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Effects of Feather Pecking and Ingestion on Intestinal Microbiota and Microbial Metabolites in Pullets and Laying Hens

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„Der Horizont ist da, wo sich die Grenze verschiebt.“

Aus „Reise zum Horizont“
ein Dokumentarfilm von Thomas Latzel

Contents

Abbreviations

ASD	autism spectrum disorders
CNS	central nervous system
DGGE	denaturing gradient gel electrophoresis
g	gravitational force
GI	gastrointestinal
GIT	gastrointestinal tract
H	high feather peckers
IBD	inflammatory bowel disease
L	low feather peckers
LSL	Lohmann-Selected Leghorn
OCD	obsessive compulsive disorders
PCR	polymerase chain reaction
SCFA	short-chain fatty acids
vs.	versus

1 General introduction

1.1 Types of feather pecking

Feather pecking is a behavioral disorder and one of the most important problems in laying hens. It can be divided into two different categories, a mild and a severe form and different motivational causal systems are underlying (Kjaer and Vestergaard, 1999; McAdie and Keeling, 2002; Newberry et al., 2007; Dixon et al., 2008). Mild feather pecking is defined as gentle repeated pecks at the tips and edges of feathers. Thereby feathers are not removed. The pecks are similar to social exploration or allo-preening (Riedstra and Groothuis, 2002; Dixon et al., 2008). The severe form of feather pecking appears as strong pecking and feathers are pulled and removed, causing plumage damage (Savory, 1995). It is generally assumed that severe feather pecking in laying hens is a form of redirected behavior influenced by the motivational system of foraging and feeding (Blokhuis, 1986; Huber-Eicher and Wechsler, 1998; Wechsler and Huber-Eicher, 1998; Dixon et al., 2008). A less supported theory is based on a frustrated dust-bathing motivation (Vestergaard and Lisborg, 1993).

1.2 Feather pecking vs. feather eating - the shift of terminology

In the middle of the last century the term “feather pulling/eating” was often used in scientific literature (e.g. Willimon and Morgan, 1953; Marsboon and Sierens, 1962). Later on, in the seventies, feather pulling/eating was replaced by the term feather pecking (e.g. Hughes and Duncan, 1972; Allen and Perry, 1975). In recent studies, the consumption of feathers by feather peckers was investigated and once more, the term “feather eating” was inducted in the scientific literature (McKeegan and Savory, 1999, 2001).

1.3 Effect of feathers in the gut

Feathers are composed of keratin, a structural protein that is almost resistant to digestion by proteolytic enzymes (Newell and Elvehejem, 1947). Normally, feathers are considered to be an almost non nutritive matter (McCasland and Richardson, 1966). Nevertheless, feather peckers pluck and eat feathers (McKeegan and Savory, 1999, 2001). Little is known about the consequences of feather eating on the gut function and on the amount of feathers eaten at all. Ingested feathers increase the speed of feed passage through the gastrointestinal tract (GIT) (Harlander-Matauschek et al., 2006; Benda et al., 2008).

1.4 The relationship of gut microbiome and behavior

The relationship between gut and brain is described as the gut-brain axis, a complex bidirectional communication system that involves brain and gastrointestinal functions (Collins and Bercik, 2009). The brain has an impact on motility and secretion of the GIT via efferent fibers and receives signals by afferent fibers from the GIT (O´Mahony et al., 2011). As the relevance of gut microbiome is further recognized and investigated, the term microbiome-gut-brain axis is increasingly used (e.g. Cryan and O´Mahony, 2011; Grenham et al., 2011).

It is known that disease of the GIT can influence behavior and in some behavior disorders alterations of the intestinal microbiome are observed. In some human patients with inflammatory bowel disease (IBD) an altered gut microbiome can be seen (Frank et al., 2007; Sartor,

2008). This observation is also made in animal models of intestinal inflammation (Lupp et al., 2007). Whether these changes are causing the disease or whether they result from inflammation is still discussed (Sekirov et al., 2010). In patients with IBD an increased prevalence of depression was observed (Walker et al., 2008).

In children affected by autism spectrum disorders (ASD) the gut microbiome can be different compared to healthy individuals. Finegold et al. (2010) found a higher microbial diversity in the feces of autistic children compared to healthy ones. Children with ASD and gastrointestinal (GI) symptoms are reported to have more severe measures of irritability, anxiety and social withdrawal than children concerned from ASD but not suffering from GI symptoms (Nikolov et al., 2009). Adams et al. (2011) found a strong correlation between the severity of autism and GI symptoms.

Not only the microbiome itself has an influence, also diet does. In mice, there is a relation between the dietary induced change in the diversity of gut microbiome and the animal behavior suggesting relevance for memory and learning (Li et al., 2009). Due to this influence of diet on the gut microbiome, it possibly plays a role in the gut-brain axis (Neufeld et al., 2011).

Some patients with fructose malabsorption show early signs of mental depression and mood disturbances (Ledochowski et al., 1998). Giving a fructose-reduced diet improved mood and early signs of depression (Ledochowski et al., 2000a). Also of relevance is the fact that in patients with this malabsorption disorder plasma tryptophan levels are decreased (Ledochowski et al., 2000b). Tryptophan is the precursor of the neurotransmitter serotonin. An increased level of tryptophan in plasma on the other hand was seen when giving *Bifidobacterium infantis* for 14 days to rats (Desbonnet et al., 2009). A probiotic formulation of *Lactobacillus helveticus* and *Bifidobacterium longum* reduced anxiety-like behavior in rat and relieved stress in humans (Messaoudi et al., 2011). Emphasizing the preliminary character of the results, the recommendation was given to extend preclinical and clinical investigations on the relation of gut microbiome and mental disorders (Messaoudi et al., 2011).

Latest findings suggest the possibility to treat certain disorders of the central nervous system (CNS) in humans by influencing the gut microbiome. Nevertheless, it has to be realized that the differentiation of gut microbiome in health and in disease is still not completely possible. Even though the recent insights present a solid base for prospective studies there is still no progressed understanding of the interactions and further research is necessary (Grenham et al., 2011).

1.5 Aim of this study

The aim of this study is to enlarge the knowledge about feather pecking by looking at this behavorial disorder from a new perspective. A better understanding of reason and impact in the animal itself was strived. The following topics were investigated:

The effect of feather intake on the gut microbiota and its metabolism in pullets (Chapter 2)

The amount and size of ingested feathers and feather parts respectively in laying hens belonging to a high (H) or low feather pecking line (L) (Chapter 3)

The intestinal microbial metabolism in these laying hens (Chapter 3)

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2 Dietary inclusion of feathers affects intestinal microbiota and microbial metabolites in growing Leghorn-type chickens

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Dietary inclusion of feathers affects intestinal microbiota and microbial metabolites in growing Leghorn-type chickens[1]

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ABSTRACT Feather pecking in laying hens is a serious behavioral problem that is often associated with feather eating. The intake of feathers may influence the gut microbiota and its metabolism. The aim of this study was to determine the effect of 2 different diets, with or without 5% ground feathers, on the gut microbiota and the resulting microbial fermentation products and to identify keratin-degrading bacteria in chicken digesta. One-day-old Lohmann-Selected Leghorn chicks were divided into 3 feeding groups: group A (control), B (5% ground feathers in the diet), and C, in which the control diet was fed until wk 12 and then switched to the 5% feather diet to study the effect of time of first feather ingestion. The gut microbiota was analyzed by cultivation and denaturing gradient gel electrophoresis of ileum and cecum digesta. Short-chain fatty acids, ammonia, and lactate concentrations were measured as microbial metabolites. The concentration of keratinolytic bacteria increased after feather ingestion in the ileum ($P < 0.001$) and cecum ($P = 0.033$). Bacterial species that hydrolyzed keratin were identified as *Enterococcus faecium*, *Lactobacillus crispatus*, *Lactobacillus reuteri*-like species (97% sequence homology), and *Lactobacillus salivarius*-like species (97% sequence homology). Molecular analysis of cecal DNA extracts showed that the feather diet lowered the bacterial diversity indicated by a reduced richness ($P < 0.001$) and shannon ($P = 0.012$) index. The pattern of microbial metabolites indicated some changes, especially in the cecum. This study showed that feather intake induced an adaptation of the intestinal microbiota in chickens. It remains unclear to what extent the changed metabolism of the microbiota reflects the feather intake and could have an effect on the behavior of the hens.

Key words: laying hen, keratinolytic bacteria, gut microbiota, ammonia, feather pecking

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INTRODUCTION

Feather pecking and cannibalism in laying hens is a serious problem for animal welfare and health. A recent epidemiological study in the United Kingdom showed that 68.5% of laying hen flocks at 25 wk of age and 85.6% of laying hen flocks at 40 wk of age showed feather pecking (Lambton et al., 2010). Similar prevalence of feather-pecking behavior can be assumed in other European countries.

Feather pecking is a multifactorial problem. It can be caused by environmental, genetic, or nutritional factors (van Krimpen et al., 2008), whereby to date, the environmental and nutritional factors have been more thoroughly investigated. Using molecular genetics, it may be possible to select against the propensity to develop feather pecking in the future (Rodenburg et al., 2004). Distinctions in feather pecking activity between different layer strains are known. Four commercial strains of hens were tested, and the highest pecking activity was found in ISA Brown, followed by Lohmann Brown, Norbrid 41, and Lohmann-Selected Leghorn chickens (Kjaer, 2000), indicating that it is possible to select hens on the basis of their feather-pecking behavior (Kjaer et al., 2001). There are also several environmental conditions that influence the occurrence of feather pecking. Pecking damage at the plumage is associated with group size and density interactions (Savory et al., 1999). At a constant stocking density, feather pecking increased with group size (Bilcík and Keeling, 2000). A temperature of less than 20°C in the stable increased the risk of feather pecking (Green et al., 2000). A high light intensity increased the frequency of severe feather pecking (Kjaer and Vestergaard, 1999). Early access to litter for growing hens reduced feather pecking (Huber-Eicher and Sebo, 2001). Feeding is considered an important factor, and feather pecking can be positively

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and negatively affected by dietary modifications (van Krimpen et al., 2005). Feeding pellets has a higher risk compared with that of mash feed, especially if hens are not housed on straw (Aerni et al., 2000). Deficiencies in protein (Ambrosen and Petersen, 1997) or minerals, such as sodium (Hughes and Whitehead, 1979), can lead to increased feather pecking and cannibalism. However, the source of protein, plant vs. animal, seemed to have no effect on prevention or alleviation of feather pecking (McKeegan et al., 2001).

In laying hens, feather pecking is often associated with feather eating (McKeegan and Savory, 1999; Harlander-Matauschek and Feise, 2009). The physiological implications for gut function are not clear, but feathers might be considered as structured, fiber-like particles and as a potential nitrogen source for the intestinal microbiota. The microbiota balance is expected to respond to the feather ingestion; however, limited knowledge is available in chickens. The intake of feathers accelerated the intestinal transit time in hens (Harlander-Matauschek et al., 2006). To our knowledge, there are no data on the influence of feather ingestion on the intestinal microbiota and their metabolism in poultry. Feathers are composed of keratin, which is almost resistant to digestion by the common proteolytic enzymes (Newell and Elvehejem, 1947). If feathers are used for animal nutrition, they have to be processed to feather meal because of hygiene reasons and to improve digestibility by hydrolysis. Comparing amino acid digestibility of hydrolyzed feather meal, the variations of amino acid digestibility were determined by standardized ileal amino acid digestibility at 7 and 21 d of age in broiler chickens and true amino acid digestibility in cecectomized roosters. The lowest digestibility was found for aspartic acid with 19.7% at 7 d of age, whereas the highest digestibility was observed for isoleucine in roosters with 89.5% (Garcia et al., 2007). Protein and amino acid intake has been shown to affect the behavior of chickens. Diets low in protein (11.1%) led to a poorer plumage condition and a higher rate of cannibalism (Ambrosen and Petersen, 1997). There are no studies investigating the effect of a diet high in total protein on the behavior of the hens. Besides total protein content, single amino acids may influence behavior. Tryptophan is a precursor for the synthesis of serotonin. In diets containing 2% instead of 0.16% tryptophan [0.17% was recommended by the NRC (1994)], the frequency of gentle feather pecking was reduced. For severe feather pecking, the same tendency was observed (Hierden et al., 2004).

Feather eating often occurs as a consequence of feather pecking (McKeegan and Savory, 1999; Harlander-Matauschek and Feise, 2009). The intake of feathers leads to an added protein intake, and although the main protein, keratin, is almost resistant to enzymatic degradation (Newell and Elvehejem, 1947), keratin might serve as a substrate for the intestinal microbiota, influencing the composition and the microbial protein fermentation. Fermentation products, such as ammonia, might affect behavior in animals, but there are no experimental data in chickens thus far to our knowledge. Emeash et al. (1997) demonstrated that a significant increase in the incidence of feather pecking and cannibalism occurred when broilers were exposed to ammonia as an air pollutant.

In the current study, we hypothesized that feathers can be used by keratinolytic microbes in the gut and that the composition of the intestinal microbiota is influenced. To detect a possible effect of the keratin intake on the microbial fermentation, we determined the pattern of resulting metabolites. This should allow us to get a better insight into the effect of feathers on the intestinal microbiota and the metabolites that might be relevant in influencing the behavior in chickens.

MATERIALS AND METHODS

Birds and Housing

In total, 61 non-beak-trimmed 1-d-old Lohmann-Selected Leghorn chicks were housed and reared in a climate-controlled room and divided into 3 feeding groups. Group A (control) and B (5% ground feathers in the diet) included 24 birds each. Group C (13 animals) was fed the control diet until wk 12 and was then switched to the 5% feather diet to study the effect of the time of feather ingestion. The animals were kept in modified floor-pens without litter to avoid uncontrolled particle intake. The pens had a ground area of 2.13 m^2 and were provided with perches, a sand bath, ropes, and laying nests. During the first 2 d, the light was on for 24 h, followed by 16 h of light per day until d 6. In the second week, the light was on for 14 h, followed by a gradual reduction of 1 h/wk down to 8 h of light in wk 8 until the end of housing at wk 18. Light intensity was about 40 lx in the first week, 30 lx in the second week, 15 lx in the third week, and 10 lx in the next 2 wk, followed by 5 lx until wk 15, and from then until the eighteenth week it was at 10 lx. During the first week, the temperature was decreased from 32 to 30°C, followed by a gradual reduction to 20°C in wk 7 and onwards. The health status was monitored daily by visual inspection. The BW gain was recorded weekly. The diet and drinking water were provided ad libitum by nipple drinkers and round troughs.

Diet Composition

The diets were based on wheat and soybean extract and fulfilled the requirements for pullets according to the recommendations of Lohmann for raising LSL pullets. The diets were produced at the University of Hohenheim, Germany. All diets were pelleted to ensure a standardized feather intake. Pellets were produced using a pellet press (Typ 14, Amandus Kahl GmbH, Reinbek, Germany); the pellet diameter was 3 mm.

The feathers used in this study were taken from white-feathered laying hens that were raised and housed at the experimental farm of the University of

Hohenheim, Germany. They were slaughtered at the end of the laying period at an age of 84 wk. The feathers were plucked manually from all body parts, except wing and tail, and stored at −10°C. The grinding of the feathers was conducted using a meat cutter (K64 DC8, Seydelmann KG, Aalen, Germany) with 2 vertical knife pairs starting at 30 s with 4,000 rotations and continuing up to 60 s with 64,000 rotations. Feathers had to be minimally wetted to facilitate cutting. The cut feathers were maximally 1 cm long in the shaft. They were dried on plastic sheets at 26°C in climatic rooms for 72 h.

The diet was also fed in a parallel trial conducted at the University of Hohenheim. The diet composition and nutrient content are described in Kriegseis et al. (2012). The starter diet was fed from wk 1 to 9 and the grower diet from wk 10 to slaughtering.

Sampling

Twelve birds of group A and 12 of B were slaughtered in wk 10 and wk 17, respectively. Group C (13 birds) was slaughtered in wk 18. Birds were anesthetized by stunning and killed by exsanguination. The digesta was collected from the ileum (Meckel's diverticulum to ostium ceci) and from both ceca. Samples for the quantitative bacterial cultivation were processed immediately. The samples for molecular biological analysis and determination of bacterial metabolism were immediately deep frozen at −80°C.

Microbiological and Molecular Biological Analysis of Ileum and Cecum Digesta

After the serial dilution of the samples, the intestinal digesta was plated on Schaedler II, MacConkey, and tryptose sulfite cycloserine agars for total anaerobic bacteria, enterobacteria, and clostridia, respectively. Plates were incubated at 39°C for 48 h under anaerobic conditions. To identify keratinolytic bacteria, an agar with feather meal as the sole nitrogen source was used. It contained 20 g/L of feather meal (1200 Hydrolysed Feather Meal (FE^{85}), GePro Geflügelproteinvertriebs-GmbH, Diepholz, Germany), 0.5 g/L of NaCl, 0.3 g/L of K_2HPO_4, 0.4 g/L of KH_2PO_4, and 15 g/L of agar (Riffel and Brandelli, 2002). The colonies were assessed semiquantitatively because of their colorless and small growth. The growth intensity of the colonies was assessed using visual scoring (strong and weak growth). A suitable score was developed in a preliminary test. Strong growth was defined as consisting of areas with very dense growth (agar was not visible anymore) combined with areas with less dense growth (agar partly visible, some singular colonies detectable). Weak growth comprised only areas with less dense growth or even fine growth (a large area of the agar without colonies was visible, a higher number of single colonies detectable). To compare the groups, strong growth was contrasted with weak growth. Single colonies were isolated, cultivated, and microscopically checked for purity on Schaedler II agar. The DNA was extracted (Nucleospin Tissue kit, Machery-Nagel, Düren, Germany) from pure cultures and amplified by PCR using Qiagen HotStarTaq Mastermix (Qiagen, Hilden, Germany) and primers 27f and 1492r (Eurofins MWG Operon, Ebersberg, Germany) to amplify the 16S rRNA gene. The following PCR program was used: 95°C for 900 s, 35 cycles at 94°C for 60 s, 54°C for 60 s, 72°C for 60 s, 72°C for 600 s, and then held at 4°C. The PCR product was sequenced by Eurofins MWG Operon (Ebersberg, Germany) using cycle sequencing technology (dideoxy chain termination/cycle sequencing). The resulting sequences were compared with the NCBI/BLAST database.

For the characterization of the digesta microbiota, a denaturing gradient gel electrophoresis (**DGGE**) was performed. Cecum digesta of 6 animals of groups A to C at wk 17 and 18 of age were used. The DGGE was performed as described previously (Kraatz et al., 2006). Briefly, the partial 16S rDNA of eubacterial species was amplified by PCR and DGGE was performed on a DCodeTM DGGE system (Bio-Rad, Irvine, CA) with 10 μL of PCR product at 60°C and 85 V for 16 h. Differing from the described method, DNA extraction was performed with a commercial kit (Qiagen Stool kit, Qiagen). Cecal digesta (200 mg) was extracted in triplicates according to the instructions of the manufacturer, except for an increase in temperature during the lysis step to 90°C. Purified DNA was then pooled per sample and the DNA was quantified. Analysis of the gels was conducted using the software program Phoretix (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). The diversity indices richness, Shannon, and evenness were calculated with Excel (Microsoft Corp., Redmond, WA). Richness is defined as the number of species in a sample. The Shannon index describes the diversity of the bacterial community, and evenness estimates the similarity of species abundance (Kwak and Peterson, 2007).

Microbial Metabolites

Short-chain fatty acids (**SCFA**), ammonia, and lactate concentrations were measured as microbial metabolites. For sample preparation, 0.5 g of digesta was diluted with 1.0 mL of ice-cold 100 m*M* 3-(*N*-morpholino) propanesulfonic acid buffer (pH 7.5), homogenized for 1 min, and incubated for 10 min on ice. Samples were then homogenized again and centrifuged at 17,000 × *g* at 4°C for 10 min. The supernatant was kept on ice, until 100 μL was taken for determination of the SCFA. The rest of the supernatant was mixed with 50 μL of Carrez-I and Carrez-II solutions and subsequently used for ammonia and lactate analysis. Samples were centrifuged and the supernatants were filtered by a 0.45-μm cellulose acetate syringe filter.

Analysis of SCFA was carried out by gas chromatography (Agilent Technologies 6890N with auto sampler

G2614A and auto injector G2613A; Santa Clara, CA). An Agilent 19095N-123 HP-INNOWAX polyethylene glycol column was used. Then, 100 μL of the sample supernatant was diluted with 900 μL of internal standard solution, containing 0.5 mmol/L of capronic acid. The standard solution contained 50 mL of 10 mmol/L stock solution (250 μL capronic acid, 2 g of oxalic acid dihydrate in 200 mL), 2.5 g of sodium azide and 10 g of oxalic acid dihydrate in 1,000 mL.

Ammonia was quantified using a Berthelot reaction assay. Twenty microliters of the sample supernatant was mixed with 100 μL of phenol nitroprusside and 100 μL of alkaline hypochlorite in a 96-well microtiter plate. After incubation for 10 min at room temperature, a photometric measurement was carried out at 620 nm with a Tecan microtiterplate reader (Tecan Austria GmbH, Salzburg, Austria).

Analysis of D- and L-lactate was carried out with HPLC using an Agilent 1100 system with Phenomenex Chirex 3126 (D)-penicillamine 150 × 4, 6-mm column and Phenomenex C18 4.0-L × 2.0 ID mm precolumn (Agilent Technologies). Two hundred microliters of sample supernatant was filled up to 1 mL with copper-II-sulfate solution (0.5 mmol). The column temperature was 35°C and the UV detector wavelength was 253 nm.

Statistical Analysis

The statistical analysis was carried out with the software SPSS 15.0 (SPSS Inc., Chicago, IL) using the Kolmogorov-Smirnov test to test normal data distribution. Normally distributed data were compared using the GLM 2-way ANOVA procedure to define the effects of diet and age and the diet by age interaction. Group A was compared with group B to determine the influence of feather intake and data from group B at the age of 17 wk was compared with data from group C to define the effect of time of feather ingestion ($P < 0.05$). Growth on keratin agar was analyzed with the chi-square test.

RESULTS

Microbiological and Molecular Biological Analysis

The concentrations of total anaerobic bacteria, enterobacteria, and clostridia in the ileal and cecal digesta are presented in Tables 1 and 2. Enterobacteria increased in group B compared with group A in the ileum ($P = 0.01$) and in the cecum ($P = 0.02$).The concentrations of clostridia were higher in the birds fed with additional feathers in the cecum contents ($P = 0.014$). The comparison of groups B and C revealed a general tendency toward higher bacterial counts in the ileum and cecum digesta in the birds with the early exposure to feathers as part of their diets. Comparing B and C, enterobacteria counts were higher in group B in the ileum ($P = 0.005$) and cecum digesta ($P = 0.007$) and clostridia in the ileum digesta ($P = 0.01$). Compared with the control group, ileal and cecal digesta from birds fed feather-containing diets showed a higher growth of colonies on the keratin agar (Tables 3 and 4). The difference was even more pronounced in the ileum at 17 wk of age. The control group showed strong growth only in 1 of 12 animals, whereas in the groups being fed diets containing feathers, almost every animal showed strong growth. In the cecum, the control group also displayed a higher proportion of high growth, especially at 17 wk of age. Thus, the results in the cecum were comparable to the ileum, but differences were not as distinctive as in the ileum. Significant effects were only observed at 17 wk of age.

Keratinolytic strains subcultivated from the keratin agar were identified as *Enterococcus faecium*, *Lactobacillus crispatus*, *Lactobacillus reuteri*-like species (97% sequence homology) and *Lactobacillus salivarius*-like species (97% sequence homology).

The analysis of the cecal microbiota in wk 17 showed a reduced richness as well as a reduced shannon index in birds of group C compared with those of birds of the

Table 1. Bacterial counts ($\log_{10}$ cfu/g) in the ileal digesta of 10- and 17-wk-old laying hens fed diets with or without 5% ground feathers

	Group[1]								
	10 wk		17 wk			*P*-value[2]			
Type	A (n = 12)	B (n = 12)	A (n = 12)	B (n = 12)	C (n = 13)	A and B	Age 10–17	Group × age	B and C
Total anaerobic bacteria	9.1 ± 0.57	8.8 ± 0.55	8.7 ± 0.49	9.0 ± 0.55	8.6 ± 0.37	0.847	0.532	0.055	0.058
Enterobacteria	$5.4^{a} \pm 0.84$[3]	$5.9^{b} \pm 0.69$[4]	$5.7^{a} \pm 0.96$	$6.4^{b,A} \pm 0.59$	$5.6^{B} \pm 0.74$	0.010	0.117	0.719	0.005
Clostridia	7.9 ± 0.97	8.1 ± 0.56	8.1 ± 0.65	$8.6^{A} \pm 0.46$	$8.1^{B} \pm 0.45$	0.099	0.068	0.380	0.010

[a,b]Different lowercase superscripts within a row indicate significant differences ($P \leq 0.05$) between groups A and B.

[A,B]Different capitalized superscripts within a row indicate significant differences ($P \leq 0.05$) between groups B and C.

[1]A = control group; B = treatment group with 5% ground feathers starting on d 1 of age; and C = treatment group with 5% ground feathers starting on wk 13.

[2]Two-way ANOVA analysis.

[3]n = 10.

[4]n = 11.

Table 2. Bacterial counts ($\log_{10}$ cfu/g) in the cecal digesta of 10- and 17-wk-old laying hens fed diets with or without 5% ground feathers

	Group[1]								
	10 wk		17 wk			*P*-value[2]			
Type	A (n = 12)	B (n = 12)	A (n = 12)	B (n = 12)	C (n = 13)	A and B	Age 10–17	Group × age	B and C
Total anaerobic bacteria	9.5 ± 0.42[3]	9.5 ± 0.62	9.7 ± 0.19	9.6 ± 0.71	9.1 ± 0.53	0.752	0.258	0.508	0.085
Enterobacteria	7.0[a] ± 0.78[4]	7.6[b] ± 0.45[5]	7.4[a] ± 0.95	7.8[b,A] ± 0.64	7.1[B] ± 0.63	0.020	0.211	0.700	0.007
Clostridia	7.8[a] ± 1.04	8.6[b] ± 0.55	8.6[a] ± 0.44	8.8[b] ± 0.80	8.6 ± 0.67	0.014	0.024	0.158	0.469

[a,b]Different lowercase superscripts within a row indicate significant differences ($P \leq 0.05$) between groups A and B

[A,B]Different capitalized superscripts within a row indicate significant differences ($P \leq 0.05$) between groups B and C.

[1]A = control group; B = treatment group with 5% ground feathers starting on d 1 of age; and C = treatment group with 5% ground feathers starting on wk 13.

[2]Two-way ANOVA analysis.

[3]n = 11.

[4]n = 8.

[5]n = 10.

2 other trial groups (Table 5). Compared with the control group, only small numerical reductions of richness and shannon index were observed in birds fed the 5% feather diet from d 1. The evenness of the cecal microbiota was similar for all trial groups.

Microbial Metabolites

The results for microbial metabolites are summarized in Tables 6 and 7. In the ileum, no significant differences were found for the different diets, except that birds fed the diet with 5% feathers from d 1 showed higher concentrations of SCFA than those of birds fed the 5% feather diet from wk 13 ($P = 0.039$). Ammonia and SCFA concentrations increased with age ($P = 0.003$ and $P < 0.001$, respectively).

Ammonia concentrations in cecal contents were significantly higher in the group that received 5% feathers from d 1 than in the control group ($P = 0.014$). Animals fed the diet with 5% feathers from d 1 showed significantly lower L-lactate concentrations in wk 10 and wk 17 compared with the control ($P = 0.001$). However, birds that received the 5% feather diet starting in wk 13 showed significantly lower D-lactate concentrations in wk 17 compared with that of the birds that received the diet from d 1 ($P = 0.045$).

Propionate, i-butyrate, i-valeriate and n-valeriate all displayed significantly higher molar ratios in the cecal contents of the animals that received 5% feathers from d 1 compared with the control group ($P = 0.049$, $P = 0.020$, $P = 0.045$, and $P = 0.030$, respectively). No differences were observed in the group that was fed the 5% feather diet starting in wk 13 compared with the control group.

DISCUSSION

Feather pecking and feather eating are important husbandry problems in laying hens. To our knowledge, no data are available regarding the effect of feather ingestion on the gut microbiota, especially on the composition and metabolic activity of the intestinal microbiota. Using a specific cultural method, we could demonstrate that intestinal bacteria can utilize keratin

Table 3. Semiquantitative assessment of keratinolytic bacteria in the ileal digesta of 10- and 17-wk-old laying hens fed diets with or without 5% ground feathers

	Group[1]					
	10 wk		17 wk			
Growth	A (n = 12)	B (n = 12)	A (n = 12)	B (n = 12)	C (n = 13)	*P*-value[2]
Strong[3]	4	8	1[a]	11[b]	11[b]	<0.001
Weaker[4]	8	4	11	1	2	

[a,b]Different superscripts within a row indicate significant differences ($P \leq 0.05$).

[1]A = control group; B = treatment group with 5% ground feathers starting on d 1 of age; and C = treatment group with 5% ground feathers starting on wk 13.

[2]Chi-square test.

[3]Areas with very dense growth (agar not visible anymore) combined with areas with less dense growth (agar partly visible, some singular colonies detectible).

[4]Areas with less dense growth combined with fine growth (a large area of the agar without colonies visible, a higher number of single colonies was seen).

Table 4. Semiquantitative assessment of keratinolytic bacteria in the cecal digesta of 10- and 17-wk-old laying hens fed diets with or without 5% ground feathers

	Group[1]					
	10 wk		17 wk			
Growth	A (n = 12)	B (n = 12)	A (n = 12)	B (n = 12)	C (n = 13)	*P*-value[2]
Strong[3]	7	9	9[a]	12[b]	13[b]	0.033
Weaker[4]	5	3	3	0	0	

[a,b]Different superscripts within a row indicate significant differences ($P \leq 0.05$).

[1]A = control group; B = treatment group with 5% ground feathers starting on d 1 of age; and C = treatment group with 5% ground feathers starting on wk 13.

[2]Chi-square test.

[3]Areas with very dense growth (agar not visible anymore) combined with areas with less dense growth (agar partly visible, some singular colonies detectible).

[4]Areas with less dense growth combined with fine growth (a large area of the agar without colonies visible, a higher number of single colonies was seen).

provided by the addition of ground feathers to the normal diet of growing hens. Whether this has an effect on animal behavior is still speculative.

Increased growth of keratinolytic bacteria was seen in groups fed the 5% feather diet, independently whether feathers were fed from hatch or from wk 13. Keratinolytic bacteria form a part of the normal intestinal microbiota in chickens. Even though there was no addition of feathers in the diet of the control group, the intestinal microbiota was able to utilize keratin as a substrate. This finding indicates that the ingestion of feathers may have an inducing effect on certain bacterial groups and this was stimulated by an increased intake of feathers. Due to the group housing conditions, it can be assumed that hens from the control group were also able to ingest feathers due to preening and contact to conspecifics. Due to the regular and standardized intake of feather particles with the diet in groups B and C, a clear adaptation to the substrate could be seen, indicated by a stronger growth of keratinolytic bacteria on the keratin agar. The differences between the 3 groups were detected already in the ileum, and they were stronger than in the ceca. The increased growth of keratinolytic bacteria in groups B and C was almost identical in the ileum and in the cecum digesta. This indicates that the time of exposure to the feather-containing diet from d 1 or after wk 12 had no influence on the adaptation of keratinolytic bacteria to ingested feather particles.

Examples of keratinolytic bacteria isolated from the poultry processing industry are *Vibrio* sp. (Sangali and Brandelli, 2000), *Flavobacterium* sp. (Riffel and Brandelli, 2002), *Cryseobacterium* sp. (Riffel et al., 2003), and *Bacillus* sp. (Joshi et al., 2007). Determined enzymes are serine proteases and metalloproteases. Serine proteases and metalloproteases are also common in enterococci (Lopes et al., 2006; Macovei et al., 2009), lactobacilli (Kunji et al., 1996), and in peptidolytic clostridia. Furthermore, cysteine aminopeptidases, which can attack the abundant cystine bonds in keratin, occur in several unrelated bacteria, such as *Streptococcus thermophilus* (Chapot-Chartier et al., 1994) or *Porphyromonas endodontalis* (Rosen et al., 2001).

Thus, it was expected that keratinolytic bacteria could be isolated from the intestine using media containing feather meal as a nitrogen source. The isolation of the *Enterococcus faecium* strain and several *Lactobacillus* spp. strains in this study confirms the ability of the intestinal microbiota to degrade keratin. However, compared with growth on nutrient-rich media, colonies were rather small for all isolates. This indicates that although these strains may be able to perform keratinolysis, keratin is not their preferred protein source.

Table 5. Bacterial diversity indices in cecal DNA extracts from laying hens fed diets with or without 5% ground feathers after 17 wk of life

	Group[1]			
Index	A	B	C	*P*-value[2]
Richness	23.7[a] ± 1.63	21.5[a] ± 1.52	17.0[b] ± 1.79	<0.001
Shannon	2.27[a] ± 0.22	2.09[a] ± 0.13	1.96[b] ± 0.19	0.012
Evenness	0.72 ± 0.06	0.74 ± 0.03	0.70 ± 0.07	0.373

[a,b]Different superscripts within a row indicate significant differences ($P \leq 0.05$).

[1]A = control group; B = treatment group with 5% ground feathers starting on d 1 of age; and C = treatment group with 5% ground feathers starting on wk 13.

[2]One-way ANOVA analysis.

Table 6. Microbial metabolites in the ileal digesta of 10- and 17-wk-old laying hens fed diets with or without 5% ground feathers

	Group[1]								
	10 wk		17 wk			*P*-value[2]			
Metabolite	A (n = 12)	B (n = 12)	A (n = 11)	B (n = 12)	C (n = 13)	A and B	Age 10–17	Group × age	B and C
Ammonia, mmol/L	0.5 ± 0.32	0.5 ± 0.14	0.7 ± 0.26	0.8 ± 0.33	0.8 ± 0.25	0.429	0.003	0.616	0.597
D-lactate, mmol/L	3.7 ± 3.29	3.0 ± 3.02[3]	2.4 ± 2.94	2.6 ± 3.45	5.0 ± 6.45	0.774	0.398	0.616	0.265
L-lactate, mmol/L	23.6 ± 14.56	21.1 ± 22.99	14.3 ± 10.66	16.9 ± 11.94	13.8 ± 9.87	0.992	0.152	0.584	0.490
Total SCFA,[4] mmol/L	1.5 ± 0.49[5]	1.6 ± 0.70[3]	3.5 ± 1.17	3.4^{A} ± 1.54	2.3^{B} ± 0.92	0.928	<0.001	0.774	0.039

[A,B]Different superscripts within a row indicate significant differences ($P \leq 0.05$) between groups B and C.

[1]A = control group; B = treatment group with 5% ground feathers starting on d 1 of age; and C = treatment group with 5% ground feathers starting on wk 13.

[2]Two-way ANOVA analysis.

[3]n = 11.

[4]SCFA = short-chain fatty acid; mainly acetate.

[5]n = 8.

The diverse diets caused only slight differences in the intestinal concentrations of total anaerobic bacteria, enterobacteria, and clostridia. Some statistically significant differences were seen for enterobacteria and clostridia in the ileum and cecum, however, a biological relevance cannot be assumed as the difference is too small.

The bacterial metabolism was slightly affected by the intake of feathers early in life. Whereas the bacterial metabolite concentrations in the ileum were mostly unchanged, cecal contents showed increased ammonia concentrations and a higher molar ratio of some SCFA concentrations. These metabolites are built while protein degradation takes place and may indicate a higher bacterial protein catabolism as a result of feather degradation. Furthermore, compared with the control group, a reduction in the L-lactate concentration was noted in animals fed the feather diet early in life. These results show that a shift occurred in the bacterial metabolism in the cecum of animals fed the feather-supplemented diet. It is therefore probable that the microbiota balance also changed depending on the feather intake. This is also illustrated by the qualitative analysis of the microbiota composition. Although the detection limit of the DGGE method only allows detection of dominant species, a change in microbiota composition could be observed. Here, the largest effect was noted in the cecum of the animals that received feathers later in life, as the number of species (richness) was clearly reduced. This seems contradictory to the concentration of enterobacteria and clostridia, which were similar compared with the control group. However, an increased total count of clostridia may be based on the selective increase of a few clostridial species. Numerical differences were also observed for animals that received feathers from d 1. This indicates that feathers modified

Table 7. Microbial metabolites in the cecal digesta of 10- and 17-wk-old laying hens fed diets with or without 5% ground feathers

	Group[1]								
	10 wk		17 wk			*P*-value[2]			
Metabolite	A (n = 6)	B (n = 6)	A (n = 8)	B (n = 6)	C (n = 10)	A and B	Age 10–17	Group × age	B and C
Ammonia, mmol/L	3.3^{a} ± 0.49	5.3^{b} ± 1.66	4.4^{a} ± 0.85	5.4^{b} ± 2.22	4.0 ± 1.10	0.014	0.313	0.435	0.101
D-Lactate, mmol/L	1.8 ± 1.17	0.6 ± 0.68[3]	1.1 ± 1.35^{4}	1.8^{A} ± 1.43	0.6^{B} ± 0.61	0.540	0.621	0.073	0.045
L-Lactate, mmol/L	3.2^{a} ± 1.85	0.5^{b} ± 0.24	$2.1^{a} \pm 1.43^{3}$	1.0^{b} ± 0.59	0.6 ± 0.64	0.001	0.474	0.124	0.325
Total SCFA,[5] mmol/L	76.2 ± 4.62	54.3 ± 10.50	78.9 ± 15.66	74.5 ± 36.66	63.7 ± 14.86	0.107	0.183	0.267	0.417
Acetate, mol%	78.1 ± 1.49	78.2 ± 3.25	81.0 ± 1.85	79.5 ± 3.13	77.7 ± 4.28	0.479	0.046	0.454	0.387
Propionate	3.3^{a} ± 0.66	4.8^{b} ± 1.93	3.7^{a} ± 0.73	4.0^{b} ± 1.56	5.1 ± 1.74	0.049	0.975	0.440	0.439
i-Butyrate	0.1^{a} ± 0.07	0.4^{b} ± 0.21	0.2^{a} ± 0.08	0.4^{b} ± 0.49	0.2 ± 0.11	0.020	0.543	0.845	0.266
n-Butyrate	17.9 ± 0.80	15.6 ± 5.38	14.3 ± 1.62	14.8 ± 1.60	15.8 ± 4.29	0.435	0.066	0.250	0.599
i-Valeriate	0.1^{a} ± 0.06	0.3^{b} ± 0.17	0.2^{a} ± 0.07	0.2^{b} ± 0.19	0.2 ± 0.13	0.045	0.739	0.164	0.603
n-Valeriate	0.5^{a} ± 0.21	0.7^{b} ± 0.08	0.6^{a} ± 0.21	0.7^{b} ± 0.21	1.0 ± 0.28	0.030	0.408	0.266	0.076

[a,b]Different lowercase superscripts within a row indicate significant differences ($P \leq 0.05$) between groups A and B.

[A,B]Different capitalized superscripts within a row indicate significant differences ($P \leq 0.05$) between groups B and C.

[1]A = control group; B = treatment group with 5% ground feathers starting on d 1 of age; and C = treatment group with 5% ground feathers starting on wk 13.

[2]Two-way ANOVA analysis.

[3]n = 5.

[4]n = 7.

[5]SCFA = short-chain fatty acid.

the diversity of the dominant microbiota in the cecum toward fewer species with possibly different physiological responses.

In conclusion, the understanding of the complex problem of feather pecking in laying hens must take into account that the feather intake has an influence on the gut microbiota. There are obviously resident bacteria able to use keratin as a substrate. The measured microbial metabolic profile was affected. It can be speculated that effects on the intestinal microbes could stimulate the release of other metabolites as hydrogen sulfide and other sulfur-containing substances or biogenic amines that are not only reactive but can have a potential effect on behavior.

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3 Differences in intestinal microbial metabolites in laying hens with high and low levels of repetitive feather-pecking behavior

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4 Summarizing Discussion

Diet and behavior relate in a circuitous manner. To evaluate the behavioral consequences of dietary components various studies have been conducted in an attempt to understand the relationships between what creatures eat and how they behave (Worobey, 2006). At the same time, studies have concentrated on various parameters - such as genetic predisposition, social environment, personality or other individual characteristics - that determine food choice (Worobey, 2006).

Feather pecking is a multifactorial problem. Nutritional, environmental and genetic factors are known as underlying reasons (van Krimpen et al., 2008). The interaction of different factors can even result in increased feather pecking behavior (van Krimpen et al., 2005). In the following the importance of the external factor nutrition as well as feed form is regarded initially followed by environmental factors.

Dietary modifications can positively and negatively affect feather pecking behavior (van Krimpen et al., 2005). Increased feather pecking and cannibalism can be the consequence of deficiency in minerals, such as sodium (Hughes and Whitehead, 1979). Deficiencies in protein (Ambrosen and Petersen, 1997) resulted in increased feather pecking and cannibalism. The intake of amino acids, especially methione and cysteine, is also of importance. These sulfur-containing amino acids are involved in the synthesis of feather keratin. The necessity of a good feather development is stressed in an experiment by McAdie and Keeling (2000) who ruffled or trimmed feathers of laying hens resulting in intensified feather pecking and even cannibalism in the birds. Diets low in methionine and cysteine (5.1 g/kg, 6.5 g/kg) led to poorer plumage condition compared to diets containing 6.7 or 7.0 g/kg (Elwinger et al., 2008). Contrary to these results, the plumage condition of laying hens was not affected by low (4.0 g/kg) versus high (8.0 g/kg) levels of methionine and cysteine (Kjaer and Sørensen, 2002). However, these two studies are not completely comparable, because the content of protein and lysine was identical for all diets in the experiment of Kjaer and Sørensen (2002) but differed between the diets in the study of Elwinger et al. (2008). Lysine deficiency was reported to induce feather pecking in broilers (Quentin et al., 2005). Increased levels of tryptophan can reduce feather pecking behavior. The amino acid tryptophan is a precursor for the synthesis of serotonin. Feeding diets containing 2% instead of 0.16%, tryptophan [0.17% was recommended by the NRC (1994)] reduced the frequency of gentle feather pecking and severe feather pecking tended to be reduced (van Hierden et al., 2004). Summarized it can be assumed that deficiencies in protein and amino acids can lead to feather pecking whereas high levels of tryptophan might decrease this behavior (van Krimpen et al., 2005). Investigating animal vs. plant protein, McKeegan et al. (2001) found that the source of protein seemed to have no effect on feather pecking behavior. A 10% energy reduction in the diet is compensated with a higher feed intake by the hens (van Krimpen et al., 2008). It has to be kept in mind that severe feather pecking is a form of redirected behavior influenced by the motivational system of foraging and feeding (Blokhuis, 1986; Huber-Eicher and Wechsler, 1998; Wechsler and Huber-Eicher, 1998; Dixon et al., 2008). More time spending on feed intake implies less time for feather pecking behavior. Feeding strategies that cause a prolonged time of feed intake could decrease the risk of feather pecking behavior (van Krimpen, 2008). A diet with higher amounts of coarsely ground nonstarch polysaccharides (195 vs. 133 g/kg in the control) also led to a prolonged time of feed intake. However, no differences in feather

pecking behavior between the feeding groups were observed in this experiment (van Krimpen et al., 2008). On the other hand, Huber-Eicher and Wechsler (1998) found feather pecking reduced by increased foraging behavior.

Not only dietary components, but also feed form is a factor influencing feather pecking. The impact of feed form can possibly be seen in the different times used for feed intake (van Krimpen et al., 2005). Feeding pellets is more likely to cause feather pecking than mash feed (El Lethey et al., 2000; Aerni et al., 2000). Savory and Mann (1997) determined no significant effect of feed form (mash or pellet) on feather pecking when the pullets were housed in pens with wood shavings as litter substrate. Another study compared mash and crumbled pellets and found no influence on plumage condition (Wahlström et al., 2001). These studies illustrate that feed form has diverse effects on feather pecking behavior and therefore an interaction with other factors, e.g. housing conditions is likely (van Krimpen et al., 2005). Interaction of foraging material and feed form was demonstrated. Feather pecking was highest in groups without straw and fed on pellets (Aerni et al., 2000; El Lethey et al., 2000). Not only quantity but also quality and thereby availability of the foraging material is of importance (Huber-Eicher and Wechsler, 1998).

In addition to the necessity of litter material to allow the birds to perform foraging behavior, other environmental factors are known to influence feather pecking behavior. Group size and stocking density influence pecking damage at the plumage (Savory et al., 1999). At a constant stocking density, feather pecking increased with group size (Bilcík and Keeling, 2000). In addition, stable temperature is of importance. A temperature of less than 20°C resulted in increased risk of feather pecking (Green et al., 2000). High light intensity increased severe feather pecking (Kjaer and Vestergaard, 1999). The relevance of the rearing period must not be overlooked. Early access to litter reduced feather pecking (Huber-Eicher and Sebo, 2001). Chow and Hogan (2005) suggest that deprivation of exploratory-rich environment led to exploratory behavior to conspecifics and pecking to pen mates can lead to the development of feather pecking.

Genetic factors also influence feather pecking. Various chicken strains are known to differ in feather pecking activity (Kjaer, 2000). There are also individual differences, as not every hen of a group develops this behavioral disorder (Jensen et al., 2005). The genetic effects on feather pecking behavior were confirmed by Kjaer et al. (2001) who successfully selected hens for and against feather pecking behavior. Many behavioral traits are genetically linked to severe feather pecking (Jensen et al., 2005).

Feather peckers grew faster, started laying earlier and were more active and investigative (Jensen et al., 2005). This could mean that nutritional requirements for growth and egg laying are higher in feather peckers (Jensen et al., 2005) which could lead to a higher general pecking tendency or to specific appetite for nutrients available in feathers (Jensen et al., 2005; Harlander-Matauschek and Bessei, 2005; Harlander-Matauschek et al., 2006). Adding chopped feathers to the diet affected feather pecking behavior in hens. Feather pecking activity was reduced, which may indicate that feathers in the diet satisfied the specific appetite for substrates otherwise obtained from feathers of other birds (Kriegseis et al., 2012).

High feather pecking birds show on one sight a high number of pecking movements towards feathers of other birds, but on the other side also eat more plucked feathers than non-peckers (Mc Keegan and Savory, 1999; Harlander-Matauschek and Feise, 2009), meaning

that feathers are part of the diet of feather pecking birds. The aim of the first study was to investigate to what extend the ingested feathers themselves could have an influence on feather pecking.

Feathers are composed of keratin, a fiber protein that is almost resistant to digestion by proteolytic enzymes (Newell and Elvehejem, 1947). Normally, feathers are considered not to be of nutritive value (McCasland and Richardson, 1966). To use feathers as feedstuff they have to be processed by heat and pressure to hydrolysed feather meal. The protein content of feather meal is about more than 80% and at least 70% have to be digestible (Becker and Nehring, 1967), but the use as feedstuff is limited due to the deficient amounts of lysine, methionine and histidine (Papadopoulos, 1985). Degradation of native feathers by the digestive system of the hens cannot be expected, but consideration was made whether there are intestinal bacteria able to use keratin as substrate.

In the poultry processing industry several keratinolytic bacteria were isolated, e. g. *Vibrio* sp. (Sangali and Brandelli, 2000), *Flavobacterium* sp. (Riffel and Brandelli, 2002), *Cryseobacterium* sp. (Riffel et al., 2003), and *Bacillus* sp. (Joshi et al., 2007). Determined enzymes are serine proteases and metalloproteases. Serine proteases and metalloproteases are also common in enterococci (Lopes et al., 2006; Macovei et al., 2009) and lactobacilli (Kunji et al., 1996). Therefore, it was expected that keratinolytic bacteria could be isolated from the intestine using media containing feather meal as a nitrogen source. This medium was used in several studies isolating bacteria from poultry processing industry (e.g. Riffel and Brandelli, 2002; Riffel et al., 2003). In a preliminary test, digesta samples were plated on this agar and bacteria were found to grow in very small and colorless colonies. This feather meal agar is a minimal cultural medium, because it may not contain other nitrogen sources when keratinolytic activity has to be confirmed reliably. Based on the results of the preliminary test a suitable score was developed to analyse the colonies semiquantitatively. Growth intensity was assigned in two different categories. To compare the groups, strong growth was contrasted with weak growth. In the groups with chopped feathers in the diet a stronger growth was seen in the ileum and cecum and the difference to the control group was significant in the older birds. These results show the better adaptation of the gut microbiota to keratin as substrate due to the feather containing diet. The results also illustrate that there are keratinolytic bacteria in all groups, also in the control group. A certain intake of feathers in this group cannot be excluded due to preening and contact to conspecifics. Growth intensity on the agar, however, was clearly stronger when the birds received a standardised feather intake via diet.

The feather meal used for the agar plates was a hydrolyzed feather meal meant for feed industry. It could be seen as a contradiction that hydrolyzed keratin was used for bacterial isolation, as native feathers were used in the diet. Practical aspects and analytical reasons had to be taken into account for performing that type of work. The clear differences in growth intensity compared to the control group demonstrate the adaptation to native keratin as substrate and prove the suitability of the method.

The isolation of the *Enterococcus faecium* strain and several *Lactobacillus* spp. strains illustrate the potential ability of the intestinal microbiota to degrade keratin. However, compared with growth on nutrient-rich media, colonies were rather small for all isolates. This indicates that although these strains may be able to perform keratinolysis, keratin is not their preferred protein source.

In the cecal contents ammonia concentration and the molar ratio of propionate, i-butyrate, i-valeriate and n-valeriate were higher in pullets with feathers in the diet compared to the controls. This suggests a higher proteolytic activity in this group and shows a shift in bacterial metabolism of the cecum. Thus, it is probable that the microbiota balance also changed due to feather intake. This is illustrated by the lowered bacterial diversity in these groups. The largest effect was seen in the cecum of the hens that received feathers later in life. Numerical differences were also observed for animals that received feathers from day one.

The aim of the first study was to investigate the impact of standardized feather intake via diet on gut microbiota and microbial metabolism in growing leghorn-type chickens. In the second study we examined adult laying hens with high (H) and low (L) levels of repetitive feather-pecking behavior. They received a commercial diet without the addition of chopped feathers. The amount of ingested feathers and feather parts in the gizzard was quantified and the intestinal microbial metabolism was investigated.

The birds originated from a selection experiment in which birds were divergently selected on high and low feather pecking activity for six generations (Kjaer et al., 2001). The birds of the present experiment were the fourth generation after this selection. A total of 120 birds, 60 belonging to the H line and 60 to the L line were studied and feather pecking behavior was analysed. As expected, H birds showed a significantly higher number of severe feather-pecking bouts than L birds. A total of 44 hens, 22 H birds with the highest and 22 L birds with the lowest feather pecking activity were chosen for further investigation.

The question arises whether or not testing the 22 highest feather peckers from the H line and the 22 lowest feather peckers from the L line biased the results when compared to a random sample of birds in the L and H lines. Although the birds from the H line differed significantly from the low line in feather pecking, there was a considerable overlap among birds of these two groups. For this reason, it was decided to choose the extreme birds of both lines. Thereby it was expected to yield somewhat larger differences among treatments than in random sampling, but the direction of the differences was expected to remain unaltered. On the other hand, it has to be taken into account that this divergent selection of the extremes is a process also conducted in the selection breeding before.

It was of interest whether the difference in feather pecking behavior would be reflected by feather intake. The complete content of the gizzard was visually analyzed. In doing so not only the quantity but also the quality was of interest. Feathers were allocated to one of three groups: entire feather (calamus identifiable, rachis with vane), feather part (rachis with barbs) and feather particle (single barbs of the vane). More feather parts and particles were found in the gizzard of H than L hens. The amount of entire feathers in the gizzard, however, did not give clear discriminative results between lines. The higher amount of particles in H birds could indicate a possible stronger diminution of feathers in H birds by a higher gizzard grinding activity as suggested by Benda et al. (2008). The static spot sampling nature has to be taken into account as well. Feather intake may vary in certain time periods and these variations could not be recorded.

The striking results of intestinal microbial metabolism are the higher amounts for lactate and total SCFA in H birds suggesting a higher microbial activity compared to hens of the L line. The lower amounts for ammonia in the H birds fit on this assumption because they might be explained by an increased utilization by the gut microbes for their own microbial protein syn-

thesis. In the first experiment, the molar ratio of propionate, i-butyrate, i-valeriate and n-valeriate and ammonia concentrations were higher in the group with chopped feathers in the diet and this was assumed to result from feather degradation. Accordingly, intestinal microbial metabolism was affected in both experiments, independently whether the feather intake was standardized or voluntary. In the second experiment, biogenic amines were determined and putrescine and cadaverine were higher in L birds. Contrary to the other metabolites this suggests a higher intestinal microbial proteolytic activity in L than in H birds. Nevertheless, knowledge on biogenic amines as microbial metabolites in laying hens is very limited.

In mammals, biogenic amines were shown to have behavioral functions. Changes in the concentration of biogenic amines in brain tissue, plasma, serum or whole blood have been found in many pathological and psychiatric conditions, including OCD`s (Fiori and Turecki, 2008). Anxiety-like behavior can result from low brain putrescine concentrations (Gupta et al, 2009) and a putrescine antidepressant like effect is suggested (Zomkowski et al., 2006). Whether the decreased gut putrescine levels in the birds of the H line compared to L birds play a role in the neurobiology of feather pecking would be an interesting question in the future.

Several studies suggest a relation of metabolites released by the gut microbiome and mental disorders in humans (e.g. Wang et al., 2010; Fiori and Turecki, 2008). Total SCFA, propionate and n-butyrate were higher in birds of the H line. Total SCFA and propionate might be interesting as these metabolites were also increased in fecal samples of children with autism (Wang et al., 2010). An increased proportion of propionate was also determined in pullet chicks feed a diet including 5 % feathers (Meyer et al., 2012 – chapter 2). Propionic acid administered by intracerebroventricular cannulation impaired cognition and social behavior in rats (Shultz et al., 2009; MacFabe et al., 2011). These changes are consistent with the symptoms of autism spectrum disorders in humans (Shultz et al., 2009). Whether propionate rich diets or small amounts of propionate entered into the CNS through different SCFA as a control treatment affect severe feather pecking or other behavioral traits in laying hens would be an intriguing question for future research. Of concern would also be the difference of propionate concentration in the blood of the portal vein and the peripheral blood regarding the metabolism in the liver.

As mentioned before not only microbial metabolism but also the microbiome itself is of relevance. Initial studies on the bacterial fecal composition of autistic children showed a higher number of clostridial species compared to the control (Finegold et al., 2002; Song et al., 2004). These studies give first insight into the topic, but a larger number of studies would be necessary to feel confident about it (Song et al., 2004). In autistic children the diversity of bacteria in the feces was higher compared to control subjects (Finegold et al., 2010). In the study of Meyer et al. (2012) the bacterial diversity in the cecal content was investigated and found being lower when feathers were included into the diet.

The microbiome-gut-brain axis is an expanding area of research in human medicine and likely only “the tip of the iceberg” was seen until now (Grenham et al., 2011). These studies give first insight in the possible relation of gut microbiome and feather pecking as behavioral disorder in laying hens and further research is necessary.

It would be of future interest to investigate a possible influence of probiotics on behavior of hens. Wang et al. (2010) found significant lower cell counts of intestinal Bifidobacteria in au-

tistic children compared to controls. A probiotic formulation consisting of *Lactobacillus helveticus* and *Bifidobacterium longum* reduced anxiety-like behavior in rats and relieved stress in humans (Messaoudi et al., 2011). The results are somewhat preliminary and extended investigations are recommended (Messaoudi et al., 2011).

It was shown that the intake of feathers lead to an altered composition of the gut microbiota and to a different metabolite spectrum in pullets and those adult laying hens, differing in their feather pecking behavior, had a different pattern of bacterial metabolites in the intestine. We could identify gut bacteria being able to use keratin as substrate. Further studies are warranted, studying the relationship between the gut microbiome and behavior in hens. To what extend a targeted manipulation of the gut microbiome, e.g. by the use of probiotics, could help to solve the problem of feather pecking will be an intriguing question in the future.

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5 Summary

Beatrice Meyer: *Effects of Feather Pecking and Ingestion on Intestinal Microbiota and Microbial Metabolites in Pullets and Laying Hens*

Feather pecking in laying hens is a serious behavioral problem that is often associated with feather eating. The aim of the first study was to investigate the influence of ingested feathers on the gut microbiome and its metabolism and to identify keratinolytic gut microbiota. Therefore two different diets, with or without 5% ground feathers, were fed to raising Lohmann-Selected Leghorn chicks. They were divided into 3 feeding groups: group A (control), B (5% ground feathers in the diet), and C, in which the control diet was fed until week 12 and then switched to the 5% feather diet to study the effect of time of first feather ingestion. The gut microbiota was analyzed by cultural methods and by denaturing gradient gel electrophoresis of the ileum and cecum digesta. Short-chain fatty acids, ammonia, and lactate concentrations were measured as microbial metabolites.

The concentration of keratinolytic bacteria increased after feather ingestion in the ileum ($P < 0.001$) and cecum ($P = 0.033$). Bacterial species that hydrolyzed keratin were identified as *Enterococcus faecium*, *Lactobacillus crispatus*, *Lactobacillus reuteri*-like species (97% sequence homology), and *Lactobacillus salivarius*-like species (97% sequence homology). Molecular analysis of cecal DNA extracts showed that the feather diet lowered the bacterial diversity indicated by a reduced richness ($P < 0.001$) and shannon ($P = 0.012$) index. The pattern of microbial metabolites indicated changes, especially in the cecum. Ammonia concentration and the molar ratio of propionate, i-butyrate, i-valeriate and n-valeriate were higher in pullets with feathers in the diet compared to the controls.

The second study was conducted with adult laying hens of a selected high and a low feather pecking line based on six generations and affirmed in a controlled behavioral study. The number of whole feathers, feather parts and feather particles in the gizzard and the intestinal microbial metabolites (biogenic amines, short-chain fatty acids, ammonia and lactate) in the ileum and ceca were determined.

In the gizzard the number of feather particles was higher in the hens with high pecking activity ($P = 0.012$). The pattern of intestinal microbial metabolites was affected. Putrescine and cadaverine concentrations were higher in the ileum of the hens with low pecking activity ($P < 0.001$, and $P = 0.012$). Ammonia was higher in the ileum and cecum in this line ($P < 0.001$, and $P = 0.004$). In the cecum of the laying hens with high pecking activity higher amounts of L-lactate, D-lactate, total lactate, SCFA and higher molar ratios of propionate and n-butyrate were determined ($P = 0.007$, $P = 0.005$, $P = 0.006$, $P < 0.001$, $P < 0.001$, and $P = 0.034$). Acetate, i-butyrate, i-valerate and n-valerate all present higher molar ratios in the cecum of the hens with low pecking activity ($P = 0.001$, $P = 0.003$, $P = 0.001$, and $P < 0.001$).

It was shown that the intake of feathers lead to an altered composition of the gut microbiota and to a different metabolite spectrum in pullets and those adult laying hens, differing in their feather pecking behavior, had a different pattern of bacterial metabolites in the intestine. We could identify gut bacteria being able to use keratin as substrate. Further studies are warranted, studying the relationship between the gut microbiome and behavior in hens. To what extend a targeted manipulation of the gut microbiome, e.g. by the use of probiotics, could help to solve the problem of feather pecking will be an intriguing question in the future.

6 Zusammenfassung

Beatrice Meyer: *Der Einfluss von Federpicken und –aufnahme auf die intestinale Mikrobiota und den mikrobiellen Metabolismus bei Jung- und Legehennen*

Federpicken ist ein bedeutendes Problem in der Haltung von Legehennen und die Federn werden dabei häufig aufgenommen. Das Ziel der ersten Studie war es, den Einfluss der aufgenommenen Federn auf die intestinale Mikrobiota und den mikrobiellen Metabolismus zu untersuchen und keratinabbauende Bakterien zu identifizieren. Dafür wurden zwei verschiedene Rationen, mit bzw. ohne 5 % zerkleinerten Federn, an Junghennen der Rasse Lohmann-Selected-Leghorn verfüttert. Die Tiere wurden einer der folgenden Fütterungsgruppen zugeordnet: Gruppe A (Kontrolle), Gruppe B (5 % zerkleinerte Federn im Futter) and Gruppe C, die zunächst das Futter der Kontrollgruppe erhielt und ab der 13. Lebenswoche das Futter mit 5 % zerkleinerten Federn. Auf diese Weise sollte der Effekt des Zeitpunkts der ersten Federaufnahme untersucht werden. Die intestinale Mikrobiota wurde mittels kultureller Nachweisverfahren und denaturierender Gradientengelelektrophorese der Ileum- und Zäkumdigesta untersucht. Als mikrobielle Metaboliten wurden kurzkettige Fettsäuren, Ammonium und Laktat bestimmt.

Die Konzentration von keratinabbauenden Bakterien war nach der Aufnahme von Federn im Ileum ($P < 0.001$) und Zäkum ($P = 0.033$) erhöht. Bakterielle Spezies, die in der Lage waren, Keratin abzubauen wurden als *Enterococcus faecium*, *Lactobacillus crispatus*, *Lactobacillus reuteri*-ähnlich (97 % Sequenzhomologie) und *Lactobacillus salivarius*-ähnlich (97 % Sequenzhomologie) identifiziert. Die molekulare Analyse von DNA-Extrakten aus der Zäkumdigesta ergab, dass die bakterielle Vielfalt nach Federaufnahme verringert war, was durch eine reduzierte Richness ($P < 0.001$) und einen niedrigeren Shannon-Index ($P = 0.012$) angezeigt wurde. Das Muster der mikrobiellen Metaboliten zeigte besonders im Zäkum Veränderungen. Die Konzentrationen von Ammonium und die molaren Anteile von Propionat, n-Butyrat, i- und n-Valeriat waren bei den Junghennen, die Federn in ihrer Ration hatten, im Vergleich zur Kontrollgruppe höher.

Die zweite Studie wurde mit adulten Hühnern aus jeweils einer stark bzw. einer schwach federpickenden Linie, selektiert über sechs Generationen, durchgeführt, die in einer kontrollierten Verhaltensstudie phänotypisch bestätigt wurden. Die Anzahl an ganzen Federn, Federteilen und Federteilchen in Muskelmagen und die mikrobiellen Metaboliten (biogene Amine, kurzkettige Fettsäuren, Ammonium und Laktat) in Ileum und Zäkum wurden bestimmt.

Im Muskelmagen war die Anzahl an Federteilchen bei den Hennen mit starker Pickaktivität größer ($P = 0.012$). Das Muster der mikrobiellen Metaboliten war verändert. Die Konzentrationen von Putrescin und Cadaverin waren im Ileum der schwachen Federpicker höher ($P < 0.001$, und $P = 0.012$). Der Gehalt an Ammonium war im Ileum und Zäkum dieser Linie höher ($P < 0.001$, and $P = 0.004$). Im Zäkum der Tiere mit starker Pickaktivität wurden höhere Konzentrationen an L-Laktat, D-Laktat, Gesamtlaktat, kurzkettigen Fettsäuren und höhere molare Anteile von Propionat und n-Butyrat gemessen ($P = 0.007$, $P = 0.005$, $P = 0.006$, $P < 0.001$, $P < 0.001$, und $P = 0.034$). Die molaren Anteile von Azetat, i-Butyrat, i-Valerat and n-Valerat waren in den Hennen mit schwacher Pickaktivität erhöht ($P = 0.001$, $P = 0.003$, $P = 0.001$, und $P < 0.001$).

Es wurde gezeigt, dass die Federaufnahme zu einer Beeinflussung der intestinalen Mikrobiota führt und der mikrobielle intestinale Metabolismus sowohl bei Junghennen als auch bei Legehennen, die genetisch auf unterschiedliches Federpickverhalten selektiert sind, beeinflusst ist. Wir konnten intestinale Bakterien identifizieren, die in der Lage sind, Keratin abzubauen. Weitere Studien wären sinnvoll, um die Beziehung von intestinalem Mikrobiom und Verhalten bei Hennen zu untersuchen. Inwieweit eine gezielte Manipulation des intestinalen Mikrobioms, z. B. durch die Anwendung von Probiotika, dazu beitragen könnte, das Problem des Federpickens zu lösen, ist eine faszinierende Frage für die Zukunft.

7 Publications

Parts of the present dissertation have been presented as poster or lecture at conferences.

Poster:

Meyer, B., Harlander-Matauschek, A., Vahjen, W., Zentek, J.
Federaufnahme von Legehennen - Konsequenzen für Zusammensetzung und Metabolismus der intestinalen Mikrobiota.
Nutztierernährung morgen: Gesunde Tiere - effiziente und nachhaltige Erzeugung - wertvolle Produkte., Zürich, 02.-04.09.2009
Schriftenreihe Institut für Nutztierwissenschaften Ernährung-Produkte-Umwelt ETH Zürich, Band 32, Zürich: Institut für Nutztierwissenschaften, 2009, pp. 236-237
ISBN: 978-3-906466-32-9

Meyer, B., Harlander-Matauschek, A., Vahjen, W., Zentek, J.
Auswirkungen der Federaufnahme bei Legehennen auf Zusammensetzung und Metabolismus der intestinalen Microbiota.
4. Doktorandensymposium am Fachbereich Veterinärmedizin der Freien Universität Berlin, Berlin 06.11.2009
Abstract Proceedings p. 72
ISBN: 978-3-86664-683-4

Meyer, B., Harlander-Matauschek, A., Vahjen, W., Zentek, J.
Impact of feather ingestion of laying hens on intestinal microbiota and microbial metabolites (Auswirkungen der Federaufnahme bei Legehennen auf die intestinale Mikrobiota und mikrobielle Metaboliten).
64. Jahrestagung der Gesellschaft für Ernährungsphysiologie,
Göttingen 09.-11.03.2012
Proceedings of the Society of Nutrition Physiology, 19, Frankfurt a.M., DLG-Verlag, 2010, p. 74
ISBN:978-3-7690-4103-3

Meyer, B., Harlander-Matauschek, A., Vahjen, W., Zentek, J.
How does feather eating affect intestinal microbiota and microbial metabolites in growing leghorn-type chickens?
46th Congress of the International Society for Applied Ethology (ISAE),
Wien 31.07.-04.08.2012
ISAE 2012 Proceedings of the 46th Congress of the International Society for Applied Ethology. Wageningen Academic Publishers, p. 172
ISBN: 978-90-8686-204-7

Oral presentation:

Meyer, B., Harlander-Matauschek, A., Vahjen, W., Zentek, J.
Feather ingestion of laying hens - influence on the intestinal microbiota and microbial metabolites
13th Congress of the European Society of Veterinary and Comparative Nutrition, Sardinien/Italien, 15.10.-17.10.2009, Congress Proceedings p.92
ISBN: 978-88-904452-55

8 Contribution of co-authors

The enumeration of the feather parts was accomplished by the working group of Dr. Alexandra Harlander-Matauschek (chapter 3).

Planning, accomplishment and analysis of the feather pecking behavior were done by the working group of Dr. Alexandra Harlander-Matauschek (chapter 3).

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10 Declaration of authorship / Selbstständigkeitserklärung

I hereby confirm that the present work was solely composed by my own. I certify that I have used only the specified sources and aids. The contributions of co-authors are listed in chapter 8.

Hiermit bestätige ich, dass ich die hier vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe. Die Beiträge von Koautoren sind in Kapitel 8 erläutert.

Berlin, den 27.02.2013

Beatrice Meyer