **5.97** | **10.1** | **9.93** | **7.52** | **6.69** | **7.76** | **7.83** |
| SD | 0.32 | 4.21 | 3.02 | 3.28 | 1.99 | 2.95 | 2.55 | 2.74 | 2.81 |
| N[23] | 3 | 4 | 3 | 3 | 3 | 3 | 4 | 4 | 27 |
| **AV20+**[1] | **7.37** | **9.91** | **7.46** | **5.63** | **9.88** | **12.41** | **4.88** | **10.8** | **8.54** |
| SD | 4,13 | 2.18 | 0.20 | 1.10 | 1.8 | 3.92 | 3.01 | 3.63 | 3.40 |
| N[23] | 2 | 3 | 2 | 2 | 2 | 2 | 3 | 3 | 19 |

[1] Results are reported as means ± SD
[2] Sample sizes vary due to sample quality
[3] Sample sizes vary because antibody was established during the course of the experiment

Regarding to the proportions of intraepithelial lymphocytes in the duodenum and jejunum, the results show no statistically significant differences among the feeding groups. Between the different intestinal segments, there were also no statistically significant differences. However, the means of the different IEL fractions were higher in the case of cells isolated from the jejunum. A three-factor analysis of variance showed no interaction and single factor effects concerning the factors mill, physical form and particle size of the diet (**Table 29**). The fractions of CD4+ and AV20+ cells were not included in the statistical analysis due to the small number of sample sizes.

**Table 29:** Interaction and single factor effects of a three-factor analysis of variance on proportions of intraepithelial lymphocytes detected for the eight feeding groups in the duodenum and jejunum

| | ***P*-value** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **CD3+** | **TCR1+** | **TCR2+** | **TCR3+** | **CD8α+** | **28-4+** | **28-4+CD3+** |
| Mill (M) | 0.628 | 0.270 | 0.958 | 0.898 | 0.830 | 0.760 | 0.874 |
| Physical form (F) | 0.234 | 0.499 | 0.258 | 0.313 | 0.073 | 0.839 | 0.841 |
| Particle size (PS) | 0.498 | 0.674 | 0.810 | 0.588 | 0.335 | 0.378 | 0.868 |
| M * F | 0.844 | 0.196 | 0.300 | 0.537 | 0.663 | 0.149 | 0.323 |
| M * PS | 0.773 | 0.106 | 0.324 | 0.904 | 0.598 | 0.749 | 0.104 |
| F * PS | 0.985 | 0.684 | 0.263 | 0.905 | 0.547 | 0.688 | 0.353 |
| M * F * PS | 0.313 | 0.220 | 0.284 | 0.721 | 0.380 | 0.692 | 0.835 |

On average, 83 % of CD45+ leucocytes were isolated from the duodenum and 88 % of CD45+ leucocytes from the jejunum. The fraction of cells expressing the CD3 receptor (T cells) ranged from 70 % in the duodenum to 75 % in the jejunum. Within the T cell population, αβ+ T cells and γδ+ T cells were detected, whereas higher percentages of γδ+T cells (30 % in the duodenum; 32 % in the jejunum) were found compared with TCRαβ1+ cells (21 % in the duodenum; 24 % in the jejunum) and TCRαβ2+ cells (15 % in the duodenum; 16 % in the jejunum).

NK cells, expressing the 28-4 glycoprotein, had percentages of about 7 % in both intestinal segments. There were also cells of about 5 %, which bore the CD3 receptor in addition with 28-4 glycoprotein (**Figure 7**). In the duodenum, 52 % of cells expressed the CD8α receptor and 56 % of CD8α+ cells were detected in the jejunum. The CD8 glycoprotein is characteristic for cytotoxic T cells as well as for NK cells, although there were proportions of 28-4+ cells, which did not bear the CD8 receptor (**Figure 7**). T helper cells, identified by their CD4 receptor, and AV20+ B cells were determined in proportions of about 8 % respectively.

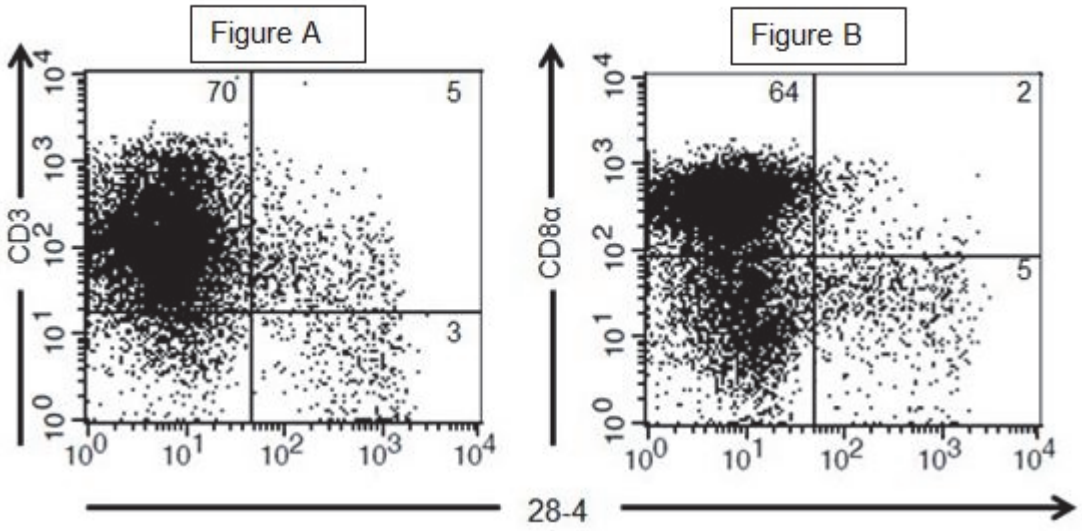


**Figure 7**: Dual immunofluorescence analysis of IELs using the 28-4 mAb together with mAbs, specific for CD3 (Figure A) and CD8α (Figure B). Percentages (in relation to CD45+ cells) of detected cells are shown in the corner of each quadrant. One representative experiment is depicted, presenting IELs isolated from jejunal tissue

In order to examine whether only IELs were isolated, tissue sections were verified by histological examination (**Figure 8**). The histological image shows that almost exclusively, cells were obtained from the epithelium, and that the lamina propria was preserved to a large extent.

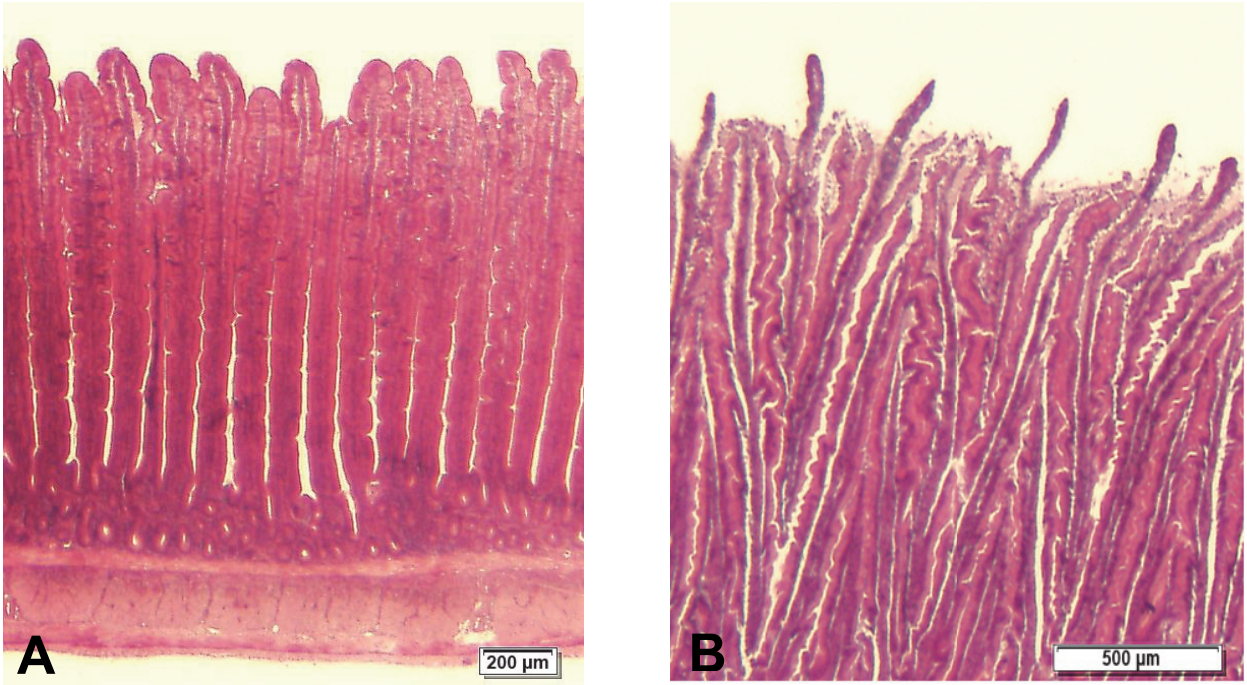


**Figure 8:** Microscopic picture of jejunal tissue before (A) and after (B) the procedure of isolation of IELs (HE)

# 5 Discussion

## 5.1 Feed processing

The eight experimental diets were produced using two different mills, the roller mill and the hammermill. By changing the grinding intensities, coarsely and finely ground feed was produced either as a mash or it was processed further to expandate. Thus, the feed structure of the different experimental diets was determined by three different factors:

1. Milling method
2. Physical form
3. Particle size

The structure of thermally processed feed can be characterized by its macro- and microstructure (Svihus, 2006). These terms can be explained using the example of expanded feed. Expandate has a diameter of e.g. 2.5 mm and has a coarsely ground macrostructure. However, by dissolving the expandate in water, the microstructure, namely the particle size distribution of the feed constituents, becomes apparent. This differentiation is not common for mash diets as thermal processing is not implemented and therefore no microstructure exists. Therefore, mash feed is characterized by its macrostructure only.

The results of the dry sieving analysis reflect the macrostructure of the diets. They showed that in the case of coarse grinding, 32 % coarse particles (> 2.5 mm) were found on average, irrespective of the physical form of the diet (mash or expandate) or the milling method used (roller mill or hammermill). The proportions of coarse particles in the finely ground diets ranged from 2 % to 11 %. Considerable amounts of medium sized particles (> 1 mm to ≤ 2.5 mm) were found in all diets, although on average, this particle fraction was larger within the finely ground diets (58 %) compared to the coarsely ground diets (46 %). The proportion of small particles (> 0.15 mm to ≤ 1 mm) were on average higher in the finely ground diets (35 %) than in the coarsely ground diets (23 %), and with regard to all diets, only a small amount of particles passed through the 0.15 mm sieve (1 - 2%).

In summary, the dry sieving analysis showed that the diets can be divided into finely and coarsely ground feed in regard to their macrostructure. In this respect, the physical form of the diets as well as the mill, which was used for grinding, had only a minor impact on the particle size distribution. This is not in accordance with the results of the wet sieving analysis. The wet sieving analysis is normally used for the determination of the particle size distribution of thermally processed feed such as pellets or expandate (Wolf et al. 2012). In order to allow for a better comparability, the mash diets were also wet sieved. By dissolving

the expanded diets in water, the microstructure was revealed and significant shifts occurred in the particle size distribution compared with the results of the dry sieving analysis.

Irrespective of the milling method used, there was a significant increase of particles that were smaller than 0.15 mm in the expanded diets. Nevertheless, differences between coarsely and finely ground expandate were observed. On average, the coarsely ground expandate had smaller proportions of particles sized < 0.15 mm (46 %) than the finely ground expandate (62 %). In addition, the proportion of particles sized > 1 mm were larger in the coarsely ground expandate (34 %) compared with the finely ground expandate (15 %).

The results of the wet sieving analysis demonstrated that the process of thermal expansion led to a further reduction of particle sizes. As a consequence, differences in the particle size distribution between the coarsely and finely ground expandate became less pronounced but were still detectable. With regard to the results of the dry sieving analysis, there was an increase of particles smaller than 0.15 mm in the mash diets, although to a lesser extent as found in the expandate. The results of the proportion of coarse particles in the coarsely ground mash diets were comparable with those of the dry sieving analysis. However, with regard to particles larger than 2.5 mm, there was an increase of about 10 % in the coarsely ground mash diets, which can be attributed to the water-related swelling of the grains (Wolf et al. 2012). The particle size distribution of the finely ground mash diets was also comparable with that analyzed by dry sieving, although a shift occurred from medium-sized particles in favor of particles smaller than 0.15 mm.

In summary, mash as well as expanded diets could be classified into coarsely and finely ground feed with regard to their macrostructure. However, after the wet sieving analysis, mainly medium and small-sized particles could be detected in the expandate, revealing its microstructure. Nevertheless, differences between the coarsely and finely ground expandate could be observed.

Therefore, it should be emphasized that effects induced by the feeding of expandate may be attributed to both its macro- and microstructure. This is especially the case regarding effects, which take place after the feed comes into contact with digestive juices in the upper digestive tract and consequently loses its macrostructure. In this context, Svihus (2006) stated that the macrostructure of thermally processed feed can affect feed intake patterns while the microstructure is able to influence the gut function.

Apart from differences in the feed structure, the electrical energy consumption (kWh/t) required during feed production also varied among the feed variants and depended mainly on three factors:

1. Mill
2. Grinding intensity
3. Physical form

The hammermill uses energy less efficiently compared to the roller mill (Koch, 1996), which was also the case in this study (**Figure 1**), particularly concerning the processing of finely ground diets. Additionally, the production of finely ground feed required more energy than the processing of coarsely ground feed. The roller mill used 2.6 times and the hammermill 6.7 times more energy, respectively. The electrical energy, which was used to produce the expandate, by far exceeded that which was required for the mash feed production.

From an environmental and economic point of view, a higher net energy consumption may be justified if simultaneous positive effects can be attained in terms of animal health, animal performance and feed hygiene and safety.

## 5.2 Effect of feed structure on animal performance

The performance data of the laying hens recorded during the experimental trial (**Figure 9**) were in accordance with values reported by the breeding company and found in the literature (Jeroch et al., 2013). This indicates that the hens were reared under optimal conditions and received an adequate diet, which led to a normal development and the maintenance of healthy animals.

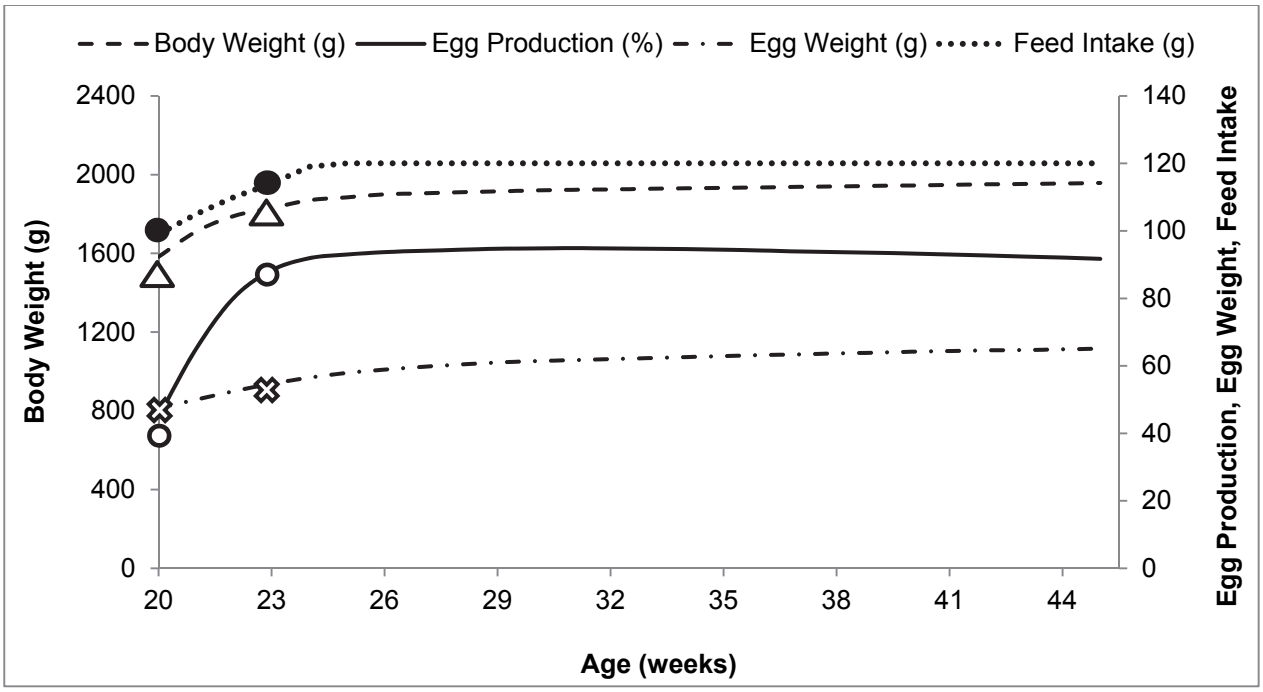


**Figure 9:** Course of the performance parameters of LOHMANN BROWN CLASSIC; modified from Jeroch et al. (2013).
Performance data of this study are marked by symbols: Body weight △; Egg production ○; Egg weight ⊠; Feed intake ●

Due to different experimental conditions including differences in particle sizes, feed forms, grinding methods and age and breed of the hens, it is not entirely possible to compare the present results with those of other studies.

As emphasized by Hamilton and Proudfoot (1995), the particle size of the feed is of less importance than the physical form of the diet in regard to the performance of laying hens. Few studies showed that the EP and the FI were influenced by the feed form, i.e. an increased EP as well as higher FIs were found for laying hens which were fed mash instead of pelleted/crumbled diets (Pepper et al., 1968; Blair et al., 1973; Deaton et al., 1987; Hamilton and Proudfoot, 1995).

This was confirmed by the results of the present study, due to the fact that the physical form of the diet had an impact on the FI of the first experimental week as well as on the overall FI, while the EP remained unaffected. Hens that were fed with mash had higher FIs than those given the expandate. It is possible, however, that observed differences in animal performance in the first experimental week were a consequence of the changeover of the feed. In the pre-experimental period, all laying hens received mash, while during the trial runs, four feeding groups were fed with mash, and four with expandate (**Figure 1**).

In this context, Jones (1986) showed that the feeding of a new diet formulation in birds resulted in a decreased FI. According to Haskell et al. (2001), the decreased FI may be the result of frustration as the broilers had different expectations regarding the feed.
In this study, the lower FI of hens fed the expandate also led to an improved FE in the first experimental week, although recorded EWs were comparable within the groups.
However, apart from the higher FIs of the first experimental week, it should also be noted that higher FIs were recorded for the mash-fed-birds regarding the total experimental trial.
The question arises why the FI behavior of hens was influenced by the physical form of the diet.
It is conceivable that the diet form mash was more attractive for the birds than the thermally processed feed, as also shown by Savory (1974), who observed a preference of mash to pellets when chicks were given a choice of feed. Additionally, it was demonstrated that birds had a pecking preference for textured feed over non-textured feed (Schiffman, 1969), and that they spent more time consuming mash than pellets, although the quantity of total ingested feed was not influenced (Jensen et al., 1962; Fujita, 1973). In contrast, other studies observed higher FIs in pelleted-fed hens compared with those given a mash-form diet (Jensen and McGinnis, 1952; Black et al., 1958).
However, it is known that the feeding of highly processed feed can impair the gut motility e.g. by the occurrence of a decreased gizzard activity, which may result in a decrease of the gut reflux and of the pancreatic enzyme secretion (Nir et al., 1994b; Engberg et al., 2002; Svihus, 2006). Therefore, it seems reasonable to suppose that the FI behavior is also influenced by such a dysfunctional gut motility.
It is also possible that the energy level of the diets had an impact on the FI behavior as it has been suggested that laying hens are able to regulate their FI with regard to the energy level (Hill and Dansky, 1954). This hypothesis was confirmed by Harms et al. (2000), who reported that the feeding of low energy diets led to a compensatory increase of the FI of laying hens. Vice versa, laying hens fed with high energy diets had decreased FIs, while the egg production was not influenced by the dietary energy content. In the present study however, the starch apparent ileal nutrient digestibility was significantly lower in hens fed with the expandate, than that of those given the mash diets (Ruhnke, I. 2013, pers. comm.), and thus the above-mentioned hypotheses could not be corroborated. In this context, Svihus (Svihus, 2006) emphasized that the use of thermally processed feed could lead to a decreased starch digestibility, which was confirmed by the results of the present study (Ruhnke, I. 2013, pers. comm.).
Regarding the effect of feeding different particle sizes, a consensus exists that laying hens perform equally, irrespective of whether coarsely ground or finely ground diets are used as feed (Berg and Bearse, 1951; Deaton et al., 1989; Hamilton and Proudfoot, 1995). However,

few studies showed that performance parameters such as BW, BWG, EW or EP were similar when fine or coarse particles were fed, but higher FIs were observed when coarse particles were fed (MacIsaac and Anderson, 2007; Safaa et al., 2009). There is evidence suggesting that laying hens prefer larger feed particles instead of finer ones (Portella et al., 1988), which may explain the increased feed consumption. In the present study, no significant differences were found regarding the influence of the factor particle size on laying performance, with the exception that in the last experimental week, the FCR was significantly higher for laying hens that were fed with coarsely ground feed compared to those that received the finely ground diet. This indicates that these hens had a lower feed conversion efficiency in the last experimental week, although this could be not confirmed for the total experimental period. While the FI of the last experimental week was comparable within the groups, hens which were fed with finely ground diets had higher weight gains on average (39.1 g/hen) than those fed with coarsely ground feed (36.4 g/hen), which explains the favorable FCR. The improved FCR of hens fed with finely ground diets was not connected with an improvement in the FE, which is of greater significance for the assessment of productive performance in laying hens. Furthermore, the factor particle size did not affect the BW, BWG, EP and EW, while the milling method had no impact on the overall animal performance.

## 5.3 Effect of feed structure on the development of digestive organs and on the microscopic structure of the small intestine

Several studies were performed in order to examine the effect of feed structure on the avian digestive tract, whereby mostly broilers were used as study animals. Thus in the following, the influence of feed structure will be discussed on the basis of scientific literature referring to laying hens as well as broilers.

In the present study, laying hens that were given coarsely ground feed had significantly higher relative gizzard weights than hens fed with the finely ground diets, whereas the weight of the proventriculus, pancreas and segments of the small intestine were not affected.

There is clear evidence that the particle size of the diet has an impact on the development of the digestive tract, which is also accompanied by changes in physiological processes. Several studies showed that the feeding of coarse particles led to an increase of the relative gizzard weight in broilers compared to the feeding of finer particles (Nir et al., 1994a; Engberg et al., 2002; Peron et al., 2005; Amerah et al., 2007c). The inclusion of whole grains in the diet led to the same effect so that broilers which received whole grains had heavier gizzards than those fed with pelleted diets (Preston et al., 2000; Plavnik, 2002; Gabriel et al., 2003; Svihus et al., 2004a). Higher gizzard weights of laying hens could be achieved when

feeding 40 % of whole wheat, incorporated into 3 mm pellets, in comparison with feeding 3 mm pellets, which did not contain whole wheat (Hetland et al., 2003b). Moreover, the addition of wood shavings to layer diets also resulted in increased gizzard weights and enhanced gastroduodenal refluxes (Hetland et al., 2003a). It can be concluded that the feeding of structural components is associated with an increase in the gizzard weight in laying hens.

Furthermore, this study showed that on average, the gizzard weights of birds fed with coarsely ground mash were higher than those of animals which received the coarsely ground expandate. This can be explained by the differences in the particle size distribution of both feed forms. The mash feed had larger proportions of coarser particles than the expandate, as illustrated by the results of the wet sieving analysis. The intake of coarse feed particles leads to an increased frequency of gizzard contractions (Roche, 1981), producing finer particles, so that an utilization of the feed is ensured.

Studies demonstrated that the development of the small intestine did not tend to be affected as strongly by the feed particle size as the gizzard (Wu et al., 2004; Peron et al., 2005; Gabriel et al., 2007), which was also consistent with results found in this study. The reason for this may be that the gizzard is able to grind feed to a consistently fine particle size irrespective of whether the ingested diet contains large or small particles (Hetland et al., 2004). However, Amerah et al. (2007a) and Lentle et al. (2006) demonstrated that both the duodenal digesta and the excreta of broilers, which were fed with coarser particles in mash diets, contained greater amounts of large particles compared with those fed fine particles in pelleted diets. Consequently, it would be conceivable that the small intestine is also affected by the feed structure, although this was not confirmed by the present study. In this context, Nir et al. (1994b) observed that the length of the jejunum and ileum was reduced when feed was offered as fine pellets. Dahlke et al. (2002) showed that the relative organ weights of the duodenum and jejunum plus ileum were positively related to increased particle size in mash diets. Moreover, Gabriel et al. (2003) observed lower duodenum weights in pellet-fed birds compared with those receiving whole grains.

Focusing on the physical form of the diet, various investigations demonstrated that feeding mash instead of thermally processed feed led to increased gizzard weights in broilers (Nir et al., 1994b; Engberg et al., 2002). Engberg et al. (2002) observed higher gizzard and pancreas weights when broilers were fed with mash diets instead of pelleted diets. Additionally, a higher activity of pancreatic enzymes including amylase, lipase, and chymotrypsin was found in pancreatic homogenates of mash-fed birds compared to pelleted-fed birds.

There seems to be a connection between the development of the gizzard and the pancreas, because increased relative gizzard weights were often found in combination with increased relative pancreas weights (Engberg et al., 2002; Gabriel et al., 2007; Williams et al., 2008; Rougiere et al., 2009).

The results of the present study corroborated these observations. Laying hens which received the mash diets had significantly higher gizzard weights as well as increased pancreas weights than those fed with the expandate. The increased gizzard weight might be a consequence of enhanced gizzard activity. The reason for the increased organ weights remains unclear. It is possible however that the expandate did not have to be ground to such an extent as mash in the gizzard due to its softer consistency and finer microstructure.

In this context, it was speculated that birds fed with pelleted diets become "overeaters" (Svihus and Hetland, 2001; Svihus, 2006). Thus, the passage rate of ingested feed increases while the gizzard activity is reduced. Ferket (2000) stated that the feeding of highly processed diets has a negative impact on the gastric reflux, which leads to proventricular hypertrophy and less efficient digestion. Vice versa, when mash feed is ingested, the retention time in the gizzard is increased, and the activity of the gizzard as well as the gut reflux enhanced. The release of CCK might consequently be stimulated, which could reinforce the pancreatic enzyme secretion (Li and Owyang, 1993; Svihus et al., 2004a; Svihus, 2006). This would explain the increased pancreas weights, which were also found in the present study, suggesting a higher activity of this organ.

Apart from the higher weights of the gizzard and the pancreas, the physical form of the diet also had an impact on the weight of the proventriculus. Mash-fed hens had increased proventriculus weights compared with expandate-fed hens. These results were not confirmed by other related studies, however, a dilatation of this organ could be determined when fine pelleted diets were fed (Jones and Taylor, 2001; Gabriel et al., 2007; Witte, 2012). Witte (2012) observed both a dilatation of the proventriculus and an underdeveloped gizzard, suggesting that an overload of the proventriculus may play a role as a consequence of reduced gizzard motility induced by fine pelleted feed.

Regarding the microstructure of the small intestine, this study showed that the physical form of the diet influenced the villus length and crypt depth. Hens fed with mash diets had longer duodenal and shorter ileal villi as well as increased duodenal villus height to crypt depth ratios than those given the expandate. Additionally, the feeding of mash tended to increase jejunal villus height, while expanded fed birds showed tendentially deeper duodenal crypts. Moreover coarsely instead of finely ground feed tended to promote duodenal villus height as well as duodenal and ileal crypt depth. In this context, increased villus to crypt length and surface ratios in the duodenum occurred when broilers were fed with whole wheat compared

to those given the mash, suggesting that the feeding of coarse diets enhances the intestinal villus and crypt development (Gabriel et al., 2007). Further investigations revealed that pellet-fed broilers had a higher number of villi per transversal duodenum cut and greater duodenal crypt depths when compared to mash-fed birds. Moreover, an increase in particle size of the diets resulted in deeper crypts and higher duodenal villi, irrespective of the physical form of the diet (Dahlke et al., 2002). With regard to the effect of feed structure on the intestinal microstructure, the mechanism that leads to the development of longer villi and deeper crypts has not yet been elucidated. However, it is known that the factor diet plays an important role in maintaining and improving gut health (Uni et al., 1998; Noy et al., 2001; Montagne et al., 2003). The general assumption exists that an enlargement of the absorptive surface can increase the absorption of nutrients and may lead to an improved feed conversion ratio (Dahlke et al., 2002; Gabriel et al., 2007).

## 5.4 Effect of feed structure on the intestinal glucose absorption

To date, there are no studies that address the question of how differently processed feed affects glucose absorption in the small intestine of poultry. An important objective of the present work was to examine the effect of different feed structures on active glucose absorption in the jejunum of laying hens. The results showed that the jejunal active glucose transport, mediated by the SGLT-1, was influenced by the physical form of the diet, while the particle size of the feed as well as the milling method used had no impact. The application of phloridzin led to a congruent decrease of the glucose absorption rate, demonstrating that the SGLT-1 was responsible for glucose transport. Mash-fed hens had a higher glucose transport rate than expandate-fed hens. In the following, possible explanations for the increased glucose transport rates and the involvement of the physical form of the diet will be discussed.

Two different hypothesizes exist, which may explain the higher glucose transport rates: Firstly, the villus surface area of mash-fed birds was increased (same SGLT-1 density, but increased villus surface area); secondly, the expression of the SGLT-1 was enhanced in the jejunal tissue of mash-fed birds (higher SGLT-1 density per villus surface area). Concerning the first hypothesis, the jejunal villi in the jejunum of hens that were fed with mash were not significantly higher than in those given the expandate. However, there was a tendency that the villus height of mash-fed hens was increased ($P$ = 0.087) compared with the villus height of expandate-fed hens, whereby the villus surface area might also be increased.

In this context, it was stated that the transport rates of glucose are closely related to the degree of villi (Calhaun, 1933) and microvilli development (Planas et al., 1987), as well as to

the availability of nutrients, i.e. to the luminal carbohydrate concentration (Ferraris and Diamond, 1986, Suvarna et al. 2005). Regarding the latter and the second hypothesis, it might be possible that the feeding of thermally processed diets resulted in a poorer nutrient utilization and consequently in a lower intestinal carbohydrate level reducing the expression of the SGLT-1 transporter. This was confirmed by the results of the present study, according to the decreased starch apparent ileal digestibilities in hens fed with the expandate (I. Ruhnke, 2013, Institute of Animal Nutrition, Freie Universität Berlin, Germany, pers. comm.). This could be explained by the occurrence of increased gizzard and pancreas weights in the mash-fed birds, which is associated with increased secretory activity of the pancreas (Hetland et al., 2003; Svihus et al., 2004). Therefore, it is possible that the feeding of mash diets enhanced digestion and nutrient absorption, whereby the expression of the SGLT-1 might be increased due to increased luminal carbohydrate concentrations.

## 5.5 Effect of feed structure on lymphocyte subpopulations in the gut-associated lymphoid tissue

Only few studies are published concerning the effect of the feed structure on the intestinal immune system, whereby they are mainly focused on interactions with the intestinal microbiota. Decreased colonization and growth of *Salmonella* in the caecum was observed when broilers were fed with coarsely ground as well as mash diets (Huang et al., 2006; Santos et al., 2008).

However, flow cytometric investigations including the characterization of IELs in laying hens and the examination of effects on the distribution and relative frequency of IELs related to the use of different feed structures are scarce.

This study showed that the protocol for the flow cytometric analysis, which was established in order to determine the different lymphocyte subsets from the gut-associated lymphoid tissue of laying hens, led to reliable and reproducible results. In this context, it should be emphasized that the comparison to results of other studies is not entirely possible due to the use of different species/chicken strains, ages of animals, sample sites as well as applied methodology (Ernst et al., 1986; Lillehoj and Chung, 1992). However, the results of this study showed several similarities to priorly published research.

The majority of IELs in the duodenum and in the jejunum were T cells expressing the CD3 receptor. These T cells consisted of a greater amount of the γδ T cells than TCRαβ1 cells and TCRαβ2 cells, which is consistent with findings of Lillehoj and Chung (1992). They could demonstrate that the amount of intraepithelial γδ T cells among the IELs increased during the postnatal development while the proportions of TCRαβ cells decreased.

With regard to the present study, most of the detected T cells also expressed the CD8 homologue and were CD4−, so that they could be identified as cytotoxic T cells. This T cell subpopulation was often found in the epithelium, whereas T helper cells (CD4+), similarly to B cells, represented a minor subpopulation among the IELs (Lillehoj and Chung, 1992; Göbel et al., 2001).

In this study, it could be demonstrated that ~ 5 % of T cells also carried the 28-4 glycoprotein, which is usually expressed by NK cells. These cells are probably comparable to NKT cells, which were previously determined in mammals as well as in chickens (Godfrey et al., 2000; Göbel et al., 2001; Godfrey and Rossjohn, 2011).

The percentages of detected NK cells (~ 7 %) were lower in the duodenum and jejunum as compared with those determined by Göbel et al. (2001). This could be explained by the use of different chicken breeds as well as the different age of the chickens, because the number and distribution of the IELs differ during the postnatal development as well as within the strains (Bäck, 1972; Lillehoj and Chung, 1992; Vervelde and Jeurissen, 1993; Göbel et al., 2001). In this context, the IELs of 19-day-old embryos almost exclusively expressed the 28-4 glycoprotein, while only few T cells were found. By contrast, IELs of 2- to 3-month-old birds contained a large proportion of T cells (~ 40 %), whereas only about 15 % of NK cells were detected (Göbel et al., 2001). Although NK cells are associated with the expression of CD8 homologue and 28-4 glycoprotein, few cells were found in this study, which carried the 28-4 receptor but did not express CD8.

With regard to the effect of feed structure on the IELs, the results showed no difference in the distribution and relative frequency among the feeding groups as well as concerning the factors particle size, physical form and grinding method of the diets. The assumption that the feed structure has an impact on the enteric immune system is based on investigations focusing on mast cells as well as on the intestinal microbiota.

Liu et al. (2006) examined the effect of particle size (coarse vs. fine) and physical form of the diet (mash vs. pellet) on the number and location of mast cells as well as on the histamine content in the small intestine of broilers. The histological examination showed that the feeding of coarsely ground diets led to an increase of the total number of mast cells, which were concentrated in the upper part of the villi of the intestinal epithelium. In contrast, the epithelium of broilers fed with finely ground diets, contained fewer numbers of mast cells, which were evenly distributed in the tissue sections. Additionally, the histamine content was higher in the jejunum when feeding coarsely ground rather than finely ground diets. With regard to the physical form of the feed, no significant differences were observed in the number and localization of mast cells and histamine content in the intestinal tissue. These results suggest that the factor particle size might particularly impact the cells of the enteric immune system. However, it could be not concluded that either finely ground nor coarsely

ground feed had positive or negative effects on the immunological response, because the parameter immune cell number within the epithelium is not solely an appropriate indicator for evaluating the immune competence of animals.

# 6 Conclusions and future perspectives

The results of the present study illustrated that the feed structure had no impact on the animal performance in general, with the exception that the physical form of the diet (mash vs. expandate) influenced the FI. Regarding the development of gastrointestinal organs and the intestinal microstructure of epithelium, positive effects were observed when hens were fed mash instead of expanded diets, which may explain the observed heightened glucose absorption in the jejunum of mash-fed birds. This study provided a characterization of different lymphocyte subpopulations from the gut-associated lymphoid tissue of Lohmann Brown hens, aged 23 weeks. However, the feed structure did not seem to influence the distribution and relative frequency of IELs in healthy hens of this age. The milling method used for the feed production had no effect on the investigated parameters.
Finally, it should be noted that based on the results of the present study, neither intensive grinding nor the use of thermal treatment processes led to improved animal performance. In contrast, the feeding of coarsely ground mash diets seemed to have positive effects on the gastrointestinal function and thus may lead to improved animal health and welfare. From an environmental and economical point of view, it should be stressed that the production of coarsely ground mash diets using energy-saving milling methods is resource-efficient and therefore contributes to climate protection.

Based on the results of this study, further investigations are required in order to clarify in more detail in which way the feed structure is influencing physiological processes in the organism of laying hens. In this context, it would be important to examine whether observed structural changes of the gastrointestinal organs are accompanied by functional changes, e.g. regarding the passage rate of ingested feed, the gut reflux and the pancreatic enzyme secretion.
With respect to the results of the Ussing chamber experiment and the detected differences in the glucose absorption, it should be elucidated whether the intestinal SGLT-1 protein content is also influenced by the feed structure. Thereby, the connection between functional glucose absorption, SGLT-1 density and absorptive surface area could be explained. In this regard, it would be interesting to assess whether the feeding of differently structure diets also influences the absorption of other nutrients such as amino acids or minerals.
The results of this study showed that the feed structure did not seem to influence the distribution and relative frequency of IELs in healthy hens. It might be conceivable that the effect of feed structure on the avian intestinal immune system will become sufficiently

apparent only in case of the occurrence of diseases. Therefore, further investigations might be important including infection experiments.

Moreover, the experimental design could be modified by extending the duration of the feeding trial. This could lead to even more significant effects particularly regarding the animal performance. In this context, the experimental design could be used focusing on the post hatch period of chicks due to the fact that most of the development occurs when the neonatal bird begins to consume feed (Uni, 2006).

With regard to the experimental diets the effect of the inclusion of whole grains could be proofed in future studies. In addition, it should be investigated whether the results of this study are robust to changes in the processing parameters (e.g. temperature and pressure) during the process of expansion.

# 7 Summary

Röhe, Ilen:

**Effects of feed structure on animal performance, gastrointestinal morphology, gut-associated lymphoid tissue and jejunal glucose transport in laying hens**

New developments in the production and processing of feed for the use in the poultry sector are associated with changes in the feed structure, which is characterized by the particle size and the particle size distribution as well as the physical form of the diet, i.e. mash, pellets or expandate. Different grinding methods, grinding intensities as well as thermal treatment processes are used in order to achieve more energy-efficient feed production and to increase the quality and safety of feed. The aim of this study was to investigate whether gastrointestinal function and consequently animal performance and health were affected by the feeding of differently structured diets. For this purpose, investigations were focused on morphological, histological and immunological parameters and on the jejunal glucose transport of the digestive tract in laying hens.

The eight experimental diets were produced using two different mills, the roller mill and the hammermill. By changing the grinding intensities, coarsely and finely ground feed was produced either as a mash or it was processed further to expandate. In total, the following eight diets were produced, which differed in their feed structure, but showed an identical botanical and chemical composition (30 % corn, 29 % wheat and 22 % extracted soybean mash): Coarsely and finely ground mash diets, coarsely and finely ground expanded diets, each of them produced by one of the two mills. In eight consecutive trial runs, a total of 384 laying hens (Lohmann Brown), aged 20 weeks were randomly allocated to eight different experimental groups (in total 48 hens per feeding group) with six animals per floor pen each representing a single experimental unit. The hens had *ad libitum* access to feed and water and were kept in barn systems. Each experimental group received differently structured diets over a period of 21 days. During the experimental trial, the laying performance, i.e. the egg production and the egg weight of each feeding group was recorded daily, and the feed intake and body weight recorded weekly. At the end of each trial run, the hens, aged 23 weeks, were slaughtered. In order to examine the effect of different feed structures on the development of the digestive organs, the pancreas, proventriculus, gizzard and the three segments of the small intestine of hens of each feeding group were extracted and subsequently weighed. For morphometric analysis, segments from the duodenum, jejunum and ileum were removed, processed histologically and finally the villi lengths and crypt

depths were determined. In order to investigate the effect of the different diets on the active glucose transport in the jejunum, Ussing chamber experiments were conducted. On the basis of flow cytometric investigations, the intraepithelial lymphocytes (IELs) of the duodenum and jejunum of laying hens were characterized, and the effect of different feed structures on their distribution and relative frequency examined. All collected data were analyzed by using a three-factorial analysis of variance in order to control for interaction and single factor effects with respect to the factors milling method, physical form and particle size of the diet.

Concerning the collected animal performance data, the physical form of the diet had an impact on the overall feed intake. Hens fed with mash diets showed significantly higher feed intakes than those given the expandate. Regarding the total experimental period, neither the particle size of the feed nor the milling method used affected the overall animal performance. However, the results showed that both the physical form as well as the particle size of the diet influenced the development of the digestive organs. Laying hens that received mash instead of expandate had significantly higher gizzard and proventriculus weights as well as increased pancreas weights. Hens fed the coarsely ground diets showed significantly higher relative gizzard weights than those fed the finely ground diets. Regarding the microscopic structure of the small intestine, the results demonstrated that only the physical form of the diet had an impact on the villus lengths and crypt depths. Laying hens fed with mash diets showed significantly longer duodenal and shorter ileal villi as well as increased duodenal villus height to crypt depth ratios than those given the expandate. Moreover, the results of the Ussing chamber experiment showed that the active glucose transport in the jejunum, mediated by the SGLT-1, was influenced by the factor physical form of the diet. Mash-fed hens had a higher glucose transport rate than expandate-fed hens. The protocol for the flow cytometric analysis, which was established in order to determine the different lymphocyte subsets from the gut-associated lymphoid tissue of laying hens, led to reliable and reproducible results. With regard to the effect of the feed structure, the results showed no difference in the distribution and relative frequency of the IELs.

To conclude, the results of this study illustrated that in general the feed structure had no impact on the animal performance, with the exception that the physical form of the diet (mash vs. expandate) influenced the FI. The feeding of mash diets compared with the feeding of expanded diets showed positive effects regarding the development of gastrointestinal organs and the intestinal microstructure of epithelium of laying hens, which may have led to the observed heightened glucose absorption of the jejunum. Furthermore, the feeding of coarsely ground diets also resulted in increased gizzard weights, but did not influence the intestinal microstructure of the epithelium and the jejunal glucose absorption. This study provides a characterization of different lymphocyte subpopulations from the gut-associated lymphoid tissue of Lohmann Brown hens. However, the feed structure did not seem to

influence the distribution and relative frequency of IELs in healthy hens of this age. The milling method used for the feed production had no effect on the investigated parameters.

Finally, it should be noted that based on the results of the present study, neither intensive grinding nor the use of thermal treatment processes led to improved animal performance. In contrast, the feeding of coarsely ground mash diets seemed to have positive effects on the gastrointestinal function and thus may lead to improved animal health and welfare. From an environmental and economical point of view, it should be emphasized that the production of coarsely ground mash diets using energy-saving milling methods is resource-efficient and therefore contributes to climate protection.

# 8 Zusammenfassung

Röhe, Ilen:

**Einfluss der Futterstruktur auf die Leistung, die gastrointestinale Morphologie, das darmassoziierte lymphatische Gewebe und auf den jejunalen Glukosetransport von Legehennen**

Neue Entwicklungen in der Herstellung und Bearbeitung von Futter im Geflügelsektor sind mit Veränderungen in der Futterstruktur verbunden. Der Begriff der Futterstruktur umfasst dabei einerseits die Partikelgröße und Partikelgrößenverteilung, andererseits die Form des Angebots, das heißt, ob beispielsweise schrotförmiges, pelletiertes oder expandiertes Futter verfüttert wird. Dabei werden unterschiedliche Vermahlungsarten und -intensitäten sowie thermische Behandlungsverfahren genutzt, um eine energieeffizientere Futterproduktion zu erzielen und die Qualität und Sicherheit der Futtermittel zu erhöhen.

Ziel der Studie war es daher zu überprüfen, ob die Fütterung unterschiedlich strukturierten Futters die Magen-Darm-Funktion sowie die damit verbundene Leistung und Gesundheit von Legehennen beeinflusst. Zu diesem Zweck wurden morphologische, histologische, transportphysiologische und immunologische Untersuchungen des Gastrointestinaltrakts bei Legehennen durchgeführt.

Die Futtervarianten wurden mittels zwei verschiedener Mühlen, der Hammermühle und dem Weizenstuhl hergestellt, grob und fein vermahlen und entweder ohne Behandlung als Schrot verfüttert oder noch zusätzlich einer thermisch-mechanischen Druckkonditionierung unterzogen und als Expandat verfüttert. Es entstanden die folgenden acht Futtervarianten, die sich in ihrer Futterstruktur unterschieden, jedoch aus botanisch und chemisch identischen Ausgangskomponenten bestanden (30 % Mais, 29 % Weizen und 22 % Sojaextraktionsschrot): grob und fein vermahlenes Schrot, grob und fein vermahlenes Expandat, jeweils produziert durch eine der beiden Mühlen. In acht konsekutiv angelegten Versuchsreihen wurden insgesamt 384 Legehennen (Lohmann Brown; Alter: 20 Wochen) acht Fütterungsgruppen (48 pro Gruppe) zugeordnet, die jeweils über einen Zeitraum von 21 Tagen das entsprechende Futter erhielten. Dabei wurden sechs Tiere zusammen in einer Gruppe in Bodenhaltung gehalten (experimentelle Einheit), denen ad libitum-Fütterungs- und Tränkeeinrichtungen zur Verfügung standen.

Während des gesamten Versuchszeitraumes wurden verschiedene Leistungsparameter der Legehennen erhoben, d.h. pro Fütterungsgruppe wurde die Legeleistung und die Eigewichte täglich, die Futteraufnahme sowie das Gewicht der Tiere wöchentlich bestimmt. Im Alter von

23 Wochen wurden die Versuchstiere nach vorheriger Betäubung durch Blutentzug getötet. Um den Einfluss der Futterstruktur auf die Entwicklung der Verdauungsorgane der Legehennen in den verschiedenen Versuchsgruppen zu untersuchen, wurden Pankreas, Drüsen- und Muskelmagen sowie die drei Dünndarmabschnitte entnommen und anschließend gewogen. Für die histologischen Untersuchungen wurden Gewebeproben vom Duodenum, Jejunum und Ileum entnommen, histologisch bearbeitet und die Länge der Darmzotten und Kryptentiefe morphometrisch beurteilt. Mit Hilfe von Ussing Kammer Versuchen wurde der aktive Glukosetransport im Jejunum der Tiere gemessen und überprüft, ob dieser durch die Fütterung der verschieden Diäten beeinflusst wurde. Mittels durchflusszytometrischer Messungen wurden intraepitheliale Lymphozyten (IEL) im Duodenum und Jejunum zum einen charakterisiert und zum anderen mögliche Effekte der Futterstruktur auf deren Verteilung und relative Häufigkeit untersucht. Die statistische Analyse der Ergebnisse erfolgte durch eine dreifaktorielle Varianzanalyse, so dass der Einfluss der einzelnen Faktoren (Mühle, Form und Partikelgröße) sowie deren Interaktionseffekte überprüft werden konnte.

Die Auswertung der Leistungsdaten ergab, dass die Form des Futters einen Einfluss auf die Futteraufnahme der Tiere hatte. Legehennen, die mit Schrot gefüttert wurden, hatten signifikant höhere Futteraufnahmen als jene, die das Expandat erhielten. Bezogen auf den gesamten Versuchszeitraum beeinflusste dagegen weder die Partikelgröße des Futters noch die verwendete Mühle die Leistung der Tiere. Allerdings hatte die Form sowie die Partikelgröße des Futters einen Einfluss auf die Entwicklung einzelner Organgewichte des Gastrointestinaltraktes. Legehennen, die mit Schrot anstelle von Expandat gefüttert wurden, hatten signifikant schwerere Drüsen- und Muskelmägen sowie höhere Pankreasgewichte. Außerdem wiesen Hennen, die mit grob vermahlenen Diäten gefüttert wurden im Vergleich zu denen, die fein vermahlenes Futter erhielten, signifikant höhere Muskelmagengewichte auf. Die Ergebnisse der histologischen Untersuchungen des Dünndarmgewebes veranschaulichten, dass einzig die Form des Futters Einfluss auf die Länge der Darmzotten und Kryptentiefe hatte. Die mit Schrot gefütterten Legehennen hatten signifikant längere duodenale und kürzere ileale Darmzotten als Hennen, die Expandat als Futter erhielten. Außerdem führte die Fütterung von Schrot zu einem größeren Verhältnis von Villuslänge zur Kryptentiefe im Duodenum. Die Resultate der Ussing Kammer Versuche ergaben, dass der durch den SGLT-1 vermittelte aktive Glukosetransport im Jejunum durch den Faktor Form des Futters beeinflusst wird. Somit wiesen die mit Schrot gefütterten Hennen höhere Glukosetransportraten auf als Hennen, die mit Expandat gefüttert wurden. Das eigens etablierte Protokoll für die durchflusszytometrische Bestimmung unterschiedlicher Lymphozytensubpopulationen aus dem darmassoziierten lymphatischen Gewebe führte zu

aussagekräftigen und reproduzierbaren Ergebnissen. Allerdings konnten keine durch Futter bedingten Effekte auf die Verteilung und relative Häufigkeit der IEL festgestellt werden.
Die Ergebnisse der vorliegenden Studie deuten darauf hin, dass die Futterstruktur im Allgemeinen keinen Einfluss auf die Leistung der Legehennen hat, mit Ausnahme der Futteraufnahme, die durch die Form des Futters (Schrot vs. Expandat) beeinflusst wurde. Die Fütterung von Schrot im Vergleich zu Expandat hatte positive Effekte auf die Entwicklung der Organe des Magen-Darm-Traktes und auf die Mikroarchitektur der intestinalen Schleimhaut, wodurch möglicherweise auch die nachgewiesenen erhöhten Glukoseaufnahmeraten im Jejunum zu erklären sind. Zudem führte die Verfütterung von grob vermahlenen Diäten zu erhöhten Muskelmagengewichten, während die Mikroarchitektur des Darmepithels sowie der intestinale Glukosetransport davon nicht beeinflusst wurde. Durch die vorliegende Arbeit konnten verschiedene Lymphozytensubpopulationen im darmassoziierten lymphatischen Gewebe von Legehennen der Rasse Lohmann Brown charakterisiert werden. Allerdings deuten die Ergebnisse daraufhin, dass die Futterstruktur keinen Einfluss auf die Verteilung und relative Häufigkeit der IELs in gesunden Legehennen dieses Alters hat. Die zur Futterproduktion genutzten Mühlen hatten insgesamt keinen Einfluss auf die untersuchten Parameter.

Auf Grundlage der Ergebnisse dieser Studie ist abschließend festzuhalten, dass weder die intensive Vermahlung noch die Nutzung thermischer Behandlungsverfahren von Futter zu verbesserten Leistungen der Legehennen führten. Vielmehr deuten die Untersuchungsergebnisse darauf hin, dass insbesondere durch die Fütterung von grob vermahlenem Schrot die Funktion des Magen-Darm-Traktes positiv beeinflusst wird und dies auch positive Auswirkungen auf die Gesundheit und das Wohlbefinden der Tiere haben könnte. Zudem ist aus ökologischer und ökonomischer Sicht zu betonen, dass eine ressourcenschonende Futtermittelproduktion durch die Herstellung von grob vermahlenem Schrot und die Verwendung energiesparender Mühlen gewährleistet wird und somit zum Klimaschutz beitragen könnte.

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# 10 Appendix

## Publications

### Oral Presentations

Röhe, I. (2012)
Überprüfung der Effekte unterschiedlicher Zerkleinerungsverfahren von Futtermitteln auf ernährungsphysiologische Parameter bei Legehennen. 7. Doktorandensymposium & DRS Präsentationssemiar „Biomedical Sciences", Freie Universität Berlin, Berlin, Germany, 13.Juli.2012.

Röhe, I., I. Ruhnke, R. Löwe, J. Zentek (2013)
Effects of grinding method, particle size, and physical form of the diet on the glucose transport of the isolated epithelium of the jejunum in laying hens.
Proceedings of the Society of Nutrition Physiology **22**: 158, Göttingen, Germany,
19.-21.März 2013.

### Journal Publications

Röhe, I., I. Ruhnke, F. Knorr, A. Mader, F. Goodarzi Boroojeni, R. Löwe, and J. Zentek (2013). Effects of grinding method, particle size and physical form of the diet on gastrointestinal morphology and jejunal glucose transport in laying hens. *Poultry Science, Submitted.*

Ruhnke, I., I. Röhe, W. Meyer, S. Kröger, K. Neumann and J. Zentek (2013).
Method for the preparation of mucosal flaps from the jejunum of laying hens for transporter studies in Ussing chambers. *Archives of Animal Nutrition* 67: 161-168.

Goodarzi Boroojeni, F., A. Mader, F. Knorr, I. Ruhnke, I. Röhe, H. Abdul, K. Männer and J. Zentek (2013).
Effect of different heat treatments and organic acid levels on nutrient digestibility in broilers. *Poultry Science, Submitted.*

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## Declaration

I hereby declare that this dissertation is my own work and has not previously been submitted anywhere for any award. Where other sources of information have been used they have been acknowledged.

Berlin, 20.09.2013

Ilen Röhe