Universitat Autònoma de Barcelona

USE OF MICROENCAPSULATED FEED ADDITIVES COMPOSED OF ORGANIC ACIDS AND ESSENTIAL OILS IN BROILER CHICKEN DIETS: EFFECTS ON GROWTH PERFORMANCE AND GUT HEALTH

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Certify:

That the thesis dissertation entitled **"Use of microencapsulated feed additives composed of organic acids and essential oils in broiler chicken diets: effects on growth performance and gut health",** presented by Nedra Abdelli to apply for Doctor degree, has been made under their direction and, considering it finished, authorize its presentation so that it is judged by the corresponding commission.

And for the record to the appropriate effect, sign those present in Bellaterra, February 18, 2022

Dr. José Francisco Pérez Hernández **Dr. David Solà-Oriol**

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“Ambition is the path to success. Persistence is the vehicle you arrive in”

Bill Bradley

To my beloved parents

Thank you for always being there for me. Everything I have and everything I am, I owe it all to you. Thank you, my two lifelines.

To my husband

Thank you for your endless support, sacrifices and tireless efforts to help me to achieve my goals.

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Summary

The increasing worldwide interest towards supporting a sustainable agricultural system requires upgrading farming practices that are profitable, environmentally friendly, good for communities, and antibiotic-free. In this frame, managing gut health is crucial for the sustainability of poultry production under antibiotic free programs, especially with the increasing pressure of intestinal diseases such as coccidiosis and necrotic enteritis. Thus, gut health has become an extremely important research topic due to its complexity with a special focus on developing feed additives which help to maintain healthy intestinal microbiota. This implies energy saving for the host which results in an improvement in productive performance of the birds. Among the used feed additives, organic acids and essential oils have been extensively used in poultry production due to proved growth stimulatory effects. The main objective of the current thesis was to study the effects of microencapsulated blends of organic acids and essential oils on growth performance and gut health of broiler chickens subjected to different challenging conditions, and to highlight the mechanisms of action behind these effects.

In order to achieve this objective a series of trials were performed and the obtained results were presented in three chapters (chapter 4 to 6).

The chapter 4 aimed to develop a custom gene expression panel, which could provide a snapshot of gene expression variation under challenging conditions. Two trials were conducted where broilers were challenged with necrotic enteritis in the first one while a coccidial challenge was applied in the second trial. Several genes involved in different functions were incorporated in the panel including barrier function, immune response, nutrient transport as well as oxidative stress and digestive hormones. The obtained results showed that the developed panel allows a global gene expression profiling which gives a greater overview of genes and pathways involved in broiler response to pathogen challenges. It also provides insights into differences of gene expression patterns and magnitude of responses under either a coccidial vaccine challenge or NE.

The objectives of **chapter 5** were (1) to show evidence of the progressive release of fumaric acid and thymol, as examples of organic acids and essential oils, when these are microencapsulated in lipid matrix microparticles under *in vitro* and *in vivo* intestinal

conditions; and (2) to evaluate the effect of microencapsulated fumaric acid and thymol on the performance and gut health of broiler chickens challenged with a short-term fasting period as a model of mucosal damage and increased GIT permeability. The obtained results showed that the used matrix was able to provide both the right timing and location for the release of active compounds. Moreover, microencapsulated fumaric acid, thymol, or their combination showed positive effects when broilers were subjected to challenging conditions, alleviating the negative effects promoted by the fasting challenge on animal performance, intestinal histomorpholgy, and microbiota.

Chapter 6 aimed to investigate the efficacy of different microencapsulated blends containing organic acids and essential oils on performance and gut health of broilers under challenging conditions of necrotic enteritis. Two experiments were carried out, in which the first trial aimed to determine the design of combinations with a high efficiency, while the second trial focused on finding the optimal dose for each combination. Results of the first trial showed that the tested blends enhanced the abundance of some beneficial families such as Ruminococcaceae and Lachnospiraceae; while reducing that of harmful ones such as Enterobacteriaceae and Helicobacteraceae. This positively influenced the intestinal histomorphology and thereby the growth performance of challenged broilers. Among the 4 tested blends, the two showing the best effects on intestinal histomorphology and growth were selected for the dose response trial. Results showed that 0.5 g/kg of the blend containing calcium butyrate and fumaric acid, and 2 g/kg of the blend containing medium chain fatty acids, calcium butyrate, fumaric and citric acid, in both cases combined with cinnamaldehyde, carvacrol, and thymol (8:1:1) improved growth performance and intestinal histomorphology of chickens on d 42 of the experiment, and decreased fecal Enterobacteriaceae and *Clostridium perfringens* counts. They also exerted similar beneficial effects to those observed in first trial on cecum microbiota.

Taking together, the obtained results provide importance that the efficacy of such feed additives depends both on designing proper combinations and doses as well as using the proper technique of protection allowing the active compounds to be released in target GIT segments. Their beneficial effects are achieved through a variety of complex and frequently interrelated mechanisms of action.

Resumen

El creciente interés mundial por apoyar un sistema agrícola sostenible requiere mejorar las prácticas agrícolas para que sean rentables, respetuosas con el medio ambiente, y a su vez utilicen un nivel muy reducido de antimicrobianos. En este marco, el manejo de la salud intestinal es clave para garantizar la sostenibilidad de la producción avícola bajo programas libres de antibióticos, especialmente con la creciente presión de enfermedades intestinales como la coccidiosis y la enteritis necrótica. Por lo tanto, la salud intestinal se ha convertido en un tema de investigación prioritario en la producción ganadera, con un enfoque práctico dirigido al desarrollo de aditivos y estrategias alimentarias que ayuden a mantener una microbiota intestinal saludable. El objetivo es que esta situación permita un ahorro energético para el huésped lo que se traduce en una mejora en el rendimiento productivo de las aves. Entre los aditivos alimentarios más utilizados, los ácidos orgánicos y los aceites esenciales se han utilizado ampliamente en la producción avícola debido a sus efectos constatados como estimulantes del crecimiento.

El objetivo principal de la presente tesis fue estudiar los efectos de mezclas microencapsuladas de ácidos orgánicos y aceites esenciales sobre el rendimiento productivo y la salud intestinal de pollos de engorde expuestos a diferentes condiciones desafiantes, y resaltar los principales mecanismos de acción responsables de estos efectos. Para lograr este objetivo diseñamos una serie de ensayos que han sido descritos junto con sus resultados en tres capítulos (capítulo 4 al 6).

El **capítulo 4** tuvo como objetivo principal desarrollar un panel de expresión génica, dirigido a proporcionar una fotografía instantánea de posibles variaciones en la expresión génica al nivel de intestino delgado en condiciones de desafío. Se realizaron dos ensayos en los que los pollos de engorde fueron desafiados con enteritis necrótica en el primero, mientras que en el segundo ensayo se aplicó un desafío con una sobredosis de vacuna de coccidia. En el panel se incorporaron varios genes implicados en diferentes funciones, incluida la función de barrera, la respuesta inmunitaria, el transporte de nutrientes, así como el estrés oxidativo y las hormonas digestivas. Los resultados obtenidos mostraron que el panel desarrollado permite la obtención de un perfil de expresión génica global que ofrece una visión más amplia de los genes y las vías involucradas en la respuesta de los pollos de engorde a los desafíos con patógenos.

También proporciona información sobre las diferencias de los patrones de expresión génica y la magnitud de las respuestas bajo un desafío con una sobre dosis de vacuna de coccidia o de enteritis necrótica.

Los objetivos del **capítulo 5** fueron (1) mostrar evidencia de la liberación progresiva de ácido fumárico y timol, como ejemplos de ácidos orgánicos y aceites esenciales, cuando estos están microencapsulados en una matriz lipídica simulando las condiciones intestinales *in vitro* y luego *in vivo*; y (2) evaluar el efecto de la mezcla encapsulada del ácido fumárico y el timol sobre el rendimiento y la salud intestinal de pollos de engorde desafiados con un ayuno de duración corta como modelo de daño de la mucosa intestinal relacionada con un aumento de la permeabilidad del tracto gastro-intestinal. Los resultados obtenidos mostraron que la matriz utilizada pudo proporcionar tanto el momento como la ubicación adecuados para la liberación de los compuestos activos. Además, el ácido fumárico microencapsulado, el timol microencapsulado o su combinación mostraron efectos positivos cuando los pollos de engorde se sometieron a condiciones desafiantes, reduciendo los efectos negativos promovidos por el desafío del ayuno sobre el rendimiento productivo, la histología intestinal y la microbiota.

El **capítulo 6** tuvo como objetivo principal investigar la eficacia de diferentes mezclas microencapsuladas que contienen ácidos orgánicos y aceites esenciales sobre el rendimiento productivo y la salud intestinal de pollos de engorde bajo un desafío de enteritis necrótica. Se llevaron a cabo dos experimentos. El primer ensayo tuvo como objetivo determinar el diseño de combinaciones con una alta eficacia, mientras que el segundo ensayo se centró en encontrar la dosis óptima para cada combinación. Los resultados del primer ensayo mostraron que las mezclas probadas aumentaron la abundancia de algunas familias consideradas como beneficiosas como Ruminococcaceae y Lachnospiraceae; en paralelo con reducir la de las dañinas como Enterobacteriaceae y Helicobacteraceae. Esta variación coincidió con cambios positivos en la histología intestinal, lo que se reflejó al nivel del rendimiento productivo de los pollos de engorde desafiados. Posteriormente, entre las 4 mezclas probadas, las dos que mostraron los mejores efectos sobre la histología intestinal y el crecimiento fueron seleccionadas para el ensayo de dosis/respuesta. Los resultados mostraron que 0,5 g/kg de la mezcla que contenía butirato de calcio y ácido fumárico, y 2 g/kg de la que contenía ácidos grasos de cadena media, butirato de calcio, ácido fumárico y cítrico, en ambos casos combinados con cinamaldehído, carvacrol y timol (8:1:1) ejercieron

efectos beneficiosos sobre el rendimiento productivo y la histología intestinal de los pollos a los 42 días del experimento. Estas mezclas también disminuyeron los recuentos fecales de Enterobacteriaceae y *Clostridium perfringens*. Los resultados del segundo experimento mostraron efectos beneficiosos similares a los observados en el primer ensayo sobre la microbiota cecal.

En conjunto, los resultados obtenidos demuestran la importancia sobre la eficacia del aditivo que tiene el diseño particular de las combinaciones de ácidos orgánicos y aceites esenciales, las dosis de suplementación, así como la posibilidad de proporcionar una protección adecuada mediante microencapsulación que permita controlar a la vez el lugar y el momento de liberación de los compuestos activos. Por otro lado, los resultados demuestran también que los efectos beneficiosos de tales aditivos se alcanzan a través de una variedad de mecanismos de acción que son complejos y frecuentemente interrelacionados.

Index of contents

Index of tables

Index of figures

Abbreviations

AC	aromatic compounds
ADFI	average daily feed intake
ADG	average daily gain
AGPs	antibiotic growth promoters
AHSA1	activator of HSP90 ATPase activity 1
AID	apparent ileal digestibility
ATTD	apparent total tract digestibility
AvBD	avian β-defensin
BB	blue brilliant
BF	barrier function
BW	body weight
BWG	body weight gain
CD	crypt depth
CLDN	claudin
COX16	cytochrome C oxidase assembly factor COX16
CP	crude protein
DM	dry matter
EE	ether extract
EIF4EBP1	eukaryotic translation initiation factor 4E-binding protein 1
EOs	essential oils
FABP	fatty acid binding protein
FCR	feed conversion ratio
FD	fat digestibility
FDR	false discovery rate
FP	fasting period
GE	gross energy
GHRL	ghrelin
GIT	gastrointestinal tract
GPX7	glutathione peroxidase 7

H	digestive hormones
HIF1A	hypoxia-inducible factor 1-alpha
HMOX2	heme oxygenase 2
HSPA	heat shock protein family A
IFNG	interferon gamma
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IR	immune response
JAM	junctional adhesion molecule
LCFAs	long chain fatty acids
LPS	lipopolysaccharide
MB	metabolism
MCFAs	medium chain fatty acids
mTOR	mammalian target of rapamycin
MUC	mucin
NC	negative control
NE	necrotic enteritis
NOS	nitric oxide synthase
NT	nutrient transport
OAs	organic acids
OCLN	occludin
OM	organic matter
OX	oxidative stress
PFAs	phytogenic feed additives
PTGES	prostaglandin E synthase
PUFA	polyunsaturated fatty acids
RPS6KB1	ribosomal protein S6 kinase B1
SCFAs	short chain fatty acids
SIgA	secretory immunoglobulin

SLC	solute carrier
SOD	superoxide dismutase
TJP	tight junction protein
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
VDR	vitamin D receptor
VH	villus height
XDH	xanthine dehydrogenase

CHAPTER 1

General introduction

Optimal gut health is of critical importance to the poultry performance as it is responsible of regulating physiological homeostasis which provides the animal with the ability to withstand infectious and non-infectious stressors. Although different definitions have been provided, gut health is considered an intricate and complex area, which encompasses a number of physiological and functional features, including nutrient digestion and absorption, barrier function, a stable microbiome, host metabolism and energy generation, mucus layer development, and mucosal immune responses. The continuous interaction among all of these components allows the bird to perform properly to reach values close to its genetic potential. Therefore, fully elucidating the interaction among all of these features will allow a better understanding of gut health.

Poultry industry also faces the growing concerns about environmental and public health risks associated with the emergence of antibiotic resistance in zoonotic bacterial pathogens, which have led to global interest in adopting a more restricted use of antibiotics in animal production. However, the severe restrictions on the use of antibiotics have put the poultry production under continuous challenges with the increased occurrence of digestive infectious diseases, especially necrotic enteritis due to *Clostridium perfringens* (Selaledi et al., 2020) as well as coccidiosis. Other unintentional consequences of the severe restrictions on the use of antibiotics include impaired performance by around 4% on average, especially in low-sanitary status flocks where higher morbidity, feed conversion ratio (FCR) and flock BW variance is observed (Cowieson and Kluenter, 2019). In order to mitigate higher risks associated with the absence of antibiotics, changes in husbandry and environmental management are also required such as reducing stocking density, increasing down-time, cleaning-out more frequently, maintaining ideal temperature, keeping strict biosecurity, reducing stress, breed selection (Cervantes, 2015; Selaledi et al., 2020).

Therefore, a great deal of current research is being focused on the development of alternatives to antibiotics, putting the poultry industry at the forefront of advances in the development of prebiotics, probiotics, organic acids, phytogenics especially essential oils, nutritional antioxidants, anti-nutritional enzymes, and immune modulators for the regulation of gut health and functionality (Dittoe et al., 2018; Suresh et al., 2018). Among the abovementioned feed additives, organic acids (OAs) and essential oils (EOs) have been used extensively in poultry production. Indeed, combining

hydrophobic EOs with lipophilic OAs in chicken diets has been recently considered among the most promising alternatives to antibiotics and has received much attention for the potential synergistic and additive benefits on growth performance and health in poultry and swine production compared to individual EOs or OAs (Li et al., 2018). However, these feed additives may present difficulties in handling and offensive odor, making them unpleasant to work with and deterring animals from consuming feed. The volatile nature in the case of EOs and their early absorption in the upper gastrointestinal tract (GIT) may also explain losses of efficacy. These challenges can be resolved by microencapsulation, also known to enhance the oxidative stability, thermo stability, photo stability, shelf-life, and biological activity of feed additives as well as to ensure their targeted delivery in to the lower intestine of animals (Stevanović et al., 2018).

On this basis, it seems opportune to study the effects of microencapsulated feed additives composed of mixtures of OAs and EOs on the performance and gut health of broiler chickens. We plan to perform this study either in broiler chicken maintained under non-challenging conditions or subjected to challenges including short-term fasting and/or *Clostridium perfringens*. This thesis will also try to clarify some of the mechanisms behind these effects with a special focus on the modulation of intestinal microbiota composition as well as intestinal expression of genes related to vital functions such as barrier function, immune response and nutrient transport.

CHAPTER 2

Hypothesis and Objectives

In this thesis we hypothesize that:

1. The supplementation of microencapsulated feed additives composed of OAs and EOs improves the growth performance of broiler chickens under compromised sanitary conditions.
2. These feed additives exert their stimulatory growth effects through modulating the intestinal microbiota which may affect the intestinal structure and thereby the gut health of challenged broiler chickens.
3. Microencapsulation of OAs and EOs, will promote a delayed release of the contained active compounds into the targeted GIT section, exerting beneficial effects on performance, immunity, and the digestive GIT functions in broiler chickens.

Therefore, the general objective of the current thesis was to evaluate the effects of microencapsulated blends of OAs and EOs on growth performance and gut health of broiler chickens subjected to different challenging conditions, and to highlight the mechanisms of action behind these effects.

The specific objectives of this thesis were:

1. To sum up the current trends in the use of phytogenic feed additives and organic acids in poultry with a special focus on their interaction with gut ecosystem, gut function, *in vivo* oxidative status and immune system (Chapter 3).
2. To develop a custom gene expression panel that could provide a snapshot of intestinal gene expression variation under challenging conditions (Chapter 4).
3. To show evidence of the progressive release of OAs and EOs, when these are microencapsulated in lipid matrix microparticles under *in vitro* and *in vivo* intestinal conditions; and to evaluate the effect of microencapsulated OAs and EOs on the performance and gut health of broiler chickens challenged with a short-term fasting period (Chapter 5).
4. To investigate the effects of different microencapsulated blends containing OAs and EOs on performance and gut health of broilers under challenging conditions of necrotic enteritis (Chapter 6).

CHAPTER 3

Literature review

Abdelli, N., Solà-Oriol, D., & Pérez, J. F. (2021). Phytogenic Feed Additives in Poultry: Achievements, Prospective and Challenges. *Animals*, *11*(12), 3471.

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animals

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Review

Phytogenic Feed Additives in Poultry: Achievements, Prospective and Challenges

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Simple Summary: Plant secondary metabolites and essential oils also known as phytogenics are biologically active compounds that have recently attracted increased interest as feed additives in poultry production, due to their ability to promote feed efficiency by enhancing the production of digestive secretions and nutrient absorption, reduce pathogenic load in the gut, exert antioxidant properties and decrease the microbial burden on the animal's immune status. However, the mechanisms are far from being fully elucidated. Better understanding the interaction of phytogenics with gastrointestinal function and health as well as other feed ingredients/additives is crucial to design potentially cost-effective blends.

Abstract: Phytogenic feed additives have been largely tested in poultry production with the aim to identify their effects on the gastrointestinal function and health, and their implications on the birds' systemic health and welfare, the production efficiency of flocks, food safety, and environmental impact. These feed additives originating from plants, and consisting of herbs, spices, fruit, and other plant parts, include many different bioactive ingredients. Reviewing published documents about the supplementation of phytogenic feed additives reveals contradictory results regarding their effectiveness in poultry production. This indicates that more effort is still needed to determine the appropriate inclusion levels and fully elucidate their mode of actions. In this frame, this review aimed to sum up the current trends in the use of phytogenic feed additives in poultry with a special focus on their interaction with gut ecosystem, gut function, in vivo oxidative status and immune system as well as other feed additives, especially organic acids.

Keywords: phytogenics; performance; digestibility; microbiota; immunity; oxidant status; organic acids; microencapsulation; poultry

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1. Introduction

Poultry production is undergoing a continuous challenge to develop management strategies to optimize chickens' efficiency while limiting food safety concerns. Traditionally, antimicrobials have been widely used for improving health and growth performance in poultry; however, the increased public awareness about the risk of developing cross-resistance of pathogens to antibiotics has resulted in the gradual removal of antibiotics for therapeutic and prophylactic uses in food animals [1]. The shift away from antibiotic supplementation has resulted in a tremendous growth in research focusing on the implementation of effective alternative control methods, management and dietary amendments aiming to improve animal health, welfare, and productivity. A wide range of feed additives including a broad spectrum of essential oils and related compounds from botanical sources to organic acids [1,2], as well as probiotics and prebiotics [3], chemicals such as aldehydes [4], bacteriophages [5], zinc oxide [6], exogenous enzymes [7] and competitive exclusion products [8] have been used in animal production. Particularly, phytogenic feed additives

(PFAs), also popularly referred as phytobiotics or botanicals, have gained an increasing interest as cost-effective feed additives with proven positive effects on broiler chickens' intestinal health. Indeed, antioxidative, immunomodulatory and growth-promoting effects have also been largely described in the literature. Therefore, the aim of the present review is to summarize the main results of some recent studies evaluating the effect of PFAs supplementation on the major components of bird gastrointestinal health and functionality, with special focus on nutrient digestibility, gut microbiota, immune system, oxidative status and growth performance of broilers and laying hens.

2. Gastrointestinal Health and Functionality

The regulation of gastrointestinal tract (GIT) function involves complex interactions among six major components, including the diet, effective digestion and absorption, normal and stable microbiota, effective immune status, gut mucosa, and neuroendocrine and motor function of the gut [9]. Both, diet composition (ingredients, nutrients and additives) and form, including structure and particle size, may affect the GIT functionality, especially through modulating the immune system and intestinal microbiota [9]. In fact, some dietary factors such as certain types of dietary fiber, trypsin inhibitors, phytate, lectins, undigested protein in the distal gastrointestinal tract, mycotoxins, as well as diets with poor nutrient balance, may affect the inflammatory process by modulating both pro-inflammatory and anti-inflammatory mechanisms [10] and thereby, disrupt the structural and functional integrity of the gut [11]. Conversely, feed additives such as phytobiotics, organic acids, enzymes, prebiotics, probiotics [12–14], functional foods and nutraceuticals [15] may play key roles in promoting overall health and growth performance.

The main attributes of an effective GIT functionality is an optimal digestion and absorption, maintenance of fluid and electrolyte balance, and elimination of waste products as well as maintenance of a barrier against antigens and pathogens [9]. The gastrointestinal compartments of healthy chickens are densely harbored by complex microbial communities which provide both nutrition and protection for the animal. Commensal microbiota may stimulate the development of immune system including the mucus layer, epithelial monolayer, the intestinal immune cells (e.g., cytotoxic and helper T-cells, immunoglobulin producing cells and phagocytic cells), and the lamina propia; thereby allowing to form a protective barrier between the host and the microbes [16]. Moreover, the microbiota of the distal gut (i.e., caeca and colon) uses the undigested feed to produce vitamins (e.g., vitamin K and vitamin B groups), amino acids, and short chain fatty acids (SCFAs: acetic acid, butyric acid and propionic acid) which eventually become available for the host. These SCFAs are considered of great interest for the host, for their bacteriostatic properties allowing to eliminate foodborne pathogens, such as *Salmonella* spp. [17], and as a source of energy which can stimulate gut epithelial cell proliferation and the gastrointestinal absorption surface [18].

On the other hand, impaired digestion and absorption results in a delivery of excess nutrients (such as starch, protein and fat) to the distal segments of the gastrointestinal tract, which induces alterations in the GIT microbial community resulting in qualitative and/or quantitative imbalance of normal microbiota in the small intestine. This imbalance is characterized by proliferation of pathogens which may lead to a sequential reaction in the GIT, including reduced intestinal barrier function (e.g., thinning of intestinal wall) and poor nutrient digestibility; and therefore, increasing the risk of bacterial translocation and inflammatory responses [19]. These negative effects on the symbiotic interactions between host and microbe lead to adverse effects on feed efficiency, productivity, and health of chickens [16].

The maintenance of GIT integrity is also crucial to ensure effective immune system as GIT is considered the largest organ of the immune system [20], that plays pivotal physiological role as barrier against antigens and pathogens. On the other hand, the GIT also possesses a neuroendocrine function through conveying neuroendocrine signals to the brain during digestion aiming to align the digestive and absorptive capacity of the GIT with

the amount and composition of ingested food [11]. Therefore, it secretes gastrointestinal peptide hormones, such as gastrin, secretin and cholecystokinin in response to nutrient, neural or hormonal stimulation as well as by metabolic products of the gut microbiota. These peptide hormones are involved in the digestive and absorptive function of the GIT such as the regulation of gastric acid and pancreatic secretion, release of bile from the gall bladder and gut motor activity [9].

The diet is a main factor modulating the composition and the metabolic activity of the GIT microbiota [21]. In this regard, several feed additives have been developed focusing on enhancing immune response, reducing pathogen load in the GIT, promoting the colonization of the GIT with beneficial bacteria and stimulating digestion and absorption. The current review will discuss the effects of PFAs on the different components of GIT functionality.

3. Phytogenics as an Alternative to Antimicrobials in Poultry Feeding

A broad range of plants derived products may fall under the category of phytogenic feed additives. They may be classified either based on their origin (the part of the plant) into herbs (products from flowering, non-woody, and non-persistent plants from which leaves and flowers are used) and spices (non-leaf parts of plants, including seeds, fruits, bark or root with intensive taste or smell); or depending on the process used to derive the active ingredients as essential oils (EOs: volatile lipophilic substances obtained by cold extraction or by steam or alcohol distillation) and oleoresins (extracts derived by non-aqueous solvents). The bioactive components of PFAs are secondary metabolites being polyphenols the main group. Other bioactive compounds include terpenoids (monoterpenes, steroids. . .), phenolics (tannins), glycosides, and alkaloids [21]. The composition and concentration of these bioactive substances may vary according to several factors including the plant, parts of the plant, geographical origin, harvesting season, climatic conditions, processing techniques such as extraction, distillation and stabilization as well as storage conditions [22,23].

In recent years, PFAs have attracted an increasing attention as natural alternative to antibiotic growth promoters (AGPs) in poultry production which can be included in feeds as dried, solid, and ground form, or as extracts (crude, concentrated and purified) [24]. A wide variety of herbs and spices (thyme, oregano, cinnamon, rosemary, marjoram, yarrow, garlic, ginger, green tea, black cumin, coriander, among others) as well as EOs (from thymol, carvacrol, cinnamaldehyde, garlic, anise, rosemary, citruses, clove, ginger) have been used in poultry, individually or mixed, for their potential application as AGP alternatives [24]. Although the repertoire mechanisms of action of PFAs is not fully elucidated in poultry, one of their primary mode of action is related to their antimicrobial effects which allow controlling potential pathogens [25]. The results obtained in some recent studies will be reported with more details in the current review. However, some authors reported no positive effects of PFAs inclusion [26]. This discrepancy may be attributed to several factors, including the inherent variability of the botanic composition, as well the variability of the animal scenarios, environmental, management and sanitary conditions (i.e., including the likely presence of a pathogen challenge). The technique of treatment (cold, steam distillation, extraction or maceration with non-aqueous solvents. . .) has been also reported to change the active substances and related compounds in the final product [22].

Among PFAs, there is a rising interest in EOs for animal nutrition as some of these feed additives have been shown to possess a much higher biological activity compared to the raw material they were extracted from [27]. EOs are complex mixtures of volatile compounds, being mainly hydrocarbons (terpenes, sesquiterpenes), oxygenated compounds (alcohol, aldehydes, ketones) and a small percentage of non-volatile residues (paraffin, wax) [28]. Chemically, EOs consist fundamentally of two classes of compounds, the terpenes and phenylpropenes. Although, the effects of a mixture of EOs rely on the additive and synergy or antagonic effects of their components, 2 or 3 components may constitute up to 85% of the total mixture [29] and thus, contribute to its primary property [2]. In fact, thymol and

carvacrol are the two main phenols which account for almost 80% of the EO of oregano and are the main contributors to its antibacterial and antioxidant activities. The compound p-cymene is another dominant component of oregano EO [30]. Even though this component is not considered as an effective antimicrobial agent, it is a precursor for carvacrol which possesses higher preference for liposomal membranes, enabling carvacrol to be more easily transported into the cell [31].

EOs are perceived as growth promoters in poultry diets with strong antimicrobial and anticoccidial activities [2]. Thus, literature shows that growth promoting effects of EOs exist both abundantly and controversially, which makes it imperative to perform more in-depth research to understand the underlying mechanisms.

4. Effects of Phytogenic Feed Additives on Chickens

The effects of PFAs on the main metabolic and physiologic process like nutrient digestibility, intestinal microbiota, immunity, oxidant status and growth performance of broilers will be discussed with details below.

4.1. Effects of Dietary PFA Supplementation on Growth Performance

Several studies have been carried out using herbs, spices, and EOs and showed inconsistent results on chicken performance. Although some studies showed that PFAs have positive effects on body weight gain and FCR in chickens [32,33], others reported either an improved chicken body weight gain without affecting FCR [34,35] or an enhanced feed conversion rate associated to a lack of effects on body weight or feed intake [36,37]. This inconsistency may be explained by several factors such as the botanical source, the concentration and the duration of supply of the active compounds, the feed composition, and the experimental challenging conditions, animal age and health status [38].

4.1.1. Chickens Maintained under Non-Challenging Conditions

Detailed results of some recent studies evaluating the effects of PFAs on growth performance of birds maintained under non-challenging conditions are presented in Tables 1–3. Dietary supplementation of EOs containing menthol, anethol and eugenol [39] as well as carvacrol alone [40], or combined with either thymol [41–43] or thymol and limonene [44] has been shown to promote growth performance of broiler chickens. Similar positive effects on production performance were obtained by supplementing laying hens diets with thymol and cinnamaldehyde [39], star anise oil [45], *Citrullus lanatus* EO [46], tea tree EO [47] and peppermint oil [48]. Several other studies have also evaluated the effects of supplementing black cumin (*Nigella sativa* L.) seeds on broilers, quails and laying hens. Although some authors failed to find any effect by supplementing Japanese quails diets with black cumin [49], others reported improved growth performance in quails [50,51] broiler chickens [52] and laying hens [53,54]. These growth-promoting effects have been attributed to the presence of a large number of pharmacologically active compounds such as thymoquinone, dithymoquinone, thymohydroquinone, nigellone, melanthin, nigilline, nigelamine, damascenone, *p*-cymene and pinene and a variety of essential nutrients including vitamins A, B, C, D and E, as well as minerals such as magnesium, calcium, phosphorus, potassium, iron, cobalt, zinc and manganese [41,55]. Moreover, curcuminoids and lipophilic turmeric extract containing curcumin and turmerones; known for their gastroprotective and anti-inflammatory activities, showed positive effects on growth performance of slow-growing [56] and fast-growing broiler chickens [57], respectively. Similar growth-promoting effects were observed by supplementing broiler chickens by a PFA of *Aerva lanata*, *Piper betle*, *Cynodon dactylon*, and *Piper nigrum* [58], *Pulicaria gnaphalodes* powder [59], *Achyranthes japonica* extract [60], *Boswellia serrata* [61] and bioactive olive pomace extract from *Olea europaea* [62] as well as laying hens by fennel seeds or red pepper [53] a mixture of *Punica granatum*, *Thymus vulgaris*, and *Allium sativum* [63] and dietary Nettle (*Urtica cannabina*) [64]. However, egg weight, laying rate and FCR were not improved by

dietary supplementation of either EO of star anise (*Illicium verum* Hook.f.) [45] or a mixture of 13.5% thymol and 4.5% cinnamaldehyde [65].

4.1.2. Chickens Maintained under Challenging Conditions

A large number of studies have been performed to investigate the positive effects of PFAs on broiler chickens subjected to challenges related to environmental conditions including heat stress, lipopolysaccharide (LPS) and pathogens such as *Clostridium perfringens*, *Eimeria* spp, *Salmonella typhimurium*, or *Escherichia coli*, among others. Results of some recent studies are illustrated in Tables 2 and 4.

Heat stress in chickens induces a tight junction disruption that may lead to increased gut permeability and eventually to a dysregulation of the body's homeostasis [66], and thereby poor nutrient absorption, increased secretion of electrolytes and water in gastrointestinal tract leading to compromised performance [67]. However, the adverse effects caused by heat stress on broiler growth performance have been shown to be alleviated by the supplementation of turmeric rhizome powder [68], enzymatically treated *Artemisia annua* [69], ginger [70] and curcumin [71]. *In ovo* injection of black cumin (*Nigella sativa*) extract improved post-hatch performance of thermally challenged broiler chickens during incubation [72]. Similarly, 59-week-old cold-stressed laying hens supplemented with oregano EO showed improved FCR and egg production from week 9 to 12 of a 12-week feeding trial [73].

Numerous studies have been also performed to discern the efficacy of PFAs in chickens subjected to coccidiosis and necrotic enteritis (NE) classified among the most significant diseases affecting the poultry industry, which have become more prominent in the wake of policies to reduce the use of antibiotics in animal production. *Clostridium perfringens*-challenged broilers supplemented with PFAs consisting of either benzo [c]phenanthridine alkaloids from *Macleaya cordata*, active component of carvacrol from oregano (4.95 g/100 g), cinnamaldehyde from cinnamon (2.97 g/100 g), and capsaicin from paprika (1.98 g/100 g), or EO of thyme and anise as leading active ingredients and other including oregano, carvacol, yucca extract and cinnamaldehyde exhibited similar growth performance as the control non-infected group [74]. Microcapsules with a blend of EOs (thyme, peppermint, savoury, and black pepper) at the dose of 0.5, 1, and 2 kg/ton in the *C. perfringens*-challenged broiler chickens results in raising final weight and total feed intake [75]. However, supplementation of EO containing 25% thymol and 25% carvacrol as active components, did not influence the growth performance during d 0 to 14 and tended to linearly reduce the FCR between 14 and 28 d of age of *Clostridium perfringens* challenged broilers [76]. Regarding coccidiosis, the supplementation of a cashew nut shell oil and commercial castor oil blend allowed a recovery in performance similar to that observed with birds receiving the ionophore monensin during the accumulated experimental period (1 to 42 d) [77]. Hussein et al. reported that combinations of peppermint, chamomile and prebiotic yeast cell wall were as effective as salinomycin in preventing the decline in the weight gain and FCR performance of coccidiosis-challenged broilers [78]. However, the effects of dietary *Yucca*-derived saponin supplementation on growth performance seem to be dependent on broiler health status. Although Su et al. [79] reported that saponin supplementation via an extract from *Y. schidigera* serves as an effective growth promoter in non-challenged broilers, Oelshlager et al. [80] found no significant influence of *Yucca* extract on growth responses of broilers during a mixed coccidian challenge. This suggests a reduction in the bioefficacy of saponins when used during an immune challenge [80].

Similarly, a 20-day experiment showed that dietary curcumin supplementation from day 12 to 20 failed to positively affect growth performance of broiler chickens challenged with *Eimeria* species on day 14 of age [81]. These authors attributed the lack of effect to the short period of supplementation and suggested that it might be beneficial if curcumin is fed in broilers for a 42-d period. This suggestion was based on the results of Rajput et al. [82], showing that birds fed curcumin-supplemented diets for 42 d exhibited significant increase

in the BW and feed efficiency during the finisher stage (22–42 d), whereas no significant difference in growth performance was observed during the starter phase (0–21 d).

PFAs supplementation has been also shown to alleviate the effects on the broilers growth performance of other pathogens, such as *Achyranthes bidentate* under *Escherichia coli* challenge [83], resveratrol under *Escherichia coli* challenge [84], a PFA consisting of various nutritional acids and four different alkaloids obtained from special plants under *Salmonella typhimurium* challenge [85], and *Allium hookeri* roots in LPS-induced young broiler chickens.

Table 1. Effects of dietary supplementation of PFAs on growth performance of broilers under non-challenging conditions.

Feed Additive	Major Components	Dose, (mg/kg Diet)	Diet	Age	Treatment Effects (%, Compared to Control)			References
					BW	ADFI/FI	FCR	
Olea europaea extract	Triterpenes (10%) polyphenols (2%)	750	Wheat-soybean meal based diet	21–42 d	NM	NS	−7.9	[62]
Achyranthes japonica extract	Flavonoid (1.15 mg/g), polyphenol (4.26 mg/g) and saponin (0.47 mg/g)	1000	Corn-soybean meal based diet	0–35 d	3.5	−2.4	−6.2	[60]
EOs	Carvacrol (20%) and thymol (25%)	200	Corn-soybean meal based diet	29–42 d	NS	−9.6	−11.8	[43]
EOs	Carvacrol (5%), cinnamaldehyde (3%), and capsicum oleoresin (2%)	100	Corn-soybean or wheat -soybean meal based diet		16.4	6.1	−9.4	[86]
Aerva lanata, *Cynodon dactylon*, *Piper nigrum* and *Piper betle*	Phenolic acid contents (10,176.8 µg/g), flavonoids (53.0 µg/g), other (220.2 µg/g)	10,000	Corn-soybean meal based diet	0–42 d	14.1	NS	−14.0	[58]
Pulicaria gnaphalodes powder	Phenolic compounds, alkaloids, terpenoids, and triterpene saponins	3000	Corn-soybean meal based diet	0–42 d	4.3	NS	−3.0	[59]
Standardized lipophilic turmeric extract	3.1% of curcuminoids content and terpenes (turmerones)	10,000	NM	0–42 d	9.0	1.6	−7.7	[57]
EOs	Carvacrol (63.5%), thymol (3.4%) and paracymene (13.1%)	400 µL	Corn-wheat-soybean meal based diet	28–43 d	4.2	NS	−3.9	[40]
Thyme powder	Major EO (thymol (50.48%), γ-terpinene (11.03%), P-cymene (9.77%), and carvacrol (4.30%)), phenolic acids (salicylic acid (2450.03 ppm), ellagic acid (1240.42 ppm)) and flavonoid compounds	5000	Corn-soybean meal-based diet	0–42 d	4.6	3.3	NS	[87]
EOs (oregano, anise, and citrus peel; CBP)	Carvacrol: 102 g of the chemical component/kg of CBP	150	Corn-soybean meal-based diet	0–42 d	NS	-5.3	NS	[88]
Combination of herbs, spices, EOs and extracts	Mainly EOs from mint, star anise and cloves	100	Corn-soybean meal-based diet	0–42 d	7.0	NS	NS	[39]
EO (powdered and matrix-encapsulated form)	-Powdered: menthol and anethole -Encapsulaed: carvacrol, thymol, and limonene	150 100	Corn-wheat-soybean meal based diet	0–42 d	NS 2.4	NS NS	NS NS	[44]
EOs	Oregano containing carvacrol (26.4 mg/kg) or thymol (13 mg/kg)	300 600	Corn-soybean meal-based diet	0–42 d	7.8 9.6	4 8	NS NS	[41]
Spices: *Nigella sativa* seeds	Thymoquinone, dithymo- quinone, thymohydroquinone, nigellone, melanthin, nigilline, nigelamine, damascenone, *p*-cymene and pinene	10,000 20,000	Corn-soybean meal-based diet	0–35 d	3	NS	5.6	[52]

NS: not significant; NM: not mentioned.

Table 2. Effects of dietary supplementation of PFAs on growth performance of laying hens.

Feed Additive	Major Components	Dose (mg/kg Diet)	Diet	Line and Age	Main Findings	References
Non-Challenging Conditions						
Mentha arvensis (MA) and *Geranium thunbergii* (GT) extracts	MA: menthol, isomenthol, neomenthol, p-cymene, d-menthone, eugenol, and cineol GT: citronellol, isomenthone, and geraniin	100, 500 and 1000	Corn-wheat-soybean meal based diet	Hy-Line Brown layers (28–44 weeks)	↑ FI, egg production and egg weight	[89]
Fermented pine (*Pinus densiflora*) needle extract	α-pinene, caryophyllene, beta-pinene and bisbenzene, camphene, borneol, phellandrene, quercetin, kaempferol, and terpene	2.5 and 5	Corn-soybean meal-based diet	Hy-Line Brown laying hens (40–46 weeks)	↑ FI, egg production and egg mass	[90]
Fermented *Schisandra chinensis* pomace (SC), fermented *Pinus densiflora* (PD) needle extract, and *Allium tuberosum (AT)* powder	SC: lignin PD: phenolics, flavonoids, and tannins AT: organosulfur compounds, polyphenols, and saponins	1000 and 3000	Corn-soybean meal-based diet	Hy-line brown laying hens (48–54 weeks)	-=Egg production, daily egg mass and FCR. -↑ FI	[91]
Dry leaf extract of peppermint (*Mentha piperita L.*)	Menthol	0, 74, 148, 222, and 296	Corn-soybean meal-based diet	Bovans Brown laying hens (32–44 weeks)	↑ FI, egg production, egg weight and egg mass	[48]
Citrullus lanatus EOs	Phenolics (1.57 mg/100 g) Sterols (600.56 mg/100 g) Flavonoids (163.5 mg RE/kg)	1000 and 2000	Corn-soybean meal-based diet	White Leghorn laying hens (18–26 weeks)	↑ Weight gain, ADFI, ADG and egg mass; ↓ FCR	[46]
Tea tree (*Melaleuca alternifolia)* EO	Terpinen-4-ol (40.0%), γ-Terpinene (23.0%) and α-Terpinene (10.4%)	40 and 80	NM	Lohmann Brown hens (55–58 weeks)	↑ Daily egg production and ↓ FCR	[47]
Thyme *(Thymbra spicata)* and Rosemary (*Rosemarinus officinalis*)	-Thyme: Carvacrol (87.81%), thymol (9.58%), L-Linalool (0.86%), borneol (0.74%) -Rosemary: 1.8 cineole (34.08%), camphor (27.95%), alpha-Pinene (14.50%), borneol l(8.65%), alpha-Terpineol (7.39%), alpha-Thujone (1.09%), camphene (0.55%)	1000 for each source	Corn-soybean meal-based diet	Bovans-White (48–56 weeks)	-No effects on FCR -↓ Egg production and egg weight	[92]
Cumin (*Cuminum cyminum L.*) seed oil	Cuminol, cuminique alcohol, cuminaldehyde, cymine, phellandrene, carvone, cymol, terpenes, α-pinene . . .	500	Corn-soybean meal-based diet	Boven hens (24–30 weeks)	=Egg production rate, egg mass and FI ↓ FCR and ↑ egg weight	[93]
Eucalyptus leaves	Polyphenols	500, 800 and 1200	Corn-soybean meal-based diet	Yueqinhuang laying hens (35–44 weeks)	↑ Egg production and egg mass	[94]
Fennel seeds (F), black cumin (BC) seeds and hot red pepper (RP)	F: trans-anethole BC: thymoquinone, anethole, carvacrol and 4-terpinol RP: Capsaicin	5000 for each	Corn-soybean meal-based diet	Lohmann Brown Lite laying hens (32–40 weeks)	↑ Egg weight, egg production, egg mass and ↓ FCR by F and RP	[53]
Green tea	Polyphenols	200	Corn-soybean meal-based diet	Hy-line Brown (65–74 weeks)	↑ Egg production and ↓ FCR	[95]
EOs	Thymol (13.5%) and cinnamaldehyde (4.5%)	50, 100 and 150	Corn-wheat-soybean meal based diet	Lohmann White (54–65 weeks)	=Egg production, egg weight, egg quality, FI and FCR	[65]

Table 2. *Cont.*

Feed Additive	Major Components	Dose (mg/kg Diet)	Diet	Line and Age	Main Findings	References
Echinacea purpurea powder	Caffeic acid and alkamids, phenolic acids, polyacetylenes	2500, 5000, 7500 and 10,000	Corn-soybean meal-based diet	Leghorn laying hens (43–53 weeks)	↑ Egg production and egg mass	[96]
Peppermint EO Thyme EO	-Menthol and menthone -Thymol, γ-Terpinen and ρ-Cymene	1000	Corn-soybean meal-based diet	Lohmann LSL-lite (40–48 weeks)	↑ Egg production and egg mass ↓ FCR	[97]
Dried grape pomace	Polyphenols	40,000 and 60,000	Corn-soybean meal-based diet	Bovans laying hens (80–92 weeks)	=Live weight, feed intake, egg production and feed efficiency	[98]
Fennel (F) and thyme (T) extracts	F: anethole, limonene T:-Thymol, γ-Terpinen and ρ-Cymene	40	Corn-soybean meal-based diet	Hy-Line White (26–38 weeks)	↑ Egg weight and egg mass	[99]
Cold stress + *Escherichia coli*						
Curcuma longa	Curcumin	200	Corn-soybean meal-based diet	Hy-Line Brown laying hens (84–90)	=Egg production, egg mass, feed intake and FCR	[100]
Cold stress						
Oregano EO	Carvacrol and thymol	50, 100, 150 and 200	Corn-soybean meal-based diet	Semi-heavy laying hens (59–71 weeks)	=FCR, egg production and egg mass	[73]
Heat stress						
Grape pomace flour	Polyphenols	10,000, 20,000 and 30,000	Corn-soybean meal-based diet	Hy-Line lineage (74–79 weeks)	↑ FI	[101]

ADG: Average Daily Gain; ADFI: Average Daily Feed Intake; FI: Feed Intake; FCR: Feed Conversion Ratio; ↑: increased; ↓: decreased; =: equal.

Table 3. Effects of dietary supplementation of PFAs on growth performance of other birds under non-challenging conditions.

Feed Additive	Major Components	Dose (mg/kg Diet)	Diet	Line and Age	Main Findings	References
Grape seed extract	Polyphenols	100 and 200	Corn-soybean meal-based diet	Duckling (Pekin-female; 0–6 weeks)	↑ ADG, and final body weight with ↓ FCR	[102]
Oregano EO	Carvacrol and thymol (85%)	100	Corn-soybean meal-based diet	Duckling (Cherry valley; 0–5 weeks)	=ADG, FCR	[103]
Eucalyptus (*Eucalyptus camaldulensis*)	p-cymene, 1, 8-cineole, b-phellandrene, spathulenol, cryptone aldehydes, cuminal, phellandral, and a-phellandrene	100 and 200	NM	Laying Japanese quails	=Productive traits	[104]
Oregano EO	Thymol (5%) and carvacrol (65%)	150 and 300	Corn-soybean meal-based diet	Duckling (Cherry valley; 11–42 days)	=Final body weight, ADG, FI, and FCR	[105]
NM	Thymol	2000, 4000 and 6250	NM	Quail (*Coturnix japonica*; 85–128 days)	=BWG, FI, egg production, and egg weight	[106]
Leaves of *Astragalus membranaceus*	Polyphenols (saponins, flavonoids)	10,000, 30,000 and 50,000	Corn-soybean meal-based diet	Japanese quail (0–35 days)	↑ FI, and weight gain	[107]
Mentha piperita (peppermint)	Phenolic compounds	10,000, 20,000, 30,000 and 40,000	Corn-soybean meal-based diet	Quail	=FI and ADG	[108]

BWG: body weight gain; ADG: Average Daily Gain; ADFI: Average Daily Feed Intake; FI: Feed Intake; FCR: Feed Conversion Ratio; ↑: increased; ↓: decreased; =: equal.

Table 4. Effects of dietary supplementation of PFAs on growth performance of broilers under challenging conditions.

Feed Additive	Major Components	Dose, (mg/kg Diet)	Diet	Age	Treatment Effects (%, Compared to Control)			References
					BW	ADFI/FI	FCR	
Clostridium perfringens								
Herb: *Macleaya cordata* plant	Four specific alkaloids mainly sanguinarine and protopine	120	Corn-soybean meal-based diet	15–35 d	12.7	NS	−14.8	[109]
Plant: *Macleaya cordata*	Benzo [c]phenanthridine alkaloids	NM	Corn-soybean meal-based diet	15–21 d	NS	NS	−8.9	[74]
Plant extracts	Carvacrol (4.95 g/100 g), cinnamaldehyde (2.97 g/100 g), and capsaicin (1.98 g/100 g)	NM			NS	NS	−10.0	
EOs	Thyme and anise, oregano, carvacol, yucca extract and cinnamaldehyde	NM			NS	NS	−11.6	
EO	Thymol (25%) and carvacrol (25%) as active components	60, 120 and 240	Wheat-soybean meal-based diet	14–28 d	NS	NS	NS	[76]
Eimeria								
Herb: *Curcuma longa*	Curcumin	100 and 200	Corn-soybean meal-based diet	12–20 d	NS	NS	NS	[81]
EOs: cashew nut shell liquid and castor oil	Cardanol, cardol, and anacardic acid Ricinoleic acid	1500	Corn-soybean meal-based diet	0–42 d	2.3	NS	NS	[77]
Escherichia coli								
Resveratrol	Polyphenols	600	Corn-soybean meal-based diet	0–42 d	6.1	2.2	−3.9	[84]
Salmonella typhimurium								
Plant: *Macleaya cordata*	Benzo [c]phenanthridine alkaloids	5000	Corn-soybean meal-based diet	8–15 d	NS	NS	−11.0	[85]
Heat-Stress								
Plant: Turmeric	Curcumin	100	Corn-soybean meal-based diet	21–42 d	NS	NS	−2.8	[71]
Herb: *Zingiber officinale*	Gingerdiol, gingerol, gingerdione, and shogaols	2000	Corn-based diet	0–42 d	3.3	NS	3.0	[70]
Herb: *Artemisia annua*	Phenolics (44.24 mg GAE/g) and flavonoids (27.8 mg RE/g)	1000	NM	21–42 d	8.2	4.1	NS	[69]
Turmeric rhizome powder	Phenolic compounds: curcuminoids	2000	Corn-soybean meal-based diet	0–42 d	10.6	NS	6.9	[68]

GAE: Total phenolic contents were expressed as Gallic Acid Equivalents (mg GAE/g); RE: total flavonoid content was expressed as Rutin Equivalents (mg RE/g); NM: Not Mentioned; NS: Not Significant; BW: Body Weight; ADFI: Average Daily Feed Intake; FI: Feed Intake; FCR: Feed Conversion Ratio.

4.2. Effects of Dietary PFA Supplementation on Digestibility

Evaluating digestibility is important as it directly contributes to the animal feed efficiency. However, improving the digestibility is crucial not only for better feed efficiency but also to reduce the amount of undigested feed in the gut, which may favour the occurrence of intestinal imbalances. These imbalances may lead to inflammatory processes and accelerated turnover of intestinal tissue, which results in poorer performance.

Numerous studies have been carried out to study the effects of PFAs inclusion on nutrient digestibility in broiler chickens, laying hens as well as ducks and showed inconsistent results.

The use of either extracts from olive leaves rich in polyphenols [110] or a bioactive olive pomace extract from *Olea europaea* [62] failed to enhance nutrient apparent total tract digestibility (ATTD) coefficients, or apparent ileal digestibility (AID) of dry matter (DM),

organic matter (OM), ether extract (EE), and gross energy (GE), respectively. However, the use of EOs such as a blend of carvacrol combined with either cinnamaldehyde and capsicum oleoresin [111] or thymol and limonene in encapsulated forms [44] increased fat digestibility (FD), the AID of crude protein (CP), phosphorus and cysteine. Similarly, a combination of over 30 essential oils and phytogenic compounds increased the digestibility of DM, CP and EE [112].

In laying hens, dietary supplementation of peppermint oil at 0, 74, 148, 222, and 296 mg/kg linearly increased digestibility of CP, EE, and phosphorus from 32 to 44 weeks of age [48], while 100 mg/kg of EOs including thymol 13.5% and cinnamaldehyde 4.5% as major active components significantly increased protein and fat digestibility from 54 to 65 weeks of age [65].

An increased nutrient digestibility was also obtained by supplementing meat-type ducks fed high nutrient density diets by a phytogenic blend containing quillaja, anise, and thyme [113]. Similar results were obtained in broiler chickens supplemented with an EO blend, a quillaja saponin blend, or a combination of both phytogenic preparations [114] or a PFA of *Aerva lanata*, *Piper betle*, *Cynodon dactylon*, and *Piper nigrum* [58]. These authors attributed the digestion-stimulating properties of the PFA basically to piperine, which has been previously reported to stimulate digestion and increase absorption of selenium, vitamin B complex, β carotene, and other nutrients.

Possible mechanisms behind improved nutrient digestibility by PFAs supplementation could be attributed to the ability of these feed additives to stimulate appetite, saliva secretion, intestinal mucus production, bile acid secretion, and activity of digestive enzymes such as trypsin and amylase as well as to positively affect the intestinal morphology [58] or to possess an overlapping mode of action including local effects at the intestinal border and systemic alterations of macronutrient metabolism by these feed additives [114].

4.3. Effects of Dietary PFA Supplementation on Intestinal Microbiota

It is well-known that farm animal performance is directly linked with gut function and health, which is determined by the continuous interaction among diet, intestinal integrity, gut microbiome and the immune system of chickens. Keeping in view the importance of intestinal microbiome [115], several authors studied the effects of PFAs on the microbiota composition (Table 5). The results showed that these effects are both phytogenic composition and inclusion level dependent [116,117], and can range from neutral with no effect [117–119] up to beneficial [120–125].

The supplementation of a PFA containing the carvacrol as main active compound [124] was shown to modulate the intestinal microbiota more at cecal rather than ileal level by increasing cecal mucosa-associated levels of *Bacteroides* spp., *Clostridium cluster IV, and Clostridium cluster XIVa.* This increase could be considered beneficial as Clostridia are not only dominant in ceca [126], but they also contribute to the maintenance of overall gut function, especially through butyrate production [127]. *Deinococcus*, Bacillaceae and Caulobacteriales were increased in ileal digesta of laying hens fed on a dietary supplementation of EOs, promoting an increase of digestive enzyme activity leading to improved feed utilization efficiency [128]. Moreover, although some authors reported a decrease of the relative abundance of beneficial commensal bacteria such as *Lactobacillus* by plant extract supplementation [43,129], several others found Clostridiales and/or Lactobacillales to be higher in broilers supplemented with EOs of carvacrol and thymol [130,131] or thymol, eugenol and piperine [121]. More precisely, addition of thymol and carvacrol to the diet changes the host ileum microbial population dynamics by increasing the abundance of *L. crispatus* and *L. agilis*, and decreasing *L. salivarius* and *L. johnsonii* [131]. *L. crispatus* is known to be a rod-shaped species of the genus *Lactobacillus* and is a hydrogen peroxide-producing beneficial microbial species that plays a key role in the protection of the host from infection [132]. A study conducted by Gudiña et al. [133] revealed that a biosurfactant produced by a *L. agilis* strain showed considerable anti-adhesive activity against *S. aureus*, as well as antimicrobial activity against *P. aeruginosa, S. aureus* and *S. agalactiae*. Green tea

and pomegranate have also been proven to modulate the intestinal microbiota [122,134], especially by promoting beneficial bacteria in the intestinal tract [125,135]. In a recent study conducted by Perricone et al. [123], broilers receiving a plant extract composed of green tea leaves (*Camellia sinensis*) and pomegranate rinds (*Punica granatum*) promoted greater relative abundance of lactic acid bacteria compared to the control group. These results are of particular interest because lactic acid bacteria are recognized to positively affect the intestine by regulating the composition of intestinal microflora, developing intestinal immunity and promoting gut health [136].

Table 5. Effects of dietary PFA supplementation on microbiota of broilers.

Feed Additive	Major Components	Dose	Duration of Supplementation	Site and Age of Sampling	Main Effects on Microbiota	References
Green tea leaves (*Camellia sinensis*) and pomegranate rinds (*Punica granatum*)	Green tea: catechins Pomegranate: tannins and flavonoids	2 mL/L in drinking water	From 0 to 4 days, 10, 11, 20, and 21.	Cecum Day 50	Family: ↑ Lactobacillaceae and Peptococcaceae Genus: ↑ *Roseburia* and ↓ *Shuttleworthia*	[123]
Aerva lanata, *Cynodon dactylon*, and *Piper nigrum* (2 kg from each) and *Piper betle* (2 L.)	Phenolic acid contents (10,176.8 µg/g), Flavonoids (53.0 µg/g), others (220.2 µg/g)	1 and 2% in the feed	42 days	Cecum 42 days	↑ *Bifidobacterium*	[58]
EOs	Carvacrol (102 g/kg PFA)	115 g/kg in the feed	42 days	Ileum and cecum 42 days	↑ Cecal Bacteroides, Clostridium cluster IV, and Clostridium cluster XIVa -Day 14: ↑ Firmicutes, Bacteroidetes and Thermi in the ileal microbiota of the HPE group ↓ Proteobacteria and Tenericutes, and 10 genera (e.g., Ruminococcus, Faecalibacterium) -Day 28: ↑ Bacteroidetes and Cyanobacteria and three genera (e.g., Alistipes) in the cecal microbiota of the HPE group ↓ Actinobacteria and two genera (Lactobacillus and unclassified Coriobacteriaceae).	[124]
EOs	Carvacrol (20%) and thymol (25%)	200 (LPE) and 400 (HPE) g/mg in the feed	42 days	Duodenum, ileum, and cecum 14 and 28 days		[43]
-Oregano essential oil (OEO) -Commercial blend of phytogenic (CBP)	5% essential oil of *Origanum vulgare subsp. Hirtum* plants -Carvacrol (102 g/kg CBP)	300 and 500 ppm 150 ppm in the feed	42 days	Ileum 21 days	↓ *Escherichia coli* for both OEO and CBP groups compared to the NC. =*Lactobacillus*	[137]
EOs	Thymol, eugenol and piperine (29%)	0.03% in the feed	35 days	Ileum 35 days	↑ *Lactobacillus* counts ↓ *Escherichia coli* counts	[121]
Tea polyphenols (TP)	Caffeine (69.8 mg), (–)-EGCG (495 mg), (–)-epicatechin gallate (112 mg), (–)-epicatechin (100 mg), (–)-epigallocatechin (78 mg) and (–)-gallocatechin gallate (96 mg/ 1000 mg TP)	0.03, 0.06 and 0.09 kg^{-1} BW in the feed	56 days	Ileum mixed with cecum 56 days	↑ Species of *Lactobacillus reuteri*, uncultured *Bacteroides* sp. and *L. crispatus*	[135]
EOs	Thymol (25%) and carvacrol (25%)	120 mg/kg in the feed	21 days	Ileum 21 days	↑ *Lactobacillus crispatus* and *Lactobacillus agilis* abundance ↓ *Lactobacillus salivarius* and *Lactobacillus johnsonii* abundance	[131]

Table 5. *Cont.*

Feed Additive	Major Components	Dose	Duration of Supplementation	Site and Age of Sampling	Main Effects on Microbiota	References
EOs	Equal mixture of thymol plus carvacrol	100 and 200 mg/kg in the feed	42 days	Duodenum, jejunum, and ileum; 24 days	↑ *Lactobacilli* counts ↓ *Escherichia coli* and *Clostridium perfringens* counts with 200 mg/kg	[120]
EOs	Thymol (25%) and carvacrol (25%)	60, 120, and 240 mg/kg in the feed	28 days	Ileum and cecum; 21 and 28 days	Ileum: ↓ *Escherichia* populations Cecum: ↓ numbers of total bacteria and Escherichia on day 28	[130]

↑: increased; ↓: decreased; =: equal.

4.4. Effects of Dietary PFA Supplementation on Immunity

An increasing number of studies have shown that health-promoting activities of phytochemicals are attributed to their ability to improve host defence against microbial infection [138]. The immune activating properties of several phytochemicals such as dandelion (*Taraxacum officinale*), mustard (*Brassica juncea*) and safflower (*Carthamus tinctorius*) [139] as well as thistle (*Silybum marianum*), turmeric (*Curcuma longa*), reishi mushroom (*Ganoderma lucidum*), and shiitake mushroom (*Lentinus edodes*) [140] have been evaluated in vitro using avian lymphocytes and macrophages. In both studies, all extracts inhibited tumour cell growth and stimulated the innate immunity in poultry. These results were further confirmed by several in vivo trials, which are giving increasing evidence that through interactions with immune system, PFAs can modulate immune responses through various mechanisms. Some of the most recent studies are synthesized in Table 6. Dietary immunomodulation is a key to enhance the productivity and immune system integrity of farm animals raised under the absence of antibiotics [141]. One of those mechanisms is the modulation of the expression of the cytokines playing a key role in both the adaptive and the innate immune system [142,143].

Table 6. Effects of dietary PFA supplementation on immunity of broilers.

Feed Additive	Major Components	Dose, (mg/kg Diet)	Experimental Conditions	Immune Response	References
Curcumin EOs (PHY)	Curcumin (72%; CU) carvacrol (21.55 mg/g), thymol (18.76 mg/g) and cinnamaldehyde (27.62 mg/g) of PHY	50 100	Corn-soybean meal-based diet	↓ Total leukocyte and heterophils number in the CU and PHY + CU groups, ↓ lymphocytes in the CU group	[144]
EOs	Oregano oil (50 g), carvacrol (10 g), thyme oil (33.33 g), eucalyptus oil (50 g), thymol (5 g), eucalyptol(10 g), and acacia (Arabic gum) surfactant (27 g) in water up to 1 L	500	Challenge with virulent Newcastle disease virus+ vaccin against Newcastle disease (ND), the avian influenza (AI), infectious bronchitis (IB), and infectious bursal disease (IBD)	↓ Hemagglutination inhibition and viral shedding titres 1 wk after challenge ↑ ELISA antibody titre for IBD virus at the 28th d of age	[145]
Resveratrol	Polyphenols	300 and 600	Corn-soybean meal-based diet and chickens challenged with *Escherichia coli*	↑ Total Ig and IgG at d22 and total Ig and IgM at d 35	[84]
Cashew nut shell liquid and castor oil	Cardanol, cardol, and anacardic acid Ricinoleic acid	1500	Broilers challenged with *Eimeria* spp.	↑ Gene expression of TNF-α, IL-6 and IFN-γ and ↓ expression of IL-1 and COX-2	[146]
Yucca schidigera	Saponins	250	Corn-soybean meal-based diet Mixed Eimeria challenge	=Lymphocyte percentages to that of unchallenged birds on d7 p.i	[80]

Table 6. *Cont.*

Feed Additive	Major Components	Dose, (mg/kg Diet)	Experimental Conditions	Immune Response	References
EOs	Carvacrol (5%), cinnamaldehyde (3%), and capsicum oleoresin (2%)	100	Two control diets based on either wheat or maize	↓ CD40LG, IFN-γ and IL-6.	[86]
EOs	Cinnamon bark oil (CNO) Clove bud oil (CLO) Ajwain seed oil (AJO)	300 600 400	Corn-soybean meal-based diet. Broilers vaccinated against NDV at 5 and 18 d of age, and IBDV at 14 d of age.	↑ Antibody titres against NDV vaccine with CNO and CLO at 35 d of age	[147]
EOs	Carvacrol, thymol and cinnamic aldehyde	5000 and 10,000	Corn-soybean meal-based diet.	↑ Total erythrocyte counts, hemoglobin content and ↓ leucocyte count	[42]
Thyme powder	Major EOs (thymol (50.48%), γ-terpinene (11.03%), P-cymene (9.77%), and carvacrol (4.30%)), phenolic acids (salicylic acid (2450.03 ppm), ellagic acid (1240.42 ppm)) and flavonoid compounds	2000, 5000 and 8000	Corn-soybean meal-based diet.	↑ Lymphocytes, white blood cells, and IgG. ↓ TNF-α, IFN-γ, NF-κBP50 by all the doses. ↓ IL-6 by the dose of 8000	[87]
Allium hookeri (AH) roots Fermented root	Phenols	10,000 and 50,000 for both	Corn-soybean meal-based diet. LPS-induced young broiler chicken	↓ IL-1b with 1% AH root and 5% fermented root, TNFSF15 expression with fermented root (1% and 5%), and IL-8 with 1% fermented root supplementation	[148]
EOs	Oregano: (5%)	300	Corn-soybean meal-based diet.	↑ Secondary antibody titer and IgG titer, ↓ H/L ratio	[88]
Artemisia annua	Phenolics (44.24 mg GAE/g) and flavonoids (27.8 mg RE/g)	1000	Heat-stressed broilers	↑ Intestinal SIgA and IgG	[69]
Turmeric rhizome	Phenolic compounds (16.2 mg/g)	2000	Corn-soybean meal-based diet and broilers kept under chronic heat stress	↑ Total secondary antibody titer, and ↓ H/L ratio	[68]
EOs	Carvacrol (60.2%) and thymol (4%)	50 and 100 in water	Corn-soybean meal-based diet and broilers vaccinated with inactivated avian influenza and Newcastle disease (NDV)	↑ Antibody titer against NDV and avian influenza virus	[149]

SIgA: secretory immunoglobulin A; p.i.: post infection; ↓: increased; ↑: decreased: =: equal.

A study conducted by Pirgozliev et al. [86] demonstrated that broilers under non-challenging conditions, supplemented by a commercial blend comprising 5% carvacrol, 3% cinnamaldehyde, and 2% capsicum oleoresin showed downregulated IFN-γ and IL-6 cytokines indicating a lower inflammation level than those in the other groups.

In broilers under challenging conditions such as necrotic enteritis, Lee et al. [150] showed that a mixture of *Capsicum* and turmeric oleoresins reduced intestinal IL-8, lipopolysaccharide-induced TNF-α factor (LITAF), IL-17A and IL-17F mRNA levels. Similarly, the expression of pro-inflammatory cytokines was reduced by the supplementation of *Allium hookeri* in LPS-induced young broiler chickens [148] and thyme powder in broilers without any challenge [87]. Nonetheless, a blend of cashew nut shell liquid (CNSL) and castor oil modulated the inflammatory response of broiler chickens against *Eimeria* spp. by increasing gene expression of TNF-α, IL-6 and IFN-γ and reducing expression of IL-1 and COX-2, one week post-infection [146] These authors concluded that although inflammation is a highly undesirable phenomenon owing to its costly effects on animal production efficiency, this increased inflammatory response observed in challenged birds

treated with CNSL–castor oil was necessary to help the immune system to effectively fight against coccidiosis and other pathogenic bacteria to prevent intestinal dysbiosis.

Other authors have rather evaluated the levels of SIgA, IgM, and IgG, three major classes of immunoglobulin in chickens that play key roles in the maintenance of immunity [151]. SIgA is involved in the protection and homeostatic regulation of intestinal mucosal epithelia by limiting the access of numerous microorganisms and mucosal antigens, while IgG directly contributes to an immune response including neutralization of toxins and viruses. A decrease of SIgA and IgG observed in the jejunum mucosa of heat-stressed broilers was counteracted by the supplementation of enzymatically treated *Artemisia annua* [69]. Similarly, an increase of IgG was observed by the supplementation of thyme powder [87] and EOs of oregano [88] in broilers raised under normal conditions or by the supplementation of resveratrol in chickens challenged with *Escherichia coli* [84].

The effect of PFAs, especially EOs, on the immune response to some viral vaccines in broiler chickens was also evaluated in several studies. A mixture containing oregano oil (50 g), carvacrol (10 g), thyme oil (33.33 g), eucalyptus oil (50 g), thymol (5 g), eucalyptol (10 g), and acacia (Arabic gum) surfactant (27 g) supplemented through the drinking water at a dose of 0.5 mL/L showed an immune-stimulating response to Newcastle disease (ND) and infectious bursal disease (IBD) vaccines, antiviral effect against ND virus, especially if administered before the challenge [145].

The supplementation of cinnamon bark oil (CNO) [147,152], clove bud oil (CLO) and ajwain seed oil (AJO) [147], cinnamaldehyde combined with formic acid [147] and Origanum essential oil containing 60.2% carvacrol and 4% thymol [149] enhanced immune response against NDV in broiler chickens. However, antibody titres against avian influenza virus and NDV were unaffected by cinnamon bark powder supplementation (2 and 4 g/kg diet) [153] or clove EO (0.15, 0.30 and 0.45 g/kg) [154], and antibody titres against NDV vaccine and sheep red blood cells were not significantly influenced by the supplementation of EOs mixture obtained from anise, oregano, and citrus peel in broiler chickens [155].

The supplementation of black cumin (*Nigella sativa*) combined with *Echinacea* enhanced the immune response after AI-H9N2 vaccination and reduced the pathogenicity of infection in dexamethasone-stressed chickens [156]. The immune-modulating effects of black cumin may be attributed to pharmacologically active constituents, such as thymol, nigellidine, nigellimine, thymoquinone, dithymoquinone and thymohydroquinone [157] which are able to induce pharmacological effects against antigenic challenge [158].

4.5. Effects of Dietary PFA Supplementation on Blood Biochemical Parameters and Oxidant Status

Several authors studied the effect of PFA supplementation on the serum biochemical indicators that help to display the nutrient's metabolism and body physiological state [159]. Serum lipid parameters have been shown to be reduced in broiler chickens by the supplementation of lavender EO (cholesterol and LDL-C; [160]), *Pulicaria gnaphalodes* powder (cholesterol and triglycerides; [59]) and a mixture of oregano, anise and citrus EOs (cholesterol; [161]). Serum cholesterol has been also reduced in laying hens supplemented by EOs [162], either black cumin or hot red pepper [53], peppermint oil [48] or nettle *Urtica cannabina* [64].

The mechanisms explaining the hypocholesterolemic effect of PFAs may be associated to the reduced activity of enzymes involved in lipid metabolism including 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (enzyme associated with cholesterol synthesis), cholesterol-7 hydroxylase fatty acid synthase and pentose phosphate pathway [147,163].

The supplementation of PFAs has been also shown to improve the antioxidant status of broilers [164–166]. A recent study performed by Paraskeuas et al. [39] revealed that a mixture of menthol, anethol and eugenol increased blood plasma total antioxidant capacity (TAC) in a linear pattern, corroborating the results previously obtained by other authors [167,168]. The activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), enzymes considered as one of the defensive mechanism of the body against the oxidative stress [169], was increased by supplementing broilers by lavender EO [160] and

laying hens by star anise oil [45] and grape pomace [101]. Malondialdehyde (MDA) levels were also decreased in laying hens supplemented by grape pomace [98] and star anise oil [45].

4.6. Effects of Dietary PFA Supplementation on Meat, Internal and External Egg Quality

Benefits of dietary PFA supplementation on the quality and shelf-life of meat products are still ambiguous. Although the supplementation of a PFA based on EOs of carvacrol, thymol and cinnamic aldehyde was unable to prevent broiler meat lipid peroxidation caused by freezing temperatures [42], other authors reported increased antioxidant levels and reduction of lipid peroxidation by *Nigella sativa* seeds [52], turmeric [57], curcuminoids [56] as well as herbal components containing curcumin, carvacrol, thymol and cinnamaldehyde [144]. Manipulating lipid peroxidation has been shown to be, in part, achieved through modulating the profile of meat fatty acids by PFAs supplementation. Total saturated fatty acid (SFA) levels were reduced and monounsaturated/polyunsaturated fatty acid (MUFA/PUFA) levels were increased by PFAs supplementation [56,144,170,171]. Particularly, SFA such as lauric, stearic, myristic and palmitic acid are undesirable due to their hypercholesterolemic properties in the form of LDL [144,170]. On the contrary, omega 3 and 6 fatty acids play key roles in human nutrition¸ being precursors of principal molecules involved in the regulation of the cardiovascular and immune system including eicosanoids, prostaglandins, leukotrienes and thromboxanes [144].

External and internal egg quality has been also shown to be influenced by PFAs supplementation. Eggshell thickness was increased by supplementation of herbal EOs mixture [162], peppermint oil [48] and nettle *Urtica cannabina* [64]. A possible mechanism behind these positive effects is the ability of PFAs such as EOs to improve uterine health and increase calcium storage as well as pancreatic secretions, resulting in the enhancement of nutrient digestion and consequently the improvement in eggshell and egg quality [48].

Regarding internal quality, the supplementation of PFAs such as black cumin or red pepper [53], star anise (*Illicium verum* Hook.f.) EO [45] grape pomace flour [101] and curcumin [100] has been shown to reduce egg lipid peroxidation and increase its antioxidant levels, thereby, generating internal stability of the stored eggs and contributing to extending egg shelf life. The Haugh unit score, known as an indicator of egg freshness and is related to shelf life, has been also shown to be increased by PFAs supplementation [46,48,63]. Similar to meat, egg yolk cholesterol content has been shown to be reduced by PFAs supplementation [48,64]. Although the egg yolks of laying hens supplemented with *Citrullus lanatus* EOs showed increased total PUFA and n-6 fatty acids and reduced n-3 fatty acids [46], nettle *Urtica cannabina* supplementation increased total n-3 PUFA concentration while reducing the ratio of n-6/n-3 [64].

5. Challenges and Prospective of Using PFAs in Animal Nutrition

5.1. Challenges of Using PFAs in Animal Nutrition

Performing systematic and comprehensive studies evaluating the efficacy and safety of PFAs is still difficult due to their complex composition [172]. In addition, inconsistency in the obtained results may be attributed to several factors related either to the enormous variability per se of PFA, including source and bioactive compounds of the PFAs which may depend on the plant, botanical origin growing locations, manufacturing methods, the storage conditions, and the effective dose; or to the environmental conditions, management and rearing conditions (challenge vs. no challenge and differences in the underlying microbial challenge if applied, age, genetics. . . [22,172]. Some authors also reported that the appropriate minimum inhibitory concentration (MIC) for most phytogenic compounds is higher than the level considered as cost-effective [172]. On the other hand, various phytogenic compounds such as EOs can evaporate rapidly due to their volatile and reactive nature, resulting in largely varied concentrations in the final feed additive. Their effectiveness in animals may also be affected by several conditions during production processes and storage. Thus, ensuring their stability presents a difficulty, as do maintaining their

biological activity and masking their strong odour [173]. Moreover, mutual interactions with other substance from feed matrix have been reported such as lower biological effects of PFA present in fibrous diets or high protein diets [174]. Several phytogenic compounds have been also shown to be largely absorbed in the upper GIT, meaning that without proper protection, the majority would not reach the lower gut where they would exert their major functions. In this context, a study conducted by Hafeez et al. [44] showed that the benefits of supplementing the broiler diet with a mixture of encapsulated EOs were higher than the tested PFA in powdered, non-protected form.

Therefore, novel delivery technologies have been developed to protect PFAs from the degradation and oxidation process during feed processing and storage, ease the handling, allow a slower release and target the lower GIT [173]. Among these techniques, microencapsulation is gaining an increasing interest where various carrier types including polymer-based particles such as polysaccharide-protein carriers and lipid-based particles such as vegetable oils and liposomes were tested. The advantages and disadvantages of both carrier materials from the encapsulation efficiency, loading capacity, and release kinetics viewpoints were previously discussed in the literature. Although advantages of polysaccharide-protein carriers include their mechanical and thermal stabilities, nutritional quality, low cost, and easy preparation procedure, they present low encapsulation efficiency, loading capacity, and release efficiency in small intestine [175]. As for lipid-based particles, they are characterized by high encapsulation efficiency, loading capacity, and release efficiency in the small intestine. However, their disadvantages include low mechanical and thermal stabilities [173]. Moreover, liposomes cannot be used for large scale production owing to complex preparation procedures, reduced production capacity, and higher cost [176].

Despite some studies showed some positive effects on chickens by using single EO, several others have rather chosen the combination of various EOs and their isolated components to take advantages from their synergistic effects [2,177]. Indeed, synergistic interactions are of great importance because they enhance the antimicrobial and antioxidant activity by maximizing the efficiencies of the combined agents in the best possible manner which results in several fold reduction in the required doses of EOs applied in situ and thereby lowering their organoleptic impacts. Interestingly, blends containing hydrocarbons and phenylpropanoids (e.g., cinnamaldehyde, eugenol, carvacrol, and capsicum oleoresin) in combination with other components were reported to enhance the bioactivities of these mixtures [178]. A special attention was placed on the interaction of phenolic monoterpenes (thymol, carvacrol) and phenylpropanoids (eugenol) with other groups of components, particularly with other phenols, phenylpropanoids and monoterpenes alcohols, whereas monoterpenes and sesquiterpenes hydrocarbons were used to a lesser extent [178]. Moreover, combining phenolics with monoterpenes alcohols has been reported to produce synergistic effects on several microorganisms, in particular, the combination of phenolics (thymol with carvacrol, and both components with eugenol) were synergistically active against *Escherichia coli* strains [178]. In ruminants, these combinations were the most effective dietary supplementation options that showed ruminal antimicrobial advantages to modulate the ruminal fermentation pathways [179,180].

5.2. Prospective of PFAs in Animal Nutrition: Combination of EOs with OAs

It has been reported that feed additives with different functions and complementary mode of actions hold the most promising solution to replace antibiotics in animal feed mainly for three reasons: (i) all the beneficial effects of antibiotics are unlikely to be covered by an individual alternative; (ii) some alternatives possess a synergetic effect that may decrease the required dose considered as cost-effective; (iii) substituting the antibiotics must be an integrated approach that includes feeding, management and biosecurity rather than a supplementation of feed additives alone [172].

As for the synergy between feed additives, the combined use of hydrophobic EOs with lipophilic OAs has been considered the most promising method to substitute antibiotics

for the potential synergistic and additive beneficial effects on the intestinal health and growth performance compared with individual EOs or OAs [181,182]. Some results of recent studies are illustrated in Table 7. The main mode of action linked to the synergic effects of OAs and EOs may be the modulation of the intestinal microbiota. However, the antimicrobial activities depend on the gram staining of bacteria as Gram-negative (G−) bacteria differ from Gram-positive (G+) bacteria with the respect to the structure of the cell wall. The cell walls of G+ bacteria are 90–95% composed of peptidoglycan allowing hydrophobic molecules (EOs) to easily penetrate the cells, acting on both the cell walls and the cytoplasm, causing a disruption of the structure and function of bacteria cell membranes [183]. This increase of the bacterial membrane permeability could facilitate the influx of OAs into the cytoplasm due the lipophilic nature of their undissociated form, disturbing the proton and associated anion concentrations in the cytoplasm [182]. As for phenolic compounds, once inside the cell, they can interfere with enzymes involved in the production of energy at lower concentrations and denature proteins at higher concentration. However, G− bacteria possess a different composition as their peptidoglycan layer is only 2–3 mm thick and composes only 20% of the dry weight of the cell. An outer membrane comprised of a double layer of phospholipids firmly linked by Braun's lipoprotein to the inner membrane, lies outside of the peptidoglycan layer, making G− bacteria less permeable by providing an extra layer of protection and thus more resistant to EOs than the G+ bacteria [183]. Moreover, the "quid" provided by the core polysaccharides and the O-side chain allows these bacteria to be more resistant to EOs and other natural extracts possessing anti-microbial activities. Although the antimicrobial properties of long chain fatty acids (LCFA) are related to their potential to incorporate themselves into target membranes of G+ bacteria, promoting leakage of cellular protons and ions due to their lipophilic nature, the lipopolysaccharide (LPS) layer in the cell wall of G− bacteria prevents medium chain fatty acids (MCFA) and LCFA from crossing the cell membrane. Moreover, G− bacteria are also able to assimilate MCFA and LCFA into the cell and subsequently metabolize them per the β-oxidation cycle (i.e., *Escherichia coli*) and utilize short chain fatty acids (SCFA) as energy sources (i.e., *Salmonella*, *Escherichia coli*). These differences in the cell membrane compositions make EOs more powerful in the control of G+ bacteria compared to G− ones. However, OAs are reported to be more effective against G− bacteria than EOs [184] as small hydrophilic solutes of OAs are able to pass through the membrane via porin proteins but not the hydrophobic polyphenol molecules [172].

Table 7. Application of mixtures of phytogenics and organic acids with major physiological responses in poultry.

Mixture of OA+EO	Study Design	Main Findings	Reference
Citric (25%) and sorbic (16.7%) acids, thymol (1.7%), and vanillin (1.0%)	Type: male breeder chickens Dose: 500 g/metric ton diet Form: microencapsulated Duration: 15 days Conditions: without challenge	-Increased *Lactobacilliaceae*, *Clostridiaceae* and *Ruminococcaceae* abundance -Decreased Staphylococcaceae,	[185]
Thyme (4%), carvacrol (4%), hexanoic acid (0.5%), benzoic acid (3.5%) and butyric acid (0.5%)	Type: male Arbor Acres broiler chickens Dose: 500 mg/kg diet Form: Encapsulated Duration: 42 days Conditions: *Eimeria* spp. and *Clostridium perfringens*	-Improved FCR -Higher villus height and villus height/crypt depth ratio. -Reduced intestinal *C. perfringens* counts, liver *C. perfringens* carriage, and gut lesion scores. -Reduced serum fluorescein isothiocyanate dextran (FITC-D) concentrations. -Upregulated claudin-1, IGF-2 and A20 mRNA expression. -Downregulated TRAF-6, TNFSF15 and TOLLIP mRNA levels	[181]

Table 7. *Cont.*

Mixture of OA+EO	Study Design	Main Findings	Reference
Citric (25%, as fed) and sorbic acids (16.7%, as fed), thymol (1.7%, as fed) and vanillin (1%, as fed)	Type: Male Ross 308 broiler chickens Dose: 5 g/kg diet Form: Encapsulated Duration: 47 days Conditions: without challenge	-Improved growth performances -Improved gut morphology -Microbial control against *Clostridium perfringens*, *Enterobacteriaceae*, *Enterococci* and *Mesophilic* bacteria	[186]
Fumaric, sorbic, malic, and citric acids, thymol, vanillin, and eugenol	Type: Cobb 500 male broilers Dose: 300 g/t diet Form: Protected Duration: 42 days Conditions: *Eimeria* spp. and *Clostridium perfringens*	-Greater body weight gain -Higher apparent ileal nutrient and energy digestibility -Improved intestinal integrity with lower blood fluorescein isothiocyanate-dextran concentration -Improved intestinal macroscopic and histologic alterations -Greater expression of MUC2, CLDN1, and OCLN genes	[182]
Citric andsorbic acids, thymol, and vanillin	Type: By-product breeder chicks Dose: 500 g/metric ton diet Form: Microencapsulated Duration: 4 days Conditions: Without challenge	-Enhanced in vitro functional activity of peripheral blood leukocytes (degranulation, oxidative burst, and nitric oxide production)	[187]
Sorbic acid (200 g/kg), fumaric acid (200 g/kg), and thymol (100 g/kg)	Type: Roman laying hens Dose: 150 and 300 mg/kg diet Form: Encapsulated Duration: 21–30 weeks Conditions: Without challenge	-Increased laying rate with 150 mg/kg. -A linear increase in ileal villus height. -Increased mRNA relative expression of aminopeptidase, sodium-glucose cotransporter 1, and Na+-independent neutral amino acid transporter in duodenum and glucose transporter 2 in jejunum with 300 mg/kg. -Higher mRNA relative expression ofmucin-2 in ileum with 300 mg/kg. -Linear decrease of the secretory immunoglobulin in ileum A.	[188]

The mechanism of inhibition to microorganisms by OAs may be affected by several factors, including the reduction in pH, the ratio of the un-disassociated form of the acid, chain length, degree of branching and cell physiology/metabolism [189]. Indeed, the lipophilic nature of weak organic acid allows them to easily penetrate the plasma membrane and thus acidify the cell's interior eventually killing the bacterium [172].

6. Conclusions

This review tried to gather the most recent available scientific information regarding the use of phytogenics in poultry nutrition along with their beneficial effects on performance, digestibility, microbiota, immune response, oxidant status, as well as egg and meat quality. Several studies have reported the promising effects of these feed additives when combined together or with organic acids; however, extra attention should be focused on the selection of active compounds to form potentially effective blends. Moreover, choosing the appropriate technique of protection as well as types and physiochemical properties of wall materials are the most critical aspects governing efficiency by controlling both the timing and location of the release of active compounds.

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CHAPTER 4

Criado-Mesas, L., Abdelli, N., Noce, A., Farré, M., Pérez, J.F., Solà-Oriol, D., Martín-Venegas, R., Forouzandeh, A., González-Solé, F., & Folch, J.M. (2021). Transversal gene expression panel to evaluate intestinal health in broiler chickens in different challenging conditions. *Scientific Reports*, *11*, *6315*.

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Transversal gene expression panel to evaluate intestinal health in broiler chickens in different challenging conditions

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There is a high interest on gut health in poultry with special focus on consequences of the intestinal diseases, such as coccidiosis and C. *perfringens*-induced necrotic enteritis (NE). We developed a custom gene expression panel, which could provide a snapshot of gene expression variation under challenging conditions. Ileum gene expression studies were performed through high throughput reverse transcription quantitative real-time polymerase chain reaction. A deep review on the bibliography was done and genes related to intestinal health were selected for barrier function, immune response, oxidation, digestive hormones, nutrient transport, and metabolism. The panel was firstly tested by using a nutritional/*Clostridium perfringens* model of intestinal barrier failure (induced using commercial reused litter and wheat-based diets without exogenous supplementation of enzymes) and the consistency of results was evaluated by another experiment under a coccidiosis challenge (orally gavaged with a commercial coccidiosis vaccine, 90× vaccine dose). Growth traits and intestinal morphological analysis were performed to check the gut barrier failure occurrence. Results of ileum gene expression showed a higher expression in genes involved in barrier function and nutrient transport in chickens raised in healthy conditions, while genes involved in immune response presented higher expression in *C.perfringens*-challenged birds. On the other hand, the *Eimeria* challenge also altered the expression of genes related to barrier function and metabolism, and increased the expression of genes related to immune response and oxidative stress. The panel developed in the current study gives us an overview of genes and pathways involved in broiler response to pathogen challenge. It also allows us to deep into the study of differences in gene expression pattern and magnitude of responses under either a coccidial vaccine or a NE.

The interest on the gut health in poultry has grown over the past two decades with special focus on consequences of the intestinal diseases. Pathogens may damage the intestinal mucosa, leading to impaired absorption of nutrients, reduced weight gain and decreased overall performance. In particular, the avian gut function is reported to be seriously threatened by coccidiosis[1] and C. *perfringens*-induced necrotic enteritis (NE)[2,3], especially under antimicrobial-free production systems. Thus, the high economic losses associated with poor feed efficiency, mortality, and medication costs have fuelled the search for biomarkers that likely associate with normal or abnormal conditions[4], and allow the understanding of events affecting the intestinal barrier, its functionality, and the ecology of the gastrointestinal microbiota[5]. Previous studies have reported that intestinal gene expression of mucins, tight junctions (TJ) and nutrients transporters may be considered as gastrointestinal biomarkers of intestinal barrier function (BF)[1,5]. It has also been reported that inflammation associated with oxidative stress

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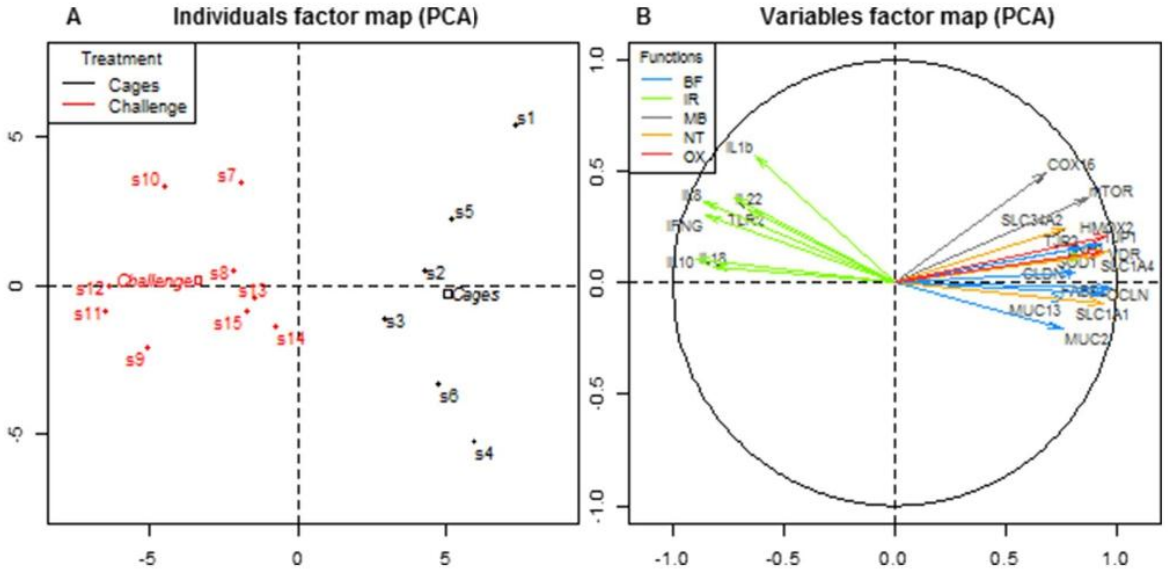

Figure 1. PCA representations of intestinal gene expression data in ileum chicken broiler samples (**A**) The samples dot plot; (**B**) variables arrow plot.

(OX) may produce physiological changes in gene expression which suggests that inflammation-induced OX plays a crucial role in intestinal function[1,6]. Thus, we hypothesized that gut health challenges may induce changes in the intestinal gene expression, and that the overall assessment of expression levels of a wide range of genes involved in several functions, such as BF, immune response (IR), and OX, among others, may provide an overall insight into the host responses during coccidiosis and NE challenges. Therefore, our objective was to develop a custom gene expression panel, which could provide a snapshot of gene expression variation under challenging conditions. Hence, a deep review of the publications related to this topic was carried out, and a list of candidate genes that act as markers in a wide range of functions in the intestine were selected, including the BF, IR, OX, digestive hormones (H), nutrient transport (NT), and metabolism (MB). The panel was firstly tested by an experiment using a nutritional/*Clostridium perfringens* model of intestinal barrier failure and the consistency of results was further evaluated by another experiment under a coccidiosis challenge where the protein expression of *OCLN* and *CLDN* was also measured.

Results

C.perfringens challenge. *Growth performance.* Supplementary Table 1 shows that *C.perfringens*-challenged chickens exhibited lower body weight (BW) on day (d) 10 ($P=0.04$), 28 and 42 ($P<0.001$) as a result of lower average daily gain (ADG; $P<0.001$) compared to the group raised in cages. The overall feed conversion ratio (FCR) was significantly increased ($P<0.001$) in challenged birds (2.07) compared to the non-challenged group (1.54).

Intestinal morphological analyses. As shown in Supplementary Table 2, chickens raised in cages presented higher villus height (VH; $P=0.016$), reduced crypt depth (CD; $P<0.001$), improved VH:CD ratio and lower intraepithelial lymphocytes/100 µm VH ($P<0.001$) than those under *C.perfringens* challenge.

Intestinal gene expression. Due to technical problems, *AvBD9* gene was withdrawn from the study because of its low mRNA expression levels in all samples. Hence, a gene expression panel of 44 target genes was used in the current study.

A principal component analysis (PCA) was performed with genes as variables and samples as cases with treatment as a qualitative variable, getting a two-dimensional (2D) representation that preserves the 62% of the total variance in the experiment. The samples dot plot (Fig. 1A) shows that the first principal component (46.36% of variance explained) separates the treatments: all the samples collected from chickens in cages are located on the right-hand side and the challenge samples on the left, with the squared points representing the means of the treatments clearly separated too. Although all the genes were used to define the principal dimensions, the variables used in the arrow plot (Fig. 1B) were restricted to the genes which showed significant expression differences between treatment means, and are well explained in the 2D-principal components space. This arrows plot combined with the samples dot plot showed a higher expression of the IR genes in chickens maintained under challenging conditions, while BF, MB, NT, and OX genes were more expressed in birds kept in cages. Moreover, a heatmap analysis showed patterns which are consistent with the PCA clusters (Fig. 2), in terms of sample expression pattern. Ileum samples were clustered each with their group (cages and challenge conditions),

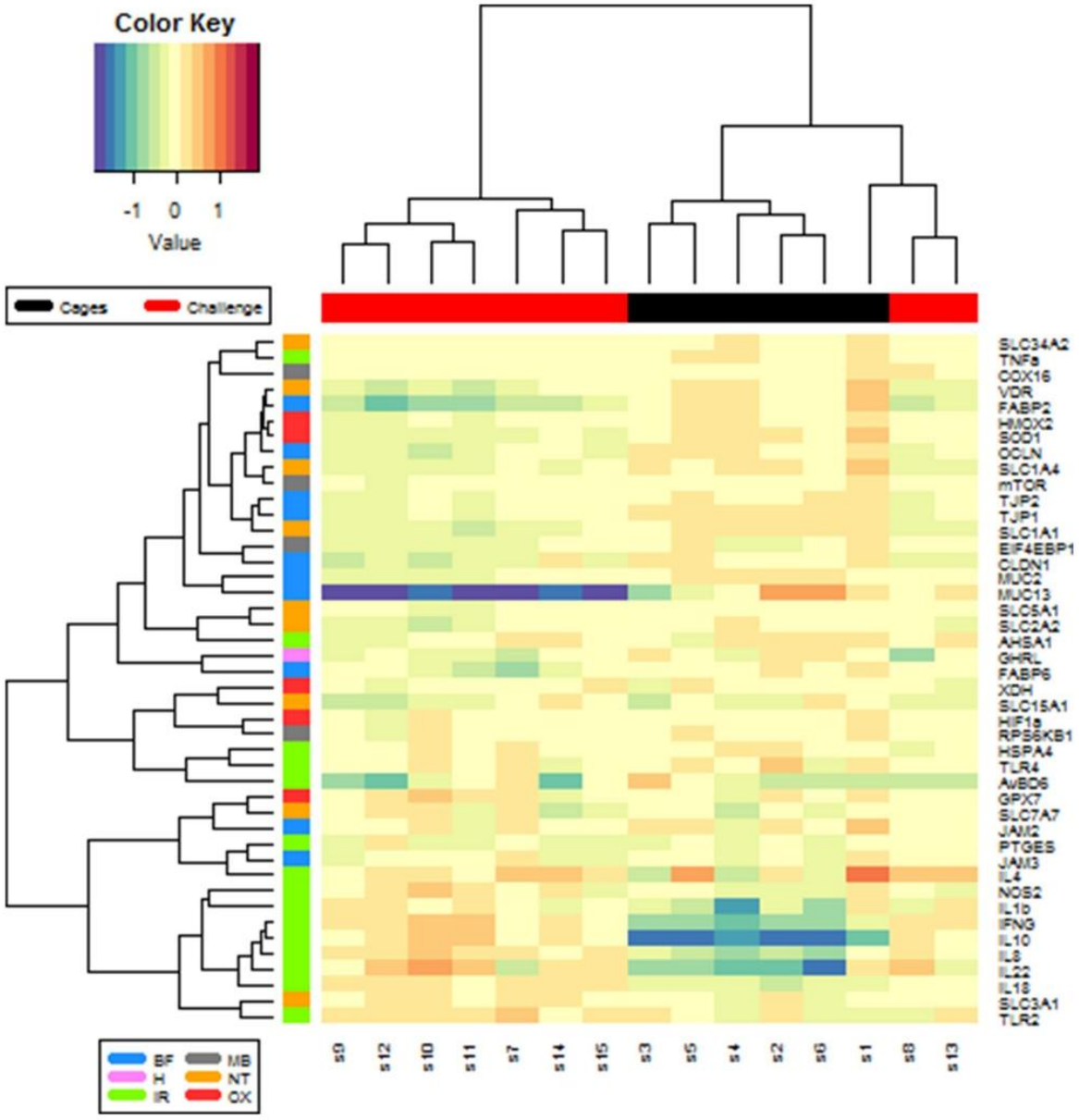

Figure 2. Heatmap of gene expression level of the 44 genes analysed in ileum. Genes were represented in the y-axis and samples in the x-axis. Experimental conditions (x-axis) were labelled in black colour for cages and red colour for challenging conditions. Groups of functions (y-axis) were labelled with different colours.

except for two samples. Genes were not grouped perfectly according to their function; however, IR genes tended to group together.

ANOVA-one-way was applied to the data of mRNA expression levels. The *P*-values obtained were adjusted for multiple testing using Benjamini–Hochberg procedure to control false discovery rate (FDR ≤ 0.05)[7] and the output is summarized in Supplementary Table 3. All genes related to BF, except *JAM2* and *JAM3* genes, showed a higher expression in cages group than challenging one, with significant differences among them. *FABP6* ($P = 0.08$) and *GHRL* ($P = 0.06$) genes tended to be more expressed in cages group. Regarding the IR genes, *TNFa* ($P = 0.001$) presented higher gene expression values and *HSPA4* ($P = 0.08$) tended to be higher in cages group, whereas *IFNG*, *IL10*, *IL18*, *IL8* ($P < 0.001$), *TLR2* ($P = 0.04$), *IL22* ($P = 0.002$), *IL1b* ($P = 0.02$) were more expressed in *C.perfirngens*-challenged chickens. In the MB function group, *mTOR* revealed higher gene expression ($P = 0.002$) while *RPS6KB1* and *COX16* showed a tendency to be more expressed ($P = 0.089$ and $P = 0.057$, respectively) in the cages group. For NT, *SLC1A1*, *SLC1A4* ($P < 0.001$), *VDR* ($P = 0.001$), *SLC34A2* ($P = 0.02$) presented significantly

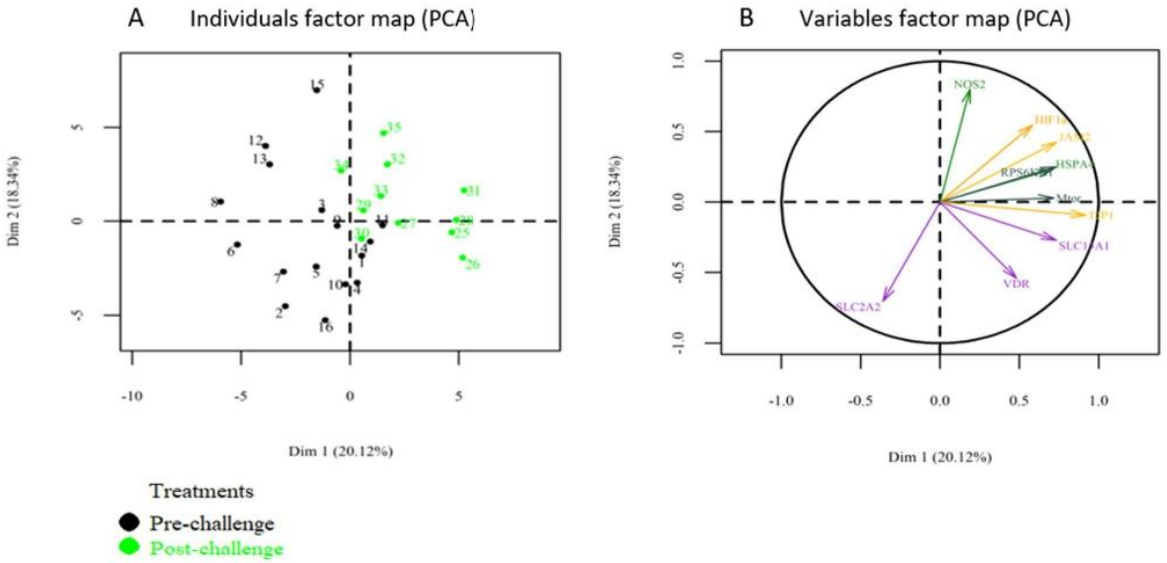

Figure 3. PCA representations of intestinal gene expression data in ileum chicken broiler samples (**A**) The samples dot plot; (**B**) variables arrow plot.

higher gene expression levels and *SLC2A2* tended to be more expressed ($P=0.09$) in chickens raised in cages. Finally, *SOD1* and *HMOX2* involved in OX showed higher mRNA levels ($P<0.001$) in the cages group.

Eimeria challenge. *Growth performance.* As shown in Supplementary Table 4, before *Eimeria* challenge (d7 and d9) no dietary treatment effect was observed on BW. After challenge, broiler chickens supplemented with the coccidostat showed higher BW15 ($P<0.001$) than the non-supplemented NC group.

Coccidia oocyst shedding. Results of oocyst counting in the excreta of chickens on d 15 are reported in Supplementary Fig. 1. Broiler chickens supplemented with coccidiostat showed significantly reduced oocyst counting ($P<0.001$) than the non-supplemented NC group.

Intestinal gene expression. A first PCA was performed to observe the effect of *Eimeria* challenge on samples clustering. Although there was no perfect separation, the samples dot plot (Fig. 3A) showed that most samples of non-challenged chickens are located on the left-hand side whereas, those of *Eimeria* challenged birds are on the right. The variable arrow plot (Fig. 3B) did not show a clear separation between both groups according to gene function. Moreover, the expression pattern and gene clusters showed in the heat map (Fig. 4) were consistent with the PCA result, in terms of sample expression pattern. Neither the samples nor the genes were perfectly clustered according to the experimental group or function, respectively. However, IR genes tended to conglomerate together in the same group. Differences of gene expression were also examined between both groups and results are reported in Supplementary Table 5. The *Eimeria* challenge affected the expression of genes related to BF by reducing mRNA levels of *CLDN1* ($P=0.007$) and *FABP6* ($P=0.01$), while increasing *JAM2* ($P=0.019$) and *TJP1* ($P=0.014$). It also increased the expression of *HSPA4* ($P=0.013$), *INFG* ($P<0.001$) and tended to increase *NOS2* ($P=0.07$) related to IR. Regarding MB, *RPS6KB1* was significantly increased ($P=0.03$) and *mTOR* tended to be increased ($P=0.07$) by the *Eimeria* challenge. The challenged chickens also showed an increased level of *HIF1a* ($P=0.01$) and *XDH* ($P<0.001$), and a tendency to increase *SOD1* ($P=0.09$) gene expression. Finally, an increase of *SLC15A1* ($P=0.007$) and a tendency to decrease *SLC2A2* ($P=0.06$) gene expressions was observed.

A second PCA was performed to study the effect of dietary coccidiostat supplementation on samples clustering. No clear separation was observed between samples collected from both groups (samples dot plot; Fig. 5A). The variable arrow plot (Fig. 5B) did not show a clear separation between both groups according to gene function. The results of a heat map developed to investigate the expression pattern and gene clusters (Fig. 6) were consistent with the PCA result in terms of sample expression pattern. Neither samples, nor genes were perfectly clustered according to the experimental group or function, respectively. Differences in gene expression were examined between both groups and results are represented in Supplementary Table 6. Supplementation of coccidiostat significantly reduced the mRNA levels of *INFG* ($P=0.006$) gene which is related to IR, while two genes involved in NT, *SLC1A1* and *SLC7A7* ($P=0.07$), tended to increase their mRNA expression levels.

Protein expression. As shown in Table 1, *Eimeria* challenge significantly reduced *CLDN1* protein expression ($P<0.001$) but not the *OCLN* protein level ($P=0.33$).

However, the supplementation of coccidiostat did not affect neither *CLDN1* nor *OCLN* protein level (Table 2).

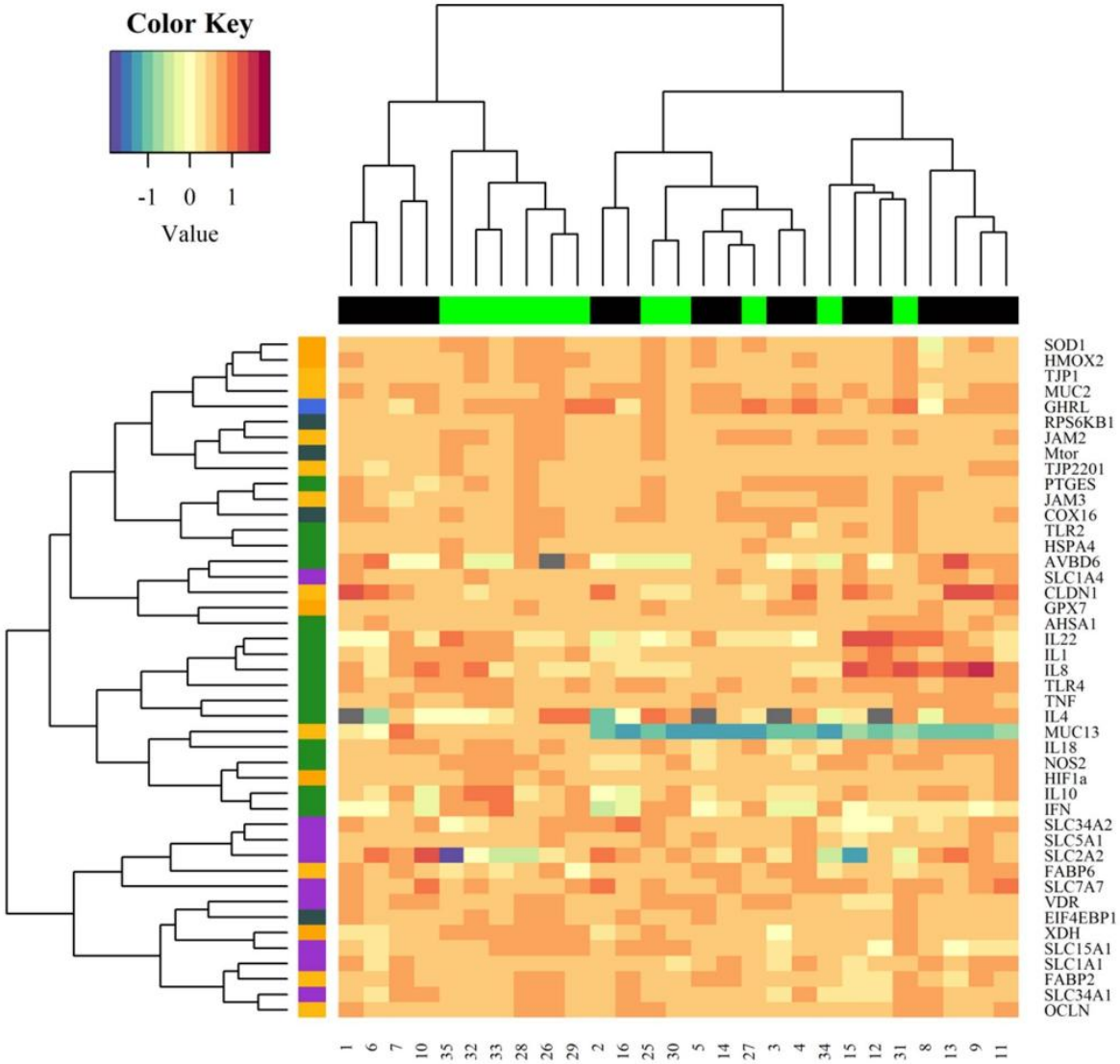

Figure 4. Heat map. The X-axis is sample expression pattern in different treatment group (black colour: before *Eimeria* challenge; green colour: after *Eimeria* challenge). The Y-axis is the gene clusters according to functions.

Discussion

In the present study, the diet/*C. perfringens* challenge was induced by allocating newly hatched broiler chicks on a recycled litter characterized for its high content of *Clostridium perfringens,* as previously reported by[8], and including wheat in the diet without xylanases, with the aim of increasing digesta viscosity and accentuating the dietary challenge. These broiler chickens showed a decrease of BW at d42 by 32% and an increase of global FCR by 26% compared to non-challenged birds. As for the *Eimeria* challenge, it resulted in a reduction of a 30% of BW at d15 compared to the standard Ross 308 values[9]. The compromised growth performance of *C.perfringens*-challenged chickens may be attributed to the damage of intestinal morphology as proven by the decreased VH:CD ratio suggesting a reduction of the absorptive capacity of the intestine[10]. This reduced ratio resulted from the decrease of VH and increase of CD indicating an increase of metabolic cost of intestinal epithelium turnover. The structural changes of the intestinal morphology induced by NE in the current study were in concordance with several authors[3,10] and suggest a gut barrier failure occurrence which was later confirmed by the down-regulation of genes involved in NT and BF associated to an up-regulation of IR genes. The *Eimeria* challenge also affected the gene expression although to a lesser extent than the obtained with diet/*C. perfringens* diet, thus confirming the difference of the severity of the caused damage and demonstrating the adaptability of the gene expression panel to different challenge conditions.

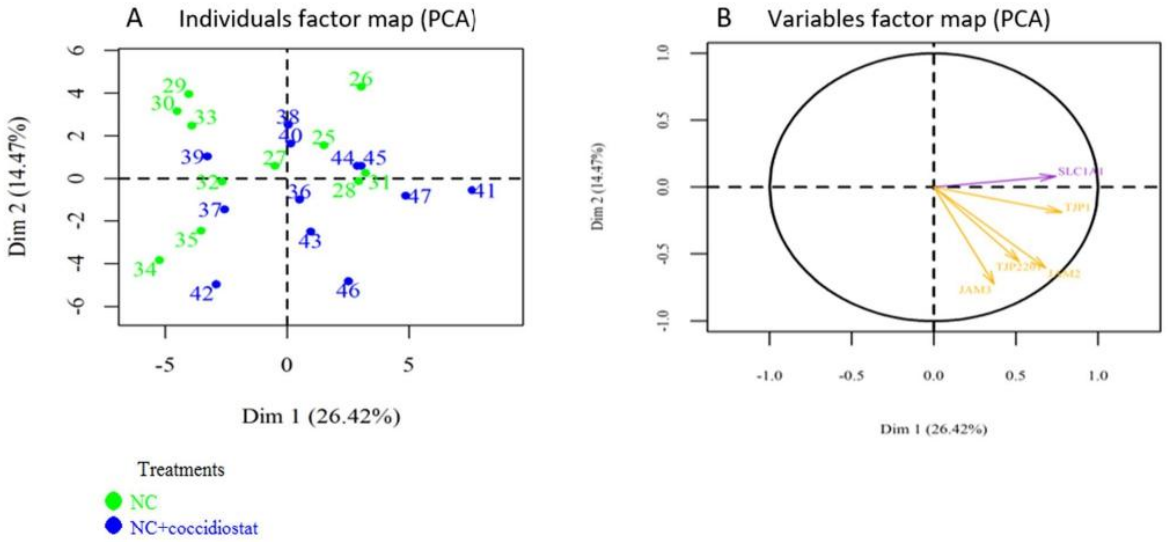

Figure 5. PCA representations of intestinal gene expression data in ileum chicken broiler samples (**A**) The samples dot plot; (**B**) variables arrow plot.

Nutrient transport. Our results showed a down-regulation of *SLC1A1* and *SLC1A4* mRNA levels of the *C.perfringens* challenged birds suggesting a reduced uptake of energy source glutamate and essential amino acids, respectively[11], which may explain the major changes observed on the intestinal microvilli morphology and growth performance. Previous studies have also showed that *Eimeria* challenge leads to a decrease of *SLC1A1* gene expression[12,13]. However, in the current study no effect was observed on the *SLC1A1* gene expression suggesting that intestinal cells of the *Eimeria* challenged chickens did not suffer a depletion of the energy source glutamate. The mRNA levels of *SLC2A2*, located at the basolateral membrane of the gut epithelium and involved in glucose efflux, were reduced by the *Eimeria* challenge in the current study, which is in agreement with other authors[14,15]. However, our results showed an increase of ileum *SLC15A1* mRNA levels of *Eimeria*-challenged birds, which was not in concordance with results obtained by Miska and Fetterer[14]. This inconsistency may be attributed to the sampling site (ileum *vs* jejunum). In agreement with this hypothesis, Su et al.[12] reported that *SLC15A1* mRNA levels were decreased in the jejunum and increased in the ileum of *Eimeria acervulina*-challenged layers. *C.perfringens*-challenged chickens showed a down-regulation of *SLC34A2* gene, which is involved in regulating both intestinal Pi (phosphate) absorption and renal Pi resorption, and of *VDR* gene, a transcription factor that mediates the vitamin D3, involved in signalling intestinal calcium and phosphate absorption[16]. This reduction of *SLC34A2* may be explained by the decrease of *mTOR*, known to stimulate many intestinal NTers including *SLC34A2*[17]. This down-regulation of *mTOR* may be attributed to the reduced amino acids and energy uptake in intestinal cells of *C. perfringens*-challenged broilers[18] and the reduced VH:CD ratio of *C. perfringens*-challenged broilers. The *mTOR/RPS6KB1* pathway is essential to the intestinal cell migration[19] and thus, could help to accelerate the healing of NE-induced intestinal damage and promote the recovery of tissues. In contrast, *Eimeria*-challenged birds showed an activation of *mTOR* complex 1 pathways indicating a higher gut cell turnover to reduce the intestinal mucosal disruption.

Barrier function. Effects on TJ are pathogen-dependant as while some pathogens can utilize these proteins as receptors for attachment and subsequent internalization, others modify or destroy them by different pathways and thereby provide a gateway to the underlying tissue[20]. Accordingly, our results showed that TJ are differently modulated with either *C.perfringens* or *Eimeria* challenge. In general, *C. perfringens*-induced subclinical NE downregulated the gene expression of *OCLN, CLDN1, FABP2, TJP1,* and *TJP2,* and there was a tendency in the downregulation of *FABP6.* Meanwhile, *Eimeria* challenge showed a downregulation of *CLDN1* and *FABP6* and an upregulation of the *JAM2* and *TJP1* mRNA levels, as well as a reduction in *CLDN1* protein expression. *C.perfringens* enterotoxin (CPE) has been reported to use claudin family proteins within the tight junction structure as binding sites/receptors and eventually cause pore formation in host cells as well as an increase in paracellular permeability and cytotoxicity as a result of this attachment[21,22]. In fact, the decrease of intestinal *CLDN1*[23,24], and *OCLN* mRNA expression[23,25–28] has already been described in *C. perfringens*-challenged birds. However, the present study demonstrates that this challenge, not only causes a disruption in transmembrane proteins, but also in cytosolic protein that contribute to the functionality of TJ. Moreover, *MUC2*, a fundamental component of the protective mucus layer which can protect the intestine against pathogens and promote tissue restitution[29], was downregulated by *C. perfringens*-induced subclinical NE, as observed by other authors, highlighting that the effects of this challenge were able to affect different components of the BF. In the case of the *Eimeria* challenge, the literature reported inconsistent results. As described by Soutter et al.[30], the variability in the results could be related to the magnitude and timing of the challenge dose and vaccine formulation, as well as, chicken breed

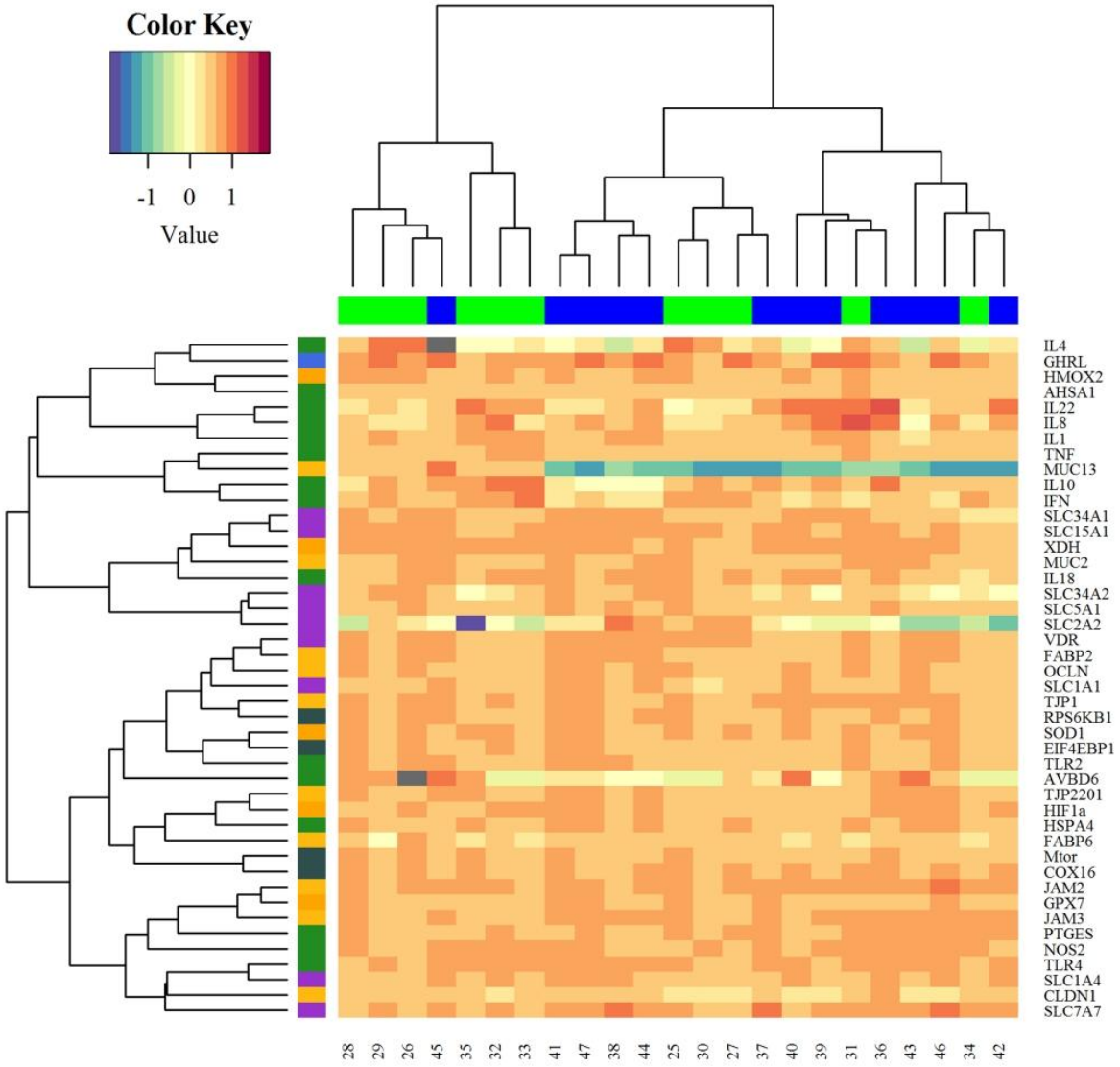

Figure 6. Heat map. The X-axis is sample expression pattern in different treatment group (green colour: negative control; blue colour: negative control + coccidiostat). The Y-axis is the gene clusters according to functions.

Protein (g/ng mucosa)	Experimental treatments		SEM	*P* value
	NC before challenge	NC after challenge		
OCLN	29.8	28.8	4.23	0.33
CLDN1	31.1	22.7	2.69	< 0.001

Table 1. Effect of *Eimeria* challenge on ileal *OCLN* and *CLDN1* protein expression (SEM: standard error of mean).

or genetic line. However, gene expression and the subsequent confirmation by protein expression that was performed in our study clearly demonstrate the participation of CLDN1 while seems to rule out the participation of OCLN in *Eimeria* challenge.

Protein (g/ng mucosa)	Experimental treatments		SEM	*P* value
	NC	NC + coccidiostat		
OCLN	28.8	31.0	2.35	0.26
CLDN1	22.7	24.2	2.98	0.29

Table 2. Effect of coccidiostat supplementation on ileal *OCLN* and *CLDN1* protein expression (SEM: standard error of mean).

Immune response. The binding mechanism of *C. perfringens* to the intestinal epithelial cell starts by the recognition and binding of pathogen-associated molecular patterns (PAMPs) by highly conserved pathogen recognition receptors, of which Toll-like receptors (TLRs) are the best characterized[31]. In particular, intestinal *TLR2* has been reported to recognize peptidoglycans in Gram-positive bacteria such as C.*perfringens,* and was upregulated in *C. perfringens* challenged broiler chickens in the current study, which corroborated the results of previous studies[23,32]. In the present work, *Eimeria* challenge showed no effect on *TLR2* and *TLR4* gene expression, which was in concordance with previous studies[33,34]. However, other authors reported that *TLR4* expression was upregulated at 12 h post infection (hpi) but not at 72 hpi in the ceum of *E.tenella* infected chickens[35]. Hence, this may explain why *TLR2* is not associated with chicken response to *Eimeria* infection, and suggest that *TLR4* is involved only in an early phase of response. Interactions between bacterial PAMPs and TLRs cause a cascade of signalling events that culminate in the secretion of various cytokines which have been reported to represent key components of innate immunity during the early phase of the host response to pathogens[36]. In the current study, we obtained an up-regulation in the *C. perfringens*-challenged group of *IL18, IL1B*, *IL22,* and *IL8.* These results indicated that *C. perfringens*-induced subclinical NE triggered an inflammatory IR in broiler chicken intestine as most pro-inflammatory cytokines were up-regulated. In this sense, the *IL8,* a CXC chemokine involved in the recruitment of leucocytes to mucosal sites of inflammation, was upregulated by *Eimeria maxima/C. perfringens* co-infection[37] and *C. perfringens* infection alone[27] or associated to high fishmeal diet[38]. Previous studies have also showed an up-regulation of *IL1B* in *C. perfringens* challenged broilers[2,23]. Moreover, both challenges significantly increased the proinflammatory cytokine *IFNG* mRNA levels compared with those in the unchallenged control, as previously reported in *C. perfringens*-[27,39], *Eimeria spp*-[40] and *C.perfringens/Eimeria maxima*-coinfected chickens[24]. The supplementation of coccidiostat resulted in a down-regulation of *IFNG* suggesting a reduced intestinal inflammation due to its ability to clear *Eimeria spp* as proven by the reduced oocyst shedding. *IFNG* has been also reported to affect the intracellular replication of *Eimeria spp* through the activation of inducible nitric oxide synthase (*iNOS*), an enzyme responsible for the production of nitric oxide (NO) proposed to be the effector molecule against *Eimeria spp*[41]. This may explain the tendency to upregulate mRNA levels of *2NOS* observed in the current study 6 d post infection (dpi) with *Eimeria.* In the case of *C. perfringens* infection, no effect on iNOS gene expression was observed, in contrast to other studies[42,43]. However, it has been reported that *iNOS* regulation is independent of *IFNG* during *C. difficile* colitis[44], suggesting that the mechanism underlying this activation needs to be further investigated.

To protect the intestinal integrity, a release of anti-inflammatory cytokines would be expected to be observed in *C.perfringens*-infected birds. Our results are in agreement with other authors that reported an increase of *IL10* in *C. perfringens*[43] and *C. butyricum* challenged birds[45]. However, a lack of response on *IL4* was observed in the current study and could be attributed to the age of chickens (42 d). Accordingly, Collier et al.[46] showed an increased gene expression of *IL4* in birds co-infected with *Eimeria* and C. *perfringens* at d22 but not at d28 of the experiment.

On the other hand, although the up-regulation of *HSPs*, in particular *HSP70*, as well as *AvBD*, are considered to be a protective mechanism[47,48] our results showed lower *HSP70* gene expression and no *AvBD* gene expression variation in NE birds. A possible explanation is that expression patterns of *AvBD* are pathogen-dependant, and AvBD8, 10, 11, and 13 are defensins that may play a key role in host intestinal defence against NE pathogens[49]. Nonetheless, none of these was incorporated in our gene expression panel.

Oxidative stress markers and digestive hormones. Several studies reported an occurrence of OX in chickens challenged with *Eimeria*[40,50] and *C. perfringens*[51] evidenced by reduced antioxidant enzyme activity. However, our results showed that although *Eimeria* challenged chickens showed an up-regulation of *HIF1A* and *XDH,* as well as a tendency to increase *SOD1*, *C.perfringens* challenge decreased *SOD1* and *HMOX2* mRNA levels. These differences confirm that the damage caused was more pronounced in case of C.*perfringens* challenge, where a decrease of oxidative enzymes indicates a failure of these chickens to combat the excess free radicals produced during the infection as well as to promote an anti-inflammatory response as proven by the reduced mRNA levels of *GHRL*[52]. In fact, *GHRL* gene expression was up-regulated in duodenum and jejunum of heat-stressed[53] and bursa of IBDV-infected broiler chickens[52], which suggests that *GHRL* may function as an anti-inflammatory factor[52].

In conclusion, the panel developed in the current study allows a global gene expression profiling which gives a greater overview of genes and pathways involved in broiler response to pathogen challenge. It also provides insights into differences of gene expression patterns and magnitude of responses under either a coccidial vaccine challenge or NE induced by the use of commercial reused litter and wheat-based diets without exogenous supplementation of enzymes. Considering these results, further studies will be performed using this panel to

explore the underlying molecular mechanisms responsible of positive effects of feed additives containing organic acids and essential oils on broiler chickens gut health and performance.

Material and methods

Ethics approval and consent to participate. All animal experimentation procedures were approved by the animal Ethics Committee of the Universitat Autònoma de Barcelona and were performed in accordance with the European Union guidelines for the care and use of animals in research (Council OFTHE. 20.10.2010. Off J Eur Union. 2010; 33–79), as well as the ARRIVE guidelines[54].

Feeding program, bird management and husbandry. *C. perfringens challenge.* A total of 148 1-d-old Ross 308 male broiler chickens were included in the study. Birds were obtained from a commercial hatchery, where they were vaccinated *in ovo* according to the standard vaccination program, against Marek disease, Gumboro disease and infectious Bronchitis. Nonetheless, all chicks used in the trial did not receive the coccidiosis vaccination.

Upon arrival, chicks were weighed and randomly assigned according to initial BW into two rooms, healthy conditions with 7 replicates (4 chicks per cage) *vs* challenging conditions with 10 replicates (12 chicks per pen) in order to get a similar initial average BW for each replicate. Both rooms share the same environmental conditions. Mean room temperature was maintained to 35 °C during the first four d post placement and then decreased progressively to 25 °C on d 14. The range of relative humidity was maintained between 50 and 70%. Light intensity and day-length were adjusted according to the producer recommendations. For the first two ds, birds were given 24 h of light which were reduced to 23 h of light and 1 h of dark from d3 to d10 and 18 h of light and 6 h of dark from d11 till the end of the experimental period. The birds were weighed by cage or pen, and feed intake was recorded at the end of each phase (10, 28 and 42 ds).

Chickens were given a 3-phase feeding program consisting of starter (0 to 10 d), grower (11 to 28 d) and finisher (29 to 42 d) phases. Supplementary Table 7 lists the composition of the antibiotic-free and coccidiostat-free basal diet used during each phase. All diets were formulated to meet the requirements for maintenance and growth for broilers (Fundación Española para el Desarrollo de la Nutrición Animal, 2008). Feed in mash form and water were available ad libitum. All diets used were sampled and stored for their subsequent analysis.

Eimeria challenge. A total of 140 one-d-old Ross 308 broiler male chickens were included in the experiment. The chicks shared the same floor pen during the first week. They also shared the same mash basal diet whose composition was similar to that used in the first phase of the experiment 1. On d 7, 120 birds were individually weighed and distributed into 30 battery brooders cage (4 chicks per cage) with the aim to get a similar initial average body weight for each cage. Dietary treatments were as follows: (1) the same basal diet used during the first week as negative control (NC) and (2) NC supplemented with (0.0033%) robenidine hydrochloride.

Challenge procedure. *C. perfringens challenge.* To promote challenging condition to the animals, the floor area of the pen (1.5 × 0.75 m) was covered with a litter consisting of 10% of clean wood shavings and 90% of reused litter material. The reused litter material was obtained from a commercial poultry flock where a clinical NE was claimed as previously characterized for its content of mesophilic aerobic bacteria ($> 10^5$/g), Enterobacteriaceae (520×10^2/g), filamentous fungi and yeasts (220×10^2/g), sulphite-reducing anaerobes and *Clostridium perfringens* ($> 10^5$/g). A challenging method consisting to expose broilers to litter contaminated by *Clostridium perfringens* has been previously described[8]. Moreover, percentages of 15, 20 and 25% of wheat were incorporated in the starter, grower and finisher diets, respectively, without exogenous enzyme supplementations with the aim to increase viscosity of intestinal digesta.

Eimeria challenge. On d 9 of the study, all birds were orally gavaged with a commercial coccidiosis vaccine (EVANT, HIPRA, Spain) containing a mixture of viable sporulated oocysts (3.5×10^4 *Eimeria acervulina*, 2.1×10^4 *Eimeria maxima*, 3.1×10^4 *Eimeria praecox*, 3.1×10^4 *Eimeria mitis* and 2.9×10^4 *Eimeria tenella*). This amount was chosen to represent a strong coccidia challenge that would induce intestinal damage, but not result in bird mortality. Housing system was also taken into account as chickens were reared in brooder cages where recycling of live oocysts by reinfection from litter is not possible as in floor pens[30].

Growth performance and sample collection. *C.perfringens challenge.* The chickens were weighed, and feed disappearance was determined at 0, 10, 28 and 42 ds of age. Mortality rate and BW of dead birds were also daily recorded. From these values, average daily feed intake (ADFI), ADG, and FCR corrected by mortality were calculated. On d 42, one bird per replicate was euthanized and samples were taken for ileum histomorphology analysis and ileum gene expression.

Eimeria challenge. To study the effect of both *Eimeria* challenge and coccidiostat supplementation sampling was performed on d 7 (pre-challenge) and 15 (post-challenge). All extra chickens (20) were euthanized on d 7 and one bird per replicate was euthanized on d 15 to collect ileum tissue for gene expression analysis and jejunum mucosa for proteomics analysis.

On d 15, faeces samples from all replicates were also collected for *Eimeria* oocyst count.

Performed analyses. *Eimeria oocyst count.* Faecal samples were sent to the Laboratory of Parasitology Service. The oocyst counting was conducted using the McMaster egg-counting technique according to Roep-

storff and Nansen, 1998. Briefly, four grams of faecal sample were transferred into a container, 56 ml of tap water were added, and the material was mixed thoroughly with a stirring device to ensure uniform suspension. Then faecal suspension was poured through a tea strainer and a 10-ml tube was filled to capacity with the filtered suspension. The tube was centrifuged for 2 min at 1800 RPM, the supernatant was removed and 4 ml of Zinc sulphate solution (SO_4Zn 33%, 1.18) was added. The sediment was then carefully resuspended and the McMaster counting chamber was filled. The number of oocyst per gram of faeces was calculated by multiplying the total number of oocysts by a coefficient of 20.

Intestinal morphological analyses. Ileum samples of about 5 cm were collected at the midpoint between Meckel's diverticulum and the ileo-cecal junction. Tissue sections (5 µm) were fixed in 4% paraformaldehyde and then embedded in paraffin. Afterwards, the sections were prepared, stained with haematoxylin eosin and analysed using a light microscope. The morphometric variables measured included villus height (VH), crypt depth (CD), the ratio of villus height to relative crypt depth (VH:CD) and intraepithelial lymphocytes (per 100 µm villus height). For the determination of goblet cell number (per 100 µm villus height), tissue slides were prepared and stained with periodic acid-Schiff. Ten villi were measured for each sample and only complete and vertically oriented villi were evaluated. The mean from 10 villi per sample was used as the average value for further analysis. All morphometric analysis was done by the same person, who was blinded to the treatments.

Tissue collection, RNA purification and cDNA synthesis. At the midpoint of ileum samples of about 1 cm were collected, snap frozen in 1 mL of RNA later (Deltalab, Spain) and stored at − 80 °C for subsequent RNA isolation and gene expression analysis.

A sample of 50 mg of ileum tissue was submerged in 1 mL of TRIzol Reagent (Thermo Fisher Scientific) and homogenized with a Polytron device (IKA, Staufen, Germany). Total RNA was obtained with the Ambion RiboPure kit (Thermo Fisher Scientific), by following the manufacturer's protocol. RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop products) and RNA purity was checked with Agilent Bioanalyzer-2100 equipment (Agilent Technologies), according to the producer's protocol. All samples showed an RNA integrity number higher than 8.

Between 0.8 and 1 µg of total RNA was reverse-transcribed into cDNA with random primers using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) in a final volume of 20 µl. The following thermal profile was applied: 25 °C 10 min; 37 °C 120 min; 85 °C 5 min; 4 °C hold. A negative control was performed with no reverse transcription (–RT control) to test the possible residual genomic DNA amplification. cDNA samples were stored at − 20 °C until use.

Primer design and testing. Genes included in this study were previously selected based on published information. Primers were designed for 48 genes using the PrimerExpress 2.0 software (Applied Biosystems) (Supplementary Table 8). Primers were designed spanning exon-exon boundaries or alternatively located at different exons. In addition, genomic DNA amplification and primer dimer formation were controlled.

All primers were tested for quantitative real-time polymerase chain reaction (RT-qPCR) performance and specificity in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) using two-fold serial dilutions (1/10, 1/100) of a pool of cDNA from all samples. –RT controls were also included.

Selection of genes for the expression panel. A list of 48 genes related to intestinal response to different environmental or dietary challenges was selected according to the bibliography[13,14,18], among others. The list included: (1) genes participating in the BF (*CLDN1*, *FABP2*, *FABP6*, *JAM2*, *JAM3*, *MUC13, MUC2*, *OCLN*, *TJP1*, and *TJP2*); (2) a gene coding for an hormone involved in energy homeostasis (*GHRL*); (3) genes that play an important role for the IR like pattern recognition receptors, host defense peptides, cytokines and stress proteins (*AHSA1*, *AvBD6*, *AvBD9*, *HSPA4*, *IFNG*, *IL10*, *IL18*, *IL1B*, *IL22*, *IL4*, *IL8*, *NOS2*, *PTGES*, *TLR2*, *TLR4*, and *TNF*); (4) genes involved in MB processes (*COX16*, *EIF4EBP1*, *mTOR*, and *RPS6KB1*); (5) genes coding for NT such as solute carriers among others (*SLC15A1*, *SLC1A1*, *SLC1A4*, *SLC2A2*, *SLC34A2*, *SLC3A1*, *SLC5A1*, *SLC7A7*, and *VDR*); (6) in addition genes implicated in OX were selected (*GPX7*, *HIF1A*, *HMOX2*, *SOD1*, and *XDH*); and finally three avian reference genes were selected according to the bibliography (*LBR, NDUFA*, and *YWHAZ*) (Table 3).

Gene expression study in chicken intestine. RT-qPCR efficiency was checked using relative standard curves which were constructed for each gene assay with three-fold serial dilutions of the pool of cDNA and it was analysed per triplicate. The dilution series consisted of 1/5, 1/15, 1/45, 1/135, and 1/405 dilutions of pooled cDNA. High throughput RT-qPCR was performed in a 48.48 microfluidic dynamic array IFC chip in a BioMark HD System (Fluidigm Corporation)[77]. After that, mRNA quantification of 48 genes (45 target genes and 3 reference genes) of 15 animals was performed per duplicate using the 1/20 final dilution and a negative control was included to check the non-specific amplification of primers.

Protein expression. Approximately 30 mg of mucosa was homogenized (Polytron, Kinematica AG, Luzern, Swiss) in phosphate saline buffer at 4 °C during 30 s. Then, the samples were submitted to 3 freeze–thaw cycles (3 min each cycle) and centrifuged (3500 g, 10 min, 4 °C). The supernatant was used to quantify OCLN and CLDN1 by ELISA following the instructions of the manufacturer (Chicken occludin ELISA Kit and chicken claudin 1, MyBioSource Inc., San Diego, CA, USA).

Function	Gene	Description	References
Barrier function	CLDN1	Transmembrane protein of TJ	55
	FABP2	Related with epithelial cell content and occurance	23,55
	FABP6	Necessary for the transport of bile acids in the gut and it was associated with bacterial presence and inflammation	55
	JAM2	Transmembrane protein of TJ	
	JAM3	Transmembrane protein of TJ	
	MUC13	Transmembrane mucine that plays a role in cell signaling pathways	23
	MUC2	Secretory mucine important in the establishment of the mucus layer	23,55
	OCLN	Protein of TJ involved in both inter-membrane and paracellular diffusion of small molecules	
	TJP1	Scaffold proteins that form part of the cytoplasmatic plaque of TJ	55
	TJP2	Scaffold proteins that form part of the cytoplasmatic plaque of TJ	
Digestive hormone	GHRL	Induces motor activity in the intestinal tract	52,53
Immune response	AHSA1	Co-chaperone activator of HSP90	12
	AvBD6	Avian defensin involved in antimicrobial functions and protecting the gut epithelium	46,56,57
	AvBD9	Avian defensin with antimicrobial properties and other cellular functions	
	HSPA4	Member of HSP proteins and play a prominent role in repair and protection of the intestinal environment	48,58,59
	IFNG	Host defense for combating against the intracellular pathogens including Salmonella	57,60,61
	IL10	Anti-inflammatory cytokine produced by activated macrophages and T cell	57,60,61
	IL18	Pro-inflammatory cytokine, primarily produced by macrophages, targeting T helper type-1 (Th1) cells	48,57,61,62
	IL1β	Mediator of the inflammatory response and involved in cellular processes	37,60
	IL22	Commonly used as marker of inflammation involved in T-lynphoocytes activation	37,60
	IL4	Cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells	57,59,61
	IL8	Secreted in response to pathogenic bacteria infection or specific inflammatory citokines	57,58,61,63
	NOS2	Induce the development of Th1 type of IR in infections	37,60
	PTGES	Intestinal inflammatory factor	64
	TLR2	Transmembrane receptor for the recongition of gram positive bacteria	47,49,60,61
	TLR4	Transmembrane receptor for the recongition of gram negative bacteria	
	TNFa	Regulation of the host immunity against multiple pathogens	57,61
Metabolism	COX16	Enzyme involved in the generation of energy by the mitochondria	65
	EIF4EBP1	mTOR pathway proteins in jejunum—protein synthesis and cell proliferation	66,67
	mTOR	mTOR pathway proteins in jejunum—protein synthesis and cell proliferation	
	RPS6KB1	mTOR pathway proteins in jejunum—protein synthesis and cell proliferation	
Nutrient transport	SLC15A1	Peptide transporter	13
	SLC1A1	Excitatory amino acid transporter	68–71
	SLC1A4	Neutral amino acid transporter by ASC system	71
	SLC2A2	Glucose transporter	70–72
	SLC34A2	n Intestinal phosphate absorption and phosphate homeostasis	66
	SLC3A1	Protetin related to neutral amino acid transporter	13
	SLC5A1	Sodium glucose transporter 1	
	SLC7A7	L amino acid transporter 2	
	VDR	Transcription factor that mediates the vitamin D3, involved in signaling intestinal calcium and phosphate absorption	65,67,73
Oxidation	GPX7	Intracellular antioxidant, and plays a great role in the detoxification of various peroxides	73
	HIF1A	Transcription factor that regulates genes involved in inflammation and cell death	23
	HMOX2	Oxidative stress marker	74
	SOD1	Antioxidant enzyme	73
	XDH	Enzyme associated to the synthesis of reactive oxygen species and is member of cellular defense system	23
Reference gene	LBR	Reference gene	75
	NDUFA	Reference gene	
	YWHAZ	Reference gene	76

Table 3. Summary of the bibliography used for the selection of the genes included in the panel and a brief description of their main function.

Statistical analysis. The performance data were analysed considering the pens of birds as the experimental unit. All data concerning ADG, ADFI, FCR and intestinal histology from *C.perfringens* challenge experiment as well as BW from *Eimeria* challenge were analysed as one-way ANOVA using the GLM procedure for the statistical package SAS. Data of oocyst counting and protein expression were analyzed with T-test. When significant treatment effects were disclosed, statistically significant differences among means were determined by a Tukey multiple comparison test of means. The level of statistical significance was set at $P \leq 0.05$ and tendency at $P \leq 0.10$. Protein expression analysis was performed.

Processing, normalization, and statistical analysis of the RT-qPCR data. Data was collected and checked with Fluidigm Real-Time PCR analysis software 3.0.2 (Fluidigm) and analysed using DAG Expression software 1.0.4.11[78]. The relative standard method curve was applied; target gene expression levels were normalized using reference genes and resulted in normalized quantity (NQ) values of each sample and assay. Statistical computations were performed using R 3.4.3 (R Development Core Team 2013). Firstly, all data were subjected to a logarithmic transformation to get closer to the Gaussian distribution. The gene expression data were subjected to one-way ANOVA using treatment as factor. The differences between treatments averages associated to P-values ≤ 0.05 were regarded as statistically significant.

The Benjamini and Hochberg FDR[7] multiple testing correction was added to the initial P-value information. The mean profiles of the treatments throughout the genes have been represented using a lines graph.

Two visualization tools based on unsupervised statistical learning methods were performed: PCA and heatmap representation. PCA for dimension reduction and visualization was applied to samples as cases and genes expressions as variables. Heatmaps are frequently used to represent expression levels and to show a double clustering for both samples and genes. In our setting, different clustering methods were used for samples (columns) and genes (rows). Indeed, a correlation-based distance $d = (1 - r)/2$, where r is the Pearson's coefficient, and the complete linkage method were chosen to classify genes proximities, while the usual Euclidean distance and the Ward's linkage were implemented to conglomerate the samples[79].

Data availability

All data generated or analysed during this study are included in this published article.

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Author contributions
N.A. and L.C.M.: Gathering information and papers, writing the outlines, writing the paper. J.F.P. and D.S.O.: Proposing the subject and experimental design, choosing the list of genes incorporated in the panel, reviewing and editing the paper. L.C.M., A.N., A.F. and J.M.F. performed the primer design and gene expression analysis. R.M.V.: performing protein expression analysis, reviewing and editing the paper. N.A. and A.F.: participating in protein expression analysis. M.F. and F.G.S.: proposed and implemented in R programming language the statistical methods and tools to perform the comparative analysis of treatments' groups based on the RT-qPCR data.

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CHAPTER 5

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Microencapsulation Improved Fumaric Acid and Thymol Effects on Broiler Chickens Challenged With a Short-Term Fasting Period

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The first objective of this study was to demonstrate the usefulness of the microencapsulation technique to protect fumaric acid and thymol, avoiding their early absorption and ensuring their slow release throughout the gastrointestinal tract (GIT). For this purpose, the release of a lipid matrix microencapsulated brilliant blue (BB) was assessed *in vitro*, using a simulated broiler intestinal fluid, and *in vivo*. *In vitro* results showed that more than 60% of BB color reached the lower intestine, including 26.6 and 29.7% in the jejunum and ileum, respectively. The second objective was to determine the effects of microencapsulated fumaric acid, thymol, and their mixture on the performance and gut health of broilers challenged with a short-term fasting period (FP). One-day-old male ROSS 308 chickens ($n = 280$) were randomly distributed into seven treatments, with 10 replicates of four birds each. Dietary treatments consisted of a basal diet as negative control (NC), which was then supplemented by either non-microencapsulated fumaric acid (0.9 g/kg), thymol (0.6 g/kg), or a mixture of them. The same additive doses were also administered in a microencapsulated form (1.5 and 3 g/kg for the fumaric acid and thymol, respectively). At day 21, chickens were subjected to a 16.5-h short-term FP to induce an increase in intestinal permeability. Growth performance was assessed weekly. At day 35, ileal tissue and cecal content were collected from one bird per replicate to analyze intestinal histomorphology and microbiota, respectively. No treatment effect was observed on growth performance from day 1 to 21 ($p > 0.05$). Microencapsulated fumaric acid, thymol, or their mixture improved the overall FCR (feed conversion ratio) and increased ileal villi height-to-crypt depth ratio (VH:CD) ($p < 0.001$) on day 35 of the experiment. The microencapsulated mixture of fumaric acid and thymol increased cecal abundance of Bacteroidetes, Bacillaceae, and Rikenellaceae, while decreasing that of Pseudomonadaceae. These results indicate that the microencapsulation technique used in the current study can be useful to protect fumaric acid and thymol, avoiding early absorption, ensure their slow release throughout the GIT, and improve their effects on fasted broiler chickens.

Keywords: microencapsulation, fumaric acid, thymol, gut health, broiler

INTRODUCTION

Intensive genetic selection has led to vast improvements in the efficiency of the poultry industry. However, a faster growth rate under intensive conditions coupled with increasing restrictions on the use of antimicrobials is pushing chicken rearing to higher prevalence of intestinal diseases. The gastrointestinal tract (GIT) is a highly complex and dynamic ecosystem involving the qualitative and quantitative equilibrium of the microbial load, morphological structure of the intestinal wall, and the adequate activity of the immune system. An optimal gastrointestinal function is crucial for a sustainable, cost-effective animal production (1). Therefore, the "gut health" concept has drawn significant attention among scientists (2) to develop nutritional strategies and natural alternatives aiming to modulate the gut function toward a satisfactory poultry performance and feed efficiency.

Among the most studied alternatives, organic acids (OAs) and phytogenic feed additives, including essential oils (EOs), may show antimicrobial potential to control dysbiosis and enhance performance of broilers raised without antimicrobials. Although most of the beneficial effects of OAs are associated with their ability to lower the pH, they may also elicit direct non-pH effects on bacterial metabolism by targeting the cell wall, and the cytoplasmic membrane, as well as function related to prokaryote replication and protein synthesis (3). Several studies reported an antimicrobial activity of OAs against most common poultry pathogens such as *Clostridium perfringens* (4), *Salmonella* (5, 6), *Campylobacter jejuni* (7), and *Escherichia coli* (8). On the other hand, there are also published reports that suggest that dietary EOs may stimulate digestive secretions for enhancing nutrient digestibility (9), regulate the gut microbiota composition (10), maintain intestinal integrity, and strengthen mucosal barrier function (11), improve cellular and humoral immunity (12, 13), as well as modulate the immunity related gene expression of chickens (14). The antimicrobial effect of EOs has been linked to their ability to affect the proteomes and cell morphology of pathogenic bacteria (15), which is able to disrupt the outer membrane lipids, and initiate cell lysis leading to an increased permeability. Moreover, combinations of EOs with OAs may show a synergetic potential (16). Nevertheless, the positive effects of OAs and EOs remain controversial (17, 18), which may be attributed to an early absorption of the active compounds that may reduce their levels in the lower GIT (19, 20). Therefore, researchers aim to develop strategies to preserve feed additives from early absorption or volatilization, and to ensure their progressive delivery along the lower GIT. Among the techniques used for protecting feed additives, microencapsulation has been widely applied. In this context, multifarious strategies have been successfully investigated to manufacture microcapsules including chemical methods, such as interfacial polycondensation, interfacial cross-linking, and matrix polymerization; physicochemical methods, such as ionotropic gelation, coacervation-phase separation, chilling, and freeze drying; and physical methods, such as pan coating, air-suspension coating, and centrifugal extrusion (21–23). Despite the benefits of these methods, they still present some drawbacks that may limit their use, such as being costly and time consuming. However, the electrohydrodynamic processes used in the current study is a technique composed of two sister technologies including electrospraying and electrospinning, which provides a broad range of benefits. It is considered as an innovative, cost-effective, and one-step method that ensures the scale-up processes for high-throughput production. Moreover, this energy-saving technique has recently emerged as a promising approach suitable for incorporation of heat-sensitive active compounds (24) by preserving their structure and efficacy upon processing, storage and delivery (21). On the other hand, vegetable oils included in the lipid matrix microparticles used in the current study are composed of long-chain triglycerides reported to possess a slower digestion than that of proteins and polysaccharides (25).

Therefore, we hypothesized that microencapsulation of fumaric acid and thymol, as examples of OAs and EOs, will promote a delayed release of the contained active compounds into the targeted GIT section, exerting beneficial effects on performance, immunity, and the digestive GIT functions in broiler chickens. We also hypothesized that these effects will be more pronounced in broilers exposed to challenging conditions that can negatively affect their gut health.

Therefore, the objectives of this study were (1) to show evidence of the progressive release of fumaric acid and thymol, as examples of OAs and EOs, when these are microencapsulated in lipid matrix microparticles under *in vitro* and *in vivo* intestinal conditions; and (2) to evaluate the effect of microencapsulated fumaric acid and thymol on the performance and gut health of broiler chickens challenged with a short-term fasting period (FP) (as a model of mucosal damage and increased GIT permeability).

MATERIALS AND METHODS

Release of Blue Brilliant (BB) Color

In vitro Screening

A simulated GIT *in vitro* test was designed to study the release of a microencapsulated BB color (E133) along the GIT of broiler chickens. This microencapsulated BB included 20% of free blue color protected with the same wall material (hydrogenated fats) used to microencapsulate the feed additives tested in the *in vivo* experiment.

The first step was the preparation of a calibration curve. Quantities of 0.0125, 0.025, 0.05, 0.075, 0.1, 0.1125, 0.15, 0.175, and 0.2 g of non-microencapsulated powdered BB color were placed in conical flasks (four for each dose). The following reagents were then added to each flask: 25 ml of phosphate buffer (0.1 M, pH 6.0), 10 ml of 0.2 M HCl, 1 ml of a freshly prepared pepsin, 0.5 ml of chloramphenicol solution, 27 ml of Trizma-Maleate buffer (0.1 M, pH 7.5), 0.5 ml of $CaCl_2$ at 325 mM, 1.5 ml of NaCl at 3.25 mM, 0.25 g of bile salts, and 3 ml of pancreatin. Flasks were closed and stirred gently for 2 h at 39°C, and then absorbance was measured using a spectrophotometer at 450 nm. The calibration equation obtained was $Y = 725.14X - 0.1887$, where "Y" was the absorbance at 450 nm and "X" was the concentration, and the R^2 was approximately 0.98.

Afterwards, the BB release was simulated under "stomach" and "small intestine" conditions. The BB release under simulated "stomach" conditions was evaluated according to a protocol adapted from Boisen and Fernández (26). A total of 28 conical flasks were used and a total of 0.5 g of microencapsulated BB containing 20% of non-microencapsulated one was placed in each. Afterwards, 25 ml of phosphate buffer (0.1 M, pH 6.0) was added to each flask, followed by a gentle magnetic stirring before adding 10 ml of 0.2 M HCl. The pH was then adjusted to pH 2.0 before adding 1 ml of freshly prepared pepsin and 0.5 ml of chloramphenicol. Finally, the flasks were closed and stirred for 90 min in a thermostatically controlled incubator at 39°C. This time was decided based on the study of Ravindran (27) reporting that the digesta spends 90 min in the upper digestive tract. The equivalent transit time per segment was also adapted from the same study being as follows: 30 min in the crop (0–30 min) and 60 min in the proventriculus/gizzard (30–90 min). Thus, during these 90 min, flasks were taken out (four per time) at 30, 60, and 90 min, and absorbance was measured using a spectrophotometer at 450 nm. The concentration was calculated using the calibration equation, which allowed to calculate the percentage of release per segment of the upper digestive tract.

The BB release was then evaluated under simulated small intestine conditions according to Martin et al. (28). After 90 min, the rest of the flasks were removed, and the rest of the previously mentioned reagents used for the preparation of calibration curve were added. Flasks were then stirred gently for 2 min before adding 3 ml of freshly prepared pancreatin. All flasks were then placed in the incubator for a total of 2 h 20 min, reported to be approximately the total transit time in the lower GIT (27). At the end of the first 30 min, the pH was adjusted to 7. Four flasks were removed at 10, 40, 110, and 140 min post-incubation considered as the equivalent transit time for the duodenum, jejunum, ileum, and cecum, respectively (27). The absorbance was measured using a spectrophotometer at 450 nm and the concentration was determined using the calibration equation, which allowed the calculation of the release percentage per segment of the lower digestive tract.

In vivo Screening

A total of seven 41-day-old Ross 308 male chickens were used to assess the *in vivo* screening of both non-microencapsulated and microencapsulated (containing 20% of E133) BB color. The chickens received the basal diet supplemented with either 0.6% of non-microencapsulated or 3% of microencapsulated BB color (one and six chickens, respectively) during 24 h before being electrically stunned and euthanized. The entire GIT was then removed for the assessment of BB release. Six chickens were used for the microencapsulated BB color to ensure that the kinetics of release were similar in all birds.

In vivo Experiment

The study was performed at the animal experimental facilities of the Servei de Granges i Camps Experimentals (Universitat Autònoma de Barcelona; Bellaterra, Barcelona, Spain). The experimental procedure received prior approval from the Animal Protocol Review Committee of the same institution (CEEAH1043R2). All animal housing and husbandry conformed to the European Union Guidelines (29).

Experimental Design, Dietary Treatments, and Animal Husbandry

A total of 280 1-day-old male ROSS 308 chickens were purchased form a local hatchery, where they received *in ovo* vaccinations for Marek disease, Gumboro disease, and infectious bronchitis. Upon arrival, chicks were weighed and allotted, according to initial body weight, to seven dietary treatments in a completely randomized design. Each dietary treatment was replicated 10 times in battery brooder cages with four chickens per replicate. A non-medicated (non-antibiotic or anticoccidials drug), corn–soybean meal-based diet was used as the basal diet for all treatments (**Table 1**). The used feed additives (Tecnovit, Alforja, Spain) including fumaric acid, thymol, and their mixture were tested either under microencapsulated or non-microencapsulated form. The microencapsulated fumaric acid included 60%, while thymol contained 20% of active compounds. The equivalent concentration of active compounds was used for the non-microencapsulated forms. Dietary treatments were then produced by supplementing the basal diet with the tested feed additives as follows: (1) basal diet, negative control group (NC); (2) NC+ 1.5 g/kg of microencapsulated fumaric acid; (3) NC+ 3 g/kg of microencapsulated thymol; (4) NC+ microencapsulated blend of fumaric acid (1.5 g/kg) and thymol (3 g/kg); (5) NC+ 0.9 g/kg non-microencapsulated fumaric acid; (6) NC+ 0.6 g/kg free thymol; and (7) NC+ mixture of non-microencapsulated fumaric acid (0.9 g/kg) and thymol (0.6 g/kg).

Chickens were given a two-phase feeding program consisting of a starter (day 0–14) and grower (day 15–35). All diets were formulated according to CVB poultry guidelines (30). All diets used were sampled, ground, and stored at 5°C until they were analyzed in duplicate.

The brooder temperature was maintained at 32°C during the first 2 days and was then gradually reduced to 25°C on day 14. Birds were provided with a 24-h light during the first 2 days, 23 h light/1 h darkness program from day 3–10, and 18 h light/6 h darkness through the remainder period (days 11–35). All birds were allowed *ad libitum* access to feed in mash form, as well as fresh water. Birds and housing facilities were inspected twice daily to control general health status, feed, and water availability, temperature, mortality, and any unexpected events, during the experimental period.

Growth Performance Evaluation

Body weight (BW) and feed intake (FI) from each replicate cage were recorded on days 0, 7, 14, 21, 28, and 35. Average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated. Mortality rates were recorded daily.

Short-Term Fasting-Induced Challenge

On day 21, after finishing the productive performance control, the smallest bird in each replicate was removed for stocking density reasons. Afterwards, a short-term FP was performed by removing feeders for 16 h and 30 min. This aimed to induce a challenge, as this practice has been reported as a model to

TABLE 1 | Dietary compositions and nutrient levels (% as fed-basis, unless otherwise indicated) of the basal diet.

	Starter	Growing
Ingredient composition, g/kg diet		
Maize	550	582
Soybean meal 48	303	350
L-lysine HCl	1.2	0.20
DL-methionine	2.4	1.3
Soy oil	8.0	19.0
Palm oil	—	17.0
Full fat soybean	100.0	—
Limestone	10.3	6.3
Dicalcium phosphate	15.7	15.5
Salt	2.0	2.0
Premix*	4.0	4.0
Sodium bicarbonate	3.4	2.7
Calculated composition (%)		
Dry matter	87.8	87.9
ME (kcal/kg)	2975	3101
Crude protein	22	21
Lysine	1.35	1.18
Methionine	0.59	0.47
Ca	0.95	0.78
Total P	0.65	0.63
Available P	0.45	0.44
Analyzed composition (%)		
Dry matter	88.5	88.2
GE, kcal/kg	4100	4300
Crude protein	21.9	21.4
Ether extract	0.43	0.54
Crude fiber	2.9	2.7
Ash	5.8	5.6

(*) Provided per kg of feed: Vitamin A (retinyl acetate) 10,000 IU; vitamin D3 (cholecalciferol) 4,800 IU; vitamin B1 (Thiamine) 3 mg; vitamin B2 (riboflavin) 9 mg; vitamin B3 (Nicotinamide) 51 mg; vitamin B6 (pyridoxine hydrochloride) 4.5 mg; vitamin B9 (folic acid) 1.8; vitamin B12 (cyanocobalamin) 0.04 mg; vitamin E (DL-α-Tocopheryl acetate): 45 mg; vitamin K3 (Menadione) 3 mg; pantothenic acid (calcium D-pantothenate) 16.5 mg, biotin [D-(+)-biotin] 0.15 mg; choline chloride 350 mg; iron ($FeSO_4$) 54 mg; iodine [$Ca(IO_3)_2$] 1.2 mg; zinc (ZnO) 66 mg; manganese (MnO) 90 mg; copper ($CuSO_4$) 12 mg; selenium (Na_2SeO_3) 0.2 mg; 6-Phytase EC 3.1.3.26: 1500 FYT; Butylated hydroxytoluene (BHT) 25 mg; Colloidal silica 45 mg, Sepiolite 1,007 mg.

increase intestinal permeability and thereby negatively affect the gut integrity (31).

Sampling Procedure and Analyses

Diet proximate analyses were performed following Association of Official Agricultural Chemists methodology (32): dry matter (Method 934.01), crude protein (Method 968.06), crude fat (Method 2003.05), and crude fiber (Method 962.09). Gross energy was determined by an adiabatic calorimeter (IKAC-4000, Janke-Kunkel; Staufen, Germany).

On day 35, the bird with the closest BW to the mean of the cage was stunned using an electrical stunner (Reference: 105523, FAF, France) before being euthanized for tissue sampling. The GIT was immediately dissected and content from the cecum was collected for microbiota sequencing. Ileal tissue was collected to perform the histomorphological analysis.

Histomorphological Analysis

Ileal samples of about 5 cm were collected at the midpoint between Meckel's diverticulum and the ileo-cecal junction. Tissue sections (5 μm) were fixed in 4% paraformaldehyde and then embedded in paraffin. Afterwards, the preparations were deparaffinized and hydrated before being subjected to PAS (Periodic acid-Schiff) staining with Schiff's reagent for morphometric analyses and goblet cells count. Samples were analyzed using a light microscope. The morphometric variables measured included villus height (VH), crypt depth (CD), villus height-to-relative crypt depth ratio (VH:CD), and number of goblet cells/100 μm VH (GC). Ten villi were measured for each sample, and only complete and vertically oriented villi were evaluated. The mean from 10 villi per sample was used as the mean value for further analysis. All morphometric analysis was done by the same person, who was blinded to the treatments.

Microbial Diversity Analysis

Bacterial DNA was extracted from cecal content samples (250 mg) using the commercial MagMAX CORE Nucleic Acid Purification 500RXN Kit (Thermo Fisher, TX, USA) and following the manufacturer's instructions. For 16S rRNA gene sequence-based analysis, the V3–V4 region of the bacteria 16S ribosomal RNA gene were amplified by PCR (95°C for 3 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and 72°C for 5 min) using primers F5′-barcode TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GCCTACGGGNGGCW GCA G-3′ and R5′-GTCTCGTGGG CTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTAT CTAATCC-3′. A negative control of the DNA extraction and a positive Mock Community control were included to ensure quality control. After 25 cycles of amplifications, 550-bp amplicons were obtained. The Illumina Miseq sequencing 300 × 2 approach was used. Raw sequencing reads were quality clipped, assembled, and compared with available genomic sequences using a Microomics Systems S.L (Barcelona, Spain) software and were validated and subsequently completed with the Kraken Metagenomics (33) and QIIME (34) software. Taxonomic assignment of phylotypes was performed using a Bayesian classifier trained with Silva database version 132 (99% Operational taxonomic units full-length sequences) (35).

Statistical Analysis

Statistical analyses were carried out on BW, ADG, ADFI, FCR, and histomorphological analysis with ANOVA using the GLM procedure of SAS software (SAS 9.4 Institute Inc., Cary, NC, USA). Normal distribution and homoscedasticity of variances were checked prior to the analysis, using the Shapiro–Wilk test and Levene's test from UNIVARIATE and GLM procedures, respectively. All data related to growth performance and intestinal histomorphology were firstly analyzed according to a according to a completely randomized design, considering treatment groups as the predictor and the number of cages

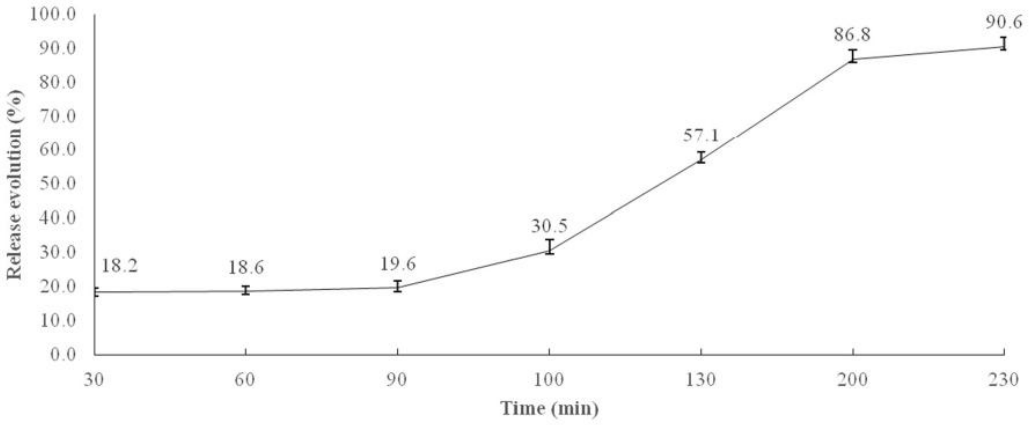

FIGURE 1 | *In vitro* microencapsulated BB release profile in simulated stomach (0–90 min) and small intestine conditions (90–230 min). Each value is the average release of four flasks at simulated conditions of the crop, 0–30 min; gizzard, 30–90 min; duodenum, 90–100; jejunum, 100–130 min; ileum, 130–200 min; and cecum, 200–230 min.

(individual broiler chickens for the histomorphology) as the experimental unit. A further analysis of contrasts excluding the NC aiming to compare the microencapsulated feed additives to the non-microencapsulated ones was also performed for the growth performance and histomorphological data. Means were compared using the Tukey multiple comparisons test and deemed significant at $p \leq 0.05$.

Biostatistical analysis for microbiota was performed using open-source software RStudio v.3.5.1. Diversity was analyzed at OTU level using a vegan package (36). Richness and α-diversity were calculated using raw counts based on Simpson, Shannon, and Inverse-Simpson estimators. β-diversity was evaluated by multivariate ANOVA. Finally, differential abundance analysis was performed with taxa relative abundances under a zero-inflated log normal mixture model, and *p*-values were corrected by false discovery rate (FDR) using a metagenomeSeq package (37).

RESULTS

Release of Blue Brilliant (BB) Color

In vitro Screening

The percentage release of microencapsulated BB was calculated taking into account the transit time (min) in the broiler GIT, adapted from (27). **Figure 1** shows the results obtained, indicating that about 19.6% of microencapsulated BB were released in times equivalent to crop and gizzard retention, 10.9% in duodenum, 26.6% in jejunum, 29.7% in ileum, and 3.8% in the cecum.

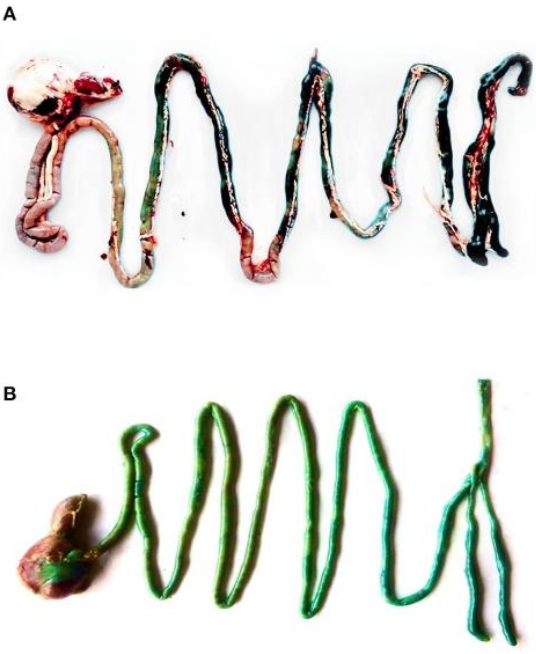

FIGURE 2 | The GIT of a 42-day-old broiler supplemented with either microencapsulated **(A)** or non-microencapsulated BB **(B)**.

In vivo Screening

Figure 2 shows the GIT of a 42-day-old broiler supplemented by either microencapsulated (A) or non-microencapsulated BB (B). The blue color was observed in the entire GIT of the broiler supplemented with non-microencapsulated BB, whereas it was observed only from the jejunum and backwards for the birds receiving the microencapsulated BB. Unfortunately, the quantification of the *in vivo* release of the microencapsulated BB was not possible. The reason behind this limitation was that once mixed with the feed mostly composed of maize in the GIT, the

TABLE 2 | Effect of dietary treatments on growth performance of broiler chickens.

Items	Experimental treatments							SEM	*p*
	NC	Microencapsulated			Non-microencapsulated				
		OA	EO	OA+EO	OA	EO	OA+EO		
BW g									
Day 0	42.8	43.2	43.0	42.9	43.3	43.1	42.9	0.16	0.350
Day 21	720	719	699	699	710	705	708	15.6	0.931
Day 28	1106^{b}	1266^{a}	1240^{a}	1260^{a}	1129^{b}	1112^{b}	1132^{b}	21.0	<0.001
Day 35	1694^{ab}	1802^{a}	1790^{a}	1804^{a}	1670^{ab}	1646^{b}	1682^{ab}	33.2	0.001
ADG g/day									
Days 0–21	32.2	32.2	31.2	31.2	31.7	31.5	31.7	0.9	0.501
Days 21–28	55.1^{b}	78.1^{a}	77.4^{a}	80.2^{a}	59.7^{b}	58.1^{b}	60.5^{b}	2.24	<0.001
Days 28–35	84.1	76.5	78.6	77.7	77.4	76.4	78.6	4.24	0.892
Days 0–35	47.2^{ab}	50.3^{a}	49.9^{a}	50.3^{a}	46.5^{ab}	45.8^{b}	46.8^{ab}	0.95	0.001
ADFI g/day									
Days 0–21	44.2	44.1	43	42.3	43.1	42.5	43.2	1.13	0.460
Days 21–28	94.2^{b}	111.9^{a}	109.2^{ab}	115.4^{a}	106.3^{b}	103.4^{b}	102.9^{b}	3.64	0.004
days 28–35	143.0	122.4	128.6	124.9	132.6	131.4	133.9	6.97	0.501
Days 0–35	73.1	73.3	73.4	73.5	73.6	72.4	73.3	1.70	0.990
FCR									
Days 0–21	1.37	1.37	1.38	1.35	1.36	1.35	1.36	0.040	0.762
Days 21–28	1.71^{a}	1.43^{b}	1.41^{b}	1.44^{b}	1.78^{a}	1.78^{a}	1.70^{a}	0.036	<0.001
Days 28–35	1.70^{a}	1.60^{b}	1.64^{ab}	1.61^{b}	1.71^{a}	1.72^{a}	1.70^{a}	0.021	0.006
Days 0–35	1.55^{a}	1.46^{b}	1.47^{b}	1.46^{b}	1.58^{a}	1.58^{a}	1.57^{a}	0.017	<0.001

NC, negative control; OA, organic acid: fumaric; EO, essential oil: thymol.
Inclusion levels: microencapsulated fumaric acid: 1.5 g/kg; microencapsulated thymol: 3 g/kg; non-microencapsulated fumaric acid: 0.9 g/kg; non-microencapsulated thymol: 0.6 g/kg.
Different letters indicate significant difference between the seven dietary treatments at $p \leq 0.05$.
Data are presented as mean (n = 10 replicates/group for all evaluated parameters).

TABLE 3 | Effect of dietary treatments on the ileal histomorphology on day 35 of age.

Items	Experimental treatments							SEM	*p*
	NC	Microencapsulated			Non-microencapsulated				
		OA	EO	OA+EO	OA	EO	OA+EO		
VH	607.8^{b}	742.7^{a}	720.6^{ab}	754.8^{a}	639.3^{ab}	657.6^{ab}	628.1^{ab}	28.32	0.040
CD	81.3^{ab}	66.6^{c}	64.6^{c}	64.0^{c}	82.9^{ab}	76.9^{b}	86.1^{a}	2.45	<0.001
VH:CD	7.5^{b}	11.1^{a}	11.2^{a}	11.8^{a}	7.8^{b}	8.6^{b}	7.3^{b}	0.42	<0.001
Goblet cells/100 μm VH	27.7^{a}	14.1^{b}	15.4^{b}	16.1^{b}	24.3^{a}	23.5^{a}	26.0^{a}	0.67	<0.001

NC, negative control; OA, organic acid: fumaric; EO, essential oil: thymol.
Inclusion levels: microencapsulated fumaric acid: 1.5 g/kg; microencapsulated thymol: 3 g/kg; non-microencapsulated fumaric acid: 0.9 g/kg; non-microencapsulated thymol: 0.6 g/kg.
Different letters indicate significant difference between the seven dietary treatments at $p \leq 0.05$.
Data are presented as mean (n = 10 replicates/group for all evaluated parameters).

digesta color turns to green, making the use of the equation of the calibration curve previously established, no longer correct.

In vivo Experiment

Growth Performance

Growth performance data are shown in **Table 2**. No treatment effect was observed on growth performance of broiler chickens before performing the short-term FP challenge on day 21. The FCR from day 21 to day 28 as well as the overall FCR were improved in the experimental groups fed the microencapsulated fumaric acid, thymol, or their mixture ($p < 0.001$). The analysis of contrasts showed an improved overall ADG and FCR by the supplementation of microencapsulated form of all the feed additives ($p < 0.001$) as compared to the non-encapsulated ones and the NC.

Histomorphological Analysis

Histomorphological analysis of the middle portion of the ileum is shown in **Table 3**. Results showed that the experimental groups receiving either the microencapsulated fumaric acid or the

microencapsulated mixture of fumaric acid and thymol exhibited higher VH ($p = 0.040$) than the NC group. All experimental groups fed the microencapsulated feed additives showed lower CD, higher VH:CD ratio, and lower count of goblet cells/100 µm VH ($p < 0.001$).

Microbial Diversity Analysis

Results of microbial diversity analysis revealed that neither α-diversity (**Table 4**) nor β-diversity (**Figure 3**) was different among experimental groups ($p > 0.05$).

At the phylum level, eight phyla were determined, including mainly Firmicutes, Bacteroidetes, Tenericutes, and Verrucomicrobia, followed by Proteobacteria, Actinobacteria, Patescibacteria, and Cyanobacteria (**Figure 4**), with no differences between dietary treatments ($p > 0.05$). The majority of Firmicutes sequences (**Figure 5**) corresponded to Ruminococcaceae and Lachnospiraceae while the majority of the Bacteroidetes sequences correlated with sequences of Bacteroidaceae and Rikenellaceae.

TABLE 4 | Effect of dietary treatments on microbiota α-diversity indices in cecal content of broiler chickens on day 35.

	Experimental treatments			SEM	*p*
	NC	Encap OA+EO	Non-encap OA+EO		
Shannon	3.72	3.67	3.60	0.028	0.751
Simpson	0.94	0.94	0.93	0.032	0.962
Inverse Simpson	18.7	16.5	16.4	1.22	0.570

NC, negative control; OA, organic acid: fumaric; EO, essential oil: thymol.
Encap, microencapsulated; Non-encap, non-microencapsulated.
Inclusion levels: microencapsulated fumaric acid: 1.5 g/kg; microencapsulated thymol: 3 g/kg; non-microencapsulated fumaric acid: 0.9 g/kg; non-microencapsulated thymol: 0.6 g/kg.
$p \leq 0.05$ was considered significant.

A more in-depth examination of the individual metagenomics profile changes was detected on the dietary treatments using $\log_2$ changes. Broilers supplemented with the microencapsulated mixture of fumaric acid and thymol compared to those fed the NC (**Figure 6**) had significant differences in the relative abundance of Firmicutes (0.39-fold decrease; $p < 0.0001$) and Bacteroidetes (0.52-fold increase; $p < 0.0001$) phylum, and some families including Rikenellaceae (1.14-fold increase; $p = 0.0034$), Tannerellaceae (3.35-fold increase; $p = 0.0067$), Bacillaceae (1-fold increase; $p = 0.0085$), Chitinophagaceae (2.45-fold decrease, $p = 0.0027$), Pseudomonadaceae (2.58-fold decrease; $p = 0.0403$), and Sphingomonadaceae (2.42-fold decrease; $p = 0.0004$).

Broiler supplementation with the non-encapsulated mixture of fumaric acid and thymol significantly changed the abundance of Bacteroidetes (0.34-fold increase; $p = 0.0060$) phyla, and families such as Clostridiaceae 1 (2.51-fold increase; $p = 0.0096$), Erysipelotrichaceae (0.53-fold increase; $p = 0.0020$), Desulfovibrionaceae (1.02-fold increase; $p = 0.0101$), Ruminococcaceae (0.27-fold decrease; $p < 0.0001$), and Chitinophagaceae (1.15-fold decrease; $p < 0.0001$) compared to the NC group (**Figure 7**). The comparison between both forms of the mixture of fumaric acid and thymol (**Figure 8**) showed that the microencapsulated one changed the abundance of Firmicutes (0.22-fold decrease; $p < 0.0001$), Bacteroidetes (0.18-fold increase; $p < 0.0001$), Tenericutes (0.11-fold increase; $p < 0.0001$), and Verrucomicrobia (0.22-fold increase; $p = 0.0003$) phylum, and some families like Bacteroidaceae (0.05-fold increase; $p = 0.0385$), Erysipelotrichaceae (0.85-fold increase; $p = 0.0021$), Clostridiaceae 1 (0.61-fold decrease; $p = 0.0006$), and Pseudomonadaceae (1.27-fold decrease; $p = 0.0003$).

DISCUSSION

In the current study, a short-term FP of 16.5 h was applied on day 21 as an experimental model to challenge gut

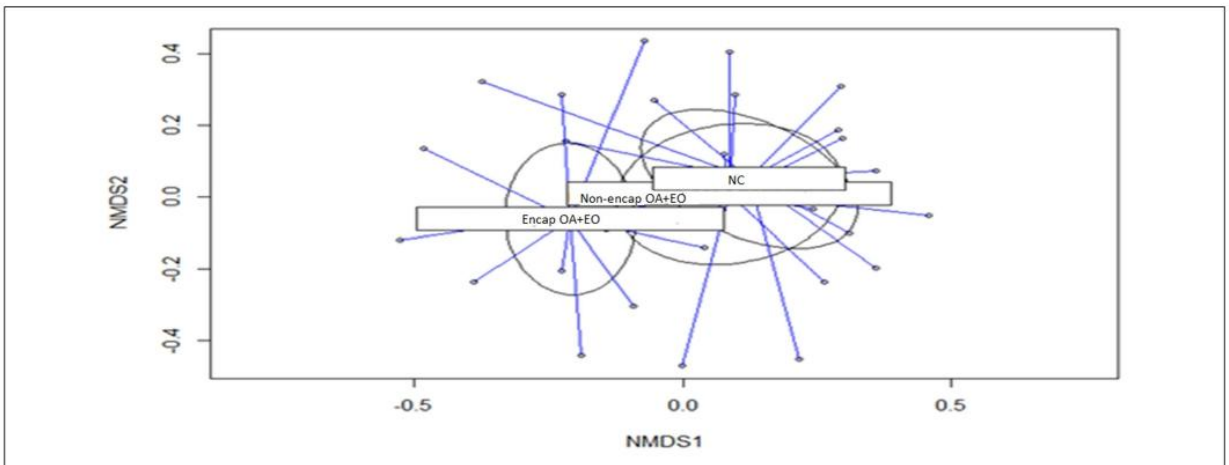

FIGURE 3 | Non-metric multidimensional scaling (NMDS) ordination plot of bacterial communities of the different treatment groups ($p = 0.430$). NC, negative control; OA, organic acid: fumaric; EO, essential oil: thymol. Encap, microencapsulated; Non-encap, non-microencapsulated. Inclusion levels: microencapsulated fumaric acid: 1.5 g/kg; microencapsulated thymol: 3 g/kg; non-microencapsulated fumaric acid: 0.9 g/kg; non-microencapsulated thymol: 0.6 g/kg.

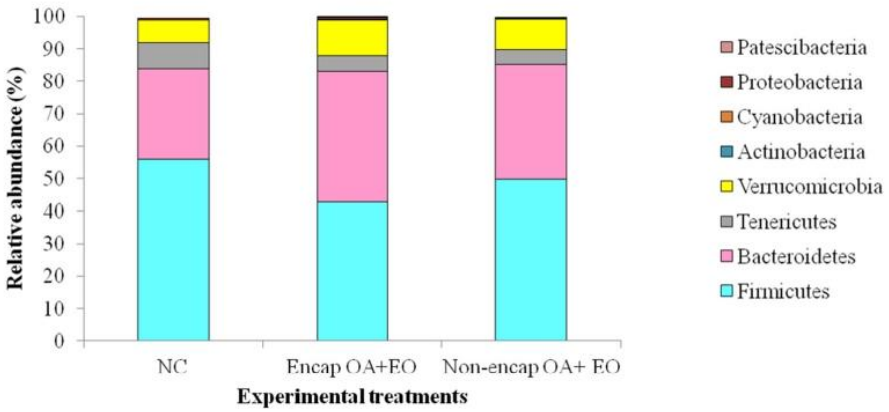

FIGURE 4 | Phyla present in the cecum microbiota of broilers from different treatment groups on day 35. NC, negative control; OA, organic acid: fumaric; EO, essential oil: thymol. Encap: microencapsulated; Non-encap, non-microencapsulated. Inclusion levels: microencapsulated fumaric acid: 1.5 g/kg; microencapsulated thymol: 3 g/kg; non-microencapsulated fumaric acid: 0.9 g/kg; non-microencapsulated thymol: 0.6 g/kg.

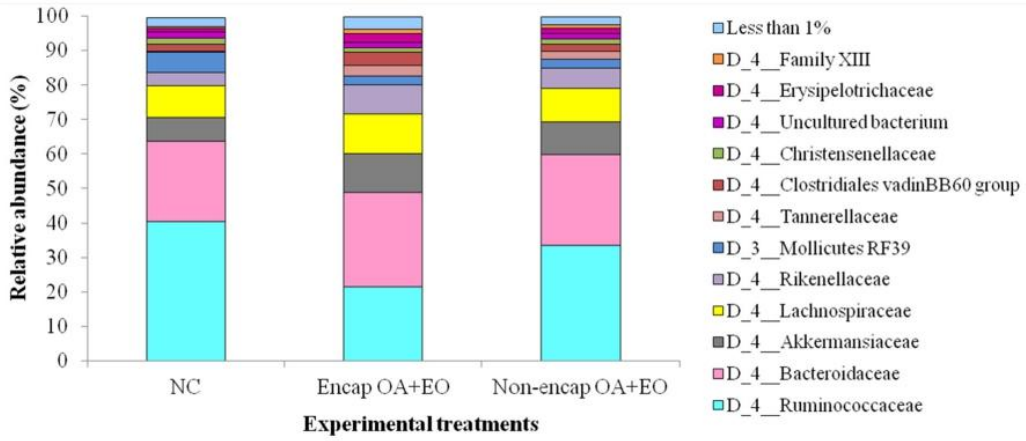

FIGURE 5 | Abundant bacterial families present in the cecum microbiota of broilers from different treatment groups on day 35. NC, negative control; OA, organic acid: fumaric; EO, essential oil: thymol. Encap: microencapsulated; Non-encap, non-microencapsulated. Inclusion levels: microencapsulated fumaric acid: 1.5 g/kg; microencapsulated thymol: 3 g/kg; non-microencapsulated fumaric acid: 0.9 g/kg; non-microencapsulated thymol: 0.6 g/kg.

health. The objective was to investigate whether the evaluated feed additives were able to alleviate the induced negative effects on growth performance, intestinal histomorphology, and microbiota 2 weeks later. Short-term FP up to 24 h has been reported to increase intestinal permeability (38), which may potentially induce bacterial translocation (39), lameness (40), and compromised growth performance (41). A recent study conducted by Herrero-Encinas et al. (31) showed that a 15.5-h short-term FP induced an increase in intestinal permeability by reducing *Claudin-1* expression, which triggered an inflammatory response, resulting in a higher CD and lower VH:CD ratio compared to control non-fasted group.

Effects of the Free Feed Additives Supplementation

Compared to the NC, the supplementation of non-microencapsulated fumaric acid, thymol, or their mixture did not show any significant effect on growth performance, neither before the short-term FP nor on day 28 or 35. A lack of effect was also obtained with the analysis of ileal histomorphology. However, the significant increase in goblet cells in the ileum of these groups may suggest a higher demand for enhanced mucin secretion, likely helping to reduce the possible damage of the small intestine epithelia (42). Other studies also reported that free OAs failed to reach the cecum in adequate concentrations and, thus, to reduce the *Campylobacter* colonization in broilers

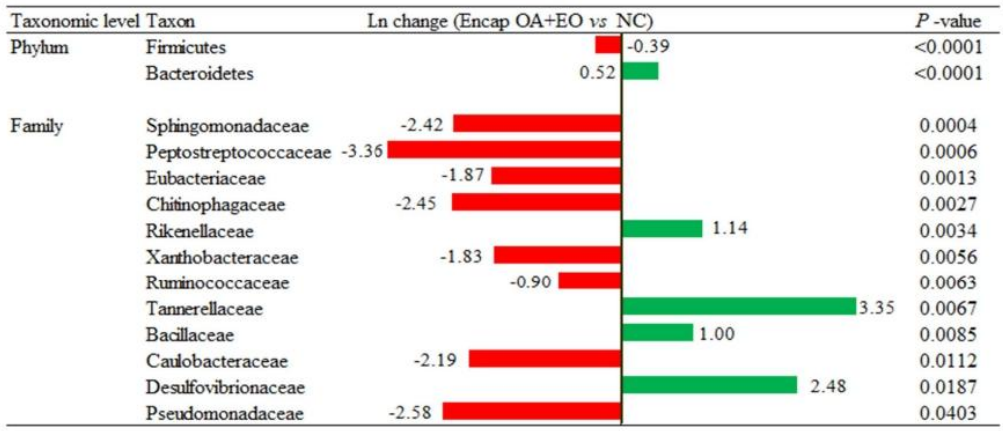

FIGURE 6 | Ln changes promoted by the supplementation of the microencapsulated mixture of fumaric acid and thymol as compared to the NC (fold discovery rate *p*-adjusted <0.05) in taxa. Positive values (■) and negative values (■) indicate greater and lower abundance. Only significant taxa are presented. Differences presented are based on all taxa detected in samples per diet. NC, negative control; OA, organic acid: fumaric; EO, essential oil: thymol. Encap: microencapsulated. Inclusion levels: microencapsulated fumaric acid: 1.5 g/kg; microencapsulated thymol: 3 g/kg.

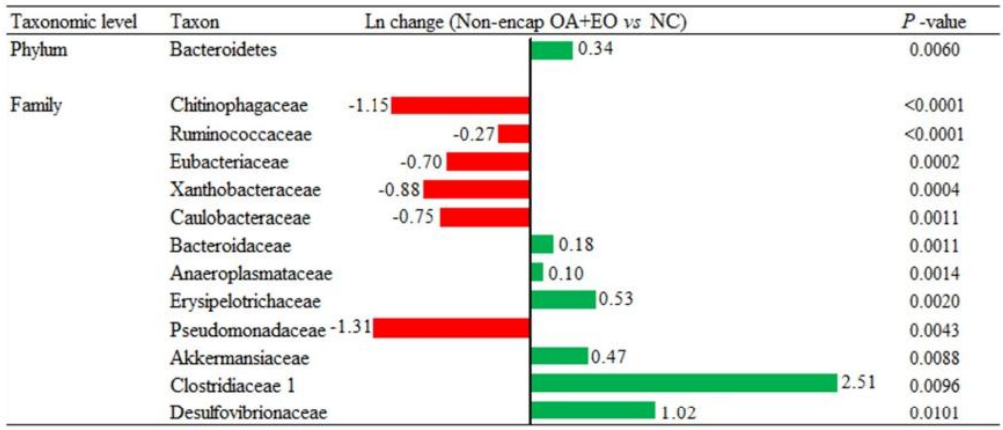

FIGURE 7 | Ln changes promoted by the supplementation of the non-encapsulated mixture of fumaric acid and thymol as compared to the NC (fold discovery rate *P*-adjusted <0.05) in taxa. Positive values (■) and negative values (■) indicate greater and lower abundance. Only significant taxa are presented. Differences presented are based on all taxa detected in samples per diet. NC, negative control; OA, organic acid: fumaric; EO, essential oil: thymol. Non-encap: non-microencapsulated. Inclusion levels: non-microencapsulated fumaric acid: 0.9 g/kg; non-microencapsulated thymol: 0.6 g/kg.

(18, 43). This lack of effect may be attributed to their rapid degradation, absorption, and metabolism in the upper section of the GIT (before or almost just entering the duodenum) as shown by the results of the *in vivo* release of non-encapsulated BB. This early absorption means that the majority would not reach the lower GIT tract where they would exert their major functions (18), which may represent a serious limitation for their efficacy.

Effects of Microencapsulated Feed Additives

The feed additives tested in the current study were microencapsulated using lipid-based particles, reported to possess high encapsulation efficiency, loading capacity, and release efficiency in the small intestine (25). This slow release throughout the GIT was confirmed by the results of the *in vitro* BB release, showing that nearly 60% was released at jejunum, ileum, and cecum equivalent retention times. Although the quantification was not possible, these findings were further supported by the results of the *in vivo* BB release, where the microencapsulated blue color was not observed in the duodenum, suggesting that the release started from the jejunum and backwards. A similar study was performed by Lee et al. (44) to evaluate the physiochemical properties and prolonged release behavior of chitosan-denatured β-lactoglobulin microcapsules

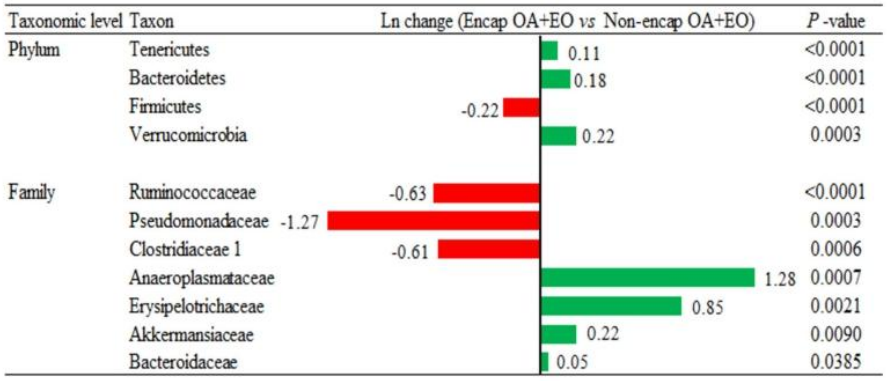

FIGURE 8 | Ln changes promoted by the supplementation of the microencapsulated mixture of fumaric acid and thymol as compared to the non-encapsulated one (fold discovery rate *P*-adjusted <0.05) in taxa. Positive values (■) and negative values (■) indicate greater and lower abundance. Only significant taxa are presented. Differences presented are based on all taxa detected in samples per diet. NC, negative control; OA, organic acid: fumaric; EO, essential oil: thymol. Encap: microencapsulated; Non-encap, non-microencapsulated. Inclusion levels: microencapsulated fumaric acid: 1.5 g/kg; microencapsulated thymol: 3 g/kg; non-microencapsulated fumaric acid: 0.9 g/kg; non-microencapsulated thymol: 0.6 g/kg.

for potential food applications. These authors obtained similar promising results, showing that their wall matrix provided both the right timing and location for the BB dye release.

Targeting the lower GIT may be advantageous to enhance the intestinal development, which helps to improve digestion and nutrient absorption, and thereby, the growth performance (19). Growth performances have been evaluated in several experiments in which broiler chickens were supplemented by OAs, EOs, or the mixture of both. However, the considerable increasing number of published articles has generated great information inconsistency. Although some studies revealed improved production performance traits by protected OA supplementation (45), EO supplementation (46), or their mixture (47–49), others reported no effect on growth performance of chickens (50). This discrepancy may be attributed to the heterogeneity of experimental conditions, such as the chemical structure of the OAs or EOs used, the dose, the supplementation form (mixed or not), the sanitary challenge conditions, the number of used chickens, the size of cages or barns, the buffering capacity of feeds, the feed nutritional dietary value, and other factors.

In the current study, the supplementation of microencapsulated fumaric acid, thymol, and their mixture improved the overall FCR during the whole experiment by 5.8% compared to the NC, and up to 7.0% compared to the non-microencapsulated ones. This improvement of growth performance may, in part, be attributed to the observed beneficial effects of these feed additives on ileal histomorphology (increased VH, reduced CD, increased VH:CD ratio, and lower goblet cells/100 μm as compared to the NC group). Similar positive effects were previously reported by other authors, where feeding a protected blend containing a minimum of 200 g/kg of sorbic acid, a minimum of 200 g/kg fumaric acid, a minimum of 100 g/kg thymol to broiler chickens reared under conventional conditions (51) or an encapsulated blend containing 4% thyme, 4% carvacrol, 0.5% hexanoic acid, 3.5% benzoic acid, and 0.5% butyric acid to broiler chickens challenged with necrotic enteritis (48) resulted in longer villi and a greater VH:CD ratio.

In the current study, all the feed additives with the same form of presentation (microencapsulated or non-microencapsulated) showed similar effects on the growth performance and histomorphological analysis. Thus, only the cecal microbiota of the chickens supplemented with the mixture, either microencapsulated or not, has been analyzed as compared to the NC group. The obtained results showed that the supplementation of the microencapsulated mixture of fumaric acid and thymol increased the relative abundance of phyla Bacteroidetes and decreased the relative abundance of phyla Firmicutes compared to the NC group. Similar results were obtained by Chen et al. (10) and Wu et al. (52) by the supplementation of broiler chickens by plant essential oil and sodium butyrate, respectively. However, the supplementation of broilers by a blend containing 4% thyme, 4% carvacrol, 0.5% hexanoic acid, 3.5% benzoic acid, and 0.5% butyric acid encapsulated in Ca-alginate and whey protein microcapsules resulted in an increase of the relative abundance of Firmicutes while the relative abundance of Bacteroidetes decreased (48). Although an increase in fecal Bacteroidetes has been associated with decreased nutrient absorption (53), this phylum composed of Gram-negative bacteria has been recently reported to be gut-friendly, being involved in many important metabolic activities. Indeed, Bacteroidetes participate in the degradation of polysaccharides and other indigestible carbohydrates to produce short-chain fatty acids (SCFAs), especially propionate *via* the succinate pathway (10, 52), utilization of nitrogenous substances, the biotransformation of bile acids, and the prevention of pathogen colonization (54). Among the Bacteroidetes, the

microencapsulated blend tested in the current study increased the abundance of Rikenellaceae whose effect on the host gut health remains inconsistent. Some studies considered a reduced abundance of this family to be beneficial (48) as it utilizes the mucin, involved in preventing adhesion of various pathogens and toxins present in the intestinal lumen, as carbon and energy source, which may decrease the intestinal mucosal barrier integrity (55). However, members of this family, such as *Alistipes*, showed to be increased in the current study, have been reported to produce propionic and succinic acids by fermentation of glucose, lactose, mannose, and melibiose, and form the iso-methyl branched-chain fatty acid or long-chain saturated acids (10). Tannerellaceae, a family belonging to Bacteroidetes, which was also increased by the supplementation of the microencapsulated blend of fumaric acid and thymol, produces acetate and succinate as its major metabolic end-products. Succinate can provide energy in two distinct ways. It can be either taken up directly by chicken intestinal cells through a sodium-dependent transport system and then introduced in the tricarboxylic acid or Krebs cycle or converted by several other Bacteroidetes bacteria into propionate after decarboxylation (56). As for Firmicutes, they are Gram-positive bacteria associated with the decomposition of polysaccharides and the production of butyrate (57). Belonging to this phylum, the abundance of Lachnospiraceae, known as butyric acid-forming bacteria (58), showed a numerical increase in the chickens fed the microencapsulated blend of fumaric acid and thymol as compared to the NC group (11.50 vs. 9.18%, respectively). Enhancing SCFA production is crucial for animal gut health. Indeed, butyrate has been reported to possess anti-inflammatory properties through the inhibition of nuclear factor-kappa B activation, leading to decreased expression of pro-inflammatory cytokines (59), which may explain the decrease in necrotic lesions induced by *C. perfringens* in the small intestine (60). It may also improve growth performance through pathogen control (17), barrier integrity enhancement by upregulating the AMP-activated protein kinase, which regulates the assembly of tight junctions (61), and the activation of goblet cells to produce mucin, which forms a protective layer on the enterocytes (62). As for propionate, it can also be used as an energy source by the epithelial cells and is known to stimulate the trypsin activity (63) and to possess health-promoting effects, including an anti-inflammatory activity, which may improve growth performance (56).

On the other hand, the increased abundance of Bacillaceae induced by the microencapsulated blend tested in the current study may be considered beneficial as a recent study showed a positive correlation between this family and total volatile fatty acids (VFAs). Bacillaceae has also been shown to play a key role in improving the immune status by enhancing different antioxidants and tight-junction genes (64).

The increase in the above-mentioned families may explain the decline observed in other families containing pathogen bacteria such as Pseudomonadaceae. The infection of broiler chickens with *Pseudomonas aeruginosa* is associated with high mortality and clinical signs including respiratory manifestations, diarrhea, and septicemia (65). Moreover, *Pseudomonas veronii* is a potential opportunistic pathogen whose abundance increased in broiler chickens challenged by *C. perfringens* (66).

Surprisingly, the microencapsulated blend tested in the current study increased the abundance of Desulfovibrionaceae, a producer of hydrogen sulfides reported to be toxic to mucosal tissue, which leads to severe inflammation of chicken GIT (67) and decreased that of Ruminococcaceae and Peptostreptococcaceae, known as butyric acid-forming bacteria (58). The decrease of Peptostreptococcaceae was not in concordance with previous studies that reported this family to be higher in broiler chickens supplemented with a blend of medium-chain fatty acids containing 0.3% capric acid and 2.7% lauric acid (68), as well as mice supplemented with 13.3 mg/ml of eugenol in drinking water for 7 days (11). However, the improved growth performance and intestinal histomorphology of the supplemented chickens indicated these birds to possess healthier intestinal microbiota compared to the NC group despite the above-mentioned unexpected changes of the gut microbiota.

Taken together, our results indicate that microencapsulating the fumaric acid and thymol using a lipid matrix prevents their absorption in the upper part of the digestive tract and directs their bioactivity toward the lower GIT, mainly the jejunum and ileum. In previous *in vitro* studies, we confirmed that the lipid base particles (empty particles *per se*) did not possess any antimicrobial activity. Therefore, it can be concluded that once released, fumaric acid and thymol enhanced intestinal microbiota balance in favor of beneficial bacteria, which may be responsible for the improvement of ileum histomorphology and thereby feed efficiency of broiler chickens. The positive effects of microencapsulated fumaric acid, thymol, or their combination were observed when broilers were under the challenging conditions of short-term fasting period, but not earlier, highlighting the usefulness of using such feed additives when sanitary conditions of animals are compromised.

CONCLUSION

In summary, the results of the current study confirmed the ability of the lipid matrix, obtained through the use of the electrohydrodynamic processes, to allow a slow release of fumaric acid and thymol throughout the broiler GIT. Microencapsulated fumaric acid, thymol, or their combination showed positive effects when broilers were subjected to challenging conditions, alleviating the negative effects promoted by the fasting challenge on animal performance, intestinal histomorpholgy, and microbiota.

DATA AVAILABILITY STATEMENT

The datasets generated for this study have been submitted to the Sequence Read Archive (SRA) database of the National Center for

Biotechnology Information as FASTQ files under study accession number PRJNA734795.

ETHICS STATEMENT

The animal study was reviewed and approved by CEEAH Universitat Autònoma de Barcelona.

AUTHOR CONTRIBUTIONS

NA, JFP, EV, and DS-O conceived and designed the study. NA and RK performed the experiments. NA and DM-D analyzed the data. NA wrote the manuscript. JFP, DS-O, EV, and IC corrected the manuscript. All authors read and approved the final manuscript.

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CHAPTER 6

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Targeted-Release Organic Acids and Essential Oils Improve Performance and Digestive Function in Broilers under a Necrotic Enteritis Challenge

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Simple Summary: Controlling digestive diseases in the poultry industry is crucial to maximize profitability. Necrotic enteritis (NE) is a real threat for poultry that leads to high financial losses. Microencapsulated blends of organic acids and essential oils have gained increasing interest as feed additives that could alleviate the effects of these diseases by controlling the intestinal microbiota and enhancing the gut function of broiler chickens. Organic acids actually used as feed additives, including short-chain fatty acids (C1-C6), medium-chain fatty acids (C7-C12), and other organic acids, may show a range of variable physiological effects in the animals when combined with different phytogenic compounds. This study was designed to understand the mechanisms of action of these feed additives, their effect on intestinal morphology and growth performance, as well as their interaction with the gut microbiome. Our results provide evidence on the importance of designing proper combinations and doses of these additives to enhance growth performance, the microbiota profile, and histomorphology. Dietary supplementation of 0.5 g/kg of BUTYTEC-PLUS and 2 g/kg of ACITEC-MC as microencapsulated blends are recommended to improve broiler chickens performance under NE challenge due to their positive effect on gut microbiome and the absorptive capacity of the intestine.

Abstract: An experiment was performed to evaluate the effect of four different microencapsulated blends of organic acids (OA) and nature-identical aromatic compounds (AC) on growth performance and gut health of broilers challenged with a recycled NE litter. A total of 600 one-day-old male Ross 308 broilers were randomly assigned to five treatments consisting of a basal diet (as negative control) supplemented with each of the tested microencapsulated blends: OA1 (malic and fumaric acid) + AC; 2.5 g/kg; OA2 (calcium butyrate+fumaric acid) + AC; 1.7 g/kg; MCFA (capric-caprylic; caproic and lauric acid) + AC; 2 g/kg; and MCFA + OA3 (calcium butyrate+fumaric and citric acid) + AC; 1.5 g/kg. The AC used was the same for all treatments; including cinnamaldehyde, carvacrol, and thymol (8:1:1), as major compounds. Three tested blends enhanced growth performance by improving intestinal histomorphology ($p < 0.001$). The tested blends enhanced the abundance of some beneficial families such as Ruminococcaceae and Lachnospiraceae; while reducing that of harmful ones such as Enterobacteriaceae and Helicobacteraceae. A further dose-response experiment showed that 0.5 g/kg of the blend 2 and 2 g/kg of the blend 4 improved growth performance and intestinal histomorphology of chickens on d 42 and decreased fecal Enterobacteriaceae and *C. perfringens* counts. Similar effects to the previous experiment were observed for cecum microbiota.

Keywords: organic acids; aromatic compounds; microencapsulation; performance; intestinal histomorphology; microbiota; gut health; broiler

1. Introduction

With the pressure of increasing awareness and changing mindsets of the consumers, poultry production is currently facing an important challenge consisting of how to deal with serious issues related to digestive diseases to maintain gut health under the antibiotic free rearing program [1]. In this context, necrotic enteritis (NE) and Eimeria coccidiosis are considered the most important digestive infectious diseases in chickens. NE is a widespread disease commonly diagnosed in poultry flocks that is caused by the overgrowth of commensal *Clostridium perfringens*, a spore forming, gram-positive, anaerobic, rod-shaped bacterium [2]. Although the primary causative agent is *Clostridium perfringens* types A and C, several additional factors have been reported as predisposing factors, such as cereal type in the diet, dietary protein levels, anti-nutritional factors, coinfection with other pathogens (particularly coccidiosis), as well as environmental and management factors such as stress, high animal density, and immunosuppression [3].

Global economic losses associated with enteric diseases in the poultry industry are estimated at US$ 6 billion year [4] due to increased mortality in case of acute clinical NE, and reduced growth performance, greater medication costs, and elevated risk of contamination of poultry products in the case of subclinical NE. The latter is more prevalent where *Clostridium perfringens* toxins, such as NetB toxin, damage the structure and function of epithelial cells leading to gut inflammation [1] accompanied by a disruption of the gut microbial community, impair gut barrier function [5] and thus, infected birds exhibit increased gut permeability and depressed growth [6].

For decades, NE and coccidiosis have been kept under control using antimicrobials and ionophore coccidiostats [7]. Antimicrobial pre-mixes and preventive or metaphylactic uses in large group of pigs and poultry are the main characteristics of those countries where antimicrobial consumption remains high. However, oral formulations generally result in higher exposure of the gastrointestinal microbiome to the antimicrobials, which is of particular concern in terms of a potential source of resistant bacteria [8]. The concern over the evolution and spread of antimicrobial resistance, which represents a potential threat for human and animal health, has led to an increasing interest in animal production schemes based on low or free antibiotic exposure.

Short- and medium-chain fatty acids, as well as essential oils (EO), can be considered promising candidates for preventing NE. EO have been reported to possess in vitro antimicrobial and antioxidant activities against a wide range of pathogenic bacteria [9], as well as to improve intestinal integrity and fortify the mucosal barrier [10], and to enhance cellular and humoral immunity [11]. On the other hand, short-chain fatty acids (SCFAs) are either simple mono-carboxylic acids such as formic, acetic, propionic, and butyric acids or carboxylic acids with the hydroxyl group such as lactic, malic, tartaric, and citric acids or short-chain carboxylic acids containing double bonds like fumaric and sorbic acids [12]. Although dietary supplementation of SCFAs may modulate microbiota through their bactericidal and bacteriostatic activity [13], they have also been shown to stimulate the expression of genes regulating growth, division, differentiation, proliferation, and apoptosis of epithelial cells [14]. They have been shown to improve performance and modulate resistance of broilers to diseases [15]. Medium-chain fatty acids (MCFAs), including caproic, caprylic, or capric acid possess a strong antibacterial activity against various gram-negative bacteria like *E. coli* and *Salmonella* and gram-positive bacteria like *Enterococcus*, *Staphylococcus*, and *Clostridia* [16] through targeting the bacterial cell membrane and the various essential processes that occur within and at the membrane. Other processes such as cell lysis, inhibition of enzyme activity, or impaired nutrient uptake may also contribute to inhibition of bacterial growth [17].

Combining EO and organic acids has shown to be efficacious due to the reported synergism between both compounds [18]. In fact, EO may increase the permeability of cell membranes which

allows organic acids to diffuse easily into the microbial cells. Recently, microencapsulation of organic acids and EO has shown to prevent their absorption in the upper part of digestive tract, while allowing a higher bioactivity towards the lower gastrointestinal tract [19].

In the present study, it is hypothesized that a microencapsulated botanical and acidifier active combination would prevent the performance decrease associated with NE by affecting the intestinal microbiota and digestive function in broiler chickens. Thus, the aim of the present study was to investigate the efficacy of different microencapsulated blends containing short and medium-chain fatty acids, calcium butyrate, and nature-identical aromatic compounds (thymol, cinnamaldehyde, and carvacrol as major compounds) on performance and gut health of broilers under challenging conditions of NE.

Two experiments were carried out, in which the first trial aimed to determine the design of combinations with a high efficiency, while the second trial focused on finding the optimal dose for each combination.

2. Materials and Methods

2.1. Ethics Statement

All animal experimentation procedures were approved by the animal Ethics Committee (CEEAH) of the Universitat Autònoma de Barcelona (number code: CEEAH 1043R2) and were performed in accordance with the European Union guidelines for the care and use of animals in research [20].

2.2. Birds and Experimental Design

2.2.1. Trial 1

A total of 600 one-day-old Ross 308 male broiler chickens obtained from a local hatchery were used in a 41-day-experiment. The room was provided with 50 floor pens (4 lines of 15, 10, 10, and 15 pens, respectively, divided by 2 central feeding aisles). Upon arrival, chicks were weighed and randomly assigned according to initial body weight (BW) into 5 experimental groups, each with 10 replicates and 12 birds per replicate, and continuously controlled over a period of 41 days. A non-medicated (no antibiotic or anticoccidial drug) wheat–corn–soybean meal-based diet was used as the basal diet for all treatments. Dietary treatments were then produced by supplementing the basal diet with the tested feed additives: Organic acids (OA) plus nature identical aromatic compounds (AC). The mixture of aromatic compounds used was the same for all treatments and contained cinnamaldehyde, carvacrol, and thymol (8:1:1). These were obtained by synthesis or isolated through chemical processes, which are chemically identical to natural flavoring substances. The experimental treatments consisted of the basal diet as a negative control (NC) and 4 commercial microencapsulated products (Tecnovit, Alforja, Spain) as follows: **1.-** NC + OA1 (malic and fumaric acid) + AC, (ACITEC-A-GR, 2.5 g/kg); **2.-** NC + OA2 (cacium butyrate + fumaric acid) + AC, (BUTYTEC PLUS, 1.7 g/kg); **3.-** NC + MCFA (capric-caprylic acid, caproic acid, lauric acid) + AC, (ACITEC-M1, 2 g/kg); and **4.-** NC + OA3 (calcium butyrate + fumaric acid + citric acid) + MCFA + AC, (ACITEC-MC, 1.5 g/kg). Experimental doses of the commercial products were included as recommended by the company in commercial conditions.

2.2.2. Trial 2

A total of 810 one-day-old Ross 308 male broiler chickens obtained from a commercial hatchery were used in a 42-day dose-response experiment. The room had 90 floor pens (2 lines of 23 and 2 lines of 22 pens each, divided by 2 central feeding aisles). Upon arrival, chicks were weighed and randomly assigned according to their initial BW into 10 experimental groups, each with 9 floor pens and 9 birds per replicate, and continuously controlled over a period of 42 days. The experimental treatments consisted of 5 increasing doses of two products selected from trial one: 0 g/kg for both products considered as negative control (NC; basal diet with recycled litter); basal diet supplemented with 0.5, 1,

2, and 4 g/kg of OA2 + AC or 1, 2, 4, and 8 g/kg of OA3 + MCFA + AC with recycled litter; and the positive control (PC) consisting of basal diet and non-contaminated new litter.

The chickens were weighed and feed disappearance was determined at 0, 10, 28, and 41 days of age (42 days for the second trial). Mortality rate and body weight of dead birds were also recorded daily. From these values, the average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratio (FCR) corrected by mortality were calculated.

2.3. Animal Husbandry

Both trials were carried out in a commercial growing poultry unit (Vila-rodona, Tarragona, Spain). Chicks were obtained from a commercial hatchery, where they were in ovo vaccinated according to the standard vaccination program, against Marek disease, Gumboro disease, and infectious bronchitis. Nonetheless, none of the chicks used in either trial received the coccidiosis vaccination. The birds were given 24 h of light for the first 2 days, which was reduced to 23 h of light and 1 h of dark from d 3 to d 10, and 18 h of light and 6 h of dark from d 11 until the end of the experimental period. The relative humidity was maintained between 50% and 70%.

2.4. Experimental Diets

The chickens were given a 3-phase feeding program in both experiments, consisting of a starter (0 to 10 d), grower (11 to 28 d), and finisher (29 to 41/42 d for first trial and second trial, respectively) diets. Table 1 lists the composition of the antibiotic-free and coccidiostat-free basal diet used during each phase. All diets were formulated to meet the requirements for maintenance and growth for broilers according to CVB poultry guidelines [21]. All chickens were given ad libitum access to feed in mash form and water. Housing facilities and birds were inspected twice daily (morning and afternoon) as regards general health status, feed and water availability, temperature, mortality, and any unexpected events. Samples were taken from all diets used, ground, and stored at 4 °C for their subsequent analysis in duplicate.

Table 1. Ingredients and nutrient composition (% as fed-basis, unless otherwise indicated) of basal diet.

Items	Starter	Growing	Finishing
Ingredient composition (%)			
Maize	41.20	40.30	40.50
Wheat	15.00	20.00	25.00
Soybean meal 48	28.80	32.60	25.50
L-lysine HCL	0.15	0.07	0.05
DL-methionine	0.23	0.13	0.14
L-threonine	-	-	0.05
Soy oil	1.10	1.40	-
Palm oil	-	2.50	6.00
Extruded soybean meal	10.00	-	-
Limestone	1.09	0.70	0.70
Dicalcium phosphate	1.49	1.44	1.08
Salt	0.20	0.20	0.20
Vitamin-mineral premix *	0.40	0.40	0.40
Sodium bicarbonate	0.34	0.26	0.27
Calculated composition (%)			
Dry matter	88.1	88.1	88.4
G.E (kcal/kg)	3009	3101	3249
Crude protein	22.0	21.0	18.0
Lysine	1.35	1.18	1.06
Methionine	0.58	0.46	0.43
Ca	0.95	0.78	0.67
Total P	0.64	0.62	0.53
Available P	0.45	0.44	0.37

Table 1. *Cont.*

Items	Starter	Growing	Finishing
Analyzed composition (%)			
Dry matter	90.2	90.4	90.6
GE, kcal/kg	4081	4332	4395
Crude protein	22.5	21.3	18.5
Ether extract	4.8	6.0	7.9
Crude fiber	3.7	4.7	3.8

(-) Ingredient not incorporated. (*) Provided per kg of feed: Vitamin A (retinyl acetate) 10.000 IU; vitamin D3 (cholecalciferol) 4.800 IU; vitamin B1 (Thiamine) 3 mg; vitamin B2 (riboflavin) 9 mg; vitamin B3 (Nicotinamide) 51 mg; vitamin B6 (pyridoxine hydrochloride) 4.5 mg; vitamin B9 (folic acid) 1.8; vitamin B12 (cyanocobalamin) 0.04 mg; vitamin E (DL-α-Tocopheryl acetate): 45 mg; vitamin K3 (Menadione) 3 mg; pantothenic acid (calcium D-pantothenate) 16.5 mg, biotin (D-(+)-biotin) 0.15 mg; Chloride of choline 350 mg; iron (FeSO4) 54 mg; iodine (Ca(IO3)2) 1.2 mg; zinc (ZnO) 66 mg; manganese (MnO) 90 mg; copper (CuSO4) 12 mg; selenium (Na2SeO3) 0.2 mg; 6-Phytase EC 3.1.3.26: 1500 FYT; Butylated hydroxytoluene (BHT) 25 mg; Colloidal silica 45 mg, Sepiolite 1007 mg.

2.5. Necrotic Enteritis Challenge Procedure

The floor area of 1.125 m^2 (1.50 × 0.75 m) and 0.96 m^2 (1.20 × 0.80 m) per pen for the first and the second trial, respectively, was covered with 10% clean wood shavings and 90% recycled litter material. The recycled litter material was selected from a commercial poultry flock where it was claimed there was clinical NE, previously characterized for its content of mesophilic aerobic bacteria (>10^5 CFU/g), Enterobacteriaceae (4.2 × 10^4 CFU/g), filamentous fungi, yeasts, sulphite-reducing anaerobes (1.2 × 10^4 CFU/g), and *Clostridium perfringens* (>10^5 CFU/g). The challenging method consisting of exposing broilers to a contaminated litter characterized by high *Clostridium perfringens* counts, was previously used [22]. Moreover, wheat was included in the starter (15%), growing (20%), and finishing (25%) diets without xylanases, with the aim of increasing digesta viscosity and accentuating the dietary challenge.

2.6. Sampling Procedure and Analyses

2.6.1. Feed

Diet proximate analyses were performed following AOAC methodology [23]: Dry matter (Method 934.01), crude protein (Method 968.06), crude fat (Method 2003.05), and crude fiber (Method 962.09). Gross energy was determined by an adiabatic calorimeter (IKA C-4000, Janke-Kunkel; Staufen, Germany).

2.6.2. Bacteria Counts

For the second trial, 3 pooled fecal samples per treatment were collected from the negative control, the positive control, the lowest and the highest dose for each product for monitoring the evolution of Enterobacteriaceae, lactic bacteria, and *Clostridium perfringens* load using a quantitative count at d 14, 28, and 42.

From the faeces samples, a bank of decimal dilutions was prepared, in sterile Ringer's solution, in order to proceed to the count and detection of: total Enterobacteriaceae, total lactic bacteria, and total *Clostrididum perfringens.*

The culture media used were: MacConkey agar in the case of Enterobacteriaceae, MRS agar for the count and detection of lactic bacteria, and TSN agar for *Clostridium perfringens* count. The incubation conditions were adequate for each determination, following the traditional methodologies in microbiology [24]. All trials were performed in triplicate.

2.6.3. Acute Phase Proteins

At the end of each experiment, the bird with the closest BW to the mean of the pen was selected and blood samples were aseptically collected from the wing vein into vacutainers. Blood samples were centrifuged at 4000× *g* for 15 min to obtain the serum that was immediately stored at −20 °C for further analysis of acute phase proteins.

The concentration of serum amyloid A (SAA) and alpha-1-acid glycoprotein (AGP) were determined using a solid phase sandwich ELISA (de Life Diagnostics, Knyperseley, UK) following the manufacturer's recommendations.

Afterwards, the bird was killed by intracardiac administration of sodium pentobarbital (30 mg/kg BW), and jugular exsanguination for tissue sampling. The gastrointestinal tract was immediately dissected and content from ileum and ceca were collected for microbiome sequencing. Ileal tissue was collected in both trials to perform the histomorphological analysis.

2.6.4. Histomorphological Analysis

At the midpoint between Meckel's diverticulum and the ileo-cecal junction, ileal samples of about 5 cm were collected. For the first trial, tissue sections (5 µm) were fixed in 4% paraformaldehyde and then embedded in paraffin. Afterwards, the sections were prepared, and stained with hematoxylin-eosin. For the second experiment, the preparations were deparaffinized and hydrated before being subjected to PAS (Periodic acid-Schiff) staining with Schiff's reagent for morphometric analyses and goblet cells count. For both trials, samples were analyzed using a light microscope. The morphometric variables measured included villus height, crypt depth, villus height to relative crypt depth ratio (V:C), and number of goblet cells and lymphocytes (only for the first trial). Ten villi were measured for each sample and only complete and vertically oriented villi were evaluated. The mean from 10 villi per sample was used as the mean value for further analysis.

2.6.5. Preparation of the 16S rRNA Gene Amplicon Library for MiSeq Sequencing

The composition and structure of the sampled microbial communities was assessed through amplifying and sequencing the V3-V4 variable regions of the 16S rRNA gene. The Illumina Miseq sequencing 300 × 2 approach was used.

Library Preparation and Sequencing

Ileal and ceca contents (250 mg) for the first and the second trial, respectively, were collected from one bird per replicate for DNA isolation using the commercial MagMAX CORE Nucleic Acid Purification Kit 500RXN (Thermo Fisher, Barcelona, Spain), following the manufacturer's instructions. Mock community DNA was included as control (Zymobiomics Microbial Community DNA).

Samples were amplified using primers specific to the V3-V4 regions of the 16S rRNA DNA (V3-V4-Forward5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3', V3-V4-Reverse 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC -3') [25]. The library preparation was carried out in Microomics Systems S.L. (Barcelona, Spain).

Amplicon Sequences Processing and Analysis

Raw demultiplexed forward and reverse reads were processed using the following methods and pipelines as implemented in QIIME2 version 2019.4 with default parameters unless stated [26]. DADA2 was used for quality filtering, denoising, pair- end merging, and amplicon sequence variant calling (ASV, i.e., phylotypes) using *qiime dada2 denoise-paired* method [27]. Q20 was used as quality threshold to define read sizes for trimming before merging (parameters: –p-trunc-len-f and –p-trunc-len-r). Reads were truncated at the position when the 75th percentile Phred score felt below Q20 for for both forward and reverse reads. After quality filtering steps, average sample size of reads was determined and phylotypes were detected. ASVs were aligned using the *qiime alignment mafft method* [28]. The alignment was used to create a tree and to calculate phylogenetic relations between ASVs using qiime phylogeny fasttree method [29]. ASV tables were subsampled without replacement in order to even sample sizes for diversity analysis using *qiime diversity core-metrics-phylogenetic* pipeline. The sample with the smallest sample size was discarded in order to take advantage of the sequencing depth of the dataset. Subsequently, subsampling to the next lowest sample size was used for each comparison. Unweighted and weighted Unifrac distances were calculated to compare community structure [30]. Taxonomic

assignment of ASVs was performed using a Bayesian Classifier trained with Silva V4 database (i.e., 99% OTUs database) using the *qiime feature-classifier classify-sklearn* method [31]. Unifrac distance matrices and ASV tables were used to calculate principal coordinates and construct ordination plots using R software package version 3.6.0

2.7. Statistical Analysis

For the first trial, statistical analyses were carried out on BW, ADG, ADFI, FCR, and histomorphological analysis with ANOVA using the GLM procedure of SAS software (SAS 9.4 Institute Inc., Cary, NC, USA). Normal distribution and homocedasticity of variances was checked previous to the analysis by using the Shapiro-wilk test and Levene's test from UNIVARIATE and GLM procedures, respectively. All performance and histomorphological data were analyzed according to a randomized complete block design, considering treatment groups as the source of variation, and the number of pens (individual broiler chickens for the histomorphology) as the experimental unit. Means were compared using the multiple comparisons Tukey test, and deemed significant at $p \leq 0.05$. A p-value situated between 0.05 and 0.10 was considered a trend towards significance.

For the second trial, the linear and quadratic contrasts were used to compare effects of increasing dietary supplementation of each tested blend. A further analysis of the following orthogonal contrasts was performed: Contrast C1, comparing the NC and the PC, contrast C2: Comparing the PC and the dose of 2 g/kg OA3 + MCFA + AC, and contrast C3: Comparing the PC and the dose of 0.5 g/kg OA2 + AC.

Biostatistical analysis for microbiota was performed in open source software RStudio v.3.5.1. Diversity was analysed at OTU level using vegan package [32]. Richness and alpha diversity were calculated with raw counts based on Simpson, Shannon, and Inverse-Simpson estimators. Beta diversity was evaluated by multivariate ANOVA. Finally, differential abundance analysis was performed with taxa relative abundances under a zero-inflated log normal mixture model and p-values were corrected by false-discovery rate (FDR) with a metagenomeseq package [33].

3. Results

3.1. First Trial

3.1.1. Growth Performance

Mortality was 0.36% and was not related to any dietary treatment. Table 2 shows the growth performance of the birds that was lower than Ross 308 standards, and confirmed that the experimental challenge impaired the growth of the animals. Birds supplemented with blends containing malic and fumaric acid (OA1), calcium butyrate and fumaric acid (OA2), or capric-caprylic acid, caproic acid, lauric acid, calcium butyrate, fumaric acid, and citric acid (MCFA + OA3) showed higher BW at d 41 and higher ADG041 ($p < 0.001$) than those fed the negative control. Chickens supplemented with the blend OA2 + AC showed the highest overall ADFI ($p = 0.02$). All tested blends improved the FCR041 ($p < 0.001$).

Table 2. Growth performance of chickens fed with experimental diets from d 1 to 41 (Trial 1).

Items	Experimental Treatments					SEM	*p*-Value
	NC	OA1 + AC	OA2 + AC	MCFA + AC	MCFA + OA3 + AC		
BW (g)							
d0	42.8	42.7	42.8	42.7	42.9	0.05	0.4008
d10	228.4 ^{c}	240.5 ^{a,b}	229.3 ^{b,c}	242.6 ^{a}	237.9 ^{a,b,c}	2.84	0.0026
d28	1020 ^{y}	1093 ^{x}	1101 ^{x}	1100 ^{x}	1141 ^{x}	28.8	0.0803
d41	1942 ^{b}	2188 ^{a}	2285 ^{a}	2085 ^{a,b}	2203 ^{a}	51.3	0.0005
ADG (g/d)							
d0–10	18.6 ^{c}	19.8 ^{a,b}	18.6 ^{b,c}	20.0 ^{a}	19.5 ^{a,b,c}	0.28	0.0022
d11–28	44.0	47.4	48.4	47.6	50.1	1.70	0.1116
d29–41	70.9	84.3 ^{a,b}	91.0 ^{a}	75.8 ^{b,c}	81.7 ^{a,b}	2.74	<0.0001
d0–41	46.3 ^{b}	52.3 ^{a}	54.6 ^{a}	49.8 ^{a,b}	52.7 ^{a}	1.268	<0.0001

Table 2. *Cont.*

Items	Experimental Treatments					SEM	*p*-Value
	NC	OA1 + AC	OA2 + AC	MCFA + AC	MCFA + OA3 + AC		
ADFI (g/d)							
d0–10	32.0 ^{x}	31.1 ^{x,y}	31.1 ^{x,y}	30.5 ^{y}	32.0 ^{x,y}	0.41	0.0721
d11–28	77.5	82.8	84.0	80.5	82.9	1.78	0.1248
d29–41	162.3 ^{a,b}	167.2 ^{a,b}	173.9 ^{a}	158.4 ^{b,c}	170.1 ^{a,b}	3.57	<0.0001
d0–41	88.5 ^{b}	96.9 ^{a,b}	99.6 ^{a}	93.0 ^{a,b}	98.1^{a,b}	1.93	0.0248
FCR							
d0–10	1.72 ^{a}	1.57 ^{a,b}	1.67 ^{a,b}	1.53 ^{b}	1.64 ^{a,b}	0.032	0.0051
d11–28	1.82	1.77	1.76	1.69	1.70	0.035	0.1203
d29–41	2.29 ^{a}	1.98 ^{b}	1.91 ^{b}	2.01 ^{a,b}	2.08 ^{a,b}	0.042	0.0007
d0–41	1.87 ^{a}	1.78 ^{b}	1.78 ^{b}	1.75 ^{b}	1.79 ^{b}	0.034	<0.0001

^{a,b,c} Values with different letters within a row indicate a significant difference at $p \leq 0.05$. ^{x,y} Values with different letters within a row indicate a trend toward statistical significance at $p \leq 0.1$. NC: negative control; OA1: malic acid + fumaric acid; OA2: calcium butyrate + fumaric acid; OA3: calcium butyrate + fumaric acid + citric acid + MCFA; MCFA: capric-caprylic acid + caproic acid + lauric acid; AC: cinnamaldehyde, carvacrol and thymol as major compounds.

3.1.2. Histomorphological Analysis

As shown in Table 3, the villus length to crypt depth V:C ratios were significantly higher ($p < 0.001$) in those birds on the OA1 + AC, OA2 + AC, and MCFA + OA3 + AC treatments compared to those birds of the negative control. All tested blends reduced crypt depth ($p < 0.001$). However, no treatment effect was observed on goblet cell and intraepithelial lymphocyte counts.

Table 3. Effect of experimental treatments on histomorphology of ileum of broiler chicken at d 41 (Trial 1).

Items	Experimental Treatments					Statistics	
	NC	OA1 + AC	OA2 + AC	MCFA + AC	MCFA + OA3 + AC	SEM	*p*-Value
Villus height (μm)	828.5 ^{c}	1044.0 ^{a,b}	1088.4 ^{a}	925.1 ^{b,c}	1054.6 ^{a,b}	35.90	<0.0001
Crypt depth (μm)	219.6 ^{a}	174.5 ^{b}	179.2 ^{b}	191.5 ^{b}	182.5 ^{b}	4.90	<0.0001
VH:CD ratio	3.79 ^{c}	6.04 ^{a}	6.10^{a}	4.89 ^{b}	5.82 ^{a}	0.22	<0.0001
Goblet cells density/ 100 μm of villus height	15.8	13.1	11.4	13.5	13.9	1.07	0.6134
Intraepithelial lymphocyte density/ 100 μm of villus height	7.9	6.6	6.0	7.1	6.8	0.37	0.6424

^{a,b,c} Values with different letters within a row indicate a significant difference at $p \leq 0.05$. NC: Negative control; OA1: Malic acid + fumaric acid; OA2: calcium butyrate + fumaric acid; OA3: Calcium butyrate + fumaric acid + citric acid + MCFA; MCFA: Capric-caprylic acid + caproic acid + lauric acid; AC: Cinnamaldehyde, carvacrol, and thymol as major compounds.

3.1.3. Acute Phase Proteins

No treatment effect was observed on the serum concentration of alpha-1-acid glycoprotein ($p = 0.97$) or on the serum concentration of serum amyloid A ($p = 0.56$), with a mean of 0.348 mg/mL and 119.8 ng/mL, respectively (data not shown).

3.1.4. Ileal Microbiota Analysis

Alpha and Beta Diversity

Alpha diversity indices showed a similar pattern distribution for all experimental treatments (within sample variability; Figure 1). None of the alpha diversity indices were statistically different among diets. The β-diversity was not affected by the experimental treatments (between sample variability; $p = 0.50$).

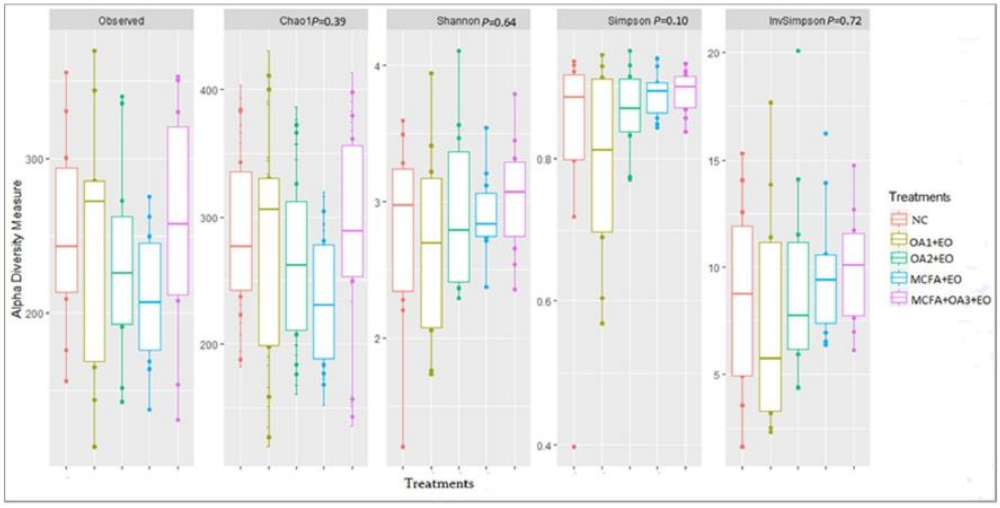
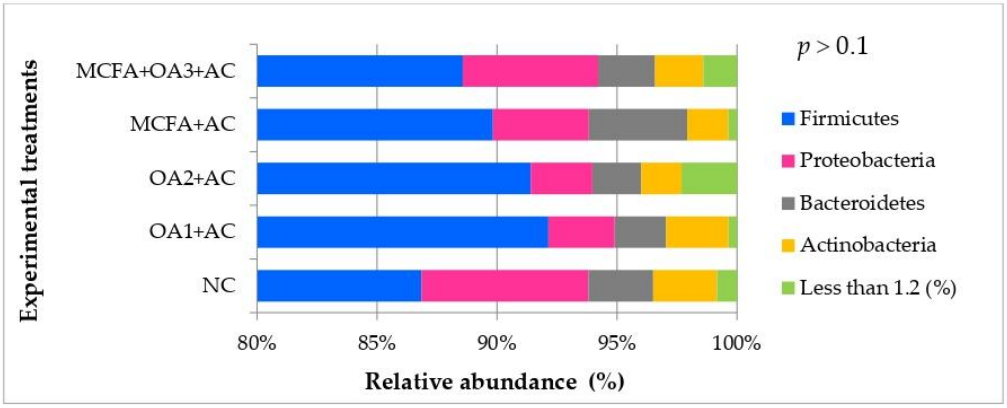


Figure 1. Alpha diversity indices of the ileal microbiota of broiler chickens at d 41 of age. (NC: Negative control; OA1: Malic acid + fumaric acid; OA2: Calcium butyrate + fumaric acid; OA3: Calcium butyrate + fumaric acid + citric acid + MCFA; MCFA: Capric-caprylic acid + caproic acid + lauric acid; AC: Cinnamaldehyde, carvacrol, and thymol as major compounds).

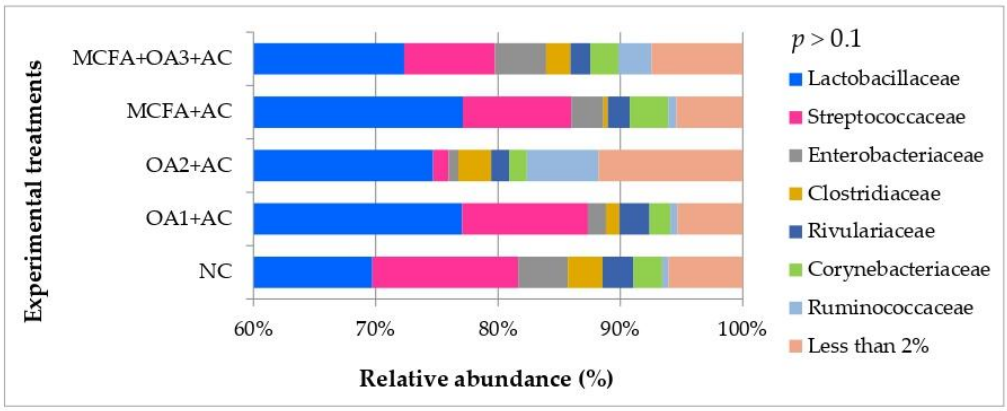
Composition of the Ileal Microbiota

No treatment effect was observed on ileal microbiota composition. The relative abundance of the main phyla, families and genera in the ileal microbiota of the birds is shown in Figure 2. Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria were the most abundant phyla with no effect due to dietary treatments. The most abundant families were Lactobacillaceae, Streptococcaceae, and Enetrobacteriaceae. *Lactobacillus* and *Streptococcus* followed by *Escherichia* and *Clostridium* were the most frequent genera.

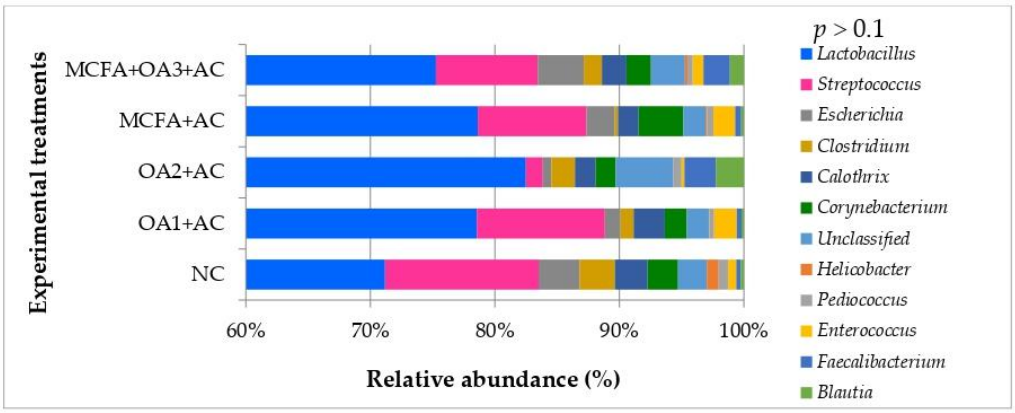
Nevertheless, although overall patterns were similar, a deeper examination of the individual metagenomics profiles was performed by means of log2 changes. Results showed some changes over 1-2 log2 individual taxa, when comparing all treatments containing microencapsulated blends of organic acids and AC with the negative control (Figure 3). Some families that contain relevant pathogenic taxa, such as Enterobacteriaceae, Helicobacteriaceae, Rickettsiaceae, and Clostridiaceae, decreased compared to the control treatment. Likewise, the abundance of some families shown to be beneficial for the host, such as Bifidobacteriaceae, Ruminococcaceae, and Lachnospiraceae, was enhanced in groups supplemented with the microencapsulated blends.



(**a**)



(**b**)



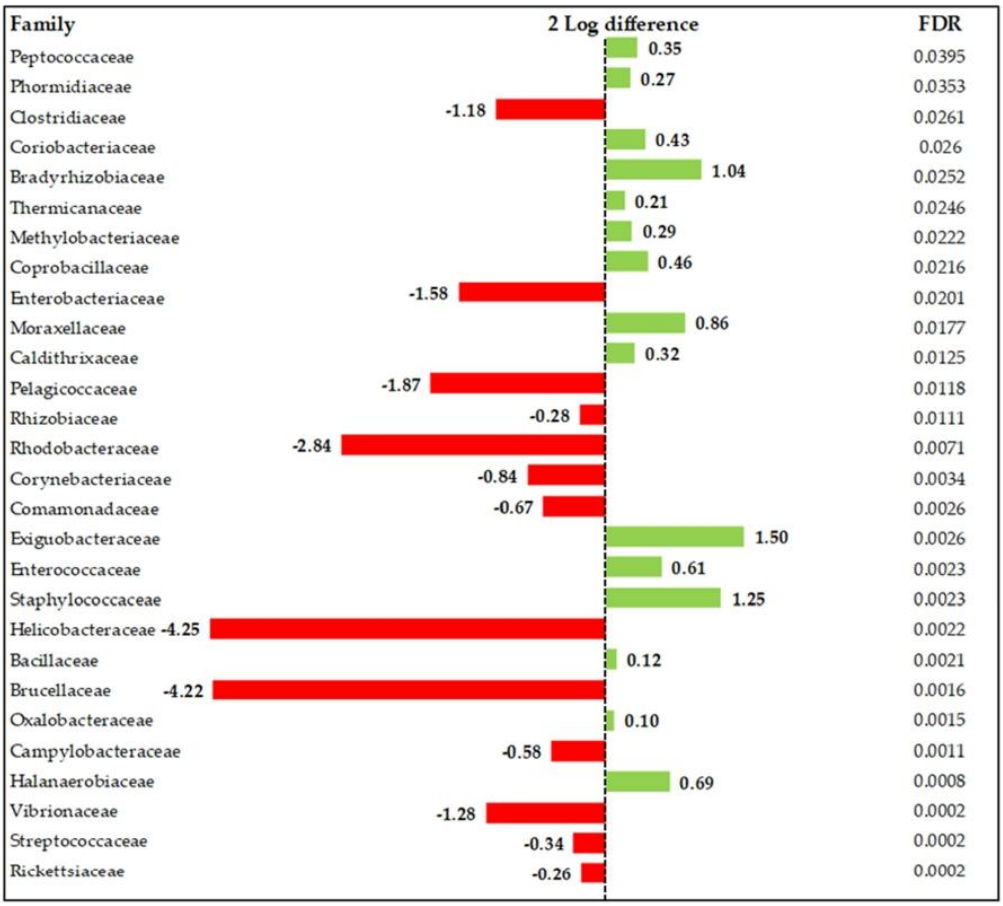
(**c**)

Figure 2. Relative abundance (%) of the main phyla (**a**), families (**b**), and genera (**c**) present in the ileal microbiota of broiler chickens at d 41 of age (Trial 1). NC: Negative control; OA1: Malic acid + fumaric acid; OA2: Calcium butyrate + fumaric acid; OA3: Calcium butyrate + fumaric acid + citric acid + MCFA; MCFA: Capric-caprylic acid + caproic acid + lauric acid; AC: Cinnamaldehyde, carvacrol, and thymol as major compounds).

3.2. Second Trial

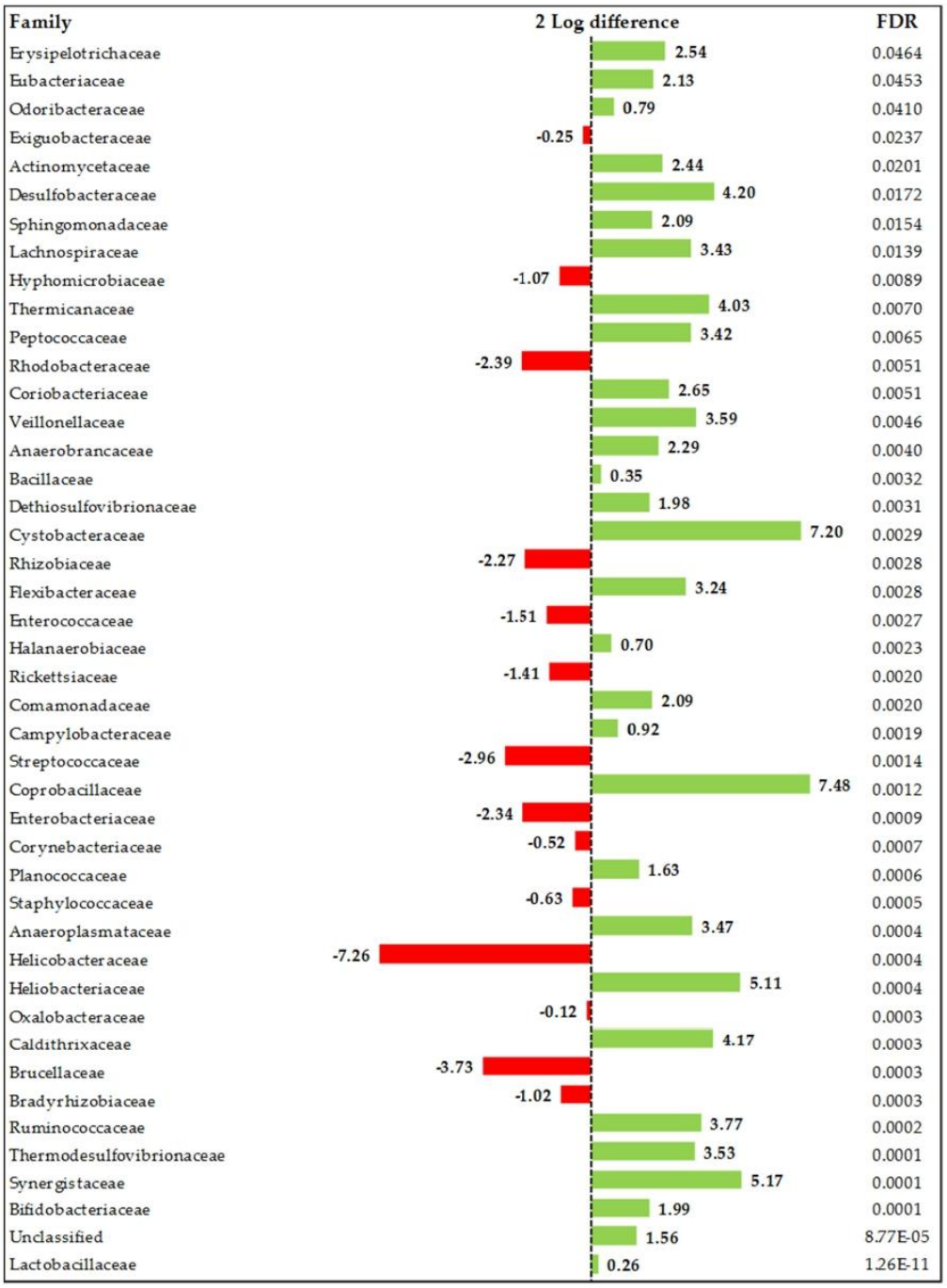
3.2.1. Growth Performance

The growth performance results are shown in Table 4. A linear-quadratic dose response analysis showed, for the overall parameters, a quadratic effect of OA2 + AC inclusion on BW, ADG, and ADFI ($p < 0.001$). These findings suggest that an improvement of growth performance could be observed up to a dose of 2 g/kg. However, higher doses may be associated with reduced growth performance. As for OA3 + MCFA + AC, a quadratic effect was observed only for BW42 ($p = 0.02$), while a linear response was observed for ADG042 ($p = 0.004$), ADFI042 ($p = 0.05$), and FCR042 ($p = 0.01$). Contrast C1 showed that chickens fed the PC had a higher BW42 ($p = 0.01$) as a result of better ADG042 ($p = 0.03$). Contrast C2 showed that supplementing 2 g/kg of OA3 + MCFA + AC improved BW10 ($p = 0.03$) and tended to improve FCR1028 ($p = 0.08$). The overall growth parameters showed no difference between PC and 2 g/kg of OA3 + MCFA + AC supplementation. Contrast C3 showed that the lowest dose (0.5 g/kg) of OA2 + AC improved the ADG042 ($p = 0.05$). No significant difference was observed for BW42 ($p = 0.28$), ADFI042 ($p = 0.13$), and FCR042 ($p = 0.28$).



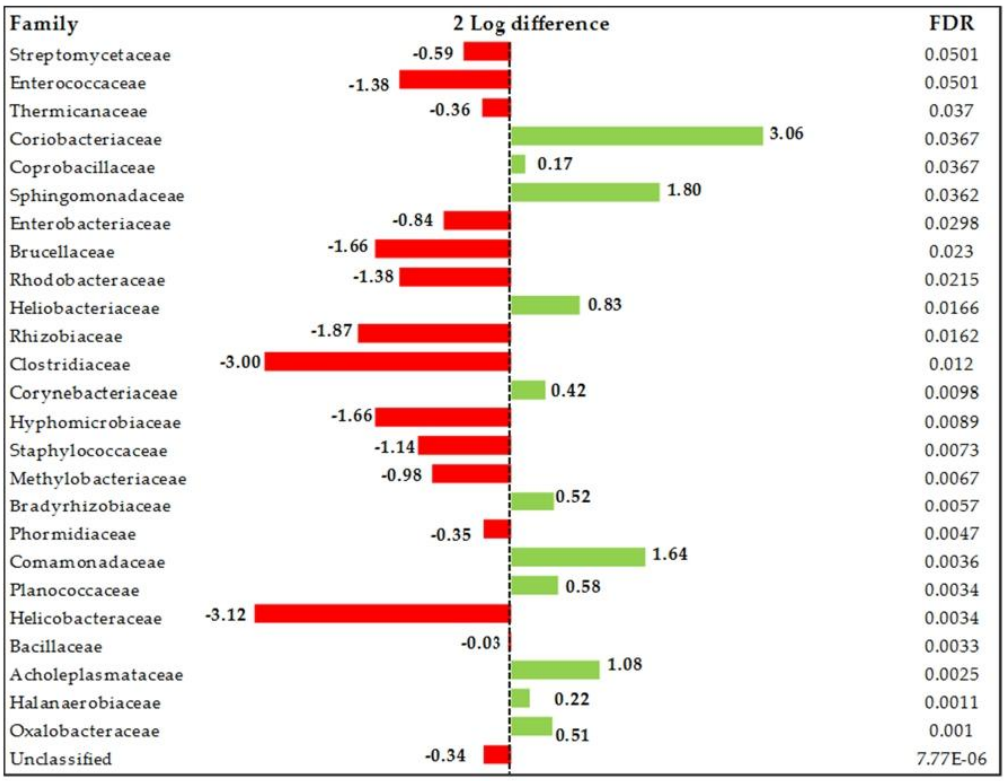
(**a**)

Figure 3. *Cont.*



(**b**)

Figure 3. *Cont.*



(**c**)

Figure 3. *Cont.*

(**d**)

Figure 3. Differentially abundant taxa (family) from the ileum (in change and FDR-adjusted, $p \leq 0.05$) on d 42 between OA1 + AC vs. NC (**a**); OA2 + AC vs. NC (**b**); MCFA + AC vs. NC (**c**); MCFA + OA3 + AC vs. NC (**d**). NC: Negative control; OA1: Malic acid + fumaric acid; OA2: Calcium butyrate + fumaric acid; OA3: Calcium butyrate + fumaric acid + citric acid + MCFA; MCFA: Capric-caprylic acid + caproic acid + lauric acid; AC: Cinnamaldehyde, carvacrol, and thymol as major compounds).

Culling and mortality rates are shown in Figure 4. Mortality was higher ($p < 0.001$), and culling rate tended to be higher ($p = 0.10$) with the highest dose of OA2+AC (4g/kg).

Table 4. Effect of dietary treatments on productive performance of chickens during the different phases and the entire experimental period (Trial 2).

Items	Experimental Treatments									SEM	*p*-Value			
	NC	OA2 + AC (A)			OA3 + MCFA + AC (B)						Linear		Quadratic	
		0.5 g/kg	1 g/kg	2 g/kg	4 g/kg	1 g/kg	2 g/kg	4 g/kg	8 g/kg		A	B	A	B
BW (g)														
d0	39.3	39.4	39.4	39.4	39.4	39.4	39.4	39.4	39.4	0.04	0.85	0.85	0.22	0.4
d10	228.5	246.3	234.6	234.3	227.2	240.5	249	243.2	239.8	5.01	0.15	0.42	0.23	0.03
d28	1132	1283	1255	1241	1203	1194	1296	1263	1247	23.1	0.78	0.01	0.001	0.001
d42	2369	2598	2561	2573	2327	2469	2549	2549	2527	42.8	0.007	0.06	<0.0001	0.02
ADG (g/d)														
d0–10	18.9	20.7	19.5	19.5	18.8	20.1	21.0	20.4	20.0	0.46	0.13	0.34	0.41	0.03
d0–42	55.5	60.9	60.0	60.3	54.5	57.8	59.8	59.8	59.2	1.07	0.006	0.004	<0.0001	0.11
ADFI (g/d)														
d0–10	26.8	27.5	26.2	27.8	26.9	26.7	28.9	27.9	27.6	0.78	0.88	0.56	0.66	0.17
d0–42	95.1	102.6	101.4	100.6	95.2	98.3	102.1	99.9	101.1	1.46	0.08	0.05	0.0002	0.1
FCR														
d0–10	1.42	1.33	1.34	1.43	1.43	1.33	1.38	1.37	1.38	0.042	0.1	0.29	0.47	0.73
d0–42	1.71	1.68	1.69	1.67	1.75	1.70	1.71	1.68	1.71	0.018	0.13	0.01	0.42	0.96

NC: Negative control; OA2: Calcium butyrate + fumaric acid; OA3: Calcium butyrate + fumaric acid + citric acid + MCFA; MCFA: Capric-caprylic acid + caproic acid + lauric acid; AC: Cinnamaldehyde, carvacrol, and thymol as major compounds.

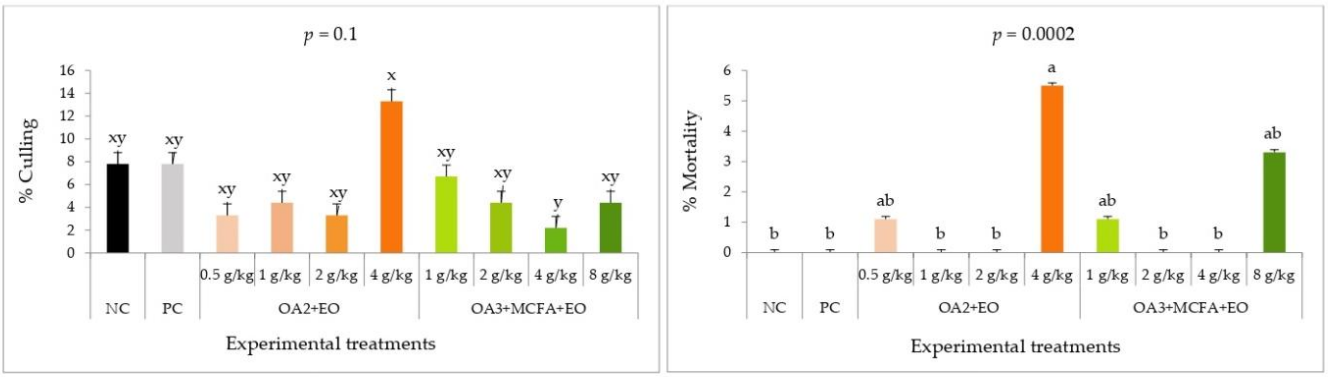


Figure 4. Culling (**a**) and mortality (**b**) calculated as a percentage from the total of chickens per treatment (90 chickens). ^{a,b} Means with different superscripts indicate significant differences ($p \leq 0.05$). ^{x,y} Means with different superscripts indicate a tendency toward significance ($p \leq 0.1$). NC: Negative control; PC: positive control; OA2: Calcium butyrate + fumaric acid; OA3: Calcium butyrate + fumaric acid+ citric acid + MCFA; MCFA: Capric-caprylic acid + caproic acid+lauric acid; AC: Cinnamaldehyde, carvacrol, and thymol as major compounds.

3.2.2. Histomorphological Analysis

Results of the histomorphologiacal analysis of the ileum are shown in Table 5. A linear-quadratic dose response analysis showed a quadratic effect of OA2 + AC supplementation on CD and the ratio VH: CD ($p < 0.001$), suggesting an improvement of these two parameters up to a dose of 2 g/kg. However, a worsening could be observed when higher doses (4 g/kg) are used.

3.2.3. Bacteria Counts

The bacteria count results are shown in Figure 5. Baseline values were determined prior to the distribution of animals by collecting eight samples of feces from the transportation cages. With regards to Enterobacteriaceae, differences between dietary treatments were observed from d 28 of the experiment, where all doses of both blends showed reduced counts compared to the negative control (NC). On d 42, this effect remained only for the dose of 0.5 g/kg of OA2 + AC. A dietary treatment effect on *Clostridium perfringens* counts was observed on d 14, when the dose of 4 g/kg of OA2 + AC showed higher values compared to PC. This had been maintained on d 42. A similar effect was observed with the blend of OA3 + MCFA + AC where the dose of 8g/kg showed high count values on d 42. An effect of day-post-infection was observed for the doses of 1 g/kg of OA3 + MCFA + AC and 0.5 g/kg of OA2 + AC, where a significant decrease of *C. perfringens* count was observed at the end of the experiment. The supplementation of 0.5 g/kg of OA2 + AC resulted in count values similar to the PC.

3.2.4. Cecal Microbiota Analysis

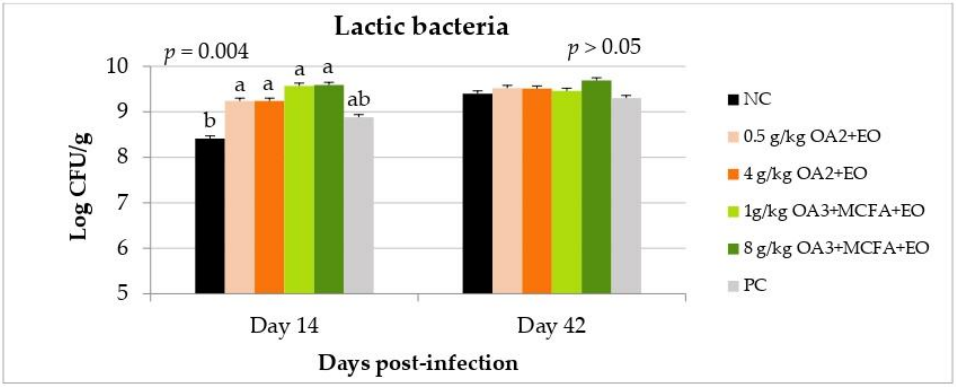
Alpha and Beta Diversity

The diversity of the cecal microbiota among dietary treatments was assessed using the α-diversity and β-diversity measurements. The challenge did not affect the α-diversity, as no difference was observed between the negative control and the positive one. However, the species richness of samples collected from chickens supplemented with both microencapsulated products was significantly higher than NC (Table 6).

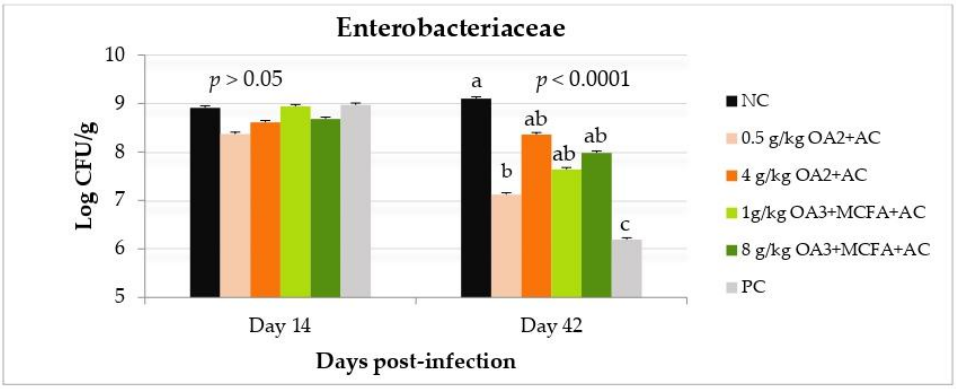
Table 5. Effect of dietary treatments on histology of the ileum at the end of the experiment (Trial 2).

Items	Experimental Treatments									SEM	*p*-Value			
	NC	OA2 + AC (A)				OA3 + MCFA + AC (B)					Linear		Quadratic	
		0.5 g/kg	1 g/kg	2 g/kg	4 g/kg	1 g/kg	2 g/kg	4 g/kg	8 g/kg		A	B	A	B
Villus height, VH (μm)	803.7	872.1	895.0	873.7	773.5	909.9	847.4	843.6	850.8	36.15	0.20	0.92	0.29	0.56
Crypt depth, CD(μm)	160.9	149.5	148.8	146.0	181.9	148.1	147.1	158.2	152.2	6.28	0.002	0.85	<0.0001	0.69
Ratio VH:CD	5.0	5.8	6.1	6.0	4.3	6.2	5.8	5.4	5.7	0.27	0.002	0.81	<0.0001	0.46
Goblet cells Density/100 μm of villus height	21.7	22.2	21.1	20.8	29.1	19.5	19.3	22.6	23.2	1.53	0.003	0.17	0.05	0.55

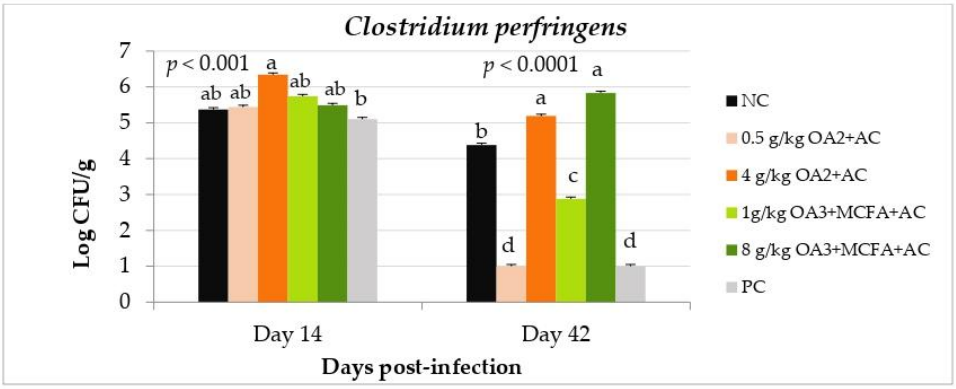
NC: Negative control; OA2: Calcium butyrate + fumaric acid; OA3: Calcium butyrate + fumaric acid + citric acid + MCFA; MCFA: Capric-caprylic acid + caproic acid + lauric acid; AC: Cinnamaldehyde, carvacrol, and thymol as major compounds.



(**a**)



(**b**)



(**c**)

Figure 5. Effect of treatments on lactic bacteria (**a**), Enteribacteriaceae (**b**), and *C.perfringens* (**c**) count (log10 CFU) in feces collected on d 14 and 42 of age. OA2: Calcium butyrate + fumaric acid; OA3: Calcium butyrate + fumaric acid + citric acid + MCFA; MCFA: Capric-caprylic acid + caproic acid + lauric acid; AC: Cinnamaldehyde, carvacrol, and thymol as major compounds; NC: Negative control; PC: Positive control. ^{a,b,c,d} Means with different superscripts for the same day indicate significant difference between treatments ($p \leq 0.05$).

Table 6. Effect of dietary treatments on alpha diversity.

Index	NC	PC	OA2 + AC	OA3 + MCFA + AC	SEM	*p*-Value
Shannon	0.91 [b]	0.93 [a,b]	0.94 [a]	0.95 [a]	0.006	0.009
Simpson	3.01 [b]	3.14 [a,b]	3.31 [a]	3.36 [a]	0.074	0.008
Invsimpson	12.43 [b]	15.60 [a,b]	19.26 [a]	19.82 [a]	1.502	0.004

[a,b] Values with different letters within a row indicate a significant difference at $p \leq 0.05$. NC: Negative control; OA2: Calcium butyrate + fumaric acid; OA3: Calcium butyrate + fumaric acid + citric acid + MCFA; MCFA: Capric-caprylic acid + caproic acid + lauric acid; AC: Cinnamaldehyde, carvacrol, and thymol as major compounds; PC: Positive control.

No treatment effect was observed on β-diversity (between sample variability; $p = 0.50$, Figure 6).

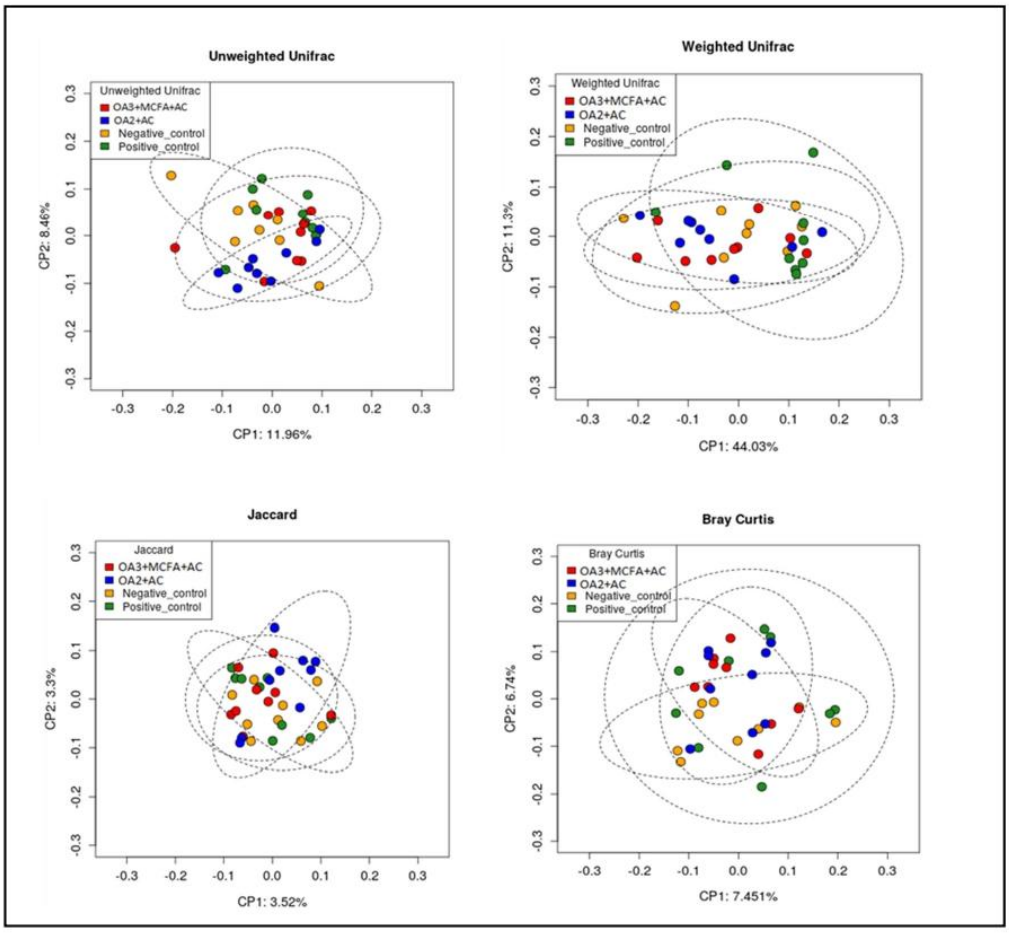
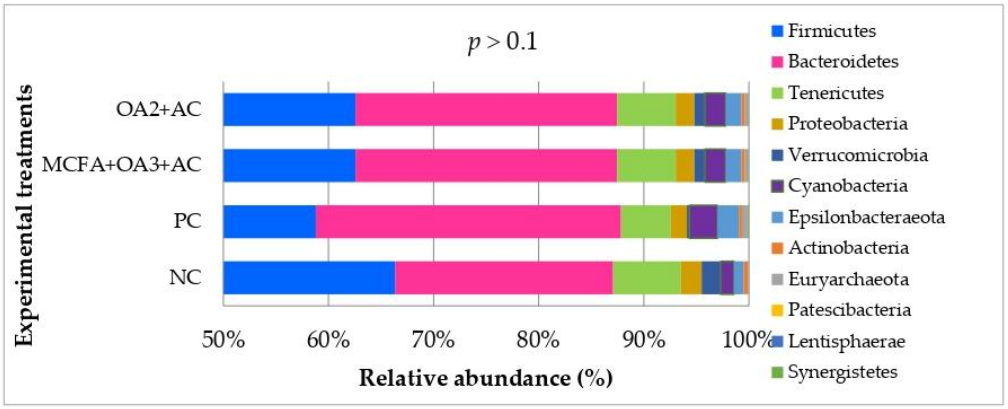


Figure 6. Effect of different dietary treatments on bacterial beta diversity on cecum of broilers on d 42 (Trial 2). (OA2: Calcium butyrate + fumaric acid; OA3: Calcium butyrate + fumaric acid + citric acid + MCFA; MCFA: Capric-caprylic acid + caproic acid + lauric acid; AC: Cinnamaldehyde, carvacrol, and thymol as major compounds).

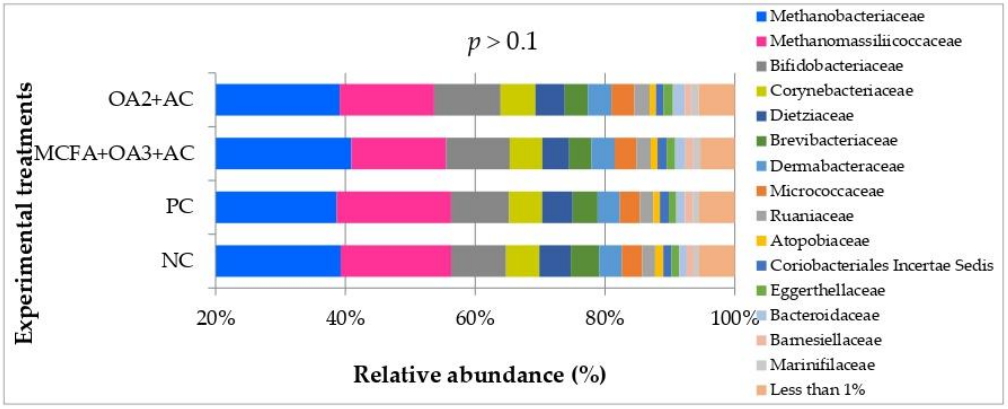
Composition of the Cecal Microbiota

No treatment effect was observed on the cecum microbiota composition. The relative abundance of the main phyla, families, and genera in the ileal microbiota of the birds is shown in Figure 7. Firmicutes, Bacteroidetes, and Tenericutes were the most abundant phyla, without any effect due to dietary

treatments. The most abundant families were Methanobacteriaceae, Methanomassiliicoccaceae, and Bifidobacteriaceae. *Ruminococcaceae UCG-014*, *Bacteroides* followed by *Barnesiella* and *Faecalibacterium* were the most frequent genera. Nevertheless, although overall patterns were similar, a deeper examination of the individual metagenomics profiles was performed by means of log2 changes (Figure 8). Results showed some changes over 1-2 log2 individual taxa when comparing OA3 + MCFA + AC treatment (2 g/kg) and OA2 + AC treatment (0.5 g/kg), to the negative control. Our results showed that the supplementation of OA3 + MCFA + AC increased Ruminococcaceae, Coriobacteriales *Incertae Sedis*, Eubacteriaceae, Lactobacillaceae, Bacillaceae, Corynebacteriaceae, and Peptostreptococcaceae, while reducing *Clostridium sp. CAG: 360*, Eggerthellaceae, and Enterobacteriaceae. OA2 + AC supplementation promoted Christensenellaceae, Ruminococcaceae, Peptostreptococcaceae, Vadin BE97, Clostridiaceae, Bifidobacteriaceae, and Coriobacteriales *Incerte Sedis*, while reducing *Clostridium sp. CAG: 360*, Clostridiales Family XIII, and Muribaculaceae.

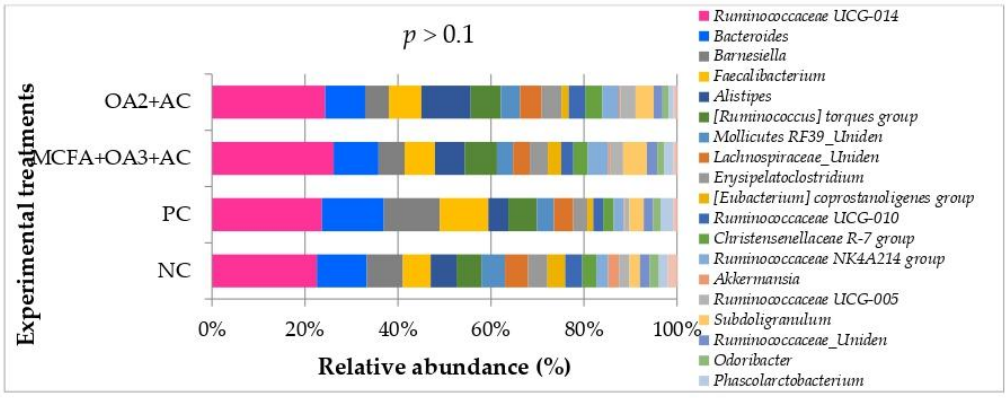


(**a**)



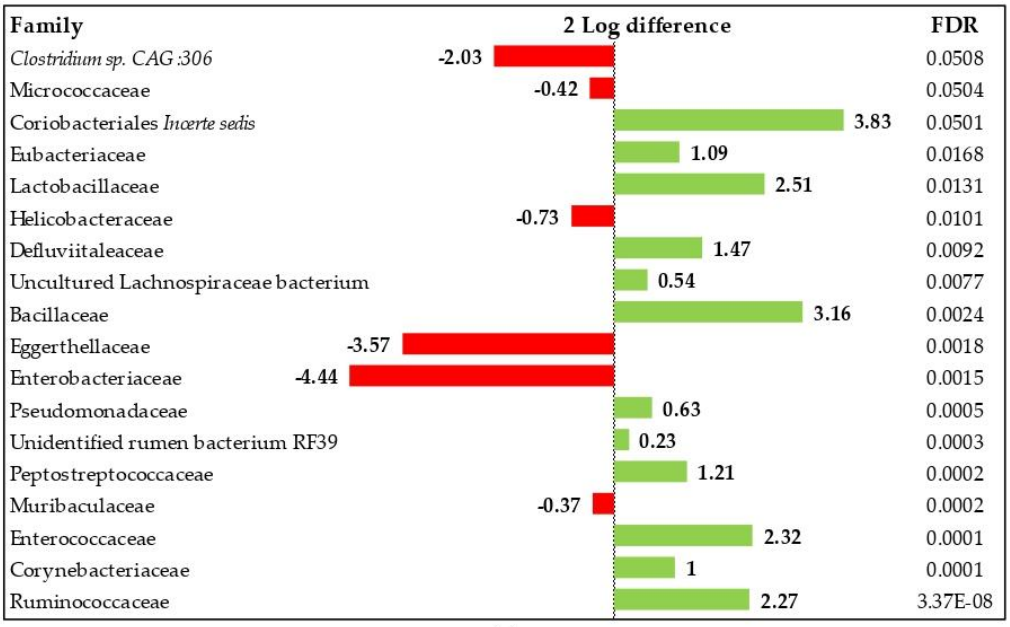
(**b**)

Figure 7. *Cont.*



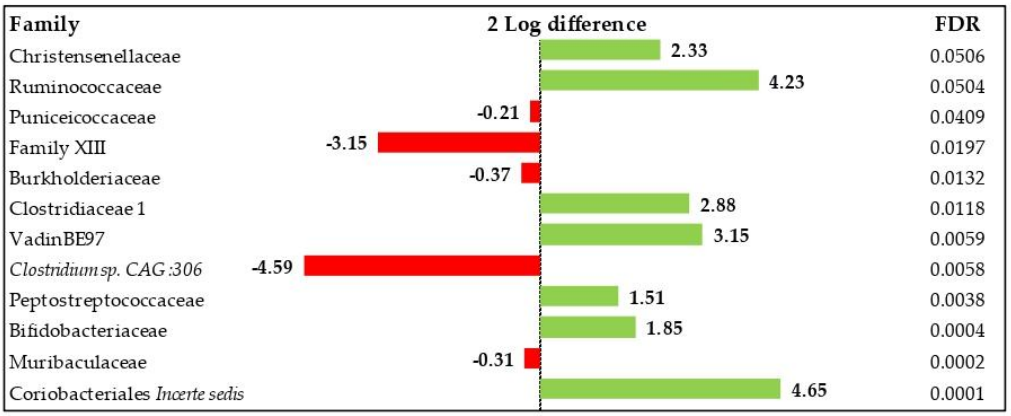
(**c**)

Figure 7. Relative abundance (%) of the main phyla (**a**), families (**b**), and genera (**c**) present in the cecum microbiota of broiler chickens at d42 of age (Trial 2). NC: Negative control; OA2: Calcium butyrate + fumaric acid; OA3: Calcium butyrate + fumaric acid + citric acid + MCFA; MCFA: Capric-caprylic acid + caproic acid + lauric acid; AC: Cinnamaldehyde, carvacrol, and thymol as major compounds; PC: Positive control.

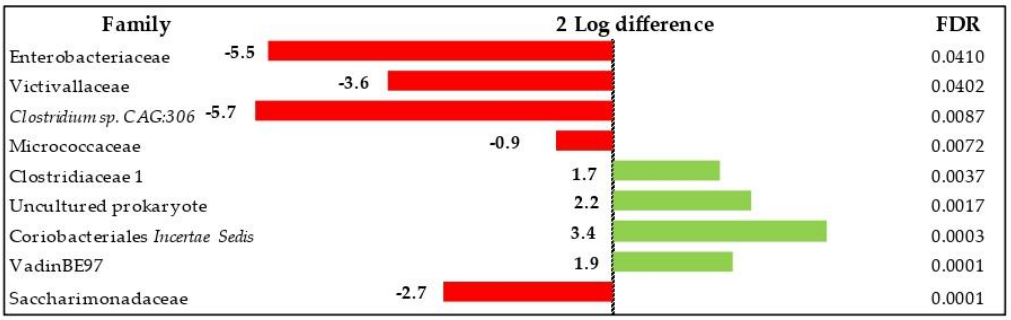


(**a**)

Figure 8. *Cont.*



(**b**)



(**c**)

Figure 8. Differentially abundant taxa (family) from the cecum (in change and FDR-adjusted, $p \leq 0.05$) on d 42 between OA3 + MCFA + AC vs. NC (**a**), OA2 + AC vs. NC (**b**), and NC vs. PC (**c**). OA2: Calcium butyrate + fumaric acid; OA3: Calcium butyrate + fumaric acid + citric acid + MCFA; MCFA: Capric-caprylic acid + caproic acid + lauric acid; AC: Cinnamaldehyde, carvacrol, and thymol as major compounds.

4. Discussion

4.1. The Relevance of Organic Acids and Essential Oils Combination on Growth Performance

Induced *Clostridium perfringens* challenge did not result in clinical signs of NE and higher mortality of chickens, but it increased the morbidity that was evidenced by reducing growth performance up to 21% compared to the standard Ross 308 values. These results confirmed that the use of 90% recycled commercial litter contaminated with *Clostridium perfringens* combined with wheat inclusion without xylanases, is a suitable model to induce subclinical NE without promoting mortality of animals. In this scenario, experimental blends improved the growth of chickens compared to the negative control, except for the blend containing only MCFA and aromatic compounds (MCFA + AC). The highest BW gains were observed for birds fed the blend containing calcium butyrate and fumaric acid (OA2 + AC), followed by the blend containing the same compounds combined with citric acid and MCFA (OA3 + MCFA + AC). These findings suggested that embedding the active substances using a continuous film of vegetable fats provides better resistance to the acidic pH, allowing a slower release of these substances further down in the intestine, which resulted in improving growth performance. The performance responses could be related to an antimicrobial activity of organic acids inhibiting harmful microbiota and favoring the proliferation of beneficial bacteria [34]. Results regarding ileal and cecal microbiota

showed a decrease of pathogenic taxa and an increase of some beneficial families in birds fed the microencapsulated blends compared to those receiving the negative control. These results were in line with those observed in feces where, for example, 0.5 g/kg of OA2 + AC significantly reduced *Clostridium perfringens* count on d 42 and Enterobacteriaceae from d 28. In fact, simple monocarboxylic acids such as formic, acetic, propionic, and butyric acids, or carboxylic acids bearing a hydroxyl group on the alpha carbon such as lactic, malic, and tartaric acids possess a strong antimicrobial activity [35]. Salts of some of these acids have been shown to enhance broilers performance, and short-chain carboxylic organic acids, such as sorbic and fumaric acids containing double bonds, also have antifungal activity [36]. The principal mode of action of organic acids is that non-dissociated forms can diffuse through lipophilic bacteria, mold membranes, disrupt the enzymatic reaction, and disorder transport systems of the bacteria [37]. Following the penetration of organic acids into bacterial cytoplasm, the non-ionized ones decompose to H (H+) ions and (A−) ions, resulting in a decline of the pH inside the bacteria. These changes are known to activate a specific mechanism (H^+ - ATPase pump) that aims to return the intracellular levels to normal pH. The process requires energy, which would result in reduced energy availability for cell proliferation leading to a reduced bacterial growth [38]. Other effects related to a low internal pH are inhibition of glycolysis, prevention of active transport, and interference with signal transduction [39].

The effects of organic acids on the growth performance of broiler chickens could be also related to their ability to enhance protein digestion, influence intestinal cell morphology, stimulate pancreatic secretions, act as a substrate for the intermediary metabolism, improve retention of many nutrients (e.g., chelating minerals), increase intestinal integrity, and affect electrolyte balance in the feed and intestine [39,40]. Several authors also reported a beneficial effect of essential oils on feed digestion through increasing bile salt secretion and stimulating the enzymatic activities of intestinal mucosa and pancreas [41]. These beneficial effects of both organic acids and essential oils may be further potentiated when these are combined [42,43] and protected to avoid the active material to be metabolized and absorbed in the proximal part of the digestive tract [44]. In the present study, organic acids combined with AC affected the histomorphology and integrity of small intestine as showed by the villus height and crypt depth of OA1, OA2, or MCFA + OA3 groups. All tested blends reduced the crypt depth of the ileum that resulted in improved VH: CD ratio, which is an indicator of the absorptive capacity of the small intestine [45]. Several authors [44,46,47] pointed out their promoting effect on the development of gastrointestinal mucosa and villus height. In fact, enteric infection may damage the epithelium and compromise villus height leading to increase crypt depth indicating greater enterocyte-cell flow, and more steady cell-renewal rate within the digestive tract usually resulted from the increased sloughing [48]. These constant renewal processes demand more energy and protein, leading to diverting nutrients away from productive purposes. Our findings are in line with several studies [49,50]. However, numerical improvement [51] or no growth performance effects have been reported by other authors [52]. Disparity among studies could depend on the specific used organic acids and AC, their combination, diet formulation, doses, or differences in the underlying microbial challenge.

4.2. High Doses of the Additives May Become Deleterious

Our results showed a dose-dependent effect on productive performance and intestinal integrity where the best results were obtained with a dose of 2 g/kg for the blend of calcium butyrate, fumaric acid, citric acid, MCFA, and AC (OA3 + MCFA + AC) and 0.5 g/kg of the blend containing calcium butyrate, fumaric acid combined with AC (OA2 + AC). Higher dietary levels were associated to compromised productive performance resulting from shorter villus height, deeper crypts, and thus, reduced VH:CD ratio. High doses were also associated with higher culling and mortality rates, and higher fecal counts of Clostridium *perfringens*. It could be argued that a high dietary inclusion of organic acids may cause a damage [53], allowing more nutrients to drain from the mucosa into the lumen, which could favor the proliferation of intestinal *Clostridium perfringens* and cause more lesions.

In the same sense, a study conducted by Timbermont et al. [54], showed that butyric acid (164.5 and 123 g/ton in starter and grower feed, respectively) combined with MCFA, mainly lauric acid (150 and 112.5 g/ton in starter and grower feed, respectively) and essential oils (thymol, cinnamaldehyde, and essential oil from eucalyptus; 90 and 67.5 g/ton in starter and grower feed, respectively) significantly reduced the number of birds with macroscopic lesions. However, the beneficial effect was lost when higher concentrations were used (330 and 250 g/ton of butyric acid combined with 360 and 270 g/ton of MCFA, and 240 and 180g/ton of essential oils for starter and grower feed, respectively). Several authors reported beneficial effect of low concentration of butyrate on promoting mucosal barrier function, while excessive butyrate disrupted it (100 mM and 8 mM of butyrate for Barcelo et al. [55]; Peng et al. [56], respectively).

4.3. The Relevance of Organic Acids and Essential Oils Combination on Ileum and Caeca Microbiota

The poultry gastrointestinal tract (GIT) is densely harbored by microorganisms, being in close and intensive interaction with the host and digesta particles. They are involved in the exchange of nutrients, and modulate the host gut morphology, physiology, and immunity [57]. The end-products of intestinal microbial fermentation are short-chain fatty acids (SCFAs), involved in the regulation of intestinal blood flow, intestinal immune responses, and mucin production as well as the stimulation of enterocyte growth and proliferation [58]. Among SCFAs, butyrate has gained a specific interest, being the main source of energy for epithelial cells and colonocytes. It also stimulates mucin synthesis and intestinal motility, cell proliferation and differentiation, while suppressing inflammatory diseases [59]. Thus, enhancing butyrate-producing bacteria would be of great interest for improving animal gut health and productivity. Unfortunately, we did not measure SCFAs in digesta and, consequently, we could not establish the correlations between these concentrations and relative abundance of bacterial taxa. However, as compared to the negative control, the tested microencapsulated blends increased the abundance of family members of Lachnospiraceae (*Coprococcus, Roseburia, Anaerostipes*) and Ruminococcaceae (*Faecalibacterium, Anaerotruncus*). These families, belonging to the Firmicutes phylum, express enzymes favoring the production of butyrate over propionate [60]. Supplementation of OA2 + AC also increased the abundance of Erysipelotrichaceae family, also known as Clostridium cluster XVI, that harbors different butyrate-producing bacteria [61,62]. It also increased the abundance of Bifidobacteriaceae that play an important role in pathogen exclusion and gut barrier maintenance due to their great production of SCFA through simple carbohydrates and oligosaccharides degradation [63]. Other studies reported that Actinobacteria, and mainly Bifidobacteria species, through inducing regulatory T-cells, can modulate the immune-inflammatory and autoimmune response [64,65]. Another family whose abundance was enhanced by OA2 + AC supplementation was Actinomycetaceae. Belonging to this family, *Streptomyces spp*, by producing Streptomycin, exerts a strong antimicrobial action against a number of gram-negative bacteria such as *Escherichia coli, Enterobacter, Salmonella*, and *Brucella* [66]. Moreover, ionophores, extensively used as anticoccidials, are fermentation products of Streptomyces and other fungi species. The abundance of Coriobacteriaceae that can produce high levels of SCFAs resulting in competitive exclusion of unfavorable microorganisms [67] was enhanced by the majority of tested blends. In addition, tested blends reduced Helicobacteraceae abundance by reducing *Helicobacter pullorum*, which has been also isolated from cecal epithelial cells [68] and shown to have a pathological outcome in the gut of chickens [69].

The first experiment of the current research showed that better performance responses were observed by the supplementation of OA2 + AC and OA3 + MCFA + AC through enhancing the abundance of beneficial families and reducing that of harmful ones in the ileum. A similar effect was observed in the cecum. The role of some families whose abundance changed in the cecum was previously discussed in the ileum microbiota.

Supplementation of OA2 + AC reduced Clostridiales Family XIII abundance that was previously found to be linked to broilers showing high FCR values [70] suggesting that their high abundance may result in compromised bird performance [71]. It also increased the abundance of Peptostreptococcaceae,

normal commensal bacteria that have been shown to be higher in gut microbiota of healthy animals compared to those experiencing dysbiosis of the intestinal microbiota. This indicates that this family is involved in the maintenance of the gut homeostasis and enhancement of the barrier function [72].

5. Conclusions

In summary, dietary supplementation of microencapsulated blends of either BUTYTEC-PLUS or ACITEC-MC alleviates the negative impact of NE infection through modulating the gut microbiome. The positive effects on gut microbiome may enhance the absorptive capacity of the intestine by increasing the VH:CD ratio, which leads to improved productive performance. Results from the second trial show that inclusion doses up to 2 g/kg of BUTYTEC-PLUS and 8 g/kg of ACTEC-MC result in similar feed utilization efficiency, survival, and growth performance as the non-challenged positive control. However, these effects are dose-dependent as high inclusion rates such as 4 g/kg of BUTUYTEC-PLUS appear to promote detrimental effects on chickens. Consecuently, dietary supplementations of 0.5 g/kg of BUTYTEC-PLUS and 2 g/kg of ACITEC-MC are recommended to improve broiler chickens performance under NE challenge.

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CHAPTER 7

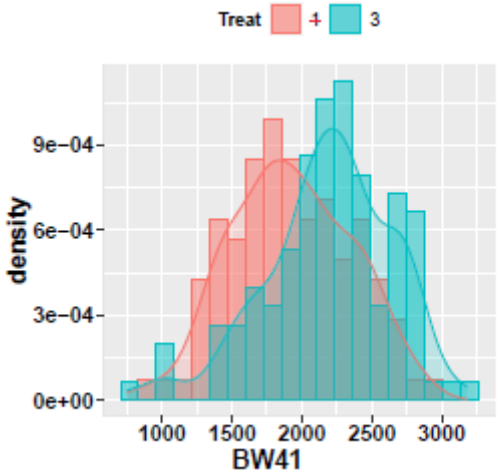
General Discussion

The main objective of the current thesis was to evaluate the effect of *in feed* microencapsulated feed additives, composed of organic acids and essential oils, on performance of broiler chickens subjected to different challenging conditions, as well as to understand the mechanisms of action behind these effects. Results have been discussed for each individual trial in the different chapters of the present thesis. Therefore, this general section will try to discuss results in a transversal way in order to provide insights into the health promoting effects of the tested feed additives and the underlying mechanisms with their implications on performance and return.

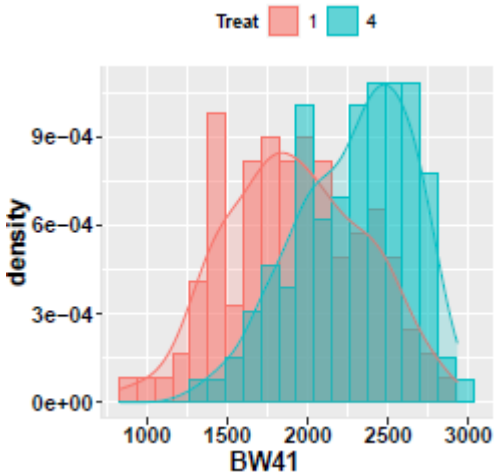
1 Effects on growth performance

We hypothesized that effects of feed additives in poultry could be higher when animals are exposed to challenging conditions. Then, two models of challenges were used to evaluate the effects of microencapsulated feed additives composed of OAs and EOs on growth performance of broiler chickens as compared to their non-supplemented counterparts. In chapter 5, the challenging conditions were induced by applying a short-term fasting period reported to promote a disruption of the intestinal barrier leading to a leaky gut, which may trigger an inflammatory response (Herrero-Encinas et al., 2020). Our results showed that the supplementation of microencapsulated fumaric acid, thymol, and their mixture improved the overall FCR during the whole experiment by 5.8% compared to the NC, and up to 7.0% compared to the non-microencapsulated ones. Similarly, the tested microencapsulated feed additives composed of OAs and EOs improved the growth performance of broilers under necrotic enteritis challenge (chapter 6). These results were in concordance with several other authors (Pham et al., 2020; Stefanello et al., 2020).

Considering our interest to understand the exact effect of these feed additives on growth performance, we performed a statistical analysis of the individual BW of our first trial (chapter 6). The idea behind this analysis was to explore if the additives may have a main target with those animals that show a slow growth or less efficient growth aiming to show the impact of the target feed additives on the reduction of morbidity due to the improvement in intestinal health status. Figure 7.1 shows BW distribution at d 41 of different supplemented blends as compared to NC or Treatment 1. The comparison includes the following blends: in figure 7.1.a: T3= NC + (malic and fumaric acid) +AC; b: T4=NC + (calcium butyrate and fumaric acid) +AC; c: NC + MCFA (capric-caprylic acid, caproic acid, lauric acid) + AC and d: NC + (calcium butyrate, fumaric acid and citric acid) + MCFA+ AC). The obtained results show a shift to the right of the histograms of supplemented groups in figures "7.1.a, 7.1.b, and 7.1.d". These findings hint an improvement of final BW of the birds of each supplemented group, regardless their individual growth potential. Moreover, it is also relevant that the mixture of calcium butyrate and fumaric acid + AC (figure "7.1.b") showed an increase of the percentage of birds with heavy BW which may suggest a higher growth promotion effect in those individual birds with a slower growth. This finding is about a great interest for poultry production as this category of chicks may be associated to several issues throughout the production cycle including reduced growth performance, higher mortality, higher sensitivity to diseases and high flock heterogeneity among others.



(a)



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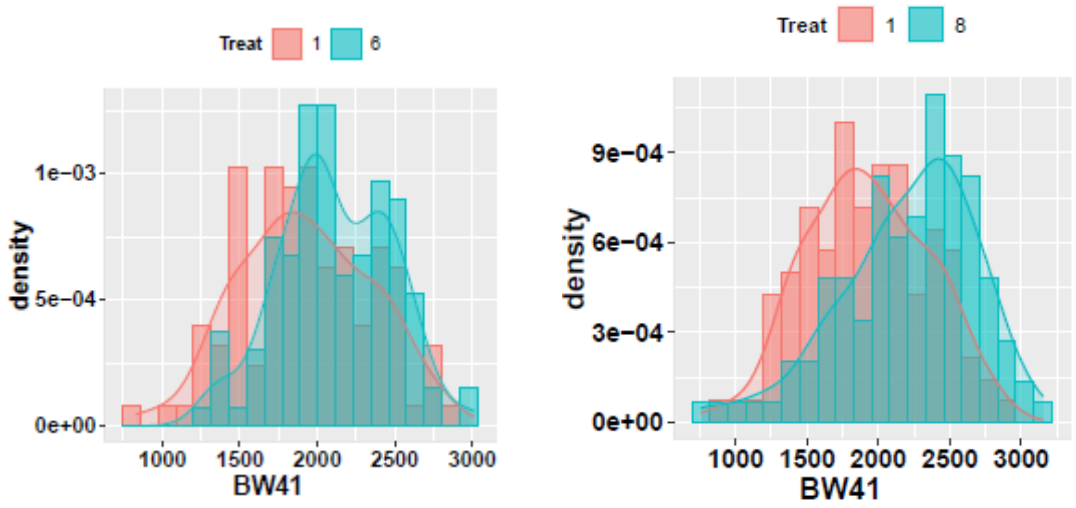

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Figure 7.1 Histograms of BW 41 of different supplemented blends as compared to NC *(a: T1=NC; T3=NC+ (malic and fumaric acid) +AC; b: T1=NC; T4=NC+(calcium butyrate and fumaric acid) +AC; c: T1=NC; T6=NC + MCFA (capric-caprylic acid, caproic acid, lauric acid) + AC and d: T1=NC; T8=NC+(calcium butyrate, fumaric acid and citric acid) + MCFA+ AC); AC: cinnamaldehyde, carvacrol, and thymol (8:1:1).*

An analysis of quartile was also performed to explore more deeply the individual BWG during the finisher phase ($BWG_{41\text{-}28d}$) (Table 7.1). Each time, the quartile corresponds to the percentage of chickens possessing a $BWG_{41\text{-}28d} \leq$ the mentioned value. For instance, focusing on Q40, 60% of the group supplemented with the mixture of (calcium butyrate and fumaric acid +AC) (figure 7.1.b) exhibited a BWG during the finisher phase higher than 1125 g, followed by the group receiving the mixture of (malic and fumaric acid +AC) (figure 7.1.a) with a $BWG_{41\text{-}28d}$ higher than 1061 g and the group supplemented with and (calcium butyrate, fumaric acid and citric acid + MCFA+ AC) ($BWG_{41\text{-}28d}$ =1045g) (figure 7.1.d) as compared to the NC where 60% of the birds showed a $BWG_{41\text{-}28d}$ greater than 772g. These results corroborate the stimulatory effects of the supplemented feed additives on growth performance of broiler chickens.

Table 7.1. Analysis of quartile of $BWG_{41\text{-}28d}$

Treat	Q20	P-value	Q30	P-value	Q40	P-value	Q75	P-value
1	503	0.003	667	0.054	772	<0.001	1221	0.058
3	819		936		1061		1381	
1	503	0.027	667	<0.001	772	0.013	1221	0.002
4	884		1012		1125		1435	
1	503	0.005	667	0.003	772	0.026	1221	0.246

6	745		849		933		1300	
1	503	0.015	667	0.001	772	0.003	1221	0.035
8	766		885		1045		1405	

T1=NC; T3=NC+ (malic and fumaric acid) +AC; T4=NC+(calcium butyrate and fumaric acid) +AC; T6=NC + MCFA (capric-caprylic acid, caproic acid, lauric acid) + AC and T8=NC+(calcium butyrate, fumaric acid and citric acid) + MCFA+ AC); AC: cinnamaldehyde, carvacrol, and thymol (8:1:1).

2 Effects on feed cost

With the aim to understand whether the improved growth performance was associated to a reduced cost of feed production, a statistical analysis was performed where the cost was calculated as follows: Cost/kg live weight (€/kg) = feed cost (€/kg) × feed intake (kg)/BWG (kg). The increase of feed price due to the incorporation of feed additives ranged from 2.4 to 7.2%/ton which is equivalent to 12.5 to 38.6 €/ton, for each phase. A reduced cost was observed by the use of the following mixtures: (calcium butyrate and fumaric acid +AC) (P=0.007) and (malic and fumaric acid +AC) (P=0.019), whereas the mixture of (calcium butyrate, fumaric acid and citric acid + MCFA+ AC) showed a tendency to decrease the cost/kg of live weight (P=0.085). This effect is linked to the ability of these feed additives to improve the intestinal health of broilers which implies saving energy for the host resulting in better growth performance. These results are also of great interest for poultry production as the improvement of the return was higher than the additional costs imposed by incorporating the tested feed additives which could increase the profit of the producers.

3 Mechanisms of action behind the growth promoting effects of the tested feed additives

The gastrointestinal tract possesses digestive, absorptive, metabolic, immunological and endocrinological functions which are on the basis of an efficient growth (Oviedo-Rondón, 2019). Therefore, any disruption of the intestinal health may affect one or several of these systemic functions. On the other hand, the trend towards antibiotic free poultry production and its sustainability requires the development of a larger understanding and practical application of concepts related to intestinal health that imply complete holistic management of the production system (Oviedo-Rondón, 2019). In this sense, intestinal diseases have been reported to be a major threat for avian

intestinal health especially under antimicrobial-free rearing programs. Indeed, as observed in chapter 4 of the current thesis and corroborating the results of several other authors, challenging conditions of coccidiosis and *C. perfringens*-induced necrotic enteritis may initiate a cascade of reactions including impaired barrier function, triggered immune response, and compromised nutrient transport among others. Under both short-term fasting period (chapter 5) and NE challenge (chapter 6) we observed that the use of the tested feed additives alleviated some of the induced negative effects on broiler gut health. In particular, supplemented feed additives improved the intestinal histomorphology of challenged broiler chickens. This improvement, especially under NE challenge (chapter 6), was evidenced by longer villi, shallower crypts and greater VH/CD ratio which was in concordance with previous studies (Jerzsele et al., 2012; Pham et al., 2020). It also suggests that the feed additives were able to alleviate the mucosal atrophy and epithelial cell necrosis due to NE induced challenge (Pham et al., 2020). Moreover, intestinal histomorphology is an important indicator of intestinal health, recovery and functionality (Pham et al., 2020) as crypt is the production site where divisions of stem cells occur to allow villus renewal. Improved intestinal histomorphology may suggest an increased surface area for digestion and absorption which may explain the improved growth performance of challenged chickens supplemented with the tested feed additives.

We also explored the likely role of the changes of the intestinal microbiota associated to the challenging conditions and experimental diets, likely derived from the antimicrobial activity of EOs (Chowdhury et al., 2018), OAs (Bedford and Gong, 2018) and blends of OAs and EOs (Cerisuelo et al., 2014; Pham et al., 2020). Indeed NE-challenged broilers supplemented with the tested feed additives showed an increase of beneficial taxa over harmful microbes, suggesting an enhancement of short-chain fatty acids (SCFAs) production, which are known to perform immune, anti-infectious and metabolic roles (Iacob et al., 2019). Particularly, these feed additives increased the abundance of some families belonging to Firmicutes, known as butyrate-producing bacteria such as Lachnospiraceae and Ruminococcaceae. The promoted butyrate production is about great interest as it is considered as the main driving force toward an optimal gut health by beneficially influencing intestinal villus structure in broiler chickens and controlling pathogens such as *Salmonella Enteritidis* and *Clostridium perfringens* (Onrust et al., 2015). Moreover, it possesses anti-inflammatory properties

by inhibiting the nuclear factor-kappa B activation, resulting in decreased expression of proinflammatory cytokines (Place et al., 2005). Butyrate is also involved in the regulation of the assembly of tight junctions by affecting the enteroendocrine L-cells secreting GLP-2, reported to exert beneficial effect on general gut health, such as the stimulation of intestinal crypt cell proliferation and the reduction of apoptosis in the crypt compartment (Mitic et al., 2000). Regarding the results of microbiota modulation with the supplementation of the mixture of thymol and fumaric acid in chapter 5, the trend was different by enhancing Bacteroidetes abundance and thereby, propionate production which can also be used as energy source by the epithelial cells and possesses anti-inflammatory activity. The difference of results observed between the supplementation of the mixture of thymol and fumaric acid under short-term fasting period (chapter 5) and the mixtures tested under the NE challenge (chapter 6) may be related to the difference of the composition of the blends tested in each case. The intestinal microbiota modulation allowing to reduce the abundance of harmful taxa under the NE challenge in chapter 6 was reflected in reduced fecal *C. perfringens* shedding. The intention was also to take full advantage from the panel of gene expression developed in chapter 4 to provide further insights into the effects of the evaluated feed additives under both short-term fasting period (chapter 5) and NE challenge (chapter 6) on the expression of genes involved in barrier function, immune response, nutrient transport. Unfortunately, this was not feasible due to insufficient number of collected samples. Therefore, it was not possible to draw a coherent conclusion from the obtained results leading to rule out the data.

On the other hand, the effective use of such feed additives requires that they reach the final portion of the GIT, considered the main site of interest for microbiota modulation as it is where the potential pathogenic bacteria are present in the greatest amount (Stefanello et al., 2020). However, the large pH variation throughout the bird's GIT may induce the dissociation of the OAs and EOs prior to the contact with the target bacteria. Therefore, the use of the adequate technology of protection which allows the gradual release of these active compounds along the entire GIT targeting its distal part, may prevent their rapid degradation in the upper segments. It may also avoid the interaction with other components of the diet, and the possible interference with palatability (Ahmad et al., 2021). The beneficial effects on broiler gut health under both short-term fasting period (chapter 5) and the NE challenge (chapter 6) as well as the

results of the BB colour release observed in chapter 5 confirmed that the major part of active compounds of the microencapsulated tested feed additives were able to reach the lower intestine. This hints that the microencapsulation technique used was able to provide both the right timing and location for the active compounds release.

CHAPTER 8

General Conclusions

From the results presented in the different chapters of the current thesis, the following conclusions can be drawn:

1. Feeding broiler chickens maintained under compromised sanitary conditions with diets containing microencapsulated feed additives, composed of OAs and EOs, exerts a growth promoting effect as compared to their challenged non-supplemented counterparts.
2. The use of some of the tested feed additives at the feed cost prices assumed in the present thesis enhanced the feed cost (/BW) which may increase the profit of producers.
3. Intestinal diseases such as coccidiosis and necrotic enteritis induce a cascade of reactions which can be evaluated by the expression of barrier function, immune response, nutrient transport and oxidative stress related genes, therefore:
    a. The gene expression pattern and magnitude of responses are different between coccidiosis and necrotic enteritis disease.
    b. Protein expression analysis allows to know if the higher mRNA expression results in higher protein expression (although the correlation of both analyses is reported to be low in the literature).
4. The tested feed additives composed of OAs and EOs exert their beneficial effects on broiler gut health especially through intestinal microbiota modulation. This modulation towards enhancing the abundance of beneficial taxa (Ruminococcaceae, Lachnospiraceae and Bifidobacteriaceae among others) beneficially affects the structure of the intestine by increasing the ratio of villus height to crypt depth, leading to better absorption and therefore higher growth performance.
5. Microencapsulation of the tested feed additives using a lipid matrix was an effective technique to provide both the right timing and location for the active compounds release, allowing them to exert their beneficial effects in the lower GIT.

CHAPTER 9

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ANNEX 1

Curriculum vitae of the author

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2017-present	**Ph.D. Student in Animal Production**
	Universitat Autònoma de Barcelona, Spain
2016-2017	**M.Sc. in Research in Animal Nutrition**
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2015-2016	**Postgraduate Specialization in Animal Nutrition**
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2007-2010	**Engineering degree, Animal Science**
	National Agronomic Institute of Tunisia
2005-2007	**Preparatory cycle in Biology and Geology**
	Higher University of Applied Sciences and Technologies of Gabès

Scientific publications

Abdelli, N., and S. Dridi. 2022. Avian Neuropeptide beyond feed intake regulation. *Submitted to veterinary sciences.*

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Courses

English course (B2.2): 2019
Servei de llengües, UAB

Training course on statistical techniqueswith R: 2018
Servei d'Estadistica Aplicada, UAB

Training Course in Laboratory Animal Science for Scientists Responsible for the Design or Conduct of Animal Experiments: 2018
Universitat Autònoma de Barcelona

Fellowships

2017-present: Pre doctoral research grant (FI-DGR 2017)
Agencia de Gestión de Ayudas Universitarias y de Investigación. Generalitat de Catalunya

2015-2017: Scholarship to attend the International Master of Sciences in Animal Nutrition
International Centre for Advanced Mediterranean Agronomic Studies (CIHEAM)

Personal skills

Languages

Mother tongue(s)	**Arabic**				
Other language(s)	***UNDERSTANDING***		***SPEAKING***		***WRITING***
	Listening	**Reading**	**Spoken interaction**	**Spoken production**	
French	C1	C1	C1	C1	C1
English	B2	B2	B2	B2	B2
Spanish	B2	B2	B2	B2	B2

Softwares

Microsoft office ●●●●●

SAS ●●●○○