LIIsimposiocientíficode avicultura MÁLAGA del 28 al 30 de octubre de 2015

ASOCIACIÓN ESPAÑOLA DE CIENCIA AVÍCOLA
Sección Española de WPSA
www.wpsa-aeca.es

COM-12

The effect of a protected sodium butyrate dietary intervention on *Campylobacter* and *Eimeria* species infection in broilers

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Campylobacteriosis by *Campylobacter coli* and *C. jejuni* is the most common cause of bacterial foodborne illness in humans resulting in widespread economic costs. Poultry meat contaminated with gut contents is the primary source of infection. Alternative methods of control are needed, as the use of prophylactic antibiotics is no longer allowed. As part of an experiment to study the effects of a protected sodium butyrate dietary intervention on the broiler gut microbiota and *Campylobacter jejuni*, we also characterised the effects on *Eimeria spp*. parasite, cause of intestinal coccidiosis. Day-old Ross 308 pullet chicks (n= 144) were split in subgroups of 12, to give 4 replicates (pens) for each of the 3 treatments. Environmental conditions in the experimental unit met the Home Office regulations during the thirty-six day trial. Diets were: control (standard commercial feed), AM3 (feed with additive at 3kg/tonne) and AM5 (feed with additive at 5kg/tonne), supplied ad libitum. *Campylobacter*-positive dirty litter was applied to the housing floors. Faeces were sampled on a weekly basis from the pen floors and McMaster faecal egg counts (FEC) were performed.On days 21, 28 and 36, 3 birds/pen were euthanized and samples collected from luminal content and gut tissue, liver, spleen and caeca. Lesion scoring for *Eimeria spp*. was performed using the method described by Johnson and Reid (1970). Caecal luminal content was used to enumerate total levels of *Campylobacter spp.* (CFU/g); then, genomic DNA was extracted to perform *Campylobacter* species-specific real-time PCR. Dietary treatment did not have overall significant effects on *Campylobacter spp*. counts in caeca, *Eimeria spp.* FEC, lesion scores or body weight gain/FCR. On day 36 all groups had similar *Campylobacter spp*. counts and control group had lower *Eimeria spp*. FEC. A significant positive correlation between *Eimeria spp.* FEC and *Campylobacterspp.* counts in caeca was found for AM3 and AM5 groups The effect of the sodium butyrate additive in the variables studied is not conclusive. Further analysis such as qPCR need to be concluded in order to support this hypothesis.

La campilobacteriosis por *Campylobactercoli* y *C. jejuni* es la causa más comúnde intoxicación alimentariade origen bacteriano en humanos, que ocasiona costes económicos generalizados. La principal fuente infección es la carne de ave contaminada por contenido intestinal. Son necesarios métodos de control alternativos puesto que el uso de antibióticos como profilaxis ya no es una práctica permitida. Como parte de un experimento para estudiar los efectos del butirato de sodio incluido en la dieta sobre la microbiota intestinal del broiler y *Campylobacterjejuni*, también caracterizamos los efectos sobre el parásito *Eimeria spp*., agente

causal de coccidiosis intestinal. Pollitas de un día Ross 308 (*n*= 144) se separaron en grupos de 12, dando 4 réplicas (corrales) para cada uno de los tres tratamientos. Las condiciones ambientales en la unidad experimental siguieron la normativa indicada por la Home Office durante los 36 días experimentales. Las dietas fueron: control (pienso comercial convencional), AM3 (pienso con el aditivo a 3kg/t) y AM5 (pienso con el aditivo a 5kg/t), administradas *ad libitum*. Cama positiva a *Campylobacter*fue distribuida en los corrales. El muestreo de heces fue semanal y se realizaron recuentos parasitarios usando el método McMaster. En los días 21, 28 and 36 se eutanasiaron 3 aves/corral y se tomaron muestras de contenido y tejido de intestino, hígado, bazo y ciegos. Las lesiones intestinales por *Eimeria spp*. fueron examinadas siguiendo el método descrito por Johnson and Reid (1970). Los niveles totales de *Campylobacterspp*. (CFU/g) fueron calculados a partir del contenido cecal y posteriormente se extrajo el ADN para realizar PCR a tiempo real específica para*Campylobacter*. El tratamiento dietético no tuvo efectos significativos en el recuento de *Campylobacter*en ciego, recuento de *Eimeria spp*. en heces, lesiones intestinales, ganancia de peso o indice de conversión. En el dia 36, todos los grupos tuvieron similares recuentos de *Campylobacter* yel grupo control registró recuentos más bajos de *Eimeria spp*. Se identificó correlación positiva entre losrecuentos de *Eimeria spp.*en heces y *Campylobacter* en ciegos para los grupos AM3 y AM5. El efecto del aditivo butirato de sodio sobre las variables estudiadas no es concluyente, pero es necesario completar análisis como la PCR a tiempo real para apoyar esta hipótesis.

Keywords: *Campylobacter*; *Eimeria*; qPCR; sodiumbutyrate.

Palabras clave: *Campylobacter*; *Eimeria*; qPCR; butirato de sodio.

Introduction

Campylobacteriosis by *Campylobacter coli* and *C. jejuni* is the most common cause of bacterial foodborne illness in humans. Poultry meat contaminated with gut contents at slaughter is the primary source of infection, which occurs by cross-contamination and direct consumption of meat. Most poultry flocks are thought to be colonized with *Campylobacter spp.* and approx. 80% of the carcasses are positive at retail (Humphrey, 2006), fact that raises concerns not only in public health due to its zoonotic potential but also in food production and animal welfare,as economic costs are high.

The interaction of *Campylobacter spp.*with other microorganisms in the chicken gut supports a relationship with the host other than commensalism, as it may act as an opportunistic pathogen in the case of a co-infection, colonising the gut more easily (Humphrey, 2006). Dose-dependent *Campylobacter spp.* counts in caeca when chicken are co-infected with *E. tenella* have been identified, and that is though to be related to the increased mucus and blood in intestinecaused by*E. tenella*infection (Macdonald *et al*., 2015). Besides, *Eimeria spp.*has also proved to be associated with other chicken pathogens providing a growth advantage to the bacterium, like for *Clostridium perfringens* (Al-Sheikhly and Al-Saieg, 1980; Collier *et al*., 2008).

Alternative methods of control are needed as the use of prophylactic antibiotics is no longer allowed.Thus, these aim to help the developmentof healthy gut microbiota that competitively excludes *Campylobacter spp.* from the caeca. Butyrate is a volatile short-chain fatty acid, acting as a biodegradable weak acid able to inhibit the proliferationof pathogen microorganisms without affecting the chicken gut microflora (Fernández-Rubio *et al.*, 2009). As part of an experiment to study the effects of a protected sodium butyrate dietary intervention on the broiler gut microbiota and

Campylobacter jejuni, we also characterised the effects on *Eimeria spp*. parasite, cause of intestinal coccidiosis.

Materials and methods

Animals and housing: day-old *Gallus gallusdomesticus*Ross 308 pullet chicks (*n*= 154) were collected from hatchery and 10 birds were euthanised to use as pre-treatment samples.

The broiler facility consists of 4 self-contained berths, each of which was split to provide 3 pens, giving a total number of pens = 12. Remaining birds (*n* = 144) were split in three groups of 48, to form the control and intervention groups (AM3 and AM5). These birds were randomly allocated to pens in subgroups of 12, with pen acting as the experimental unit. As a result, each treatment was applied to 4 pens with 12 birds per pen. All birds were exposed to *Campylobacter* positive top-litter (~100g/pen)sourced by the hatchery; the dirty litter was applied to the housing floors in two occasions during the trial, at days 1 and 14.

Temperature of the top litter in the poultry housing was adjusted at 32°C prior to arrival of chicks, then gradually reduced by around 0.5°C per day from day 2 until day 29; thereafter the temperature was maintained at 18°C. The chicks' behaviour was used as a guide when setting the infrared heat lamp temperature and height. Humidity was kept above 65% to a maximum of 75% and ventilation set at 60m per minute from around 2h post-arrival of chicks.

Hours of light and darkness followed the plan: 1 hour of darkness (day 1), 2h (day 2) then increasing by 1h per day until day 6,when 6h of darkness was reach(from 10pm to 4am). This was maintained between days 6 and 32, and reduced to 2h of darkness between days 33 and 36 (depletion).

The trial lasted for thirty-six days, when broilers often reach a finish weight of 2.2 kg.Environmental conditions and stocking density satisfied the regulations detailed by the Home Office Code of Practice.

Diet: control and intervention feed was supplied and formulated by Wynnstay Group Plc. (Powys, Llansantffraid, UK) as a starter, grower and finisher ration, with added coccidiostats. Analysis of the butyrate (measuring butyric acid with GC-FID) was performed to confirm inclusion of the additive in the intervention feeds.The control group birds were fed standard commercial feed, AM3 group had commercial feed containing AdiMix30C at 3kg/tonne and AM5 group had commercial feed containing AdiMix30C at 5kg/tonne (additive supplied by NutriAd International NV). Diets were offered from day 0 and food consumption was recorded daily. Throughout the trial birds had access to feed and water *ad libitum*, via pan feeders and nipple drinkers that were gradually adjusted to the size of the birds.

Live weights of all birds were recorded on a weekly basis.

Faecal sampling: faeces were sampled on a weekly basis from day 14, taking 1 sample per pen in a zip bag. Pen floors were examined following a W shape and fresh faeces were collected. Bags were closed with care to eliminate all air contained inside. Samples were stored at +4°C.

Faecal egg counts (FEC) were performedin fresh faeceswithin 24-48h of faecal collection, using the McMaster method (sensitivity: 25 eggs/g faeces). This method included an initial step of mixing faeces with tap water and straining through muslin plus a centrifugation step, which should help to release the oocysts into the water solution and to reduce faecal debris of the sample (grass, fibrous material, feathers, etc.). The salt saturated flotation solution was made up in the laboratory and adjusted to a specific gravity of 1.18-1.20. Only few samples were prepared simultaneously for microscopy screening and the waiting time indicated for the samples to stand in the final step was entirely respected. Oocysts were examined with the 12.5x microscope objective lens, counted and recorded.

Tissue, lumen and viscera sampling and exams: on days 21, 28 and 36, 3 birds from each pen wereeuthanised, weighed and eviscerated. The following samples were collected: luminal content and gut tissue from the duodenum/jejunum, ileum, caeca, large intestine and crop (only on day 36); liver, spleen and caeca. Luminal content from the caecal samples was subsampled into test tubes containing maximum recovery diluent (MRD) and the subsample transferred to the laboratory for microbial analysis. This involved performing four additional logarithmic serial dilutions from each initial 1:10 subsample, then plating out 100µl of each dilution (-1 to -5) in triplicate on selective media (mCCDA) to detect and enumerate total levels of *Campylobacter spp.* as CFU/g.All collected samples were flash-frozen, stored at -80°C and later freeze-dried (except gut sections)

Intestines were also subjected to lesion scoring by the method described by Johnson and Reid (1970), which provides a numerical ranking of the gross lesions caused by each *Eimeria* species. It was performed by macroscopic examination of the whole intestine divided in four areas (duodenum and upper intestine, middle intestine, lower intestine/ileum and rectum, caeca). A score from 0 to 4was assigned to each region depending on the severity of the lesions. Scrapings of tissue were screened for oocysts microscopically. Gut sections were stored in tubes containing 10% neutral buffered formalin.

DNA extraction and molecular biology work: total genomic DNA was extracted from freeze-dried day 0 whole-gut luminal content samples, day 14 faecal samples and days 21, 28 and 36 caecal luminal content samples. Selected samples were tested using *Campylobacter* species-specific real-time PCR and changes in microbial populations will also be profiled in selected samples using Illumina whole metagenome shotgun sequencing.

Body scans: in addition, 1 bird from each pen was euthanised and frozen on day 36 to perform a whole body scan, in order to get the body composition, fat content and bone density data using a dual energy X-ray absorptiometry (DEXA) densitometry scanner.

Statistical analysis: data reported were analysed using SPSS software package version 22 (SPSS, Chicago, IL, USA). T-tests, two and one-way ANOVAand Pearson's product-moment tests were carried out. Data from counts were transformed by $\log_{10}$ before statistical analysis. Significance was accepted when $p<0.05$.

Results

No significant differences were identified for chicken performance parameters(body weight gain and FCR),body composition (fat content, weight of heart, liver and spleen, breast and leg) or bone density among the dietary treatment groups. Overall mortality rate was 6.9%.

The microscopic analysis revealed a mixed species infection of *Eimeria* parasites.

Dietary treatment did not have a significant effect on *Eimeria spp.*FEC ($p>0.05$) (Table 1); neither was significance in the interaction diet*time. Time was highly significant ($p<0.01$)

*Eimeria spp.*FEC increased with the time for all treatments, while both AM5 and AM3 were negative on day 14 (*Figure 1*). AM5 treatment was the one giving higher FEC at the middle of the trial (day 21 and day 28), and finishing with similar FEC as AM3. The latter followed a similar pattern throughout the trial but had lower FEC compared to AM5. Control group was positive to *Eimeria spp.* earlier and, even if followed an increasing trend in FEC, these values kept lower throughout the last third of the trial (day 28 and day 36).

Table 1. Average values of *Eimeria spp.* FEC (log_{10} oocysts/g faeces) by dietary treatment and positive replicates (pens) across the time.

	Day 14		Day 21		Day 28		Day 36	
Diet	**FEC**	**% positive replicates**	**FEC**	**% positive replicates**	**FEC**	**% positive replicates**	**FEC**	**% positive replicates**
Control	0.817	25	1.373	50	2.938	100	3.678	100
AM3	0	0	0.942	50	3.490	100	4.676	100
AM5	0	0	1.809	75	4.181	100	4.593	100

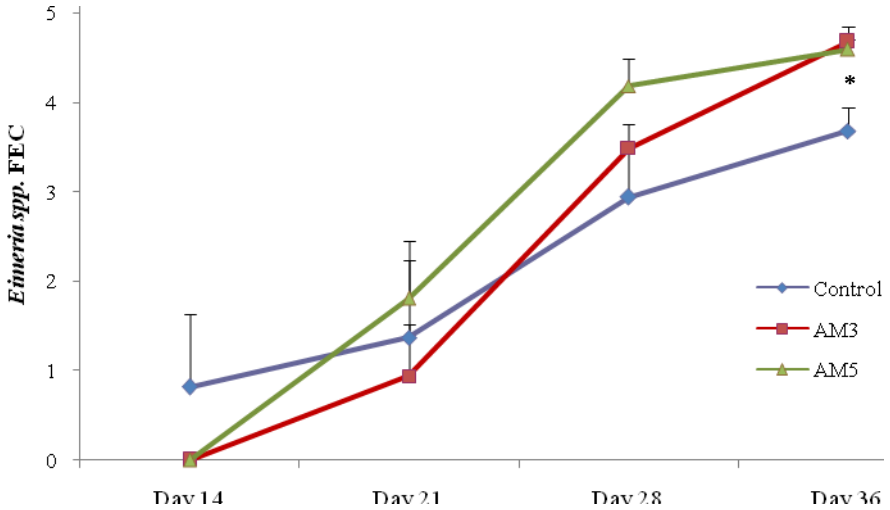

Figure 1. Average values of *Eimeria spp.* FEC (log_{10} oocysts/g faeces) by dietary treatment across the time. Significant differences between treatments are indicated by asterisks (*)

The gross lesion exam of the intestins for lesion scoring also showed the presence of several *Eimeria* species infecting the chicken's guts. The region with highest scores, therefore more affected on its function, was mid intestine (jejunum) followed by low intestine (ileum, large intestine, rectum), identified for all treatments.For the purpose of data analysis, an average lesion score per bird was calculated as the sum of the values of positive scores/*n* positive gut regions; this way the dietary treatment did not have an effect on lesion scores (p>0.05).Thenumber of replicates (birds) positive to lesion scoring increasedfor all treatmentsas the trial advanced, being less gradual for AM5 treatment (*Table 2*). AM5 showed a similar trend as control group (Figure 2). Among all the treatments, it was control group who accounted the lowest lesion scores at day 36.

Table 2. Average values of lesion scoresby dietary treatment and positive replicates (birds) across the time.

	Day 21		Day 28		Day 36	
Diet	Lesion score	% positive replicates	Lesion score	% positive replicates	Lesion score	% positive replicates
Control	0.69	62.5	1.25	75	1.25	100
AM3	0.56	37.5	0.94	75	1.9	100
AM5	0.69	50	1.5	100	1.56	100

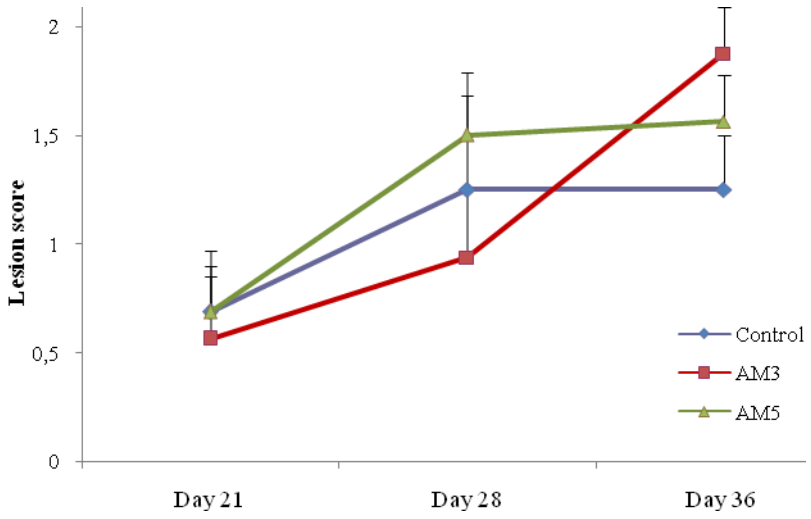

Figure 2. Average values of lesion scores by dietary treatment across the time.

As for *Eimeria spp.*, dietary treatment did not have a significant effect on*Campylobacter spp*. counts in caeca(p>0.05).AM5 treatment kept lower counts until day 21, hence it appeared to delay the onset of the infection (*Figure 3*).While AM3 maintained similar counts at the middle of the trial the rest of treatment groups increased, yet on day 36 all treatment groups had similar values.

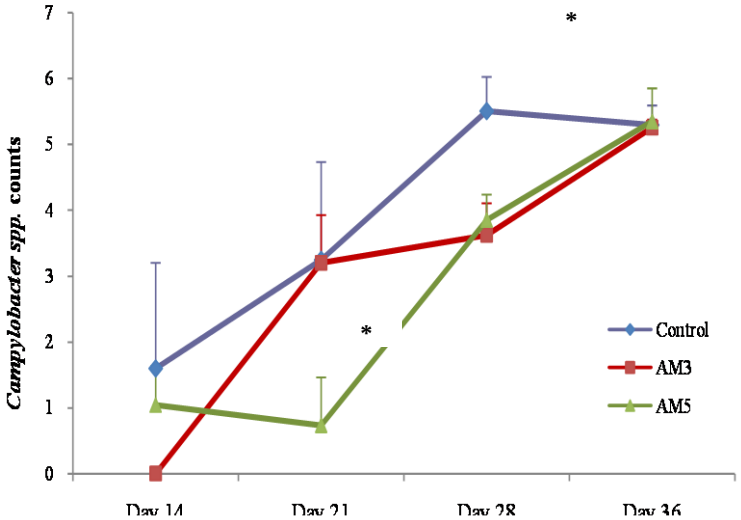

Figure 3.Enumeration of *Campylobacter spp.* in caeca(log_{10} CFU/g) by dietary treatment across the time. Significant differences between treatments are indicated by asterisks (*)

A significant positive correlation ($p<0.01$) between *Eimeria spp*. FEC and*Campylobacter spp.* counts in caeca was found for AM3 (r= 0.755, r^2=0.57) and AM5 treatments (r= 0.792, r^2=0.63) (*Figure 4*)

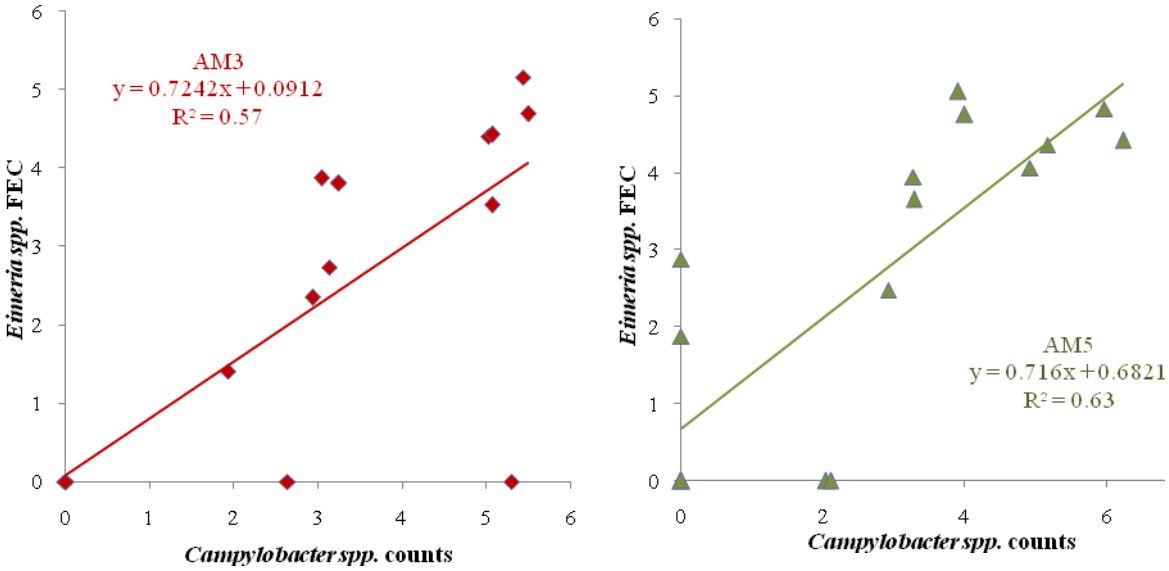

Figure 4. Pearson's product-moment test for *Eimeria spp.* FEC (log_{10} oocysts/g faeces) and *Campylobacter spp.* in caeca(log_{10} CFU/g)

Discussion

The infection by *Eimeria spp.*encountered in this trial was assumed to be caused by the dirty litter, which was spread on purpose on the pen floors to favorise the infection with *Campylobacter spp.*

It was observed that the parasitic infection started at different time points among the pens, being control group the first to be positive on FEC, and it got spread as FEC increased considerably when the birds were aged between 3 to 4 weeks, reaching peak infection by week 5 (day 36).

Control group maintained higher *Campylobacter spp*. counts in caeca for the length of the trial. AM5 treatment delayed the onset of the infection and AM3 managed some control over it during the middle of the trial. Nevertheless, dietary treatments failed to reduce the counts at day 36, when it would be desired to get lower counts in order to reduce the chance of contamination by faecal spillage at slaughter. In addition, both intervention groups achieved higher *Eimeria spp.*FEC and lesion scores compared to control group at the end of the trial.This could possibly be explained by the dynamics of *Eimeria spp.*infections, as they are self limited in durationand in broilers follow a pattern from low litter oocyst counts in their first weeks of life to peak excretion around four to five weeks of life, decreasing afterwards (Conway and McKenzie, 2007).

The study of the relationship between the two microorganisms in a co-infection revealed that when the parasite oocyst shedding increased,caecal colonisation by *Campylobacter spp*. was elevated as welland this was significant for the dietary treatments AM3 and AM5. This has been previously reported in studies of co-infection by *E. tenella* and *C. jejuni*(Macdonald*et al*., 2015),which also revealedthat concomitant infectionappear to decrease the bacterial contamination in liver and spleen tissues.

The effect of the sodium butyrate additive in the variables studied is not conclusive. The analysis of *Campylobacterspp.* in caeca byreal-time PCR and the study of changes in chicken microbial using whole metagenome sequencing techniques are to be concluded in order to support this hypothesis.

Acknowledgements

This research was supported by funds from the EU's Convergence European Regional Development Fund through the Welsh Assembly Government KESS program. The author express her thanks to Dr Gabriel de la Fuente and Dr Emilio del Cacho for advice given, as to the Royal Veterinary College London for the hospitality provided during a short training period, notably to Dr Damer Blake, Dr Virginia Marugán-Hernández and Dr Matthew Nolan for support and guidance.

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