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Comfrey (Symphytum spp.) as an alternative field crop contributing to closed agricultural cycles in chicken feeding

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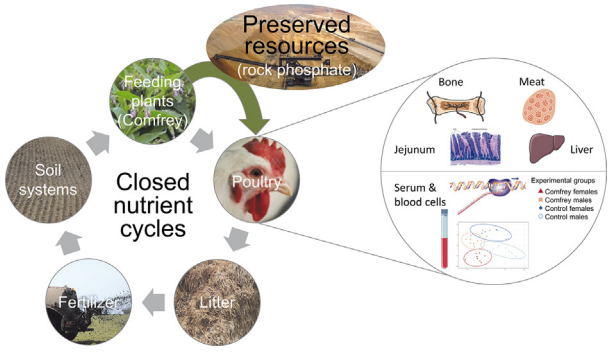
HIGHLIGHTS

- Comfrey as a regionally adapted feed crop could be used in poultry farming.
- Intake of comfrey leaves showed no aberration of health and tissue integrity.
- Comfrey leaves in poultry feed can effectively replace rock mineral supplements.
- Close nutrient cycles by reactivating phosphorus from over-fertilized soils

GRAPHICAL ABSTRACT



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ABSTRACT

Local cultivars of comfrey (Symphytum spp.) have been used to cover protein and mineral requirements of farm animals in low-input systems. Due to its known health-promoting (e.g. allantoin), but also anti-nutritive ingredients (e.g. pyrrolizidine alkaloids), multidisciplinary approaches are essential in order to quantify the nutritional value and the potential of its use in poultry and farm animals in terms of meeting animal needs, using local resources as well as remediating over-fertilized soils. Focusing on animal effects, here one-day old sexed Cobb500 broiler chickens were subjected to either a standard control diet or a standard diet supplemented with 4% dried comfrey leaves for 32 days. Performance traits indicate good acceptance of supplementation with comfrey leaves. Parameters for liver function, mineral homeostasis, bone mineral density as well as intestinal microanatomy revealed no signs of impairment. Quantified pyrrolizidine alkaloids were below the detection limit in liver and breast muscle (<5 μg/kg tissue). Comfrey supplemented male broiler chickens showed higher ash content in breast muscle and revealed altered gene expression profiles for metabolic pathways in blood cells. In healthy broiler chickens, the transcriptome analyses revealed no aberrations in the immune-related pathways due to comfrey supplementation. The results imply that the use of comfrey leaves in a high-performance broiler line seems feasible and offers the potential for closed nutrient cycles in site-adapted local agricultural systems. Further analyses need to focus on possible growth-promoting and health-improving components of comfrey and the safe use of chicken products for human consumption.

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1. Introduction

Current feeding systems for highly productive poultry are usually based on a few plant varieties, where maize, soybeans and wheat are dominating to cover energy and protein requirements (Ravindran, 2013). At the same time, finite resources and the increasing environmental burdens, due to eutrophication, demand closed nutrient cycles and the prevention of irreversible losses (Campbell et al., 2017). To lower vulnerability and to increase options for sustainable livestock and poultry farming, conservation of agricultural biodiversity and the implementation of regional nutrient cycles have been proposed as a strategic goal (Collette et al., 2011). In fact, there is a growing body of literature on plant and insect species, which offer an attractive profile of macro- and micronutrients and exhibit traits that are beneficial for the adaptation of feed production to the risks of climate change. Varieties of grain legumes (Abraham et al., 2019; Olukosi et al., 2019) and black soldier fly larvae (Spranghers et al., 2017; Onsongo et al., 2018) were identified as promising alternatives for local use in poultry farming.

Among the cultivation of locally produced feed crops, comfrey (*Symphytum spp.*) is of interest for use in animal nutrition [Vogl et al., 2016]. Comfrey varieties are members of the *Boraginaceae* family and appear as fast growing, perennial and drought resistant plants with an extensive root system (Robinson, 1983). While the usage of comfrey root has a long tradition to treat e.g. osteoarthritis and skin lesions [Grube et al., 2007, Barna et al., 2007, Smith and Jacobson, 2011, Stucki et al., 2019], also comfrey leaves show a valuable profile of nutrients and phytochemicals. Comfrey leaves contain approximately 14% dry matter (DM) with up to 25% crude protein, 12.5% crude fiber and 23% minerals [Heitman and Oyarzun, 1971, Bareeba et al., 1992]. In particular, comfrey mobilizes considerable amounts of minerals, with its leaves retaining potassium (5.9%), calcium (1.4%), and phosphorus (0.5%) (Robinson, 1983). Indeed, comfrey is designated as a dynamic accumulator plant and is known for its capacity to provide minerals in bioavailable form, which might be beneficial when cultivated on over-fertilized soils. This has the potential to achieve sparing effects to effectively replace e.g. calcium phosphate supplements in feed formulation. The phytochemical composition of comfrey includes a wide variety of bioactive compounds, such as allantoin, phenolic acids and tannins (Staiger, 2012). Allantoin naturally origins from purine catabolism and subsequent uric acid oxidation and is known for its pharmacological effects for topical application improving wound healing (Araújo et al., 2010; Savić et al., 2015) and reducing inflammation (Lee et al., 2010; Frikeche et al., 2015). However, comfrey also contains anti-nutritive components such as pyrrolizidine alkaloids (e.g. lycopsamine, 7-*O*-acetyllycopsamine, echimidine, intermedine, and their *N*-oxides), which are mainly present in the root but also appear in the leaves (Awang et al., 1993; Couet et al., 1996). When administered in purified form at high levels, pyrrolizidine alkaloids derived from comfrey root induced hepatotoxic events in a dose-dependent manner in chickens (Brown et al., 2016). Potential biotechnological approaches could be used to develop pyrrolizidine alkaloid-depleted varieties (Kruse et al., 2019).

The agricultural nutrient cycle requires great attention due to its importance for the production of high-quality food and the environmental challenges associated with animal emissions. Forage crop production and farm animal husbandry (i.e. monogastrics) have a significant impact in this context. It is hypothesized that comfrey leaves contribute to the dietary protein and mineral requirements in broiler nutrition as a locally produced feed component. However, the currently available information is not sufficient to adequately assess the benefits and risks of supplementing comfrey leaves in chicken husbandry. The aim of the present study is to evaluate whether a supplementation of 4% comfrey leaves in broiler diets proves feasible to effectively replace mineral supplements. The comprehensive phenotyping of Cobb500 broilers comprised measurements for performance, tissue integrity and mineral homeostasis in serum, bone, small intestine, liver, and breast muscle as well as metabolic and regulatory effects at the transcriptional level of blood cells.

2. Materials and methods

2.1. Animals, diets and sample collection

The trial was approved by the Animal Welfare Committee of the Leibniz Institute for Farm Animal Biology (FBN) and was generally licensed by the Ethics Committee of the federal state of Mecklenburg-Western Pomerania, Germany (LALLF 7221.3-1.1-042/17). The protocol comprised 128 one-day old sexed Cobb500 broiler chickens, which were randomly assigned to eight floor pens located in two equally equipped rooms (four pen replicates per dietary group, each pen 3.8 square meters). Animals were kept in groups each consisting eight males and eight females. The birds were raised either on a standard control diet based on wheat, soy and maize or on a control diet supplemented with 4% dried comfrey leaves (Tables 1, A.1), with the nutrient composition of the experimental diets being analyzed by an external laboratory (LMS LUFA, Rostock, Germany). Starter diets (day 1–11 of life) and grower diets (day 12–32 of life) were offered ad libitum in pelleted form with 2 mm and 4 mm in diameter, respectively. Dried comfrey leaves were obtained from green house cultivars (OxyGenesis GmbH, Kalkar, Germany). During the grounding of plant material and the pelleting process, the temperature of the feed stuff was raised to approximately 58–60 °C (Kahl, Reinbek, Germany). Formulated diets met requirements (NRC, 1994) and contained phytase supplements. Water in automatic drinking cups was supplied ad libitum. The broilers were inspected daily. At day 32, a subset of 16 male and 16 female individuals balanced for dietary group and pen was randomly selected for tissue sampling. Broilers were stunned by electronarcosis and subsequently slaughtered by exsanguination in the experimental abattoir of the FBN. Sampling comprised trunk blood, femora, jejunum, liver, and breast muscle. Veterinary inspection of the broilers before and the carcasses and organs after slaughter confirmed the lack of any impairments, disease symptoms and pathological signs. Serum was prepared by centrifugation of clotted blood (20 min; 4 °C; 3500 × g) and stored at −80 °C until further analyses. The clotted blood was stored at −80 °C until further usage for total RNA extraction. The left femora were removed and stored at −20 °C until further analyses. The jejunal sections were approximately 4 cm in length and were taken 3 cm proximal to the Meckel's diverticulum.

2.2. Quantification of allantoin and pyrrolizidine alkaloids

Allantoin contents of ground comfrey leaves and experimental grower diets were analyzed via HPLC (PhytoLab GmbH, Vestenbergsgreuth, Germany). The pyrrolizidine alkaloids were quantified via SPE-LC-MS/MS (Institute Kirchhoff Berlin GmbH, Berlin, Germany). As liver and breast muscle represent main product tissues in broiler farming, pooled samples per dietary group and tissue were subjected to pyrrolizidine alkaloid quantification. The analyses comprised 28 pyrrolizidine alkaloids such as 7-*O*-acetylintermedine, 7-*O*-acetyllycopsamine, 7-*O*-acetylintermedine N-oxide, 7-*O*-acetyllycopsamine N-oxide, echimidine, echimidine N-oxide, intermedine, intermedine N-oxide, lycopsamine, lycopsamine N-oxide which are listed in Table A.2 (detection limit at 5 μg/kg).

2.3. Broiler performance data

Total body weight (BW) was recorded pen-wise at day 1, day 9, day 16, day 23, day 30, and day 32. Daily feed intake (DFI), daily weight gain (DWG), and feed conversion ratio (FCR) were calculated for the entire experimental period. At day 32, individual live weight and carcass weight were recorded for the selected representative animals. For meat characteristics, individual pH of breast muscle (*M. pectoralis*)

was recorded at 24 h (pH24h) post mortem via a probe electrode (pH-Star, Matthäus, Pöttmes, Germany). The meat colors L* (lightness), a* (redness), b* (yellowness) were measured in breast muscle samples using a CR-300 device (Minolta AG, Langenhagen, Germany) after chilling at 4 °C for 24 h. The ash content of breast muscle samples was determined in triplicate by calcination in a muffle furnace at 600 °C using established protocols (Helvich, 1990).

2.4. Bone characteristics

Femora were thawed at room temperature overnight ($n = 12$, balanced for diet, sex and pen). Once thawed, each bone was weighed and bone length and diameter were measured using digital callipers. Dual energy x-ray absorptiometry (DXA) scans were performed using a STRATOS dR (DMS group France). The region of interest was selected and contained both trabecular and cortical bone. The scans were analyzed using the software 3D DXA, Medix DR for measurements of bone mineral content (BMC) and bone mineral density (BMD). Moreover, a breaking strength test was conducted on each bone (Crenshaw et al., 1981), which involved subjecting the bones to a specific load using a 3-point bending jig (Instron 3366, Instron, High Wycombe, Bucks, UK). The load cell was 100 kg and a crosshead speed of 25 mm/min was applied. The force of an attached anvil measuring 50 mm in length and 10 mm in width was applied to the midpoint of the same facial plane of each bone until the bones fails (determined automatically by the Bluehill software, version 3, Instron).

2.5. Intestinal histology

The dissected segments of the jejunum ($n = 32$) were washed in phosphate-buffered saline and were fixed overnight with 3.7% buffered paraformaldehyde. After fixation, sub-segments of approximately 1 cm in length were cut from each segment. These jejunal samples were dehydrated via a series of increasing concentrations of ethanol, transferred to xylol and embedded in paraffin. With a microtome (LEICA RM2255) 5 μm thin serial sections were prepared. The histological sections were deparaffinized and stained with hematoxyline and eosin (HE). Microphotographs were taken with a bright field microscope (Zeiss Axio Imager M2). To quantify morphological parameters, microphotographs were measured using Fiji, the image processing-package of ImageJ (https://imagej.net/Fiji). The jejunal villus height, villus width, and crypt depth were assessed by 10 individual measurements (Fig. 1). Furthermore, the intestinal villus to crypt ratio (VCR) was calculated (Jeurissen et al., 2002).

2.6. Serum parameters

Serum aspartate transaminase (AST), γ-glutamyltransferase (GGT), bilirubin, albumin and triacylglycerides ($n = 32$) were analyzed to obtain measurements of liver function and integrity with commercial assays using Fuji DriChem 4000i (FujiFilm, Minato, Japan). For analyses of mineral homeostasis and growth, serum inorganic phosphorus and calcium (FujiFilm) as well as serum parathyroid hormone (CSB-E118880Ch, CusaBio, Houston, USA) and triiodothyronine (EIA-4569, DRG, Marburg, Germany) were determined. The hormones were analyzed in duplicate using commercially available enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's protocols.

2.7. Transcriptome profiling of blood cells

Blood samples ($n = 32$) were ground into powder in liquid nitrogen and subjected to RNA extraction using TRIzol reagent (Invitrogen, Karlsruhe, Germany) as previously described (Mewis et al., 2014). Subsequently, DNaseI treatment was applied to RNA extracts (Roche Diagnostics, Mannheim, Germany). Samples were purified using the column-based NucleoSpin Kit (Macherey-Nagel, Düren, Germany). RNA quality and quantity was assessed with a NanoDrop ND-2000 (Peqlab, Erlangen, Germany). All samples showed an absorbance ratio of 260/280 greater than 2.1, indicating high RNA integrity. All RNA samples were stored at −80 °C until further use. For hybridization on genome-wide ChiGene-1_0-st arrays (Affymetrix, Santa Clara, CA, USA), total RNA samples were used to generate biotinylated DNA targets with the GeneChip WT PLUS Regent Kit (Affymetrix). Processing of arrays ($n = 32$) followed manufacturer's instructions according to the GeneChip Hybridization, Wash, and Stain Kit (Affymetrix). Raw data was generated using a GeneChip Scanner 3000 (Affymetrix) and was deposited in a MIAME-compliant database, the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo; accession number: GSE147575). Corresponding annotation data were based on an updated assignment of probes and respective probe-sets via the R package rePROBE (Hadlich et al., 2020). Genomic information was retrieved from RefSeq (*Gallus gallus* release 103; http://ftp.ncbi.nih.gov/genomes) and Ensembl (Gallus_gallus.GRCg6a release 97; ftp://ftp.ensembl.org/pub). In total, the workflow revealed 17,894 probe-sets for gene expression profiling.

2.8. Data analyses

For performance data referring to the whole broiler population, a linear model was applied including dietary group (R language, version 3.6.2, package stats). Individual data obtained at slaughter were analyzed by a linear model considering dietary group, sex and slaughter order (R language). Differences were considered significant at $P \leq 0.05$.

The quality of all microarrays was assessed via the R package arrayQualityMetrics (version 3.42.0) [Kauffmann et al., 2009]. Raw intensities were normalized using the RMA (Robust Multichip Average) approach. Mean and standard deviation of each probe-set were calculated, probe-sets showing a mean below the 5th percentile among all samples and a standard deviation above the 90th percentile within each experimental group were discarded from further analyses to improve statistical power (Bourgon et al., 2010). Expression data were interrelated by dietary groups and sexes using a sparse Partial Least Squares Discriminant Analysis (sPLS-DA; package 'mixOmics', version 6.10.6) (Rohart et al., 2017). The variable selection and classification process considered three components. Moreover, relative changes of mRNA abundances were estimated via a linear model (package limma, version 3.42.0) including dietary group, sex and slaughter order (Ritchie et al., 2015). To correct for multiple testing, false discovery rates (q-values) were calculated (package qvalue, version 2.18.0) (Storey and Tibshirani, 2003; Storey et al., 2019). The level of significance was set at $q \leq 0.15$ which corresponds to $P \leq 0.014$. The identified genes were used for enrichment analyses employing the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using the KEGG Orthology-Based Annotation System (KOBAS) (Kanehisa and Goto, 2000; Xie et al., 2011). The level of significance was set at false discovery rate (FDR) ≤0.05.

3. Results

3.1. Dietary specifications

The used comfrey leaves contained 186 g/kg crude ash, 352 g/kg crude protein (285 g/kg digestible protein), 27 g/kg crude fat, 126 g/kg crude fiber, 10.8 g/kg calcium (Ca), 6.9 g/kg phosphorus (P), and 64.9 g/kg potassium (K) in DM. The grower diet supplemented with 4% comfrey leaves showed an allantoin content of 0.76 mg/g DM, while allantoin was not detectable in the control diet. The analyzed concentrations of pyrrolizidine alkaloids in ground comfrey leaves were summed up to 137.0 μg/g DM (Table A.2) which corresponds to approximately 5.5 μg/g DM in comfrey starter and grower diets. Due to a DFI in broiler chickens of <100 g/day, the ingested pyrrolizidine alkaloid concentration therefore corresponds to a dose of about 50% of the toxic

Table 1
Analyzed nutrient composition of the experimental diets during starter and grower periods. FM – fresh matter; DM – dry matter; ME – metabolizable energy.

Item	Unit	Starter control	Starter + 4% comfrey leaves	Grower control	Grower + 4% comfrey leaves
DM	g/kg FM	902	905	904	898
Crude ash	g/kg DM	56	60	54	59
Crude protein	g/kg DM	221	237	216	218
Crude fat	g/kg DM	48	71	42	41
Crude fiber	g/kg DM	26	62	36	36
Starch	g/kg DM	357	306	371	360
Calcium (Ca)	g/kg DM	3.8	3.9	3.8	3.9
Phosphorus (P)	g/kg DM	3.0	2.9	3.1	3.0
Ca: P ratio		1.27	1.34	1.23	1.30
Sodium (Na)	g/kg DM	1.2	1.0	1.2	0.7
Magnesium (Mg)	g/kg DM	0.9	0.9	0.9	0.9
Potassium (K)	g/kg DM	5.9	6.9	5.3	6.4
ME (poultry)	MJ/kg DM	11.7	11.8	11.6	11.4

amount estimated for humans (1000 μg/day) at medium and long-term exposure (BfR, 2013).

3.2. Broiler performance

Performance was recorded pen-wise with each pen containing 8 male and 8 female broilers. In the starter phase, broilers supplemented with comfrey leaves showed lower BW at day 9 ($P = 0.036$). From day 16 on, there were no significant differences among dietary groups (Table 2). Considering the entire experimental period, values for DFI, DWG, and FCR remained unaltered between the dietary groups.

3.3. Carcass traits, bone development and intestinal microanatomy

At slaughter, live weight and carcass weight were unaltered between broilers supplemented with 4% comfrey leaves compared to control fed animals at day 32 (Table 3). In breast muscle, the comfrey supplementation prompted significantly lower values for redness (a*) and increased values for yellowness (b*) in male broiler chickens. Moreover, comfrey-supplemented male animals showed increased ash content of breast muscle compared to control animals. In contrast, supplemented females showed higher values for b*, but remained unchanged for a* and ash content compared to controls. The measurements for pH24h and lightness (L*) in breast muscle were unaffected by diet in both sexes. No statistically significant differences in bone characteristics including femur maximal load, bone mineral density and bone mineral content were observed between the dietary groups within sexes (Table 3). The intestinal parameters such as villus height, villus width, crypt depth, and VCR remained unaltered between the dietary groups in both sexes (Table 3).

3.4. Quantification of pyrrolizidine alkaloids

The analyzed pyrrolizidine alkaloids were not detectable in liver and breast muscle tissues of the examined broiler chickens (Table A.2).

3.5. Serum parameters of liver integrity, mineral homeostasis and growth

No signs of impairment were detected for parameters related to liver function and integrity (Fig. 2) such as serum AST (males: $P = 0.503$; females: $P = 1.000$), GGT (males: $P = 0.954$; females: $P = 0.574$), bilirubin (males: $P = 0.997$; females: $P = 0.996$), albumin (males: $P = 0.847$; females: $P = 0.591$), and triacylglycerides (males: $P = 0.966$; females: $P = 0.986$). For mineral homeostasis and growth, serum concentrations of inorganic phosphorus (males: $P = 0.917$; females: $P = 0.395$), calcium (males: $P = 0.997$; females: $P = 0.910$), parathyroid hormone (males: $P = 0.871$; females: $P = 1.000$) and triiodothyronine (males: $P = 0.341$; females: $P = 0.834$) were unaltered between the dietary groups in both sexes.

3.6. Transcriptome profiling of blood cells

The analyses comprised 12,225 probe-sets which represented 10,393 genes. Due to the pronounced sex dimorphism in broiler

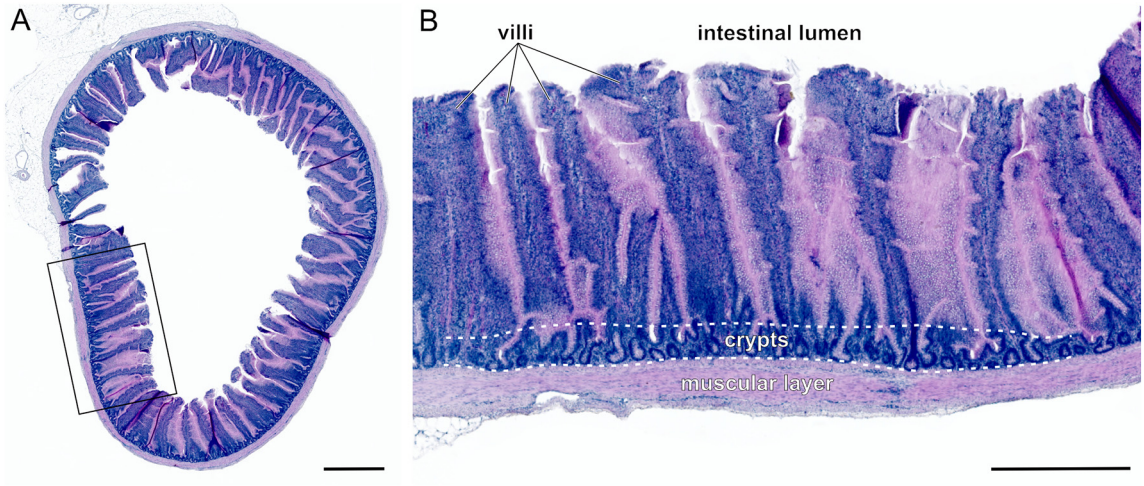

Fig. 1. Histological cross section of a representative hematoxylin and eosin (HE)-stained slice of the jejunum (A). Rectangle indicates a representative area used for analyzing intestinal morphology traits including villus height, villus width and crypt depth (B). Enterocytes lining the villi and the crypts appear in blue color. Muscle cells, fibroblasts and mucin appear in pink. Dashed lines indicate the crypt depth. Scale bar = 1 mm (A), 500 μm (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Performance traits between broiler chickens fed a standard control diet and a standard diet supplemented with 4% comfrey leaves recorded pen-wise at the respective days. Data represent the entire experimental period per capita (mean ± SE).

Trait	Unit	Control diet	Control diet + 4% comfrey leaves	*P* value
BW (day 1)	g	39.3 ± 0.1	39.3 ± 0.3	0.901
BW (day 9)	g	204.5 ± 3.7	192.9 ± 2.2	0.036
BW (day 16)	g	588.4 ± 7.1	556.5 ± 12.0	0.062
BW (day 23)	g	1153.0 ± 8.0	1126.1 ± 13.8	0.143
BW (day 30)	g	1871.4 ± 19.4	1831.8 ± 24.8	0.255
BW (day 32)	g	2101.3 ± 19.1	2065.3 ± 31.5	0.365
Daily feed intake (DFI)	g	88.8 ± 0.9	89.5 ± 1.2	0.651
Daily weight gain (DWG)	g	65.2 ± 0.5	64.2 ± 1.0	0.406
Feed conversion ratio (FCR)	g/g	1.36 ± 0.01	1.39 ± 0.03	0.272

chickens, the sPLS-DA approach identified the first out of three components to differentiate between male and female animals (Table A.3). The respective probe-sets included in the second and third components differentiated the dietary responses within sexes (Fig. 3). The second component mainly differentiates between the dietary groups and the third component mainly differentiates between the sexes within the dietary groups. Corresponding transcripts were displayed in Table A.3. Genes identified with the highest loadings were HSPA13, RP11-434D12.1 and EMC2 for component 2 and CACNA1H, PCCB and LOC107054910 for component 3.

The differentially expressed gene analysis revealed 681 probe-sets to be significantly altered between male comfrey-supplemented animals and male control animals (Table A.4). The corresponding genes were subjected to an enrichment analysis (Tables 4, A.5). Expression profiles of supplemented females remained unchanged compared to controls considering the significance threshold.

4. Discussion

Modern poultry production relies on global resource cycles which have become increasingly fragmented with associated ecological burdens and economic constrains (Tóth et al., 2014; Jarvie et al., 2015). Due to its extensive root system, the comfrey plant could facilitate regional nutrient cycles when cultivated on currently over-fertilized soils. In fact, preventing run-off of e.g. phosphorus is one of the major challenges in agricultural practice (Campbell et al., 2017). Comfrey plants can be cultivated in large quantities and harvested multiple times a year (Robinson, 1983).

Compared to commercial feed crops with respect to the crude ash (186 g/kg DM) and crude protein contents (352 g/kg DM), dried comfrey leaves are superior to alternative protein sources such as alfalfa, Medicago sativa, (116 g/kg, 209 g/kg; dehydrated) and also show a remarkable profile of micro- and macronutrients compared to soybean, Glycine max., (52 g/kg, 348 g/kg; full-fat, extruded) or blue lupine, Lupinus angustifolius, (35 g/kg, 318 g/kg; toasted) (Sauvant et al., 2004). Hence, the use of comfrey leaves contributes to meeting the requirements of bioavailable minerals and protein in chicken feed (Table 1). Based on the retrieved DFI values throughout the entire experimental period and the analyzed profile of macro- and micronutrients of comfrey leaves, a dietary supplementation of 4% comfrey leaves accounts for approximately 9.3% of the phosphorus intake (calcium: 11.1%; crude protein: 6.25%). As chicken rely on a daily supply of calcium phosphates via diet, this offers potential for an effective reduction in mineral supplements of e.g. calcium phosphates in poultry nutrition. The usage of comfrey varieties targets the important issue of lowering the environmental impact of poultry husbandry in terms of phosphorus emissions.

When considering the entire experimental period in this study, the supplementation of comfrey leaves was well accepted by the broiler chickens as reflected in the unaltered daily feed intake and daily weight gain (Table 2). This suggests that the supplementation with comfrey leaves was effective. The performance of broilers in both the control group and the comfrey-supplemented group matched the standard values of Cobb500 chicken (Cobb-Vantress, 2018). The exploited performance of the dietary groups is reflected in constant levels of serum triiodothyronine (Fig. 2), which indicate unaffected superior growth processes at day 32 of life.

As the representative histological cross-section of an HE-stained section of the jejunum in Fig. 1 illustrates, there was no evidence of impairment for the intestinal microanatomical parameters in comfrey-supplemented male and female broilers (Table 3). The values of villus height, crypt depth and VCR were similar to those of previous work done in broiler chickens (Calik et al., 2019; Santin et al., 2001). Any intolerances or inflammatory processes due to comfrey ingredients would have resulted in e.g. intestinal atrophy and malabsorption. The observations made in this study indicate that the jejunum of the comfrey-supplemented chickens were likely functionally equivalent to those chickens fed the control diets suggesting intestinal integrity in all animals. As shown in Fig. 2, serum markers of liver integrity and function such as GGT, AST, bilirubin, albumin and triacylglycerides remained unaltered by comfrey supplementation in all animals.

Table 3
Final live weight, carcass traits, bone characteristics and parameters of intestinal histology between male and female animals fed a standard control diet and a standard diet supplemented with 4% comfrey leaves. Data were retrieved from individual broilers at day 32 (mean ± SE).

Trait	Unit	Males			Females		
		Control diet	Control diet + 4% comfrey leaves	P value	Control diet	Control diet + 4% comfrey leaves	P value
Live weight	g	2214.1 ± 33.9	2191.4 ± 42.3	0.884	1940.3 ± 37.2	1819.6 ± 36.8	0.184
Carcass weight	g	1487.5 ± 25.6	1426.8 ± 33.7	0.406	1295.0 ± 37.7	1191.3 ± 31.6	0.178
pH 24 h (M. pectoralis)		5.85 ± 0.04	5.84 ± 0.03	0.982	5.73 ± 0.04	5.70 ± 0.02	0.984
L* (lightness; *M. pectoralis*)		50.27 ± 1.37	49.92 ± 0.82	0.999	52.00 ± 0.8	51.65 ± 0.86	0.974
a* (redness; *M. pectoralis*)		6.40 ± 0.46	5.41 ± 0.25	0.027	6.12 ± 0.19	5.25 ± 0.32	0.447
b* (yellowness; *M. pectoralis*)		0.46 ± 0.42	5.04 ± 0.36	<0.001	0.35 ± 0.25	6.52 ± 0.45	<0.001
Ash (M. pectoralis)	%	1.13 ± 0.01	1.17 ± 0.01	0.024	1.14 ± 0.01	1.17 ± 0.02	0.580
Femur weight	g	11.6 ± 0.1	11.5 ± 0.6	0.997	10.5 ± 0.3	9.6 ± 0.6	0.822
Femur length	mm	65.0 ± 1.5	65.3 ± 1.2	0.999	63.7 ± 2.4	60.0 ± 1.5	0.588
Femur diameter	mm	8.4 ± 0.6	8.9 ± 0.5	0.872	7.9 ± 0.2	7.6 ± 0.4	0.997
Femur max. Load	kg	28.9 ± 1.2	22.4 ± 3.8	0.246	22.0 ± 2.1	23.2 ± 0.8	0.967
Bone mineral density	g/cm^2	0.43 ± 0.02	0.38 ± 0.02	0.368	0.45 ± 0.01	0.45 ± 0.02	0.963
Bone mineral content	g	0.85 ± 0.04	0.74 ± 0.04	0.360	0.89 ± 0.03	0.89 ± 0.04	0.974
Villus height (*Jejunum*)	µm	1549.5 ± 68.6	1513.9 ± 85.4	0.994	1324.9 ± 97.9	1507.6 ± 108.1	0.840
Villus width (*Jejunum*)	µm	272.0 ± 20.8	252.7 ± 27.0	1.000	234.9 ± 19.6	259.1 ± 31.1	1.000
Crypt depth (*Jejunum*)	µm	136.9 ± 7.1	114.2 ± 10.4	0.529	112.5 ± 7.6	107.6 ± 11.4	0.721
VCR (Jejunum)		11.5 ± 0.8	14.0 ± 1.5	0.508	11.9 ± 0.8	14.8 ± 1.3	0.173

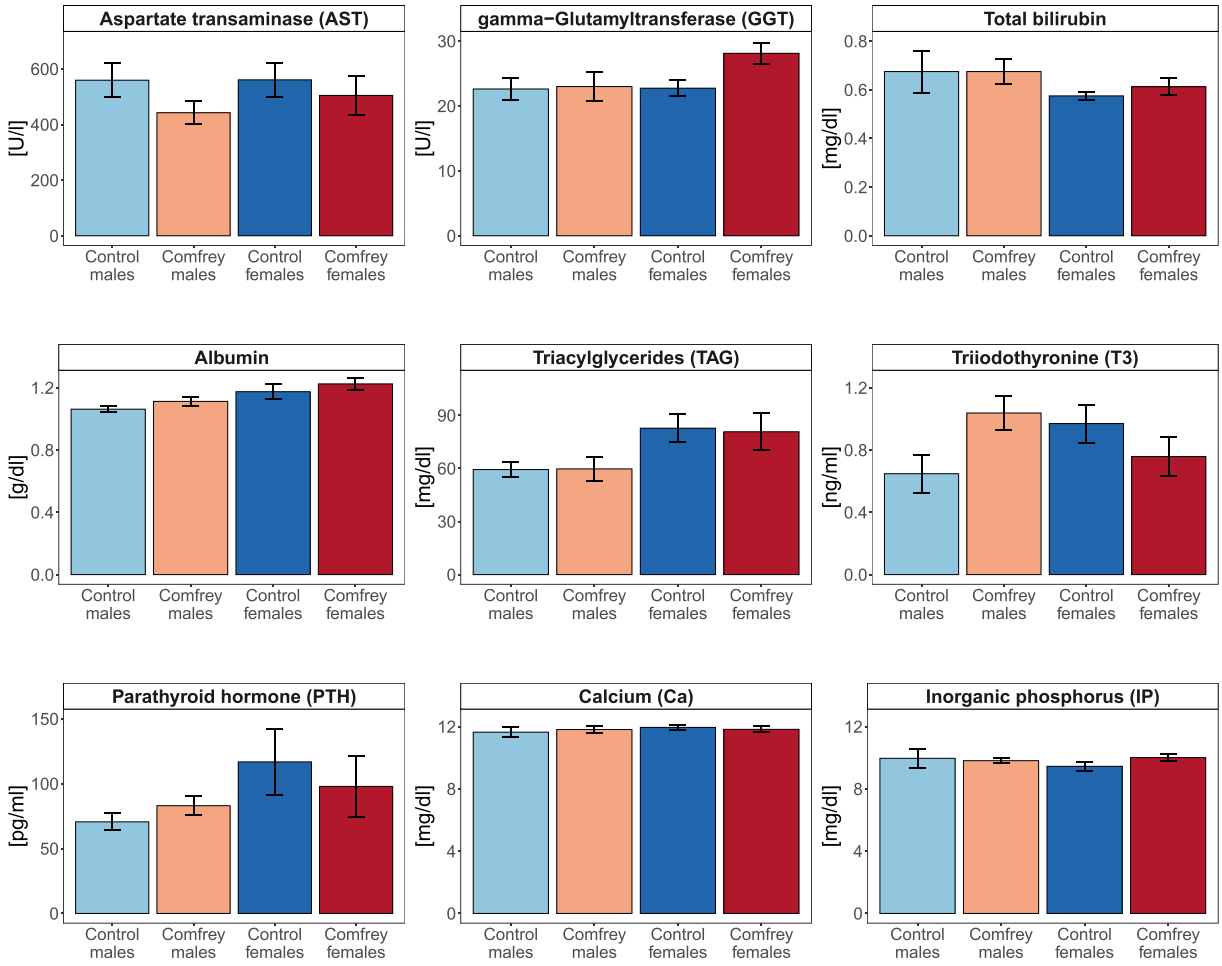

Fig. 2. Serum parameters reflecting liver function and integrity, mineral homeostasis and growth of male and female animals fed a standard control diet and a standard diet supplemented with 4% comfrey leaves. Values are displayed as mean ± SE.

This observation matches to previous reports when a number of serum parameters, including GGT and AST, were unaffected by the intake of comfrey alkaloid root extracts in California White chickens (Brown et al., 2016). Also previously shown, the consumption of moderate amounts of comfrey leaves seems not to prompt any signs of impaired liver function in humans (Anderson and McLean, 1989). In the current study, serum parathyroid hormone and mineral concentrations as well as bone characteristics were equally unaffected by diet in both sexes. This indicates a balanced supply and excretion of minerals to maintain bone development in comfrey supplemented broilers.

Interestingly, the ash content of the breast muscle was increased in male comfrey-supplemented broiler chickens compared to controls (Table 3), which might be attributed to the quantities and bioavailability of calcium, phosphorus and potassium in comfrey leaves (Table 1). Moreover, comfrey contains considerable amounts of sulfur-rich amino acids which might have contributed to the higher ash content (Robinson, 1983). As meat and a variety of animal products are important sources of minerals for human nutrition (Pereira and Vicente, 2013), an increased ash content in the breast muscle of broilers is to be considered beneficial. Regarding meat color, values indicating the yellowness of breast muscle were increased in supplemented male and female broiler chickens compared to control fed animals (Table 3). Overall, the retrieved meat color data correspond to long-term analyzes in Ross508 and Cobb500 broiler chickens (Petracci et al., 2004). The current study shows that the composition of the poultry feed is crucial for breast meat color development, as has been revealed by plant supplements using spirulina (cyanobacteria) which altered values of yellowness due to the pronounced zeaxanthin content (Toyomizu et al., 2001). Although a quality criteria both in the selection and acceptance of raw or cooked meat products by the consumer, the observed yellowness of breast muscle resembles values known from organic farming and might be associated with fatty acid metabolism (Laudadio et al., 2011; Gálvez et al., 2020). Interestingly, a favorable fatty acid profile with reduced levels of saturated fatty acids (SFA) and increased levels of monounsaturated fatty acids (MUFA) was observed in pigs after supplementation with comfrey leaves in both *Musculus longissimus dorsi* and back fat (Bee et al., 1999).

Currently there is little data on in-vivo consumption of comfrey leaves and the molecular mechanisms of efficacy or toxicity are not yet fully understood. In this study, gene expression profiles from blood cells served as a proxy to assess the effects of comfrey on metabolism and immune status. In order to maximize discrimination of the experimental groups based on gene expression profiles of whole blood cells, the resulting components derived by sPLS-DA were shown in Table A.3. Clearly, male and female broilers were separated by component 1 (Table A.3), which resembles the pronounced sex dimorphism in broiler chickens as described elsewhere (Van der Heide et al., 2016). However, the variable selection approach showed the separation of broilers fed on control vs. comfrey-supplemented diets particularly by component 2, which explained 11% of the variance and discriminated between males (Fig. 3). Correspondingly, the differential gene

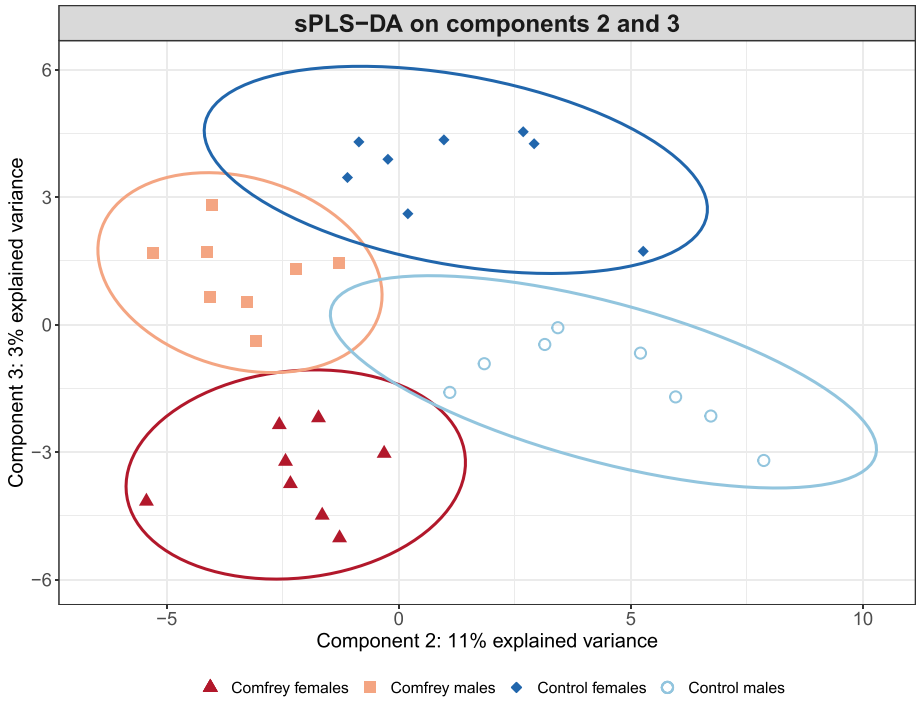

Fig. 3. The applied sPLS-DA identified component 2 and component 3 to differentiate between male and female animals fed a standard control diet and a standard diet supplemented with 4% comfrey leaves. Corresponding symbols represent individual animals, circles indicate 95% confidence interval for each group.

expression analysis confirmed dietary effects at the transcriptional level which were particularly pronounced in male broilers (Table 4). The appearance of altered 'metabolic pathways' and specifically the 'valine, leucine and isoleucine degradation' pathway might be attributed to the shifts in the dietary supply of distinct amino acids and effects of bioactive compounds. Against the background of isoenergetic diets, the catabolism of micro- and macronutrients has to be balanced and respective downstream products are likely to cover the energy demand via entering the citric acid cycle. The transcriptional patterns also indicate shifts in fat metabolism, which may be associated with increased formation of acetyl-CoA during fatty acid beta-oxidation (ACAA2) and decreased cholesterol biosynthesis (ACAT2, Table A.4). Furthermore, the 'Fanconi anemia pathway' is a network known to maintain DNA integrity (Kim and D'Andrea, 2012). Its appearance in the list of pathways after comfrey supplementation could explain the endogenous ability to process anti-nutritive compounds. Interestingly, the enrichment analysis has revealed differences in the mRNA profile for genes associated with 'melanogenesis' (Tables 4, A.5). The lowered expression of MC1R (melanocortin 1 receptor) in blood cells of comfrey supplemented male broilers might be attributed to the body's immune responses. Indeed, bioactive comfrey ingredients including allantoin are known to mediate pharmacological mechanisms, e.g. reducing inflammation (Lee et al., 2010; Frikeche et al., 2015). However, the mRNA profiles revealed no clear status of enriched immune pathways in this study. It would be of interest to investigate whether any molecular signatures for immune-promoting effects of comfrey leaves can be observed when exposed to challenging conditions, e.g. feather pecking, high parasitic load, or inflammation.

Table 4
Significantly enriched pathways (KEGG, FDR-value ≤0.05) deduced from genes found to be differentially expressed between male broiler chickens fed a standard control diet and a standard diet supplemented with 4% comfrey leaves.

Pathway	p-Value	FDR	# molecules
Metabolic pathways	7.52E−05	0.009	50
Valine, leucine and isoleucine degradation	6.28E−04	0.037	6
Fanconi anemia pathway	1.15E−03	0.037	6
Melanogenesis	1.21E−03	0.037	8

The total amounts of analyzed pyrrolizidine alkaloids in ground comfrey leaves were 137.0 μg/g DM (Table A.2). This corresponds to approximately 20.6 μg/g fresh leaf material which matches previous reports (Couet et al., 1996). Chickens are considered sensitive to pyrrolizidine alkaloids (Rode, 2002), however, in this study none of the animals showed any signs of illness and consequently the content of pyrrolizidine alkaloids in muscle and liver tissue was below the detection limit (Table A.2). In contrast, rats showed a sensitivity to comfrey pyrrolizidine alkaloids applied in high dosages while exhibiting impaired liver function and carcinogenic potential (Culvenor et al., 1980). However, besides dosages and specific experimental settings, the murine metabolism does not necessarily resemble avian conditions and the risk of cancer derived from a murine model due to exposure to pyrrolizidine alkaloid extracts does not seem to be generally transferable to other species (Prakash et al., 1999; Rode, 2002). On the other hand, the cytochrome P450 enzyme family is ubiquitously expressed in different species including chickens and pyrrolizidine alkaloids could be metabolized by biotransformation to reactive pyrroles with pronounced toxicity (Stickel and Seitz, 2000). Detailed knowledge of species-specific detoxification mechanims may be useful for the assessment of pyrrolizidine alkaloid exposure to other members of the *Borginaceae* family. In fact, there is a conceivable risk from contaminated cereals or the uptake of pyrrolizidine alkaloid containing plants in free-range farming systems. To further exploit comfrey leaves as an alternative feed component for broiler nutrition, possible interactions of different organs and cell types in the context of an allostatic load after variable supply of nutrients and anti-nutritive ingredients need to be investigated in low-intake studies. A compilation of case reports from the last 30 years shows that the evidence of harm due to comfrey is rather weak (Avila et al., 2020). In fact, pyrrolizidine alkaloid-depleted comfrey products are considered non-hazardous and are used for therapeutic purposes (Benedek et al., 2010). To breed for new comfrey varieties with low or depleted content of pyrrolizidine alkaloids would be therefore advantageous.

5. Conclusions

Taken together, standard broiler diets supplemented with 4% comfrey leaves prove feasible in terms of performance and could effectively replace calcium phosphate supplements. The analyzed set of parameters obtained in jejunum, serum, bone, liver and breast muscle indicated maintenance of high tissue function and integrity in all animals. Comfrey cultivation has considerable potential to establish regional value chains and closed nutrient cycles for locally produced poultry feed. Although animal health is an essential prerequisite for the use of comfrey as a feeding crop for the future, a bio-economic modelling would be useful with regard to the potential for mineral phosphorus savings and the remediation of over-fertilized soils.

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CRediT authorship contribution statement

Michael Oster: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization, Project administration. **Henry Reyer:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - review & editing, Visualization. **Jonas Keiler:** Methodology, Investigation, Resources, Writing - review & editing, Visualization. **Elizabeth Ball:** Methodology, Resources, Writing - review & editing. **Christina Mulvenna:** Methodology, Resources, Writing - review & editing. **Eduard Muráni:** Methodology, Resources, Writing - review & editing. **Siriluck Ponsuksili:** Methodology, Resources, Data curation, Writing - review & editing. **Klaus Wimmers:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Abraham, E.M., Ganopoulos, I., Madesis, P., Mavromatis, A., Mylona, P., Nianiou-Obeidat, I., Parissi, Z., Polidoros, A., Tani, E., Vlachostergios, D., 2019. The use of lupin as a source of protein in animal feeding: genomic tools and breeding approaches. Int. J. Mol. Sci. 20 (4), 851.

Anderson, P.C., McLean, A.E.M., 1989. Comfrey and liver damage. Hum. Toxicol. 8, 68–69.

Araújo, L.U., Grabe-Guimarães, A., Mosqueira, V.C.F., Carneiro, C.M., Silva-Barcellos, N.M., 2010. Profile of wound healing process induced by allantoin. Acta Cir. Bras. 25 (5), 460–461.

Avila, C., Breakspear, I., Hawrelak, J., Salmond, S., Evans, S., 2020. A systematic review and quality assessment of case reports of adverse events for borage (Borago officinalis), coltsfoot (Tussilago farfara) and comfrey (Symphytum officinale). Fitoterapia, 104519.

Awang, D.V., Dawson, B.A., Fillion, J., Girad, M., Kindack, D., 1993. Echimidine content of commercial comfrey (Symphytum spp.-Boraginaceae). J Herbs Spices Med Plants 2 (1), 21–34.

Bareeba, F.B., Odwongo, W.O., Mugerwa, J.S., 1992. The potential of Russian comfrey (*Symphytum officinale*) as an animal feedstuff in Uganda. In: Stares, J.E., Said, A.N., Kategile, J.K. (Eds.), The Complementarity of Feed Resources for Animal Production in Africa. Proceedings of the Joint Resources Networks Workshop Held in Gaborone, Botswana. African Feeds Research Network. ILCA (International Livestock Centre for Africa), Addis Ababa. Ethiopia (430pp).

Barna, M., Kucera, A., Hladícova, M., Kucera, M., 2007. Der wundheilende Effekt einer Symphytum-Herba-Extrakt-Creme (Symphytum × uplandicum Nyman): Ergebnisse einer randomisierten, kontrollierten Doppelblindstudie. Wien Med Wochenschr 157 (21−22), 569–574.

Bee, G., Seewer Lötscher, G.J., Dufey, P.A., 1999. Effect of comfrey (Symphytum peregrinum) fed to pigs on meat quality traits. EAAP Publication No 100, Zürich, 25 August, AFO1 126.

Benedek, B., Ziegler, A., Ottersbach, P., 2010. Absence of mutagenic effects of a particular Symphytum officinale L. liquid extract in the bacterial reverse mutation assay. Phytother. Res. 24 (3), 466–468.

BfR Opinion No. 018/2013 of 5 July, 2013. Pyrrolizidine alkaloids in herbal teas and teas. latest accessed on 23 March 2020. https://www.bfr.bund.de/cm/349/pyrrolizidine-alkaloids-in-herbal-teas-and-teas.pdf.

Bourgon, R., Gentleman, R., Huber, W., 2010. Independent filtering increases detection power for high-throughput experiments. Proc. Natl. Acad. Sci. U. S. A. 107 (21), 9546–9551.

Brown, A.W., Stegelmeier, B.L., Colegate, S.M., Gardner, D.R., Panter, K.E., Knoppel, E.L., Hall, J.O., 2016. The comparative toxicity of a reduced, crude comfrey (Symphytum officinale) alkaloid extract and the pure, comfrey-derived pyrrolizidine alkaloids, lycopsamine and intermedine in chicks (Gallus gallus domesticus). J. Appl. Toxicol. 36 (5), 716–725.

Calik, A., Omara, I.I., White, M.B., Li, W., Dalloul, R.A., 2019. Effects of dietary direct fed microbial supplementation on performance, intestinal morphology and immune response of broiler chickens challenged with coccidiosis. Front Vet Sci 6, 463.

Campbell, B., Beare, D., Bennett, E., Hall-Spencer, J., Ingram, J., Jaramillo, F., Ortiz, R., Ramankutty, N., Sayer, J., Shindell, D., 2017. Agriculture production as a major driver of the Earth system exceeding planetary boundaries. Ecol. Soc. 22, 8.

Cobb-Vantress, 2018. Cobb500™ broiler performance & nutrition supplement. accessed on 23 March 2020. https://cobbstorage.blob.core.windows.net/guides/5a171aa0-6994-11e8-9f14-bdc382f8d47e.

Collette, L., Hodgkin, T., Kassam, A., Kenmore, P., Lipper, L., Nolte, C., Stamoulis, K., Steduto, P., 2011. Save and Grow. Food and Agriculture Organization of the United Nations, Rome, Italy Accessed on 23 March 2020. www.fao.org/docrep/014/i2215e/i2215e.pdf.

Couet, C.E., Crews, C., Hanley, A.B., 1996. Analysis, separation, and bioassay of pyrrolizidine alkaloids from comfrey (Symphytum officinale). Nat. Toxins 4 (4), 163–167.

Crenshaw, T.D., Peo Jr., E.R., Lewis, A.J., Moser, B.D., 1981. Bone strength as a trait for assessing mineralization in swine: a critical review of techniques involved. J. Anim. Sci. 53 (3), 827–835.

Culvenor, C.C.J., Clarke, M., Edgar, J.A., Frahn, J.L., Jago, M.V., Peterson, J.E., Smith, L.W., 1980. Structure and toxicity of the alkaloids of Russian comfrey (Symphytum x Uplandicum nyman), a medicinal herb and item of human diet. Experientia 36 (4), 377–379.

Frikeche, J., Couteau, C., Roussakis, C., Coiffard, L.J., 2015. Research on the immunosuppressive activity of ingredients contained in sunscreens. Arch. Dermatol. Res. 307 (3), 211–218.

Gálvez, F., Domínguez, R., Maggiolino, A., Pateiro, M., Carballo, J., De Palo, P., Barba, F.J., Lorenzo, J.M., 2020. Meat quality of commercial chickens reared in different production systems: industrial, range and organic. Ann. Anim. Sci. 20 (1), 263–285.

Grube, B., Grünwald, J., Krug, L., Staiger, C., 2007. Efficacy of a comfrey root (Symphyti offic. radix) extract ointment in the treatment of patients with painful osteoarthritis of the knee: results of a double-blind, randomised, bicenter, placebo-controlled trial. Phytomedicine 14 (1), 2–10.

Hadlich F, Reyer H, Oster M, Trakooljul N, Muráni E, Ponsuksili S, Wimmers K (2020). rePROBE: workflow for revised probe assignment and updated probe-set annotation in microarrays. bioRxiv. https://doi.org/https://doi.org/10.1101/2020.03.10.985119.

Heitman, H., Oyarzun, S., 1971. Comfrey as a feed for swine. Calif. Agric. 25 (1), 7–8.

Helvich, K., 1990. Official Methods of Analysis. 15th ed. Assoc Off Anal Chem, Washington, DC.

Jarvie, H.P., Sharpley, A.N., Flaten, D., Kleinman, P.J., Jenkins, A., Simmons, T., 2015. The pivotal role of phosphorus in a resilient water–energy–food security nexus. J. Environ. Qual. 44 (4), 1049–1062.

Jeurissen, S.H., Lewis, F., Mroz, Z., Rebel, J.M., 2002. Parameters and techniques to determine intestinal health of poultry as constituted by immunity, integrity, and functionality. Curr Issues Intest Microbiol 3 (1), 1–14.

Kanehisa, M., Goto, S., 2000. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 28 (1), 27–30.

Kauffmann, A., Gentleman, R., Huber, W., 2009. Arrayqualitymetrics—a bioconductor package for quality assessment of microarray data. Bioinformatics 25, 415–416.

Kim, H., D'Andrea, A.D., 2012. Regulation of DNA cross-link repair by the Fanconi anemia/BRCA pathway. Genes Dev. 26 (13), 1393–1408.

Kruse, L.H., Stegemann, T., Jensen-Kroll, J., Engelhardt, A., Wesseling, A.M., Lippert, A., Ludwig-Müller, J., Ober, D., 2019. Reduction of Pyrrolizidine alkaloid levels in comfrey (Symphytum officinale) hairy roots by RNAi silencing of Homospermidine synthase. Planta Med. 85 (14/15), 1177–1186.

Laudadio, V., Ceci, E., Tufarelli, V., 2011. Productive traits and meat fatty acid profile of broiler chickens fed diets containing micronized fava beans (*Vicia faba* L. var. minor) as the main protein source. J. Appl. Poult. Res. 20 (1), 12–20.

Lee, M.Y., Lee, N.H., Jung, D., Lee, J.A., Seo, C.S., Lee, H., Kim, J.H., Shin, H.K., 2010. Protective effects of allantoin against ovalbumin (OVA)-induced lung inflammation in a murine model of asthma. Int. Immunopharmacol. 10 (4), 474–480.
Mewis, J.L., Sun, X., Zuidhof, M.J., Guan, L.L., 2014. Research note: methodology for high-quality RNA extraction from poultry whole blood for further gene expression analysis. Br. Poult. Sci. 55 (2), 194–196.
National Research Council, 1994. Nutrient Requirements of Poultry. Ninth Revised edition. The National Academies Press, Washington, DC.
Olukosi, O.A., Walker, R.L., Houdijk, J.G., 2019. Evaluation of the nutritive value of legume alternatives to soybean meal for broiler chickens. Poult. Sci. 98 (11), 5778–5788.
Onsongo, V.O., Osuga, I.M., Gachuiri, C.K., Wachira, A.M., Miano, D.M., Tanga, C.M., Ekesi, S., Nakimbugwe, D., Fiaboe, K.K.M., 2018. Insects for income generation through animal feed: effect of dietary replacement of soybean and fish meal with black soldier fly meal on broiler growth and economic performance. J. Econ. Entomol. 111 (4), 1966–1973.
Pereira, P.M.D.C.C., Vicente, A.F.D.R.B., 2013. Meat nutritional composition and nutritive role in the human diet. Meat Sci. 93 (3), 586–592.
Petracci, M., Betti, M., Bianchi, M., Cavani, C., 2004. Color variation and characterization of broiler breast meat during processing in Italy. Poult. Sci. 83 (12), 2086–2092.
Prakash, A.S., Pereira, T.N., Reilly, P.E., Seawright, A.A., 1999. Pyrrolizidine alkaloids in human diet. Mutat. Res. 443 (1–2), 53–67.
Ravindran, V., 2013. Poultry feed availability and nutrition in developing countries. Poultry Development Review. Food and Agriculture Organization of the United Nations, Rome, Italy , pp. 60–63 Accessed on 23 March 2020. http://www.fao.org/3/a-i3531e.pdf#page=66.
Ritchie, M.E., Phipson, B., Wu, D.I., Hu, Y., Law, C.W., Shi, W., Smyth, G.K., 2015. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 43 (7), e47.
Robinson, R.G., 1983. Comfrey—A Controversial Crop; University of Minnesota. Agricultural Experiment Station, St. Paul, MN, USA.
Rode, D., 2002. Comfrey toxicity revisited. Trends Pharmacol. Sci. 23 (11), 497–499.
Rohart, F., Gautie, B., Singh, A., Lê Cao, K.A., 2017. mixOmics: an R package for 'omics feature selection and multiple data integration. PLoS Comput. Biol. 13 (11), e1005752.
Santin, E., Maiorka, A., Macari, M., Grecco, M., Sanchez, J.C., Okada, T.M., Myasaka, A.M., 2001. Performance and intestinal mucosa development of broiler chickens fed diets containing Saccharomyces cerevisiae cell wall. J. Appl. Poult. Res. 10 (3), 236–244.
Sauvant, D., Perez, J.M., Tran, G. (Eds.), 2004. Tables of Composition and Nutritional Value of Feed Materials: Pigs, Poultry, Cattle, Sheep, Goats, Rabbits, Horses and Fish. Wageningen Academic Publishers.
Savić, V.L., Nikolić, V.D., Arsić, I.A., Stanojević, L.P., Najman, S.J., Stojanović, S., Mladenović-Ranisavljević, I.I., 2015. Comparative study of the biological activity of allantoin and aqueous extract of the comfrey root. Phytother. Res. 29 (8), 1117–1122.
Smith, D.B., Jacobson, B.H., 2011. Effect of a blend of comfrey root extract (Symphytum officinale L.) and tannic acid creams in the treatment of osteoarthritis of the knee: randomized, placebo-controlled, double-blind, multiclinical trials. J Chiropr Med 10 (3), 147–156.
Spranghers, T., Ottoboni, M., Klootwijk, C., Ovyn, A., Deboosere, S., De Meulenaer, B., Michiels, J., Eackhout, M., De Clercq, P., De Smet, S., 2017. Nutritional composition of black soldier fly (Hermetia illucens) prepupae reared on different organic waste substrates. J. Sci. Food Agric. 97 (8), 2594–2600.
Staiger, C., 2012. Comfrey: a clinical overview. Phytother. Res. 26 (10), 1441–1448.
Stickel, F., Seitz, H.K., 2000. The efficacy and safety of comfrey. Public Health Nutr. 3 (4a), 501–508.
Storey, J.D., Tibshirani, R., 2003. Statistical significance for genomewide studies. Proc. Natl. Acad. Sci. U. S. A. 100 (16), 9440–9445.
Storey, J.D., Bass, A.J., Dabney, A., Robinson, D., 2019. qvalue: Q-value estimation for false discovery rate control. R package version 2.18.0. Available online. http://github.com/jdstorey/qvalue.
Stucki, K., Dal Cero, M., Vogl, C.R., Ivemeyer, S., Meier, B., Maeschli, A., Hamburger, M., Walkenhorst, M., 2019. Ethnoveterinary contemporary knowledge of farmers in pre-alpine and alpine regions of the Swiss cantons of Bern and Lucerne compared to ancient and recent literature–is there a tradition? J. Ethnopharmacol. 234, 225–244.
Tóth, G., Guicharnaud, R.A., Tóth, B., Hermann, T., 2014. Phosphorus levels in croplands of the European Union with implications for P fertilizer use. Eur. J. Agron. 55, 42–52.
Toyomizu, M., Sato, K., Taroda, H., Kato, T., Akiba, Y., 2001. Effects of dietary Spirulina on meat colour in muscle of broiler chickens. Br. Poult. Sci. 42 (2), 197–202.
Van der Heide, E.M.M., Lourenco, D.A.L., Chen, C.Y., Herring, W.O., Sapp, R.L., Moser, D.W., Tsuruta, S., Masuda, Y., Ducro, B.J., Misztal, I., 2016. Sexual dimorphism in livestock species selected for economically important traits. J. Anim. Sci. 94 (9), 3684–3692.
Vogl, C.R., Vogl-Lukasser, B., Walkenhorst, M., 2016. Local knowledge held by farmers in Eastern Tyrol (Austria) about the use of plants to maintain and improve animal health and welfare. J Ethnobiology Ethnomedicine 12 (1), 40.
Xie, C., Mao, X., Huang, J., Ding, Y., Wu, J., Dong, S., Kong, L., Gao, G., Li, C.Y., Wei, L., 2011. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res. 39 (suppl_2), W316–W322.