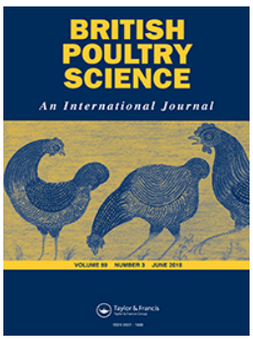

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Tall oil fatty acid inclusion in the diet improves performance and increases ileal density of lactobacilli in broiler chickens

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ABSTRACT

1. Studies were conducted with tall oil fatty acids (TOFA) to determine their effect on broiler chicken performance and ileal microbiota. TOFA, a product originating from coniferous trees and recovered by fractional distillation of side-streams from pulp production, mainly comprises free long-chain fatty acids (~90%) and resin acids (~8%). Conjugated linolenic acids and pinolenic acid are characteristic fatty acid components of TOFA.
2. TOFA products at 750 mg/kg feed were tested in two 35-day broiler chicken trials, each using a wheat soya-based diet and with 12 replicate pens per treatment. In both trials, TOFA improved body weight gain at all time points ($P < 0.001$) and feed conversion efficiency during the first 21 days ($P < 0.01$). Two different dry TOFA formulations (silica carrier and palm oil coating) were tested and showed performance effects similar to liquid TOFA.
3. Ileal digesta of the broiler chickens was analysed for total eubacteria, *Lactobacillus* spp., *Enterococcus* spp., *Escherichia coli* and *Clostridium perfringens* on days 14 and 35. TOFA significantly increased total eubacteria and lactobacilli density on day 14 ($P < 0.05$). There was a significant positive correlation between these bacterial groups and broiler body weight on day 14 ($P < 0.01$).
4. A numerical reduction in *C. perfringens* was observed. *In vitro* growth inhibition studies showed that *C. perfringens* was strongly inhibited by 10 mg/l TOFA ($P < 0.001$), while common lactobacilli were resistant to >250 mg/l. The *in vitro* results were thus in line with *in vivo* observations.
5. The mechanisms behind the bacterial shifts and their role in performance improvement are unknown. Further purification of TOFA components is needed to identify the effective agents.

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Broilers; ileal microbiota; tall oil fatty acids

Introduction

The European Union started banning individual antibiotic growth promoters (AGPs) in 1997 and the ban of all AGPs was implemented in 2006. The consequences of the AGP removal on animal welfare vary depending on the geographical location of production sites and the quality of farm management (Casewell et al. 2003; Grave et al. 2004). Some AGPs suppress growth of Gram-positive lactobacilli in the small intestine and stimulate less sensitive Gram-negative bacteria such as *E. coli* (Apajalahti and Kettunen 2006a).

Plants synthesise compounds that are part of their defence system against microorganisms and which might offer benefits when introduced into animal feeds. Well-known antibacterial components include phenolic compounds, which are found in all plants, and resin acids, which are common in coniferous trees (Pearce 1996; Savluchinske-Feio et al. 2006). Many free long-chain fatty acids have antibacterial properties (Desbois and Smith 2010). The present paper deals with tall oil fatty acids (TOFA), a plant-derived material less studied as a feed component. TOFA is a high-volume by-product from wood processing industries utilising coniferous trees as the raw material. In the Kraft process, acylglycerols are hydrolysed under alkaline conditions into free fatty acids and glycerol. After acidification, the by-product is referred to as crude tall oil. Fractional distillation of crude tall oil produces a TOFA product, the fatty acid profile of which is dependent on the distillation conditions, the type of trees used as raw materials and the climate in which they grow (Logan 1979). The raw material for the TOFA used in the present study originated from northern coniferous trees, mainly Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) grown in Finland, and consisted primarily of free fatty acids (~90%) and resin acids (~8%). Table 1 compares the fatty acid composition of TOFA to common fat sources used in broiler chicken diets. The proportion of saturated fatty acids in TOFA is significantly lower than in any other fat source. The other feature clearly differentiating TOFA from other fat sources is the presence of conjugated linoleic acids and pinolenic acid.

TOFA has been previously studied in limited broiler chicken trials, where it was found to improve body weight in two trials conducted under a necrotic enteritis challenge (Kettunen et al. 2015; Kettunen et al. 2017). In a trial with no challenge, performance improvements were observed in the presence of a chemical coccidiostat (Kettunen et al. 2017).

The present paper describes the effect of TOFA on the performance of broiler chickens in two independent 35-day trials in the absence of coccidiostats or a specific challenge. The effects of TOFA on small intestinal microbiota were studied by monitoring microbial shifts during the trials and determining the susceptibility of key bacteria to TOFA in *in vitro* bacterial growth studies.

Materials and methods

Animals and housing

Two 35-day feeding trials with broiler chickens were conducted in the research facility of Alimetrics Ltd in Southern Finland, in accordance with EU Directive 2010/63/EU. Trial

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Table 1. Fatty acid characteristics of selected dietary fat sources, % of total fatty acids.

Degree of fatty acid saturation	Tallow	Soybean oil	Sunflower oil	TOFA[1]
Saturated	43.0	20.5	10.6	2.8
Monounsaturated	50.0	22.5	20.3	31.1
Diunsaturated	3.0	50.7	64.9	55.2
- conjugated linoleic acids	*0.3*	*0.0*	*0.0*	*8.6*
Triunsaturated	1.0	6.4	0.3	10.2
- pinolenic (columbinic) acid	*0.0*	*0.0*	*0.0*	*7.1*
Others	3.0	0.0	3.9	0.7

Data from Hilditch and Williams (1964), Chin et al. (1992), O'Quinn et al. (2000b), Fritsche et al. (2000) and Sauvant et al. (2004).
[1]Values from analysis report of the manufacturer.

1 was started in June 2014 and Trial 2 in December 2015. Newly hatched chicks were obtained from a commercial hatchery (DanHatch, Mynämäki, Finland) and the birds were not given vaccinations or commercial inoculants. Birds used in Trial 1 were Ross 508 breed and in Trial 2 Ross 308 (Aviagen). In both trials, 540, 1-day-old sexed male broilers were used.

The temperature of the house was raised to 32°C 2 days before the chicks arrived. The chicks were randomly allocated to 36 open floor pens of 1.125 m^2 with wood shavings litter. Luminosity was adjusted to 20 L and air humidity to 60%. Brooder lamps were adjusted to provide extra heating during the first week. The temperature was gradually decreased to 22°C during the first 2 weeks of the trial. Temperature, ventilation and humidity were monitored continuously throughout the trials. From day 1, the dark hours were increased daily by 1 h from 24 h light until the light-dark cycle was 18 h light and 6 h dark daily. Feed and water were freely available at all times. Weight of birds and feed consumption (FC) per pen were measured on days 0, 14, 21 and 35 of the trial period. Dead birds and birds euthanised because of health problems were weighed and daily mortality was recorded.

Dietary treatments

The basal starter and grower diets were wheat-soy based pelleted feeds, the composition of which are shown in Table 2. Two-phase feeding was used: the starter diet was fed from days 0 to 14 and the grower diet on days 14–35. The dietary treatments employed in each trial are shown in Table 3. Both trials had TOFA treatments, but different dry TOFA formulations, with dry TOFA used in Trial 1, referred to as TOFA-S which had 50% TOFA and 50% silica, while that in Trial 2, referred to as TOFA-P, contained 47% TOFA, 47% hydrogenated palm oil and 6% silica. The TOFA dry products were mixed with wheat before inclusion in the diets. The TOFA was mixed with sunflower oil before inclusion in the treatments. TOFA-oil replaced a corresponding amount of sunflower oil in the control diets. Test products were obtained from Hankkija Ltd (Hyvinkää, Finland) and were produced at the tall oil refinery of Forchem Ltd (Rauma, Finland). The TOFA-oil used is a commercial product (Progres™) and the dry TOFA formulations were specifically produced by Hankkija Ltd for the trials. The TOFA products used contained about 90% free fatty acids (of which 50%–55% various linoleic acids, 25%–30% oleic acid and 6%–8% pinolenic acid) and 8%–9% resin acids (mainly abietic, dehydroabietic and pimaric acid). The feeds were manufactured at Natural Resources Institute Finland (Jokioinen, Finland).

Table 2. Composition of basic diets used in the trials.

	Trial 1		Trial 2	
	Starter	Grower	Starter	Grower
Added ingredients (g/kg)				
Wheat	555.2	658.2	578.4	688.5
Soybean meal	360.0	275.0	338.0	247.0
Sunflower oil	40.0	25.0	39.0	23.0
Monocalcium phosphate	17.5	17.0	16.0	17.0
Limestone	15.2	13.5	16.0	12.6
Sodium chloride	4.2	4.2	4.2	4.2
Mineral premix[1]	2.0	2.0	2.0	2.0
Vitamin premix[2]	2.0	2.0	2.0	2.0
DL-methionine	2.0	1.6	2.2	1.8
L-lysine	1.9	1.5	2.2	1.9
Calculated nutrient concentrations				
Metabolisable energy (MJ/kg)	12.24	12.22	12.24	12.25
Crude protein	230.20	199.50	230.60	199.50
Lysine	13.30	10.86	13.30	10.80
Methionine	5.30	4.50	5.50	4.70
Threonine	8.50	7.20	8.40	7.10
Methionine + cysteine	8.50	7.60	9.00	8.00
Calcium	10.00	8.70	10.00	8.67
Non-phytate phosphorus	4.50	4.30	4.18	4.30

[1]Containing: calcium 296.9 g/kg, zinc 32.5 g/kg, manganese 25.0 g/kg, iron 12.5 g/kg, copper 4.0 g/kg, iodine 225 mg/kg, selenium 100 mg/kg.
[2]Containing: calcium 331.3 g/kg, all-rac-α-tocopheryl acetate 30.0 g/kg, niacin 20.1 g/kg, panthotenic acid 7.51 g/kg, riboflavin 3.0 g/kg, pyridoxine 2.01 g/kg, retinol 1.8 g/kg, menadione 1505 mg/kg, thiamine 1257 mg/kg, folic acid 504 mg/kg, biotin 75.0 mg/kg, cholecalciferol, 56.3 mg/kg, cobalamin 12.5 mg/kg.

Table 3. Treatment groups in the trials.

	Trial 1	Trial 2
Treatment	g/kg	g/kg
Control	-	-
TOFA-Oil	0.75	0.75
TOFA-S (TOFA content)	1.50 (0.75)[1]	-
TOFA-P (TOFA content)	-	1.60 (0.75)[2]

[1]Dry TOFA formulation in Trial 1 contained 50% silica and 50% TOFA.
[2]Dry TOFA formulation in Trial 2 contained 47% hydrogenated palm oil, 6% silica and 47% TOFA.

Sampling

On days 14 and 35, two birds from each pen were weighed and euthanised by cervical dislocation. The abdominal cavity was opened, the ileum was removed and the ileal digesta samples were snap-frozen in dry ice and stored frozen until analysis.

Analysis of bacteria in intestinal digesta

The samples from Trial 1 were pooled by pen (12 replicates), while in Trial 2 samples from individual birds were analysed (24 replicates). Bacteria in ileal digesta were lysed and bacterial DNA purified as described previously by Kettunen et al. (2017). Bacteria were quantified by quantitative real-time polymerase chain reaction (PCR) using an ABI Prism Sequence Detection System 7500 instrument (Life Technologies, USA). PCR conditions and primers for analysis of different bacteria were as described by Nadkarni et al. (2002) for total eubacteria, by Rinttilä et al. (2004) for *Lactobacillus* spp. and *Enterococcus* spp., by Malinen et al. (2005) for *Escherichia coli* and by Tansuphasiri (2001) for *C. perfringens*. Primers for *C. perfringens* analysis were designed to amplify the phospholipase C gene of the

pathogen, while all other assays quantified 16S rDNA genes of the bacterial groups. Bacterial densities are expressed as $\log_{10}$ gene copies/g fresh digesta.

Growth inhibition studies with pure bacterial cultures

The effect of TOFA on the growth of bacteria commonly found in the small intestine of broiler chickens was tested using the strains and growth media listed in Table 4. TOFA was diluted in ethanol and 0.5 ml of an appropriate dilution was applied in 20 ml serum bottles. The ethanol was evaporated to dryness, 15 ml of culture medium were added and the bottles sealed with rubber stoppers and aluminium crimps and autoclaved at 121°C for 20 min. The final TOFA concentrations were 0, 10, 50, 250 and 750 mg/l. Bacteria used as inocula were grown overnight at 37°C in a gyratory shaker. Each test culture was inoculated with 1% of a culture grown overnight and incubated for 24 h with continuous shaking. Growth was monitored by measuring turbidity at 6 and 24 h against the fresh growth medium with the Eppendorf BioSpectrometer at 600 nm wavelength in 1 cm cuvettes. Highly turbid cultures were diluted to a corresponding growth medium prior to measurement to reach an absorbance within the linear range and calculated back to the original undiluted culture.

Statistical analysis

Data were subjected to one-way ANOVA using SPSS software (IBM, version 22). Confidence limits were set at 95%, with $P < 0.05$ considered statistically significant. Means from treatment groups were compared by Tukey's Honestly Significant Difference (HSD) test. Pearson correlations between body weight and bacterial densities were analysed by the SPSS software.

Results

Effect of TOFA products on broiler chicken performance

In Trial 1, liquid TOFA oil and the dry TOFA-S (TOFA-silica mixture) both improved mean body weight gain (BWG) of broiler chickens at all time points (Table 5). The magnitude of the increase in body weight (BW) was approximately 50, 100 and 200 g at 14, 21 and 35 days, respectively. The TOFA products increased FC during the periods 0–14, 0–21 and 0–35 days by 1, 2 and 5 kg/pen, respectively. Feed conversion ratio (FCR) improved significantly with TOFA-S and nearly significantly with TOFA oil, during the first 3 weeks. When the weight of dead birds was included in the calculation, the effect of the TOFA products on FCR was even more significant (mortality-corrected FCR). Overall the mortality was relatively high, but there were no differences between the treatments (Table 5).

Table 5. Effect of the liquid (TOFA-Oil) and dry (TOFA-S) TOFA products on performance of broiler chickens in Trial 1.

Parameter	Control	TOFA-Oil	TOFA-S	SE[1]	*P*-value
d 0 to d 14					
Initial BW (g)	43.9	43.9	44.1	0.27	0.812
BWG (g)	399[a]	**446[b]**	**454[b]**	6.35	**0.000**
FC (kg/pen)	7.3[a]	**8.1[b]**	**8.0[b]**	0.13	**0.000**
FCR	1.23	1.23	1.21	0.01	0.360
Mortality (%)	1.1	1.1	2.2	0.92	0.636
Mortality-corrected FCR	1.23	1.21	1.19	0.01	0.166
d 0 to 21					
BWG (g)	860[a]	**962[b]**	**967[b]**	13.25	**0.000**
FC (kg/pen)	17.5[a]	**19.4[b]**	**18.9[b]**	0.33	**0.001**
FCR	1.42[b]	1.36[ab]	**1.36[a]**	0.02	**0.028**
Mortality (%)	3.8	1.1	3.6	1.68	0.526
Mortality-corrected FCR	1.39[b]	**1.35[a]**	**1.34[a]**	0.01	**0.004**
d 0 to 35					
BWG (g)	2286[a]	**2512[b]**	**2498[b]**	22.16	**0.000**
FC (kg/pen)	52.3[a]	**57.2[b]**	56.4[ab]	1.13	**0.015**
FCR	1.65	1.67	1.63	0.03	0.716
Mortality (%)	6.1	7.2	6.7	2.07	0.933
Mortality-corrected FCR	1.59	1.57	1.57	0.01	**0.046**

[ab]Values within rows with different superscripts are significantly different ($P < 0.05$) by Tukey's HSD test.
[1]Pooled SE.

In Trial 2, BW of birds at all measuring points was markedly higher than in Trial 1, the difference between the control birds from each trial being 100 g and 180 g at 21 and 35 days, respectively. Both TOFA oil and the dry product TOFA-P (palm oil-coated TOFA) increased BWG significantly by day 14. At the later time points TOFA-P increased BWG significantly, while the increasing effect of TOFA oil was only numerical (Table 6). No differences between treatments were observed in FC or mortality. Both TOFA oil and TOFA-P improved FCR and mortality-corrected FCR during the first 2 weeks. With both test products, mortality-corrected FCR was significantly improved during the period 0–21 days (Table 6).

Analysis of small intestinal bacteria

TOFA is known to contain antibacterial components and thus may have an impact on bacterial composition in the small intestine. In both broiler chicken trials, selected bacteria were analysed in ileal digesta samples (see Tables 7 and 8 for Trials 1 and 2, respectively). Digesta samples from day 14 showed consistent results in the two trials, with both lactobacilli. and total eubacteria increasing. In Trial 2, the increase was statistically significant for TOFA-Oil, while TOFA-P showed only a numerical increase in the density of these bacterial groups. The magnitude of increase in bacterial density with TOFA-Oil was more than 200% for lactobacilli. and 140% for total eubacteria, while for TOFA-P, the corresponding numerical increase was 130% and

Table 4. Bacterial strains and growth media.

Bacterial species	DSMZ strain number	Growth medium
Lactobacillus crispatus	DSM 20584	MRS broth (Difco, product 288130)
Lactobacillus salivarius	DSM 20555	MRS broth (Difco, product 288130)
Lactobacillus reuteri	DSM 20016	MRS broth (Difco, product 288130)
Escherichia coli	DSM 1103	LB broth (LAB M, product LAB173)
Clostridium perfringens	DSM 756	TSGY broth[1]
Enterococcus faecalis	DSM 20478	MRS broth (Fluka, product 69966)

[1]Trypticase soy broth 30g, yeast extract 3g, glucose 5g, made up to 1 l with distilled water. The medium was boiled and cooled down under flow of anoxic N_2 gas.

Table 6. Effect of the liquid (TOFA-Oil) and dry (TOFA-P) TOFA products on performance of broiler chickens in Trial 2.

Parameter	Control	TOFA-Oil	TOFA-P	SE[1]	*P*-value
d 0 to d 14					
Initial BW (g)	47.4	47.3	47.5	0.42	0.971
BWG (g)	449[a]	**491[b]**	**497[b]**	5.06	**0.000**
FC (kg/pen)	8.5	8.7	8.7	0.08	0.254
FCR	1.27[b]	**1.21[a]**	**1.21[a]**	0.01	**0.000**
Mortality (%)	0.0	2.8	2.4	0.77	0.088
Mortality-corrected FCR	1.27[b]	**1.20[a]**	**1.19[a]**	0.01	**0.000**
d 0 to 21					
BWG (g)	956[a]	1001[ab]	**1043[b]**	13.55	**0.001**
FC (kg/pen)	20.2	20.0	20.8	0.29	0.194
FCR	1.46[b]	1.44[ab]	**1.38[a]**	0.02	**0.008**
Mortality (%)	3.2	6.0	3.1	1.24	0.190
Mortality-corrected FCR	1.44[b]	**1.40[a]**	**1.37[a]**	0.01	**0.001**
d 0 to 35					
BWG (g)	2467[a]	2557[ab]	**2610[b]**	33.21	**0.017**
FC (kg/pen)	57.2	57.0	60.2	1.05	0.083
FCR	1.68	1.65	1.62	0.02	0.219
Mortality (%)	6.7	8.3	4.2	1.62	0.260
Mortality-corrected FCR	1.62	1.60	1.60	0.02	0.807

[ab]Values within rows with different superscripts are significantly different ($P < 0.05$) by Tukey's HSD test.
[1]Pooled SE.

Table 7. Effect of dietary TOFA on the density of bacteria in ileal digesta of 14- and 35-day-old broiler chickens in Trial 1.

Bacterium/group	Control	TOFA-Oil	TOFA-S	SE[1]	*P*-value
Day 14					
Total eubacteria	11.1	11.4	11.3	0.13	0.272
Lactobacillus spp.	10.1[x]	**10.5[y]**	10.4[xy]	0.13	**0.097**
Enterococcus spp.	7.8	8.3	7.8	0.18	0.147
E. coli	6.2	6.7	6.3	0.27	0.513
C. perfringens	7.3[ab]	7.3[b]	**6.0[a]**	0.37	**0.034**
Day 35					
Total eubacteria	11.5	11.5	11.5	0.11	0.909
Lactobacillus spp.	10.5	10.5	10.6	0.13	0.821
Enterococcus spp.	8.7	8.6	8.5	0.19	0.692
E. coli	5.9	6.0	6.2	0.28	0.735
C. perfringens	7.3	7.0	6.8	0.28	0.460

Bacterial densities are expressed as $\log_{10}$ gene copies/g fresh digesta.
[ab]Values within rows with different superscripts are significantly different ($P < 0.05$) by Tukey's HSD test.
[xy]Values within columns with different superscripts exhibit a near-significant trend ($0.05 < P < 0.10$) by Tukey's HSD test.
[1]Pooled SE.

100%, respectively. In Trial 1, the effects were similar but less significant, possibly due to the lower number of replicates analysed. No dietary effects were observed on the densities of enterococci and *E. coli* (Tables 7 and 8). The samples from day 35 showed no significant diet effects for the bacteria analysed.

To gain an understanding of the link between BW and small intestinal bacteria, correlation analysis was performed whereby bacterial densities in each chicken were compared against its BW, regardless of treatment group. The results indicated that on day 14, there was a statistically significant, positive correlation between BW and the density of total eubacteria and lactobacilli. in both trials (Table 9). In Trial 2 the correlation persisted and was still significant on day 35.

Effect of TOFA on growth of intestinal bacteria in vitro

In order to explain the bacterial effects described above, an *in vitro* growth inhibition study was carried out for TOFA and common small intestinal bacteria. *C. perfringens* proved highly sensitive to TOFA, its growth being completely

Table 8. Effect of dietary TOFA on the density of bacteria in ileal digesta of 14- and 35-day-old broiler chickens in Trial 2.

Bacterium/group	Control	TOFA-Oil	TOFA-P	SE[1]	*P*-value
Day 14					
Total eubacteria	10.9[a]	**11.3[b]**	11.1[ab]	0.10	**0.044**
Lactobacillus spp.	10.0[a]	**10.5[b]**	10.3[ab]	0.12	**0.015**
Enterococcus spp.	7.7	8.1	7.9	0.16	0.294
E. coli	5.9	5.7	5.7	0.18	0.727
C. perfringens	6.0	5.5	5.6	0.25	0.355
Day 35					
Total eubacteria	11.4	11.3	11.5	0.10	0.611
Lactobacillus spp.	10.4	10.4	10.6	0.13	0.584
Enterococcus spp.	8.0	7.7	7.9	0.15	0.395
E. coli	5.8	5.7	5.9	0.22	0.680
C. perfringens	6.4	6.3	6.6	0.26	0.750

Bacterial densities are expressed as $\log_{10}$ gene copies/g fresh digesta.
[ab]Values within rows with different superscripts are significantly different ($P < 0.05$) by Tukey's HSD test.
[1]Pooled SE.

inhibited by the 10 mg/l concentration during the first 6 h (Table 10). During the following 18 h, the bacterium started to grow in the presence of the lowest TOFA concentration tested, but no growth took place at 50 mg/l or higher concentration. Onset of *Enterococcus faecalis* growth was delayed by the highest concentrations of TOFA, but no inhibition was detected after 24 h. *E. coli*, the only Gram-negative bacterium tested, proved resistant to TOFA and no inhibition was detected even at the highest concentrations used. Among the lactobacilli included in the study, *L. crispatus* was most susceptible to TOFA. At 250 mg/l concentration, some growth suppression was detected at the first time point. At the concentration of 750 mg/l the inhibitory effect was evident still after 24 h of growth. *L. reuteri* and *L. salivarius* were affected by TOFA only at the highest concentration and during the first 6 h of incubation. At the 24-h time point, no differences in the growth of these bacterial species were detected between the treatments (Table 10).

Discussion

The introduction of TOFA into broiler chicken diets improved performance of the birds in two independent trials, one of which was conducted in summertime with Ross 508 and the other 18 months later (in wintertime) with Ross 308 birds. In both trials, the beneficial effect on BWG was detectable throughout the 35-day trial period and amounted to 250 g and 100 g by the end of Trial 1 and 2, respectively. The improvement in FCR was only significant during the first 21 days of the trials and ranged between four and seven points on day 21. The results suggest that TOFA played a role in early development of the broiler chickens. It is worth noting, however, that if the FCR for the period 0–35 days was corrected for the major differences in the final BW then the FCR improvement by TOFA would likely be evident also for 0–35 days. In Trial 2, the birds in the control treatment were heavier than the control group in Trial 1 from day old to 35 days. Thus, the greater scope for improvement in Trial 1 birds may be one of the reasons why the positive effect of TOFA products was more pronounced in that trial. Trial 1 birds had lower FC which seemed to be more tightly linked to BWG than in Trial 2. The conditions in both trials appeared to be challenging,

Table 9. Pearson correlation between body weight of broiler chickens and densities of selected bacteria in small intestinal digesta.

	Total eubacteria		*Lactobacillus* spp.		*Enterococcus* spp.	
Origin of broiler chicken	Correlation[1]	Significance[2]	Correlation	Significance	Correlation	Significance
Trial 1, BW day 14	**0.38**	0.026	**0.38**	0.023	**0.43**	0.010
Trial 1, BW day 35	0.84	0.844	0.58	0.581	0.17	0.173
Trial 2, BW day 14	**0.34**	0.004	**0.31**	0.009	0.12	0.341
Trial 2, BW day 35	**0.25**	0.038	**0.31**	0.012	0.08	0.492

[1]Pearson correlation coefficient.
[2]Two-tailed significance of the correlation.

Table 10. Effect of TOFA concentration on the growth of intestinal bacteria, measured as absorbance at 600 nm after 6 and 24 h of incubation.

Bacterial species	0 mg/l	10 mg/l	50 mg/l	250 mg/l	750 mg/l	SE[1]	*P*-value
Growth at 6 h							
C. perfringens	4.29^{b}	**0.00^{a}**	**0.00^{a}**	**0.02^{a}**	**0.01^{a}**	0.018	**0.000**
Ent. faecalis	0.20^{c}	0.21^{c}	0.18^{c}	**0.13^{b}**	**0.07^{a}**	0.006	**0.000**
E. coli	1.18^{a}	1.25^{a}	1.34^{ab}	1.30^{ab}	**1.48^{b}**	0.035	**0.003**
L. crispatus	0.20^{c}	0.21^{c}	0.21^{c}	**0.16^{b}**	**0.09^{a}**	0.006	**0.000**
L. reuteri	1.41^{b}	1.33^{b}	1.16^{b}	0.99^{b}	**0.41^{a}**	0.051	**0.001**
L. salivarius	2.62^{b}	2.24^{b}	2.18^{b}	1.94^{b}	**0.28^{a}**	0.147	**0.000**
Growth at 24 h							
C. perfringens	6.55^{b}	6.27^{b}	**0.00^{a}**	**0.03^{a}**	**0.03^{a}**	0.0351	**0.000**
Ent. faecalis	1.20^{ab}	**1.33^{c}**	1.26^{bc}	1.20^{ab}	1.15^{a}	0.0102	**0.000**
E. coli	1.27^{a}	1.24^{a}	1.19^{a}	1.34^{a}	**1.57^{b}**	0.0283	**0.001**
L. crispatus	9.56^{b}	9.66^{b}	9.57^{b}	9.30^{b}	**4.45^{a}**	0.1595	**0.000**
L. reuteri	6.85	6.75	6.72	6.80	7.14	0.0810	0.066
L. salivarius	8.16	8.19	8.28	8.32	8.39	0.1303	0.771

[abc]Values within rows with different superscripts are significantly different ($P < 0.05$) by Tukey's HSD test.
[1]Pooled SE.

judging from the high bird mortality (5%–7%) during the trials.

The TOFA oil used in the trials was from different production batches, but the effects obtained with the liquid oil products were still consistent. In Trial 1, the formulation of TOFA was a mixture with silica (TOFA-S), the rationale being to render the product dry and easier to apply in feed. The results with the two formulations (oil or dry) of TOFA in Trial 1 were nearly identical, indicating that the silica carrier in TOFA-S was inert and had no effect on the effectiveness of the product. The dry TOFA-P product in Trial 2 was a palm oil-coated TOFA-silica cocktail. The coating appeared to improve performance, resulting in slightly better performance with TOFA-P than TOFA-Oil. It is possible that the effective agents in the coated TOFA-P were released at a lower rate, thus retaining higher product concentrations in distal sections of the intestinal tract.

Published literature on the use of TOFA in animal feeds is surprisingly scarce, especially when taking into account the high volume of TOFA locally available in countries performing wood processing and pulping. Kettunen et al. (2015, 2017) studied the effect of a similar TOFA product to that used in the present study in necrotic enteritis challenge trials. They found that TOFA increased BW significantly but had no effect on FCR, possibly due to the challenge imposed. TOFA was found to improve performance in the presence of a chemical coccidiostat (Diclazuril) but only tended to improve performance when an ionophoric coccidiostat (Salinomycin) was used. Ionophoric coccidiostats may have stronger effects on intestinal microbiota than chemical coccidiostats, which may partly explain the findings (Kettunen et al. 2017).

Analysis of key bacteria in the ileum of broiler chickens showed consistent results in both trials. The density of total lactobacilli in ileal digesta increased with the TOFA oil diet, significantly in Trial 2 and as a strong trend in Trial 1. The results for total bacteria were similar, most likely because lactobacilli comprise a large proportion of total bacteria in small intestine (Apajalahti and Kettunen 2006a; Apajalahti and Kettunen 2006b). qPCR assays used are designed to amplify genes for which the chromosomal copy number is not necessarily the same in different bacterial groups. Therefore, the comparison of abundance of different bacteria is not accurate, while it is safe to compare dietary effects within each bacterial group. The correlation analysis of lactobacilli and BW in individual birds (independent of treatment) showed a statistically significant correlation on day 14. This is surprising, since increasing small intestinal bacterial density is generally reported to reduce the availability of dietary nutrients for the host (Lin 2014; Apajalahti and Vienola 2016). This does not exclude the possibility that enrichment of specific species of the genus *Lactobacillus* in the presence of TOFA promoted BWG. It is possible that TOFA promoted BWG and enriched lactobacilli independently, with no causal relationship between the two. The density of *E. coli* and *Enterococcus* spp. did not show consistent numerical differences between treatments, while the density of *C. perfringens* showed a trend for a reduction in the presence of TOFA products at day 14.

Most of the components of TOFA, including the long-chain fatty acids and resin acids, have been shown to inhibit the growth of bacteria. There are many studies on the antibacterial potency of free fatty acids and, although it is difficult to compare various studies due to differences in methodology, some trends have been found. In general, unsaturated fatty acids are more antibacterial than saturated fatty acids and there is a positive correlation between the number of double bonds and antibacterial efficacy (Desbois and Smith 2010). In TOFA, more than 95% of all fatty acids are unsaturated, the degree of unsaturation being higher than in the common fat sources used in animal feeds. Up to 10% of all fatty acids in TOFA are triunsaturated, while

the abundance of triunsaturated fatty acids in the sunflower oil used in the base diet in the trials described above is only 0.3% (Sauvant et al. 2004). About 8% of TOFA is composed of resin acids (mainly abietic, dehydroabietic and pimaric acids), which are hydrophobic diterpene carboxylic acids with antimicrobial, antifungal and antiparasitic activities (San Feliciano et al. 1993; Rubio et al. 2005; Savluchinske-Feio et al. 2006).

The main bacterial taxa inhabiting the upper intestine of broiler chickens are species of the genera *Lactobacillus, Enterococcus* and *Escherichia* (*E. coli*). The most common *Lactobacillus* spp. in the upper intestine of broiler chickens are *L. crispatus, L. salivarius* and *L. reuteri* (Abbas Hilmi et al. 2007). Although these are often considered a homogeneous group of bacteria, in reality the species differ markedly from each other. *L. reuteri* is the only heterofermentative species in chicken intestine, meaning that in addition to lactic acid it also produces acetic acid, ethanol and CO_2 from its metabolism. *L. crispatus* and *L. salivarius* are homofermentative and produce mainly lactic acid (Wood and Holzapfel 1995). *L. salivarius* strains isolated from chicken intestine have been found to produce a bacteriocin that effectively inhibits the growth of many pathogens, such as *Campylobacter jejuni*, various serotypes of *Salmonella enterica*, pathogenic strains of *E. coli* and *C. perfringens* (Svetoch et al. 2011). *L. reuteri* also produces a bacteriocin called reuterin, using glycerol as a precursor (Doleyres et al. 2005). It is possible that TOFA does not enrich all lactobacilli, but selects for specific species. The results of the *in vitro* growth inhibition studies with the relevant bacterial species indicated that the three *Lactobacillus* species tested tolerated at least 50-fold higher TOFA concentration than *C. perfringens*. There were slight differences in the tolerance of different species, *L. crispatus* being somewhat more sensitive than *L. salivarius* and *L. reuteri. E. coli*, the only Gram-negative bacterium tested, was not inhibited by TOFA. The results of the pure culture studies were in line with the results of bacterial analysis of the broiler chicken digesta.

The mechanism influencing increased feed intake and growth by TOFA is not clear. Consistent shifts in microbiota were detected in the two trials, but the direct reasons for these shifts and their role in performance improvement are not known. TOFA contains specific and potent antimicrobial components, pinolenic acid, conjugated linoleic acids and resin acids, all at about the same concentration, 50–70 mg/kg of feed. These acids have been shown to inhibit growth of some bacteria already at one-tenth of the concentration added to the trial diets (Galbraith et al. 1971; Kettunen et al. 2015). Differing sensitivity of bacterial species to active agents may well offer a competitive advantage to resistant bacteria, whose numbers may be elevated, as was observed in this study for lactobacilli. The mechanism for performance enhancement may lie in systemic effects of the TOFA components. Revealing the potential systemic effects was beyond the scope of this paper, but such mechanisms are known for several components of TOFA, e.g. conjugated linoleic acids, pinolenic acid and other free fatty acids. These compounds have been shown to affect carbohydrate and lipid metabolism, immune functions and the regulation of hormone production (Houtsmuller 1981; O'Quinn et al. 2000a; Pariza et al. 2001; Christiansen et al. 2015; Xie et al. 2016; Mielenz 2017).

Future studies should be conducted with purified fractions of TOFA to identify the role of different components and determine whether performance and microbial effects can be obtained with a purified compound or if a mixture of several acids is required. The fate of TOFA components in broiler chickens need to be studied, to determine any residual concentrations of the most potent acids in the intestinal tract and how rapidly and in which part of the digestive tract they are taken up by the host.

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