TESIS DE LA UNIVERSIDAD DE ZARAGOZA

2018

47

Carolina Pineda Quiroga

Assessment of dry whey powder as prebiotic in the feeding of laying hens and broilers

Departamento

Producción Animal y Ciencia de los Alimentos

Director/es

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ISSN 2254-7606

Prensas de la Universidad Universidad Zaragoza

ISSN 2254-7606

Universidad Zaragoza
1542

Tesis Doctoral

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Neiker Tecnalia, Instituto Vasco de Investigación y Desarrollo Agrario

Departamento de Producción Animal

Universidad de Zaragoza

Facultad de Veterinaria

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ASSESSMENT OF DRY WHEY POWDER AS PREBIOTIC IN THE FEEDING OF LAYING HENS AND BROILERS

CAROLINA PINEDA QUIROGA

December, 2017

Assessment of dry whey powder as prebiotic in the feeding of laying hens and broilers

Evaluación del lactosuero en polvo como prebiótico en la alimentación de gallinas de puesta y pollos de engorde

Doctoral thesis presented by

Carolina Pineda Quiroga

Directed by

Dr. Aser García Rodríguez

and Dr. Roberto Ruiz Santos

in order to obtain the doctoral degree within the

Doctorate Program "Animal Production"

of University of Zaragoza, Faculty of Veterinary Medicine

Zaragoza, December 2017

Neiker Tecnalia, Instituto Vasco de Investigación y Desarrollo Agrario
Departamento de Producción Animal

Dr. Aser García Rodríguez y **Dr. Roberto Ruiz Santos**, investigadores principales del Departamento de Producción Animal de Neiker Tecnalia, Instituto Vasco de Investigación y Desarrollo Agrario.

Informan:

Que la presente memoria de tesis titulada **"Assessment of dry whey powder as prebiotic in the feeding of laying hens and broilers"**, que se corresponde con el proyecto de tesis doctoral aprobado el 08 de julio de 2014, y de la que es autora **Carolina Pineda Quiroga,** ha sido realizada bajo su dirección y cumple con las condiciones exigidas para que sea presentada y defendida como Tesis Doctoral.

Fdo. Dr. Aser García Rodríguez Fdo. Dr. Roberto Ruiz Santos

Vitoria-Gasteiz, 11 de Diciembre de 2017

The present Thesis comprises a compendium of four Published Scientific Manuscripts together with a fifth manuscript submitted to publication.

Pineda-Quiroga, C., Atxaerandio, R., Zubiria, I., Gonzalez-Pozuelo, I., Hurtado, A., Ruiz, R., Garcia-Rodriguez, A., 2017. Productive performance and cecal microbial counts of floor housed laying hens supplemented with dry whey powder alone or combined with *Pediococcus acidilactici* in the late phase of production. Livestock Science 195, 9-12. doi.org/10.1016/j.livsci.2016.11.007.

Pineda-Quiroga, C., Atxaerandio, R., Ruiz, R., García-Rodríguez, A., 2017. Effects of dry whey powder alone or combined with calcium butyrate on productive performance, duodenal morphometry, nutrient digestibility, and ceca bacteria counts of broiler chickens. Livestock Science 206, 65-70. doi.org/10.1016/j.livsci.2017.10.001

Pineda-Quiroga, C., Camarinha-Silva, A., Borda-Molina, D., Atxaerandio, R., Ruiz, R., García-Rodríguez, A., 2017. Feeding broilers with dry whey powder and whey protein concentrate affected productive performance, ileal digestibility of nutrients and cecal microbiota community. Animal, 1-9. doi:10.1017/S1751731117002208

Pineda-Quiroga, C., Camarinha-Silva, A., Atxaerandio, R., Ruiz, R., García-Rodríguez, A., 2017. Changes in broiler performance, duodenal histomorphometry, and caeca microbiota composition in response to wheat-barley based diets supplemented with non-antibiotic additives. Animal Feed Science and Technology 234, 1-9. doi.org/10.1016/j.anifeedsci.2017.09.002

Pineda-Quiroga, C., Borda-Molina, D., Chaves-Moreno, D., Ruiz, R., Atxaerandio, R., Camarinha-Silva, A., García-Rodríguez, A. 2017. Microbial and functional profile of the laying hens' ceca fed with prebiotic, probiotic and synbiotic: A preliminary report. Submitted to Environmental Microbiology Reports

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Certifican:

Que la presente memoria de tesis titulada **"Assessment of dry whey powder as prebiotic in the feeding of laying hens and broilers"**, de la que es autora **Carolina Pineda Quiroga,** ha sido realizada bajo su dirección, y que considerándola finalizada, autorizan su presentación en la modalidad de **Compendio de Publicaciones** para que sea juzgada por la comisión correspondiente.

Fdo. Dr. Aser García Rodríguez Fdo. Dr. Roberto Ruiz Santos

Vitoria-Gasteiz, 11 de Diciembre de 2017

The experiments comprising the present thesis have been financed by the LIFE program of the European Union (LIFE11 ENV/ES/639, European Commission, Brussels, Belgium) and the Department of Economic Development and Competitiveness (Basque Government, Vitoria-Gasteiz, Spain). **Carolina Pineda Quiroga** has received a predoctoral grant from the Department of Education, Language Policy and Culture (PRE/2013/1/1077, Basque Government, Vitoria-Gasteiz, Spain).

I gratefully thank my family for all their love and encouragement. For my parents, who have always supported me in all my pursuits.

I would like to express my sincere gratitude to my PhD supervisors, Dr. Aser García Rodríguez and Dr. Roberto Ruiz Santos, for their support during these past four years. My sincere thanks also to the Animal Production Department staff, who helped me during the experiments that compose this Thesis.

RESEARCH ACTIVITIES

CONTRIBUTIONS TO CONFERENCES

Pineda-Quiroga, C., Camarinha-Silva, A., Borda-Molina, D., Atxaerandio, R., Ruiz, R., García-Rodríguez, A (2017). Suplementación con lactosuero en polvo y concentrado proteico de lactosuero en dietas de pollos de engorde: Efecto sobre el rendimiento productivo y la composición de la microbiota cecal. AIDA, XVII Jornadas sobre Producción Animal, Zaragoza, España. Libro de Resúmenes, pp 210-212.

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Camarinha-Silva, A., **Pineda-Quiroga, C**., Borda-Molina, D., Atxaerandio, R., Ruiz, R., García-Rodríguez, A (2017). Effect of supplementing broiler diets with dry whey powder and whey protein concentrate on the productive performance, digestibility of nutrients and cecal microbiota composition. 71th Jahrestagung der Gesellschaft für Ernährungsphysiologie (GfE), Göttingen, Germany. Book of Abstracts, Research 73.

Borda-Molina, D., **Pineda-Quiroga, C**., Camarinha-Silva, A., Atxaerandio, R., Ruiz, R., García-Rodríguez, A (2017). Caecal microbial community response to prebiotic and probiotic supplementation in laying hens diets. 71th Jahrestagung der Gesellschaft für Ernährungsphysiologie (GfE), Göttingen, Germany. Book of Abstracts, Research 74.

Borda-Molina, D., **Pineda-Quiroga, C**., Camarinha-Silva, A., Atxaerandio, R., Ruiz, R., García-Rodríguez, A (2017). Probiotic and prebiotic effects in the cecal microbial metagenome of laying hens. VAAM Annual Meeting, 69th Annual Meeting of the German Society for Hygiene and Microbiology, Wurzburg, Germany. Book of Abstracts, Research 32.

Pineda-Quiroga, C., Atxaerandio, R., Ruiz, R., García-Rodríguez, A (2016). Duodenal morphometry and digestibility of broilers fed with dry whey alone or combined with

butyrate. 67th Annual Meeting of the European Federation of Animal Science, Belfast, Ireland. Book of Abstracts, pp 247.

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Pineda-Quiroga, C., Atxaerandio, R., Ruiz, R., García-Rodríguez, A (2016). Production and cecal microbial counts of broilers fed with wheat-barley based diets and dry whey. 66th Annual Meeting of the European Federation of Animal Science, Warsaw, Polony. Book of Abstracts, pp 422.

Pineda-Quiroga, C., Atxaerandio, R., Ruiz, R., García-Rodríguez, A (2016). Production, duodenal histomorphometry, and cecal microbial counts of broilers fed with dry whey. 66th Annual Meeting of the European Federation of Animal Science, Warsaw, Polony. Book of Abstracts, pp 423.

Pineda-Quiroga, C., Atxaerandio, R., Zubiria, I., Ruiz, R., García-Rodríguez, A (2015). Suplementación con lactosuero en polvo y concentrado proteico de lactosuero en dietas de iniciación de broilers: Efectos sobre el rendimiento productivo. AIDA, XVI Jornadas sobre Producción Animal, Zaragoza, España. Libro de Resúmenes, pp 269-271.

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RESEARCH INTERNSHIP

- Institute of Animal Science, Microbial Ecology Research Group, University of Hohenheim, Stuttgart, Germany. From 1st to 30th of October 2017.

- Institute of Animal Science, Microbial Ecology Research Group, University of Hohenheim, Stuttgart, Germany. From 1st of April to 30th of June 2016.

COURSES

- Genomics in foodborne pathogen surveillance and outbreak investigation (20 hours). University of the Basque Country, Vitoria-Gasteiz, Spain. From 12sd to 13th of July 2017.

- Preparation of metagenomics and amplicon libraries for Illumina platforms (20 hours). FISABIO, Valencia, Spain. From June 14th to June 16th 2017.

- Seminario de introducción al uso de la supercomputación aplicado a la bioinformática (8 horas). Fundación Centro de Supercomputación de Castilla y León, León, España. 24 de Octubre de 2016.

- Curso práctico del uso de la supercomputación aplicado a la metagenómica y genómica comparada (28 horas). Fundación Centro de Supercomputación de Castilla y León, León, España. Del 25 al 28 de Octubre de 2016.

- Environmental impact assessment of livestock systems (40 horas). Wageningen University, Wageningen, Netherlands. From 9th to 13th of Februray 2015.

- Curso intensivo de estadística, nivel intermedio (28 horas). Universidad del País Vasco, Vitoria-Gasteiz, España. Del 21 de Mayo al 24 de Junio de 2014.

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ABBREVIATIONS

5-CHIT	5 g/kg of CHIT
20-INU	20 g/kg of INU
60-DWP	60 g/kg of DWP
80-WPC	80 g/kf of WPC
ADG	average daily gain
AGV	ácidos grasos volátiles
AGPs	antibiotics as growth promoters
AID	apparent ileal digestibility
AMEn	apparent metabolizable energy corrected by N
ANOSIM	one-way analysis of similarity
BCa	butirato de calcio recubierto de grasa
BW	body weight
CaB	fat-coated calcium butyrate
CFU	colony forming units
CHIT	chitosan
CP	crude protein
CPL	concentrado proteico de lactosuero
DIA	digestibilidad ileal aparente
DM	dry matter
DWP	dry whey powder
DWP-CHIT	mixture of DWP plus CHIT
EU	European Union
FCR	feed conversion ratio
FI	feed intake
FOS	fructo-oligosaccharide
F/B	firmicutes/bacteroidetes ratio
GIT	gastrointestinal tract
GOS	galacto-oligosaccharides
ICA	índice de conversión alimenticia
INU	inulin
LP	lactosuero en polvo

NSP	non-starch polysaccharides
MOS	mannan-oligosaccharides
OTUs	operational taxonomic units
PA	*Pediococcus acidilactici*
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
PERMANOVA	permutational analysis of variance
RD	real decreto
RDP	ribosomal database project
SCFAs	short chain fatty acids
SIMPER	similarity percentage analysis
SIMPROF	similarity profile permutation test
SOP	standard operative procedure
WPC	whey protein concentrate

ABSTRACT

During the last decades, the interest to search for non-antibiotic fed alternatives to improve the performance of laying hens and broilers through gastrointestinal microbial modulations without causing antimicrobial resistance has increased considerably. From the nutritional perspective, one of the approaches is based on the dietary inclusion of prebiotics to benefit the intestinal microbial composition, and therefore animal health and performance. The main objective of the present thesis was to evaluate the prebiotic potential of the inclusion of dry whey powder (DWP), as a lactose source, in the formulation of laying hens and broilers' feed. In this context, five studies were conducted to determine the feasibility of the use of DWP when added to corn-soybean or whey-barley based diets. Moreover, DWP effectiveness through its simultaneous supplementation with other non-antibiotic feed additives was also assessed.

The first study determined the effect of supplementing corn-soybean based diets of laying hens with DWP, *Pediococus acidilactici* (PA), and the combination of both as synbiotic (DWP-PA) on the productive performance, egg quality traits, and cecal microbial counts. The results showed that cecal counts of *Bifidobacterium* spp. were increased with the addition of DWP, while an interaction between DWP and PA levels was found on egg production and on cecal counts of *Clostridium perfringens*, so that the addition of DWP increased egg production and reduced *C. perfringens* colony counts only when PA was not used. According to these results, the addition of DWP modulated the target cecal bacteria and increased egg production.

The second study was carried out with the same animals used in study 1. The aim of this was to analyze the cecal microbial composition, using Illumina amplicon sequencing of the 16S rRNA gene, and the cecal microbial functional profile, using DNA sequencing through Illumina HiSeq2500 platform, of laying hens fed with DWP, PA, and DWP-PA. The results revealed that microbial communities of hens fed with control and PA were different from those fed with DWP and DWP-PA, while no differences were found between control and PA, and between DWP and DWP-PA. Feeding with DWP and DWP-PA mainly promoted the presence of *Olsenella* spp. *Lactobacillus crispatus*, and *Megamonas* spp. in comparison with the remaining diets. Metagenomics approach revealed that a core of main functions was shared between all metagenomes (45.5%), although DWP stimulated that microbiota encoded more unique functions (22.5%) compared with control, which showed the lowest percentage (1.6%).

Major presence of genes encoding the metabolism of butanoate, propanoate, galactose, and inositol phosphate were especially stimulated by DWP. Results from this experiment indicated that each dietary supplementation influenced the cecal microbial community, but these changes did not imply a disturbance in their main biological roles. However, some specific metabolic functions encoded by the community, were present or absent depending on the source of supplementation.

In the third study, apparent ileal digestibility (AID), pH of gastrointestinal content at various segments, duodenal histomorphometry, cecal microbial counts, and productive performance of broilers were studied in response to DWP and fat-coated calcium butyrate (CaB) supplementation to corn-soybean based diets. The results indicated that with the addition of DWP, the AID of dry matter, crude protein, Ca and P increased, and cecum pH decreased only when CaB was also added. Similarly, with the addition of DWP, villus height, villus height to crypt depth ratio, and villus surface area were increased only when CaB was also added, while the supplementation of WP increased *Bifidobacterium* spp. colony counts only when CaB was no added. In relation to performance results, it was observed that with the dietary supplementation of DWP, the average daily gain (ADG) and feed intake (FI) increased during starter-grower finisher periods, and the entire feeding period only when CaB was also added. However, with the addition of DWP, feed conversion ratio (FCR) decreased in broilers fed without CaB, but it increased in those fed with CaB during the grower-finisher and entire feeding periods. These findings suggest that the supplementation of DWP without CaB addition improve FCR of broilers. However, the joint supplementation of DWP and CaB improve duodenal development, increases nutrient AID, and the weight and feed ingestion of broilers.

The fourth study was carried out to assess the influence of supplementing corn-soybean broiler diets with DWP and whey protein concentrate (WPC) on AID, productive performance and cecal microbiota composition at the end of the productive period, using Illumina amplicon sequencing of the 16S rRNA gene. The results showed that 60-DWP increased the AID of Ca, while 80-WPC improved both AID of Ca and P when compared to control diet. Feeding broilers with 60-DWP and 80-WPC increased their BW, ADG, and FI during the starter and grower-finisher periods, and during the entire feeding period. Supplementing 60-DWP and 80-WPC reduced FCR during the starter period, while 60-DWP reduced this parameter during the entire feeding period.

Cecal microbial communities of broilers fed with 60-DWP and 80-WPC differed from those fed with control diet. The abundance of *Bacteroides fragilis*, *Bacteroides* spp., *Escherichia coli/Shigella flexneri* and *Megamonas furniformis* increased when 60-DWP and 80-WPC were included, while the presence of *Helicobacter pullorum* decreased. *Lactobacillus salivarius* consistently increased in chickens with better FCR, which were those fed with 60-DWP. These results indicate that growth of chickens is improved by 60-DWP and 80-WPC supplementation because of a higher mineral digestibility, increased FI, and modulation of cecal microbiota communities.

The fifth study was conducted to investigate the effect of supplementing wheat-barley based diets with 60-DWP, chitosan (5-QUIT), DWP-QUIT, and Inulin (20-INU) on duodenal histomorphometry, productive performance and cecal microbiota composition at days 21 and 42 of age, using Illumina amplicon sequencing of the 16S rRNA gene. The results indicated that feeding chickens with any of the tested additives diminished their BW, ADG, and FI during the starter period. This was also observed during the entire feeding period, except for INU supplementation that showed similar values to control. At day 21, no differences in microbiota composition of control, 60-DWP, 5-CHIT and 20-INU birds were found, which ceca were highly harboured by *Lactobacillus gallinarum*, although only control promoted greater BW, ADG, and FI. Control and 60-DWP treatments did not differ in their ceca communities at day 42, although only control increased BW, ADG, and FI. In both cases, ceca showed higher abundance of *Lactobacillus gallinarum* and *Bacteroides vulgatus*, and lower abundance of *Escherichia coli/Shigella flexneri* and *Bacteroides fragilis*. The present findings indicate that chicken growth is reduced by supplementing wheat-barley based diets with DWP, CHIT, DWP plus CHIT, and INU, at the tested doses, as a consequence of a reduction in FI. Moreover, the results revealed that cecal microbiota composition was influenced by diet at every stage of life, although no clear association between microbiota and performance was detected.

RESUMEN

Durante las últimas décadas se ha observado un incremento en la búsqueda de alternativas alimenticias diferentes a los antibióticos que mejoren el rendimiento productivo de gallinas de puesta y pollos de engorde mediante la modulación de las poblaciones microbianas del tracto gastrointestinal. Desde la perspectiva del manejo alimenticio, uno de los enfoques se centra en la inclusión de prebióticos en la dieta que actúen modulando la composición microbiana de manera beneficiosa, y por ende el estatus sanitario y productivo de los animales. De este modo, el principal objetivo de la presente tesis fue evaluar el potencial prebiótico del lactosuero en polvo (LP), como fuente de lactosa, en gallinas de puesta y pollos de engorde. Para ello se desarrollaron cinco estudios con el fin de determinar la factibilidad de incluir LP en dietas con matrices cereales de maíz-soja y trigo-cebada. Además, la presente tesis también evaluó alternativas para aumentar la efectividad del LP mediante su suplementación simultánea con otros aditivos de naturaleza no antibiótica.

El primer estudio determinó el efecto de la inclusión de LP, *Pediococus acidilactici* (PA), y la combinación de los dos como simbiótico (DWP-PA) en dietas para gallinas ponedoras con base cereal de maíz y soja sobre el rendimiento productivo, la calidad de los huevos y los recuento microbianos cecales. Los resultados mostraron que los recuentos cecales de *Bifidobacterium* spp. incrementaron con la inclusion de LP. Del mismo modo, se observó una interacción entre los niveles de LP y PA en la producción de huevos y en los recuentos cecales de *Clostridium perfringens*, ya que la adición de LP incrementó la producción de huevos y redujo las unidades formadoras de colonia de *C. perfringens* sólo cuando PA no fue adicionado a la dieta. De acuerdo con estos resultados, se puede inferir que la adición de LP moduló la composición bacteriana cecal e incrementó la producción de huevos.

El segundo estudio fue llevado a cabo empleando los mismos animales del primer estudio. En este caso, el objetivo fue la evaluar la composición microbiana cecal mediante la secuenciación del gen ribosomal RNA 16S, así como el perfil funcional microbiano mediante la secuenciación completa del ADN bacteriano de gallinas de puesta alimentadas con LP, PA y LP-PA. Los resultados mostraron que las comunidades microbianas de gallinas alimentadas con la dieta control y PA fueron diferentes de aquellas alimentadas con LP y LP-PA, mientras que no se observaron diferencias entre la dieta control y PA, ni entre LP y LP-PA. La suplementación de LP y LP-PA en la dieta incrementó la presencia de *Olsenella* spp. *Lactobacillus crispatus*, y

Megamonas spp. en comparación con los tratamientos restantes. En relación con el perfil funcional, se observó que los todos los metagenomas bacterianos comparten un núcleo de funciones comunes (45.5%). Sin embargo, fue evidente que la adición de LP en la dieta causó que la microbiota codificara más funciones únicas (22,5%) en comparación con la dieta control, la cual mostró el porcentaje más bajo (1.6%). La dieta que contenía LP favoreció el incremento de las funciones microbianas relacionadas con el metabolismo del butanoato, propanoato, galactosa e inositol fosfato. Los resultados de este experimento indican que cada uno de los suplementos empleados influenció la comunidad microbiana cecal, pero que estos cambios no implicaron una alteración en los principales roles funcionales de la misma. Sin embargo, algunas funciones metabólicas microbianas estuvieron presentes o ausentes dependiendo de la fuente de suplementación.

En el tercer estudio se evaluó la digestibilidad ileal aparente (DIA), el pH del contenido gastrointestinal de varios segmentos, la histomorfometría duodenal, los recuentos microbianos cecales y el rendimiento productivo de pollos de engorde en respuesta a la adición de LP y butirato de calcio recubierto con grasa (BCa) en dietas con base de maíz y soja. Los resultados muestran que con la adición de LP se incrementó la DIA de la materia seca, proteína cruda, Ca y P, y se redujo el pH cecal sólo cuando BCa también fue adicionado a la dieta. También observamos que la suplementación de LP incrementó la altura de las vellosidades, el ratio altura de la vellosidad:profundidad de la cripta, y el área de la superficie de las vellosidades solamente cuando BCa fue suplementado de manera conjunta, mientras que la suplementación de LP incrementó los recuentos de colonias cecales de *Bifidobacterium* spp. sólo cuando BCa no fue suplementado. En relación con los resultados de rendimiento productivo, se observó que la ganancia media diaria (GMD) y la ingestión de alimento (IA) incrementó durante los periodos de arranque y finalización, y durante todo el periodo de alimentación cuando LP fue suministrado de manera conjunta con BCa. Sin embargo, el índice de conversión alimenticia (ICA) se redujo con la adición de LP en ausencia de BCa y viceversa. Estos resultados sugieren que la suplementación de LP en ausencia de BCa, o de BCa en ausencia de LP mejora el ICA de los pollos de engorde. Sin embargo, la suplementación conjunta de LP y BCa mejoró el desarrollo duodenal, incrementó la digestibilidad de los nutrientes, y el peso e ingestión alimenticia de los animales.

El cuarto estudio fue desarrollado con el fin de evaluar la inclusión de LP y concentrado proteico de lactosuero (CPL) en dietas para pollos de engorde con base cereal de maíz y soja, sobre la DIA, el rendimiento productivo y la composición de la microbiota cecal empleando la secuenciación del gen ribosomal RNA 16S en la plataforma de Illumina. Los resultados mostraron que la dieta 60-LP incrementó la DIA del Ca, mientras que la dieta 80-CPL mejoró la DIA del Ca y P en comparación con la dieta control. Del mismo modo, las dietas 60-L y 80-CP incrementaron el peso vivo (PV), la GMD y la IA durante los periodos de arranque, de crecimiento y finalización, y durante el periodo completo de alimentación. Las dietas 60-LP y 80-CP redujeron el ICA durante el periodo de arranque, mientras que 60-LP redujo este parámetro durante el periodo completo de alimentación. Las composición de comunidades microbianas cecales de los pollos alimentados con 60-LP y 80-CP fueron diferentes de aquellos alimentados con la dieta control. La abundancia de *Bacteroides fragilis*, *Bacteroides* spp., *Escherichia coli/Shigella flexneri* y *Megamonas furniformis* incrementó por alimentar a los animales con 60-LP y 80-CP, mientras que la presencia de *Helicobacter pullorum* se vio disminuida. La abundancia de *Lactobacillus salivarius* incrementó de manera consistente en los pollos con mayor ICA, que fueron aquellos alimentados con 60-LP. Los resultados de este estudio permiten concluir que el crecimiento de los pollos se puede mejorar por la suplementación con 60-LP y 80-CP debido a la mayor digestibilidad de los minerales, al incrementó en la IA y a la modulación en la composición de la microbiota cecal.

El quinto y último estudio se realizó con el fin de investigar el efecto de suplementar 60-LP, quitosano (5-CHIT), DWP-CHIT e inulina (20-INU) en dietas para pollos de engorde con base cereal de trigo y cebada, sobre la histomorfometría duodenal, el rendimiento productivo y la composición de la microbiota cecal a los 21 y 42 días de vida, mediante la secuenciación del gen ribosomal RNA 16S en la plataforma Illumina. Los resultados de este estudio indicaron que la alimentación de los animales con cualquiera de los aditivos suministrados redujo el PV, la GMD y la IA durante el periodo de arranque. Esto también fue observado durante todo el periodo de alimentación, excepto para el tratamiento 20-INU, que mostró valores similares al grupo control. En relación a las comunidades microbianas cecales, no se observaron diferencias entre el grupo control, 60-LP, 5-QUIT y 20-INU al día 21 de vida, en los cuales el ciego estuvo principalmente colonizado por *Lactobacillus gallinarum*, aunque

sólo el control promovió un mayor PV, GMD e IA. El grupo control y 60-LP no difirieron en sus comunidades cecales al día 42, aunque sólo el tratamiento control incrementó el PV, la GMD, y la IA. En ambos casos, el ciego tuvo una alta abundancia de *Lactobacillus gallinarum* and *Bacteroides vulgatus*, y una baja de *Escherichia coli/Shigella flexneri* and *Bacteroides fragilis*. Estos resultados indican que el crecimiento de los pollos de engorde se redujo por la suplementación de LP, QUIT, LP más QUIT e INU a las dosis evaluadas, como consecuencia de una disminución en la IA. Además, los resultados ponen en evidencia que la composición de la microbiota cecal fue modulado por la dieta en cada una de las edades muestreadas, aunque no se observó una asociación clara entre esta y el rendimiento productivo.

GENERAL INTRODUCTION

1. BACKGROUND OF THE USE OF ADDITIVES IN POULTRY FEEDING AS GROWTH PROMOTERS IN THE POST-ANTIBIOTIC ERA

Antibiotics at sub-therapeutic doses have been widely included in the animal diet formulation as growth promoters (AGPs) from early 1950's. Noticeably, AGPs made it possible to improve animal health and performance, therefore increasing the profitability of production systems (Huyghebaert et al., 2011). However, supplementation of antibiotics at low levels during extended periods of time has also contributed to the appearance of antimicrobial resistance in some pathogenic and non-pathogenic strains (Redondo et al., 2014). As a result, the transference of resistance genes from animal to human microbiota occurred, compromising the therapeutic effectiveness of antibiotics in veterinary and human medicine (Brown et al., 2017). This risk led to the ban of the use of AGPs in the European Union since 2006 (European Commission Regulation No. 1831/2003), and to the exhaustive control of antibiotic supplies in USA (FDA, 2015). The impact of its gradual elimination on poultry farms resulted in the increment of the incidence of enteric diseases, with the associated increase in the use of therapeutic antibiotics and subsequent economic cost (Mateos et al., 2002; Patterson and Burkholder, 2003). Consequently, poultry farmers are subjected to the pressure of having to use safe feed additives that guarantee profitable yields, similar to those obtained with AGPs (Huyghebaert et al., 2011).

Therefore, the challenge for modern animal nutrition is to develop and implement new alternatives to improve the performance of the animal farming industry through GIT microbial modulations that do not result in antimicrobial resistance (Brown et al., 2017). One way is to use specific feed additives or dietary raw materials that benefit GIT microbiota composition or its metabolism (Tuohy et al., 2005; Gaggìa et al., 2010). Indeed, diet formulation focused on specific effects on the gut microbiota ecosystem is gaining importance in the monogastric animal industry (Redondo et al., 2014; Angelakis, 2017). In this scenario, the addition of enzymes to the diet, the inclusion of whole grains, or the supplementation with organic acids, probiotics, prebiotics, phytobiotics, and immunostimulants are considered as a feasible alternative (Huyghebaert et al., 2011; Angelakis, 2017). The special interest of the present work lies on the use of prebiotics and its effects on the performance of laying hens and broilers.

2. THE USE OF PREBIOTICS IN THE FEEDING OF LAYING HENS AND BROILERS

With the industrialization of poultry farming systems, the world production of laying eggs and broiler meat has grown, from 2003 to 2013, at a rate of 2.3% and 4.1% per year respectively, as a result of the parallel increment on per capita consumption (FAO, 2017). The appropriate nutritional management of birds is one of the key factors to reach the goal of production (Callaway, 2012). Higher productive performance is usually the result of higher feed intake, better digestion and absorption of nutrients, as well as of a certain balance in the qualitative and quantitative microbial load of the animal gut (Huyghebaert et al., 2011). The appropriate maintenance and possible modulation of microbial populations with new additives such as prebiotics has become an attractive alternative that is creating new nutritional possibilities. Although knowledge about the role and effects of prebiotics on animal production has increased, essential information concerning their mechanisms of action is still required to better comprehend their role on animal physiology and metabolism.

2.1 Prebiotics definition and their mode of action

The most recent definition of prebiotics refers to a substrate that is selectively metabolized by commensal microorganisms, conferring health benefits to the host through (Gibson et al., 2017). Prebiotics act as growth substrates (Patrascu et al., 2017) to enhance the activity of bacterial genera (Scott et al., 2015) such as bifidobacterial and butyrate-producing clostridia (Riviére et al., 2016). However, commensal microorganisms have a differential preference for prebiotics, since their genome determines the enzymes that they produce, and therefore their ability to utilize the prebiotic substrate (Wilson and Whelan, 2017). Previously, the effect of dietary supplementation with prebiotics was determined using the increase in *Lactobacillus* spp. and *Bifidobacterium* spp. counts as an standard reference (Gibson and Roberfroid, 1995). Nevertheless, by using novel sequencing techniques, it is now known that, through a cross-feeding process, prebiotics modulate a wider range of microorganisms other than *Lactobacillus* spp. and *Bifidobacterium* spp. (Gibson et al., 2017).

To exclude antibiotics from the prebiotic concept, because they also induce gut microbiota changes and positive host effects when are added to the fed, it is essential to clarify that the prebiotic compound is metabolized by bacteria to organic molecules that can be later used by the host, whereas antibiotics do not. Likewise, restricting the prebiotic concept to compounds that exert their action through their metabolization by resident microbiota is important. Otherwise, any compound, medicament or feed ingredient that affects the gut microecosystem could be considered a prebiotic (Bindels et al., 2015).

A prebiotic compound also needs to be resistant to gastric acidity, enzymatic hydrolysis and gastrointestinal absorption, must be selectively metabolized by beneficial commensal bacteria, and its fermentation should induce local or systemic benefits to the host (Gibson and Roberfroid, 1995). According to recent studies, only inulin and inulin-derived fructo-oligosaccharide products (FOS) fulfill all the criteria for prebiotic classification in livestock (Angelakis, 2017). However, galacto-oligosaccharides (GOS), mannan-oligosaccharides (MOS), glycol-oligosaccharides, malto-oligosaccharides, xylo-oligosaccharides (XOS), gluco-oligosaccharides, pectins, lactose and its derivatives, including lactulose and lactosucrose, have also been recognized as candidate prebiotics (EFSA, 2017). However, it has been anticipated that this list will continue to expand as more knowledge about the interaction between feed components and GIT microbiome becomes available (Hutkins et al., 2016).

The underlying mechanisms by which prebiotics improve poultry performance are mainly based on prebiotic-mediated changes in the GIT commensal microbiota (Rinttilä and Apajalahti, 2013; Pourabedin and Zhao, 2015). Given that prebiotics are usually fermentable substrates, their most obvious effect is their ability to modify the GIT microbiota composition towards the enrichment with resident microbial groups able to use prebiotic compounds as an energy substrate for their fermentative processes (Valcheva and Dieleman, 2016). Fermentation of prebiotics results in short-chain fatty acids (SCFAs) production, which reduce luminal pH, provide energy for epithelial cells and for the host. Moreover, a balanced bacterial population confers metabolic, protective, and trophic functions to the host through a wide range of products accessible to host cell, influencing host physiological processes locally and systemically (Verbeke et al., 2015; Ajuwon, 2016; Valcheva and Dieleman, 2016; Fig 1).

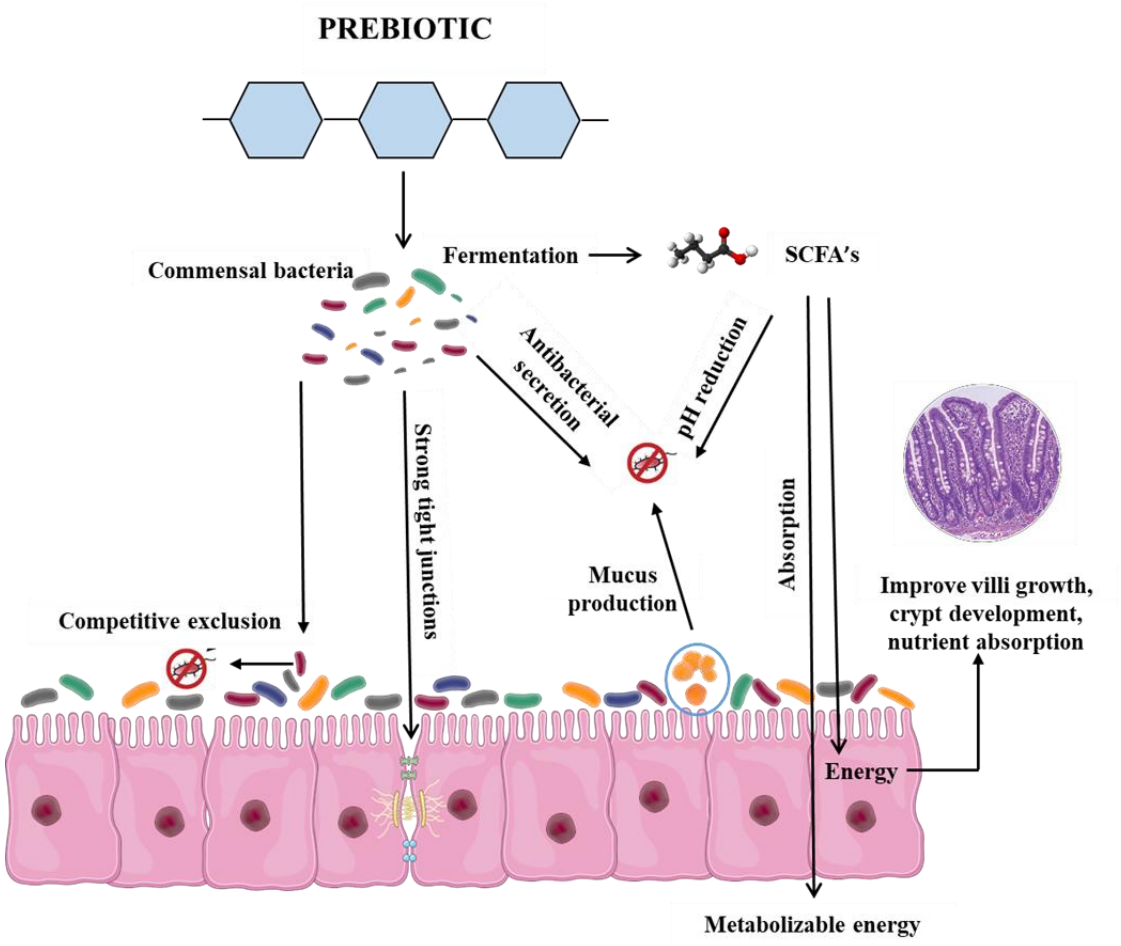

Fig 1. Potential mechanism of action of in-feed prebiotics. (Figure based on Pourabedin and Zhao, 2015).

2.1.1 Prebiotics and host nutritional benefits

Host nutritional benefits due to prebiotics supplementation are mainly related to its fermentation into SCFAs in the hindgut (mainly acetic, propionic, and butyric acids, as well as lactate). SCFAs are absorbed across the cecal epithelium by passive diffusion, providing up to 11% of metabolizable energy for mature birds (Annison et al., 1968). SCFAs could also improve mineral and protein availability because they reduce intestinal pH, promoting their solubilization (Feng et al., 2005; Resta, 2009).

Microbial prebiotic-mediated changes also affect the synthesis of vitamins and nitrogen compounds, the degradation of dietary indigestible components, and facilitate the removal of undesirable dietary components. Broilers' cecum microbiome encodes up to 5% of genes for co-factor and vitamin synthesis, and 10% for protein and amino

acid metabolism (Danzeisen et al., 2011) that could be used by the host or for the microbial metabolism itself (Pan and Yu, 2014). Moreover, metagenomics analysis in broilers revealed the presence of genes encoding lactase, cellulase, hemicellulase, and arabinoxylanse activity, contributing to the microbial digestion of these indigestible dietary components for SCFAs production (Qu et al., 2008; Sergeant et al., 2014), as well as amylase and protease activity (Xu et al., 2003). Microbial action also contributes to remove anti-nutritional factors such as saponins (García-Amado et al., 2007) and mycotoxins (Young et al., 2007), increasing the nutritional value of feedstuffs.

2.1.2 Prebiotics-mediated changes on intestinal morphology, health status, and immune system

Increased proliferation of enterocytes, increment of villus height, villus:crypt ratio, and improvement of intestinal epithelial barrier by strengthening of tight-junctions are promoted by the fermentation of prebiotics into SCFAs, especially butyric acid (Abdelqader et al., 2013; Pourabedin et al., 2014). Improvements in gut morphology lead to positive effects on feed utilization, and also create a protective barrier against enteric diseases since an adequate epithelial integrity decreases both the risk of pathogens invasion and the permeability to endotoxins (Hooper and Gordon, 2001).

Dietary prebiotics promote a balanced commensal GIT microbiota that acts protecting the host against the establishment of enteric pathogens. Commensal communities colonize the intestinal mucosa and form a dense layer of bacteria covering the mucosal surface. This layer of bacteria occupies diverse niches, blocking the attachment sites and subsequent colonization by enteric pathogens by means competitive exclusion (Nurmi et al., 1992). Bacteria also produce bacteriostatic and bactericidal substances that control the population of pathogens. Previous studies in broilers have shown that lactic acid and other SCFAs produced by commensal bacteria inhibit the growth of *E. coli*, *Salmonella Typhimurium*, and *Clostridium perfringens* by means of pH reduction and the bactericidal effect of undissociated form of SCFAs (van der Wielen et al., 2002a; Murry et al., 2004). Moreover, it has been determined that various strains of bacteria isolated from chicken GIT tract can also produce bacteriocins with inhibitory effects against *Salmonella Enteritidis*, *Campylobacter jejuni* (Stern et al., 2006), and *Listeria monocytogenes* (Shin et al., 2008).

Resident bacteria also stimulate mucosa mechanisms of defense, increasing the number of goblet cells and mucus production (Gaggìa et al., 2010). Moreover, microorganisms act as an antigenic stimulus for the maturation of the gut-associated lymphoid tissue, contribute to increase the number of intraepithelial lymphocytes and the immunoglobulin producing cells (Umesaki, 2014).

2.2 Dietary factors affecting the effectiveness of prebiotics

The type of cereal used in diet formulations is one of the factors influencing the effects of prebiotics, so that the commensal microbial profile of poultry fed with a corn-based diet largely varies in diversity and community composition from those fed with wheat, barley or rice (Jia et al., 2009; Hammons et al., 2010; Rodríguez et al., 2012). In consequence, the resident gut bacteria able to ferment and use prebiotics as a growth substrate differ, as well as their metabolites, widely influencing the prebiotic-mediated effects. In particular, cereals containing high levels of indigestible, water-soluble, non-starch polysaccharides (NSP) such as wheat, barley or rice, favor the proliferation of *Clostridium perfringens* or *E. coli*, whereas those cereals poorer in NSP do not (Jia et al., 2009). Moreover, even a slight variation in the type of cereal grain can affect intestinal bacteria at the strain level, as demonstrated by Hammons et al. (2010), who found that a standard corn-soybean ration with or without wheat middlings influence the strain of *Lactobacillus agilis*. Therefore, to properly evaluate the prebiotics efficacy and their usefulness in a poultry feeding context, they should be tested using different cereal matrixes.

The efficacy of prebiotics could also be affected by the presence of other diet additives. Their dietary combination with probiotics (directly fed microorganisms), turning them into synbiotics, confers benefits beyond those achieved by prebiotics alone (Awad et al., 2009). When prebiotics are combined with probiotics in diets, they benefit the host by improving the survival and implantation of live microbial dietary supplements in the GIT by acting as their substrate (Gibson and Roberfroid, 1995). A more efficient probiotic implantation as well as the prebiotics stimulating effect on resident bacteria contribute to maintaining intestinal homeostasis and the general health status of the host. Indeed, performance and microbial GIT results are favored, in poultry, by the joint use of prebiotics and probiotics (Awad et al., 2009; Abdelqader et

al., 2013; Wang et al., 2016), although findings are inconsistent (Willis et al., 2007; Jung et al., 2008). Prebiotics efficacy could also be maximized by combine them with organic acids or natural antimicrobials, that reduce the gastrointestinal load of potentially pathogenic bacteria (Adil et al., 2010; Kong et al., 2010). Under these conditions, prebiotics could be selectively fermented and used as a substrate for beneficial commensal bacteria, conferring multiple benefits to the host as reported by Çinar et al. (2009) and Taherpour et al. (2012). These evidences indicate that it is worth evaluating the prebiotics combinations with other dietary substances to determine potential synergism.

3. AN INSIGHT OF THE MICROBIOTA COMPOSITION OF THE AVIAN GASTROINTESTINAL TRACT

The avian GIT is densely populated with microorganisms. Bacteria are by far the main colonizers, although archea, fungi, and viruses have also been identified (Wei et al., 2013). Microorganisms are found across the entire length of the GIT, where different sections are colonized by specialized microbiota adapted to the physicochemical conditions, host physiology, and nutrient availability (Borda-Molina et al., 2016). Nonetheless, microbial communities are tightly interconnected between GIT organs, influencing microbiota both up and down-stream (Stanley et al., 2014). Moreover, each microbial community exhibits a wide variation in their genome content, affecting their roles within the overall ecosystem (Mohd Shaufi et al., 2015).

The crop and gizzard, where feed is temporally stored, fermented, and mechanically grinded, are highly dominated by lactic acid producing bacteria belonging mainly to *Lactobacillus* species (Witzig et al., 2015; Borda-Molina et al., 2016). The duodenum and ileum, where most of nutrient enzymatic digestion and absorption occurs, are mainly colonized by *Lactobacillus* species and, to a lesser extent, by *Clostridium*, *Streptococcus*, and *Enterococcus* (Stanley et al., 2014; Borda-Molina et al., 2016). The cecum, where complex non-digested substrates such as cellulose and other polysaccharides are fermented, consist of two blind pouches that have the longest residence time of digesta (12-20 hours) of all digestive organs (Pan and Yu, 2014). It is by far the most densely colonized organ in birds, and its bacterial diversity is much higher than that found in the upper GIT tract (Stanley et al., 2014; Mohd Shaufi et al.,

2015). This organ can harbor more than 2,300 operational taxonomic units (OTUs; 95% sequence identity; Danzeisen et al., 2011), with the most abundant bacterial families belonging to Ruminococcaceae, Lachnospiraceae, Anaeroplasmataceae, Erysipelotrichaceae, Peptoccaceae, and Lactobacilaceae (Borda-Molina et al., 2016), although a significant proportion belonging to Bifidobacteriaceae and Coriobacteriaceae have also been identified (Apajalahti and Vienola, 2016). However, ceca microbial community description is still ongoing, what makes it the target organ to evaluate responses associated to poultry feeding practices (Rinttilä and Apajalahti, 2013; Stanley et al., 2014).

Significant changes in the taxonomical composition of the GIT occur during the lifespan of laying hens and broilers (Oakley et al., 2014; Videnska et al., 2014; Ranjitkar et al., 2016). As animals grow, the complexity of the GIT increases. Certain bacteria may disappear or emerge in the intestinal microbiota of older animals over time, while others remain stable throughout their entire life. Members belonging to Firmicutes phylum are consistently identified in the ceca of hens and broilers during their life, whereas Proteobacteria members are abundant during the first week, but decrease at expenses of Bacteroidetes in older animals (Videnska et al., 2014; Ranjitkar et al., 2016). Despite this dynamic microbiota succession, Ranjitkar et al. (2016) suggested that a mature microbiota in broilers is stablished after 22 days of age, whereas Videnska et al. (2014) reported extensive successional changes during the lifespan of hens, identifying a stable microbiota composition only from 7 months of age onwards. Nevertheless, Oakley et al. (2014) reported that age-related microbiota changes can be strongly influenced by diet variations, suggesting that it would be relevant to study the diet effects at different poultry ages. This is because diet is also one of the major factors that shape the microbial profile and their encoded functions, having the potential to influence it towards a desired direction (Apajalahti et al., 2004; Rehman et al., 2007). However, the diet is usually formulated to meet poultry nutritional requirements, but its ability to influence the metabolically active microbiota is often overlooked (Apajalahti and Vienola, 2016). Microorganisms derive most of their energy from dietary compounds, which are either resistant to the attack of digestive enzymes and acids, or are absorbed so slowly by the host that bacteria can successfully compete for them (Apajalahti et al., 2004; Pan and Yu, 2014). As bacterial species have different nutrient preferences for maintenance and growth, the GIT microbial profile, and largely the

cecal profile, is commonly considered a reflection of the ingested feed, and the digested and absorbed nutrients in the proximal intestine (Apajalahti et al., 2004).

4. DRY WHEY POWDER AS AN ALTERNATIVE PREBIOTIC SOURCE

4.1 Whey production and end disposal management

Whey is the residual liquid obtained after casein precipitation by action of acids or enzymes during cheese-making and casein manufacture processes (Smithers, 2008). The former, whose pH is ≤ 5, is called acid whey and it is obtained by direct acidification of milk, while the latter, whose pH is around 6, is called sweet whey and it is produced by the enzymatic coagulation of milk (Illanes, 2016). Usually, the production of one kilogram of cow cheese is associated with the generation of 9 liters of liquid whey, although it is almost half of that amount for sheep or goat cheese (Guimarães et al., 2008).

The steady growth in the production of dairy products has led to a concomitant increase in the volume of whey produced. Recent statistics have shown that the total worldwide cheese production has increased at a rate of 2.3% per year from 2004 to 2014. In parallel, liquid whey has grown at a rate of 2.2% per year in the same period of time (FAO, 2017). In Spain, the evolution of cheese production during those years showed a fluctuant increase of 0.5%, while registers for whey are scarce but show an increase of 2.4% from 2012 to 2014 (Fig 2). In any case, whey is the major co-product of dairy industries, and is usually considered a nuisance (Smithers, 2015).

Dairy companies have sought out the most economical end disposal methods for whey, which are usually based on the premise that whey is a waste product with little value (Gajendragadkar and Gogate, 2016). Such strategies have included spraying it in open fields, discharging into water bodies or treatment through municipal sewage systems (Smithers, 2008), without considering that whey has more polluting power than a typical sewage effluent (Ryan and Walsh, 2016). While these practices provided at least a partial and quick solution, all of them lead to legislative punishments for contamination and therefore low economical return (Smithers, 2008; Gajendragadkar and Gogate, 2016). Moreover, these methods have never provided a sustainable solution for whey management.

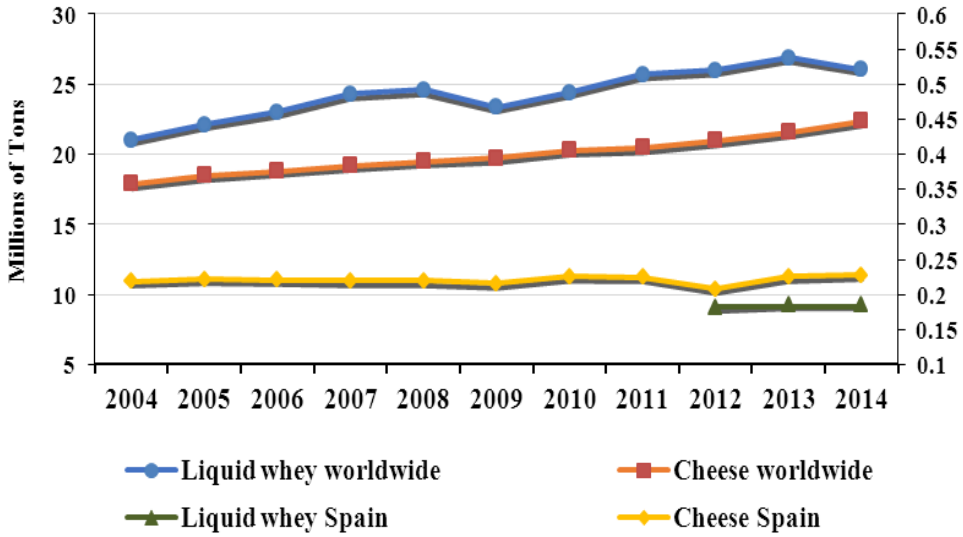

Fig 2. Worldwide and Spanish liquid whey and cheese production. Data of liquid whey includes all dairy sources of whey (FAO, 2017).

Legislative restrictions on whey disposal have encouraged the exploration of its physical, chemical, nutritional and biological properties, showing that whey is a relevant source of nutrients. Liquid whey retains about 55% of the milk solids (Siso, 1996), of which the great majority are lactose (accounting to 70-72%), followed by whey proteins (12%), and minerals (10%; Table 1). Therefore, its inadequate end disposal means a loss of nutrients as well (Gajendragadkar and Gogate, 2016). In fact, whey valorization commenced with approaches concerning to lactose utilization as a primary whey solid component (Gänzle et al., 2008). Afterwards, the possibility of taking advantage of whey proteins gained importance because of their remarkable biological value, which exceeds that of other common edible proteins such as eggs by 5%, and meat or soybean by up to 30% (Smithers, 2008). As a result of the intrinsic value of whey components, the challenge consists in turning it from a waste product into a valuable raw material to be used by the animal feeding, agri-food, biotechnology, or pharmacological industries (Smithers, 2015; Chen and Gänzle, 2017).

Table 1. Chemical composition of liquid and dry whey.

Component (%)	Liquid whey	Sweet dry whey [a]
Lactose	5.0	70
Total protein [b]	1.0	12
Fat	0.2	2.9
Sodium chloride	0.3	3.0
Ash	0.7	8.6
Calcium	0.05	1.55
Phosphorous	0.04	1.00

[a] Consolidated data from Shingoethe (1975), Smithers (2015), and Neiker-Tecnalia analysis. [b] Whey protein comprises ~50% of β-lactoglobulin, ~20% α-lactalbumin, ~20% glycomacropeptide, and ~10% among immonuglobulins, lactoferrin, lactoperoxidase, serum album, lysozyme, and growth factors.

4.2 Use of whey in animal feeding

Whey has been considered as a highly nutritive co-product that can be used to feed farm animals in liquid, condensed or dried form, or as dried whey products (Schingoethe, 1975; Siso, 1996). However, since whey contains more than 90% of water and high amounts of readily fermentable components, it is difficult to supply it under sanitary acceptable conditions (Illanes, 2016). As a result, the use of dry whey powder is widely preferred, as well as the use of sweet whey because it has better flavor and less salt content than acid whey (Siso, 1996).

The valorization of whey for animal feeding has been mainly focused on the use of lactose as the major solid component (Gänzle et al., 2008; Smithers, 2008), representing an energy source. Its energy value for ruminants and pigs is comparable to the energy value of corn and wheat, and slightly higher than barley (Table 3). In newborn calves, dry whey is frequently included in the milk replacer formula as the major carbohydrate component (Pantophlet et al., 2016), while in dairy cows at late stage of lactation, both whey and pure lactose have successfully replaced cornstarch (DeFrain et al., 2004). Similarly, dry whey has been included in weanling and growing pigs' diets (Grinstead et al., 2000; Lutz et al., 2017). However, for laying hens and broilers, whey represents less energy supply than other cereals, but the positive properties rely on the prebiotic-lactose effects (Morishita et al., 1982; van der Wielen et al., 2002a). Moreover, the use of other whey derivatives in poultry feeding, like whey proteins, has also recently been explored (Szczurek et al., 2013).

Table 2. Energy content of dry whey, corn, wheat, and barley [1]

	Dry whey	Corn	Wheat	Barley
Dairy cows [2]	1.07	1.06	1.05	1.00
Beef [3]	1.09	1.08	1.06	1.00
Growing pigs [4]	14.11	14.94	14.94	14.11
Broilers and laying hens [5]	8.58	14.00	13.46	11.64

[1] Data obtained from FEDNA (2010). [2] Energetic values expressed as Feed Units for Lactation. [3] Energetic values expressed as Feed Units for growth. [4] Energetic values expressed as Digestible Energy (MJ/kg). [5] Energetic values expressed as Apparent Metabolizable Energy corrected by N (EMAn, MJ/kg).

4.2.1 Lactose as prebiotic in poultry and its effects on performance

Lactose (4-0-b-galactopyranosyl-D-glucopyranose, $C_{12}H_{22}O_{11}$) is a disaccharide consisting of galactose bound to glucose. Lactose can be considered as a prebiotic-like compound because it is non-digested by poultry host, but may promote the growth of beneficial intestinal bacteria. To be metabolized, lactose requires the hydrolysis by the lactase-brush border enzyme in the intestine (Deng et al., 2015). However, lactase activity in the proximal intestine of birds is negligible (Denbow, 2000), entering to the large intestine at higher concentrations than other sugars (Morishita et al., 1982). Thus, undigested lactose is fermented by large intestinal microbiota (Siddons, 1972), affecting the intestinal microorganisms composition and their fermentation products (Atkinson et al., 1957; van der Wielen et al., 2002a; Venema, 2012). It is known that inadequate high amounts of lactose might result in formation of gas causing intestinal discomfort, bloating, and osmotic diarrhea (Morishita et al., 1982; Shariatmadari and Forbes, 2005). Nevertheless, microbial lactose fermentation is considered beneficial to the poultry host if side effects are avoided by supplementing appropriate amounts to the diet (Venema, 2012).

Studies about the influence of lactose on the GIT microbiota composition of chickens began about 90 years ago, reporting the consistent finding that lactose acts as prebiotic, increasing *Lactobacillus* spp. and *Bifidobacterium* spp. cecal counts (Ashcraft, 1933; Atkinson et al., 1957; Samli et al., 2007; Radfar and Farhoomand, 2008). Moreover, other findings reinforced the lactose-prebiotic effects, showing that it reduces *Salmonella enteriditis* (Tellez et al., 1993) and *Salmonella typhimurium* counts (DeLoach et al., 1990; Hinton et al., 1991). However, a more comprehensive research

about the detailed effects of lactose on the GIT microbiota composition using culture-independent methods are lacking so far. One option to expand the knowledge is to use the technical progress in the field of next-generation sequencing and bioinformatics analysis, which overcome the cultivation biases and offer detailed information on microbial composition, diversity structure, and functionality of biological samples (Qu et al., 2008; Deusch et al., 2015).

Regarding the performance effects promoted by lactose, results consistently indicate that its supplementation to corn-soybean based diets improve broilers' body weight gain (Gulsen et al., 2002; Kermanshahi and Rostami, 2006; Radfar and Farhoomand, 2008; Khani et al., 2015) and laying hens' egg production (Aghaei et al., 2010), together with the increase on *Lactobacillus* spp. and *Bifidobacterium* spp. cecal counts, although knowledge about the lactose-prebiotic effects using a cereal matrix other than corn have not yet been reported. Further lactose-induced effects such as the maintenance of the immune system, and the improvement on ileum morphology (Gulsen et al., 2002) have also been attributed, while little is known about its effect on nutrient digestibility and duodenal histomorphology. Moreover, to the best of our knowledge, studies related to the search of synergies between lactose and other feed additives on poultry feeding have not been described up to now.

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OBJECTIVES

MAIN OBJETIVE

To evaluate the prebiotic potential of dry whey powder on laying hens and broilers feeding, and its influence on productive performance, cecal microbiota profile and digestive parameters.

Specific objectives

1. To assess the effect of supplementing corn-soybean based diets for laying hens with dry whey powder alone or combined with a probiotic on the productive performance, egg quality traits, and cecal microbial counts (Chapter 1).

2. To provide an insight into the cecal microbiome changes in response to dietary inclusion of dry whey powder alone or combined with a probiotic (Chapter 2).

3. To evaluate the effect of supplementing corn-soybean based diets for broilers with dry whey powder alone or combined with calcium butyrate on the productive performance, duodenal morphometry, nutrient digestibility, and cecal bacteria counts (Chapter 3).

4. To evaluate the effect of supplementing corn-soybean based diets for broilers with dry whey powder and whey protein concentrate on the productive performance, nutrient digestibility, and ceca microbiota community (Chapter 4).

5. To evaluate the effect of supplementing wheat-barley based diets for broilers with dry whey powder and other non-antibiotic feed additives on performance, duodenal histomorphometry, and cecal microbiota community (Chapter 5).

CHAPTER 1

PRODUCTIVE PERFORMANCE AND CECAL MICROBIAL COUNTS OF LAYING HENS SUPPLEMENTED WITH DRY WHEY POWDER ALONE OR COMBINED WITH *PEDIOCOCCUS ACIDILACTICI*

Published in the scientific Manuscript: Pineda-Quiroga, C., Atxaerandio, R., Zubiria, I., Gonzalez-Pozuelo, I., Hurtado, A., Ruiz, R., Garcia-Rodriguez, A., 2017. Productive performance and cecal microbial counts of floor housed laying hens supplemented with dry whey powder alone or combined with *Pediococcus acidilactici* in the late phase of production. Livestock Science 195, 9-12. doi.org/10.1016/j.livsci.2016.11.007

ABSTRACT

Probiotics, prebiotics, and synbiotics have been proposed as safe additives in animal feeding. The purpose of this study was to assess the effect of supplementing corn-soybean diets of laying hens with dry whey powder (prebiotic), *Pediococcus acidilactici* (probiotic), and the combination of both (synbiotic) on the productive performance, egg quality traits, and cecal microbial counts. A total of 300 laying hens, 57 wk of age, were randomly allocated to floor pens for 70 d. Pens were assigned to 1 of 4 experimental diets with 5 pens per treatment and 15 laying hens per pen. The experiment consisted of a 2×2 factorial arrangement of treatments with 2 levels of inclusion of dry whey powder (DWP, 0 and 60 g/kg of diet) and 2 levels of *P. acidilactici* (PA, 0 and 2 g/kg of diet). Cecal counts of *Bifidobacterium* spp. were increased with the addition of DWP (8.4 vs. 6.5 log10 cfu/g cecal content, P = 0.012). An interaction between levels of DWP and PA was found on egg production (P = 0.008) and on cecal counts of *Clostridium perfringens* (P=0.047), so that the addition of DWP increased egg production (82.5 vs. 75.6%) and reduced *Clostridium perfringens* colony counts (4.3 vs. 5.8 log10 cfu/g cecal content) only when PA was not used. In conclusion the joint addition of DWP and PA in hens' diets during the late stage of production did not improve productive performance or change the cecal microbial population. However, the addition of DWP increased *Bifidobacterium* spp. cecal counts and only reduced the *Clostridium perfringens* counts together with an increase on egg production, when PA was not added.

1. INTRODUCTION

It is well known that nutrition, age, and health status, as well as housing system, are key factors influencing the productive performance of laying hens (Ahmadi and Rahimi, 2011). Their health status, egg production, and quality will decrease after the laying peak (Liu et al., 2013). Because the use of medication is being minimized to avoid potential residues in eggs, producers rely on nutritional measures to improve the persistency on egg production and the resistance against intestinal disorders (Lensing et al., 2012; Yörük et al., 2004). As a consequence, the use of prebiotics, probiotics, and synbiotics in diets could be a safe alternative to improve animal performance and health

(Janczyk et al., 2009; Patterson and Burkholder, 2003). Whey is a coproduct of cheese-making process, with lactose being its major component (about 70% of dry matter; Aghaei et al., 2010). Lactose can be used as a prebiotic in non-mammalian animals, because they lack the enzyme lactase (Allaart et al., 2013). Lactose is not digested, and is thus fermented by the cecal microflora, which could decrease pH, promote lactic-acid bacteria growth, and suppress pathogenic bacteria (Gülsen et al., 2002; van der Wielen et al., 2002). Probiotics, such as *Pediococcus acidilactici*, are beneficial live microorganisms that confer health benefits to the host mainly through the regulation of the intestinal microbial homeostasis (Gaggìa et al., 2010). Synbiotic is known as the combination of probiotics and prebiotics. Prebiotics beneficially affect the host because improve the survival and implantation of probiotics in the gastrointestinal tract (Awad et al., 2009).

We hypothesize that the beneficial effect of *Pediococcus acidilactici* could be enhanced by the simultaneous addition of dry whey powder so as to obtain additional benefits beyond those achieved when provided alone. Therefore, the purpose of this study was to assess the effect of diets supplemented with dry whey powder (prebiotic), *P. acidilactici* (probiotic), or their combination (synbiotics), on the productive performance, egg quality traits, and cecal microbial populations of floor-housed laying hens during the late phase of production.

2. MATERIALS AND METHODS

2.1 Animal housing and experimental diets

The experiment followed the European Union (2010/63/EU) and Spanish regulations (RD 53/2013) for animal experimentation, and was conducted at the experimental facilities of Neiker-Tecnalia (Vitoria-Gasteiz, Spain). A flock of 300, 57 wk-old hens (ISA Brown strain, Avigán Terralta S.A, Tarragona, Spain) with uniform body weight (2,035.4 ± 52 g) was used in an experiment lasting 70-d. Hens were randomly allocated, in groups of 15, to 2.5 m^2 floor pens with wood shavings. They had been fed with the same commercial diet, and had been subjected to the light program established by commercial guidelines previous to the experiment (ISA, 2010).

Pens were randomly assigned to 1 of 4 experimental treatments, each with 5 replicates, consisting in 4 dietary treatments: no supplementation of dry whey powder (DWP) or *P. acidilactici*/kg of diet (PA), inclusion of 60 g of DWP/kg of diet, 2 g of PA/kg of diet, or a mixture of 60 g of DWP and 2 g of PA/kg of diet. Dry whey powder was a commercial sweet powder (Sueromancha S.L, Toledo, Spain; 703 g of lactose/kg of product). The commercial probiotic (Bactocell, Lallemand, France) contained a live culture of *P. acidilactici* (strain MA 18/5, 1010 cfu/g). All experimental diets were formulated to meet laying hens' requirements (FEDNA, 2008), with ingredients and composition shown in Table 1. Feed and water were provided ad libitum and the light cycle program was 16 L:8D throughout the experiment.

2.2 Sample collection

The feeder from each pen was weighted weekly to calculate feed intake. Total eggs produced per pen were recorded daily to calculate egg production. Last day of the experiment, 3 hens per treatment were randomly selected and slaughtered by CO_2 inhalation. The cecal content was collected from each hen for bacterial counts.

2.3 Calculations and measurements

Feed intake was calculated as the difference between initial and final feeder weight. Feed conversion ratio (FCR) was expressed as the amount of feed (kg) to produce 1 kg of eggs or 12 eggs. Egg measurements started 2 wk after the beginning of the experiment. Egg production was calculated weekly as described by Ajakaiye et al. (2010). Eggs laid during the last day of each week were individually weighed and graded according to European Commission (2008). Egg quality traits were measured on 12 eggs per pen laid on three consecutive days. Measurements of egg weight, shell thickness and albumen height were made according to Keener et al. (2006). Yolk index was calculated as the ratio of yolk height to yolk diameter, while egg-shape index was calculated as the ratio of egg width to egg length. Haugh units score were estimated using the formula Haugh=100×log (T–1.7× W0.37+7.57), where H=height of the albumen (mm) and W=egg weight (g). *Escherichia coli* culture and counts were determined on chromogenic medium (ChromID coli, BioMérieux, France) and

Clostridium perfringens on tryptone sulphite neomycine agar (Scharlab, Spain). *Bifidobacterium* spp. and *Lactobacillus* spp. were cultured and enumerated on the man, rogosa and sharpe agar (Becton, Dickinson and Company, New Jersey, USA) according to O'Sullivan et al. (2011).

Table 3. Dietary ingredients and composition of the experimental diets (as-fed basis).

Item	PA [1] (g/kg)	0	0	2	2
	DWP [2] (g/kg)	0	60	0	60
Ingredients (g/kg)					
Yellow corn		413	455	409	451
Soybean meal		252	250	253	251
Wheat		100	100	100	100
Barley		100	0	100	0
Soybean oil		23.1	25.1	24.4	26.3
DWP		0	60	0	60
PA		0	0	2	2
Dicalcium phosphate		17.5	15.8	17.5	15.8
Sodium chloride		3.3	2.2	3.3	2.2
Vitamin-mineral premix and pigments [3]		6	6	6	6
Other components[4]		85.2	85	85.2	85
Chemical composition					
AMEn [5], MJ/kg		11.5	11.5	11.5	11.5
Crude protein, g/kg		173	173	173	173
Ca, g/kg		38	38	38	38
Available P [6], g/kg		3.7	3.7	3.7	3.7

[1] PA = *Pediococcus acidilactici*. Bactocell (strain MA 18/5, 10^{10} cfu/g; Lallemand, Blagnac, France). [2] DWP = dry whey powder. Dry sweet powder (703 g of lactose/kg of product; Sueromancha S.L, Toledo, Spain Toledo). [3] Providing the following per kilogram of diet: vitamin A, 8,000 IU; vitamin D_3, 1,600 IU; vitamin E, 16 mg; thiamine, 1 mg; riboflavin, 3 mg; pyridoxine, 1 mg; vitamin B_{12}, 0.01 mg; vitamin K, 1 mg; pantotenic acid, 7 mg; nicotinic acid, 16 mg; Mn, 70 mg; ZnO, 50 mg; Fe ($FeSO_4$ H_2O), 30 mg; Cu ($CuSO_4$ $5H_2O$), 4 mg; I (KI), 1 mg; Co, 0.2 mg; Se (Na_2SeO_3), 0.1 mg; CL, 240 mg; phytase, 300 units; ethoxyquin, 110 mg; xanthine, 0.6 mg, and canthaxanthin, 0.4 mg. [4] Providing the following per kilogram of diet: L-Methionine, 1.2; Sodium bicarbonate, 0.6; Calcium carbonate, 83.4. [5] AMEn: Apparent metabolizable energy corrected by N, calculated according to FEDNA (2010). [6] Calculated value.

2.4 Statistical analysis

Pen was considered the experimental unit. Data were analyzed considering a 2×2 factorial arrangement of treatments using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, US). The results were considered significant if $P < 0.05$.

3. RESULTS

No deaths occurred during the experiment. Results relative to productive performance, egg quality traits and cecal microbial counts are shown in Table 2. An interaction between levels of DWP and PA was found on egg production ($P = 0.008$) and on cecal counts of *Clostridium perfringens* ($P = 0.047$), so that the addition of DWP increased egg production (82.5 vs. 75.6%) and reduced *C. perfringens* (4.3 vs. 5.8 log10 cfu/g cecal content) only when PA was not used. Cecal counts of *Bifidobacterium* spp. was increased with the addition of WP ($P = 0.012$). However, these microbial results should be viewed with caution because of the reduced number of replicate pens with 1 hen per pen. The remaining performance results, including egg quality traits, and cecal counts were not affected by the evaluated levels additives.

4. DISCUSSION

To the best our knowledge no studies until today have reported the joint use of DWP and PA in poultry diets. The mixture of DWP and PA did not result in a synergic effect leading to a positive modulation of cecal bacteria and to better performance, as previously reported with different mixtures of prebiotics and probiotics in poultry diets (Gaggìa et al., 2010). PA are usually found in the gastrointestinal tract of healthy chickens (Ghareeb et al., 2012), and thus their adaptation to gut host conditions should not be a problem when is externally supplemented. However, the simultaneous presence of DWP could stimulate the development of other bacteria populations that could compete with PA for sites of attachment to the gut or for the usage of growth substrates, limiting the PA growth and their beneficial function on hens. Other possible reasons for the lack of a synergic effect during the present study would be the type of microorganisms chosen as probiotics, the evaluated dose, the prebiotic sources, or a combination of all these aspects (Chambers and Gong, 2011). Therefore, further

research is necessary to determine the adequate additive doses, or to find another complementary additive with a better synergistic effect with those tested in this study.

The inclusion of DWP in the diets, however, increased egg production by 9% together with a concomitant reduction of 26% in *C. perfringens* counts only when PA was not added. We consider that lactose contained in DWP promoted the observed changes. Lactose is not absorbed in the intestine of poultry, but is fermented to short chain fatty acids (SCFAs) instead (Gülsen et al., 2002). High energy metabolites such as SCFAs would supply more energy for poultry metabolism (Józefiak et al., 2004) and stimulate greater egg production. It is also known that the absence of detrimental bacteria may improve performance in poultry (Torok et al., 2011). Therefore, the favourable modulation of cecum bacteria populations may also explain the enhanced egg production.

Feeding with DWP increased Bifidobacterium spp. counts by 30%. Lactose is used by Bifidobacterium spp. as a source of energy (Goodfellow et al., 2012), what could explain their increase. Larger amounts of health-promoting bacteria such as Bifidobacterium spp. might suppress other potentially pathogenic by competitive exclusion (Gaggìa et al., 2010), improving then the health status of hens.

Table 2. Effect of the experimental diets on animal performance, egg quality, classification, and cecal microbial counts.

Item	PA [1] (g/kg)	0	0	2	2	SEM [3]	P-value		
	DWP [2] (g/kg)	0	60	0	60		DWP	PA	WP×PA
Performance (n = 5 pens/treatment; 15 hens/pen)									
Weight gain (g/d)		1.5	2	2	1.1	0.2	0.123	0.329	0.424
Egg production (%)		75.6	82.5	82.6	76.9	2.0	0.771	0.684	0.008
Egg weight (g)		66	66.2	65	65.7	0.8	0.720	0.915	0.581
Feed intake (g DM/d)		86	97	99	100	6.9	0.268	0.468	0.478
Feed conversion ratio (kg/kg eggs)		1.7	1.8	1.8	2	0.3	0.485	0.598	0.632
Feed conversion ratio (kg/dozen eggs)		1.4	1.5	1.4	1.6	0.1	0.52	0.666	0.392
Egg quality (n = 5 pens/treatment; 12 eggs/pen)									
Yolk index		0.48	0.47	0.47	0.48	0.01	0.899	0.896	0.389
Egg-shape index		74.5	74.3	74.2	74.6	0.2	0.720	0.954	0.384
Shell thickness (mm)		0.4	0.4	0.4	0.4	0	0.447	0.645	0.341
Haugh units		90.7	88.7	88.5	89.3	1.1	0.551	0.211	0.179
> 73 g		5.8	8.8	9.7	9.2	2.1	0.335	0.561	0.435
63 to 73 g		64.8	58.7	65.3	55.3	4.2	0.876	0.918	0.669
53 to 62		28.9	31.4	24.9	35	4.4	0.101	0.79	0.371
< 53		0.4	0.2	0.9	0.5	0.1	0.542	0.637	0.432
Cecal microbial counts (Log_{10} cfu/g of cecal content) (n = 3 pens/treatment; 1 hen/pen)									
Bifidobacterium spp.		6.5	8.4	5.2	7.2	6.8	0.012	0.074	0.996
Lactobacillus spp.		9	9	9.8	9.2	9.3	0.310	0.111	0.277
Clostridium perfringens		5.8	4.3	4.8	4.9	4.9	0.071	0.631	0.047
E. coli		6.3	6.6	7.0	6.5	6.6	0.819	0.448	0.264

[1] PA = *Pediococcus acidilactici*; [2] DDWP = dry whey powder; [3] SEM: Standard error of the mean.

5. CONCLUSION

In conclusion the joint addition of DWP and PA in hens' diets during the late stage of production did not improve productive performance or change the cecal microbial population. However, the addition of DWP increased *Bifidobacterium* spp. cecal counts and only reduced the *Clostridium perfringens* counts together with an increase on egg production, when PA was not added.

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CHAPTER 2

MICROBIAL AND FUNCTIONAL PROFILE OF THE LAYING HENS' CECA FED WITH PREBIOTIC, PROBIOTIC, AND SYNBIOTIC

Submitted to Environmental Microbiology Reports : Pineda-Quiroga, C., Borda-Molina, D., Chaves-Moreno, D., Ruiz, R., Atxaerandio, R., Camarinha-Silva, A., García-Rodríguez, A. 2017. Microbial and functional profile of the laying hens' ceca fed with prebiotic, probiotic, and synbiotic: A preliminary study

ABSTRACT

Diet plays an important role in modulating the cecal microbiome. On this study we aim to assess the effects, on the ceca of laying hens, of supplementing diets with dry whey powder as prebiotic, *Pediococcus acidilactici* as probiotic, and the combination of both as synbiotic for 70 days. Cecal microbiota composition was determined using 16S rRNA Illumina amplicon gene sequencing, while functional profiling was studied by whole DNA sequencing. Targeted sequencing results showed a clear grouping of the samples per diet at operational taxonomic unit (OTU) level. Bacteroidaceae and Ruminococcaceae were the most representative families in all diets followed by Porphyromonadaceae, while Lachnospiraceae and Coriobacteriaceae were notably more detected in the synbiotic diet. Moreover, prebiotic and synbiotic diets promoted the presence of *Olsenella* spp. and *Lactobacillus crispatus*. In relation to the metagenomics approach, it showed that a core of main functions was shared between all metagenomes (45%). The prebiotic diet promoted the presence of more unique functions (22.5%) compared with control, which showed the lowest percentage (1.6%). Major presence of genes encoding the metabolism of butanoate, propanoate, galactose, and inositol phosphate were especially stimulated by prebiotic diet. On the other hand, probiotic increased the abundance of ascorbate and aldarate metabolism-related genes, while synbiotic increased those related to starch and sucrose metabolism. Control showed higher presence of genes related to β-lactam resistance, being those absent in synbiotic. The results indicated that each dietary supplementation influenced the cecal microbial composition, but these changes did not imply a disturbance in their main biological roles. However, some specific functions were evident depending on the source of supplementation.

1. INTRODUCTION

It is widely recognized that diet is one of the contributing factors shaping the composition and functions encoded by the microbiota in the gastrointestinal tract (Borda-Molina et al., 2016). Dietary supplements such as prebiotics, probiotics, and synbiotics have frequently been used in poultry industry as a feasible alternative to increase animal health and performance (Angelakis, 2017). It has been proposed that

underlying mechanisms by which these additives improve animal conditions are mainly mediating changes in the gastrointestinal microbiota (Angelakis, 2017). Successful productive results in laying hens have been reported due to the inclusion of dry whey powder (DWP) as prebiotic (Pineda-Quiroga et al., 2017), and *Pediococcus acidilactici* MA 18/5 (PA) as probiotic (Mikulski et al., 2012), while productive results were inconclusive when DWP and PA were used as synbiotic (Pineda-Quiroga et al., 2017). However, to the best of our knowledge not many efforts have been done to explore the GIT microbial community and its corresponding functionality in laying hens receiving these types of additives.

The ceca are the primary site of fermentation of the avian GIT and it harbor the most complex and yet not fully characterize microbial community. Ceca resident microorganisms are responsible for a wide range of catabolic pathways, resulting in the synthesis of a range of products that are accessible to the host (Stanley et al., 2014). A better understanding of the phylogenetic structure and functional capacity of the ceca microbial consortia, in response to dietary interventions is essential to elucidate their roles in the host physiology and productivity. Sequencing technologies and the development of bioinformatic tools allow us to investigate in depth such challenges by avoiding the cultivation biases (Sergeant et al., 2014). Therefore, this study aims to give a preliminary insight into the microbiota changes in response to the dietary inclusion of DWP, PA, and a mixture of both through the analysis of the microbial community structure and function usign high throughput sequencing technologies.

2. MATERIALS AND METHODS

2.1 Animals, experimental diets, sample collection, and DNA extraction

The experiment followed the European Union (2010/63/EU) and Spanish regulations (RD 53/2013) for the care and use of animals for experimental and other scientific purposes. The study was conducted at the experimental farm of Neiker-Tecnalia in Arkaute (Vitoria-Gasteiz, Spain). A total of 300 laying hens (ISA Brown strain, Avigán Terralta S.A, Tarragona, Spain) with 57 weeks of age were managed and fed during 10 weeks as described in Pineda-Quiroga et al. (2017). The treatments involved a control diet (corn-soybean basal diet without supplementation of DWP or

PA), prebiotic (60 g/kg of inclusion of DWP), probiotic (2 g/kg of inclusion of PA), and synbiotic (a mixture of 60 g/kg of DWP and 2 g/kg of of PA). The DWP was a commercial sweet powder (Sueromancha S.L, Toledo, Spain; 703 g of lactose/kg of product) and the probiotic was a commercial probiotic "Bactocell" (Lallemand, France), containing a live culture of P. acidilactici (strain MA 18/5, 10^{10} CFU/g). Feed and water were provided ad libitum throughout the experiment.

On the last day of the experiment, 12 hens (three per treatment) were randomly selected from different pens and euthanized by CO_2 inhalation to isolate cecal digesta content. Samples were immediately stored at -80°C until further analysis. Total nucleic acid was extracted using the PowerSoil DNA extraction Kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer recommendations. DNA was quantified with Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, DE, USA) and DNA integrity was checked through agarose gel electrophoresis.

2.2 16S rRNA gene amplification, Illumina sequencing, and bioinformatics analysis

Cecal DNA of all sampled hens was used for Illumina amplicon library preparation. PCR amplifications of the V1-2 hypervariable region of the 16S rRNA gene were performed using PrimeSTAR HS DNA Polymerase (Clontech Labotatories, Mountain View, CA, USA) according to Camarinha-Silva et al. (2014). Libraries were sequenced using paired-end sequencing on an Illumina MiSeq platform.

Bioinformatic analysis followed the Mothur Miseq SOP (Kozich et al., 2013). Primers and barcodes were trimmed and resulting sequences were aligned using SILVA-based bacterial reference alignment. Chimera sequences were checked and removed using UCHIME. Finally, 647.359 sequence reads, with a mean number of reads per sample of 53.947 ± 3.821 were obtained. These reads were then clustered into operational taxonomic units (OTUs) using a ≥ 97% sequence identity threshold. Singletons were deleted and OTUs with less than 10 reads per sample were removed from the analysis (< 0.002% of the total). Finally, a total of 934 OTUs were taxonomically assigned using the naive Bayesian RDP classifier. Sequences are

available at the European Nucleotide Archive, under the accession number PRJEB21237 in http://www.ebi.ac.uk/ena/data/view/PRJEB21237.

Relative abundances of the OTUs were analyzed using multivariate statistical routines in PRIMER (version 7.0.9, PRIMER-E; Plymouth Marine Laboratory, Plymouth UK). Data was standardized by total, and a resemblance matrix was generated using Bray-Curtis similarity coefficient. The microbial community structure was explored with non-metric multidimensional scaling (nMDS) plots and the statistical comparison between diets was determined by means of a permutational analysis of variance (PERMANOVA, 999 permutations). Differences were studied based on pair-wise tests using a permutation method, being considered significant if $P \leq 0.05$. Individual OTUs contributing to dissimilarity for each comparison were identified by a similarity percentage analysis (SIMPER). SIMPER was also used to determinate the average of similarity in ceca microbial community composition among the replicates. Pielou's evenness index and Shannon-weaver index of diversity (H') were determined and analyzed using a Kruskal- Wallis test (R environment,V 3.3.3).

2.3 Metagenomics sequencing and analysis

Cecal DNA from one laying hen per diet was sequenced through Illumina HiSeq2500 platform. The sequencing generated an average number of 7.528.949 sequences, with a length of 100 base pairs, which were cleaned and assembled using the CLC Main Workbench software version 9.0.1 (CLCbio®, Maryland, USA). Subsequently, genes annotations were done through the metagenomics RAST server version 4 (http://metagenomics.anl.gov/). The annotation considered the KEGG database for proteins and KEGG categories (KO) for taxonomy, working with default parameters from MG-RAST (minimum percentage identity cutoff of 60%, maximum e-value cutoff of 1e−5, and minimum alignment length cutoff of 15 bp). The metagenome sequences are publicly available under the MG-RAST project mgp21245 (Metagenome IDs: control [mgm4730023.3], probiotic [mgm4730065.3], prebiotic [mgm4730022.3] and synbiotic [mgm4730024.3]).

For the data analysis, the $\log_2$fold change (LFC) in the presence of genes was calculated based on normalized reads with the DESeq R package (Love at al., 2014). Genes meeting the cut-off criteria of P-value ≤ 0.05 (Wald test), LFC ≥ 1, or LFC ≤ -1

were considered as differentially present. Venn diagram was depicted with those genes shared between each diet and their interactions, using the online tool Venny 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

3. RESULTS

3.1 Microbial community analysis based on 16S rRNA gene amplicon sequencing

Exploring the global bacterial community structure using a nMDS revealed that biological replicates grouped per diet (Fig 1). A clear separation was observed between samples from control and synbiotic, and also between probiotic and synbiotic diets. The average similarity within replicates of ceca samples was 67% in control diet, 69% in prebiotic and probiotic, and 59% in synbiotic. PERMANOVA analysis indicated that microbial communities of hens fed with control and probiotic were different from those fed with prebiotic and synbiotic ($P = 0.001$), while no differences were found between control and probiotic, and between prebiotic and synbiotic. Moreover, the lowest Pielou's evenness and Shannon diversity were detected in synbiotic when compared to control ($P < 0.030$), while no differences were observed between the remaining diets (Fig 1).

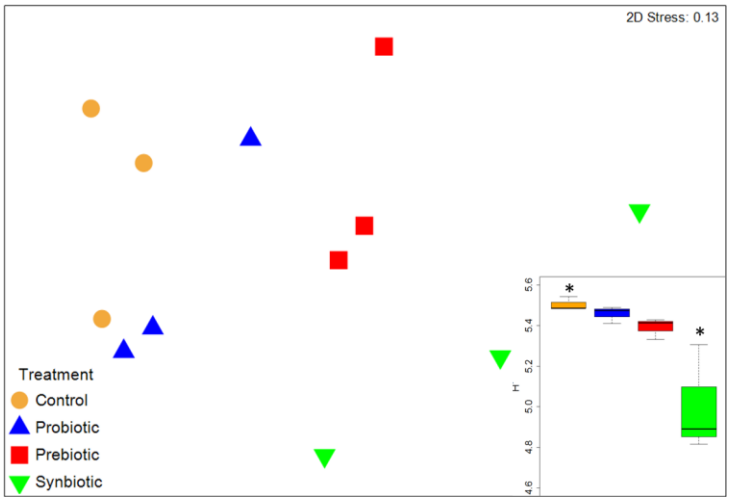

Fig 1. nMDS plot showing the distribution of the biological replicates on the diets. The diversity calculated with the Shannon index is plotted in the boxplot on the right bottom side.

Bacteroidetes was the most abundant phylum identified in all diets (46% on average), followed by Firmicutes (34% on average). Prebiotic and synbiotic diets promoted the increase on Actinobacteria (14 and 9% on average, respectively) and a decrease on Proteobacteria (2.5 and 4% on average, respectively) in comparison to control diet (4 and 3.5% on average, respectively), and probiotic (5 and 4% on average, respectively; Fig 2A).

At family level, Bacteroidaceae was dominant in all diets (16% on average), followed by Ruminococcaceae (11.5% on average). In prebiotic and synbiotic treatments, Coriobacteriaceae and Lactobacillaceae abundance were higher in comparison to control and probiotic (10.5 and 4.2% on average for both treatments, respectively), while Prevotellaceae was lower (5.6% on average for both treatments) (Fig 2B). Porphyromonadaceae and Lachnospiraceae families were identified in similar abundances in all diets (8.11 and 6.5% on average, respectively), as well as members of less representative families of Spirochaetaceae, Rikenellaceae, Erysipelotrichaceae, and Elusimicrobiaceae (3.5, 2.5, 2.4 and 1.2% on average, respectively). At genus level, *Bacteroides* was the most abundant in all diets (14% on average; Fig 2A), whilst *Olsenella* and *Lactobacillus* were more abundant in prebiotic (6 and 11.5% on average, respectively) and synbiotic (4.1 and 4.4% on average, respectively) in comparison to control (2.5 and 3.0% on average, respectively) and probiotic (3.0 and 2.5% on average, respectively). Representatives of *Parabacteroides*, *Alistipes*, and *Ruminococcus2* genera were detected in all diets with similar abundance (3.3, 2.5, and 2.0% on average, respectively), while *Barnesiella*, *Sphaerochaeta*, *Faecalibacterium*, *Paraprevotella*, *Elusimicrobium*, and *Collinsella* accounted for nearly 1.2% of the total abundance in all treatments.

The percentage of dissimilarity of cecal microbiota from hens fed with prebiotic and control was 46%, prebiotic and probiotic was 38%, synbiotic and control was 39%, and synbiotic and probiotic was 43%. These results were mainly due to the higher abundance of OTUs associated to *Olsenella* spp. (OTU 1) and *Lactobacillus crispatus* (OTU 3) in prebiotic and synbiotic diets. *Megamonas* spp. (OTU 28) was also detected in both diets at 1.1% of abundance, although it was less present in control (0.02%) and probiotic diets (0.07%). In addition, OTU 2 was one of the most abundant phylotypes detected in prebiotic and synbiotic diets in comparison to the control and probiotic, but it was not possible to classify it taxonomically (Fig 3).

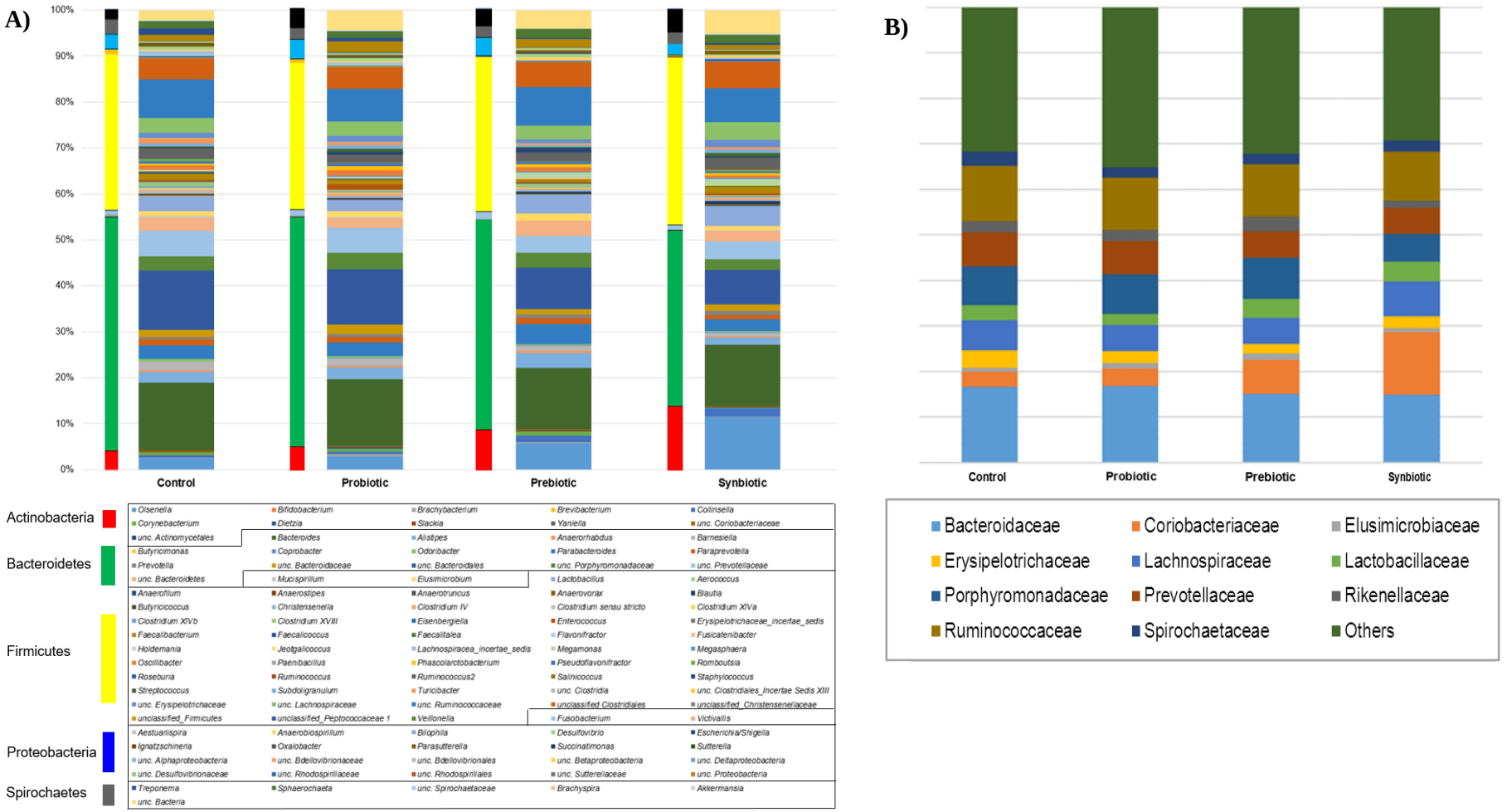

Fig 2. Taxonomical information related to the 16S rRNA gene amplicon sequencing (average relative abundance > 1%). **A)** The small bars indicate information at phylum level and the wider bars indicate information at the genus level. **B)** Abundance information at family level.

OTUs 11, 16, 19, 20, 35 and 28 were detected with nearly 1% of abundance in all treatments, but there were not possible to classify them at genus or specie taxonomical levels.

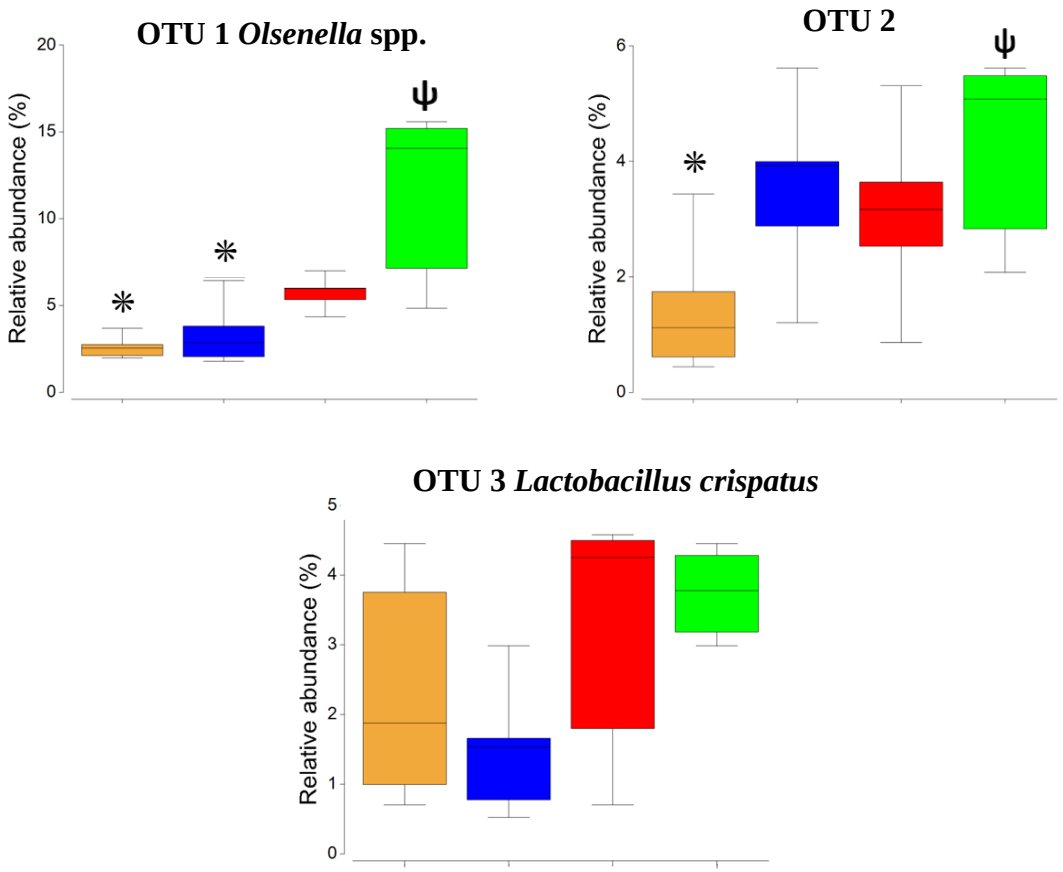

Fig 3. Box-plots showing the most abundant OTUs. The color convention indicates yellow for control, blue for the probiotic, red for the prebiotic and green for the synbiotic treatment. Different symbols indicate statistical significance ($P \leq 0.05$).

3.2 Metagenome analysis

The proportion of bacterial sequences from the metagenome data-set was 99%, with the remainder of the reads belonging to Archaea and Eukaryotes. The main phyla in all diets were Bacteroidetes (between 46 and 60%) and Firmicutes (between 26 and 33%), while less than 8% of the reads belong to Actinobacteria and Proteobacteria. The annotation analysis of the reads obtained from the KEGG categories identified 4265 total genes encoding microbial functions, of which a core of 1764 functions (45.5%)

were shared between all metagenomes (Fig. 4). Microbial communities from the supplemented diets encoded more functions in comparison with the control, being prebiotic those with higher classified unique functions. Moreover, it was observed that the microbial consortia of hens fed with control and probiotic diets shared more functional information between them (76.2%), while hens fed with prebiotic shared less common functions with both control and synbiotic (approx. 57%).

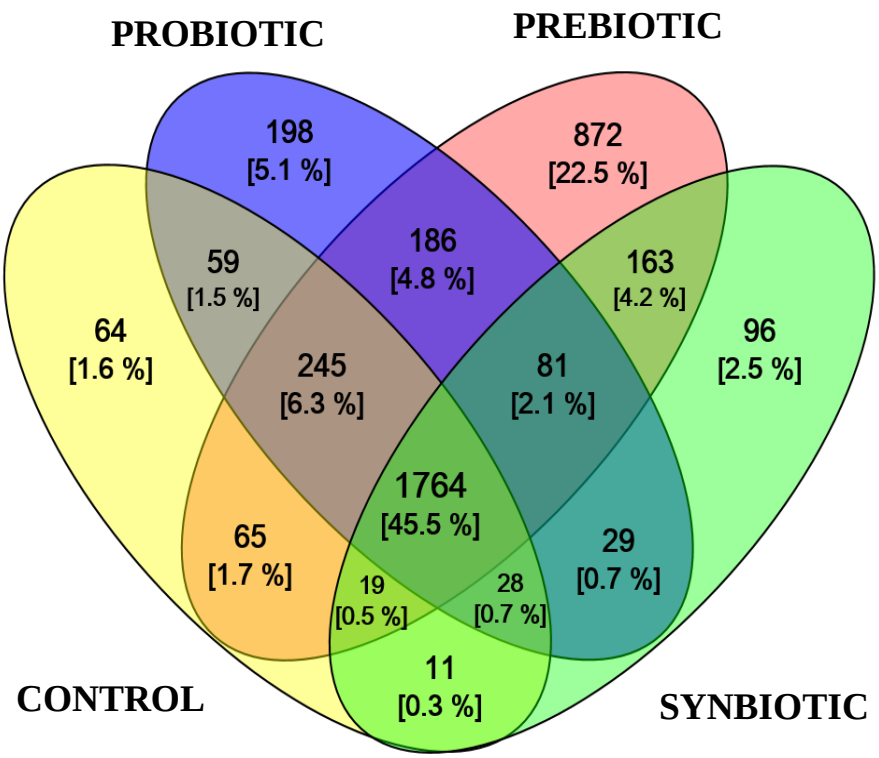

Fig 4. Venn diagram depicting the percentage of genes assigned and shared between the four metagenomes (the color convention indicates, yellow for control, blue for the probiotic, red for the prebiotic and green for the synbiotic).

The fold change analysis showed that control diet mainly increased the microbial pathways related to lysine degradation, whereas supplemented diets increased the microbial genes related to starch, sucrose, pyruvate, citrate cycle, and glycerophospholipids metabolism. Other microbial functions that were also affected by control diet are shown in Table 1. Prebiotic supplementation increased the pathways related to butanoate, propanoate, fructose, mannose, inositol phosphate metabolism, and galactose metabolism in comparison to the others (Table 2). It was also observed an

increase in the abundance of genes related to fatty acid biosynthesis as well as in the pathways for the thiamine metabolism in comparison to the other diets. Some specific genes related to fatty acids metabolism and retinol metabolism was also exclusively identified when prebiotic was offered. On the other hand, feeding with probiotic resulted in an increase on the abundance of ascorbate and aldarate metabolism-related genes when compared to the remaining diets. This diet also increased the microbial genes encoding nicotinate and nicotinamide metabolism in comparison to control and prebiotic. Additionally, probiotic was the only diet showing more genes related to glyoxylate and dicarboxylate metabolism (Table 3). Synbiotic supplementation augmented the pathways for starch and sucrose metabolism in comparison to the others (Table 4). This treatment also increased the abundance of genes related to retinol and glycolysis/gluconeogenesis metabolism, and with the steroid hormone biosynthesis in comparison to other diets. More pathways for amino sugar and nucleotide sugar metabolites were promoted by synbiotic compared to probiotic and prebiotic, as well as for nicotinate and nicotinamide in relation to control.

Some functions related to amino acid metabolism and degradation were also affected by each dietary treatment, and are shown in Table 1, 2, 3, and 4. Information for the biosynthesis of other secondary metabolites did not show remarkably differences between the diets. Regarding antibiotic resistance, it was observed higher expression of genes for β-lactam resistance (methicillin resistance protein) in control treatment in comparison to prebiotic and probiotic diets, whereas it was absent in synbiotic (Table 1).

Table 1. Microbial genes more present in the cecal of laying hens fed with control diet. The functions shown correspond to the third level of the KEGG categories assignation. Negative values indicate that the control has a value of 0% of abundance for the corresponding function.

E.C [1]	Functions affected	Control *vs.* Probiotic [2]	Control *vs.* Prebiotic	Control *vs.* Synbiotic	Control [3]
		Log_2FC	Log_2FC	Log_2FC	Abundance (%)
Starch and sucrose metabolism					-
EC:5.4.2.6	Beta-phosphoglucomutase [K01838]	-1.64	-1.72	-2.33	-
Piruvate metabolism					
EC:1.1.2.4	D-lactate dehydrogenase (cytochrome) [K00102]	-3.48	-4.13	-3.69	-
Citrate cycle (TCA cycle)					-
EC:4.2.1.3 4.2.1.99	2-methylisocitrate dehydratase [K01682]	-1.40	-2.05	-1.84	-
Glycerophospholipid metabolism					-
	Glycerol-3-phosphate dehydrogenase subunit B				-
EC:1.1.5.3	[K00112]	-1.88	-1.12	-2.69	
Glycine, serine and threonine metabolism					-
EC:2.1.4.1	Glycine amidinotransferase [K00613]	-1.38	-1.88	-2.56	-
EC:4.4.1.1	cystathionine gamma-lyase [K01758]	-1.63	-1.63	-2.56	-
EC:2.1.1.20	glycine N-methyltransferase [K00552]	AB	1	AB	0
EC:3.5.3.3	creatinase [K08688]	1	1	AB	0
EC:2.3.1.178	Diaminobutyric acid acetyltransferase[K06718]	1	1	AB	0
EC:2.1.1.5	betaine-homocysteine S-methyltransferase [K00544]	AB	1	AB	0
Arginine and proline metabolism					-
EC:3.5.2.10	Creatinine amidohydrolase [K01470]	-1.20	-1.44	-1.52	-
EC:4.1.1.17	Ornithine decarboxilase [K01581]	-1.80	-1.22	-1.89	-
Cysteine metabolism					-
EC:2.6.1.57	Aromatic-amino-acid transaminase [K00832]	-1.21	-1.37	-2.18	-
Tyrosine metabolism					-

	2-hydroxyhepta-2,4-diene-1,7-dioate isomerase				-
EC:4.1.1.68 5.3.3-	[K05921]	-1.99	-1.22	-2.10	
Pantothenate and CoA biosynthesis					-
EC:2.7.1.33	Type I pantothenate kinase [K00867]	-1.02	-1.20	-1.68	-
EC:4.1.1.36	Phosphopantothenoylcysteine decarboxylase [K01598]	-1.79	-1.80	-1.10	-
Folate biosynthesis					
EC:2.6.1.85					
4.1.3.38	para-aminobenzoate synthetase [K01247]	-3.57	-3.72	-2.10	-
Riboflavin metabolism					-
	Low molecular weight phosphotyrosine protein				-
EC:3.1.3.2 3.1.3.48	phosphatase [K14394]	-1.31	-1.22	-3.10	
Lysine degradation					
EC:2.6.1.48	5-aminovalerate aminotransferase [K14268]	AB	AB	AB	2.08
EC:1.2.1.20	glutarate semialdehyde dehydrogenase [K14269]	AB	AB	AB	2.08
β-lactam resistance					
	methicillin resistance protein [K02547]	2.82	2.17	AB	-

[1] E.C: Enzyme Commission numbers. [2] Control: no additive supplementation; Probiotic: 2 g/kg of *P. acidilactici*; Prebiotic: 60 g/kg of dry whey powder, Synbiotic: 2 g/kg of *P. acidilactici* and 60 g/kg of dry whey powder. [3] Microbial genes only present in control diet. AB: Genes that were absent in the diet against which the comparison was made.

Table 2. Microbial genes more present in the ceca of laying hens fed with prebiotic. The functions shown correspond to the third level of the KEGG categories assignation.

E.C.[1]	Functions affected	Prebiotic *vs.* Control[2]	Prebiotic vs Probiotic	Prebiotic vs Synbiotic	Prebiotic[3]
		Log_2FC	Log_2FC	Log_2FC	Abundance (%)
Butanoate metabolism					
EC:2.8.3.12	Glutaconate CoA-transferase, subunit A [K01039]	1.40	AB	2.62	-
EC:1.1.1.61	4-hydroxybutyrate dehydrogenase [K00043]	/	1.09	2.44	-
EC:1.1.1.30	3-hydroxybutyrate dehydrogenase [K00019]	AB	AB	AB	13.7
EC:1.1.1.4 1.1.1.303	Butanediol dehydrogenase / diacetyl reductase [K00004]	AB	AB	AB	11.3
Propanoate metabolism					
EC:3.5.99.7	1-aminocyclopropane-1-carboxylate deaminase [K01505]	1.22	/	1.8	-
EC:4.2.1.79	2-methylcitrate dehydratase [K05608]	1.57	1.61	AB	-
EC:3.5.99.7	1-aminocyclopropane-1-carboxylate deaminase [K00923]	1.22	/	1.70	-
EC:2.3.3.5	2-methylcitrate synthase [K01659]	/	/	1.73	-
EC:4.1.3.30	methylisocitrate lyase [K03417]	/	/	2.44	-
Fructose and mannose metabolism					
EC:1.1.1.67	mannitol 2-dehydrogenase [K00045]	/	/	1.19	-
EC:2.7.1.105	PFK; 6-phosphofructo-2-kinase [K00900]	AB	1.01	1.70	-
EC:3.2.1.80	fructan beta-fructosidase [K03332]	1.63	1.32	AB	-
Inositol phosphate metabolism					

EC:2.7.1.92	5-dehydro-2-deoxygluconokinase [K03338]	1.06	1.20	AB	-
EC:5.3.1.-	iolB; 5-deoxy-glucuronate isomerase [K03337]	AB	1.76	1.70	-
Galactose metabolism					
EC:3.2.1.108 3.2.1.62	lactase-phlorizin hydrolase [K01229]	3.22	2.23	AB	-
EC:1.1.1.251	galactitol-1-phosphate 5-dehydrogenase [K00094]	1.37	2.38	AB	-
EC:2.7.1.144	tagatose 6-phosphate kinase [K00917]	/	/	1.17	-
EC:4.1.2.21	2-dehydro-3-deoxyphosphogalactonate aldolase [K01631]	/	2.97	1.12	-
EC:4.2.1.6	galactonate dehydratase [K01684]	/	/	1.35	-
EC:3.2.1.20 3.2.1.3	maltase-glucoamylase [K12047]	AB	AB	AB	1.65
Phenylalanine, tyrosine and tryptophan biosynthesis					
EC:4.1.3.27 2.4.2.18	anthranilate synthase/phosphoribosyltransferase [K13497]	4.96	1.80	/	-
EC:4.1.1.48 5.3.1.24	indole-3-glycerol phosphate synthase / phosphoribosylanthranilate isomerase [K13498]	1.42	1.26	1.26	-
EC:2.7.1.71 4.2.3.4	shikimate kinase / 3-dehydroquinate synthase [K13829]	1.09	1.78	2.99	-
EC:5.4.99.5 4.2.1.51	chorismate mutase / prephenate dehydratase [K14170]	/	/	1.27	-
Glycine, serine and threonine metabolism					
EC:1.1.99.1	choline dehydrogenase [K00108]	2.11	1.23	3.81	-
EC:2.1.1.20	glycine N-methyltransferase [K00552]	AB	AB	AB	1.61
Fatty acid metabolism					
EC:2.3.1.40 6.2.1.20	long-chain-fatty-acid--[acyl-carrier-protein] ligase [K05939]	2.17	1.55	/	-

	cytochrome P450, family 4, subfamily A				2.42
EC:1.14.15.3	[K07425]	AB	AB	AB	
EC:2.3.1.21	carnitine O-palmitoyltransferase 2 [K08766]	AB	AB	AB	4.04
Fatty acid biosynthesis					
EC:2.3.1.-	fatty acid synthase, bacteria type [K11533]	/	1.22	4.56	
Thiamine metabolism					
EC:2.5.1.3 2.7.1.50	hydroxyethylthiazole kinase [K14154]	/	2.23	1.11	-
EC:2.7.1.49 2.7.4.7					-
2.5.1.3	thiamine-phosphate diphosphorylase [K14153]	1.22	/	AB	
Retinol metabolism					
EC:1.1.1.-	retinol dehydrogenase 16 [K11154]	AB	AB	AB	2.42
	diacylglycerol O-acyltransferase 2-like protein 4	AB	AB	AB	
EC:2.3.1.75 2.3.1.76	[K11156]				3.23

[1] E.C: Enzyme Commission numbers. [2] Control: no additive supplementation; Probiotic: 2 g/kg of *P. acidilactici*; Prebiotic: 60 g/kg of dry whey powder; Synbiotic: 2 g/kg of *P. acidilactici* and 60 g/kg of dry whey powder. [3]Microbial genes only present in hens fed with prebiotic. Slash indicates that gene did not pass the filters stablished to be considered for comparisons. AB: Genes that were absent in the diet against which the comparison was made.

Table 3. Microbial genes more present in the ceca of laying hens fed with probiotic. The functions shown correspond to the third level of the KEGG categories assignation.

E.C.[1]	Functions affected	Probiotic vs Control[2]	Probiotic vs Prebiotic	Probiotic vs Synbiotic	Probiotic[3]
		Log_2FC	Log_2FC	Log_2FC	Abundance (%)
Ascorbate and aldarate metabolism					
EC:1.1.1.122	D-threo-aldose 1-dehydrogenase [K00064]	1.23	/	2.13	-
EC:5.1.3.22	L-ribulose-5-phosphate 3-epimerase [K03079]	1.10	3.05	1.58	-
Glyoxylate and dicarboxylate metabolism					
EC:1.2.1.2	formate dehydrogenase [K00122]	AB	2.94	/	-
EC:4.1.1.8	oxalyl-CoA decarboxylase [K01577]	/	2.40	1.07	-
EC:3.5.1.56	N,N-dimethylformamidase [K03418]	AB	AB	AB	1.03
EC:1.2.1.2	formate dehydrogenase-N, gamma subunit [K04509]	AB	AB	AB	1.55
K11472	glycolate oxidase FAD binding subunit	AB	AB	AB	1.03
EC:4.1.1.2	Oxalate decarboxylase [K01569]	AB	AB	AB	0.6
EC:1.1.1.37	Malate dehydrogenase [K00025]	AB	0.15	AB	0.5
Alanine, aspartate and glutamate metabolism					
EC:4.1.1.12	aspartate 4-decarboxylase [K09758]	/	1.17	2.61	-
EC:1.5.1.12	1-pyrroline-5-carboxylate dehydrogenase [K00294]	/	/	1.01	-

EC:6.3.5.5	carbamoyl-phosphate synthase [K01954]	/	1.27	1.97	-
EC:1.5.99.8 1.5.1.12	delta 1-pyrroline-5-carboxylate dehydrogenase [K13821]	1.59	/	2.30	-
Phenylalanine metabolism					
EC:1.2.1.39	phenylacetaldehyde dehydrogenase [K00146]	2.47	2.94	/	-
EC:4.2.1.80	2-keto-4-pentenoate hydratase [K02554]	1.57	1.94	/	-
EC:1.14.12.19	cinnamic acid dioxygenase subunit alpha [K05708]	1.31	2.67	/	-
Nicotinate and nicotinamide metabolism					
EC:3.1.3.5 3.6.1.45	UDP-sugar diphosphatase [K11751]	1.49	/	/	-
EC:3.1.3.5	5'-nucleotidase [K01081]	/	1.05	/	-
EC:1.6.1.1	NAD(P) transhydrogenase [K00322]	/	1.81	/	-
EC:2.7.7.1	nicotinamide-nucleotide adenylyltransferase [K00952]	/	3.16	/	-

[1] E.C: Enzyme Commission numbers.[2] Control: no additive supplementation; Probiotic: 2 g/kg of *P. acidilactici*; Prebiotic: 60 g/kg of dry whey powder; Synbiotic: 2 g/kg of *P. acidilactici* and 60 g/kg of dry whey powder. [3] Microbial genes only present in hens fed with probiotic. Slash indicates that gene did not pass the filters stablished to be considered for comparisons. AB: Genes that were absent in the diet against which the comparison was made.

Table 4. Microbial genes more present in the ceca of laying hens fed with synbiotic. The functions shown correspond to the third level of the KEGG assignation.

E.C.[1]	Functions affected	Synbiotic vs Control[2]	Synbiotic vs Probiotc	Synbiotic vs Prebiotic	Synbiotic [3]
		Log_2FC	Log_2FC	Log_2FC	Abundance (%)
Starch and sucrose metabolism					
EC:2.4.1.7	sucrose phosphorylase [K00690]	1.82	/	/	-
EC:2.4.1.8	maltose phosphorylase [K00691]	1.11	/	/	-
EC:3.2.1.122	maltose-6'-phosphate glucosidase [K01232]	1.03	/	/	-
EC:5.4.2.6	beta-phosphoglucomutase [K01838]	2.34	/	/	-
EC:3.2.1.54	cyclomaltodextrinase [K01208]	1.23	/	2.32	-
EC:3.2.1.39	glucan endo-1,3-beta-D-glucosidase [K01199]	1.69	2.11	3.05	-
EC:2.4.1.12	cellulose synthase (UDP-forming) [K00694]	/	/	2.37	-
Glycolysis / Gluconeogenesis					
EC:4.1.1.1	pyruvate decarboxylase [K01568]	1.10	2.12	/	-
EC:6.2.1.13	acetyl-CoA synthetase (ADP-forming) [K01905]	2.10	3.17	1.47	-
Amino sugar and nucleotide sugar metabolism					
EC:3.5.1.41	chitin deacetylase [K01452]	/	2.12	2.47	-

Cysteine and methionine metabolism					
EC:2.1.1.10	homocysteine S-methyltransferase [K00547]	1.76	/	/	-
EC:2.6.1.57	aromatic-amino-acid transaminase [K00832]	2.18	/	/	-
EC:1.1.1.272	(R)-2-hydroxyacid dehydrogenase [K05884]	1.69	/	1.05	-
Valine, leucine and isoleucine biosynthesis					
EC:2.6.1.66	valine--pyruvate aminotransferase [K00835]	3.69	/	4.05	-
Fatty acid biosynthesis					
EC:3.1.2.14	oleoyl-[acyl-carrier-protein] hydrolase [K01071]	1.70	1.24	/	-
Steroid hormone biosynthesis					
EC:5.3.3.1	steroid delta-isomerase [K01822]	/	1.17	1.68	-
EC:3.1.6.2	steryl-sulfatase [K01131]	1.10	2.12	2.45	-
Nicotinate and nicotinamide metabolism					
EC:3.2.2.1	purine nucleosidase [K01239]	1.08	/	/	-
EC:1.6.1.2	NAD(P) transhydrogenase subunit alpha [K00324]	1.10	/	/	-
Retinol metabolism					
EC:1.3.99.23	all-trans-retinol 13,14-reductase [K09516]	1.10	1.83	1.76	-
Galactose metabolism					
EC:3.5.1.25	N-acetylgalactosamine-6-phosphate	AB	AB	AB	6.71

	deacetylase [K02079]				
Phenylalanine, tyrosine and tryptophan biosynthesis					
EC:4.2.1.51 4.2.1.91	cyclohexadienyl dehydratase [K01713]	AB	AB	AB	4.47

[1] E.C: Enzyme Commission numbers.[2] Control: no additive supplementation; Probiotic: 2 g/kg of *P. acidilactici*; Prebiotic: 60 g/kg of dry whey powder; Synbiotic: 2 g/kg of *P. acidilactici* and 60 g/kg of dry whey powder. [3] Microbial genes only present in hens fed with synbiotic. Slash indicates that gene did not pass the filters stablished to be considered for comparisons. AB: Genes that were absent in the indicated diets.

4. DISCUSSION

As previously described in laying hens older than 7 months by Videnska et al. (2014), we found with both 16S rRNA gene sequencing and metagenomics datasets, that Bacteroidetes and Firmicutes dominated the cecal microbiota composition, followed in a lesser extent by Proteobacteria and Actinobacteria. Feeding with synbiotic showed a decrease in the diversity indexes in comparison to control diet, which may be due to an increase of some specific microbial groups. These findings could imply less richness of the cecal microbiome, which is undesirable due to the negative impact it has on poultry performance (Stanley et al., 2014). Indeed, a reduction on egg production was observed when this synbiotic diet was offered to laying hens (Pineda-Quiroga et al., 2017). On the other hand, feeding hens with probiotic diet resulted in similar microbiota composition to the control, which was unexpected because of the well-known effects of probiotics to modulate the poultry cecal microbial composition (Lee et al., 2007; Djezzar et al., 2012). A possible explanation for this finding could be attributed to the late supplementation of probiotic, which did not give enough time of exposition to *P. acidilactici* to the hens. Thus, it is necessary to start with the dietary treatment at early stages of age, because the cecal microbiota community of hens older than 7 months is quite stable (Videnska et al., 2016). However, the current results revealed that prebiotic and synbiotic diets promoted favorable changes in the ceca microbiota composition towards an increase in both lactic-acid producing bacteria and bacteria able to improve the efficiency of short chain fatty acids synthesis (SCFAs). Both diets increased the abundance of *Olsenella* spp. (OTU 1), which is an anaerobic bacterium that ferments carbohydrates to lactic acid. This has been identified in the GIT of laying pullets (Chalvatzi et al., 2016), and some reports indicate that is involved in lipid and cholesterol metabolism (Goodfellow et al., 2012). An increase in *Lactobacillus crispatus* (OTU 3) was also promoted, which has been classified as potentially probiotic in poultry (Rezvani et al., 2016). Moreover, an aside finding is that its supplementation in broiler diets reduces the colonization of *Campylobacter jejuni* (Neal-McKinney et al., 2012), and exerts an inhibitory effect against *Salmonella enterica* serovar Enteriditis (van der Wielen et al., 2002). With a lower relative abundance overall, a propionic acid-producer bacteria identified as *Megamonas* spp. (OTU 28; De Vos et al., 2009) increased in abundance when feeding the above-mentioned diets. This bacterium acts as

hydrogen sink in the ceca, increasing the SCFAs production, which bring benefits to the energy metabolism of the host (Sergeant et al., 2014).

The metagenomics results suggest that diverse taxon's in the gut microbiota maintain a conserved core of genes, sharing almost half of them in the four diets. Among those functions differentially encoded, the cecal microbiota of the prebiotic group had more unique functions assigned than the remaining diets. Prebiotic exhibited more abundance of genes related to enzymes for galactose metabolism, causing the hydrolysis of lactose to SCFAs. It is probable due to the presence of lactose from dry whey powder as the only feed additive in the prebiotic diet. This finding might imply that hens fed with prebiotic had more energy availability, because of the extensive lactose fermentation to SCFA's (Józefiak et al., 2004). Moreover, prebiotic was the diet exhibiting higher abundance of specific genes related to metabolism of butanoate, as well as more pathways for butanoate and propanoate metabolism. These SCFA's represent several beneficial effects in the host related to mineral metabolism, the maintenance of the sanitary status, and the energy metabolism. In this sense, their absorption by ceca mucosa provides up to 11% of metabolizable energy for mature birds (Annison et al., 1968), which could be used for productive purposes. Indeed, when the same prebiotic than those used in the current study was offered to laying hens, it promoted higher egg production (Pineda-Quiroga et al., 2017).

More pathways related to mineral, vitamin, and fatty acids metabolism were also promoted by prebiotic diet. Regarding mineral metabolism, this diet increases the abundance of inositol phosphate (InsP) metabolism-related genes, which is in agreement with previous reports, indicating that degrading activities of InsP are mainly carried out in the ceca of laying hens (Marounek et al., 2010). Our results support the role of the cecal microorganisms in InsP degradation and phosphorous release, which was previously reported by Rodehutscord and Rosenfelder (2016). It is a positive finding for laying hens, because InsP contains a considerable amount of available phosphorus, which could be used for egg shell formation, hen skeletal integrity, and bone mineralization (Rodehutscord and Rosenfelder, 2016). In regards to vitamins and fatty acid metabolism, prebiotic diet increased the microbial pathways for thiamine metabolism and fatty acids biosynthesis, which could positively affect the hen. Thiamin (vitamin B1) improves the efficiency of metabolizable energy in broilers. As an active coenzyme (thiamine diphosphate), it is part of pyruvate dehydrogenase and α-

ketoglutarate dehydrogenase, both enzymes are indispensable for carbohydrate metabolism and energy production (Bäckermann et al., 2008).

Interestingly, supplemented diets exhibited less presence of β-lactams resistance related genes. Specifically, our results showed less abundance of methicillin resistance protein-related genes in prebiotic and probiotic supplementation, and absence of it in synbiotic supplementation, indicating that the tested additives interfere with the presence or tranference of this resistance. However, the reasons why DWP, PA or the combination of both might have inhibited the transfer of antibiotic resistance genes are unclear.

5. CONCLUSION

On this study we analyzed taxonomical and functional changes in the ceca microbiota of laying hens fed with prebiotics (dry whey powder), probiotics (*P. acidilactici*), and synbiotic (a mixture of both). The results exposed that supplementation of prebiotics and synbiotics modified the microbiota composition, whereas feeding with probiotic and without additives (control diet), did not affect it. Nevertheless, all dietary supplementations induced modulations in the abundance or specific presence of microbial functional genes, although these did not imply a disturbance in their main biological roles.

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CHAPTER 3

EFFECTS OF DRY WHEY POWDER ALONE OR COMBINED WITH CALCIUM BUTYRATE ON PRODUCTIVE PERFORMANCE, DUODENAL MORPHOMETRY, NUTRIENT DIGESTIBILITY, AND CECA BACTERIA COUNTS OF BROILER CHICKENS

Published in the Scientific Manuscript: Pineda-Quiroga, C., Atxaerandio, R., Ruiz, R., Garcia-Rodriguez, A. 2017. Effects of dry whey powder alone or combined with calcium butyrate on productive performance, duodenal morphometry, nutrient digestibility, and ceca bacteria counts of broiler chickens. Livestock Science 206, 65-70. doi.org/10.1016/j.livsci.2017.10.001

ABSTRACT

Prebiotics and organic acids have been proposed as safe additives in poultry feeding to promote performance and health. The purpose of this study was to assess the influence of supplementing corn-soybean diets of broiler chickens with dry whey powder (DWP), fat-coated calcium butyrate (CaB), and a mixture of both on apparent ileal digestibility (AID), pH of gastrointestinal content at various segments, productive performance, duodenal histomorphometry, and ceca microbial counts. The experiment consisted of a 2 × 2 factorial arrangement, with 2 DWP inclusion rates (0 and 60 g/kg of diet) and 2 CaB rates (0 and 1 g/kg of diet). One-day-old male broiler chickens were randomly allocated to floor pens and assigned to 1 of 4 treatments. In Trial 1, 120 broiler chickens were allocated to 4 treatments with 3 pens per treatment and 10 broiler chickens per pen during 21 d. With the addition of DWP, the AID of dry matter, crude protein, Ca, and P increased, and cecum pH decreased only when CaB was also added (CaB × DWP, $P < 0.046$). In Trial 2, 1,200 broiler chickens were allocated to the 4 treatments with 10 pens per treatment and 30 broiler chickens per pen during 42 d. With the dietary supplementation of DWP, average daily gain and feed intake of broiler chickens increased during starter, grower-finisher periods, and the entire feeding period only when CaB was also added ($P < 0.047$). However, with addition of DWP, feed conversion ratio (FCR) decreased in broiler chickens fed the diet without CaB, but it increased in those fed with CaB during the grower-finisher and entire feeding periods ($P < 0.001$). Duodenal histomorphometry measurements were evaluated using hematoxylin and eosin stains, and cecal microbial counts were determined by selective culture media. With the addition of DWP, villus height, villus height to crypt depth ratio, and villus surface area were increased only when CaB was also added (CaB × DWP, $P < 0.017$), while the supplementation of DWP increased *Bifidobacterium* spp. counts only when CaB was not added (CaB × DWP, $P = 0.049$). Results obtained in the present study indicate that the supplementation of DWP without CaB addition improved the FCR of broiler chickens. However, the supplementation of DWP together with CaB improve duodenal development, increases nutrient AID, and the weight and ingestion of broiler chickens.

1. INTRODUCTION

The use of prebiotics and organic acids in poultry feeding are in force as a result of the banning on the use of in-feed antibiotics as growth promoters in the EU (European Commission, 2003), and their restricted use in other countries (Huyghebaert et al., 2011). Prebiotics are defined as a non-digestible dietary compounds that modulate the composition, activity or both of gut microbiota, conferring a beneficial physiological effect on the host (Bindels et al., 2015). They promote the growth of specific species such as bifidobacteria and lactobacilli at the expenses of potentially pathogenic bacteria (Macfarlane et al., 2006; Vicente et al., 2007), and generate positive changes in gut morphology and digestive enzymes secretion in broiler chickens (Xu et al., 2003). Dry whey is a co-product of cheese industry, with lactose being its major component (about 70% of dry matter). Because of negligible lactase activity in the gastrointestinal tract of broiler chickens (Denbow, 2000), lactose can be used as a prebiotic. Most of non-digested lactose reaches the ceca, becoming an available substrate for beneficial bacteria such as *Bifidobacterium* spp. (Goodfellow et al., 2007). Moreover, lactose fermentation can lead to a decrease of cecum pH, and to a reduction of potentially pathogenic bacteria such as *Salmonella enteriditis* (Stringfellow et al., 2009; Tellez et al., 1993). However, to our knowledge, the effect of dry whey powder on the duodenal histomorphometry, pH of digestive organs, and nutrient ileal digestibility of broiler chickens has not been studied so far.

Organic acids and their salts are considered as safe feed additives, being their use in animal diets approved by most EU member states (Adil et al., 2010). Their general mode of action relates to their antimicrobial activity, their ability to reduce gastrointestinal tract pH, and to improve nutrient digestion (Dibner and Buttin, 2002). Butyric acid, a short chain organic acid, has been used in poultry diets in its free form (Adil et al., 2010), as glyceride, or as lipid coated sodium butyrate (Lesson et al., 2005). Although benefits of butyric acid on broiler chickens' performance (Adil et al., 2010), pH of digestive organs (Mahdavi and Torki, 2009), and intestinal morphometry (Lesson et al., 2005) have been reported, little is known about their effect on ceca microbial counts and nutrient ileal digestibility. Similarly, results concerning the use of fat-coated calcium butyrate in broiler chickens' diets are still lacking.

Studies about the joint utilization of prebiotics and organic acids in poultry feeding are scarce (Bozkurt et al., 2009; Taherpour et al., 2012). Furthermore, we are not aware of any study assessing the combined effect of dry whey powder and fat-coated calcium butyrate. We hypothesized that butyric acid could reduce the gastrointestinal load of potentially pathogenic bacteria. Under these conditions, lactose could be selectively used as a substrate for the growth of beneficial ceca bacteria in detriment of pathogenic bacteria, thus improving the sanitary status of broiler chickens. In addition, given the reported benefits of both additives, we expected a synergistic activity that would improve gut development and nutrient digestibility. Thus, this study was conducted to assess the effect of supplementing broiler chickens' diets with dry whey powder, fat-coated butyric acid, and the combination of both on apparent ileal digestibility, duodenal histomorphometry, productive performance, pH of gastrointestinal content at various segments, and ceca bacterial populations.

2. MATERIALS AND METHODS

2.1 Animals, housing, and experimental diets

The experiment followed the European Union (2010/63/EU) and Spanish regulations (RD 53/2013) for the care and use of animals for experimental, and was conducted at the experimental facilities of Neiker-Tecnalia (Vitoria-Gasteiz, Spain). One-day old male broiler chickens (Ross 308 strain) were obtained from a local commercial hatchery (AN Avícola Melida, S.A., Zumaia, Spain), and were randomly allocated to floor pens, at a stock density of 30 kg/m2. Pens were equipped with a manual feeder, nipple drinkers, and wood shavings as litter material. Room temperature and lighting program were implemented according to the strain guidelines (Aviagen, 2014).

Diets were corn-soybean based, and were formulated to meet broiler chickens' requirements during the starter and grower-finisher stages (FEDNA, 2008). Starter diets (from d 0 to 21) were offered in a crumbles form, and grower-finisher diets (from d 21 to 42) in a pelleted form. Chromium oxide (5 g Cr_2O_3/kg) was added to starter diets as external indigestible marker. The ingredient composition and the analyzed nutritional value of experimental diets are shown in Table 1. Animals had ad libitum access to one

of the following experimental diets: no supplementation of dry whey powder (DWP) or fat-coated calcium butyrate (CaB), inclusion of 60 g/kg of DWP, inclusion of 1 g/kg of CaB, or inclusion of a mixture of 60 g/kg of DWP and 1 g/kg of CaB. The DWP was a commercial sweet powder (703 g/kg lactose; Sueromancha S.L, Toledo, Spain). The CaB was a commercial product composed by 860 g/kg of salt (160 g Ca and 700 g butyric acid) and 140 g/kg of lipids (Globamax Performant, Global Nutrition International, Fougères, France).

2.2 Experimental design, measurements, and sampling

2.2.1 Trial 1: Apparent ileal digestibility (AID) and pH of gastrointestinal content at various segments

In this trial, 120 one-day-old broiler chickens were randomly allocated to 1.0 x 0.83 m floor pens, and assigned to receive 1 of the 4 experimental diets formulated for the starter period (Table 1). Each treatment comprised 3 pens with 10 broiler chickens each. At 21 d of feeding, 6 broiler chickens per pen were slaughtered by CO_2 inhalation. For nutrient AID determination, ileal digesta was collected. Ileum was considered to be the portion of the small intestine from the Meckel's diverticulum to the ileocecal junction, and the digesta of the last two-thirds of this section were gently collected as described by Kluth (2005). Ileal samples from broiler chickens within the same pen were pooled, stored in plastic containers, frozen at -20ºC, and lyophilized. Dry ileal digesta samples were ground to pass through a 0.5-mm screen, and stored in airtight containers at room temperature until chemical analyses. The AID of dry matter (DM), crude protein (CP), starch, Ca, and P were estimated using Cr_2O_3 as indigestible external marker.

For pH measurements, crop, proventriculus, gizzard, ileum, and cecum were carefully ligated and removed. Approximately 0.4 g of digesta from each section were collected, diluted in 1.6 ml of distilled water and gently agitated. Measurements were made using a calibrated electronic pH meter (Basic 20, Crison, Barcelona, Spain).

Table 1. Dietary ingredient and composition of the experimental diets of Trial 1 and 2 "as fed-basis".

Item	Starter (d 0 to 21)				Grower-finisher (d 21 to 42)			
CaB [1] (g/kg)	0	0	1	1	0	0	1	1
DWP [2] (g/kg)	0	60	0	60	0	60	0	60
Ingredients, g/kg								
Yellow corn	499	501	535	520	500	540	500	538
Soybean meal, 44% CP [4]	300	298	303	278	316	317	316	318
Wheat	153	101	115	110	107	0	105	0
CaB	0	0	1	1	0	0	1	1
DWP	0	60	0	60	0	60	0	60
Soybean oil	14	19	10	10	42	51	42	51
Dicalcium phosphate	5	3	5	3	20	19	20	19
Vitamin and mineral premix [5]	4	4	4	4	4	4	4	4
Calcium carbonate	14	3.8	16	3.8	4	3.8	4	3.8
Sodium chloride	3.8	2.7	3.8	2.7	3.8	2.7	3.8	2.7
DL-Met	0.4	0.4	0.4	0.4	1.8	1.7	1.8	1.7
L-Lys	1.7	1.7	1.7	1.7	0.6	0.1	0.6	0.1
Chromium oxide	5	5	5	5	0	0	0	0
Chemical composition								
AMEn [6], MJ/kg	12.3	12.2	12.3	12.3	13.2	13.1	13.1	13.1
CP, g/kg	199	193	192	196	189	184	185	182
Starch, g/kg	413	362	438	420	399	382	399	392
Ether extract, g/kg	3.8	4.6	4.6	3.8	7.7	7.5	6.7	7.6
Ca, g/kg	7.0	6.8	7.6	7.1	8.2	7.9	8.5	8.2
Available P, g/kg	6.2	6.7	6.3	6.1	4.2	4.0	4.2	4.2

[1] CaB: fat-coated calcium butyrate. Globamax Performant (Global Nutrition International, Fougères, France). [2] DWP: dry whey powder. Dry sweet powder (703 g of lactose/kilogram of product; Sueromancha S.L, Toledo, Spain). [4] CP: crude protein. [3]Providing per kg of diet: vitamin A, 8,000 IU; vitamin D_3, 1,600 IU; vitamin E, 16 mg; thiamine, 1 mg; riboflavin, 3 mg; pyridoxine, 1 mg; vitamin B_{12}, 0.01 mg; vitamin K, 1 mg; nicotinic acid, 16 mg; pantotenic acid, 7 mg; Mn, 70 mg; ZnO, 50 mg; Fe ($FeSO_4 \cdot H_2O$), 30 mg; Cu ($CuSO_4$ $5H_2O$), 4 mg; I (KI), 1 mg; Co, 0.2 mg; Se (Na_2SeO_3), 0.1 mg; choline, 240 mg; phytase, 300 units, and ethoxyquin, 110 mg. [4] AMEn: Apparent metabolizable energy corrected by N, calculated according to FEDNA (2010).

2.2.2 Trial 2: Growth performance, duodenal histomorphometry, and ceca bacteria counts

For this trial, 1,200 one-day-old broiler chickens were used during 42 d. They were randomly allocated to 2.5 x 1.0 m floor pens, and assigned to 1 of the 4 experimental diets (Table 1). Starter diets were offered from d 0 to 21, and grower-finisher diets from 21 d to 42. Each treatment consisted of 10 replicate pens, and 30 broiler chickens each. To determine productive performance, all broiler chickens and feeders in each pen were weighted weekly. Body weight (BW), average daily gain (ADG), feed intake (FI), and feed conversion ratio (FCR) were recorded on a pen basis. Mortality was recorded daily.

To determine ceca microbial counts, 3 broiler chickens from each treatment were randomly selected on d 21 and slaughtered by CO_2 inhalation, as previously was done in Pineda-Quiroga et al (2017). The gastrointestinal tract was dissected, cecum was collected under sterile conditions, and 1 g of digesta content, resulting from the mixture of both ceca pouches, was diluted in 9 mL of buffered peptone water (BioMérieux, France) and immediately 10-fold serially diluted in sterile saline solution. Dilutions were tested in duplicate for enumeration of *E. coli*, *Clostridium perfringens*, *Bifidobacterium* spp., and *Lactobacillus* spp. For *E. coli*, dilutions to 10^{-9} were plated on a selective chromogenic agar medium (ChromID Coli, BioMérieux, Marcy-L'etoile, France) and incubated at 37 ± SD 1ºC for 24 h; only glucuronidase-positive red-pink colonies were counted. For *C. perfringens*, dilutions to 10^{-7} were plated on tryptone sulphite neomycine agar (Scharlab, Barcelona, Spain) and incubated at 45 ± SD 1ºC for 24 h. For *Lactobacillus* spp., the selective medium Lactobacilli man rogosa and sharpe (MRS) agar (BD, Franklin Lakes, New Jersey, US) with 50 U/mL of nystatin (Sigma-Aldrich, St. Louis, Missouri, US) was used. The same MRS Agar (BD, US) supplemented with L-Cysteine hydrochloride (0.5 g/kg) (Oxoid, Basingstoke, UK), 100 µg/mL mupirocin (Oxoid, UK), and 50 U/mL nystatin (Sigma-Aldrich, US) was used for *Bifidobacterium* spp. In both cases, dilutions to 10^{-11} were tested and plates were incubated at 37 ± SD 1ºC for 48 h; 5 colonies from the last dilutions with 30 to 150 colony-forming units (cfu) were then selected for Gram staining and catalase test to identify catalase-negative, Gram-positive bacilli. Plates for *E. coli* were incubated aerobically, whereas all other incubations were carried out under anaerobic conditions

(Genbox Anaer, BioMérieux, France). To confirm the species, selected colonies from each culture were subjected to an automated system to bacterial identification, using appropriated identification cards (ANC for anaerobic species and GN for fermenting and no-fermenting Gram-negative bacilli). Counts were expressed as the mean cfu/g of dilution duplicates that gave 30 to150 cfu per plate.

For duodenal histomorphometry measurements, duodenum from the same broiler chickens that were sampled for ceca counts was extracted. Samples were preserved in 10% buffered formalin saline before preparing the histological sections. Tissue samples were dehydrated by immersion in alcohols of increasing grade, infiltrated in xylene, and embedded in paraffin. Transversal sections were cut and stained with hematoxylin and eosin, and embedded in paraffin. Slides were then examined with an optical microscope (Nikon Eclipse-80*i*; Nikon Corporation, Tokyo, Japan) coupled with a camera (DS-R*i*1; Nikon Corporation, Japan), and images were analyzed using the image software NIS elements 3.1 (Nikon Corporation, Japan). Villus height and crypt depth were measured on 10 well-oriented villus and crypt, according to the protocol described by Liu et al. (2011). The apparent villus surface area was estimated according to Iji et al. (2001). Five measurements of each structure were performed per sample.

2.3 Chemical analysis and calculations

Feeds and ileum samples were analyzed in triplicate for DM (Method 930.15), ash (Method 942.05), CP (Method 990.03), ether extract (Method 920.39), and starch by spectrophotometry according to Association of Official Analytical Chemists (2007). Measurements of Ca, P, and Cr were made by spectroscopy plasma atomic emission.
Calculation of the AID of nutrients was made using the following formula:

$$\text{AID of diet component} = \frac{\left(\text{Diet component}/Cr_2O_3\right)\text{d} - \left(\text{Diet component}/Cr_2O_3\right)\text{i}}{\left(\text{Diet component}/Cr_2O_3\right)\text{d}}$$

where (Diet component/Cr_2O_3)d is the ratio of the diet component to the Cr_2O_3 content of the diet, and (Diet component/Cr_2O_3)i is the ratio of the component to Cr_2O_3 in the ileal digesta.

2.4 Statistical analysis

Pen was considered the experimental unit in both trials. After normality and homoscedasticity of data were confirmed, data from Trial 1, as well as performance results, and duodenal histomorphometry from Trial 2 were analyzed considering a Gaussian distribution. Initial BW was included as a covariate in statistical models of performance data. Cecal bacteria counts and mortality data from Trial 2 were analyzed assuming a lognormal distribution and a binomial distribution respectively. All models included the pen as a random effect. All data were subjected to a two-way ANOVA, in a 2 × 2 factorial arrangement using the GLIMMIX procedure of SAS (V 9.3, SAS Inst. Inc., Cary, NC). The results were considered significant if $P < 0.05$.

3. RESULTS

3.1 Apparent ileal digestibility (AID) and pH of gastrointestinal content at various segments

Results relative to nutrient AID and pH of gastrointestinal content are shown in Table 2. With the dietary supplementation of DWP, the AID of DM, CP, Ca, and P increased, and cecum pH decreased only when CaB was also added (CaB × DWP, $P < 0.046$).

3.2 Productive performance

Results relative to productive performance are shown in Table 3. With the dietary supplementation of DWP, a higher ADG, and FI of broiler chickens were observed during the starter period only when CaB was also added. When DWP was not added to diets, FI increased in those broiler chickens fed the diet without CaB (CaB × DWP, $P < 0.001$). Mean mortality values were 1.33%, which remained unaffected by treatments and their interaction. During grower-finisher period, higher FI were observed by addition of DWP only when CaB was also added. Similarly, when DWP was not added to diets, FI increased in those broiler chickens fed the diet without CaB (CaB × DWP, $P < 0.001$). The addition of DWP reduced FCR by feeding without CaB. Also,

the addition of CaB reduced FCR in those broiler chickens fed the diet without DWP (CaB × DWP, P < 0.001). During this period the mean mortality percentage was 1%, not differing according to treatments and their interaction. For the entire feeding period, the addition of DWP increased ADG and FI of broiler chickens only when CaB was also added. When DWP was not added to diets, FI increased in those broiler chickens fed the diet without CaB (CaB × DWP, P < 0.047). The addition of DWP reduced FCR by feeding without CaB. Similarly, the addition of CaB reduced FCR when DWP was not added to diets (CaB × DWP, P < 0.001). Mean mortality values were 2.25%, remaining unaffected by treatments and their interaction.

Table 2. Influence of diet supplementation with dry whey powder (DWP) and fat-coated calcium butyrate (CaB) on apparent ileal digestibility (AID) and pH of gastrointestinal content at various segments of broiler chickens at 21 d (Trial 1) [1]

Item	CaB [2] (g/kg)	0	0	1	1	SEM [4]	P-value		
	DWP [3] (g/kg)	0	60	0	60		CaB	WP	CaB × DWP
Nutrient AID									
Dry matter		0.932	0.931	0.950	0.954	0.003	0.003	0.704	0.035
Crude protein		0.707	0.707	0.757	0.815	0.023	0.004	0.704	0.041
Starch		0.954	0.960	0.944	0.962	0.012	0.113	0.133	0.128
Ca		0.628	0.630	0.723	0.764	0.009	< 0.001	0.046	0.042
P		0.505	0.525	0.614	0.702	0.023	< 0.001	0.045	0.004
pH of gastrointestinal content									
Crop		4.91	5.15	4.92	4.74	0.40	0.968	0.654	0.588
Proventriculus		3.23	3.47	3.22	2.78	0.39	0.811	0.412	0.424
Gizzard		2.98	2.90	2.81	2.71	0.12	0.510	0.143	0.968
Ileum		5.68	6.07	5.58	6.27	0.27	0.590	0.869	0.591
Cecum		6.01	5.90	6.35	5.40	0.19	0.014	0.706	0.046

[1] Data presented the means based on 3 replicate pens per treatment and 5 broiler chickens per replicate. [2] CaB: fat-coated calcium butyrate. [3] DWP: dry whey powder. Dry sweet powder. [4] SEM: Standard error of the mean.

3.3 Duodenal histomorphometry and cecal bacteria counts

As shown in Table 4, with addition DWP, higher villus height, villus height to crypt depth ratio, and villus surface area were observed only when CaB was also added (CaB × DWP, P < 0.017). With the dietary supplementation of CaB, crypt depth increased and villus height to crypt depth ratio decreased only when DWP was not added (CaB × DWP, P = 0.017). In relation to cecal bacteria counts, the

supplementation of DWP increased *Bifidobacterium* spp. counts only when CaB was not added (CaB × DWP, P = 0.049).

Table 3. Influence of diet supplementation with dry whey powder (DWP) and fat-coated calcium butyrate (CaB) on productive performance of broiler chickens (Trial 2) [1]

Item	CaB [2] (g/kg)	0	0	1	1	SEM [4]	P-value		
	DWP [3] (g/kg)	0	60	0	60		CaB	DWP	CaB × DWP
Starter period (d 0 to21)									
Body weight (g)		677	625	633	782				
Average daily gain (g/d)		30	28	28	35	0.6	< 0.001	< 0.001	< 0.001
Feed intake (g /d)		48	43	43	54	1.2	0.003	0.011	< 0.001
Feed conversion ratio		1.57	1.55	1.54	1.52	0.03	0.252	0.370	0.988
Grower-finisher period (d 21 to 42)									
Body weight (g)		2.616	2.540	2.673	2.785				
Average daily gain (g/d)		92	91	97	95	1.2	< 0.001	0.234	0.798
Feed intake (g /d)		148	133	137	148	1.8	0.155	0.180	< 0.001
Feed conversion ratio		1.61	1.46	1.42	1.56	0.03	0.069	0.828	< 0.001
Entire feeding period (d 0 to 42)									
Average daily gain (g/d)		62	63	61	66	0.9	< 0.001	0.477	0.047
Feed intake (g /d)		103	92	97	107	1.2	< 0.001	0.898	< 0.001
Feed conversion ratio		1.67	1.53	1.54	1.65	0.03	0.723	0.533	< 0.001

[1] Data presented means based on 10 replicate pens per treatment and 30 broiler chickens per replicate. [2] CaB: fat-coated calcium butyrate. [3] DWP: dry whey powder. [4] SEM: Standard error of the mean.

Table 4. Influence of diet supplementation with dry whey powder (DWP) and fat-coated calcium butyrate (CaB) on duodenal histomorphometry and cecal bacteria counts of broiler chickens (Trial 2) [1]

Item	CaB [2] (g/kg)	0	0	1	1	SEM [4]	P-value		
	DWP [3] (g/kg)	0	60	0	60		CaB	WP	CaB ×DDWP
Duodenal histomorphometry									
Villus height (μm)		1.151	1.278	738	1.409	58	0.023	< 0.001	< 0.001
Crypt depth (μm)		154	169	221	161	13.2	0.033	0.102	0.008
Villus height to crypt depth ratio		7.7	8.6	3.4	8.7	0.9	0.024	0.001	0.017
Villus surface area (μm^2)		54	53	49	83	6.3	0.054	0.012	0.012
Cecal bacterial count (Log_{10} cfu/g cecal content)									
Bifidobacterium spp.		7.01	10.20	8.28	9.77	0.38	0.202	0.002	0.049
Lactobacillus spp.		11.75	9.01	11.68	11.52	0.96	0.523	0.458	0.591
Clostridium perfringens		2.33	1.39	1.78	4.24	1.00	0.625	0.333	0.138
E. coli		11.84	13.1	12.09	11.97	1.04	0.609	0.691	0.535

[1] Data presented means based on 10 replicate pens per treatment and 3 broiler chickens per treatment. [2] CaB: fat-coated calcium butyrate. [3] DWP: dry whey powder. [4] SEM: Standard error of the mean.

4. DISCUSSION

The current study shows that supplementing broiler chickens diets with DWP or CaB separately has resulted in a reduction on their weight, FI, and FCR at every stage of the productive period. Results indicate that supplementation with either DWP or CaB limited the growth of broiler chickens as a consequence of reduced feed intake. Opposed to our results with DWP supplementation, Gülsen et al. (2002) and Kermanshahi and Rostami (2006) found that supplementing broiler chicken diets with 40 or 38.5 g/kg of whey powder improved weight without affecting FI during the starter and grower-finisher periods. With respect to CaB supplementation, Levy et al. (2015) found an improvement of broiler chicken's weight without affecting FI, during the grower stage and for the entire feeding period, by supplementing diets with 0.4 and 0.5 g/kg of fat-encapsulated microbeads of Ca butyrate. As it can be noted, the amounts of DWP and CaB used in the mentioned studies were smaller than those of our experiment. This could indicate that the quantity required to enhance weight without affecting FI should be smaller. As for CaB, this assumption should be taken with caution, because the amount to be reduced depends on the type of organic acid, supply form (free, salt or coated; van den Borne et al., 2015), salt to organic acid ratio, amount and type of coating fat, and the technical coating process among others (Guilloteau et al. 2010; van den Borne et al., 2015). The reasons for the FI reduction are not clear. However, it could be attributed to the reported increase in digesta osmolarity promoted by lactose in broiler chickens (Morishita et al., 1982), because an augmented osmotic pressure in the duodenum reduces their voluntary feed intake (Ferket and Gernat, 2006). For CaB supplementation, the reduction of FI could be linked to a negative modification in the feed odour, as subjectively observed in our case. It is known that olfactory system also regulates the FI in poultry (El Boushy and Poel, 1994), and that short-chain organic acids could negatively affect feed odour, reducing palatability (Andreopoulou et al., 2014).

In addition to reducing broiler chickens' weight, supplementing DWP in absence of CaB increased *Bifidobacterium* spp. counts. This finding should be viewed with caution because of the reduced number of replicates used in this experiment. However, it would confirm that some members of this genus are able to use lactose as an energy substrate for growth (Goodfellow et al., 2012). Although it is expected that greater

populations of health-promoting bacteria in cecum, such as *Bifidobacterium*, contribute to enhance the weight of broiler chickens (Gaggìa et al., 2010), our results did not evidence this. In this way, Geier et al., (2009) confirmed that diet-induced changes in ceca microbiota do not always translate into altered broiler chickens performance, probably because to differences at strain level, and hence to the functional level of bacteria (Torok et al., 2013).

Our results also revealed that feeding with CaB in absence of DWP decreased broiler chickens' weight together with a reduction of villus height, villus surface area, and villus height to crypt depth ratio, and an increment in crypt depth. Again, these results should be taken with caution because of the reduced number of replicates. Deeper crypts and shorter villus could indicate a higher rate of enterocyte-cell migration and more constant cell renewal rate within the gut (Miles et al., 2006), likely caused by increased sloughing (Yamauchi et al., 2010). These constant renewal processes demand more energy and protein, that would be less available for growth of other body tissues. Present findings relative to duodenal morphometry disagree with Dibner and Buttin (2002) and Lesson et al. (2005), who pointed out the promoting effect of butyric acid on gastrointestinal mucosa development and villus length. However, it is known that high inclusion rates of dietary organic acids could reduce cellular proliferation and differentiation, and promote intestinal mucosa damage (Mariadason et al., 1999).

Contrary to that observed when DWP and CaB were supplied separately, their simultaneous inclusion increased weight and FI of broiler chickens during every stage of the productive cycle, without affecting FCR. According to our results, we consider the increase in FI one of the reasons that explain the weight improvement. The addition of DWP could mitigate the odour of CaB, as indeed subjectively detected, which might have made feed more palatable. However, we do not know whether CaB reciprocally reduced the DWP effects associated with digesta osmolarity. Our results also showed that DWP addition protected the duodenal mucosa and increased nutrient AID when CaB was also added. These favourable effects might also explain the improved weight of broiler chickens. Accurate explanations for the protective effect of DWP, and for the positive result when combined with CaB, remain unclear. However, it could be hypothesized that the organic Ca also contained in DWP could form a complex with the acidic anion of butyric acid released in the duodenum. As a result, the amount of free butyric acid could be smaller, avoiding an excess reaching the enterocytes and

preventing their damage. A better mucosa integrity and gut development is usually accompanied by higher expression of brush border enzymes and by an increase of total intestinal transporters (Ruhnke et al., 2015), which might explain the higher nutrient AID. A greater nutrient AID, together with the observed increase in FI, could promote a higher digestible nutrient intake, and consequently enhanced animal weight.

The simultaneous addition of DWP and CaB also reduced cecum pH. It has been reported that lactose fermentation decreases cecum pH in poultry because of lactic acid and volatile fatty acids production during fermentation (Stringfellow et al., 2009; van der Wielen et al., 2002). Organic acids also have an effect on pH of ceca digesta, lowering it (Dibner and Buttin, 2002). It would be expected that reduced cecal pH induced changes in their microbial composition. Contrary, this reduction did not lead to bacterial modulations. However, this does not mean that other non-studied ceca bacteria populations might have been affected, what could have contributed to enhance the health status and weight of broiler chickens.

5. CONCLUSION

Results obtained in the present study indicate that the supplementation of DWP without CaB addition improved the FCR of broiler chickens. However, the supplementation of DWP together with CaB improve duodenal development, increases nutrient AID, and the weight and ingestion of broiler chickens.

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CHAPTER 4

FEEDING BROILERS WITH DRY WHEY POWDER AND WHEY PROTEIN CONCENTRATE AFFECTED PRODUCTIVE PERFORMANCE, ILEAL DIGESTIBILITY OF NUTRIENTS, AND CECAL MICROBIOTA COMMUNITY

Published in the scientific Manuscript: C. Pineda-Quiroga, A. Camarinha-Silva, D. Borda-Molina, R. Atxaerandio, R. Ruiz and A. García-Rodríguez.. Feeding broilers with dry whey powder and whey protein concentrate affected productive performance, ileal digestibility of nutrients and cecal microbiota community. Animal. 1-9 doi: 10.1017/S1751731117002208

ABSTRACT

Dietary interventions are a common practice in the poultry industry to promote optimal performance and health of animals. Here, we aim at assessing the influence of supplementing corn-soybean diets of broilers with dry whey powder (DWP) and whey protein concentrate (WPC) on nutrient apparent ileal digestibility (AID) and productive performance. Cecal microbiota composition was also determined using Illumina amplicon sequencing. Dietary treatments were control diet (no supplementation of DWP or WPC), 60-DWP (60 g/kg of DWP), and 80-WPC (80 g/kg of WPC). One-day-old male broilers were randomly assigned to one of three treatments and housed in floor pens. In Trial 1, 90 1-day-old chicks were allocated to three pens/treatment, with 10 birds/pen, during 21 days for AID evaluation. Diet 60-DWP increased Ca AID ($P = 0.041$), while diet 80-WPC improved Ca and P AID ($P < 0.001$ and 0.002, respectively) when compared with control diet. In Trial 2, 810 one-day-old chicks were allocated to nine pens/treatment, with 30 birds/pen, during 42 days. Feeding chickens with 60-DWP and 80-WPC increased their body weight (BW), average daily gain (ADG), and feed intake (FI) during the starter ($P < 0.001$ for all variables) and grower-finisher periods ($P < 0.001$ for BW and FI, and $P=0.048$ for ADG), and during the entire feeding period ($P < 0.05$), when compared with control diet. Diets 60-DWP and 80-WPC reduced the feed conversion ratio (FCR) of chickens during the starter period ($P < 0.001$ and 0.003, respectively), while 60-DWP reduced this parameter during the entire feeding period ($P = 0.048$), when compared to control diet. At day 42, cecal microbial communities of chickens that were fed with 60-DWP and 80-WPC differed from those fed with control diet ($R = 0.776$, $P = 0.008$; and $R = 0.740$, $P = 0.008$, respectively). The abundance of *Bacteroides fragilis*, *Bacteroides* spp., *Escherichia coli/Shigella flexneri* and *Megamonas furniformis* increased when 60-DWP and 80-WPC diets were offered, while the presence of *Helicobacter pullorum* decreased. *Lactobacillus salivarius* consistently increased in chickens with better FCR, which were those fed with 60-DWP. The results obtained in the present study indicate that growth of chickens is improved by DWP and WPC supplementation because of a higher mineral digestibility, increased feed intake, and modulation of cecal microbiota communities.

1. INTRODUCTION

The main purpose of modern intensive broiler production is to promote a high growth rate, feed efficiency, and optimal health status of animals. Such desirable conditions can be achieved through different dietary interventions that, during the last decades, have focused on the addition of low levels of antibiotics, as growth promoters, for an extended period of time (Huyghebaert et al., 2011). One of the advantages of the use of antibiotics as growth promoters is the improvement of weight gain and feed efficiency through their impact on gut microbiota (Danzeisen et al., 2011). However, the ban on their use in the European Union (European Commision, 2003) and the potential restriction in other countries (Huyghebaert et al., 2011), have resulted in a concomitant reduction of animal performance and in an increase of the incidence of enteric pathologies. This situation has led to an increasing interest in non-resistant, non-residual feeding alternatives that benefit productivity and health status through a favorable modulation of the gut microbiota (Gaggìa et al., 2010).

Animal performance might be associated with changes in gut microbiota. Promotion of a balanced gut microbiota population is basic to protect the host against pathogenic bacteria, enhancing the intestinal integrity and morphology, as well as the absorption of nutrients (Rinttilä and Apajalahti, 2013). One way to benefit gut balance is the use of specific dietary components that serve as substrate to a selective group of bacteria, such as the case of prebiotics (Gaggìa et al., 2010). Prebiotics are defined as a non-digestible, dietary compounds that modulate the composition and/or activity of the gut microbiota, conferring a beneficial physiological effect on the host (Bindels et al., 2015). Dry whey powder (DWP) is a co- product of cheese industry with high lactose content (~70% of dry matter). Given that birds have a negligible lactase activity in the gut (Denbow, 2000), lactose can be used as a prebiotic. Lactose is not digested, being thus fermented by the cecal microflora,which can lead to a decrease on cecum pH and to a reduction of potential pathogenic bacteria such as *Salmonella enteritidis* (Tellez et al., 1993). The benefits of feeding broilers and laying hens with DWP, as lactose source, on their performance and cecal bacteria counts has been reported (Radfar and Farhoomand, 2008; Pineda-Quiroga et al., 2017). However, to our knowledge, results on the effects of DWP on broilers concerning nutrient ileal digestibility and cecal microbiota community are lacking so far.

The protein source used in feed formulation influences the productive performance and the gut microbiota composition of broilers. The benefits of the selected protein on animal growth are related to their inclusion level, digestibility and amino acid profile (Beski et al., 2015). Gut microbiota could also be affected, as diet non-digested proteins might reach the hind gut, promote a proteolytic fermentation and stimulate an increase in the microbiota that use amino acids as energy source (Qaisrani et al., 2015). Whey protein concentrate (WPC) is a co-product of cheese or rennet casein industries with relevant protein (30% CP of dry matter) and lactose (52.5% of dry matter) contents. The WPC is considered as an excellent amino acid source in bird nutrition and is composed of biologically active proteins such as β-lactoglobulin, α-lactalbumin and immunoglobulins (Szczurek et al., 2013). These proteins have a higher biological value compared with soybean meal (Smithers, 2015), the main protein source in poultry feed. The lactose of WPC might promote broiler performance by stimulating the growth of beneficial cecal bacteria. The benefits of the inclusion of WPC on the performance and protein ileal digestibility of broiler diets have been reported (Szczurek et al., 2013), but little is known about its effects on digestibility of other nutrients and on cecal microbiota community.

We hypothesize that lactose from supplemented DWP and WPC could modulate the cecal microbiota composition towards beneficial bacteria, in detriment of pathogens, therefore enhancing the sanitary status and the productive performance of broilers. In the case of WPC supplementation, we additionally hypothesize that the benefits of lactose on performance could be further potentiated by simultaneously providing a high nutritional value protein in the diet. Thus, the aim of this study was to evaluate the effect of supplementing broiler diets with DWP as lactose source, and WPC as protein and lactose source, on the productive performance, nutrient ileal digestibility, and cecal microbial community of broiler chickens.

2. MATHERIALS AND METHODS

2.1 Animals, housing and experimental diets

This experiment followed the European Union (2010/63/EU) and Spanish regulations

(RD 53/2013) for the care and use of animals for experimental and other scientific purposes. The study was carried out at the experimental farm 126 of Neiker-Tecnalia in Arkaute (Vitoria-Gasteiz, Spain). One-day-old male broiler chicks (Ross 308 strain), with an average body weight of 42 ± 0.99 g, were obtained from a local commercial hatchery (AN Avícola Melida, S.A, Zumaia, Spain). At arrival, birds were randomly allocated to their floor pens, with stocking density being 30 kg/m2. Pens were equipped with a manual feeder, nipple drinkers, and wood shavings as litter material. Room temperature and lighting program were adjusted following the guidelines for Ross 308.

Diets were corn-soybean based, and were formulated to meet broilers' requirements during the starter and grower-finisher stages (FEDNA, 2008). Starter diets were offered from day one to 21, and grower-finisher diets were offered from day 22 to 42. Chromium oxide (Cr_2O_3, 5 g/kg of diet) was added to starter diets as an external indigestible marker. Birds had *ad libitum* access to one of the following experimental diets: control (no supplementation of DWP or WPC), 60-DWP (60 g/kg of inclusion of DWP) and 80-WPC (80 g/kg of inclusion of WPC). Diets with DWP and WPC were formulated to provide 42 g/kg of lactose. The ingredient composition and the analyzed nutritional value of experimental diets are shown in Table 1. The used DWP was a commercial sweet powder (Sueromancha S.L, Toledo, Spain; 703 g/kg of lactose, 126 g/kg of crude protein). Renylat 3300 (Industrias Lácteas Asturianas, Spain; 520 g/kg lactose content, 350 g/kg of crude protein) was used as WPC.

2.2 Experimental design, measurements, and sampling

2.2.1 Trial 1: Apparent ileal digestibility of nutrients (AID)

In this trial, 91 one-day-old chicks were randomly allocated to 1.0 m x 0.83 m floor pens, which were assigned to one of the three experimental diets formulated for the starter period (Table 1). Each treatment comprised three pens with 10 chickens each. At 21 days of feeding, five chickens per pen were euthanized by CO_2 inhalation, and their ileal digesta was collected. Ileum was considered as the portion of the small intestine from the Meckel's diverticulum to the ileocecal junction, and digesta of the last two-thirds of this section was gently collected. Ileal samples from birds within the same pen were pooled, frozen at -20ºC, and lyophilized. Dried ileal digesta samples

Table 1. Ingredients and chemical composition of the experimental diets (Trials 1 and 2)

Item[1]	Starter period (1 to 21 days)			Grower-Finisher period (22 to 42 days)		
	Control	60-DWP	80-WPC	Control	60-DWP	80-WPC
Ingredients (g/kg, *as fed* basis)						
Yellow corn	510	502	513	500	547	520
Wheat	115	96	104	93	0	24
Soybean meal (470 g CP/kg)	312	283	247	314	300	280
Palm oil				37	36	40
Soybean oil	25	26	27	29	28	24
Dry whey powder	0	60	0	0	60	0
Whey protein concentrate	0	0	80	0	0	80
Vitamin and mineral premix[2]	4	4	4	4	4	4
Dicalcium phosphate	9.5	4	4	5	3.5	3
Calcium carbonate	18	18	14	13	16	11
Sodium chloride	2.7	2.7	2.7	2.7	2.7	2.7
DL-Methionine	1.3	1.3	1.3	1	1	1
L-Lysine	1.8	1.8	1.8	1	1	1
Salmocid	0.5	0.5	0.5	0.5	0.5	0.5
Chromium oxide	0.5	0.5	0.5	0	0	0
Analyzed chemical composition unless otherwise indicated						
AMEn (MJ/kg)[3]	11.81	11.87	11.89	12.92	12.91	12.95
CP (g/kg)	203	198	199	195	193	185
Lys (g/kg)[3]	11.9	11.5	11.1	11.2	11.2	11.3
Met (g/kg)[3]	4.4	4.3	4.2	4.1	4	4
Ca (g/kg)	10.5	9.6	9.7	8.5	8.2	8.3
Available P (g/kg)[3]	5.7	5.0	5.4	4.7	4.5	4.9
Lactose (g/kg)	0	0	42	0	42	42

[1] Control: no supplementation of dry whey powder or whey protein concentrate; 60-WP: 60 g/kg of dry whey powder; 80-WPC: 80 g/kg of whey protein concentrate. [2] Providing the following per kg of diet: of diet: 8 000 IU vitamin A (*trans*-retinyl acetate), 1 600 IU vitamin D_3 (cholecalciferol), 16 mg vitamin E (DL-α-tocopherol), 1 mg thiamine (thiamine-mononitrate), 3 mg riboflavin, 1 mg pyridoxine (pyridoxine. HCL), 0.01 mg vitamin B_{12} (cyanocobalamin), 1 mg vitamin K_3 (menadione nicotinamide bisulphite), 16 mg niacin (nicotinic acid), 7 mg pantotenic acid (D-Ca pantothenate), 70 mg Mn (manganese oxide), 50 mg Zn (zinc sulfate), 30 mg Fe (iron sulfate monohydrate), 4 mg Cu (copper sulphate), 1 mg I (calcium iodate), 0.2 mg Co (cobalt sulfate), 0.1 mg Se (sodium selenite), 240 mg choline (choline choride), 300 units phytase, 110 mg ethoxyquin. [3] Calculated value.

were ground to pass through a 0.5 mm screen, and stored in airtight containers at room temperature until chemical analyses. The AID of dry matter (DM), crude protein (CP), calcium (Ca) and phosphorous (P) was estimated using Cr_2O_3 as external indigestible marker.

2.2.2 Trial 2: Determination of productive performance and cecal microbial community

In this trial 810 one-day old chicks were randomly allocated to 1.0 x 2.5 m floor pens, which were assigned to one of the three experimental diets (Table 1) for a period of 42 days. Each treatment comprised nine replicates of 30 chickens each. To determine productive performance, all chickens and feeders from each pen were weighted weekly. Body weight (BW), average daily gain (ADG), feed intake (FI), and feed conversion ratio (FCR) were recorded on a pen basis. Mortality was recorded daily.

2.2.2.1 Cecal sample collection and DNA extraction

At 42 days, five chickens from each experimental treatment were randomly selected from different pens and euthanized by CO_2 inhalation. The gastrointestinal tract was dissected, the two ceca were opened longitudinally, and digesta samples were collected with a sterile spoon. Samples were immediately stored at -80°C until further analysis. Total nucleic acid was extracted from samples using the PowerSoil DNA extraction Kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer recommendations. DNA was quantified in Nanodrop (ND-1000 Spectrophotometer, Nanodrop Technologies, DE, USA) and DNA integrity was checked through agarose gel electrophoresis.

2.2.2.2 16S rRNA gene amplification, Illumina sequencing, and bioinformatics sequence analysis

The V4 hypervariable region of the 16S rRNA gene was used to prepare Illumina amplicon libraries (Caporaso et al., 2011). Two PCR were performed to

incorporate Illumina adapters and barcodes for sample identification. The PCR products were purified using Agencourt AMPure®XP kit (Agencourt Bioscience Corporation, USA) according to the manufacturer instructions, and eluted in 20µl of water. Amplicons were quantified using Qubit fluorometric quantitation (Qubit® 3.0, Thermo Fisher Scientific Inc., USA), and the Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA, USA) in order to pool the samples and sequence them on an Illumina MiSeq platform.

Illumina reads were analyzed using Mothur Miseq SOP (Kozich et al., 2013). Primers and barcodes were trimmed prior to analysis. All samples comprised 3.912.932 sequence reads, with a mean number of reads per sample of 261.202 ± 62.590. Sequences were aligned using SILVA-based bacterial reference alignment obtained from Mothur. Chimera sequences were checked and removed using UCHIME. A final dataset was then clustered into operational taxonomic units (OTU) at ≥ 97% similarity. OTUs with less than 10 reads in one sample were removed from the analysis (< 0.0013% of the total). A total of 855 OTUs were taxonomically assigned using the Seqmatch function in Ribosomal Database Project. Sequences are available at the European Nucleotide Archive under the accession number PRJEB17510 in http://www.ebi.ac.uk/ena.

2.3 Chemical analysis and calculations

Experimental diets and ileal samples were analyzed in triplicate for DM (method 934.01), total ash (method 942.05), CP (method 990.03), ether extract (method 920.39), according to the Association of Official Analytical Chemists (2007). Measurements of Ca, P and Cr_2O_3 oxide were determined by spectroscopy plasma atomic emission.

Calculation of nutrients CAID was made using the following formula:

$$\text{CAID of diet component} = \frac{(\text{Diet component}/Cr_2O_3)\text{d} - (\text{Diet component}/Cr_2O_3)\text{i}}{(\text{Diet component}/Cr_2O_3)\text{d}}$$

Where (Diet component/Cr_2O_3)d is the ratio of the diet component to the Cr_2O_3 content in the diet, and (Diet component/Cr_2O_3)i is the ratio of the component to Cr_2O_3 in the ileal digesta.

2.4 Statistical analysis

For analysis of productive performance and nutrient AID, pen was considered the experimental unit. Performance data were evaluated during the starter (day 1 to 21) and grower-finisher periods (day 22 to 42), as well as for the entire feeding period (day 1 to 42). One-way ANOVA was performed considering the experimental diet as the fixed effect, and the pen as the random effect. Performance and AID were analyzed using a Gaussian distribution, whereas survival data were analyzed considering a binomial distribution. Initial BW was used as a covariate in performance analysis. Data were analyzed using the GLIMMIX procedure of SAS V 9.3 (SAS Inst. Inc., Cary, NC). In case of a significant effect ($P < 0.05$) multiple comparisons were carried out to detect differences between treatments using the Tukey's range test.

For sequencing analysis, relative abundances of the OTUs were analyzed by means of multivariate statistical routines using PRIMER (version 7.0.9, PRIMER-E, Plymouth Marine Laboratory, Plymouth, UK). Data were standardized in relation to their total amount, and a sample similarity matrix was created using Bray-Curtis coefficient. Community structures ordination was explored by hierarchical clustering, and similarity profile permutation test (SIMPROF) was used to seek for statistically significant clusters. Statistical comparisons of microbial communities between treatments were determined using one-way analysis of similarity (ANOSIM, 999 permutations), and significant differences were considered if $P < 0.05$. Subsets of OTUs summarizing the overall differences in microbial community composition were identified using BEST routine. Individual OTUs contributing to dissimilarity between treatments were identified by similarity percentage analysis (SIMPER), as well as the average of similarity in cecal microbial community composition among birds fed with the same diet.

3. RESULTS

3.1 Nutrient coefficient of AID

Results relative to nutrient AID are shown in Table 2. An increase on the AID of Ca was promoted by 60-DWP diet compared to control diet (P = 0.041) while no differences were found between 60-DWP and 80-WPC (P = 0.695) or 80-WPC and control diet (P = 0.119). Chickens fed with 60-DWP and 80-WPC showed an increase in the AID of P when compared to control diet (P < 0.001 and P = 0.002, respectively), while no differences were found between 60-DWP and 80-WPC (P = 0.406). Feeding birds with experimental diets did not influence the AID of DM and CP (P = 0.402 and P = 0.201, respectively).

Table 2. Influence of diet supplementation with dry whey powder and whey protein concentrate on nutrient apparent ileal digestibility in broilers at 21 d (Trial 1).

Item	Treatment [1]				
	Control	60-DWP	80-WPC	SEM [2]	P-value
Dry matter	0.928	0.932	0.927	0.003	0.402
Crude Protein	0.841	0.861	0.862	0.008	0.201
Calcium	0.699[b]	0.743[a]	0.731[ab]	0.009	0.043
Phosphorus	0.656[b]	0.786[a]	0.762[a]	0.012	<0.001

[1] Control: no supplementation of dry whey powder or whey protein concentrate; 60-DWP: 60 g/kg of dry whey powder; 80-WPC: 80 g/kg of whey protein concentrate.
[2] Standard error of the mean. [a-b] Means followed by different superscript in a column indicate differences between treatments (P < 0.05).

3.2 Performance parameters

Broilers fed with 60-DWP and 80-WPC diets showed the highest values for growth parameters in the analyzed periods (Table 3). At the end of the starter period, birds fed with 80-WPC showed higher BW, ADG and FI values when compared to the control diet (P < 0.001) and 60-DWP (P < 0.001). The FCR of 80-WPC diet was lower than that of control diet (P = 0.003), and did not differ from that of 60-DWP (P = 0.078). Feeding with 60-DWP promoted an increase in BW and ADG, as well as a reduction in FCR (P < 0.001) when compared to control diet. However no differences were observed for FI (P = 0.494). Mean mortality values were 1.75 %, remaining unaffected by treatments.

At the end of the grower-finisher period, animals fed with 80-WPC and 60-DWP increased their BW (P < 0.001), ADG (P = 0.048) and FI (P < 0.001) as compared to those fed the control diet, whereas no significant differences were found on FCR (P =

0.793). There were no statistical differences in BW, ADG, FI, and FCR between birds fed with 60-DWP and 80-WPC ($P > 0.05$). Mean mortality values were 1.22 %, and remained unaffected by treatments.

For the entire feeding period, birds fed with 60-DWP and 80-WPC showed higher ADG (P = 0.006 and P < 0.001, respectively) and FI (P < 0.001 and P = 0.001, respectively) than those fed control diets. A decrease in FCR was observed by feeding with 60-DWP compared to control diet (P = 0.048), while no difference were found between 80-WPC and control diet (P = 0.096). When comparing 60-DWP and 80-WPC, no differences were detected for ADG (P = 0.061) and FCR (P = 0.974), although FI was lower in 60-DWP (P = 0.005). Mean mortality values were 2.25%, remaining unaffected by treatments.

Tabla 3. Influence of diet supplementation with dry whey powder and whey protein concentrate on productive performance (Trial 2).

Item	Treatment [1]				
	Control	60-DWP	80-WPC	SEM [2]	P-value
Starter period (d 0 to 21)					
Body weight (g)	464^{c}	582^{b}	677^{a}	5.45	< 0.001
Average daily gain (g/d)	21^{c}	26^{b}	31^{a}	0.27	< 0.001
Feed intake (g /d)	41^{b}	43^{b}	54^{a}	1.37	< 0.001
Feed conversion ratio	1.94^{b}	1.61^{a}	1.65^{a}	0.045	< 0.001
Grower-finisher period (d 21 to 42)					
Body weight (g)	1822^{b}	2078^{a}	2231^{a}	44.24	< 0.001
Average daily gain (g/d)	62^{b}	69^{ab}	72^{a}	2.23	< 0.001
Feed intake (g /d)	117^{b}	129^{a}	128^{a}	1.94	< 0.001
Feed conversion ratio	1.88	1.86	1.82	0.117	0.793
Entire feeding period (d 0 to 42)					
Average daily gain (g/d)	41^{b}	47^{a}	52^{a}	1.22	< 0.001
Feed intake (g /d)	78^{c}	85^{b}	91^{a}	84.83	< 0.001
Feed conversion ratio	1.90^{b}	1.80^{a}	1.85^{ab}	0.059	0.039

[1] Control: no supplementation of dry whey powder or whey protein concentrate; 60-WP: 60 g/kg of dry whey powder; 80-WPC: 80 g/kg of whey protein concentrate. [2] Standard error of the mean. [a-b] Means followed by different superscript in a column indicate differences between treatments (P < 0.05).

3.3 Microbial community analysis

Bacteroidetes, Firmicutes, and Proteobacteria were the most representative phyla in all diets (Fig 1). Control samples showed 45% of the sequences associated to Bacteroidetes, 40% to Firmicutes, and 14% to Proteobacteria. Bacteroidetes and Proteobacteria decreased their abundance in birds fed 60-DWP diets (31% and 6%, respectively) and 80-WPC diets (39% and 5%, respectively), while Firmicutes increased their abundance (63% in 60-DWP and 55% in 80-WPC). A change in the Firmicutes/Bacteroidetes (F/B) ratio was also observed between treatments, being the ratio 0.89 in samples from control diet, 2.03 in 60-DWP, and 1.43 in 80-WPC.

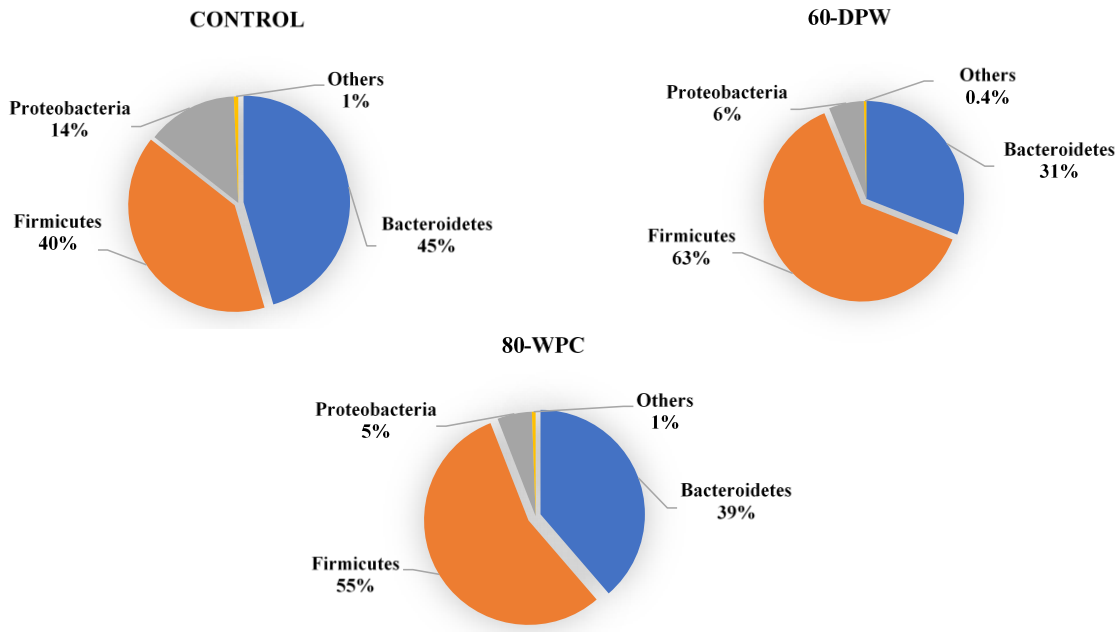

Fig 1. Composition of bacteria in cecal samples at phylum level. Relative abundance (> 1% on average) at different phylum in response to experimental diets. Control: no supplementation of dry whey powder or whey protein concentrate; 60 g/kg of dry whey powder, 80-WPC: 80 g/kg of whey protein concentrate.

The most abundant families harbouring the ceca are shown in Fig 2. Cecal samples from control diet were mainly colonized by Ruminococcaceae, Bacteroidaceae, Porphyromonadaceae, and Helicobacteraceae (24%, 22%, 17% and 10% respectively), while samples from 60-DWP and 80-WPC diets were represented by Ruminococcaceae (38% and 34%, respectively), Bacteroidaceae (17% and 27%, respectively),

Lachnospiraceae (15% and 11%, respectively), and Porphyromonadaceae (10% and 8%, respectively). *Bacteroides* was the most representative genus in all samples, accounting for 22% of total community in samples from control diet, 17% in 60-DWP, and 27% in 80-WPC (Fig 3), followed by *Barnesiella* and *Helicobacter* (15% and 10%) in control diet. In 60-DWP and 80-WPC diets *Faecalibacterium* accounts for 12% of total bacteria, *Barnesiella* for 9% and 6%, respectively, and *Ruminococcus* for 6% and 4%, respectively.

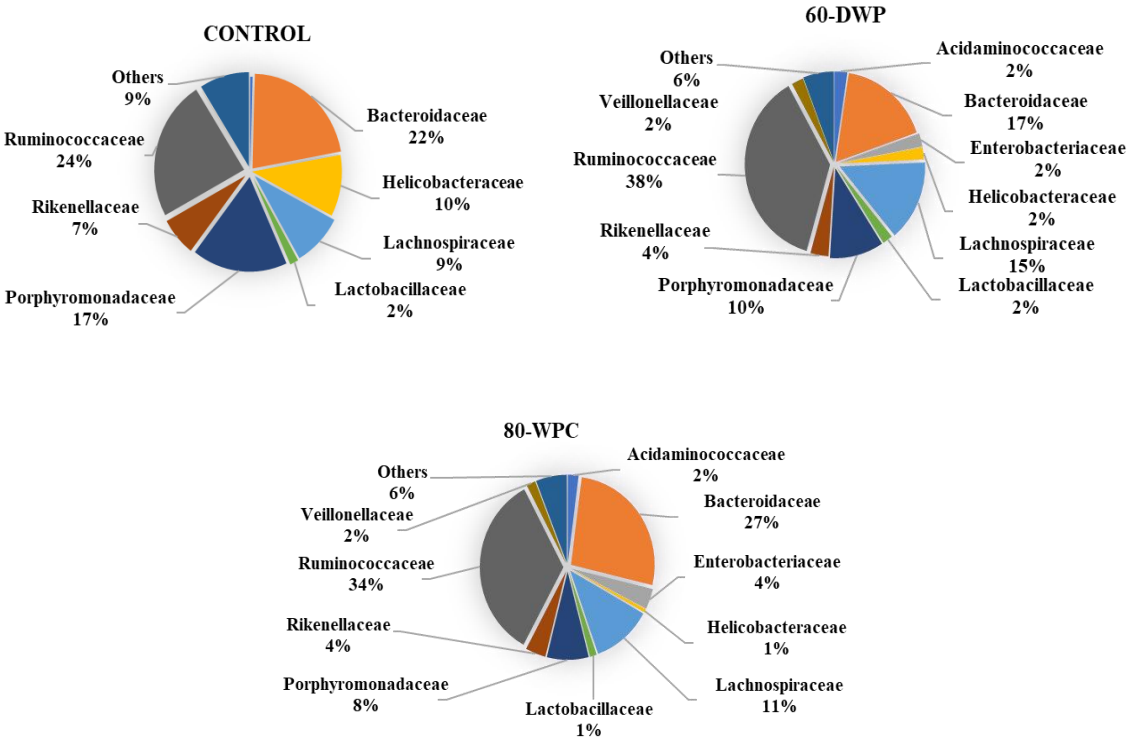

Fig 2. Composition of bacteria in cecal samples at family level. Relative abundance (> 1% on average) at different families in response to experimental diets. Control: no supplementation of dry whey powder or whey protein concentrate, 60-DWP: 60 g/kg of dry whey powder, 80-WPC: 80 g/kg of whey protein concentrate.

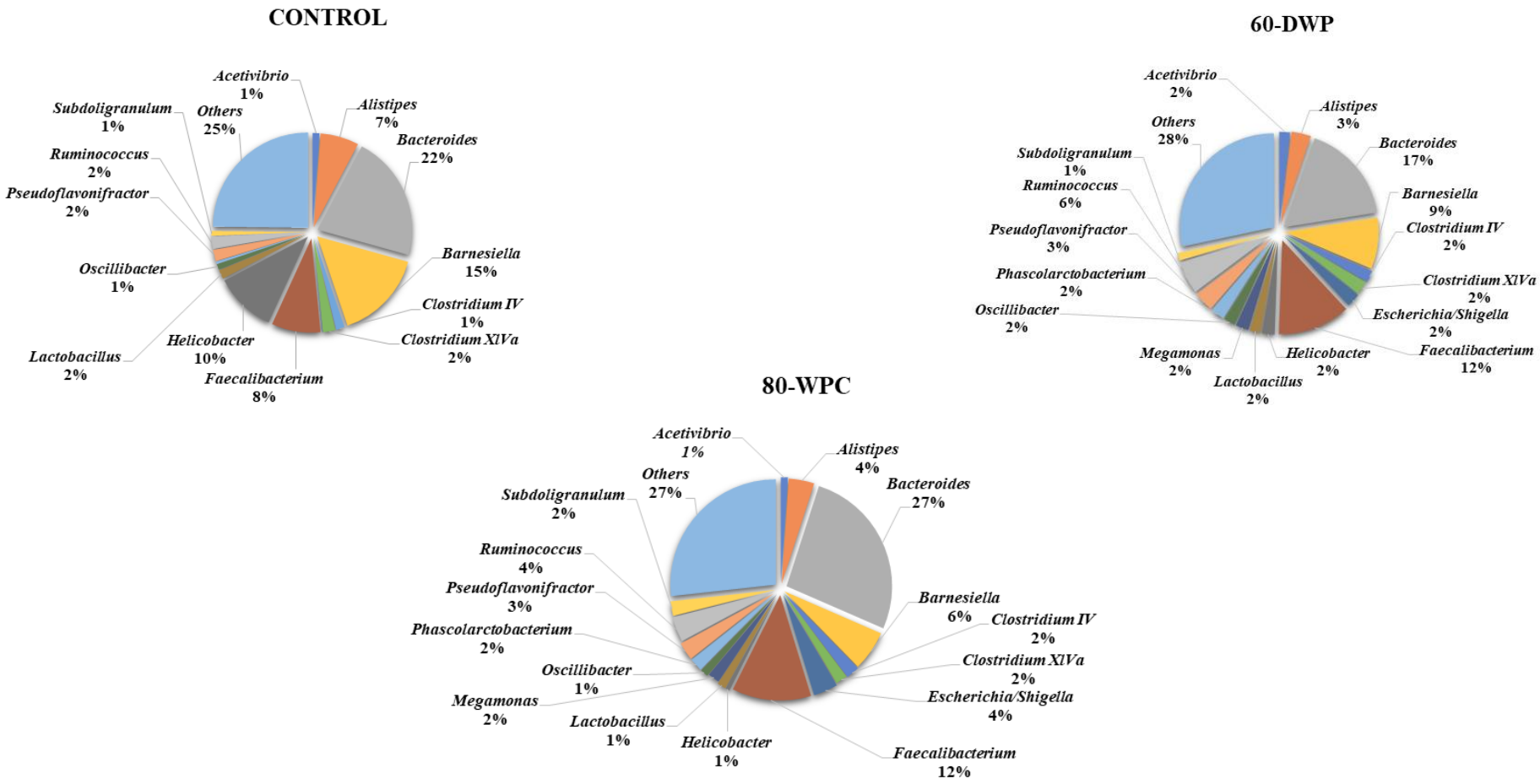

Fig 3. Composition of bacteria in cecal samples of broilers at genus level. Relative abundance (>1% on average) of genus in response to experimental diets. Control: no supplementation of dry whey powder or whey protein concentrate, 60-DWP: 60 g/kg of dry whey powder, 80-WPC: 80 g/kg of whey protein concentrate

Exploratory analysis of the microbial community revealed two main clusters that separate the cecal samples of chickens fed with control diet from those fed with 60-DWP and 80-WPC (Fig 5). Within each cluster microbial community similarities between 55-60% were observed. The average similarity within replicates of cecal samples was 62% in control diet, 61% in 60-DWP, and 56% in 80-WPC.

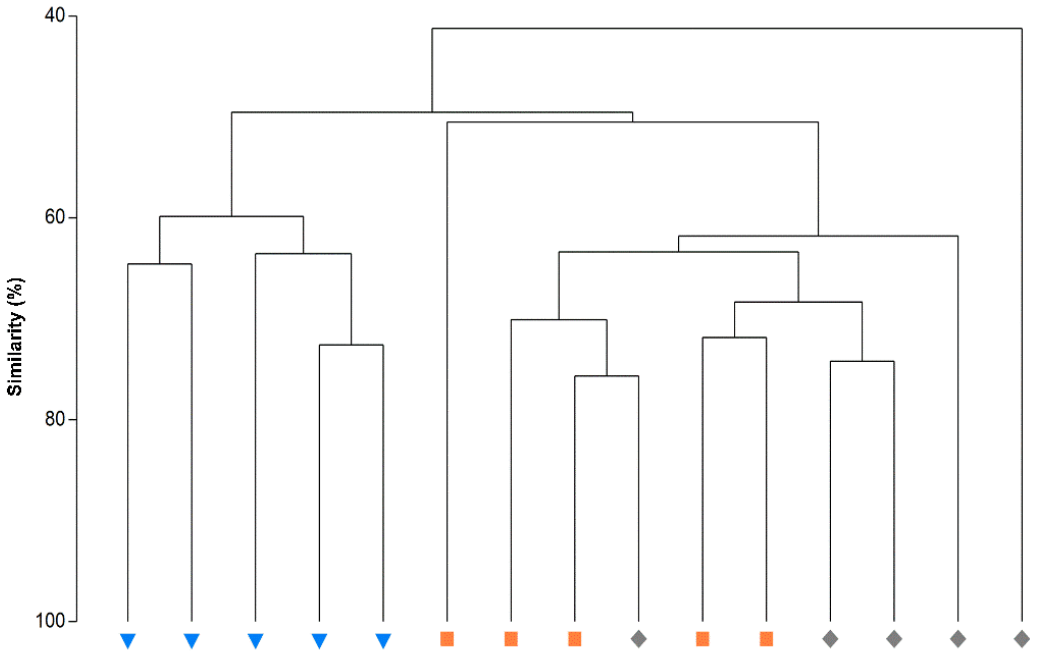

Fig 4. Hierarchical cluster representing relationships among microbial communities of cecal samples from chickens fed with the experimental diets. On y-axis: similarity percentage based on Bray Curtis matrix, on x-axis: individual samples of cecal digesta. Experimental diets: ▼ Control: no supplementation of dry powder whey or whey protein concentrate ■ 60-DWP: 60 g/kg of dry whey powder ◆ 80-WPC: 80 g/kg of whey protein concentrate.

Significant differences in the composition of the bacterial community were observed between experimental diets (R = 0.476, P = 0.001; Table 4). A subset of 6 OTUs summarizes the overall differences (Table 5), which are associated to *Faecalibacterium* spp. (OTU 1), *Bacteroides* spp. (OTU 2), unclassified Porphyromonadaceae (OTU 3), *Bacteroides fragilis* (OTU 5), *Helicobacter pullorum* (OTU 7), and *Escherichia coli/Shigella flexneri* (OTU 9).

Table 4. One-way ANOSIM of cecal microbial communities of broilers associated with experimental diets.

Treatment[1]	Control	60-DWP	80-WPC
Control	-	**0.776**	**0.740**
60-DWP	*0.008*	-	**-0.024**
80-WPC	*0.008*	*0.508*	-

[1] Control: no supplementation of dry whey powder or whey protein concentrate; 60-DWP: 60 g/kg of dry whey powder; 80-WPC: 80 g/kg of whey protein concentrate. The R statistic (indicated in boldface) and significance level (indicated in italics) are shown for each comparison between experimental diets.

Microbial communities from cecal samples of chickens fed with control diet were different from those of 60-DWP (R = 0.776, P = 0.008). The percentage of dissimilarity was 51%, mainly due to the decrease in the abundance of OTUs associated to *Bacteroides* spp. (OTU 2), unclassified Porphyromonadaceae (OTU 3), *Helicobacter pullorum* (OTU 7), *Barnesiella intestinihominis* (OTU 17), and unclassified Rikenellaceae (OTU 13) in 60-DWP treatment. An increase in the abundance of phylotypes associated to *Faecalibacterium* spp. (OTU 1), *Bacteroides fragilis* (OTU 5), *Bacteroides* spp. (OTU 6), *Escherichia coli/Shigella flexneri* (OTU 9), *Megamonas funiformis* (OTU 12), *Lactobacillus salivarius* (OTU 33), *Ruminococcus* spp. (OTU 4), unclassified Lachnospiraceae (OTU 10), and *Phascolarctobacterium* spp. (OTU 11) in 60-DWP treatment also contributed to the dissimilarity between both groups.

Microbial communities of samples from control diet were significantly different from those from 80-WPC (R = 0.740, P = 0.008). The percentage of dissimilarity was 52%, being this attributed to the decrease in the abundance of OTUs associated to *Bacteroides* spp. (OTU 2), unclassified Porphyromonadaceae (OTU 3) *Helicobacter pullorum* (OTU 7), unclassified Rikenellaceae (OTU 13), and *Barnesiella intestinihominis* (OTU 17) in 80-WPC samples. In contrast, *Faecalibacterium* spp. (OTU 1), *Bacteroides fragilis* (OTU 5), Bacteroides spp. (OTU 6), *Escherichia coli/Shigella flexneri* (OTU 9), unclassified Lachnospiraceae (OTU 10), *Phascolarctobacterium* spp. (OTU 11), *Megamonas funiformis* (OTU 12), and *Subdoligranulum variabile* (OTU 22) were more abundant in 80-WPC samples compared to control samples.60-DWP and 80-WPC microbial communities did not differ (R = -0.024, P = 0.508). The most common OTUs in both diets were associated to *Bacteroides* spp. (OTU 6), *Escherichia coli/Shigella flexneri* (OTU 9), *Megamonas*

funiformis (OTU 12), *Faecalibacterium* spp. (OTU 1), unclassified Rikenellaceae (OTU 13), and *Phascolarctobacterium* spp. (OTU 11).

Table 5. Abundance of OTUs that contribute to dissimilarity in cecal microbial communities of broilers associated with experimental diets

	Relative abundance (%)			Taxonomy	RDP Score	Reference
	Control[1]	60-DWP	80-WPC			
OTU1*	7.56	12.46	12.00	*Faecalibacterium* spp.	98.4	Scupham (2007)
OTU2*	18.28	8.58	14.84	*Bacteroides* spp.	98.1	Eckburg et al. (2005)
OTU3*	12.29	8.67	5.27	unclassified Porphyromonadaceae	82.8	Sakamoto et al. (2007)
OTU4	2.13	5.66	3.89	*Ruminococcus* spp.	98.4	Turnbaugh et al. (2009)
OTU5*	1.90	2.38	6.54	*Bacteroides fragilis*	97.7	Cerdeño-Tárraga et al. (2005)
OTU6	1.47	6.24	5.22	*Bacteroides* spp.	95.3	Bakir et al. (2006)
OTU7*	10.5	2.20	0.63	*Helicobacter pullorum*	98.0	Dewhirst et al. (2005)
OTU9*	0.06	2.39	3.75	*Escherichia coli/Shigella flexneri*	97.7	Wang et al. (1997)
OTU10	0.37	1.96	1.37	unclassified Lachnospiraceae	94.2	Bjerrum et al. (2006)
OTU11	0.42	2.39	2.05	*Phascolarctobacterium* spp.	98.8	Scupham (2007)
OTU12	0.01	2.27	1.77	*Megamonas funiformis*	97.6	Sakon et al. (2008)
OTU13	3.68	1.36	1.60	Unclassified Rikenellaceae	87.1	Song et al. (2006)
OTU17	3.19	0.20	0.91	*Barnesiella intestinihominis*	97.6	Morotomi et al. (2008)
OTU22	0.04	-	1.71	*Subdoligranulum variabile*	98.0	Holmstrøm et al. (2004)
OTU23	0.29	0.96	1.36	Unclassified Ruminococcaceae	88.0	Krogius-Kurikka et al. (2009)
OTU31	0.38	-	1.17	*Odoribacter* spp.	98.0	Li et al. (2012)
OTU33	0.59	1.10	-	*Lactobacillus salivarius*	98.0	Groisillier and Lonvaud-Funel (1999)

[1] Control: no supplementation of dry whey powder or whey protein concentrate; 60-DWP: 60 g/kg of dry whey powder; 80-WPC: 80 g/kg of whey protein concentrate. * These OTUS were identified by BEST routine as summarizing the overall differences in microbial community.

4. DISCUSSION

The current study shows that including 60 g/kg of DWP or 80 g/kg of WPC in diets improves broiler growth and feed efficiency, from early growth stages to later ages. These dietary supplementations improve ileal digestibility of calcium and phosphorus, and promote a higher ratio of Firmicutes/Bacteroidetes in ceca. Similarly, Gulsen et al. (2002) showed that adding 38.5 g/kg of DWP as lactose source at starter and grower-finisher diets improved BW, whereas Kermanshahi and Rostami (2006) reported that supplying 40 g/kg of DWP did not affect BW during the starter period, but BW and FCR were improved both during the grower-finisher and the entire feeding periods. Authors attributed the weight increase to a higher absorption of protein and some minerals such as calcium and phosphorous, although they did not present such results. Regarding the use of proteins from whey in broiler diets, Szczurek et a.. (2013) reported an increase in BW, and a reduction of FCR when adding 8 or 32 g/kg of WPC during both the starter and grower-finisher periods. These authors attributed the improved performance to the observed increase in ileal digestibility of protein measured on broilers at 26 days of age. Digestibility results differ from ours, possibly due to age differences, number of animals sampled, and ingredient and nutrient composition of diets. According to our findings, we consider the improvement of AID of Ca and P, and the increase in FI as one of the reasons explaining the improved performance of broilers fed with DWP or WPC. Both feed ingredients contain a considerable amount of Ca and P, which led to a reduction in dicalcium phosphate during diet formulation, and to lesser extent in calcium carbonate, with respect to control diet. It is known that mineral organic sources have higher intestinal absorption than inorganic sources (Nollet et al., 2007), and that lactose greatly stimulates the absorption of Ca and P (Matin et al., 2013), which would explain the observed results. The higher Ca and P digestion influences the growth of animals because these minerals promote bone development and mineralization (Proszkowiec-Weglarz and Angel, 2013). Similarly, we consider that higher Ca and P digestion, together with the observed increase in FI, promotes a higher digestible mineral intake, and in consequence an improvement in growth.

We also consider that the observed changes in ceca microbiota would enhance broiler performance. Therefore, birds with a higher weight showed a greater F/B ratio. These results are in agreement with Singh et al. (2013), who found that broilers with greater weight exhibited higher F/B ratio in ceca. Representatives of the Firmicutes

phylum are known for harvesting energy from diets and transfering calories to the host, with the subsequent weight gain (Turnbaugh et al., 2006; Turnbaugh et al., 2009). Some of the microorganisms belong to the Ruminococcaceae and Lachnospiraceae families, which were more abundant in broilers with higher weight. Both families break down complex, plant-derived carbohydrates and resistant starch, which are found in diet grains, into saccharides and make them available for microbial fermentation to high energy metabolites (Biddle et al., 2013). It was therefore expected that the microorganisms that play an important role in this process were more abundant in birds with higher weight, indicating improved efficiency in extracting energy from the diet.

Our results also revealed that ceca from those chickens with a higher weight was colonized to a larger extent with bacteria able to ferment lactose, among other carbohydrates, and produce short chain fatty acids (SCFAs) as fermentation products. The abundance of *Faecalibacterium*, *Clostridium XIVa* and *IV*, which are linked to butyric acid production, and *Ruminococcus* associated to acetic acid production (De Vos et al., 2009) increased in supplemented diets. Stanley et al. (2016) showed greater abundance of *Faecalibacterium* and *Ruminococcus* in broilers with higher growth, together with a major abundance of *Bacteroides fragilis* in those birds with a better energy metabolism. In our study, *B. fragilis* (OTU 5), *Bacteroides* spp. (OTU 6), and *Megamonas funiformis* (OTU 12), known to produce acetic acid and propionic acid as fermentation product (Krieg et al., 2010), were also detected in the ceca of chickens fed with supplemented diets. An increase on *Escherichia coli/Shigella flexneri* (OTU 9) was noted as well. Torok et al. (2011) showed an increase of *E. coli* in broilers with improved feed effciency. However, to the best of our knowledge no studies have so far reported an association between OTU 6 and OTU 12 and any performance parameter in poultry. OTU 6 is a common inhabitant of layer hens' ceca (Prasai et al., 2016), and a previous study has reported that *Megamonas* acts as hydrogen sink in the ceca of broilers, thereby increasing the SCFAs production (Sergeant et al., 2014). Ceca colonization by efficient SCFA's-producing microbiota provides extra energy for host metabolism (Józefiak et al., 2004; Rinttilä and Apajalahti, 2013), promoting their growth.

In the present study higher abundance of *Lactobacillus salivarius* (OTU 33) was identified in broilers with better FCR results, which were those fed with 60-DWP. The same findings were also reported by Stanley et al. (2013). *L. salivarius* is a lactic-acid-

producing bacteria able to ferment lactose (De Vos et al., 2009), and it has been used as probiotic in poultry nutrition (Saint-Cyr et al., 2016). We hypothesize that these bacteria improved feed efficiency, possibly through the improvement of the sanitary status and gut microbiota balance of broilers. *L. salivarius* produces effective bacteriocins against some potentially pathogenic bacteria, and has the ability to stimulate butyrate producing bacteria, together with the establishment of good microflora balance (Messaoudi et al., 2013).

Broilers with higher weight showed lower cecal abundance of *Helicobacter pullorum* (OTU 7), being this a positive finding because *H. pullorum* is considered a potential zoonotic pathogen, constituting an important health problem both for poultry and humans. Low abundance of this bacteria reduces the chickens' risk of enteric disease and vibrionic hepatitis (Borges et al., 2015). Therefore, productivity is not threatened by disease and birds are able to gain weight more efficiently. This bacterial reduction also minimizes the risk of contamination of chicken products for human consumption, thereby reducing the risk of several human digestive pathologies (Borges et al., 2015). We hypothesize that the reduction in the abundance of *H. pullorum* could be related to the observed parallel increase of SCFAs-producing bacteria, and to the effectiveness of SCFAs as an inhibitor of some harmful bacteria due to their ability to damage pathogen cell walls.

The current study also showed that inclusion of WPC in diets did not result in improved performance compared with diets supplemented with DWP, despite being both formulated with a similar amino acid profile. The reason could lay on the fact that lactose, provided in equal quantities by both diets, promoted a similar mineral digestibility. The microbial community composition is therefore modulated similarly, leading to chickens reaching comparable productive performance results.

Future work should also include the characterization of different sections of the upper gastrointestinal tract to determine the microbial community modulations that might occur due to the dietary interventions. Also, it would be relevant to investigate the gut bacterial function through metagenomics studies to elucidate the biochemical properties of microbiota, and potential metagenomics modulation by feeding chickens with different dietary treatments.

5. CONCLUSION

Data presented here indicate that both DWP and WPC are viable ingredients in poultry feeding because they improve chicken growth, ileal digestibility of Ca and P, and modulate the cecal microbiota. Of special interest is the association of a greater abundance of *Bacteroides fragilis*, *Bacteroides* spp., *Escherichia coli/Shigella flexneri* and *Megamonas furniformis* with improved weight, as well as a greater abundance of *Lactobacillus salivarius* with better FCR. The reduction of the pathogenic phylotype *Helicobacter pullorum*, linked to higher chicken weight, is especially important since DWP and WPC could improve broiler sanitary status and reduce foodborne pathogen contamination risk.

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CHAPTER 5

CHANGES IN BROILER PERFORMANCE, DUODENAL HISTOMORPHOMETRY, AND CECA MICROBIOTA COMPOSITION IN REPONSE TO WHEAT-BARLEY BASED DIETS SUPPLEMENTED WITH DRY WHEY POWDER AND OTHER NON-ANITIBIOTIC ADDITIVES

Published in the scientific Manuscript: C. Pineda-Quiroga, A. Camarinha-Silva, R. Atxaerandio, R. Ruiz and A. García-Rodríguez. 2017. Changes in broiler performance, duodenal histomorphometry, and caeca microbiota composition in response to wheat-barley based diets supplemented with non-antibiotic additives. Animal Feed Science and Technology 234, 1-9. doi.org/10.1016/j.anifeedsci.2017.09.002

ABSTRACT

The present study was conducted to investigate the effect of supplementing wheat-barley based diets with dry whey powder (DWP), chitosan (CHIT), a mixture of DWP-CHIT, and inulin (INU) on productive performance, duodenal histomorphometry and ceca microbial composition of chickens. A total of 1500 one-day-old male birds were allocated to floor pens and assigned to one of the following treatments: control diet (no additive supplementation), 60-DWP (60 g/kg of DWP), 5-CHIT (5 g/kg of CHIT), DWP-CHIT (60-DWP plus 5-CHIT), and 20-INU (20 g/kg of INU). Each treatment had 10 replicate pens, with 30 birds per pen. Measurements of productive performance were made during the starter period (d 1- 21) and for the entire feeding period (d 1- 42), while duodenal measurements were registered at d 21. Ceca microbiota composition was determined using Illumina amplicon sequencing at d 21 and 42. During the starter period, feeding chickens with any of the tested additives diminished their body weight (BW), average daily gain (ADG) and feed intake (FI) as compared to control diet ($P < 0.05$). This was also observed during the entire feeding period ($P < 0.05$), except for INU supplementation that showed similar values to control birds. None of treatments affected duodenal histomorphometry. Ceca microbiota composition was influenced by diet at every stage of the productive period ($P = 0.001$), although no clear association between microbiota and performance was detected. At d 21, no differences in microbiota composition of control, 60-DWP, 5-CHIT, and 20-INU birds were found, which ceca were highly harboured by *Lactobacillus gallinarum*, although only control promoted greater BW, ADG, and FI. Control and 60-DWP treatments did not differ in their ceca communities at d 42, although only control increased BW, ADG, and FI. In both cases, ceca showed higher abundance of *Lactobacillus gallinarum* and *Bacteroides vulgatus*, and lower abundance of *Escherichia coli/Shigella flexneri* and *Bacteroides fragilis*. DWP-CHIT diet promoted an increase of *Klebsiella pneumoniae* at d 21, and of *Streptococcus gallolyticus* at d 42, together with a performance reduction as compared to control diet. The present findings indicate that chicken growth is reduced by supplementing wheat-barley based diets with DWP, CHIT, DWP plus CHIT, and INU, at the tested doses, as a consequence of a reduction in FI. Ceca microbiota composition and diversity varied in a diet-dependent manner during both sampled ages, although a linkage between microbiota and performance was not clear.

1. INTRODUCTION

Dietary interventions during the last decades, implemented to improve broiler growth, included the addition of low levels of antibiotics as growth promoters in feed for an extended period of time (Dibner and Richards, 2005). The impact on their use has received considerable attention due to the strong changes on the gut microbiota, intestinal wall and the enhancement of bird weight and feed efficiency (Niewold, 2007; Danzeisen et al., 2011). However, their ban in the European Union (EC Regulation, No. 1831/2003), and their potential restriction in other countries, have resulted in a reduction of animal performance and in an increase of enteric pathologies (Dibner and Richards, 2005). In consequence, non-antibiotic feed additives are a subject of increasing interest (Roberts et al., 2015). In this sense, it is advisable to identify which additives are best suitable for poultry, in part due to conflicting evidences about the fact that their use does not always improve broiler productivity and health (Geier et al., 2009b). These inconsistencies might be related to the main cereal used in diet formulation (Rodríguez et al., 2012), being therefore essential to evaluate additive effectiveness in wheat and barley based diets, which are two of the most common energy sources in poultry nutrition (Amerah, 2015).

Prebiotics are defined as non-digestible dietary compounds that modulate the composition and/or activity of gut microbiota, conferring a beneficial effect on the host (Bindels et al., 2015). Successful productive results have been reported in poultry by using dry whey powder (DWP; Pineda-Quiroga et al., 2017) and inulin (Velasco et al., 2010) as prebiotics in corn based diets, although results about the response of chickens to diets based on cereal high in soluble non-starch polysaccharides (NSP) are scare. Ceca fermentation of lactose from DWP, and fructo-oligosaccharides from inulin, promote the growth of selective beneficial bacteria populations in detriment of potentially pathogens (Flickinger et al., 2003; Gulsen et al., 2002). Similarly, natural antimicrobials such as chitosan, which has a wide spectrum of activity against Gram-positive and Gram-negative bacteria (Kong et al., 2010), have been used in corn based broilers diets with positive performance results (Khambualai et al., 2009), though it is possible that their inclusion increase gut digesta viscosity.

Knowledge about the effect of supplementing chicken wheat-barley diets with non-antibiotic feed additives can give an insight into how these influence bird

performance through duodenal development and ceca microbial modulations when these diets are used. A more comprehensive research of the specific effects of dry whey powder, chitosan, and inulin is therefore essential to identify their adequacy in broiler feeding. Similarly, potential synergies between prebiotics and natural antimicrobials should be explored, and the combined use of dry whey powder and chitosan is proposed as a first approach. In this sense, it could be expected that antimicrobials reduce the gastrointestinal load of potentially pathogenic bacteria and, under these conditions, prebiotics could be selectively used as substrate for the growth of beneficial bacteria. Therefore, the aim of this study was to investigate the influence of the above cited additives, and of the mixture of dry whey powder plus chitosan, on broiler performance, duodenal histomorphometry, and cecal microbial composition during the lifespan of the broilers.

2. MATHERIALS AND METHODS

The experiment was performed in accordance with the European Union (2010/63/EU) and Spanish regulations (RD 53/2013) for the care and use of animals for experimental and other scientific purposes.

2.1 Test substances

The additives tested in the present study were dry whey powder (DWP), chitosan (CHIT), and inulin (INU). The DWP was a commercial sweet powder composed by a mixture of ovine and bovine whey (Sueromancha S.L, Toledo, Spain) composed by 703 g of lactose/kg of product. The CHIT was the commercial product ChitoClear® fg 95 (Trades, S.A., Tarragona, Spain), prepared from chitin of shrimp shells with a degree of deacetylation ≥ 95%. The INU was a commercial product Orafti®GR (Trades S.A, Spain) obtained from chicory roots, containing 900 g of inulin/kg of product.

2.2 Animals, housing, and experimental diets

The study was conducted at the experimental facilities of Neiker-Tecnalia in Arkaute (Vitoria-Gasteiz, Spain). A total of 1500 one-day-old male broiler chickens

(Ross 308 strain) were obtained from a local commercial hatchery (AN Avícola Melida, S.A, Zumaia, Spain). At arrival, birds were randomly allocated to floor pens at a stocking density of 30 kg/m^2. Pens were equipped with one manual feeder, nipple drinkers, and wood shavings as litter material. Room temperature and lighting program were implemented following the guidelines for Ross 308.

Wheat and barley based diets were formulated to meet broilers' requirements during the starter and grower-finisher stages (FEDNA, 2008). Starter diets were offered from day one to 21 as crumbles, and grower-finisher diets were offered from d 22 to – 42 as pellets. Each treatment comprised 10 pens, with 30 chickens each. Birds had ad libitum access to one of the following experimental diets: control (no additive supplementation), 60-DWP (60 g/kg of inclusion of dry whey powder), 5-CHIT (5 g/kg of inclusion of chitosan), DWP-CHIT (60 g/kg of inclusion of dry whey powder plus 5 g/kg of inclusion of chitosan), and 20-INU (20 g/kg of inclusion of inulin). β-glucanases and β-xylanases were included in the same amount in all diets (Nutralzyme, Nutral S.A., Madrid, Spain). A commercial biocide product (Salmocid-F®, Adiveter S.L., Tarragona, Spain) was also added to diets during the industrial making process to control their microbiological charge. The ingredient composition and nutritional value of experimental diets are shown in Table 1.

2.3 Measurements and sampling

2.3.1 Productive performance

To determine productive performance, all chickens and feeders from each pen were periodically weighted. Body weight (BW), average daily gain (ADG), feed intake (FI), and feed conversion ratio (FCR) were recorded on a pen basis. Mortality was recorded daily.

Table 1. Ingredients and chemical composition of experimental diets

	Starter period (1 to 21 d)					Grower-Finisher period (22 to 42 d)				
Item	[1] Control	60-DWP	5-CHIT	DWP-CHIT	20-INU	Control	60-DWP	5-CHIT	DWP-CHIT	20-INU
Ingredients (g/kg, *as fed* basis)										
Barley	325	311	330	317	320	409	400	445	443	411
Wheat	262	235	270	227	259	303	262	281	220	300
Yellow corn	110	110	110	110	110	0	0	0	0	0
Soybean meal	264	245	245	245	250	235	223	218	214	211
Soybean oil	3.0	5.0	4.1	3.0	5.0	17	23	15	26	23
Dry whey powder	0	60	0	60	0	0	60	0	60	0
Chitosan	0	0	5.0	5.0	0	0	0	5.0	5.0	0
Inulin	0	0	0	0	20	0	0	0	0	20
Dicalcium phosphate	7.2	4.3	7.2	4.3	7.2	7.2	4.3	7.2	4.3	7.2
Calcium carbonate	18	18	18	18	18	18	18	18	18.0	18
Vitamin and mineral mix [2]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4
Sodium cloride	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7
DL-Methionine	0.7	0.7	0.7	0.9	0.5	0.9	0.8	0.9	0.9	0.8
L-Lysine	2.6	2.3	2.6	2.3	2.2	1.4	0.9	1.2	0.80	1.10
Enzyme [3]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Salmocid	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Analyzed chemical composition										
CP (g/kg)	21.80	22.00	21.75	21.86	21.60	20.00	20.70	20.00	20.00	20.00
EE (g/kg)	23	24	25	23	24	40	42	41	41	40
Ca (g/kg)	11.3	11.1	11.2	11.1	11.4	9.0	8.5	9.0	10.0	11.0
Calculated chemical composition										
AMEn (MJ/kg)	12.10	12.79	13.01	12.73	12.81	13.39	13.36	13.36	13.39	13.36
Available P (g/kg)	6.1	5.9	6.0	5.7	6.2	5.5	5.6	5.1	5.0	5.5
Met+Cys (g/kg)	0.41	0.40	0.40	0.42	0.40	0.41	0.40	0.40	0.40	0.41
Lys (g/kg)	1.35	1.31	1.29	1.30	1.28	1.16	1.12	1.10	1.11	1.08

[1] Control: no additive supplementation; 60-DWP: 60 g/kg of dry whey powder; 5-CHIT: 5 g/kg of chitosan, DWP-CHIT: 60 g/kg of dry whey powder plus 5 g/kg of chitosan; 20-INU: 20 g/kg of inulin. [2] Providing the following per kg of diet: of diet: 8 000 IU vitamin A, 600 IU vitamin D3, 16 mg vitamin E, 1 mg thiamine, 3 mg riboflavin, 1 mg pyridoxine, 0.01 mg vitamin B12, 1 mg vitamin K_3, 16 mg niacin, 7 mg pantotenic

acid, 70 mg Mn, 50 mg Zn, 30 mg Fe, 4 mg Cu, 1 mg I, 0.2 mg Co, 0.1 mg Se, 240 mg choline, 300 units phytase, 110 mg ethoxyquin. [3] Nutralzyme (Endo1-4 β-glucanase 500 TGU/g, endo 1-4 β-xylanase 1120 TXU/g, Nutral S.A, Madrid, Spain).

2.3.2 Duodenal histomorphometry

At d 21, 6 chickens from each treatment were randomly selected from different pens and euthanized by CO_2 inhalation. The gastrointestinal tract was dissected, the duodenum was removed, and the tissue samples were collected and preserved in 10% buffered formalin saline before preparing the histological sections. The cecum from these birds was sampled for microbial community analysis. Duodenal tissue samples (3 per bird) were dehydrated by immersion in alcohols of increasing grade, infiltrated in xylene, and embedded in paraffin. Transversal sections were cut (4–6 μm), placed on glass slides and stained using the haematoxiline and eosine technique. Slides were examined using a Nikon Eclipse-80i optical microscope (Nikon Corporation, Japan) coupled with a DS-Ri1 Nikon camera (Nikon Corporation, Japan), and images were analyzed using the image software NIS elements 3.1 (Nikon Corporation, Japan). Villus height and crypt depth were measured on 5 well-oriented villus and crypt per slide, according to the protocol described by Liu et al. (2011). The apparent villus surface was calculated according to Iji et al. (2001).

2.3.3 Cecal microbial composition: DNA extraction, 16S rRNA amplification, Illumina sequencing, and bioinformatics sequence analysis

At d 21 and 42, 6 chickens from each experimental treatment were randomly selected from different pens and euthanized by CO_2 inhalation. The gastrointestinal tract was dissected, the two ceca were opened longitudinally, and digesta samples were collected with a sterile spoon. Samples were immediately stored at −80 °C until further analysis. Total nucleic acid was extracted from samples using the PowerSoil DNA extraction Kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer recommendations. DNA was quantified in Nanodrop (ND-1000 Spectrophotometer, Nanodrop Technologies, DE, USA) and DNA integrity was checked by agarose gel electrophoresis.

The V4 hypervariable region of the 16S rRNA gene was used to prepare Illumina amplicon libraries (Caporaso et al., 2011). Two PCR were performed to

incorporate Illumina adapter sequences and barcodes for sample identification. The PCR products were purified using Agencourt AMPure®XP kit (Agencourt Bioscience Corporation, USA) according to the manufacturer instructions, and eluted in 20 µl of water. Amplicons were quantified using Qubit fluorometric quantitation (Qubit® 3.0, Thermo Fisher Scientific Inc., USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA, USA) to pool the samples and sequence them on an Illumina MiSeq platform.

Illumina reads were analyzed using Mothur Miseq SOP (Kozich et al., 2013). Primers and barcodes were removed prior to analysis. One replicate from 5-CHIT group was removed from the analysis because no reads were registered. All samples comprised 4.460.848 sequence reads, with a mean number of reads per sample of 78.260 ± 13.525. Sequences were aligned using SILVA-based bacterial reference alignment obtained from Mothur. Chimera sequences were checked and removed using UCHIME (Edgar et al., 2011). A final dataset was then clustered into operational taxonomic units (OTU) at ≥ 97% similarity. OTUs with only 1 read appearing in one sample were removed from the analysis (< 0.0004% of the total). A total of 652 OTUs were taxonomically assigned using the naïve Bayesian RDP classifier (Wang et al., 2007). OTUs were then manually evaluated against the Ribosomal Database Project using the Seqmatch function. Sequences are available at the European Nucleotide Archive, under the accession number PRJEB20379 in http://www.ebi.ac.uk/ena/data/view/PRJEB20379

2.4 Chemical analysis

Samples of experimental diets were analyzed in triplicate for dry matter (method 934.01), nitrogen (method 990.03), and ether extract (method 920.39) according to the Association of Official Analytical Chemists (2007). Ca was measured by spectroscopy plasma atomic emission.

2.5 Statistical analysis

For the analysis of productive performance, duodenal histomorphometry, and ceca microbiota, pen was considered as the experimental unit. Performance data were

evaluated separately for the starter (d 1 to 21) and for the entire feeding period (d 1 to 42). One-way ANOVA was performed, with the experimental diet being the fixed effect and with the pen included as a random effect. Performance and duodenal measurements were analyzed considering a Gaussian distribution, whereas mortality data were analyzed using a binomial distribution. Initial BW was included as a covariate on performance analysis. Data were analyzed using the PROC GLIMMIX in SAS V 9.3 (SAS Inst. Inc., Cary, NC). Statistical significance of the experimental diet effect was declared at $P < 0.05$. In case of a statistically significant effect, the Dunnett range test was used to compare each treatment to the control.

For sequencing analysis, relative abundances of the OTUs obtained from ceca content were analyzed by multivariate statistical routines using PRIMER (version 7.0.9, PRIMER-E; Plymouth Marine Laboratory, Plymouth, UK; Clarke and Warwick, 2001). Data were standardized, and a sample resemblance matrix was generated using Bray-Curtis similarity coefficient. Microbial community structures were explored by Principal Coordinate Analysis (PCoA) showing the centroids for each diet-age point (control, 60-DWP, 5-CHIT, DWP-CHIT and 20-INU at d 21 and 42 of age, respectively). The effect of diet was tested within each time point (d 21 and 42) by means of a Permutational analysis of variance (PERMANOVA, 999 permutations). Differences were studied based on pair-wise tests using a permutation method, being considered significant if $P < 0.05$. Individual OTUs contributing to dissimilarity for each comparison were identified by similarity percentage analysis (SIMPER). Pielou's evenness index and Shannon-weaver index of diversity (H') were used to calculate OTU evenness and diversity.

3. RESULTS

3.1 Productive performance and duodenal histomorphometry

Results of productive performance are described in Table 2. In general, control diet promoted the highest BW, ADG, and FI values during the starter period and for the entire feeding period in comparison to 60-DWP, 5-CHIT, and DWP-CHIT. On the other hand, control showed higher values for these variables during the starter period than 20-INU, while no differences were observed for the entire feeding period. For FCR, no

differences were found between control and 60-DWP during the starter period, while control presented significantly lower values than 5-CHIT, DWP-CHIT, and 20-INU. For the entire feeding period, FCR did not differ between diets. Mortality mean values during the experiment were 5%, remaining unaffected by treatments.

None of the treatments affected duodenal histomorphometry variables, with overall (mean ± SEM) being 1523 ± 97.6 µm for villus height, 191 ± 18.4 µm for crypt depth, 8.8 ± 1.0 for villus:crypt ratio and 52.4 ± 4.3 µm2 for villus surface area.

Table 2. Effect of experimental diets on broiler performance at different periods of feeding.

Item	Treatments [1]						
	Control	60-DWP	5-CHIT	DWP-CHIT	20-INU	SEM[2]	P-value[3]
Starter period (d 0 to 21)							
Body weight (g)	617	504***	521***	588*	559***	8.1	<0.001
Average daily gain (g/d)	28	22***	23***	26**	25***	0.4	<0.001
Feed intake (g/d)	45	36***	38***	40*	41**	0.6	<0.001
Feed conversion ratio	1.56	1.61	1.63*	1.65*	1.64*	0.019	0.024
Entire feeding period (d 1 to 42)							
Body weight (g)	2010	1692***	1828**	1848**	1934	35.0	<0.001
Average daily gain (g/d)	46	40***	43**	44*	45	0.9	<0.001
Feed intake (g/d)	89	78***	74***	76***	89	1.5	<0.001
Feed conversion ratio	1.92	1.95	1.89	1.92	1.99	0.043	0.235

[1] Control: no additive supplementation; 60-DWP: 60 g/kg of dry whey powder; 5-CHIT: 5 g/kg of chitosan, DWP-CHIT; 60 g/kg of dry whey powder plus 5 g/kg of chitosan; 20-INU: 20 g/kg of inulin. [2] SEM: standard error of the mean. [3] P-value refers to the overall treatment effect. Statistical significance of each "experimental diet *vs* control" individual comparison, obtained using the Dunnett test, is coded as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.2 Microbial community analysis

At the phylum and family levels, changes were observed in their composition irrespective of the experimental diets (Fig 1A, 1B). Firmicutes was the most abundant phylum at d 21 and 42 of life (90% and 60% on average, respectively), while Proteobacteria was the second most abundant at d 21 (9.7% on average), and Bacteroidetes and Actinobacteria were so at d 42 (30% and 3.6%, on average,

respectively). At a family level, Lachnospiraceae, Lactobacillaceae, and Enterobacteriacaecae were the most representative at d 21 (41%, 20%, and 10%, on average, respectively), while Bacteroidaceae was the most abundant at day 42 (31% on average). Bifidobacteriaceae increased in all diets at d 42 in relation to d 21 of age (3.5% *vs.* 0.78%, on average, respectively), while Ruminococcaceae remained unaffected at both sampled ages (23%, on average, at d 21, and 22%, on average, at d 42).

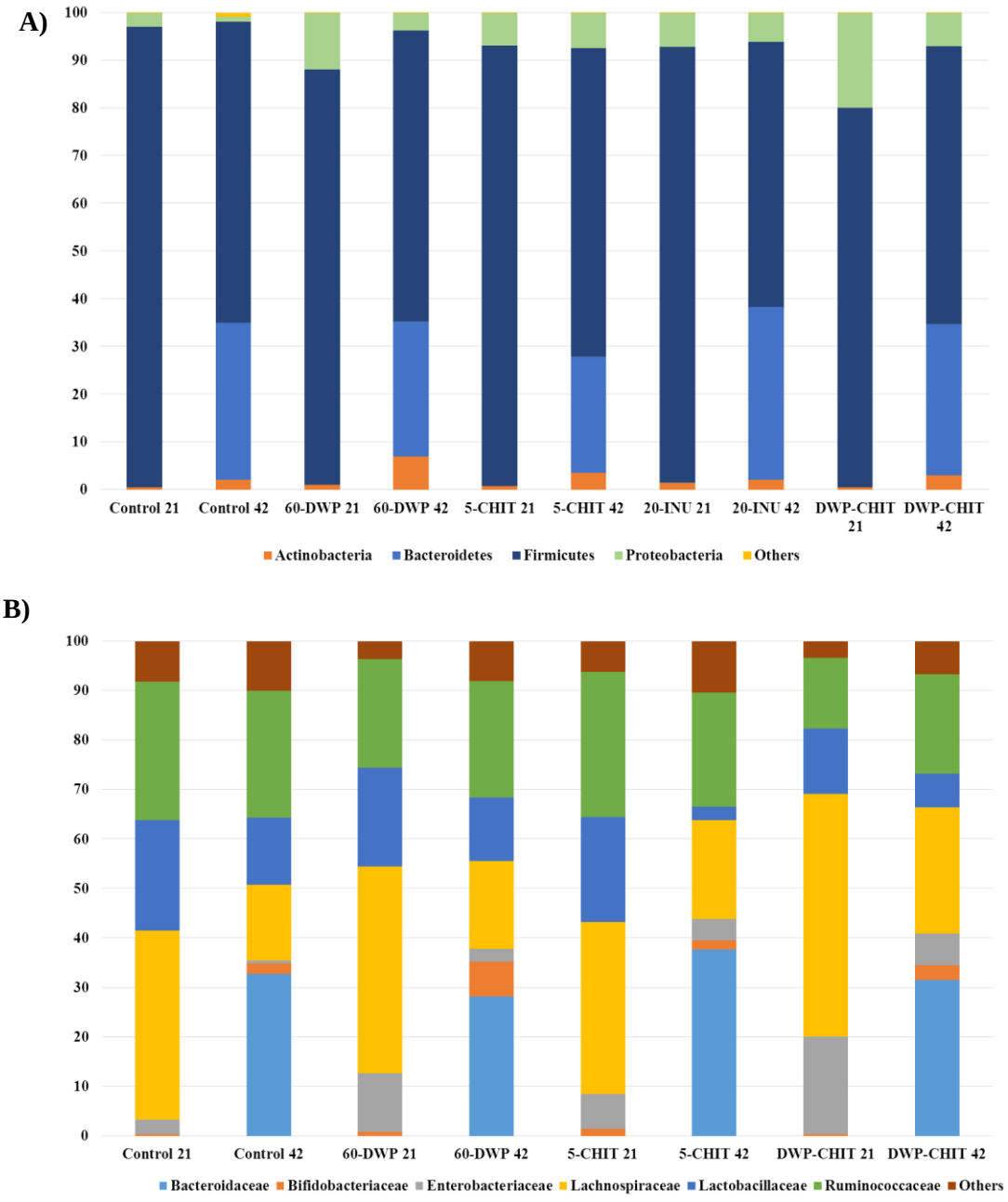

Fig 1. Composition of bacteria in cecal samples at phylum **A)** and family level **B)**. Relative abundance >1% on average, in response to experimental diets.

At genus level, ceca samples were dominated by *Lactobacillus* at d 21 in all diets (22% on average), except for DWP-CHIT, which showed higher abundance of *Escherichia/Shigella* (18% on average). *Ruminococcus2* was the second most representative genus in all diets (15% on average), with the exception of 5-CHIT, which showed higher abundance of *Clostridium XlVa* (9.5% on average). At d 42, *Bacteroides* was the major genera (31% of average relative abundance), followed by *Lactobacillus* (11% on average; Fig 2).

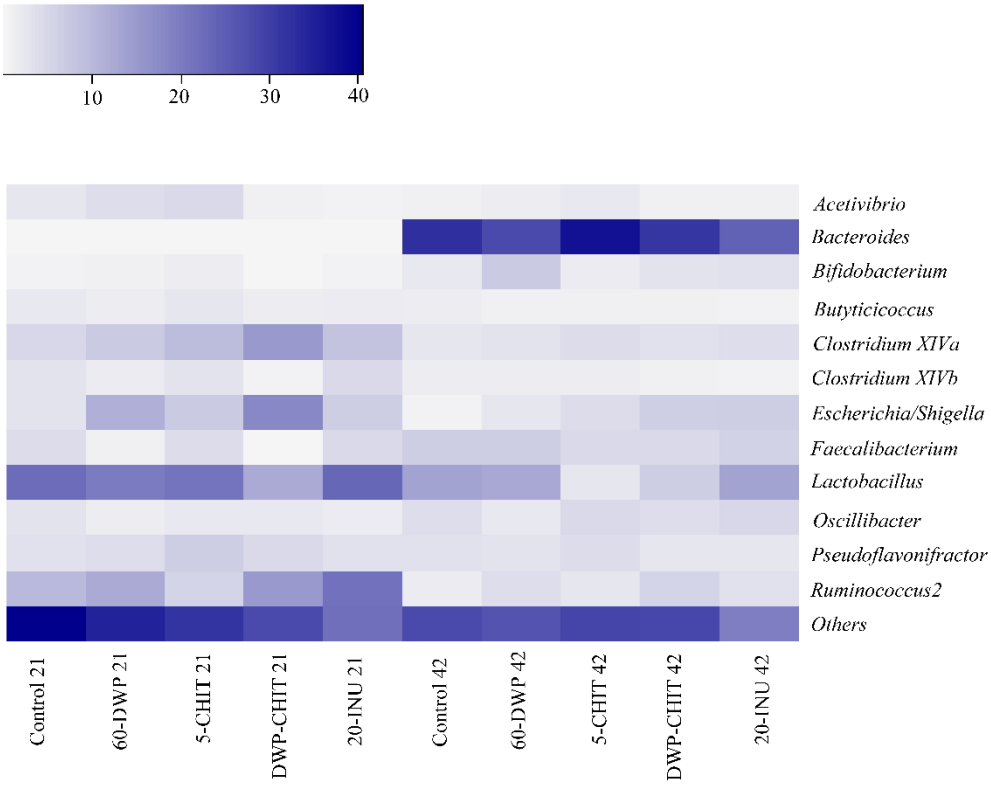

Fig 2. Composition of cecal bacteria samples at genus level. Relative abundance (>1% on average) of genus in response to experimental diets, at d 21 and 42 of age. Experimental diets: Control: no additive supplementation; 60-DWP: 60 g/kg of dry whey powder; 5-CHIT: 5 g/kg of chitosan; DWP-CHIT: 60 g/kg of DWP plus 5 g/kg of CHIT; 20-INU: 20 g/kg of inulin.

The exploratory analysis of bacteria community structure using a PCoA revealed a clear separation of communities at d 21 from those at d 42. The principal coordinate axis explained 79.2% of the total variation, indicating that it properly reflects the bacterial structure differences (Fig 3).

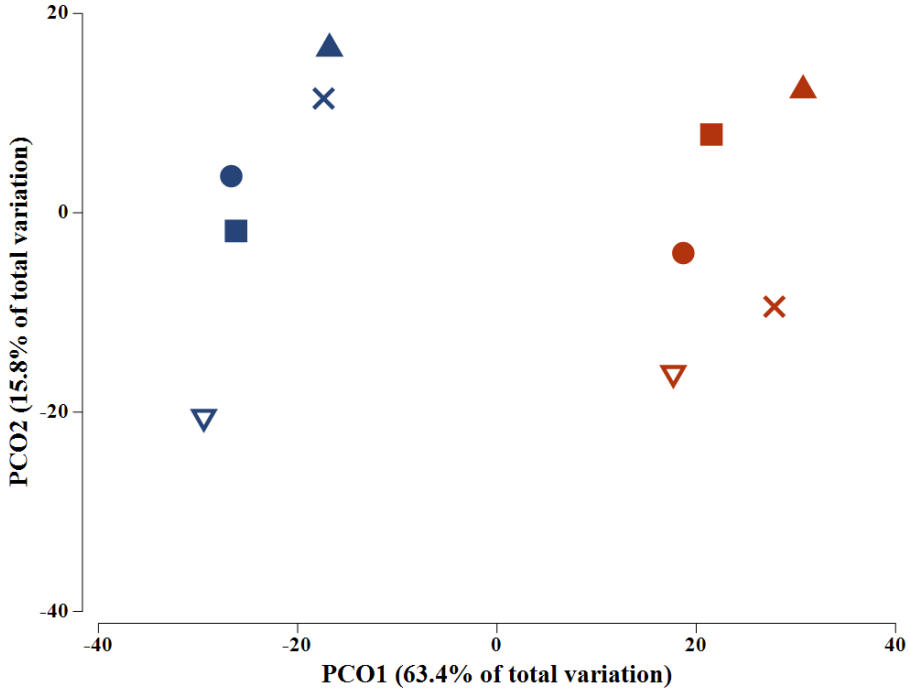

Fig 3. Principal coordinate analysis (PCoA) depicting the centroids of the ceca microbial communities from chickens fed with the experimental diets, at d 21 (blue color) and 42 (brown color). Experimental diets: ▲ Control: no additive supplementation; ■ 60-DWP: 60 g/kg of dry whey powder; **X** 5-CHIT: 5 g/kg of chitosan; ▼ DWP-CHIT: 60 g/kg of DWP plus 5 g/kg of CHIT; ● 20-INU: 20 g/kg of inulin.

3.2.1 Ceca microbiota on d 21 of age

Statistical differences due to diets were detected on ceca microbial composition at d 21. Microbiota from chickens fed with DWP-CHIT was different from those fed with control, 5-CHIT, and 20-INU, whereas no differences were found between DWP-CHIT and 60-DWP (Table 3). Microbial composition did not differ for the remaining diet comparisons. Diversity indices showed, on average, lower Pielou's evenness and Shannon diversity for DWP-CHIT (0.55 and 2.97, respectively) in comparison to control (0.63 and 3.68, respectively), 5-CHIT (0.65 and 3.46, respectively), and 20-INU (0.58 and 3.15, respectively). These results were mainly attributed to the higher abundance of *Escherichia coli/Shigella flexneri* (OTU 3), and the lower abundance of *Lactobacillus gallinarum* (OTU 1) in DWP-CHIT diet in comparison with the remaining experimental diets (Fig 4). Similarly, higher abundance of *Lactobacillus* spp.

(OTU 13) was observed in DWP-CHIT in relation to control and 5-CHIT diets, while *Klebsiella pneumoniae* (OTU 66) was particularly detected in DWP-CHIT diet (Table 4).

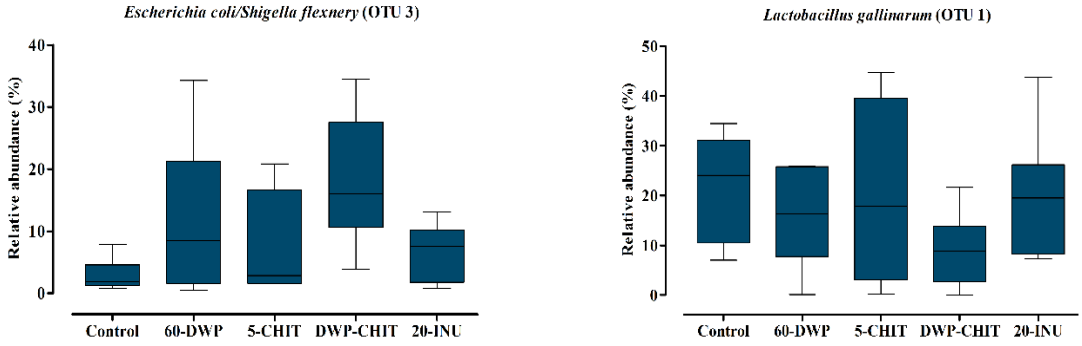

Fig 4. Relative abundance of the most relevant OTUs contributing to differences between ceca microbial communities of chickens fed with experimental diets at day 21 of age. Experimental diets: Control: no additive supplementation; 60-DWP: 60 g/kg of dry whey powder, 5-CHIT: 5 g/kg of chitosan; DWP-CHIT:60 g/kg of DWP plus 5 g/kg of CHIT; 20-INU: 20 g/kg of inulin.

3.2.2. Ceca microbiota on day 42 of age

Statistical differences in ceca microbial composition at d 42 were found between diets. Microbial composition of broilers fed with control differed from those fed with 5-CHIT, DWP-CHIT, and 20-INU, whereas no differences were found between control and 60-DWP (Table 3). Diversity indices showed, on average, similar Pielou's evenness and Shannon diversity values for control (0.60 and 3.67 respectively) in relation to 5-CHIT (0.61 and 3.68 respectively), DWP-CHIT (0.61 and 3.59 respectively), and 20-INU (0.62 and 3.71 respectively). These results were because of the increase in the abundance of *Lactobacillus gallinarum* (OTU 1) and *Bacteroides vulgatus* (OTU 4) in control diet, and the reduction in the abundance of *Escherichia coli/Shigella flexneri* (OTU 3) and *Bacteroides fragilis* (OTU 7) in comparison to 5-CHIT, DWP-CHIT, and 20-INU (Fig 5). *Streptococcus gallolyticus* (OTU 39) were absent in samples from control but it appears, in low abundance, in DWP-CHIT (Table 4).

Differences were also observed between microbial communities from samples of broilers fed with 60-DWP from those fed with 5-CHIT, and DWP-CHIT, whereas no differences were found between 60-DWP and 20-INU (Table 3). Diversity indices showed, on average, lower Pielou's evenness and Shannon diversity for 60-DWP (0.58 and 3.62 respectively) in comparison to 5-CHIT (0.61 and 3.68, respectively) and to DWP-CHIT (0.61 and 3.59, respectively). These results were mainly a consequence of an increase in the abundance of *Lactobacillus gallinarum* (OTU 1), *Bacteroides vulgatus* (OTU 4), and *Bifidobacterium saeculare* (OTU 11) in 60-DWP, as well as of the reduction of *Escherichia coli/Shigella flexneri* (OTU 3), and *Bacteroides fragilis* (OTU 7) when compared to 5-CHIT, DWP-CHIT, and 20-INU (Fig 4). OTUs associated to *Lactobacillus* spp. (OTU 13), *Anaerostipes butyraticus* (OTU 27), and *Streptococcus gallolyticus* (OTU 39) were less abundant in 60-DWP in comparison to DWP-CHIT (Table 4).

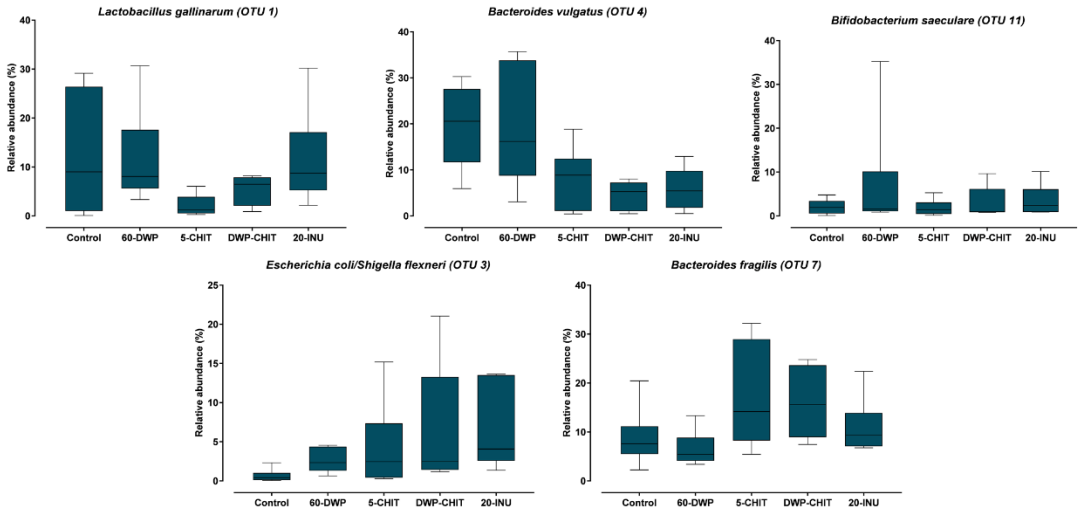

Fig 5. Relative abundance of the most relevant OTUs contributing to differences between ceca microbial communities of chickens fed with experimental diets at day 42 of age. Experimental diets: Control: no additive supplementation; 60-DWP: 60 g/kg of dry whey powder, 5-CHIT: 5 g/kg of chitosan; DWP-CHIT:60 g/kg of DWP plus 5 g/kg of CHIT; 20-INU: 20 g/kg of inulin.

Table 3. Statistical differences between diets at d 21 and 42 based on PERMANOVA and SIMPER results.

Treatment [1]	Groups	Day 21 of age				Day 42 of age			
		t value	P (perm)	Unique permutations	% Dissimilarity	t value	P (perm)	Unique permutations	% Dissimilarity
Control	60-DWP	1.159	0.156	410	58	1.003	0.414	410	44
	5-CHIT	0.931	0.624	206	57	1.595	0.008	412	51
	DWP-CHIT	1.921	0.004	404	66	1.935	0.001	404	55
	20-INU	1.232	0.116	392	56	1.494	0.025	419	49
60-DWP	5-CHIT	0.898	0.672	206	61	1.538	0.006	408	56
	DWP-CHIT	1.034	0.346	309	59	1.559	0.013	413	55
	20-INU	0.935	0.566	411	58	1.185	0.152	410	51
5-CHIT	DWP-CHIT	1.510	0.034	210	69	0.942	0.540	410	49
	20-INU	1.173	0.177	209	62	1.174	0.198	414	51
DWP-CHIT	20-INU	1.416	0.034	404	61	0.912	0.634	412	48

[1] Control: no additive supplementation; 60-DWP: 60 g/kg of dry whey powder; 5-CHIT: 5 g/kg of chitosan, DWP-CHIT: 60 g/kg of dry whey powder plus 5 g/kg of chitosan; 20-INU: 20 g/kg of inulin. Each line shows the results of the comparison between each treatment (treatment column) and each of the rest of treatments (groups columns). P (perm) values ≤ 0.05 between comparisons were considered significantly different.

Table 4. Relative abundance of OTUs that contribute to dissimilarity in the caeca microbial communities at d 21 and 42.

	Treatments [1]							
	Control	60-DWP	5-CHIT	DWP-CHIT	20-INU	Taxonomy	RDP [2] score	Reference
Day 21 of age								
OTU 13	0.25	3.95	0.39	2.46	3.12	*Lactobacillus* spp.	95.8	Roos et al. (2005)
OTU 21	1.44	1.44	-	1.51	0.01	*Clostridium lactifermentans*	97.7	van der Wielen et al. (2002)
OTU 66	0.01	0.03	-	1.74	0.08	*Klebsiella pneumoniae*	99.6	Boye and Hansen (2003)
Day 42 of age								
OTU 13	0.77	0.78	-	1.40	1.49	*Lactobacillus* spp.	95.8	Roos et al. (2005)
OTU 27	0.08	0.89	-	1.98	-	*Anaerostipes butyarticus*	97.0	Eeckhaut et al., 2010
OTU 39	-	0.62	-	1.00	-	*Streptococcus gallolyticus*	97.7	Snaidr et al., 1997

[1] Control: no additive supplementation; 60-DWP: 60 g/kg of dry whey powder; 5-CHIT: 5 g/kg of chitosan, DWP-CHIT: 60 g/kg of dry whey powder plus 5 g/kg of chitosan; 20-INU: 20 g/kg of inulin. [2] Ribosomal data base project.

4. DISCUSSION

The current study shows that supplementing broiler diets with 60 g/kg of DWP, 5 g/kg of CHIT, a mixture of DWP and CHIT, or 20 g/kg of INU resulted in decreased weight at early growth stages, as a consequence of a reduction in feed intake. Lower weight and feed intake were also observed at later growth stages, except for INU supplementation that resulted in similar values to those of birds fed with control diet (no supplementation). Poor performance results obtained in the present study were unexpected, since an improvement in growth and intake has been reported in broilers fed with similar amounts of DWP (Kermanshahi and Rostami, 2006), CHIT (Khambualai et al., 2009), and INU (Velasco et al., 2010; Mirza Aghazadeh and Nabiyar, 2015). Conflicting findings could be related to differences in the basal diet composition. Contrary to corn-soybean based diets used in the above-cited reports, we used wheat and barley based diets with exogenous enzymes. Both cereals contain a substantial amount of soluble non-starch polysaccharides that increase the digesta viscosity due their water holding capacity (Munyaka et al., 2016). It is assumed that a high digesta viscosity depresses broiler feed intake and nutrient digestibility (Rodríguez et al., 2012). We consider that supplementing wheat and barley diets with DWP and CHIT could have resulted in worse digesta characteristics, heightening their deleterious effects on broiler metabolism. However, reasons explaining the poor results observed by INU supplementation are unclear, since inulin effectiveness has been reported in wheat and barley diets, at every growth stage, when supplied at the same amount than in the current study (Rebolé et al., 2010). Regarding DWP, lactose supplied in inadequate doses increases digesta osmolarity in broilers (Morishita et al., 1982). In relation to CHIT, high amounts increase the viscosity of the intestinal content (Razdan and Pettersson, 1994). It is known that increased osmotic pressure in bird gut and high digesta viscosity stimulate the satiety center of the brain, diminishing bird voluntary feed intake (Ferket and Gernat, 2006; Khambualai et al., 2009). It is also known that undesirable alterations of digesta physicochemical properties markedly decrease nutrient digestion and absorption, resulting in a reduction of broiler performance (Józefiak et al., 2004; Amerah, 2015).

The present study reflected changes in ceca microbial communities throughout broiler lifespan. Firmicutes was the most predominant *phylum* at d 21 and 42 of age, irrespective of the dietary treatment, which is in accordance with previous reports (Danzeisen et al., 2011; Mohd Shaufi et al., 2015; Ranjitkar et al., 2016). Proteobacteria was the following most abundant phyla in all dietary treatments in young birds, but it was replaced by Bacteroidetes and Actinobacteria at older ages. Bacteria belonging to the genus *Lactobacillus* were the early colonizers of the cecum in the young birds, followed by *Ruminococcus2*, which is consistent with the findings of Tannock (2004) and Ranjitkar et al. (2016), while *Bacteroides* became the most representative genus in older birds. According to Apajalahti et al (2004), microbiota changes at different chicken productive stages are mainly attributed to the rapid growth of the gastrointestinal tract, which concomitantly leads to strong modulations of its microecosystem. Microbial colonization of the digestive tract begins immediately after hatching, being rapidly populated by microorganisms from the surrounding environment and from food and water (Pedroso and Lee, 2015). Their establishment is quick, and a slow transition takes place until a stable and more diverse adult community is achieved (Kohl, 2012). During this process changes in the microbiota structure occur, that are largely influenced by diet composition, with certain bacteria disappearing or emerging in the gut while others remain stable throughout the chicken life (Pourabedin and Zhao, 2015), which is in agreement with our observations.

Our results also revealed that, as expected, the ceca microbiota composition was influenced by diet at every stage of the productive period, although an association between microbiota and performance was not clear. At d 21 of age, ceca from birds fed with control, DWP, CHIT, and INU did not differ in their microbiota communities, although only control promoted a greater weight and feed intake. All of these ceca samples were highly harboured by *Lactobacillus gallinarum* (OTU 1). At d 42 of age, control and DWP did not differ in their ceca microbiota, although only control birds increased their weight and feed intake. Both ceca showed higher abundance of OTU 1 and *Bacteroides vulgatus* (OTU 4), and lower of *Escherichia/Shigella* (OTU 3) and *Bacteroides fragilis* (OTU 7). It should be noted that birds with similar microbial composition differed in their performance results. These findings are in agreement with those reported by Geier et al. (2009b), who found that diet-induced changes in broiler gut microbiota do not always translate into changes in performance, as well as with

Owens et al. (2008) and Mountzouris et al. (2010), who found that performance differences in response to feed additives are not always associated to differences in the gut microbiota.

Studies about the association of OTU 1, OTU 4, and OTU 7 with broiler performance are ambiguous and scarce. Borda-Molina et al. (2016) found higher abundance of OTU 1 in the ceca of broilers with better weight, which were fed with inorganic sources of Ca and P without phytase, whereas Stanley et al. (2016) linked *Lactobacillus* spp. to poor performance in broilers fed with commercial diets. OTU 7 has not been consistently linked with changes in chicken performance either (Stanley et al., 2013; Torok et al., 2013; Stanley et al., 2016). Inconsistencies in the association between gut microbial composition and animal performance might be partly due to differences at the strain level, and hence to the functional level of bacteria (Torok et al., 2013). However, based on our results, additional factors, independent of the presence or absence of certain bacteria, would have been crucial for animal performance. Some of these factors are related to a reduction in feed intake and nutrient digestibility, due to worse physicochemical properties of the digesta caused by the NPS content of the diets.

This study also showed that an increase of *Klebsiella pneumoniae* (OTU 66) at d 21 of age, and of *Streptococcus gallolyticus* (OTU 39) at d 42, were particularly promoted by feeding with a mixture of DWP and CHIT, together with a reduction in performance. These bacteria can be part of the gut microbiota of clinically healthy broilers (Sekizaki et al., 2008), but they also represent a high sanitary risk because they are opportunistic pathogens in both animals and humans (Schulz et al., 2015; Wu et al., 2016). OTU 66 is one of the respiratory pathogens causing high mortality in poultry farms (Aly et al., 2014), while OTU 39 causes septicemia in birds (Sekizaki et al., 2008). Unfavourable shifts in the intestinal microbiota are likely to result in relevant losses for poultry industry, either by a reduction in animal performance or by an increased risk of illness and death (Geier et al., 2009a). These findings suggest that the joint supplementation of DWP and CHIT at the tested doses did not act synergistically, as initially hypothesized. For a better understanding of the effects of the joint use of DWP and CHIT in wheat and barley based diets, further investigations evaluating them at lower doses are advisable to determine their feasibility in this type of diets.

Further work should not only focus on the ceca bacterial profile in response to additive supplementation at some time points of the broiler lifespan, but should also

include the characterization of different sections of the upper gastrointestinal, as well as a more continuous temporal sampling regimen, to accurately determine the microbial community succession. It would also be relevant to investigate gut bacterial functions, through metagenomics, to elucidate the functional properties of the microbiota, as well as the potential metagenomics modulation when broilers are fed with different non-antibiotic additives.

5. CONCLUSION

Results indicate that wheat and barley based diets supplemented with DWP, CHIT, and DWP plus CHIT reduced broiler performance during the entire productive period as a consequence of lower feed intake, whereas INU supplementation was not disadvantageous. Ceca microbiota composition and diversity varied at d 21 and 42 of age in a diet-dependent manner. However, a clear association between productive results and microbiota profile was not evident.

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GENERAL DISCUSSION

As it has been described in the introductory section, the global production of laying eggs and broiler meat is growing in parallel to consumers' demand. This situation demands that accurate management practices are implemented by poultry farmers to improve animal health and performance in order to enhance the competitiveness and efficiency of the activity. During past years, the poultry industry massively adopted the dietary use of AGPs at sub-therapeutic doses as an effective measure to increase production (Patterson and Burkholder, 2003; Castanon, 2007). However, implications about the development of antimicrobial resistance in the animals' GIT microbiota and the transference to human microbiota were not considered in depth. Indeed, the antimicrobial resistance is a huge concern nowadays. From the nutritional perspective, one of the challenges is to search for safe feed additives that improve animal performance through GIT microbial modulations without causing antimicrobial resistance (Brown et al., 2017). In this scenario, prebiotics have been proposed as part of the solution.

The main objective of this Thesis was to evaluate the prebiotic potential of dry whey powder, as a lactose source, in laying hens and broilers' feeding. With this aim, four experiments were carried out to determine the feasibility of this dairy co-product to enhance productive yields when supplemented in corn-soybean and whey-barley based diets. It was hypothesized that lactose is selectively fermented by cecal microbiota because of the negligible lactase activity in poultry (Denbow, 2000), leading to compositional and metabolic microbial changes that might positively affect laying hens and broiler chickens. In addition, the present Thesis also explored ways to increase dry whey powder effectiveness through its simultaneous supplementation with other non-antibiotic feed additives. The results of each study making up this dissertation were analyzed and discusse in the corresponding Chapter, and so this section summarize and integrate the most relevant findings, and discusses the main differences detected. Results concerning dry whey powder supplementation as the sole additive will be discussed in separate of those referring to its joint combination with other feed additives.

1. The effect of DWP supplemented as the sole additive

Performance effects caused by the addition of 60 g/kg of DWP to corn-based diets, in the absence of other additives, were consistent between laying hens and broilers. Supplementation resulted in an increase on egg production of hens only when PA was not added (Chapter 1), while DWP addition improved broilers' FCR during the entire productive cycle only when CaB was not included (Chapter 3). Moreover, DWP enhanced broiler growth and FCR when it was supplemented alone (Chapter 4). In contrast, the addition of DWP to wheat-barley based diets negatively influenced broilers performance (Chapter 5), what could be attributed to differences in the basal diet composition. Contrary to the satisfactory results observed when corn was used (Chapter 1, 3, and 4), the last experiment used wheat and barley as the cereal base. Both grains contain a substantial amount of soluble non-starch polysaccharides which increase digesta viscosity due to their water holding capacity (Munyaka et al., 2016). In addition, it is known that lactose could increase broiler digesta osmolarity when is inappropriately supplied (Morishita et al., 1982). Therefore, the present results indicate that supplementing wheat and barley diets with DWP, at the tested dose, could have resulted in high osmolarity and viscosity. Undesirable alterations of digesta physicochemical properties markedly decrease nutrient digestion and absorption (Józefiak et al., 2004; Amerah, 2015), resulting in a reduction of broiler performance. In order to stablish more solid conclusions regarding the suitability and interest of adding DWP to diets rich in NSP, future studies should test the formulation of diets with lower doses of DWP than those used in the experiments that compose the present dissertation.

Cecal microbial effects caused by DWP supplementation differed depending on the type of cereal matrix used. When DWP was added to corn-based diets for broilers, a higher abundance of *Faecalibacterium* spp. *Alistipes* spp., *Barnesiella* spp., *Clostridium IV*, and *Subdogranulum* spp. genera was observed (Chapter 3). On the other hand, when DWP was added to wheat-barley diets, an increase in the abundance of *Lactobacillus* spp., *Bacteroides* spp., and *Oscillibacter* spp was noted. These divergent findings were not surprising because it has been reported that the consistency in the microbial changes induced by in-feed prebiotics widely varies between trials when the cereal basis change, even when the same prebiotic is used (Torok et al., 2013; Stanley et al., 2014; Crisol-Martínez et al., 2017). This is mainly because bacteria differ from each other according to their substrate preferences and growth requirements (Apajalahti et al., 2004), and the cereal used substantially affects the GIT environment and the digesta characteristics

(Apajalahti, 2005; Pan and Yu, 2014). Since the present thesis only determined the DWP-induced changes in the cecal microbiota when corn, wheat or barley was used, it would be desirable to conduct further studies taking into account other cereals commonly used in poultry feeding, such as sorghum, oats, rice, or millet (Batonon-Alavo et al., 2015).

A clear association between performance and microbial composition was evidenced by feeding with DWP and corn-based diets, so that birds with higher performance had different microbiota composition than those with lower yields. It was possible to identify that *Bifidobacterium* spp., *Olsenella* spp., and *Lactobacillus crispatus* were more abundant in the ceca of hens with higher egg production (Chapter 1 and 2), while *Bifidobacterium* spp. *Bacteroides fragilis*, *Escherichia coli/Shigella flexneri*, *Megamonas funiformis*, and *Lactobacillus salivarus* were the main phylotypes harboring the ceca of broilers with improved performance (Chapter 4). Moreover, the microbial functional profile of hens with high egg production showed more unique functions and pathways involved in SCFAs production and phosphorous degradation than those with lower productivity (Chapter 2), and ceca from broilers with higher weight showed lower abundance of *Helicobacter pullorum*. On the contrary, a relation between performance and microbiota when DWP was added to wheat-barley based diets could not be clearly established because the microbial composition of broilers with poorer performance did not differ from those of broilers exhibiting better productive results (Chapter 5). In fact, the cecal microbiota of both group of birds showed higher abundance of *Lactobacillus gallinarum* and *Bacteroides vulgatus*, and lower of *Escherichia coli/Shigella flexneri* and *Bacteroides fragilis*. It is worth remarking that microbial results of the current Thesis were obtained using 16S amplicon sequencing and an adapted pipeline for bioinformatics analysis, so other bacterial groups could possibly have been identified if different procedures had been applied. The inconsistent association between microbiota and performance between trials agrees with the findings of Geier et al. (2009) and Mountzouris et al (2010), who reported that GIT microbial composition is not always linked to changes in poultry performance. This might be partly due to differences at the strain level, and hence, to the functional level of bacteria (Torok et al., 2013). However, in some cases additional factors would have been crucial for animal performance, such as animal genetics, environmental conditions, flock sanitary status, or intrinsic diet features (Stanley et al., 2013a). In the specific case of

this dissertation, the lack of association between performance and microbiota composition evidenced in the Chapter 5 could be due to the fact that the physicochemical properties of the digesta had a larger effect on the productive results than the cecal microbial communities.

The results of the present thesis also revealed differences in the cecal microbial composition between hens (Chapter 2) and broilers (Chapter 4) fed with corn-based diets supplemented with DWP, indicating that the genetic line the of animals and their productive stage largely determine this composition. *Elusimicrobium* spp., and *Parabacteroides* spp., genera were particularly identified in hens, while *Oscillibacter* spp., *Pseudoflavonifractor* spp., *Megamonas* spp., and *Barnesiella* spp. were specially detected in broilers. These results were already expected, because animals that have had a longer production period, such as the hens used for the trial of Chapter 1, have a mature and well stablished microbiota community (Videnska et al., 2014). In contrast, broilers of 42 days of age, such as those from Chapter 4, do not have an stable microbial community yet (Donaldson et al., 2017). In any case, microbial results from hens and broilers would hardly be similar even if both lines of animals were at the same stage of the productive life, due to the intrinsic variation in intestinal development rates, feed intake, and SCFAs production between them (Walugembe et al., 2015).

2. The effect of DWP supplemented simultaneously with other feed additives

In an attempt to enhance the DWP efficacy as prebiotic, its simultaneous supplementation with probiotics and organic acids to corn-soybean based diets were evaluated. The use of WPC as source of lactose and whey proteins was also tested as a manner to potentiate the lactose-performance effects by providing a high nutritional value protein in diet.

Performance effects produced by the simultaneous addition of DWP with other dietary components to corn-based diets showed inconsistencies by comparing broilers and laying hens results. The results achieved indicated that there was a synergic effect between DWP and CaB in broilers (Chapter 3), and that WPC could be successfully added to the diet (Chapter 4) because of the improvement on BW, ADG, and FI. In contrast, the joint supplementation of DWP and PA as synbiotic to laying hens did not improve egg production (Chapter 1). Conversely, the addition of PA suppressed the

observed enhancer effect of DWP, indicating that the synergism between both additives did not occur. In broilers, the favorable performance results could be related to the improvement on duodenal histomorphometry and the increase on AID of DM, CP, Ca, and P (Chapter 3 and 4). Moreover, a concomitant modulation on the cecal microbial composition were caused by WPC supplementation, which promoted major abundance of *Bacteroides fragilis*, *Escherichia coli/Shigella flexneri*, *Megamonas funiformis*, and *Subdoligranulum variabile* (Chapter 4). In opposite, laying hens with lower performance fed with DWP-PA did not present changes in their cecal microbial composition with respect to the more productive hens fed with DWP, although a reduction on the total number and type of cecal microbial functions were observed (Chapter 2). In this sense, reductions on microbial genes coding SCFAs production and phosphorous metabolism, which are essential for egg production, were observed, which could explain the productive differences. However, it would have also been appropriate to measure the digestibility of nutrients and mineral retention in order to stablish whether factors other than the microbial functionality profile affected hens' production. Anyway, based on the existing results, it could be inferred that the type of probiotic evaluated in the present thesis, at the tested doses, was not the proper one to be mixed with DWP. Therefore, different doses of PA or other probiotics strains should be evaluated in a synbiotic mixture containing DWP as prebiotic in laying hens feeding. Furthermore, it should be advisable to test this type of DWP-containing synbiotics on broilers feeding in order to expand its feasibility to poultry meat lines.

Potential synergies between DWP and other additives in broilers were also explored using wheat and barley as main cereals (Chapter 5). Contrary to the positive effects observed when DWP was used simultaneously with other compounds using corn-based diets (Chapter 3 and 4), unsatisfactory performance results were evidenced when DWP was mixed to CHIT using diets rich in NSP (Chapter 5). Moreover, these poor results were accompanied by the reduction on microbial community diversity and by the increase of *Klebsiella pneumoniae* (OTU 66) and *Streptococcus gallolyticus* (OTU 39). These disagreeing findings between studies are mainly attributed to differences in the base cereal used as stated earlier in the current general discussion. Moreover, the use of DWP-CHIT mixture on wheat and barley diets promoted the presence of potentially pathogenic bacteria, which could also negatively influence broilers performance results.

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CONCLUSIONS

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First: The inclusion of DWP to corn-based diets for broilers and laying hens improved their productive performance, whereas adding DWP into wheat-barley based diets for broilers resulted in poor performance results.

Second: A clear association between performance and cecal microbial composition was evidenced in laying hens and broilers fed with DWP added to corn-based diets, since higher abundance of *Bifidobacterium* spp., *Olsenella* spp. and *Lactobacillus crisptaus* were observed in hens with better egg production, and more *Bifidobacterium* spp., *Bacteroides fragilis, Megamonas funiformis,* and *Lactobacillus salivarus* were detected in broilers with improved performance.

Third: The inclusion of DWP to corn-based diets reduced the cecal presence of potentially pathogenic bacteria, lowering *Clostridium perfringens* colony counts in more productive hens and decreasing *Helicobacter pullorum* abundance in more productive broilers.

Fourth: Supplementing DWP to corn-based diets promoted higher cecal abundance of *Faecalibacterium* spp. *Alistipes* spp., *Barnesiella* spp., *Clostridium IV*, and *Subdogranulum* spp. genera in both laying hens and broilers, while its supplementation to wheat-barley diets increased the abundance of *Lactobacillus* spp., *Bacteroides* spp., and *Oscillibacter* spp.

Fifth: The inclusion of DWP to corn-based diets for laying hens resulted in an increase in the total cecal microbial encoded functions, with the community showing greater number of encoded functions and more metabolic pathways related to the metabolism of SCFA, fructose, mannose, and inositol phosphate.

Sixth: A linkage between the performance and the microbiota composition when DWP was added to wheat-barley based diets was not evidenced, given that the cecal microbial composition of broilers with lower productive results was not different from those achieving higher yields.

Seventh: The supplementation of WPC to corn-based diets for broilers improved their performance, the mineral AID, and concomitantly modulated the cecal microbial composition, increasing the cecal abundance of *Bacteroides fragilis*, *Megamonas funiformis*, and *Subdoligranulum variabile*.

Eighth: Supplementing DWP simultaneously with CaB in corn-based diets for broilers improved bird weight, duodenal development, and AID of nutrients, whereas its joint supplementation with PA as synbiotic to laying hens neither result in better performance results nor cecal microbial composition or functionality changes.

Nineth: The simultaneous supplementation of DWP and CHIT to wheat-barley based diets reduced broilers' performance concomitantly with a reduction on the microbial community diversity, and with the increment on undesirable, potentially pathogenic *Klebsiella pneumoniae* and *Streptococcus gallolyticus* bacteria.

CONCLUSIONES

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Primera: La inclusión de LP en dietas para pollos de engorde y gallinas ponedoras con base cereal de maíz mejoró el rendimiento productivo, mientras que su inclusión en dietas con base cereal de trigo y cebada para pollos de engorde redujo el rendimiento productivo animal.

Segunda: Se observó una asociación entre el rendimiento productivo y la composición de la microbiota cecal en gallinas ponedoras y pollos de engorde alimentados con LP y dietas con base cereal de maíz, pues hubo una mayor abundancia de *Bifidobacterium* spp., *Olsenella* spp. y *Lactobacillus crisptaus* en gallinas con más producción de huevos, mientras que *Bifidobacterium* spp., *Bacteroides fragilis*, *Megamonas funiformis*, y *Lactobacillus salivarus* se detectaron en mayor abundancia en pollos con mejor rendimiento productivo.

Tercera: La inclusión de LP en dietas con base cereal de maíz redujo la presencia cecal de bacterias potencialmente patógneas, disminuyendo los recuentos de *Clostridium perfringens* en gallinas más productivas, así como la abundancia de *Helicobacter pullorum* en pollos con mejor rendimiento.

Cuarta: La suplementación de LP en dietas con base cereal de maíz incrementó la abundancia cecal de *Faecalibacterium* spp. *Alistipes* spp., *Barnesiella* spp., *Clostridium IV*, and *Subdogranulum* spp. en gallinas y pollos, mientras su suplementación en dietas con base cereal de trigo y cebada incrementó la abundancia de *Lactobacillus* spp., *Bacteroides* spp., y *Oscillibacter* spp.

Quinta: La inclusión de LP en dietas con base cereal de maíz aumentó la funcionalidad de la microbiota cecal, mostrando un número mayor de funciones relacionadas con el metabolismo de la fructosa, manosa, del inositol fosfato y de los AGV.

Sexta: No se evidenció una relación entre el rendimiento productivo y la microbiota cecal al suplementar LP en dietas con base cereal de trigo y cebada, pues la

composición microbiana de los pollos menos productivos no fue diferente de aquellos con mejor rendimiento.

Séptima: La suplementación de CPL en dietas para pollos de engorde con base cereal de maíz mejoró el rendimiento productivo, la digestibilidad ileal de los minerales, y moduló la composición de la microbiota cecal, incrementando la abundancia de *Bacteroides fragilis*, *Megamonas funiformis*, y *Subdoligranulum variabile*.

Octava: La suplementación conjunta de LP y BCa in dietas con base cereal de maíz para pollos de engorde mejoró el peso de los animales, el desarrollo duodenal y la digestibilidad ileal de los nutrientes, mientras que la suplementación de LP junto con PA como simbiótico en gallinas ponedoras no resultó en un mejor rendimiento productivo ni en cambios de la composición y funcionalidad de la microbiota cecal.

Novena: La suplementación conjunta de LP y QUIT en dietas con base cereal de trigo y cebada redujo el rendimiento productivo de los pollos de engorde de manera conjunta con una reducción en la divesidad de las comunidades microbianas cecales, y con el incremento en *Klebsiella pneumoniae* and *Streptococcus gallolyticus*, consideradas bacterias potencialmente patógenas.