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Effects of a riboflavin source suitable for use in organic broiler diets on performance traits and health indicators

C. Lambertz[1†], J. Leopold[1], K. Damme[2], W. Vogt-Kaute[3], S. Ammer[4a] and F. Leiber[4]

[1]*Research Institute of Organic Agriculture (FiBL), Kasseler Strasse 1a, 60486 Frankfurt am Main, Germany;* [2]*Poultry Competence Centre of the Bavarian Institute for Agriculture, Mainbernheimer Strasse 101, 97318 Kitzingen, Germany;* [3]*Naturland e.V., Kleinhaderner Weg 1, 82166 Gräfelfing, Germany;* [4]*Research Institute of Organic Agriculture (FiBL), Ackerstrasse 113, 5070 Frick, Switzerland*

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*Riboflavin (vitamin B_2) is essential for monogastric animals. It is mainly produced by recombinant microorganisms (*Candida famata, Bacillus subtilis *and* Ashbya gossypii *). The availability of genetically modified organism (**GMO**)-free riboflavin, obligatory in European organic agriculture, is a major issue. Besides, requirements for organic livestock might differ from conventional production because other genotypes and feed formulations are used. The effects of a fermentation suspension with a high native content of riboflavin produced with unmodified* A. gossypii *by fermentation were investigated at graded dosages as an alternative to conventional (GMO-based) riboflavin in slow-growing broilers on performance traits and health and welfare indicators. In 2 runs with 800 animals each, Ranger Gold™ broilers were fed with 4 dietary treatments. For starter diets (day 1 to 18), treatments included a basal diet (1) without any riboflavin supplementation (negative control, **N-C**), (2) with conventional riboflavin supplementation (Cuxavit B2 80% riboflavin) at 9.6 mg/kg (positive control, **P-C**), (3) with riboflavin supplementation from the alternative source at 3.5 mg/kg (**A-low**) and (4) with riboflavin supplementation from the alternative source at 9.6 mg/kg (**A-high**). For the finisher diet (day 29 until slaughtering), P-C and A-high were supplemented with 8.0 mg/kg and A-low with 3.5 mg/kg. Diets were formulated according to organic regulations. Animals were kept in floor pens with 20 chickens per pen. Weekly, BW, feed and water consumption were recorded. Every second week, animal-based health and welfare indicators (feather score and footpad dermatitis) were scored. Slaughter traits were assessed for five males and females per pen at 62/63 days of age. Final body weight of A-high differed from N-C and A-low, but not from P-C. From week 2 until six years of age, A-high had a higher daily weight gain when compared to all other groups. With 74.4%, dressing percentage was higher in A-high compared with all other groups (73.3%). Breast percentage of A-low was lower than that of both control groups but did not differ from A-high. The highest frequency of liver scores indicating fatty liver syndrome was found in P-C, followed by N-C and A-low. Feather scores did not respond to treatment; the highest frequency of mild footpad dermatitis was observed in A-high, however at a low prevalence. In conclusion, the tested fermentation suspension with a high native content of riboflavin derived from fermentation of* A. gossypii *can be used at levels of commercial recommendations as alternative to riboflavin produced from GMO in broiler feeding. Further studies must verify whether riboflavin can be reduced without inducing riboflavin deficiency in slow-growing broilers.*

Keywords: vitamin B_2, chicken, fattening, supplementation, organic

Implications

Currently, riboflavin (vitamin B_2) for use as feed additive in monogastrics is produced by genetically modified micro-organisms. This bears the risk of the spread of antibiotic resistant genes, and alternative sources of this vitamin from non-genetically modified microorganism sources are needed. Findings of the present study, using fermentation suspension with a high native content of riboflavin produced from *Ashbya gossypii* without genetic modifications, proved its feasibility to be used in broiler diets when supplemented at commercial levels.

Introduction

Riboflavin (vitamin B_2) is an important co-factor for multiple flavin enzymes. Deficiency in broilers results in decreased growth and diminished appetite as a consequence of mucosa

[a]Present address: Department of Animal Sciences, Georg-August-University, 37075 Göttingen, Germany.
† E-mail: christian.lambertz@fibl.org

inflammations and epithelial irritations (Ogunmodede, 1977; Chung and Baker, 1990). Curled-toe paralysis is also a frequently observed clinical symptom in riboflavin-deficient chicken (Wyatt *et al*., 1973; Johnson and Storts, 1988). Further relevant symptoms in birds lacking riboflavin are nervous malformations (Cai *et al*., 2006) and footpad dermatitis (Shepherd and Fairchild, 2010). Symptoms of riboflavin deficiency are therefore relevant in terms of animal welfare and economy of poultry farmers. Broiler chickens are most sensible to riboflavin deficiency in the period between 7 and 14 days of age (Olkowski and Classen, 1998). Deyhim *et al*. (1992) reported the NRC recommendation to be satisfactory when growing broilers are fattened for 4 weeks but found benefits of greater riboflavin supplementation when animals were raised for 8 weeks. Native contents of feed components used in broiler diets are variable, and only a few of these contain enough riboflavin to meet the requirements of young growing chicken (NRC, 1994; McDowell, 2008). Highly variable riboflavin contents in organically grown cereals (0.62 to 1.58 mg/kg) and legumes (1.00 to 3.84 mg/kg) are reported, which in turn are not able to meet requirements in diets without further vitamin supplementation or use of feedstuffs other than grains and legumes (Witten and Aulrich, 2018 and 2019). Currently, it is unknown to which extent a sufficient supply is reached natively in organic diets, which usually constitute of more components than conventional diets. Furthermore, it is unknown whether the different growth performance of slower- and faster-growing broiler genotypes may affect riboflavin requirements, given that there is still no common definition in the EU of the maximum daily weight gain (**DWG**) of slow-growing genotypes. Concluding, vitamin requirements in organic livestock production may differ from those in conventional systems, but no specific data are currently available.

A second aspect of this issue is the source of riboflavin for supplementation: at industrial scale, riboflavin is manufactured by microorganisms like *Bacillus subtilis*, *A. gossypii* and *Candida famata* (Stahmann *et al*., 2000). In many of the production strains, genetic modifications were applied to increase exploitation potential (Stahmann *et al*., 2000). These have replaced synthetic production (Revuelta, 2017). Genetic modifications in *A. gossypii*, for example, were also used to produce recombinant proteins, microbial lipids, nucleosides and aroma alcohols (Aguiar *et al*., 2017). With these modifications, antimicrobial resistance genes are introduced into the production strains. Taking the example of *B. subtilis* strain KKCM-10445, the European Food Safety Authority recently pointed out the risk for the spread of antimicrobial resistance when using riboflavin from this source as feed additive (Rychen *et al*., 2018). In organic production, the use of vitamins produced with the help of genetic modifications is not allowed (EC, 2007 and 2008), making the availability of GMO-free vitamins a highly important issue of livestock feed security in this sector. With optimized production strains and fermentation conditions, *A. gossypii* is feasible for GMO-free riboflavin manufacturing.

On this background, it was the aim of this study to investigate the utility of a fermentation suspension with a high native content of riboflavin produced with *A. gossypii* by fermentation without genetic modification at graded dosages as alternative to riboflavin produced from GMO in slow-growing broilers on performance and health traits. Further, the inclusion of a negative control (**N-C**) without supplemented riboflavin in the experiment aimed at investigating whether or not a typical organic broiler diet would cause deficiency symptoms if this vitamin is not supplemented.

Materials and methods

Birds and housing

The study was conducted at the Poultry Competence Centre of the Bavarian Institute for Agriculture, Germany, from April to October 2018.

In 2 runs with 800 animals each, Ranger Gold™ (Aviagen Epi GmbH, Cuxhaven, Germany) mixed-sex broilers were raised under conventional floor husbandry conditions. Day-old chicks were randomly allocated to 40 groups of 20 birds per group at a stocking density of 4 animals/m^2. Chickens were hatched from parent stock kept at the institute (average weight at hatch = 39 g). Straw granulate (1 kg/m^2) was used as litter and feed, and water was available *ad libitum*. The lighting scheme was as follows: day 1–3: 23 h light, 1 h dark; day 4 until 3 days prior to slaughtering: 18 h light, 6 h dark; last 3 days prior to slaughtering: 23 h light, 1 h dark. At the first day of life, animals were vaccinated against infectious bronchitis and at day 16 against infectious bronchitis and Newcastle's disease and received a combination of vitamins A, D_3 and E via drinking water.

Diets

The basal diet was an organic diet formulation produced according to current nutritional recommendations of NRC (1994) by an organically certified feed mill (Kaisermühle Gänheim Otmar Kaiser GmbH, Arnstein-Gänheim, Germany). The premix was formulated without riboflavin supplementation. The riboflavin content of single feed components was analyzed by HPLC (method DIN EN 14152:2006) and calculated as riboflavin content (Table 1). Experimental diets were prepared by adding riboflavin from a commercially available GMO-based and an alternative non-GMO source. The conventional riboflavin source was Cuxavit B_2 80% included as spray-dried powder containing 80% riboflavin (Kaesler Nutrition GmbH, Cuxhaven, Germany). The alternative riboflavin source was a suspension derived from fermentation of non-genetically modified *A. gossypii*. This aseptic fermentation process was conducted by the company AGRANO GmbH & Co. KG (Riegel am Kaiserstuhl, Germany). In detail, an *A. gossypii* strain from a publicly available collection was used and fermentation was carried out using a substrate of organically certified sugar, oil and nitrogen. The final product was not purified. It was pasteurized at 65°C for 1 h. It is registered in the EU Feed Materials

Table 1 *Riboflavin content of the feed components and composition of the starter and finisher diets of broilers*

Component	DM (%)	Riboflavin (mg/kg OM)	Starter diet (%)	Finisher diet (%)
Maize (whole grain)	86.8	0.99	15.0	15.0
Wheat (whole grain)	86.9	0.80	16.4	11.0
Wheat (powder)	85.9	0.76	–	7.7
Triticale (whole grain)	86.3	0.89	6.0	18.0
Peas (whole bean)	85.7	1.57	12.0	12.0
Soy (whole beans)	93.8	2.47	1.8	–
Soy cake (pellet)	92.3	2.95	13.9	13.4
Wheat gluten feed (pellet)	92.2	3.07	8.7	–
Wheat bran	86.1	2.00	8.0	6.0
Rapeseed cake (pellet)	93.1	2.86	–	4.0
Maize gluten feed (pellet)	92.4	2.67	4.6	4.6
Linseed cake (pellet)	91.4	1.91	4.0	–
Sesame cake (pellet)	91.6	4.21	3.0	–
Beer yeast (powder)	94.2	20.40	2.5	–
Grass meal (powder)	90.2	8.81	–	4.0
Sunflower oil	99.9	[2]	0.5	–
Premix[1]	96.2	[2]	0.6	0.5
$CaCO_3$	[3]	[3]	1.8	2.0
Monocalcium phosphate	[3]	[3]	0.8	1.2
Diatomaceous earth	[3]	[3]	0.3	0.3
Salt	[3]	[3]	0.1	0.2

OM = organic matter.
[1]The premix was prepared by the company Miavit GmbH (Essen, Germany) without riboflavin supplementation.
[2]Below the lower limit of detection.
[3]Not analyzed.

Register (08290-EN) and is certified organic. It proved to be stable at temperatures between 2°C and 8°C for at least 6 months and can be stabilized using formic acid for storage at ambient temperature.

From day 1 to 28, a starter diet and, from day 29 until slaughtering, a finisher diet were offered. For starter diets, treatments evaluated included a basal diet (1) without riboflavin supplementation (N-C), (2) with conventional riboflavin supplementation at 9.6 mg riboflavin/kg (positive control, **P-C**), (3) with riboflavin supplementation from the alternative source at 3.5 mg/kg (**A-low**) and (4) with riboflavin supplementation from the alternative source at 9.6 mg/kg (**A-high**). For the finisher diet, P-C and A-high were supplemented with 8.0 mg/kg and A-low with 3.5 mg/kg. The composition of starter and finisher basal diets is presented in Table 1. Diets were allocated randomly to one of the 40 groups (10 replications per treatment with a total of 200 animals per run).

Representative samples of starter and finisher diets were analyzed for nutrient contents (Method VO(EG) 152/2009) and riboflavin content as described above by LUFA-ITL GmbH (Kiel, Deutschland) (Table 2).

Data collection

Individual body weight was recorded weekly starting from the first day of age until slaughtering at 62 and 63 days of age when an average final BW of more than 2 kg was reached. With these data, DWG was calculated at pen level. Feed consumption was measured at weekly intervals at pen level by subtracting feed residuals from offered feed amounts. Each pen was equipped with a recorder that measured water consumption at pen level. At days 14, 28, 42 and 56, plumage cleanliness (score 0 = clean to 3 = heavily soiled) and foot pad dermatitis (score 0 = no foot pad dermatitis to 2 = severe foot pad dermatitis) and hock burn (score 0 = no evidence of hock burn to score 2 = evidence of hock burn) were scored for 5 to 10 animals/pen according to the Welfare Quality® Assessment protocol for poultry (Welfare Quality®, 2009). The number of diseased and treated animals was recorded.

At 62 and 63 days of age, animals were slaughtered after electrical stunning. Per run, 200 broilers, namely, 5 randomly selected males and 5 females of each pen, were subjected to assessment of slaughter traits. For these animals, dressing percentage and weights of abdominal fat, liver, heart and gizzard were recorded. In addition, liver color (score 0 = dark red to score 2 = yellowish, disrupted structure) was scored in run 2.

With the data collected, the European broiler index (**EBI**) was calculated according to Marcu *et al.* (2013):

$$\text{DWG (g)} \times \text{survival rate (\%)/feed conversion (kg feed/kg BW gain)} \times 10$$

Table 2 *Composition of the dietary starter and finisher treatments of broilers and the riboflavin suspension*

Item	Dietary treatments – starter diet[1]				Dietary treatments – finisher diet[2]				Riboflavin suspension
	N-C	P-C	A-low	A-high	N-C	P-C	A-low	A-high	
DM (g/kg)	885	882	878	877	875	874	872	865	54
Riboflavin (mg/kg OM)	3.00	9.36	5.51	11.40	1.99	9.15	5.43	11.00	741.00
Crude protein (g/kg of OM)	215	222	216	227	187	181	182	178	17
Ether extract (g/kg of OM)	57	56	57	60	42	40	42	40	18
Crude fiber (g/kg of OM)	33	41	44	42	43	41	41	40	<3
Saccharose (g/kg of OM)	38	38	37	40	40	40	43	40	<10
Starch (g/kg of OM)	346	341	340	329	398	399	402	404	<10
Nitrogen-free extracts (g/kg of OM)	510	499	498	477	533	542	537	541	11
Crude ash (g/kg of OM)	70	64	63	71	70	70	70	66	8
ME (MJ/kg of OM)	11.6	11.5	11.5	11.6	11.5	11.4	11.5	11.4	0.9
Amino acids									
Lysine (g/kg of OM)	9.3	9.4	9.2	9.8	8.2	8.4	8.3	7.9	0.6
Methionine (g/kg of OM)	3.4	3.5	3.4	3.6	2.9	2.9	2.8	2.8	<0.5
Cysteine (g/kg of OM)	3.8	3.6	3.8	3.9	3.2	3.3	3.1	3.1	<0.5
Threonine (g/kg of OM)	7.7	7.6	7.6	8.1	6.7	6.7	6.6	6.4	<0.5

OM = organic matter; ME = metabolizable energy.
[1]N-C, without riboflavin supplementation; P-C, conventional riboflavin supplementation at 9.6 mg/kg; A-low, riboflavin supplementation from alternative source at 3.5 mg/kg; A-high, riboflavin supplementation from alternative source at 9.6 mg/kg.
[2]N-C, without riboflavin supplementation; P-C, conventional riboflavin supplementation at 8.0 mg riboflavin/kg; A-low, riboflavin supplementation from alternative source at 3.5 mg/kg; A-high, riboflavin supplementation from alternative source at 8.0 mg riboflavin/kg.

and income over feed costs (**IOFC**) as:

$$\text{Body weight (kg)} \times 2.65\ € - \text{feed consumption (kg)} \times 0.56\ €.$$

For the economic evaluation of the alternative riboflavin source in comparison to both control diets, IOFC was corrected for estimated riboflavin costs. For this estimation, product costs for the alternative riboflavin source were set at 2.0 €/kg at a riboflavin concentration of 700 mg/kg. For the conventional riboflavin source of treatment P-C, product costs were set at 42.50 €/kg (Cuxavit B_2 80%).

Uniformity was calculated using individual BWs at the end of the fattening period.

Statistical analysis

The statistical analysis was conducted with the software package SAS, version 9.3 (Statistical Analysis Systems, Cary, NC, USA) with α set at 0.05. In the ANOVA (GLM procedure), the treatment group and the run as well as its interaction served as fixed effects. Pen was considered as the statistical unit. Schematic box plots and proc univariate were used to test normal distribution of data. Performance and slaughter traits that fulfilled ANOVA requirements were analyzed with the following statistical model:

$$y = \mu + T_i + R_j + T_i * R_j + e_{ijk}$$

where T is the treatment (1,2,3,4) and R is the run (1,2). As the interaction term did not show a significant effect on any of the included traits, it was deleted from the final model.

For data of footpad dermatitis, feather condition, hock burn and liver color scoring a χ^2 test was applied to determine differences between dietary treatments with α set at 0.05.

Results

Performance traits

Animals receiving the A-high diet showed a higher final BW than animals fed either without riboflavin supplementation (N-C) or with riboflavin from the alternative source at low level (A-low) ($P < 0.05$; Table 3). When supplemented at the higher dosage (A-high and P-C), no difference between riboflavin sources was detected ($P > 0.05$). Similarly, DWG of A-high and P-C with 36.1 and 35.3 g did not differ from each other ($P > 0.05$) but was higher when compared to both diets with lower riboflavin supplementation ($P < 0.05$). Details on the development of DWGs during the fattening period are presented in Table 4. In the first week of age, A-high but not P-C showed higher DWG than the un-supplemented N-C group ($P < 0.05$). In subsequent weeks until the sixth week of age, A-high had greater DWGs than all other treatments ($P < 0.05$). From the seventh week on, treatment differences were not observed ($P > 0.05$). Uniformity at the end of the fattening period of run 1 was 40.8%, 49.5%, 42.4% and 46.3% for N-C, P-C, A-low and A-high, respectively. Respective values for the second run were 40.7%, 44.1%, 46.2% and 44.1%.

Animals of groups A-high and P-C consumed more feed than both other groups ($P < 0.05$; Table 3). In contrast, feed conversion rate did not differ at the end of the fattening period ($P > 0.05$). Water consumption of A-high and P-C

Table 3 *Performance traits of broilers fed with a basal diet without riboflavin supplementation (N-C), with riboflavin supplementation from a conventional source (P-C) or from an alternative source at two different levels (A-low and A-high) (LS means,* n = *400 animals per treatment, 2 runs)*

Item	Dietary treatment				SE
	N-C	P-C	A-low	A-high	
Final BW (g)	2173^{bc}	2246^{ab}	2150^{c}	2299^{a}	32.8
Daily weight gain (g)	34.1^{bc}	35.3^{ab}	33.8^{c}	36.1^{a}	0.5
Feed consumption (g/animal)	5101^{c}	5256^{ab}	5018^{c}	5430^{a}	82.0
Feed conversion rate (kg feed/kg gain)	2.40	2.38	2.38	2.41	0.02
Water consumption (ml)	9971^{bc}	10 151^{ab}	9748^{c}	10 441^{a}	138
Water : feed ratio (ml/g)	1.96	1.94	1.95	1.93	0.03
Mortality (%)	2.09	4.20	5.24	4.38	1.41
Mortality day 0 to 7 (%)	1.31	1.84	3.45	1.87	1.25
EBI (points)	143^{ab}	145^{ab}	137^{b}	147^{a}	3
IOFC (€/animal)	2.58	2.67	2.56	2.71	0.05
IOFC corrected for additional riboflavin costs (€/animal)	2.58	2.67	2.51	2.58	0.05

N-C = without riboflavin supplementation; P-C = conventional riboflavin supplementation at 9.6 mg/kg in starter and 8.0 mg/kg in finisher diet; A-low = riboflavin supplementation from alternative source at 3.5 mg/kg in starter and finisher diet; A-high = riboflavin supplementation from alternative source at 9.6 mg/kg in starter and 8.0 mg/kg in finisher diet; SE = standard error; EBI = European broiler index: daily weight gain (g) × survival rate (%)/feed conversion (kg feed/kg BW gain) × 10; IOFC = income over feed costs: BW (kg) × 2.65 € − feed consumption (kg) × 0.56 €.
[a,b,c]Different superscripts within rows indicate significant differences between treatments at $P < 0.05$.

Table 4 *Development of the daily weight gain (g/d) from week 1 to 9 of broilers fed with a basal diet without riboflavin supplementation (N-C), with riboflavin supplementation from a conventional source (P-C) or from an alternative source at two different levels (A-low and A-high) (LS means,* n = *400 animals per treatment, 2 runs)*

Week	Dietary treatment				SE
	N-C	P-C	A-low	A-high	
1	6.2^{bc}	6.6^{ab}	6.1^{c}	6.7^{a}	0.2
2	11.3^{b}	11.6^{b}	10.9^{b}	12.4^{a}	0.3
3	17.1^{b}	17.9^{b}	17.2^{b}	19.8^{a}	0.5
4	27.0^{b}	27.4^{b}	26.0^{b}	30.3^{a}	0.7
5	34.5^{b}	36.3^{b}	33.6^{b}	39.8^{a}	1.2
6	46.3^{b}	45.3^{b}	45.1^{b}	52.1^{a}	1.9
7	48.1	45.9	43.3	46.7	2.9
8	60.5	65.5	65.8	62.1	2.8
9	58.1	63.6	57.9	56.9	3.3

N-C = without riboflavin supplementation; P-C = conventional riboflavin supplementation at 9.6 mg/kg in starter and 8.0 mg/kg in finisher diet; A-low = riboflavin supplementation from alternative source at 3.5 mg/kg in starter and finisher diet; A-high = riboflavin supplementation from alternative source at 9.6 mg/kg in starter and 8.0 mg/kg in finisher diet; SE = standard error.
[a,b,c]Different superscripts within rows indicate significant differences between treatments at $P < 0.05$.

was greater than that of A-low, and that of A-high also differed to N-C ($P < 0.05$). Water : feed ratio, however, varied in a small range between 1.93 and 1.96 ml/g ($P > 0.05$). Mortality ranged between 2.09% and 5.24% without differences ($P > 0.05$). In all groups, more than 40% of the total mortality rate occurred during the first week of age. EBI only differed between A-low and A-high ($P > 0.05$). Numerically A-high (2.71 €/animal) and P-C (2.67 €/animal) had greater IOFC than N-C (2.58 €/animal) and A-low (2.56 €/animal) ($P > 0.05$). When corrected for the additional riboflavin costs of the alternative riboflavin source, which was calculated at 0.1287 €/animal for the higher dosage, IOFC of A-low decreased to 2.51 €/animal and A-high to 2.58 €/animal ($P > 0.05$).

Slaughter traits

Due to random selection, live and slaughter weight of the animals selected for the assessment of slaughter traits differed from that presented above. In the sub-sample of 100 animals per treatment, A-low weights were lower compared to all other groups ($P < 0.05$; Table 5). With 74.4%, dressing was higher in A-high compared with all other groups (73.3%; $P < 0.05$). Breast percentage of A-low was lower than that of both control groups ($P < 0.05$) but did not differ to A-high ($P > 0.05$). For the ratio of thigh, wings and carcass, no difference between treatments was found ($P > 0.05$). With 2.01%, A-high animals had higher percentages of abdominal fat than P-C and A-low ($P < 0.05$). N-C and A-high showed a greater liver ratio than both other groups ($P < 0.05$). The highest frequency of livers scored as 2, indicating fatty liver syndrome, was found in P-C, followed by N-C and A-low ($P < 0.01$, Table 6). Groups did not differ in heart percentage ($P > 0.05$, Table 5). Relative to slaughter weight, A-high had lower gizzard weights than the other treatments ($P < 0.05$).

Health and welfare traits

Severe foot pad dermatitis (score 2) was not recorded, and more than 90% of the animals in all groups did not show foot pad dermatitis (Table 6). Given the low prevalence, A-high had a greater prevalence of mild dermatitis (8%) than all other treatments ($P < 0.01$).

Table 5 *Slaughter traits of broilers fed with a basal diet without riboflavin supplementation (N-C), with riboflavin supplementation from a conventional source (P-C) or from an alternative source at two different levels (A-low and A-high) slaughtered at 62/63 days of age (LS means;* n = *100 animals per treatment, 2 runs)*

	Dietary treatment				
Item	N-C	P-C	A-low	A-high	SE
Final BW at day 56 (g)	2315[a]	2305[a]	2175[b]	2316[a]	29.4
Slaughter weight (SW, g)	1694[a]	1691[a]	1595[b]	1725[a]	24.0
Dressing (%)	73.3[b]	73.3[b]	73.3[b]	74.4[a]	0.3
Breast (% of SW)	23.7[a]	23.6[a]	22.9[b]	23.4[ab]	0.2
Thigh (% of SW)	30.9	31.1	31.1	30.7	0.17
Wings (% of SW)	11.4	11.5	11.6	11.4	0.08
Carcass (% of SW[1])	28.3	28.0	28.3	28.3	0.17
Abdominal fat (% of SW)	1.85[ab]	1.77[b]	1.75[b]	2.01[a]	0.07
Liver (% of SW)	2.66[b]	2.79[a]	2.83[a]	2.67[b]	0.04
Heart (% of SW)	0.48	0.49	0.49	0.49	0.01
Gizzard (% of SW)	1.54[a]	1.63[a]	1.62[a]	1.43[b]	0.04

N-C = without riboflavin supplementation; P-C = conventional riboflavin supplementation at 9.6 mg/kg in starter and 8.0 mg/kg in finisher diet; A-low = riboflavin supplementation from alternative source at 3.5 mg/kg in starter and finisher diet; A-high = riboflavin supplementation from alternative source at 9.6 mg/kg in starter and 8.0 mg/kg in finisher diet; SE = standard error.
[1]Including neck.
[a,b]Different superscripts within rows indicate significant differences between treatments at $P < 0.05$.

Table 6 *χ^2 test of liver color and foot pad scoring at slaughter of broilers fed with a basal diet without riboflavin supplementation (N-C), with riboflavin supplementation from a conventional source (P-C) or from an alternative source at two different levels (A-low and A-high)*

	Dietary treatment			
Item	N-C	P-C	A-low	A-high
Liver color[1]	$n = 99$	$n = 99$	$n = 96$	$n = 97$
0	28	19	39	46
1	57	64	48	45
2	14	16	9	6
				$\chi^2 = 22.58$; $P < 0.01$
Food pad dermatitis[2]	$n = 198$	$n = 198$	$n = 192$	$n = 194$
0	191	192	186	178
1	7	6	6	16
2	0	0	0	0
				$\chi^2 = 8.65$; $P < 0.05$
Plumage cleanliness[3]	$n = 100$	$n = 100$	$n = 100$	$n = 100$
0	69	70	67	70
1	31	30	33	30
2	0	0	0	0
3	0	0	0	0
				$\chi^2 = 0.20$; $P > 0.05$

N-C = without riboflavin supplementation; P-C = conventional riboflavin supplementation at 9.6 mg/kg in starter and 8.0 mg/kg in finisher diet; A-low = riboflavin supplementation from alternative source at 3.5 mg/kg in starter and finisher diet; A-high = riboflavin supplementation from alternative source at 9.6 mg/kg in starter and 8.0 mg/kg in finisher diet.
[1]Score 0 = dark red to score 2 = yellowish, disrupted structure.
[2]Score 0 = no foot pad dermatitis to score 2 = severe foot pad dermatitis.
[3]Score 0 = clean to 3 = heavily soiled.

Regarding plumage cleanliness, scores 2 and 3 were not observed and differences between treatments were not found at neither of the assessments during the fattening period ($P > 0.05$).

Discussion

Owing to a lack of data, recommendations for vitamin supplementation of poultry cannot be factorially derived but are based on results of dose-response trials. Worldwide,

recommendations of the NRC (1994) are most widely used for conventional and organic production alike. Nevertheless, they are far from representing the current broiler production and need to be updated (Leeson, 2007). In practice, excessive safety margins surpass these thresholds as vitamin production using genetically modified bacteria, fungi and yeast strains is very efficient and cost-effective. Native vitamin contents of dietary ingredients are usually not considered when formulating diets, although they may differ considerably, depending on the components (NRC, 1994; Witten and Aulrich, 2018). Supplementations were also increased because of the immense development of performance of fast-growing broiler genotypes during the last decades (Siegel, 2014). For slower-growing broiler genotypes and organic production conditions, vitamin recommendations were not adjusted, yet.

In view of the need for GMO-free riboflavin sources as alternative to the use of genetically modified bacteria, fungi and yeast strains, which pose the risk for the spread of antimicrobial resistance (Rychen *et al.*, 2018), this study investigated riboflavin produced with *A. gossypii* by fermentation without genetic modification in slow-growing broilers. The riboflavin suspension from this newly developed fermentation process was offered at graded dosages and compared to a diet with native riboflavin contents only and a diet containing a conventional riboflavin product derived from the currently, most widely used *B. subtilis* strain KKCM-10445. We hypothesized that (1) the riboflavin source does neither affect performance nor health and welfare traits when supplemented at the same level and (2) an alteration of riboflavin supplementation will affect these traits.

The fermentation suspension as such did not contain nutrients in relevant quantities due to the high water content. Given the low supplementation amounts added to the basal diet, an effect on the nutrient composition can therefore be ignored. Nevertheless, it reduced the DM content. For practical use, supplementation in the form of the solution is applicable, though its addition as a dry product in premixes is preferable. Although the dietary treatments were prepared from one batch of basal diet, nutrient composition differed between treatments, especially in CP, CF, starch and ash content, which can only be explained by sampling and analysis bias. When interpreting results, it must be considered that diets were mixed under practical conditions in a commercial feed mill.

Native riboflavin contents amounted 3.05 and 1.99 mg/kg in the starter and finisher diet (N-C), respectively. These contents can be assumed to be higher than that in conventional diets and be referred to the use of different ingredients in the diets formulated according to organic regulations. In particular, beer yeast (20.40 mg/kg) in the starter diet and grass meal (8.81 mg/kg) in the finisher diet contributed native riboflavin. This in addition to the slow growth explains that N-C animals did not show any clinical signs of riboflavin deficiency or increased mortality rate when compared to riboflavin-supplemented diets.

In addition to performance, which can be considered as a very sensitive indicator of riboflavin deficiency (Wyatt *et al.*, 1973), the riboflavin content in brain, liver and heart tissues in relation to growth rate needs further investigation. Sampling these tissues, Olkowski and Classen (1998) detected an association of riboflavin supplementation and its status in tissues with the most pronounced effect found at 7 and 14 days of age. Feeding a basal diet with a native riboflavin content of 2.3 mg/kg, no clinical signs but few instances of mild ('leg weakness') deficiency were detected by Olkowski and Classen (1998). At 1.8 mg/kg of dietary riboflavin for broiler chicken, Cai *et al.* (2006) found malformation of nerves from the 11th day of life onward. However, slow-growing genotypes are less prone to neuropathies (Chung and Baker, 1990), so that clinical symptoms of riboflavin deficiency may be less prevalent than in fast-growing genotypes. Signs of suboptimal riboflavin supply may also be observed by animal-based welfare measures. For example, diminished feather condition or an increase of footpad dermatitis related to 'leg weakness' (Shepherd and Fairchild, 2010) may result from undersupply. However, although the native riboflavin supply, especially in the second phase, for treatment N-C of the present experiment was not far above the referred critical thresholds, the lack of differences between riboflavin dosages in terms of health and welfare traits (liver, feather and footpad scores) emphasizes that animals of group N-C were not riboflavin-deficient.

Riboflavin supplementation at 1 to 2 mg/kg can be sufficient for fast-growing broilers to reach their maximum weight gain (Olkowski and Classen, 1998). This contrasts findings from a tropical environment with lower growth rates, in which optimum growth was reached when 5.1 mg/kg of the vitamin was added (Ogunmodede, 1977). Riboflavin supplementation in the present study increased BW when supplemented at 10 mg/kg, but growth did generally not reach performance objectives of the breeding company, which is 48 g/day when slaughtered at 63 days of age (Aviagen, 2018). At this supplementation level, the effect of the alternative riboflavin source did not differ from the conventional one. This treatment differences in weight gain were due to differences in feed intake as feed conversion was not affected. In contrast, Olkowski and Classen (1998) did not find an effect of increasing riboflavin supplementation from 1 to 10 mg/kg on feed consumption. In view of the EBI that summarizes the main performance traits (DWG, feed conversion and mortality), findings reflected differences in final BW and the advantage of the higher compared with the lower dosage of the alternative riboflavin source. Modern broiler genotypes under conventional production conditions reach EBI values twice as large (Marcu *et al.*, 2013). In general, growth performance in the present study was comparable to weight gains reported by Rezaei *et al.* (2018) for the slow-growing broiler hybrid Rowan Ranger.

Results of the slaughter traits are biased by the fact that live and slaughter weights of the sub-sample subjected to slaughter assessment slightly differed from that of the study population. The superior dressing percentage of A-high compared with all other treatments was not related to greater proportions of valuable cuts. Riboflavin supplementation

at high dosages independent of the riboflavin source decreased liver proportions, while highest indications for fatty liver syndrome were only found when the conventional riboflavin was offered. The relationship between riboflavin source and effects on liver metabolism however needs further clarification. Determining the riboflavin status in different tissues may be the most promising way to validate thresholds for riboflavin deficiency apart from growth performance.

From an economic point of view, a reduction of riboflavin supplementation becomes relevant when the vitamin is not produced with GMOs but from fermentation processes that are much more cost intensive. With the development of the DWGs in mind, a reduction of riboflavin supplementation without negative effects on performance seems to be feasible in the latter fattening period. The first days and weeks of age need special attention, and undersupply during this period results in diminished growth and clinical signs of leg paralysis (Johnson and Storts, 1988; Cai *et al*., 2006).

In summary, the first hypothesis that the riboflavin source does neither affect performance nor health and welfare traits when supplemented at the same level than conventional sources was accepted based on the findings of the present study. The second hypothesis that a decrease of riboflavin supplementation affects these traits could neither be accepted nor rejected given the high native riboflavin concentrations and the low growth performance of the animals, which in turn did not induce riboflavin deficiency.

In conclusion, findings of the present study clearly indicate that the tested riboflavin derived from fermentation of *A. gossypii* can be used as alternative to riboflavin produced from GMO in broiler feeding. Findings are limited to the study conditions, in particular to slow-growing broilers and diets rich in native riboflavin. The supplementation at levels of commercial recommendations did neither affect performance nor health and welfare traits when compared with its commercially available counterpart. When used at lower dosage, growth was reduced as a result of decreased feed intake. Further studies are needed to verify whether riboflavin levels can be further reduced especially in finishing diets without inducing riboflavin deficiency in slow-growing broilers. This will reduce costs as alternative GMO-free riboflavin production is more cost-intensive than its production with GMO.

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C. Lambertz 0000-0001-7786-4163

Declaration of interest

The authors declare that they have no competing interests.

Ethics statement

Following the Guide for the Care and Use of Agricultural Animals in Research and Teaching, animals were treated in a way avoiding any unnecessary discomfort. Birds were observed daily for behavioural and clinical abnormalities.

Software and data repository resources

The model was not deposited in an official repository.

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