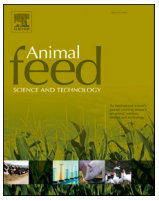
Animal Feed Science and Technology 309 (2024) 115888

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci



Effect of the dietary administration pattern of silver nanoparticles on growth performance, biodiversity of digestive microbiota and tissue retention in broiler chickens

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ARTICLE INFO

Keywords:
Silver nanoparticles
Broiler feeding
Additives
Caecal microbiota
Growth performance
Tissue retention

ABSTRACT

The effects of dietary inclusion of silver nanoparticles (NanoAg, <100 nm diameter) on growth performance, gut microbiota, and silver tissue retention was assessed in broilers from 1 to 42 days of age. A total of 870 1-day-old male broilers (Ross 308) were weighted and distributed in 36 floor pens (n = 24) in an environmentally controlled room. The feeding program consisted of two periods (1–21 days and 22 to 42 days of age) and two experimental diets with the same ingredient composition including 2 g kaolin/kg with or without adsorbed NanoAg (10 mg/g kaolin). The experimental design was completely randomized, and the effects of the length of the period in which the birds received NanoAg (none, Ag0; from 1 to 21 days, Ag21; or from 1 to 35 days, Ag35) on growth performance, biodiversity of digestive microbiota and silver retention in body tissues were studied. A common feed without NanoAg was provided to all pens from 36 days onwards. At 21 and 42 days of, one random bird per pen (n = 12) was slaughtered and cecal samples were collected from 9 birds per treatment randomly selected to analyse gut microbiota. Besides, samples of liver and breast muscle were collected to determine silver tissue retention. From 1 to 21 days of age, NanoAg supplementation tended to improve feed conversion ratio (FCR, P = 0.070). From 22 to 35 days of age, FCR tended to be lower (P = 0.072) and average daily gain (ADG) was greater (P < 0.001) in broilers fed Ag21 and Ag35 than in those fed Ag0. Cumulatively, ADG was greater (P < 0.001) for Ag21 and Ag35 than for Ag0, but FCR was unaffected. Caecal microbiota was affected by age of birds, but dietary supplementation with NanoAg did not modify bacterial community structure, diversity and taxa distribution in the caecum neither at 21 nor at 42 days. At 21 days of age, silver retention in liver was 0.591 mg/kg dry tissue in all broilers supplemented with NanoAg. However, at 42 days of age, silver retention in the liver was only detected in two (0.139 mg/kg, n = 2) and 8 (0.183 mg/kg, n = 8) birds fed Ag21 and Ag35, respectively. Silver retention in muscle was exclusively detected at 42 days in two birds (0.124 mg/kg, n = 2) fed Ag35. Irrespective of the supplementation period, NanoAg increased ADG from 1 to 42 d. No silver retention was detected in the liver nor in breast muscle after 21 days of the end of treatment. However, traces of silver might remain in the liver 7 days after removal of

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio; NanoAg, silver nanoparticles.

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https://doi.org/10.1016/j.anifeedsci.2024.115888
Received 26 May 2023; Received in revised form 11 January 2024; Accepted 12 January 2024
Available online 25 January 2024

NanoAg. Silver supplementation might be a promising strategy to improve growth performance in broilers without expecting any changes in the gut microbiota nor tissue retention after 21 days of NanoAg withdrawal.

1. Introduction

The ban on the use of ZnO as growth promoter in the European Union and the need to reduce the therapeutic use of antibiotics in animal feeding have increased the interest for alternative solutions to improve animal performance. Under these circumstances, nanoscale materials have emerged as new antimicrobial agents because of their high surface-to-volume ratio and unique chemical and physical properties (Morones et al., 2005). In this respect, different types of nanometals like copper, zinc, titanium (Schabes-Retchkiman et al., 2006), magnesium, gold (Gu et al., 2003), and silver (Byers and Gong, 2007) have been proposed as potential antimicrobial additives in animal feeding. Silver nanoparticles (NanoAg, <100 nm diameter) have a wide range of applications, such as food storage, household products, disinfectants, textiles, and medical equipment (Furno et al., 2004; Poynton et al., 2012). In addition, NanoAg may exert an antimicrobial effect, are resistant to gastric inactivation, and have a low intestinal absorption rate. As a result, the potential risk of toxicity is reduced (Fondevila et al., 2009; Fondevila, 2010). The doses that promote physiological and productive effects in pig feeding are low and range from 20 to 40 mg NanoAg/kg diet (Fondevila et al., 2009).

The objective of this study was to evaluate the growth-promoting effects of using NanoAg in broiler feeding and to determine its potential influence on digestive microbial population and silver retention in target tissues (liver and breast muscle) depending on the time of offering. It was hypothesised that an early removal of the additive might reduce the risk of silver retention in tissues at slaughter age, without compromising the benefits of NanoAg in the diet on growth performance.

Table 1
Ingredient (g/kg) and chemical (g/kg dry matter) composition of the experimental diets.

	Starter (1–21 days)	Growing (22–42 days)
Ingredient composition	407	388
Wheat	154	255
Corn	357	283
Soybean meal, 470 g CP/kg	40	40
Soybean oil	17.1	11.9
Dicalcium phosphate	9.6	8.3
Calcium carbonate	3.7	3.4
Sodium chloride	4.0	4.0
Vitamin-mineral premix[1]	2.7	2.1
DL-methionine	2.0	1.7
-lysine HCl	0.6	0.6
-threonine	2.0	2.0
Kaolin[2]		
Analysed composition	895	894
Dry matter, g/kg as fed	934	944
Organic matter	246	213
Crude protein (CP)	57	62
Ether extract	96	89
Neutral detergent fibre	335	409
Starch		
Estimated composition	2970	3065
AMEn, kcal/kg	10.0	8.0
Calcium	4.8	3.9
Digestible phosphorous		
Digestible amino acids:	12.0	10.0
Lysine	5.6	4.8
Methionine	8.8	7.6
Methionine + Cystine	7.6	6.6
Threonine	2.5	2.1
Tryptophan	8.5	7.2
Isoleucine	9.4	8.1
Valine		

[1] Included per kg of feed: vitamin A, 9500 IU; vitamin D_3, 3400 IU; vitamin E, 30 mg; vitamin K_3, 2.5 mg; vitamin B1, 2 mg; vitamin B2, 6.5 mg; vitamin B6, 3 mg; vitamin B12, 0.02 mg; pantothenic acid (d-calcium pantothenate), 12 mg; folic acid, 1 mg; biotin, 0.13 mg; choline, 270 mg; niacin, 45 mg; manganese ($MnSO_4 \cdot H_2O$), 90 mg; zinc (ZnO), 75 mg; iron ($FeSO_4 \cdot H_2O$), 33 mg; copper ($CuSO_4.5\ H_2O$), 8 mg; iodine [$Ca(IO_3)_2$], 1.1 mg; selenium (Na_2SeO_3), 0.35 mg; BHT, 2 mg; 6-phytase (EC 3.1.3.26), 1500 FYT

[2] Including or not 10 mg NanoAg/g kaolin depending on dietary treatment

2. Material and methods

2.1. Animals, diets and experimental design

The procedures used in this research were approved by the Animal Ethics Committee of the University of Zaragoza, Spain (procedure PI55/18) and were in compliance with the Spanish Policy for Animal Protection RD 53/2013 (Spanish Law, 2013), which complies with EU Directive 2010/63 (EU, 2010) on the protection of animals used for experimental and other scientific purposes. A total of 870 one-day old male broilers (Ross 308) were obtained from a commercial hatchery. Chicks were vaccinated against Marek's disease virus and infectious bronchitis. At the arrival to the experimental farm, the chicks (individual weight ranging from 37 to 41 g) were allotted at random into 36 floor pens (1.0 × 2.0 m^2) in groups of 24 broilers each (average weight 945 ± 22 g per pen). The floor pens were provided with sawdust bed, and a feeder and nipple drinkers were installed. The experimental barn was environmentally controlled, and the light schedule consisted in 23 h light from 1 to 4 days of age, followed by a gradual decrease to reach 18 h light per day from day 7 onwards. Room temperature was maintained at 30–32ºC on days 1 and 2 and then, it was gradually reduced and maintained at 20 ºC from 28 to 42 days of age.

The feeding program consisted of two periods (starter phase, from 1 to 21 days of age, and grower phase, from 22 to 42 days of age). For each period, a feed in mash form based on wheat, corn, and soybean meal, was formulated to meet bird requirements (FEDNA, 2018). Each diet was supplemented, or not, with 20 mg NanoAg (27 nm average particle size) per kilogram, provided by Laboratorios ENOSAN (Zaragoza, Spain). The NanoAg particles were adsorbed onto kaolin (Al2Si2O5(OH)4, 10 mg NanoAg/g kaolin) as a carrier to facilitate a homogeneous distribution and included at 2 g NanoAg kaolin per kilogram to reach the silver dose. Further details on the chemical characteristics of the product have been previously published (Rodríguez-Garraus et al., 2022). The same amount of kaolin but without NanoAg was also included in the control feed to avoid any nutrient dilution effect. Feed and water were provided for ad libitum consumption throughout the experiment. Ingredient and chemical composition of the experimental feeds are presented in Table 1.

The experimental design was completely randomized, with three treatments differing in the length of the period in which the birds were supplemented with NanoAg. There was a control group (Ag0), in which the chicks did not receive the tested additive throughout the experiment, and two experimental groups which received the diet with NanoAg from 1 to 21 days (Ag21) or from 1 to 35 days (Ag35) of age. Each treatment was replicated 12 times. From 36 to 42 days of age, all birds were fed with a common growing diet free of additives.

Feed disappearance and BW of the birds were recorded by pen at 21, 35 and 42 days of age. From these data, average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were determined by feeding phase and cumulatively. Mortality was recorded and weighed as produced and the data was used to correct FCR. In addition, at end of the starter and grower phases, one bird per pen (12 from Ag0 and 6 from Ag21 and Ag35 at 21 days, and 12 per treatment at 42 days) was selected at random, euthanized under CO_2 atmosphere, and weighed individually. Samples of liver and breast muscle were collected from these birds to determine silver tissue retention. In addition, 9 of these birds per treatment were selected at random and the caecal contents (approximately 5 g) were sampled, immediately frozen in liquid N, and stored at –80 ºC for further analysis of gut microbiota. At the end of the experiment all animals were slaughtered with the same procedure.

2.2. Analytical procedures

Representative sample of the experimental diets were ground through a 1-mm sieve and analysed for dry matter, ash, crude protein, and ether extract following the AOAC procedures ref. 934.01, 942.05, ref. 976.05 and 2003.05 (AOAC, 2005), respectively. Besides, the concentration of neutral detergent fibre was analysed as described by Mertens (2002) with an Ankom 200 Fibre Analyser (Ankom Technology, New York, USA), using α-amylase and sodium sulphite, and the results were expressed exclusive of residual ashes. Total starch content was determined enzymatically in samples ground to 0.5 mm by using a commercial kit (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland). In addition, composition in other nutrients (apparent metabolizable energy, amino acids and minerals) in the feeds were calculated according to FEDNA (2021).

The bacterial community of the caecal samples collected at 21 and 42 d of age was analysed by amplicon sequencing using a MiSeq V3 (600 cycles) kit (Illumina Inc., San Diego, CA, USA) at the Genomics Service of the Instituto de Parasitología y Biomedicina López Neyra (IPBLN-CSIC, Granada, Spain). Data were analysed as described by (Palma-Hidalgo et al., 2021). Briefly, samples of caecal content were freeze-dried and bead beaten for 1 min (Mini-BeadBeater, BioSpec Products, Bartlesville,OK, USA), and DNA was extracted using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen Ltd., Barcelona, Spain). Negative controls for the DNA extraction and sequencing were also included. The prokaryotic universal primers used for the amplification were Pro341F 5′-CCTACGGGAGGCAGCAG-3′ and Pro805R 5′-GACTACNVGGGTATCTAATCC-3′ targeting the V3_V4 prokaryotic hypervariable region of the 16 S rRNA gene (Takahashi et al., 2014). Primer-sorted and demultiplexed paired-end reads were performed, and downstream processing was performed using QIIME 2 (Bolyen et al., 2019). Low-quality reads ($Q < 25$) and chimeras were identified and removed using chimera.vsearch. Denoising was performed using DADA2 and Amplicon Sequence Variants (ASV) were aligned against Greengenes 13_8 97 % for bacteria (DeSantis et al., 2006). Once alignment was performed, data from each of the two major microbial groups were processed separately. The number of sequences per sample for each microbial group was normalized across all the samples, singletons were removed and the diversity indexes (Richness, Shannon and Simpson indexes) were calculated. The treatments effects on the bacterial community structure was visualized using Principal Coordinate Analysis (PCA) based on the Bray-Curtis dissimilarity metrics. Logarithm transformed data were analysed by non-parametric permutational analysis of variance

(PERMANOVA) after 999 random permutations based on the Monte Carlo test using the Primer and PERMANOVA software (PRIMER-E Ltd., Plymouth, UK).

Total silver retained in liver and breast muscle tissues was determined by ICP-MS technology. The tissues (2 g sample) were cut into small pieces and ground using a stainless-steel ball mill provided with 25 mm-diameter balls for 3 min at 25 Hz. Then, 200 mg of the ground sample were added to a 3 mL of 38 % HCl and 7 mL of 69 % HNO_3 solution and attacked by microwaves for 30 min at 200 °C and 800 psi. The samples were brought to 50 mL MilliQ water with a 3 % HCl solution. The digested samples were analysed by ICP-MS with a Perkin Elmer NexION 2000 ICP-MS equipment (Perkin Elmer, Toronto, Canada). Finally, a calibration curve ranging from 0.3 to 15 μg L-1 was constructed and an internal standard of Rh 10 ppb was also used and prepared in 1 % HNO_3 to calculate the total silver concentration in the tissues. The accuracy of ICP-MS analyses was assessed by the use of the certified reference material DOLT-4 (National Research Council Canada, Ottawa, Canada) with a known silver concentration, and by the evaluation of the analytical recovery assays by spiking the involved samples. A recovery of 1.04 and 0.98 was obtained in lyophilised breast muscle and liver, respectively (n = 10).

2.3. Statistical analysis

Data on growth performance and gut microbiota were analysed as a completely randomized design by ANOVA with three treatments according to the length of the period of NanoAg supplementation (Ag0, Ag21, and Ag35) using the GLM procedure of SAS (SAS Institute, 2018). In addition, a non-orthogonal contrast was also planned to study the effect of dietary inclusion of NanoAg (comparison of Ag0 vs. Ag21 and Ag35). Each treatment was replicated 12 times, except for microbiological studies (n = 9), and for all measurements the experimental unit was the floor pen. Data describing mortality, the relative abundance of bacterial taxa and silver retention were explored for normality using the Shapiro-Wilk test. Relative abundance of bacterial taxa did not meet normality requirements according to the Shapiro-Wilk test and consequently, data were analysed by the Kruskall-Wallis non-parametric test. Differences were considered significant at $P < 0.05$ and a trend to significance was considered at $P < 0.10$. Tukey's test was used for comparison among means.

3. Results

The analysed silver concentration in the starter feeds was 0.04 ± 0.01 and 12.33 ± 1.41 mg Ag/kg for the non-supplemented and the supplemented diets, respectively (n = 6). In the growing phase, these concentrations were 0.08 ± 0.00 and 15.96 ± 2.31 mg Ag/kg, respectively. The Ag concentration in the experimental feeds is considered in agreement with expected target doses.

Overall mortality along the experiment was low (0.013 respect to the total initial number) and no mayor differences among treatments were detected (P = 0.695). The effects of dietary supplementation of NanoAg on growth performance in broilers from 1 to 42 days of age are presented in Table 2. From 1 to 21 days of age, ADG tended to be greater in broilers fed Ag35 than in those fed Ag0 or Ag21 (P = 0.084) but ADFI was not affected. As a result, broilers fed Ag35 showed better FCR than those fed Ag0 (P < 0.05), with Ag21 broilers being intermediate. In fact, broilers supplemented with NanoAg (Ag21 and Ag35) tended to show better FCR than Ag0 birds according to the contrast analysis (P = 0.070). At 35 days of age, body weight of the birds was greater (P = 0.001) for birds given NanoAg (Ag21 and Ag35) than for those fed Ag0. As a result, from 22 to 35 days of age, ADG was greater (P < 0.001) and FCR tended to be better (P = 0.072) when NanoAg were included in diet respect to Ag0. From 36 to 42 days of age, a period in which all the birds

Table 2
Effect of offering control feed (Ag0) or feed added with 20 mg NanoAg/kg for 1 to 21 days (Ag21) or 1 to 35 days (Ag35) on individual body weight (g), average daily feed intake (ADFI, g/d), average daily gain (ADG, g/d) and feed conversion ratio (FCR, g/g) of broiler chickens.

Phase		Ag0	Ag21	Ag35	SEM	P-value
Body weight		39.6	39.2	38.9	0.31	0.317
day 1		907	910	932	8.4	0.084
day 21[1]		2313b	2394a	2448a	17.3	<0.001
day 35[2]		3098b	3200a	3258a	23.2	<0.001
day 42[2]						
1 to 21 days	ADFI	61.8	61.6	60.4	0.55	0.168
22 to 35 days	ADG	41.3	41.5	42.5	1.00	0.084
	FCR[1]	1.499a	1.486ab	1.421b	0.0192	0.018
	ADFI	172.4	180.3	174.3	3.18	0.206
36 to 42 days	ADG[2]	100.4b	106.0a	108.2a	1.00	<0.001
	FCR	1.718	1.704	1.616	0.0306	0.072
	ADFI	286.0	292.1	316.5	13.72	0.285
	ADG	112.2	115.1	115.7	1.87	0.392
1 to 42 days	FCR	2.558	2.551	2.750	0.1369	0.529
	ADFI	136.1	139.6	136.1	2.14	0.423
	ADG[2]	72.8b	75.3a	76.6a	0.55	<0.001
	FCR	1.868	1.858	1.778	0.0324	0.124

SEM: standard error of means. The pen (n = 12) was considered as the experimental unit
[1] Contrast Ag0 vs. Ag21, Ag35, P < 0.10
[2] Contrast Ag0 vs. Ag21, Ag35, P < 0.001. Within a row, letters indicate treatment differences (P < 0.05)

receive a common diet, growth performance parameters were not affected by treatment but differences on broiler BW were maintained ($P < 0.001$). Cumulatively, ADG over the entire production period was greater ($P < 0.001$) for the Ag21 and Ag35 groups than for the Ag0 group, but FCR was not affected.

Bacterial sequencing of caecal contents generated a total of 839,185 high quality sequences. The sequencing depth was normalized to 10,000 sequences per sample allowing a high coverage (average 0.98). Principal Coordinate Analysis and Permanova (Fig. 1A) identified a clear effect of the age of the chicken on the bacterial community ($P < 0.001$). In particular, bacterial diversity increased with the age of the birds in terms of richness (221 vs 310 ASVs, $P < 0.001$), Shannon Index (4.95 vs 5.37, $P = 0.027$) and Simpson Index (0.988 vs 0.993, $P < 0.001$) for animals sampled at 21 and 42 days of age, respectively. The caecal bacterial community was dominated by Firmicutes (65.1 %, Table 3) and Bacteroidetes (23.9 %) and other minority phyla such as Verrucomicrobia (4.57 %) and Proteobacteria (1.90 %). Animals sampled at 21 days of age had higher abundance of Actinobacteria ($P = 0.05$) and Bacteroidetes ($P = 0.034$), whereas those sampled at 42 days of age had higher levels of Cyanobacteria ($P = 0.032$), Proteobacteria ($P = 0.004$) and Verrucomicrobia ($P = 0.004$).

No differences on the bacterial community structure were observed across dietary treatments at 21 and 42 days of age (Fig. 1B and C, respectively) nor on the bacterial diversity (Table 3). Dietary treatments also had minor effects on the bacterial taxa abundance since only 3 % of the taxa differed significantly. The genus *Bacteroides* ($P = 0.029$) and the species *Bacteriodes cellulosolvens* ($P = 0.067$) were the only taxa increased with NanoAg supplementation when chickens ere sampled at 21 days of age ($P = 0.007$). On the contrary, at 42 days of age the chicken in the treatment Ag21 tended to have higher abundances of the phylum Actinobacteria ($P = 0.041$), the family Corynebacteriaceae ($P = 0.055$), the genus *Corynebacterium* ($P = 0.055$), and the species *Brevibacterium casei* ($P = 0.065$) and *Helicobacillus massiliensis* ($P = 0.005$), than those receiving the treatments Ag0 or Ag35.

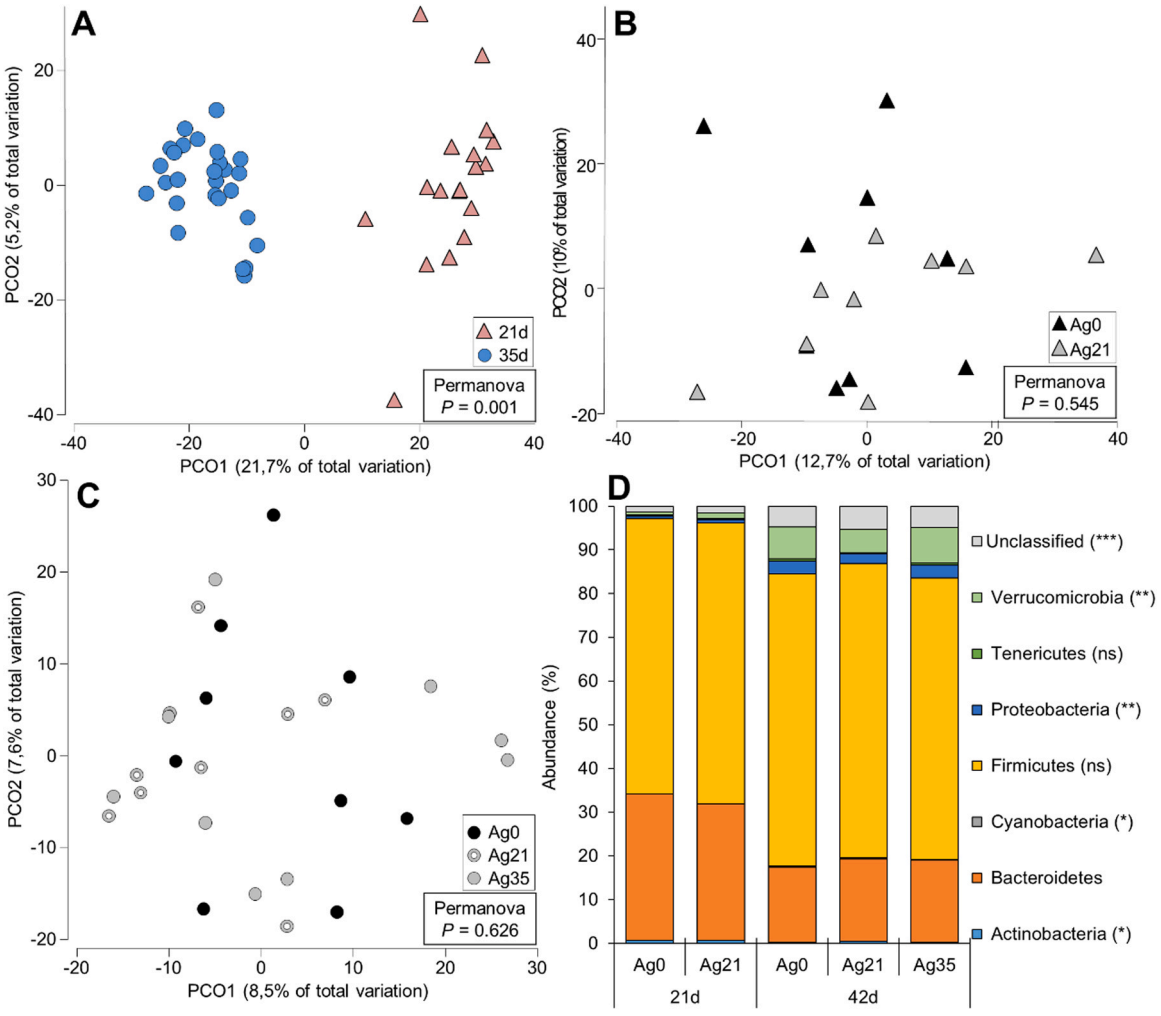

Fig. 1. : Principal co-ordinate (PCoA) analysis showing the effect of age (A) and NanoAg dietary supplementation on the caecal bacterial community in broilers at 21 (B) and 35 days of age (C). PERMANOVA values are provided based on the Bray-Curtis dissimilarity. Bacterial taxa distribution (D) is provided indicating the effects of the age (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS not significant). Broilers were diets without (Ag0) or with 20 mg NanoAg/kg from day 1 to 21 (Ag21) or 35 (Ag35) days of age.

Table 3
Effects of NanoAg supplementation on the caecal bacterial diversity and taxa abundance in broilers of 42 days of age fed diets without NanoAg (Ag0) or with 20 mg Nano Ag/kg from 1 to 21 days (Ag21) or from 1 to 35 days (Ag35) of age.

Treatment[1]	Ag0	Ag21	SEM	P-value
Diversity				
Richness (ASV)	218	224	9.288	0.895
Shannon Index	4.93	4.97	0.051	0.627
Simpson Index	0.99	0.99	0.001	0.566
Abundance (%)				
Firmicutes / Bacteroidetes ratio	2.140	2.347	0.2714	0.508
Bacteroides spp.	0	0.028	0.0069	0.029
Bacteroides cellulosolvens	0	0.026	0.0069	0.067

SEM: standard error of means. One bird per pen (n = 9) was considered as the experimental unit

Analytical values of silver retention (as silver concentration in dry tissues) and number of positive results (N; out of 12 birds) in breast muscle and liver in broilers at 21 and 42 days of age (end of the starter and grower phase, respectively) are presented in Table 4. Irrespective of age or tissue, results on silver retention in samples from the Ag0 group were in all cases below the detection limits (<0.112 mg Ag/kg). At 21 days of age, only one out of 12 broilers supplemented with NanoAg from 1 to 21 days of age retained silver in breast muscle (individual concentration 0.187 mg/kg dry tissue). However, silver was detected in all the livers (n = 12) collected form broilers fed NanoAg at this age with greater values ($P < 0.001$) compared to the control group. At 42 days of age, silver retention in the breast muscle was not detected in broilers from the Ag21 group, and only in two birds from the Ag35 group (individual values of 0.118 and 0.129 mg Ag/kg dry tissue). At this age, silver retention in the liver was detected in two birds from Ag21 (individual values of 0.127 and 0.151 mg/kg dry tissue) and in 8 birds from those fed Ag35. The contrast analysis detected differences between Ag35 and the other two treatments ($P < 0.05$), but no differences were detected between the Ag21 and the Ag0 groups.

4. Discussion

The beneficial role of metallic silver nanoparticles on health of the gastrointestinal tract through its selective effect on potentially pathogenic microorganisms has been documented (Sondi and Salopek-Sondi, 2004; Wadhera and Fung, 2005). In fact, NanoAg supplementation has been used with a minimal risk of toxicity (Lansdown, 2006; Bergin et al., 2016) but there is not much literature dealing with the potential responses on animal growth performances. In a study with a small number of weaned piglets (n = 5 per treatment), Fondevila et al. (2009) observed an increase in weight gain when adding 20 or 40 mg/kg NanoAg, but this was not validated in the subsequent trial at a larger scale.

Published results on the inclusion of NanoAg in poultry feeds are inconsistent. Elkloub et al. (2015) observed a 0.12 to 0.15 higher growth rate, and improved overall FCR in 0.08 units in broilers supplemented with 2 to 10 mg NanoAg/kg of feed compared to non-supplemented birds. However, other studies reported no significant responses on growth performance to the inclusion of NanoAg in a range of 20 to 60 mg/kg (Fondevila, 2010; Ahmadi, 2012) or 4 to 12 mg/kg (Ahmadi et al., 2013). Pineda et al. (2012) observed numerical improvements on growth performance in broilers fed 10 and 20 mg NanoAg/kg from 7 to 36 days of age compared to the control, although these differences were not significant. The lack of significant differences might be partly related to a low number of replicates used per treatment, with four to six cages per treatment and 4 to 15 birds per cage in most studies. In the present experiment, ADG from 1 to 42 days of age increased by 0.04 with NanoAg supplementation. Furthermore, NanoAg improved FCR when included in the diet in the starter and the grower periods. The relevance of this result relies on the high number of replicates (n = 12, with 24 birds per pen), resulting in a low coefficient of variation (0.032 for ADG). These results suggest that potential benefits on growth

Table 4
Effect of offering control feed (Ag0) or feed added with 20 mg NanoAg/kg for 21 (Ag21) or 35 (Ag35) days of age on the caecal bacterial diversity and taxa abundance in broilers of 42 days of age.

Treatment[1]	Ag0	Ag21	Ag35	SEM	P-value
Diversity					
Richness (ASV)	311	326	293	6.788	0.107
Shannon Index	5.36	5.41	5.35	0.019	0.236
Simpson Index	0.99	0.99	0.99	0.001	0.361
Abundance (%)					
Firmicutes / Bacteroidetes ratio	3.967	3.614	3.544	0.1275	0.408
Phylum Actinobacteria	0.246ab	0.429a	0.232b	0.0389	0.041
Fam. Corynebacteriaceae	0.004	0.060	0.018	0.0105	0.055
Gen. *Corynebacterium*	0.004	0.060	0.018	0.0105	0.055
Brevibacterium casei	0	0.017	0.009	0.0039	0.065
Helicobacillus massiliensis	0a	0.092b	0.014a	0.0153	0.005
Clostridium colinum	0.024	0.004	0	0.0050	0.098

SEM: standard error of means. One bird per pen (n = 9) was considered as the experimental unit. Within a row, letters indicate treatment differences ($P < 0.05$)

Table 5
Average silver retention (mg Ag/kg dry tissue), median and number of positives (N, out of 12 sampled birds per treatment) in breast muscle and liver in broilers at 21 and 42 days of age, given diets without (Ag0) or with 20 mg NanoAg/kg for 21 (Ag21) or 35 (Ag35) days of age.

Bird age	Tissue		Ag0	Ag21	Ag35	SEM	P-value
21 days	Muscle	Ag conc.	0.000	0.016		0.0110	0.328
		Median	0.000	0.000			
		N	0	1			
	Liver	Ag conc.	0.000	0.591		0.0470	<0.001
		Median	0.000	0.523			
		N	0	12			
42 days	Muscle	Ag conc.	0.000	0	0.021	0.0080	0.127
		Median	0.000	0.000	0.000		
		N	0	0	2		
	Liver	Ag conc.	0.000b	0.023b	0.122a	0.0209	<0.001
		Median	0.000	0.000	0.130		
		N	0	2	8		

Individual values below the quantification limit (0.112 mg Ag/kg) are assumed as 0.0
SEM: standard error of means. One bird per pen (n = 12) was considered as the experimental unit
Within a row, letters indicate treatment differences (P < 0.05)

performance might be expected by supplementing broiler diets with 20 mg NanoAg/kg feed.

The study of the caecal bacterial community showed that broilers experienced a progressive microbial development driven by the age, consisting of an increase in bacterial diversity (from 221 to 310 ASV at 21 and 35 days of age, respectively) and a shift in the overall community structure with a substantial increase in the Firmicutes to Bacteroidetes ratio. Previous studies have suggested that dietary supplementation with nanoparticles can also have a broad effect on the bacterial community accelerating the gut microbial development leading to increases in the Firmicutes to Bacteroidetes ratio in mice (Williams et al., 2015; van den Brule et al., 2016). This response may be due to the fact that aerobic bacteria, more typical from immature gut microbiomes such as those present in young broilers, are more sensitive to NanoAg than anaerobic bacteria (Lu et al., 2013). The present study did not agree with this observation since no differences were noted between treatments on the overall bacterial community structure, the abundance of the main microbial taxa nor on the Firmicutes to Bacteroidetes ratio. However, it has also been suggested that silver intake may exert positive responses associated to subtle and more selective antimicrobial effects against selective pathogens. This effect may occur by altering membrane functions (Percival et al., 2005; Lok et al., 2006) and inhibiting cell replication (Morones et al., 2005; Yang et al., 2009). In this respect, an antibacterial effect of low concentrations of silver nanoparticles on potential pathogens such as *Escherichia coli, Salmonella spp., Staphylococcus aureus,* and others has been reported in vitro (Baker et al., 2005; Shrivastava et al., 2007; Smekalova et al., 2016). In the current study, broilers remained in good health status throughout the experiment and the caecal abundance of *E. coli* and *Salmonella spp*. were under the detection limits, while genus *Staphylococcus* represented negligible abundances (below 0.01 %). In a previous dose-response study, our team observed that NanoAg supplementation tended to promote a decrease of coliforms and a quadratic decrease in the proportion *Clostridium perfringens/Clostridium histolyticum* ratio in weaned pigs (Fondevila et al., 2009). Similarly, Sawosz et al. (2009) reported an increase in the *Lactobacillus* concentration of caecal content from quails supplemented with NanoAg at 25 mg/kg, whereas others showed increasing levels of the Lactobacilllaceae and Lachnospiraceae families in mice fed nanoparticles (Williams et al., 2015; van den Brule et al., 2016). In the present study, the average abundances of *Lachnospiraceae*, *Clostridium* and *Lactobacillus* were 16 %, 10 % and 1.4 %, respectively, and were unaffected by the experimental treatments. However, increasing exposure to NanoAg tended to promote a progressive decrease in the caecal abundance of the pathogen *Clostridium colinum*, associated to ulcerative enteritis in chickens (Ononiwu et al., 1978). The observed increase in the caecal abundance of certain minoritarian taxa at 21 days (*Bacteroides cellulosolvens*) and 42 days of age (*Brevibacterium casei* and *Helicobacillus massiliensis*) in broilers fed NanoAg may suggest an indirect effect associated to a niche replacement by these microbes. These findings suggest a minor effect of NanoAg on the gut microbiota, although it cannot be discarded that more positive effects could appear when broilers are exposed to higher pathogen loads as a result of more challenging environmental conditions.

One of the main characteristics that an additive may comply is to be innocuous for both animals and consumers. This lack of toxicity is linked to its potential tissue retention. In a study with mice, Bergin et al. (2016) observed that 70 to 100 % of the metallic silver (20 and 110 nm particle size) ingested for 3 days was excreted in faeces. Moreover, the tissue retention in the liver, that was below 0.01 % of the total silver dosed, was similar to the non-supplemented mice, and similar responses were recorded in spleen and kidneys. In growing pigs dosed with 20 or 40 mg NanoAg/kg feed from 1 to 35 days post-weaning, Fondevila et al. (2009) did not detect any silver retention in muscle or kidneys (n = 18 piglets per treatment), whereas some degree of silver retention was observed in the liver of these pigs. In laying hens receiving colloidal silver nanoparticles (20 nm particle size) for 20 days, Gallocchio et al. (2017) observed silver retention in the liver (from 0.141 to 0.269 mg/kg) and egg yolks, but not in muscles or kidneys compared with non-supplemented hens. In any case, silver retention in tissues is expected to be affected by the dose, particle size and its presentation form (Bergin et al., 2016; Frohlich and Frohlich, 2016; Abad-Álvaro et al., 2019).

In a previous study (Fondevila, 2010), silver retention in muscle of 42 days-old broilers supplemented with 20 to 30 mg NanoAg/kg of feed from 1 to 35 days of age was detected in half of the sampled birds (n = 10 out of 20). In the present experiment, such proportion dropped to 0.17 of birds fed with 20 mg NanoAg/kg for 35 days, and was not detected in those supplemented for 21 days and maintained non-supplemented for another 21 days. These results demonstrate the low retention capacity of silver particles in broiler

muscles, and their ability to reduce silver recovery to non-detectable levels after 21 days of withdrawal. As observed (Fondevila et al., 2009; Bergin et al., 2016), liver is more susceptible to retain silver, but in this case, 0.83 and 0.33 of animals given NanoAg for 21 and 35 days were able to detoxify this organ at the end of the growth period (42 days of age).

5. Conclusions

The supplementation of broiler diets with silver nanoparticles at an inclusion rate of 20 mg/kg can be considered as a promising strategy to improve growth performance without expecting substantial changes in the gut microbiota. Besides, silver retention in muscle tissue was not detected after 21 days of withdrawal and was only detected in 2 out of 12 birds after 7 days of withdrawal. However, silver retention in liver, though of low magnitude, was detected in several animals. In any case, further studies are required to confirm specific levels of silver retention in body tissues in farm conditions with a higher number of animals and a more challenging environment.

Declaration of Competing Interest

None.

Acknowledgements

This work has been supported by the European Fund for Regional Development Interreg POCTEFA EFA-183-16 (UE), with the participation of the Department of Industry and Innovation of the Government of Aragón (Spain) and the European Social Fund. K. Ben-Jeddou was financed by the EU Horizon 2020 programme under the Marie Sklowdowska-Curie grant agreement no. 801586, and A. Belanche was contracted by the Spanish State Research Agency (AEI: 10.13039/501100011033) through the Ramón y Cajal program (RYC2019-027764-I, Government of Spain).

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