Fachbereich Veterinärmedizin der Freien Universität Berlin

Institut für Tierernährung

Impact of dietary protein and fiber on the nutritional physiology of poultry

Habilitationsschrift
zur Erlangung der Lehrbefähigung
am Fachbereich Veterinärmedizin
der Freien Universität Berlin

Eingereicht von
Dr. med. vet. Ilen Röhe

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Meiner Familie

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List of abbreviations

AB	Alcian blue
ANF	Antinutritional factors
CD (+; -)	Cluster of differentiation (positive; negative)
CP	Crude protein
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FCR	Feed conversion ratio
HPLC	High performance liquid chromatography
IEL	Intraepithelial leukocytes
Isc	Short-circuit current
NBF	Neutrally buffered formalin
NSP	Non-starch polysaccharides
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
SCFA	Short chain fatty acids
SGLT-1	Sodium-dependent glucose cotransporter 1

Preface

Nutrition is one of the most important factors determining the health, welfare and productivity of poultry. A nutritionist's vision is to formulate diets that maintain or strengthen gastrointestinal health ensuring optimal overall health and maximum productivity. This presupposes, on the one hand, that nutritional physiological processes in the organism are precisely known and, on the other hand, that dietary components are identified that can directly trigger health-related effects. Fundamental advances have been achieved in the field of poultry nutrition and nutritional physiology over the past few decades, although various gaps in knowledge still remain.

The present thesis is based on ten peer-reviewed publications that were published between 2013 and 2021 and focus on **i)** the establishment and further development of analytical methods characterizing physiological features and processes in the intestinal tract and **ii)** the significance of dietary protein and **iii)** dietary insoluble fiber on the nutritional physiology of poultry. **Chapter I** comprises three studies aimed at developing and refining laboratory techniques that will provide better insight into the physiological properties and functions of the intestinal tract. Analytical methods will shed light on the intestinal nutrient transport in chickens, the intestinal mucus layer formation in pigs and the distribution and frequency of intraepithelial immune cells in the gut of chickens. **Chapter II** includes three publications that examined the effect of qualitative or quantitative differences in dietary protein on the nutritional physiology of chickens. In particular, the impact of feeding differently processed grain legumes on immunological, morphological and functional alterations in the gastrointestinal tract of broilers are investigated. Furthermore, studies regarding protein requirements of male dual purpose chickens were performed investigating the effect of dietary protein reduction on digestive physiology and growth performance. **Chapter III** is based on a series of four publications evaluating the impact of dietary lignocellulose as an insoluble fiber source in poultry nutrition. Feeding experiments with laying hens and broilers are presented investigating the effect of varying dietary lignocellulose concentrations on chicken's productive performance, digestive physiology and intestinal microbiota. Finally, a review article summarizes and evaluates studies on the effects of lignocellulose in poultry nutrition and compares the results with those observed in feeding trials using traditional insoluble fiber sources. In the **general discussion** the most important results from Chapters I-III are highlighted and intensively discussed in comparison with previous literature findings. Furthermore, open research questions are identified and future research perspectives addressed.

Introduction

Nutrition is one of the key factors that affect gastrointestinal health in poultry. Hence, there is an increased interest in maintaining or enhancing gut health through optimal feeding as this has a determining effect on chicken's overall health and productivity (Choct 2009, Gadde et al. 2017). But what exactly does the term "gut health" mean? An accurate definition of "gut health" is still missing (Kogut and Arsenault 2016, Celi et al. 2017). While in human medicine the term "health" refers to the "state of complete physical, mental, and social well-being" according to the World Health Organization (WHO 1948), in the livestock sector this term implies physically healthy but also high-performing animals (Kogut and Arsenault 2016, Celi et al. 2017). This emphasizes that in production animals such as poultry an optimal gastrointestinal health is strongly related to an optimal gastrointestinal functionality. For example, a healthy intestine should digest and absorb dietary nutrients via the intestinal epithelium as efficiently as possible so that they can be optimally utilized by the chicken. However, the gastrointestinal tract not only has digestive and absorptive tasks, but also takes on a variety of functions, such as immunological, metabolic or endocrine functions (Perry 2006). As highlighted in Figure 1, key components that affect intestinal functionality and health are the gut microbiota, the intestinal mucus layer and the gut immune system. Each of these components and their interactions have a decisive impact on maintaining gastrointestinal homeostasis and are strongly related to the host's nutrition (Figure 1). In order to be able to assess the effects of feeding on gastrointestinal health, it is important to have a precise knowledge of characteristics and physiological processes of the gastrointestinal tract. The idea of identifying certain biomarkers in the gastrointestinal tract that would enable the assessment of health and functionality of the gastrointestinal system in farm animals has been raised (Celi et al. 2019). Over the last few decades, intensive research has contributed to improve the understanding of gastrointestinal physiology in humans and animals (Borda-Molina et al. 2018, Pluske et al. 2018, Camilleri 2019). Recently, however, large gaps have been identified in the literature in relation to research fields on gastrointestinal permeability, gastrointestinal barrier function, the endocrine gut system or gut microbiome-host interaction (Celi et al. 2017, Celi et al. 2019). Particularly with regard to chickens, there are still many knowledge gaps in the area of nutritional physiology. On the one hand, this is due to the fact that fewer research studies have been and are carried out on poultry than in rodents or pigs. On the other hand, there is a lack of analytical methods that assess physiological characteristics and functions of the avian gastrointestinal tract. In addition, analytical methods that are established for investigations on the mammalian intestine cannot be extrapolated directly to the chicken's intestine. Part of the present thesis therefore is focused on the establishment and further

development of analytical tools that make physiological features and processes in the intestine visible and thus measurable. Addressed analytical methods relate to the intestinal nutrient transport and barrier function in chickens via Ussing Chamber experiments, the intestinal mucus formation in pigs characterized by histological methods and the gut immune system, whose intraepithelial immune cells are investigated in the chicken's intestine by immunohistochemical techniques.

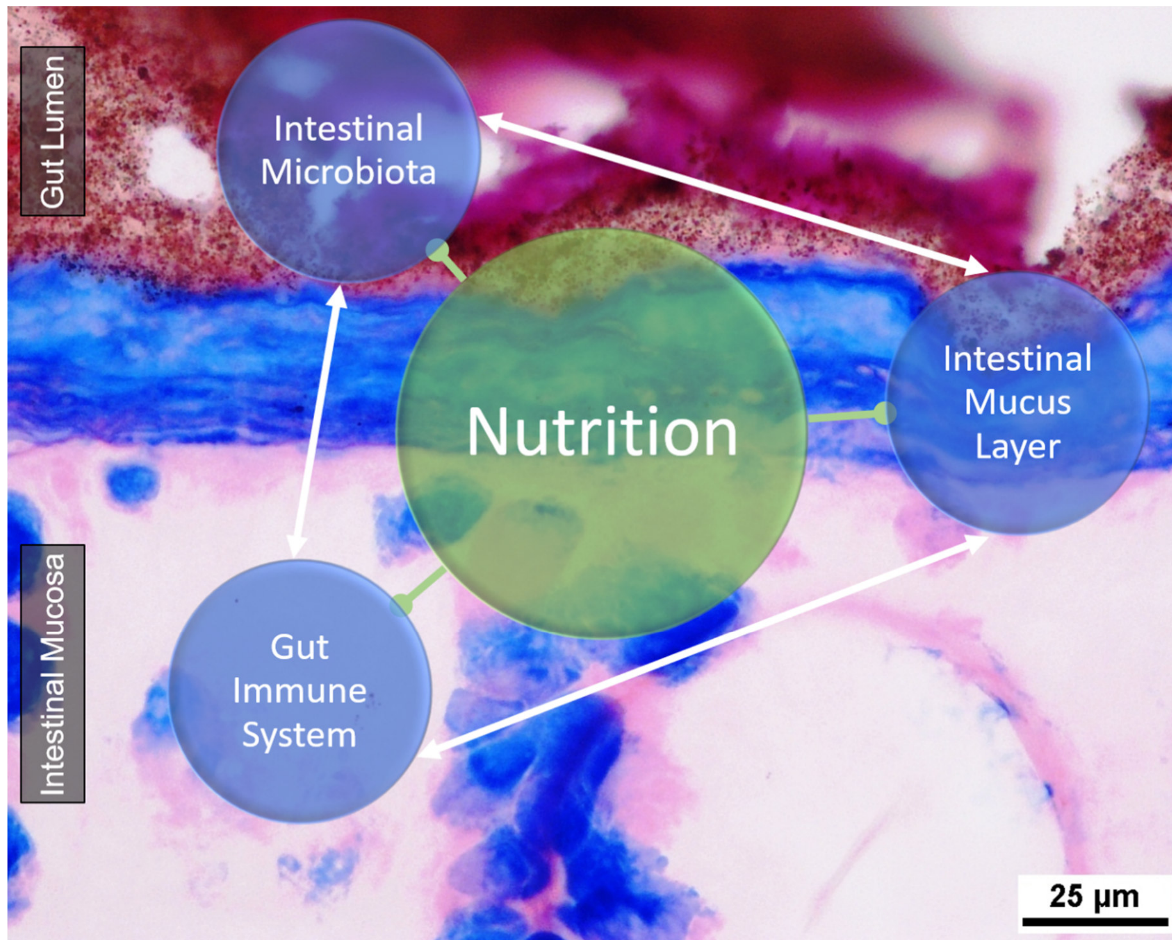

Figure 1. Histological cross-section of the large intestine (Röhe at al. 2018) with key components that are crucial for maintaining intestinal functionality and health. There is a close interaction between the intestinal microbiota and the host's intestinal epithelium resulting in effects on the host's immune system including the intestinal mucus layer as the first physical and immunological barrier of the gut mucosa. The host's diet is of central importance as the dietary composition has a decisive impact on the intestinal microbial composition. Moreover, the host's nutrition can have a direct impact on the intestinal mucosa modulating intestinal immune responses and intestinal mucus layer formation.

In addition to carbohydrates, proteins are the most important nutritional components in poultry feed. Proteins, that are composed of individual amino acids, are needed for optimal chicken health and productivity. Protein compounds are important for metabolic processes

in the body, as they are required for the production of e.g. enzymes, hormones or antibodies (Beski et al. 2015, Wu 2018). Due to its high quantity of protein and its amino acid profile close to “ideal”, soybean meal is the most important and preferred protein source in poultry feed. However, in relation to various environmental, economic and social problems associated with cultivating and importing soybeans, significant efforts are being made in Europe to promote the use of locally grown legumes as protein source in poultry feed (Iji et al. 2017, Jouan et al. 2020). In addition to locally grown soy varieties, native peas, lupins or faba beans are of particular interest, as they might partially replace imported soybean meal as a protein source in poultry feed. However, grain legumes contain several secondary plant substances known as antinutritional factors (ANF) that can impair poultry health and productivity (Gatel 1994, Mikić et al. 2009). Protease inhibitors, which are found in abundance in raw soybeans (Van Eys et al. 2004, Yasothai 2016), represent one of the most prominent examples of ANFs, which are known to reduce protein digestibility and productivity in chickens (Choct et al. 2010, Palliyeguru et al. 2011). In addition, it is supposed that other legume-associated ANFs, such as lectins or antigenic proteins, can affect chicken’s productivity by directly impairing the intestinal health of farm animals. Studies in calves and piglets showed that the feeding of different legumes containing lectins or antigenic proteins led to intestinal mucosal damage and local as well as systemic immune responses (Pusztai et al. 1979, Bush et al. 1992, Dreau et al. 1995, Lallès et al. 1996). It was not yet known whether feeding such ANF containing legumes triggers similar reactions to the intestinal wall and the immune system in chickens. Different feed processing methods are available that can decrease the amount of certain ANF and thus increase the nutritional value of legumes (Van der Poel et al. 1990, Zentek and Goodarzi Boroojeni 2020). However, ANFs differ in their chemical structure, so that not every feed processing method is suitable for successfully eliminating all relevant ANFs. Thus, in the present work the effects of feeding different grain legumes in combination with the application of different feed processing methods on the nutritional physiology of chickens are examined with a special focus on the gut immune system, intestinal nutrient absorption and intestinal morphology. In addition to the protein quality, the amount of protein provided by feeding is also critical to achieve optimal poultry health and productivity. The dietary concentration of protein and single amino acids must be precisely adapted to the requirements of the chicken. A lack or excess of dietary protein would have negative effects on chicken’s performance and health (Morris et al. 1999, Gous 2010). In addition, the feeding of dietary protein concentrations exceeding chicken’s needs is accompanied with economic losses and environmental pollution (Nahm 2002, Costantini et al. 2021). Thus, the amino acid pattern of the dietary protein should correspond to the specific amino acid requirement of the chicken following the concept of ideal protein (Martens et al. 2012, Chrystal et al. 2020). The chicken’s protein

requirement is of course closely linked to the productivity of the respective chicken genetic, so that, for example, broilers of a rapidly-growing line have a much higher protein requirement than those of a slow-growing breed. The protein and amino acid content in the feed must therefore be tightly tailored to the breed of chickens used in order to guarantee efficient production. Although worldwide poultry production is based primarily on the use of intensively-growing broiler genetics and high-performing laying hen lines, there is a niche market for alternative chicken breeds. Interest in alternative chicken lines such as dual purpose breeds is driven by diverse region-specific consumer demands, particularly with respect to raising animal welfare standards (Fernyhough et al. 2020, Rayner et al. 2020). In contrast to conventional broiler and laying hen hybrid breeds, recommendations for protein and nutrient requirements are largely lacking for dual purpose chickens. Therefore, studies presented in the current work address the protein requirements of broilers of a newly bred dual purpose genetic. Investigations should clarify whether these chickens have different dietary protein needs than conventional hybrid broilers and whether a dietary protein reduction affects digestive physiology and growth performance.

In contrast to dietary proteins, dietary fiber has long been viewed as an antinutritional factor in poultry nutrition. Due to its low nutritional value, the dietary inclusion of fiber-rich feed components can reduce the energy- and nutrient content of feed which is associated with an undesirable increase in voluntary feed intake of chickens (Choct 2015). In addition, feeding certain fiber sources, which are high in soluble non-starch polysaccharides (NSP), could result in an increased digesta viscosity in chickens, which is related to an impaired nutrient digestibility and productivity (Annison et al. 1991, Choct 1997). However, dietary fiber represents one of the most diverse and complex groups of molecules found in nature (Hamaker and Tuncil 2014) so that various fiber sources can differ significantly in their chemical and physicochemical properties. In recent years, intensive research into different types of dietary fibers has proven that the feeding of fiber sources mainly consisting of insoluble NSPs can have a positive impact on poultry health and productivity. Several experiments showed that the feeding of insoluble fiber sources such as oat hulls, soybean hulls or wood shavings positively affected digestive tract development and function in chickens improving nutrient digestibility and productive performance (González-Alvarado et al. 2007, Amerah et al. 2009, Jiménez-Moreno et al. 2009, González-Alvarado et al. 2010). Moreover, it is well established that dietary fiber can modify the growth and composition of intestinal bacteria which in turn might have consequences on avian intestinal health (Makki et al. 2018, Jha and Mishra 2021). In addition to classic fiber feed components, which arise as by-products during industrial processing of food and feed such as oat hulls, soybean hulls or wheat bran, studies on an "innovative" source of dietary fiber, lignocellulose, have been increasing in recent years. Lignocellulose is a constituent of plant cell walls and is

mainly composed of the insoluble carbohydrate polymers cellulose and hemicelluloses as well as the phenolic polymer lignin (Collard and Blin 2014, Liu et al. 2014). In the last decade, few studies were performed in order to investigate the effect of dietary lignocellulose on productive performance, digestive physiology, and intestinal microbiota in broilers and laying hens (Bogusławska-Tryk et al. 2015, Farran et al. 2017, Kheravii et al. 2017, Makivić et al. 2019, Bogusławska-Tryk et al. 2020, Sun et al. 2020). The results obtained are contradictory and show either beneficial, indifferent or adverse effects of feeding lignocellulose on the parameters examined. What these studies have in common is that lignocellulose was used as a diet diluent and that relatively low dietary lignocellulose inclusion levels of up to 2% were applied. In order to provoke clearer effects, studies of the present work investigate the effect of feeding relatively high levels of dietary lignocellulose of up to 10% on productive performance, digestive physiology and intestinal microbiota in laying hens and broilers. In addition, different feed formulations are tested in which the effect of lignocellulose is investigated either on the basis of energy- and nutrient-reduced diets or in isoenergetic and isonitrogenous feed variants. The thesis concludes with a review article that summarizes and evaluates previous results on the effects lignocellulose in poultry nutrition. In particular, effects of dietary lignocellulose on productive performance, nutrient digestibility, gastrointestinal tract development and intestinal microbiota in poultry are presented and compared with those observed in feeding experiments using traditional insoluble fiber sources.

Chapter I

Analytical methods investigating physiological characteristics and functions of the intestinal tract

The gastrointestinal tract is an organ with a multitude of functions that are very complex and subject to great dynamism. The gastrointestinal tract assumes digestive, absorptive, metabolic, immunological and endocrine functions. Because of its complexity, the intestinal tract is still considered as a “black box”, with many fundamental and important knowledge gaps that have yet to be filled. In order to address these gaps, the establishment and further development of analytical methods is of crucial importance. **CHAPTER I** comprises three studies that aimed to elaborate and refine laboratory methods that allow a better insight into physiological characteristics and functions of the intestinal tract. Investigations were focused on the characterization of the intestinal nutrient transport in chickens via Ussing Chamber studies, the intestinal mucus layer in pigs using histological techniques and on intestinal intraepithelial immune cells in chickens detected by immunohistochemical methods. The establishment and further development of the methods described would help to improve the understanding of the physiological processes occurring in the intestinal tract. In addition, the application of these analytical tools could serve to clarify the importance of intestinal mucosal permeability, the intestinal barrier function and the intestinal immune system for maintaining intestinal health.

PUBLICATION 1 originated from a research project intended to evaluate whether and to what extent dietary alterations modulate the intestinal nutrient transport in laying hens. Intestinal nutrient transport processes can be investigated using the Ussing chamber technique. This technique was devolved by the Danish scientists Ussing and Zerahn (1951) and allows the examination of barrier function and transepithelial transport processes along the gastrointestinal tract *ex vivo*. In principle, an intestinal tissue sample is vertically placed between two halves of a chamber, so that the mucosal membrane is facing one chamber half and the serosal membrane is targeted towards the other half-chamber (Clarke 2009). By using different buffer solutions and an automatic voltage clamp, epithelial osmotic and electrochemical gradients can be eliminated facilitating measurements of active transepithelial transport processes (Clarke 2009). In order to gain representative and reproducible results special attention should be paid with regard to used tissue preparation technique. However, precise information on how intestinal tissue samples of chickens are prepared prior to Ussing chamber studies are rarely described in the literature. Hence, a study was performed aiming to develop and describe a specific preparation method for

jejunal tissue samples of laying hens in order to carry out valid electrophysiological examinations via Ussing chamber experiments (PUBLICATION 1). For this purpose, jejunal tissue samples of ten laying hens, aged 20 weeks, were subjected to a special preparation technique. The jejunum resected was cut open longitudinally and subsequently stripped with the aid of microscopic slides to remove the Tunica serosa, the Tunica muscularis and the Lamina muscularis mucosae. The remaining Tunica mucosa was then mounted into Ussing chambers, which were additionally supported with closely meshed nets to preserve tissue integrity and to prevent tissue destruction. In order to prove tissue integrity, histological and histomorphometric analyses were performed, both on stripped samples not mounted in Ussing chambers and on those after they had been used in Ussing chamber experiments. Electrophysiological measurements were focused on the assessment of the transepithelial resistance respectively its reciprocal transepithelial conductance and on the short-circuit current in connection with the administration of various substrates, particularly glucose, phloridzin and carbachol. Differences in the short circuit current (ΔIsc) upon substrate stimulation were used as indirect measure for alterations in the active transepithelial transport processes. A further aim of this study was to evaluate the impact of glutamine application in the experimental buffers used for tissue maintenance. It was hypothesized that glutamine addition would support the vitality of intestinal cells and result in enhanced transport ability. Therefore, half of the tissue samples used were treated with a conventional Krebs–Henseleit buffer, while the other half was treated on the basis of the same buffer, but with the addition of 5 mM glutamine. Most importantly, results of this study showed that the tissue preparation and handling applied were capable to display active nutrient transport across living jejunal mucosal tissue of laying hens. Histological analyzes showed that the stripping method used was successful in separating the Tunica mucosa from the Tunica serosa, the Tunica muscularis and the Lamina muscularis mucosae without damage. Moreover, histological examinations showed that the mucosal specimens of the jejunum, after having been used in the Ussing chamber experiments, basically retained their morphological integrity, although intestinal villi were shorter and wider than those of untreated tissue samples. Electrophysiological measurements displayed that the addition of glucose to the mucosal side of the epithelium clearly increased the short circuit current. Furthermore, changes in the ΔIsc after glucose addition were negatively correlated to changes in ΔIsc after phloridzin application indicating a SGLT-1 mediated glucose transport. The addition of carbachol to the serosal side of the mucosa towards the end of the experiment again led to an increase in ΔIsc proving functionality and vitality of jejunal tissue. Tissue conductance was stable and showed no significant differences between the tissue samples examined. With respect to the different experimental buffers used, the glutamine addition had neither an impact on tissue conductance nor on ΔIsc after substrate

application. In conclusion, tissue preparation as performed in combination with the use of net-supported Ussing chambers enabled investigations into jejunal ion and nutrient transport processes in laying hens. Histological and electrophysiological examinations proved the integrity, viability and functionality of stripped tissue samples during Ussing chamber tests while the use of glutamine in the experimental buffer showed no beneficial effect.

The importance of an accurate and precise methodological approach in order to achieve reproducible and reliable results was also the starting point of **PUBLICATION 2**. This study was focused on the intestinal mucus layer, which serves as the first physical and immunological barrier of the intestinal mucosa interacting with the external environment of the gut lumen (Gendler and Spicer 1995, Cone 2009, Cai et al. 2020). Due to the unstable gel structure of the mucus layer, which consists mainly of water, the histological characterization and quantification is difficult from a methodological point of view (Johansson et al. 2011). The aim of this study was to establish and investigate different histological protocols for the preparation, description and quantification of the intestinal mucus layer in the jejunum and colon of piglets. Therefore, an animal experiment was employed using ten weaned piglets fed on a commercial complete diet that was mainly based on wheat, barley, corn and soybean meal. After two weeks of feeding, piglets were sacrificed and samples of the mid-jejunum and the ascending colon were collected. Intestinal tissue samples were either cryopreserved using liquid nitrogen or chemically fixed using a methacarn solution (60% methanol, 30% chloroform, and 10% glacial acetic acid, vol/vol/vol). Cryopreserved serial sections were then postfixed using three different postfixation methods: paraformaldehyde (PFA) vapor, neutrally buffered formalin (NBF) solution or precooled ethanol (EtOH) solution. Methacarn fixed samples were dehydrated and embedded in paraffin wax to produce serial tissue sections. In order to assess whether pH variation in the staining solution might have an impact on the qualitative and quantitative depiction of the intestinal mucus, serial sections of cryopreserved samples were stained with Alcian blue (AB)-PAS using either pH 2.5 or pH 0.5. In addition to the evaluation of the effect of fixation method and pH value of the staining reagent, two different measuring methods for assessing the thickness of the intestinal mucus layer of cryopreserved samples were compared: The “integral” method, measuring the mucus area and the length of the underlying mucosa as well as the “10 point” measuring method, assessing the mucus thickness from the luminal surface of the epithelium to the beginning of intestinal contents at 10 randomly chosen points. Moreover, histomorphological parameters, in particular the crypt depth, the crypt area, the absolute and relative number of goblet cells as well as the absolute and relative mucin staining area of methacarn fixed tissue samples were detected and correlated with the mucus layer thickness determined in cryopreserved tissue. The

results demonstrated that cryopreservation in combination with chemical postfixation preserved the mucus layer in the colon, but not entirely in the jejunum. Chemical fixation using methacarn solution was not successful in maintaining the intestinal mucus layer, neither in jejunal nor in colonic tissue samples. Thus, a microscopic determination of the mucus layer thickness was only performed in cryopreserved colonic tissue samples. The choice of postfixation on cryopreserved samples had no effect on the measurability of the mucus thickness, but influenced the visual appearance of intestinal mucus in the histological image: PFA fixation resulted in a laminar mucus appearance allowing a clear demarcation of the mucus layer while NBF and EtOH postfixation led to a slight displacement of the mucus over the epithelial borders and in the digesta. Neither varying pH values in the staining solution nor the choice of method for measuring the mucus layer thickness had an effect on the results of the mucus thickness determination. Pearson correlation analyses showed that the crypt depth and crypt area correlated with the absolute number of goblet cells and the absolute mucin staining area indicating that the density of goblet cells and the relative proportion of the mucin staining area did not differ between crypts of different sizes. In addition, the absolute number of goblet cells correlated with the absolute and relative mucin staining area, which suggests a relationship between the number of intestinal goblet cells and the amount of tissue-associated mucus produced. Interestingly, the relative mucin staining area determined in chemically fixed colon samples correlated positively with the mucus layer thickness measured in histological sections of cryopreserved colon tissue. To conclude, reliable mucus preservation enabling the measurement of the mucus layer thickness in the colon of piglets can be achieved by following the established histological protocol of this study, which includes tissue cryopreservation, chemical postfixation and AB-PAS staining. Furthermore, the relative mucin staining area which can be determined on conventional, chemically fixed tissue samples might be considered as a suitable histomorphological parameter for the assessment the intestinal mucus layer thickness.

Various antigens can pass the intestinal mucus layer and are confronted with the mucosal epithelium, which is at the center of interactions between the host and the luminal intestinal environment (Turner 2009). The single-layered mucosal epithelium is mainly composed of enterocytes but also contains various specialized cells, such as mucus-secreting goblet cells, enteroendocrine cells and intraepithelial leukocytes (IEL). The IEL present a heterogeneous group of cells consisting of T cells, natural killer cells, B cells, monocytes, macrophages, dendritic cells and heterophils (Vervelde and Jeurissen 1993, Kaspers and Schat 2012). Based on studies in humans and pigs, it is known that the topological distribution of IEL can differ along the intestinal villi (Vega-López et al. 2001, Goldstein 2004). However, no information was available with regard to the spatial distribution of IEL within the small intestine of broilers. Therefore, an experiment was conducted targeting to

establish an immunohistochemical protocol for displaying and localizing IEL in microscopic tissue sections of the small intestine of broilers (**PUBLICATION 3**). Analyses were focused on the determination of intraepithelial $CD3^+$ T cells and intraepithelial $CD45^+$ leukocytes. This experiment was part of a wider group of research activities aiming to explore whether the feeding of different plant protein sources influences the gut immune system of chickens. In a feeding trial, one-day-old male broiler chicks were allocated to six pens and fed a commercial complete diet based on corn, wheat and soybean meal. After 35 days of feeding, two animals per pen were sacrificed (n=six) and subsequently samples from the mid-jejunum taken. Tissue samples were then fixed in a 4% phosphate-buffered formaldehyde solution for 48 hours. Afterwards tissue samples were embedded in solidified paraffin wax in order to produce serial tissue sections. An immunohistochemical protocol was established using the indirect detection method including steps of antigen retrieval with citrate buffer, peroxidase blocking, antibody labeling using specific antibodies, chromogenic detection and counterstaining with hematoxylin. Regarding the primary antibodies, a rat anti-human CD3 antibody was chosen as previous experiments using flow cytometry proved cross-reaction with the chicken CD3 complex (Göbel 2000). A mouse anti-chicken CD45 was used for the detection of intraepithelial $CD45^+$ leukocytes. In order to prove the specificity of primary antibody binding different isotype control antibodies were applied. Microscopic evaluation was only performed on vertically oriented villi and crypts and intraepithelial immune cells were determined in three different regions of the villi (tip and mid region as well as crypt area) in order to display their topological distribution. The results showed that the established protocol was successful in the immunohistochemical determination of intraepithelial immune cells in the jejunum of broilers. Brown-stained $CD3^+$ and $CD45^+$ cells could be clearly depicted in the microscopic picture and isotype control antibodies confirmed the specificity of used antibodies. In addition, hematoxylin-staining of the epithelial cell nuclei helped to quantify the intraepithelial immune cells per 100 enterocytes. It could be demonstrated that the majority of IELs present in the intestinal villi of broilers were represented by intraepithelial T cells, which was confirmed by the results of flow cytometric analyzes carried out in the same experiment. Moreover, the current study displayed that the topological distribution of intraepithelial immune cells differed along the jejunal villi of broilers. Intraepithelial leukocytes were most densely localized in the mid region of the villi, while smaller numbers were observed in the villus tip. Lowest amounts of intraepithelial immune cells could be found in the crypt region. Moreover, regarding the villus tip and the villus mid region, more than 60% of intraepithelial leukocytes were T cells, expressing the CD3 receptor. Interestingly, in the crypt region, only about 40% of $CD45^+$ leukocytes represented $CD3^+$ T cells suggesting that in addition to T cells, other immune cells such as monocytes or B cells might be of greater importance in this region. In

conclusion, the established and described protocol allowed a reliable immunohistochemical determination of IEL and intraepithelial T cells in the jejunum of broilers. The application of antibodies against both, the CD3 T cell receptor and the CD45 leukocyte receptor, enabled a quantitative assessment of the jejunal intraepithelial T cell population within the overall IEL population. Moreover, the present experiment provides for the first time information on the topological distribution and characterization of intraepithelial immune cells in tissue samples of the jejunum of broilers.

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Chapter II

Qualitative and quantitative differences of dietary protein affecting the nutritional physiology of poultry

Due to its high quantity of protein and its favorable amino acid pattern, soybean meal is the dominating protein source in poultry feed. In Europe, however, there is an increased interest in becoming economically independent from soy imports from overseas and also in satisfying consumer demand for locally grown, non-genetically modified food and feed. Peas are indigenous legumes that are rich in protein, essential amino acids and starch, but peas also contain several ANFs, which, when fed to piglets and calves, are known to affect intestinal morphology and digestive function (Bush et al. 1992, Dreau et al. 1995). Whether similar effects regarding the feeding of peas can also be induced in chickens was not yet known and therefore the starting point for the first study of this chapter. Furthermore, two experiments were focused on the dietary protein requirements of broilers of a dual purpose chicken genetic. Interest in the use of dual purpose chickens has been revived in recent years as the common practice of killing male layer-type chicks is not justifiable for animal welfare reasons (Bruijnis et al. 2015, Reithmayer and Mußhoff 2019). Dual purpose breeds can be used both for meat and egg production, although their productivity is considerably lower compared to those of conventional hybrid broilers or laying hens (Damme and Ristic 2003, Rizzi and Chiericato 2010, Stehr et al. 2019). However, data on the nutritional requirements of dual purpose chickens are limited. Therefore, two studies were carried out to specifically examine the protein requirements of male dual purpose chickens and to investigate whether a reduction in dietary protein would affect growth performance, nutrient digestibility and body composition.

The starting point for investigations in **PUBLICATION 4** was the observation that the feeding of differently processed peas instead of commercial soybean meal induced an accumulation of $CD3^+$ T cells in the intestinal mucosa of broilers (see PUBLICATION 3). The occurrence of mucosal immune reactions might be accompanied with mucosal damage and increased intestinal permeability (Lallès et al. 1996, Mekbungwan and Yamauchi 2004) which could impair the intestinal nutrient absorption (Musch et al. 2002). The aim of present study was therefore to investigate whether the feeding of different protein sources (pea vs. soybean) and differently processed peas (raw, fermented or enzymatically pre-digested) affect intestinal morphology and function in broilers. In addition, data from PUBLICATION 3 and 4 were correlated to check whether there is a direct connection between the observed immunological alterations in the jejunal mucosa and the measured morphological and

functional properties of the jejunum of broilers. The feeding experiment was based on the preparation of four different diets: A control diet, containing toasted soybean meal, as well as three treatment diets containing raw peas, fermented peas and enzymatically pre-digested peas, each supplying 30% of diet's crude protein (CP). Experimental diets were fed to a total of 360-day-old broiler chicks, which were randomly allocated to 24 pens resulting in six replicates per feeding group. After 35 days of feeding, broilers were sacrificed and jejunal tissue collected in order to perform histomorphological, electrophysiological and gene expression analyses. Histological examinations were performed on formalin-fixed jejunal tissue and focused on the assessment of intestinal villus and crypt morphology. According to PUBLICATION 1, stripped jejunal mucosa samples were placed in net-supported Ussing chambers assessing transepithelial conductance and differences in the short-circuit current upon glucose, phloridzin and carbachol addition. Furthermore, jejunal expression of glucose transporter genes, tight junction protein genes and genes related to apoptosis and cell maturation were detected via quantitative real-time polymerase chain reaction (PCR). With respect to the study results, histomorphological measurements proved that the dietary protein source affected the mucosal microarchitecture in the jejunum of broilers. The villus surface area was larger in broilers fed the soybean meal-based diet compared to those fed the pea-containing feed. Ussing chamber experiments revealed that significant differences in ΔIsc upon glucose addition were detected exclusively between broilers fed the soybean meal-based diet and those receiving the enzymatically pre-digested peas. Feeding the different diets had no effect on transepithelial conductance respectively transepithelial resistance. The expression of the Na^+-dependent SGLT-1, which is located in the small intestinal brush border, was down-regulated in broilers fed diets containing raw and fermented pea. The jejunal expression of tight junction and cell maturation genes was not influenced in broilers fed the different diets. Combining and correlating data of PUBLICATION 3 and 4 on immunological, morphological and functional parameters of the jejunum in broilers showed remarkable coherences. Correlation analyzes showed that the development of the villus morphology in the jejunum of broilers was related to the appearance of jejunal intraepithelial immune cells. Hence, the jejunal villus length was negatively correlated to the number of jejunal, intraepithelial $CD3^+$ T cells. Moreover, intestinal morphology was related to intestinal, transepithelial absorption processes. Correlation analyses revealed that the jejunal villus surface area was positively correlated to the jejunal glucose transport measured during the Ussing chamber experiments. Interestingly, the occurrence of immune cells in the jejunal mucosa of broilers seem to be also associated with the functional jejunal glucose transport. Results showed that there was a negative correlation between the mucosal number of $CD45^+$ leukocytes and the jejunal electrogenic glucose transport. In conclusion, the results of this study showed that both

factors, the dietary protein sources and the application of feed processing, can have an impact on the mucosal development and nutrient transport in the jejunum of broilers. Moreover, it could be demonstrated that alterations in the occurrence of intestinal intraepithelial immune cells are related to changes in the intestinal morphology and functional glucose transport of broilers.

PUBLICATION 5 was based on the hypothesis that slow-growing male dual purpose chickens have a lower requirement for dietary protein compared to conventional high-performing hybrid broilers. Therefore, a feeding experiment was performed in order to evaluate whether the feeding of diets with a protein content below the recommendation for conventional hybrid broilers affects growth performance, nutrient digestibility and whole body composition of male dual purpose chickens. In total, 480-day-old Lohmann Dual chicks (Lohmann Tierzucht GmbH, Cuxhaven) were randomly allocated to 24 pens receiving *ad libitum* access to feed and water. Based on a phase feeding concept, broilers were offered a starter (1 to 14 days of age), a grower (15 to 30 days of age) and a finisher diet (31 to 77 days of age). In each phase, three different experimental diets were fed, which were largely isocaloric, but differed in their dietary protein concentration. The control diet was mainly based on corn, soybean and wheat meal and was formulated to meet the nutritional recommendations for conventional hybrid broilers. Control feed contained 23% CP in the starter diet, 22% CP in the grower diet and 20% CP in the finisher diet. In the two other feed variants, the dietary CP content was reduced by either 5% or 10% by partially replacing the protein source soybean meal with corn meal. Diets were balanced with the specific amino acids L-lysine, DL-methionine and threonine in order to prevent amino acid related deficiencies. Feeding three different diets to broilers located in 24 pens resulted in eight replicates per feeding group. During the whole trial, growth performance parameters such as feed intake, body weight gain and feed conversion ratio (FCR) were assessed. On day 77, broilers were sacrificed and digestive samples of the ileum and colon were collected in order to assess the apparent ileal and the total digestibility of nutrients. In addition, one animal per pen was used in order to analyze the chemical composition of the whole body. In general, growth performance of dual purpose chickens was not affected by feeding protein-reduced diets. Broilers had an average final body weight of 2052 to 2232 g and an FCR between 2.96 and 3.19. Interestingly, a dichotomy of the broiler population in high-performing and low-performing birds was detected. The total body weight gain in the high-performance group was around 2500 g while broilers of the low-performance group only showed a mean weight gain of about 1400 g. The apparent ileal and total digestibility of nutrients was increased in slow-growing broilers fed the reduced dietary protein concentrations. Broilers fed the protein-reduced diets had a higher ileal and total digestibility of protein, calcium and phosphorus than those receiving the control diet. The lower

digestibility in control-fed chickens might indicate an inefficient utilization of dietary nutrients. Differences in the nutrient digestibility's did not affect the chemical body composition of dual purpose chickens showing a similar body content of CP, ether extract and crude ash. In conclusion, the results of this study revealed that male dual purpose chickens seem to have a lower requirement for dietary protein than conventional high-performing hybrid broilers. Neither a 5% nor a 10% decrease in dietary protein had a negative impact on growth performance or carcass composition.

Hence, the question arose whether the protein content in the feed can be reduced even further without having negative effects on productivity or body composition of male dual purpose chickens. A reduction in dietary protein would have the advantage that feed costs could be lowered and at the same time the nitrogen input into the environment could be decreased. Beside the protein content of feed, dietary phosphorus is another cost factor with environmental impact in poultry production. Therefore, a follow-up study was conducted to examine the effect of both factors, reducing dietary protein concentrations and lowering dietary phosphorus levels in dual purpose chickens (**PUBLICATION 6**). For this purpose, day-old male Lohmann Dual chicks (Lohmann Tierzucht GmbH, Cuxhaven) were allocated to 24 pens and fed four different experimental diets resulting in six replicated pens. The experimental setup was a 2 x 2 factorial design with two concentrations of dietary CP and two different calcium-phosphorus ratios. The protein content of the first treatment diet was 10% lower compared to that of the control feed of this study and 15% lower than that of conventional broiler feed used in PUBLICATION 5. A third set of diet contained the same amount of protein as the control feed, but showed a reduced calcium-phosphorus ratio. The fourth experimental diet contained a reduced protein concentration as well as lower calcium and phosphorus concentrations. Experimental diets were composed to be isoenergetic and a three-phase feeding concept was applied. During the 63-day feeding trial, growth performance parameters of body weight gain, feed intake and FCR were determined. According to PUBLICATION 5, analyses of apparent ileal and total digestibility of nutrients and measurements of whole body composition were performed. Regarding the entire fattening period, broiler performance was not influenced by either the reduction of dietary protein or the reduction in dietary calcium and phosphorus concentrations. This seems to indicate that both the maintenance and performance requirements of dual purpose chickens were adequately met in terms of dietary protein, amino acid, calcium and phosphorus concentration. However, analyses of the whole body composition showed that the factor dietary protein content affected the protein and fat composition of dual purpose chickens. Whole bodies of chickens fed lower dietary protein concentrations contained lower amounts of protein and higher amounts of fat, while the different calcium and phosphor concentrations in the diet had no impact on body composition. The nutrient digestibility in

chickens was not influenced by the dietary protein content, but there were differences due to the choice of the calcium-phosphorus ratio in the feed. The apparent ileal digestibility of crude ash, phosphorus and protein was negatively affected by reducing calcium and phosphorus concentrations in the diets. However, as these differences were not reflected in the total digestibility of nutrients, it can be assumed that the overall nutrient supply of the chickens remained unaffected by feeding the different diets. In conclusion, this study showed that low-performing dual purpose chickens seem to have a lower nutritional requirement compared to high-performing hybrid broilers as the reduction of dietary protein, calcium and phosphorus concentrations did not impair their growth performance.

Chapter III

Impact of dietary insoluble fiber on the nutritional physiology of poultry

In recent years there has been an increased interest in the use of dietary fiber in poultry nutrition. Particular attention was paid to various insoluble fiber sources, which mainly accrue as by-products during industrial processing of food, but also feed, such as oat hulls, soybean hulls or wheat bran. Insoluble fibers exhibit various physicochemical properties that could have an impact on avian digestive physiology may affecting chicken's health and productivity (Hetland et al. 2004, Mateos et al. 2012). In addition, fibers are not degraded by intestinal endogenous enzymes and could therefore potentially serve as nutrient substrates for intestinal microorganisms. As a result, fiber feeding could have an impact on intestinal microbial composition and the production of bacterial metabolites, which in turn could affect avian intestinal health (Walugembe et al. 2015, Mahmood and Guo 2020). This chapter includes a series of four publications, which evaluate the effect of feeding insoluble fibers on the nutritional physiology of poultry, with a special focus on the fiber source lignocellulose. The first two studies investigated the effect of feeding a nutrient-reduced diet containing 10% lignocellulose on the productive performance, digestive physiology and intestinal microbiota of dual purpose laying hens. In contrast, the third experiment was based on isoenergetic and isonitrogenous feed variants and examined the effects of different dietary lignocellulose concentrations on the growth performance, digestive physiology and intestinal microbiota of slow-growing broilers. The chapter concludes with a review that summarizes and evaluates studies on the effect of dietary lignocellulose in poultry and compares these studies with those that have used other insoluble fiber sources.

Data and information on the nutritional requirements including feeding recommendations for chickens of dual purpose genotypes are scarce. Studies showed that dual purpose hens, fed on commercial layer diets, developed higher body weights and a higher body fat content than commercial hybrid hens (Rizzi and Chiericato 2010, Mueller et al. 2018), which may indicate a dietary nutrient oversupply of fed dual purpose chickens. Therefore, **PUBLICATION 7** aimed to examine the effects of feeding an energy- and nutrient-reduced diet containing 10% lignocellulose on performance, body composition as well as external and internal egg quality parameters of dual purpose hens. As there is a negative correlation between body weight and reproductive efficiency (Reddy and Siegel 1977, Robinson et al. 1993), it was hypothesized that the feeding of lignocellulose containing energy- and nutrient-reduced diets results in a lower body weight and body fat content of dual purpose hens, which would be accompanied with a higher laying performance. Moreover, as studies

have shown that dietary lignocellulose could also affect avian digestive physiology and the gut microbiota (Yokhana et al. 2015, Makivić et al. 2019), it should be investigated whether the feeding of lignocellulose would influence the gastrointestinal tract traits, intestinal microbiota and excreta quality of dual purpose chickens (**PUBLICATION 8**). It was hypothesized that dietary fiber inclusion would have a clear impact on digestive tract characteristics, the bacterial composition and activity in the hindgut, and the excreta properties of dual purpose laying hens. Thus, to evaluate the working hypotheses, a feeding experiment was conducted using day-old female chicks of a dual purpose genetic (Lohmann Dual, Lohmann Tierzucht GmbH, Cuxhaven), which were randomly assigned to twelve pens. Chickens were offered two different experimental diets in mash form resulting in six replicates per feeding group: a basal control diet and a treatment diet, based on control feed, but diluted with 10% lignocellulose (ARBOCEL® R, J. Rettenmaier & Söhne GmbH + Co KG, Rosenberg, Germany). Based on a phase-feeding concept, chickens received diets for a period of 52 weeks in order to assess potential effects within the growing (1-22 weeks of age) and the laying period (23-52 weeks of age). During the growing period, weekly body weight, feed intake and FCR was recorded and, in addition, weekly egg weight, egg mass and egg production were determined during the laying period. At three, six, 17 and 52 weeks of age, respectively one animal per pen, with a representative average body weight, was sacrificed in order to analyses the chemical body composition. External and internal egg quality parameters, such as shell thickness, shell breaking strength or yolk and albumen mass, were determined at 27, 33, 42 and 52 weeks of age. Analyses of excreta dry matter and viscosity were performed at ten, 17, 22 and 52 weeks of age. At the end of the experiment, remaining hens were slaughtered and gastrointestinal organs removed to determine the relative organ weights. In addition, tissue samples of the colorectum were fixed in formalin, dehydrated and then infiltrated with solidified paraffin wax followed by AB/PAS staining of prepared tissue sections to perform histomorphological analyses. Moreover, digesta samples of the cecum were analyzed for bacterial metabolites and composition using gas chromatography, high performance liquid chromatography (HPLC), photometry, and quantitative real-time PCR. Results presented in PUBLICATION 7 clearly revealed that chicken's performance was affected by feeding energy- and nutrient-reduced diets containing 10% lignocellulose. Lignocellulose-fed hens developed lower body weights compared to those fed the control diet, with poor flock uniformity in terms of individual body weight in both feeding groups. Interestingly, chickens fed the different diets showed a comparable feed intake in the growing phase, while lignocellulose-fed hens compared to control-fed hens had a superior feed consumption during the laying period. With respect to the laying performance, hens fed with lignocellulose produced lighter eggs, but had a higher egg production and egg mass resulting in an improved feed efficiency than hens offered the

control feed. Performance data indicate, that lignocellulose-fed hens had a lower dietary energy-and nutrient intake than control-fed chickens and consequently developed lower body weights during the growing phase. The decrease in body weight of lignocellulose-fed chickens was positively associated with their laying performance. Difference in productive performance were related to the chemical body composition of hens. With respect to the growing period, no differences in the body composition between pullets of both feeding groups could be detected. However, analyses showed that laying hens fed with lignocellulose had a higher body protein, but a lower body fat content compared to hens fed the control diet at 52 weeks of age. Moreover, correlation analyses indicated that the body fat content of hens was negatively correlated to the hen's egg production. Egg quality analyses confirmed that lignocellulose-fed hens produced eggs showing a lower egg size and egg surface area resulting in lower egg weights compared to hens fed the control feed. Differences in egg size and egg weight were also reflected in the results of the internal egg quality analyzes. The reduced egg weight of hens fed with lignocellulose could be attributed to the lighter egg yolk and egg albumen, although the egg yolk and albumen ratio was not affected by feeding. In conclusion, the results of PUBLICATION 7 confirmed the assumption that dual purpose laying hens have different nutritional requirements compared to conventional hybrid hens. Results revealed that *ad libitum* feeding of energy- and nutrient-reduced diets containing 10% lignocellulose reduced body fat content of dual purpose hens, which was directly associated with an improved laying performance.

The aim of PUBLICATION 8 was to investigate whether differences in dual purpose hen productivity and body composition observed in PUBLICATION 7 were also accompanied with effects on the gastrointestinal tract development, intestinal histomorphology, intestinal microbiota and excreta characteristics. Results showed that the relative weight of gastrointestinal organs was affected by feeding the different diets. Lignocellulose-fed hens had, in relation to the body weight, increased weights of the gizzard, small and large intestine compared to control-fed chickens, while relative proventriculus and liver weights were not affected by feeding. Histomorphological analyses showed that the colorectal villus area and the mucosal enlargement factor of villi and crypts, as a suitable indicator for the assessment of the epithelial surface area (Wiese et al. 2003), was increased in hens fed lignocellulose compared to those receiving the conventional layer diet. To examine whether fiber feeding affected intestinal mucus production, the number of intestinal goblet cells and the intestinal mucin staining area were determined. However, both parameters were not affected by feeding the different diets. Regarding the gut microbiota, analyses of bacterial metabolites in the cecum of hens showed that the luminal concentration of ammonia, acetic acid, propionic acid and the total amount of the short chain fatty acids (SCFA) were higher in hens offered the control diet compared to those receiving the lignocellulose containing

feed. Results also showed that generally neither the counts nor the activity of detected bacterial populations differed between hens of both feeding groups. Interestingly, correlation analyses demonstrated that the concentration of SCFA in the cecum of hens was negatively related to the colorectal mucosal enlargement factor of the villi. This observation could indicate an effect of feeding on the intestinal absorption of bacterial metabolites if similar levels of bacterial SCFA were produced in the gut of hens in both feeding groups. Analyses of excreta showed that the feeding of lignocellulose generally increased the excreta dry matter while no effects of feeding were observed regarding the excreta viscosity. To conclude, this study showed that feeding of nutrient-reduced diets containing 10% lignocellulose had an impact on the growth performance of dual purpose hens, which was associated with the appearance of greater relative weights of the gastrointestinal organs and the enlargement of the mucosal epithelium. Moreover, feeding the different diets affected the concentration of bacterial metabolites in the hindgut of dual purpose chickens, which might be related to changes in the absorption of microbial SCFA via the intestinal epithelium. In addition, the feeding of lignocellulose lowered the moisture content of excreta and hence improved the excreta quality of hens.

The influence of dietary lignocellulose on chicken productivity and digestive physiology was also examined in **PUBLICATION 9**. In contrast to PUBLICATION 7 and 8, feed variants were balanced to be isocaloric and isonitrogenous and different dietary lignocellulose concentrations were used. The aim of this study was to investigate the impact of feeding increasing concentrations of dietary lignocellulose on performance, nutrient digestibility, excreta dry matter and intestinal microbiota in slow-growing broilers. As diets contained similar levels of energy and protein, it was assumed that despite increasing concentrations of dietary lignocellulose, the animal performance and nutrient digestibility would not be impaired. Furthermore, it was hypothesized that the dry matter of excreta could be increased with increasing concentrations of dietary lignocellulose. Moreover, it was expected that the inclusion of dietary lignocellulose would affect cecal microbial composition and the bacterial production of cecal metabolites. In a feeding experiment 60 slow-growing broilers of an intercross line (New Hampshire x White Leghorn) were allocated to individual cages at the age of 10 weeks. Over a period of 23 days, broilers received *ad libitum* access to three different experimental diets in mash form resulting in 20 replicates per feeding group. The experimental diets were mainly based on wheat, soybean meal, and corn and contained either 0.8%, 5% or 10% of a lignocellulose product (ARBOCEL R, J. Rettenmaier & Söhne GmbH + Co. KG, Rosenberg, Germany). During the experimental trial, body weight, feed intake and FCR of broilers were determined weekly. On the last three days of the experiment, excreta samples were collected in order to detect the apparent excreta digestibility of ether extract, organic matter, gross energy, as well as the excreta dry matter

content. At the end of the trial, broilers were sacrificed and digesta samples were collected from the ileum and cecum. Ileal digesta samples were analyzed for the apparent ileal digestibility of CP. Cecal digesta samples were analyzed for bacterial metabolites and bacterial cell counts using gas chromatography, HPLC, photometry, and quantitative real-time PCR. With respect to animal performance, feeding increasing concentrations of dietary lignocellulose did not affect body weight gain, feed intake or the FCR of slow-growing broilers. However, apparent ileal digestibility of CP was impaired by feeding 10% dietary lignocellulose. Furthermore, increasing concentrations of dietary lignocellulose lowered the apparent excreta digestibility of organic matter and gross energy in broilers. Thus, results of digestibility analyses suggest that dietary lignocellulose, on the one hand, restricted protein digestibility and, on the other hand, was largely excreted undigested by the chickens. Excreta quality seemed to be not affected by lignocellulose feeding, as moisture content of excreta samples were comparable among the different feeding groups. As expected, the feeding of dietary lignocellulose had an impact on the microbial composition and on bacterial fermentation patterns. Among the measured bacterial populations, a significant difference in cecal bacterial counts was detected for the *Escherichia/Hafnia/Shigella* group. Moreover, the feeding of 10% dietary lignocellulose decreased the concentration of SCFA, in particular that of propionic acid, butyric acid and i-valeric acid, as well as the concentration of lactate and ammonia in the cecum of broilers. Correlation analyses revealed that dietary lignocellulose was negatively related to the total concentration of cecal bacterial metabolites. In conclusion, the feeding isocaloric, isonitrogenous diets containing increasing concentrations of lignocellulose did not affect growth performance, but impaired nutrient digestibility of slow-growing broilers. While minor changes in cecal microbial composition were observed, cecal bacterial metabolite concentrations were significantly reduced with increasing levels of dietary lignocellulose.

PUBLICATION 10 gives a comprehensive overview on lignocellulose as a dietary component in poultry nutrition. The first part of the current review refers to the physicochemical properties of lignocellulose. In addition, reference is made to methodological aspects of the incorporation of lignocellulose in experimental diets, as this can have a decisive influence on the study results. The main focus of the current review is on the comparative presentation of study results related to the effects of lignocellulose feeding on growth performance, nutrient digestibility, the development of the gastrointestinal tract and the intestinal microbiota in poultry. Moreover, these results are compared with those observed in feeding experiments using other insoluble fiber sources. In particular, the potential mode of action of insoluble dietary fiber on the digestive physiology of chickens is discussed. Starting with the chemical composition, lignocellulose consists mainly of the carbohydrate polymers cellulose and hemicelluloses as well as the phenolic polymer lignin

(Collard and Blin 2014, Liu et al. 2014). Lignocellulosic products used as additives in animal feed are usually derived from forest residues, and their proportional composition of carbohydrates and aromatic polymers can vary depending on the type and quality of wood being processed. As these differences in the composition could result in potential effects on chickens after feeding, the products used in the cited studies of this review are presented in a supplementary table. Regardless of the product, lignocellulose has a significantly higher proportion of insoluble NSP (over 90% in the dry matter) compared to other insoluble fiber sources that are used in animal nutrition, such as wheat bran, oat hulls or sunflower hulls (Slama et al. 2019). In addition, lignocellulose differs from insoluble fiber sources mentioned in terms of its physicochemical properties such as swelling capacity, water holding- and binding capacity (Jiménez-Moreno et al. 2013, Slama et al. 2019). Another characteristic of dietary fibers concerns the particle size and the physical shape of the fiber particles, properties which after feeding can have a crucial influence on digestive physiological processes in poultry. To investigate the effect of dietary fiber in chickens, several feeding experiments were carried out using different feed formulations. As shown in figure one of the current review, there are basically three different options to include insoluble fiber sources in experimental diets: First, diets can be “diluted” with the fiber source of interest; second, dietary fiber sources are included in isoenergetic and isonitrogenous diets; third, an insoluble ash source is substituted by the fiber source of interest. Hence, the dietary inclusion of an insoluble fiber source is coupled with differences in the feed- and nutrient composition of the experimental diets. During the past decade, several studies examined the effect of feeding lignocellulose on chicken’s performance using different feed formulations and lignocellulose inclusion levels. Regarding the effect of dietary lignocellulose on chicken’s productivity, this review includes results from ten studies in broilers and six studies in laying hens. Results obtained in trials using relatively low dietary concentrations of 0.25 to 2% lignocellulose are contradictory. Regarding the feeding of lignocellulose to broilers and laying hens, most studies showed no effects on growth or laying performance (Bogusławska-Tryk et al. 2015, Miloševic et al. 2015, Yokhana et al. 2015, Kheravii et al. 2017, Zeitz et al. 2019, Sun et al. 2020), few studies reported positive effects on the broiler weight gain (Sarikhan et al. 2010, Rahmatnejad and Saki 2016), and one study demonstrated a negative effect on the FCR of broilers (Abdollahi et al. 2019). In three further studies, an increase in the dietary concentration of lignocellulose (Sozcu 2019, Sozcu and Ipek 2020) or a change in the feed composition using the same lignocellulose inclusion level (Makivić et al. 2019) led to contradicting results. In studies using relatively high dietary fiber inclusion levels of up to 15%, broiler productivity was negatively affected when the feed was diluted with lignocellulose (Oikeh et al. 2019), whereas no adverse effects were observed when lignocellulose was included in isoenergetic and isonitrogenous

diets (see PUBLICATION 9). In studies with broiler breeder and dual purpose hens, results showed that the feeding of nutrient-reduced, lignocellulose-enriched diets reduced the body fat accumulation of hens, which was directly associated with an improved laying (Mohiti-Asli et al. 2012, see PUBLICATION 7). Chicken's productivity is largely related to how efficiently the feed is digested, so that dietary nutrients can be absorbed through the intestine and thus used by the organism for maintenance and performance. However, studies on the impact of dietary lignocellulose on the nutrient digestibility in chickens are scarce. On the basis of the literature research carried out, only six studies could be identified which had performed digestibility measurements. The results of these feeding experiments, in which different feed formulations and lignocellulose inclusion levels were used, did not show a clear picture, so that a final assessment regarding the influence of dietary lignocellulose on nutrient digestibility in chickens is not yet possible. Regarding the development of gastrointestinal organs in chickens, most studies showed that feeding lignocellulose did not affect the relative weight of the gizzard as well as that of the small and large intestine (Kheravii et al. 2017, Makivić et al. 2019, Zeitz et al. 2019, Sun et al. 2020). However, the feeding of lignocellulose resulted in alterations of the intestinal mucosa, which were observed in the small intestine (Sarikhan et al. 2010, Makivić et al. 2019, Sozcu 2019, Bogusławska-Tryk et al. 2020, Sozcu and Ipek 2020). Some of these studies also proved a dose-response effect of dietary lignocellulose on the development of the intestinal epithelium (Makivić et al. 2019, Bogusławska-Tryk et al. 2020, Sozcu and Ipek 2020). Few studies investigated the impact of dietary lignocellulose on the microbial composition and bacterial metabolites in the intestine of chickens. In general, results are conflicting, which may be explained by differences in the used experimental design, in particular regarding the used feed formulation and lignocellulose inclusion level. Some studies have shown that the use of lower concentrations of dietary lignocellulose reduced cecal counts of *Escherichia* spp. and *Clostridium* spp. (Bogusławska-Tryk et al. 2015, Makivić et al. 2019), while an increase in the intestinal number of *Lactobacillus spp* and *Bifidobacterium* spp. was observed (Bogusławska-Tryk et al. 2015, Makivić et al. 2019). Alterations in the cecal microbial composition, however, were not accompanied by changes in the concentration of cecal bacterial metabolites (Bogusławska-Tryk et al. 2015). In another study, dietary lignocellulose increased cecal microbial diversity in male free-range chickens (Hou et al. 2020), while, on the contrary, no alterations in the overall cecal microbial diversity were found in broilers and laying hens (Zeitz et al. 2019, Sun et al. 2020). In accordance, the feeding of 1 and 2% dietary lignocellulose increased the concentration of cecal SCFA in broilers (Sun et al. 2020), while no changes in cecal bacterial metabolites were observed in a broiler study using similar dietary lignocellulose inclusion levels (Zeitz et al. 2019). In two further studies the feeding of relatively high concentrations of dietary lignocellulose had

generally no impact on cecal microbial composition in dual purpose hens, but reduced the cecal concentration of microbial metabolites (see PUBLICATION 8 and 9). In summary, based on the studies performed so far, the feeding of lignocellulose appears to have neglectable effects on the productivity and gross morphology of gastrointestinal organs in poultry. However, dietary lignocellulose seemed to affect intestinal mucosal development and gut microbiota in chickens, although it cannot be ruled out that these effects were related to differences in study design used rather than to the fiber inclusion.

General discussion

Feeding is one of the major factors that have an impact on the functionality and health of the avian gastrointestinal tract. In order to be able to assess the effects of feeding on digestive function and gastrointestinal health, analytical tools are required that can make potential effects measurable and thus visible. The following section begins with a detailed discussion of the results described in Chapter I on the establishment and further development of analytical methods that enables examinations on physiological characteristics and functions of the intestinal tract. Then the results presented in Chapter II on the nutritional importance of protein in poultry nutrition are addressed and discussed in a broader, overarching context. Finally, the importance of dietary fiber in poultry nutrition is examined and the results presented in Chapter III on the influence of dietary lignocellulose on the nutritional physiology of chickens are discussed in depth. During the entire discussion, open research questions are identified and future research perspectives addressed.

Establishment and refinement of analytical methods that allow an assessment of physiological characteristics and functions of the intestinal tract

In general, the implementation of Ussing Chamber experiments is a technically challenging process (Barthe et al. 1998). The type of preparation of intestinal tissue prior to Ussing chamber studies has a decisive impact on the reliability of gained results (Polentarutti et al. 1999). Samples should be carefully prepared and handled in order to reduce tissue stress and damage, which could affect tissue viability and integrity (Polentarutti et al. 1999, Bajka et al. 2003, Neirinckx et al. 2011). Ussing Chamber experiments of PUBLICATION 1 confirmed that the tissue preparation technique used was suitable for carrying out transport physiological studies on the jejunum of laying hens. The stripping process did not negatively affect tissue integrity as demonstrated by electrophysiological measurements and histological examinations. The tissue conductance assessed during Ussing chamber experiments is a useful indicator for proving intestinal integrity (Clarke 2009, Metzler-Zebeli et al. 2017). The results showed that tissue conductance of stripped jejunal samples delivered stable values and were in accordance with those reported before (Amat et al. 1999, Rehman et al. 2006). Histological analyses of jejunal samples displayed that the Tunica mucosa could be entirely preserved before and after stripped tissue samples had been used for the Ussing Chamber studies. In this regard, it can be suggested that the use of the net supported Ussing chamber helped to maintain the intestinal tissue integrity during the experiments. From a histological point of view, the organization and structure of the intestinal wall is very similar between various animal species (Balimane et al. 2000,

Pelkonen et al. 2001), but there are great differences in the thickness and strength of the individual intestinal layers. Thus, depending on the species of interest, different tissue preparation techniques need to be applied before tissue samples are suitable for examinations in Ussing chambers. With respect to mice and rats, studies demonstrated that the native intestinal tissue can be used for Ussing Chamber tests without additional preparation (Nejdfors et al. 2000, Sjögren et al. 2016). Traditionally, however, the removal of the Tunica serosa and Tunica muscularis is the preferred preparation technique as it diminishes the effect of the intrinsic neuromuscular system (Clarke 2009) and may facilitate the diffusion of nutrients and oxygen into the tissue (Sjögren et al. 2016). Thus, seromuscular stripping has been successfully used for Ussing chamber experiments on the intestines of humans, pigs or horses (Neirinckx et al. 2011, Sjöberg et al. 2013, Pieper et al. 2016). The preparation of the intestinal wall of Galliformes is a particular challenge due to the poorly developed Lamina submucosa (Bacha and Bacha 2000). Consequently, studies failed to perform Ussing Chamber experiments with intestinal tissue of chickens and turkeys as tissue preparation resulted in fragile and perforated mucosal samples (Neirinckx et al. 2011). The maintenance of the intestinal integrity after tissue preparation and during the Ussing chamber experiments was a prerequisite for investigations on active transepithelial transport processes. All stripped jejunal samples responded to the mucosal glucose and phloridzin application as indicated by changes in the ΔIsc. This proved the involvement of the SGLT-1 transporter in the glucose absorption process across the brush border membrane. A functional response could also be observed when testing the substrate carbachol at the end of the experiments. The serosal addition of the cholinergic agent carbachol significantly induced an increase in the short-circuit current. This response indicated a carrier-mediated chloride secretion (Dharmsathaphorn and Pandol 1986) and produced evidence that the epithelial cells showed vitality and functionality even towards the end of the experiment. This agreed with the results of the histological evaluation showing no evidence of mucosal damage from stripping or from handling during the Ussing chamber testing. However, the morphological appearance of the intestinal villi of jejunal samples changed after they had been used in the experiments showing villi shortening. The reason for this observation remains unknown. It can be speculated that the use of different substrates, in particular the application of carbachol, may have altered villus appearance, as this substrate is known to induce intestinal muscle contractions when applied to the basolateral side of the mucosa (Li et al. 1991). Furthermore, it is conceivable, that the histological preparation itself affected tissue morphology, as particularly the fixative solution can contribute to tissue shrinkage (McInnes 2005). Whether this observation was attributed to the handling, the addition of the different substrates during the Ussing Chamber experiments or the histological preparation method needs further clarification. With regard

to tissue vitality, it must be emphasized that the samples of the current study were examined in the chambers over a short period of only 30-45 minutes. In studies with pigs, the duration of Ussing chamber experiments is significantly longer, so that intestinal tissue can be examined over a period of more than 180 minutes (Kern et al. 2017, Romanet et al. 2020). The buffer supplementation of glutamine, representing a principal energy source for enterocytes, showed no advantages or disadvantages with regard to the measurements taken. The effect of glutamine was tested as the application of this amino acid might improve intestinal energy metabolism and viability of the intestinal mucosa in *in vitro* studies (Yang et al. 2000). Future studies should therefore investigate whether, after using the tissue preparation technique described, investigations into the intestinal nutrient transport can also be carried out in Ussing chambers over a longer period of time and how a "longevity" of the tissue could be achieved. Previous studies that have successfully implemented the Ussing chamber technique for investigating nutrient transport processes across the chicken's intestine lack detailed information on how intestinal samples were prepared prior to the experiments. A few studies used non- stripped intestinal tissue samples (Awad et al. 2007), while most studies stripped intestinal tissue specimens prior to Ussing chamber studies (Amat et al. 1999, Awad et al. 2005, Röhe et al. 2014, Metzler-Zebeli et al. 2017, Tang et al. 2019). Another difficulty in describing and comparing tissue preparation methods applied is that the definition of the technical term "stripping" is interpreted differently in the literature. While most authors understand stripping as the removal of the Tunica serosa together with the Lamina muscularis mucosa (Clarke 2009, Metzler-Zebeli et al. 2017, Tang et al. 2019, Baskara et al. 2021), some authors use this term as a synonym for the removal of the Tunica serosa alone (Awad et al. 2008, Yunus et al. 2010). Differences in the used preparation technique might also results in differences regarding the electrophysiological responses detected during Ussing chamber studies. Any removal of individual layers of the intestine is accompanied with a reduction in the thickness of the gut wall, which could result in changes of the transepithelial resistance that may affect intestinal permeability (Stevens 1964, Neirinckx et al. 2011). This could explain the large differences in the reported data on transepithelial conductivity respectively transepithelial resistance of intestinal tissue samples obtained from laying hens of the same breed and of similar age. Tissue conductance of samples from the proximal jejunum was twice as high in the study of Rehman et al. (2006) compared to those assessed in the study of Metzler-Zebeli et al. (2017). In line with this, measured ΔIsc responses due to mucosal addition of 5 mmol/l glucose widely differed between both studies: ΔIsc values after glucose application ranged between 0.1 µA/cm^2 (Metzler-Zebeli et al. 2017) to 61 µA/cm^2 (Rehman et al. 2006). To conclude, based on the experience of the current study in combination with published literature data, it should be noted that stripping off the Tunica serosa, the Lamina muscularis

mucosae and the Lamina muscularis mucosae seems to be the most suitable preparation technique for successfully carrying out Ussing chamber experiments on the intestinal epithelium of laying hens. Whether this specific preparation technique is also successful for tissue from other intestinal sections such as the large intestine needs further clarification. Regardless of the preparation technique chosen, care should be taken to ensure that the individual steps of tissue handling and preparation prior the Ussing chamber tests are precisely described in future publications. As a result, better comparability and improved reproducibility of results obtained could be achieved.

The intestinal epithelium is usually covered by a dense, adherent mucus layer shielding the mucosa and the underlying immune system against potentially harmful, external influences (Johansson et al. 2008, van der Post et al. 2019). Disturbances in mucus production and mucus layer formation are associated with various diseases such as ulcerative colitis and Crohn’s disease (Braun et al. 2009, Hong et al. 2017, van der Post et al. 2019) highlighting the importance of the intestinal mucus in maintaining gut health. Based on this knowledge, there is an increased interest in developing methods that enable a qualitative and quantitative assessment of the intestinal mucus layer in humans and animals. In addition to complex *in vivo* examinations using intravital microscopy (Holm et al. 1990, Atuma et al. 2001) or the cultivation of mucosal biopsies to study mucus synthesis and secretion *in vitro* (MacDermott et al. 1974, Finnie et al. 1996), histological techniques in particular are of great importance with regard to intestinal mucus depiction. However, most histological methods are unsuitable for visualizing the intestinal mucus, since the fragile mucus layer is lost through the common processes of tissue fixation and preparation (Strugala et al. 2003). By comparing different histological protocols, it was possible to explore a certain method that allowed an accurate representation of the mucus layer in the colon of piglets (PUBLICATION 2). Cryopreservation followed by various postfixations led to reliable mucus conservation in the colon of the piglets. Using this specific protocol, the mucus layer of the colon could be preserved in its original state, which means that the layer was firmly attached to the underlaying intestinal epithelium and covered by intestinal colonic content. In addition to issues regarding the mucus shrinkage after histological tissue processing (Ermund et al. 2013), methodological problems were often referred to the detachment of the mucus layer from the epithelium impeding an accurate measurement of the mucus layer thickness (Johansson et al. 2008, Callies 2012, Wlodarska et al. 2015). In order to maintain the mucus layer in its physiological position, it seems of great importance that intestinal sections are filled with digesta prior to fixation. Thus, rinsing the intestinal tube before fixation seems to be contraindicated, as this could lead to a certain loss of mucus and tissue stability due to the lack of a physical demarcation provided by the intestinal content (Johansson et al. 2011). This assumption would also explain why it was only partially possible to display the

jejunal mucus layer in the current study, as digesta was largely missing in the small intestinal lumen. Moreover, it is conceivable that jejunal mucus preservation using cryopreservation and subsequent postfixation was ineffective as the biochemical composition of jejunal and colonic mucus may differ (Holmén Larsson et al. 2013, Faderl et al. 2015). In this regard, further studies are needed in order to evaluate mucin chemotypes of different intestinal sections and their impact on the intestinal mucus layer formation in pigs. In line with results of studies in mice (Johansson et al. 2008, Wlodarska et al. 2015), the current study confirmed that chemical methacarn fixation was unsuitable for intestinal mucus preservation in piglets as only residues of intestinal mucus were observed in the jejunum while the colonal mucus layer was detached from the epithelium. A shrinkage or removal of the adherent mucus layer might be attributed to the physical impact of liquid fixatives (Johansson et al. 2008) or related to the dehydration process during tissue processing (Strugala et al. 2003, Ermund et al. 2013). However, it must be emphasized that the chemical fixation of tissues, for example by means of formalin, is inexpensive, practicable and therefore used very frequently worldwide. Hence, the question arose whether histomorphological parameters, which are determined in conventional, chemically fixed tissue samples, may correlate with the mucus layer thickness assessed in cryopreserved tissue samples. In order to verify this hypothesis, various histomorphological parameters, such as the goblet cell number or the mucin staining area, were determined in methacarn fixed colon specimens. The subsequent correlation analyzes revealed two relevant coherences: First, the number of goblet cells were not related to the measured mucus layer thickness indicating that mucus layer formation is less dependent on the density of goblet cells per crypt than on the secretory capacity of each goblet cell; second, the relative mucin staining area was positively related to the thickness of the mucus layer emphasizing that the higher the proportion of mucins per crypt area the thicker the colonal mucus layer. These results suggest that the secretory activity level of goblet cells is decisive for the amount of produced mucins in the crypt and thus for the thickness of the mucus layer. However, results of PUBLICATION 2 are based on tissue samples from a total of ten piglets. Thus, in order to confirm this observation and hypothesis, further studies including large-scale experiments are needed. For future studies aiming to estimate the thickness of the mucus layer on the basis of chemically fixed intestinal samples, it can therefore be recommended to determine both, the intestinal mucin staining area and the number of intestinal goblet cells. Moreover, it would be worthwhile to study the secretory activity of goblet cells e.g. by assessing gene expression patterns of single mucins. This would allow correlation analyzes of histomorphological and molecular-biological parameters, which would provide important information on mucus synthesis and secretion. The ability of displaying the intestinal mucus layer in the histological image in combination with information on the secretory activity of

goblet cells contributes to better characterization of the dynamic process regarding intestinal mucus layer formation and depletion. In this context, current research questions regarding the effect of feeding on intestinal mucus formation might be better answered. In this regard, the role of dietary fiber or various feed additives such as probiotics or phytobiotics on the formation of the intestinal mucus layer could be examined more closely. As pigs are often used as experimental models for humans due to their anatomical, genetic and physiological similarities (Meurens et al. 2012), the histological method described for the assessment of the colonic mucus layer could also be helpful in developing medicaments in connection with the treatment of various inflammatory bowel diseases such as ulcerative colitis or Crohn’s disease.

Inflammatory bowel diseases or food and feed allergies are accompanied with inflammatory processes of the gut, which are often characterized by a massive accumulation of immune cells in the intestinal mucosa (Drut et al. 2004, Carmack et al. 2009, Kreft et al. 2020). In this regard, endoscopy followed by histological evaluation presents the gold standard for diagnostic modality (Flynn et al. 2019). In order to verify immunological alterations in the intestine, it is essential to know the “normal state” of the resident immune cell population of the mucosa of healthy humans and animals. However, with regard to the avian digestive tract, information on the distribution and frequency of intraepithelial leukocytes are scarce. In PUBLICATION 3, the establishment of an immunohistochemical protocol allowed the specific characterization of intraepithelial immune cells in the jejunum of broilers. It could be demonstrated that T lymphocytes were the most dominant population among the detected leukocytes in the mucosa of healthy broilers. In addition, a topological description of the distribution of IEL in the intestinal section was carried out and it was confirmed that there are differences in the IEL distribution between the villi and the crypt region. A direct comparison of these observations with those from previous experiments is challenging, as on the one hand the overall data availability is limited and on the other hand a wide variety of methods for mucosal immune cell characterization is used. In general, two basic methodological approaches are applied to characterize intraepithelial leukocytes of the chicken’s intestine: Flow cytometric analyses and histological methods. The flow cytometry is based on fluorescence-activated cell sorting (FACS) displaying the relative distribution and frequency of intraepithelial leukocytes. In contrast, histological examinations are carried out on tissue sections so that a precise morphological evaluation can be carried out. The use of flow cytometry has the advantage that previously isolated mucosal cells of a larger section of the intestine can be analyzed, so that representative information about the distribution of intestinal immune cells can be obtained, but only on a relative basis. In contrast to this, histological observations enable the determination of the absolute number and precise localization of immune cells in tissue sections, although only a relatively small

region of the intestine could be examined. Regarding the literature, there are mainly data on the relative distribution and frequency of mucosal immune cells assessed on flow cytometric analyzes. In accordance with the current study, the majority of detected leukocytes were $CD3^+$ T cells, expressing the γδ T cell receptor (Lillehoj and Chung 1992, Göbel et al. 2001, Hong et al. 2006, Fenzl et al. 2017, Meijerink et al. 2021). With respect to histological techniques, intestinal intraepithelial leukocytes of chickens were characterized either by classical histological staining using e.g. HE-staining solution (Arias and Koutsos 2006, Zhai et al. 2014, Rehman et al. 2018) or by immunohistochemical staining using specific antibodies (Jeurissen et al. 1989, Vervelde and Jeurissen 1993, Schwarz et al. 2011). While HE-staining only allows the characterization of immune cells on the basis of cell morphology, immunohistochemistry enables the selective identification of immune cell surface antigens by specific antibody binding and should therefore be used in preference. However, the few studies in chicken that used immunohistochemical staining for IEL evaluation showed contradictory results. In two laying hen experiments either no intraepithelial T cells at all (Jeurissen et al. 1989) or fewer intraepithelial T cells compared to B cells were immunohistochemically determined (Vervelde et al. 1993), which is in clear contrast to the results of the present study. In line with the current results, intraepithelial T cells could be immunohistochemically determined in the jejunum of broilers (Noujaim et al. 2008). The antibodies used were suitable for the delimitation of T cell subpopulations, such as $CD4^+$ or $CD8^+$ T cells, but the entirety of the intraepithelial immune cells was not recorded (Noujaim et al. 2008). Therefore, a quantitative assessment of the T cell population in relation to other intraepithelial leukocytes was not possible. However, this was possible in the current experiment and in a recently published study, which methodologically referred to PUBLICATION 3 (Pascual et al. 2020). The results confirmed that most of the determined intraepithelial $CD45^+$ leukocytes represented $CD3^+$ T cells (Pascual et al. 2020). However, in contrast to the present study, the intraepithelial immune cells were not recorded topologically. Apart from the current experiment and based on recent literature research, no information on the topological distribution of avian intraepithelial immune cells are present. The evaluation of intraepithelial leukocyte density patterns of human jejunal biopsies showed, however, a similar distribution compared to those observed in PUBLICATION 3 (Goldstein 2004). In contrast, a homogeneous distribution of intraepithelial immune cells was found in the small intestine of pigs (Vega-López et al. 2001, Rieger et al. 2015). In another study, the distribution of intraepithelial T cells was immunohistochemically determined in clinically healthy dogs. In contrast to data from humans (Goldstein 2004), pigs (Vega-López et al. 2001) and broilers (PUBLICATION 3), a progressive increase in the intraepithelial T cell density from the crypt to the villus tip was observed (Elwood et al. 1997). Further research is needed to clarify the apparent differences in the distribution pattern of

intraepithelial immune cells in the intestine between humans and different animal species. The reasons that there are only limited literature data on the intestinal immune cell distribution of various animal species are certainly that immunohistochemical investigations are time-consuming, complex and thus expensive. One possibility of reducing the effort and cost of these investigations would be to combine the results of elaborate immunohistochemical analyses with those of time-efficient flow cytometric analyses, as recently shown by Ferrara et al. (2017) in pigs. According to this procedure, one immune cell type is determined by means of immunohistochemistry in intestinal sections as a reference for flow cytometric examinations allowing a wider differentiation of IEL. Whether this method can also be used successfully in the chicken's intestine must be investigated in the future.

Impact of qualitative and quantitative differences in dietary protein on the nutritional physiology of poultry

The protein quality of grain legumes is primarily dependent on their amino acid composition and the type and amount of ANFs present. Few specific ANF such as lectins or antigenic proteins are suspected of triggering intestinal immune reactions and provoking intestinal mucosal damage as observed in piglets and calves (Bush et al. 1992, Dreau et al. 1995). In accordance with this, results of PUBLICATION 3 revealed that pea-fed broilers showed a quantitative increase of intraepithelial T cells in the jejunum compared to those receiving a commercial soybean-based feed. Various feed processing methods can be used in order to reduce the ANF content in grain legumes, such as heat treatment or fermentation processes using exogenous enzymes or microbes (Hejdysz et al. 2020, Zentek and Goodarzi Boroojeni 2020). However, ANFs differ in their chemical structure, so that not every feed processing method is suitable to successfully reduce all ANFs. In this context, results of PUBLICATION 3 demonstrated, that broilers regardless of whether they were fed raw, fermented, or enzymatically pre-digested peas showed a jejunal, mucosal accumulation of T cells compared to those fed the control diet. These findings suggest that the type of feed treatment was not adequate to prevent supposed ANF-mediated immune reactions. Based on this observation, it was hypothesized that the examined immune reactions could be associated with alterations of the intestinal morphology and function in these broilers. Results of PUBLICATION 4 confirmed this assumption as both, the jejunal histomorphology and functional glucose transport were related to the detected number of jejunal intraepithelial immune cells. A major key finding was that an increase in the density of intestinal intraepithelial T cells was associated with a decrease in the intestinal villus length, as shown by correlation analyzes. This observation could indicate that the local intestinal immune system might have had a direct impact on the development of intestinal

villus morphology. In this regard, it is known that patients with inflammatory bowel disease commonly show an intestinal immune cell infiltration, which is associated with atrophy and damage of the gut mucosa (Iwamoto et al. 1996, Di Sabatino et al. 2003). This can be explained by an enhanced cytokine production of activated IEL contributing to mucosal destruction and apoptosis (Croitoru and Zhou 2004, Takashima et al. 2019). However, pea-fed broilers of the present study showed an accumulation of intestinal IEL and only shortened, non-destroyed intestinal villi, which should not be equated with an inflamed and damaged gut. In line with this, results of the Ussing chamber experiments demonstrated that tissue conductance, as an excellent indicator of mucosal integrity, was similar between broilers of the different feeding groups. Moreover, jejunal expression of tight junction proteins and genes related to apoptosis and cell maturation were not affected by feeding the different diets. Consistent with this, broilers of all feeding groups were clinically healthy and showed a comparable growth performance. Therefore, it can be assumed that the observed changes in the intestinal immune system, intestinal morphology and functionality in pea-fed broilers did not have a significant adverse effect on intestinal health. However, with respect to gut functionality, the results of the Ussing Chamber investigations also showed that the intestinal glucose absorption was impaired as the intestinal intraepithelial immune cells increased. At the same time, an increase in the intestinal surface area was associated with an increased rate of transepithelial active glucose transport. Therefore, with regard to the underlying mode of action, it can be speculated that pea feeding induced immune reactions in the jejunum of broilers, which negatively affected the development of the intestinal epithelium resulting in a restricted mucosal nutrient transport. However, this did not appear to have had a significant impact on broiler health and performance. In this context, it would have to be evaluated whether an increase in the dietary proportion of peas to over 30% would result in more pronounced effects on the intestine impairing gut health and broiler productivity. The observation of PUBLICATION 4 that the transepithelial SGLT-1 mediated glucose transport in the small intestine of broilers is dependent on the mucosal intestinal surface makes an important contribution to basic research. It has already been shown in several studies with pigs and chickens that an increase in the intestinal villus surface area is accompanied with an improved growth performance (Mekbungwan and Yamauchi 2004, Thanh et al. 2009, Jazi et al. 2017, Yi et al. 2018). From this it was deduced that the expansion of the intestinal villus surface has a decisive influence on the intestinal nutrient absorption. It was suggested that an enlargement of the intestinal villus surface area is associated with an increase in the absorptive intestinal function ensuring an optimal intestinal nutrient transport from mucosal to serosal compartments (Caspary 1992, Mekbungwan and Yamauchi 2004, Adil et al. 2010). Conversely, it has been speculated that a shortening of the intestinal villi and thus a reduction in the mucosal intestinal surface

area could be associated with a reduced intestinal absorptive function (Park et al. 1998, Yamauchi et al. 2006). In the current study it could be clearly demonstrated that the jejunal active glucose transport measured during the Ussing Chamber studies was dependent on the jejunal villus surface area assessed by histomorphometric analyses. Hence, an increased mucosal surface area in the jejunum of broilers was accompanied by a larger jejunal glucose transport capacity. In addition to the mucosal absorptive surface, the number of expressed intestinal nutrient transporters can also have an influence on the nutrient absorption capacity (Röder et al. 2014, Lehmann and Hornby 2016). However, the results of the present publication suggest that the size of the absorptive mucosal surface had a more decisive influence on the transepithelial nutrient transport than the number of expressed nutrient transporters as demonstrated by jejunal SGLT-1 gene expression analyses. In summary, it can be stated that, regardless of the feed treatment carried out, the feeding of peas influenced both the intestinal morphology and the epithelial nutrient transport in broilers, which was associated with the observed mucosal immune cell accumulation. This suggests that certain ANF present in both the raw and processed peas may have induced these effects. In this regard and based on previous studies, in particular, lectins, antigenic proteins or various NSPs might be relevant. It is assumed that lectins and antigenic proteins can interact with the intestinal mucosa and initiate intestinal immune reactions and mucosal alterations as observed in rats, calves and piglets (Lorenz-Meyer et al. 1985, Kik et al. 1990, Dreau et al. 1995, Lallès et al. 1996). Moreover, peas contain about 25% soluble NSP (Englyst and Hudson 1996, Adamidou et al. 2011) which have also shown to affect the intestinal mucosal development and the gut immune system (Teirlynck et al. 2009). As ANFs were not determined in the current study, further experiments are necessary in order to chemically characterize those substances, which might lead to observed effects on intestinal morphology, function and immune system. In addition to different feed processing methods for reducing ANFs in grain legumes, research should also be focused on the development of analytical methods, with which various ANFs such as lectins can be reliably determined. The use of different feed processing methods in combination with the analytical detection of different ANFs in the feed before and after processing could clarify, which ANFs may affect the intestinal health of chickens and how a broader use of peas in poultry feed would be possible through application of suitable feed technologies.

PUBLICATION 5 and 6 were not focused on different dietary protein sources, but examined the influence of varying dietary protein concentrations on the growth performance, nutrient digestibility and whole body composition of male dual purpose chickens. An optimal dietary protein and amino acid supply is a basic requirement for optimal chicken's productivity. Falling short or exceeding the protein requirement of chickens, however, would have

negative consequences on animal health and performance (Morris et al. 1999, Gous 2010). A lack of protein or amino acids is accompanied with interferences of enzyme synthesis, antibody formation and growth performance while a protein or amino acids excess is associated with liver and kidney stress as well as additional energy and water requirements of chickens (Jeroch et al. 2019). Moreover, an optimal dietary protein supply is related to lower feed costs and reduced nitrogen excretion and ammonia emissions in poultry production (Sterling et al. 2005, Kamran et al. 2010). Results of PUBLICATION 5 and 6 revealed that male Lohmann Dual chickens seem to have a lower dietary protein and amino acid requirement than conventional broilers. A reduction in the protein content of up to 15% in relation to conventional broiler feed did not impair body weight development or the FCR of these chickens. In accordance, studies using different dual purpose genotypes also proved that a reduction in dietary protein had no negative impact on growth performance parameters (Koreleski and Świątkiewicz 2008, Koreleski and Świątkiewicz 2009). In a recent study, male Lohmann Dual chickens were fed two different diets, containing either 21.5% or 17.1% of CP. Although no phase feeding concept was implemented in this study, chickens from both feeding groups showed a similar growth performance over a period of nine weeks (Kreuzer et al. 2020). Thus, the current and the few studies cited above allow the rough assessment that male dual purpose chickens have a lower protein requirement than conventional hybrid broilers. However, open questions, for example on effects of different dietary energy-protein ratios or with regard to requirements for certain amino acids and their relationship to one another, must be addressed in future studies. Another main recognition of the present studies was related to flock uniformity of Lohmann Dual chickens. Based on their individual body weight, chickens could be divided into those showing poor growth rates and those with high growth rates. On average, chickens in the high performing group were more than twice as heavy as those in the low performing group. Poor flock uniformity was also observed in Lohmann Dual laying hens (see PUBLICATION 7). The low herd uniformity must be primarily attributed to the specific genotype of dual purpose chickens, as genetic selection is based on both, high growth rates and high reproductive performance. Due to the negative correlation of both characteristics, an optimal implementation of both breeding goals in one genotype is challenging (Rizzi et al. 2007, Rizzi and Chiericato 2010, Steenfeldt and Hammershøj 2015). However, a high level of flock uniformity is a prerequisite for establishing an optimal feeding concept that is geared towards covering the nutrient requirements of the entire herd. Therefore, future research should be directed towards improving the flock uniformity of dual purpose genotypes through breeding measures. In accordance with data on growth performance, the whole body composition of dual purpose chickens was generally not affected by reducing dietary protein. Surprisingly, feeding the lowest protein concentration led to a shift in the body

protein and body fat percentage of the chickens (PUBLICATION 6). In connection with the feeding of high-energy and low-protein diets, studies have shown that chickens increased their feed consumption in order to satisfy their protein and amino acid requirements (Emmans 1987, Aletor et al. 2000, Belloir et al. 2017). Due to the higher energy intake, chickens showed an increased body fat content, while no changes in body protein content were detected (Aletor et al. 2000, Belloir et al. 2017). As in the current study growth performance and particularly the feed consumption was comparable between the feeding groups, observed effects on body composition might be explained by the selection of chickens that were used for analyzing whole body composition. In this context it must be emphasized that only one chicken per pen was examined for body composition and that the flock uniformity, as already discussed, was extremely low. Thus, the differences in body composition can be explained by the poor uniformity rather than by feeding the different diets. In the current studies, dietary protein reduction had no negative impact on apparent ileal and total digestibility of nutrients in dual purpose chickens. Rather, chickens that received the control feed with the highest protein content even showed a poorer nutrient digestibility. In this regard it is conceivable, that the feeding of the control diet resulted in an intestinal excess of proteins that could escape intestinal enzymatic degradation (Awad et al. 2016). Consequently, some of the dietary protein could have been excreted undigested by the chickens (Kamran et al. 2004), which would explain the inferior protein digestibility. Intestinal protein degradation and absorption might also dependent on individual factors such as the development of the gastrointestinal tract. In this context, a recent study compared the macroscopic and microscopic characteristics of the gastrointestinal tract of Lohmann Dual chickens with those of conventional Ross 308 broilers (Alshamy et al. 2019). Interestingly, marked differences in gastrointestinal morphology between both genetics were observed. In chickens of the same body weight, dual purpose chickens had for example a shorter intestine and a smaller intestinal mucosal surface area compared to hybrid broilers (Alshamy et al. 2019). This supports the hypothesis that possibly the protein concentration in the control feed was too high and at the same time the intestinal amino acid absorption capacity too low, so that the surplus of dietary protein could have been excreted by the chickens. In summary, based on both present studies, it can be assumed that chickens of the dual purpose genotype examined have a lower protein requirement than conventional hybrid broilers. This would offer the opportunity to reduce dietary protein content or to use alternative protein sources with a lower protein quality and biological value. From an economic and ecological point of view, that sounds positive at first glance. However, due to their poor feed conversion ratio, male dual purpose chickens have to be fattened more than twice as long to achieve the same body weight as hybrid broilers. Thus, dual purpose chickens consume significantly more feed per kg gain resulting in a higher

resource expenditure per unit meat compared to conventional broilers (Damme et al. 2015). Consequently, production costs of fattening dual purpose chickens are much higher compared to conventional hybrid broilers. Hence, these additional costs must be reflected in the meat price and it is questionable whether consumers are willing to pay for these products, as recent surveys have shown (Brümmer et al. 2018, Busse et al. 2019).

Impact of feeding insoluble fiber on the nutritional phsiology of poultry

In the last decade, research has concentrated on the use of a "novel" insoluble dietary fiber source, lignocellulose. In contrast to other insoluble fiber sources, such as wheat bran, which contains around 20% residual starch and 16% CP in the dry matter (Bach Knudsen 1997), lignocellulose consists of about 95% of insoluble fiber (Slama et al. 2019), so that its nutritional value can be assessed as low. Therefore, in PUBLICATION 7, lignocellulose was used as a "diet diluent" in order to specifically reduce the energy and nutrient content of a commercial laying hen feed. Hence, it should be tested whether dual purpose hens require a lower dietary energy and nutrient content than conventional hybrid hens. Results of PUBLICATION 7 confirmed the hypothesis that the body weight gain of dual purpose hens can be slowed down by the addition of 10% dietary lignocellulose and that laying performance of those hens can even be increased by nutrient-reduced feeding. A crucial point of this study was that the feed intake behavior of chickens was significantly influenced by the supplementation of lignocellulose. This, in turn, had a considerable effect on overall productivity, but also on the digestive tract development of dual purpose hens (see PUBLICATION 8). It is generally accepted, that the dietary energy content has a decisive impact on the feed intake behavior of chickens. It is suggested that chickens are able to meet their metabolic energy requirements by increasing or decreasing their feed intake, depending on the energy content of feed (Fisher and Weiss 1956, Leeson et al. 1996, Van Krimpen et al. 2007). In this regard, pullets and laying hens increased their feed intake when fed diets that has been diluted with up to 20% sand or oat hulls (Van Krimpen et al. 2007, Van Krimpen et al. 2009). Consequently, no differences were observed in terms of energy intake and average weight gain between hens fed the undiluted or diluted diets (Van Krimpen et al. 2007, Van Krimpen et al. 2009). However, this hypothesis was not confirmed by results of the current study. With respect to the growing period, chickens of both feeding groups had a comparable feed consumption so that lignocellulose-fed hens had a significantly lower energy and nutrient intake than hens fed the control feed. Consequently, control-fed hens developed higher body weights than hens receiving the lignocellulose-containing diet. This suggests that other factors may have played a role in regulating the chicken's feed intake. In this context, a more recent review emphasized that the historical perception that the feed intake behavior of chickens is mainly determined by the dietary

energy content has to be put into perspective (Classen 2017). It was stated that the regulation of feed intake by chickens is complex and that several factors can affect the adequate adjustment of feed intake such as the chicken's genotype, the feed composition or feed processing (Classen 2017). Regarding the chicken's genotype, Classen (2017) highlighted that modern laying hen strains might be unable of accurately altering feed intake in response to dietary energy concentrations. Therefore, it is questionable whether dual purpose laying hens used in the current study possessed this ability. However, lignocellulose-fed chickens showed an increased feed intake compared to control-fed hens during the laying period. This could be interpreted as a specific response of hens to compensate for the higher energy demands required for egg production. Apart from the dietary energy content, the feed structure, which is characterized by the particle size and the physical form of feed, appears to have a decisive influence on chicken's feed intake. It is well established that multiple sensory factors, particularly visual and textural feed characteristics, can have a strong impact on the avian feed intake behavior (Latshaw 2008, Classen 2017). There is evidence that chickens prefer larger feed particles to finer ones and that feed with a very fine particle size distribution is poorly ingested (Portella et al. 1988, Ferket and Gernat 2006, Neves et al. 2014). Feed structure of diets used in the current study strongly differed in terms of textural properties, as the supplemented lignocellulose product was finely ground and had a powdery, voluminous appearance. Thus, it is conceivable that pullets, fed the lignocellulose containing diet, were not able to compensate their energy deficiency by increasing the feed intake due to the physical form of fine lignocellulose fibers and its physical bulking effect. A similar observation was made in connection with broiler breeder hens, whose feed contained 3% powdered cellulose (Mohiti-Asli et al. 2012). Another key finding from the current study was that the feeding of lignocellulose affected the body composition of dual purpose hens, which in turn was related to the hens laying performance. Lignocellulose-fed hens had a lower body fat content than hens that were fed the conventional diet which was directly linked to an improved laying performance, as demonstrated by correlation analyses. It is well established that a high body fat accumulation can be accompanied with ovarian dysfunction and reduced reproduction effectiveness in humans and animals (Ezeh et al. 2014, Liu et al. 2016, Sirotkin et al. 2017, Kannan et al. 2019). Regarding the avian reproduction, the paradox of rapid muscle growth and body fat accumulation versus reproduction performance and health is a phenomenon often described for broiler breeder hens (Decuypere et al. 2010). As broiler breeder hens are genetically selected for meat production and rapid growth rates, they tend to become obese when fed *ad libitum* which is accompanied with an inferior laying performance (Yu et al. 1992, Chen et al. 2006, Liu et al. 2016). Therefore, feed restriction is a common feeding concept used for broiler breeder hens in order to avoid an excessive

energy intake and body fat accumulation (Mench 2002, Decuypere et al. 2010). However, as highlighted by Decuypere et al. (2010), applying feed restriction induces a second paradox: satisfactory reproduction versus hunger stress and impaired animal welfare. Based on the results of the present study, it would be interesting to investigate whether broiler breeding hens also show an improved laying performance, if they were fed with reduced energy and nutrient dense, lignocellulose-containing diets and how this would affect hunger stress. Although the feeding of nutrient-reduced diets containing lignocellulose basically improved hens' productivity, a negative impact on the individual egg weights was observed. Analyses of the external egg quality confirmed that lignocellulose-fed chickens produced smaller and therefore lighter eggs than hens receiving the control feed. However, due to the higher laying performance, the total egg mass was nevertheless higher in lignocellulose-fed hens. All other important egg quality parameters such as shell thickness, shell stability or yolk and albumen ratio were not affected by feeding. In this regard, it is well established that at the onset of lay, the body weight of hens is the most important factor that determines the egg weight respectively egg size throughout the laying period (Festing and Nordskog 1967, Du Plessis and Erasmus 1972, Summers and Leeson 1983). As lignocellulose-fed hens had a lower energy and nutrient intake, body weight development was reduced compared to control-fed hens, which might explain the lower egg weights. The question therefore arises as to which feeding strategies and concepts could be applied to positively influence both, the laying performance and the individual egg weight of dual purpose laying hens. Future studies could examine whether feeding lower concentrations of insoluble fiber sources, for example the use 6% or 8% lignocellulose, are more suitable in order to reduce the body development of hens more moderately. Another interesting option would be to feed the chickens with a conventional feed at the early growing period in order to ensure optimal growth rates. In a later stage of growth, it would be conceivable to switch to an energy-reduced, lignocellulose-containing diet in order to prevent increased fat accumulation in the chickens. A suitable time to change feed could be the 12. or 16. weeks of age, as this is the phase in which the greatest increases in hens body fat content can be observed (Grandhaye et al. 2019). Whether this proposed feeding concept actually leads to optimal body weight development with a reduced body fat content in dual purpose hens and whether this is accompanied by improved laying performance and heavier egg weights needs to be clarified in further studies.

Results of PUBLICATION 8 confirmed the hypothesis that feeding energy- and nutrient-reduced diets supplemented with 10% lignocellulose has an impact on digestive tract development of dual purpose chickens. The relative weight of gastrointestinal organs was increased in hens fed the lignocellulose containing diet compared to those fed the conventional layer diet. This effect must primarily be attributed to the use of energy- and

nutrient-reduced diets rather than to the use of dietary fibers. In this regard, similar observations were made in chickens that had restrictive access to feed (Susbilla et al. 1994, Zubair and Leeson 1994, Palo et al. 1995). Broilers offered 50% less feed compared to those of a control group developed lower body weights and, at the same time, higher relative digestive tract weights (Susbilla et al. 1994). This is comparable to the current study, as dual purpose chickens fed with dietary lignocellulose had lower energy and nutrient intakes, lower body weights and, consequently, higher relative digestive tract weights than chickens that were fed the control diet. In order to specifically examine the effect of dietary fiber on the development of digestive organs, feeding experiments would have to be carried out on the basis of feed formulations that have a similar energy and nutrient content. Feeding effects were observed not only with regard to the gross morphology, but also in terms of the intestinal histomorphology. The results showed that hens fed dietary lignocellulose developed a larger mucosal surface area in the large intestine than hens fed the control diet. With this finding, too, the question arises whether the effects are more related to feeding of nutrient-reduced diets or to the use of dietary fibers. Studies on the influence of dietary lignocellulose on avian intestinal histomorphology are scarce and results obtained are contradicting. It could be demonstrated that lignocellulose-fed broilers had a higher villus height, villus apparent surface area and villus height to crypt depth ratio in the jejunum and ileum compared to those fed a control diet (Sarikhan et al. 2010, Sozcu 2019). On the contrary, in a broiler study using lignocellulose inclusion levels of 0.25, 0.5 and 1.0%, results showed that villus height was reduced in the duodenum and jejunum of chickens fed 0.5% dietary lignocellulose compared to those offered the control feed (Bogusławska-Tryk et al. 2020). In another study, broilers fed 0.6% lignocellulose, which was included in the diet at the expense of soybean meal, had longer intestinal villi and deeper crypts in the duodenum and jejunum compared to those fed a control feed (Makivić et al. 2019). Interestingly, no effects on duodenal and jejunal histomorphology were observed in broilers that were fed the same dietary lignocellulose inclusion level, but supplemented at the expense of 0.3% soybean meal and 0.3% corn (Makivić et al. 2019), indicating that mucosal development was affected by feed composition rather than dietary lignocellulose inclusion. A direct comparison with results of the current study is difficult, because significantly lower dietary lignocellulose inclusion levels of 0.05 to 1% were used (Sarikhan et al. 2010, Makivić et al. 2019, Sozcu 2019, Bogusławska-Tryk et al. 2020) provoking a smaller "dilution effect" on dietary energy and nutrient content. In this regard, it was speculated that chickens, fed high-fiber, low-nutrient diets, increase the intestinal mucosal surface, in order to compensatory enhance the absorption of nutrients (Brenes et al. 1993, Bedford 2000). In the current study, transepithelial nutrient transport was not investigated, but there was a remarkable correlation between the concentration of bacterial metabolites and the mucosal villus

surface area in the large intestine of hens. Results showed that the lower the concentration of bacterial metabolites in the intestinal lumen, the larger the absorptive mucosal surface area. This might indicate, that lignocellulose-fed chickens, which may have suffered from nutritional deficiencies, enhanced the resorption of energy yielding bacterial metabolites by increasing the intestinal mucosal surface. However, this presupposes that similar amounts of metabolites have been produced by microorganisms in the large intestines of the chickens, regardless of whether hens received the lignocellulose-containing feed or the control diet. As the current study only determined the intestinal concentration of bacterial metabolites, but not the direct resorption of these via the intestinal epithelium, further studies would have to be carried out to prove this hypothesis. Analyses of microorganisms residing in the hen's large intestine may support the hypothesis. Results showed that there were basically no differences in the number or activity of the bacterial groups measured, which suggests that the amount and composition of the bacterial metabolites produced were similar in chickens of both feeding groups. Studies on the impact of dietary lignocellulose on the intestinal microbiota in chickens are scarce drawing no clear picture. Results of a few studies showed that inclusion of dietary lignocellulose increased the luminal concentration of bacterial metabolites in chickens (Bogusławska-Tryk et al. 2015, Sun et al. 2020), but others showed no impact (Zeitz et al. 2019, Hou et al. 2020). At this point, it should be emphasized that these studies differed in the lignocellulose products and the dietary lignocellulose inclusion levels used. Moreover, different feed formulations were chosen to test the effect of dietary lignocellulose on intestinal microbial composition and bacterial metabolites. On the one hand, this makes it difficult to interpret the observed effects and, on the other hand, prevents a direct comparison between the results of different studies. This important aspect is taken up again in PUBLICATION 10 and discussed intensively there. Another important observation of this study was that the hens' excrement quality was positively influenced by the use of dietary lignocelluloses. Lignocellulose-fed chickens had generally a lower excreta moisture content compared to control-fed hens. In this context, it has been speculated that feeding lignocellulose leads to a longer digesta transit time in chickens, which could have a positive effect on the excreta dry matter content (Kheravii et al. 2017). However, studies in broilers showed no effect of feeding lignocellulose on excreta scoring (Zeitz et al. 2019) or excreta moisture content (see PUBLICATION 9). Therefore, further investigations are necessary in order to examine whether lignocellulose, due to its physicochemical properties, actually has an impact on excreta quality in poultry. High moisture contents of excreta and litter are directly associated with the occurrence of foot-pat dermatitis and breast burns in chickens representing a serious problem in modern poultry production (Shepherd and Fairchild 2010, Swiatkiewicz et al. 2017). Reducing the excreta moisture content by feeding insoluble fiber sources would

be an important approach to prevent these diseases and thereby improving animal welfare and productivity in poultry.

PUBLICATION 9 confirmed that increasing concentrations of dietary insoluble fiber do not appear to have a negative impact on the productivity of chickens if isoenergetic and isonitrogenous feed variants are chosen, even when using higher supplements of 10% lignocellulose. As the feed intake of slow-growing broilers was similar among the different feeding groups, the energy and nutrient intake of chickens was also comparable. Therefore, no adverse effects on broiler weight gain or FCR from feeding lignocellulose were observed. In contrast, studies have shown that the feeding of high-fiber, nutrient-diluted diets might be accompanied with a lower energy and nutrient intake of broilers, which has resulted in an impaired growth performance (Oke and Oke 2007, Oikeh et al. 2019). Thus, it becomes obvious that, regardless of the fiber inclusion, the dietary nutrient content is a particularly decisive factor influencing chicken's productivity. However, results of PUBLICATION 9 also revealed that the apparent ileal digestibility of CP was decreased with increasing dietary lignocellulose concentrations. Usually, a poor digestibility of nutrients is associated with negative effects on growth performance in broilers (Li et al. 2010, Gopinger et al. 2014, Hejdysz et al. 2019). Slow-growing broilers of the current study showed a comparable growth performance despite reduced protein digestibility. This might be explained by the fact that slow-growing broilers have lower nutrient needs to meet maintenance and performance requirements (Morris and Njuru 1990, Wang et al. 2013), which was also demonstrated by results of PUBLICATION 5 and 6. In the current study, the CP content in the feed was relatively high at around 22%, which corresponds to the recommendations for fast-growing broilers (GfE, 1999). Therefore, it is conceivable that despite higher dietary lignocellulose concentrations and lower protein digestibilities, the amount of amino acids absorbed through the intestinal tract was sufficient to ensure optimal growth performance in slow-growing broilers. Nevertheless, the question arises as to why the feeding of increasing dietary lignocellulose concentrations impaired the apparent ileal digestibility of CP of broilers. In this regard, it was suggested that the dietary inclusion of insoluble fibers could directly impede the digestion and absorption of nutrients in the small intestine of poultry and pigs (Bach Knudsen et al. 1993, Jørgensen et al. 1996, Williams et al. 2019). Furthermore, it was suggested that the feeding of lignocellulose might have an abrasive effect on the intestinal mucosa (Bogusławska-Tryk et al. 2015, Makivić et al. 2019), thus enhancing endogenous amino acid losses. In addition to the inferior ileal protein digestibility, apparent total tract digestibility of organic matter and gross energy was also reduced with increasing dietary lignocellulose concentrations in broilers of the current study. Digestibility of organic matter and gross energy was simultaneously reduced in broilers by increasing dietary lignocellulose concentrations. This seems to indicate that lignocellulose was neither

degraded by endogenous enzymes in the small intestine nor largely fermented by microbes in the intestinal tract of chickens. Thus, dietary lignocellulose was probably excreted undigested, so that the digestibility of the organic matter and the gross energy decreased with increasing levels of dietary lignocellulose. This assumption is also supported by results regarding analyzed bacterial metabolites in the large intestine of broilers. Correlation analyses revealed that dietary lignocellulose inclusion was negatively related to the total concentration of SCFA, lactate, and ammonia in the cecum of broilers. This implies a poor lignocellulose degradability by bacteria that colonize the large intestine of chickens. Moreover, the feeding of lignocellulose generally did not have a significant effect on the bacterial composition in the hindgut of chickens, with the exception that increasing concentrations of dietary lignocellulose lowered cecal counts of *Escherichia/Hafnia/Shigella*. A few studies have investigated the effect of feeding dietary lignocellulose on gut microbiota in poultry showing contradicting results. Ileal and cecal counts of *Escherichia coli* and *Clostridium* spp. were also reduced in broilers receiving 0.25 and 0.5% dietary lignocellulose while the cecal SCFA profile was generally not affected by dietary lignocellulose inclusion (Bogusławska-Tryk et al. 2015). In another study, cecal microbial diversity and the abundance of butyrate-producing bacteria was increased in free-range chickens fed 2 and 4% dietary lignocellulose, although no effects on the luminal concentration of SCFA were observed (Hou et al. 2020). On the contrary, broilers and laying hens fed diets containing 0.8 and 1% lignocellulose showed no alterations in the overall cecal microbial diversity (Zeitz et al. 2019, Sun et al. 2020). As already shown, feeding of diets diluted with 10% dietary lignocellulose did not alter cecal microbial composition in dual purpose hens, but reduced the cecal concentration of SCFA and ammonia (see PUBLICATION 8). Thus, due to the limited amount of data and the contradicting results in connection with the effects of dietary lignocellulose on the intestinal microbiota, a final assessment is not yet possible. However, there is evidence that insoluble fibers and lignified material are generally poorly degraded by microorganisms residing in the avian digestive tract (Choct et al. 1996, Hetland et al. 2004, Jha et al. 2019). A special feature of poultry is the short digestive tract, which causes a fast digesta transit time and thus restricts bacterial fermentation processes of fibrous plant material. In addition, several studies have shown that only small and soluble fiber fractions may reach the cecum (Bjornhag and Sperber 1977, Vergara et al. 1989, Rougière and Carré 2010), which appears to be the main location for bacterial fermentation of dietary fiber (Jørgensen et al. 1996, Józefiak et al. 2004). Thus, it can be assumed that dietary lignocellulose, which mainly consists of insoluble NSPs and lignin, would not enter the cecum avoiding possible bacterial degradation. Moreover, there is an ongoing controversy whether microbes, colonizing the large intestine of chickens, might be able to sufficiently degrade complex NSP such as lignocellulose or cellulose.

Some scientists assume that the cellulolytic activity of bacteria in the avian hindgut is low (McNab 1973, Mead 1989, Waite and Taylor 2014), others have identified certain bacteria of the phylum Bacteroidetes in the cecum of broilers that are probably able to break down complex fiber ingredients such as cellulose (De Maesschalck et al. 2019). Therefore, further studies are necessary to clarify the extent to which fibrous feed components consisting mainly of insoluble NSPs can be utilized by intestinal microorganisms and whether this has an impact on gut physiology and health in poultry.

The knowledge gained in PUBLICATION 7 to 9 formed the basis for creating a review article in order to identify similarities and differences between previous studies with regard to the effects of dietary lignocellulose in poultry. In the past, various reviews on the subject of fiber feeding in chickens have already been carried out (Hetland et al. 2004, Mateos et al. 2012, Kheravii et al. 2018, Jha et al. 2019). The conclusion of these research works was basically similar, namely that dietary fiber can have an effect on digestive physiology and chicken's health, but that this is explicitly dependent on the fiber source used. Therefore, the current review focused specifically on one particular fiber source, lignocellulose, which can be viewed as a kind of "novel" source of fiber, as its use as a component of poultry feed has only been studied more intensively in the last ten years. Right at the beginning, PUBLICATION 10 addresses one of the crucial points in connection with studies on the effects of insoluble fiber feeding, which concerns the conception of feed formulations used. In principle, there are three different options to include fiber sources in experimental diets as illustrated in the first figure. In all three possible experimental designs, a control feed is compared with a treatment diet, which contains the fiber source to be investigated. Regardless of which feed formulation is chosen to examine the effect of dietary fiber in a feeding experiment, control feed and treatment diet differ not only in the fiber content, but also in the feed and nutrient composition. Therefore, the effects of feeding these diets can be traced back to both, the dietary fiber and/or the feed and nutrient composition. This important aspect needs to be considered when interpreting results of studies investigating the impact of dietary fiber in animals in order to avoid drawing wrong conclusions. With regard to the influence of dietary lignocellulose on productivity and nutrient digestibility in broilers and laying hens, the results of previous studies have shown basically negligible effects. Noteworthy effects were only observed when using nutrient-reduced diets that contained relatively high dietary lignocellulose inclusion levels resulting in a lower energy and nutrient intake of chickens impairing their growth performance. However, positive effects on the productivity and nutrient digestibility in chickens could be demonstrated in several studies using other insoluble dietary fiber sources such as oat hulls, pea hulls or wood shavings (González-Alvarado et al. 2007, Amerah et al. 2009, Jiménez-Moreno et al. 2011). This observation was mainly explained by the fact, that feeding those fibers had a

stimulating effect on digestive tract development and function. The feeding of coarsely ground fiber sources such as oat, soybean and pea hulls, or wood shavings increased the gizzard size and weight (Hetland et al. 2003, González-Alvarado et al. 2007, Amerah et al. 2009) indicating an enhanced gizzard function (Roche 1981, Jiménez-Moreno et al. 2009, Svihus 2011). Furthermore, some studies observed an increased intestinal weight and length in chickens fed structural insoluble fiber sources (Sklan et al. 2003, González-Alvarado et al. 2007, Jiménez-Moreno et al. 2009, González-Alvarado et al. 2010). Moreover, it is suggested that the feeding of moderate amounts of coarsely ground dietary fiber increases the digesta retention time in chickens (Hetland et al. 2003, Mateos et al. 2012). Most studies that used powdery lignocellulose as dietary fiber source, however, showed that the development of gastrointestinal organs (Kheravii et al. 2017, Makivić et al. 2019, Zeitz et al. 2019, Sun et al. 2020) and the feed passage rate in chickens (Abdollahi et al. 2019) was not influenced by dietary fiber inclusion. In summary, the feeding of coarsely ground, structural fiber sources seem to enhance digestive tract development and function may improving nutrient digestibility and growth performance of chickens. The question arises whether the fiber inclusion itself, the particle size of the fiber source or a combination of both factors are responsible for observed effects on gizzard development and digestive function. In this regard, Jiménez-Moreno et al. (2010) could demonstrate that the feeding of 3% coarsely, ground oat hulls increased gizzard weights and lowered gizzard pH in broilers, while the dietary inclusion of the same amount finely ground cellulose had no impact the digestive tract parameters surveyed. Thus, it seems obvious that the particle size of the fiber source, rather than the fiber inclusion itself, is the determining factor that stimulates digestive tract development and thus potentially has a positive effect on nutrient digestibility and productivity in chickens. On this basis, it can be assumed that finely ground, powdery lignocellulose, similar to cellulose, has little influence on the development and function of the digestive tract, which is also supported by the results of most previous studies. As lignocellulose is also available in pelleted or crumbled form, it would be interesting to investigate whether the physical form of lignocellulose might have an impact on gastrointestinal morphology and function in chickens. It is well established that dietary fiber can affect the gut microbiota in humans and animals, which in turn might have consequences on the intestinal health (Makki et al. 2018, Jha et al. 2019). Studies focusing on the influence of dietary lignocellulose on the intestinal microbiota also showed that the incorporation of fiber in poultry feed was accompanied with alterations of the intestinal bacterial composition and intestinal microbial metabolites (Bogusławska-Tryk et al. 2015, Kheravii et al. 2017, Makivić et al. 2019, Zeitz et al. 2019, Sun et al. 2020). However, the results do not reflect a uniform picture, neither with regard to the impact on specific bacterial groups, nor concerning the occurrence of certain microbial metabolites. In principle, dietary

fibers can serve as a fermentative substrate for bacteria colonizing the intestinal tract, but there are differences in the microbial fermentability of different fiber sources depending on the type of fiber, fiber form and fiber-specific physicochemical properties (Jha et al. 2019). As already discussed (see PUBLICATION 9), there are different scientific opinions as to whether and, if so, to what extent dietary lignocellulose can be fermented by microbes residing in the avian digestive tract. Based on the research results to date, this question cannot yet be definitively answered. There is, however, another essential problem with regard to the interpretation of those results, which in turn is related to the feed formulation chosen to study the effect of dietary fiber on gut microbiota. Depending on the amount of dietary fiber added, there are corresponding changes in the nutrient composition between the control feed and the fiber-containing diet. Therefore, variations in nutrient composition caused by dietary fiber inclusion should not be ignored in terms of effects on internal microbial diversity (Hou et al. 2020). Alterations in the nutritional composition of the feed result in changes of the amount of substrate that reaches the large intestine and can be fermented by resident bacteria. Consequently, changes in the dietary nutrient composition particularly in relation to the carbohydrate fraction, may affect the gut microbiota and bacterial fermentation pathways. Future studies should take this important aspect into account. It would be conceivable, for example, to investigate the fiber source of interest with the aid of different feed formulations in order to consider this factor. In addition, animal experiments could be accompanied by *in vitro* experiments in order to get a more precise insight into the bacterial fermentation of different fiber sources.

Final conclusion

This thesis focuses on the influence of dietary proteins and fibers on the nutritional physiology of poultry. In addition, the current work contributes to basic research in the field of nutritional physiology by elaborating and further developing analytical methods that enable the characterization of physiological properties and functions of the intestinal tract.

The modification and further development of the Ussing chamber technique allowed the determination of the transepithelial nutrient transport and the characterization of the barrier function in the small intestine of chickens. In addition, a histological protocol could be established that achieved a reliable intestinal mucus preservation and thus enabled the measurement of the mucus layer thickness in the hindgut of piglets. Moreover, the establishment of an immunohistochemical protocol allowed the visualization of intraepithelial immune cells in intestinal tissue sections of chickens, so that their topological distribution along the avian small intestine could be characterized for the first time. The combination of these methods is of particular value, as the interactions between intestinal mucosal nutrient transport, barrier function and immune system can be investigated at the same time. These methods can be used to collect data on transepithelial nutrient uptake, mucus formation and the immune system of the intestinal tract in both healthy and sick animals. This allows the characterization of physiological and pathophysiological processes that take place in the digestive tract, and creates a basis for a better understanding of the underlying mechanisms of action. This knowledge forms the foundation for future research projects aimed at examining the effects of, for example, feeding specific dietary components or the administration of drugs on the digestive tract. Another important point is that the more precisely physiological states and complex processes that take place in an organism can be characterized and understood, the sooner alternative methods to animal experiments can be implemented. For example, *in silico* models can only produce accurate and valid results if the biological data on which they are built also meet these criteria. Therefore, the ascertainment of high-quality biological data from animal models with the help of suitable analytical methods is of particular importance in order to reduce and replace animal experiments in the future.

Another important finding of this thesis was that the feeding of different plant protein sources can have an influence on the gut immune system, the digestive tract morphology and intestinal functionality in chickens. Results from the present studies revealed that the feeding of peas instead of soybeans increased the density of intraepithelial immune cells in the small intestine of broilers. Particularly noteworthy was the fact that the accumulation of intraepithelial immune cells was directly associated with a reduced absorptive mucosal surface area and a decreased transepithelial nutrient transport in the small intestine of

broilers. On the one hand, this observation reveals that the intestinal immune system is closely linked to morphological and functional properties of the intestinal epithelium in chickens. On the other hand, the results suggest that certain pea-related ANFs may have induced observed effects. Future studies should be centered on the identification of those bioactive secondary plant substances in order to clarify whether a wider use of peas in poultry feeding could be realized considering suitable feed technologies. In addition to protein sources of different origins, quantitative differences in the dietary protein content can also influence digestive physiology and growth performance of chickens. Studies presented in the current thesis demonstrated that a significant reduction in the dietary protein content did not adversely affect nutrient utilization and growth performance in chickens of a dual purpose breed. Hence, dual purpose chickens seem to have a much lower protein requirement than conventional hybrid chickens. However, male dual purpose chickens have to be fattened and fed for about twice as long as compared to conventional hybrid broilers. The justification for the use of dual purpose genetics is therefore not an economic one, but is derived from social demands and political decisions that have arisen in connection with the practice of killing day-old male chicks. If there should be a market for dual purpose chickens in the future, alternative feeding concepts would have to be developed in which both economic and ecological aspects are considered. As shown, one approach would be to reduce the protein content in feed. Instead of using valuable, imported soybeans as a feed component, it would also be conceivable to feed dual purpose chickens with alternative plant protein sources such as native peas or lupins, which have both a lower protein content and a lower biological value of the protein.

With respect to the development of concepts to promote intestinal health, there has been an increased interest in the use of dietary insoluble fiber sources in poultry nutrition. Studies of the present work centered on evaluating the effects of feeding lignocellulose on the nutritional physiology and productivity of laying hens and broilers. Results confirmed that the targeted use of lignocellulose as a dietary nutrient diluent was an effective method to decrease body fat content of dual purpose hens, which was directly associated with an improved laying performance. In addition, effects on the morphology of gastrointestinal organs and the development of the intestinal mucosa were observed, which were also related to the feeding of high-fiber, low-nutrient diets. A further study demonstrated that increasing concentrations of dietary lignocellulose up to 10% did not show any negative effects on growth performance of broilers, when diets were balanced to be isoenergetic and isonitrogenous. However, increasing dietary lignocellulose concentrations decreased the ileal protein digestibility in broilers suggesting an inhibiting effect of fiber-feeding on protein utilization. Moreover, data on the digestibility of organic matter and intestinal microbiota suggest that dietary lignocellulose might be not a suitable nutrient substrate for bacteria

residing in the intestinal tract of chickens. Future studies should clarify the extent to which intestinal microorganisms colonizing the avian intestinal tract can use dietary insoluble fibers as a source of nutrients and energy and whether this has been linked to effects on the intestinal physiology and health of poultry. In order to be able to clearly differentiate between fiber-associated effects and those related to the feed and nutrient composition, special attention should be paid to the conception of the feed formulations in advance of feeding experiments. In addition, the precise characterization of physicochemical properties of fiber sources to be examined should be of particular importance, as these properties significantly determine physiological processes in the gastrointestinal tract of chickens.

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List of publications and authors contribution

PUBLICATION 1

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

Designed the study: Ruhnke, Röhe, Meyer, Zentek
Performed experiments: Ruhnke, Röhe, Kröger
Analyzed the data: Ruhnke, Röhe, Neumann
Prepared manuscript: Ruhnke, Röhe, Meyer, Zentek

PUBLICATION 2

Röhe, I.*, Hüttner, F. J.*, Plendl, J., Drewes, B. and J. Zentek. 2018. Comparison of different histological protocols for the preservation and quantification of the intestinal mucus layer in pigs. Eur. J. Histochem. 62: 2874.

Designed the study: Röhe, Hüttner, Zentek
Performed experiments: Hüttner, Röhe, Drewes
Analyzed the data: Röhe, Hüttner
Prepared manuscript: Röhe, Hüttner, Zentek, Plendl, Drewes
* shared first authorship

PUBLICATION 3

Röhe, I., Göbel, T., Goodarzi Boroojeni, F. and J. Zentek. 2017. Effect of feeding soybean meal and differently processed peas on the gut mucosal immune system of broilers. Poult. Sci. 96: 2064-2073.

Designed the study: Röhe, Zentek
Performed experiments: Röhe, Goodarzi Boroojeni
Analyzed the data: Röhe, Göbel
Prepared manuscript: Röhe, Zentek, Göbel

PUBLICATION 4

Röhe, I., Goodarzi Boroojeni, F. and J. Zentek. 2017. Effect of feeding soybean meal and differently processed peas on intestinal morphology and functional glucose transport in the small intestine of broilers. Poult. Sci. 96: 4075-4084.

Designed the study:	Röhe, Zentek
Performed experiments:	Röhe, Goodarzi Boroojeni
Analyzed the data:	Röhe
Prepared manuscript:	Röhe, Zentek

PUBLICATION 5

Urban, J., **Röhe, I.** and J. Zentek. 2018. Effect of protein restriction on performance, nutrient digestibility and whole body composition of male Lohmann Dual chickens. Europ. Poult. Sci. 82: 221.

Designed the study:	Röhe, Zentek
Performed experiments:	Urban, Röhe
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PUBLICATION 6

Urban, J., **Röhe, I.** and J. Zentek. 2018. Effect of dietary protein, calcium and phosphorus concentrations on performance, nutrient digestibility and whole body composition of male Lohmann Dual chickens. Europ. Poult. Sci. 82: 231.

Designed the study:	Röhe, Zentek
Performed experiments:	Urban, Röhe
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Prepared manuscript:	Urban, Röhe, Zentek

PUBLICATION 7

Röhe, I., Urban, J., Dijkslag, A., te Paske, J. and J. Zentek. 2019. Impact of an energy-and nutrient-reduced diet containing 10% lignocellulose on animal performance, body composition and egg quality of dual purpose laying hens. Arch. Anim. Nutr. 73: 1-17.

Designed the study: Röhe, Zentek
Performed experiments: Röhe, Urban
Analyzed the data: Röhe
Prepared manuscript: Röhe, Zentek, Dijkslag, te Paske

PUBLICATION 8

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Designed the study: Röhe, Zentek
Performed experiments: Röhe, Metzger
Analyzed the data: Röhe, Vahjen
Prepared manuscript: Röhe, Zentek

PUBLICATION 9

Röhe, I., Metzger, F., Vahjen, W., Brockmann, G. A. and J. Zentek. 2020. Effect of feeding different levels of lignocellulose on performance, nutrient digestibility, excreta dry matter, and intestinal microbiota in slow growing broilers. Poult. Sci. 99: 5018-5026.

Designed the study: Zentek, Röhe
Performed experiments: Metzger
Analyzed the data: Metzger, Röhe, Vahjen
Prepared manuscript: Röhe, Metzger, Zentek, Brockmann

PUBLICATION 10

Röhe, I. and J. Zentek. 2021. Lignocellulose as an insoluble fiber source in poultry nutrition: a review. J. Anim. Sci. Biotechnol. 12: 82.

Designed the study: Röhe
Prepared manuscript: Röhe, Zentek

Complete list of publications (in chronological order)

1. Ohlhoff, B., Savvateeva, D., Leisner, J., Hartmann, F., Südekum, K.-H., Bernsmann, T., Spolders, M., Jahnke, A., Lüth, A., **Röhe, I.**, Numata, J. and R. Pieper. 2022. Transfer of Non-Dioxin-Like Polychlorinated Biphenyls (ndl-PCBs) from Feed and Soil into Hen Eggs. J. Agric. Food Chem. 70: 8955-8962.

2. Ellner, C., Martínez-Vallespín, B., Saliu, E.-M., Zentek, J. and **I. Röhe**. 2021. Effects of cereal and protein source on performance, apparent ileal protein digestibility and intestinal characteristics in weaner piglets. Arch. Anim. Nutr. 75: 263-277.

3. **Röhe, I.** and J. Zentek. 2021. Lignocellulose as an insoluble fiber source in poultry nutrition: a review. J. Anim. Sci. Biotechnol. 12: 82.

4. **Röhe, I.**, Metzger, F., Vahjen, W., Brockmann, G. A. and J. Zentek. 2020. Effect of feeding different levels of lignocellulose on performance, nutrient digestibility, excreta dry matter, and intestinal microbiota in slow growing broilers. Poult. Sci. 99: 5018-5026.

5. **Röhe, I**., Vahjen, W., Metzger, F. and J. Zentek. 2020. Effect of a “diluted” diet containing 10% lignocellulose on the gastrointestinal tract, intestinal microbiota, and excreta characteristics of dual purpose laying hens. Poult. Sci. 99: 310-319.

6. Alshamy, Z., Richardson, K. C., Harash, G., Hünigen, H., **Röhe, I.**, Hafez, H. M., Plendl, J. and S. Al. Masri. 2019. Structure and age-dependent growth of the chicken liver together with liver fat quantification: A comparison between a dual-purpose and a broiler chicken line. Plos One 14: e0226903

7. **Röhe, I.**, Urban, J., Dijkslag, A., te Paske, J. and J. Zentek. 2019. Impact of an energy-and nutrient-reduced diet containing 10% lignocellulose on animal performance, body composition and egg quality of dual purpose laying hens. Arch. Anim. Nutr. 73: 1-17.

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9. Urban, J., **Röhe, I.** and J. Zentek. 2018. Effect of protein restriction on performance, nutrient digestibility and whole body composition of male Lohmann Dual chickens. Europ. Poult. Sci. 82: 231.

10. **Röhe, I.**, Hüttner, F. J.*, Plendl, J., Drewes, B. and J. Zentek. 2018. Comparison of different histological protocols for the preservation and quantification of the intestinal mucus layer in pigs. Eur. J. Histochem. 62: 2874.

11. Keimer, B., Kröger, S., **Röhe, I.**, Pieper, R., Simon, A. and J. Zentek. 2018. Influence of differently processed yeast (*Kluyveromyces fragilis*) on feed intake and gut physiology in weaned pigs. J. Anim. Sci. 96: 194-205.

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particle size, milling method, and thermal treatment of feed on performance, apparent ileal digestibility, and pH of the digesta in laying hens. Poult. Sci. 94: 692-699.

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

Acknowledgments

First and foremost, I would like to express my sincere gratitude to my mentor, Professor Jürgen Zentek for his constant encouragement, invaluable advice, and continuous support throughout my academic research.

Further, I would sincerely like to thank Professor Klaus Männer, Professor Marcus Doherr and Professor Thomas Göbel for providing very helpful academic support and personal advice.

I also like to extend my thanks to my colleagues at the Institute of Animal Nutrition for the dedicated and successful cooperation. Many thanks also to the numerous doctoral candidates as well as the technical staff and animal keepers who have supported my research projects.

Finally, my deepest gratitude goes to my family and friends for their encouragement and patience during the years. In particular, I would like to thank my parents, Monika and Peter, and my brother, Oke, for their unconditional support at all times.

Annex – Original publications on which the present thesis is based

PUBLICATION 1

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

DOI: https://doi.org/10.1080/1745039X.2013.776328
You have to purchase this part online.

PUBLICATION 2

Röhe, I., Hüttner, F. J., Plendl, J., Drewes, B. and J. Zentek. 2018. Comparison of different histological protocols for the preservation and quantification of the intestinal mucus layer in pigs. Eur. J. Histochem. 62: 2874.

DOI: https://doi.org/10.4081/ejh.2018.2874

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Comparison of different histological protocols for the preservation and quantification of the intestinal mucus layer in pigs

Ilen Röhe,[1] Friedrich Joseph Hüttner,[1] Johanna Plendl,[2] Barbara Drewes,[2] Jürgen Zentek[1]

[1]Department of Veterinary Medicine, Institute of Animal Nutrition, Freie Universität Berlin
[2]Department of Veterinary Medicine, Institute of Veterinary Anatomy, Freie Universität Berlin, Germany

Correspondence: Ilen Röhe, Department of Veterinary Medicine, Institute of Animal Nutrition, Freie Universität Berlin, Königin-Luise-Straße 49, 14195 Berlin, Germany.
Tel. +49.30.83852256 – Fax: +49.30.838458299.
E-mail: ilen.roehe@fu-berlin.de

Key words: Mucus layer; mucin; intestine; pig; fixation; goblet cell; histochemistry.

Contributions: IR, FJH, literature review, study and research design, data collection, data analysis and interpretation, draft of manuscript, final manuscript revision; JP, data interpretation, critical manuscript revision; BD, technical analysis, data collection, critical manuscript revision; JZ, study and research design, data interpretation, critical manuscript revision; IR, FJH, contributed equally to this work.

Conflict of interest: The authors indicate no conflict of interest.

Acknowledgements: The support by T. Fuhrmann-Selter is gratefully acknowledged.

Received for publication: 20 November 2017.
Accepted for publication: 18 January 2018.

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European Journal of Histochemistry 2018; 62:2874
doi:10.4081/ejh.2018.2874

Abstract

The histological characterization of the intestinal mucus layer is important for many scientific experiments investigating the interaction between intestinal microbiota, mucosal immune response and intestinal mucus production. The aim of this study was to examine and compare different fixation protocols for displaying and quantifying the intestinal mucus layer in piglets and to test which histomorphological parameters may correlate with the determined mucus layer thickness. Jejunal and colonal tissue samples of weaned piglets (n=10) were either frozen in liquid nitrogen or chemically fixed using methacarn solution. The frozen tissue samples were cryosectioned and subsequently postfixed using three different postfixatives: paraformaldehyde vapor, neutrally buffered formalin solution and ethanol solution. After dehydration, methacarn fixed tissues were embedded in paraffin wax. Both, sections of cryopreserved and methacarn fixed tissue samples were stained with Alcian blue (AB)-PAS followed by the microscopically determination of the mucus layer thickness. Different pH values of the Alcian Blue staining solution and two mucus layer thickness measuring methods were compared. In addition, various histomorphological parameters of methacarn fixed tissue samples were evaluated including the number of goblet cells and the mucin staining area. Cryopreservation in combination with chemical postfixation led to mucus preservation in the colon of piglets allowing mucus thickness measurements. Mucus could be only partly preserved in cryosections of the jejunum impeding any quantitative description of the mucus layer thickness. The application of different postfixations, varying pH values of the AB solution and different mucus layer measuring methods led to comparable results regarding the mucus layer thickness. Methacarn fixation proved to be unsuitable for mucus depiction as only mucus patches were found in the jejunum or a detachment of the mucus layer from the epithelium was observed in the colon. Correlation analyses revealed that the proportion of the mucin staining area per crypt area (relative mucin staining) measured in methacarn fixed tissue samples corresponded to the colonal mucus layer thickness determined in cryopreserved tissue samples. In conclusion, the results showed that cryopreservation using liquid nitrogen followed by chemical postfixation and AB-PAS staining led to a reliable mucus preservation allowing a mucus thickness determination in the colon of pigs. Moreover, the detected relative mucin staining area may serve as a suitable histomorphological parameter for the assessment of the intestinal mucus layer thickness. The findings obtained in this study can be used for the implementation of an improved standard for the histological description of the mucus layer in the colon of pigs.

Introduction

Intestinal mucus is produced by goblet cells and forms a dynamic interface between the external environment and the epithelial surface referred to as the intestinal mucus layer.[1] The intestinal mucus layer serves as the first physical and immunological barrier of the mucosa.[2,3] Mucus contains more than 85% water, its major components are proteins and lipids. The gel structure of intestinal mucus layer is based on secreted mucins. These are complex, hydrophilic proteins with a high number of O-glycosylations forming a substantial part of the mucus protein fraction.[4,5] Due to the labile structure of mucus, a histological description and quantification of the intestinal mucus layer is difficult from a methodological point of view.[6,7] An optimal histological fixation should preserve the original structure of the mucus providing a realistic picture of the natural situation.[8] A large number of methods have been used for mucus depiction including cryopreservation,[9] and common chemical fixation using Carnoy´s solution,[7] methacarn[10,11] and buffered paraformaldehyde solution.[12,13] As the fixation process has a strong impact on the preservation of the mucus in histological sections,[14] data regarding the dimension of the mucus layer thickness in humans and animal species differ considerably depending on the applied fixation method. Thus, the mucus thickness of cryopreserved colonal samples of pigs was on average 31.9±17.6 µm although information on the process of postfixation were not provided.[9] Chemical fixation by using Carnoy´s solution indicated a mucus thickness of 215±24 µm.[15] Similarly, measured mucus thicknesses in the colon of humans ranged from 34.4±8.9 µm after fixation with Carnoy´s solution[7] to 107±48 µm in unfixed sections.[16] Due to the difficulties in the direct histological determination of the intestinal mucus layer thickness, different methodological approaches exist to ascertain histological parameters, which may correlate with the thickness of the mucus layer. Thus, the number of goblet cells or the extent of mucin staining area per villus or crypt have been evaluated.[17-19] Apart from methodological variations, the sites of tissue sampling from the alimentary tract[14] as well as factors such as feed composition[1] can influence the thickness of the mucus layer, thus limiting a meaningful comparison between studies. A standard histological methodology for tissue preparation and measurement of the intestinal mucus layer in pigs could facilitate the direct comparison of results between different studies. Moreover, pigs are often used as an animal model for humans in terms of surgical procedures but also for the investigation of cases of human diseases as they have great

similarities to humans regarding their anatomy, genetics and physiology.[20] Studies on mice showed that the intestinal mucus layer seems to play a decisive role in course of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease.[11,21] Hence, the development of a histological protocol for the description of the intestinal mucus layer in pigs as animal model for humans might also help to elucidate causes of such inflammatory diseases in the human gut. Thus, the aim of this study was to examine different histological protocols for the preparation, description and quantification of the intestinal mucus layer in the jejunum and colon of weaned piglets. Different tissue fixations, staining solutions and measuring methods of the mucus layer thickness were included. Moreover, several histomorphological parameters were determined including the intestinal goblet cell number or the intestinal mucin staining area in order to examine correlations between those parameters and the detected mucus layer thickness.

Materials and Methods

Animals and housing

Ten weaned piglets (25±2 days of age) were part of a two weeks feeding trial, approved by the local state office of health and social affairs, Landesamt Berlin (LaGeSO Reg. Nr. A 0100/13). The animals were housed in conditioned flat deck pens, each containing a male and a female piglet. Feed and water were offered *ad libitum*. Main components of the complete feed included wheat, barley, corn and soy. At the end of the trial, piglets (39±2 days of age) were anesthetized with 0.25 mL/kg body weight of ketamine hydrochloride (Ursotamin®, 10%ig, Serumwerk Bernburg AG, Bernburg, Germany) and 0.05 mL/kg body weight azaperone (Stresnil®, Jansen-Cilag, Neuss, Germany) and then euthanized with an intracardial injection of 10 mg/kg body weight tetracaine hydrochloride, mebezonium iodide and embutramide (T61®, Intervet, Unterschleißheim, Germany).

Sampling, prefixation, fixation and histochemical staining

From each of the piglets, samples of the mid jejunum and the ascending colon were either instantly frozen in liquid nitrogen or chemically fixed using methacarn solution (60% methanol, 30% chloroform, and 10% glacial acetic acid, vol/vol/vol). Cryopreserved tissue samples were included into TissueTek (Sakura Finetechnical Co., Ltd, Torrance, CA, USA), cut with a microtome-cryostat (Microm HM 560, Microm International GmbH, Dreieich, Germany) and serial sections were mounted on silanized slides and dehydrated using a heating plate at 50°C for at least 30 min. Cryopreserved tissue samples were then postfixed using three different postfixations: paraformaldehyde vapor (PFA; Merck KGaA, Darmstadt, Germany) incubated six hours at 60°C or four percent neutrally buffered formalin solution (NBF; Carl Roth, Karlsruhe, Germany) for 2 h or precooled 100% ethanol solution (EtOH; Berkel GmbH, Germany) for one hour at -80°C. Methacarn fixed samples were dehydrated by 100 % methanol (2 x 30 min) followed by 100% ethanol (2 x 20 min), cleared in xylene (2 x 15 min) and infiltrated with solidified paraffin wax.[22] Obtained paraffin blocks were cut in sections using a sledge microtome (type 1400, Leitz, Oberkochen, Germany). Serial sections of cryopreserved samples were stained with AB/PAS (Chroma, Waldeck, Germany) using either pH 2.5 or pH 0.5 in order to evaluate whether pH variation might have an impact on the qualitative and quantitative depiction of the intestinal mucus. The methacarn fixed sections of the colon were deparaffinized with xylene (2 x 10 min) and hydrated by descending concentrations of ethanol (100% - 70%, 3 min) and also stained with AB/PAS pH 2.5.

Estimation and measurement of the intestinal mucus layer thickness

Mucus thickness was determined by measuring three sites on each section. Each site had at least a 100 µm continuous length of mucus layer with an intact layering of epithelium, mucus and intestinal contents. The mucus layer thickness was determined by comparing the "integral" with the "10 point" measuring method. With respect to the "integral" method, the mucus area was determined by encircling the mucus forming an irregular polygon. In addition, the length of the mucosa, which was located beneath the polygon, was measured. The mucus layer thickness was subsequently calculated by dividing the mucus area by the length of the underlying mucosa. Using the "10 point" method the mucus thickness was measured from the luminal surface of the epithelium to the beginning of intestinal contents at 10 randomly chosen points (30 points per section). Mucus thickness measurements were performed using a light microscope (Axioskop 50, Zeiss, Jena, Germany) equipped with a digital camera (DS-Ri1, Nikon, Tokyo, Japan) and a corresponding analysis program (NIS-Elements 3.22.15, Nikon).

Evaluation of histomorphological parameters of methacarn fixed colonal tissue samples

Histomorphological analyses were performed in order to correlate histomorphological parameters of methacarn fixed tissue with the mucus layer thickness of cryopreserved tissue. As the mucus layer thickness could not be measured in the jejunum of cryopreserved samples, histomorphological parameters were detected in colonal tissue samples only. In total, 15 vertically oriented crypts per section were analyzed. For each crypt, the crypt depth and crypt area were measured. Furthermore, the absolute number of goblet cells (total number of cells per crypt) and the relative number of goblet cells (goblet cells per 1mm basement membrane of crypts) were determined. Moreover, the absolute mucin staining area (total mucin staining area per crypt) and the relative mucin staining area (mucin staining area in % of total crypt area) were detected. According to Hedemann *et al.*[23] all mucus cells (goblet cells and crypt secretory cells), their apical secretion as well as the mucus material present in the crypt lumen were taken into account for the determination of the mucin staining area. Histological analyses were conducted with a light microscope (BX 43, Olympus, Hamburg, Germany), which was equipped with a digital camera (DP72, Olympus) and an image analysis program (Cell Sense software, Olympus).

Statistics

Statistical analyses were performed by using SPSS 22 (IBM, Chicago, IL, USA). Based on serial sections, the different postfixation methods, AB staining solutions and thickness measurement methods were statistically evaluated using the Kruskal-Wallis test because data were not normally distributed based on Shapiro-Wilk test. As mucus thickness determination was not possible following methacarn fixation, the methodological comparison between cryopreservation followed by different postfixations and methacarn fixation was assessed descriptively. Based on Shapiro-Wilk test, data on colonal crypt depth, crypt area, goblet cell number and mucin staining area followed a normal distribution. Pearson correlation analyses were performed illustrating correlations between colonal crypt depth, crypt area, goblet cell number, mucin staining area determined in methacarn fixed samples and the thickness of the mucus layer examined in cryopreserved specimens. Differences at $P<0.05$ were considered significant.

Results

Cryopreservation using liquid nitrogen followed by the application of different chemical postfixations partly preserved the

mucus layer in the jejunum of piglets (Figure 1A). Hence, a microscopic determination of the mucus layer thickness in those samples was not feasible. By using PFA and EtOH as postfixations the jejunal mucus appeared as a patchwork with a decreasing staining intensity (Figure 1A). Mucus patches were not observed by the application of NBF as a postfixative. Chemical fixation by methacarn solution did not preserve the mucus layer in the jejunum. Merely single mucus patches were observed in the close proximity of the epithelium (Figure 1B). In the colon, cryopreservation in combination with chemical postfixation led to an intact layering of mucosa, mucus and colon contents allowing thickness measurements of the mucus layer (Figure 1 C,D). Following methacarn fixation, a detachment of the mucus layer from the intestinal epithelium was generally observed (Figure 1E). Consequently, thickness measurements of the mucus layer could not be performed.

With regard to the effect of the different postfixations, PFA fixation resulted generally in a laminar appearance of the mucus allowing a clear demarcation of the mucus layer (Figure 1D). NBF and EtOH postfixation slightly led to a displacement of the mucus layer so that mucus fragments exceeded epithelial borders and were found within the digesta (Figure 1F). However, PFA, NBF and EtOH as postfixation had no impact on the thickness of mucus layer (P=0.406 and P=0.226, Figure 2). The mucus in the sections stained with AB pH 2.5 had a darker blue color compared to AB pH 0.5. Varying pH values did not affect the mucus layer thickness in the colon (P=0.226, Figure 2). The use of the "integral" and the "10 point" measuring method led to comparable results regarding the mucus layer thickness determination (P=0.605, Figure 2 A,B).

Pearson correlation analyses showed a strong positive correlation between the crypt depth and the crypt area (Table 1) determined in histological slides of methacarn fixed colonal tissue (P≤0.001). Moreover, the crypt depth and crypt area correlated with the absolute number of goblet cells (P≤0.05) and the absolute mucin staining area (P≤0.001). Furthermore, the absolute number of goblet cells and the absolute and relative mucin staining area was correlated (P≤0.01; P≤0.05). With respect to the mucus layer thickness, measured in histological sections of cryopreserved tissue, correlation analyses showed that neither the crypt depth (Pearson coefficient: 0.272; P=0.447) and crypt area (Pearson coefficient: 0.360; P=0.307) nor the absolute or relative number of goblet cells correlated with the mucus layer thickness (Figure 3 A,B). However, correlation analyses revealed that, in contrast to the absolute mucin staining area, the relative mucin staining area was positively correlated with the mucus layer thickness (P≤0.05; Figure 3 C,D).

Discussion

The aim of this study was to develop an improved histological protocol for the preservation of intestinal mucus in histological sections, allowing a reliable determination of the mucus layer thickness in the intestine of piglets. Based on the ascertained mucus layer thickness data, correlation analyses were included in this study evaluating whether histomorphological parameters of chemically fixed tissue samples such as the number of intestinal goblet cells or the intestinal mucin staining area can be used as indirect quantitative indicator for the mucus layer thickness. The histological fixation of intestinal mucus is generally difficult due to its unstable structure.[6] A large number of histological methods exist, aiming to depict the intestinal mucus layer, which impedes the direct comparison of results gained from different studies. The results of this study proved that tissue fixation had a fundamental impact on the intestinal mucus layer visualization. Cryopreservation followed by different postfixations led to reliable mucus preservation in the colon of piglets while mucus was only partly preserved in jejunal samples. Mucus could not be sufficiently preserved by chemical fixation using methacarn neither in the jejunum nor in the colon. Cryopreservation preserves the original state of tissue samples[8] and retained mucus in its position between epithelium and intestinal content. It seems of great importance that intestinal sections are filled with digesta prior to fixation as the intestinal content protects the mucus from being washed off.[24] In this study, tissue samples were not rinsed before cryopreservation or chemical fixation in order to minimize the risk of washing away intestinal content, which might be accompanied with a loss of intestinal mucus.[16] In a former study examining different regions of the gastrointestinal tract of pigs, cryopreservation led to mucus preservation despite washing the intestinal sections in NaCl solution prior to freezing albeit a considerable variability in the measured mucus thickness within the same region was observed.[9] In a previous study comparing cryopreservation and chemical fixation using Carnoy´s solution, both procedures did not lead to reliable mucus preservation,[15] although information regarding the process of postfixation of cryopreserved samples were not mentioned. In the current study, chemical fixation using methacarn solution proved to be unsuitable for mucus preservation as only residues of intestinal mucus were observed in the jejunum while the colonal mucus layer was detached from the epithelium impeding any mucus thickness determination. A detachment of the mucus layer from the epithelium following methacarn fixation was also observed in the colon of mice.[10,11] With regard to mucus losses, similarly, Carnoy´s solution has been described to be insufficient for a reliable mucus fixation in the small intestine of mice.[25] The physical impact of liquid fixatives might cause the

Table 1. Pearson correlation coefficients of histomorphological parameters including crypt depth (µm), crypt area (mm²), absolute number of goblet cells (total number of goblet cells per crypt), relative number of goblet cells (goblet cells per 1mm basement membrane of crypts), the absolute mucin staining area (total mucin staining area per crypt in µm²) and the relative mucin staining area (mucin staining area in % of total crypt area), determined in histological slides of methacarn fixed colonal tissue of piglets (n=10).

	Crypt area	Absolute goblet cell number	Relative goblet cell number	Absolute mucin staining area	Relative mucin staining area
Crypt depth	0.970***	0.649*	-0.613	0.877***	0.178
Crypt area		0.673*	-0.544	0.941***	0.297
Absolute goblet cell number			0.194	0.797**	0.674*
Relative goblet cell number				-0.281	0.510
Absolute mucin staining area					0.599

*P≤0.05; **P≤0.01; ***P≤0.001.

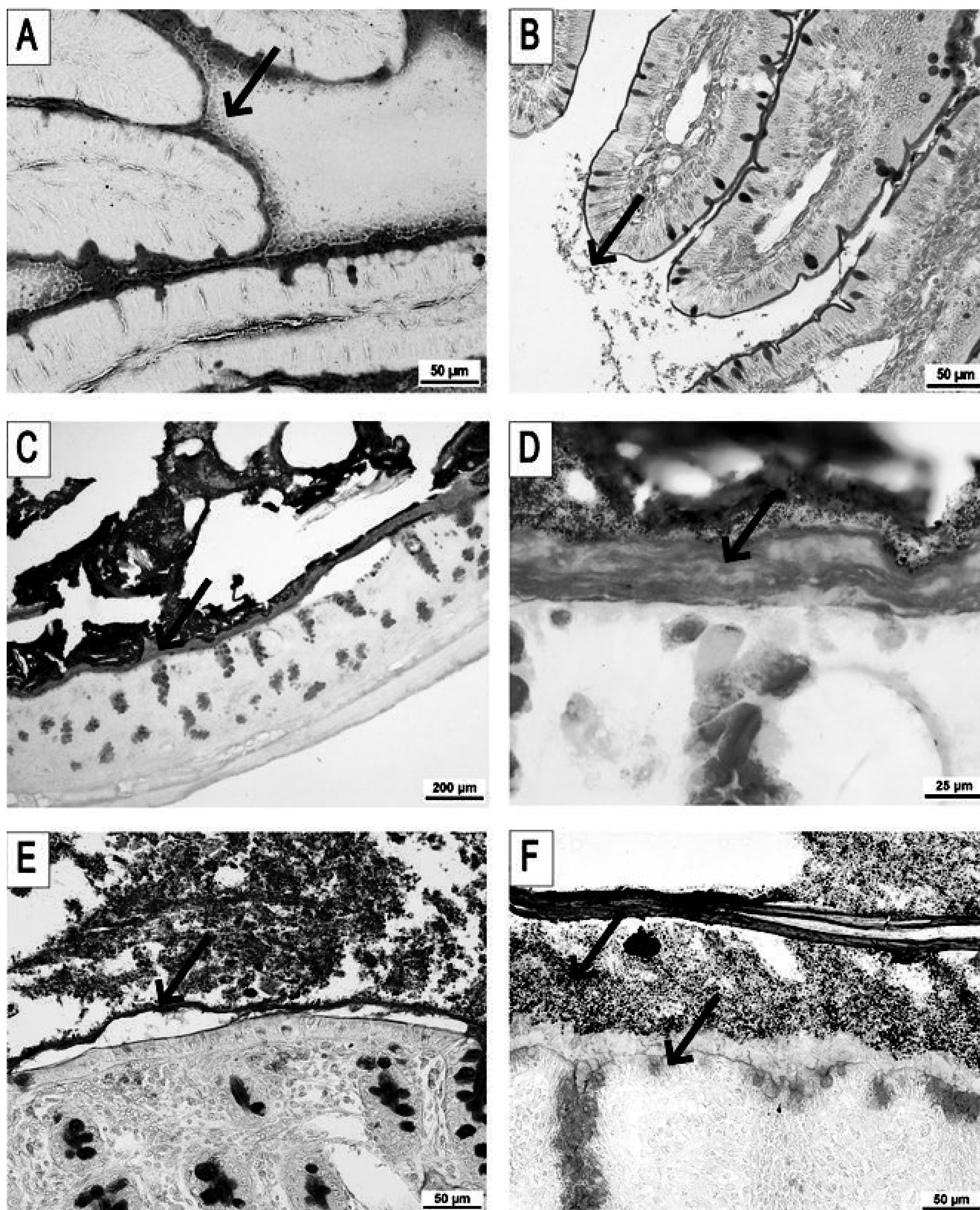

Figure 1. A) Patchy looking mucus in the jejunum; prefixation: cryopreservation; postfixation: PFA; staining: AB pH2.5-PAS. B) Single mucus patches in the jejunum; fixation: methacarn; staining: AB pH2.5-PAS. C,D) Laminar appearance of the mucus in the colon; prefixation: cryopreservation; postfixation: PFA; staining: AB pH2.5-PAS. E) Detachment of the mucus layer in the colon; fixation: methacarn; staining: AB pH2.5-PAS. F) Slight displacement of the mucus over the epithelial borders and in the digesta; prefixation: cryopreservation; postfixation: NBF; staining: AB pH2.5-PAS.

loss of mucus or intestinal content that stabilizes the mucus layer.[24] Moreover, tissue samples fixed in water-containing fixatives have to be dehydrated by ascending concentrations of alcohol and xylene in order to allow the tissue embedding in paraffin wax. This dehydration process also contributes to a shrinkage and removal of the adherent mucus layer.[26,27] Very few studies have explicitly investigated the effect of different postfixation methods on the histological depiction of the intestinal mucus layer. Postfixation of cryopreserved samples proved necessary for reliable mucus preservation in this study. PFA postfixation preserved the laminar arrangement of the colonal mucus in all sections in contrast to NBF and EtOH where partly mucus displacement over epithelial borders and in the intestinal content occurred. This could be explained by the absence of water and minor physical influence of the PFA vapor compared to the liquid fixatives NBF and EtOH. PFA has also successfully been used as a postfixative in a previous study investigating the mucus layer of the rat's stomach.[28] The use of NBF and EtOH as a postfixative can lead to protein cross-linking,[8,29] which might cause modifications of the single mucus layer. The patchy mucus distribution in the jejunum following PFA and EtOH postfixation was not observed in the colon. This difference might be explained by the different biochemical composition of jejunal and colonal mucus.[30] Rapid dehydration rates might also cause mucus patches in the jejunum. In a previous study on mice, chemical fixation using Carnoy´s solution led to threadlike mucus in the small intestine, caused by shrinkage due to the fixative solution.[27] The staining process can also have an impact on the mucus depiction.[9] Various pH levels of the AB staining solution can be used for the differentiation of mucins.[8] By using AB pH 2.5-PAS the intestinal mucus was stained in a darker blue color compared to AB pH 0.5-PAS. AB solutions with a pH of 2.5 generally stain carboxyl and sulfate groups of acid mucopolysaccharides while complex sulfated mucins are selectively stained using a pH of 0.5.[31-32] Varying pH values of the AB staining solution showed no impact on the measured mucus layer thickness. Thus, a quantification of intestinal mucus is possible using both AB pH values although a pH of 2.5 is preferred due to the higher staining intensity. In this study, variation in the mucus layer thickness measuring methods showed no impact on the quantification of the mucus thickness. In most studies, mucus thickness is determined by measuring the mucus layer at 10 to 40 randomly chosen

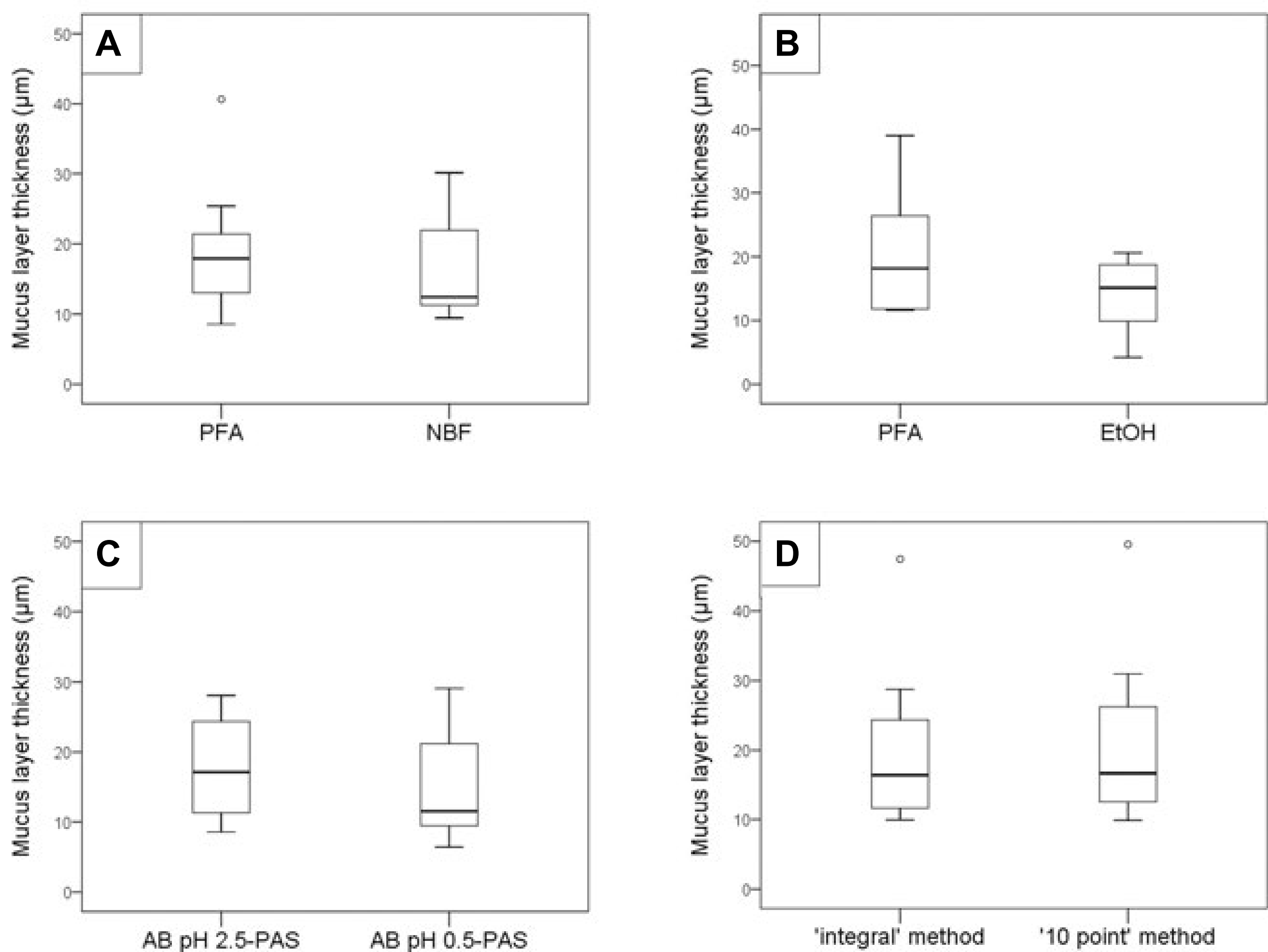

Figure 2. Comparison of different postfixations, staining solutions and measuring methods for the depiction of the mucus layer thickness in cryopreserved colonal samples(n=10). A) PFA and NBF postfixation. B) PFA and EtOH postfixation. C) AB staining with pH values 2.5 and 0.5. D) "Integral" and the "10 point"measuring method.

points.[28,33] The “integral” mucus thickness measuring method has slightly higher time expenditure, but was preferred in this study owing to higher precision of a polygon compared to 10 randomly selected points.

Correlation analyses revealed that the colonal crypt depth and crypt area were positively correlated with the absolute number of goblet cells and the absolute mucin staining area while both parameters were not related to the relative number of goblet cells and the relative mucin staining area. On the one hand this indicates that more goblet cells and hence more mucus can be measured in deeper crypts with a larger crypt area but on the other hand that the density of goblet cells and the relative proportion of the mucin staining area do not vary between crypts of different sizes. Furthermore, the absolute measured mucin area corresponded with the absolute number of goblet cells but interestingly there was no relationship between the relative number of goblet cells and the relative mucin staining area suggesting that mucus secretion is less dependent on the density of goblet cells per crypt than on the secretory capacity of each goblet cell. Due to this fact, most, but not all studies, which are focused on the histological evaluation of the goblet cell secretory activity, report data on the area of mucin granules or on the mucin staining area rather than to refer to the number of intestinal goblet cells.[17,34-36] Methodological studies evaluating the relationship between goblet cells, mucus secretion and mucus layer thickness in the intestine are scarce. To our knowledge, investigations on the histological description of the intestinal mucus layer in combination with the determination of histomorphological parameters, which may serve as indicator for the mucus layer thickness in the intestine of pigs are not available. The results of this study showed that neither the dimension of the crypt (crypt depth and crypt area) nor the absolute or relative number of goblet cells were related to the measured mucus layer thickness underlining that particularly the determination of the goblet cell number in histological slides is not an eligible tool for the assessment of the mucus layer thickness. However, the results clearly showed that, in contrast to the absolute mucin staining area, the relative mucin staining area was positively correlated with the thickness of the mucus layer emphasizing that the higher the proportion of mucins per crypt area the thicker the colonal mucus layer. As the relative mucin staining area was not dependent on the density of goblet cells per crypt length these results indicate that the secretory activity level of goblet cells is decisive for the number of produced mucins in the crypt and thus for the thickness of the mucus layer. The results of a study evaluating the effect of dietary fiber on pigs showed, that a small relative mucin staining area was observed in crypts of the small intestine of pigs fed on pectin-containing diets.[23] It was speculated that a small relative mucin staining area is associated with a lower production and secretion of mucins implying a decreased susceptibility to certain intestinal infections,[23] as conversely a large goblet cell area accompanied with an increased mucin secretory activity might correspond to a thick mucus layer.[17-37]However, the validity of this hypothesis could be not verified as investigations were focused on the qualitative and quantitative determination of the mucin staining area but not on the quantitative examination of the mucus layer thickness.[23] In 2009, Hedemann *et al.* evaluated the effect of non-digestible carbohydrates on the intestinal mucus layer in the colon of rats deter-

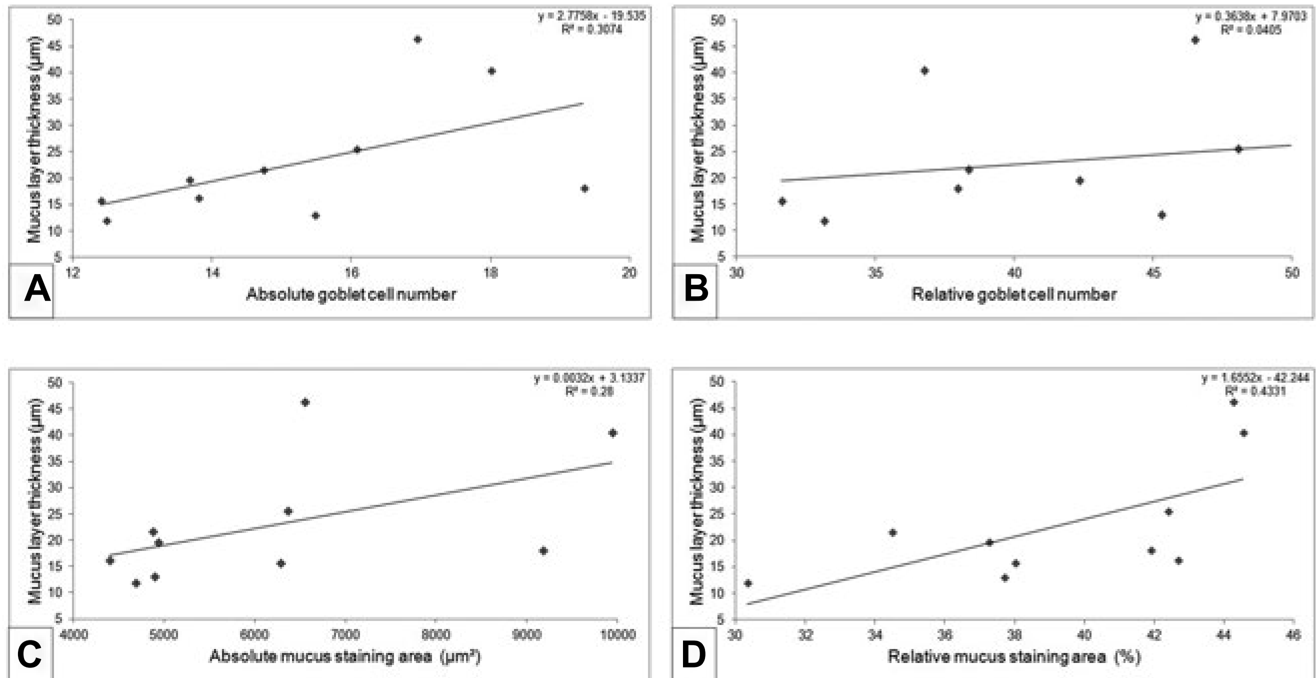

Figure 3. Correlation analyses of different histomorphological parameters, determined in histological slides of methacarn fixed tissue, and the mucus layer thickness, measured in histological sections of cryopreserved tissue, in the colon of piglets (n=10). A) Relationship between the absolute number of goblet cells (total number of goblet cells per crypt) and the mucus layer thickness (Pearson coefficient: 0.554; P=0.096). B) Relationship between the relative number of goblet cells (goblet cells per 1mm basement membrane of crypts) and the mucus layer thickness (Pearson coefficient: 0.201; P=0.577). C) Relationship between the absolute mucin staining area (total mucin staining area per crypt) and the mucus layer thickness (Pearson coefficient: 0.529; P=0.116). D) Relationship between the relative mucin staining area (mucin staining area in % of total crypt area) and the mucus layer thickness (Pearson coefficient: 0.658; P=0.039).

mining different parameters such as the mucin staining area and the mucus layer thickness.[36] The mucin staining area was qualitatively and quantitatively ascertained in histological slides of NBF fixed caecal and colonal tissue while the mucus thickness layer was measured *in vivo* using a micropipette technique. In accordance with the results of the present study, correlation analyses showed that the staining area of neutral mucins, representing the major part of mucins in goblet cells, were positively correlated with the mucus layer thickness implying that a large mucin staining area is related to an increased mucin secretion and a thick intestinal mucus layer in rats.[36] Based on the results of the current study, further analyses are needed in order to evaluate the different mucin chemotypes and their impact on the mucus layer formation in the intestine of pigs. Investigations should be focused on the determination of mucin gene expression patterns in order to ascertain the effect of intestinal mucus synthesis and secretion on the mucus layer thickness.

In conclusion, the current study contributes to the establishment of an improved standard for the histological description of mucus in the colon of pigs. The results showed that immediate cryopreservation using liquid nitrogen followed by chemical postfixation and AB-PAS staining led to a reliable mucus preservation allowing a mucus thickness determination in the colon of pigs. Moreover, the results of this study proved that the detected relative mucin staining area was correlated with the measured mucus layer thickness and thus may serve as a suitable histomorphological parameter for the assessment of the intestinal mucus layer thickness in the colon of pigs.

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PUBLICATION 3

Röhe, I., Göbel, T., Goodarzi Boroojeni, F. and J. Zentek. 2017. Effect of feeding soybean meal and differently processed peas on the gut mucosal immune system of broilers. Poult. Sci. 96: 2064-2073.

DOI: https://doi.org/10.3382/ps/pew491

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Effect of feeding soybean meal and differently processed peas on the gut mucosal immune system of broilers

I. Röhe,*,[1] T. W. Göbel,† F. Goodarzi Boroojeni,* and J. Zentek*

*Institute of Animal Nutrition, Department of Veterinary Medicine, Freie Universität Berlin, Königin-Luise-Str. 49, 14195 Berlin, Germany; and †Institute for Animal Physiology, Department of Veterinary Sciences, University of Munich, Veterinärstr 13, 80539 Munich, Germany

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ABSTRACT Peas are traditionally used as a protein source for poultry. However, peas contain antinutritional factors (ANF), which are associated with the initiation of local and systemic immune reactions. The current study examined the effect of feeding raw or differently processed peas in comparison with feeding a soybean meal (SBM) based control diet (C) on the gut mucosal immune system of broilers in a 35 day feeding trial. In six replicates, a total of 360 one-day-old male broilers were randomly allocated to four different groups receiving C, or three treatment diets containing raw, fermented, and enzymatically pre-digested peas, each supplying 30% of required crude protein. After slaughtering, jejunal samples were taken for immunohistochemical, flow cytometric, and gene expression analyses. Investigations were focused on the topological distribution of intraepithelial leukocytes (villus tip, villus mid, and crypt region) as well as on the further characterization of the different intraepithelial lymphocytes (IEL) and concomitant pro- and anti-inflammatory cytokines. Broilers receiving the raw or processed pea diets had higher numbers of intraepithelial $CD45^+$ leukocytes in the tip ($P = 0.004$) and mid region ($P < 0.001$) of villi than birds fed C. Higher numbers of intraepithelial $CD3^+$ lymphocytes were found in the villus tip ($P = 0.002$) and mid region ($P = 0.003$) of birds fed raw or processed pea containing diets in comparison with those fed C. The flow cytometric phenotyping showed a similar relative distribution of IEL among the feeding groups. The expression of intestinal pro- and anti-inflammatory cytokines was affected by feeding the different diets only to a minor extent. To conclude, feeding of diets formulated with raw and processed peas in comparison with feeding a SBM control diet initiated mucosal immune responses in the jejunum of broilers indicated by a quantitative increase of intraepithelial T cells. Further research is needed in order to ascertain the specific factors which are responsible for observed local immune reactions and how these local reactions might affect the immune status and health of broilers.

Key words: broiler, pea, feed processing, mucosal immune system, intraepithelial lymphocytes

2017 Poultry Science 96:2064–2073
http://dx.doi.org/10.3382/ps/pew491

INTRODUCTION

As the demand for protein-rich feedstuffs rapidly increases there is a growing interest in using home-grown legumes as feed protein. Peas are locally grown legumes representing a valuable energy and protein source for poultry. The nutritional composition and quality of peas differ depending on factors such as variety, location, and climate conditions (Nikolopoulou et al., 2007; Wang et al., 2010). Hence, the crude protein content of peas can range from 208 to 264 g/kg (Igbasan et al., 1997). Peas contain protein and starch as major nutrient fractions, but also antinutritional factors **(ANF)**, which can be classified in proteinaceous ANF, including antigenic proteins, trypsin inhibitors, α-amylase inhibitors, and lectins, as well as in non-proteinaceous ANF, such as alkaloids, tannins, and saponins. While trypsin and α-amylase inhibitors are known to reduce the nutrient digestibility of feed associated with reduced animal performance (Bush et al., 1992; Cowieson et al., 2003; Frikha et al., 2013), antigenic proteins and lectins interact with the intestinal mucosa and thus may initiate immune reactions (Greer, 1983; Huisman and Tolman, 1992; Lalles et al., 1996; Pusztai and Bardocz, 1996). Concerning the effect of antigenic proteins, studies showed that the feeding of peas led to systemic immune responses demonstrated by increasing antibody titers against pea proteins in the blood of calves and piglets (Nunes et al., 1987; Le Guen et al., 1991; Bush et al., 1992; Salgado et al., 2002). Specific studies on local, gut associated immune reactions are scarce and were mainly focused on immunological effects initiated by feeding different soya protein sources to calves and piglets (Kilshaw and Sissons, 1979; Li et al., 1990). The feeding of heated soybean meal **(SBM)**, which contained high levels of the antigenic proteins glycinin

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Received July 15, 2016.
Accepted December 19, 2016.
[1]Corresponding author: ilen.roehe@fu-berlin.de

and β-conglycinin, led to local mucosal T cell and B cell infiltration in piglets and calves (Dreau et al., 1995; Lalles et al., 1996). Apart from antigenic proteins, legumes contain lectins, which may also trigger immune reactions by interacting with the intestinal mucosa leading to villus atrophy as well as a destruction of intestinal microvilli and enterocytes (Pusztai et al., 1979; King et al., 1980; Kik et al., 1990). Consequently, intestinal nutrient absorption is decreased and local as well as humoral immune responses may be initiated (Donatucci et al., 1987; Kik et al., 1990; Huisman and Tolman, 1992). Based on these observations, a reduction of immunogenic factors might increase the nutritional value of legumes and attenuate immunological reactions in the host. Processing methods are available to improve the nutritional value of feed compounds by increasing the nutrient digestibility and decreasing ANF content. Several studies showed that proper thermal treatment of legumes effectively reduced the lectin content (Van Der Poel et al., 1990; Alonso et al., 1998). Furthermore, fermentation processes using bacteria or exogenous enzymes degraded antigenic proteins in SBM (Yamanishi et al., 1995; Feng et al., 2007; Wang et al., 2011).

To our best knowledge, data on chicken concerning the effect of feeding raw peas or differently processed peas on the intestinal immune system are not available. Therefore, the current study aims to prove the effect of feeding different protein sources (soya vs. pea) as well as differently processed peas on the gut mucosal immune system in broilers focusing on jejunal intraepithelial leukocytes. It was hypothesized, that the feeding of peas might modulate mucosal immune reactions and that the feeding of fermented or enzymatically predigested peas might differently affect mucosal immune responses.

MATERIAL AND METHODS

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 G. Nr. 0203/14).

Animals, Rearing Conditions and Experimental Diets

In six replicates, a total of 360 one-day-old male broiler chicks (Cobb 500) were randomly allocated to four different experimental groups of 15 birds per pen. The birds had ad libitum access to feed and water and were reared on litter-floor pens (softwood shavings). The pen represented the experimental unit. The ambient temperature was maintained at 33°C for the first experimental week, after which the temperature was gradually reduced by 3°C per week until reaching 24°C. The lighting program was carried out as follows: From day 0 to day three 24 h of light, from day 4 until day seven 20 h of light, and from day 8 to the end of the trial 16 h of light. Over a period of five weeks birds received four different experimental diets in mash form, whereby a two-phase feeding schedule of starter (1 to 21 days) and grower (21 to 35 days) feed was implemented. Four experimental diets were produced and differed in terms of protein source and treatment conditions: A control diet **(C)**, based on corn, wheat and toasted SBM (at 110°C for 3 min), and three diets containing raw peas **(RP)**, fermented peas **(FP),** and enzymatically pre-digested peas **(EP)**, each supplying 30% of required crude protein. Peas (*Pisum sativum L. Madonna*) were treated either with a probiotic or enzyme product. Ground peas, intended for the FP diet, were mixed in an equal proportion with water containing 2.57×10^8 *Bacillus subtilis* spores/kg pea (GalliPro, Chr. Hansen, Denmark). Fermentation was done for 48 h at 30°C. Ground peas, intended for the EP diet, were soaked in water (at the ratio of 1:1) containing three commercial enzyme preparations: AlphaGal^{TM} (0.1 g/kg pea - Kerry, Beloit, Wisconsin, USA) containing an α-galactosidase, RONOZYME® ProAct (0.2 g/kg pea - DSM, Kaiseraugst, Switzerland) containing a protease, and RONOZYME® VP (0.2 g/kg pea - DSM, Kaiseraugst, Switzerland) containing a mixture of pectinase and β-glucanase. Enzymatic pre digestion was done for 24 h at 30°C. After both processes, the different pea products were dried (at 75°C for less than 3 sec) and ground using a dryer mill (Ultra-Rotor Type U III a, Jäckering Mühlen- und Nährmittelwerke GmbH, Hamm, Germany). Finally, the processed pea products were used to produce the experimental diets. The feed composition and the nutrient content of the experimental diets were analyzed and are shown in Table 1. All diets were formulated to be isoenergetic and isonitrogenous and met the respective nutrient recommendations for broilers (GfE, 1999). During the experimental trial broilers were clinically inspected daily, the bodyweight (BW) and feed intake was recorded weekly and the feed conversion ratio (FCR) was calculated. At the end of trial, broilers were slaughtered by stunning and cervical decapitation, followed by exsanguination.

Sampling and Analyses

Immunohistochemical Analyses Immunohistochemical analyses **(IHC)** were focused on the determination of $CD3^+$ intraepithelial T- cells as well as $CD45^+$ intraepithelial leukocytes in the jejunal tissue of broilers fed the different experimental diets. Jejunal tissue of 12 randomly selected birds (two animals per pen of six replicate pens) per feeding group was used for IHC analyses. Immediately after slaughtering, 8 to 10 cm segments were removed from the mid-jejunum, defined as tissue located in the midway between the point of entry of the bile ducts and Meckel's diverticulum. Tissue sections were cut

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Table 1. Feed composition (% unless noted) and analyzed nutrient content of experimental starter (St: 1-21-day-old) and grower (Gr: 22-35-day-old) broiler diets.[1]

Diets	C[2]		RP		FP		EP	
	St	Gr	St	Gr	St	Gr	St	Gr
Ingredient (%)								
Pea product	0	0	31.8	26.7	30.2	25.4	31.3	26.3
Maize	31.5	34.8	7.6	14.7	9.8	16.7	8.3	15.3
Wheat	20	30.0	20.0	30.0	20.0	30.0	20.0	30.0
Soybean meal (CP 44%)	36.7	24.9	25.0	15.0	24.5	14.6	24.8	14.9
Soybean oil	7.40	5.70	10.0	7.9	9.8	7.7	9.9	7.8
Premix[3]	1.2	1.20	1.2	1.2	1.2	1.2	1.2	1.2
MCP	1.42	1.13	1.6	1.3	1.6	1.3	1.6	1.3
Limestone	1.46	1.12	1.4	1.1	1.4	1.1	1.4	1.1
L-Lysine HCL	0.09	0.28	0.59	0.70	0.60	0.71	0.59	0.71
DL-Methionine	0.26	0.25	0.51	0.46	0.51	0.45	0.51	0.46
L-Threonine	0.02	0.12	0.32	0.37	0.32	0.37	0.32	0.37
L-Tryptophan		0.05	0.08	0.12	0.08	0.12	0.08	0.12
TiO_2[4]		0.50		0.50		0.50		0.50
Analyzed Nutrients (g/kg)								
Crude Protein	233	189	229	185	223	187	222	190
Crude Fat	91.9	81.2	120	95.8	111	88.5	117	91.5
Crude Fiber	28.7	25.1	37.6	34.3	51.4	35.4	61.5	37.0
Starch	261	359	259	340	248	346	267	323
Phosphorus	7.21	6.07	7.28	5.74	7.60	6.10	7.20	6.02
Calcium	9.16	7.38	9.26	7.13	9.37	7.44	9.17	7.66
Sodium	1.81	1.78	2.00	1.86	1.84	1.80	1.96	1.98
Potassium	8.31	6.57	7.60	6.67	7.61	6.11	7.35	6.17
Calculated								
AME_N (MJ/kg)[5]	12.57	12.65	12.57	12.65	12.57	12.65	12.57	12.65

[1]As-fed basis.
[2]C = control diet; EP = enzymatically pre-digested pea diet; FP = fermented pea diet; RP = raw pea diet.
[3]Contents per kg premix: 400,000 IU vit. A; 40,000 IU vit. D3; 8,000 mg vit. E (α-tocopherol acetate); 300 mg vit. K3; 250 mg vit. B1; 250 mg vit. B2; 2,500 mg nicotinic acid; 400 mg vit. B6; 2000 μg vit. B12; 25,000 μg biotin; 1,000 mg calcium pantothenic acid; 100 mg folic acid; 80,000 mg choline chloride; 5,000 mg Zn (zinc oxide); 2,000 mg Fe (iron carbonate); 6,000 mg Mn (manganese oxide); 1,200 mg Cu (copper sulfate-pentahydrate); 45 mg I (calcium Iodate; 30 mg Co (cobalt- (II)-sulfate-heptahydrate); 35 mg Se (sodium selenite); 130 g Na (sodium chloride); 55 g Mg (magnesium oxide).
[4]Indigestible marker (Sigma Aldrich, St. Louis, MO).
[5]Estimated according to equation of the World's Poultry Science Association (1984).

open longitudinally and placed on cork boards by using hedgehog spines and subsequently fixed in a 4% phosphate-buffered formaldehyde solution for 48 h. After dehydration and infiltration with solidified paraffin wax, the samples were embedded. The paraffin blocks were cut at 5 μm with a sledge microtome (Typ 1400, Leitz, Wetzlar, Germany), and the obtained sections were mounted on glass slides. IHC analyses were performed by the indirect immunhistochemical method using the EnVision system (EnVision+System-HRP, mouse K4007, Dako, Golstrup, Denmark) following the manufacturer's recommendations. A staining protocol was established allowing a reliable immunohistochemical characterization of $CD3^+$ T cells as well as $CD45^+$ leukocytes in jejunal tissue of broilers. Briefly, in order to enhance staining intensity, antigen retrieval was performed by heating tissue sections in citrate buffer (pH: 6.2) for 20 min at 96 to 98°C. After applying the peroxidase block solution of the EnVision system, tissue sections were incubated with primary antibodies against the CD3 T cell receptor and against the CD45 leukocyte receptor overnight at 4°C using humidity chambers (Table 2). Preliminary tests showed that the mouse-anti chicken CD3 antibody, which was used for flow cytometric phenotyping, was not suitable for IHC as cells could be not stained. Alternatively, the rat-anti human CD3 antibody was used as it cross-reacts with the chicken CD3 complex (Göbel et al., 2000). Tissue slides were then incubated with secondary antibodies conjugated with horseradish peroxidase (Table 3) and finally immune cells were visualized using a chromogenic dye. Additionally, slides were counterstained with hematoxylin (Carl Roth GmbH&Co. KG, Karlsruhe, Germany). Isotype control antibodies were used confirming the specificity of primary antibody binding. After the staining procedure, tissue slides were analyzed with a light microscope (BX 43, Olympus, Germany), which was equipped with a digital camera (DP72, Olympus, Germany) and an image analysis program (Cell Sense software, Olympus, Germany). Intraepithelial immune cells of only vertically oriented villi and crypts were examined (15 villi and 15 crypts per tissue slide, 4 slides per animal). Intraepithelial immune cells were quantified in the tip and mid region of the villi per 100 enterocytes and in the crypt region per 10.000 μm^2.

Flow Cytometric Analyses Flow cytometric analyses **(FC)** were performed in order to further

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Table 2. Primary antibodies used for phenotyping of intraepithelial immune cells.

Primary antibody	Isotype	Dilution	Antigen specificity	Used for
Rat anti-human CD3[1] Clone: CD3-12	IgG1	1:100	CD3	IHC
Mouse anti-chicken CD45[1] Clone: UM 16-6	IgG2a	1:200, 1:400	CD45	IHC, FC
Mouse anti-chicken CD3[1] Clone: CT-3	IgG1	1:100	CD3	FC
Mouse anti-chicken CD8α:RPE[1] clone: 11–39	IgG1	1:10	CD8 alpha	FC
Mouse anti-chicken TCRγ/δ[1] clone: TCR-1	IgG1	1:50	γ/δ T cell receptor	FC
Mouse anti-chicken TCR$\alpha\beta$/Vβ1 clone: TCR 2	IgG1	1:100	α/β T cell receptor	FC
Mouse anti-chicken TCR$\alpha\beta$/Vβ2 clone: TCR3	IgG1	1:100	α/β T cell receptor	FC
Mouse anti-chicken 28-4	IgG3	1:100	NK cell receptor	FC
Mouse anti-chicken CD4-FITC[1] clone: 2–35	IgG2b	1:200	CD 4	FC

[1]AbD Serotec, Düsseldorf, Germany.

Table 3. Secondary antibodies used for phenotyping of intraepithelial immune cells.

Secondary antibody	Primary antibody	Dilution	Used for
Goat anti-rat IgG HRP[2]	Rat anti-human CD3[1]	1:500	IHC
Goat anti-mouse IgG HRP[3]	Mouse anti-chicken CD45[1]	As provided	IHC
Goat anti-mouse IgG2a[4] Alexa Fluor 647	Mouse anti-chicken CD45[1]	1:400	FC
Goat F(ab)$_2$ anti-mouse IgG1[2] R-PE	Mouse anti-chicken CD3[1] mouse anti-chicken TCRγ/δ[1] mouse anti-chicken TCR$\alpha\beta$/Vβ1 mouse anti-chicken TCR$\alpha\beta$/Vβ2	1:200	FC
Human anti-mouse IgG3[1] FITC	Mouse anti-chicken 28-4	1:100	FC

[1]AbD Serotec, Düsseldorf, Germany.
[2]Southern Biotech, Birmingham, AL.
[3]Dako, Golstrup, Denmark.
[4]Life Technologies, Darmstadt, Germany.

characterize the different intraepithelial lymphocytes (**IEL**) of the jejunal epithelium of broilers fed with the different diets. At first, a protocol was established allowing the determination of lymphocyte populations in the jejunal epithelium of broilers. Jejunal tissue of 12 randomly selected birds (two animals per pen of six replicate pens) per feeding group was used for FC analyses. The entire jejunum of birds was resected and adhering fat and mesenteries were removed. Afterwards, segments were opened longitudinally at the mesenteric side, flushed with PBS buffer to remove fecal contents, and immediately transferred to PBS buffer.

In order to isolate and purify the intraepithelial leukocytes from the intestinal epithelium, incubation and centrifugation steps were performed as follows. Jejunal tissue samples were transferred into 100 mL of medium containing dithiothreitol (**DDT** 2 mM) and ethylenediamine tetraacetic acid (**EDTA** 1 mM) and incubated in an orbital shaker (MaxQShakers, Fisher Scientific GmbH, Schwerte, Germany) at 110 rpm for 3 × 30 min at 4°C. After every 30 min, tissue sample containing media were filtered through synthetic gauze (210-mm nylon mesh) to collect the cell suspensions. Cell suspensions were centrifuged (Heraeus®Megafuge1.0R, Thermo Scientific, Karlsruhe, Germany) at 600 × g for 10 min at 4°C and obtained cell pellets were resuspended in Hanks-balanced salt solution (**HBSS** without $Ca2^+$ and $Mg2^+$). Samples were then incubated in 60 μL DNAse (1:500 dilution) at room temperature for 5 min and centrifuged at 600 × g for 10 min at 4°C. In order to eliminate remaining cellular debris and mucus obtained cell pellets were resuspended in 25% Percoll solution and subsequently centrifuged at 600 x g for 20 min at 4°C. Cell pellets were resuspended in Roswell Park Memorial Institute Medium No. 1640 (**RPMI 1640**) and centrifuged at 300 × g for 10 min at 4°C. Finally, obtained cells were assessed according to their vitality via trypan blue (1:10 dilution) exclusion staining and adjusted in RPMI 1640 at 1 × 10^6 cells/100 μL.

For FC analyses, the purified immune cell preparations were transferred into test tubes (Falcon, Becton Dickinson, Franklin Lakes, New Jersey, USA) and respective primary antibodies were added (Table 2) After an incubation period on ice for 25 min, intraepithelial immune cells were washed with PBS with 0.5% bovine serum albumin (**BSA**) and 2 mM EDTA. After centrifugation at 300 × g for 5 min., 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 (Table 3). After an incubation period of 25 min at 0°C, the centrifugation and washing steps were repeated as described above. Finally, the test tubes were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) and the data were evaluated with BD CellQuest Pro Software. According to fluorescence characteristics, the $CD45^+$ leukocyte population could be identified constituting

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Table 4. List of primers used in this study.

Targets	Sequences of primers (5′ to 3′)	A_T (°C)[1]	Reference
β-Actin	GAGAAATTGTGCGTGACATCA CCTGAACCTCTCATTGCCA	60	Li et al. (2005)
GAPDH	GGTGGTGCTAAGCGTGTTA CCCTCCACAATGCCAA	60	Li et al. (2005)
β-2 Microglobulin	AAGGAGCCGCAGGTCTAC CTTGCTCTTTGCCGTCATAC	60	Li et al. (2005)
IFN-γ	CTCCCGATGAACGACTTGAG CTGAGACTGGCTCCTTTTCC	60	Sadeyen et al. (2004)
IL-12p40	AGACTCCAATGGGCAAATGA CTCTTCGGCAAATGGACAGT	60	Hong et al. (2006)
IL-1β	TGGGCATCAAGGGCTACA TCGGGTTGGTTGGTGATG	60	Hong et al. (2006)
IL-17	CTCCGATCCCTTATTCTCCTC AAGCGGTTGTGGTCCTCAT	60	Hong et al. (2006)
IL-2	TCTGGGACCACTGTATGCTCT ACACCAGTGGGAAACAGTATCA	60	Hong et al. (2006)
IL-4	AACATGCGTCAGCTCCTGAAT TCTGCTAGGAACTTCTCCATTGAA	60	Avery et al. (2004)
TGF-β2	TGCACTGCTATCTCCTGA ATTTTGTAAACTTCTTTGGCG	60	Sundaresan et al. (2008)

[1] A_T = annealing temperature.

a gate. Within the gated leukocyte population, the distribution and relative frequency of the different IEL were evaluated and expressed as percentages.

Gene Expression Analyses

Gene expression analyses were performed as described previously (Villodre-Tudela et al., 2015). Jejunal tissue of 12 randomly selected birds (two animals per pen of six replicate pens) per feeding group was used for gene expression analyses. Total RNA was extracted from 30 mg of jejunal tissue using the NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The mRNA quality and quantity were determined in a Bioanalyzer (Agilent 2100, Agilent, Waldbronn, Germany). Afterwards, reverse transcription of 100 ng of total RNA into cDNA in a final volume of 20 μL was carried out using the Super Script III Reverse Transcriptase First-Strand cDNA Synthesis System (Invitrogen, Carlsbad, CA). Primers for *interferon gamma (IFN-γ), interleukin 12p40 (IL-12p40), interleukin 1 beta (IL-1β), interleukin 17 (IL-17), interleukin 2 (IL-2), interleukin 4 (IL-4), and transforming growth factor-β2 (TGF-β2)* were used (Table 4). The real-time quantitative PCR was performed on a Stratagene MX3000p (Stratagene, Amsterdam, The Netherlands). The reference genes *β-Actin, Glycerinaldehyde-3-phosphat-Dehydrogenase (GAPDH) and β2-microglobilin* were used for normalization and times-fold expression was calculated based on mean cycle threshold values of the housekeeping genes with the aid of the relative expression software tool REST (Pfaffl et al., 2002).

Statistical Analyses

Statistical analyses were conducted using SPSS (version 22.0, Chicago, IL). Means and standard deviation of the means are reported for the four experimental groups (mean ± SD). The pen represented the experimental unit. The Shapiro-Wilk Test was used to test normal distribution. Due to normal distribution, statistical analyses were conducted by ANOVA and posthoc Tukey's test. The software tool REST was used for group-wise comparison and statistical analysis of relative gene expression results. Differences at $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

The current study investigated the effect of feeding different protein sources (soya vs. pea) as well as differently processed peas on the jejunal mucosal immune system in broilers. Throughout the whole experiment, broilers remained in good health. With respect to the bird performance, the final BW as well as the FCR of broilers fed the C (BW: 1884 g; FCR: 1.478), RP (BW: 1737 g; FCR: 1.469), FP (BW: 1722g; FCR: 1.463), and EP (BW: 1767g; FCR: 1.447) were comparable ($P > 0.05$). It was hypothesized that the feeding of peas modulates mucosal immune reactions and that the feeding of processed peas might alter mucosal immune responses, influencing the frequency of jejunal intraepithelial leukocytes and the expression of pro- and anti-inflammatory cytokines.

The protocols, which were established for the IHC analyses as well as the FC measurements, could be successfully applied. The gained results present basic information regarding the topological distribution of intraepithelial leukocytes and allow a further characterization of the different IEL in the jejunum of broilers. Irrespective of the experimental diets, IHC analyses revealed that intraepithelial leukocytes were most densely localized in the mid region of the villi, while smaller numbers were found in the villus tip and crypt region (Table 5; Figure 1). Furthermore, the results showed

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Table 5. Number, localization and relation of jejunal CD3$^+$ and CD45$^+$ cells/100 enterocytes in the tip and mid region of villi and in crypts/10.000 μm^2 of broilers fed with the different experimental diets.[1, 2, 3]

Phenotype	Region	C[4]	RP	FP	EP	P-value
CD3$^+$	Villus tip	13.3^b ± 4.67	21.9^a ± 2.24	21.2^a ± 4.12	23.0^a ± 4.01	0.002
	Villus mid	20.1^b ± 5.22	31.6^a ± 5.44	30.2^a ± 4.42	30.7^a ± 4.98	0.003
	Crypt	6.61 ± 3.96	12.0 ± 5.82	11.0 ± 3.05	11.7 ± 4.31	0.165
CD45$^+$	Villus tip	22.2^b ± 8.12	32.8^a ± 4.85	32.6^a ± 3.82	35.4^a ± 4.40	0.004
	Villus mid	32.3^b ± 8.25	48.7^a ± 6.43	47.1^a ± 2.66	49.0^a ± 5.26	<0.001
	Crypt	16.6 ± 2.95	19.4 ± 3.21	21.5 ± 1.57	22.0 ± 5.26	0.075
CD3$^+$/CD45$^+$ (%)	Villus tip	62.0 ± 20.0	68.1 ± 14.4	64.8 ± 6.97	64.9 ± 6.52	0.904
	Villus mid	63.4 ± 12.9	65.6 ± 14.3	63.9 ± 7.07	62.9 ± 8.54	0.981
	Crypt	38.8 ± 19.5	62.5 ± 30.0	51.7 ± 16.0	52.4 ± 12.4	0.309

[1]Results are reported as means of six replicate pens ± SD.
[2]Statistical analyses were conducted by ANOVA and posthoc Tukey's test.
[3]Means with different superscripts are significantly different ($P < 0.05$).
[4]Animals fed with: C = control diet; RP = raw pea diet; FP = fermented pea diet; EP = enzymatically pre-digested pea diet.

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that the majority of the intraepithelial leukocytes were T cells, expressing the CD3 receptor, which is consistent with data found in the literature (Lillehoj et al., 2004). On average, 64.8% of leukocytes in the tip and 64.0% of leukocytes in the mid region of the villi represented CD3$^+$ lymphocytes while in the crypt region 50.8% of leukocytes were CD3$^+$. To our best knowledge, no information is available concerning the topological distribution of intraepithelial leukocytes in the villi of the chicken small intestine. However, results of the present study are in accordance with examinations of intraepithelial leukocyte density patterns of human jejunal biopsies demonstrating that leukocyte density is greatest along the lower and middle third of the villi and decreases in the tip region (Goldstein, 2004). By contrast, IEL were homogeneously distributed in the upper, middle and base of the villus in the small intestine of pigs (Vega-Lopez et al., 1995).

The results of the IHC quantification of CD45$^+$ and CD3$^+$ cells in the jejunum of broilers showed differences between the control and the treatment groups (Table 5; Figure 1). Birds receiving the pea diets had higher numbers of intraepithelial CD45$^+$ cells in the tip ($P = 0.004$) and mid region ($P < 0.001$) of villi than broilers fed the control diet. Moreover, higher numbers of intraepithelial CD3$^+$ cells were found in the villus tip ($P = 0.002$) and mid region ($P = 0.003$) of birds fed the pea containing diets in comparisons with those fed the control diet. The number of detected immune cells in the crypt region as well as the relation of CD3$^+$ to CD45$^+$ cells in the tip, mid and crypt region of the villi were comparable among the different feeding groups.

On the basis of FC analyses, the relative frequency of various intraepithelial lymphocyte populations was determined in the jejunum using the CD45$^+$ leukocytes as reference (Table 6). Irrespective of the experimental diets, results of the immunohistochemical staining were confirmed by those of the FC analyses as the majority of leukocytes were characterized as CD3$^+$ T cells ranging from 47% in the control group to 57.1% in the treatment groups. Within the T cell population, $\gamma\delta^+$ T cells dominated compared with TCR$\alpha\beta$1$^+$ cells and TCR$\alpha\beta$2$^+$ cells, which is in accordance with previous data in chicken (Lillehoj and Chung, 1992). The majority of cells could be identified as CD8α^+ cytotoxic cells, whereas CD4$^+$ T helper cells represented only a minor subpopulation among the intraepithelial immune. Natural killer (**NK**) cells, expressing the 28-4 receptor, were the second largest immune cell population, which was also shown by Göbel et al. (2001) demonstrating that NK cells and T cells can be considered as the two dominating IEL populations in the chicken intestine. Regarding the effect of feeding the different diets the results of the FC showed that the relative frequency of IEL did not differ among the groups emphasizing that there was no shift in the lymphocyte patterns.

The expression of intestinal pro- and anti-inflammatory cytokines was generally not distinctively influenced towards "pro" or "anti" inflammatory patterns by feeding the different diets. Solely, the expression of TGF-β2 mRNA was upregulated ($P = 0.048$) and the expression of IL-1β mRNA downregulated ($P = 0.034$) in the jejunum of broilers fed with the enzymatically pre-digested pea diet, while jejunal gene expression of IFN-γ, IL-12p40, Il-17, IL-2, and IL-4 was not affected (Table 7). The cytokine TGF-β2 has anti-inflammatory potential inhibiting the activation and proliferation of different immune cells (Grande, 1997). Studies showed that cytokines of the TGF-β family are particularly involved in the regulation of T-cell homeostasis inhibiting proliferation and differentiation of T cells (Kehrl et al., 1986; Lucas et al., 2000). On the other hand, relative gene expression of the pro-inflammatory cytokine IL-1β was increased in birds of the EP group. IL-1β is particularly produced by stimulated macrophages and induces several immunological processes including T-cell proliferation and regulation of acute-phase response (Weining et al., 1998). Based on these results and the minor differences in the gene expression patterns it is not clear if other cytokines were involved in the process of observed intraepithelial T cells expansion or whether

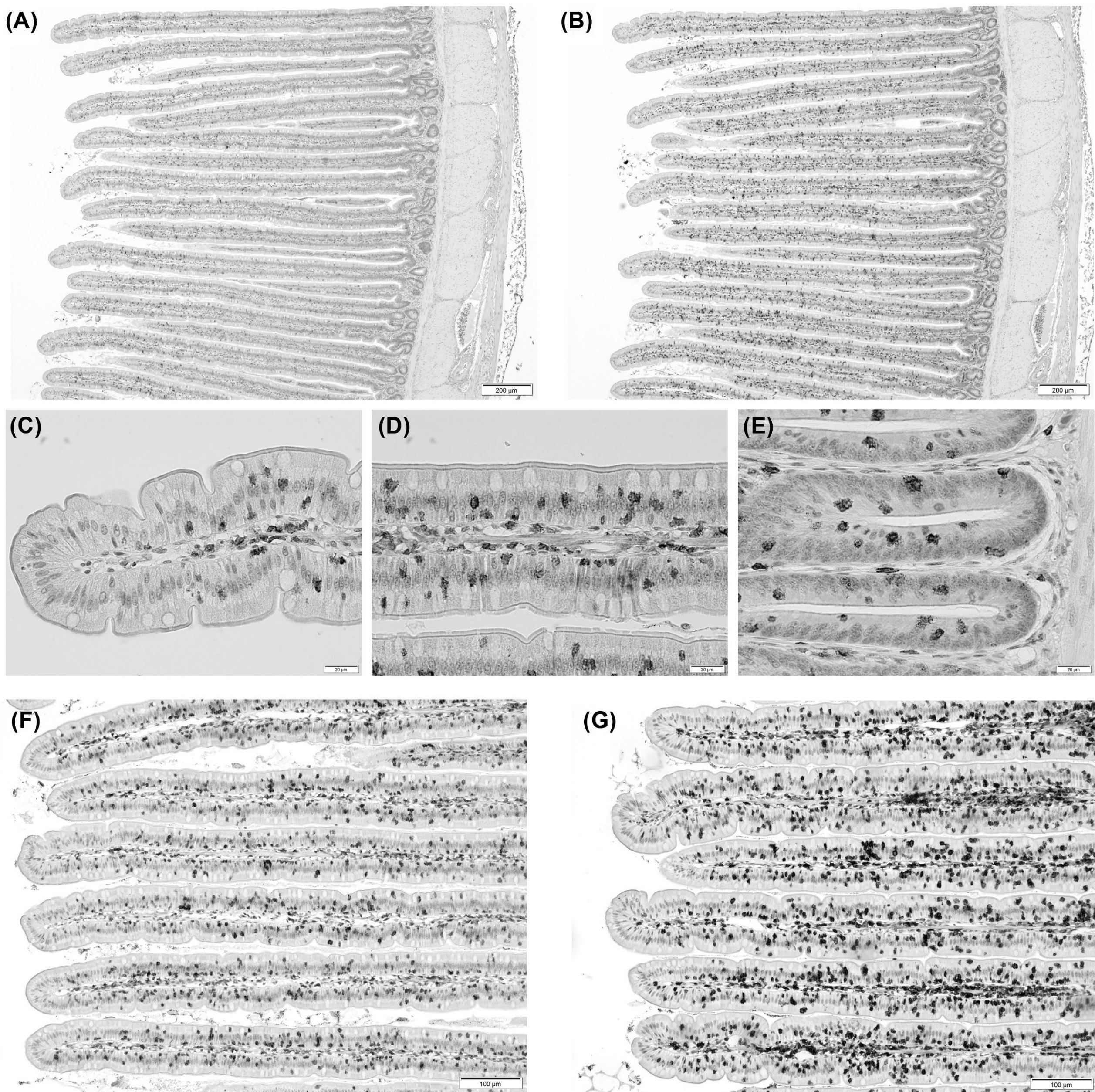

Figure 1. Light microscopic images of brown stained CD45^{+} and CD3^{+} intraepithelial cells in jejunal tissue of broilers; A: Intraepithelial CD3^{+} T cells; B: Intraepithelial CD45^{+} leukocytes (Control group); C, D, E: CD45^{+} leukocytes in the villus tip (C), villus mid (D) and crypt region (E); F, G: CD45^{+} leukocytes of broilers fed the control diet (F) or the fermented pea diet (G).

Table 6. Flow cytometric characterization of intraepithelial lymphocyte populations in the jejunum using the CD45+ leukocytes as reference (%) of broilers fed the different experimental diets. Intraepithelial lymphocyte were characterized by their specific surface antigens.[1, 2]

Phenotype	C[3]	RP	FP	EP	*P*-value
CD3+	47.0 ± 15.2	57.4 ± 13.7	59.3 ± 18.8	54.7 ± 12.0	0.530
TCR1+	35.4 ± 5.22	38.2 ± 9.66	35.2 ± 6.79	38.0 ± 10.9	0.885
TCR2+	7.32 ± 2.33	10.4 ± 6.26	9.91 ± 4.05	9.53 ± 3.41	0.620
TCR3+	6.24 ± 1.66	8.32 ± 2.26	8.68 ± 2.69	6.53 ± 0.84	0.106
CD8α+	36.8 ± 10.8	35.4 ± 8.97	38.4 ± 10.6	40.0 ± 14.8	0.907
28-4+	17.8 ± 4.70	16.5 ± 7.60	12.2 ± 5.06	16.0 ± 10.9	0.609
CD4+	12.3 ± 7.05	9.58 ± 3.11	12.0 ± 6.78	12.2 ± 4.99	0.812

[1]Results are reported as means of six replicate pens ± SD.
[2]Statistical analyses were conducted by ANOVA and posthoc Tukey's test.
[3]Animals fed with: C = control diet; RP = raw pea diet; FP = fermented pea diet; EP = enzymatically pre-digested pea diet.

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Table 7. Relative mRNA expression of pro- and anti-inflammatory cytokines in jejunal tissue of broilers fed with the different diets.[1, 2, 3]

Gene	Group[4]	Relative expression[5]	P-value
IFN-γ	RP	1.948	0.068
	FP	1.566	0.202
	EP	1.137	0.671
IL-12p40	RP	1.236	0.393
	FP	1.231	0.549
	EP	0.826	0.513
IL-1β	RP	1.091	0.655
	FP	0.903	0.595
	EP	0.747	0.034
IL-17	RP	0.837	0.670
	FP	0.600	0.242
	EP	0.643	0.353
IL-2	RP	0.918	0.714
	FP	0.847	0.533
	EP	0.875	0.583
IL-4	RP	0.906	0.517
	FP	1.003	0.985
	EP	1.120	0.471
TGF-β2	RP	1.213	0.090
	FP	1.074	0.562
	EP	1.291	0.048

[1]Results are reported as means of six replicate pens ± SD.
[2]Statistical analyses performed by a pairwise fixed reallocation randomization test using the software tool REST.
[3]Reference genes were used for normalization of the real-time PCR data.
[4]Animals fed with: RP = raw pea diet; FP = fermented pea diet; EP = enzymatically pre-digested pea diet.
[5]Gene expression of the control group was set to 1.

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the higher lymphocyte density has not influenced the expression patterns at all.

The results of this study show that the feeding of raw and processed peas initiated similar mucosal immune responses in the jejunum of broilers. This observation reveals two aspects: firstly, in comparison with the SBM based control diet, the fed pea products contained substances, which lead to a T-cell accumulation in the gut mucosa; secondly, neither fermentation nor enzymatic pre-digestion of peas had an influence on the efficacy of these immunomodulating compounds. In literature, legumes containing ANF are discussed to be responsible for immunomodulating effects focusing on the one hand on antigenic proteins, such as legumin and vicilin in peas or glycinin and β-conglycinin in soya, and on the other hand on the glycoprotein lectin. With respect to antigenic proteins, only few studies examined the effect of feeding peas on the gut immune system, whereas solely systemic immune responses were investigated. Increased anti-legumin and anti-vicilin plasma titers were observed in calves and piglets after feeding raw peas (Nunes et al., 1987; Le Guen et al., 1991; Bush et al., 1992; Salgado et al., 2002). Data concerning the effect of feeding peas on the intestinal immune system of chickens are not available to our best knowledge. However, the potential role of antigenic proteins as immunomodulating compounds were examined in several studies approaching the effect of feeding soybean proteins on the local and systemic immune system (Kilshaw and Sissons, 1979; Li et al., 1990; Lallès et al., 1995; Feng et al., 2007).With respect to local immune reactions, mucosal T-cell and B-cell infiltration as well as an increase of IgA and IgG positive plasma cells were found in the duodenum and jejunum of piglets and calves after feeding heated SBM (Dreau et al., 1995; Lalles et al., 1996). The heated soybean diets contained high levels of glycinin and β-conglycinin; alcohol-treated soya protein concentrate (Dreau et al., 1995) or hydrolyzed soya protein isolate (Lalles et al., 1996) were used as control diets being virtually free of those storage proteins. Hence, the authors conjectured that glycinin and β-conglycinin might be responsible for inducing hypersensitivity reactions in the intestinal mucosa, although the effect of other ANF, such as lectins, could not be ruled out (Dreau et al., 1995). In this study, the feeding of the control diet containing toasted SBM led to the lowest frequency of mucosal intraepithelial T cells. Considering immune reactions caused by food or feed components it seems of great importance to define terms used in this area. As reviewed, the terms hypersensitivity, hypersensitivity reaction and allergy should be used in connection with immune reactions initiated by a defined stimulus and a specific immunologic mechanism in individuals, which are susceptible to a certain food or feed component (Johansson et al., 2001; Johansson et al., 2004; Cianferoni and Spergel, 2009). In the present work as in previous studies most of the animals, which were fed with pea or soya proteins showed immune reactions, which underline that the majority of animals were susceptible. Therefore, it can be assumed that the observed immune reactions after feeding of legume proteins are not be equated with hypersensitivity reactions as described e.g., for humans in relation to allergic reactions caused by peanut or wheat consumption.

Apart from specific antigenic pea or soya proteins, plant lectins are associated with the initiation of local and systemic immune response. Lectins are carbohydrate-binding, non-digestible proteins, which can be found in varying quantities in several legume seeds e.g. peas, beans, lupins, or soybeans. Lectins interact with the intestinal mucosa accompanied by a destruction of enterocytes, an alteration of villi and crypts and a decreasing nutrient absorption may followed by local as well as humoral immune response (Pusztai et al., 1979; Donatucci et al., 1987; Kik et al., 1990; Huisman and Tolman, 1992). Lectins and lectin peptides showed mitogenic activity leading to an activation and proliferation of T lymphocytes and inflammatory response in mice and rats (Greer, 1983; Licastro et al., 1993; Benjamin et al., 1997; Lima et al., 1999). By feeding fish with either lectin containing SBM or pure soybean agglutinin, pathological changes in the intestinal tract were detected. A destruction of villus integrity and cellular infiltrations into the lamina propria were observed indicating mucosal immune reactions (Buttle et al., 2001). It was suggested that soybean agglutinin rather than antigenic soya proteins were responsible for observed intestinal damage, as the feeding of both pure

soybean agglutinin and SBM showed comparable effects (Buttle et al., 2001). Lectins can be eliminated by proper heat treatment (Van Der Poel et al., 1990; Alonso et al., 1998). The effectiveness of lectin reduction depends on the processing temperature and time. The lectin haemagglutinating activity of beans was inactivated by steaming at 100°C for 15 min (Rodriguez and Bailey, 1987) or at 136°C for 1.5 min while the heating of beans at 102°C for 5 min did not sufficiently reduce lectin content (Van Der Poel et al., 1990). In the current study, the lectin content of the used diets was not analyzed as there is a lack of standardized and validated bioassays. Therefore, it is questionable whether lectins were completely eliminated by processing, particularly with regard to the pea products.

Legumes containing ANF could also indirectly affect the intestinal immune system by influencing the intestinal microbiota leading to changes in the microbial composition due to enhanced fermentation of oligosaccharides or non-starch polysaccharides (Choct et al., 1996; Rubio et al., 1998; Olkowski et al., 2010). As reviewed by Brisbin et al. (2008) it is presumed that, similar to mammals, commensal bacteria and bacterial fermentation products directly interact with IEL triggering innate and adaptive immune reactions in chickens. Further research is required in order to prove whether and which pea related ANF might be responsible for observed local immune reactions. In this context, it is essential to develop analytical methods allowing a reliable determination of the different ANF present in legume seeds. Moreover, it would be important to examine whether these local immune reactions are accompanied with systemic immune responses and in which way the intestinal microbiota might be involved.

CONCLUSION

In conclusion, the present study provides information on the topological distribution of intraepithelial leukocytes as well as allowed a characterization of the different IEL in the jejunum of broilers. Furthermore, the results of this work showed that the implementation of peas in broiler diets resulted in an increase of jejunal intraepithelial T cells suggesting an immune modulating effect of pea containing ANF. Neither bacterial fermentation nor the usage of exogenous enzymatic pre-digestion of peas influenced the frequency of detected immune cells in the jejunal epithelium. Whether legume derived antigenic proteins, lectins or other ANF might be responsible for observed mucosal immune responses and whether these reactions have a beneficial or detrimental impact on the immune status and health of broilers needs further clarification.

ACKNOWLEDGMENTS

This work has been funded by CORNET through AiF Projekt GmbH (106 EN/1 (OE142/13a).

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PUBLICATION 4

Röhe, I., Goodarzi Boroojeni, F. and J. Zentek. 2017. Effect of feeding soybean meal and differently processed peas on intestinal morphology and functional glucose transport in the small intestine of broilers. Poult. Sci. 96: 4075-4084.

DOI: https://doi.org/10.3382/ps/pex199

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

I. Röhe,[1] F. Goodarzi Boroojeni, and J. Zentek

Institute of Animal Nutrition, Department of Veterinary Medicine, Freie Universität Berlin, Königin-Luise-Str. 49, 14195 Berlin, Germany

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ABSTRACT Peas are locally grown legumes being rich in protein and starch. However, the broad usage of peas as a feed component in poultry nutrition is limited to anti-nutritional factors, which might impair gut morphology and function. This study investigated the effect of feeding raw or differently processed peas compared with feeding a soybean meal-based control diet (C) on intestinal morphology and nutrient transport in broilers. A total of 360 day-old broiler chicks were fed with one of the following diets: The C diet, and 3 diets containing raw peas (RP), fermented peas (FP) and enzymatically pre-digested peas (EP), each supplying 30% of dietary crude protein. After 35 d, jejunal samples of broilers were taken for analyzing histomorphological parameters, active glucose transport in Ussing chambers and the expression of genes related to glucose absorption, intestinal permeability and cell maturation. Villus length ($P = 0.017$) and crypt depth ($P = 0.009$) of EP-fed broilers were shorter compared to birds received C. The villus surface area was larger in broilers fed C compared to those fed with the pea-containing feed ($P = 0.005$). Glucose transport was higher for broilers fed C in comparison to birds fed with the EP diet ($P = 0.044$). The sodium-dependent glucose co-transporter 1 (SGLT-1) expression was down-regulated in RP ($P = 0.028$) and FP ($P = 0.015$) fed broilers. Correlation analyses show that jejunal villus length negatively correlates with the previously published number of jejunal intraepithelial T cells ($P = 0.014$) and that jejunal glucose transport was negatively correlated with the occurrence of jejunal intraepithelial leukocytes ($P = 0.041$). To conclude, the feeding of raw and processed pea containing diets compared to a soybean based diet reduced the jejunal mucosal surface area of broilers, which on average was accompanied by lower glucose transport capacities. These morphological and functional alterations were associated with observed mucosal immune reactions. Further studies are required elucidating the specific components in peas provoking such effects and whether these effects have a beneficial or detrimental impact on gut function and animal health.

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

2017 Poultry Science 96:4075–4084
http://dx.doi.org/10.3382/ps/pex199

INTRODUCTION

In Europe, considerable efforts are being made to promote the usage of locally grown legumes as a protein source for animal feed. The reasons for these attempts are diverse, ranging from the economic interest of being independent of soybean imports to social demands and consumer expectations regarding the absence of genetically modified feed. Legumes can be produced in an ecologically and environmentally friendly manner by local farmers. Peas are a traditional grown protein source rich in essential amino acids and starch, although the nutritional composition and quality may vary based on variety, location, and climate conditions (Nikolopoulou et al., 2007; Barac et al., 2010). Thus, the protein content can range from 208 to 264 g/kg (Igbasan et al., 1997). In comparison with soybeans, peas contain higher amounts of lysine, similar concentrations of threonine, but lower proportions of sulfur amino acids and tryptophan (Lallès, 1993; Gatel, 1994). The nutritional value of peas is limited due to several anti-nutritional factors (**ANF**) that could impair the nutrient digestibility, and thus animal performance (Cowieson et al., 2003; Meng and Slominski, 2005; Moschini et al., 2005). In this regard, studies showed that feeding raw peas influenced the development of the intestinal microstructure in pigs leading to villus atrophy and alterations of the villus morphology (Mekbungwan et al., 2003; Mekbungwan and Yamauchi, 2004). Moreover, the feeding of raw and differently processed peas resulted in a quantitative increase of intraepithelial T cells in the jejunum of broilers, suggesting an immune-modulating effect of peas (Röhe et al., 2017). Mucosal damage and the occurrence of mucosal immune

© 2017 Poultry Science Association Inc.
Received March 16, 2017.
Accepted June 16, 2017.
[1]Corresponding author: ilen.roehe@fu-berlin.de

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

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

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MATERIAL AND METHODS

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

Animals and Rearing Conditions

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

Experimental Diets

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

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

Diets	C[2]		RP		FP		EP	
	St	Gr	St	Gr	St	Gr	St	Gr
Ingredient (%)								
Pea product	0	0	31.8	26.7	30.2	25.4	31.3	26.3
Maize	31.5	34.8	7.6	14.7	9.8	16.7	8.3	15.3
Wheat	20	30.0	20.0	30.0	20.0	30.0	20.0	30.0
Soybean meal (CP 44%)	36.7	24.9	25.0	15.0	24.5	14.6	24.8	14.9
Soybean oil	7.40	5.70	10.0	7.9	9.8	7.7	9.9	7.8
Premix[3]	1.2	1.20	1.2	1.2	1.2	1.2	1.2	1.2
MCP	1.42	1.13	1.6	1.3	1.6	1.3	1.6	1.3
Limestone	1.46	1.12	1.4	1.1	1.4	1.1	1.4	1.1
L-Lysine HCL	0.09	0.28	0.59	0.70	0.60	0.71	0.59	0.71
DL-Methionine	0.26	0.25	0.51	0.46	0.51	0.45	0.51	0.46
L-Threonine	0.02	0.12	0.32	0.37	0.32	0.37	0.32	0.37
L-Tryptophan		0.05	0.08	0.12	0.08	0.12	0.08	0.12
TiO_2[4]		0.50		0.50		0.50		0.50
Analyzed Nutrients (g/kg)								
Crude Protein	233	189	229	185	223	187	222	190
Crude Fat	91.9	81.2	120	95.8	111	88.5	117	91.5
Crude Fiber	28.7	25.1	37.6	34.3	51.4	35.4	61.5	37.0
Starch	261	359	259	340	248	346	267	323
Phosphorus	7.21	6.07	7.28	5.74	7.60	6.10	7.20	6.02
Calcium	9.16	7.38	9.26	7.13	9.37	7.44	9.17	7.66
Sodium	1.81	1.78	2.00	1.86	1.84	1.80	1.96	1.98
Potassium	8.31	6.57	7.60	6.67	7.61	6.11	7.35	6.17
Calculated								
AME_N (MJ/kg)[5]	12.57	12.65	12.57	12.65	12.57	12.65	12.57	12.65

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

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

Sampling and Analyses

Histomorphological Analyses. Histological examinations were focused on the characterization of the jejunal microstructure of broilers fed the different experimental diets. Jejunal tissue of 12 randomly selected birds (2 animals per pen of 6 replicate pens) per feeding group was used for histological and morphometric analyses. Subsequently after slaughtering, 8 to 10 cm segments were taken from mid-jejunum, defined as tissue located in the midway between the point of entry of the bile ducts and Meckel's diverticulum. Tissue sections were cut open longitudinally and placed on cork boards by using hedgehog spines and fixed in a 4% phosphate-buffered formaldehyde solution for 48 h. After dehydration and infiltration with solidified paraffin wax, the samples were embedded. The paraffin blocks were cut at 5 μm with a sledge microtome (Typ 1400, Leitz, Wetzlar, Germany), and the obtained sections were mounted on glass slides. Tissue slides were stained with hematoxylin-eosin (Merck KGaA, Darmstadt, Germany) and analyzed with a light microscope (Photomicroscope III, Zeiss, Germany), which was equipped with a digital camera (DP72, Olympus, Germany). By using an image analysis software (CellSense software, Olympus, Germany), the following histological parameters were examined: the villus length (measured from the tip of the villi to the villus crypt junction), the crypt depth (defined as the depth of the invagination between adjacent villi), the villus length-to-crypt depth ratio, the villus area (calculated by multiplying the individual villus area by the number of villi per 1,000 μm intestinal cross-section) and the villus surface area (calculated by multiplying the

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Table 2. List of primers used in this study.

Targets	Sequences of primers (5′ to 3′)	A_T (°C)[1]	Reference
β-Actin	GAGAAATTGTGCGTGACATCA CCTGAACCTCTCATTGCCA	60	Li et al. (2005)
GAPDH	GGTGGTGCTAAGCGTGTTA CCCTCCACAATGCCAA	60	Li et al. (2005)
β2-Microglobulin	AAGGAGCCGCAGGTCTAC CTTGCTCTTTGCCGTCATAC	60	Li et al. (2005)
SGLT-1	GCCATGGCCAGGGCTTA CAATAACCTGATCTGTGCACCAGTA	60	Gilbert et al. (2007)
GLUT-2	TGTTCAGCTCCTCCAAGTACC ACAACGAACACATACGGTCC	60	Humphrey et al. (2004)
ZO-1	CTTCAGGTGTTTCTCTTCCTCCTC CTGTGGTTTCATGGCTGGATC	60	Osselaere et al. (2013)
CLDN-5	GCACAAAGCTCTCCCTC CATCACTTCTCCTTCGTCAGC	60	Osselaere et al. (2013)
ALP	CTGCTGCCTGTAGTCCTT CTGGCCCCTAAGAAAGCGAA	60	This study
CASP-3	TGCAGAAGTCTAGCAGGGAAACCC AAGTTTCCTGGCGTGTTCCTTCAG	60	Brisbin et al. (2008b)

[1]A_T = annealing temperature.

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individual villus surface area by the number of villi per 1,000 μm intestinal cross-section).

Intestinal Electrogenic Glucose Transport. Jejunal tissue of 8 randomly selected birds (2 animals per pen of 4 replicate pens) per feeding group was used investigating the active glucose transport in Ussing chambers. Ussing chambers were equipped with microcomputer-controlled voltage/current clamps (K. Mussler Scientific Instruments, Aachen, Germany) detecting transepithelial potential and conductance (**Gt**) during the experiment. As previously described (Ruhnke et al., 2013), the *Tunica mucosa* was stripped from jejunal samples and placed vertically in net-supported Ussing chamber with an exposed area of 0.79 cm^2. Tissue was bathed in modified Krebs-Henseleit buffer containing (mmol/L) NaCl, 115; $NaHCO_3$, 25; mannitol, 20; KCl, 5; Na_2HPO_4, 2.4; $CaCl_2$, 1.5; $MgCl_2$, 1.2; and NaH_2PO_4, 0.6 (pH adjusted to 7.4). The buffer solution was continually stirred, heated to 38°C, and oxygenated with carbogen. After equilibration for approximately 8 to 10 min under open-circuit conditions, the tissue was short-circuited by clamping the voltage at 0 mV. After a tissue stabilization period of about 10 min, 10 mmol/L D-glucose (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was added to the buffer solution of the mucosal side and 10 mmol/L mannitol (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to the buffer solution of the serosal side of the chamber maintaining osmotic balance across the mucosa. Since a baseline of the short-circuit current (Isc) was reached 100 μmol/L phloridzin (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany) was applied to the mucosal side. Phloridzin presents a specific inhibitor of the SGLT-1 and binds to the transporter but is not transferred, preventing glucose absorption into the epithelial cell (Ferraris and Diamond, 1986; Awad et al., 2007). Finally, after a stable Isc baseline was reached, 100 μmol/L carbamylcholine chloride (carbachol; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was applied to the serosal side of chambers. The electrical response was observed as the peak response, obtained approximately 3 min after addition of the different substrates. The electrogenic ion movements by active transport were displayed by the difference between the peak Isc/Gt and the basal Isc/Gt expressed by ΔIsc values.

Gene Expression Analyses. Gene expression analyses were performed from jejunal tissue of 12 randomly selected birds (2 animals per pen of 6 replicate pens) per feeding group. From 30 mg of jejunal tissue total RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and the mRNA quality and quantity were analyzed by a Bioanalyzer (Agilent 2100, Agilent, Waldbronn, Germany). Subsequently, reverse transcription of 100 ng of total RNA into cDNA in a final volume of 20 μL was executed using the Super Script III Reverse Transcriptase First-Strand cDNA Synthesis System (Invitrogen, Carlsbad, CA). Primers for the sodium-dependent glucose co-transporter 1 (***SGLT-1***), glucose transporter 2 (***GLUT-2***), zona occludens 1 (***ZO-1***), claudin 5 (***CLDN-5***), alkaline phosphatase (***ALP***) and caspase 3 (***CASP-3***) were used (Table 2). Primers for ALP were developed at the Institute of Animal Nutrition. The real-time quantitative PCR was conducted with a Stratagene MX3000p (Stratagene, Amsterdam, The Netherlands). The reference genes β-actin, glycerinaldehyde-3-phosphate-dehydrogenase (***GAPDH***) and β2-microglobulin were used for normalization and times-fold expression was determined based on mean cycle threshold values of the housekeeping genes using the relative expression software tool REST© (Pfaffl et al., 2002).

Statistical Analyses

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

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

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

[1]Results are reported as means of 6 replicate pens ± SD.
[2]Statistical analyses were conducted by ANOVA and post hoc Tukey's test.
[3,a,b]Means with different superscripts are significantly different ($P < 0.05$).
[4]Animals fed with: C = control diet; RP = raw pea diet; FP = fermented pea diet; EP = enzymatically pre-digested pea diet.

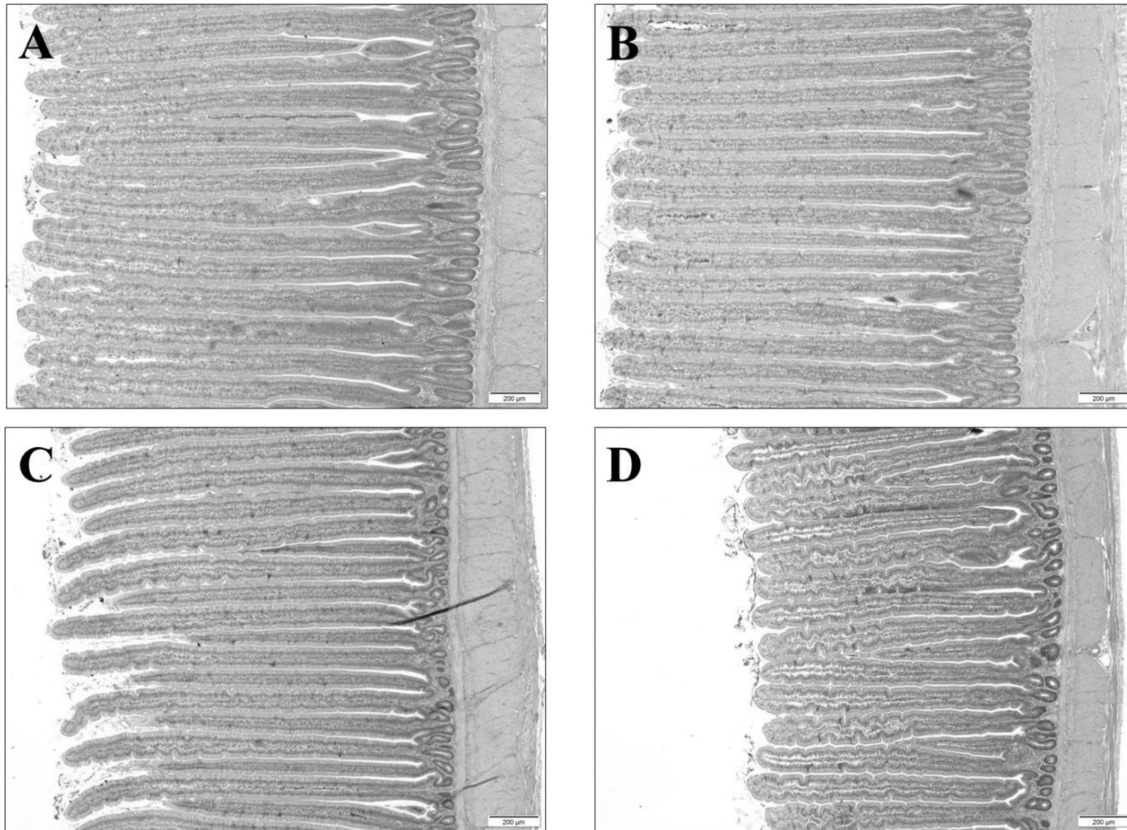

Figure 1. Typical light microscopic images of jejunal tissue of broilers fed with the control diet (A), raw peas (B), fermented peas (C) and enzymatically pre-digested peas (D). Tissue sections were stained with hematoxylin and eosin.

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

RESULTS

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

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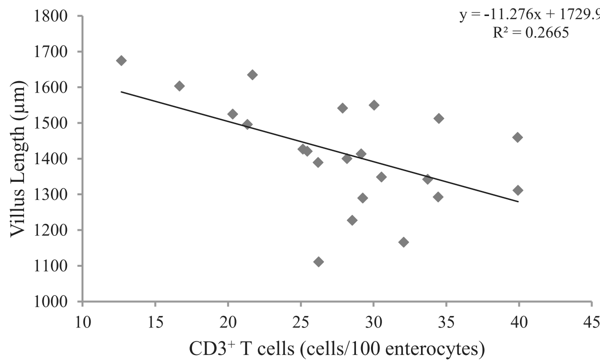

Figure 2. Relationship between jejunal villus length (n = 6) and number of jejunal intraepithelial CD3$^+$ T cells (n = 6), determined in the villus mid (Röhe et al. 2017) (Pearson coefficient: –0.516, $P < 0.05$).

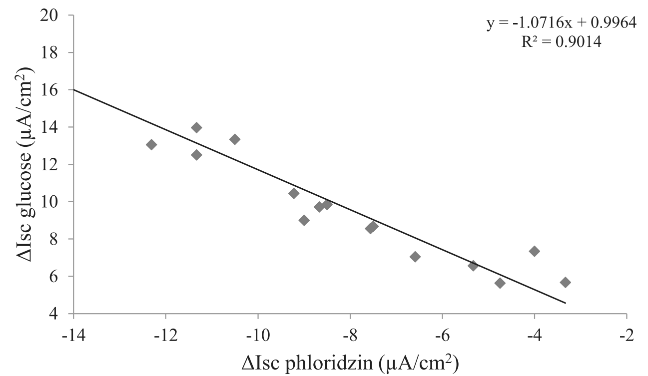

Figure 3. Relationship between the electrical responses after glucose and phloridzin addition during the Ussing chamber experiments (n = 4). Pearson coefficient: –0.949, $P < 0.01$.

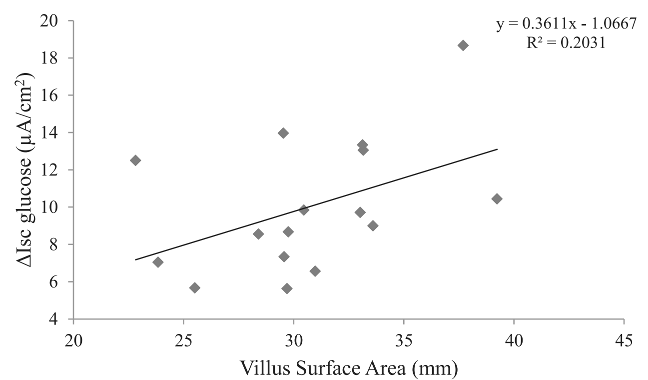

Figure 4. Relationship between the jejunal, SGLT-1 mediated glucose transport (n = 4) and the jejunal villus surface area (n = 6). Pearson coefficient: 0.451; $P = 0.08$.

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

The results of the Ussing chamber experiments are displayed in Table 4. Basal values for Gt were uniform among the feeding groups. Pearson correlation analyses show that there is a strong relationship between ΔIsc glucose and ΔIsc phloridzin (Figure 3) revealing that SGLT-1 transporters were inhibited by the addition of phloridzin and that measured values of ΔIsc glucose representing the active glucose transport across the intestinal epithelia (Pearson coefficient: –0.949, $P < 0.01$). Broilers receiving the EP diet showed lower values for ΔIsc glucose ($P = 0.044$) and ΔIsc phloridzin ($P = 0.002$) than birds fed with C. The addition of carbachol led to comparable ΔIsc responses among the feeding groups. Figure 4 displays that there is a relationship between ΔIsc glucose and the intestinal surface area, although correlation analysis showed a trend towards significance (Pearson coefficient: 0.451; $P = 0.08$). Taking into account the recently published data regarding the distribution of intraepithelial immune cells (Röhe et al. 2017), correlation analyses (Figure 5) show that the glucose uptake is negatively correlated with the occurrence of jejunal intraepithelial CD45$^+$ leucocytes in the villus mid (Pearson coefficient: –0.516; $P = 0.041$).

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

Table 4. Effect of D-Glucose, phloridzin and carbachol on short-circuit current (ΔIsc)[1] in isolated jejunal mucosa of broilers fed with the different diets.[2,3,4]

Item	C[5]	RP	FP	EP	*P*-value
Gt (mS/cm^2)	7.48 ± 2.33	7.88 ± 1.73	6.75 ± 1.23	6.01 ± 1.63	0.485
ΔIsc glucose (μA/cm^2)	12.8^a ± 4.26	11.0^{a,b} ± 2.26	9.88^{a,b} ± 2.96	6.30^b ± 0.82	0.044
ΔIsc phloridzin (μA/cm^2)	–11.3^a ± 2.64	–9.51^{a,b} ± 1.71	–8.48^{a,b} ± 2.05	–4.35^b ± 0.87	0.002
ΔIsc carbachol (μA/cm^2)	3.69 ± 1.42	5.51 ± 2.98	4.70 ± 1.25	5.89 ± 1.30	0.399

[1]ΔIsc is the difference between the basal and the maximal value obtained after adding substances.
[2]Results are reported as means of 4 replicate pens ± SD.
[3]Statistical analyses were conducted by ANOVA and post hoc Tukey's test.
[4,a,b]Means with different superscripts are significantly different ($P < 0.05$).
[5]Animals fed with: C = control diet; RP = raw pea diet; FP = fermented pea diet; EP = enzymatically pre-digested pea diet.

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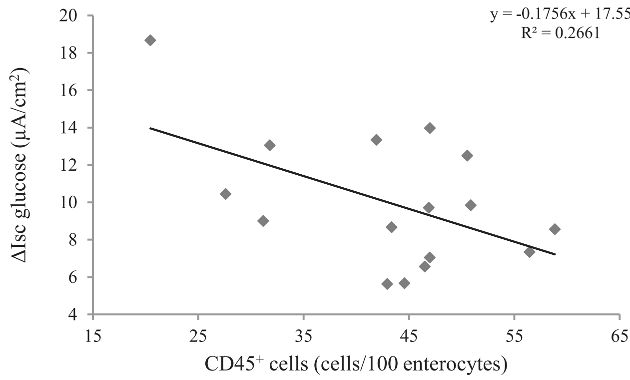

Figure 5. Relationship between the active glucose transport (n = 4) and the number of jejunal intraepithelial CD45$^+$ leucocytes (n = 6), detected in the villus mid (Röhe et al. 2017). Pearson coefficient: –0.516, $P = 0.041$.

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

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

[1]Results are reported as means of 6 replicate pens ± SD.
[2]Statistical analyses performed by a pairwise fixed reallocation randomization test using the software tool REST©.
[3]Reference genes were used for normalization of the real-time PCR data.
[4]Animals fed with: C = control diet; RP = raw pea diet; FP = fermented pea diet; EP = enzymatically pre-digested pea diet.
[5]Gene expression of the control group was set to 1.

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

DISCUSSION

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

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

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

Viewing the results, the question arises, which components in the used processed and unprocessed pea diets might lead to observed effects on gut function, intestinal morphology and immune system. Peas contain heat-labile lectins which could interact with the intestinal mucosa leading to villus atrophy and a destruction of intestinal microvilli and enterocytes may followed by mucosal immune responses (Pusztai et al., 1979; Rossi et al., 1984; Kik et al., 1990; Buttle et al., 2001). In the present study, peas were not heat treated. Although the lectin content of the different experimental diets were not determined, it seems conceivable that lectins were not eliminated by both processing methods. As highlighted by Lallès (1993), information regarding the lectin content of peas as well as soybeans is scarce due to the lack of analytical methods providing a reliable determination of functional lectins. Lectins can be directly or indirectly measured by methods such as the hemagglutination test or enzyme-linked immunosorbent assay (ELISA) techniques (Kik et al., 1990; Alonso et al., 1998; Wang et al., 2011). However, those tests do not allow a differentiation between non-toxic lectins, which have no negative impact on the gut wall, and toxic lectins (Van Der Poel et al., 1990; Huisman and Tolman, 1992), which are associated with mucosal destruction and intestinal immune responses. Apart from lectins, antigenic proteins, present in peas but also in soybeans, are considered to be responsible for intestinal villus atrophy and intestinal immune reactions in calves and piglets (Seegraber and Morrill, 1986; Bush et al., 1992; Dreau et al., 1995; Lallès et al., 1996). Antigenic proteins could be effectively degraded by fermentation using bacteria such as *Bacillus subtilis* (Kiers et al., 2000; Visessanguan et al., 2005; Song et al., 2010). Feeding of *Bacillus subtilis* fermented SBM compared to feeding conventional heat treated SBM resulted in increased villus heights in the small intestine of weaned piglets (Feng et al., 2007). In this study, the histomorphological analyses of intestinal villi and crypts showed comparable results between birds fed with diets containing fermented or unprocessed peas. This might be explained by species differences regarding pigs and birds or by the level of antigenic epitopes in the fed pea products. Apart from lectins and antigenic proteins, legumes contain also different non- digestible carbohydrates, including α-galactosides and dietary fiber, which could have an impact on the digestion of nutrients in general and by that on the intestinal microbiota and gut function (Choct et al., 1996; Teirlynck et al., 2009). In the present study, the crude fiber content of grower diets was on average higher in the pea-containing diets (ranging from 3.4% to 3.7%) compared to the SBM based diet (2.8%). Dietary fiber underwent different definitions, one is that it includes any polysaccharide reaching the large intestine as resistant starch, lignin, soluble and insoluble NSP (Montagne et al., 2003). The NSP content of peas usually range between 14% and 20% (Englyst and Hudson, 1996; Bach Knudsen, 1997) depending on variety, location, and growing conditions while about 25% of NSP represent soluble and 75% insoluble NSP (Englyst and Hudson, 1996; Periago et al., 1996; Nikolopoulou et al., 2007; Adamidou et al., 2011). Soluble NSP are known to increase gut viscosity resulting in a reduced feed passage rate (Van der Klis and Van Voorst, 1993b; Almirall and Esteve-Garcia, 1994; Choct et al., 1996), which could affect the diffusion rate of substrates, intestinal enzymatic activity, and consequently the nutrient absorption (Ikeda and Kusano, 1983; Fengler and Marquardt, 1988; Annison, 1993; Smits et al., 1997). Moreover, soluble NSP might also affect the development of intestinal mucosa as well as mucosal immune responses (Teirlynck et al., 2009). It is hypothesized that viscous digesta may enhances the rate of mucosal cell losses due to increased shear forces of the digesta (Montagne et al., 2003; Teirlynck et al., 2009). Apart from physiochemical effects it could be demonstrated that the feeding of diets supplemented with isolated soluble NSP increased both the digesta viscosity and the concentration of volatile fatty acids in the ileum of broilers suggesting a proliferation of the fermentative microbiota in the small intestine (Choct et al., 1996). Intestinal bacteria and its fermentation products may influence the development of the intestinal morphology and the mucosal immune system (Sakata, 1987; Chichlowski et al., 2007; Brisbin et al., 2008a; Awad et al., 2009). Alterations of the intestinal mucosa in connection with a mucosal T cell accumulation were also seen in this study. However, it might be expected that applied feed processing methods led to potential changes in the diet's NSP content accompanied by changes in the intestinal viscosity and intestinal microbial composition. By contrast, the observed effects on intestinal villus surface area and mucosal immune system were seen both in birds receiving the raw and processed peas. Thus, further studies are required in

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

CONCLUSIONS

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

ACKNOWLEDGMENTS

This work has been funded by CORNET through AiF Projekt GmbH (106 EN/1 (OE142/13a)).

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PUBLICATION 5

Urban, J., **Röhe, I.** and J. Zentek. 2018. Effect of protein restriction on performance, nutrient digestibility and whole body composition of male Lohmann Dual chickens. Europ. Poult. Sci. 82: 221.

DOI: https://doi.org/10.1399/eps.2018.221

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DOI: https://doi.org/10.1399/eps.2018.231
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PUBLICATION 7

Röhe, I., Urban, J., Dijkslag, A., te Paske, J. and J. Zentek. 2019. Impact of an energy-and nutrient-reduced diet containing 10% lignocellulose on animal performance, body composition and egg quality of dual purpose laying hens. Arch. Anim. Nutr. 73: 1-17.

DOI: https://doi.org/10.1080/1745039X.2018.1551950
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PUBLICATION 8

Röhe, I., Vahjen, W., Metzger, F. and J. Zentek. 2020. Effect of a “diluted” diet containing 10% lignocellulose on the gastrointestinal tract, intestinal microbiota, and excreta characteristics of dual purpose laying hens. Poult. Sci. 99: 310-319.

DOI: https://doi.org/10.3382/ps/pez492

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Effect of a "diluted" diet containing 10% lignocellulose on the gastrointestinal tract, intestinal microbiota, and excreta characteristics of dual purpose laying hens

I. Röhe,[1] W. Vahjen, F. Metzger, and J. Zentek

Institute of Animal Nutrition, Department of Veterinary Medicine, Freie Universität Berlin, Königin-Luise-Str. 49, 14195 Berlin, Germany

ABSTRACT Low performing dual purpose hens have different nutritional requirements compared to conventional hybrid hens. Lignocellulose is a low fermentable polymer, acting as a diet diluent and may influence physiological and digestive processes. This study investigated the effect of a 10% dietary lignocellulose dilution on the development of gastrointestinal organs, intestinal morphology, intestinal microbiota, and excreta characteristics of dual purpose hens. One-day-old female Lohmann Dual chicks were allocated to 12 pens and fed two different diets: A standard control diet (**CON**) and a treatment diet (**LC**), based on CON but diluted with 10% lignocellulose (ARBOCEL®). At 52 wk of age, gastrointestinal organs were extracted and weights determined. Colorectal tissue samples were chemically fixed and stained for histomorphological examinations. Cecal digesta samples were analyzed for bacterial metabolites and composition using gas chromatography, HPLC, photometry, and PCR. Excreta dry matter and viscosity was consistently assessed during the trial. LC-fed hens showed increased weights of the gizzard ($P = 0.003$), small ($P < 0.001$), and large intestine ($P = 0.048$) compared to hens fed CON. LC-fed hens had a larger colorectal villus area ($P = 0.049$), a higher mucosal enlargement factor of villi ($P = 0.016$) and crypts ($P = 0.030$) than CON-fed hens. The concentration of short-chain fatty acids (**SCFAs**) ($P = 0.017$) and ammonia ($P = 0.013$) was higher in CON-fed hens compared to LC-fed hens. Bacterial composition and activity was generally not affected by feeding the different diets. LC-fed hens had a higher excreta dry matter content than hens fed CON at 10 ($P < 0.001$), 17 ($P < 0.001$), and 22 ($P = 0.002$) wk of age. Correlation analyses revealed a negative relationship between the concentration of SCFAs in the cecum and the colorectal villus surface area ($P < 0.01$). In conclusion, the feeding of high levels of lignocellulose increased gastrointestinal organ weights and colorectal surface area in dual purpose laying hens. A higher intestinal surface area in combination with lower concentrations of SCFAs might indicate a compensatory reaction of hens fed LC enhancing the absorption of bacterial metabolites by increasing the intestinal mucosal surface.

Key words: dual purpose chicken, lignocellulose, digestive tract, gastrointestinal morphology, microbiota

2020 Poultry Science 99:310–319
http://dx.doi.org/10.3382/ps/pez492

INTRODUCTION

New approaches to avoid the killing of day-old male chicks of the layer type are necessary. The use of dual purpose chicken might be one possible solution using both sexes, the male for meat and the female for egg production. Studies showed that dual purpose hens fed with standard layer diets developed higher bodyweights and an increased body fat percentage accompanied with lower productivity in comparison with commercial hybrid hens (Rizzi et al., 2002; Rizzi et al., 2007; Rizzi and Chiericato, 2010; Steenfeldt and Hammershøj, 2015). From this, it can be concluded that low performing hens have different nutritional requirements compared to conventional hybrid laying hens. Recently published data showed that the feeding of an energy- and nutrient-reduced diet containing 10% lignocellulose reduced body fat content and improved laying performance in dual purpose laying hens (Röhe et al., 2019). The question arose whether a high concentration of dietary fiber might be accompanied with alterations of the chickens intestinal tract and microbiota. It is well known that the feeding of dietary fiber could affect the digestive tract development, intestinal morphology, and gut microbiota in poultry. Known changes depend on the used fiber source, inclusion

Received May 29, 2019.
Accepted August 15, 2019.
[1]Corresponding author: ilen.roehe@fu-berlin.de

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level, and its chemical and physical characteristics such as particle size, solubility, and degree of lignification (Hetland and Svihus, 2001; Montagne et al., 2003; De Vries et al., 2012). The term "dietary fiber" underwent different definitions, one is that it includes any polysaccharide reaching the large intestine such as resistant starch, lignin, soluble and insoluble non-starch polysaccharides (**NSP**) (Montagne et al., 2003). Lignocellulose, a constituent of plant cell walls, is mainly composed of the insoluble NSP cellulose (40 to 47 wt%) and hemicellulose (25 to 35 wt%) as well as the biopolymer lignin (16 to 31 wt%) (Liu et al., 2014). Lignocellulose is the most abundant and bio-renewable biomass on earth and has gained particular attention as potential resource for sustainable production of chemicals and fuels (Zhou et al., 2011). Moreover, research has been focused on the use of lignocellulose as a dietary component for livestock and companion animals with potential effects on digestive physiology and function. Studies showed that dietary lignocellulose at low inclusion levels up to 0.8% might stimulate the development of the digestive tract in pullets and laying hens (Yokhana et al., 2015) and enhance mucosal development in broilers (Sarikhan et al., 2010; Makivic et al., 2019). In general, chickens show a low bacterial capacity to ferment insoluble NSP because they have a high feed passage rate, a short digestive tract and limited microbial cellulolytic activity in the hindgut (McNab, 1973; Carré et al., 1990; Jørgensen et al., 1996; De Vries et al., 2012; Waite and Taylor, 2014). However, it was reported that the dietary inclusion of low concentrations of lignocellulose might modulate bacterial populations and metabolites in the small and large intestine of broilers (Sarikhan et al., 2010; Bogusławska-Tryk et al., 2015; Kheravii et al., 2017; Makivic et al., 2019). Moreover, some studies showed that dietary lignocellulose at low inclusion levels might have a beneficial effect on litter quality by lowering the excreta moisture content (Farran et al., 2013; Kheravii et al., 2017; Makivic et al., 2019).

The goal of the current study was to investigate the effect of feeding diets containing high levels of lignocellulose on the development of gastrointestinal organs, intestinal histomorphology, intestinal microbiota, and excreta characteristics in dual purpose laying hens. We hypothesized that the diet dilution by 10% lignocellulose (ARBOCEL® R, J. Rettenmaier & Söhne GmbH + Co KG, Rosenberg, Germany) would have a clear impact on the digestive tract traits, the bacterial composition and activity in the hindgut ,and excreta characteristics of dual purpose laying hens.

MATERIAL AND METHODS

All procedures involving handling and treatments of animals were approved by the local state office of occupational health and technical safety (Landesamt für Gesundheit und Soziales, Berlin, Germany, LaGeSo G 0171/16).

Animals, Rearing Conditions, and Experimental Diets

In total, one hundred thirty two 1-day-old female chicks of a dual purpose breed (Lohmann Dual, Lohmann Tierzucht, Cuxhaven) were randomly allocated to 12 pens. The birds were kept on litter-floor pens (*Miscanthus* shavings) and had *ad libitum* access to feed and water. The ambient temperature was adjusted as follows: for the first 2 d of age the ambient temperature was 35°C and was then gradually decreased to 19 ± 1°C by 35 d of age and maintained at a constant to the end of the experiment. The lighting regime was 24 h during the first 2 d, followed by a gradually reduction to 9 h of light per d until 17 wk and followed by an increase to 14 h of light per d until the end of the trial. Two different experimental diets were offered in mash form resulting in 6 replicates per feeding group: the basal control diet (**CON**) and a treatment diet (**LC**), based on CON but diluted with 10% lignocellulose (ARBOCEL® R, J. Rettenmaier & Söhne GmbH + Co KG, Rosenberg, Germany). The used lignocellulose source was produced by means of mechanical processing of fresh natural dried wood and had an average fiber length of 200 to 300 μm and a bulk density of 60 to 105 g/l, per supplier information. Detailed information on the feed composition, nutrient content, and feeding schedule are described in Röhe et al. (2019). Feed composition and analyzed nutrient content of a grower and a layer diet are displayed in Table 1. In addition, the feed discrete mean particle size (**dMean**) is indicated based on the results of the conducted dry-sieve analysis.

Sampling and Analyses

Dry-Sieve Analyses Analyses of the feed particle sizes of diets were conducted with a grower diet (6 to 12 wk of age) and a layer diet (32 to 42 wk of age). A representative 100-g sample of each diet was passed through a sieve stack situated on sieve shaker (Analysette 3, Fritsch, Idar Oberstein, Germany) for 10 min at an amplitude of 7. The sieve stack (Analysensiebe, Retsch GmbH, Haan, Germany) was composed of 9 sieves with screens of different mesh sizes (4, 2.5, 2.0, 1.6, 1.25, 1.0, 0.63, 0.40, and 0.15 mm). 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. The dMean was calculated as described earlier (Fritz et al., 2012).

Determination of Organ Weights The proventriculus, gizzard, small intestine, large intestine, and the liver were extracted from the chickens carcass and subsequently intestinal content, adhering fat, and mesenteries removed. The organs were weighed, and organ-to-BW ratios calculated. The total gastrointestinal tract weight was determined by summing the single intestinal segments.

Table 1. Feed composition (%) and analyzed nutrient content of grower (6 to 12 wk of age) and layer diets (32 to 42 wk of age).

	CON[1]		LC[1]	
	Grower	Layer	Grower	Layer
Ingredient (%)				
Wheat	22.56	39.75	20.30	35.78
Maize	30.09	21.58	27.08	19.42
Soybean meal. extracted	5.00	5.00	4.50	4.50
Rapeseed meal. extracted	4.87	4.50	4.38	4.05
Rapeseed expeller		5.50	0.00	4.95
Sunflower meal. extracted	10.00	9.80	9.00	8.82
Triticale	5.00		4.50	0.00
Barley	5.00		4.50	0.00
Wheat bran	10.00		9.00	0.00
Oat bran	3.00	1.00	2.70	0.90
ARBOCEL®R[2]			10.00	10.00
Calcium carbonate	1.39	8.51	1.25	7.66
Sodium bicarbonate	0.27	0.15	0.24	0.14
Common salt	0.18	0.23	0.16	0.21
Monocalcium phosphate	0.10	0.10	0.09	0.09
Choline chloride	0.05	0.05	0.05	0.05
Premix[3]	0.30[1]	0.30[2]	0.27	0.27
L-Lysin HCL	0.48	0.23	0.43	0.21
DL-Methionine	0.13	0.11	0.12	0.10
L-Threonin	0.08		0.07	0.00
Plant oil	1.50	3.19	1.35	2.87
Analyzed Nutrients (g/kg)				
Crude Protein	186	182	170	165
Crude Fat	39.9	64.4	35.5	56.1
NDF	134	104	195	198
ADF	67.3	62.1	154	120
ADL	14.6	20.8	45.2	37.7
Starch	430	372	375	326
Crude Ash	46.3	120	39.7	109
Calcium	6.98	33.5	6.33	29.3
Phosphorus	5.09	4.74	4.66	4.41
Sodium	1.80	1.76	1.59	1.97
Potassium	5.82	4.92	5.37	4.51
Calculated				
AME_N (MJ/kg)[4]	13.15	12.70	11.65	11.15
dMean[5]	1.47	1.54	1.36	1,37

[1]CON = control-fed birds; LC = lignocellulose-fed birds.
[2] J. Rettenmaier & Söhne GmbH+CO. KG, Rosenberg, Germany.
[3]provided per kg grower (layer): 10,000 (9,000) IU vitamin A; 2,500 (2,500) IU vitamin D3; 40.0 (20.0) mg vitamin E (α-tocopherol acetate); 1.50 (2.00) mg vitamin K3; 2.50 (1.00) mg vitamin B1; 5.00 (4.00) mg vitamin B2; 25.0 (20.0) mg nicotinic acid; 3.00 (2.00) mg vitamin B6; 25.0 (25.0) μg vitamin B12; 75 (100) μg biotin; 8.00 (6.52) mg calcium pantothenic acid; 0.80 (0.50) mg folic acid; 80.0 (50.0) mg Zn (zinc oxide); 40.0 (5.00) mg Fe (iron carbonate); 80.0 (50.0) mg Mn (manganese oxide); 15.0 (12.0) mg Cu (copper sulfate-pentahydrate); 1.00 (1.00) mg I (calcium iodate); 0.25 (0.20) mg Se (sodium selenite).
[4]AME_N (MJ/kg) = nitrogen-corrected apparent metabolizable energy estimated according to WPSA (1984).
[5]dMean = discrete mean particle size (based on dry-sieve analysis) according to equation of Fritz et al. (2012).

Histomorphological Analyses Tissue sections from the colorectum were cut open longitudinally and placed on cork boards by using hedgehog spines and fixed in a 4% phosphate-buffered formaldehyde solution for 24 h. After dehydration and infiltration with solidified paraffin wax, the samples were embedded. The paraffin blocks were cut at 4 μm with a sledge microtome (Typ SM 2000 R, Leica, Nussloch, Germany). Obtained sections were mounted on glass slides. Tissue slides were stained with AB/PAS (Chroma, Waldeck, Germany) at pH 2.5 and analyzed with a light microscope (Photomicroscope III, Zeiss, Germany), which was equipped with a digital camera (DP72, Olympus, Germany). Histomorphometric parameters were measured by using an image analysis software (CellSense software, Olympus, Germany). In total, 15 vertically oriented villi and crypts per section were analyzed. The villus length (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) was measured and based on that the villus length-to-crypt depth ratio calculated. Furthermore, villus and crypt area was assessed by multiplying the individual villus respectively crypt area by the number of villi respectively crypts per 1,000 μm intestinal cross-section. In order to estimate the enlargement of the intestinal surface epithelium by villi and crypts, the mucosal enlargement factor of the villus and crypt was determined by dividing the total villus respectively crypt surface length by the length of corresponding lamina muscularis mucosae) as described earlier (Wiese et al., 2003; Rieger et al., 2015). Furthermore, the absolute number of goblet cells (total number of goblet cells per villus respectively crypt) and the relative number of goblet cells (goblet cells per 100 μm basement membrane of villus respectively crypt) were counted. Moreover, the absolute mucin staining area (total mucin staining area per villus respectively crypt in mm^2) and the relative mucin staining area (mucin staining area per villus area respectively crypt area in %) was determined for the assessment of the intestinal mucus layer thickness (Röhe et al., 2018).

Determination of Bacterial Metabolites in the Cecum Digesta After sampling, cecal digesta was frozen in liquid nitrogen and stored at −80°C. The determination of bacterial metabolites was conducted as described by Kröger et al. (2017). Short-chain fatty acids (**SCFAs**) were analyzed by gas chromatography (Agilent Technologies 6890 N, auto sampler G2614A, and injection tower G2613A; Network GC Systems, Böblingen, Germany) equipped with a flame ionization detector. D- and L-lactate was measured by HPLC (Agilent 1100; Agilent Technologies, Böblingen, Germany) with a pre-column (Phenomenex C18 4.0 4.0 × 2.0 mm; Phenomenex Ltd., Aschaffenburg, Germany) and an analytical column (Phenomenex Chirex 3126 (D)-penicillamine 150 × 4.6 mm; Phenomenex Ltd.) Ammonia was analyzed calorimetrically by the Berthelot reaction in microtitration plates using a Tecan Sunrise microplate reader (Tecan Austria GmbH, Grödig, Austria).

Analyses of Bacterial Cell Concentration and Activity in the Cecum Digesta Digesta samples were taken from the cecum, instantly frozen in liquid nitrogen and stored at −80°C. The quantification and qualification of selected representatives of the microbiota were carried out from DNA and RNA extracts in order to assess bacterial concentration and activity. The bacterial groups were examined by seven group primers: clostridial cluster I, IV, and XIVa,

Table 2. Primers used for quantification of bacterial 16S copy numbers in cecal contents.

Specificity	Primer	Primer sequences (5′ to 3′)	Product (bp)	A_T[1]	Reference
Clostridial Cluster XIVa	g-Ccoc-F	AAATGACGGTACCTGACTAA	440	60	(Matsuki et al., 2002)
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGAA			(Matsuki et al., 2002)
Clostridial Cluster I	CI-F1	TACCHRAGGAGGAAGCCAC	231	63	(Song et al., 2004)
	CI-R2	GTTCTTCCTAATCTCTACGCAT			(Song et al., 2004)
Clostridial Cluster IV	sg-Clept-F	GCACAAGCAGTGGAGT	239	60	(Matsuki et al., 2002)
	sg-Clept-R	CTTCCTCCGTTTTGTCAA			(Matsuki et al., 2002)
Lactobacillus spp.	Lac-1	AGCAGTAGGGAATCTTCCA	341	58	(Walter et al., 2001)
	Lac-2	CACCGCTACACATGGAG			(Heilig et al., 2002)
Bifidobacterium spp.	g-BIFID-F	TCGCGTCYGGTGTGAAAG	243	58	(Rinttilä et al., 2004)
	g-BIFID-R	CCACATCCAGCRTCCAC			(Rinttilä et al., 2004)
Bacteroides-Prevotella-Porphyromonas Cluster	BPP1	GGTGTCGGCTTAAGTGCCAT	140	55	(Rinttilä et al., 2004)
	BPP2	CGGAYGTAAGGGCCGTGC			(Rinttilä et al., 2004)
E. coli/Hafnia/Shigella group	Entero-F	GTTAATACCTTTGCTCATTGA	340	55	(Malinen et al., 2003)
	Entero-R	ACCAGGGTATCTAATCCTGTT			(Malinen et al., 2003)

[1] A_T = annealing temperature (°C).

Lactobacillus spp., *Bifidobacterium* spp., the *Bacteroides-Prevotella-Porphyromonas* cluster and the E. coli/ Hafnia/ Shigella group (Table 2). DNA and RNA Extraction was performed with a commercial NucleoSpin® RNA Kit (REF 740955, Macherey-Nagel GmbH & Co. KG, Düren, Germany) in combination with the NucleoSpin® RNA/DNA Buffer Set (REF 740944, Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer, except for the use of 100 mg sample. Quantification of bacterial DNA and rRNA was performed with a Stratagene MX3000p (Stratagene, Amsterdam, The Netherlands) using a commercial master mix (Brilliant II SYBR® Green QPCR Master Mix with Low ROX (Stratagene, Amsterdam, Netherlands). Primer sequences and annealing temperatures are given in Table 2. All primers were purchased from MWG Biotech (Straubing, Germany). A calibration series of PCR products with known copy numbers per ng DNA was used to calculate copy numbers/ g sample. With respect to the quantification of bacterial 16S rRNA, total RNA was transcribed into cDNA using a commercial kit (Superscript III, ThermoFisher Scientific, Berlin, Germany) and subsequently amplified as described before.

Analyses of Dry Matter and Viscosity in Excreta

Excreta dry matter and viscosity were measured from samples taken at different time points (at weeks 10, 17, 22, and 52 of the trial). With respect to dry matter analyses, fresh excreta was weighed into aluminum jars of known weight. Samples were dried in an incubator at 103°C and weighed again after weight constancy to detect loss of water.

Viscosity was determined by adding 10 ml of water to 5 g of excreta. Samples were continuously stirred for 30 min at 30°C, followed by centrifugation for 15 min at 1854 × g at 4°C. In total, 2 ml of the supernatant was centrifuged for further 10 min at 17500 × g. Afterwards 532 μl of that supernatant was used for viscosity analysis (DV-II Viscometer, Brookfield Eng Labs inc., Stoughton, MA, USA).

Statistical Analyses

Statistical analyses were conducted using SPSS (version 25.0, Chicago, IL). Results are reported as means and standard error of the means (mean ± SEM). The normally distributed data were analyzed by using Students t test. Spearman correlation analyses were performed displaying correlations between the mucosal enlargement factor of colorectal villi and the relative weight of the gastrointestinal organs as well as between the concentration of SCFA in the cecum and the mucosal enlargement factor of colorectal villi. Non-normally distributed data from microbiological data was analyzed via Kruskal-Wallis test and subsequent Mann-Whitney-U test, where appropriate. Differences were considered significant at $P < 0.05$.

RESULTS

With respect to the particle size distribution of the experimental diets results of the dry-sieve analyses showed that the inclusion of lignocellulose led to an increase of the proportion of smaller particles resulting in a lower dMean of the LC diet compared to CON diet (Table 1). During the whole feeding trial, birds were healthy and showed no clinical evidence of disease. Results on the animal performance of dual purpose hens are display in Röhe et al. (2019).

Gastrointestinal Organ Weights

The inclusion of dietary lignocellulose affected the relative weight of gastrointestinal organs (Table 3). LC-fed hens showed increased relative weights of the gizzard ($P = 0.003$), small intestine ($P < 0.001$) and large intestine ($P = 0.048$) resulting in a higher weight of the total gastrointestinal organs ($P = 0.002$) compared to hens fed CON.

Table 3. Impact of dietary lignocellulose on the relative weight of the gastrointestinal organs (%) and liver weight (%) of dual purpose hens.[1]

Item	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
Proventriculus	0.32	0.35	0.01	0.264
Gizzard	2.36	3.51	0.22	0.003
Small intestine	1.95	2.29	0.06	<0.001
Large intestine	0.61	0.74	0.03	0.048
Total gastrointestinal tract	5.25	6.84	0.30	0.002
Liver	2.20	2.45	0.12	0.313

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Students t test.

Table 4. Impact of dietary lignocellulose on the villus length (VL), crypt depth (CD), villus length-to-crypt depth ratio (VL/CD), villus area (VA), villi mucosal enlargement factor (VMEF), crypt area (CA), crypts mucosal enlargement factor (CMEV) and ratio between enlargement factors (EFV/EVC) in the colorectum of dual purpose hens.[1]

Item	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
VL (μm)	302	331	14.5	0.329
CD (μm)	65.0	76.1	3.35	0.101
VL/CD	4.94	4.66	0.22	0.535
VA (mm^2)	0.21	0.28	0.02	0.049
VMEF	3.86	5.52	0.37	0.016
CA (mm^2)	0.036	0.043	0.002	0.214
CMEF	1.57	2.14	0.14	0.030
VMEF/CMEF	2.51	2.62	0.14	0.718

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Students *t* test.

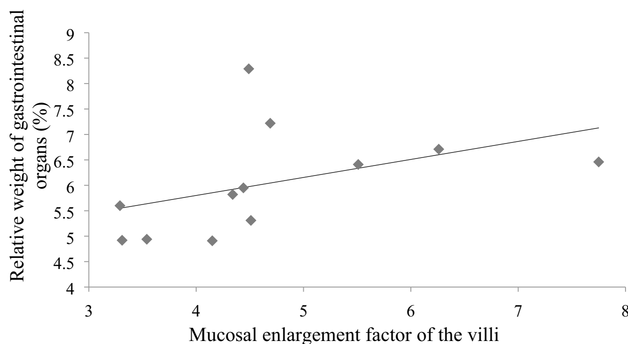

Figure 1. Correlation analyses of the mucosal enlargement factor of colorectal villi and the relative weight of gastrointestinal organs of dual purpose hens (Spearman coefficient: 0.699; $P = 0.011$).

Histomorphological Analyses

LC-fed hens had a larger colorectal villus area ($P = 0.049$) than CON-fed hens (Table 4). The mucosal enlargement factor of villi ($P = 0.016$) and crypts ($P = 0.030$) were higher in LC-fed hens compared to those fed CON. Correlation analyses revealed that the mucosal enlargement factor of colorectal villi was positively related to the relative weight of the gastrointestinal tract ($P = 0.011$; Figure 1). No differences in the number of goblet cells and the detected mucin staining area of the colorectum could be detected among the feeding groups (Table 5).

Bacterial Metabolites and Cell Counts

Analyses of bacterial metabolites in the cecum showed that the absolute concentration of acetic acid ($P = 0.018$), propionic acid ($P = 0.010$), n-valeric acid ($P = 0.001$), and the total amount of the SCFA ($P = 0.017$) were higher in CON-fed hens compared to those receiving LC (Table 6). With respect to the molar ratio, the proportion of SCFAs in the cecum was not influenced by feeding the different diets. The cecal concentration of ammonia was higher in CON-fed hens than in LC-fed hens ($P = 0.013$). Correlation analyses showed that the mucosal enlargement factor of colorectal villi ($P = 0.022$) was negatively related to the absolute concentration of SCFA in the cecum (Figure 2). Bacterial copy numbers of 16S rDNA as well as bacterial activity as measured via 16S rRNA were similar among the feeding groups (Table 7 and 8), except that the 16S rRNA of *Lactobacillus* spp. was significantly higher in CON-fed hens compared to LC-fed hens ($P = 0.002$).

Excreta Dry Matter and Viscosity

Analyses of the excreta dry matter content showed that LC-fed hens had a lower excreta water content than hens fed CON at 10 ($P < 0.001$), 17 ($P < 0.001$), and 22 ($P = 0.002$) wk of age while viscosity of excreta samples of both feeding groups were comparable during the trial (Table 9).

DISCUSSION

The bodyweight and body fat percentage of dual purpose hens was reduced by feeding a nutrient reduced diet containing a 10% lignocellulose which was accompanied with an increased laying performance (Röhe et al. 2019). The aim of the current study was to examine whether the dietary addition of high levels of lignocellulose might also influence gastrointestinal organ weights, intestinal morphology and microbiota as well as excreta characteristics of dual purpose laying hens.

Gizzard Development

The results showed that hens fed with lignocellulose had increased weights of the gizzard compared to those fed the basal diet. Several studies investigated the effect of dietary fiber on the gizzard development of chicken (Jørgensen et al., 1996; Hetland and Svihus, 2001; González-Alvarado et al., 2007; Jiménez-Moreno et al., 2009). However, only a few studies used the non-fermentable fiber source lignocellulose as feed ingredient. In line with the results of the present study, increased relative weights of the gizzard were observed in pullets fed diets containing 1% lignocellulose

Table 5. Impact of dietary lignocellulose on the absolute and relative number of goblet cells as well as the absolute and relative mucin staining area in the colorectum of dual purpose hens.[1]

Item	Region	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
Absolute goblet cell number	Villus	49.3	47.0	1.51	0.468
Relative goblet cell number	Villus	16.4	14.4	0.62	0.111
Absolute goblet cell number	Crypt	11.9	12.5	0.64	0.668
Relative goblet cell number	Crypt	18.2	16.6	0.85	0.367
Absolute mucin staining area (mm^2)	Villus	0.60	0.80	0.006	0.102
Relative mucin staining area (%)	Villus	28.2	28.2	1.09	0.982
Absolute mucin staining area (mm^2)	Crypt	0.012	0.011	0.001	0.879
Relative mucin staining area (%)	Crypt	31.6	26.1	1.54	0.070

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Students t test.

Table 6. Impact of dietary lignocellulose on the absolute and relative concentration of bacterial metabolites in the cecum digesta of dual purpose hens.[1]

Item	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
Acetic acid (μmol/g)	37.5	23.2	3.22	0.018
Propionic acid (μmol/g)	3.79	2.19	0.34	0.010
i-butyric acid (μmol/g)	0.29	0.34	0.09	0.759
n-butyric acid (μmol/g)	4.83	2.89	0.53	0.061
i-valeric acid (μmol/g)	0.51	0.28	0.12	0.355
n-valeric acid (μmol/g)	0.61	0.31	0.05	0.001
Total SCFA (μmol/g)	47.5	29.2	4.11	0.017
Acetic acid (mol. %)	78.8	80	0.85	0.530
Propionic acid (mol. %)	8.15	7.76	0.46	0.692
i-butyric acid (mol. %)	0.64	1.05	0.22	0.372
n-butyric acid (mol. %)	9.95	9.28	0.68	0.644
i-valeric acid (mol. %)	1.19	0.91	0.25	0.604
n-valeric acid (mol. %)	1.32	1.10	0.06	0.066
D-Lactate (μmol/g)	0.51	0.57	0.11	0.813
L-Lactate (μmol/g)	2.87	4.23	0.41	0.099
Ammonia (μmol/g)	8.38	4.54	0.84	0.013

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Students *t* test.

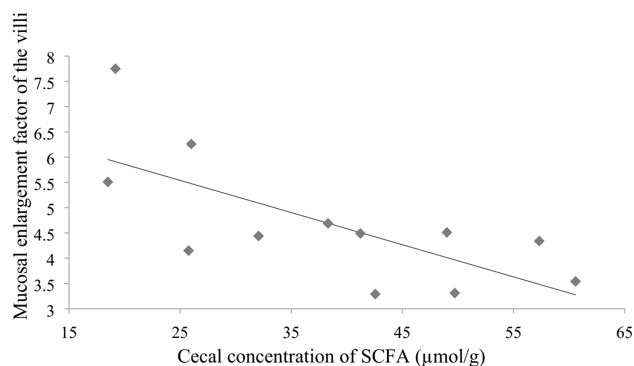

Figure 2. Correlation analyses of the concentration of short-chain fatty acids (SCFA) in the cecum and the mucosal enlargement factor of colorectal villi of dual purpose hens (Spearman coefficient: −0.650; $P = 0.022$).

(ARBOCEL® RC FINE) over a period of 10 wk (Yokhana et al., 2015). Similarly, laying hens, aged 31 wk, developed heavier gizzards when fed diets diluted with 0.8% lignocellulose (ARBOCEL® RC FINE) compared to hens fed a control diet after 12 wk of feeding (Yokhana et al., 2015). Interestingly, no effects on gizzard weight development were found in younger hens fed those diets for a shorter period of 3, 6, and 9 wk (Yokhana et al., 2015) which suggests that the length of the feeding period of lignocellulose might be relevant for the development of effects in the digestive tract. In contrast, feeding diets supplemented with 0.4 or 0.6% lignocellulose (ARBOCEL® R) did not affect gizzard weights in 42 d old broilers (Makivic et al., 2019). In another study, relative gizzard weights of broilers were also not influenced by feeding diets supplemented with 1 or 2% lignocellulose (OptiCell®, Agromed Austria GmbH) over a period of 35 d (Kheravii et al., 2017).

Several studies showed that the feeding of dietary fiber or so-called "structural components" could stimulate gizzard development in chickens. The feeding of mostly insoluble NSP sources, such as hulls of pea, oat, and soy or wood shavings, increased the gizzard weight of broilers (Jørgensen et al., 1996; González-Alvarado et al., 2007; Amerah et al., 2009; Jiménez-Moreno et al., 2009). Furthermore, insoluble NSP stimulated the gizzard function as indicated by a lower gizzard digesta pH (González-Alvarado et al., 2007; Jiménez-Moreno et al., 2009; Jiménez-Moreno et al., 2011; Makivic et al., 2019). An increase of dietary fiber was often accompanied with an increase in the proportion of coarser particles in the diets (Amerah et al., 2009; Jiménez-Moreno et al., 2009). It is well known that the feeding of coarsely ground as well as mash diets can increase the relative gizzard weights of broilers and laying hens compared to feeding finer particles and thermally processed diets (Engberg et al., 2002; Peron et al., 2005; Amerah et al., 2007; Rougière et al., 2009; Röhe et al., 2014). Consequently, it might be difficult to distinguish between the effect of fiber inclusion and that of the feed particle size. In the present study, the inclusion of lignocellulose did not increase the proportion of coarser particles in the diet, but on the contrary increased the fraction of smaller feed particles. Thus, observed effects regarding an enhanced gizzard development seem to be not connected with an increase in feed particle size. Independent of the particle size, fiber particles are harder to grind and thus accumulate in the gizzard lumen (Hetland et al., 2003), which in

Table 7. Impact of dietary lignocellulose on bacterial cell count (log10 16S rDNA copy number/g) in cecal digesta of dual purpose hens.[1]

Item	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
Clostridial cluster I	10.1	9.66	0.15	0.180
Clostridial cluster IV	10.3	10.5	0.05	0.093
Clostridial cluster XIVa	10.2	10.2	0.05	0.394
Lactobacillus spp.	8.48	8.25	0.07	0.093
Bifidobacterium spp.	8.72	8.98	0.11	0.485
Bacteroides/Prevotella/Porphyromonas-Cluster	9.92	10.1	0.08	0.310
E. coli/ Hafnia/ Shigella group	7.44	7.97	0.14	0.132

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Mann-Whitney-U-Test.

Table 8. Impact of dietary lignocellulose on the bacterial activity (log10 copy number 16S rRNA/g) in cecal digesta of dual purpose hens.[1]

Item	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
Clostridial cluster I	10.2	9.79	0.15	0.093
Clostridial cluster IV	12.8	12.8	0.05	0.818
Clostridial cluster XIVa	13.4	13.6	0.06	0.132
Lactobacillus spp.	10.2	9.58	0.11	0.002
Bifidobacterium spp.	9.87	10.0	0.11	0.589
Bacteroides/Prevotella/Porphyromonas Cluster	11.7	11.35	0.08	0.937
E. coli/ Hafnia/ Shigella group	6.95	7.15	0.13	0.905

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Mann-Whitney-U-Test.

Table 9. Impact of dietary lignocellulose on the excreta dry matter (DM) and -viscosity of dual purpose hens at different time points of the trial.[1]

Weeks of age	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
Excreta DM (%)				
Week 10	19.5	22.7	0.53	<0.001
Week 17	22.1	25.5	0.59	<0.001
Week 22	21.1	25.5	0.85	0.002
Week 52	22.1	22.7	0.50	0.596
Viscosity (mPas)				
Week 10	2.05	1.76	0.13	0.287
Week 17	1.55	1.35	0.06	0.095
Week 22	1.28	1.30	0.05	0.815
Week 52	1.33	1.36	0.07	0.862

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Students t test.

turn might stimulate organ development and function (Hetland et al., 2005; Mateos et al., 2012). Moreover, as average daily feed intake of LC-fed hens was higher during the laying period (Röhe et al., 2019), it would be also conceivable that an increased dry matter intake stimulated gizzard activity leading to an increased gizzard weight.

Intestinal Tract and Histomorphology

In the present study, relative weights of the small and large intestine were higher in lignocellulose fed hens compared to those receiving the control diet. Similarly, pullets showed increased small intestinal weights after feeding diets containing 1% lignocellulose (ARBOCEL® RC FINE) over a period of 10 wk compared to pullets fed the control (Yokhana et al., 2015). By contrast, feeding diets supplemented with 0.4 or 0.6% lignocellulose (ARBOCEL® R) did not affect relative weights of the intestine in 42 d old broilers (Makivic et al., 2019). Several studies showed that dietary inclusion of insoluble fiber sources such as oat hulls and sawdust was accompanied with an increase in length and weight of the small and large intestine in chicken (Welch et al., 1988; Jørgensen et al., 1996; Hetland and Svihus, 2001; Sklan et al., 2003; Oke and Oke, 2007; Jiménez-Moreno et al., 2009). In general, it is suggested that an increase in the intestinal size and length but also an enlargement of the intestinal mucosa contributes to a higher intestinal weight (Uni et al., 2003). In this study, correlation analyses proved that an increase of the gastrointestinal weight was related to the enlargement of the intestinal mucosa.

Furthermore, the results showed that dietary lignocellulose inclusion enhanced the mucosal development of the large intestine indicated by a greater villus area and a higher villus and crypt mucosal enlargement factor in the colorectum. However, the number of mucus producing goblet cells and the relative mucin staining area were not affected by feeding lignocellulose. Studies on the effect of feeding lignocellulose on intestinal histomorphology and intestinal mucus production are

scarce but are generally in line with the results of this study. Broilers fed 0.6% lignocellulose (ARBOCEL® R) at the expense of soybean meal showed an increased villus height and width as well as crypt depth in the duodenum, jejunum and ileum compared to those receiving the control diet (Makivic et al., 2019). Similarly, an increase of dietary insoluble fiber by adding lignocellulose up to 0.75% (ARBOCEL®) led to an increase of the villus height and crypt depth in the ileum of broilers after 42 d of feeding (Sarikhan et al., 2010). The dietary inclusion of 1.25% lignin resulted in an increased jejunal villus length in 42-day-old broilers while a higher inclusion level of 2.5% lignin (Alcell®, Alcell Technologies Inc., Canada) decreased villus length (Baurhoo et al., 2007). In the same study, the number of jejunal goblet cells per villus were not affected by feeding the different lignin inclusion levels (Baurhoo et al., 2007). Apart from lignocellulose or lignin as fiber source, only few studies exist investigating the effect of other insoluble fiber sources on intestinal morphology in chickens providing contradictory results. An elevation of the dietary crude fiber content from 1.61 to 4.44% by adding pea hulls, mainly consisting of insoluble fiber, tended to reduce linearly villus height and significantly lowered crypt depth in the jejunum of broilers (Jiménez-Moreno et al., 2011). In contrast to that but in line with our findings, the jejunal villus height and the villus surface area of 98-day-old turkeys increased as the concentration of dietary crude fiber was heightened from 3 to 9% (Sklan et al., 2003). It was speculated that an increase in the digestive tract weight accompanied with an enlargement of the intestinal mucosa displays a compensatory reaction of chickens due to the feeding of high fiber, low nutrient diets (Bedford, 2000). Thus, nutrient absorption might be enhanced by increasing the digestive capacity (Brenes et al., 1993; Bedford, 2000). On the other hand, Amerah et al. (2009) reported a decrease in weight and length of the small intestine of broilers fed increasing levels of dietary fiber. It was suggested that the lower nutrient density in the intestine of birds fed diets containing insoluble fiber might reduce the intestinal surface area although histomorphometric parameters were not determined (Amerah et al., 2009).

Bacterial Metabolites and Microbiota

The hypothesis of Bedford (2000) that chickens fed with nutrient reduced diets enhance the absorption of nutrients by increasing the intestinal mucosal surface area is supported by the results of this study. Hens fed the nutrient reduced diet had lower cecal concentrations of SCFAs, particularly lower levels of acetic acid, propionic acid and n-valeric acid, compared to hens fed with the control diet. Coincidentally, those hens had a higher colorectal mucosal enlargement factor, a histomorphometric parameter reflecting the mucosal surface (Wiese et al., 2003). This might indicate a compensatory reaction of birds fed lignocellulose enhancing the absorption of bacterial metabolites by developing a higher intestinal mucosal surface area. Accordingly, correlation analyses in this study have shown that the concentration of SCFAs in the cecum of hens was negatively correlated with the colorectal mucosal enlargement factor of the villi, in other words: the lower the concentration of SCFA in the gut lumen, the higher the absorptive villus surface area. Thus, the hypothesis on a compensatory reaction to increase resorption of energy yielding SCFA may hold true.

In chickens, the bacterial fermentation of insoluble fiber sources and lignified material such as lignocellulose is low (McNab, 1973; Carré et al., 1990; Jørgensen et al., 1996; Montagne et al., 2003; De Vries et al., 2012; Waite and Taylor, 2014). Lower concentrations of SCFAs and ammonia were also detected in the cecum of LC-fed hens compared to those fed CON suggesting a diet dilution effect of the lignocellulose inclusion. However, the results also showed that generally neither the number nor the activity of detected bacterial populations differed between hens of both feeding groups, which favors the idea that the reduced SCFA concentrations are due to an increase in the villus surface accompanied with a higher SCFA absorption. Uniquely, the activity of *Lactobacillus* spp. was significantly higher in CON-fed hens compared to LC-fed hens. There is a lack of studies investigating the impact of feeding lignocellulose on the microbiota in laying hens. Some studies on broilers showed that the feeding of lower inclusion levels of lignocellulose might modulate bacterial populations and metabolites in the small and large intestine (Sarikhan et al., 2010; Bogusławska-Tryk et al., 2015; Kheravii et al., 2017; Makivic et al., 2019). The feeding of broilers with diets containing lignocellulose up to 1% (ARBOCEL® RC) increased counts of *Lactobacillus* spp. in the ileum and *Bifidobacterium* spp. in the ileum and caeca, and decreased counts of ileal and cecal *Escherichia coli* and *Clostridium* spp. (Bogusławska-Tryk et al., 2015). The concentration of ileal and cecal SCFAs was higher in broilers fed 0.5% lignocellulose compared to those fed the control diet. Interestingly, a higher dietary inclusion level of 1% lignocellulose showed no effect on cecal SCFAs concentration (Bogusławska-Tryk et al., 2015). As lignocellulose was included in the diet as an expense of wheat it could be speculated that observed effects on intestinal microbiota and metabolites could be also attributed to a varying nutrient composition of the feed. Diets diluted with 1 and 2% lignocellulose had in general no effect on detected bacteria counts except that *Clostridium* spp. counts were reduced in the cecum of broilers fed 2% lignocellulose (OptiCell®) (Kheravii et al., 2017).

Excreta Characteristics

Analyses of the excreta revealed that hens fed LC had generally a higher dry matter content than hens fed CON, although hens of both feeding groups showed a comparable excreta dry matter content at 52 wk

of age. Few studies display that dietary lignocellulose inclusion might have a positive effect on litter quality observed during broiler trials. Broilers fed with diets supplemented with 0.6, 0.8, 1, and 2% lignocellulose had a lower moisture content in the litter compared to broilers fed control diets (Farran et al., 2013; Kheravii et al., 2017; Makivic et al., 2019). It was hypothesized that the dietary inclusion of lignocellulose might increase digesta retention time and water holding capacity, which might enhance the water absorption in the digestive tract and thus elevates the excreta dry matter content (Kheravii et al., 2017). With respect to the excreta viscosity, differences could be not observed between LC- and CON-fed hens. It is well known that dietary soluble fiber can increase gut viscosity resulting in a reduced feed passage rate while an opposite effect is supposed for dietary insoluble fiber (Van der Klis and Van Voorst, 1993; Almirall and Esteve-Garcia, 1994; Choct et al., 1996).

In conclusion, the results of this study show that feeding of high levels of lignocellulose increased the weights of gastrointestinal organs of dual purpose laying hens, which was accompanied with the development of an increased colorectal mucosal surface. The amount of cecal SCFAs and ammonia was reduced in lignocellulose-fed hens compared to those fed the basal diet. Interestingly, the concentration of SCFAs in the cecum of hens was negatively correlated with the colorectal villus surface. This might indicate a compensatory reaction of birds fed lignocellulose enhancing the absorption of energy yielding bacterial metabolites by increasing the intestinal mucosal surface. In order to prove this, further studies are needed including experiments in metabolism cages investigating the energy requirements for maintenance and production of dual purpose hens in relation to feeding diets with varying energy- and nutrient levels. Cecal microbial composition and activity was generally not influenced by feeding the different diets supporting the hypothesis that the mucosal absorption rate of cecal SCFAs was increased in lignocellulose-fed hens due to an increased mucosal surface area. Moreover, a lower excreta moisture content could be detected in lignocellulose fed hens, which might have positive effects on litter quality under practical conditions. In connection with recently published data on animal performance and body composition (Röhe et al., 2019) results of this study suggest that the feeding of nutrient reduced diets containing high levels of fibre might be an interesting possibility to feed dual purpose chickens, maintain animal health and simultaneously improve economic viability.

ACKNOWLEDGMENT

This work was supported by funds of the German Government's Special Purpose Fund held at Landwirtschaftliche Rentenbank (grant number 28RZ372049).

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PUBLICATION 9

Röhe, I., Metzger, F., Vahjen, W., Brockmann, G. A. and J. Zentek. 2020. Effect of feeding different levels of lignocellulose on performance, nutrient digestibility, excreta dry matter, and intestinal microbiota in slow growing broilers. Poult. Sci. 99: 5018-5026.

DOI: https://doi.org/10.1016/j.psj.2020.06.053

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Effect of feeding different levels of lignocellulose on performance, nutrient digestibility, excreta dry matter, and intestinal microbiota in slow growing broilers

I. Röhe,*[,1] F. Metzger,* W. Vahjen,* G. A. Brockmann,[†] and J. Zentek*

*Department of Veterinary Medicine, Institute of Animal Nutrition, Freie Universität Berlin, Berlin, Germany; and [†]Albrecht Daniel Thaer-Institute for Agricultural and Horticultural Sciences, Humboldt-Universität zu Berlin, Berlin, Germany

ABSTRACT Lignocellulose is a constituent of plant cell walls and might be used as a fiber source in poultry nutrition. The current study investigated the impact of increasing dietary levels of lignocellulose on performance, nutrient digestibility, excreta DM, intestinal microbiota, and bacterial metabolites in slow growing broilers. At an age of 10 wk, 60 male broilers of an intercross line (New Hampshire × White Leghorn) were allocated to cages and fed isoenergetic and isonitrogenous diets containing 0.8% (**LC1**), 5% (**LC2**), or 10% (**LC3**) lignocellulose. After 23 D of feeding, broilers were killed and digesta samples of ileum and excreta analyzed for nutrient digestibility and DM. Cecal contents were analyzed for microbial composition and metabolites. Broiler performance was not affected by feeding dietary lignocellulose. LC3 fed broilers showed reduced ileal digestibility of protein compared to chickens fed LC1 ($P = 0.003$). Moreover, increasing levels of dietary lignocellulose reduced apparent digestibility of organic matter and gross energy ($P < 0.001$). Feeding of lignocellulose had no impact on the excreta DM of broilers. Increasing levels of dietary lignocellulose lowered cecal counts of *Escherichia/Hafnia/Shigella* ($P = 0.029$) and reduced the total concentration of short-chain fatty acids ($P < 0.001$), lactate ($P < 0.05$), and ammonia ($P = 0.009$). The molar ratio of cecal acetic acid was higher in LC3 fed broilers ($P < 0.001$), while the proportions of cecal propionic acid and n-butyric acid were higher in LC1 and LC2 fed chickens ($P < 0.001$). Correlation analyses indicated that dietary lignocellulose was negatively related to the total concentration of cecal bacterial metabolites ($P < 0.001$). In conclusion, the feeding of lignocellulose did not affect growth performance, but impaired nutrient digestibility of slow growing broilers. While minor changes in cecal microbial composition were detected, cecal bacterial metabolite concentrations were significantly reduced with increasing levels of dietary lignocellulose. These findings suggest that lignocellulose is not extensively degraded by bacteria residing in the large intestine of broilers.

Key words: lignocellulose, broiler, performance, digestibility, microbiota

2020 Poultry Science 99:5018–5026
https://doi.org/10.1016/j.psj.2020.06.053

INTRODUCTION

In recent years, intensive research regarding the use of different fiber sources in poultry nutrition has contributed to the understanding that dietary fiber is more than a simple diet diluent. Studies have shown that diets supplemented with fibrous components can have effects on the digestive tract development and function resulting in improved chicken health and performance (Hetland and Svihus, 2001; Montagne et al., 2003; González-Alvarado et al., 2007; Jiménez-Moreno et al., 2011). However, as the term "dietary fiber" includes a large and heterogeneous group of compounds, observed effects are strongly dependent on the used fiber source, its physicochemical characteristics (i.e., solubility, viscosity, particle size, degree of lignification, hydration capacity), and the inclusion level (Bach Knudsen, 2001). Dietary fiber can be defined differently, although most commonly a physiological or chemical definition is used. With respect to the physiological definition, dietary fiber is any polysaccharide and lignin, which is not degraded by endogenous enzymes in the digestive tract, thus reaching the large intestine (Trowell et al., 1976; Bach Knudsen, 2001). Chemically, dietary fiber can be considered as the sum of non-starch polysaccharides (**NSP**) and lignin (Theander et al., 1994). Due to their

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Received April 6, 2020.
Accepted June 20, 2020.

[1]Corresponding author: ilen.roehe@fu-belin.de

water solubility, NSP can be divided into soluble and insoluble NSP showing different effects in the digestive tract when fed to the animal. The feeding of soluble NSP sources is related to negative effects on animal performance and health, as gut viscosity might be increased resulting in a decreased feed passage rate, while the contrary effect is observed for dietary insoluble fiber (Van der Klis and Van Voorst, 1993; Almirall and Esteve-Garcia, 1994; Choct et al., 1996). Moreover, soluble NSP might be fermented to a larger extent by gut microbes while insoluble NSP are not degraded extensively by microbial fermentation in poultry (Montagne et al., 2003; Hetland et al., 2004; De Vries et al., 2012).

Lignocellulose, a constituent of plant cell walls, is mainly composed of insoluble NSP cellulose and hemicellulose as well as phenolic lignin (Liu et al., 2014). In the last decade, few studies examined the use of dietary lignocellulose for different farm and companion animals revealing potential effects on digestive physiology and function (Abad et al., 2013; Kröger et al., 2017; Slama et al., 2020). With respect to poultry nutrition, the feeding of lignocellulose at inclusion levels below 1% could improve broiler performance (Sarikhan et al., 2010; Makivic et al., 2019). However, other studies, using similar inclusion levels, showed that dietary lignocellulose had no impact (Bogusławska-Tryk et al., 2015; Kheravii et al., 2017; Zeitz et al., 2019) or, by using higher inclusion levels, an adverse effect on performance parameters of chickens (Oke and Oke, 2007; Oikeh et al., 2019; Röhe et al., 2019). There is a lack of studies investigating the impact of dietary lignocellulose on nutrient digestibility. While the feeding of 1 to 2% dietary lignocellulose had no effect on nutrient digestibility (Kheravii et al., 2017), the apparent total tract digestibility of fat and fatty acids was increased in broilers fed 1% lignocellulose (Bogusławska-Tryk et al., 2016). Few studies showed that microbial composition (Bogusławska-Tryk et al., 2015; Kheravii et al., 2017; Makivic et al., 2019; Zeitz et al., 2019) and bacterial metabolites (Bogusławska-Tryk et al., 2015; Zeitz et al., 2019) might be affected by feeding lignocellulose at inclusion levels below 2%. Moreover, it could be demonstrated that litter moisture content was reduced when broilers were fed with dietary concentrations of lignocellulose up to 2% (Farran et al., 2013; Kheravii et al., 2017; Makivic et al., 2019).

In all of the above-mentioned studies, lignocellulose was supplemented on top thus diluting dietary energy and nutrient content and, in most cases, relatively low dietary lignocellulose levels were fed. In the current study, experimental diets were isoenergetic and isonitrogenous and relatively high dietary lignocellulose inclusion levels of 5 and 10% were used. The aim of this study was to investigate the impact of feeding increasing concentrations of dietary lignocellulose on performance, nutrient digestibility, excreta DM, cecal microbiota, and cecal bacterial metabolites in slow growing broilers. It was hypothesized that animal performance and nutrient digestibility might be not affected by increasing levels of lignocellulose as the diets were isoenergetic and isonitrogenous. Furthermore, it was expected that the excreta DM content might be increased with increasing levels of dietary lignocellulose and the cecal microbiota could be changed by feeding different diets.

MATERIALS AND METHODS

All procedures involving handling and treatments of animals were approved by the local state office of occupational health and technical safety (Landesamt für Gesundheit und Soziales, Berlin, Germany, LAGeSo G0165/14).

Broilers, Rearing Conditions, and Experimental Design

In this study, 60 male broilers of generation F12 of an advanced intercross line (AIL_{12}) were used. This line was a breed from an initial cross of the inbred lines New Hampshire (NHI, high growth) and White Leghorn (WL77, low growth) followed by repeated random intercrossing (Nassar et al., 2012). Broilers were raised on floor pens and fed a commercial broiler diet until 5 wk of age. In an adaption phase, broilers were divided into 3 groups (20 animals per pen) and fed the 3 experimental diets until 10 wk of age. Afterwards, broilers were transferred into individual cages and fed the same experimental diets as during the adaption phase for a period of 23 D. The cage represented the experimental unit resulting in 20 replicates per feeding group. The single cages (61 × 50 × 35 cm) were equipped with a nipple drinker system, a feeder, and a sitting perch. Broilers were offered ad libitum access to mash feed and water. Ambient temperature was adjusted to 21°C and broilers had 12 h light per day. During the experimental trial, body weight and feed intake of broilers were recorded weekly. From these data, the average daily weight gain, average daily feed intake, and feed conversion ratio were calculated. At the end of the trial, broilers were sacrificed by stunning and cervical decapitation. Subsequently, ileal contents and excreta were collected for analyses of nutrient digestibility, and cecal digesta were used for the determination of bacterial metabolites and microbiota composition.

Experimental Diets

The experimental diets were mainly based on wheat, soybean meal, and corn and contained different concentrations of a lignocellulose product (ARBOCEL R, J. Rettenmaier & Söhne GmbH + Co. KG, Rosenberg, Germany). In the first treatment diet, dietary lignocellulose was included at a level of 0.8% (**LC1**), the second treatment diet contained 5% lignocellulose (**LC2**), and the third treatment 10% dietary lignocellulose (**LC3**). The used lignocellulose source had a crude fiber content of 65%, an average fiber length of 200 to 300 µm, and a bulk density of 60 to 105 g/L, per supplier information.

Titanium dioxide (3 g of TiO_2/kg of diet) was used as an indigestible marker for the ileal and apparent excreta digestibility measurements. The nutrient content of the diets was determined by classical Weende procedures

(Naumann and Bassler, 2004). Phosphorus content was measured using the ammonium vanadate/molybdate method (Gericke and Kurmies, 1952) and calcium content was determined using atomic absorption spectrometry (AAS Vario 6 Spectrometer, Analytik Jena, Jena, Germany). The feed composition and analyzed nutrient content of the experimental diets are displayed in Table 1.

Sampling and Analyses

Nutrient Digestibility and Excreta DM Nutrient digestibility and excreta DM were analyzed for 20 broilers per treatment group (n = 20). For the determination of the apparent ileal digestibility of crude protein, digesta was taken from the distal two-thirds of the ileum. On the last 3 D of the trial, excreta samples were collected in order to detect the apparent excreta digestibility of ether extract, organic matter, gross energy, and excreta DM. Ileal digesta and excreta were immediately frozen (−80°C) until further analysis. Samples were freeze-dried before chemical analyses. The following equation was used for apparent ileal (AID) and excreta digestibility (AD) calculation:

$$AID\,/\,AD = 100 - \left[\frac{\%\ Marker\ in\ feed}{\%\ Marker\ in\ ileum/excreta} \times \frac{\%\ Nutrient\ in\ ileum/excreta}{\%\ Nutrient\ in\ feed}\right] \times 100$$

Excreta DM was determined by weighing frozen excreta in aluminum jars of known weight and subsequent drying in an incubator at 103°C, in order to detect the loss of water.

Analyses of Bacterial Cell Counts in the Cecum Digesta The paired ceca were removed and squeezed in order to collect cecal digesta. Digesta samples of 10 broilers per treatment group (n = 10) were subsequently frozen in liquid nitrogen and stored at −80°C. The quantification of selected representatives of the microbiota was carried out from DNA, detecting bacterial 16S rDNA copy numbers as an equivalent to bacterial cells of specific bacterial groups. Seven group primers were used to analyze important bacterial groups: clostridial cluster I, IV, and XIVa, *Lactobacillus* spp., *Bifidobacterium* spp., the *Bacteroides-Prevotella-Porphyromonas* cluster, and the *Escherichia/Hafnia/Shigella* group (Table 2). DNA was extracted with a commercial NucleoSpin RNA Kit (REF 740955, Macherey-Nagel GmbH & Co. KG, Düren, Germany) in combination with the NucleoSpin RNA/DNA Buffer Set (REF 740944, Macherey-Nagel GmbH & Co. KG) according to the manufacturer, except for the use of the 100 mg sample. Quantification of bacterial groups was done using a Stratagene Mx3000P system (Stratagene, Amsterdam, The Netherlands) with a commercial master mix (Brilliant II SYBR Green QPCR Master Mix with Low ROX, Stratagene). Primer sequences and annealing temperatures are shown in Table 2. All primers were obtained from MWG Biotech (Straubing, Germany). A calibration series of PCR products with known copy numbers per ng DNA was observed to calculate copy numbers/g sample.

Analyses of Bacterial Metabolites in the Cecum Digesta Cecal digesta from 10 chickens per treatment group (n = 10) were collected, instantly frozen in liquid nitrogen, and stored at −80°C. The analysis of the total concentration of bacterial metabolites was performed as described earlier (Kröger et al., 2017). The total concentration of short-chain fatty acids (**SCFAs**) was determined by gas chromatography (Agilent 6890N, Agilent Technologies, Böblingen, Germany, autosampler G2614A, and injection tower G2613A, Network GC Systems, Böblingen, Germany) equipped with a flame ionization detector. In order to detect shifts in the SCFA profile, the molar proportions of SCFAs were calculated. The total concentrations of D- and L-lactate were measured by HPLC (Agilent 1100, Agilent Technologies) with a pre-column (Phenomenex C18, 4.0 × 2.0 mm, Phenomenex Inc., Aschaffenburg, Germany) and an analytical column (Chirex 3126 (D)-penicillamine, 150 × 4.6 mm, Phenomenex Inc.) Ammonia was detected colorimetrically by the Berthelot reaction in microtitration plates using a Tecan Sunrise microplate reader (Tecan Austria GmbH, Grödig, Austria).

Statistical Analyses

Statistical analyses were performed using SPSS (version 26.0, SPSS Inc., Chicago, IL). Means ± SEM are reported. The cage represented the experimental unit. Regarding animal performance, nutrient digestibility, and bacterial metabolites, 20 replicates per treatment were used. In the LC3 group, one chicken was identified as female and thus excluded resulting in 19 replicates. Analyses of bacterial cell counts and overall activity were based on 10 broilers/replicates per treatment. The normally distributed data were analyzed by using ANOVA followed by post hoc Tukey's test. Microbiological data failed the normality test and were analyzed via Kruskal-Wallis test and subsequent Mann-Whitney U test, where appropriate. Spearman correlation analyses were conducted displaying correlations between dietary lignocellulose content and total concentration of cecal bacterial metabolites. Differences were considered significant at $P < 0.05$.

RESULTS

During the whole feeding trial, broilers were healthy and showed no clinical evidence of disease. Feeding different dietary levels of lignocellulose did not affect overall animal performance (Table 3).

Table 1. Feed composition and analyzed nutrient content of diets.

Diet composition	LC1[1]	LC2[2]	LC3[3]
Ingredient (%)			
Wheat	40.7	34.9	23.0
Soybean meal extracted	29.0	30.6	34.5
Corn	19.0	19.0	18.0
Lignocellulose	0.80	5.00	10.0
Soya oil	2.50	2.50	6.50
Premix[4]	4.00	4.00	4.00
Calcium carbonate	4.00	4.00	4.00
TiO_2[5]	0.30	0.30	0.30
Analyzed nutrients (%)			
Crude protein	22.7	22.8	22.0
Ether extract	4.34	4.35	7.58
Crude fiber	4.15	6.24	9.52
NDF	14.1	16.4	18.9
ADF	4.83	7.51	10.6
ADL	1.09	2.30	2.94
Crude ash	6.69	6.54	6.44
Calcium	1.00	0.96	0.90
Phosphorus	0.68	0.65	0.64
Sodium	0.22	0.22	0.22
Potassium	0.79	0.76	0.84
Calculated AME_n (MJ/kg)[6]	14.5	14.3	14.5

[1]LC1 = 0.8% dietary lignocellulose.
[2]LC2 = 5% dietary lignocellulose.
[3]LC3 = 10% dietary lignocellulose.
[4]Provided per kg diet: 10,000 IU vitamin A; 3,000 IU vitamin D3; 70.0 mg vitamin E (α-tocopherol acetate); 50.0 mg Fe (iron carbonate); 60.0 mg Zn (zinc oxide); 20.0 mg Zn (Zinc chelate of glycine hydrate); 60.0 mg Mn (manganese oxide); 20.0 mg Mn (manganese chelate of glycine hydrate); 4.00 mg Cu (copper sulfate pentahydrate); 4.00 mg Cu (copper chelate of glycine hydrate); 0.80 mg I (calcium iodate); 0.40 mg Se (sodium selenite).
[5]TiO_2 = titanium (IV) oxide (Sigma-Aldrich Co., St. Louis, MO).
[6]AME_n calculated according to WPSA (1989) equation.
Abbreviations: ADF, acid detergent fiber; ADL, acid detergent lignin; NDF, neutral detergent fiber.

With respect to nutrient digestibility (Table 4), increasing levels of dietary lignocellulose impaired apparent ileal digestibility of crude protein. LC3 fed broilers showed reduced digestibility of crude protein compared to those fed with LC1 ($P = 0.003$). Apparent excreta digestibility of ether extract was not affected by feeding different diets. Increasing levels of dietary lignocellulose proportionately reduced the apparent excreta digestibility of organic matter and gross energy in broilers ($P < 0.001$). The DM content of excreta was not influenced by feeding increasing levels of dietary lignocellulose (Table 4).

Feeding increasing levels of lignocellulose affected the total concentration of bacterial metabolites and relative proportions of SCFAs in the cecum digesta of broilers (Table 5). Broilers fed LC3 showed lower total SCFA concentrations in the cecum than LC1 and LC2 fed chickens ($P < 0.001$). In particular, lower amounts of propionic acid ($P < 0.001$), i-butyric acid ($P = 0.001$), n-butyric acid ($P < 0.001$), and i-valeric acid ($P = 0.001$) were detected in the cecal content of LC3 fed broilers in comparison with those fed LC1 and LC2. With respect to the molar ratio, the relative proportion of acetic acid was higher in LC3 fed chickens ($P < 0.001$), while the relative proportions of propionic acid and n-butyric acid were higher in LC1 and LC2 fed broilers ($P < 0.001$). Moreover, the total concentration of D-lactate ($P = 0.037$), L-lactate ($P = 0.001$), and ammonia ($P = 0.009$) was found to be lower in broilers fed LC3 compared to those fed LC1 and LC2. Spearman correlation analyses revealed that dietary lignocellulose inclusion was negatively related to the total concentration of SCFAs ($P < 0.001$) in the cecum of broilers (Table 6). With regard to dietary lignocellulose, a moderate negative relationship was observed for propionic acid ($P < 0.001$) and n-butyric acid ($P < 0.001$) and low negative correlations were found for i-butyric acid ($P = 0.001$) and i-valeric acid ($P = 0.008$). The total cecal concentrations of D-lactate, L-lactate, and ammonia were negatively correlated to the lignocellulose levels of the diets ($P = 0.029$, $P < 0.001$, $P = 0.003$).

Analyses of bacterial copy numbers of 16S rDNA showed that bacteria of the *Escherichia*/*Hafnia*/*Shigella* group were numerically and significantly reduced in the cecal content of broilers fed increasing dietary lignocellulose levels (Table 7). LC3 fed chickens had lower numbers of *Escherichia*/*Hafnia*/*Shigella* compared to LC1 fed broilers ($P = 0.029$). Bacteria of clostridial cluster IV and *Bacteroides*/*Prevotella*/*Porphyromonas* cluster were higher in LC1 and LC2 fed broilers compared to those fed LC 3 ($P = 0.001$, $P = 0.009$). Bacterial copy numbers of the clostridial cluster XIVa and *Bifidobacterium* spp. were higher in LC2 and LC3

Table 2. Primers used for quantification of bacterial 16S copy numbers in cecal contents.

Specificity	Primer	Primer sequences (5′-3′)	Product (bp)	A_T[1]	Reference
Clostridial cluster XIVa	g-Ccoc-F	AAATGACGGTACCTGACTAA	440	60	Matsuki et al., 2002
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGAA			Matsuki et al., 2002
Clostridial cluster I	CI-F1	TACCHRAGGAGGAAGCCAC	231	63	Song et al., 2004
	CI-R2	GTTCTTCCTAATCTCTACGCAT			Song et al., 2004
Clostridial cluster IV	sg-Clept-F	GCACAAGCAGTGGAGT	239	60	Matsuki et al., 2002
	sg-Clept-R	CTTCCTCCGTTTTGTCAA			Matsuki et al., 2002
Lactobacillus spp.	Lac-1	AGCAGTAGGGAATCTTCCA	341	58	Walter et al., 2001
	Lac-2	CACCGCTACACATGGAG			Heilig et al., 2002
Bifidobacterium spp.	g-BIFID-F	TCGCGTCYGGTGTGAAAG	243	58	Rinttilä et al., 2004
	g-BIFID-R	CCACATCCAGCRTCCAC			Rinttilä et al., 2004
Bacteroides-Prevotella-Porphyromonas cluster	BPP1	GGTGTCGGCTTAAGTGCCAT	140	55	Rinttilä et al., 2004
	BPP2	CGGAYGTAAGGGCCGTGC			Rinttilä et al., 2004
Escherichia/*Hafnia*/*Shigella* group	Entero-F	GTTAATACCTTTGCTCATTGA	340	55	Malinen et al., 2003
	Entero-R	ACCAGGGTATCTAATCCTGTT			Malinen et al., 2003

[1]A_T = annealing temperature (°C).

Table 3. Impact of different levels of dietary lignocellulose on performance of slow growing broilers (n = 20).[1]

Days	LC1[2]	LC2[3]	LC3[4]	SEM	*P*-value[5]
Body weight (g)					
1	1,204	1,172	1,186	14.1	0.651
7	1,342	1,310	1,308	14.5	0.556
14	1,483	1,460	1,450	16.7	0.722
23	1,658	1,639	1,618	19.2	0.700
Average daily weight gain (g)					
1–7	19.7	19.6	17.5	0.56	0.199
7–14	20.0	21.6	20.2	0.84	0.725
14–23	19.5	19.9	18.7	0.60	0.717
1–23	20.6	21.2	19.6	0.53	0.484
Average daily feed intake (g)					
1–7	37.7	39.7	41.4	1.96	0.748
7–14	35.0	39.3	33.1	1.75	0.341
14–23	43.3	48.8	42.6	1.65	0.245
1–23	39.1	43.1	39.3	1.22	0.315
Feed conversion ratio (g feed/g weight gain)					
1–7	1.89	2.10	2.47	0.12	0.142
7–14	1.95	1.89	1.85	0.12	0.938
14–23	2.27	2.55	2.31	0.08	0.325
1–23	1.98	2.15	2.10	0.05	0.298

[1]LC3: n = 19.
[2]LC1 = 0.8% dietary lignocellulose.
[3]LC2 = 5% dietary lignocellulose.
[4]LC3 = 10% dietary lignocellulose.
[5]Statistical analyses were conducted by ANOVA.

fed broilers in comparison with chickens fed LC1 ($P = 0.006$, $P = 0.008$).

DISCUSSION

In the last decade, few studies were performed in order to investigate whether dietary lignocellulose affects performance, digestive physiology, and intestinal microbiota in chickens (Bogusławska-Tryk et al., 2015; Farran et al., 2017; Kheravii et al., 2017; Makivic et al., 2019; Zeitz et al., 2019). One aspect common to these studies is that lignocellulose was used as a diet diluent resulting in energy- and nutrient-reduced experimental diets. Furthermore, relatively low concentrations of dietary lignocellulose were included in these experiments. Contrary to this, the broiler feed of the present trial was not diluted with lignocellulose producing isoenergetic and isonitrogenous diets with relatively high dietary lignocellulose inclusion levels of up to 10%.

Table 4. Impact of different levels of dietary lignocellulose on AID CP, AD EE, AD OM, and AD GE as well as on the excreta DM of slow growing broilers (n = 20).[1]

Item	LC1[2]	LC2[3]	LC3[4]	SEM	*P*-value[5]
AID CP (%)	82.3^{a}	81.7^{a}	78.5^{b}	0.50	0.003
AD EE (%)	94.9	93.8	95.6	0.19	0.058
AD OM (%)	70.0^{a}	66.0^{b}	63.4^{c}	0.55	<0.001
AD GE (%)	75.2^{a}	71.4^{b}	68.7^{c}	0.04	<0.001
Excreta DM (g/kg)	299	299	311	4.09	0.386

[a–c]Means with different superscripts are significantly different ($P < 0.05$).
Abbreviations: AD EE, apparent excreta digestibility of ether extract; AD GE, apparent excreta digestibility of gross energy; AD OM, apparent excreta digestibility of organic matter; AID CP, apparent ileal digestibility of CP.
[1]LC3: n = 19.
[2]LC1 = 0.8% dietary lignocellulose.
[3]LC2 = 5% dietary lignocellulose.
[4]LC3 = 10% dietary lignocellulose.
[5]Statistical analyses were conducted by ANOVA and post hoc Tukey's test.

Table 5. Impact of different levels of dietary lignocellulose on the concentration of bacterial metabolites and the relative proportions of SCFA in the cecum digesta of slow growing broilers (n = 20).[1]

Item	LC1[2]	LC2[3]	LC3[4]	SEM	*P*-value[5]
Acetic acid (µmol/g)	38.1	39.1	33.9	0.99	0.220
Propionic acid (µmol/g)	8.89^{a}	9.83^{a}	4.82^{b}	0.38	<0.001
i-Butyric acid (µmol/g)	0.41^{a}	0.39^{a}	0.25^{b}	0.02	0.001
n-Butyric acid (µmol/g)	8.45^{a}	8.04^{a}	5.17^{b}	0.28	<0.001
i-Valeric acid (µmol/g)	0.28^{a}	0.28^{a}	0.16^{b}	0.02	0.001
n-Valeric acid (µmol/g)	0.59	0.62	0.54	0.02	0.292
Total SCFA (µmol/g)	56.8^{a}	58.2^{a}	44.9^{b}	1.44	<0.001
Acetic acid (mol. %)	66.8^{b}	67.1^{b}	75.5^{a}	0.67	<0.001
Propionic acid (mol. %)	16.0^{a}	16.8^{a}	10.9^{b}	0.52	<0.001
i-Butyric acid (mol. %)	0.76	0.69	0.59	0.04	0.451
n-Butyric acid (mol. %)	14.9^{a}	13.8^{a}	11.4^{b}	0.32	<0.001
i-Valeric acid (mol. %)	0.52	0.50	0.38	0.04	0.318
n-Valeric acid (mol. %)	1.07	1.08	1.32	0.06	0.465
D-Lactate (µmol/g)	1.03^{a}	1.06^{a}	0.42^{b}	0.14	0.037
L-Lactate (µmol/g)	1.70^{a}	1.34^{a}	0.67^{b}	0.13	0.001
Ammonia (µmol/g)	9.46^{a}	8.72^{a}	7.71^{b}	0.44	0.009

[a,b]Means with different superscripts are significantly different ($P < 0.05$).
Abbreviation: SCFA, short-chain fatty acids.
[1]LC3: n = 19.
[2]LC1 = 0.8% dietary lignocellulose.
[3]LC2 = 5% dietary lignocellulose.
[4]LC3 = 10% dietary lignocellulose.
[5]Statistical analyses were conducted by ANOVA and post hoc Tukey's test.

Broiler Performance and Nutrient Digestibility

In this study, male broilers of an advanced intercross line were used based on an initial cross between the dual purpose New Hampshire and White Leghorn chicken. Thus, growth performance was expectedly much lower compared to commercial broiler hybrids.

Results showed that broiler performance was not affected by feeding varying concentrations of lignocellulose. The feeding of diets with similar energy and protein content, containing 0.8 and 5% lignocellulose, also did not affect the performance parameters of 13-week-old slow growing broilers (Nassar et al. 2019). Studies in broilers and laying hens have shown that feeding energy- and nutrient-reduced diets containing relatively high levels of lignocellulose of 10 to 15% clearly reduced body weight (Oikeh et al., 2019; Röhe et al., 2019). Few studies showed that feeding diets supplemented with lignocellulose at lower inclusion levels of up to 2% had no effect on broiler performance (Bogusławska-Tryk et al., 2015; Kheravii et al., 2017; Zeitz et al., 2019). On the contrary, it could be demonstrated that body weight gain and feed conversion ratio were improved when broilers were fed with diluted diets containing lignocellulose at a maximum of 0.75% (Sarikhan et al., 2010; Makivic et al., 2019). Thus, it becomes obvious that the inclusion level and particularly the nutrient content of diets are crucial factors influencing the performance of chickens. Feeding diets diluted with

Table 6. Spearman correlation analyses of dietary lignocellulose and bacterial metabolites in the cecum of slow growing broilers.

Item	Spearman coefficient	*P*-value
Acetic acid (μmol/g)	−0.204	0.122
Propionic acid (μmol/g)	−0.599	<0.001
i-Butyric acid (μmol/g)	−0.435	0.001
n-Butyric acid (μmol/g)	−0.630	<0.001
i-Valeric acid (μmol/g)	−0.341	0.008
n-Valeric acid (μmol/g)	−0.133	0.316
Total SCFA (μmol/g)	−0.440	<0.001
D-Lactate (μmol/g)	−0.322	0.029
L-Lactate (μmol/g)	−0.488	<0.001
Ammonia (μmol/g)	−0.385	0.003

Abbreviation: SCFA, short-chain fatty acids.

relatively high fiber inclusion levels could result in lower energy and nutrient intake of chickens, which impairs growth performance (Oke and Oke, 2007; Oikeh et al., 2019; Röhe et al., 2019). On the other hand, chicken performance seems to be unaffected by relatively high dietary fiber inclusion levels when the diets are isoenergetic and isonitrogenous, as in the current study. Regarding the positive effects of dietary lignocellulose at lower inclusion levels, it was speculated that lignocellulose might improve growth performance due to an increase in nutrient digestibility in combination with improved gizzard function (Sarikhan et al., 2010; Makivic et al., 2019). In the present study, however, increasing levels of dietary lignocellulose proportionally decreased the apparent ileal digestibility of crude protein and apparent excreta digestibility of organic matter and gross energy. With respect to protein digestibility, this might indicate that dietary lignocellulose directly impeded the digestion and absorption of amino acids in the small intestine of broilers. Furthermore, it could be suggested that the feeding of lignocellulose might have an abrasive effect on the intestinal mucosa (Bogusławska-Tryk et al., 2015; Makivic et al., 2019), thus increasing endogenous amino acid losses. Results regarding organic matter and gross energy digestibility show that lignocellulose was neither degraded by endogenous enzymes in the small intestine nor largely fermented by intestinal microbes in the large intestine, as discussed later. Similarly, Jiménez-Moreno et al. (2010) showed that 3% dietary cellulose supplementation was generally insufficient in improving nutrient utilization at the exception of fat retention. It was speculated that dietary cellulose might have an effect on micelle formation and lipid absorption enhancing bile acids recycling and fat absorption (Jiménez-Moreno et al., 2010). In line with the latter, the apparent ileal fat digestibility and total tract digestibility of total fatty acids were increased in broilers fed 1% dietary lignocellulose (Bogusławska-Tryk et al., 2016). With respect to the current study, the total tract digestibility of ether extract was not affected by increasing levels of lignocellulose. This could be explained by the relatively high dietary inclusion levels of 5 and 10% lignocellulose, which might diminish potential positive effects on intestinal fat resorption.

Excreta DM

The results of the current study showed that excreta DM was not influenced by feeding increasing levels of lignocellulose. Few studies in chickens have demonstrated that the feeding of dietary lignocellulose might increase excreta DM content of chickens, which is associated with improved litter quality. Feeding broilers with varying concentrations of lignocellulose up to 2% resulted in lower moisture content of the litter (Farran et al., 2013; Kheravii et al., 2017; Makivic et al., 2019). In another study with dual purpose hens, the feeding of 10% lignocellulose temporally reduced excreta DM content while excreta viscosity was not affected (Röhe et al., 2020). Compared to other insoluble NSP sources, lignocellulose shows a high water holding capacity (Slama et al., 2019). The higher the water holding capacity of a dietary component, the lower the free water content in the digesta (Takahashi et al., 2009). Thus, it was suggested that both, water holding capacity and digesta retention time, might be increased in broilers fed lignocellulose resulting in increased luminal water absorption and higher excreta DM content (Kheravii et al., 2017). Whether differences in the diet composition, feeding duration, chickens genetics, or age might lead to diverging results in the current study needs further clarification.

Table 7. Impact of different levels of dietary lignocellulose on bacterial cell count ($\log_{10}$ 16S rDNA copy number/g) in cecal digesta of slow growing broilers (n = 10).

Item	LC1[1]	LC2[2]	LC3[3]	SEM	*P*-value[4]
Clostridial cluster I	9.28	9.03	9.15	0.14	0.756
Clostridial cluster IV	9.96[a]	10.1[a]	9.79[b]	0.04	0.001
Clostridial cluster XIVa	9.86[b]	10.1[a]	10.1[a]	0.03	0.006
Lactobacillus spp.	7.11	7.35	7.17	0.09	0.585
Bifidobacterium spp.	9.39[b]	9.65[a]	9.69[a]	0.05	0.008
Bacteroides/Prevotella/Porphyromonas cluster	9.94[a]	10.0[a]	9.68[b]	0.05	0.009
Escherichia/Hafnia/Shigella group	7.42[a]	6.85[a,b]	6.24[b]	0.17	0.023

[a,b]Means with different superscripts are significantly different ($P < 0.05$).
[1]LC1 = 0.8% dietary lignocellulose.
[2]LC2 = 5% dietary lignocellulose.
[3]LC3 = 10% dietary lignocellulose.
[4]Statistical analyses are based on Mann-Whitney U test.

Intestinal Microbiota and Bacterial Metabolites

A few studies have investigated the effect of feeding dietary lignocellulose on gut microbiota in poultry (Bogusławska-Tryk et al., 2015; Kheravii et al., 2017; Makivic et al., 2019; Zeitz et al., 2019; Röhe et al., 2020). A direct comparison of results is difficult as lignocellulose products of different suppliers were used with varying dietary inclusion levels. In addition, different methods were applied for detecting intestinal bacteria performing either culture-based techniques or 16S rDNA sequencing. In this study, a significant difference in cecal bacterial counts could be detected for the *Escherichia/Hafnia/Shigella* group. Those bacteria were reduced by >0.5 log10 with increasing dietary concentrations of lignocellulose. With respect to the other measured bacterial groups, only minor differences were observed with no direct effect of dietary lignocellulose levels. Generally, literature data regarding the potential effect of dietary lignocellulose on cecal microbial composition of broilers are contradictory. The feeding of increasing concentrations of dietary lignocellulose up to 0.6% reduced microbial counts of *Escherichia coli* in the cecal content of broilers (Makivic et al., 2019). Similarly, cecal counts of *E.coli* were reduced in broilers fed 0.25 and 0.5% dietary lignocellulose while a further increase of dietary lignocellulose of 1% showed no effect on *E.coli* counts (Bogusławska-Tryk et al., 2015). This observation contradicts our results, as cecal counts of *Escherichia/Hafnia/Shigella* decreased further with increasing dietary lignocellulose contents of up to 10%.

In the current study, the feeding of 10% dietary lignocellulose reduced the concentration of SCFAs, lactate, and ammonia in the cecum of broilers implying decreased general bacterial activity. On the contrary, the cecal concentration of lactic acid and total SCFA was increased in broilers fed 0.5% lignocellulose, compared to those fed a control diet (Bogusławska-Tryk et al., 2015). The authors suggested that SCFAs were increased in lignocellulose fed broilers due to more intense bacterial fermentation of lignocellulose (Bogusławska-Tryk et al., 2015). In another study, the effect of 2 different lignocellulose products on intestinal bacterial metabolites was investigated in broilers (Zeitz et al., 2019). The used lignocellulose products had the same chemical composition, but it was assumed that they differed in terms of susceptibility to microbial fermentation (Zeitz et al., 2019). Results showed that dietary inclusion of both the lignocellulose products at an inclusion level of 0.8% had no effect on the total concentration of SCFA (Zeitz et al., 2019), suggesting that both lignocellulose products seemed to have comparable susceptibility to cecal microbial fermentation. The molar proportion of acetic acid was increased and that of butyric acid decreased in broilers fed the potential fermentable lignocellulose compared to those fed the non-fermentable lignocellulose or the control diet (Zeitz et al., 2019). Interestingly, in the current trial, a similar shift in the relative proportions of SCFAs was observed showing higher relative levels of acetic and propionic acid and lower levels of butyric acid in LC3 fed broilers.

Basically, an interpretation of results regarding the effects of feeding different fiber sources on intestinal microbiota is challenging. If intestinal microbes and bacterial metabolites are modified by the dietary inclusion of a fiber source, it could be assumed that these modifications are clearly caused by the dietary fiber itself. However, in all of the above-mentioned studies including the current study, experimental diets differed in both, the lignocellulose inclusion level and the nutrient composition of diets. Changes in the nutrient composition of the feed lead to variations in the amount of substrate that enters the large intestine and could be fermented by residing bacteria. Consequently, changes in the dietary nutrient composition may influence intestinal microbial composition and bacterial fermentation pathways. Thus, with respect to the results of the present study, the decrease in cecal counts of *Escherichia/Hafnia/Shigella* and cecal microbial metabolites with increasing dietary lignocellulose might refer to both, an insufficient bacterial degradation of lignocellulose and a reduction of bacterial substrate availability due to changes in diet composition.

Concerning the fermentability of lignocellulose or cellulose, there is still an intensive debate, of whether or to what extent intestinal microbes in monogastrics might be able to degrade such complex polysaccharides (McNab, 1973; Józefiak et al., 2004; Waite and Taylor, 2014; De Maesschalck et al., 2019). In principle, chickens are less capable of degrading insoluble NSP in the large intestine compared to pigs or rats (Carré et al., 1990; De Vries et al., 2012). The reasons for this might be attributed to both, the anatomical and physiological characteristics of the chicken's gastrointestinal tract and the gut microbial composition. The avian digestive tract is relatively short resulting in a high feed passage rate, which generally limits intense fiber degradation. Moreover, several studies showed that only small and soluble fiber fractions might be able to enter the cecum (Bjornhag and Sperber, 1977; Vergara et al., 1989; Rougière and Carré, 2010), which appears to be the main location for bacterial fermentation of dietary fiber (Jørgensen et al., 1996; Józefiak et al., 2004). As lignocellulose is mainly composed of insoluble NSP, it is questionable whether large amounts of this material would enter the cecum. Apart from chicken-specific digestive tract characteristics, there is an ongoing controversy whether microbes, residing in the large intestine of chickens, might be able to sufficiently degrade complex NSP such as lignocellulose or cellulose. There is evidence that microbial cellulolytic activity in the avian hindgut appears to be minimal (McNab, 1973; Mead, 1989; Waite and Taylor, 2014). However, a recent study with broilers showed that bacteria of the phylum *Bacteroidetes*, more specifically *Alistipes* spp., might be able to degrade amorphous cellulose producing energy yielding metabolites (De Maesschalck et al., 2019). Nevertheless, depending on the dietary fiber source, contributions of

bacterial SCFA to energy requirements of chickens are estimated to be low, ranging from 3.5 to 8% (Jørgensen et al., 1996; Jamroz et al., 2002; Józefiak et al., 2004). Finally, authors speculate that lignocellulose could have an abrasive effect on the intestinal mucosa and adhering bacteria (Bogusławska-Tryk et al., 2015; Makivic et al., 2019) or that phenolic monomers of lignocellulose might possess antimicrobial properties (Bogusławska-Tryk et al., 2015; Zeitz et al., 2019). However, the reported antimicrobial effects are associated with the feeding of purified lignin (Ricke et al., 1982; Baurhoo et al., 2007), which clearly differs in terms of the chemical structure from the native lignin as a component of lignocellulose (Zemek et al., 1979; Makivic et al., 2019).

In conclusion, performance parameters of slow growing chickens were not affected by feeding isoenergetic and isonitrogenous diets containing lignocellulose up to 10%. However, increasing levels of dietary lignocellulose impaired protein digestibility indicating that lignocellulose might interfere with the digestion and absorption of amino acids in the small intestine of broilers. Moreover, the apparent excreta digestibility of organic matter and gross energy were proportionally reduced with increasing levels of dietary lignocellulose indicating that lignocellulose could be neither degraded by intestinal endogenous enzymes nor sufficiently fermented by intestinal microbes, as supported by data on bacterial metabolites. Contrary to our expectations, excreta DM was not affected by feeding varying concentrations of dietary lignocellulose. Considerable changes in cecal microbes were limited to bacteria of the group *Escherichia*/*Hafnia*/*Shigella*. Cecal bacterial metabolites were reduced with increasing levels of dietary lignocellulose suggesting that lignocellulose was not extensively degraded by intestinal bacteria in the large intestine of slow growing chickens. As diet composition was altered by the inclusion of lignocellulose, further research is needed in order to clarify whether or to what extent lignocellulose might be degraded by intestinal microbes in chickens.

ACKNOWLEDGMENTS

Conflict of Interest Statement: The authors did not provide a conflict of interest statement.

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PUBLICATION 10

Röhe, I. and J. Zentek. 2021. Lignocellulose as an insoluble fiber source in poultry nutrition: a review. J. Anim. Sci. Biotechnol. 12: 82.

DOI: https://doi.org/10.1186/s40104-021-00594-y

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Röhe and Zentek *Journal of Animal Science and Biotechnology* (2021) 12:82
https://doi.org/10.1186/s40104-021-00594-y

Journal of Animal Science and Biotechnology

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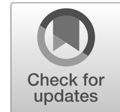

Lignocellulose as an insoluble fiber source in poultry nutrition: a review

Ilen Röhe* and Jürgen Zentek

Abstract

Extensive research in recent years into the use of various fiber sources in poultry nutrition has led to the perception that dietary fiber is more than a simple diet diluent. Several studies showed that the feeding of insoluble fiber sources such as oat hulls, sunflower hulls or wood shavings may affect digestive physiology and function improving chickens health and growth performance. In this context, the effect of lignocellulose as an insoluble dietary fiber source is increasingly being investigated. Lignocellulose is a component of plant cell walls and consists mainly of the insoluble carbohydrate polymers cellulose and hemicelluloses as well as the phenolic polymer lignin. Lignocellulose is chemically and physicochemically different from other insoluble fiber sources and thus possibly has different effects on poultry compared to traditional fiber sources. Several studies investigated the effect of dietary lignocellulose on growth performance, nutrient digestibility, gastrointestinal tract development and intestinal microbiota in broilers and laying hens. Studies differed in terms of feed formulation and lignocellulose inclusion level as well as products of different suppliers were used. The results obtained are inconsistent; beneficial, indifferent or detrimental effects of feeding lignocellulose were observed, so that a final assessment of lignocellulose as a "novel" insoluble fiber source is difficult. This review article summarizes the results of studies in connection with the feeding of lignocellulose to poultry, compares them with those that have used other insoluble fiber sources and illuminates the possible mechanisms of action.

Keywords: Fiber, Growth performance, Gut health, Gut morphology, Lignocellulose, Microbiota, Nutrient digestibility, Poultry

Introduction

In recent years, there have been increasing scientific reports that dietary fiber can have a positive effect on animal health and productivity. Fiber as feed component in poultry nutrition has traditionally been given little consideration as it has only a low nutritional value from a chemical point of view. However, due to its unique physicochemical properties, several studies showed that insoluble fiber sources may affect digestive tract development and function resulting in improved chicken health and growth performance [1–4]. Feeding experiments were mainly carried out with insoluble fiber sources that arise as by-products during industrial production such as oat hulls, sunflower hulls, soybean hulls, wheat bran or wood shavings. In the last decade, research has concentrated on the use of an "innovative" insoluble dietary fiber source, lignocellulose (LC). LC is a constituent of plant cell walls and thus the most abundant and bio-renewable biomass on earth [5]. Studies in farm and companion animals showed that dietary LC may have potential effects on digestive physiology and function [6–10]. This review gives a comprehensive overview of the effects of dietary LC in poultry. First, the physicochemical properties of LC are described and reference is made to methodological aspects of the incorporation of LC into feed, as this can have a decisive influence on the study results. Next, the results of studies on the effects of dietary LC on growth performance, nutrient digestibility, gastrointestinal tract development

* Correspondence: ilen.roehe@fu-berlin.de
Department of Veterinary Medicine, Institute of Animal Nutrition, Freie Universität Berlin, 14195 Berlin, Germany

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and intestinal microbiota are summarized and compared with those observed in feeding experiments using other insoluble fiber sources. In particular, the potential mode of action of insoluble dietary fiber on the digestive physiology of chickens is discussed. In addition, some considerations regarding future research directions and methodological challenges are presented and discussed.

Chemical composition and physicochemical properties of LC

Dietary fiber comprises a significant part of plant feedstuffs and is chemically defined as the sum of non-starch polysaccharides (NSP) and lignin [11]. From a physiological point of view, dietary fiber comprises any polysaccharide and lignin that is not degraded by endogenous enzymes in the digestive tract, hence reaching the hindgut [12, 13]. Different types of plants contain different amounts and chemical structures of fibers with varying physical properties [14]. Therefore, fiber sources differ in their content of soluble and insoluble NSP. Fiber sources with high levels of insoluble NSP are for example LC, oat hulls, sunflower hulls or wheat bran, while sugar beet pulp or apple pomace contain higher concentrations of soluble NSP [3, 13]. Lignocellulosic biomass refers to plant dry matter of different origin and is mainly composed of the carbohydrate polymers cellulose and hemicelluloses as well as the phenolic polymer lignin [15, 16]. The proportional composition of carbohydrate and aromatic polymers of LC may vary depending on the type of lignocellulosic biomass used [17, 18]. LC applied in animal nutrition is usually derived from forest residues containing different proportions of hard and soft wood as well as bark. A recent study analyzed the chemical composition of three LC products used as fiber additives in animal feed [19]. Table 1 shows the chemical and physicochemical characteristics of these LC products compared to that of other insoluble fiber sources, in particular, oat hulls, sunflower hulls and wheat bran. The LC products showed a similar crude fiber content, but differences in the detergent fibers, which allow a rough assessment of the insoluble fractions of cellulose, hemicelluloses and lignin [13, 21]. Two products (LC2 and LC3) showed a similar cellulose, hemicelluloses and lignin content of ~ 415, ~ 150 and ~ 330 g/kg dry matter (DM), respectively, which are close to that reported by Zeitz et al. [22]. The LC1 product, however, contained significantly more lignin (~ 650 g/kg DM) and lower amounts of cellulose (~ 78 g/kg DM). All three LC products comprised high proportions of more than 90% insoluble fibers and only small amounts of soluble fibers [19, 22]. Similarly, oat hulls were mainly composed of insoluble dietary fiber [20] and contained primarily hemicelluloses and cellulose. Sunflower hulls showed slightly lower values for crude and detergent fiber, but the relative distribution of cellulose, hemicelluloses and lignin was similar when compared to LC2 and LC3. Sunflower hulls contained mostly insoluble fiber, but about twice as much soluble fiber compared to LC. Wheat bran had the lowest crude fiber content of all the fiber sources shown and was mainly composed of hemicelluloses. In addition, the proportion of soluble to insoluble dietary fibers in wheat bran was slightly higher compared to LC. With respect to the physicochemical properties, there is a positive correlation between dietary soluble fiber content and digesta viscosity in monogastric animals [13]. Due to the low proportion of soluble fibers such as pectins, insoluble dietary fiber sources have little effect on digesta viscosity [3, 14]. Another important physicochemical feature of dietary fiber is their hydration capacity, which can be characterized by the swelling capacity, the water holding and binding capacity [13]. The hydration capacity of a dietary component affects the bulking effect of digesta [23], which in turn could have consequences on digesta retention time and nutrient digestibility [24]. LC showed higher

Table 1 Chemical and physicochemical characterization of different lignocellulose products in comparison with other insoluble fiber sources

Item	Unit	LC1[a,b]	LC2[a,b]	LC3[a,b]	Oat hulls[c]	Sunflower hulls[a]	Wheat bran[a]
Crude fiber	g/kg DM	579	559	561	302	535	145
Neutral detergent fiber ($aNDF_{OM}$)		926	919	874	750	843	585
Acid detergent fiber (ADF_{OM})		728	757	737	357	679	181
Acid detergent lignin (ADL_{OM})		650	329	335	40	255	70
Total dietary fiber		953	945	949	762	897	612
Insoluble dietary fiber		942	933	938	754	871	579
Soluble dietary fiber		11	13	12	8	27	34
Water holding capacity	mL/g DM	5.21	4.63	7.43	3.9	4.35	5.51
Water binding capacity		7.29	6.30	6.35	N/A	5.88	5.09
Swelling property	%	205	150	185	2.1[d]	65	55

[a] According to Slama et al. [19]; [b] Information on the LC product used, if specified, is given in additional file 1; [c] According to Jiménez-Moreno et al. [20]; [d] Indicated as mL/g DM

hydration capacities and significantly greater swelling properties compared to oat hulls, sunflower hulls and wheat bran [19, 20]. Finally but yet importantly, the particle size of a fiber source is another key characteristic, which may influence digestive function [23]. After processing and fiber breakdown, LC is a powdery material with an average particle size of 80 to 300 μm [22, 25]. This material can then be further processed, so that various LC products are commercially available, e.g. in powdery, crumbled or pelleted form. The particle size of other insoluble fiber sources is usually larger depending on the degree of grinding.

Inclusion of insoluble fiber sources in experimental diets

In order to investigate the effect of dietary insoluble fiber in chickens, several feeding experiments were designed using different feed formulations. In principle, there are three different options to include fiber sources in diets, as displayed in Fig. 1. In "feed formulation 1", a "control" feed is compared with a "fiber dilution" diet. A "control" poultry diet usually consists of grains, protein sources, plant oils and a premix. The nutrient composition of the "control" diet should meet the nutrient recommendations for chicken diets. The treatment diet "fiber dilution" is based on the "control" diet, but supplemented with the fiber source of interest. As insoluble fiber sources, such as LC, cellulose or wood shavings, have a low nutritive value, the energy and nutrient content of the diet is diluted by the added fiber source. In "feed formulation 2", the same "control" diet is used, but the fiber containing diet is balanced to be isoenergetic and isonitrogenous. To achieve this, the "iso" diet must be formulated to have increased proportions of fat and protein at the expense of the carbohydrate source. Consequently, the "control" and the "iso" diet show remarkable differences in the feed- and nutrient composition, but show similar energy and protein concentrations. In "feed formulation 3", a "control sand" and a "fiber dilution" diet is used. The "control sand" diet is based on the "control" feed, but contains a certain percentage of an insoluble ash source, e.g. sand or sepolite. The "fiber dilution" diet is based on the "control sand" diet, but the insoluble ash source is substituted by the insoluble fiber source of interest. Thus, the feed composition is similar with the exception of the components sand

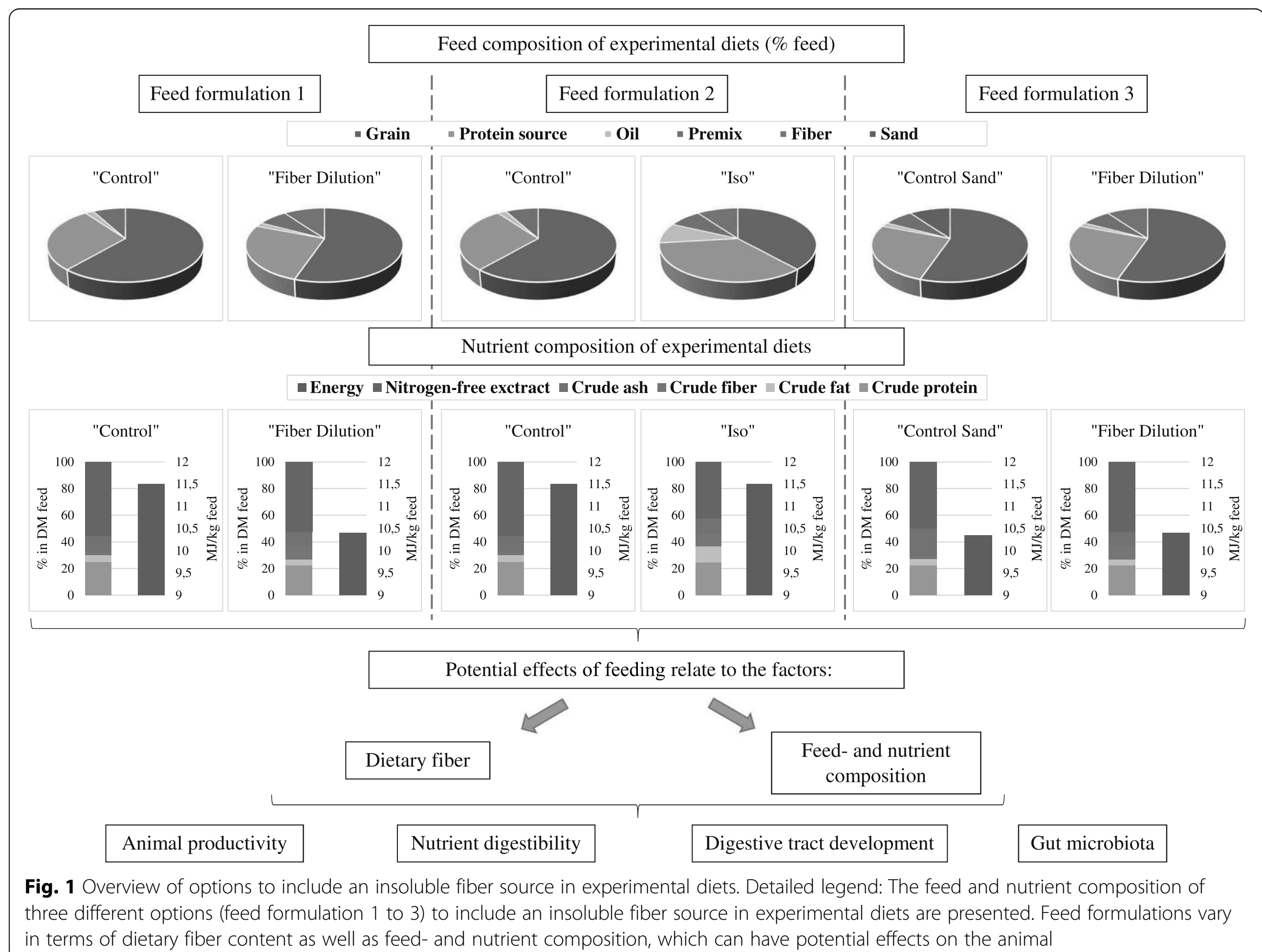

Fig. 1 Overview of options to include an insoluble fiber source in experimental diets. Detailed legend: The feed and nutrient composition of three different options (feed formulation 1 to 3) to include an insoluble fiber source in experimental diets are presented. Feed formulations vary in terms of dietary fiber content as well as feed- and nutrient composition, which can have potential effects on the animal

and fiber. As a result, the nutrient composition of both diets differs significantly only in terms of crude ash and crude fiber. In summary, the dietary inclusion of an insoluble fiber source is coupled with differences in the feed- and nutrient composition of the experimental diets. Therefore, the effects of feeding these diets can be attributed to both, the factor "dietary fiber" and the factor "feed and nutrient composition" (Fig. 1). Thus, alterations in animal productivity, nutrient digestibility, digestive tract development or gut microbiota might be related to differences in dietary fiber and/or feed and nutrient composition. Reference is made to this issue in the respective sections of this review.

Impact of dietary LC on productivity of chickens

During the last decade, several studies examined the effect of feeding LC on the productivity of broilers (Table 2) and laying hens (Table 3). Productivity parameters include body weight (BW), average weight gain (AWG), average feed intake (AFI) or average daily feed intake (ADFI), feed conversion ratio (FCR), egg production (EP) and egg weight (EW). Studies differed in terms of feed formulation and LC inclusion level used. In most studies, dietary LC was supplemented on top of feed ("feed formulation 1", Fig. 1); only few used experimental diets based on "feed formulation 2 and 3" (Fig. 1). Commonly, lower dietary LC inclusion levels in the range of 0.05% to 2% have been used, while few experiments were performed using relatively high concentrations of dietary LC of 5% to 15%.

Results obtained in broiler trials using relatively low dietary concentrations of LC are contradictory (Table 2). The feeding of diets supplemented with 0.25% to 2% LC positively affected the FCR of broilers due to an increase in weight gain [26, 28]. In contrast, broilers fed diets supplemented with 1% LC had an impaired FCR compared to those receiving the control diet [29]. However, several studies using similar LC inclusion levels showed no impact of dietary LC on broiler growth performance [22, 24, 27]. Results of a recent study demonstrated that dietary LC concentrations of 0.05% to 0.1% improved FCR of broilers, while the supplementation of 0.2% LC showed no effect on FCR [32]. Broilers fed 0.6% LC, which was added at the expense of soybean meal and corn, showed higher BW after 42 d of feeding compared to those fed the control and 0.4% LC [30]. Interestingly, the feeding of the same LC concentration of 0.6%, but which was added at the expense of soybean meal only, did not affect final BW of broilers [30] suggesting that the feed composition had a greater impact on BW development than the LC addition. Broilers fed diets diluted with relatively high concentrations of LC of 5% to 15% showed a marked decrease in average daily gain with increasing concentrations of dietary LC, while feed intake tended to increase with increasing concentrations of LC [31]. On the contrary, broiler productivity seems to be unaffected by relatively high dietary LC inclusion levels up to 10% when diets were composed to be isoenergetic and isonitrogenous [25, 39].

With respect to feeding experiments with commercial hybrid pullets and laying hens (Table 3), most studies showed that growth and laying performance were not affected by dietary LC inclusion levels of 0.8% to 2% [33–35]. However, Sozcu and Ipek [36] demonstrated that the supplementation of 0.05% and 0.1% dietary LC increased BW, ADFI, EP, and EW of laying hens between 18 to 38 weeks of age compared to hens fed the control diet. A further increase in the dietary LC concentration to 0.2%, however, led to a decrease in EP and EW [36]. In two other studies, the effect of dietary LC was investigated in broiler breeder hens [37] during the laying phase (43 to 55 weeks of age) and in dual purpose hens [38] during the growing (1–22 weeks of age) and laying period (23–52 weeks of age). Broiler breeder and dual purpose hens tended to overconsume feed leading to an increased body fat content, which in turn might be related to the observed lower productive efficiency [40–42]. Thus, the hypothesis in both studies was that BW and body fat percentage of hens can be reduced by feeding a nutrient-reduced LC-containing diet and that this is accompanied with an improved reproductive performance [37, 38]. The results showed that dietary LC reduced BW, body fat content [38] and abdominal fat weight of hens [37], which was directly associated with an improved laying performance.

In principle, due to the use of different feed formulations and inclusion levels, it is difficult to make a conclusive statement about the effect of LC on chicken productivity. Few studies showed that similar insoluble fiber sources such as cellulose or wood shavings could have a positive impact on broiler growth performance [43, 44]. It was suggested that a combination of improved gut function and enhanced nutrient digestibility was responsible for observed beneficial effects [43, 44]. With regard to dietary LC and lower inclusion levels, it was also hypothesized that digestive physiology and nutrient digestibility might be affected leading to improved growth performance [26, 28, 30]. However, data on the effects of dietary LC on digestive tract development and nutrient digestibility were inconclusive, as described later. Studies generally showed that animal productivity was impaired when diets were supplemented with higher dietary LC concentrations. This observation is explained by the fact that the energy- and nutrient content of diets was considerably reduced by the LC inclusion ("feed formulation 1", Fig. 1) resulting in a lower energy and nutrient intake of chickens impairing growth performance. However, animal productivity seems to be not affected

Table 2 Impact of dietary lignocellulose on broiler growth performance

Feed[1]	Age, d	LC Inclusion[2], %	Final BW[3], g	AFI[4], g	AWG[5], g	FCR	Reference
1	21	0		1057	582	1.81^{a}	[26]
		0.25		1049	596	1.75^{a}	
		0.50		1033	615	1.67^{b}	
		0.75		1030	618	1.66^{b}	
	1–42	0		4016	1915^{b}	2.1^{a}	
		0.25		4086	2084^{a}	1.96^{b}	
		0.50		4030	2073^{a}	1.94^{b}	
		0.75		4156	2147^{a}	1.93^{b}	
1	1–42	0	2422		80.2	1.865	[27]
		0.25	2423		80.7	1.876	
		0.5	2436		81.0	1.854	
		1.0	2429		81.0	1.875	
3	7–14	0		161	69.2^{b}	2.33^{a}	[28]
		1		150	78.0^{a}	1.92^{b}	
		2		150	71.7^{b}	2.09^{ab}	
	14–21	0		326	128	2.55^{a}	
		1		299	152	1.98^{b}	
		2		291	145	2.03^{b}	
1	1–10	0		294	277	1.059	[24]
		1		292	271	1.078	
		2		294	276	1.065	
	1–35	0		3770	2741	1.376	
		1		3778	2716	1.392	
		2		3797	2719	1.397	
1	1–21	0		1085	860	1.265^{b}	[29]
		1		1054	815	1.297^{a}	
1	1–7	0		129^{b}	121^{c}	1.07^{a}	[30]
		0.4		132^{b}	128^{ab}	1.03^{b}	
		0.6[6]		140^{a}	132^{a}	1.06^{a}	
		0.6[7]		130^{b}	126^{bc}	1.03^{b}	
	14–21	0		590	396^{b}	1.49	
		0.4		588	402^{b}	1.46	
		0.6[6]		621	432^{a}	1.44	
		0.6[7]		594	403^{b}	1.47	
	28–42	0	2428^{b}	2295^{a}	1284	1.79^{a}	
		0.4	2423^{b}	2084^{b}	1245	1.67^{b}	
		0.6[6]	2611^{a}	2186^{c}	1310	1.67^{b}	
		0.6[7]	2495^{b}	2182^{bc}	1292	1.69^{b}	
1	1–35	0	2431	3459	2390	1.42	[22]
		0.8[8]	2370	3386	2329	1.42	
		0.8[8]	2490	3452	2448	1.39	
1	13–25	0	1080^{a}	0.250[9]	0.173[10]		[31]
		5	995^{ab}	0.258[9]	0.164[10]		
		10	928^{bc}	0.258[9]	0.153[10]		

Table 2 Impact of dietary lignocellulose on broiler growth performance *(Continued)*

Feed[1]	Age, d	LC Inclusion[2], %	Final BW[3], g	AFI[4], g	AWG[5], g	FCR	Reference
		15	836[c]	0.270[9]	0.149[10]		
1	1–35	0	2154[bc]	1293[ab]	650	1.58[a,11]	[32]
		0.05	2201[b]	1266[bc]	631	1.51[b,11]	
		0.1	2305[a]	1314[a]	667	1.50[b,11]	
		0.2	2142[c]	1266[c]	626	1.55[ab,11]	
2	70–91	0.8	1658	899	454	1.98	[25]
		5	1639	992	467	2.15	
		10	1618	905	432	2.10	

[1] Feed formulation according to Fig. 1; [2] Information on the LC product used, if specified, is given in additional file 1;[3] BW = body weight; [4]AFI = average feed intake; [5] AWG = average weight gain; [6] LC was included in the diet at the expense of 0.3% soybean meal and 0.3% maize; [7] LC was included in the diet at the expense of 0.6% soybean meal; [8] Two different lignocellulose products were compared; [9] Indicated as average daily feed intake in (g/d)/BW; [10] Indicated as average daily gain in g/d/BW; [11] Indicated as g weight gain/g feed consumption; [a,b,c]Means with different superscripts are significantly different

when higher concentrations of LC are included in isoenergetic and isonitrogenious diets ("feed formulation 2", Fig. 1). This phenomenon is already known from former studies, which showed that the crude fiber concentration did not influence growth performance unless it affected the energy content in the diets [45–47]. Chickens usually have the ability to cover their metabolic energy requirement to a certain degree by increasing or decreasing the feed consumption [48, 49]. Chickens receiving diets diluted by sand or oat hulls up to 20% showed an increased feed intake resulted in a similar energy intake and average daily gain in comparison to those receiving an undiluted control diet [49, 50]. With respect to higher dietary inclusion levels of powdery LC, this

Table 3 Impact of dietary lignocellulose on hen productivity

Species	Feed[1]	Age, week	LC inclusion[2], %	Final BW[3], g	ADFI[4], g	EP[5], %	EW[6], g	Reference
Laying hen	1	30	0		126	94.7	65	[33]
			1		131	95.5	66	
		36	0		144	93.4	66	
			1		144	93.7	66	
Pullet	1	8–18	0	1580				[34]
			1	1678				
Laying hen	1	22–31	0	1902				[34]
			0.8	1919				
Pullet	1	1–8	0	766	50.6			[35]
			1	776	52.4			
			2	764	51.6			
Laying hen	1	18–38	0	1655[c]	96.3[b]	78.0[c]	55.3[c]	[36]
			0.05	1693[b]	98.0[a]	80.8[b]	56.4[b]	
			0.1	1719[a]	99.2[a]	81.8[a]	57.3[a]	
			0.2	1636[c]	95.4[b]	78.6[d]	54.4[d]	
Broiler breeder hen	3	43–55	0	~4500[a7]	174[a]	56.9[b]	69.9	[37]
			3	~4300[b7]	172[b]	62.9[a]	69.2	
Dual purpose hen	1	1–22	0	1835[a]	61.1			[38]
			10	1694[b]	61.2			
		23–52	0	1996[a]	101[b]	63.4[b]	60.6[a]	
			10	1791[b]	107[a]	72.4[a]	58.0[b]	

[1] Feed formulation according to Fig. 1; [2] Information on the LC product used, if specified, is given in additional file 1; [3] BW = body weight; [4] ADFI = average daily feed intake; [5] EP = egg production; [6] EW = egg weight; [7] Data on BW are estimated because they were taken from a figure; [a,b,c] Means with different superscripts are significantly different

regulatory mechanism seem to be partially restricted [31, 38], which might be attributed to the physical form of fine LC fibers and its physical bulking effect [37, 38]. Accordingly, the use of higher concentrations of powdery LC in feed for broiler breeder and dual purpose hens, might be a reasonable dietary strategy to control feed intake and weight gain ensuring an optimal productive performance.

Impact of dietary LC on the nutrient digestibility in chickens

Data regarding the effect of dietary LC on nutrient digestibility in chickens are scarce displaying no clear picture. A direct comparison of results is difficult: on the one hand, diets differed in their feed composition and nutrient content; on the other hand, different dietary LC concentrations were used. The feeding of isoenergetic and isonitrogenous diets containing 0.8% LC increased the true digestibility of protein as well as the apparent and true dietary amino acid digestibility in roosters compared to those fed the control diet [51]. Similarly, the same authors observed that the apparent protein digestibility was increased by 5.5% in broilers fed 0.8% dietary LC compared to those receiving the control diet [52]. In contrast, the supplementation of 1% or 2% dietary LC did not affect protein and gross energy digestibility in broilers [24, 29]. Feeding of isoenergetic and isonitrogenous diets with higher LC inclusion levels of 5% and 10% led to a decrease in the apparent ileal digestibility of crude protein and apparent excreta digestibility of organic matter and gross energy while the total tract digestibility of ether extract was not affected [25]. The apparent ileal fat digestibility and total tract digestibility of total fatty acids in broilers was also not influenced by the supplementation of 0.25% and 0.5% LC while the feeding of 1% LC resulted in an increased apparent fat digestibility [53].

If dietary LC has an impact on the digestibility of nutrients, either beneficial or detrimental, the question arises as to how LC might affect the digestive physiology of chickens. Regarding the beneficial effects, it is well known that the feeding of structural components, such as coarse fiber particles, may stimulate digestive function, which is associated with an improved nutrient digestibility [23, 54]. LC consists of very small fiber particles and thus it remains unclear whether dietary LC may affect the digestive physiology and thus the nutrient digestibility in chickens, as discussed later. Another positive effect of feeding LC might be related to the fat digestibility. Jiménez-Moreno et al. [55] speculated that dietary cellulose might have an effect on micelle formation and lipid absorption, enhancing bile acids recycling and fat absorption. Further research is needed in order to evaluate this hypothesis. With respect to the potential adverse effects, it was assumed that dietary LC might have an abrasive effect on the intestinal mucosa [27, 30], thus enhancing endogenous amino acid losses. In this regard, Kluth and Rodehutscord [56] showed that the feeding of increasing concentrations of cellulose up to 8% significantly elevated the inevitable losses of crude protein and amino acids in broilers. Whether this observation also applies to the feeding of increasing concentrations of LC requires further clarification. In summary, based on the studies carried out so far, no statement can be made about whether LC has an impact on nutrient digestibility in chickens.

Impact of dietary LC on gastrointestinal tract development, intestinal morphology and excreta characteristics of chickens

Few studies exist evaluating the effect of dietary LC on the gastrointestinal tract development and digestive physiology of broilers and laying hens. Investigations were focused on the gastrointestinal gross morphology, the intestinal histomorphology, and digesta as well as excreta characteristics.

Effect on gizzard development and function

In broilers, most studies showed that the feeding of lower dietary LC concentrations up to 2% did not affect the relative weight of the gizzard [22, 24, 28–30]. However, feeding of isoenergetic and isonitrogenous diets containing 5% LC resulted in an increased gizzard weight of slow growing broilers [39]. Similarly, the relative gizzard weight was affected by feeding LC in laying hens and pullets [34]. Pullets fed 1% LC over a period of 10 weeks showed increased relative weights of the gizzard. Moreover, laying hens, aged 31 weeks, developed heavier gizzards when fed diets diluted with 0.8% LC after 12 weeks of feeding [34]. Interestingly, these effects were not observed in chickens that received these diets for a shorter period, suggesting a time-dependent effect of LC. Studies in quails also showed that the feeding of isoenergetic and isonitrogenous diets containing 3% LC increased the relative gizzard weights [57]. Few studies investigated the effect of dietary LC on gizzard pH showing conflicting results. The feeding of diets supplemented with 0.4% and 0.6% LC decreased the gizzard pH of broilers [30], while the feeding of 0.8%, 1% and 2% dietary LC had no impact on gizzard pH [22, 24]. Broilers fed diets containing 0.05% to 0.2% LC showed also a similar gizzard pH compared to those fed the control diet [32].

In general, coarsely ground fiber sources such as oat, soybean and pea hulls, or wood shavings, containing primarily insoluble NSP, are known to increase gizzard size and weight [43, 58, 59]. An increased gizzard weight might be an indicator of enhanced gizzard function [23,

54, 60, 61]. Several feeding experiments using different coarsely ground fiber sources showed that an increased gizzard weight was accompanied with a lower gizzard pH [59, 61–63] suggesting an enhanced proventricular secretion of hydrochloric acid. Moreover, an increased gizzard activity is related to an increased gastrointestinal reflux and pancreatic enzyme secretion [64–67]. Furthermore, the feed passage rate might be affected by feeding structural fiber components improving nutrient digestibility and growth performance of chickens. In this regard, it is generally accepted that the feeding of coarsely ground, insoluble fibers increases the feed passage rate [23]. However, in poultry it is suggested that the feeding of moderate amounts of structural dietary fiber reduces the digesta transit time, as fiber particles may accumulate in the gizzard [23, 58]. A recent study proved that digesta transit time of broilers was not affected by feeding diets containing finely ground LC or oat hulls [29]. The question arises whether the fiber inclusion itself, the particle size of the fiber source or a combination of both factors are responsible for observed effects on gizzard development and digestive function. In this regard, Jiménez-Moreno et al. [55] investigated the impact of type and particle size of dietary fiber on gizzard weight of broilers (Fig. 2). Diets contained different fiber sources, in particular cellulose, oat hulls and sugar beet pulp at inclusion levels of 3%. Furthermore, diets differed in terms of particle size distribution indicated by different geometric mean diameters (GMD). Results indicated that broilers fed diets showing the highest GMD, namely coarsely ground oat hulls and sugar beet pulp, developed the greatest gizzard weights, while broilers fed diets having the lowest GMD, cellulose and finely ground sugar beet pulp, showed the lowest gizzard weights (Fig. 2). It was concluded that dietary cellulose did not stimulate gizzard function due its lack of physical structure [55]. Thus, it seems obvious that particle size of the fiber source, rather than fiber inclusion itself, is the determining factor that stimulates gizzard development. In this regard, it has been suggested that feed particles should be at least larger than 1 mm to enhance gizzard development [54, 68]. Based on this, it can therefore be assumed that fine-fiber LC, similar to cellulose, has little effect on gizzard development and function. In this context, it would be interesting to investigate whether the physical form or the macrostructure of LC might have an impact on gizzard development. Therefore, future studies should investigate the effects of the physical form of LC and that of the overall feed structure on digestive physiology in chickens.

Effect on the intestinal gross morphology

Only few studies investigated the impact of dietary LC on the development of the small and large intestine, but most of them showed no effect of LC feeding on the intestinal gross morphology. In broilers, the feeding of relatively low dietary LC inclusion levels of 0.4% and 0.6% did not affect the relative weight of the intestine [30]. In agreement with this, broilers fed diets supplemented with 1% LC had similar relative duodenal, jejunal and cecal weights compared to those fed the control diets [29]. Relative lengths of small intestinal segments were also comparable between broilers fed 0.25% to 1% dietary LC and those offered the control diet [69]. Similarly, the absolute length of the small intestine and cecum of broilers was not influenced by feeding isoenergetic and isonitrogenous diets containing 2% and 4% LC. In another study, the feeding of isoenergetic and isonitrogenous diets containing 3% LC increased the relative weight of the jejunum and ileum of quails [57]. Pullets fed 1% LC over a period of 10 weeks

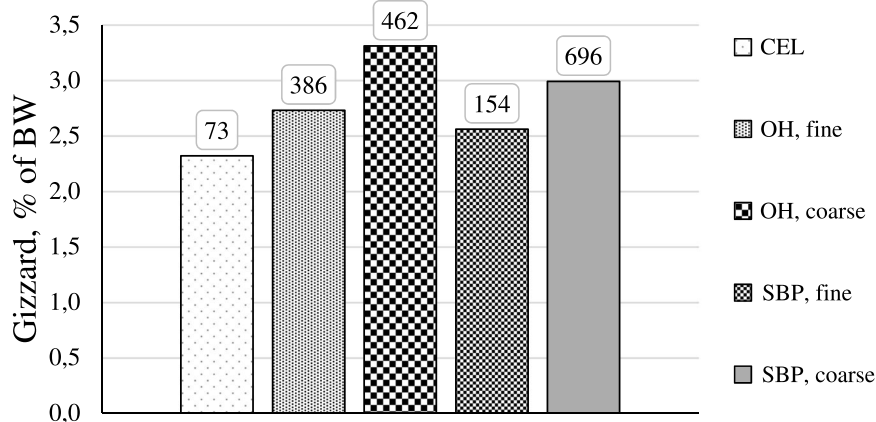

Fig. 2 Impact of dietary fiber and particle size on the gizzard weight of broilers. Detailed legend: Effect of type and particle size of dietary fiber on the relative gizzard weight of broilers according to Jiménez-Moreno et al. [55]; CEL = cellulose-fed broilers; OH = oat hulls-fed broilers; SBP = sugar beet pulp-fed broilers; the geometric mean diameter of diets is indicated above the respective bar

showed comparable relative weights of the small intestine compared to those fed the control diet [34]. The feeding of diets supplemented with LC concentrations of 1% to 2% also showed no impact on the absolute cecal weight and length of laying hens [35] nor on the relative cecal weight of pullets [34]. The feeding of isoenergetic and isonitrogenous diets containing higher concentrations of LC up to 5% also had no impact on the absolute length of the small intestine and cecum of slow growing broilers [39]. In contrast, the feeding of energy- and nutrient reduced diets containing 10% LC, led, in relation to the BW, to increased weights of the small and large intestine [70].

Reasons for alterations in length or weight of intestinal organs due to feeding insoluble fiber sources are not fully understood. In general, it is supposed that an increase in the intestinal size and length but also an enlargement of the intestinal mucosa contributes to a higher intestinal weight [71]. Several studies demonstrated that chickens fed different insoluble fiber sources at varying inclusion levels showed increased relative digestive tract weights [63, 70, 72] implying a fiber-related effect on intestinal organ development [63, 72]. However, in those studies dietary fiber inclusion also led to a decrease of the chicken's BW [63, 70, 72]. Therefore, the hypothesis that dietary fiber influenced organ weight development in those chickens is not valid as data on intestinal weight and length are related to the BW. Only considering studies in which chickens had similar empty BW, results on the effect of fiber on intestinal tract development are conflicting. On the one hand, it has been reported that feeding of isoenergetic and isonitrogenous diets containing 3% insoluble fiber sources, such as oat and soybean hulls, increased the digestive tract weight of broilers [59, 61, 73, 74]. On the other hand, the feeding of isoenergetic and isonitrogenous diets containing 3% oat hulls, soybean hulls or cellulose did not affect the relative weight of intestinal organs [55, 75, 76]. Similarly, the feeding of diets, supplemented with 10% oat hulls or cellulose, did also not affect the relative weight of intestine of broilers [77, 78]. Regarding possible fiber-effects on intestinal tract development, it was suggested that an enlargement of the digestive tract might be a consequence of physical distension caused by luminal swelling of the ingested fiber sources [59, 63, 72]. Further research is needed in order to clarify whether and why dietary insoluble fiber may have an impact on gut gross morphology.

Effects on the intestinal mucosal development

Few studies showed that dietary LC might affect the morphology of the intestinal mucosa of chickens. Sarikahn et al. [26] showed that ileal villus height and villus height to crypt depth ratio were increased in broilers fed diets containing 0.25%, 0.5% and 0.75% LC. Similarly, broilers receiving diets supplemented with up to 2% LC had an increased jejunal villus height, villus apparent surface area and villus height to crypt depth ratio compared to those fed the control diet [32]. In contrast, duodenal and jejunal villus height and crypt depth were reduced in broilers fed diets supplemented with 0.5% LC while the inclusion of 1% LC showed no effect on villus morphology [69]. Interestingly, a different observation was made in the ileum of those chickens as increasing concentrations of dietary LC led to an increase in villus height and crypt depth [69]. The feeding of 0.6% LC, which was included in the diet at the expense of soybean meal, resulted in an increased villus height and width as well as crypt depth in the duodenum, jejunum and ileum of broilers [30]. However, the same inclusion level of 0.6% dietary LC, supplemented at the expense of 0.3% soybean meal and 0.3% corn, did not affect duodenal and jejunal villus height and villus width of broilers [30] implying that intestinal mucosal development was influenced by feed composition rather than dietary LC inclusion. Abdollahi et al. [29] showed that the supplementation of 1% dietary LC had no impact on histomorphological parameters in the duodenum and jejunum of broilers. In laying hens, the feeding of 0.05% and 0.1% dietary LC increased jejunal villus height and width, villus apparent surface area and villus height to crypt depth ratio, while a further increase in dietary LC concentration of 0.2% decreased observed histomorphological parameters [36]. Dual purpose hens fed diets supplemented with 10% LC showed an enhanced mucosal development of the colorectum indicated by a greater villus area and a higher villus and crypt mucosal enlargement factor [70]. Interestingly, the colorectal villus surface was negatively correlated with the short chain fatty acid (SCFA) concentration in the cecum of hens [70]. Another study showed that the feeding of 3% dietary LC included in isoenergetic and isonitrogenous diets increased the villus height and villus height to crypt depth ratio in the jejunum and ileum of quails [57].

An enlargement of the intestinal surface area due to longer or increased numbers of intestinal villi is generally associated with an increased intestinal nutrient absorption [79, 80] and thus an improved nutrient utilization. However, the development of the intestinal microarchitecture strongly depends, among other things, on the concentration of enteral nutrients and thus on the nutrient content of the diet [81, 82]. It has been suggested that chickens fed high-fiber diets suffer from a nutrient deficiency and thus try to enhance nutrient and bacterial metabolite absorption by increasing the mucosal surface area [70, 82, 83]. Thus, it has to be emphasized that both the dietary nutrient content and/or the fiber inclusion might affect the mucosal development of

the intestine (Fig. 1). However, effects on intestinal mucosal development were also observed in studies using isoenergetic and isonitrogenous diets or lower dietary fiber inclusion levels [26, 30, 74], so that similar enteral nutrient concentrations can be expected. The potential mode of action of dietary insoluble fiber on intestinal mucosal development in chickens is still unknown. Whether specific chemical and physicochemical properties of the fiber source or changes in the intestinal microbiota due to fiber feeding could be responsible requires further clarification.

Effects on excreta quality

Studies in broilers indicated that dietary LC inclusion might have a positive effect on litter quality. Litter moisture content was lower in broilers fed diets supplemented with 0.6%, 0.8%, 1%, and 2% LC compared to litter of control-fed broilers [24, 30, 52]. Similarly, litter moisture content was also reduced in quails fed 3% dietary LC [57]. The litter DM content usually correlates with the DM content of the excreta. Consistent with the latter, laying hens fed 10% LC for 52 weeks had a higher excreta DM content at 10, 17 and 22 weeks of age compared to those fed the control diet [70]. However, studies in broilers showed no impact of dietary LC on excreta scoring or excreta DM [22, 25].

In comparison with other insoluble fiber sources, LC has a moderate to high water holding capacity [19]. It has been speculated that the water holding capacity and the digesta retention time might be increased in LC fed chickens resulting in increased luminal water absorption and higher excreta DM content [24]. In addition to the hydration capacity, digesta- and excreta DM might be also affected by the particle size of the fed fiber source. Excreta score was improved in broilers fed coarsely ground wood shavings at a ratio of 6:100 (w/w), while finely ground cellulose- and control-fed broilers showed comparable excreta scores [43]. Authors speculated that coarsely ground fibers might hold larger amounts of water reducing the solubilisation of NSP than finely ground fiber particles [43]. Further research is needed in order to clarify whether and why insoluble fiber sources might reduce excreta DM and thus improve litter quality.

Impact of dietary LC on the gut microbiota

It is well known that dietary fiber can modulate the gut microbiota in humans and animals, which in turn might have consequences on the intestinal health [84, 85]. Thus, few studies evaluated the impact of dietary LC on the intestinal microbiota in chickens. Alterations in the microbial composition can be accompanied with changes in the production of bacterial metabolites; vice versa, shifts of the intestinal bacterial metabolite profile are a clear indicator for a modification of the composition and activity of intestinal bacteria. Therefore, investigations were focused on both, bacteria residing in the avian intestinal tract and the concentration of intestinal bacterial metabolites. Table 4 shows the impact of dietary LC on the concentration of SCFA in the intestine of chickens. In general, results are conflicting, which may be explained by differences in the used study design, in particular regarding the used feed formulation, LC inclusion level and LC product.

Few studies used the same LC product, but different LC inclusion levels and feed formulations [25, 27, 30, 70]. The feeding of diets supplemented with LC at relatively low inclusion levels of 0.25% to 0.6% reduced counts of *Escherichia coli* and *Clostridium perfringens* and increased those of *Bifidobacterium* spp. and lactic acid bacteria in the ileum and cecum of broilers [30]. Similarly, ileal counts of *Lactobacillus* spp. as well as ileal and cecal counts of *Bifidobacterium* spp. were elevated in broilers fed diets supplemented with 0.25%, 0.5% and 1% LC [27]. Ileal and cecal counts of *Escherichia coli* and *Clostridium* spp. were also reduced in broilers receiving 0.25% and 0.5% dietary LC [27]. In the same experiment, however, the intestinal SCFA profile was generally not affected by LC feeding (Table 4), with the exception of broilers receiving 0.5% LC, which showed increased total SCFA concentrations in the ileum and cecum [27]. Two further studies evaluated the effect of relatively high concentrations of dietary LC on bacterial composition and metabolites [25, 70]. The feeding of diets diluted with 10% dietary LC had generally no impact on cecal microbial composition in dual pupose hens, but reduced the cecal concentration of SCFAs and ammonia [70]. Similarly, cecal bacterial metabolites were reduced in broilers fed isoenergetic and isonitrogenous diets containing 10% LC [25]. Moreover, increasing concentrations of dietary LC decreased counts of Escherichia/Hafnia/Shigella [25]. Four recent studies using a potential more fermentable LC product also displayed conflicting results regarding the effect of dietary LC on the gut microbiota in broilers and laying hens [22, 24, 35, 86]. The feeding of isoenergetic, isonitrogenous diets containing 2% and 4% LC increased the cecal microbial diversity and the abundance of butyrate-producing bacteria in free-range chickens, while the luminal concentration of butyrate, acetate and propionate was not affected [86]. In contrast, broilers and laying hens fed diets which were supplemented with 0.8% and 1% LC, showed no alterations in the overall cecal microbial diversity [22, 35]. Sun at al [35]. showed that the feeding of 1% LC increased the relative abundance of lactate- and butyrate-producing bacteria in the cecum of laying hens, which was accompanied with higher concentrations of cecal SCFAs. In contrast, the total amount

Table 4 Impact of dietary lignocellulose on the concentration of intestinal short chain fatty acids (SCFA)

Species	Feed[1]	Part[2]	LC inclusion[3], %	Unit	Total SCFA	Acetate	Propionate	Butyrate	Reference
Broiler	1	Ile	0	µmol/g	53[b]	9.16[bc]	3.78[b]	4.54	[27]
			0.25		61.1[ab]	7.16[c]	9.72[ab]	5.22	
			0.5		86.3[a]	20[a]	17.4[a]	5.33	
			1		69.6[ab]	18.3[ab]	9.18[ab]	4.54	
Broiler	1	Cec	0	µmol/g	124[b]	29.3	22.0	6.70	[27]
			0.25		150[ab]	30.1	25	6.58	
			0.5		162[a]	32.8	26.1	6.36	
			1		129[b]	26	23.8	5.79	
Broiler	1	Cec	0	µmol/g	126	101	5.98	17.8[a]	[22]
			0.8[4]		127	104	4.92	17.0[a]	
			0.8[4]		119	99	5.28	13.1[b]	
Broiler	2	Cec	0	mmol/L		5.20	1.82	0.80	[86]
			2			4.28	1.30	0.64	
			4			4.09	1.50	0.58	
Broiler	2	Cec	0.8	µmol/g	56.8[a]	38.1	8.89[a]	8.45[a]	[25]
			5		58.2[a]	39.1	9.83[a]	8.04[a]	
			10		44.9[b]	33.9	4.82[b]	5.17[b]	
Laying hen	1	Cec	0	mmol/100 g		2.42[b]	0.62[b]	0.19[b]	[35]
			1			4.07[a]	0.96[a]	0.28[a]	
			2			3.01[b]	0.72[b]	0.20[b]	
Laying hen	1	Cec	0	µmol/g	47.5[a]	37.5[a]	3.79[a]	4.83	[70]
			10		29.2[b]	23.2[b]	2.19[b]	2.89	

[1] Feed formulation according to Fig. 1; [2] Intestinal part, Ile = Ileum, Cec = Cecum; [3] Information on the LC product used, if specified, is given in additional file 1; [4]Two different lignocellulose products were compared; [a,b,c] Means with different superscripts are significantly different

of cecal SCFAs was not affected in broilers fed diets supplemented with 0.8% of the same LC product [22]. Moreover, LC-fed broilers had a lower cecal abundance of the bacteria families Ruminococcaceae and Lactobacillaceae as well as a higher abundance of Clostridiaceae, Enterobacteriaceae, Peptostreptococcaceae and Erysipelotrichaceae [22]. Diets diluted with 1% and 2% LC had in general no effect on detected bacteria except that counts of *Ruminococcus* spp. were increased and those of *Clostridium* spp. reduced in the cecum of broilers fed 2% LC [24].

Based on the studies carried out so far, no uniform picture can be drawn as to whether and to what extent dietary LC influences the intestinal microbiota in chickens. It is generally agreed that insoluble fiber sources such as LC, cellulose or wood shavings, are not extensively degraded by intestinal bacteria residing in the avian digestive tract [14, 85, 87]. On the one hand, this is due to the anatomical peculiarities of the chicken's digestive tract, which is relatively short, resulting in a short feed passage rate. In addition, several studies suggest that only small and soluble fiber fractions can enter the cecum [66, 88, 89], which appears to be the main site for bacterial fermentation of fiber in chickens [90, 91]. On the other hand, there is evidence that the cellulolytic activity of bacteria in the chicken's hindgut seems to be low [92–94]. Consequently, it is assumed that the impact of insoluble fiber on intestinal bacterial composition and activity appears to be minimal [14, 95]. However, some authors speculated that LC could be fermented in the cecum of chickens as intestinal bacterial composition or SCFA profile had changed due to dietary LC inclusion [22, 27, 35]. Furthermore, some studies used an "eubiotic" LC product, which might have a higher susceptibility to microbial fermentation than the standard LC product [22, 35, 86, 96, 97]. Moreover, it was suggested that dietary LC may have an abrasive effect on the intestinal mucosa and adhering bacteria [27, 30] or that phenolic compounds of lignin exhibit antimicrobial properties [22, 27].

The major problem in answering the question of whether dietary insoluble fibers generally have an effect on the intestinal microbiota of chickens refers to the experimental diets chosen to study that effect. With reference to Fig. 1, most studies that investigated the effect of insoluble fiber on gut microbiota, chose experimental diets based on "feed formulation 1", and a few those based

on "feed formulation 2". Depending on the amount of dietary fiber added, there are corresponding changes in the nutrient composition between the control and the fiber containing diet (Fig. 1). Alterations in the nutritional composition of the feed result in changes of the amount of substrate that reaches the large intestine and can be fermented by resident bacteria [25]. As a consequence, changes in the dietary nutrient composition may influence the gut microbiota and bacterial fermentation pathways [25], making it difficult to distinguish between nutrient composition- and fiber related effects. The best way to study the effect of insoluble fiber on gut microbiota is possibly to use feed variants according to "feed formulation 3" (Fig. 1). Feed and nutrient composition of control and fiber diets are very similar, although it cannot be ruled out that even the inclusion of an insoluble ash sources might affect the gut microbiota.

Conclusions

In conclusion, several studies were performed in order to evaluate the effect of dietary LC as an insoluble fiber source in poultry nutrition. Data on the impact of LC on growth performance, nutrient digestibility, digestive tract development and gut microbiota in chickens are inconsistent and do not allow a conclusive assessment. One of the reasons for this is that a direct comparison of results is difficult as studies differed in terms of feed formulations, LC inclusion levels and LC products. In future research, more attention should be paid to the type of feed formulation used in order to better distinguish the effects of dietary fiber from those of the feed and nutrient composition. In addition, the mode of action of LC in the digestive tract should be examined more closely, with particular reference to its chemical and physicochemical properties.

Abbreviations

LC: Lignocellulose; NSP: Non-starch polysaccharides; BW: Body weight; AWG: Average weight gain; AFI: Average feed intake; ADFI: Average daily feed intake; FCR: Feed conversion ratio; EP: Egg production; EW: Egg weight; DM: Dry matter; GMD: Geometric mean diameter; SCFA: Short chain fatty acids

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40104-021-00594-y.

Additional file 1. Overview on LC products used in the different studies. Description of data: Additional information on LC used in the different studies including supplier information and product name.

Acknowledgements

Not applicable.

Authors' contributions

IR and JZ wrote and approved the manuscript.

Funding

Open Access funding enabled and organized by Projekt DEAL.

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that we have no competing interests.

Received: 11 December 2020 Accepted: 11 April 2021
Published online: 17 June 2021

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Druck: Mensch und Buch Verlag