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Safety assessment of the functional feed additive phenylcapsaicin in a commercial broiler diet

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Abstract

Introduction: Intestinal colonisation of Salmonella is a major concern in the poultry industry, and a low dose of the high-purity synthetic capsaicin analogue phenylcapsaicin (PheCap) has the potential to be a phytobiotic alternative to antibiotics in reducing floor Salmonella in commercial broiler chicken houses. In this study we present the first safety assessment of PheCap at doses relevant for the poultry industry.

Methods: In a completely randomized block design, Ross 308 male broilers were offered feed containing 0, 10, 15, or 150 mg PheCap/kg. Growth rates, mortality, haematology, clinical chemistry, foot pad lesions, litter quality and gross pathological examination of organs and tissues were evaluated for signs of toxicity over a two-phase, 35-day growth period.

Results: No differences in feed intake and broiler growth were found, with broilers in the control group having the highest mortality. There was a statistically significant increase in the European Production Efficiency Factor (EPEF) for the 10 ($p = 0.02$) and 15 mg PheCap/kg feed ($p = 0.003$) treatment doses. No dose dependent adverse effects were found for any of the treatment doses. The No Observed Adverse Effect Level (NOAEL) of PheCap is probably higher than that of the highest weekly averaged daily intake of 36.3 mg/kg BW/day observed in the present study.

Conclusions: The inclusion of PheCap in broiler feed at doses relevant for the commercial poultry industry is assumed not have any negative effects on broiler health.

Keywords

High-purity phenylcapsaicin, growth performance, mortality, blood chemistry, footpad lesions, litter quality

Introduction

Capsaicin (8-methyl-*N*-vanillyl-6-nonemide) is a good candidate for a functional feed additive as it has been found to both increase broiler body weight (BW) and reduce gut *Salmonella enteritidis*, *Escherichia coli* and *Clostridium perfringens* in broiler chickens.[1–8] Capsaicin is the alkaloid giving chili its pungency. It is part of the fruit defense chemistry in members of the genus *Capsicum* (peppers) and ensures seed dispersal by birds by deterring mammalian fruit predators.[9–11] Capsaicin accounts for c. 48% of the active substances in capsaicinoids,[1,7] and the deterrent effect in mammals is caused by the mammalian vanilloid receptor TRPV1, which is able to detect capsaicin-like inflammatory substances.[12,13] However, high-purity capsaicin has not been available at the cost and volumes needed for the poultry industry due to the limited production of red peppers and challenges related to capsaicin purification. These challenges were solved by the development of the commercially available synthetic capsaicin analogue phenylcapsaicin (PheCap; 7-phenylhept-6-yne-acid-hydroxy-3-mathoxylbenzylamide,

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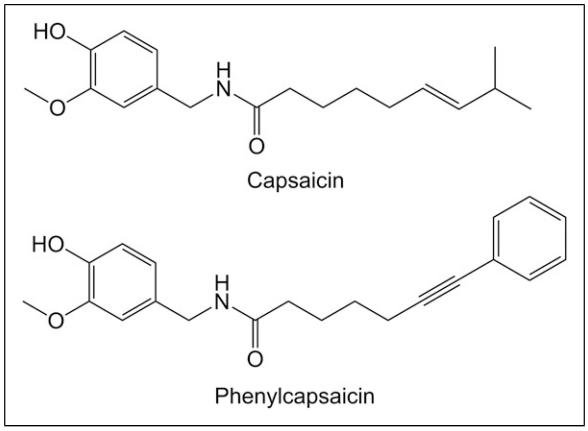

Figure 1. Chemical structure of capsaicin and phenylcapsaicin.

CAS no 848127-67-3) (aXichem AB, Malmö, Sweden), Figure 1.

Few studies on the performance and toxicological effects of high-purity capsaicin on poultry are available in the literature[14] as most studies examined the effect of capsaicinoid powder or capsicum oleoresin. The results from these studies might not be directly transferable to the effects of high-purity capsaicin and PheCap in poultry because of effects related to differences in active substances between pepper breeding lines, purity profiles, harvest time and drying methods.[15] For a comprehensive summary of the effect of hot red pepper on carcass traits, organ weights, blood parameters, antimicrobial and intestinal histomorphology, see Abd El-Hack ME et al.[1]

Nevertheless, the results from the few studies available seem to have reproduced results from studies using hot red peppers. In Arbor Acres broilers, using a purified natural capsaicin extract (2% capsaicin and 98% stearic acid as diluent), final feed concentrations of 2 and 4 mg capsaicin/kg feed were found to improve the feed to body weight ratio, breast meat quality, and small intestine, liver and immune organ development, and digestive enzyme activities. These effects were not found for 6 mg capsaicin/kg feed.[14] A concentration of 80 mg/kg feed improved meat quality, nutrient digestibility, growth performance as well as antioxidant status and immune function.[16] In female Longyan laying ducks, at a concentration of 150 mg/kg feed, egg production increased due to improved follicular growth and maturation, and higher antioxidant capacity was found.[17]

To the best of our knowledge, only a single study is available in the literature on the toxicity of high-purity PheCap. In Wistar rats, degenerative, but reversible changes in the liver at 250 mg/kg BW/day, and local irritating effects in the stomach at 100 and 250 mg PheCap/kg BW/day, were found in a 90-days repeated dose oral gavage study with a 28-days recovery period. In the same study, high-purity PheCap was non-mutagenic at concentrations up to 5000 µg in the tester stains *Salmonella typhimurium* TA 98, TA 100, TA 102, TA 1535, or TA 1537**,** using standard plate incubation and preincubation assay procedures with and without S9 activation. No biologically relevant increase in micronucleated cells was found in a human Lym micronucleus assay up to 130 µg/mL PheCap for short-term exposure without metabolic activation, 140 µg/mL for the short-term exposure with metabolic activation, and up to 20 µg/mL for the long-time exposure without metabolic activation.[18]

Here we present the first safety assessment of high-purity PheCap at doses of 10, 15, and 150 mg/kg feed in broiler chicken diets in a controlled laboratory experiment.

Materials and methods

The present study was carried out to provide an evaluation of the tolerance of PheCap (aXiphen-feed®, aXichem AB, Malmö, Sweden) over a period of 35 days in commercial male broilers. The broilers received a diet with inclusion of either 10, 15, or 150 mg PheCap/kg feed to be compared to a diet without PheCap (control). The 10 and 15 mg PheCap/kg feed concentrations were chosen based on findings from a full-scale farm pilot where 10 and 15 mg PheCap/kg feed showed significant reduction in floor *Salmonella*. In addition, 150 mg PheCap/kg feed was included as a tolerance level. A completely randomized block design comprising four treatments (0, 10, 15, 150 mg PheCap/kg feed) in two rooms was used. Within each room there were 16 pens (floor space: 2.15 m^2) in four blocks where each block consisted of 4 adjacent pens. Each treatment was replicated eight times and the experimental unit was a pen with 20 male broilers.

Study compliance

The experiment was conducted by Wageningen Livestock Research (ISO 9001:2015 certified) at the Research Facility Carus (Wageningen, The Netherlands) according to Animal and Human Welfare Codes and laboratory practice codes relevant in The Netherlands. The experimental protocol was approved by the Animal Welfare Body of Wageningen University (IvD-WU), Wageningen, The Netherlands (2019.D-0033.001). The study was carried out according to the EFSA guidelines for the assessment of the safety of feed additives for the target species.[19] Permission number 2020428 was granted by the Ministry of Agriculture, Nature and Food Quality, The Netherlands, prior to the study for the preparation, availability or stocking, delivery, transport and feeding of test feed and the product Phenylcapsaicin incorporated therein in accordance with Regulation EC 1831/2003.

Test substance

A two-phase starter and grower diet program were provided from 0-14 and 14-35 days of age, respectively. The experimental diets were formulated by Wageningen Livestock Research and

produced by Research Diet Services (Wijk bij Duurstede, The Netherlands). The diets were formulated to meet or exceed the CVB recommendation for Ross 308 broiler chickens[20] and were prepared without inclusion of anticoccidial.

The synthetically produced natural capsaicin analog PheCap (CAS no 848127-67-3) was supplied as the commercial product aXiphen-feed® (lot no. 2936918, aXichem AB, Malmö, Sweeden) with 15 g/kg PheCap, 15 g/kg lecithins (emulsifier), 38.8 mg/kg E310 Propyl gallate and 3 mg/kg E320 Butylhydroxyanisol in 970 g/kg rapeseed oil carrier. The capsaicin analog PheCap was supplemented on top to the basal diets. For each feeding phase a basal diet was produced and split into four batches. No PheCap was supplemented to the first batch (control) and PheCap was supplemented on top to the second, third, and fourth batch according to the incicated PheCap concentrations of 10, 15, or 150 mg PheCap/kg feed. All diets were pelleted, and the starter and grower diets were pelletized by using a 2.5 mm and 3.2 mm die, respectively. Maximum pellet temperature was 70°C. The diets were bagged in bags of 20 kg and transported to the trial facility where they were stored at cool conditions prior to feeding. At the trial facility, diet bags were pooled to form a composite, representative sample for each treatment group. A 500 g subsample was sent to Agrolab LUFA (Kiel, Germany) for dry matter, ash, crude protein (6.25 x N), crude fat, crude fibre, phosphorus and calcium analyses and a 500 g subsample was sent to Q&Q labs (Mölndal, Sweden) to determine the realized concentrations of PheCap in the feeds. Ingredient composition and calculated nutrient contents are presented in Tables 1 and 2, respectively, and realized concentrations of PheCap were found to be 8.8, 13.8, and 143.3 mg/kg for the starter diet and 8.6, 13.4, and 140.0 mg/kg for the grower diet for the 10, 15, and 150 mg/kg feed treatment groups, respectively.

Oral toxicity

A total of 640 1-day-old male Ross 308 broilers were purchased from a commercial hatchery (Kuikenbroederij Morren B.V., Lunteren, The Netherlands) and allocated to the 32 floor pens according to a weight class system where the mean body weight of the 20 birds per pen had to be within 3% of the mean body weight of all birds. The pens were bedded with wood shavings (±3 kg/m^2) and provided with a perch, a feeding pan (Valenta, Ø 335 mm, VDL Agrotech BV, AW Eindhoven, The Netherlands) and six nipple drinkers with drip cups (Impex Barneveld BV, MA Barneveld, The Netherlands). Feed and water were provided *ad libitum*. The temperature at placement of the broilers was 33°C and the temperature was gradually decreased to 20°C at 28 days of age and retained until the end of the experiment. During the first 3 days, light was continuous (24L:0D) and thereafter a day/night schedule of 18 h light and

Table 1. Ingredient composition and nutrient content of starter (1-14 days) and grower (15-35 days) basal diets.

Item	1–14 days	14–35 days
Ingredients		
Wheat %	40.32	37.43
Corn %	20.00	25.00
Soya bean meal HP %	25.02	20.38
Sunflower seed meal %	2.98	7.50
Potato protein ash <10%	3.00	0.00
Soya oil %	4.25	3.21
Palm oil %	0.50	3.00
Limestone (fine) %	1.47	1.08
Monocalcium phosphate %	0.94	0.43
Salt %	0.15	0.09
Sodium bicarbonate %	0.31	0.39
Premix broiler 5 g/kg %	0.50	0.50
L-Lysine HCL %	0.23	0.43
DL-Methionine %	0.26	0.27
L-Threonine %	0.05	0.13
L-Valine %	0.00	0.09
L-Arginine %	0.00	0.05
Ronozyme WX5000CT; 0.05 g/kg %	0.01	0.01
Ronozyme HiPhos GT; 0.05 g/kg %	0.01	0.01
Total %	100	100
Nutrients		
Calcium (Ca) g/kg	9.0	6.8
Phosphate (P) g/kg	5.9	4.9
Ino. Phosphate g/kg	2.5	2.8
Magnesium g/kg	1.4	1.6
Sodium g/kg	1.6	1.6
Potassium g/kg	8.5	8.1
Chloride g/kg	2.0	2.0
Electrolyte balance (dEB) mEq/kg	226	218
Dry matter g/kg	882	883
Crude ash g/kg	56	48
Crude protein g/kg	217	194
Crude fat g/kg	73	88
Crude fibre g/kg	28	33
Starch am g/kg	350	364
Sugar g/kg	42	39
Non-starch polysaccharide g/kg	150	157
Retainable P (rP) g/kg	4.00	3.00
Ca : rP	2.25	2.25
MEpoultry MJ/kg	12.93	13.26
MEbro MJ/kg	12.00	12.30
dLYSp g/kg	11.50	10.70
dMETp g/kg	5.68	5.36
dCYSp g/kg	2.95	2.66
dM+Cp g/kg	8.63	8.00
dTHRp g/kg	7.36	6.80
dTRPp g/kg	2.30	1.91
dILEp g/kg	8.14	6.70
dARGp g/kg	12.30	11.45

(continued)

Table 1. (continued)

Item	1–14 days	14–35 days
dPHEp g/kg	9.75	8.10
dHISp g/kg	4.79	4.20
dLEUp g/kg	15.01	12.52
dTYRp g/kg	6.84	5.31
dVALp g/kg	8.97	8.24
Dig. Crude protein g/kg	185	164

Table 2. The analyzed (g/kg) and calculated nutrient contents of the starter and grower diets for the 0, 10, 15, 150 mg/kg phenylcapsaicin treatment groups.

Item %	0	10	15	150	Calculated
Starter diet					
Moisture	12.15	12.00	11.70	12.10	11.80
Dry matter	87.85	88.00	88.30	87.90	88.20
Crude protein	21.75	21.40	21.40	21.90	21.70
Crude fat	7.10	7.20	7.30	9.30	7.30
Crude fiber	2.90	3.20	2.70	3.10	2.80
Crude ash	5.15	5.20	5.40	5.00	5.60
Calcium	0.98	0.89	0.82	0.79	0.90
Phosphorus	0.59	0.58	0.57	0.56	0.59
Grower diet					
Moisture	12.10	12.00	12.10	12.10	11.70
Dry matter	87.90	88.00	87.90	87.90	88.30
Crude protein	18.90	19.40	19.10	19.00	19.40
Crude fat	8.50	8.30	8.80	9.30	8.80
Crude fiber	3.85	3.50	3.60	3.90	3.30
Crude ash	4.40	4.30	4.40	4.30	4.80
Calcium	0.60	0.55	0.58	0.54	0.68
Phosphorus	0.48	0.47	0.48	0.45	0.49

6 h dark (18L:6D) per 24 h was given. Light intensity was 20 lux during the entire experimental period.

Day-old broilers were vaccinated against Infectious Bronchitis (IB) in the hatchery (IB primer, Zoetis B.V., Capelle a/d IJssel, The Netherlands) and against coccidiosis (Paracox 5, spray, MSD Animal Health, Boxmeer, The Netherlands) at the trial facility. At 14 days of age all broilers were vaccinated against New Castle Disease (NCD, Clone 30, spray vaccination, MSD Animal Health, Boxmeer, The Netherlands).

Body weight and residual feed were recorded per pen at 0, 7, 14, 21, 28, and 35 days of age to determine average BW, BW gain, and feed consumption on weekly basis, per feeding phase (0-14 days and 14-35 days) and cumulative over the entire experimental period. Culling, mortality, and weight of the removed broilers were recorded daily.

The feed conversion ratio (FCR) was calculated as gross feed intake/total gross BW gain for a given time period and the European Production Efficiency Factor (EPEF) was calculated as (mean daily body weight gain (g)/FCR x10) x (100 - mortality (%)). Daily growth rate (%) was calculated using the compounded growth rate formula $r = \sqrt[n]{K_n/K_0} - 1$ where n is number of days in the time period, and K_n and K_0 is broiler weight at the end and start of the time period, respectively, and was used to estimate daily increase in broiler bw (g). Daily feed consumption was estimated as daily increase (g) x feed conversion ratio and daily intake of mg PheCap/kg bw was estimated as daily feed consumption (g)/bw (g) x PheCap feed concentration. Realized concentrations of PheCap in the provided feeds were used when estimating daily intake of PheCap.

Litter quality was observed on day 14, 27, and 34 by an experienced assessor and friability and wetness of the litter in each pen was scored on a 1-to-10-point scale. Score 1 is complete caked litter: wet litter, total area by pressure on the litter water is appearing; Score 10 is friable litter, no caked litter particles: very dry litter (only observed at start of the experiment).

The occurrence of footpad lesions and their severity were determined at day 34 by an experienced assessor. All broilers per pen were assessed. Footpad dermatitis was scored for both feet according to the 'Swedish' classification,[21] i.e. score 0: no lesions or very small discoloration; score 1: discoloration but no deep lesion; score 2: deep lesion with ulcers or scabs, bumble foot.

The severity of footpad lesions was expressed as footpad score (FPS) per pen calculated as 100% x [(0.5 x the total number of birds with score 1) + (2 x the total number of birds with score 2)]/the total number of scored birds. The flock FPS ranges from 0 (all birds having no lesions) to 200 (all birds having score 2).

On day 35, two broilers with average weight were removed per pen, weighed, individually marked with wing tags and transported to Royal GD (Deventer, The Netherlands) for haematology, blood chemistry analysis, pathology and histology of different intestinal tissues. At Royal GD, the broilers were euthanized one by one by electrocution and 1 × 5 mL NaF blood, 1 × 5 mL EDTA blood and 1 × 5 mL serum blood were immediately drawn from the jugular vein. The blood samples were analyzed on packed cell volume (haematocrit), haemoglobin, mean corpuscular haemoglobin concentration, and total and differential counts for leukocytes. The blood plasma/serum samples were analyzed on sodium, potassium, chloride, calcium, phosphate, magnesium, total protein, albumin/globulin ratio, glucose, urea, cholesterol, creatinine, triglycerides, glutamate dehydrogenase (GLDH), haemolyse-index, bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP) and creatine kinase (CPK) concentration. Gross pathology of the liver, kidneys, spleen, lung,

stomach, small intestine, colon, cecum, heart, pancreas, adrenal gland, thymus and thyroid gland was determined in the same broilers from which blood samples were taken.

Statistical analysis

All statistics were calculated using R, version 4.2.1.[22] For the response variables growth performance, feed intake, feed conversion ratio and EPEF, we used linear mixed effects models (LME) to account for the clustering of observations in the four blocks within each of two rooms. Each block consisted of four adjacent pens. The mean of 20 broilers within each pen was used in the analysis, making the pen the unit of observation. The R syntax for these models was: lme(Response ~ PheCap, random = ~+1|Room/Block) where 'lme' is a function from the nlme library of R,[23, 24] 'Response' represents the response variable analyzed, and 'PheCap' was the predictor, treated as a categorical variable with the four levels; 0, 10, 15 and 150 mg PheCap/kg feed. The statement random = ~+1|Room/Block sets the intercept of each room to be a random effect factor as well as each block nested under a room.

We used the same modelling for the blood chemistry and haematology datasets but with the nesting structure Room/Block/Pen for the random effect factors. We did this since two broilers per pen were examined separately, making a broiler the unit of observation. Due to the binary nature of the response variables of the gross pathology data, they were analyzed using generalized linear mixed-effects models using the glmmTMB library of R.[25] Room, block, and pen were included as random effect factors since two broilers per pen were examined individually. The R syntax for these models was: glmmTMB(Response ~ PheCap+(1|Room)+(1|Block)+(1|Pen), family = binomial)

Mortality was analyzed using the survival library of R.[26,27] We used a parametric survival model with a Weibull distribution and censoring. The R syntax for this model was: survreg(Surv(Age,Status) ~ PheCap, cluster = RoomPen, dist = 'weibull') where 'survrg' is the function for performing parametric survival modelling, 'Surv' is a function used to create the survival object, i.e., the response variable. 'Age' represents the age at death (in days) except when the status indicator 'Status' is zero, then the individual was still alive at the end of the experiment. The smallest cell size of a cluster group was used as a cluster indicator.

For all the above models, we first analyzed for an overall effect of PheCap using the ANOVA function of R. When an overall effect was found, treatment contrasts from the summary output of R was used to compare the different concentrations of PheCap against the 0 mg PheCap/kg feed group.

The Kruskal-Wallis test was used to analyze for differences in litter moisture and friability due to the non-normality of these data, and, due to low variance resulting from low occurrences, the Fisher's exact test was used to analyze for the effect of dietary PheCap on footpad lesions.

Results

No differences in body weight gain were observed between the experimental treatment groups in either the starter phase (df = (3, 21), $F = 1.256$, $p = 0.31$), the grower phase (df = (3, 21), $F = 1.163$, $p = 0.35$) or overall (df = (3, 21), F = 0.194, $p = 0.34$), Table 3.

No differences in feed intake were observed between the experimental treatment groups in either the starter phase (df = (3, 21), F = 0.293, $p = 0.83$), the grower phase (df = (3, 21), F = 1.413, $p = 0.27$) or overall (df = (3, 21), F = 1.730, $p = 0.19$), Table 3.

No effect on feed conversion ratio (FCR) was found for either the starter diet (df = (3, 21), F = 0.380, $p = 0.77$), grower diet (df = (3, 21), F = 0.181, $p = 0.91$) or overall (df = (3, 21), F = 0.533, $p = 0.66$), Table 3.

A total of 22 birds died or were euthanized over the course of this study, with statistically significant lower mortality for all three treatment diets compared to the control. Half of the dead broilers (11, 6,9%) were in the control group while 5 (3.1%), 1 (0.6%) and 5 (3.1%) broilers were in the 10 (SE = 0.251, z = 2.18, $p = 0.03$), 15 (SE = 0.708, z = 2.33, $p = 0.02$) and 150 (SE = 0.251, z = 2.18, $p = 0.03$) mg/kg PheCap groups, respectively. In the control group, 10 of 11 deaths occurred during the growth period, Table 3.

This reduced mortality due to dietary PheCap resulted in a statistically significant increase in the EPEF (df = (3, 21), $F = 4.652$, $p = 0.01$), and a statistically significantly increase was found in both the 10 mg/kg feed (df = 21, $t = 2.579$, $p = 0.02$) and the 15 mg/kg feed groups (df = 21, $t = 3.363$, $p = 0.003$), but not for the 150 mg/kg feed group (df = 21, $t = 0.958$, $p = 0.35$) compared to the control group, Table 3.

In all treatment groups, and because the ratio of feed consumption to body weight decreases as the broilers get older, the daily PheCap intake was highest during the first week and decreased gradually in the following weeks. The highest daily PheCap intake was seen in the 150 mg PheCap/kg feed group during the first week (D 0–7) with 36.3 mg/kg bw/day. In the following 4 weeks the intake in this treatment group decreased to 26.8, 20.1, 15.9 and 12.4 mg/kg bw/day, respectively, Table 4. A corresponding proportionate decrease was also seen for the two other feed groups with a decrease from 3.5 to 2.5, 1.9, 1.5 and 1.2 mg PheCap/kg bw/day for the 15 mg PheCap/kg feed group, and a decrease from 2.2 to 1.6, 1.2, 1.0 and 0.7 for the 10 mg PheCap/kg feed group, during weeks 1–5, respectively, Table 4.

Table 3. Growth performance results for days 1-14, 14-34 and 1-34.

	PheCap (mg/kg feed)			
	0	10	15	150
Starter diet				
Body weight D0 (g)	38	38	38	38
Body weight D14 (g)	483	486	490	476
Body weight gain (g)	445	447	452	438
Mortality (%)	0.6	1.2	0	1.2
Feed conversion ratio (FCR)	1.106	1.097	1.096	1.112
Feed intake (g)	493	491	495	487
Daily growth rate (%)	19.9	20.0	20.0	19.8
Grower diet				
Body weight D14 (g)	483	486	490	476
Body weight D35 (g)	2556	2590	2565	2538
Body weight gain (g)	2073	2105	2075	2062
Mortality (%)	6.4	1.9	0.6	1.9
Feed conversion ratio (FCR)	1.387	1.381	1.380	1.403
Feed intake (g)	2874	2906	2863	2892
Daily growth rate (%)	8.3	8.3	8.2	8.3
All				
Body weight D0 (g)	38	38	38	38
Body weight D35 (g)	2556	2590	2565	2538
Body weight gain (g)	2518	2552	2526	2500
Mortality (%)	7.0	3.1	0.6	3.1
Feed conversion ratio (FCR)	1.337	1.331	1.329	1.352
Feed intake (g)	3367	3397	3358	3379
Daily growth rate (%)	12.8	12.8	12.8	12.8
European production efficient factor (EPEF)	501	531	540	512

In the control group, three dead birds were diagnosed with septicaemia and two with Rachitis**,** while septicaemia or rachitis was not seen in the PheCap treatment groups. This was considered to be an incidental finding since the diets were identical with the exception of the added PheCap and is not toxicologically relevant. No other differences were observed as reasons for deaths/culling between treatments, Table 5.

Litter quality assessment showed no differences between treatment groups. At day 14, all pens had the same scores for friability and moisture of the litter and no statistical analyses could be performed. No statistically significantly effects of dietary phenylcapsaicin between treatment groups were found for either friability or moisture at days 27 and 34, Table 6.

The severity of footpad lesions was very low under the present conditions with 2, 3, 1 and 0 observations for the 0, 10, 15, and 150 mg/kg treatment groups, respectively. No differences were observed in footpad lesions between treatment groups ($p = 0.33$).

Twenty two of the 64 blood samples were not suitable for analysis of the red and white blood cell parameters. Some blood samples clotted immediately but most unsuitable blood samples were visually not clotted, and it is not clear why these samples could not be analyzed. This reduced the sample sizes from 16 per treatment to 10, 11, 11, and 10 for the 0, 10, 15, and 150 mg/kg PheCap treatment groups, respectively.

Dietary inclusion of PheCap had negligible effect on red and white blood cell parameters and no statistically significant dose dependent effects between treatment groups were found**,** although a numerically dose dependent decrease was seen for haemoglobin, Table 7. A numeric decrease in the eosinophilic granulocytes, mean corpuscular haemoglobin, and monocytes was also seen in the 150 mg PheCap/kg feed group compared to the control, Table 7.

With the exception of creatinine, no statistically significantly dose dependent effects of dietary PheCap were found in the blood biochemical parameters, Table 8. For creatinine, increasing dietary concentrations of PheCap dose dependently reduced blood levels. This decrease was statistically significantly different only between the control and the 150 mg/kg feed treatment group, Table 8. The blood sodium

Table 4. Weekly growth performance results.

	PheCap (mg/kg feed)			
	0	10	15	150
0 – 7 days				
Body weight D0 (g)	38	38	38	38
Body weight D7 (g)	165	166	166	161
Body weight gain (g)	126	127	128	123
Mortality (%)	0.6	0.6	0.0	0.6
Feed conversion ratio (FCR)	1.095	1.082	1.125	1.106
Feed intake (g)	138	138	144	136
Daily growth rate (%)	23.3	23.4	23.4	22.9
PheCap intake (mg/kg bw/day)	0.0	2.2	3.5	36.3
7 – 14 days				
Body weight D7 (g)	165	166	166	161
Body weight D14 (g)	483	486	490	476
Body weight gain (g)	319	320	324	315
Mortality (%)	0.0	0.6	0.0	0.6
Feed conversion ratio (FCR)	1.112	1.103	1.084	1.115
Feed intake (g)	354	353	351	351
Daily growth rate (%)	16.6	16.6	16.7	16.7
PheCap intake (mg/kg bw/day)	0.0	1.6	2.5	26.8
14 – 21 days				
Body weight D14 (g)	483	486	490	476
Body weight D21 (g)	972	976	968	957
Body weight gain (g)	488	490	478	481
Mortality (%)	2.5	0.6	0.6	0.6
Feed conversion ratio (FCR)	1.365	1.361	1.389	1.373
Feed intake (g)	667	667	663	661
Daily growth rate (%)	10.5	10.5	10.2	10.5
PheCap intake (mg/kg bw/day)	0.0	1.2	1.9	20.1
21 – 28 days				
Body weight D21 (g)	972	976	968	957
Body weight D28 (g)	1698	1717	1697	1674
Body weight gain (g)	726	742	730	716
Mortality (%)	2.6	0.6	0.0	0.6
Feed conversion ratio (FCR)	1.355	1.338	1.338	1.370
Feed intake (g)	984	992	975	981
Daily growth rate (%)	8.3	8.4	8.4	8.3
PheCap intake (mg/kg bw/day)	0.0	1.0	1.5	15.9
28 – 35 days				
Body weight D28 (g)	1698	1717	1697	1674
Body weight D35 (g)	2556	2590	2565	2538
Body weight gain (g)	858	873	867	865
Mortality (%)	1.2	0.6	0.0	0.6
Feed conversion ratio (FCR)	1.427	1.430	1.414	1.446
Feed intake (g)	1223	1247	1225	1249
Daily growth rate (%)	6.0	6.0	6.1	6.1
PheCap intake (mg/kg bw/day)	0.0	0.7	1.2	12.4

Table 5. Mortality and culling reasons (in numbers of broilers) per treatment based on pathological examination.

	PheCap (mg/kg feed)			
	0	10	15	150
Arthritis				1
Ascites syndrome	1	1		
Sudden death syndrome	2	2		
Heart failure syndrome				1
Airsacculitis	2	1		1
Pericarditis	1	1		2
Rickets (rachitis)	2			
Septicemia	3			
Could not be determined			1	

Table 6. Visual litter quality.

	PheCap (mg/kg feed)						
	0	10	15	150	H	df	*p*
Day 14							
Friability	9.0	9.0	9.0	9.0	NA	NA	NA
Wetness	9.0	9.0	9.0	9.0	NA	NA	NA
Day 27							
Friability	7.1	6.9	6.5	7.4	4.971	3	0.17
Wetness	7.4	7.0	7.0	7.3	2.358	3	0.50
Day 34							
Friability	7.0	6.5	6.4	6.8	6.389	3	0.09
Wetness	7.0	6.9	6.9	7.0	0.972	3	0.81

concentration also seems to be slightly reduced by PheCap in the 150 mg/kg dietary treatment group although the overall effect of PheCap on sodium blood levels was not statistically significant ($p = 0.07$), Table 8. A numeric, but not statistically significant, dose dependent decrease was also found for ALT, CPK, GLDH, Haemolyse index, and LDH, Table 8.

Gross pathology

During gross pathological examinations of the individual birds on day 35 no abnormalities were observed for liver, kidneys, spleen, lung, stomach, small intestine, colon, cecum, heart, pancreas, adrenal gland, thymus, thyroid gland, beak, oesophagus, crop, pancreas or gall bladder. Some birds from all treatment groups showed mild to minor dilatation of the proventriculus but severe dilatation was only found in the 150 mg/kg group, and mild erosion of the gizzard was also seen in all treatment groups. The gross pathological findings are summarized in Table 9.

Discussion

Dietary inclusion of PheCap at levels up to 150 mg/kg feed did not affect BW, BWG, FI and FCR compared to the non-supplemented control group. This contrasts the effects found in Arbor Acre male broilers where the addition of 80 mg/kg pure natural capsaicin extract increased both averaged daily weight gain and final body weight in both the starter (d 1–21) and grower (d 22–42) periods,[16] and both 2 and 4 mg/kg decreased the FCR in the entire growth period (d 1–42).[14]

Dietary inclusion of 10, 15, and 150 mg PheCap/kg feed resulted in lower mortality rates. Due to these lower mortality rates, EPEF of the 10 and 15 mg PheCap/kg feed treatment groups was significantly higher than the control group, with the highest numerical value in the 15 mg/kg feed group.

The reduced mortality found in the present study contrast the findings summarized by Abd El-Hack ME et al.[1] Although under dissimilar experimental conditions with hot red pepper (HRP) added to broiler feed at different concentrations, none of the five studies summarized showed reduced mortality. Reduced mortality was, however, found following a diet supplemented with both 0.1 g HRP, 1 g thyme and 1 g garlic per kg feed.[1]

It is worth noticing that three dead broilers in the control group were diagnosed with septicaemia while no septicaemia was found in the PheCap treatment groups. Since capsaicin is used as a phytobiotic in broiler nutrition as a possible alternative to antibiotics in poultry, and the number of dead broilers in general was small in this study, it should be worthwhile to address in future studies to examine if PheCap could reduce septicaemia in broiler chicken and as such act as a phytobiotic.

Although no statistically significant differences were found between treatment groups and control at day 35, the average body weight of control group broilers was 180 g higher (2556 vs 2376), and the average feed conversion ratio 0.146 g/g lower (1.337 vs 1.463) than the Ross 308 male broiler performance objectives,[28] probably caused by a lower stocking density and better rearing conditions compared to farming conditions.

Dietary inclusion of Phenylcapsaicin did not negatively affect litter quality or footpad lesions.

No statistically significant effects of PheCap on the red and white blood cell parameters were found under the conditions of the present study. However, the numerical dose dependent decrease in the eosinophilic granulocytes and monocytes seen in the 150 mg PheCap/kg feed group compared to the control (Table 7.) could indicate positive effects on the immune system of higher doses of dietary

Table 7. Haematology findings on day 35 of pre-selected broilers following a diet containing 0, 10, 15 or 150 mg phenylcapsaicin (PheCap) per kilo feed.

Parameter	Unit	PheCap	Mean	SD	n	df	F or t	*p*
Basophil granulocytes	10^9/L							
		0	0		10			
		10	0		11			
		15	0		11			
		150	0		10			
Eosinophilic granulocytes	10^9/L					3, 12	0.15	0.93
		0	0.70	0.35	10			
		10	0.63	0.24	11			
		15	0.70	0.84	11			
		150	0.57	0.46	10			
Haematocrit	L/L					3, 12	3.13	0.07
		0	0.34	0.04	10			
		10	0.35	0.02	11			
		15	0.35	0.02	11			
		150	0.32	0.01	10			
Haemoglobin	mmol/L					3, 12	1.78	0.20
		0	5.38	0.65	10			
		10	5.30	0.45	11			
		15	5.26	0.35	11			
		150	4.95	0.28	10			
Leucocytes	10^9/L					3, 12	0.57	0.64
		0	8.94	2.50	10			
		10	10.39	3.57	11			
		15	8.61	3.49	11			
		150	9.38	1.85	10			
Lymphocytes	10^9/L					3, 12	0.89	0.47
		0	2.22	0.69	10			
		10	2.53	0.69	11			
		15	2.12	0.53	11			
		150	2.44	0.62	10			
Mean corpuscular haemoglobin consentration	mmol/L					3, 12	2.74	0.09
		0	16.08	1.07	10			
		10	15.06	0.90	11			
		15	15.23	0.59	11			
		150	15.48	0.77	10			
Monocytes	10^9/L					3, 12	1.83	0.20
		0	0.23	0.25	10			
		10	0.25	0.20	11			
		15	0.26	0.15	11			
		150	0.09	0.09	10			
Neutrophil granulocytes	10^9/L					3, 12	0.54	0.66
		0	5.84	1.90	10			
		10	7.01	3.02	11			
		15	5.53	2.85	11			
		150	6.18	2.39	10			

PheCap in broilers, and this should be addressed in future studies. These effects were also seen following dietary red hot pepper supplementation.[1] A numeric dose dependent decrease was also seen for haemoglobin and mean corpuscular haemoglobin, and further research should explore whether high doses of PheCap can lead to anemia.

No toxicological relevant dose dependent effects were found for basophil granulocytes, haematocrit, leucocytes, lymphocytes, or neutrophil granulocytes.

Table 8. Clinical biochemistry findings on day 35 of pre-selected broilers following a diet containing 0, 10, 15 or 150 mg phenylcapsaicin (PheCap) per kilo feed.

Parameter	Unit	PheCap	Mean	SD	n	df	F or t	p
Albumin	g/L					3, 21	1.29	0.30
		0	14.45	1.14	16			
		10	13.79	1.21	16			
		15	14.07	1.08	16			
		150	13.81	1.16	16			
Albumin/Globulin ratio						3, 21	0.63	0.61
		0	0.84	0.06	16			
		10	0.85	0.05	16			
		15	0.84	0.05	16			
		150	0.86	0.05	16			
ALP (alkaline phosphatase)	IU/L					3, 21	1.39	0.27
		0	6,630	3,570	16			
		10	6,538	5,462	16			
		15	7,278	4,247	16			
		150	9,440	5,864	16			
ALT (alanine aminotransferase)	g/L					3, 21	1.56	0.23
		0	8.19	1.97	16			
		10	7.74	2.32	16			
		15	7.50	2.22	16			
		150	6.69	1.40	16			
AST (aspartate aminotransferase)	IU/L					3, 21	1.23	0.32
		0	558.2	176.3	16			
		10	486.4	132.9	16			
		15	496.8	159.6	16			
		150	462.8	172.1	16			
Bilirubin, total	μmol/L					NA	NA	NA
		0	1		16			
		10	1		16			
		15	1		16			
		150	1		16			
Calcium	mmol/L					3, 21	1.02	0.40
		0	2.64	0.09	16			
		10	2.62	0.10	16			
		15	2.61	0.09	16			
		150	2.59	0.10	16			
Chloride	mmol/L					3, 21	1.16	0.35
		0	113.9	1.61	16			
		10	112.7	2.47	16			
		15	112.9	2.00	16			
		150	112.7	2.68	16			
Cholesterol, total	mmol/L					3, 21	0.65	0.59
		0	3.84	0.40	16			
		10	3.88	0.37	16			
		15	3.78	0.30	16			
		150	3.73	0.36	16			
CPK (creatine kinase)	IU/L					3, 21	1.05	0.39
		0	46,443	21,203	16			
		10	37,900	18,144	16			
		15	37,286	18,059	16			
		150	35,490	22,820	16			

(continued)

Table 8. (continued)

Parameter	Unit	PheCap	Mean	SD	n	df	F or t	p
Creatinine	µmol/L					3, 21	3.87	**0.02**
		0	44.94	16.30	16			
		10	48.00	19.21	16	21	0.057	0.57
		15	38.25	14.64	16	21	1.24	0.23
		150	31.22	16.61	16	21	2.56	**0.02**
GGT (gamma-glutamyltransferase)	IU/L					3, 21	0.28	0.84
		0	17.31	4.50	16			
		10	17.56	3.58	16			
		15	18.13	4.87	16			
		150	16.81	3.66	16			
GLDH (glutamate dehydrogenase)	mmol/L					3, 21	2.11	0.13
		0	3.79	0.99	16			
		10	3.56	1.00	16			
		15	3.26	0.86	16			
		150	3.14	0.59	16			
Glucose	mmol/L					3, 19	0.52	0.68
		0	13.50	1.50	11			
		10	13.73	1.07	15			
		15	13.90	0.76	15			
		150	13.91	0.82	14			
Haemolyse index	mmol/L					3, 21	0.20	0.89
		0	0.008	0.006	16			
		10	0.008	0.004	16			
		15	0.007	0.006	16			
		150	0.006	0.005	16			
LDH (lactate dehydrogenase)	IU/L					3, 21	1.16	0.35
		0	6,902	3,426	16			
		10	5,888	2,807	16			
		15	5,489	2,584	16			
		150	5,362	2,401	16			
Magnesium	mmol/L					3, 21	0.55	0.65
		0	0.96	0.05	16			
		10	0.98	0.06	16			
		15	0.97	0.08	16			
		150	0.95	0.07	16			
Phosphate	mmol/L					3, 21	1.14	0.36
		0	2.38	0.18	16			
		10	2.33	0.13	16			
		15	2.36	0.10	16			
		150	2.29	0.14	16			
Potassium	mmol/L					3, 21	0.76	0.53
		0	7.27	0.83	16			
		10	7.38	1.02	16			
		15	7.14	0.80	16			
		150	7.53	1.05	16			
Protein, total	g/L					3, 21	2.31	0.11
		0	31.88	2.73	16			
		10	30.13	2.25	16			
		15	30.75	2.21	16			
		150	29.94	2.64	16			
Sodium	mmol/L					3, 21	2.8	0.07
		0	152.81	1.47	16			
		10	152.13	1.50	16			
		15	152.25	1.84	16			
		150	151.00	2.39	16			

(continued)

Table 8. (continued)

Parameter	Unit	PheCap	Mean	SD	n	df	F or t	p
Triglycerides	mmol/L					3, 21	0.76	0.53
		0	0.19	0.08	16			
		10	0.22	0.09	16			
		15	0.23	0.09	16			
		150	0.21	0.07	16			
Urea	mmol/L					NA	NA	NA
		0	<2.0		16			
		10	<2.0		16			
		15	<2.0		16			
		150	<2.0		16			

Table 9. Findings from the gross pathology at day 35.

	PheCap (mg/kg feed)						
	0	10	15	150	χ^2	df	p
Dilation proventriculus (minor/mild/severe)	5/2/0	1/4/0	6/6/0	2/3/2	6.931	3	0.07
Erosion of the gizzard (minor/mild)	4/2	0/3	7/2	0/4	4.178	3	0.24
Feathers or wood shavings in the gizzard	5	2	2	0	2.859	3	0.41
Intestinal disorders (minor/mild)	0/1	1/0	0/0	1/0	1.822	3	0.61
Epiphysiolysis of the femoral head (unilateral/bilateral)	1/3	1/2	4/4	3/1	4.236	3	0.24
Wooden breast (minor/severe)	2/1	0/1	0/1	0/1	0.249	3	0.97
Air sac inflammation (minor/severe)	1/1	1/0	1/0	1/2	NA	NA	NA
Hydropericardium (minor/severe)	0/0	0/0	0/0	1/1	NA	NA	NA
Hock inflammation	0	1	0	0	NA	NA	NA
Periarthritis	0	0	1	0	NA	NA	NA

Of the clinical biochemistry parameters, statistically significantly dose dependent response was only found for creatinine. Being a byproduct of skeletal muscle phosphocreatine breakdown, creatinine is an indicator of protein metabolism and an increase in serum creatinine is often used as an indicator of kidney damage.[29] This is not the case here, as an increase in dietary PheCap is associated with a decrease in serum creatinine. A decrease in creatinine levels has been reported during fasting and feed restriction in yellow-legged gulls (*Larus cachinnans*)[30] and fasting in red-legged partridge (*Alectoris rufa*).[31] In the present study, there are no indications of fasting or feed restrictions according to recorded feed intake and body weight. We have no explanation as to why dietary PheCap should decrease creatinine levels and future studies should address this.

The dose dependent decrease found for ALT, CPK, GLDH, Haemolyse index, and LDH is not considered toxicologically relevant, and no toxicologically relevant dose dependent effects were found for sodium, potassium, chloride, calcium, phosphate, magnesium, total protein, albumin/globulin ratio, glucose, urea, cholesterol, triglycerides, bilirubin, AST, GGT or ALP.

During gross pathology, the examined broilers of the 15 mg/kg treatment group had the numerically highest occurrences of proventriculus dilatations, gizzard erosions and locomotion disorders. Because the pen was the experimental unit, individual PheCap consumption is not available to allow for comparison with the individual pathological findings under the present design. Furthermore, the occurrence of these findings is not consistent with the occurrence of these findings in the 10 and 150 mg/kg PheCap treatment groups. Thus, it is assumed that the numerically highest occurrences in the 15 mg/kg feed treatment group are coincidental and not caused by the inclusion of PheCap.

Only male broilers were used in the present study. However, we argue that because feed intake, and as such also the intake of the test substance, is higher in male than in female broilers, the toxicological conclusions presented are also valid for female broilers.

Because no adverse effects were found in either the performance results, haematology and clinical chemistry blood parameters or pathology, it is concluded that inclusion of phenylcapsaicin in broiler feed at doses up to 15 mg/kg feed has no negative effect on broiler chicken health, and

that the tolerance dose of 150 mg/kg feed also has no negative effect on broiler chicken health. The No Observed Adverse Effect Level (NOAEL) of PheCap for systemic toxicity in Ross 308 broilers is therefore considered to be higher than 36.3 mg/kg BW/day, the highest weekly averaged daily intake found in the first week for the 150 mg PheCap/kg feed group in the present study, Table 4.

It is not surprising that no adverse effects were observed even in the tolerance level group receiving 150 mg PheCap/kg feed (150 ppm). The amounts used in the present study are much lower than the 1000 ppm capsaicin found in wild capsicum peppers fruits, fruits that birds commonly feed on.[10,32]

Intestinal colonisation of *Salmonella* is a major concern in the poultry industry, and low dose PheCap has shown promising effects as a phytobiotic alternative to antibiotics. In an unpublished full-scale pilot using a pre- and posttest design with paired-sample, the inclusion of 15 mg PheCap/kg feed in a regular starter diet reduced the the number of *Salmonella*-positive broiler chicken houses from 57 to 18 during the test period. If this result is confirmed in future studies, low dose inclusion of PheCap in regular poultry feed is a simple and efficient alternative to antibiotics for reducing *Salmonella* in the poultry industry.

Based on the low incidence of global findings, the non-pathological character of findings, and the absence of significant dose-response relationships for main parameters, it can be assumed that the inclusion of PheCap in broiler feed does not have any negative effects on animal health even at the tolerance dose level of 150 mg/kg.

Author contributions

JvH and TV conducted the study. TRP, JvH and TV drafted the manuscript and KHJ performed the data analyses. All contributed in the presentation and interpretation of the data and critical revisions of the manuscript.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: TRP hold shares in aXichem at time of submission.

Data availability

Data sharing is not applicable; the data is the property of aXichem AB.

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Ethical approval

Not applicable, the study is a broiler feeding trail. The experimental protocol was approved by the Animal Welfare Body of Wageningen University (IvD-WU), Wageningen, The Netherlands (2019.D- 0033.001).

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