UNIVERSIDAD POLITÉCNICA DE MADRID
ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA,
ALIMENTARIA Y DE BIOSISTEMAS

USE OF OLIVE POMACE EXTRACTS
IN BROILER CHICKEN DIETS:
EFFECTS ON GROWTH PERFORMANCE
AND GUT HEALTH

PhD THESIS

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DEPARTAMENTO DE PRODUCCIÓN AGRARIA
ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA,
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LOS VOCALES

EL SECRETARIO

*A mi **familia***; Rosa Ana, Carlos José y Ana

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DISCLAIMER

Some results of this PhD. Thesis are already published:

- I. Experiment 1: Herrero-Encinas, J., M. Blanch, J. J. Pastor, A. Mereu, I. R. Ipharraguerre, and D. Menoyo. 2020. Effects of a bioactive olive pomace extract from *Olea europaea* on growth performance, gut function, and intestinal microbiota in broiler chickens. *Poultry Science*, 99: 2-10.
<https://doi.org/10.3382/ps/pez467>
- II. Experiment 2: Herrero-Encinas, J., M. Blanch, J. J. Pastor, and D. Menoyo. 2020. Diet supplementation with a bioactive pomace extract from *Olea europaea* partially mitigates negative effects on gut health arising from a short-term fasting period in broiler chickens. *Animals*, 10, 349.
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The third experiment will be submitted for publication to:

- III. Experiment 3: Herrero-Encinas, et al. Response of broiler chickens fed diets supplemented with a bioactive olive pomace extract from *Olea europaea* to an experimental coccidial vaccine challenge. *Poultry Science*

ABBREVIATIONS

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ADFI	Average daily feed intake
ADG	Average daily gain
AGPs	Antibiotic growth promoters
AGP	Alpha-1-acid glycoprotein
AID	Apparent ileal nutrient digestibility
AJC	Apical junctional complex
AMP	Antimicrobial peptides
ASBT	Apical sodium dependent bile acid transporter
AVCA	Avicholic acid
BA	Bile acid
Bu-1	Chicken B-cell marker chb6
BW	Body weight
BWG	Body weight gain
CA	Cholic acid
CAR	Carotenoid
CD	Crypt depth
CD3 $\gamma\delta$	Marker of active avian T lymphocytes
CD4+	Helper lymphocytes (Cluster differentiation 4)
CD8+	Cytotoxic lymphocytes (Cluster differentiation 8)
CDCA	Chenodeoxycholic acid
ChREBP	Carbohydrate responsive element-binding protein
CP	Crude protein
CVAR	Coxsackie virus and adenovirus receptor
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DM	Dry matter
EBI	European broiler index
EE	Ether extract
EMA	European medicines agency
EO	Essential oil
FABP2	Fatty acid binding protein 2
FABP6	Fatty acid binding protein 6
FCR	Feed conversion ratio
FI	Feed intake
FITC-d	Fluorescein isothiocyanate dextran

G:F	Gain/feed ratio
GALT	Gut-associated lymphoid tissue
GE	Gross energy
H/L	Heterophil/lymphocyte ratio
HSP-70	Heat shock protein 70
IELs	Intraepithelial lymphocytes
IFN γ	Interferon-gamma
IgA	Immunoglobulin A
IGF1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
IL	Interleukin
IL-1 β	Interleukin 1 beta
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8 (CXCLi2)	Interleukin 8 (cxcli2 former)
iNOs	Nitric oxide synthase
IP	Intestinal permeability
JAM	Junctional adhesion molecule
L	Lactulose sugar
L/M	Lactulose to mannitol ratio
LITAF	Lipopolysaccharide induces TNF factor
LP	Lamina propria
LS	Lesion score
LXR	Liver X receptor
M	Mannitol sugar
NDV	Newcastle disease virus
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OC	Oocyst count
OE	Olive pomace extract
OM	Organic matter
OPG	Oocyst per gram of excreta
OTU	Operational taxonomic unit
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PCU	Population correction unit
PFA	Phytogenic feed additives

PRRs	Pathogen recognition receptors
R	Rhamnose sugar
SCFAs	Short-chain fatty acids
SREBP1	Sterol regulatory element-binding protein 1
TCA	Taurocholic acid
TCDCa	Tauroursodeoxycholic acid
TGF- β	Transforming growth factors
TGF- β 4	Transforming growth factor beta 4
TLCA	Taurolithocholic acid
TLR	Toll like receptor
TLR-2 β	Toll like receptor 2 β
TLR4	Toll like receptor 4
TNFSF15	Tumor necrosis factor superfamily member 15
TNF- α	Tumor necrosis factor alpha
UB	Ubiquitin
VH	Villus height
VH/CD	Villus height to Crypt depth ratio
ZO	Zonula occludens

SUMMARY

SUMMARY

The need to find alternatives to the use of antibiotic growth promoters in poultry has led to an active research during the last years with the aim to develop different management and nutritional strategies to improve animal performance. In this context, feed additives such as phytochemicals are gaining interest as they promote growth and health because of their antioxidant, antimicrobial and anti-inflammatory effects. The positive properties of phytochemicals are related to certain components present in plants such as phenolic compounds, flavonoids or terpenoids. Olive oil from *Olea europaea* is an integral ingredient of the Mediterranean area with known health promoting effects. Bioactive compounds of olive fruit and olive oil include polyphenols (oleuropein and hydroxytyrosol) and triterpenes (oleanolic and maslinic acids) among others. Different extracts derived from olive mill wastes have shown positive effects on growth performance and intestinal health in food-producing animal species. However, the potential positive effects of bioactive extracts derived from olive oil industry in poultry are scarcely identified and remain to be investigated.

The main objective of this Doctoral Thesis is to evaluate the effects of supplementing broiler chicken diets with a bioactive olive pomace extract from *Olea europaea* (OE) rich in polyphenols and triterpenes on animal growth performance and gut health. Three experiments were conducted with the following objectives: 1) study the effects of supplementing grower diets with OE on broiler performance and gut health; 2) evaluate the effects of supplementing starter and grower diets with the OE on performance and gut health after a challenge of intestinal permeability induced by a short-term fasting period; 3) investigate an experimental procedure of coccidial vaccine challenge in battery cages and the anticoccidial effect of the OE in broiler chickens.

In trial 1 animals were fed with a standard non-medicated starter diet for 21 d, and from 22 to 42 d of age with their respective experimental diet: a negative control with no additives (Control), a positive control with 100 ppm of Monensin and the basal diet supplemented with 750 ppm of an OE (Lucta S.A., Spain) containing 2% of polyphenols and 10% of triterpenes. From 21 to 42 d of age, no significant differences in average daily feed intake (ADFI) were observed among dietary treatments; however, lower average daily gain (ADG) and higher feed conversion ratio (FCR) ($P < 0.05$) was observed in birds fed the Control compared to Monensin and OE groups. No significant changes on bacterial composition and diversity at a family level were observed in the caeca of birds

fed the experimental diets. Moreover, no significant differences on plasma and intestinal bile acid composition were observed among treatments. Birds fed the OE showed a significant decrease of interleukin 8 (IL-8) expression in the ileum ($P < 0.05$). Additionally, the expression of transforming growth factor beta 4 (TGF- β 4), and chicken B-cell marker (Bu-1) was significantly upregulated ($P < 0.01$) in broilers fed the OE and Monensin diets compared to those fed the Control. In conclusion, the inclusion of 750 ppm of the OE improved animal growth likely as result of its anti-inflammatory properties.

In trial 2, treatments included a control diet with no additives (CF), and diets supplemented with 100 ppm of monensin (MF) or with 500 (OE500F) or 1500 ppm (OE1500F) of an OE (Lucta S.A., Spain) containing 1% of polyphenols and 6% of triterpenes. At 14 d, all birds were submitted to a 15.5 h short-term fasting period to induce intestinal permeability (IP) increase. In addition, a control group was included, fed the control diet and no challenged with the fasting period (CNF). No significant differences were observed in ADG, ADFI and FCR among treatments before fasting period (from 1 to 14 d of age). Fasting increased ($P < 0.05$) lactulose/mannitol ratio and Alpha 1 Acid Glycoprotein concentration in plasma and reduced ($P < 0.001$) duodenum villus/crypt ratio (VH/CD). Moreover, a down-regulation of Claudin-1 ($P < 0.05$), an up-regulation of toll-like receptor 4 (TLR4) and IL-8 ($P < 0.05$) ileal gene expression was observed in CF birds compared to CNF. The OE500F treatment reduced duodenal crypt depth compared to CF ($P < 0.05$; OE linear effect). Mannitol concentration and ileal IL-8 expression were reduced in OE500F compared to CF and OE1500F ($P = 0.05$). Fasting challenge induced an increase in IP triggering an inflammatory response. Supplementation of OE up to 1500 ppm did not affect growth performance in birds fed the extract up to 32 days of age but alleviated some of the negative effects of the fasting challenge.

In trial 3, broiler chickens were randomly assigned to 5 experimental treatments. One group was fed the control diet without any additives and no challenged (NCU). The other 4 groups were challenged and fed the control diet with no additives (NCC), or supplemented with 500 ppm of coccidiostat (PCC) or with 500 (OE500C) and 1500 ppm (OE1500C) of OE (Lucta S.A., Spain) containing 1% of polyphenols and 6% of triterpenes. At 0, 7 and 14 d, all birds, except NCU group, were challenged with an oral gavage of a live *Eimeria* spp oocyst vaccine at 1x, 4x, and 16x of the manufacturer's recommended dose, respectively. Coccidial vaccine challenge depressed body weight (P

< 0.05) throughout the trial, as well as a reduction ($P < 0.05$) of FI and BWG except from 20 to 28 d, and FCR from 0 to 7, 0 to 14 and 0 to 20 d. Birds in the NCC group had higher ($P < 0.05$) oocyst counts and lower ($P < 0.05$) carotenoid plasma concentration and VH/CD ratio in duodenum and jejunum compared with NCU birds. Overall, coccidia challenge caused the expected reductions in growth performance and gut integrity. While the coccidiostat reduced oocysts excretion, dietary OE or coccidiostat had no effects on performance or gut integrity. The attenuated inflammatory response observed for all the treatments following the third infection can be attributed to the adaptation or immunization to the repetitive exposure to *Eimeria* spp.

In summary: a) Feeding broiler diets supplemented with an olive pomace extract under standard conditions did not affect performance up to 32 days of age. However, in the grower-finisher phase (from 35 to 42 days of age) the addition of 750 ppm of the extract providing 15 ppm polyphenols and 75 ppm triterpenes improved ADG and FCR.; b) The positive effects of the olive pomace extract on animal growth might be related to the anti-inflammatory properties of the olive triterpenoids and polyphenols reflected by the immunosuppressant effect on IL-8 pro-inflammatory interleukin and the increase of anti-inflammatory expression of TGF- β 4; c) A single short-term fasting period of 15.5 h in birds housed in floor pens induced an increase in intestinal permeability triggering an inflammatory response; d) The addition of 500 ppm of the olive pomace extract providing 5 ppm polyphenols and 30 ppm triterpenes alleviated some of the negative effects of increased permeability associated with the short-term fasting challenge.; e) The experimental model of coccidial vaccine challenge used induced growth depression and gut integrity reduction as indicated by increased oocyst excretion, reduced plasma carotenoid concentration, and intestinal morphology alteration.; f) Under the experimental coccidiosis challenge, the inclusion of 500 ppm or 1500 ppm of the olive pomace extract providing up to 15 ppm of polyphenols and 90 ppm of triterpenes showed no effects on performance or gut integrity. This suggests that the potential anticoccidial effects of olive pomace extracts depend on the concentration and type of the bioactive compounds and/or the *Eimeria* strain.

RESUMEN

RESUMEN

La necesidad de encontrar alternativas al uso de antibióticos como promotores de crecimiento en avicultura ha llevado, durante los últimos años, a una investigación activa con el objetivo de desarrollar diferentes estrategias de manejo y nutricionales para mejorar el rendimiento productivo. En este contexto, aditivos de piensos como los fitogénicos están ganando interés, ya que promueven el crecimiento y la salud debido a sus efectos antioxidantes, antimicrobianos y antiinflamatorios. Estas propiedades están relacionadas con ciertos componentes activos presentes en las plantas como los compuestos fenólicos, flavonoides o triterpenos. El aceite de oliva (*Olea europaea* L.) es un ingrediente básico en los países del Mediterráneo y es conocido por sus propiedades funcionales con efectos favorables sobre la salud. Los compuestos bioactivos, tanto de la oliva y como su aceite, incluyen polifenoles (oleuropeína e hidroxitirosol) y triterpenos (ácido oleanólico y máslinico), entre otros. Diferentes extractos derivados de los subproductos de la industria oleícola han mostrado efectos positivos sobre el crecimiento y salud intestinal en animales de producción. Sin embargo, los posibles efectos positivos de estos extractos bioactivos en avicultura son escasamente conocidos y faltan por ser investigados.

El objetivo principal de esta Tesis Doctoral es evaluar los efectos de la suplementación de dietas de pollos de engorde con un extracto bioactivo de pulpa de oliva (*Olea europaea*) (OE) rico en polifenoles y triterpenos sobre el crecimiento animal y la salud intestinal. Se realizaron tres experimentos con los siguientes objetivos: 1) estudiar los efectos de la suplementación con OE en dietas de crecimiento sobre los parámetros productivos y salud intestinal de pollos de engorde; 2) evaluar los efectos de la suplementación con OE en dietas de iniciación y crecimiento sobre la salud intestinal y crecimiento de pollos broiler después de un desafío de permeabilidad intestinal inducido por un ayuno de corta duración; 3) investigar un desafío experimental de coccidiosis y el efecto anticoccidial del OE en pollos broiler alojados en jaulas en batería.

En el experimento 1, los animales fueron alimentados con una dieta de iniciación estándar no medicada hasta el día 21, y del día 22 al 42 de edad con sus respectivas dietas experimentales: un control negativo sin aditivos (Control), un control positivo con 100 ppm de Monensina y una dieta basal suplementada con 750 ppm de OE (Lucta S.A., España) con 2% de polifenoles y 10% de triterpenos. No se observaron diferencias significativas en el consumo medio diario de pienso (CMD) del día 21 al 42; sin embargo, se observó una menor ganancia media diaria (GMD) y un peor índice de conversión (IC)

($P < 0,05$) en los animales alimentados con la dieta Control comparado con los grupos OE y Monensina. No se observaron cambios significativos en la composición y diversidad bacteriana a nivel familia en el contenido cecal de los pollos alimentados con las dietas experimentales. Además, la composición del plasma y de los ácidos biliares intestinales no cambió significativamente entre tratamientos. Los animales alimentados con OE redujeron significativamente la expresión de la interleucina IL-8 en el íleon ($P < 0,05$). Así mismo, la expresión del factor de crecimiento transformante $\beta 4$ (TGF- $\beta 4$) y el marcador de células B (Bu-1) fueron significativamente mayores en los pollos alimentados con las dietas que contenían OE y Monensina comparado con aquellos alimentados con la dieta Control. En conclusión, la inclusión de 750 ppm de OE mejoró el crecimiento de pollos de engorde como resultado de sus propiedades anti-inflamatorias.

En el experimento 2, los tratamientos experimentales incluyeron una dieta control sin aditivos (CF) y dietas suplementadas con: 100 ppm de monensina (MF) o con 500 (OE500F) o 1500 (OE1500F) de un OE (Lucta S.A., Spain) que contenía 1% de polifenoles y 6% de triterpenos. A los 14 días, los pollos fueron sometidos a un ayuno de corta duración de 15,5 h para inducir un aumento de la permeabilidad intestinal (PI). Además, se incluyó un grupo control no ayunado y alimentado con la dieta control (CNF). No hubo diferencias significativas en GMD, CMD e IC entre los tratamientos antes del ayuno (de 1 a 14 días de edad). El ayuno aumentó ($P < 0,05$) la relación lactulosa/manitol y la concentración de la Alfa 1 Glicoproteína Ácida en plasma y redujo ($P < 0,001$) la relación longitud vellosidad y profundidad de cripta (LV/PC) del duodeno. Además, se observó una reducción ($P < 0,05$) de la expresión génica en íleon de Claudin-1 y un aumento de receptor de tipo toll 4 (TLR4) e IL-8 ($P < 0,05$) en los animales del grupo CF respecto a CNF. En el tratamiento OE500F se observó una reducción de la profundidad de las criptas comparado con CF ($P < 0,05$; efecto lineal de OE). La concentración de manitol en sangre y la expresión de IL-8 se redujo en el tratamiento OE500F comparado con CF y OE1500F ($P = 0,05$). Se puede concluir que el ayuno indujo un aumento en la PI desencadenando una respuesta inflamatoria. Además, la suplementación de OE hasta 1500 ppm no afectó al crecimiento de los animales hasta los 32 días de edad y alivió alguno de los efectos negativos del ayuno.

En el experimento 3, los pollos de engorde fueron aleatoriamente asignados a 5 tratamientos experimentales. Un grupo fue alimentado con la dieta control sin aditivos y no se le sometió al desafío (NCU). Los otros 4 grupos fueron desafiados y alimentados con una dieta control sin aditivos (NCC), o suplementados con 500 ppm de un coccidiostato (PCC) o con 500 (OE500C) o 1500 ppm (OE1500) de un OE (Lucta S.A.,

España) que contenía un 1% de polifenoles y 6 % de triterpenos. A los días 0, 7, y 14, todos los animales, excepto los del grupo NCU, fueron desafiados respectivamente con una dosis oral de vacuna que contenía ooquistes vivos de *Eimeria* spp a 1x, 4x y 16x veces la dosis recomendada por el fabricante. El desafío produjo una reducción significativa ($P < 0,05$) del peso corporal a lo largo de todo el ensayo, al igual que una reducción ($P < 0,05$) del consumo de pienso y de la ganancia de peso corporal, de los 20 a los 28 días, y del IC de 0 a 7, 0 a 14 y 0 a 20 días. Comparados con el tratamiento NCU, los pollos del tratamiento NCC mostraron mayor ($P < 0,05$) recuento de ooquistes y menor ($P < 0,05$) concentración de carotenoides en plasma y LV/PC en duodeno y yeyuno. El desafío de coccidiosis produjo la esperada reducción de los parámetros productivos e integridad intestinal. Mientras que el coccidiostato redujo la producción de ooquistes, la inclusión de OE o del coccidiostato no produjo efectos en el crecimiento y la integridad intestinal. La respuesta inflamatoria atenuada observada en todos los tratamientos después de la tercera infección puede ser indicativo de adaptación o desarrollo de inmunidad a la repetida exposición a *Eimeria* spp.

En resumen: a) Alimentar a pollos de engorde utilizando dietas suplementadas con un extracto de pulpa de oliva bajo condiciones de producción estándar no tuvo efectos sobre el crecimiento desde el día 1 hasta los 32 días de edad. Sin embargo, en la fase de crecimiento-acabado (de 35 a 42 días de edad) la inclusión de 750 ppm del extracto aportando 15 ppm de polifenoles y 75 ppm de triterpenos mejoró la GMD y el IC.; b) Los efectos positivos del extracto de pulpa de oliva sobre el crecimiento animal podrían estar relacionados con las propiedades anti-inflamatorias de los triterpenos y polifenoles reflejadas en una inmunosupresión de la IL-8 y el incremento del marcador anti-inflamatorio TGF- β 4.; c) Un desafío de ayuno de 15,5 h horas en pollos alojados en suelo indujo un aumento de la PI desencadenando una respuesta inflamatoria.; d) La inclusión de 500 ppm del extracto de pulpa de oliva aportando 5 ppm de polifenoles y 30 ppm de triterpenos alivió alguno de los efectos negativos del desafío de ayuno.; e) El modelo experimental de desafío de coccidiosis indujo una reducción del crecimiento e integridad intestinal en pollos de engorde reflejado en una mayor excreción de ooquistes, reducción de la concentración de carotenoides en plasma y alteraciones en la morfología intestinal.; f) Bajo el desafío experimental de coccidiosis, la inclusión de 500 o 1500 ppm del extracto de pulpa de oliva aportando hasta 15 ppm de polifenoles y 90 ppm de triterpenos no produjo efectos en el crecimiento y la integridad intestinal, indicando que el potencial efecto anticoccidial de los extractos puede depender de la concentración, el tipo de compuestos bioactivos y/o la cepa de *Eimeria*.

INTRODUCTION AND OBJECTIVES

1. INTRODUCTION AND OBJECTIVES

The ban of antibiotics as growth promoters (AGPs) in the EU-25 in 2006 has led to the need to find viable alternatives to improve animal performance (Diaz-Sanchez et al., 2015). Active research on this issue has been conducted for all the livestock species during the past years with management and nutritional strategies targeting the intestinal environment and health (De Blas et al., 2012; Cheng et al., 2014). The alternatives to AGPs might be primarily based on an efficient management and optimal hygienic conditions to reduce the stress and pathogenic exposure of animals which boost the intestinal health (Oviedo-Rondon, 2019; Mateos et al., 2002). Moreover, intestinal health can be enhanced by nutritional strategies including the combination of diverse natural feed additives but their mechanism of action is not completely understood (Lillehoj et al., 2018). Alternatives to AGPs should be developed on the mechanism of action of antibiotics used at sub-therapeutic doses to achieve non-antibiotic surrogates (Gadde et al., 2017). It has been shown that AGPs might exert their function through the reduction of gut microbiota and therefore increasing animal nutrient utilization. With a lower concentration of pathogenic microbiota there is a reduction of subclinical infections and the subsequent sparing of energy cost of the immune system. Also, there is a reduction of metabolites produced by pathogenic microbiota that reduce the animal growth (Dibner and Richards, 2005; Page, 2006). Furthermore, Niewold (2007) suggested that antibiotic substances fed at a sub-therapeutic dosage reduce the host inflammatory response by decreasing pro-inflammatory processes and consequently, saving energy which promotes animal growth promotion (Niewold 2007, 2014).

Widely researched alternatives to AGPs with potential to influence the intestinal health and function and improve performance include probiotics, prebiotics, exogenous enzymes and phytogenic based feed additives (Settle et al., 2014). Phytochemicals comprise a group of substances obtained from aromatic plants, volatile compounds (essential oils) and plant extracts. The positive properties of phytochemicals are related to certain components present in plants such as phenolic compounds, flavonoids or terpenoids. According to Windisch et al. (2008), phytochemicals have shown different beneficial properties in animals such as antimicrobial effects, gut immunoregulation, antioxidant activity or better intestinal functionality.

Nowadays, the concerning of climate change is creating new concepts in the agro-industry like the agricultural eco-innovation. This concept claims to base the economy on the “zero waste” production, opening new opportunities and markets to by-products obtained from the different agro-industries (Mirabella et al., 2014). In this regard, olive industry is one of the main agricultural sectors in Spain reaching 1.3 million tonnes of olive oil which makes Spain the largest olive oil producer worldwide (International Olive Council, 2017; Romero et al., 2017). This industry generates annually million tonnes of wastes such as olive pomace and leaves which can be harnessed for animal feeding (Berbel and Posadillo, 2018). Olive oil from *Olea europaea* is an integral ingredient of the Mediterranean diet with known health promoting effects. Bioactive compounds of olive fruit and olive oil include polyphenols (oleuropein and hydroxytyrosol) and triterpenes (oleanolic and maslinic acids) among others. In this regard, it is being generating an interest in the production of extracts derived from olive by-products which contain bioactive compounds to improve performance and health in food-producing animal species (King et al., 2014; Liehr et al., 2017; Leskovec et al., 2018). Therefore, purified olive extracts with a high content of polyphenols, triterpenic acids, long-chain fatty alcohols, unsaturated hydrocarbons, tocopherols and sterols with anti-oxidative and anti-inflammatory properties might be of interest to enhance performance, and gut health in poultry (King et al., 2014; Gerasopoulos et al., 2015). Previous studies conducted with extracts of similar composition to the one used in this study showed better animal performance related to its anti-inflammatory function rather than the antioxidant one (Gisbert et al., 2017; Liehr et al., 2017; Tedó et al., 2018). This made us explore in the present Thesis only the potential reduction of the inflammatory status of the animals. Besides these beneficial potential positive effects, bioactive compounds of *Olea europaea* including maslinic acid, quercetin and oleuropein have shown promising results as natural substitutes to coccidiostats in broiler chickens (de Pablos et al., 2010; Debbou-Iouknane et al., 2019).

Antibiotic growth promoters and their alternatives have shown different effects depending on the hygienic and environmental conditions (Ferket, 2003; Lee et al., 2003; Gunal et al., 2006) optimal stocking density (Sarica et al., 2005) or without any stressor (Alali and Rieke, 2012). Thus, alternatives to AGPs should be tested in poultry under different experimental conditions, including standard, under stress or a challenge that compromises gut health. Recent studies have shown that fasting broilers up to 24 h

triggers intestinal permeability increase and might be used as an experimental model to challenge gut health (Gilani et al., 2017, 2018a). Coccidiosis causes large economic losses in the poultry industry by reducing growth performance, increasing mortality, and driving up production costs associated with the use of antibiotics and coccidiostats (Peek and Landman, 2011). Previous studies have used coccidiosis models based on single, large doses of *Eimeria* spp. vaccines to induce subclinical coccidiosis and elicit growth depression and immune responses in broiler chickens (Adedokun and Adeola, 2017; Osho et al., 2019). However, such experimental models, especially when conducted in battery cages that reduce exposure to excreted and sporulated oocysts, do not reflect the multiple cycles of *Eimeria* infection and reinfection that occur under field conditions. Therefore, it may be advantageous to utilize models that better mimic the natural cycling of *Eimeria* by inoculating the birds several times with increasing doses of vaccine (Williams, 2002).

The general objective of this Doctoral Thesis is to investigate the use of an olive pomace extract (OE) from *Olea europaea* rich in polyphenols and triterpenes as feed additive in broiler chickens. The main hypothesis is that feeding broilers with the OE might improve animal performance because of the potential antimicrobial, anti-inflammatory and positive gut function effects of olive polyphenols and triterpenes.

The first objective was to study the effects of supplementing grower diets with an olive pomace extract on broiler performance and gut health under standard conditions.

The second objective was to evaluate the effects of supplementing starter and grower diets with an olive pomace extract on broiler performance and gut health after a challenge of intestinal permeability increase induced by a short term fasting period.

The third objective was to investigate an experimental procedure of coccidial vaccine challenge in battery cages and the anticoccidial effect of an olive pomace extract in broiler chickens.

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. EUROPEAN POULTRY INDUSTRY: AN OVERVIEW

2.1.1. EUROPEAN MEAT PRODUCTION AND FUTURE

In 2018, the global poultry meat production was 95.5 million tonnes (FAO, 2019). The largest poultry meat producer is United States of America followed by China, the European Union and Brazil with 21.9, 18.2, 14.6, 14.2 million tonnes, respectively (Figure 1).

Figure 1. Poultry meat production of main regions in 2017 (adapted from FAO, 2019).

Within the EU-28, Poland (16.8%), the United Kingdom (12.9%), France (11.4%), Spain (10.7%), Germany (10.4%), and Italy (8.5%) are the largest producers (Figure 2). These six countries together accounted for more than 70% of the EU production in 2018. The position of this sector and the relevance in the worldwide economy is due to the increment of productivity in most of the European countries, being the best examples Poland and UK which have increased 41.1% and 19.2% respectively the poultry meat production from 2014 to 2018 (Figure 2). In addition, the global European production of poultry increased 3.2 million tonnes since 2010 (Eurostat, 2019). Chicken represents 83% of the poultry meat market followed by 13% of turkey, 3% of duck and 1% of others (i.e., quail, goose) (EC-CIRCAB, 2019).

Figure 2. Main EU poultry meat producers from 2014 to 2018 (adapted from Eurostat 2019).

European countries exported 1779 thousand tonnes mainly to Philippines (9.5%), Ghana (9.3%), Ukraine (10.0%), Hong Kong (7.2%), and South Africa (4.7%) in 2018 (EC-CIRCAB, 2019). Imports of poultry meat reached 813 thousand tonnes to the EU-28 mainly were from Thailand (38.3%), Brazil (36.7%), Ukraine (15.2%), Chile (4.4%) and China (2.4%) in 2018. Thus, the European Union is a net export region during the last recent years, and this trend is expected to increase, supplying poultry meat to other countries (EC-CIRCAB, 2019).

Spain, with 1624 thousand tonnes of poultry meat was the fourth producer country in the EU-28 in 2018 (Figure 2). In the same year, data of Spanish poultry meat external trades showed that exports increased and import decreased, returning to the positive balance observed since 2014, reaching 267 thousand tonnes of exports and 234 thousand tonnes of imports (Figure 3). The majority of imports to Spain come from European countries such as France, Germany, United Kingdom or Poland, and extra UE-28 countries such as Brazil (Figure 4). Furthermore, the main destinations of exports are to Portugal, France, Denmark and extra European countries such as Benin, Hong Kong, Guinea or South Africa.

Figure 3. Development of external trade of poultry meat in Spain from 2010 to 2018 (adapted from MAPA, 2019).

Figure 4. Main destinations of exports and import countries of poultry meat to Spain in 2018 (adapted from MAPA, 2019).

Based on the expected higher demand for protein consumption, global meat production is projected to grow around 16% over 2019 by 2025. Moreover, poultry meat is projected to be the main source of meat but will grow at lower rate compared to the previous years (OECD-FAO, 2018). Thus, the importance of the poultry industry is key to meet the future protein requirements for human nutrition.

2.1.2. ANTIBIOTICS IN POULTRY PRODUCTION

Antibiotics have been used widely in poultry production to improve growth performance, enhance feed efficiency and reduce mortality during the last half-century (Castanon, 2007). According to Briz (2006), antibiotics utilization improves by 5-10% growth and 2-3% conversion index in young birds. Antibiotics might protect health of birds against infections by modulating the immune status and controlling the microbial population in the intestinal tract (Lee et al., 2012; Torok et al., 2011). However, the overuse of antibiotics increases the number of bacteria resistant to the commonly used antibiotics in human medicine as well as the transfer of cross-resistance among microorganisms (EMEA, 1999; Landers et al., 2012). The world's concern for the development and transmission of these resistant strains has increased significantly triggering the regulation and restriction of antibiotics as growth promoters in the EU-25 (EC 2001, 2003a; Dibner and Richards, 2005).

According to the European Medicines Agency (EMA) the total of antimicrobial sales in Europe achieved 6634 tonnes in 2017. This means that sales of veterinary antibiotics fell by more than 14% compared to 2016 (EMA, 2019). Within these countries, Spain is the region that commercialized more antibiotics reaching 1769.5 tonnes for food-producing animals followed by Italy with 1,057.8, Germany with 766.6 and Poland with 749.6 tonnes (EMA, 2019). A more accurate approach to determine the use of antibiotics in food-producing animals is obtained by correcting the total volume of antibiotics sales per animal population (mg/PCU, milligrams of antibiotics per population correction unit) (EMA, 2019). In this regard, the average of the 31 countries that participate in EMA reached 107 mg/PCU in 2017, being Cyprus the country with most sales per PCU with 423.1 mg/PCU, followed by Italy, Spain, Hungary and Poland with 273.8, 230.3, 191, and 165.2 mg/PCU, respectively, in 2017 (Figure 5).

Focusing on Spanish antibiotic sales for food-producing animals, poultry is the fourth most numerous species with 820 PCU of a total of 7684 total PCU, which means that poultry industry consumed around 188 tonnes of active substance (10.7% of total sales) in 2017 (EMA, 2019). Moreover, premix (around 48% of total) and oral solution (around 47% of total) were the main form that antibiotics supplied to animals in Spain. This delivery form differed to the average of European countries with 50.7% as oral solution, 28.8% as premix, 9.9% as oral powder and 9.7% as injection (EMA, 2019).

Figure 5. Antimicrobial sales for food-producing animal species, in mg/PCU (Population Correction Unit), of the main European customer countries, in 2017 (adapted from EMA, 2019).

It is noticeable that the use of antimicrobials is decreasing in most of European countries such as Spain, Hungary, Belgium or Italy from 2014 to 2017 (Figure 5). This trend is reflected also in the total sales of antibiotics in Europe which fell by more than 32% (from 162 to 109 mg/PCU) between 2011 and 2017. Moreover, each member countries of EMA are developing strategies to further reduce the antibiotic utilization in food-producing animals to decrease the resistant bacteria emergence. However, scientists and industry have to address the necessity to increase meat production and the reduction or removal of antibiotic in poultry (Founou et al., 2016).

2.1.2.1. ESTRATEGIES TO REDUCE ANTIBIOTIC USE

The need to find alternatives to the antibiotic use in poultry has led to an active research during the past years with the aim to develop different management and nutritional strategies to improve gut health and performance (Oviedo-Rondón, 2019).

To reduce pathogen load and diminish the antimicrobials use it is essential to implement good sanitary practices such as pest control, biosecurity practices, good barn sanitation, water quality control and appropriate litter management (Ferket, 2004).

Moreover, the reduction of stressors, vaccination program implementation and improved environmental and hygienic conditions management at commercial farms should be included in the poultry industry to reduce the antibiotic use (Mateos, et al. 2002). Furthermore, different nutritional strategies to minimize the consequences of elimination of AGPs in poultry diets should be developed based on enhancement of nutrient digestibility or availability. The use of high quality raw materials, feed processing and sterilization or feed with the appropriate particle size are good approaches to maintain optimal digestion in poultry (Bedford, 2000; Amerah et al., 2007). Also, quality control of dietary fat type and the inclusion of enzymes in feed added improvements to reach good digestibility rates (Oviedo-Rondón, 2019). Additionally, the employment of additives in feed is another approach to control and enhance intestinal health in poultry (Oviedo-Rondón, 2019).

Nutritional strategies to replace the sub-therapeutic antibiotic utilization in poultry should be developed based on the mechanisms of action of antibiotics (Gadde et al., 2017; Lillehoj et al., 2018). Animal growth promotion mediated by antibiotics is explained by different mechanisms of action. Firstly, subclinical infections are inhibited by antimicrobial growth promoters generating a reduction of the metabolic costs of the immune system (Gaskins et al., 2002; Dibner and Richards, 2005). The second mechanism is based on the reduction of growth-depressing metabolites by AGPs such as ammonia or bile degradation products system (Gaskins et al., 2002; Dibner and Richards, 2005). Furthermore, AGPs might reduce the microbial use of nutrients due to a reduction or control of microbial population in the gut (Dibner and Richards, 2005; Page, 2006). Finally, the last mechanism of action of AGPs is based on the enhancement of nutrient utilization and absorption because animals supplemented with AGPs show thinner intestinal wall (Gaskins et al., 2002).

In addition, Niewold (2007) suggested that sub-therapeutic levels of antimicrobial drugs in feed decrease the inflammatory response by reducing the production of pro-inflammatory cytokines, and hence saving energy from the immune function. Alternatives to antibiotics seem to control the intestinal environment by modulating the immune response or controlling the microbiota population (Huyghebaert et al., 2011). During the recent years, different alternatives to AGPs are being developed such as probiotic/prebiotic (synbiotics), enzymes, organic acids, phytogenics and metals trying to reach similar effects as with AGPs in poultry (Gadde et al., 2017) (Figure 6). Moreover,

novel alternatives are being investigated, including hyperimmune egg yolk IgY, antimicrobial peptides (AMP), bacteriophages and clay minerals (Gadde et al., 2017).

Figure 6. Different alternatives to antibiotic utilization in feed in poultry production (source: Gadde et al., 2017).

2.1.3. PHYTOGENICS: AN ALTERNATIVE TO AGPs IN POULTRY FEEDING

2.1.3.1. General overview

Phytogenic feed additives (PFAs) comprises a large variety of plants which are classified as herbs or spices (fruits, seeds, or root with intensive aromatic or flavoring characteristics) or as plant-derived products from the food industry (Windisch et al., 2008; Upadhaya and Kim, 2017). PFAs can be included in animal feeds as dried, solid, and ground form or as extracts (crude, concentrated and purified) (Gadde et al., 2017). Moreover, they can be classified as herbs (products from flowering, non-woody, and non-perennial plants), essential oils (EOs) (hydro-distilled volatile compounds extracts of plants), botanicals (whole plants or processed parts), and oleoresins (extracts derived from non-aqueous solvents) (Windisch et al 2008; Clavijo and Flórez, 2017). The bioactive components of PFAs are secondary metabolites being polyphenols the most abundant (Windisch et al., 2008). Likewise, other bioactive substances are present in

these additives, including terpenoids (monoterpenes, steroids...), phenolic (tannins), glycosides, and alkaloids (Huyghebaert et al., 2011). Nevertheless, the composition and concentration of these metabolites are very heterogeneous depending on different factors (species and parts of the plant, geographical origin, environmental factors, and harvesting conditions), manufacturing (processing technique such as extraction, distillation and stabilization) and storage conditions (Windisch et al., 2008; Applegate et al., 2010; Anadón et al., 2019).

In recent years, phytochemicals have been used as natural alternative to AGPs in poultry feeding. The utilization of herbs, spices (thyme, oregano, yarrow, garlic, ginger, rosemary, cinnamon, coriander, among others) and EOs (from garlic, anise, rosemary, citrus, clove, ginger, thymol, carvacrol, cinnamaldehyde) have been used individually or in blends with potential applications as AGPs alternatives (Gadde et al., 2017). Several authors have shown positive effects on growth performance including PFAs such as black cumin seeds (Khalaji et al., 2011), blend of *Cynodon dactylon*, *Aerva lanata* and *Piper nigrum* (Oso et al., 2019), and *Ginkgo biloba* leaves (Cao et al., 2012) in broiler chicken diets. However, other studies showed no positive results on poultry growth performance with the inclusion of different EOs (Basmacioglu et al., 2004; Hernández et al., 2004; Brenes and Roura, 2010). Supplementation with several other OEs such as thymol and carvacrol (Hashemipour et al., 2013), oregano and rosemary (Mathlouthi et al., 2012), and blend of different herbal EOs (Khattak et al., 2014) were shown to improve growth performance.

Plant extracts have also shown to enhance broiler chicken performance. Zhao et al. (2013) showed a significant body weight gain and lower FCR when broilers were given diets supplemented with *Portulaca oleracea*. Similar results were observed with the addition of oregano extract (Forte et al., 2018), aniseed extract (Durrani et al., 2007) and bamboo leaf extract (Shen et al., 2019) in broiler chicken diets. In contrast, research trials conducted with other extract from grapes (Viveros et al., 2011; Chamorro et al., 2013), broccoli (Mueller et al., 2012) were unable to find improvements in growth performance. The disparity in the effects of plant extract supplementation can be influenced by the source and bioactive compounds of the PFAs, the method of extraction, the environmental conditions and the rearing conditions among studies (challenge vs. no challenge, cleaning, management).

2.1.3.2. Mechanism of action of phytonics in poultry

The mechanism of action of the PFAs are diverse and are not fully elucidated in poultry. Principally, the mode of action depends on the active molecules of the PFAs as each ingredient has specific properties (Yitbarek, 2015). However, the growth-promoting effects of PFAs are generally attributed to their antimicrobial, antioxidant properties, the beneficial effects in the immune system or enhancing the intestinal functionality (Windisch et al., 2008).

In this regard, PFAs supplementation in broiler diets reduce the toxic metabolites developed by some undesirable microorganisms of gut microbiota through the antimicrobial properties of PFAs against some specific pathogenic bacteria (Gadde et al., 2017). For example, supplementation with capsaicin extract reduced the presence of *S. Enteritidis* (Tellez et al., 1993) or *C. perfringens* (Jamroz et al., 2003) in broiler chicken gut. Also, supplementation with thymol reduced the prevalence of *E. coli* and *Clostridium* (Tiihonen et al., 2010), coliforms (Jang et al., 2007), and controlled necrotic enteritis (Timbermont et al., 2010).

Phytonic feed additives also improve health by exerting an enhancement of antioxidant activity by reducing of oxidative stress in specific tissues (Lee et al., 2017). This beneficial effect has been shown when broiler chickens were supplemented with oregano and rosemary EO reduced the lipid oxidation in breast meat (Basmacioglu et al., 2004). In addition, Zhang et al. (2013) showed that including *Forsythia suspensa* extract in broiler diets reduced the oxidative stress by increasing the serum total antioxidant capacity, and superoxide dismutase activity. Antioxidant activity was also shown when grape pomace concentrate is included in broiler diets reaching similar antioxidant capacity than vitamin E (Brenes et al., 2008).

Another beneficial effect of PFAs is the improvement of growth performance increasing the apparent ileal digestibility (AID) of nutrients (Pandey et al., 2019). PFAs dietary inclusion stimulate the enzyme production and the bile acids (BAs) flow (Platel and Srinivasan, 2000; Gadde et al., 2017). In this regard, BAs play two essential functions in the metabolism. Firstly, BAs help to the absorption of dietary fat through emulsification and micelle development (Green and Kellogg, 1986). Also, BAs seem to modulate the microbial communities in the gastrointestinal tract influencing the enzyme secretions of bacteria and getting the capacity to modulate the metabolic pathways of the host (Joyce

et al., 2014). Thus, several authors observed higher fat digestibility in broiler chickens supplemented with PFAs. Mountzouris et al. (2011) showed an improvement of apparent ileal digestibility of fat in broiler chickens supplemented with an oregano, anise, and citrus blend (125 mg/kg of diet). Moreover, inclusion of an herbal blend at 150 mg/kg in broiler chicken diets increased the apparent total tract fat digestibility.

Several authors have shown the enhancement of the digestive enzyme activities including trypsin, amylase or maltase in broiler chickens supplemented with diverse PFAs in diets (Lee et al., 2003; Hashemipour et al., 2014; Gao et al., 2019). In a similar way, Murugesan et al. (2015) reported higher apparent ileal digestibility of nutrients because of the beneficial properties of PFAs on the gut morphology. These authors showed an improvement of villus height in the duodenum, jejunum, and ileum in broiler chickens supplemented with a PFA which contained a blend of over 30 essential oils and other components. In a similar way, Abudabos et al. (2018) showed a gut histology improvement in broiler chickens supplemented with a PFA based on oregano, carvacrol, yucca extract, and cinnamaldehyde in animals infected with *C. perfringens*. In addition, Ghazanfari et al. (2015) showed a significant improvement on gut histology parameters when broilers were given diets supplemented with coriander essential oil. Also, inclusion of eugenol and thymol essential oils in broiler chicken diets improved villus height to crypt depth ratio (VH/CD) which is a useful ratio that indicate higher digestive capacity (Du et al., 2016).

It has been shown that PFAs exert immunomodulatory effects in poultry and enhance the defenses against microbial infections of the host (Gadde et al., 2017). Several authors have documented these immune effects on broiler chickens reared in standard conditions or under different microbial infections and a recompilation of these studies is reported in Table 1. Different phytochemicals supplementation exerts immunomodulatory effects by mainly increasing the concentration of immunoglobulins and cytokines in serum, reducing the oxidative stress or modulating several interleukin gene expressions in broiler chickens gut. Also, the immune response depends on whether animals are subjected to an immune stimuli (challenge) or not. For example, Hashemipour et al. (2013) showed better performance in healthy broiler chickens supplemented with an oregano extract which contains thymol and carvacrol. The improvement in BWG and FCR with oregano extract was related to an increment of IgG and reduction in heterophil and lymphocyte ratio. Kim et al. (2011) observed that inclusion of curcuma longa extract

(Polyphenol phytonutrient and curcumin) at 35 mg/kg in diets of broilers challenged with coccidia (*E. maxima* and *E. tenella* inoculum) improved BWG, FI, and FCR, reduced oocyst count and produced anti-inflammatory effects in the gut. Therefore, the immune response of phytogenics utilization in poultry depends on their active compounds and concentrations, the mode of supplementation, and the immune status of birds.

Table 1. Effect of different phytogetic feed additives on the immune response of broiler chickens reared in standard experimental conditions or under microbial infectious challenges.

Phytogenic	Active compounds	Concentration	Mode of supplementation	Challenge model	Effect on Performance	Immune response ¹	Reference
Plant blend (<i>Phlomis umbrosa</i> Turez, <i>Cynancum wilfordii</i> Hem, <i>Zingiber officinale</i> Rosc, <i>Platycodi Radix</i>)	-	0.10%	Feed	No	Improve BW Decrease Abdominal fat	Increase IgG and IGF-1 in serum	Begum et al., 2014.
<i>Yucca schidigera</i> extract	Steroidal saponin	100 mg/kg	Powder in feed	NDV	Improve EBI and BWG	Stimulation of IL-6 and IFN- γ serum concentration Increase Antibody titers against NDV	Sun et al., 2017.
<i>Camellia oleifera</i> seed	-	300 mg/kg	Extract in feed	No	No effect	Reduction of TNF- α in the jejunum and ileum	Dong et al., 2016.
<i>Macleaya cordata</i> extract	1.5% of chelerythrine and sanguinarine	25, 50 and 100 mL/L	Extract in drinking-water	NDV	Improve BW, FI and G:F	Reduce iNOs and IL-1B expression in the jejunum	Khadem et al. 2014.
<i>Pinus radiata</i> extract	Proanthocyanidins	5, 10, 20 mg/ kg of BW	Oral gavage	No	No effect	Increase immune cells (Splenocytes, bursal cells, tymocytes) Increase expression of IFN γ (Th1 helper) and reduce IL-6 (Th2 helper)	Park et al., 2011.
Cinnamon (<i>Cinnamomum cassia</i>)	Cinnamaldehyde	14.4 mg/kg	Feed	Coccidiosis (<i>E. tenella</i> , <i>E. acervulina</i> and <i>E. maxima</i>)	Increase BW	Higher expression of IL-1 β , IL-6, IL-15 and IFN- γ in intestinal lymphocytes (compared to no-supplemented and no infected) Coccidiosis effect: reduction of oocyst count and increased parasite antibody response against <i>E. tenella</i>	Lee et al., 2011.
Broccoli extract	Sulforaphane	0.075 g /kg	Feed	No	No effect	Reduction of oxidative stress by higher jejunum expression of xenobiotic and antioxidant enzymes	Mueller et al., 2012.
Oregano extract	Thymol and carvacrol	60, 100 and 200 ppm	Powder in feed	No	Improve BWG, FCR	Increase hypersensitivity, total and IgG anti-sheep red blood cell titers. Decrease H/L ratio.	Hashemipour et al., 2013.

Phytogenic	Active compounds	Concentration	Mode of supplementation	Challenge model	Effect on Performance	Immune response ¹	Reference
Oregano essential oil	25% thymol and 25% of carvacrol	60, 120 and 240 mg/kg	Feed	<i>C. perfringens</i>	No effect	Reduce gut lesion and improve VH/CD ratio. Increase antibody titers against NDV. Linearly reduce TLR2 and TNF- α in the ileum.	Du et al., 2016.
<i>Echinacea purpurea</i> L.	-	5 and 10 g/kg	Powdered plant parts in feed	NDV	Improve BWG, FI and FCR	Increment of antibody titers against NDV and sheep red blood cell titers.	Landy et al. 2011.
<i>Curcuma longa</i> extract (Turmeric)	Polyphenol phytonutrient and curcumin	35 mg/ kg	Feed	Coccidiosis (<i>E. maxima</i> and <i>E. tenella</i>)	Improve BW	Reduction OC infected with <i>E. tenella</i> . Increase antibody level against <i>Eimeria</i> microneme protein, MIC2. Changes gene expression exerting anti-inflammatory effects in the gut.	Kim et al., 2013b.
Thyme-oil extract	-	100 ppm	Oil in feed	SRCB vaccine	-	Increase total immunoglobulin	Alipour et al., 2015.
Anethole	99%	15 mg/kg	Feed	<i>E. acervulina</i>	Improve BW	Reduce fecal oocyst excretion Increase IL-6, IL-8, IL-10 and tumor necrosis TNFSF15	Kim et al., 2013c.
Garlic extract (Garlicon40®)	40% of bioactive compounds (67% of prophythiosulphinate oxide and 33% of prophythiosulphinate	10 ppm	Feed	<i>E. acervulina</i>	Improve BW	Reduce fecal oocyst excretion Reduce serum antibody titers against profiling. Reduction of TLR3, TLR5 and NF-KB and IL-10 Uninfected treatment supplemented with extract: Increase IFN- γ and IL-4	Kim et al., 2013a.
<i>Capsicum</i> and turmeric oleoresins	-	4 mg/kg turmeric oleorin 4 mg/kg <i>Capsicum</i> oleosin	Feed	<i>E. maxima</i> and <i>C. perfringens</i>	Improve BW	Reduce gut LS in the intestine and spleen. Reduce serum α -toxin levels Reduce intestinal IL-8, LITAF, IL-17A and IL-17F mRNA intestinal level Increase cytokine/chemokine in splenocytes	Lee et al., 2013

¹ BW, body weight; Ig, immunoglobulin; IGF1, insulin-like growth factor 1; NDV, Newcastle disease virus; EBI, European broiler index; BWG, Body weight gain; IL, interleukin; TNF- α , Tumor necrosis factor alpha, FI, feed intake; G:F, gain/feed ratio; iNOs, nitric oxide synthase; H/L, heterophil/lymphocyte ratio; VH/CD, villus height/crypt depth ratio; TLR, toll like receptor; OC, oocyst count; TNFSF15, tumor necrosis factor superfamily member 15; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells, LS, lesion score; LITAF, lipopolysaccharide induces TNF factor.

2.1.3.3. Bioactive compounds derived from by-products of olive industry as phytogetic feed additives

Agricultural eco-innovation is based on circular economy and cradle to cradle concepts, aiming at “zero waste” economy generating new products and utilities (Mirabella et al., 2014). With 1.6 million tons, Spain is the largest olive oil (*Olea europaea* L.) producer globally which produces olive and leaves wastes reaching more than 4 and 0.2 million tons per year, respectively (International Olive Council, 2018; Romero et al., 2017). These by-products can be recycled for different purposes such as energy production or organic fertilizer, but may also be used in animal feed because of its positive nutritional properties (Roig, et al., 2006).

The inclusion of olive leaves in broiler chicken diets showed positive effects on FCR, reducing the feed intake without affecting ADG (Varmaghany et al., 2013). However, Shafey et al. (2013) observed lower growth performance in birds fed olive leaves. Besides, during the extraction of olive oil it is generated the called “olive cake”, which contains bioactive compounds including polyphenols and oleuropeosides with antioxidant, antibacterial, and anti-inflammatory properties (Benavente-García et al., 2000). Thus, several authors have tested the inclusion of olive cake or pulp in broiler chicken diets. In recent studies, Al-Harthi (2017) and Sayehban et al. (2016) showed no detrimental effects of dried pulp inclusion in broiler diets on performance. However, Papadomichelakis et al. (2019) observed negative effects on growth performance in grower and finisher phase. These contradictory results might be as a consequence of the olive leaves and pulp which contain substances that potentially can actuate as an anti-nutritional factors reducing the palatability and digestibility of feed (Paiva-Martis et al., 2009; Sicuro et al., 2010; Aggoun et al., 2014). Therefore, during, recent years, it is being generating an interest in the purification of the compounds with bioactive properties from olive by-products to enhance animal health and performance (King, et al., 2014; Liehr et al., 2017; Leskovec et al., 2018).

In this regard, Sarica and Ürkmez (2016) showed that supplementation with 100 or 200 mg/kg of an olive leaf extract rich in oleuropein and phenols in broiler chicken diets improved growth performance and gut microflora. Oke et al. (2017) showed that water supplementation with other olive leaf extract up to 15 mL/L in broiler chickens, improved growth performance and protected against oxidative stress under hot humid tropical

climate conditions. Besides, Branciari et al. (2016) showed that supplementation with olive mill wastewater polyphenolic extract reduced the prevalence of *Campylobacter* spp in broiler chickens. By contrast, King et al. (2014) were unable to find positive results on growth performance and antioxidant activity in broilers fed purified olive extract (contained 8.82% polyphenols and a minimum of 2.5% of hydroxytyrosol) in drinking water. Moreover, Leskovec et al. (2018) showed no significant differences on growth performance and apparent total tract digestibility of broiler chickens supplemented with an olive leaf extract (6 mL/kg of feed). Also, studies carried out in pigs showed that different olive by-product extracts exerted positive effect on growth performance, immune system and gut barrier functions (Liehr et al., 2017; Tedó et al., 2018)

Bioactive compounds derived from *Olea europaea* (quercetin and oleuropein at 0.023 to 0.371 mg/mL concentration) showed promising results as anticoccidial agent in broiler chickens against *Eimeria* spp. (Debbou-louknane et al., 2019). Also, de Pablos et al. (2010) showed better body weight and reduction of lesion score and oocyst index in *E. tenella* infected broiler chickens supplemented with 90 ppm of maslinic acid in feed. Thus, bioactive compounds derived from *Olea europaea* might affect positively depending on the animal species, part of the plant and its processing and storage conditions, the amount and mode of administration (water or in feed), and the possible interaction with other feed nutrients or ingredients.

2.2. GUT HEALTH AND IMMUNE SYSTEM IN POULTRY

Gut health might be defined as the ability to maintain homeostasis and to perform gut normal physiological activities, preserving the capacity to defy infectious and noninfectious agents (Kogut et al., 2017). Within this definition, gut should meet several functions to maintain gut physiology and animal well-being and productivity such as effective digestion and absorption of feed or adequate structure and functionality of the gut barrier (Kogut et al., 2017). In addition, gut health concept encompasses a stable and balanced gut microbiota and efficient functions of the intestinal immune system.

2.2.1. AVIAN GUT IMMUNE SYSTEM

The main function of the intestinal tract is to digest and absorb the nutrients from the feed. Furthermore, the gut is the largest area between host and environment, which has to be sufficiently permeable to absorb the nutrients but, also sufficiently selective to keep out the undesirable microorganisms (Broom and Kogut, 2018). Thus, the gut must reach an equilibrium between the digestion and the local immune functions. In this context, the intestinal immune system is made up of the gut-associated lymphoid tissue (GALT) which is considered the largest immune component of the immune system with innate and adaptive diverse responses (Forchielli and Walker, 2005).

The intestinal epithelium is composed by absorptive enterocytes, mucus-secreting goblet cells, endocrine cells, and Paneth cells (Shira and Friedman, 2018). The intestinal epithelium separates the lumen substances from the lamina propria (LP) and other underlying layers (Kim and Lillehoj, 2019). The first gut barrier defense is constituted by the mucus layer that links the intestinal cells lining and the luminal material (nutrients and pathogens) (Broom and Kogut, 2018). This layer is mainly constituted by mucins secreted by goblet cells and protects the mucosa against physical damage, dehydration and, it can be differentiated in external layer that is in contact with the luminal material and the inner layer (Broom and Kogut, 2018). This latter is highly concentrated on IgA and AMP which are produced by intestinal B cells and Paneth cells respectively, in the intestinal epithelium (Broom and Kogut, 2018; Kim and Lillehoj, 2019).

In the gut, the innate immune response starts with the activation of pathogen recognition receptors (PRRs) including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain proteins, and nucleotide-binding domain leucine-rich repeat-containing receptors (Keestra et al., 2013). The PRRs are found in epithelial cells, lamina

propria and macrophages, recognizing the pathogen-associated molecular patterns (PAMPs) (Kim and Lillehoj, 2019) or the damage-associated molecular patterns (DAMPs) such as uric acid, ATP, DNA fragments and mitochondrial contents (Garg et al., 2010; Broom and Kogut, 2018).

After PRRs activation by the presence of PAMPs, the AMPs and diverse cytokines (including IL-10 and TGF- β) are produced by epithelial cells followed by the activation of the lymphocytes such as dendritic cells and macrophages which cope with the infection (Kim and Lillehoj, 2019). Also, the activation of PRRs produces a stimulation of B cells which generate the secretory IgA. This type of IgA helps to combat the pathogens in the LP and its concentration is increased by TLR4 activation which promotes B cell recruitment and transformation to secretory IgA (Kim and Lillehoj, 2019). Moreover, the previous secretion of AMP also is useful to inhibit the microbial invasion by permeabilization of intestinal cell walls and killing wide variety of bacteria, protozoa, fungi and viruses (Brisbin et al., 2008; Kim and Lillehoj, 2019). Furthermore, heterophils are an important cellular component of poultry innate defenses, which help to maintain intestinal homeostasis by expressing cytokines and chemokines as a response to bacteria (Kogut et al., 2006; Brisbin et al., 2008).

Innate immune response against a pathogen can be insufficient and an adaptive response can be triggered to reach a more specific and efficient response. According to Kim et al. (2019), gut plays a major role in the protection process against infections because it is the largest deposit of B cells and T lymphocytes. Lymphocytes are immune cells involved in adaptive immune response that are predominantly T cells and located in the LP and intraepithelial layer, namely intraepithelial lymphocytes (IELs). These T lymphocytes recognize the antigens and induce a response by secreting cytokines at cellular (Th1) or humoral (Th2) levels (Delgado et al., 2017). Furthermore, to regulate the inflammatory activity of T cells and maintain the intestinal homeostasis, there are specific cells namely regulatory T cells that suppress the inflammatory response via production of IL-10 and TGF- β anti-inflammatory cytokines (Kim and Lillehoj, 2019).

2.2.2. INTESTINAL PERMEABILITY AND MORPHOLOGY

Intestinal permeability plays also an essential role in the maintenance of gut health and homeostasis. According to Travis and Menzies (1992), intestinal permeability can be defined as “the facility with which intestinal epithelium allows molecules to pass through by non-mediated passive diffusion”.

Figure 7. Paracellular and transcellular translocation pathways in the intestinal epithelium with tight junction proteins. JAM, junctional adhesion molecule; CVAR, coxsackie virus and adenovirus receptor; ZO, zonula occludens. (Adapted from Awad et al., 2017)

Intestinal barrier permeability depends on the connections and junctions among the epithelial cells which are defined by the apical junctional complexes (AJCs) including, tight junctions, desmosomes and adherent junctions (Figure 7) (Suzuki, 2013; de Santis et al., 2015). These AJCs control the paracellular transport pathway of molecules by the bonds among IECs (Ducatelle et al., 2018). More concretely, tight junctions are transmembrane proteins that are differentiated in claudins, occluding, tricellulin, junctional adhesion molecules, zonulae occludens and cingulin (de Santis et al., 2015).

The digestive tract is the principal pathway where the enteric pathogens or non-desirable feed compounds can disrupt in the host, but at the same time is the main physical barrier against pathogens that is helped by the activation of the immune system (Awad et al., 2017). Changes in the molecular structure of the junctions or the down regulation of

genes related with the junctional structural proteins has been observed when chickens were submitted under different stresses such heat or short-term fasting period showing mucosa damages and increment of intestinal permeability (Song et al., 2013; Gilani et al., 2017, 2018a). These stress situations and pathogens can stimulate the secretion of pro-inflammatory cytokines which open epithelial TJs by the contraction of epithelial cells produced by the phosphorylation of myosin light chain (Awad et al., 2017). Besides, according to Ducatelle et al. (2018) intestinal inflammation is directly linked with the loss of intercellular junction integrity.

Gut permeability in poultry can be measured by different methods. The most common technique is by the administration of an oral solution of fluorescein isothiocyanate dextran (FITC-d) which evaluates the paracellular transport and mucosal barrier dysfunction (Baxter et al., 2017). Other biomarkers are used to analyze intestinal permeability including, TJs protein or gene expression, bacterial translocation or lactulose (L), L-rhamnose (R) and mannitol (M) sugars (Gilani et al., 2016). The latter biomarker are non-digestible oligosaccharides which provide information of the transcellular (M and R, small molecular weight) and paracellular (L, high molecular weight) transport (Gilani et al., 2016). When an animal suffers leaky gut, the lactulose pass through the TJs and travels through the bloodstream and then is excreted via excreta in chickens (Gilani et al., 2016; Celi et al., 2019).

The morphology of the intestine plays a crucial role in the concept of gut health and the measurement of the intestinal histology has been widely use as gut health biomarker in poultry (Chen et al., 2015; Ducatelle et al., 2018; De Meyer et al., 2019). Furthermore, the intestinal mucosa is in charge of nutrient absorption, and disruptions in the epithelial layer would decrease the efficiency of the metabolism of nutrients and hence the animal performance (Laudadio et al., 2012). These authors also suggested that improved VH and VH/CD ratio for different gut chicken segments were in agreement with increased nutrient absorption and growth performance. Additionally, Adibmoradi et al. (2006) suggested that higher VH and villus width, which mean a greater villus surface area is associated with better body weight of broiler chickens. By contrast, when intestinal crypts are deeper is a signal of faster tissue turnover to regenerate the villus cells, suggesting that the host's intestinal response mechanism is trying to compensate for normal atrophy of the villi due to inflammation of the pathogens and their toxins (Gao et al., 2008; De Meyer et al., 2019). Additionally, this cellular turnover process is associated with an

energy that is not used for chicken growth causing lower growth rates (Markovi et al., 2009). Furthermore, Awad et al. (2009) suggested that shorter VH is associated with poor gut health in broiler chickens. It can be concluded that intestinal histology is related to a good intestinal health status when villus height is higher, shallower crypt which derives in higher VH/CD ratio in poultry.

2.2.3. INTESTINAL MICROBIOTA AND PHYTOGENICS IN BROILER CHICKENS

Stable gut microbiota is one of the main factors of gut health in poultry, being an essential issue the knowledge of the microbial population and its functions (Kogut et al., 2017). Microbiota helps to maintain a correct nutrient digestion and absorption, the intestinal epithelium structure and the development of gut immune system (Oakley et al., 2014, Kogut et al., 2017). Thus, it is essential to recognize the composition and diversity of intestinal microbiota to enhance the productivity and health of chickens.

Firmicutes and Proteobacteria are the main phylum of bacteria in broiler chicken digestive tract (Yeoman et al., 2012). The predominant genera of bacteria in the small intestine are *Lactobacillus*, *Enterococcus* and *Clostridium* (Yeoman et al., 2012). The density and diversity of microbiota is variable throughout the intestinal tract due to the secretions of enzymes and antimicrobials, high oxygen pressure or the high rate of the digesta (Thompson and Applegate, 2006; Yeoman et al., 2012). Thus, the density of bacteria ranges from 10^8 bacteria per gram of digesta in the gizzard to over 10^{11} bacteria per gram of digesta in the ceca (Oakley et al., 2014).

Gut microbiota acts as a defense against pathogens probably due to the competition for the available nutrients with the undesirable bacteria, stimulation of the immune system, and production of antimicrobials and short-chain fatty acids (SCFAs) (Oakley et al., 2014). The later substances provide around 10% of the energy that chickens metabolize and also enhance the renewal of the intestinal epithelium (Kogut, 2013). In addition, SCFAs reduce the pathogenic bacteria by reducing the pH or having a direct impact on non-desirable bacteria, and help to maintain homeostasis controlling the anti-inflammatory activity which is essential to maintain digestive function in the gut (Sugiharto, 2016). Moreover, microbiota brings benefits to the host nutrition as the production of vitamins (vitamin K and B) and other nutrients (polysaccharides, amino acids, and SCFA) that are absorbed by the host, enhancing animal growth (Oakley et al., 2014). Furthermore, gut microbiota modulates the immune response by affecting T-cell

and B-cell activity, regulating the cellular permeability, modulating the production of AMPs, and mucus secretions in the IECs (Laparra and Sanz, 2010; Oakley et al., 2014).

Intestinal microbiota plays also an important role in nutrient utilization and growth in poultry, and it is essential to know the factors that modulate microbial population (Pan and Yu, 2014). The gastrointestinal tract of chickens is nearly sterile at hatch and the development of the microorganism in the gut stems from exogenous microbial population (Pan and Yu, 2014). Aside from the age of animal, composition of the diet, host features such as breed, sex or maternal factors affect directly to the intestinal microbiota development, but also the environmental factors (location, housing, temperature), hygiene, litter quality, and medication (Pan and Yu, 2014; Oakley et al., 2014; Kers et al., 2018). Thus, the correct management of environmental factors is key to reach a balanced gut microbiota in broiler chickens which helps the host to get an optimal nutrient digestion and absorption.

The supplementation of feed additives such as probiotics, prebiotics, and phytogenics in poultry have shown the capacity to modulate the gut microbiota (Clavijo and Flórez, 2017). Bioactive components of phytogenics can exert positive effects on animal growth and health due to their antimicrobial and anti-inflammatory properties. In this context, the management of intestinal microbiota using phytogenics has been reported in several studies, which showed the reduction of pathogens in the gut such as *Salmonella* (Vicente et al., 2007), *Eimeria* (Lee et al., 2011; Kim et al., 2013a, b, c; Lee et al., 2013) or *C. perfringens* (Du et al., 2016). Nevertheless, other authors were not able to find positive effects of phytogenics on poultry pathogens (Cross et al., 2007; Hermans et al., 2011; Kurekci et al., 2014). Thus, the utilization of useful phytogenics or an optimal blend of these additives along with good management of animals can be an alternative to antibiotic utilization for the maintenance of optimal gut health and maximizing growth performance in poultry.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. EXPERIMENTAL DESIGN

All experimental procedures concerning animals were accepted by the animal Ethics Committee of the Universidad Politécnica de Madrid and were in compliance with the Spanish Guidelines for the Care and Use of Animals in Research (Boletín Oficial del Estado, 2013) for Experiment 1 and 2. All animal and experimental procedures of Experiment 3 were approved by the University of Arkansas Institutional Animal Care and Use Committee before initiation of the experiment. In the respective experiments, environmental animal conditions (temperature, humidity, ventilation, illumination) were controlled and monitored according to the respective broiler chicken strain guidance (Cobb Broiler Management Guide, 2017; Ross Broiler Management Handbook, 2018).

3.1.1. OBJECTIVE 1: Study the effects of supplementing grower diets with an olive pomace extract on broiler performance and gut health

A feeding trial was carried out with three hundred and six 1-day-old mixed sex broiler chickens (Ross 308) with 40.6 ± 0.7 g initial live weight, obtained from a commercial hatchery (Avimosa Group, Toledo, Spain). Chicks were assigned to 18 floor pens ($1.1 \text{ m} \times 1.1 \text{ m}$) with 17 birds per pen. Pens were bedded with wood shaving and provided with a hopper feeder and a bell drinker. All birds were raised with a standard non-medicated starter diet based on wheat and soybean meal in crumble form for the first 3 weeks (Table 2). After that, 252 animals with 21 d of age (84 birds/treatment) and similar BW were used in the feeding trial ($14 \text{ birds/pen} \times 6 \text{ pens/treatment} \times 3 \text{ treatments}$). The design was completely randomized with 3 treatments, a negative control with no additives (Control), a positive control with 100 ppm of monensin (Monensin; Elanco Valquimia S.A.), and the basal diet supplemented with 750 ppm of an olive extract (OE, Lucta S. A.; Spain) which consisted of an olive pomace extract standardized to contain a minimum of 10% total triterpenes and 2% polyphenols. The theoretical concentration of OE (750 ppm, with 2% of polyphenols and 10% of triterpenes) was similar to the concentration found in the experimental diet (722 ± 23 ppm, with $2.4 \pm 0.25\%$ of polyphenols and $12.9 \pm 0.54\%$ of triterpenes). This analysis developed with ultra-high performance liquid chromatography-mass spectroscopy (UPLC-MS) technique indicated that extract mixed procedure and stability of the product fitted with the expected values.

The experimental diets were formulated to have similar nutritive value (Table 2) according to FEDNA (2010) and manufactured at IRTA (Mas de Bover, Constantí, Spain). Celite was added in the feed at 2% as acid insoluble ash marker for apparent ileal digestibility (AID) determination. Animals were fed *ad libitum* the experimental diets as pellets with 3 mm diameter from 21 to 42 d of age.

Body weight and feed consumption were determined by pen at 21, 28, 35, and 42 d of age, to calculate the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR). Mortality was recorded and weighed as produced. At the end of the experiment at 42 d of age, birds were slaughtered by asphyxiation in CO₂ atmosphere. To obtain enough ileal content to determine nutrient digestibility and to sample all animals in the fed state birds were deprived of food for 2 h, and then were refed under *ad libitum* conditions for 1.30 h to achieve as homogeneous feed intake as possible.

Two birds per pen were randomly selected and sampled to run microbial, gene expression, blood and bile acid analysis. To determine the relative abundance of bacterial families approximately 1 g of caecal content was sampled, immediately placed in dry ice and stored at -80°C. For gene expression analysis around 200 mg of ileal (approximately 4 cm from the Meckel diverticulum) mucosal scrapings were sampled in RNA later (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, and further stored at -80°C. Blood samples were collected from the heart using sterile syringes and needles. To obtain the plasma blood was collected into tubes containing EDTA and aprotinin (BD Vacutainer), held in ice for 30 min, centrifuged at 2000 × g for 10 min and stored at -80°C to later analyze bile acids. Finally, 5 g of ileal content were collected and stored at -80°C to analyze bile acid concentrations.

The ileal digesta from the remaining birds (10 to 12 broiler per pen) was collected as indicated in Mandalawi et al. (2014). Samples were pooled, homogenized, frozen at -20°C, and freeze-dried. Then, the dried samples were ground using a mortar and pestle to pass through a 0.5 mm screen and maintained in airtight containers at room temperature until determination of nutrient AID. The AID of dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE), and gross energy (GE) was estimated using the indigestible marker method (De Coca-Sinova et al., 2011).

Bile acids analysis was performed by UPLC-MS chromatography in an AQUITY I-Class (Waters Corp., Milford, MA) connected to a Xevo-G2 QToF MS detector. Separation was run on an ACQUITY UPLC BEH C18 1.7 mm column (2.1×100 mm, Waters Corp.) using water and acetonitrile as mobile phases, both containing a 0.1% of formic acid. Mass spectroscopy detection was performed in full-scan negative mode (100 to 1200 Da). Concentration of bile acid was determined based on standard curves with QuanLynx software (Waters Corp.). Bile acids were extracted using the following methodologies and using chenodeoxycholic acid-d4 (CDCA-d4) as internal standard: lyophilized ileal digesta samples were homogenized in absence of solvent on a TissueLyzer II (QIAGEN, Hilden, Germany) and 20 mg of homogenate were extracted with 840 μ L of H₂O: ACN (1:1) including internal standard, respectively. After homogenization, the mixture was centrifuged ($15000\text{ g} \times 10\text{ min}$, 4°C) and the supernatant diluted in H₂O: ACN (1:50) for UPLC-MS analysis. Plasma proteins were precipitated by addition of 200 μ L of ACN with 5 μ L internal standard to 50 μ L of plasma. After centrifugation, supernatants were directly analyzed by UPLC analysis.

The bacterial DNA extraction and sequence analysis were performed using the methodology described by Andreano et al. (2017). Briefly, bacterial DNA was isolated of caecal content samples to obtain the microbiome profile by massive sequencing of the 16S rRNA gene regions. Amplicons of the V1 to V2 16S rRNA region were amplified by PCR with F27 forward and R338 reverse primers with the sequences and conditions indicated in Andreano et al. (2017). For each amplicon, quality and concentration were analyzed using Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Samples were massive sequenced with the Ion 318 Chip Kit v2 (Life Technologies) under manufacturer's conditions on an Ion Torrent Personal Genome Machine.

The ileal content of broilers were analyzed following the standard methods of AOAC (2000) using the methods described in the Chemical Analysis (3.2) section. Moreover, in the Gene Expression Analysis (3.3) section is detailed the procedure used to analyze the expression of the following target genes: liver X receptor (LXR), carbohydrate responsive element-binding protein (ChREBP), sterol regulatory element-binding protein 1 (SREBP1), apical sodium dependent bile acid transporter (ASBT), fatty acid binding protein 2 (FABP2) and 6 (FABP6), interleukin 2 (IL-2) and 6 (IL-6), claudin 1 (Claudin1), transforming growth factor beta 4 (TGF- β 4), toll like receptor 4 (TLR4)

and 2 β (TLR-2 β), chicken B-cell marker chB6 (Bu-1), marker of active avian T lymphocytes (CD3 $\gamma\delta$), interleukin 8 (former CXCLi2), interferon-gamma (IFN- γ).

Table 2. *Ingredients and chemical composition (% , as fed basis, unless otherwise indicated) of pre-experimental (1 to 21 d) and experimental control diet (22 to 42 d).*

	Pre-experimental diet	Control diet
Ingredient		
Wheat (10.2% PB)	46.9	35.5
Soy bean meal (47% PB)	37.3	30.7
Barley	6.51	20.5
Fat ¹	5.50	8.00
Celite	-	2.00
Dicalcium phosphate	1.40	1.33
Calcium carbonate	1.15	0.98
Vitamin and mineral premix ²	0.30	0.30
Sodium chloride	0.40	0.35
L-Lys HCl (78%)	0.14	0.12
DL-met (99%)	0.26	0.23
L-Thr (98%)	0.05	0.05
Etoxiquin 66%	0.02	0.02
Endofeed DC	0.01	0.01
Phyzyme XP 5000	0.01	0.01
Calculated values		
Dry matter	88.3	89.0
AMEn (Kcal/Kg)	2950	3050
Crude protein	21.9	19.2
Ether extract	7.11	9.60
Crude fiber	3.03	2.90
Neutral Detergent Fiber	10.4	11.0
Starch	32.0	32.3
Sugars	4.75	4.20
Ca	1.00	0.90
Total P	0.61	0.55
Digestible P	0.36	0.34
Na	0.17	0.15
Ash	6.40	7.80

¹ Animal fat and soybean oil blend. ² Provided the following (per kilogram of diet): vitamin A (transretinyl acetate), 10000 IU; vitamin D₃ (cholecalciferol), 2000 IU; vitamin E (all-rac-tocopherol acetate), 20 IU; vitamin K (bisulfate menadione complex), 3 mg; riboflavin, 5 mg; pantothenic acid (D-calcium pantothenate), 10 mg; nicotinic acid, 30 mg; pyridoxine (pyridoxine·HCl), 3 mg; thiamine (thiamine-mononitrate), 1 mg; vitamin B₁₂ (cyanocobalamine), 12µg; D-biotin, 0.15 mg; choline (choline chloride), 300 mg; folic acid, 0.5 mg; Se (Na₂SeO₃), 0.1 mg; I (KI), 2.0 mg; Cu (CuSO₄·H₂O), 10 mg; Fe (FeSO₄·7H₂O), 30 mg; Zn (ZnO), 100 mg; Mn (MnSO₄·H₂O), 100 mg; and ethoxyquin, 110 mg.

3.1.2. OBJECTIVE 2: Evaluate the effects of supplementing starter and grower diets with an olive pomace extract on broiler performance and gut health after a challenge of intestinal permeability induced by a short-term fasting period

The trial was conducted on 660 one-day-old male broiler chicks (Cobb 500) from a commercial hatchery (Incubadora Uvesa, Tudela, Navarra, Spain). Animals were randomly assigned to 30 floor pens (1.1 m x 1.1 m) with 22 chickens per pen (6 pen/treatment x 5 treatments) provided with a hopper feeder, bell drinker and bedded with wood shaving.

The feeding program consisted of a starter feed (23.0% of crude protein (CP) and 2974 kcal/kg of apparent metabolizable energy (AME)), fed in crumbles from 1 to 21 d of age; and a grower feed (CP = 21.8%, AME = 3088 kcal/kg), fed from 21 to 32 d of age as pellets with 3 mm diameter. The composition of the starter and grower basal diets is shown in Table 3. The basal diet with no medication and no additives was used as a negative control. The other three diets were obtained by adding on top of the basal diet 500 (OE500F) or 1500 ppm (OE1500F) of an olive pomace extract provided by Lucta S. A. (Madrid, Spain), or 100 ppm of monensin (MF) (Elanco Valquimia S. A.) and used as a positive control. Supplemented OE was a standardized olive pomace extract containing $\geq 6\%$ of triterpenes and $\geq 1\%$ of polyphenols, quantified by HPLC-UV as oleanolic acid and hydroxytyrosol equivalents, respectively. Diets were manufactured at IRTA (Mas de Bover, Constantí, Spain).

At the beginning of the trial, chickens were divided at random into 5 groups. One group was fed the control diet *ad libitum* throughout the feeding trial from 1 to 32 days of age (treatment control no fasted, CNF). The other 4 groups were also fed *ad libitum* from 1 to 32 days of age except for a short-term fasting period (F) of 15.5 h at 14 d of age. They received the control diet (treatment control and fasted, CF) or any of the supplemented diets (OE500F, OE1500F and, MF).

From 1 to 14 d of age, animals were fed their respective experimental diets. At day 14, birds from all treatments, except those of CNF, were feed deprived for 15.5 h. Intestinal permeability was measured by the lactulose/mannitol (L/M) test following the protocol described by Gilani et al. (2018a). To this end, following the 15.5 h fasting period (15 d of age), two birds per pen (12 chicks per treatment) were randomly selected and administered an oral gavage of lactulose and mannitol. Lactulose and mannitol solution

contained 25 g lactulose and 5 g of mannitol (Sigma Aldrich, Alcobendas, Madrid, Spain) dissolved in 100 mL Milli-Q water at 25°C. Birds were slaughtered (CO₂ atmosphere asphyxiation) and sampled 90 min after the oral gavage. After this short-term fasting period, 16 chickens per pen were fed their respective diets *ad libitum* until the end of the trial at 32 d of age. The experimental feed was analyzed following the methodology described in the Chemical Analysis (3.2) section.

To calculate the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR), body weight and feed consumption were recorded per pen at 7, 14, 21, 28 and, 32 d of age. At 15 days of age (after the 15.5 h oral fasting period), the two birds per pen randomly selected for the intestinal permeability study were slaughtered by asphyxiation in CO₂ and sampled. For gene expression analysis, 200 mg of ileal mucosal scraping were taken in RNA later (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, and further stored at -80°C. For intestinal morphology, 3 cm length segments for the middle part of duodenum were collected and stored in 10% neutral buffered formaldehyde solution. To obtain the plasma, blood samples were collected immediately post-mortem via cardiac puncture into tubes containing EDTA and aprotinin (BD Vacutainer®, Plymouth, UK), held in ice for 30 min, centrifuged at 2000 x g for 10 min and stored at -80°C to later analyze alpha-1-acid glycoprotein (AGP), cytokines and lactulose and mannitol concentration. At the end of the trial (32 d of age), remaining animals were slaughtered by asphyxiation in CO₂ atmosphere.

Determination of plasma lactulose and d-mannitol concentrations were performed in an Acquity UPLC (Waters Corp., Mildford, MA, EEUU) connected to a Xevo-G2 Qtof mass spectrometer (Waters Corp., Mildford, MA, EEUU) operating in full scan negative mode (100 to 1200 m/z). Chromatographic separation was achieved with a linear gradient using a BEH amide column (21 mm x 100 mm, 1.7 µm, Waters Corp., Mildford, MA, EEUU) and mobile phases comprising A = 10 mM NH₄AC in CAN:Water (9:1) and B = 10 mM NH₄Ac in ACN:Water (4:6). Flow rate was set to 0.5 mL/min and oven temperature to 40°C. Leucine-enkephalin (200 ng/mL) was used as lock mass. Data processing was performed with QuanLynx software (Waters Corp., Mildford, MA, EEUU). Ion areas were used for quantification based on standard curves prepared using authentic standards and raffinose as internal standards (IS). Plasma samples (25 µL) were submitted to protein precipitation with 56 µl of IS solution. Four independent replicates per sample were prepared and analyzed.

Cytokine quantification in plasma was performed with commercial ELISA according to manufacturer's instructions (IL-1 β , SEA565Ga; IL-8, SEA080Ga, Wuhan USCN Business Co. Ltd., Wuhan, China). Kit standards and test samples optical densities were read at 450 nm by an ELISA plate reader (Sunrise Microplate Reader, Tecan Trading AG, Switzerland). AGP plasma concentration was measured using ELISA Kit (ab157690; Abcam, Cambridge MA) according to the manufacturer's instructions.

Gene expression was analyzed using the procedures detailed in the Gene Expression Analysis (3.3) section of the following functional target genes: tight junction proteins (Claudin-1 and 3), enterocyte protection (HSP-70), immune function (TLR-2 β , TLR4, TGF- β 4, IL-8, and Bu-1) and carbohydrate (ChREBP) and lipid (FABP6) metabolism.

Samples collected at 15 d of age were embedded in paraffin using tissue processor. Sections of 2.5 μ m were stained with haematoxylin and eosin analyzed with an Olympus BX-40 (Olympus Optical Co., Ltd., Tokyo, Japan) digital camera and the Soft software version 3.2 C4040 Z (Soft Imaging System, Olympus, GmbH, Hamburg, Germany). Images were analyzed eye blinded by the same person. Villus height (VH) and crypt depth (CD) of 9 intact villi per section were recorded for each animal.

Table 3. *Ingredients and chemical composition (% as fed basis, unless otherwise indicated) of starter (1 to 21 d) and grower diets (21 to 32 d).*

	Starter diet	Grower diet
Ingredient composition		
Maize	54.28	56.41
Soybean meal (48% CP)	38.43	35.73
Soybean oil	3.50	1.44
Animal fat	-	3.00
Dicalcium phosphate	1.82	1.74
Calcium carbonate	0.76	0.71
Salt	0.40	0.38
DL-methionine	0.29	0.22
L-lysine HCl	0.13	0.03
L-threonine	0.02	-
Minerals & Vitamins ¹	0.30	0.30
Choline chloride	0.05	0.02
Noxyfeed ²	0.02	0.02
Calculated composition		
AME, Kcal/Kg	2974	3088
Crude protein	23.0	21.8
Ash	6.30	5.90
Ether extract	6.10	7.10
Lysine	1.29	1.20
Methionine	0.62	0.55
Methionine + cysteine	0.97	0.90
Threonine	0.84	0.82
Tryptophan	0.25	0.25
Calcium	0.95	0.90
Total phosphorus	0.70	0.67
Non phytate phosphorus	0.45	0.43
Sodium	0.17	0.16
Analyzed composition (% DM)		
Dry matter	88.4	89.2
Ash	6.30	6.10
Crude protein	23.8	21.2
Ether Extract	6.94	7.50
Gross energy (Kcal/kg)	4030	4082

¹ The premix provides per kg feed: vitamin A (E672) 10000 IU; vitamin D₃ (E671) 4800 IU; vitamin E (alfa tocopherol) 45 mg; vitamin B₁ 3 mg; vitamin B₂ 9 mg; vitamin B₆ 4.5 mg; vitamin B₁₂ 40 µg; vitamin K₃ 3 mg; calcium panthotenate 16.5 mg; nicotinic acid 51 mg; folic acid 1.8 mg; biotin 0.15 mg; Fe (E1) (from FeSO₄·H₂O) 54 mg; I (E2) (from Ca(IO₃)₂) 1.2 mg; Cu (E4) (from CuSO₄·5H₂O) 12 mg; Mn (E5) (from MnO) 90 mg; Zn (E6) (from ZnO) 66 mg; Se (E8) (from Na₂SeO₃) 0.18 mg. ² ITPSA, Barcelona, Spain. Contains BHT+ propyl galate (56%) and citric acid (14%).

3.1.3. OBJECTIVE 3: Investigate an experimental procedure of coccidial vaccine challenge in battery cages and the anticoccidial effect of an olive pomace extract in broiler chickens

A feeding trial was carried out at the University of Arkansas Poultry Research Farm (Department of Poultry Science and The Center of Excellence for Poultry Science). A total of four hundred 1-d old male by-product chicks from a Cobb 500 female line were obtained from a commercial hatchery (Cobb Vantress Hatchery, Fayetteville, Arkansas, USA). Chicks were randomly distributed among 50 battery cages (0.61 × 0.61m) with 8 birds each (37.6 ± 0.4 g) in thermostatically-controlled rooms. Cages were equipped with wire flooring, 2 nipple drinkers, and a trough style feeder. The temperature of rooms was set at 33°C at the placement and gradually decreased to 22°C by 28 d post-hatch. A photoperiod of 23 hours light and 1 h dark was set from the first 7 d of age and 18 hours light and 4 hours dark until the end of the experiment at 28 d of age.

Birds were fed experimental diets *ad libitum* across 2 feeding phases consisting of a starter phase from 1 to 14 d of age and a grower phase from 15 to 28 d of age. Test additives were added to basal starter and grower diets that were corn and soybean meal-based and formulated to meet NRC (1994) and in mash form. Feeds were mixed at the University of Arkansas Poultry Feed Mill (Fayetteville, Arkansas, USA). Basal diets were supplemented with 0, 500 or 1500 ppm of a bioactive olive pomace extract (OE, Lucta S.A.; Spain) or with 500 ppm of a commercial coccidiostat (Clinacox, Huvepharma, Peachtree City, GA; 0.2% diclazuril) in powdered form (Table 4).

At the beginning of the trial, all birds were weighed and randomly allotted to 5 experimental treatments (10 cages/treatment, with 8 chicks/cage) in a randomized complete block design. One group was fed the control diet without any additives and was not challenged (NCU). The other 4 groups were orally challenged with *Eimeria* spp. and fed their respective experimental diets as follows: control basal diet with no additives or coccidiostat (NCC), basal diet supplemented with 500 ppm of a commercial coccidiostat (PCC; Clinacox, 0.2% diclazuril) or with 500 (OE500C) or 1500 ppm (OE1500C) of an olive pomace extract. Supplemented OE was a standardized olive pomace extract containing $\geq 6\%$ of triterpenes and $\geq 1\%$ of polyphenols, quantified by HPLC-UV as oleanolic acid and hydroxytyrosol equivalents respectively.

Upon arrival, all birds except those in the NCU group were orally-gavaged with a live oocyst vaccine (Coccivac-B52, Merck Animal Health, Intervet Inc. Millsboro, DE, USA). An oral gavage (0.25 mL/bird diluted in distilled water) was used to provide uniform administration and animals of NCU group received 0.25 mL of distilled water. At 7 and 14 d of age, birds in the challenge treatments (NCC, OE500C, OE1500C, and PCC) were orally inoculated with the same vaccine (1 mL) at 4 and 16x, respectively, of the manufacturer's recommendation, while chickens in the NCU group received 1 mL of distilled water (Figure 8). According to the manufacturer, the vaccine contained live sporulated oocysts of *E. maxima*, *E. maxima* MF, *E. acervulina*, *E. tenella*, and *E. mivati*.

Figure 8. Diagram of the experimental design and *Eimeria* challenge of the trial.

Body weight and feed consumption were determined by cage at 7, 14, 20, and 28 d of age to calculate the body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR). At the end of the experiment at 28 d of age, chickens were slaughtered by inhalation of CO₂. To determine the number of oocysts shed in excreta, feces from each cage (excreted feces 24 h before sampling) were sampled at 7, 14 and 21 d post hatch. At 20 d of age, two birds per pen were randomly selected and slaughtered to analyze ileal mucosa gene expression, duodenal and jejunal morphology and plasma concentrations of carotenoids (CAR), interleukin 1 β (IL-1 β) and interleukin 8 (IL-8), and alpha-1 glycoprotein (AGP). For gene expression analysis, 200 mg of ileal mucosal scrapings were collected in RNA stabilization solution (RNAlater®, Invitrogen, Carlsbad, CA) following the manufacturer's instruction and subsequently stored at -80°C. For intestinal morphology, 3 cm long segments of the middle part of duodenum and jejunum (proximal to the Meckel's diverticulum) were stored in 10% neutral buffered formaldehyde solution

for 24 hours and then transferred into 70% of ethanol solution. Blood samples were collected immediately post-mortem via cardiac puncture into tubes containing K2 EDTA (BD Vacutainer), held in ice, and centrifuged at 1300 x g for 15 min and 4°C to collect plasma. Samples were stored at -80°C for further analysis of carotenoids, IL-1 β , IL-8 and AGP plasma concentrations. At the end of the trial (28 d of age), the remaining animals were slaughtered by CO₂ inhalation and two birds per cage were randomly selected to analyze carotenoid plasma concentrations as previously described.

A representative sample of 30 g of excreta from each cage were sampled and stored at 4°C. All samples were processed within 1 week of collection. After recording the standardized excreta weight (10 g), 40 mL of water was added and samples were soaked overnight. Then, 60 mL of water was added and mixed for approximately 10 min. The solubilized sample (1 mL) was added to 9 mL of a saturated salt solution in a test tube and mixed. The solution was pipetted (1 mL) into two chambers of a McMaster counting slide. The McMaster slide was examined under light microscope at 10x magnification. A total of two slides were counted and averaged, and the oocyst per gram (OPG) was calculated based on the following formula:

$$OPG = \frac{OAC \times DF \times FSV}{CVV \times WE},$$

where, OAC is the oocyst average count, DF is the dilution factor (10), FSV is the fecal sample volume (mL), CVV is the counting chamber volume (0.15 mL) and, WE is the weight of excreta (g).

Samples collected at 20 d of age were embedded in paraffin using a tissue processor. Sections of 2.5 μ m were stained with haematoxylin and eosin and subsequently imaged with an Olympus BX-40 (Olympus Optical Co., Ltd., Tokyo, Japan) digital camera and the Soft software version 3.2 C4040 Z (Soft Imaging System, Olympus, GmbH, Hamburg, Germany). Images were analyzed by the same person who was blinded to sample treatment. Villus height and crypt depth of 9 intact villi per section were recorded for each animal.

Carotenoids plasma concentrations of 2 birds per cage were analyzed at 20 and 28 days of age. Blood processing and carotenoid analysis were conducted under yellow light. Plasma was pooled, aliquoted, and stored at -80°C until further analysis. CAR was analyzed following the spectrophotometry method as described by Allen (1987).

Quantification in plasma of cytokines was performed with commercial ELISA according to manufacturer's instructions (IL-1 β , SEA565Ga; IL-8, SEA080Ga, Wuhan USCN Business Co. Ltd., Wuhan, China). Kit standards and test sample optical densities were read at 450 nm by an ELISA plate reader (Sunrise Microplate Reader, Tecan Trading AG, Switzerland). Plasma concentration of AGP was measured using ELISA Kit (ab157690; Abcam, Cambridge MA) according to the manufacturer's instructions.

Gene expression was analyzed using the procedures detailed in the Gene Expression Analysis (3.3) section of the following functional target genes: ubiquitin (UB) (housekeeping), toll like receptor 4 (TLR4), interleukin 8 (IL-8) and 1 β (IL-1 β), interferon gamma (IFN- γ), B cell marker (Bu-1), and Claudin-1 (Claudin1) were obtained from the literature (Table 5).

Table 4. Ingredients and chemical composition (% , as fed basis, unless otherwise indicated) of starter (1 to 14 d) and grower diet (15 to 28 d).

Item	Starter diet				Grower diet			
	NCU/ NCC	OE500C	OE1500C	PCC	NCU/ NCC	OE500C	OE1500C	PCC
Ingredients								
Corn	60.87	60.87	60.87	60.87	64.80	64.80	64.80	64.80
Soybean meal	34.32	34.32	34.32	34.32	29.77	29.77	29.77	29.77
Soy oil	1.15	1.15	1.15	1.15	2.03	2.03	2.03	2.03
Limestone	1.11	1.11	1.11	1.11	1.07	1.07	1.07	1.07
Dicalcium phosphate	1.05	1.05	1.05	1.05	0.92	0.92	0.92	0.92
Sodium chloride	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39
DL-methionine	0.32	0.32	0.32	0.32	0.27	0.27	0.27	0.27
L-lysine·HCl	0.21	0.21	0.21	0.21	0.18	0.18	0.18	0.18
Inert filler (sand)	0.20	0.15	0.05	0.15	0.20	0.15	0.05	0.15
Trace mineral premix ¹	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix ²	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
L-threonine	0.085	0.085	0.085	0.085	0.073	0.073	0.073	0.073
Choline chloride (60%)	0.05	0.05	0.05	0.05	0.050	0.050	0.050	0.050
Phytase ³	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Selenium premix, 0.06%	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Clinacox®	-	-	-	0.050	-	-	-	0.050
OE	-	0.050	0.15	-	-	0.050	0.15	-
Calculated composition								
AME, Kcal/Kg	3008	3008	3008	3008	3100	3100	3100	3100
Crude protein	21.45	21.45	21.45	21.45	19.5	19.5	19.5	19.5
Ash	2.73	2.73	2.73	2.73	2.50	2.50	2.50	2.50
Ether extract	2.83	2.83	2.83	2.83	2.88	2.88	2.88	2.88
dLys	1.18	1.18	1.18	1.18	1.05	1.05	1.05	1.05
dTSAA	0.89	0.89	0.89	0.89	0.80	0.80	0.80	0.80
dThr	0.77	0.77	0.77	0.77	0.69	0.69	0.69	0.69
Calcium	0.90	0.90	0.90	0.90	0.84	0.84	0.84	0.84
Available P	0.45	0.45	0.45	0.45	0.42	0.42	0.42	0.42
Sodium	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18

¹ Supplied the following per kg of diet: manganese, 100 mg; zinc, 100 mg; copper, 10.0 mg; iodine, 1.0 mg; iron, 50 mg; magnesium, 27 mg. ² Supplied the following per kg of diet: vitamin A, 6173 IU; vitamin D3, 4409 ICU; vitamin E, 44 IU; vitamin B12, 0.01 mg; menadione, 1.20 mg; riboflavin, 5.29 mg; d-pantothenic acid, 7.94 mg; thiamine, 1.23 mg; niacin, 30.86 mg; pyridoxine, 2.20 mg; folic acid, 0.71 mg; biotin, 0.07 mg; manganese, 24 mg; zinc, 14.4 mg; selenium, 0.04 mg; copper, 0.68 mg; iodine, 0.47 mg. ³ OptiPhos 2000PF (Huvepharma, Peachtree City, GA) added to provide 250 FTU/kg of phytase.

3.2. CHEMICAL ANALYSIS

The AOAC (2000) standard methods were used to analyze the concentration of dry matter (934.01), total ash in muffle (942.05), ether extract (920.39), crude protein by combustion method (968.06) using the FP-528 nitrogen analyzer (LECO®, St. Joseph, MI, EE.UU). The concentration of gross energy was analyzed by adiabatic bomb calorimeter (PARR, 1356 model; Parr Instrument Company, Moline, IL, EE.UU). Acid insoluble ash of diets, and ileal contents were determined as indicated by De Coca-Sinova et al. (2011).

3.3. GENE EXPRESSION ANALYSIS

For gene expression analysis, 200 mg of ileal (approximately 4 cm from the Meckel diverticulum) mucosal scraping were sampled in RNA later (Invitrogen, Carlsbad, CA) following the instructions of the manufacturer, and further stored at -80°C. Total RNA was extracted from approximately 50 mg of mucosal scraping with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), disrupted with a mixer mill MM-400 (Retsch, Stuttgart, Germany) and isolated by using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Corporation, St. Louis, MO, USA). To prevent genomic DNA contamination an “in column” DNase step was performed by using the RNase-Free DNase Set (Quiagen, Australia). Extracted RNA yield and quality were measured by spectrophotometry (Epoch™, BioTek, Winoosky, VT, USA) combined with the Take3™ Micro-Volume Plate (BioTek, Santa Barbara, CA, USA) by absorbance at wavelengths of 260 and 280 nm.

Reverse transcription of around 2400 ng of extracted RNA was performed with the SuperScript VILO Master Mix (Invitrogen, Carlsbad, CA, USA). The quantitative Real-Time PCR (qRT-PCR) analysis was performed in a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) with already tested and published primer conditions (Table 5). Primers for chicken interleukin 2 (IL-2) were designed from the GenBank sequence with accession number AF000631.1 using Primer Express v.2 software (Applied Biosystems, Foster City, CA, USA). Samples were analyzed in triplicate using the right amount of each primer, ultra-purified water, and SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA).

Table 5. Genes, forward and reverse primers, and efficiency for gene expression analysis by qRT-PCR.

Gene ¹	5'-Primer sequence Forward- 3'	5'-Primer sequence Reverse- 3'	Efficiency		
			Exp 1	Exp 2	Exp 3
UB ²	GGGATGCAGATCTTCGTGAAA	CTTGCCAGCAAAGATCAACCTT	1.88	1.94	1.94
β Actin ³	GTGATGGACTCTGGTGATGG	TGGTGAAGCTGTAGCCTCTC	1.91	1.88	-
LXR ⁴	CCCAAGTCCCTGACCCTAAT	GGCTTCCACATAGGTGTGCT	2.00	-	-
ASBT ⁵	GTGATGATCATGGGATGCTG	TGCAAGAAGTGTGGAGCAAG	1.81	-	-
ChREBP ⁴	CTGAGCGATCGAAGGTGAA	TCTCCATCTTGCTGGAGTCA	1.94	2.06	-
SREBP1 ⁴	GAGGAAGGCCATCGAGTACA	GGAAGACAAAGGCACAGAGG	1.99	-	-
IFN γ 1 ⁶	GTGAAGAAGGTGAAAGATATCATGGA	GCTTTGCGCTGGATTCTCA	1.80	-	-
IFN γ 2 ¹¹	AGCTGACGGTGGACCTATTATT	GGCTTTGCGCTGGATTTC	-	-	1.94
FABP2 ⁷	AAAGATAATGGAAAAGTACTCACAGCAT	CCTTCGTACACGTAGGTCTGTATGA	1.96	-	1.76
FABP6 ⁵	TGATTTCCCTGGACTCAGC	CCCACCTTCCATTTTGACTG	1.92	1.84	1.86
IL-1 β ¹¹	TGGGCATCAAGGGCTACA	TCGGGTTGGTTGGTGATG	-	-	2.01
IL-2	CAAGATTCATCTCGAGCTCTACACA	CCCAGGTAACACTGCAGAGTTTG	1.89	-	-
IL-6 ⁷	GAGGGCCGTTCGCTATTTG	ATTGTGCCCCGAACATAAACATTC	2.00	-	-
IL-8 (CXCLi2) ⁸	CCTGGTTTTAGCTGCTCTGT	GCGTCAGCTTCACATCTTGA	1.95	1.88	1.98
Claudin1 ⁷	TGGCCACGTCATGGTATGG	AACGGGTGTGAAAGGGTCATAG	1.90	1.93	1.96
Claudin3 ¹⁰	GCCAAGATCACCATCGTCTC	CACCAGCGGGTTGTAGAAAT	-	1.98	-
HSP-70 ¹⁰	GGCTGGAGAGAAGAATGTGC	CAGCTGTGGACTTCACCTCA	-	1.92	-
TGF- β 4 ⁷	CGGCCGACGATGAGTGGCTC	CGGGGCCCATCTCACAGGGA	2.08	2.11	-
Bu-1 ⁹	GGTGTCCAGTGAAGGTGTG	GATGCAAAGGATGGGTGTC	1.91	1.92	1.94
TLR-2 β ⁷	CGCTTAGGAGAGACAATCTGTGAA	GCCTGTTTTAGGGATTTTCAGAGAATTT	1.89	1.92	-
CD3 γ δ ⁹	CAGGGATTGTGGTCGCAGAT	TACTGTCCATCATTCGGCTCAC	1.92	-	-
TLR4 ⁷	AGTCTGAAATTGCTGAGCTCAAAT	GCGACGTTAAGCCATGGAAG	2.00	1.97	1.94

¹ UB, ubiquitin; LXR, liver X receptor; ASBT, apical sodium dependent bile acid transporter; ChREBP, carbohydrate responsive element-binding protein; SREBP1, sterol regulatory element-binding protein 1; IFN- γ , interferon-gamma; FABP2, fatty acid binding protein 2; FABP6, fatty acid binding protein 6; IL-1 β , interleukin 1 beta; IL-2, interleukin 2; IL-6, interleukin 6; CXCLi2 former, interleukin 8; Claudin 1 and 3; HSP-70, heat shock protein 70; TGF- β 4, transforming growth factor beta 4; Bu-1, chicken B-cell marker chB6; TLR-2 β , toll like receptor 2 β ; CD3 γ δ , marker of active avian T lymphocytes; TLR4, toll like receptor 4. ² De Boever et al., 2008; ³ Wang et al., 2009; ⁴ Proszkowiec-Weglarz et al., 2009; ⁵ Mcquaid 2012; ⁶ Rothwell L. et al., 2004; ⁷ Chen J. et al., 2015; ⁸ Rasoli et al., 2015; ⁹ Bar-Shira et al., 2003 ; ¹⁰ Gilani et al., 2018b ; ¹¹ Hong et al., 2006.

3.4. STATISTICAL ANALYSIS

Statistical analysis was performed using SAS procedures (release 9.2; SAS Institute) in all three experiments. Shapiro-Wilk and Levene's test were used to analyze the normality of data of all experiments and variance homogeneity, respectively.

In Experiment 1, dietary effects on performances, AID and bile acids was analyzed by ANOVA by using a mixed-model with diet as fixed factor and pen as the experimental unit. When the ANOVA was significant, Tukey test ($\alpha = 0.05$) was applied to separate differences among means. For microbiota analysis, to de-multiplex, quality-filter, and analyze the microbial raw sequencing reads QIIME 1.9.1 was utilized (Caporaso et al., 2010). Then, for taxonomy analyses, reads were clustered into operational taxonomic units (OTUs) and taxonomic assignment of representative OTUs was performed using RDP classifier (Wang et al., 2007). Representative sequences were aligned by Py-Nast as default in quantitative insights into microbial ecology (QIIME) pipeline (Casparoso et al., 2010) and 0.005% of total OTUs were discarded. Shannon index was assessed to analyze alpha diversity and the nonparametric Kruskal-Wallis test was applied to evaluate the statistical significance ($P < 0.05$). Within gene expression data, the comparison of chickens fed Monensin and OE treatments relative to the control group were determined using a mixed-model in which a gene-specific effect and a sample-specific effect were treated as random variables and treatment was considered fixed (Steibel et al., 2009). For genes displaying efficiencies different from 2 ($E \neq 2$), cycle threshold (Ct) values were adjusted according to the model described by Steibel et al. (2009). The standard error was used to recalculate the lower and upper 95% confidence intervals for each fold change.

In Experiment 2, diets were the main fixed effect in the model. For performance analysis, the experimental unit was the pen and for the intestinal morphology, lactulose and mannitol plasma concentration and gene expression analysis the animal was the experimental unit. The effect of short-term fasting on the studied variables was determined by a contrast comparing CNF and CF groups. Polynomial contrasts were used to test the linear and no linear effect of OE inclusion. The positive control group (MF) was compared with the rest of the fasted groups by using the Dunnett's test at $\alpha = 0.05$. Results are presented in tables as mean and the standard error of means (SEM). For gene

displaying efficiencies different from 2 ($E \neq 2$), Ct values were adjusted according to the model described by Steibel et al. (2009).

In Experiment 3, all data were subjected to ANOVA using the GLM procedure of SAS (release 9.2; SAS Institute) with diets and block as fixed effects. For growth performance and oocyst counts, the experimental unit was the cage and for the intestinal morphology, gene expression and plasma concentration of AGP, IL-1 β , IL-8 and CAR the animal was the experimental unit. The effect of *Eimeria* spp challenge on the studied variables was determined by a contrast comparing NCU and NCC groups. Proc IML of SAS was used to generate the polynomial coefficients for the unequally spaced treatments which were included in polynomial contrasts to test the linear and no linear effect of the OE inclusion. To analyze differences among challenge groups, the Tukey's test at $P < 0.05$ was used. Oocyst per gram were analyzed using a Poisson model (GENMOD procedure and considering a Poisson distribution). Results are presented in tables as mean and the standard error of means (SEM) except in oocyst counting results, which results are presented as means of natural logarithmic transformation and the standard error. For gene displaying efficiencies different from 2 ($E \neq 2$), Ct values were adjusted according to the previous model described by other authors (Steibel et al., 2009). Cytokine IL-8 and IL-1 β plasma concentrations were natural log transformed before being statistically analyzed to fulfill the population normality and variance homogeneity assumptions.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. OBJECTIVE 1: Study the effects of supplementing grower diets with an olive pomace extract on broiler performance and gut health

The effects of experimental diets on growth performance data are shown in Table 6. From 28 to 35 d of age, broilers fed the OE and Monensin diets showed better FCR than those fed the Control diet ($P < 0.05$). During the last period, from 35 to 42 d of age, no significant differences were observed in ADFI. However, animals fed with Control showed lower ADG ($P < 0.01$) than those fed Monensin and OE diets resulting in a worse FCR ($P < 0.001$) for Control diet. In the global period, from 21 to 42 d of age, a lower ADG ($P < 0.05$) was observed in birds fed Control compared to Monensin and OE groups. Furthermore, no significant differences in ADFI were reported among treatments and hence broilers in Control diet showed worse FCR than animals fed the Monensin and OE ($P < 0.001$). These results are in agreement with Sarica and Ürmez (2016) who reported also higher final BW and better FCR in broilers fed olive leaf extracts. By contrast Leskovec et al. (2018) and King et al. (2014) were unable to detect significant differences in productive parameters after supplementing the feed and water with olive leaf and pomace extracts, respectively. However, the specified examples were performed with extracts from olive leaves (Leskovec et al., 2018) or olive pulp (King et al., 2014) rich in polyphenols, while in the present experiment extract contained a higher prevalence of triterpenes over the polyphenolic fraction. Contradictory results on broiler performance with other plant extracts rich in bioactive compounds have also been described (Leskovec et al., 2018) and it has been proven that not only the different botanical origin but also plant location, harvesting conditions, processing, and storage can affect the extract composition and activity (Huyghebaert et al., 2011). Therefore, the different concentration of the bioactive substances in the extracts (i.e., percentage of total triterpenes and polyphenols) and/or the route of administration (feed vs. water) might be behind the different results on performance among olive byproduct extract studies.

Table 6. Effect of experimental diets on broiler chicken growth performance from 21 to 42 days of age.¹

Item ²	Control	Monensin	OE	SEM ³	P value
21 - 28 d					
ADG (g/bird)	103	103	100	2.69	0.77
ADFI (g/bird)	149	146	146	3.06	0.59
FCR (g/g)	1.46	1.42	1.46	0.014	0.12
28 - 35 d					
ADG (g/bird)	105	107	108	2.46	0.60
ADFI (g/bird)	185	182	184	4.16	0.87
FCR (g/g)	1.77 ^a	1.71 ^b	1.71 ^b	0.018	0.039
35 - 42 d					
ADG (g/bird)	77.8 ^b	103 ^a	97.2 ^a	4.18	0.002
ADFI (g/bird)	193	202	205	4.68	0.22
FCR (g/g)	2.50 ^a	1.98 ^b	2.12 ^b	0.083	0.001
21 - 42 d					
ADG (g/bird)	95.1 ^b	104 ^a	102 ^a	2.03	0.018
ADFI (g/bird)	176	177	178	3.37	0.89
FCR (g/g)	1.91 ^a	1.70 ^b	1.76 ^b	0.029	<0.001

^{a,b} Means within a column and main effect not sharing a common superscript are significantly different by Tukey's test ($P \leq 0.05$). ¹ Control, negative control with no additives; Monensin, basal diet supplemented with 100 ppm of monensin; OE, basal diet supplemented with 750 ppm of OE. ² ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio. ³ SEM, standard error of means (n = 6 replicates with 14 birds each).

Plant extracts might improve broiler growth and feed efficiency by increasing nutrient digestibility as they can affect digestive enzyme function, intestinal morphology gastrointestinal tract motility, or bile acid secretion (Lee et al., 2003; Brenes and Roura, 2010; Bozkurt et al., 2016; Leskovec et al., 2018). In the present study, no significant differences on GE, OM, and, EE apparent ileal digestibility were observed among treatments (Table 7). However, birds fed Monensin showed higher CP AID ($P < 0.05$) than those fed Control diet, and OE group showed intermediate values. On the other hand, no significant differences were observed in conjugated or unconjugated bile acid concentration in ileal digesta and plasma (Table 8). However, the sum of the total bile acids tended ($P = 0.055$) to be higher in birds fed the Monensin and the predominant bile acid in ileal digesta was CDCA while tauroursodeoxycholic acid (TCDCA) was the predominant in plasma. These results are aligned the study of Leskovec et al. (2018) who reported no significant differences in nutrient apparent total tract digestibility coefficients among broilers fed the olive leaf extract and the no supplemented control. However, in

the present experiment birds fed the Monensin supplemented diet showed a better protein AID than the control but similar to those fed the OE. Recent studies support that the positive effects of Monensin on broiler growth and FCR might partially be explained by a rise in intestinal digestive enzyme activities including chymotrypsin (Bozkurt et al., 2016). Other studies have also reported improvement on broiler FCR with Monensin due to an increase of conjugated bile acids (TCA and TCDCA) and hence better dietary fat digestibility (Guban et al., 2006). Moreover, studies performed in rats have shown the ability of plant extracts rich in polyphenols to affect lipid metabolism and bile acid composition (Fotschki et al., 2017). In the present experiment, the lack of significant differences on intestinal or plasma bile acid composition among treatments is in agreement with an absence of effect on dietary fat digestibility.

Table 7. Effect of experimental diets on the apparent ileal digestibility (AID, %) of nutrients in broilers at 42 d of age.¹

Item	Control	Monensin	OE	SEM ²	P value
Digestibility					
Dry matter	65.4	68.6	66.2	0.99	0.095
Gross energy	69.5	72.5	70.2	1.04	0.13
Ether extract	83.0	84.2	82.1	1.54	0.62
Organic matter	68.5	71.7	69.2	1.06	0.12
Crude protein	72.1 ^b	77.8 ^a	73.8 ^{ab}	1.41	0.035

^{a,b} Means within a column and main effect not sharing a common superscript are significantly different by Tukey's test ($P \leq 0.05$). ¹ Control, negative control with no additives; Monensin, basal diet supplemented with 100 ppm of monensin; OE, basal diet supplemented with 750 ppm of OE. ² SEM, standard error of means ($n = 6$).

In addition, the ileal expression of genes encoding proteins involved in bile acid and lipid metabolism such as LXR, ASBT, ChREBP, SREBP1, FABP2, and FABP6 was not affected by dietary treatment (Figure 11). However, as mentioned previously, in the present study total bile acid concentration tended to be higher in birds fed the Monensin supplemented diet. Besides their role as physiological detergents that facilitate the absorption of lipids bile acids act as “hormone-like” molecules involved in several signaling pathways including the regulation of lipid, glucose, and energy metabolism (Li and Chiang, 2015). The composition of the bile acids pool is a balance between the primary (unconjugated) bile acids synthesized in the liver and their modifications to secondary (conjugated) bile acids in the intestine generated by the microbiome (Li and Chiang, 2015). After the transport of bile acids back to the liver via portal blood the

synthesis of new primary bile acids takes place according to the physiological needs. This link between gut microbiota and hepatic bile acid synthesis might be useful to detect functional changes in the microbiome or potential changes in microbial populations impacting the animal energy metabolism. Results from this study suggest a better performance in birds fed the Monensin supplemented diets because of a better protein digestibility and higher ileal total bile acid concentrations. By contrast, our results show that the positive effects of OE on performance seem not to be due to improvement in nutrient digestibility or bile acid pool modifications.

Table 8. Effect of experimental diets on bile acid composition in ileal contents and plasma of broilers at 42 d of age.¹

Item ²	Control	Monensin	OE	SEM ³	P value ⁴
Ileal content (μmol/g of sample)					
TCA	0.094	0.15	0.11	0.042	0.93
TCDCA	1.21	3.84	1.27	0.99	0.14
AVCA	0.17	0.31	0.23	0.055	0.19
CA	0.56	1.39	1.01	0.27	0.094
CDCA	4.61	9.63	8.07	2.14	0.12
Σ Conjugated	1.30	3.98	1.37	1.01	0.14
Σ Unconjugated	5.35	11.3	9.31	2.24	0.097
Total BA	6.65 ^b	15.3 ^a	10.7 ^{ab}	2.89	0.055
Plasma (nmol/mL of plasma)					
TCA	0.41	0.72	0.77	0.19	0.37
TCDCA	14.2	13.6	14.8	2.59	0.97
TLCA	0.29	0.29	0.29	0.022	0.95
CDCA	0.31	0.36	0.64	0.21	0.24
Σ Conjugated	14.9	14.6	15.9	2.75	0.96
Σ Unconjugated	0.31	0.36	0.64	0.21	0.24
Total BA	15.2	14.9	16.5	2.9	0.72

^{a,b} Means within a column and main effect not sharing a common superscript are significantly different by Tukey's test ($P \leq 0.05$). ¹ Control, negative control with no additives; Monensin, basal diet supplemented with 100 ppm of monensin; OE, basal diet supplemented with 750 ppm of OE. ² TCA, Taurocholic acid; TCDCA, Tauroursodeoxycholic acid; TLCA, Taurolithocholic acid; AVCA, Avicholic acid; CA, Cholic acid; CDCA, chenodeoxycholic acid. ³ SEM, standard error of means (n = 6). ⁴ P value are from square root data transformation analysis.

Gastrointestinal microbiome plays an important role for gut health and nutrition in poultry production (Xiao et al., 2016). Phytochemicals, probiotics, prebiotics, or exogenous enzymes are commonly used to modulate gut microbiome (Page and Richards, 2005; Oakley et al., 2014). In this context, bioactive compounds of olive pomace such as

oleuropein and hydroxytyrosol are good candidates to modulate the composition of gut microbiota and enhance gut integrity (Sarica and Ürmez, 2016). It is known that triterpenes offered through the diet are only partially absorbed in the upper digestive tract (Yin et al., 2012) and are also present along the GIT tract (Lozano-Mena et al., 2016). Therefore, coating technologies are not required in this kind of extracts to reach the gastrointestinal tract and perform its activity. In the present study, no significant ($P > 0.05$) differences among dietary treatments were observed in relative abundance of the main bacteria family (Figure 9). *Firmicutes* was the predominant phylum in the caeca which is in agreement with Danzeisen et al. (2011) and numerically, the most abundant families were *Clostridiaceae* in Control (41.7%) and Monensin (51.1%) treatments, and *Lactobacillaceae* in OE (65.5%) treatment. Also, the diversity of caecal microbiota measured by the Shannon index was similar ($P > 0.05$) among treatments (Figure 10). This in agreement to results obtained by Liehr et al. (2017) who observed no differences in composition and diversity of gut microbiome, at the phylum level, in pigs fed diets supplemented with an olive oil bioactive extract similar than the one used in this study. However, our results partially agree with Sarica and Ürmez (2016) who reported no changes in total aerobic bacteria but significant differences on *E. coli* and *Lactobacilli* counts in ileal samples of birds fed olive leaf extracts or a control diet with no additives. Again, different extracts origin (leaf vs. pomace) and composition (oleuropein vs. triterpenes) might potentially explain these discrepancies. On the other hand, monensin inclusion in chicken diets has been described to decrease *Lactobacillus* species (Guban et al., 2006; Danzeisen et al., 2011). No evidence was found that could justify these contradictory results; however, the limited number of replicates in the present study, or factors such as diet, broiler breed, the environment of the experimental farm, or technical methodologies might be behind discrepant results among microbiome studies in poultry (Danzeisen et al., 2011). Further analysis to confirm the lack of effect of the OE on bacteria at the genus or species level is needed.

Figure 9. Effects of feeding broiler chicken diets supplemented with 100 ppm of monensin or 750 ppm of OE on the relative abundance of bacteria families in caecal content ($n = 6$). Control, negative control with no additives; Monensin, basal diet supplemented with 100 ppm of monensin; OE, basal diet supplemented with 750 ppm of OE.

Figure 10. Effects of feeding broiler chicken diets supplemented with 100 ppm of monensin or 750 ppm of OE on the relative diversity of caecal microbiota (Shannon index) ($n = 6$). Control, negative control with no additives; Monensin, basal diet supplemented with 100 ppm of monensin; OE, basal diet supplemented with 750 ppm of OE.

Plant extracts may exert their beneficial effects on growth performance among others because of their antioxidant and/or immunomodulatory effects (Lillehoj et al., 2018). Previous studies conducted with extracts of similar composition to the one used in this study showed better animal performance related to its anti-inflammatory function rather than the antioxidant one (Gisbert et al., 2017; Liehr et al., 2017; Tedó et al., 2018). This made us explore only the potential reduction of the inflammatory status of the animals. Some plant extracts have shown immunomodulatory effects in studies in which poultry and pigs were experimentally infected (Zeng et al., 2015; Sugiharto, 2016). These effects include increase in lymphocyte proliferation, serum level antibodies, decrease of pro-inflammatory, and increase of anti-inflammatory cytokines (Zeng et al., 2015; Sugiharto, 2016). In a recent study, Liehr et al. (2017) reported a beneficial effect of an OE extract in pigs challenged with LPS on immune response by reducing the pro-inflammatory IL-1 β in plasma leading also to a better animal performance. Despite inflammation is normally associated with pathogen outbreaks there might be also a moderate intestinal inflammation in response to the high energy diets used under normal productive circumstances (Niewold, 2014). Moreover, as suggested by Niewold (2014)

plant extracts, like antibiotics, might increase broiler performance because of their anti-inflammatory effects at intestinal level. Regarding the gene expression results related to immune system of the present study, the expression of intestinal IL-2, IL-6, CD3 γ δ , Claudin1, TLR-2 β and IFN γ was not affected ($P > 0.05$) by dietary treatments (Figure 11). However, birds fed the OE showed a significant ($P < 0.05$) decrease of IL-8 expression in the ileum. This chemokine plays an important role in the chicken inflammatory response recruiting heterophil at a local and systemic level in response to bacterial presence (Kogut, 2002). Moreover, the expression of TGF- β 4 was significantly up-regulated in birds fed Monensin ($P < 0.05$) and OE treatments ($P < 0.01$) compared to C. The expression of Bu-1 showed a similar pattern with a significant ($P < 0.001$) up-regulation in Monensin and OE compared Control. Therefore, it is plausible that the better performance observed in birds fed the Monensin and OE diets in our experiment might be related to a lower intestinal inflammatory response explained by the immunosuppressant effect on IL-8 and the increase anti-inflammatory expression of TGF- β 4 under on-acute inflammatory conditions.

In conclusion, the present experiment shows that the inclusion of 750 ppm of an olive pomace extract containing a minimum of 10% total triterpenes and 2% polyphenols positively affects growth in broiler chickens. Among the possible mechanism of action studied so far in this work improvement in performance is likely related to the extract anti-inflammatory properties.

Figure 11. Effects of feeding broiler chicken diets supplemented with 100 ppm of monensin (Monensin) or 750 ppm of OE (OE) on the expression of selected genes in the ileum. Gene expression values are fold change relative to the mRNA levels in the control diet (C) set to be 1.0 (horizontal axis). Bars indicate the 95% confidence interval (Fold change up – Fold change low) ($n = 12$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$).

4.2. OBJECTIVE 2: Evaluate the effects of supplementing starter and grower diets with an olive pomace extract on broiler performance and gut health after a challenge of intestinal permeability induced by a short-term fasting period

4.2.1. FASTING PERIOD EFFECTS

The effects of the short-term fasting period on growth performance are shown in Table 9. In the present study birds fasted for 15.5 h at 14 d of age showed similar ADFI and FCR from 14 to 21 d of age than no fasted birds; however, the ADG tended to be lower in fasted birds ($P = 0.06$). From 21 to 31 d of age, short-term fasted and no fasted showed similar performance. Chamblee et al. (1989) observed a rapid compensatory growth in birds upon refeeding after short-term (12 h) fasting period. Modern fast growing broiler breeds might need more time to fully compensate growth performance after a short-term (less than 24 h) fasting period.

Table 9. Effect of experimental diets on broiler chickens growth performance from 14 to 32 days of age.

Item ²	Treatment ¹					SEM ⁴	P value		
	CNF	CF	OE500F	OE1500F	MF ³		CNF vs CF	OE linear	OE no linear
14-21 d									
ADG, g/bird	72.4	68.9	70.0	69.4	71.7	1.24	0.06	0.80	0.61
ADFI, g/bird	91.0	87.6	89.0	88.4	88.8	1.46	0.11	0.69	0.59
FCR, g/g	1.26	1.27	1.27	1.28	1.24	0.012	0.49	0.79	0.96
21-32 d									
ADG, g/bird	113	117	117	117	114	2.65	0.32	0.96	0.90
ADFI, g/bird	165	170	169	168	164	3.48	0.28	0.62	0.95
FCR, g/g	1.45	1.45	1.44	1.44	1.43	0.010	0.67	0.39	0.84

¹ CNF group, basal diet no fasted; CF, OE500F, OE1500F group, basal diet containing 0, 500 and 1500 ppm of olive pomace extract and fasted, respectively; MF, basal diet containing 100 ppm of monensin and fasted. ² ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio. ³ Fasted treatments were not significantly different to MF treatment by Dunnett's test at 5%. ⁴ SEM, standard error of mean (n = 6).

Intestinal health is determined by factors such as nutrition, microbiome and environment and their effects on the host immunity and mucosal barrier permeability (Ducatelle et al., 2018). The latter has been proven to be impaired when broiler chickens

are short-term fasted (less than 24 h), as measured by FITC-d, lactulose, mannitol and L/M ratio markers (Gilani et al., 2017, 2018a). Lactulose is a high-molecular-weight sugar that is absorbed via enterocyte tight junctions (paracellular transport) and mannitol is a lower-molecular-weight absorbed straight into the blood (transcellular transport) (Arrieta et al., 2006; Gilani et al., 2017, 2018a). In the present experiment, lactulose and mannitol test results showed that fasting period (CF vs CNF) significantly ($P < 0.001$) reduced mannitol plasma concentration and lactulose and L/M ratio were significantly ($P < 0.001$) increased on day 15 (Table 10). These results confirm the detrimental effects on intestinal permeability observed in chickens after short-term fasting period as Gilani et al. (2018) reported. The observed increment in paracellular transport of fasted animals was associated with the gene expression results of this experiment (Table 11). The expression of tight junction Claudin-1 was significantly reduced ($P < 0.05$) but not Claudin-3 in the ileum. Also, the expression of FABP6 was significantly ($P < 0.01$) and that of ChREBP tended ($P < 0.10$) to be down-regulated in fasted birds. Claudins are important structural components of the tight junctions and Claudin-1 and 3 are directly involved in the paracellular permeability (Ulluwishewa et al., 2011). In a recent study, Gilani et al. (2018b) reported a decreased expression of Claudin-3 but no effect on Claudin-1 and other tight junction proteins in short-term fasted birds. Despite a decreased expression of Claudin-1 has been related with an increased permeability in intestinal disorders, there seems not to be a clear pattern for Claudin-3 expression, which may or may not be affected (Lu et al., 2013). On the other hand, fasting period of the present experiment significantly ($P < 0.01$) reduced FABP6 expression in the ileum, corroborating results showed by Gilani et al. (2018b). This protein is involved in bile acids transport across the epithelium, and, according to Gilani et al. (2018b), FABP6 downregulation in fasted birds might indicate a reduced bile acid production as a consequence of dietary fatty acid absence. Considering the described effect of several dietary components (i.e., glutamine, fatty acids) and bacteria on claudin expression (Ulluwishewa et al., 2011), it seems plausible that the lack of nutrients during fasting might affect Claudin-1 expression and increase permeability.

Table 10. Effect of a short-term (15.5 h) fasting period and experimental diets on lactulose, mannitol and lactulose/mannitol ratio in chicken plasma at 15 d of age.

	Treatment ^a					SEM ³	P value		
	CNF	CF	OE500F	OE1500F	MF ²		CNF vs CF	OE linear	OE no linear
Mannitol (µg/mL)	33.0	22.1	27.6	25.5	25.7	1.54	< 0.001	0.13	0.053
Lactulose (µg/mL)	3.79	17.4	20.3	16.1	19.0	1.91	< 0.001	0.62	0.13
L/M ⁴	0.11	0.76	0.73	0.64	0.69	0.065	< 0.001	0.19	0.77

¹ CNF group, basal diet no fasted; CF, OE500F, OE1500F group, basal diet containing 0, 500 and 1500 ppm of olive pomace extract and fasted, respectively; MF, basal diet containing 100 ppm of monensin and fasted. ² Fasted treatments were not significantly different to MF treatment by Dunnett's test at 5%. ³ SEM, standard error of mean (n = 12). ⁴ L/M, Lactulose : Mannitol ratio.

Table 11. Effect of a short-term (15.5 h) fasting period and experimental diets on ileum gene expression.¹

	Treatment ²					SEM ⁴	P value		
	CNF	CF	OE500F	OE1500F	MF ³		CNF vs CF	OE linear	OE no linear
Claudin1	10.6	11.4	11.6	11.5	11.5	0.24	0.031	0.73	0.68
Claudin3	7.39	7.43	7.42	7.46	7.47	0.16	0.86	0.88	0.89
ChREBP	11.0	11.9	11.8	11.6	11.5	0.35	0.063	0.50	0.94
FABP6	0.15	1.08	1.22	1.07	0.82	0.19	0.001	0.96	0.53
TLR-2β	7.89	8.14	7.89	7.69	7.90	0.21	0.40	0.13	0.91
TLR4	11.4	10.8	10.8	10.6	10.7	0.20	0.032	0.50	0.47
TGF-β4	13.7	13.4	13.4	13.5	13.4	0.16	0.19	0.66	0.63
IL-8	6.84	5.80	6.68	5.86	6.54	0.35	0.041	0.90	0.053
HSP-70	7.60	8.10	8.09	7.99	7.85	0.22	0.12	0.73	0.88
Bu-1	8.88	8.61	8.18	7.92	8.37	0.22	0.39	0.032	0.78

¹ Values are increments of cycle threshold (ΔCt) of target gene relative to the housekeeping. Ct values are inversely related to them RNA abundance. Claudin-1, Claudin1; Claudin-3, Claudin3; ChREBP, carbohydrate responsive element-binding protein; FABP6, fatty acid binding protein 6; TLR-2β, toll like receptor 2β; TLR4, toll like receptor 4; TGF-β4, transforming growth factor beta 4; IL-8, interleukin 8 (former CXCLi2); HSP-70, heat shock protein 70; Bu-1, chicken B-cell marker chB6. ² CNF group, basal diet no fasted; CF, OE500F, OE1500F group, basal diet containing 0, 500 and 1500 ppm of olive pomace extract and fasted, respectively; MF, basal diet containing 100 ppm of monensin and fasted. ³ Fasted treatments were not significantly different to MF treatment by Dunnett's test at 5%. ⁴ SEM, standard error of mean (n = 12).

On the other hand, an increase in intestinal permeability leads to an increase of food and bacterial antigens that might trigger an inflammatory response (Spreeuwenberg et al., 2001). Regarding ileum gene expression results of this experiment, the gene expression of TGF- β 4, HSP-70 and Bu-1 markers were not significantly ($P > 0.05$) affected in birds feed deprived for 15.5 h (Table 11). However, fasting period significantly upregulated ($P < 0.05$) TLR4 and IL-8 expression but not that of TLR2- β ($P > 0.05$). Both TLR are regulators of the innate immune system that induce an inflammatory response after detecting pathogenic and non-pathogenic molecules (Chen et al., 2015). LPS produced by gram-negative bacteria or dietary fatty acids have shown to activate TLR4 and the inflammatory response after a reduced integrity of the intestinal barrier in obesity models (Velloso et al., 2015). Moreover, IL-8 chemokine recruits heterophils at a local level in response to the presence of bacteria or inflammatory cytokines (Kogut, 2002; Chen et al., 2015). A higher IL-8 expression related to the inflammatory response was observed in a gut barrier failure model with diet and coccidiosis (Chen et al., 2015). In the present study, the expression of the anti-inflammatory cytokine TGF- β 4, and that of HSP-70 included in tissue protection and repair were not affected by the short-term fasting. This is in agreement with Gilani et al. (2018b) who showed the absence of changes in HSP-70 gene expression in the jejunum and ileum of up to 19.5h fasted chickens. Taken together with these results, it seems that feed withdrawal for 15.5 h induced an intestinal inflammatory response through TLR4 signaling.

The effects of fasting period in broiler chickens on plasma cytokines and AGP concentration are shown in Table 12. Broilers fasted during 15.5 h showed no significant ($P > 0.05$) differences in IL-1 β and IL-8 plasma concentration compared to non-fasted group. However, CF group showed significantly higher ($P < 0.05$) AGP serum concentration compared to CNF animals. Similar findings were observed by Najafi et al. (2016) in broilers after a longer period of food deprivation (30 h). This acute phase protein has been also reported to increase in serum of birds, with increase of intestinal permeability caused by a rich NSP diet plus a coccidian challenge (Chen et al., 2015). Therefore, it is plausible that the high AGP values reported in this study are closely related to the observed intestinal inflammation and permeability increase and/or the stress induced by feed deprivation.

Table 12. Effect of a short-term (15.5 h) fasting period and experimental diets on AGP, IL-1 β and IL-8 concentration in chicken plasma at 15 d of age.

	Treatment ¹					SEM ³	P value		
	CNF	CF	OE500F	OE1500F	MF ²		CNF vs CF	OE linear	OE no linear
AGP ⁴	191	256	221	217	186	15.7	0.006	0.081	0.43
IL-1 β	2.66	2.14	2.47	1.95	2.59	0.53	0.47	0.90	0.78
IL-8	69.1	52.9	19.2	46.0	43.3	16.8	0.76	0.80	0.46

¹ CNF group, basal diet no fasted; CF, OE500F, OE1500F group, basal diet containing 0, 500 and 1500 ppm of olive pomace extract and fasted, respectively; MF, basal diet containing 100 ppm of monensin and fasted. ² Values in bold in fasted treatments are significantly different to MF treatment by Dunnett's test at 5%. ³ SEM, standard error of mean (n = 12). ⁴ AGP, alpha-1-acid glycoprotein ($\mu\text{g/mL}$); IL-1 β , interleukin (ng/mL) 1 β ; IL-8, interleukin 8 (pg/mL).

Studies in broilers have addressed the effects of a short-term (< 24 h) fasting period on intestinal morphology by measuring VH and CD (Thompson and Applegate, 2006; Gilani et al. 2018b). Changes in intestinal morphology seem to be related to the hours of feed withdrawal and to the intestinal section, with duodenum and jejunum responding faster than ileum (Thompson and Applegate, 2006; Gilani et al., 2018b). Higher VH and CD have been reported in the jejunum or in the ileum of short-term fasted birds (Thompson and Applegate, 2006). According to Thompson and Applegate (2006) increased CD in short term-fasted birds suggests a need of higher tissue turnover to maintain VH in order to maximize absorption when nutrients become available again. However, there is no clear relationship between such changes and intestinal permeability increase (Thompson and Applegate, 2006; Gilani et al., 2018b). In this experiment, fasted control birds showed significantly ($P < 0.001$) higher CD and lower VH/CD ratio compared to CNF treatment (Table 13) which might be associated to the increase of intestinal permeability effect showed in fasted birds. Similar results were observed by Chen et al. (2015) in chickens at 28 d of age, with intestinal permeability increase caused by a rich NSP diet plus a coccidian challenge. This duodenal morphology changes triggered by short-term fasting period could have produced in an attempt to enhance cell proliferation to maintain gut barrier integrity (Chen et al., 2015). If short-term fasting effects on VH and CD are a consequence to maintain the epithelial structure, to maximize nutrient absorption or to maintain gut integrity deserves future experiments.

Table 13. Effect of a short-term (15.5 h) fasting period and experimental diets on villus height, crypt depth and, villus/crypt ratio in broiler chickens at 15 d of age.

Item	Treatment ¹					SEM ³	P value		
	CNF	CF	OE500F	OE1500F	MF ²		CNF vs CF	OE linear	OE no linear
Villus height (µm)	1956	1907	1875	1748	1726	45.2	0.44	0.019	0.40
Crypt depth (µm)	149	181	162	158	168	6.04	< 0.001	0.014	0.33
Villus/Crypt	13.3	10.8	11.6	11.3	10.4	0.50	< 0.001	0.45	0.34

¹ CNF group, basal diet no fasted; CF, OE500F, OE1500F group, basal diet containing 0, 500 and 1500 ppm of olive pomace extract and fasted, respectively; MF, basal diet containing 100 ppm of monensin and fasted. ² Values in bold in fasted treatments are significantly different to MF treatment by Dunnett's test at 5%. ³ SEM, standard error of mean (n = 12).

4.2.2. DIETARY EFFECT OF OLIVE POMACE EXTRACT

Before fasting period, dietary treatments did not affect significantly ADG, ADFI, and FCR (from 1 to 14 d of age), being on average 31.7 g/bird/d, 37.4 g/bird/d and 1.20 g/g, respectively. Additionally, in the last period, from 21 to 32 days of age, no significant differences in performance were observed among groups (Table 9). This is in agreement with Leskovec et al. (2018) who observed no significant differences in broiler chickens performance fed diets supplemented with an olive leaf extract. Furthermore, King et al. (2014) reported no significant differences in broiler performance after supplementing an olive pomace extract in water. By contrast, Herrero-Encinas et al. (2020a) were able to detect higher ADG and better FCR in broiler fed 750 ppm of a bioactive extract from *Olea europaea* in broiler chickens from 35 to 42 d of age. Moreover, utilization of olive leaf extracts in diet of broilers improved FCR and body weight in the study of Sarica and Ürmez (2016). Thus, the potential beneficial effects of the bioactive compounds of extracts from *Olea europaea* on productive parameters might depend on the composition and concentration of the bioactive substances, bird age, and mode of supplementation (water vs. feed).

The results from lactulose, mannitol and L/M ratio showed no significant ($P > 0.05$) differences among fasted treatments (Table 10). However, a non-linear trend ($P = 0.053$) on plasma mannitol was observed with the inclusion of OE in the diet, showing higher concentration in birds fed OE500F diet compared to CF and OE1500F groups. These results are in agreement with Liehr et al. (2017), who showed an intestinal functionality

improvement by an increment of plasma mannitol concentration in pigs fed an olive oil extract and chronically challenged with lipopolysaccharides. Therefore, the higher mannitol concentration observed in birds supplemented with 500 ppm of OE in our study could be related to a lower transcellular transport damage when they were short-term fasted. However, the concentration of lactulose in the plasma and the expression of Claudin-1 and 3 and FABP6 in the ileum were similar ($P > 0.05$) among dietary treatments after 15.5 h of feed withdrawal (Table 11). This might be indicative of a lack of effect of OE on paracellular transport increase after the short-term fasting period.

Bioactive compounds of olive pomace have shown immunomodulatory effects in broiler chickens, pigs, calves and fish by reducing the production of pro-inflammatory cytokines and increasing the anti-inflammatory cytokines (Liehr et al., 2017; Morrison et al., 2018; Gisbert et al., 2017; Herrero-Encinas et al., 2020a). Regarding the inflammatory markers in plasma, the cytokine IL-1 β and IL-8 concentrations were not significantly ($P > 0.05$) affected among fasted treatments (Table 12). However, CF treatment showed significantly ($P < 0.05$) higher AGP plasma concentration compared to MF treatment. Moreover, regarding the results of ileum gene expression, a non-linear tendency ($P = 0.053$) of ileal IL-8 expression was observed with OE inclusion in the experimental diets, displaying birds fed the OE500F diet lower expression levels than those of the CF and the OE1500F groups (Table 11). Also, the expression of Bu-1 linearly increased ($P < 0.05$) with OE inclusion levels, suggesting a stimulatory effect on humoral immunity. In this regard, an immunosuppressant effect on IL-8 and a stimulatory effect on Bu-1 have been observed in our previous study with broilers fed an olive pomace extract in the growing phase (Herrero-Encinas et al., 2020a). Furthermore, increased B-cell markers has been previously described in broiler chickens fed plant extracts rich in polyphenolic compounds (Park et al., 2013). This immunomodulatory effect of OE deserves future research in a context of experimentally-induced disease.

The effects of dietary treatments on intestinal morphology are shown in Table 13. Among fasted treatments, the MF group showed significantly ($P < 0.05$) shorter VH compared to the CF and OE500F treatments, but similar than those of OE1500F. Moreover, the inclusion of OE in diets decreased ($P < 0.05$), linearly, VH and CD. These results are in agreement with other studies in broilers that have shown reduced jejunal CD in birds fed with garlic extract compared to a control (Lee et al., 2016). According to these authors, a reduced CD suggests a lower turnover rate to maintain VH and, hence, lower

energy needs to maintain gut integrity. In the present experiment, this might be the case with birds fed the OE500F, as they seem to maintain VH with lower CD. However, it was not observed with the highest dose of the extract.

Under the conditions of the present experiment, the inclusion up to 1500 ppm of an extract rich in bioactive compounds from *Olea europaea* in starter and grower broiler diets did not affect performance. The 15.5 h fasting period significantly increased intestinal permeability triggering an inflammatory response mediated by TLR4. The inclusion of 500 ppm of the OE attenuated some of the negative effects of increased intestinal permeability associated to this short-term fasting challenge.

4.3. OBJECTIVE 3: Investigate an experimental procedure of coccidia challenge in battery cages and the anticoccidial effect of an olive pomace extract in broiler chickens

4.3.1. *EIMERIA* SPP CHALLENGE

Vaccination programs against coccidiosis using live *Eimeria* oocysts is a current practice in poultry production. However, such programs can cause temporary reduction in growth performance and gut intestinal health (Williams, 2002). In this context, it is necessary to establish repeatable experimental models to mimic vaccinal oocyst cycling to aid in the development of nutritional strategies that can ameliorate the transient intestinal disruption derived from coccidiosis vaccine in poultry. Thus, the first objective of this study was to evaluate the impact of a novel experimental model of the vaccinal oocyst cycling in broiler chickens on growth performance and gut health and function. The selected timing of the repeated inoculations and increased oocyst number with each inoculation were targeted to mimic time required for oocyst shedding (4-6 days) and the sporulation in the environment (1-2 days) as well as oocysts amplification that occurs in the field (Chapman et al., 2010).

Growth performance and oocyst count in litter or excreta are the main parameters to evaluate the coccidiosis prevalence in poultry (Holdsworth et al., 2004). In the current experiment, body weight was significantly lower ($P < 0.05$) in birds of NCC group compared with those of the NCU group at 7, 14, 20 and 28 d of age (Table 14). Throughout the experimental period, NCC birds had lower ($P < 0.05$) FI and BWG compared to NCU, except from 20 to 28 d of age. Furthermore, FCR was higher ($P < 0.05$) in NCC birds compared with NCU birds during the periods from 0 to 7, 0 to 14 and 0 to 20 d, but there was no difference ($P > 0.05$) in FCR between the NCC and NCU groups from 20 to 28 d. Besides, the OPG was significantly ($P < 0.001$) higher in NCC birds compared to NCU on day 7, 14 and 21 (Table 15).

Table 14. Effect of experimental diets on *Eimeria* spp challenged broiler chickens growth performance from 0 to 28 d of age.¹

Item ²	NCU	NCC	OE500C	OE1500C	PCC	SEM ³	<i>P</i> value ⁴		
							NCU vs NCC	OE linear	OE no linear
BW (g/bird)									
0d	38.8	38.4	38.4	38.4	38.7	0.15	0.075	0.76	0.98
7d	145	126	130	128	128	4.10	0.003	0.87	0.53
14d	405	327	355	345	347	14.5	< 0.001	0.52	0.22
20d	779	646	683	685	663	23.6	< 0.001	0.32	0.41
28d	1435	1301	1327	1311	1242	36.9	0.016	0.93	0.62
FI (g/bird/d)									
0-7d	126	114	114	116	117	3.42	0.027	0.63	0.72
7-14d	313	268	297	277	275	11.7	0.011	0.86	0.082
14-20d	503	440	458	461	443	12.5	0.001	0.30	0.48
20-28d	995	951	972	969	919	26.5	0.25	0.69	0.64
0-14d	439	383	410	393	393	13.9	0.008	0.79	0.16
0-20d	942	823	869	855	835	25.0	< 0.001	0.50	0.26
0-28d	1937	1774	1841	1824	1754	45.1	0.016	0.54	0.37
BWG (g/bird/d)									
0-7d	106	87.6	91.3	89.2	89.3	4.09	0.004	0.88	0.53
7-14d	260	201	225	217	219	11.0	< 0.001	0.44	0.17
14-20d	374	319	328	340	316	11.9	0.003	0.23	0.89
20-28d	656	655	644	626	580	22.2	0.97	0.37	0.95
0-14d	366	288	317	306	308	14.5	< 0.001	0.52	0.22
0-20d	741	608	645	647	624	23.6	< 0.001	0.32	0.41
0-28d	1397	1263	1289	1272	1203	36.9	0.016	0.93	0.62
FCR (g/g)									
0-7d	1.20	1.34	1.25	1.31	1.34	0.034	0.006	0.80	0.068
7-14d	1.26	1.36	1.32	1.29	1.27	0.038	0.066	0.18	0.82
14-20d	1.36	1.41	1.42	1.45	1.43	0.027	0.22	0.29	0.85
20-28d	1.52	1.46	1.52	1.55	1.60	0.038	0.22	0.10	0.50
0-14d	1.24	1.35	1.30	1.29	1.29	0.031	0.012	0.21	0.43
0-20d	1.30	1.38	1.36	1.37	1.36	0.024	0.017	0.79	0.51
0-28d	1.39	1.41	1.43	1.43	1.46	0.024	0.49	0.53	0.67

¹ NCU, negative control with no additives and no challenged; NCC, OE500C, OE1500C, basal diet containing 0, 500 and 1500 ppm of olive pomace extract and challenged, respectively; PCC, basal diet containing 500 ppm of Clinacox® and challenged. ² BW, body weight; FI, feed intake; BWG, body weight gain; FCR, feed conversion ratio. ³ SEM, standard error of the mean ($n = 10$). ⁴ Challenged treatments were not significantly different by Tukey's test at 5%.

These results are in agreement with Leung et al. (2019) who observed a reduction in BWG and BW and increased oocyst shedding in animals challenged with *E. maxima* and *E. acervulina* at 7 d post-challenge. In addition, Wang et al. (2018) reported impaired FCR (from 15 to 40 d post hatch) in broiler chickens challenged at 21 d of age with 10x of Coccivac-B52 vaccine. Furthermore, Persia et al. (2006) showed a reduction in FI and BWG in broiler chickens from 9 to 22 d of age challenge with multiple *E. acervulina* inoculations (5.0×10^5 sporulated oocyst) at 9, 12, 15, and 18 d of age. Thus, it can be concluded that the novel replicable model of coccidia challenge used in this experiment exerted the expected response of growth depression and high OPG counts. Further, the reduction in BWG between NCU and NCC where increases in oocysts shedding were slightly less from 14 to 20 d of age following the 16 x inoculation than from 7 to 14 d following the 4 x inoculation, possibly reflecting that some degree of immunity was developing with the repeated inoculations.

Table 15. Effect of experimental diets on *Eimeria* spp challenged broiler chickens on oocyst per gram of excreta at 7, 14, and 21 days of age.¹

Item ²	NCU	NCC	OE500C	OE1500C	PCC	P value		
						NCU vs NCC	OE linear	OE no linear
7d	0.95 ± 0.95	5.11 ± 0.12 ^a	5.40 ± 0.10 ^a	5.29 ± 0.11 ^a	1.75 ± 0.62 ^b	< 0.001	0.47	0.092
14d	0.25 ± 0.97	5.46 ± 0.08 ^a	5.50 ± 0.07 ^a	5.30 ± 0.08 ^a	0.23 ± 1.00 ^b	< 0.001	0.10	0.29
21d	0.41 ± 0.92	4.42 ± 0.13 ^a	4.17 ± 0.14 ^a	4.30 ± 0.13 ^a	-0.34 ± 1.27 ^b	< 0.001	0.65	0.17

¹ Values expressed as Ln (x 1000 oocyst per gram of excreta) ± standard error. NCU, negative control with no additives and no challenged; NCC, OE500C, OE1500C, basal diet containing 0, 500 and 1500 ppm of olive pomace extract and challenged, respectively; PCC, basal diet containing 500 ppm of Clinacox[®] and challenged. ^{a, b} Challenged treatments were significantly different by Tukey's test at 5%.

Plasma carotenoid concentrations are an indicator of intestinal mucosa integrity in poultry and can be markedly impacted by coccidiosis infections in chickens (Conway et al., 1993; Holdsworth et al., 2004). In previous studies, vaccinated birds against coccidiosis or experimentally infected with *Eimeria* showed a reduction in CAR plasma concentration (Rochell et al., 2016; Leung et al., 2019; Gautier et al., 2020). These previous findings agree with results of the present experiment. The NCC showed lower ($P < 0.001$) CAR concentration compared to NCU group after 6 d of the last oral gavage vaccine (20 d of age) (Table 16). However, CAR concentration was similar ($P > 0.05$) at 28 d of age (14 d post-challenge), which may indicate that the gut integrity of challenged birds was restored.

Table 16. Effect of experimental diets on AGP and IL-8 at 20 d of age and carotenoids at 20 and 28 d of age in broiler chicken plasma. ¹

Item ³	NCU	NCC	OE500C	OE1500C	PCC	SEM ²	P value ⁴		
							NCU vs NCC	OE linear	OE no linear
IL-8	82.7	58.2	67.3	83.1	88.0	16.0	0.37	0.32	0.63
AGP	117	116	110	113	109	6.36	0.91	0.81	0.51
Carotenoids									
20d	1.82	1.24 ^b	1.30 ^{ab}	1.31 ^{ab}	1.57 ^a	0.10	< 0.001	0.67	0.74
28d	1.23	1.01	1.10	1.20	1.19	0.084	0.080	0.13	0.81

¹ NCU, negative control with no additives and no challenged; NCC, OE500C, OE1500C, basal diet containing 0, 500 and 1500 ppm of olive pomace extract and challenged, respectively; PCC, basal diet containing 500 ppm of Clinacox® and challenged. ² SEM, standard error of mean (n = 20). ³ IL-8, interleukin 8; AGP, alpha-1-acid glycoprotein. ⁴ P values of IL-8 are from natural logarithmic data transformation analysis. ^{a, b} Challenged treatments were significantly different by Tukey's test at 5%.

An increase of CD and lower VH/CD ratio has been observed during coccidiosis vaccination or infection, presumably due to increased epithelial turnover and to replace damage enterocytes (Morris et al., 2004). In this regard, Leung et al. (2019) observed morphological damage in the jejunum with a reduction of VH, CD and VH/CD ratio in chickens challenged with *Eimeria* spp. (5d post challenge). Furthermore, Wang et al. (2018) showed deeper crypts in the ileum and higher VH/CD ratio after 5 days post-challenge (26 d of age) with Coccivac-B52. These previous reports are in line with the results of in the current experiment as VH/CD ratio in duodenum and jejunum was significantly ($P < 0.05$) reduced in NCC group compared to NCU after 6 d post-challenge (20 d of age) but not in the villus height and crypt depth ($P > 0.05$) (Table 17). This response combined with the reduced plasma CAR concentration reflect a damaged mucosa in response to the *Eimeria* spp. challenge employed in the current experiment.

Table 17. Effect of experimental diets and *Eimeria* spp challenged in broiler chickens intestinal morphology at 20 d of age.¹

Item ²	NCU	NCC	OE500C	OE1500C	PCC	SEM ³	P value ⁴		
							NCU vs NCC	OE linear	OE no linear
Duodenum									
Villus height, μm	1851	1774	1719	1795	1770	61.5	0.40	0.71	0.40
Crypt depth, μm	148	178	196	195	159	13.7	0.14	0.47	0.49
VH/CD	13.2	10.1	9.50	9.62	11.6	0.90	0.026	0.78	0.70
Jejunum									
Villus height, μm	993	942	995	973	948	60.8	0.55	0.79	0.58
Crypt depth, μm	128	152	149	153	145	12.1	0.17	0.91	0.84
VH/CD	8.06	6.41	6.85	6.67	6.74	0.48	0.021	0.78	0.57

¹ NCU, negative control with no additives and no challenged; NCC, OE500C, OE1500C, basal diet containing 0, 500 and 1500 ppm of olive pomace extract and challenged, respectively; PCC, basal diet containing 500 ppm of Clinacox® and challenged. ² VH/CD, villus height: crypt depth ratio. ³ SEM, standard error of mean (n = 10). ⁴ Challenged treatments were not significantly different by Tukey's test at 5%.

Coccidiosis in poultry induces an immune response in the host that results in inflammation and activation of intraepithelial T lymphocytes in which, an up-regulation of IFN- γ expression is a hallmark response to *Eimeria* infection (Rothwell et al., 2000; Yun et al., 2000). Moreover, an upregulation of different markers occurs in coccidia infected chickens, including IL-1 β and other several cytokines of the interleukin family (ILs), toll-like receptors (TLRs), and transforming growth factors (TGF- β 1-4) (Hong et al., 2006; Kim et al., 2019). The host gut immune response depends on *Eimeria* species and strain, the age of the flocks, and the environmental conditions and feed quality in poultry (Conway and McKenzie, 2007). In this context, the utilization of an overdose (20x) of Coccivac-B52 in broiler chickens (at 14 d of age) caused increased gene expression of IFN- γ and IL-10 after 6 d post challenge (Oxford and Selvaraj, 2019). Moreover, other authors similarly showed that a single overdose of Coccivac-B52 vaccine in broilers induced an up-regulation of several interleukins including IL-1 β , IL-6, IL-10, IFN- γ , TLR4 and down-regulation of other gene markers related to gut integrity such as Claudin-1 or Occludin, (Osho et al., 2019; Osho and Adeola, 2019). According to Williams (2002) a single *Eimeria* spp. exposure might activate the primary immune response but does not generate an acquired immunization. However, regarding results of

the present experiment, no differences ($P > 0.05$) in plasma concentrations of IL-1 β , IL-8 and AGP were observed among NCC and NCU experimental treatments at 20 d of age. Moreover, the expression of TLR4, IL-8, IL-1 β , IFN- γ , Bu-1 and Claudin1 in the ileum was not different ($P > 0.05$) between NCU and NCC groups at 20 d of age. These results could indicate an attenuated inflammatory response which along with the reduced impact in growth performance following the third infection can be attributed to the adaptation or immunization to the repetitive exposure to *Eimeria*.

*Table 18. Effect of experimental diets and Eimeria challenge on broiler chicken ileum gene expression at 20 d of age.*¹

Item ³	NCU	NCC	OE500C	OE1500C	PCC	SEM ²	P value ⁴		
							NCU vs NCC	OE linear	OE no linear
TLR4	9.49	9.76	9.59	10.1	9.75	0.24	0.44	0.25	0.36
IL-8	8.27	8.73	7.90	8.29	8.51	0.43	0.46	0.63	0.20
IFN- γ	12.1	11.2	10.3	10.1	10.7	0.38	0.11	0.077	0.27
Bu-1	6.45	5.98	5.85	6.01	6.36	0.26	0.22	0.86	0.67
IL-1 β	12.5	12.0	12.3	11.9	12.1	0.26	0.24	0.68	0.38

¹ NCU, negative control with no additives and no challenged; NCC, OE500C, OE1500C, basal diet containing 0, 500 and 1500 ppm of olive pomace extract and challenged, respectively; PCC, basal diet containing 500 ppm of Clinacox® and challenged. ² SEM, standard error of mean (n = 10). ³ TLR4, toll like receptor; IL-8, interleukin 8; IFN- γ , interferon gamma; Bu-1, b cell marker; IL-1 β , interleukin 1 beta. ⁴ Challenged treatments were not significantly different by Tukey's test at 5%.

4.3.2. EFFECT OF OLIVE POMACE EXTRACT AND COCCIDIOSTAT

Natural alternatives to anticoccidial chemical drugs and ionophores are being developed to reduce coccidiosis prevalence in poultry industry (Quiroz-Castañeda and Dantán-González, 2015). Several products have been described as potential alternatives against coccidiosis but their mechanism of action and effectiveness have been not completely described or validated (Muthamilselvan et al., 2016). The second objective of this study was to evaluate the potential anticoccidial effect of an olive pomace extract in broiler chickens.

Dietary supplementation with different concentrations of OE did not affect significantly ($P > 0.05$) BW, FI, BWG and FCR in birds challenged with the three cycles of live oocyst vaccine throughout the trial (Table 14). These findings are not aligned with

de Pablos et al. (2010) who observed better BWG but no FI differences at 21 d post infection with *E. tenella* in chickens feed diets supplemented with 90 ppm of maslinic acid, a bioactive compound derived from olive oil by-products. These authors reported that the OPG from 6 to 10 d post infection were significantly reduced with maslinic acid. In addition, a recent *in vitro* study showed that quercetin and oleuropein substances derived from *Olea europaea* exerted anticoccidial activity against *Eimeria* spp. by reduction of oocyst number (Debbou-Iouknane et al., 2019). In addition, in our previous studies OE inclusion in broiler diets exerted a positive effect on performance because of its anti-inflammatory properties (Herrero-Encinas et al., 2020a). In the present study, the OPG were not significantly ($P > 0.05$) affected by OE (Table 15).

Duodenum and jejunum morphology were not affected ($P > 0.05$) by OE (Table 17). Also, no significant differences ($P > 0.05$) were observed in plasma concentration of IL-1 β , IL-8 and AGP marker with the OE inclusion in diets (Table 16). Besides, the ileum gene expression at 20 d of age of TLR4, IL-8, IL-1 β , IFN- γ , Bu-1 and Claudin1 was not significantly ($P > 0.05$) affected among challenged treatments (Table 18). Additionally, PCC treatment (Clinacox, 0.2% diclazuril) did not improve growth performance during the experiment despite the significant ($P < 0.05$) lower OPG compared to NCC, OE500C and OE1500C at 7, 14 and 21 d of age and the higher ($P < 0.05$) plasma CAR concentrations compared to NCC treatment at 20 d of age (6 d post third oocyst cycling). As with the OE, no significant effects of Clinacox were observed on the selected markers of immune response and the intestinal morphology in duodenum and jejunum (Table 17 and 18, respectively). In this regard, the active substance of Clinacox is diclazuril, which at 0.5 ppm is almost entirely effective against *E. tenella*, *E. acervulina* and *E. mitis* (McDougald et al., 1990). However, in case of *E. maxima* infection, diclazuril is not effective until the sexual stages after some intestinal damage already occur, allowing some growth depression even if the *Eimeria* cycle is subsequently disrupted (McDougald et al., 1990). These findings might explain the lack of expected beneficial effects of Clinacox on broiler chicken growth performance. Moreover, the beneficial coccidiostat activity of bioactive compounds derived from olive byproducts in broiler chickens, including the OE extract, seems to depend on the concentration and type of the bioactive compounds, the *Eimeria* strain, and the age and developed immune system of birds.

It can be summarized that under the present study conditions, the coccidiosis challenge decreased growth performance and gut integrity as indicated by oocyst

excretion, reduced plasma CAR and VH/CD ratio. Bearing in mind the effects of *Eimeria* spp. challenge and the lack of beneficial effects of inclusion of OE or Clinacox on performance, gut integrity and immune system might be indicative an immunity development response against *Eimeria* spp. Considering our previous results of OE positive immunomodulatory effects, it is plausible that this extract could be used in combination with a vaccine program for OE anti-inflammatory effects without compromising the intended development of immunity with the vaccination.

GENERAL DISCUSSION

5. GENERAL DISCUSSION

Antibiotics have long been used in poultry production to improve growth and feed efficiency (Broom et al., 2018). Antibiotics at sub-therapeutic doses exert their beneficial effects by reducing the gut microbiota and hence subclinical infections and undesirable metabolites. Also, they may enhance the nutrient utilization by a reduction of intestinal wall depth or decrease the inflammatory response of the animals (Dibner and Richards, 2005; Niewold, 2007). However, the well-known AGPs use restrictions triggered by the antimicrobial (cross)-resistance development and the intensification of the poultry production during last decades, has stimulated the use of other alternatives to reach the potential benefits of AGPs (Gadde et al., 2017). Besides, to replace AGPs by natural alternatives, these should be based on the different mechanism of action of AGP (Lillehoj et al., 2018).

Within the antibiotics surrogates, the use of phytogenic additives in poultry feeds have shown positive results on performance (Windisch et al., 2008; Gadde et al., 2017). Frequently, these improvements observed in better feed intake and body weight are related to the enhancement in flavor, palatability and odor of feed (Windisch et al., 2008). Nevertheless, PFAs are associated to the enhancement of nutrient digestibility because they promote enzyme and metabolites secretions and the maintenance of the gut morphology (Platel and Srinivasan, 2000; Hashemipour et al., 2014). Also, bioactive compounds of PFAs have shown positive effects in growth because of their antimicrobial, antioxidant properties or immunomodulatory effects (Windisch et al., 2008; Gadde et al., 2017; Lee et al., 2017).

Regarding plants extracts and according to a search performed in the PubMed database on 25th of April, 2020, the scientific interest of their use in broiler chickens is getting more consideration reaching 49 publications in 2019 (Figure 12).

Figure 12. Number of publication of plant extracts use in broiler chickens during last 20 years (PubMed database).

Among these search, the most studied plant extracts in broiler chickens and their effects on growth performance are garlic, oregano, mushrooms (different species), aniseed, cinnamon, ginger, thyme, grapeseed, rosemary and turmeric extracts. Phenolic compounds and organic acids are the main bioactive components derived from these plants including thymol, carvacrol, proanthocyanidin, cinnamic acid, turmeric acid or curcumin, among others. The effectiveness and the mechanism of action of these substances are very diverse and depends in several factors such as the type of bioactive compound, the plant and crop conditions or the mechanism of extraction (Applegate et al., 2010).

By-products derived from olive industry including olive pomace are a relevant source of bioactive substances including triterpenes, sterols, tocopherols or phenolic compounds with significant properties to enhance health (Mateos et al., 2019). Maslinic acid, an abundant triterpene in the olive fruit and leaves of *Olea europaea*, has been studied because of its anti-tumoral activity *in vitro* and *in vivo* (Juan et al., 2008; Reyes-Zurita et al., 2009; Lozano-Mena et al., 2014). In addition, this molecule has shown antioxidant and anti-inflammatory activity by inhibiting the inflammatory response induced by LPS, reducing the pro-inflammatory cytokine TNF- α , iNOs and COX-2 expression (Lozano-Mena et al., 2014). In a recent study, Peng et al. (2017) analyzed the effects of different bioactive substances at different concentrations (from 0.05 to 4%) in human macrophages and intestinal cells challenged with different foodborne bacterial

pathogens. These authors showed that maslinic and oleanolic acids (derived from the olive pomace) positively enhanced *Lactobacillus* growth, hydroxytyrosol and oleuropein reduce the growth and virulence of *Salmonella* in human intestinal cells. As well, same authors showed that these bioactive compounds exhibited anti-inflammatory effects by up-regulation of IL-10 and down regulation of IL-1 β , CXCL-8 or TNF- α gene expression. Furthermore, hydroxytyrosol found in olive pomace showed *in vitro* anti-inflammatory properties by inhibiting the secretion of several cytokines and chemokines on stimulated LPS cells (Richard et al., 2011). These findings are aligned with the results of the present Thesis. In the first experiment, we were able to find positive results in growth performance and the reduction of pro-inflammatory cytokine IL-8 and the increase of TGF- β 4 anti-inflammatory marker in healthy broiler chickens supplemented with 750 ppm of OE at 42 d of age. Similar results were obtained in the second experiment. In this case, after feed stress induction by 15.5 h short-term fasting period, birds supplemented with 500 ppm tended to reduce the expression of IL-8 with no growth alterations in broiler chickens. The anti-inflammatory effects of extracts derived from *Olea europaea* at similar concentrations used in this Thesis have been observed in other food-producing animal species. In this regard, Liehr et al. (2017) showed that feeding pigs (challenged with LPS repeated injection) with an extract derived from pomace olive-oil containing 10% of maslinic acid, 4% of oleanolic acid and 2% of hydroxytyrosol at 500 mg/kg depressed the production of pro-inflammatory IL-1 β plasma concentration and improved intestinal integrity. In addition, the same extract was tested in weaned beef heifers at 0.04 and 0.16% of diet showing a reduction of IL-6 and haptoglobin inflammatory circulating concentration markers and mitigating the immune activation after intravenous repeated LPS challenge (Cangiano et al., 2019).

Bioactive compounds from *Olea europaea* have been reported antimicrobial activity (Mateos et al., 2019). In this regard, oleanolic acid and ursolic acid have shown activity against several species of protozoa, viruses and pathogenic bacteria in human (Jesus et al., 2015). Oleuropein and hydroxytyrosol are effective inhibiting several bacteria associated with respiratory and intestinal tract infection in humans (Bisignano et al., 1999). Similarly, Debbou-louknane et al. (2019) showed antiprotozoal activity *in vitro* of oleuropein and quercetin against *Eimeria* spp. in broiler chickens. Also, de Pablos et al. (2010) showed that maslinic acid supplemented in diet reduce the prevalence of coccidiosis in broiler chickens infected with *Eimeria tenella*. However, in the first and

third experiment of the present thesis, the OE supplementation at different levels did not exert antimicrobial or anticoccidial activity, as might have been expected. In our third study, the experimental coccidial vaccine challenge included live sporulated oocysts of *E. maxima*, *E. maxima* MF, *E. acervulina*, *E. tenella* and *E. mivati*. Therefore, it is plausible that the coccidiostat activity of olive byproducts in broiler chickens might depend on the concentration and proportions of the different bioactive compounds, and the *Eimeria* spp. to be targeted. Future studies are needed to explore the potential use of olive pomace extracts as coccidiostat in poultry.

GENERAL CONCLUSIONS AND IMPLICATIONS

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6.1. GENERAL CONCLUSIONS

1. Feeding broiler diets supplemented with an olive pomace extract under standard conditions does not affect performance up to 32 days of age. However, in the grower-finisher phase (from 35 to 42 days of age) the addition of 750 ppm of the extract providing 15 ppm polyphenols and 75 ppm triterpenes improved ADG and FCR.
2. The positive effects of the olive pomace extract on animal growth might be related to the anti-inflammatory properties of the olive triterpenoids and polyphenols reflected by the immunosuppressant effect on IL-8 pro-inflammatory interleukin and the increase of anti-inflammatory expression of TGF- β 4.
3. A single short-term fasting period of 15.5 h in birds housed in floor pens induced an increase in intestinal permeability triggering an inflammatory response mediated by TLR4.
4. The addition of 500 ppm of the olive pomace extract providing 5 ppm polyphenols and 30 ppm triterpenes alleviated some of the negative effects of increased permeability associated with the short-term fasting challenge. These positive effects include lower intestinal transcellular transport damage, reduction of IL-8 pro-inflammatory marker, enhancement of Bu-1 expression which might be indicative of humoral immunity stimulation, and maintenance of intestinal morphology.
5. The experimental model of coccidial vaccine challenge used induced growth depression and gut integrity reduction as indicated by oocyst excretion reduced plasma carotenoid concentration and intestinal morphology alteration.
6. The attenuated inflammatory response observed for all the treatments following the third infection can be attributed to the adaptation or immunization to the repetitive exposure to *Eimeria* spp.
7. Under the experimental coccidial vaccine challenge, the inclusion of 500 ppm or 1500 ppm of the olive pomace extract providing up to 15 ppm of polyphenols and

90 ppm of triterpenes showed no effects on performance or gut integrity. This suggest that the potential anticoccidial effects of olive pomace extracts depend on the concentration and type of the bioactive compounds and/or the *Eimeria* strain.

6.2. IMPLICATIONS

Considering the results of experiment 1 of the present Thesis the supplementation of broiler grower-finisher diets with 750 ppm of an olive pomace extract providing 15 ppm polyphenols and 75 ppm triterpenes might be useful to improve animal performance in absence of AGPs. As depicted from experiment 1 and 2 the positive effects of the olive pomace extracts used are related to the anti-inflammatory properties of their bioactive compounds polyphenols and triterpenes. It is plausible that the olive pomace extract might be used in combination with a vaccine program for its anti-inflammatory effects without compromising the intended development of immunity with the vaccination.

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