Journal of Applied Animal Nutrition, Vol. 5; e3; page 1 of 8 doi:10.1017/jan.2016.10
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Journal of Applied Animal Nutrition

Original Research

Dietary resin acid composition as a performance enhancer for broiler chickens

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Summary

Resin acid composition (RAC) has previously been shown to inhibit the growth of the Gram-positive bacterial species *Clostridium perfringens in vitro* and to modulate the ileal microbiota of broiler chickens. The following trials examined the effect of RAC on broiler chickens in two experiments. In experiment 1, 1400 one-day-old Ross 308 broilers were divided into two coccidiostat treatments: chemical (CC) and ionophore (IC), which were further divided into two RAC dosages: 0 and 0.5 g/kg. All diets were supplemented with xylanase, β-glucanase and phytase feed enzymes. The birds were raised in a commercial-type environment without additional microbial challenge during the 42-day trial. RAC improved the body weight gain by 3.3% and feed conversion ratio by 5.7% with CC, and improved footpad lesion scores with IC but had no effect on the litter quality. Experiment 2 was a 35-day subclinical necrotic enteritis (NE) challenge trial with 510 male Ross 308 chickens. The dietary treatments included a non-challenged, non-supplemented control and four NE challenged treatments with dietary RAC supplementation at 0, 1, 2, and 3 g/kg. The birds were challenged with *Eimeria maxima* on day nine and *C. perfringens* on day 14. While RAC at 1 g/kg significantly increased bird weight gain during the challenge, it did not affect the microbial or short chain fatty acid (SCFA) profiles. In contrast, RAC at 3 g/kg reduced the abundance of the *Lactobacillus* group and tended to reduce the abundance of genus *Bifidobacterium* and the total numbers of eubacteria. These experiments suggest that dietary RAC at a moderate dose positively affected broiler performance. However, changes in caecal microbiota populations may not have influenced the observed performance effects of RAC.

Keywords: **resin acids: broiler chicken: performance: necrotic enteritis: microbiota: short chain fatty acids**

(Received 7 November 2016 – Accepted 23 December 2016)

Introduction

Coccidiosis control is essential in broiler production, because even subclinical infections decrease the performance of chickens and makes them susceptible to necrotic enteritis (NE) or other secondary enteric infections (Dalloul and Lillehoj, 2006; Van Immerseel, 2009). The bacterial species *Clostridium perfringens* is the causative agent of NE. This natural inhabitant of chicken caecum can, under predisposing conditions such as coccidial infection or sudden change in the diet, increase colonisation in the small intestine leading to necrosis of the intestinal epithelium (Van Immerseel *et al.*, 2009; Timbermont *et al.*, 2011). Emerging resistance problems against anticoccidial drugs in *Eimeria* species, as well as demands or even regulations to limit the use of antibiotic growth promoters (AGPs) or ionophore coccidiostats in poultry

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https://doi.org/10.1017/jan.2016.10 Published online by Cambridge University Press

Journal of Applied Animal Nutrition

diets have raised interest in alternative products and practices that can limit the risk of enteric pathogen outbreaks in commercial broiler operations.

A novel dietary product extracted from coniferous Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) trees described as the resin acid composition (RAC), may improve gastrointestinal health of broiler chickens through microbial modulation (Kettunen *et al.*, 2015). Resin of coniferous trees contains a mixture of diterpene resin acids such as abietic, dehydroabietic, pimaric, neoabietic, levopimaric and palustric acid (Fallarero *et al.*, 2013). These compounds have antimicrobial, antifungal and antiparasitic activity and protect the tree against the attacks of insects, fungi, bacteria and other pathogens (San Feliciano *et al.*, 1993; Savluchinske-Feio, 2006). The antibacterial effect of resin acids are likely related to solubilisation of the bacterial cell wall (Söderberg, 1996) or altered cell membrane proton gradients (Jokinen and Sipponen, 2016), and is mainly directed against Gram-positive bacteria. Many of the opportunistic pathogenic bacteria of human and animals, like *C. perfringens*, are Gram-positive. In fact, the potential of resin acids in wound-healing was recently reported in medical science (Jokinen and Sipponen, 2016).

In a previous study, RAC inhibited the *in vitro* growth of *C. perfringens*, favourably modulated ileal microbiota of broiler chickens, and increased their weight gain under a NE challenge (Kettunen *et al.*, 2015). As the effect of any potential modulator of intestinal microbiota is likely to be affected both by diet and environment, these initial findings on the positive effects of RAC warranted further supporting data from other locations, different dietary formulations and a longer rearing period. The present trial was conducted to study the effects of dietary RAC on broiler chickens in two established European research institutes, using local feed formulations and bird management practices. Experiment 1, conducted in Poland, measured bird performance, footpad dermatitis (FPD) lesions and litter quality over a 42-day rearing period. Experiment 2 was a 35-day NE challenge study in the Netherlands which studied the effects of RAC on bird performance and on the caecal microbial community and short chain fatty acid (SCFA) profiles.

Materials and Methods

Test Product

The test product RAC (Progres®, Hankkija Ltd/Suomen Rehu, Hyvinkää, Finland, patent no FI124918) consisted of 8.5% resin acids, 88.5 to 89.5% free fatty acids, and 2 to 3% neutral components, and was in liquid format. The fatty acid profile of the product was 4 to 6% saturated acids, 27 to 31% monounsaturated fatty acids, and 63 to 69% of polyunsaturated fatty acids with an n-3 to n-6 -ratio of approximately 1:4. The resin acid fraction of the product contained abietic acid (33%), dehydroabietic acid (18%), and pimaric acid (14%) as well as smaller concentrations of other resin acids of Scotch pine and Norway spruce trees.

Experiment 1

Experiment 1 was conducted at the poultry facility of the Department of Animal Nutrition and Feed Science, University of Warmia and Mazury, Olsztyn, Poland. The Animal Ethical Review Committee of the University of Warmia and Mazury approved the experiment. The trial design was completely randomised as a 2×2 factorial design, with two dietary RAC and two coccidiostat treatments: T1 - RAC 0 mg/kg + chemical coccidiostat (CC) diclazuril 1 mg/kg (Clinacox®, Janssen Pharmaceutica N.V., Beerse, Belgium); T2 - RAC 0.5 mg/kg + CC 1 mg/kg; T3 - RAC 0 mg/kg with ionophoric coccidiostat (IC) salinomycin (Sacox®, Huvepharma AD, Sofia, Bulgaria) 70 mg/kg; and T4 - RAC 0.5 mg/kg + IC 70 mg/kg.

A total of 1400 one-day-old, mixed sex Ross 308 broiler chickens were randomly allocated into the treatments to give seven replicate pens of 50 chicks per treatment. The chickens were reared in floor pens measuring 4.4 m^2, on straw bedding, in a commercial broiler shed. The lighting schedule was 24L:0D on day one, 23L:1D from day two to seven, 20L:4D from day eight to 11 and 18L:6D from day 12 to 42. The ambient temperature gradually decreased from 34 °C on day one until 20 °C on day 42. The birds had unrestricted access to water throughout the experiment, and unrestricted access to feed until day 41. They were slaughtered on day 42.

Mash-form diets (Table 1) in four dietary phases included a starter (days one to 10), grower one (days 11 to 22), grower two (days 22 to 35) and finisher (days 36 to 41). Coccidiostats were included in starter and grower premixes but not in the finisher premixes. All the premixes contained feed enzymes: endo-1,4-β-xylanase (to provide 1,100 visco units/kg feed) and endo-1,3(4)-β-glucanase (to provide 100 AGL units/kg feed; both from Rovabio® Excel AP, Adisseo, Antony Cedex, France), as well as phytase to

https://doi.org/10.1017/jan.2016.10 Published online by Cambridge University Press

Table 1. Composition and nutritional value of diets in Experiment 1

Ingredients, g/kg	Starter 1–10 days	Grower 1 11–21 days	Grower 2 22–35 days	Finisher 36–41 days
Wheat	402.5	438.0	452.9	422.3
Maize	250.0	200.0	150.0	150.0
Triticale	–	50.0	100.0	150.0
Soybean meal	293.1	250.3	224.6	201.3
Soybean oil	17.1	26.8	39.8	45.8
Limestone	10.6	10.7	10.4	10.0
Calcium phosphate	10.7	8.4	6.5	5.1
Salt	2.1	2.0	2.0	2.2
Sodium bicarbonate	2.5	2.0	1.9	1.6
L-lysine HCL	3.1	3.4	3.5	3.6
DL-Methionine	2.6	2.4	2.2	2.0
L-Threonine	0.7	1.0	1.0	1.0
Premix1	5.0	5.0	5.0	5.0
Nutritional value, g/kg:				
AME, MJ/kg	12.4	12.8	13.2	13.5
Crude protein	215.0	200.0	190.0	180.0
Digestible lysine	11.6	10.9	10.5	10.0
Digestible Methionine + cysteine	8.4	7.9	7.6	7.2
Calcium	8.5	8.0	7.5	7.0
Available phosphorus	4.5	4.0	3.6	3.3
Sodium	1.8	1.6	1.6	1.6

[1] provide per kg diet in a 0.5% dose: vitamin A 12,000 IU; vitamin ID3 2,400 IU; vitamin E 50 mg; vitamin K3 1.5 mg; vitamin B1 2.0 mg; vitamin B2 7.5 mg; vitamin B6 3.5 mg; vitamin B12 20 mcg; Niacin 35 mg; D-pantothenic acid 12 mg; Choline chloride 460 mg; Folic acid 1.0 mg; Biotin 0.2 mg; Fe 80 mg (as $FeSO_4 \cdot H_2O$); Cu 12 mg (as $CuSO_4 \cdot 5H_2O$); Zn 120 mg (as $ZnSO_4 \cdot H_2O$); Mn 85 mg (as MnO); Co 0.4 mg (as $CoSO_4 \cdot 7H2O$); I 0.8 mg (as KI); Se 0.15 mg (as Na_2SeO_3)

provide 500 FTU/kg feed (Phyzyme® XP, Danisco Animal Nutrition, Marlborough, Wiltshire, UK). Furthermore, all diets contained antioxidants BHA (E320), BHT (E321) and ethoxyquin (E324).

Body weight (BW) per bird, feed intake (FI) per pen and the visual health condition of the birds were recorded on a weekly basis, and body weight gain (BWG) and feed conversion ratio (FCR; feed/weight gain) were calculated. The European Efficiency Index (EEI) was calculated as follows:

$$\text{EEI} = \left(\text{liveability} \times \text{BW, kg/length of rearing period, days} \times \text{FCR}\right) \times 100.$$

Footpad dermatitis (FPD) lesions were scored at days 14, 28 and 42. For each pen, a random sample of 25 birds feet were macroscopically examined and scored according to the classification of Ekstrand *et al.* (1997) as follows: 0 - No lesions; no or very small superficial lesions, slight discoloration on a limited area, mild hyperkeratosis; 1 - Mild lesion; discoloration of the foot pad, superficial lesions, dark papillae; 2 - Severe lesion; ulcers or scabs, signs of haemorrhages or swollen foot pads. To calculate the FPD score the following formula, presented in the EU proposal COM (2005), was used:

$$\text{FPD score} = \begin{pmatrix}\text{number lesion class } 1{*}0.5 \\ +\text{number lesion class } 2{*}2\end{pmatrix} \times 100)/25.$$

Thus, the feet with score 0 did not contribute to the FPD score.

Litter quality in each pen was scored by visual inspection at day 14, 28, and 42 as a standard procedure at the experimental farm using Welfare Quality® (2009) scoring scale as follows: 0 - Completely dry and flaky, i.e. moves easily with the foot; 1 - Dry but not easy to move with foot; 2 - Leaves imprint of foot and will form a ball if compacted, but ball does not stay together well; 3 - Sticks to boots and sticks readily in a ball if compacted; 4 - Sticks to boots once the cap or compacted crust is broken. At each assessment, a score was given for five separate sites in each pen and for an overall score per pen average of the five scores was calculated.

The data were analysed statistically by analysis of variance (ANOVA) using the statistical software STATISTICA 10 (StatSoft Inc., Dell Software, Tulsa, Oklahoma, USA), with pen as the experimental unit. Treatment means were separated and compared using Duncan's test. Effects were considered statistically significant at $P < 0.05$ and near-significant trends at $P < 0.10$.

Experiment 2

The experiment was performed at the poultry facility of Schothorst Feed Research (Lelystad, the Netherlands), and was approved by the Animal Ethical Review Committee of Schothorst Feed Research. A total of 510 male hatchling Ross 308 broilers with an average body weight of 42.3 g were randomly divided into 30 metabolic cages with wire mesh floors and a floor space of 1.0 m^2. Each cage had one feeder and three drinking nipples, and started with 17 birds per cage, but was standardised to 16 birds per cage on day nine, to correct for mortality that had occurred during the first nine days. The lighting schedule was 24 light (L):0 dark (D) on day one and 22L:2D on day two. From day 3 to 35 the birds had 3L:1D:3L:1D:3L:1D:3L: 1D:4L:4D. The ambient temperature gradually decreased from 34 °C on day one until 20 °C on day 35. The birds had unrestricted access to feed and water throughout the experiment.

The experiment had a completely randomised design with five dietary treatments, each replicated six times, comprising a non-challenged, non-supplemented control treatment, and four NE challenged treatments with 0 (challenged control), 1, 2, and 3 g/kg of RAC. The

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https://doi.org/10.1017/jan.2016.10 Published online by Cambridge University Press

Table 2. Composition and nutritional value of diets in Experiment 2

Ingredients, g/kg	Starter diet 0–9 days	Grower diet 9–35 days
Barley	–	250.0
Limestone	17.1	14.7
Maize	384.3	–
Maizegluten meal	–	19.0
Soybean oil	13.2	42.1
Soybean meal	319.3	261.7
Wheat	200.0	350.0
Monocalcium phosphate	12.7	9.2
Salt	3.3	2.7
Animal Fat	28.4	33.0
Vitamin and mineral premix	10.0	5.0
L-lysine HCl (79%)	2.4	2.9
DL-Methionine (99%)	2.7	2.6
L-Threonine (98%)	0.6	0.8
Sodium bicarbonate	–	0.5
Diamol	6.0	6.0
Nutritional value, g/kg		
AME, MJ/kg	12.04	12.35
Crude protein	207.94	204.12
Digestible lysine	11.10	10.50
Digestible methionine + cysteine	8.22	7.98
Calcium	9.55	8.05
Available phosphorus	4.09	3.31
Sodium	1.45	1.32

dietary composition and nutrient profiles of the diets are shown in Table 2. The starter diet was given from day one to nine (pellet diameter 2.3 mm) and the grower diet from day nine to 35 (pellet diameter 3.0 mm). RAC was included in both feeding phases. For this experiment, RAC was absorbed into silicon dioxide (Tixosil 38X, Rhodia Group, Collonges au Mont d'Or, France) at 1:1 ratio before mixing to the feed. The inclusion rate of RAC has been given as the weight of the liquid product without carrier throughout the text. In the formulation, RAC was exchanged for diamol as an inert carrier, thus preventing dilution of energy and nutrients. The grower diet contained high levels of barley and wheat, which contain soluble NSPs, which increase intestinal viscosity which is considered to provide a beneficial environment for *C. perfringens* growth.

Feed intake and BW were recorded per cage on days nine, 20, and 35. From this data, BWG and FCR were calculated per cage for the periods from day nine to 20 (challenge period) and from day nine to 35 (overall experimental period). On day nine the birds in the non-challenged control group were orally placebo-treated with 1.0 ml phosphate buffered saline solution (PBS), whereas all other birds were orally inoculated with 1.0 ml PBS containing 10^4 sporulated *Eimeria maxima* oocysts. On day 14, the birds in the non-challenged control group were orally placebo-treated with 1.0 ml sterile liver broth, whereas all other birds were inoculated with 1.0 ml liver broth containing 10^8 colony forming units (cfu) of *C. perfringens*. The inocula were prepared by the GD Animal Health Service Ltd. (7400 AA Deventer, The Netherlands).

On day 15, four birds per cage were randomly selected and euthanised by intracardiac injection of T61® (Intervet, Boxmeer, the Netherlands) for visual examination of the intestinal lining and collection of caecal digesta material for microbial profiling and SCFA analysis. The intestines were lesion scored according to the method of Johnson and Reid (1970). Caecal contents were collected and pooled per cage, giving six replicated pooled samples per treatment for both days. The samples were stored at -20°C until analysis.

The caecal samples from day 15 were analysed for the abundance of total eubacteria (domain *Bacteria*), the butyrate-producing Clostridial clusters IV and XIVa, *Lactobacillus* group, genus *Bifidobacterium, Escherichia coli* and *C. perfringens* with real-time qPCR. The target gene was the phospholipase c (*plc*) gene for *C. perfringens* and 16S rRNA for all the other microbial groups or species. The annealing temperatures, target gene sequences and references to previously published methods are presented in Table 3. Microbial DNA of the caecal digesta samples was extracted and the real-time PCR analysis was conducted as described in Kettunen *et al.*, (2015). From the same samples, the SCFA profiles consisting of the concentrations of acetic, propionic, butyric, *iso*-butyric, 2-methyl-butyric, valeric, *iso*-valeric and lactic acid were analysed with gas chromatography following the previously published procedure (Kettunen *et al.*, 2015). The total SCFAs were calculated as the sum of all aforementioned SCFA classes. The total concentration of branched chain volatile fatty acids (BCVFAs) was calculated as the sum of *iso*-butyric, 2-methyl-butyric and *iso*-valeric acids.

Performance data were analysed by ANOVA using GenStat® statistical software (17th edition, VSN International Ltd., Hemel Hempstead, UK), with Fisher's Least Significant Difference as a *post-hoc* test, with cage as the experimental unit. For the real-time PCR results, the number of gene copies (or genomes in the case of *C. perfringens*) was initially log10 transformed to achieve a normal distribution. Subsequently, the average and standard error of the mean from both microbial and SCFA results were calculated for each treatment, respectively. Comparisons between the infected control group and other treatments were calculated with Dunnett's two-sided post-hoc test using IBM

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https://doi.org/10.1017/jan.2016.10 Published online by Cambridge University Press

Table 3. Real-time PCR assays of Experiment 2. The target gene was the phospholipase c -gene for Clostridium perfringens and 16S rRNA for the other groups or species

Target group or microorganism	Annealing temp. (°C)	Primer sequence (5′–3′)	Reference
Domain *Bacteria*	60	F: TCCTACGGGAGGCAGCAGT R: GGACTACCAGGGTATCTAATCCTGTT	Nadkarni *et al.* (2002)
Clostridial cluster XIVa	60	F: CGGTACCTGACTAAGAAGC R: AGTTTYATTCTTGCGAACG	Rinttilä *et al.* (2004)
Clostridial cluster IV	62	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTTGTCAA	Matsuki *et al.* (2004)
Lactobacillus group	61	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	Rinttilä *et al.* (2004)
Genus *Bifidobacterium*	65	F: CGGGTGAGTAATGCGTGACC R: TGATAGGACGCGACCCCA	Furet *et al.* (2009)
Escherichia coli	65	F: GTTAATACCTTTGCTCATTGA R: ACCAGGGTATCTAATCCTGTT	Malinen *et al.* (2005)
Clostridium perfringens	62	F: TTACCTTTGCTGCATAATCCC R: ATAGATACTCCATATCATCCTGCT	Tansuphasiri (2002)

SPSS 22 software (SPSS Inc., Chicago, IL). Effects were considered statistically significant at $P < 0.05$ and trends at $P < 0.10$.

Results and discussion

Experiment 1. Dietary RAC had a positive effect on the bird performance over the 42-day rearing period. As shown in Table 4, combined with CC, RAC significantly improved the final BW by 3.3% and FCR by 5.7%. When combined with IC, RAC tended to improve FCR by 3.0% ($P < 0.1$). Moreover, RAC significantly improved the EEI value by 6% in combination with CC and a trend towards the same direction was observed when RAC was combined with IC ($P < 0.1$). Feed intake and mortality were similar in all treatments. The EEI calculation indicated the efficacy of broiler production, as it took into account the feed consumption, final body mass, mortality and the length of the rearing period, and the result suggested that dietary RAC may bring economic benefits to broiler production. Experiment 1 was the first trial to demonstrate the positive performance effects of RAC in a mixed-sex flock of broiler chickens in a commercial-type broiler operation environment.

One of the indicators of welfare in broiler operations is the litter quality, which is affected *e.g.* by diarrhoea. Wet litter is one of the causes of necrotic lesions in the footpads, referred to as foot pad dermatitis (FPD; Shepherd and Fairchild, 2010). Dietary RAC did not

Table 4. Effect of dietary supplementation of resin acid composition (RAC) and either chemical coccidiostat (CC) or ionophoric coccidiostat (IC) on the performance of broilers, the foot pad dermatitis (FPD) score and litter quality score in Experiment 1

	CC, Diclazuril		IC, Salinomycin			ANOVA
	T1, RAC 0 g/kg	T2, RAC 0.5 g/kg	T3, RAC 0 g/kg	T4, RAC 0.5 g/kg	SEM	P-value
Performance, days 0–42						
BWG, kg	2.72^{a}	2.81^{b}	2.76^{ab}	2.81^{b}	0.011	0.014
FCR, kg/kg	1.75^{a}	1.65^{b}	1.69^{ab}	1.64^{b}	0.012	0.002
FI, kg	4.77	4.64	4.68	4.62	0.030	0.269
Mortality, %	2.86	2.29	3.14	2.57	0.427	0.916
EEI value	361.2^{a}	384.4^{b}	367.4^{ab}	383.2^{b}	3.265	0.015
FPD score, scale 0–200						
Day 14	2.00	2.57	1.14	2.57	0.430	0.629
Day 28	69.14^{a}	70.29^{a}	64.00^{a}	52.86^{b}	2.383	0.028
Day 42	98.00^{a}	91.43^{a}	91.14^{a}	74.29^{b}	2.510	0.002
Litter quality score, scale 0–4						
Day 14	0.20	0.20	0.20	0.20	–	–
Day 28	0.77	0.80	0.85	0.80	0.192	0.473
Day 42	1.11	1.05	1.23	1.17	0.035	0.370

FPD score = (number lesion class 1 * 0.5 + number lesion class 2 * 2) x 100)/25. The mean value for each treatment, the pooled standard error of the mean (SEM) and ANOVA P-value are given. Different superscripts (a,b) in same row indicate significant difference between the treatments ($P < 0.05$).

https://doi.org/10.1017/jan.2016.10 Published online by Cambridge University Press

affect litter quality, but it significantly improved FPD scores on days 28 and 42 in the IC group (Table 4). Further research is needed to reveal whether RAC affects footpad health through microbial modulation or some other mechanism.

Experiment 2. During the starter diet period (days one to nine), the treatments did not affect the production variables (data not shown). During the challenge period, the challenged control birds had a 15% lower feed intake, 20% lower body weight gain and 6% impaired FCR compared to the non-challenged control birds (Table 5). However, the challenge induced only a few clinical signs of illness to the birds, and the intestinal lesion scoring revealed only few, mild lesions with no difference between the treatments (data not shown). The challenge significantly decreased FI by 11% and BWG by 14% in the overall study period, compared to the non-challenged control birds.

Dietary RAC partly compensated for the negative effects of the NE infection during the challenge period: the BWG in the RAC 1 g/kg group was significantly higher than in the challenged control group (Table 5). The beneficial effects of RAC continued in the overall study period: RAC at 1 and 2 g/kg increased BWG, and RAC at 1 g/kg increased the BW on day 35. FCR or FI were not significantly affected by RAC, but it is still noteworthy that FI was numerically higher in the RAC-treated groups than in the non-supplemented challenged group both during the challenge and overall study period. In fact, it seems that FI in the (challenged) RAC 1 g/kg group was not depressed by the infection at all. Reduction of FI is indicative of a compromised health status, due to anorexic effects of pro-inflammatory cytokines (Shen *et al.*, 2010).

Supporting earlier findings of Kettunen *et al.* (2015) and Vuorenmaa (2015), the positive effect of RAC on the challenge period performance of the chickens was dose-dependent. Birds in the RAC 1 and 2 g/kg groups performed better than their counterparts in the RAC 3 g/kg group. In comparison, in experiment 1, RAC at 0.5 g/kg improved both BWG and FCR. Thus, there seems to be a relatively low optimum dose of RAC, and exceeding it decreased rather than increased bird performance.

The microbial and SCFA results are presented in Table 6. The abundance of *C. perfringens, E. coli* and Clostridial clusters IV and XIVa and the SCFA profiles were unaffected by dietary treatment. RAC at 1 and 2 g/kg did not affect any microbial groups selected for the study. However, RAC at 3 g/kg significantly decreased the abundance of the *Lactobacillus* groups and tended to decrease that of total eubacteria and the genus *Bifidobacterium,* compared with the non-supplemented control. The SCFA profile was unaffected by the treatments. Previously it has been observed that RAC at 1 g/kg reduced the NE-associated peak of microbial lactic acid production in ileal contents (Kettunen *et al.*, 2015). In the present experiment, RAC at 2 and 3 g/kg numerically decreased caecal lactic acid concentration. Thus, the present data lends little support to the hypothesis that performance effects of RAC are mediated through any influence on caecal microbiota. It was noteworthy that performance effects did not seem to be directly related to the caecal abundance of *C. perfringens*, a finding supported by earlier work on ileal digesta of NE-challenged broiler chicks (Kettunen *et al.*, 2015). However, the data did not preclude the possibility that some other variables of caecal microbiota might have a role in the process.

Table 5. Effect of dietary supplementation of resin acid composition (RAC) on the performance of birds during the challenge period (days 9 to 21) and the overall study period (days 9 to 35) in Experiment 2

	No challenge	Necrotic enteritis challenge with RAC at					
	RAC at 0 g/kg	0 g/kg	1 g/kg	2 g/kg	3 g/kg	SEM	ANOVA P-value
Days 9–21							
BWG, g	757^{c}	609^{a}	689^{bc}	657^{ab}	637 ^{ab}	24.3	0.005
FI, g	1050^{b}	892^{a}	996^{ab}	934^{a}	912^{a}	37.6	0.05
FCR, g/g	1.362^{a}	1.447^{b}	1.436^{b}	1.409^{b}	1.411^{b}	0.0137	0.004
BW, g, d21	986^{b}	828^{a}	913^{ab}	872^{a}	850^{a}	28.9	0.009
Days 9–35							
BWG, g	2190^{c}	1875^{a}	2070^{bc}	2047^{bc}	2016^{ab}	56.9	0.02
FI, g	3269^{bc}	2896^{a}	3357^{c}	3035^{ab}	2986^{ab}	107.3	0.04
FCR, g/g	1.484	1.522	1.627	1.479	1.476	0.0494	0.20
BW, g, d35	2418^{c}	2094^{a}	2294^{bc}	2261^{abc}	2229^{ab}	60.7	0.03

The mean value for each treatment, the pooled standard error of the mean (SEM) and ANOVA P-values are given. Different superscripts (a, b, c) in same row indicate significant difference between the treatments ($P < 0.05$).

https://doi.org/10.1017/jan.2016.10 Published online by Cambridge University Press

Table 6. Effect of RAC on the abundance of microbial species or groups and on the short chain fatty acid (SCFA) profiles in the caecal digesta samples in Experiment 2, expressed as the mean ± S.E. (standard error of the mean)

Variable	No challenge RAC at 0 g/kg	Necrotic enteritis challenge with RAC at			
		0 g/kg	1 g/kg	2 g/kg	3 g/kg
Microbial species/groups[1]					
Domain Bacteria	11.90 ± 0.039	11.98 ± 0.055	11.84 ± 0.035	11.94 ± 0.060	11.76 ± 0.077~
Clostridial cluster XIV	11.38 ± 0.090	11.27 ± 0.101	11.25 ± 0.055	11.34 ± 0.066	11.24 ± 0.094
Clostridial cluster IV	11.15 ± 0.105	11.08 ± 0.076	11.03 ± 0.049	11.07 ± 0.135	10.85 ± 0.085
Escherichia coli	9.54 ± 0.114	9.56 ± 0.118	9.64 ± 0.190	9.70 ± 0.261	9.48 ± 0.114
Genus *Bifidobacterium*	9.94 ± 0.328	9.19 ± 0.476	9.50 ± 0.174	8.69 ± 0.314	7.91 ± 0.119~
Lactobacillus group	11.33 ± 0.070*	11.59 ± 0.059	11.47 ± 0.067	11.43 ± 0.037	11.21 ± 0.052*
Clostridium perfringens	6.71 ± 0.103	7.31 ± 0.149	7.37 ± 0.195	7.54 ± 0.281	7.16 ± 0.208
SCFAs, mmol/l digesta (ww)					
Total SCFAs	95.18 ± 2.70	97.68 ± 3.98	92.89 ± 5.65	86.39 ± 5.33	95.17 ± 3.78
Acetic acid	60.40 ± 1.33	55.98 ± 2.49	55.25 ± 4.28	52.90 ± 3.74	60.20 ± 2.86
Propionic acid	3.54 ± 0.28	3.87 ± 0.61	3.81 ± 0.82	4.61 ± 0.74	5.02 ± 0.75
Butyric acid	18.15 ± 1.70	18.59 ± 1.52	18.65 ± 12.45	18.05 ± 1.48	18.78 ± 1.44
Lactic acid	10.50 ± 1.97	16.15 ± 5.01	12.27 ± 5.80	8.05 ± 1.13	7.97 ± 3.16
Valeric acid	1.14 ± 0.09	1.33 ± 0.17	1.17 ± 0.09	1.27 ± 0.11	1.24 ± 0.19
Total BCVFAs[2]	1.74 ± 0.20	1.44 ± 0.14	1.65 ± 0.13	1.51 ± 0.18	1.95 ± 0.23

Statistical difference from the challenged nonsupplemented (RAC 0 g/kg) treatment is expressed with asterisks: * - $p < 0.05$; ~ - $p < 0.1$ (Dunnett's two-sided post-hoc test). [1]Log_{10} number of the target gene sequence copies/g digesta wet weight (ww) [2]Branched chain volatile fatty acids (sum of *iso*-butyric, 2-methyl-butyric and *iso*-valeric acids).

Future studies are needed on the role of intestinal microbiota in the effects of RAC on poultry.

Conclusions

The present study showed that dietary RAC improved the performance of broiler chickens both under intentional NE challenge and under natural background challenge of a commercial setting. Moreover, the study implied that performance benefits of RAC can be seen throughout the entire five to six-week duration of a broiler grow-out period, using commercial-type diets containing feed enzymes and anticoccidial drugs. Furthermore, the study suggested that the performance benefits of RAC were not associated with a certain microbiological and/or dietary environment, and can be reproduced under varying dietary regimens and environments. The caecal microbial parameters selected for the study did not explain observed performance effects of RAC. The mechanism by which RAC improves the performance of broiler chickens remains to be unveiled in future investigations.

Acknowledgements

The research was funded by Hankkija Ltd.

Declaration of interest

J. Vuorenmaa and E. Valkonen are employed by Hankkija Ltd.

References

Ekstrand C., Algers B., and Svedberg J. (1997) Rearing conditions and foot-pad dermatitis in Swedish broiler chickens. *Preventive Veterinary Medicine*, **31**: 167–174.

Dalloul R.A., and Lillehoj H.S. (2006). Poultry coccidiosis: recent advancements in control measures and vaccine development. *Expert Review of Vaccines*, **5**: 143–163.

Fallarero A., Skogman M., Kujala J., Rjaratnam M., Moreira V.M., Yli-Kauhaluoma J., and Vuorela P. (2013) (+)-Dehydroabietic acid, an abietane-type diterpene, inhibits *Staphylococcus aureus* biofilms *in vitro*. *International Journal of Molecular Sciences*, **14**: 12054–12072.

Furet J.P., Firmesse O., Gourmelon M., Bridonneau C., Tap J., Mondot S., Doré J., and Corthier G. (2009) Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. *FEMS Microbiology Ecology*, **68**: 351–362.

Johnson J., and Reid W.M. (1970) Anticoccidial drugs: Lesion scoring techniques in battery and floor-pen experiments with chickens. *Experimental Parasitology*, **28**: 30–36.

Jokinen J.J., and Sipponen A. (2016) Refined Spruce Resin to Treat Chronic Wounds: Rebirth of an Old Folkloristic Therapy. *Advances in Wound Care*, **5**: 198–207.

Kettunen H., Vuorenmaa J., Rinttilä T., Grönberg H., Valkonen E., and Apajalahti J. (2015) Natural resin acid –enriched composition as a modulator of intestinal microbiota and performance enhancer in broiler chicken. *Journal of Applied Animal Nutrition*, **3**: e2.

Malinen E., Rinttilä T., Kajander K., Mättö J., Kassinen A., Krogius L., Saarela M., Korpela R., and Palva A. (2005). Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *American Journal of Gastroenterology*, **100**: 373–382.

Matsuki T., Watanabe K., Fujimoto J., Takada T., and Tanaka R. (2004) Use of 455 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of 456 predominant bacteria in human feces. *Applied and Environmental Microbiology*, **70**: 7220–7228.

Nadkarni M.A., Martin F.E., Jacques N.A., and Hunter N. (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology*, **148**: 257–266.

Rinttilä T., Kassinen A., Malinen E., Krogius L., and Palva A. (2004) Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in fecal samples by real-time PCR. *Journal of Applied Microbiology*, **97**: 1166–1177.

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https://doi.org/10.1017/jan.2016.10 Published online by Cambridge University Press

Savluchinske-Feio S., Curto M.J., Gigante B., and Roseiro J.C. (2006) Antimicrobial activity of resin acid derivatives. *Applied Microbiology and Biotechnology*, **72**: 430–436.

San Feliciano A., Gordaliza M., Salinero M.A., and Del Corral J.M. (1993) Abietane acids; Sources, Biological Activities, and Therapeutic Uses. *Planta Medica*, **59**: 485–490.

Shen Y.B., Piao X.S., Kim S.W., Wang L., and Liu P. (2010) The effects of berberine 484 on the magnitude of the acute inflammatory response induced by Escherichia coli 485 lipopolysaccharide in broiler chickens. *Poultry Science*, **89**: 13–19.

Shepherd E.M. and Fairchild B.D. (2010) Footpad dermatitis in poultry. *Poultry Science*, **89**: 2043–2051.

Söderberg T.A., Johansson A., and Gref R. (1996) Toxic effects of some conifer resin acids and tea tree oil on human epithelial and fibroblast cells. *Toxicology*, **107**: 99–109.

Tansuphasiri U. (2002) Development of duplex PCR assay for rapid detection of enterotoxigenic isolates of Clostridium perfringens. *Southeast Asian Journal of Tropical Medicine and Public Health*, **32**: 105–113.

Timbermont L., Haesebrouck F., Ducatelle R., and Van Immerseel F. (2011) Necrotic enteritis in broilers: an updated review on the pathogenesis. *Avian Pathology*, **40**: 341–317.

Van Immerseel F., Rood J.I., Moore R.J., and Titball R.W. (2009) Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. *Trends in Microbiology*, **17**: 32–36.

Vuorenmaa J. (2015) Natural resin acid contributes to improved broiler performance. *International Poultry Production*, **23**: Number 6, pp. 17–19.

Welfare Quality® (2009) Welfare Quality® assessment protocol for poultry (broilers, laying hens). Welfare Quality® Consortium, Lelystad, The Netherlands.

Journal of Applied Animal Nutrition

https://doi.org/10.1017/jan.2016.10 Published online by Cambridge University Press